STUDY OF PHENOTYPIC MODULATION OF *BRUCELLA ABORTUS* UPON INFECTION OF MACROPHAGES

By

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STUDY OF PHENOTYPIC MODULATION OF *BRUCELLA ABORTUS* UPON INFECTION OF MACROPHAGES

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LIST OF ABBREVIATIONS

PMN	Polymorphonuclear Leukocytes
MPO-H_2O_2-Halide	Myeloperoxidase-Hydrogen-peroxide-Halide
LPS	Lipopolysaccharide
S-LPS	Smooth Lipopolysaccharide
HtrA	High Temperature Requirement
DMEM	Dulbecco's Modified Eagle Medium
HBSS	Hanks' Balance Salt Solution
PAGE	Polyacrylamide Gel Electrophoresis
IEF	Isoelectric Focusing
MIP	Macrophage Induced Proteins
KD	Kilodalton
PCR	Polymerase chain reaction
RAP-PCR	RNA fingerprinting using arbitrary primed PCR
AP-PCR	Arbitrary Primed PCR
OMP	Outer Membrane Proteins
Mab	Monoclonal Antibodies

Min	Minute(s)
Hr	Hour(s)
2-D PAGE	Two Dimensional Polyacrylamide Electrophoresis Gel
Sod	Superoxide Dismutase
mRNA	Messenger RNA
cDNA	Complementary DNA
PBS	Phosphate Buffered Saline
MMLV-RT	Moloney murine Leukemia Virus Reverse Transcriptase
UV	Ultraviolet Light
bp	Base Pair
TBP-1	Transferrin Binding Protein 1
2,3-DHBA	2,3-Dihydroxybenzoic Acid

CHAPTER I

INTRODUCTION

Genus *Brucella*

The genus *Brucella* is a gram negative, cocci, coccobacilli or short rod shaped, nonmotile and aerobic bacterium. Most *Brucella* strains behave as slow growing fastidious organisms which require complex media containing several amino acids, thiamine, nicotinamide and magnesium ions. The optimum temperature for growth is 37°C but growth occurs at temperatures between 20° to 40° C. The optimum pH for growth is 6.6-7.4. *Brucella* species are pathogenic for a wide variety of animals including man (15).

The infection usually results in a bacteremic phase followed by localization in the reproductive organs and the reticuloendothelial system. Infection in the pregnant animal often results in placental and fetal infection and this frequently causes abortion. The organisms may localize in the mammary tissue and can be excreted in the milk. Typically growth *in vivo* is intracellular and the organisms can grow within both granulocytes and monocytes (15).

Brucella is a good model to demonstrate tissue and host specificity of pathogenic bacteria. In many animals such as, humans and rats, brucellosis may cause a mild chronic disease. The pathogen parasitizes the reticuloendothelial cells (63) and has no marked affinity for particular tissues. In contrast, in pregnant cows, sheep, goats and sows, the

pathogen localizes in the placenta and the fetal fluids and results in the characteristic climax of the disease - abortion (65). Studies have indicated that erythritol is present in the susceptible tissues of animals that suffer from the acute infectious abortion and that this sugar is responsible for the tissue specificity (63) of this pathogenic microorganism.

Distribution of Brucellosis in the World

In terms of public health and economic importance, brucellosis is a world problem due to direct and indirect transmission of the disease from infected animals to man in addition to diminution of food supply, especially animal proteins. The transmission of the disease to animals and man is directly related to animal production practice, methods of processing milk, local food habits, environmental hygiene, movement of animals, etc. Brucellosis is mostly a problem of underdeveloped countries. Brucellosis caused by *B. melitensis* is a problem in the Mediterranean basin of Europe and Africa, Mongolia, the Middle East and central and South American countries such as Argentina, Mexico and Peru. Swine brucellosis caused by *B. suis* is found mostly in Latin and North America. *B. ovis* infection is a significant problem in sheep raising countries. Bovine brucellosis is a problem in South America, Africa, Asia and Australia (51). In the United States of America bovine brucellosis is a problem in six southern states including Texas, Oklahoma, Arkansas, Louisiana, Mississippi and Florida (34).

Fate of Bacteria within Phagocytic Cells

Brucellosis is transmitted in animals through the oropharynx, mouth, skin, and mucous membranes of the eyes and nose (34). After entering the host, bacterial cells are ingested by phagocytic cells such as macrophages and polymorphonuclear leukocytes

(PMN). Endocytosis of intracellular pathogens by host cells serves these microorganisms as a vehicle and provides the bacterium with protection from host immune components. After endocytosis by phagocytic cells, bacterial cells find themselves in a vacuole called a phagosome. The enclosed environment within the vacuoles deprives the microorganisms of nutrients and subjects them to unfavorable conditions. For example, phagocytosis is followed with an acidification of the phagosome to approximately pH 6.0 (41).

Furthermore, phagocytic cells contain structures called primary granules or lysosomes. The primary granules migrate through the cytoplasm and fuse with the phagosome and release their enzymes. The granules contain various enzymes such as myeloperoxidase or catalase, lysozyme, neutral proteases, elastase, acid hydrolase and β-glucuronidase. These enzymes can digest bacterial walls and kill most microorganisms. The susceptibility of bacteria to these enzymes varies. For example gram positive bacteria are more susceptible to lysozyme as compared to gram negative bacteria such as *E. coli*. On the other hand, intracellular bacteria such as *Brucella* and *Salmonella* have developed ways by which they can survive within phagocytic cells (14).

A second bactericidal mechanism of phagocytic cells is an oxygen-dependent microbicidal mechanism called the respiratory burst. The respiratory burst is activated by physical stimuli such as when phagocytes adhere to a surface, or chemical stimuli such as opsonization of a microorganism with complement or antibody molecules. The opsonized microorganism is recognized by the receptors on the surface of the phagocytes (35). The oxygen-dependent antimicrobial mechanism results in the production of highly reactive oxidants such as superoxide anion, H_2O_2, hydroxyl radicals, hypochlorite ion and

singlet oxygen. The hypochlorite ions kill bacteria by oxidizing their proteins. Singlet oxygen and hydroxyl radicals are very reactive compounds which react with lipids to form bactericidal hydroperoxides (14).

Intracellular bacteria that are capable of survival within phagocytic cells have developed mechanisms by which they can prevent fusion of phagosome and lysosome (primary granules), survive within the phagolysosome or escape into the cytoplasm after phagolysosome formation. For example, lysosomes do not fuse with phagosomes containing *Mycobacterium tuberculosis* due to the presence of acidic sulfatides whereas *Mycobacterium microti* blocks degranulation by increasing the level of intracellular cyclic AMP. A third mechanism is used by *Mycobacterium lepraemurium* and *Salmonella typhimurium* which multiply within phagolysosomes despite extensive degranulation (59). The mechanism by which *B. abortus* survives within phagocytic cells is not well understood.

Study of Survival of *Brucella* within Phagocytic Cells

There is very little known about the survival mechanism of *B. abortus* within phagocytic cells. In an attempt to characterize the mechanism of intracellular survival of these bacteria, several groups (37, 59) have examined the ingestion and killing of rough and smooth strains of *B. abortus* by PMNs. These studies have indicated: a failure of phagocytic cells to generate lethal oxygen intermediates allows *Brucella* to survive intracellularly (59); degranulation was significantly less in PMNs incubated with smooth or rough strains of *B. abortus* as compared to that observed for an extracellular parasite (37, 59); smooth strains of *B. abortus* were more resistant to intracellular killing than the

rough strains (37, 59); viable *B. abortus* cells were not required to inhibit degranulation (59); both rough and smooth strains showed sensitivity to the oxidative killing mechanism when they were incubated in the presence of the lysates of PMNs supplemented with halides (37); finally, after ingestion of *B. abortus* by PMNs there was no stimulation of the hexose monophosphate pathway which is normally seen after ingestion of extracellular parasites (37). These observations have led these groups to the conclusion that *B. abortus* achieves intracellular survival by preventing the degranulation and oxidative killing mechanism after ingestion by phagocytic cells. This inhibition may be due to lack of proper stimulation or active inhibition of degranulation (37, 59).

Further attempts in unraveling the mechanism used by *B. abortus* to survive intracellularly led Canning et al., (9) to identify nucleotide-like compounds with molecular weight less than 1000. These compounds are involved in the inhibition of myeloperoxidase (MPO)- H_2O_2- halide antibactericidal system of PMNs by inhibition of degranulation. Synthesis of low molecular compounds that would inhibit the antibactericidal ability of phagocytic cells have been observed in other pathogenic bacteria such as *Rhodococcus equi* (17). Electronmicroscopic studies indicated that *B. abortus* inhibited the degranulation of both primary and secondary granules in neutrophils (9). Studies conducted by Kreutzer et al.; (36) indicated that ingestion of *B. abortus* did not stimulate the oxidative metabolic burst and therefore H_2O_2 required by MPO-H_2O_2-halide system was not produced. In addition, Canning et al.; (9) and Kreutzer et al.; (36)

observed that once the oxidative metabolism of PMNs is stimulated by other bacteria, *B. abortus* is not able to prevent the formation of reactive oxygen intermediates.

The Role of Cell Wall Components in Intracellular Survival

Studies of the behavior of rough and smooth strains of *B. abortus* within phagocytic cells have indicated that the rough, less virulent strains of *B. abortus* are more sensitive to the bactericidal mechanism of the phagocytic cells than the smooth, more virulent strains (37, 59). Furthermore, rough strains of *B. abortus* are more sensitive to the killing mechanism of bovine serum than the smooth strains (16). These differences in resistance to the bactericidal mechanism of phagocytic cells and serum are believed to be due to the differences in the cell surface components of the rough and smooth strains of *B. abortus*. Comparison of chemical composition of cell surface components of the smooth and rough strains of *B. abortus* revealed only quantitative minor differences in total lipids, proteins and murein layer (34).

Kreutzer et al.; (36) have found that the smooth strains of *Brucella* contain both phenol-soluble and water soluble lipopolysaccharides (LPS), whereas the rough strains contain only the water soluble components (36). The phenol-soluble component has been shown to be toxic to mice (36). Additional studies of differences of cell surface components of various *B. abortus* strains indicated that they all have the same membrane protein profiles (16). However, the smooth strains had O-antigen whereas the rough strains did not (16). Animals inoculated with the cell wall envelope of the rough strain of *B. abortus* produced a strong antibody response to the outer membrane proteins and no detectable response to S-LPS. In contrast, inoculation of animals with the cell wall

envelope of the smooth, virulent strain showed only low levels of antibodies to outer membrane proteins, and a strong response to S-LPS (16).

Ficht and Smith (72) found that the primary host cells for *B. abortus* are mononuclear phagocytes, but neutrophils are capable of phagocytosis of *B. abortus* (59). Macrophages have various receptors on their surfaces which are often used by intracellular parasites to gain entry into host phagocytes (20). Of particular interest are integrins which bind ligands through interaction with an Arginine-Glycine-Aspartic acid (RGD) amino acid sequence on the ligand surface. Intracellular parasites could use any of the several integrins as an organism specific mechanism of host cell entry (8). The presence of an outer membrane protein with a region containing RGD in *B. abortus* has been shown (20). Campbell et al., (8) have shown that *B. abortus* may bind the integrins on the surface of host macrophages in the absence of serum and thereby gain entry into the host cell.

The comparison of monoclonal antibodies (MAbs) against several outer membrane proteins (OMP) of *B. abortus* has shown them not to be as effective in protection against splenic infection in mice as anti-LPS MAb (31). Comparison of the antibody response of cattle to OMP and S-LPS of *Brucella* revealed that the response to OMP occurred later and was less intense than that to S-LPS (43). In addition, vaccination of mice with LPS provided protection against brucellosis whereas vaccination with a recombinant 31 KD *Brucella* cell surface protein (rBCSP) did not provide any protection (57).

The Role of Heat Shock Proteins in Pathogenicity

Invasion of mammalian cells by pathogenic parasites such as bacteria results in physiological changes in both the host and the pathogen which allow their adaptation to and recognition of one another. The environmental conditions that most bacterial pathogens have to cope with, in particular the intracellular pathogens, suggest that heat shock proteins may play a role in their survival within host cells. The multiple gene products that are required for the interaction between host and the bacterial pathogens are considered as virulence factors. This definition includes a wide range of molecules such as toxins, which are important in causing the disease and adhesins which mediate entry of the bacteria into the host cell (39). This definition includes mainly factors that are present only during host-pathogen interaction and are not required for survival of bacteria at other times. Therefore, housekeeping genes such as the genes involved in the synthesis of aromatic amino acids are not accepted as virulence factors (25, 30).

Due to this definition of virulence factors the role of DnaK and GroE as virulence factors has been questioned. On one hand, these proteins have important housekeeping functions and therefore should not be considered virulence factors. The fact that no null GroE mutant has ever been isolated and only temperature sensitive mutants of DnaK are available indicates that these proteins play an essential role in the bacterial survival. On the other hand, both GroE and DnaK bind to and prevent aggregation of unfolded polypeptides. Therefore, any time that the level of unfolded polypeptides increases, the increased level of heat shock proteins is required for removal of denatured polypeptides. It can be rationalized that any stress that can result in increased levels of unfolded

proteins by altering patterns of protein synthesis or translocation, or by causing denaturation of proteins within the cell, will be countered by the induction of heat shock proteins. If the level of the unfolded polypeptide increases in the living bacterium then it would be possible to consider the chaperons as virulence factors (39). In fact there are several reports that indicate increased expression of DnaK and GroE in pathogenic bacteria such as *Salmonella* and *Legionella* during intracellular survival (7, 46).

A second group of stress proteins are those induced only during various stress conditions and survival within host cells. Protease La (25) and high temperature requirement (HtrA) (48) both function as proteases and their induction may play a role in the degradation of polypeptides unfolded by stress. In contrast to the chaperons, the proteolytic heat shock proteins are required only for survival in the presence of specific stress conditions. Mutation in the *htrA* gene results in creation of avirulent strains of *Salmonella typhimurium* which demonstrates the importance of the heat shock proteins in response to host (46).

Comparison of Intracellular Survival with Various Stress Response Proteins

In order to understand the intracellular survival mechanism, the protein synthesis pattern of several bacterial pathogens during intracellular survival has been studied by two-dimensional polyacrylamide gel electrophoresis. Furthermore, the protein synthesis pattern during intracellular survival of these bacteria has been compared to the pattern obtained when the same bacteria are exposed to various stress conditions.

Two dimensional protein synthesis of *S. typhimurium* during intracellular survival within murine macrophage-like J744 cells and different stress conditions that

resemble the intracellular conditions have been compared. These comparisons have shown that macrophage induced proteins could be divided into three groups. Group 1 included those that were induced under several of the stress conditions under investigation. Group two included those that were induced only by one or two of the stress conditions. Group 3 included those that were only induced during intracellular survival. These comparisons have led to the conclusion that the macrophage-induced response was not a simple sum of individual stress responses displayed during extracellular stress (1). The study of protein synthesis pattern of *S. typhimurium* by Buchmeier and Heffron (7) indicated that GroE and DnaK are among the proteins that are induced during intracellular survival.

The two-dimensional protein synthesis pattern of *Legionella pneumophila* has also been compared to the pattern obtained under several stress conditions. These studies have revealed that 13 out of 35 macrophage-induced proteins were induced under several other stress conditions. Among these 13 proteins GroEL and GroES have been identified (38). Furthermore, study of *Chlamydia trachomatis* protein synthesis during intracellular survival has indicated that major outer membrane proteins, GroE and DnaK are among the proteins induced during intracellular survival (49).

Finally, a comparison of the two dimensional protein synthesis in *Listeria monocytogenes* with those obtained during upshift of temperature and oxidative stress has indicated that there is no overlap between these responses. It is thought that the induction of the stress proteins is not required because *L. monocytogenes* escapes rapidly from the stressful phagosome into the cytoplasm of the macrophages (21).

CHAPTER II

IDENTIFICATION AND COMPARISON OF MACROPHAGE-INDUCED PROTEINS WITH PROTEINS INDUCED UNDER VARIOUS STRESS CONDITIONS IN *Brucella abortus*

Introduction

Brucella abortus is a facultative intracellular pathogen that survives ingestion by macrophages and neutrophils. 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* (16). 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 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. In addition,

intracellular bacteria have to cope with other conditions present inside the host cell such as nutritional deprivation. 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. (37) and Riley and Robertson (59) 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/stress conditions which may resemble the environment in macrophages during intracellular growth. Two-dimensional polyacrylamide gel electrophoresis has been 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 stress were identified. Finally, the pattern of

protein synthesis under each stress condition was carefully compared to 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 the intracellular growth is not just a simple sum of the responses to various stress conditions that may resemble intracellular conditions.

Materials and Methods

Bacterial Strain:

B. abortus strain 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/60 ml blood). Aliquots of 30 ml of anticoagulant-treated blood were centrifuged for 30 min at 2500 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 (HBSS, Sigma), underlaid with Ficoll-Paque (Pharmacia) and centrifuged for 20 min at 2000 rpm in the Sorvall SH-3000 rotor (27). Mononuclear cells were isolated from the interface between diluted plasma and Ficoll-Paque and were washed with HBSS 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 $5x10^7$ cells in 100 mm tissue culture-treated petri dishes (Corning) in a humidified incubator at 37°C, and 5% CO_2 for 2 hr. Non-adherent cells were removed by washing once with DMEM containing 15% serum and twice with HBSS. Adherent cells were cultured in DMEM with 15% serum in an atmosphere containing 5% CO_2. The culture medium was changed every four days. Monocytes were allowed to mature into macrophages for one week.

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 grown on tryptose agar plates for 72 hours was harvested by centrifugation at 2080 x 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, 5% CO_2 and 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/ml streptomycin, 50 μg/ml cycloheximide and 50 μCi/ml [^{35}S]-protein labeling mix (DuPont) were 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% (V/V) ice cold Triton X-100 for 30 min. The plates were washed and bacterial cells were collected by centrifugation at 2080 x g using a benchtop centrifuge for 15 min. The pellet was washed twice with saline and was resuspended in one 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* grown intracellularly and in the tissue culture medium due to treatment of macrophages with cycloheximide, *B. abortus* was labeled in the tissue culture medium in the presence of 50 μg/ml of cycloheximide and 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/ml cycloheximide and their protein pattern were analyzed.

Radiolabeling of *B. abortus* proteins during 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 100-120 (green filter). Cells were labeled with 50 μCi/ml [^{35}S]-protein labeling mix for 20 or 60 min. Labeled cells were chased with 200 μg/ml cold methionine. All 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. (44) was followed. *B. abortus* was grown in minimal medium (26) overnight at 37°C to the mid-log growth phase. The bacterial culture was centrifuged at 2080 x 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

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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 by Fitzgeorge, et al. (21) was followed with a few minor modifications. *B. abortus* was grown on tryptose agar plates for 18 hr. Bacterial cells were harvested and transferred to saline. The concentration of H_2O_2 (Sigma) in the culture was adjusted to 0.005% and 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. However, the concentration of menadione sodium bisulfite in the culture was adjusted to 10 mM. In the case of nutritional deprivation, *B. abortus* grown on tryptose plates were harvested with saline and were washed twice with saline. The culture was divided into three equal aliquots and cells were centrifuged. The pellets were resuspended in methionine-free RPMI 1640 medium with 15% serum, minimal medium (26) or saline. 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 1-5 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 hr. The number of viable cells was determined by counting the number of colonies on the tryptose plates (12).

Two-dimensional PAGE analysis of proteins:

To prepare labeled proteins for two dimensional polyacrylamide gel electrophoresis (PAGE), pulse labeled cells were mixed with one volume of 10% TCA and the precipitate was collected by centrifugation. The precipitate was washed once with 5% TCA and once with acetone (77). The precipitate was solubilized in SDS and urea solubilization buffer (3). Two-dimensional PAGE was performed as described by O'Farrell (53) using the Investigator system of Millipore. Isoelectric focusing gels were cast in capillary tubes (Millipore Co.). The IEF gels were prepared with ampholyte solution (Millipore Co.) with the 3-8 pH. The IEF gels were prefocused for about 1 hr and then an equal number of counts were loaded on each IEF gel. The first dimensional gels were run at 18000 volt-hours. 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 DTT and 0.01% bromophenol blue (Millipore) and were loaded on top of the polyacrylamide gels (12.5%). Polyacrylamide gels were electrophoresed at 1200 mW. Along with each gel a standard (Biorad) for pI and molecular weight 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 using the PDQuest software package designed for the analysis and databasing of 2-D gels and films. Each sample was electrophoresed twice and each experiment was performed twice except for intracellular growth which was performed twice but each sample was electrophoresed only once. The changes in the protein expression were

accepted if they were observed in three out of four autoradiographs for each stress condition studied and in both autoradiographs for intracellular survival experiments.

Immunization of rabbit with DnaK:

Female New Zealand white rabbits were immunized with purified recombinant *E. coli* DnaK protein (Epicentre Technologies) emulsified in Hunter's TiterMax (CytRx Corporation). The emulsion was prepared according to manufacturer's instructions and contained 100 μg protein/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 at 4 and 8 weeks after the primary immunization. Twelve weeks after the primary immunization, the rabbits were anesthetized with parental Ketamine (35 mg/Kg) 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 (1000 x g) for 20 min at 4°C, aliquoted and stored at -80°C.

Western blotting:

Brucella were grown on tryptose plates and harvested after 72 hr. Cells were washed twice with saline and were transferred to RPMI 1640 medium prewarmed to 37°C containing 15% serum, and 50 μCi/ml [^{35}S]-protein labeling mix. *B. abortus* was labeled for 4 hr at 37°C and 5% CO_2. Cells were then washed and labeled proteins were solubilized as described above. Two concentrations of labeled proteins (36 and 27 μg)

were separated by 2-dimensional PAGE as described above. Proteins were transferred to a PVDF membrane (Micron separation Inc.) using a Polyblot transfer system (American Bionetics, Inc.) and following the instructions recommended by the manufacturer. The blot was air dried overnight and was then 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 hr 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 rabbit. The antibodies against GroEL, SodC, HtrA were generously provided by Dr. R. W. Hendrix, Dr. F. Tatum, and Dr. M. Roop II, 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 alkaline phosphatase conjugated with anti-rabbit IgG (Sigma) containing TBS, 0.125% skim milk for 2 hr 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 100 mM Tris-HCl, pH 9.5, 100mM NaCl, 100 mM $MgCl_2$, containing 300 μg/ml nitro blue tetrazolium (Sigma) and 150 μg/ml 5-bromo-4-chloro-3-indoyl phosphate (Sigma) 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 to that of cells grown under identical culture conditions in the absence of macrophages. The analysis of protein synthesis pattern 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 pattern between the two conditions. These differences include both induction and repression of several proteins (Fig. 1). The expression of 43 spots is induced during intracellular growth. Of these, 24 appear to be uniquely expressed in the macrophage environment (Fig.1B & C); these proteins are hereafter called macrophage-induced proteins (MIPs). In contrast, the synthesis of 73 spots is decreased after engulfment by macrophages (Fig. 1A).

Protein synthesis and response of *B. abortus* to heat:

The appropriate temperature for heat shock experiments and physiological response of *B. abortus* to an upshift of temperature was determined by subjecting *B. abortus* to temperature shifts of 42°C or 45°C (Fig.2). The survival plot indicated that the growth pattern of *Brucella* was not greatly changed when temperature is increased from 37°C to 42°C. However, an increase from 37°C to 45°C was lethal to the bacteria within 4 hr. The temperature that was not lethal to *Brucella* but still high enough to lead to a response was chosen for labeling experiments (44, 45). 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* to a higher temperature (Fig. 3B & C). The expression of about 70 spots increased after exposure to increased temperature. The major proteins expressed during temperature upshift in *Brucella* fall into groups with approximate molecular weights of about 20, 40, 60 and 70.

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 4 indicates that although exposure to this level of H_2O_2 is lethal to *B. abortus*, the killing occur slowly: 40% of the cells survived after 6 hr. 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. exposure to H_2O_2 and the rest of these proteins were completely repressed in the presence of 0.005% H_2O_2 (Fig. 5A). This comparison has also revealed that 21 spots were detected only in response to H_2O_2. The molecular weight of the major proteins that are expressed only in response of H_2O_2 fall in the range of 15, 20, 30, 40 and 70 (Fig. 5B & C). In addition, the expression of 16 spots increased in *B. abortus* in the presence of H_2O_2. The molecular weights of these proteins are between 10-45 KD (Fig. 5B & C).

Protein synthesis and response of *B. abortus* to menadione:

Various concentrations of menadione, which induces superoxide (12), were used to determine the effect of superoxide on growth of *B. abortus*. Our results indicated that high (50-150 mM) concentrations of menadione had immediate effects on *B. abortus*.

These concentrations were able to decrease the percentage survival to approximately 10% within 6 hrs. In contrast, more than 50% of the cells survived the presence of 10 mM menadione for 6 hrs (Fig.6). The protein synthesis pattern of *B. abortus* in the presence of 25, 50 and 75 mM menadione was investigated. Menadione concentrations of 25 mM and above resulted in very low labeling of proteins. Therefore, a 10 mM concentration of menadione was used. Comparison of the two-dimensional protein synthesis pattern of *B. abortus* in the presence and absence of 10 mM menadione indicated that a large number 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. 7B & C). The molecular weights of most of the proteins induced in response to menadione were above 35 KD.

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 was monitored for several hr. in minimal medium. Citrate was added to the minimal medium for buffering. Figure 8 indicates that at pH 4.5 *B. abortus* rapidly loses viability but at pH 5.5 viability is still 40% after 8 hr. The protein synthesis pattern of *B. abortus* was determined in response to growth at pH 5.5 and was compared to that obtained at pH 7.0 by two-dimensional polyacrylamide gel electrophoresis. 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. 9B & C). The molecular weight of the major proteins induced in response to reduced pH were in the range of 15-20, and

60 kD. Finally, expression of more than 100 spots was found to be repressed in *B. abortus* as a response to pH 5.5 (Fig. 9A).

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 containing 15% serum, saline, minimal (26) and tryptose medium. The response of the cells was followed by determining the number of viable cells at various time points. Figure 10 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 and tryptose medium. With these two media, the number of viable cells starts to increase after 1.5 hr 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 with a slow decrease in the number of viable cells which continued for 5 hr. 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. 11B), with one major protein with molecular weight of 34 KD. 8 new spots were induced when *B. abortus* was labeled in saline (Fig. 11C). The molecular weight of major proteins were about 67, 51, 42 and 14 kD. In contrast, over 70 new spots

were detected when *B. abortus* was labeled in RPMI 1640 medium (Fig. 11A), 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 induced proteins during intracellular survival and other stress conditions. Several proteins with the same molecular weight as DnaK and GroEL cross reacted with the antibodies against these proteins. The protein spot 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 I and Figure 1). In contrast, the expression of SodC and HtrA were repressed during intracellular growth. Table I 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 nutritional stress. DnaK and GroEL were also induced during reduced pH conditions along with SodC. SodC was induced in response to H_2O_2 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 to that of various stress conditions that were thought to resemble the intracellular conditions. A summary of these comparisons is presented in Table II. The comparisons indicated that there are

very few proteins induced both during intracellular survival and 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 Ficht et al. (44) who have shown that the pattern of gene expression of *B. abortus* changes during intracellular survival in macrophage-like cells by using SDS-PAGE. 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 and that of cells grown in bovine macrophages. Our results, like those obtained by Ficht et al. (44), 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 those patterns induced during 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 due to 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 *S. typhimurium* (7) and *Legionella pneumophilia* (2) increases during growth in macrophages *in vitro*.

Comparison of the protein synthesis pattern of *B. abortus* during intracellular survival with those obtained under 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. (33) have shown that macrophages infected with *B. abortus* are able to kill intracellular bacteria over the first 12 to 24 hr. following 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 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* (18). Western blot analysis and two-dimensional protein synthesis patterns of *Brucella* during intracellular survival indicated that HtrA is expressed in response to extracellular and intracellular survival. 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 profile of *B. abortus* protein synthesis during intracellular survival and the stress conditions indicated that the expression of the proteins of the same

molecular weight that stained with antibody against DnaK or GroEL were increased during intracellular survival and all stress conditions except starvation. These proteins may represent different charge forms of DnaK and GroEL.

The role played by Sod in protecting intracellular pathogens against the oxidative antimicrobial mechanism of phagocytic cells is unclear. Papp-Szabo and coworkers (55) 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 (69). 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 (24). *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 to be able to colonize the spleen of BALB/c mice (40). 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 (68). 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.

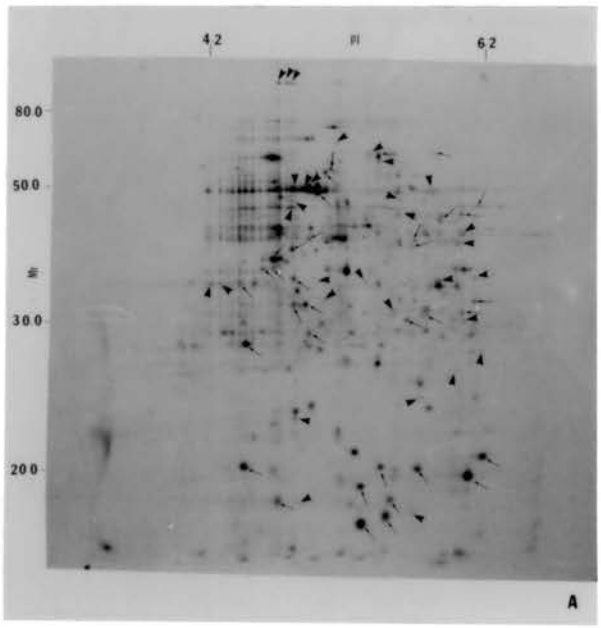
The ability of *B. abortus* to prevent the fusion of the phagosome with the primary granules (9) has been observed by electron microscopy. In addition, Canning et al. (10) have identified guanosine monophosphate (GMP) and adenine synthesized by *Brucella* during intracellular survival which inhibit the oxidative antimicrobial mechanism of

bovine PMNs by inhibition of the MPO-H_2O_2-halide system (10). Jiang et al. (33) 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* opsonized with sera containing complement is readily phagocytosed by bovine macrophages as opposed to *B. abortus* cells oponized with sera lacking complement. Thus, *B. abortus* like *Legionella* (56) , *Mycobacterium* (62) spp., and *Salmonella* (69) may enter phagocytic cells via complement receptors. This method of entry 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. Therefore, *B. abortus* may avoid the oxidative burst by choosing complement receptors for entering macrophages and thereby making the function of Sod unnecessary for survival within the host cells.

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 characterization of this response by comparison of the protein synthesis pattern to those obtained from defined environmental stress conditions. Our data represents the first comparison of *B. abortus* proteins on a whole-cell level which respond to various environmental conditions. It also provides a picture of the *B. abortus* response to an early stage of infection.

Figure 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. intracellular survival have been marked with → or ➤, respectively. (B) & (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 any of the stress conditions studied have been marked with letters. The numbers and circles present GroEL (1), HtrA (2), Dnak (3) and SodC (4).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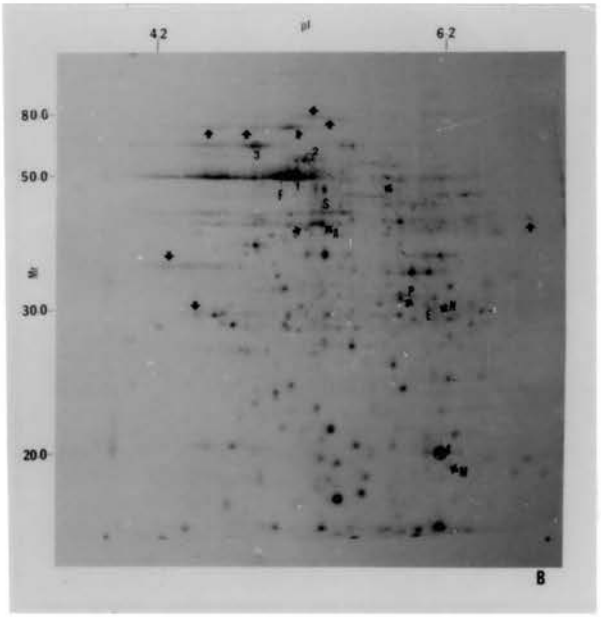
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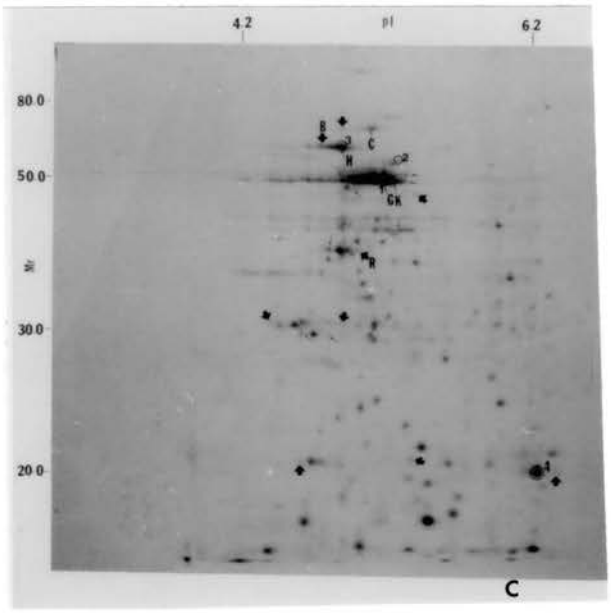
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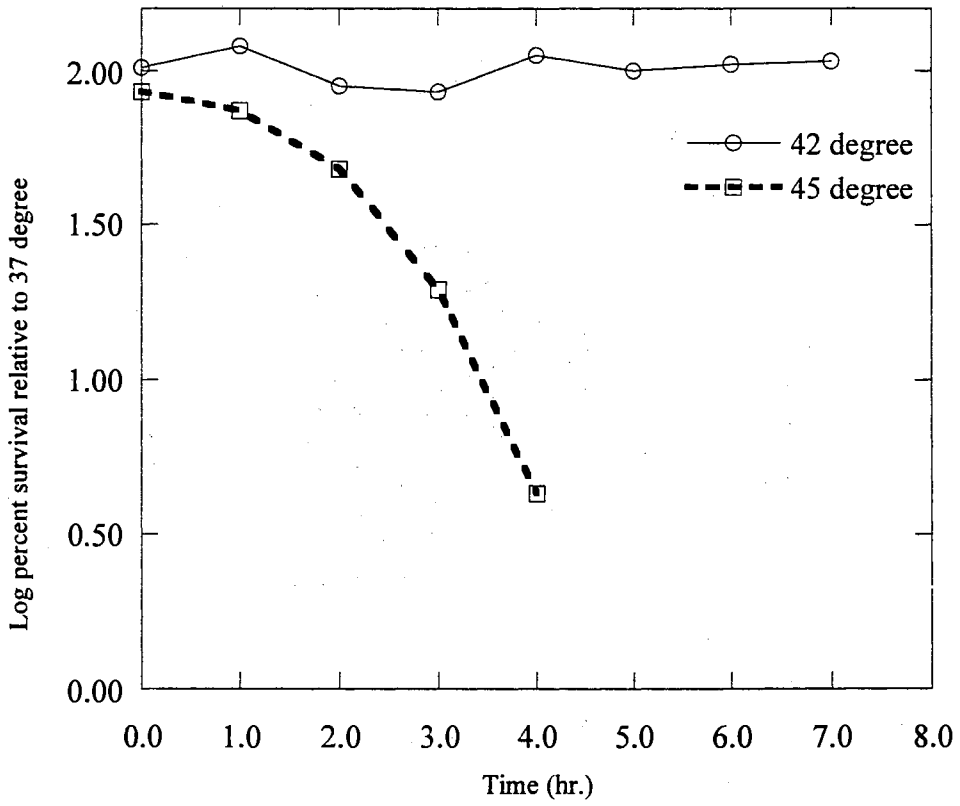
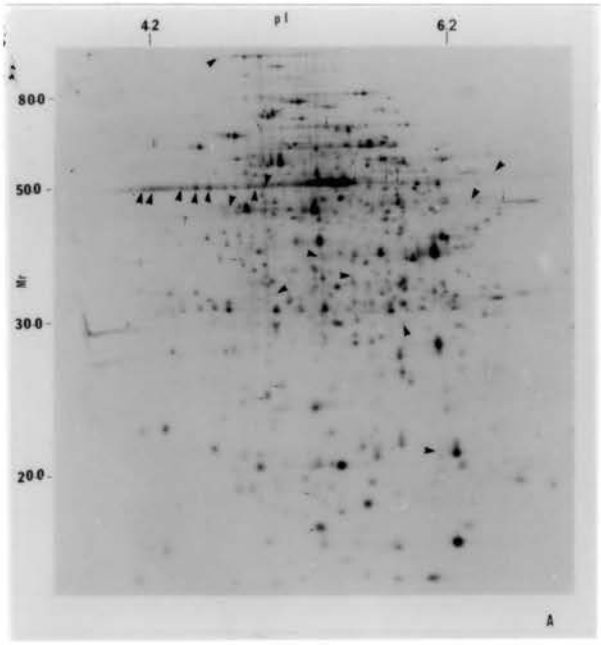


Figure 2. Survival at Different Temperatures

Figure 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 minutes. Proteins whose expression has decreased or have not been expressed in response to increased temperature after 20 min. or 60 min. have been marked with → or ➤ , respectively. (B) & (C) *B. abortus* labeled in minimal medium at 42°C for 20 min. 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 during both intracellular survival and survival at 42°C have been marked with letters.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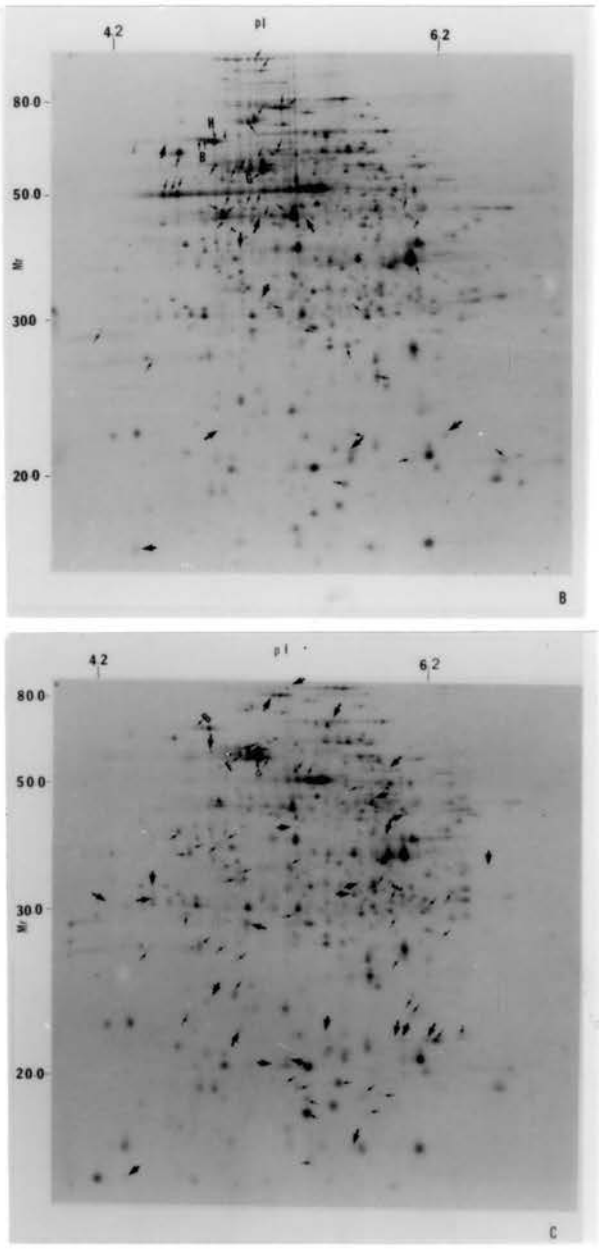
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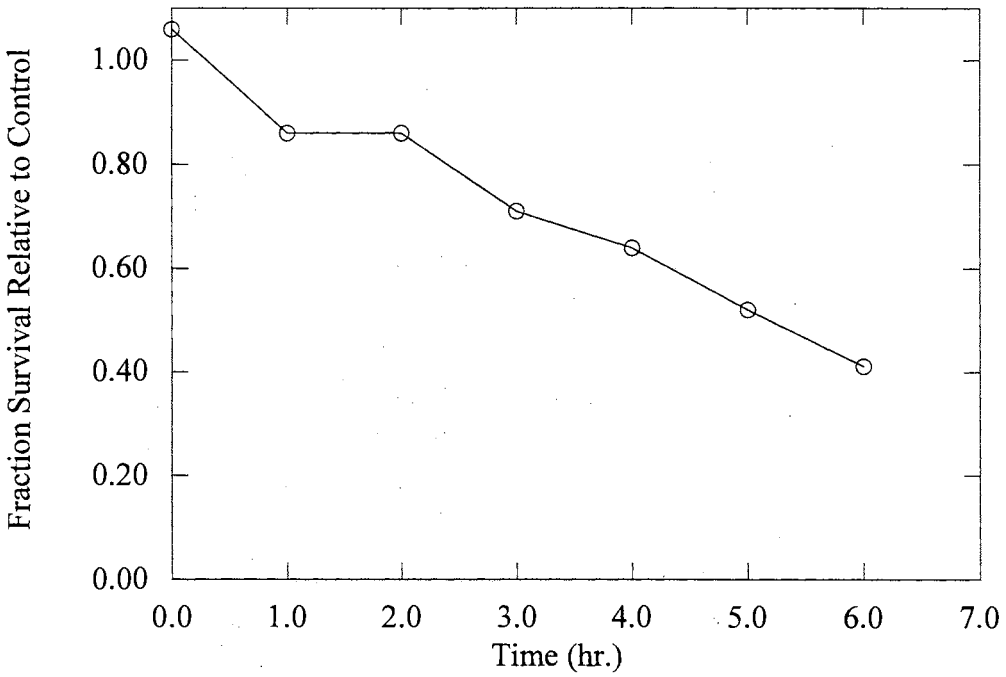
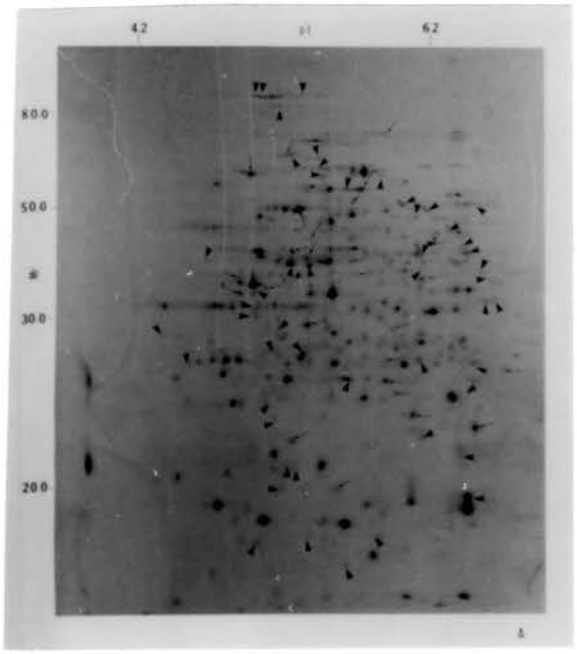


Figure 4. Survival in the Presence of Hydrogen Peroxide

Figure 5. 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. exposure to H_2O_2 have been marked with →. Proteins that have been repressed after 20 or 60 min. exposure to H_2O_2 have been marked with ➤ . (B) & (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 induced in response to H_2O_2 have been marked with → and ➧ , respectively. Proteins whose expression has been increased or induced during both intracellular survival and in the presence of H_2O_2 have been marked with letters.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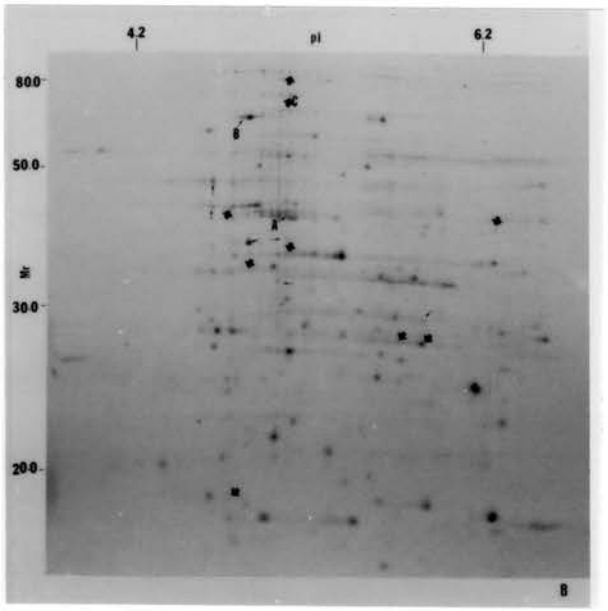
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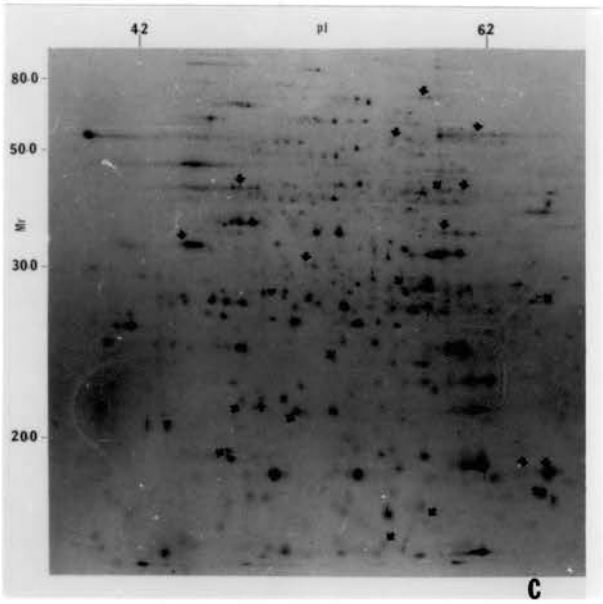
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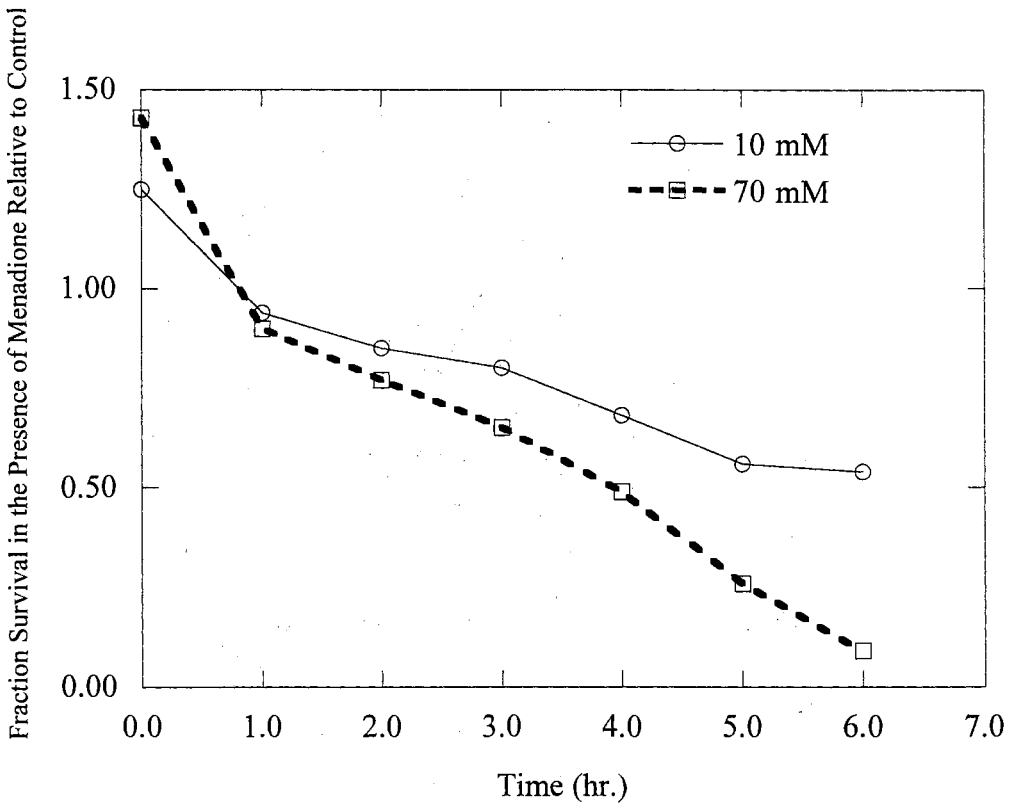
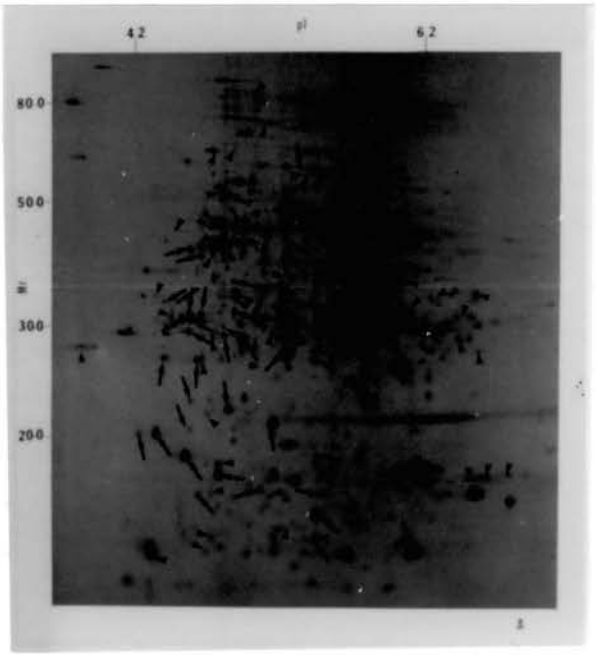


Figure 6. Survival in The Presence of Menadione Sodium Sulfite

Figure 7. 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) & (C) *B. abortus* labeled in the presence of 10 mM of 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 during both intracellular and in the presence of menadione have been marked with letters.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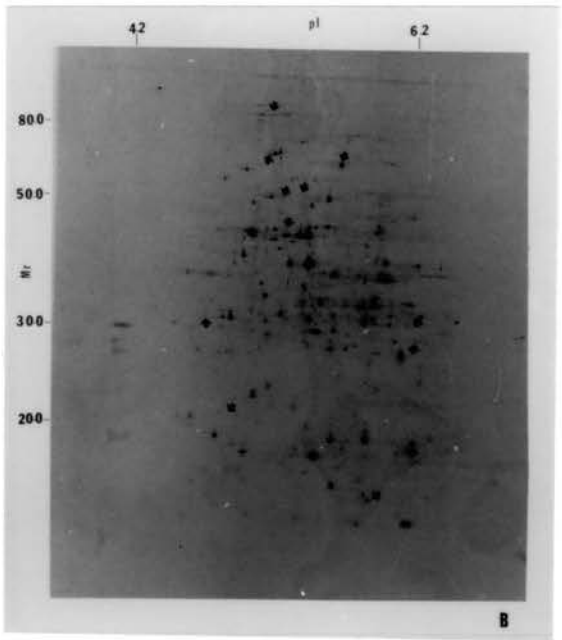
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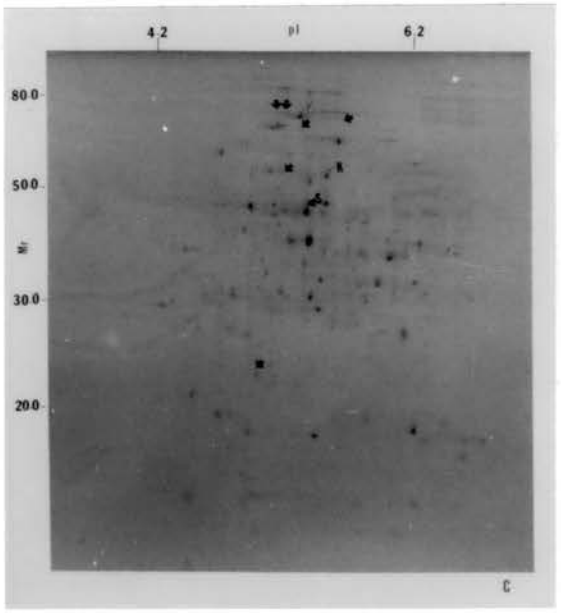
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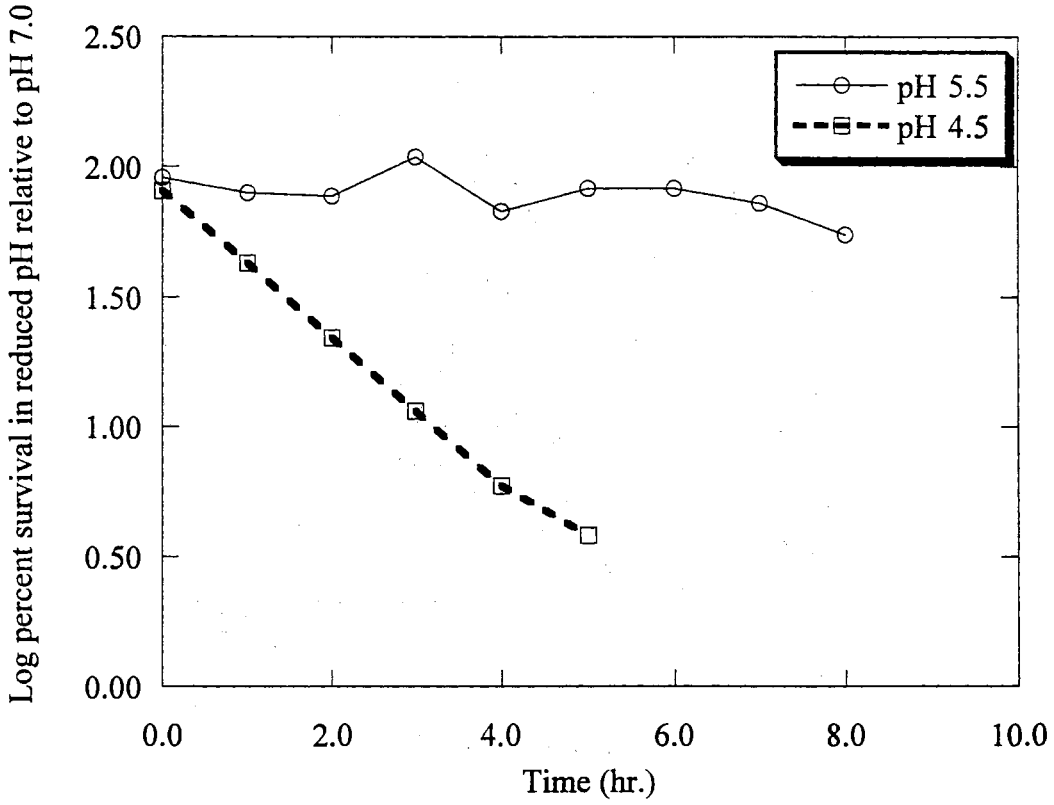
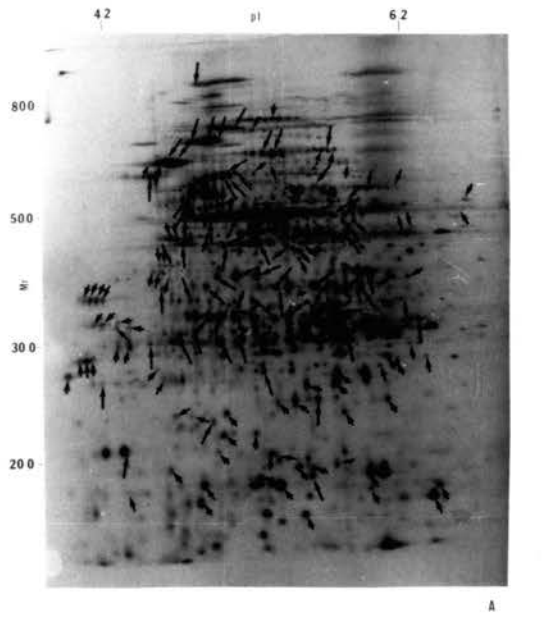


Figure 8. Survival during Exposure to Reduced pH

Figure 9. 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. survival at reduced pH have been marked with ➡ and ➡ , respectively. (B) & (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.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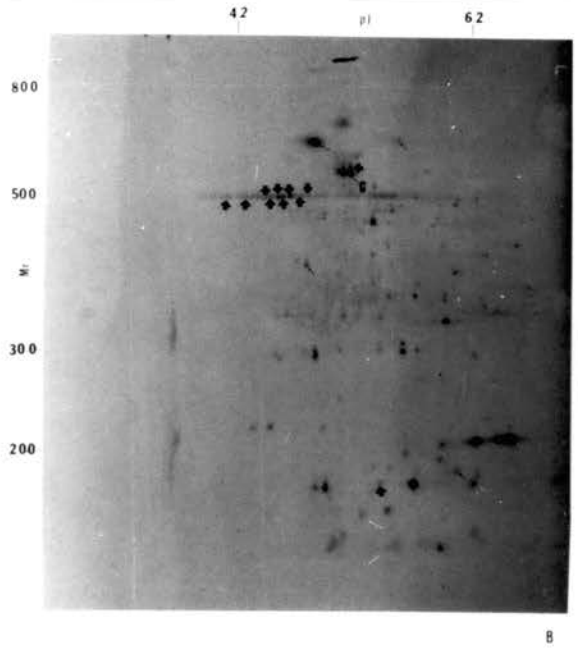
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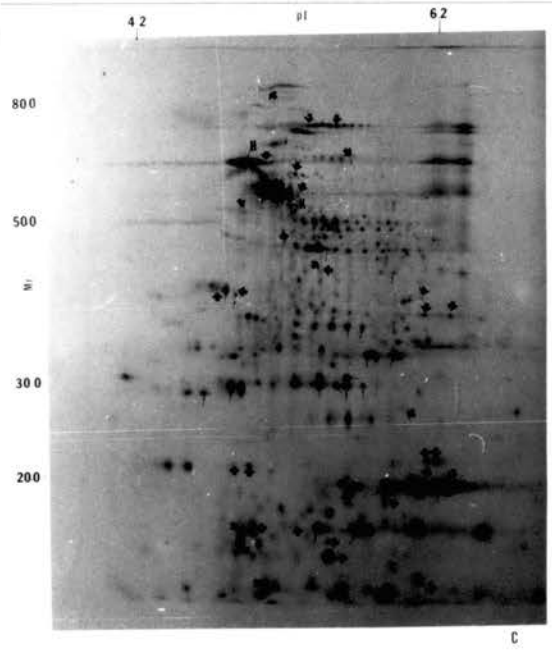
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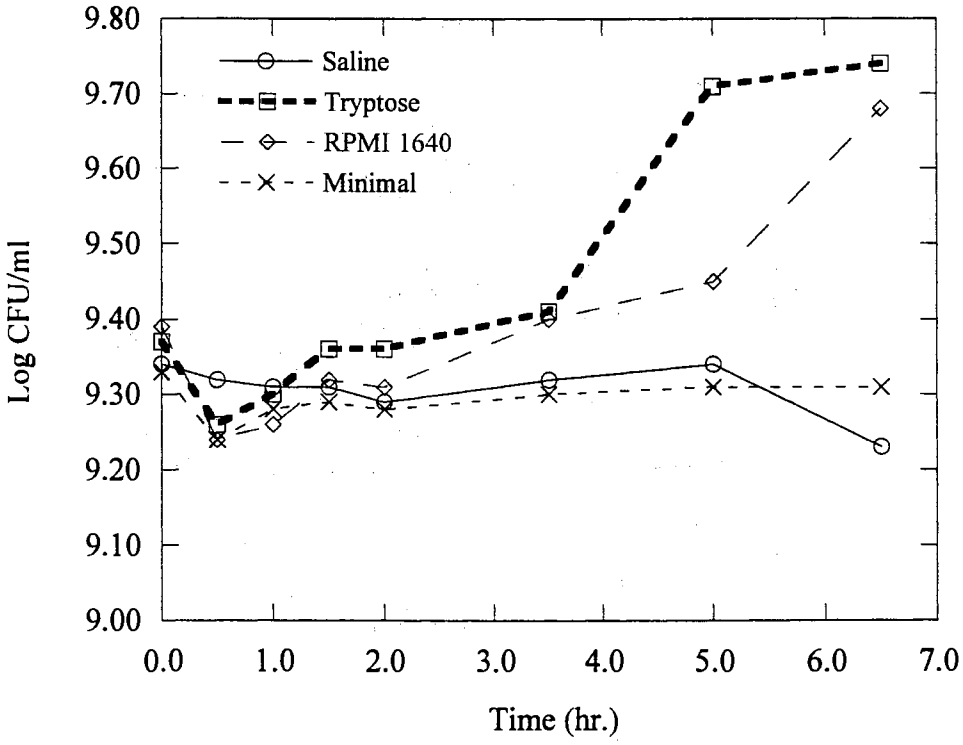
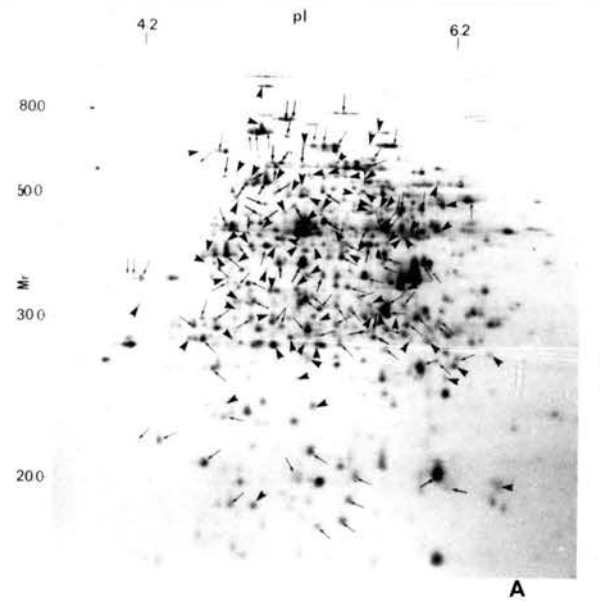


Figure 10. Survival in Various Medium

Figure 11. 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 has been 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 to 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 induce or increased during intracellular survival and nutritional stress have been marked with letters.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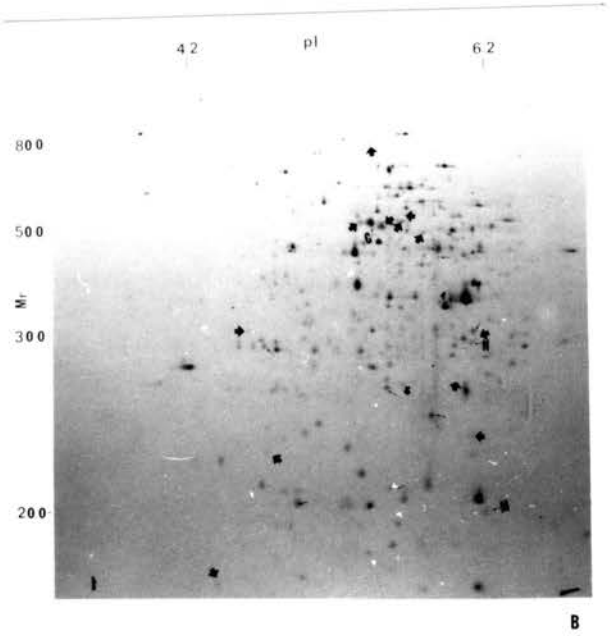
pI
6.2
800
500
Mr
300
200
A


4.2
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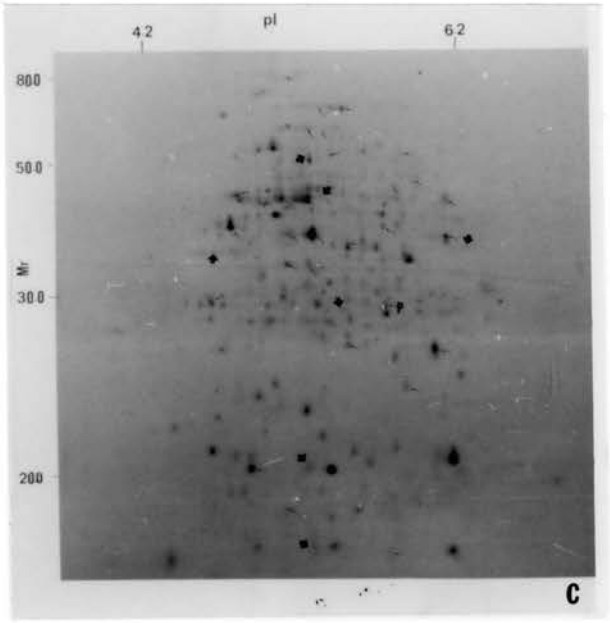
4.2
pI
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Mr
300
200
C

Table I. B. abortus stress proteins which had their expression altered by various stress conditions and macrophage infection.

	SodA	DnaK	GroEL	HtrA
Intracellular				
60 min.	↓	—	↓	↓
120 min.	↓	↑	↑	↓
H_2O_2				
20 min.	—	↓	↓	—
60 min.	↑	↓	↓	—
Menadione				
20 min.	↓	↓	—	—
60 min.	↓	↓	↓	—
Starvation				
Saline	↓	↓	↓	↓
Minimal	↓	↓	↑	↓
Heat				
20 min.	—	↑	↑	↑
60 min.	—	—	↑	—
Low pH				
20 min.	↓	↓	↑	↓
60 min.	↑	↑	↑	↑

symbols: ↑, increase; ↓, decrease; —, no change.

Table II. *B. abortus* proteins induced by stress conditions and by growth within bovine macrophages

Stress condition	Total number of proteins induced	Number of proteins induced during short exposure to stress[1]	Number of proteins induced during long exposure to stress[2]	Proteins induced under stress condition and intracellular growth	Number of proteins induced under stress conditions and intracellular growth
Intracellular	39	26	17	—	—
Heat	117	53	74	G, B, H, F, E	5
H_2O_2	35	18	19	A, B, C	3
Menadione	26	16	12	S, K	2
Reduced pH	68	17	63	G, K, H	3
Starvation	54	25	33	G, N, P, M	4

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

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

* The numbers in the table correspond to the number of spots that could be detected on the picture and not the number of spots detectable on the autoradiographes (appendixes)

CHAPTER III

IDENTIFICATION OF MACROPHAGE INDUCED GENES IN *Brucella abortus* BY RNA FINGERPRINTING USING ARBITRARILY PRIMED PCR

Introduction

Several methods have been developed for the study of differentially expressed genes in eukaryotic systems (42,66,71). Two of these methods described by Liang and Pardee (42) and Sokolov and Prockop (66) rely on the poly (A) tail of mRNA. Both of these methods involve the synthesis of a cDNA from mRNA in the presence of an arbitrary primer and oligo(dT). Neither of these methods can be used for study of differentially expressed genes in prokaryotic systems because poly(A)-tailed RNA are rarely found in bacteria (11). In addition to the above methods Welsh et al., (71-73) have developed a method for RNA fingerprinting that can be used for both eukaryotes and prokaryotes (76). This technique utilizes an arbitrary chosen primer for both first strand and second strand synthesis of cDNA.

Intracellular pathogens such as, *Brucella abortus,* have to cope with various stress conditions that may include low pH, nutritional deprivation, and various antimicrobial products such as proteolytic enzymes and reactive oxygen intermediates. Pathogens respond to these environmental conditions by a change in gene expression. During survival within macrophages, several new proteins were induced which are referred to as macrophage-induced proteins. Several of these proteins have been

identified as members of known heat shock proteins. To understand the mechanism used by *B. abortus* to survive within the bovine macrophage, we have made an attempt to identify some of the genes induced in response to intracellular survival by RNA fingerprinting using arbitrarily primed (RAP) PCR. The exact roles of the genes induced during intracellular survival are still unknown. This report describes the application of RAP-PCR to directly study the genes induced in *B. abortus* during intracellular survival within bovine macrophages.

Materials and Methods

Bacterial Strains

B. abortus strain 2308 was obtained and prepared as described in chapter 2. *Escherichia coli* strain XL1-Blue Stratagene was used for the transformation procedures (28).

Isolation of RNA from *B. abortus* Grown within Bovine Macrophage

Monocytes were isolated from peripheral blood obtained from the jugular vein of healthy heifers as described in chapter 2. Infection of macrophages and phagocytosis of *B. abortus* was achieved as described in the previous chapter. *B. abortus* was allowed to survive intracellularly for 2 hr. As a control, *B. abortus* was transferred to RPMI 1640 (ICN) containing 15% heat-inactivated fetal bovine calf and equine serum (HyClone), and was incubated at 37°C and 5% CO_2 for 2 hr. Macrophages were lysed with 1% (V/V) Triton X-100 and distilled, sterile water. Bacterial cells were collected by centrifugation 2980 x g using a benchtop centrifuge. *Brucella* cells were washed twice in phosphate buffered saline (PBS) pH 7.2, and resuspended in PBS. To kill the bacterial cells, one

volume of acetone was added to the cells suspended in PBS. The cell suspension was stirred overnight at room temperature (61).

Cells were centrifuged at 16300 x g for 10 min at 4°C. Cells were washed 3 times in 10 mM Tris-HCl, pH 8.0. The pellets were resuspended in 10 ml of protoplasting buffer (15 mM Tris-HCl pH 8.0, 0.45 M sucrose, 8 mM EDTA). The cell wall was digested by addition of 4 mg of lysozyme (Sigma) to each pellet and cells were incubated at room temperature for 60 min followed by addition of 2.5 mg of Proteinase K (Gibco, BRL) to each sample. Samples were incubated at 55°C for 60 min to allow digestion of proteins (4).

To obtain protoplasts, samples were centrifuged at 7000 rpm in a Sorvall SS-34 for 20 min at 4°C. Immediately, 0.6 ml of ice cold 10 mM KCl, 5 mM $MgCl_2$ and 10 mM Tris-HCl pH 7.4 was added, followed by 0.6 ml hot lysis buffer (0.4 M NaCl, 40 mM EDTA, 1% 2-mercaptoethanol, 1% SDS, 20 mM Tris-HCl pH 7.4) containing 100 µl of water saturated phenol. Samples were immediately transferred to a boiling waterbath for 40 sec. Cell debris was removed by centrifugation at 16300 x g for 10 min. The supernatants were treated with Rnase free Dnase for 60 min at 37°C, were extracted with phenol: chloroform 3-5 times and once with chloroform (13). RNA was precipitated in ethanol and ammonium acetate overnight at -20°C. Total RNA was resuspended in sterile water.

Fingerprinting of RNA

RNA fingerprinting by arbitrarily-primed PCR was performed by using the RAP-PCR kit from Stratagene. The first strand synthesis was carried out using Moloney murine

leukemia virus reverse transcriptase (MMLV-RT) at 37°C for 60 min as recommended by the manufacturer (Stratagene). After cDNA synthesis, PCR amplification was carried out as recommended by the manufacturer in the presence of 10 μCi/μl [α-^{32}P]dCTP. The thermal cycler parameters used were as follows: 1 low-stringency cycle; 94° C (1 min), 36° C (5 min), 72° C (5 min), followed by 40 high-stringency cycles; 94° C (1 min), 50° C (2 min), 72° C (2 min) and 1 cycle of 72° C (10 min). Four primers were used with the following sequences; primer A2, AATCTAGAGCTCCAGCAG, primer A3, AATCTAGAGCTCTCCTGG, primer A4, AATCTAGAGCTCTCCAGC, and primer A5, AATCTAGAGCTCCCTCCA (Stratagene). Following the RAP-PCR reaction 5 μl of each reaction was mixed with 5 μl stop buffer containing 80% deionized formamide, 50 mM Tris-HCl (pH 8.3) 1 mM EDTA, 0.1% (w/v) xylene cyanol dye and 0.1% (w/v) bromophenol blue dye. Samples were heated to 80° C for 2 min, 3 μl of each sample was loaded on a 4% polyacrylamide/50% urea sequencing gel (4) prepared in 1X TBE (90 mM Tris, 64.6 mM boric acid, 2.5 mM EDTA pH 8.3). Samples were electrophoresed at 45 watts until the xylene cyanol dye was at the bottom of the gel. The gel was dried under vacuum at 80° C onto a filter paper. The RAP-PCR products were visualized by autoradiography.

Isolation and Cloning of RAP-PCR Products

The autoradiogram was aligned with the gel using fluorescence markers and bands were cut out of the gel using a razor blade. The piece of acrylamide gel was placed in a microfuge tube and the DNA was eluted in 50-100 μl of TE (10 mM Tris-HCl pH 7.5, 1

mM EDTA) buffer for 1 hr at 65° C followed by overnight incubation at room temperature. An aliquat (1-2 μl) of the eluent was PCR-amplified in 50 μl using the 1-2 μl of the same primer in the absence of [α-^{32}P] dCTP and in the presence of 3 mM $MgCl_2$, 200 μM dNTPs, 1X PCR buffer and 0.5 units of *Taq* DNA polymerase (BRL) using the high- stringency conditions as described for RNA fingerprinting. The size of the amplified fragments was checked by agarose gel electrophoresis. The amplified products were cloned into pBluescript SKII vector (Stratagene) digested with *EcoRV* and T-tailed according to the procedure described by Marchuk et al., (49), and transformed into the XL1-Blue strain of *E. coli* (27). The cloned RAP products were sequenced by using the Prism ready reaction dideoxy terminator cycle sequencing kit and read on 373A DNA sequencer (Applied Biosystems Foster City, CA). Automated sequencing was provided as a service by the recombinant DNA/protein facilities at Oklahoma State University. The sequences were analyzed using several databases including Brookhaven protein data bank, Swissprot, GeneBank, and EMBL databases.

Preparation of the Probes for Northern and Southern Blot analysis

The probes were prepared by PCR amplification of eluted fragments as described previously but in the presence of 10 μCi/μl [α-^{32}P]-dCTP. Free nucleotides and unincorporated [α-^{32}P]-dCTP were not removed. The size of the probe was checked on a 4% polyacrylamide/ 50% urea gel as described in RNA fingerprinting. The probe was placed in a boiling water bath for 5 min and was immediately placed on ice before it was added to the hybridization solution. The same probe was used for both northern and southern blot analysis.

Northern Blot Analysis of RNA

Equal amounts of RNA isolated from *B. abortus* grown in the absence of macrophages, and from *B. abortus* grown within bovine macrophages were separated on formaldehyde-agarose gels (4), and transferred to a nylon membrane (Magna) by capillary transfer using 10X SSC (1.5 M NaCl, 0.15 M NaCitrate pH 7.0) as the transfer buffer. The RNA was immobilized on the nylon membrane by UV crosslinking. Each membrane was prehybridized for 2-4 hr in 50% deionized formamide (Sigma), 5X Denhardt's reagent (0.1% ficoll, 0.1% Polyvinylpyrrolidone, 0.1% BSA), 5X SSPE (0.75 M NaCl, 50 mM NaPO4 pH 7.7, 5 mM EDTA), 0.1% SDS, 100 μg./ml salmon sperm DNA at 42°C. The probe was hybridized to the membrane in the prehybridization buffer containing 10% dextran sulfate at 42°C overnight. Membranes were washed twice in 6X SSPE/0.5% SDS for 15 min at room temperature and twice in 1X SSPE/0.5% SDS for 15 min at 37°C. The probe was removed for reprobing by incubating the membrane in 50% deionized formamide/6X SSPE for 5-6 hr at 65°C, and was briefly rinsed in 2X SSPE. To ensure the complete removal of the probe, the membrane was exposed to an X-ray (Fuji) film for at least 2 days. The same membrane was used for hybridization of several RAP products.

Isolation of Genomic DNA and Southern Blot Hybridization

Genomic DNA from *B. abortus* was isolated as described by Essenberg and Sharma (19). The genomic DNA was digested by *EcoRI*, *BamHI*, and *HindIII* restriction enzymes (BRL). The genomic DNA was separated on 0.8% agarose gel. It was

fragmented in 0.25 M HCl and denatured in an alkaline solution consisting of 1.0 M NaCl /0.5 M NaOH twice for 15 min each. It was transferred to a Nylon membrane by capillary transfer using 10X SSC as the transfer buffer. Genomic DNA was immobilized on the membrane by UV crosslinking. Prehybridization and hybridization was performed in the same buffers as described above at 42° C. Removal of the probe was achieved as described above.

Results

RNA Fingerprinting

Each primer was used for fingerprinting of RNA from *B. abortus* grown in the tissue culture medium and within macrophages. The RAP products for the extracellular and intracellular samples were run side by side on the sequencing gel and each produced a ladder of fragments (Figure 1). Comparison of the RNA fingerprints for each primer for *B. abortus* grown intracellularly and extracellularly indicated that during intracellular survival several new genes were induced. This comparison found 22 (Figure 1) bands that were present only in the RNA fingerprint of *B. abortus* grown intracellularly or were increased under this condition.

Northern And Southern Blot Analysis

In order to confirm the induction of the genes identified by the RNA fingerprinting and to ensure that these products were not the result of any contamination caused by the presence of macrophages, Northern and Southern blot analyses were used, respectively. Out of 15 fragments analyzed (Figure 2 & 4) so far, fragments A4-3, A4-1, A4-6, A3-8 and A3-2 identify genes only expressed during intracellular survival (Figure

2). Northern blot analysis of fragments A2-2 and A2-6 in figure 1 panel B indicated that the level of induction of these genes was only slightly higher than that observed for control (figure 2 & 3). Further studies were not pursued for these two fragments. Northern blot analysis of the rest of these fragments indicated that there was a several-fold increase in the expression of each of these RAP products (Figure 2 and 3). Southern blot analysis of the RAP-PCR fragment indicated that all of these fragments are from the *B. abortus* 2308 genome (Figure 4) and the differences observed are not due to a contamination caused by the presence of macrophages.

Sequence Analysis of RAP-PCR Fragments

In order to understand the mechanism by which *B. abortus* survives within bovine macrophages the RAP-PCR products were cloned and were subjected to automated sequencing. The sequences of 13 of the RAP fragments induced during intracellular survival were analyzed using several databases. The sequence analysis indicated that the RAP products studied so far have shown either no homology with any previously sequenced DNA or proteins or up to 60% of similarity, over stretches longer than 200 nucleotides with hypothetical proteins in other prokaryotic systems. In several cases, there was some similarity with known proteins in several bacterial species. For example, comparison of the 360 bp sequence of fragment A3-9 revealed 53.6% similarity over 267 nucleotides with transferrin binding protein 1 precursor (TBP-1) of *Neisseria meningitidis*. The same fragment showed 51.6 % similarity over 217 nucleotides with the cell envelope protein (*oapA*) in *Haemophilus influenza*. In addition, comparison of

fragment A3-8 has shown 52% over 369 nucleotides similarity with α-glucan phosphorylase of *E. coli*.

Discussion

RNA fingerprinting using AP-PCR can be used for the study of differentially expressed genes under a variety of conditions. Use of a single primer allows this method to be used for the study of gene expression in bacteria. In contrast to specific primers which allow amplification and study of specific genes, this method allows the study of genes obtained from a cDNA mixture at random. Amplification with one primer is feasible because the first amplification step is performed under low stringency conditions so that the same primer can start chain elongation from multiple sites on the DNA template and some mismatches are allowed (73).

Some of the RAP products represent the structural RNA molecules present in *B. abortus*. However, these structural RNAs are present in both the *B. abortus* cells grown extracellularly and intracellularly. Furthermore, our studies indicated that the presence of macrophages during the intracellular studies did not cause any contamination since the southern blot analysis has indicated that the fragments studied so far all belong to the genome of *B. abortus*. The RNA and DNA molecules of the macrophages are mostly left in the supernatant during the slow centrifugations that achieve sedimentation of bacterial cells. In addition, the RNA from macrophages is most likely degraded after lysis of the macrophages since the solutions used in these steps were not RNAse-free.

The importance of the use of more than one concentration of RNA can be observed in this study. Two of the fragments thought to be induced during intracellular

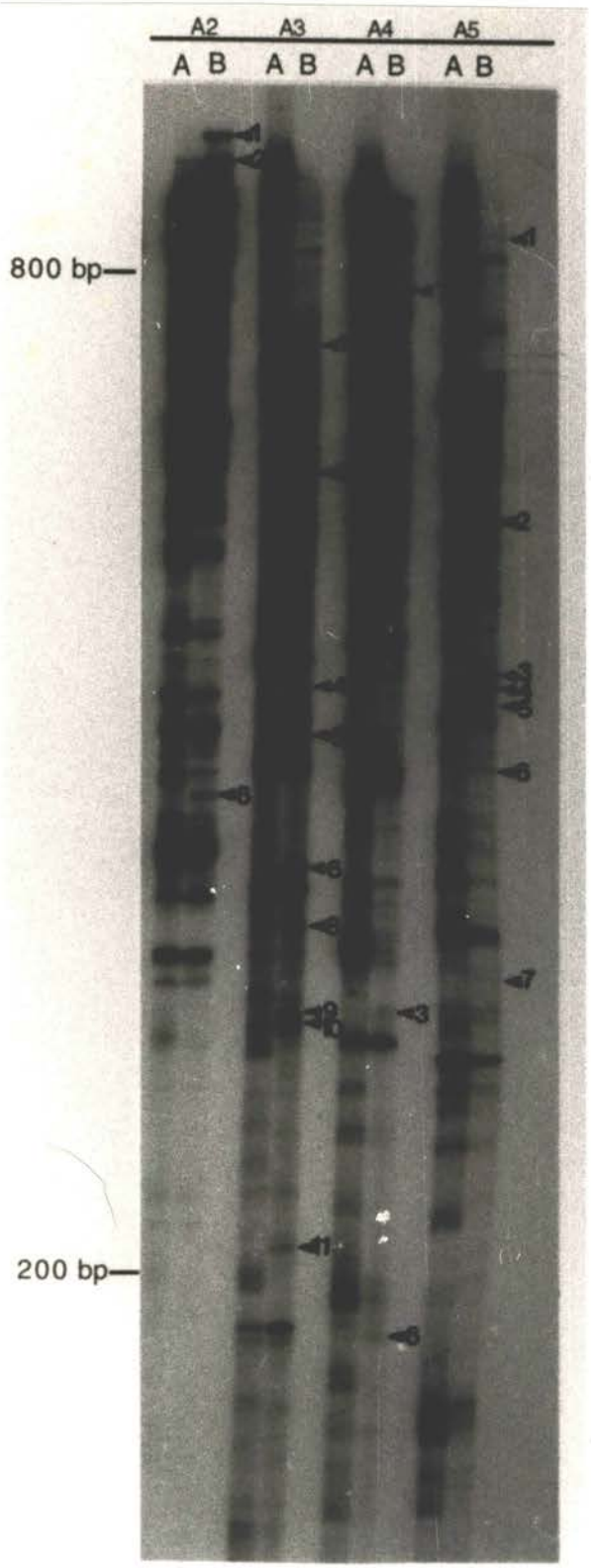
survival were only slightly induced. In addition, some of the bands that seem to be new have shown only several fold of induction. Welsh et al., (73) and Wong and McClelland (76) both have emphasized the use of at least two different concentrations of RNA to control slight concentration differences.

In the cell and body, iron is not present in free form, but is withheld from microorganisms by iron binding proteins such as transferrin and lactoferrin. Many microorganisms have developed systems to obtain iron. It has been postulated that iron acquisition systems are necessary for bacterial pathogenicity (52). Sequence analysis of one of the fragments has shown similarity to TBP-1 and outer membrane protein in *N. meningitidis* and *H. influenzae*, respectively. The product of this gene may contribute to the pathogenicity of *B. abortus* since both *tbp-1* (54) and *oapA* (75) gene products are involved in virulence of *N. meningitidis* and *H. influenzae*, respectively. However, studies of the *B. abortus* response to low iron concentration indicated that these bacteria produce 2,3-dihydroxybenzoic acid (2,3-DHBA) which acts as a siderophore. Further studies by the same group have not shown the induction of any proteins in the cell envelope of *B. abortus* that would act as a receptor for the uptake of iron from 2,3-DHBA or as TBP-1 (48). It is possible that expression of TBP-1 is induced under conditions other than low iron. construction of mutant strains of this gene for in vitro and vivo studies and characterization of this gene would be of interest .

In summary, RAP-PCR can be used to identify and study genes that are induced during intracellular survival in *B. abortus* and other intracellular bacteria. RAP requires only a very small amount of RNA. In contrast, the use of more traditional methods such

as 2-D PAGE (53) to obtain N-terminal sequences of stress-inducible proteins and differential hybridization of cDNA from cells grown under different conditions (13) require large amounts of protein or RNA and both are very time consuming.

Figure 1. RAP fingerprinting of RNA isolated from *B. abortus* grown extracellularly (lanes A) or intracellularly (lanes B) using 4 different primers. The arbitrary primer used for each fingerprint is marked at the top. A portion of gel has been shown.. Arrowheads and numbers indicate differentially expressed genes. The size range in base pairs has been shown on the left.


A2
A3
A4
A5
A B
A B
A B
A B
800 bp
200 bp

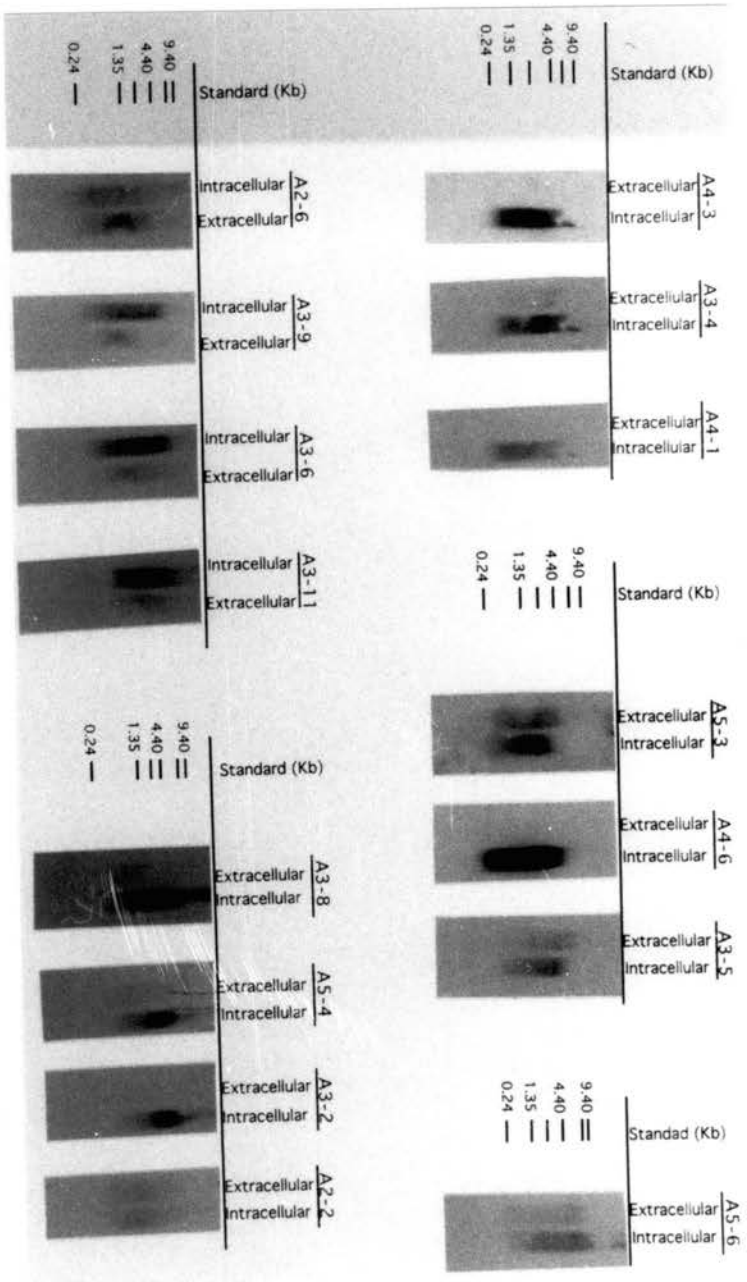


Figure 2. Northern blot analysis of total RNA isolated from B. abortus 2308 during extracellular survival in tissue culture medium and intracellular survival within bovine macrophages. Radiolabeled RAP-PCR fragments were used as probes. The letters and numbers at the top of each blot correspond to the primer and the RAP-PCR product used as the probe.

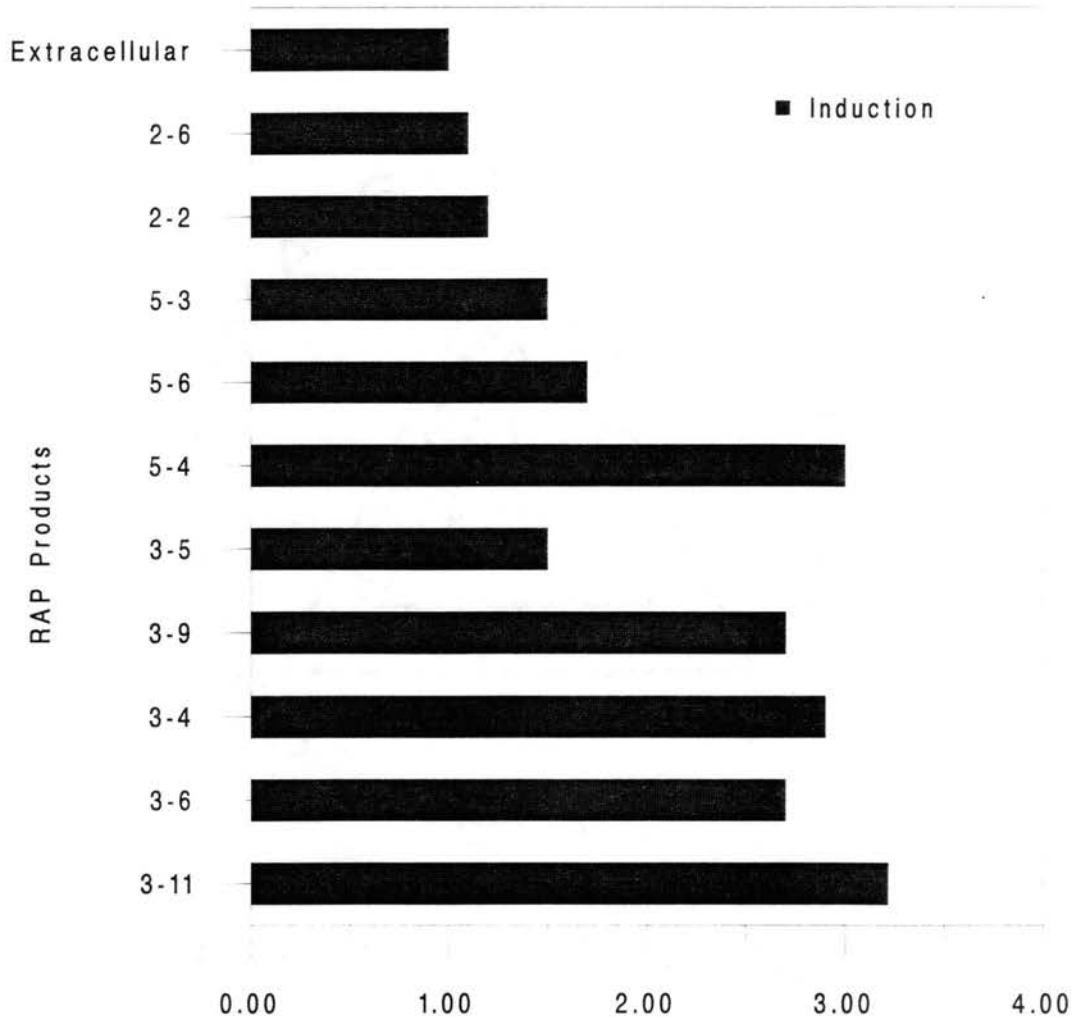
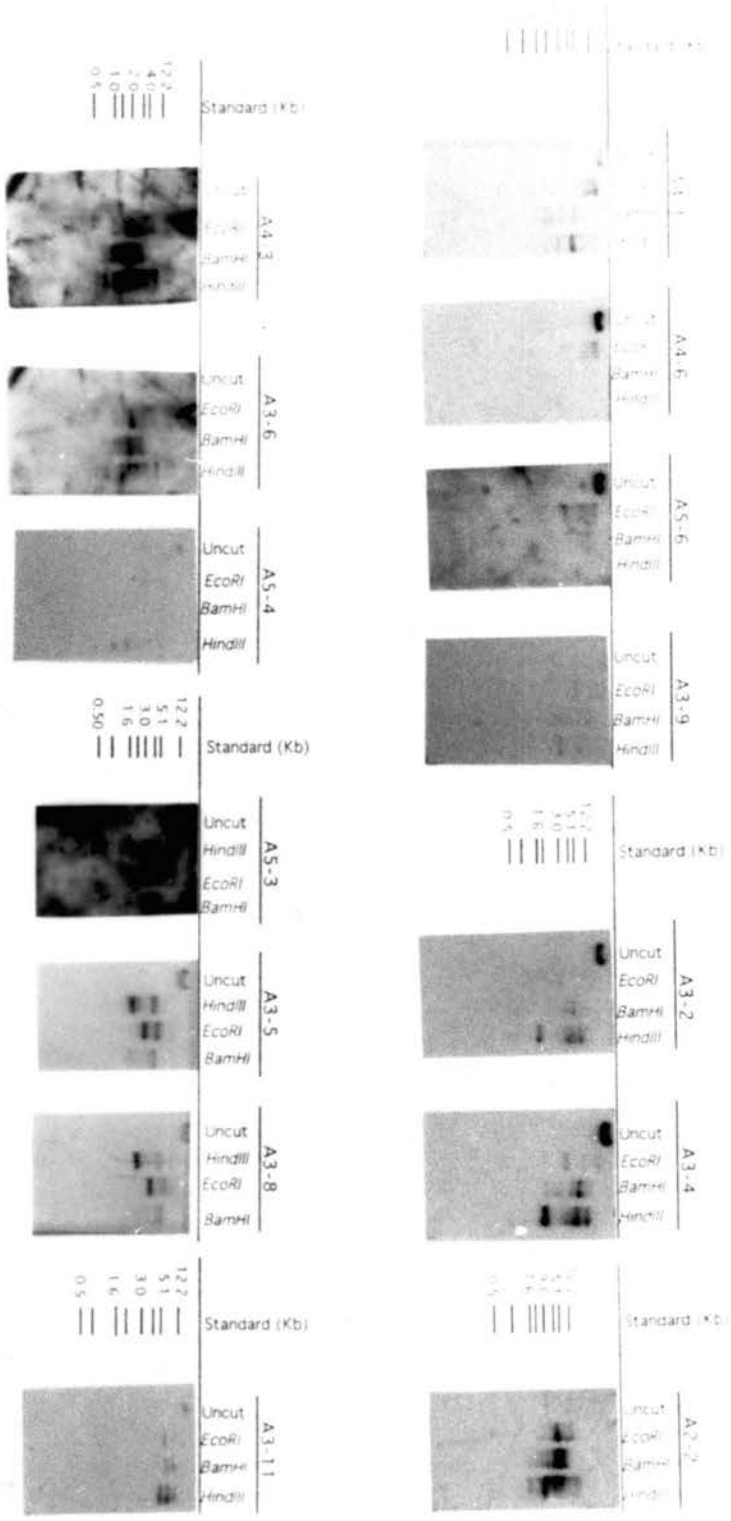


Figure 3. Induction of RAP-PCR fragments during intracellular survival as compared to induction during extracellular survival. Induction ratios were obtained by densitometric analysis of northern blots

Figure 4. Southern Blot analysis of genomic DNA isolated from *B. abortus* hybridized with RAP products. The letter and number at the top of each blot correspond to the primer and RAP-PCR product used as the probe.


Standard (Kb)
12.2
5.1
3.0
1.6
0.5
Uncut
EcoRI
BamHI
HindIII
A4-3
A3-6
A5-4
A5-3
A3-5
A3-8
A3-11
A4-6
A5-6
A3-9
A3-2
A3-4
A2-2

CHAPTER IV

CONCLUDING REMARKS

Summary

The phenotypic modulation of *Brucella abortus* strain 2308 during survival within macrophages has been studied. Two different methods have been used to study the differential gene expression in this microorganism. Both methods have shown that there are differences in the gene expression of *B. abortus* grown within macrophages and those grown in tissue culture medium and in the absence of macrophages.

To understand the mechanism by which *B. abortus* copes with the intracellular conditions, the two dimensional protein profile of *Brucella* during intracellular survival was compared to those obtained when *B. abortus* was subjected to various stress conditions. These stress conditions were chosen so that they would resemble the conditions that *B. abortus* might cope with during intracellular survival. These studies indicated that some of the known stress proteins such as DnaK and GroEL are induced during intracellular survival. In addition, several proteins have been identified which are expressed during both intracellular survival and each stress condition studied. The overall outcome of this study however, indicated that the response of *B. abortus* to an intracellular environment is not just the sum of various stress conditions but is evidently far more complex.

RNA fingerprinting studies have provided further evidence that the response to intracellular survival is a complex and uncharacterized response. The sequencing analysis of several RAP products obtained by using four arbitrary primers indicated that the genes induced during intracellular survival have not been characterized or studied previously. Our studies have shown that RNA fingerprinting using arbitrarily-primed PCR is a simple and ideal method for differential gene expression studies. This method allows the cloning and sequencing of genes induced during intracellular survival for further investigation of the intracellular mechanism.

Future Directions

Both methods used in this study have opened new doors in exploration of the mechanism by which *B. abortus* survives within macrophages. Further comparison of the two-dimensional protein synthesis pattern of the smooth, virulent strain of *B. abortus* used in these studies with the rough nonvirulent strains may be useful in targeting the virulence factors that are required for survival within macrophages. The development of mutants for the genes that are only expressed in the smooth strain may allow for the production of vaccine strains. The same type of studies are feasible using the RNA fingerprint of the smooth and rough strains. The RAP products present only in the RNA fingerprint of a smooth strain grown intracellularly and not in the RNA fingerprint of the rough strain would be of interest.

The two-dimensional protein map of *B. abortus* 2308 can be used to further identify the location of various proteins on the map. This can be achieved by a comparison of the two-dimensional protein profile of various *B. abortus* null mutants

with that of the wild type strain. Further identification of the proteins on the map is possible by obtaining the antibodies produced against various bacterial proteins and western blot analysis.

Finally, the role of the RAP product can be identified and characterized by using each product as a probe to obtain the entire gene from the genomic library. Determination of the complete nucleotide sequence of each gene, creation of null mutants and the examination of their ability to survive intracellularly, should allow a better understanding of the importance of these genes during intracellular survival.

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APPENDIXES

APPENDIX A

MOLECULAR WEIGHTS AND ISOELECTRIC POINTS OF PROTEINS INDUCED DURING SURVIVAL OF *Brucella abortus* WITHIN BOVINE MACROPHAGES

Table III. Molecular weights and isoelectric points of proteins whose expression has increased in response to 60 min intracellular survival in *B. abortus*.

SSP*	Mr (KD)	pI
4603	51.5	5.2
5701	58.5	5.3
5710	54.5	5.3
5511	42.6	5.4
7205	36.4	6.2
7303	37.9	5.9
7409	40.4	5.9
8103	27.0	6.6
8201	37.4	6.3
9206	37.2	6.8
8305	40.2	6.5

* SSP numbers are assigned automatically when a standard for a Matchset is created. To assign SSP numbers PDQuest divides the screen into rows and columns, placed so that there are approximately the same number of spots in each section and no section has over 100 spots. The spots in each section are numbered sequentially.

Table IV. Molecular weights and isoelectric points of proteins whose expression has increased in response to 120 min of intracellular survival in *B. abortus*.

SSP	Mr (KD)	pI
1201	36.3	4.8
4803	82.1	5.2
2404	40.4	5.0
3613	51.9	5.1
3612	52.0	5.1
1208	34.4	4.9
5407	41.9	5.3

Table V. Molecular weights and isoelectric points of new proteins synthesized in response to intracellular survival for 60 min in *B. abortus*.

SSP	Mr (KD)	pI
213	36.2	4.8
406	38.9	4.6
1810	81.8	4.9
3307	38.7	5.1
3825	81.8	5.1
4410	41.9	5.3
4813	81.4	5.2
5311	38.2	5.4
5518	42.1	5.4
5812	98.1	5.3
5814	92.4	5.4
6606	50.6	5.7
8022	20.2	6.6
8310	36.8	6.4
9504	42.6	7.7

Table VI. Molecular weights and isoelectric points of new proteins synthesized in response to intracellular survival for 120 min in *B. abortus*.

SSP	Mr (KD)	pI
211	36.4	4.7
1004	20.7	4.9
2714	64.8	5.0
3206	36.4	5.1
3307	38.7	5.1
3411	40.7	5.1
3820	81.8	5.1
5009	20.9	5.3
5608	49.2	5.4
8023	20.7	6.7

APPENDIX B

MOLECULAR WEIGHTS AND ISOELECTRIC POINTS OF PROTEINS INDUCED IN *Brucella abortus* IN RESPONSE TO A TEMPERATURE UPSHIFT

Table VII. Molecular weights and isoelectric points of proteins whose expression has increased during 20 min exposure of *B. abortus* to increased temperature.

SSP	Mr (KD)	pI
10	17.6	4.6
109	33.3	4.8
1010	18.2	5.1
1303	37.6	5.3
1514	42.9	5.2
2616	49.7	5.3
3205	35.9	5.7
4204	34.8	5.9
5007	19.7	6.3
5009	21.3	6.4
5105	33.7	6.3
5106	23.6	6.4
5401	39.5	6.1
5416	42.1	6.2
5610	50.0	6.3
5703	71.6	6.1
5713	56.7	6.2
5806	76.5	6.3
6111	31.9	6.6
6502	42.5	6.4
6503	42.8	6.4
6613	54.5	6.5
6712	70.0	6.5
6803	74.3	6.6
7008	20.7	6.8
7107	26.7	6.9
7312	38.5	6.7
7315	37.5	6.8
7323	39.0	6.8
7601	54.6	6.6
7703	64.7	6.6
7705	64.6	6.7
8404	39.6	7.0
8419	40.0	6.9
8502	42.4	6.9
8521	43.0	6.9
8701	58.0	6.9

Table VIII. Molecular weights and isoelectric points of proteins whose expression has increased after 60 min exposure of *B. abortus* to increased temperature.

SSP	Mr (KD)	pI
408	41.0	5.0
411	41.0	4.9
413	39.7	4.9
1101	26.6	5.0
1104	33.5	5.1
1112	25.7	5.2
1202	35.3	5.1
1401	39.7	5.3
1410	39.9	5.3
1702	63.4	5.1
2104	32.8	5.4
2108	24.3	5.4
2413	40.9	5.3
2605	53.9	5.4
2614	54.4	5.5
2721	56.7	5.5
2725	57.1	5.4
2726	57.1	5.4
3503	42.5	5.6
3604	54.3	5.7
3616	55.5	5.6
4006	19.6	6.0
4008	18.5	6.0
4013	19.1	6.0
4104	34.3	5.9
4206	35.2	6.0
4304	38.5	5.9
5007	19.7	6.3
5610	50.0	6.3
5613	48.8	6.1
7101	31.4	6.6
7107	26.7	6.9
7108	30.5	6.9
7203	35.4	6.6
7213	36.2	6.8
7312	38.5	6.7
7505	42.5	6.6
8022	22.5	7.2
8115	33.9	7.5

Table IX. Molecular weights and isoelectric points of new proteins synthesized in response to exposure of *B. abortus* to increased temperature for 20 min.

SSP	Mr (KD)	pI
1004	20.1	5.1
1112	25.7	5.2
1612	48.7	5.3
1615	48.6	5.1
2513	43.0	5.5
2515	43.8	5.3
3906	94.7	5.6
3907	94.5	5.6
4502	42.6	6.0
4612	54.9	5.9
4719	70.4	5.9
4805	74.3	5.8
4912	94.4	5.9
4914	94.0	6.0
5703	71.6	6.1
5722	56.3	6.3
5807	76.4	6.4
6111	31.9	6.6
7008	20.7	6.8
8022	22.5	7.2

Table X. Molecular weights and isoelectric points of new proteins synthesized after 60 min exposure of *B. abortus* to increased temperature.

SSP	Mr (KD)	pI
21	16.0	4.7
224	35.7	4.6
227	35.2	4.9
228	36.2	4.8
1121	30.2	5.2
2209	34.7	5.5
2419	41.3	5.3
2619	55.7	5.3
3840	73.9	5.7
5015	22.7	6.1
5733	63.2	6.1
5736	65.2	6.2
6012	17.1	6.4
6210	35.0	6.5
6212	35.2	6.4
7009	20.4	6.6
7015	22.7	6.8
7429	42.3	6.9
7624	50.9	6.7
8022	22.5	7.2
8026	22.4	6.9

APPENDIX C

MOLECULAR WEIGHTS AND ISOELECTRIC POINTS OF PROTEINS INDUCED IN *Brucella abortus* IN RESPONSE TO EXPOSURE TO H_2O_2

Table XI. Molecular weights and isoelectric points of proteins whose expression has increased after 20 min exposure of *B. abortus* to H_2O_2

SSP	Mr (KD)	pI
603	41.1	4.9
704	46.0	4.9
1505	35.6	5.1
1801	62.9	5.0
2403	30.9	5.4
2504	36.1	5.5
2603	39.7	5.5
6301	28.8	5.9

Table XII. Molecular weights and isoelectric points of new proteins synthesized during 20 min exposure of *B. abortus* to H_2O_2.

SSP	MR (KD)	pI
109	20.0	4.9
1306	28.1	5.0
1412	32.3	5.1
1612	40.8	5.0
2412	33.9	5.5
2823	92.6	5.5
2911	77.2	5.4
3823	91.6	5.6
5303	27.5	5.9
7515	36.7	-

Table XIII. Molecular weights and isoelectric points of proteins whose expression has increased in *B. abortus* during 60 min exposure to H_2O_2.

SSP	Mr (KD)	pI
105	21.2	4.6
4005	17.6	5.8
4404	29.5	5.8
5401	30.8	5.9
6304	27.9	6.0
6611	39.8	6.1

Table XIV. Molecular weights and isoelectric points of proteins induced in *B. abortus* during 60 min exposure to H_2O_2.

SSP	Mr (KD)	pI
109	20.0	4.9
416	32.7	4.8
1114	22.5	5.2
1115	22.4	5.0
1308	28.6	5.2
1602	41.0	5.0
1614	48.8	5.2
2103	21.9	5.5
3202	24.4	5.6
3310	30.4	5.6
5003	16.5	5.8
5312	29.0	5.9
5705	51.1	5.9
6007	17.2	6.0
6608	40.3	O.R.*
6804	50.5	6.0
7005	18.4	O.R.
7509	34.4	O.R.
7516	39.1	O.R.
7517	39.5	O.R.
7713	52.6	O.R.
8102	19.2	O.R.
8111	18.8	O.R.
8307	28.3	O.R.
8604	40.1	O.R.

* O.R. means that the isoelectric point for that spot was out of the range specified by the standard

APPENDIX D

MOLECULAR WEIGHTS AND ISOELECTRIC POINTS OF THE PROTEINS INDUCED IN *Brucella abortus* DURING EXPOSURE TO MENADIONE

Table XV. Molecular weights and isoelectric points of proteins whose expression has increased in *B. abortus* after 20 min exposure to menadione.

SSP	Mr (KD)	pI
1611	41.1	5.0
2809	81.1	5.1
3503	35.6	5.1
3609	40.9	5.3
8504	35.3	6.1

Table XVI. Molecular weights and isoelectric points of new proteins synthesized induced in *B. abortus* after 20 min exposure to menadione.

SSP	Mr (KD)	pI
406	30.7	4.8
1106	19.6	5.0
2808	81.8	5.1
3712	41.9	5.2
3719	50.8	5.1
3812	75.4	5.3
3818	51.4	5.3
5821	65.8	5.5
6008	13.4	5.7
8215	27.5	6.0
8303	30.5	6.0

Table XVII. Molecular weights and isoelectric points of proteins whose expression increased in *B. abortus* during 60 min exposure to menadione.

SSP	Mr (KD)	pI
3802	80.7	5.1
3811	99.8	5.3
3817	65.9	5.2
4310	32.1	5.3
4716	50.0	5.4
4825	106.7	5.3
8504	35.3	6.1

Table XVIII. Molecular weights and isoelectric points of new proteins synthesized in *B. abortus* in response to exposure to menadione for 60 min.

SSP	Mr (KD)	pI
2202	22.2	5.1
2806	110.8	5.1
2905	164.3	5.0
3801	108.5	5.1
3812	75.4	5.3
5809	105.9	5.6
3719	50.8	5.1
4616	41.1	5.3

APPENDIX E

MOLECULAR WEIGHTS AND ISOELECTRIC POINTS OF PROTEINS INDUCED IN *Brucella abortus* IN RESPONSE TO REDUCED pH

Table XIX. Molecular weights and isoelectric points of proteins whose expression has increased during exposure to *B. abortus* pH 5.5 for 20 min.

SSP	Mr (KD)	pI
1406	35.3	5.0
1823	61.8	5.0
3704	51.4	5.3
4015	16.9	5.6
5310	32.7	5.7

Table XX. Molecular weights and isoelectric points of new proteins synthesized in response to exposure of *B. abortus* to pH 5.5 for 20 min.

SSP	Mr (KD)	pI
603	46.3	4.9
604	46.2	4.9
605	45.8	4.8
606	45.9	4.8
607	45.9	4.8
608	45.8	4.7
609	45.8	4.6
613	46.2	4.9
1611	46.0	5.1
1612	46.0	5.0
1613	46.2	5.0
2315	33.3	5.2
2629	45.8	5.2
2822	58.9	5.2
3313	31.7	5.3
3507	39.6	5.4
3703	53.0	5.3
3713	54.9	5.3
4025	17.1	5.5
4026	15.8	5.5
4608	45.5	5.6
4817	65.3	5.6
5013	18.9	5.6
5804	43.9	5.6
7020	18.9	6.1
7021	17.6	5.8
7022	18.2	5.8
7122	20.2	5.9
8123	20.2	6.4

Table XXI. Molecular weights and isoelectric points of proteins whose expression has increased in *B. abortus* in response to exposure to pH 5.5 for 60 min.

SSP	Mr (KD)	pI
115	26.4	4.8
1105	26.7	5.0
1401	35.9	4.9
1406	35.3	5.0
1823	61.8	5.0
1830	62.3	5.0
2707	52.5	5.1
2709	52.2	5.2
2713	54.1	5.2
2714	52.2	5.2
2719	51.0	5.2
3704	51.4	5.3
4008	17.3	5.5
4105	27.3	5.5
4108	27.3	5.6
4501	41.1	5.5
5003	17.2	5.7
5101	28.0	5.6
5102	27.3	5.6
5107	27.4	5.7
5205	30.2	5.7
5304	32.8	5.6
5310	32.7	5.7
5905	85.3	5.6
6005	20.1	5.8
6405	38.4	5.7
7202	30.0	5.8
8001	20.0	6.1
8305	30.9	6.2

Table XXII. Molecular weights and isoelectric points of new proteins synthesized in response to exposure of *B. abortus* to pH 5.5 for 60 min.

SSP	Mr (KD)	pI
312	34.1	4.8
419	36.0	4.9
422	35.7	4.7
1019	17.3	5.0
1020	18.4	5.0
1115	21.3	5.0
1418	35.9	5.0
1612	46.0	5.0
1812	61.8	5.1
2019	18.4	5.1
2029	14.9	5.2
2101	21.4	5.1
2708	54.3	5.2
2724	54.2	5.1
2822	58.9	5.2
2909	119.4	5.1
3012	18.9	5.4
3507	39.6	5.4
3510	38.7	5.4
3514	41.0	5.4
3621	42.8	5.3
3703	53.0	5.3
3803	57.9	5.3
3913	84.1	5.4
4026	15.8	5.5
4027	16.7	5.5
4418	37.8	5.5
4425	35.9	5.5
4605	44.0	5.5
4905	84.6	5.5
5116	20.7	5.6
5122	19.7	5.6
5819	64.9	5.6
6018	16.3	5.8
6019	19.1	5.8
6021	14.4	5.7
7015	17.9	5.9
7112	25.4	5.8
7121	20.2	5.9
7122	20.2	5.9

Table XXII continued.

SSP	Mr (KD)	pI
7123	21.2	6.0
7321	33.1	6.0
7322	33.9	6.0
7419	35.3	5.8
8123	20.2	6.4
8124	21.1	6.2
8513	39.0	6.2
8525	38.7	O.R.

APPENDIX F

MOLECULAR WEIGHTS AND ISOELECTRIC POINTS OF PROTEINS INDUCED IN *B. abortus* IN RESPONSE TO NUTRITIONAL DEPRIVATION

Table XXIII. Molecular weights and isoelectric points of proteins whose expression increased when *B. abortus* was transferred from tryptose to minimal medium and was labeled for 60 min.

SSP	Mr (KD)	pI
106	27.5	4.5
212	29.1	5.0
2003	19.3	5.2
4714	48.4	5.5
5001	20.3	5.7
5102	26.6	5.6
6106	24.8	5.8
6506	41.2	5.7
7309	31.0	5.9
7315	30.3	5.9
8006	20.2	6.3

Table XXIV. Molecular weights and isoelectric points of new proteins synthesized when *B. abortus* was transferred from tryptose to minimal medium and was labeled for 60 min.

SSP	Mr (KD)	pI
5	15.5	4.8
317	30.1	4.9
1111	22.1	5.0
3717	49.1	5.5
4616	47.3	5.6
4814	78.4	5.6
5210	26.7	5.7
5609	52.2	5.6
6414	34.8	5.8
6619	46.9	5.7
7209	27.5	5.8
7315	30.3	5.9
8109	23.9	6.0
9604	44.1	O.R.

Table XXV. Molecular weights and isoelectric points of new proteins synthesized when *B. abortus* was transferred from tryptose plates to saline and was labeled for 60 min.

SSP	Mr (KD)	pI
313	30.6	4.8
1008	19.2	5.1
2510	40.5	5.3
2712	61.8	5.3
2720	52.4	5.1
3605	51.7	5.5
3831	69.1	5.4
4001	19.5	5.5
4002	16.0	5.6
4213	29.1	5.6
4406	36.7	5.6
4614	42.8	5.6
5210	26.7	5.7
5509	35.7	5.6
5514	38.2	5.7
5802	66.1	5.6
6402	34.3	5.7
6812	61.3	5.7
7519	36.3	5.9
7627	42.9	5.9

Table XXVI. Molecular weights and isoelectric points of proteins whose expression has increased when *B. abortus* was transferred from tryptose plates to saline and was labeled for 60 min.

SSP	Mr (KD)	pI
7	15.6	4.7
212	29.1	5.0
1406	37.3	5.1
1506	42.0	5.1
2003	19.3	5.2
2503	19.3	5.2
2504	40.6	5.3
2904	40.6	5.3
29.4	94.2	5.3
3302	32.7	5.4
3306	32.3	5.5
3407	37.3	5.5
3411	36.4	5.4
3501	41.5	5.4
3707	60.9	5.5
3812	69.1	5.5
4604	52.3	5.6
4615	47.5	5.6
4702	61.3	5.6
4810	67.6	5.6
5102	26.5	5.7
5509	35.7	5.6
6001	15.9	5.8
6210	29.2	5.7
6401	37.8	5.7
7104	26.6	5.9
7407	37.4	5.9
7606	50.1	5.8
7627	42.9	5.9
7703	60.9	5.8

VITA

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