ERYTHRITOL UTILIZATION OF *BRUCELLA ABORTUS*

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ERYTHRITOL UTILIZATION OF *BRUCELLA*

ABORTUS

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NOMENCLATURE

PMN	Polymorphonuclear leukocytes
PCR	Polymerase chain reaction
RAP-PCR	RNA fingerprinting using arbitrarily primed PCR
mRNA	Messenger RNA
rRNA	Ribosomal RNA
cDNA	Complementary DNA
UV	Ultraviolet Radiation
bp	Base pair
min	Minute(s)
hr	Hour(s)
RPM	Revolutions per minute
CRP	cAMP receptor protein
PTS	Phosphotransferase System
PBS	Phosphate-buffered saline
IPTG	Isopropyl-β-D-thiogalactopyranoside

CHAPTER I

INTRODUCTION

Genus *Brucella*

Members of the genus *Brucella*, which are nonmotile, aerobic, and Gram-negative bacteria, are facultative intracellular pathogens that cause brucellosis in animals and humans. According to conventional criteria such as phage sensitivity, CO_2 requirement, agglutination tests with monospecific antisera, etc., the genus is divided into six species: *B. melitensis*, *B. abortus*, *B. suis*, *B. ovis*, *B. neotomae*, and *B. canis*. These six species have their naturally preferred hosts as follows: *B. melitensis* for sheep and goats, *B. abortus* for cattle, *B. suis* for swine, *B. ovis* for sheep, *B. neotomae* for rats, and *B. canis* for dogs (11). A more recent classification based on comparisons of 16 S rRNA sequence and lipid A composition, Moreno et al. (41) found that *Brucella* species belong to the α-2 subdivision of the class *Proteobacteria*, along with *Rochalimaea quintana*, *Agrobacterium tumefaciens*, and *Rhizobium leguminosarum*, which also live in close association with eukaryotic cells. Like *B. melitensis* that has two circular chromosomes with sizes of about 2.05 and 1.15 Mb, each of the other five *Brucella* species has two chromosomes as its genome (38). By comparing the restriction and genetic maps of six species, Michaux et al. (38) demonstrated that the genomes of *Brucella* species were highly conserved, except for numerous small insertions or deletions in the chromosomes of the six species and a 640-kb inversion in the small chromosome of *B. abortus*.

Some biovars of *B. abortus* and *B. ovis* require CO_2 as a nutritional factor (11). It is fixed into glycine and alanine of proteins and pyrimidines of nucleic acids. *Brucella* species grow in chemically defined media. When erythritol is used as the carbon and energy source in undefined or defined media, it gives the shortest lag phase (21). The optimum pH and temperature for *Brucella* growth are between 6.6 and 7.4, and 37.5°C, respectively (21).

Since Smith et al. (62) demonstrated high erythritol contents in the placenta and foetal tissues and fluids such as chorion, cotyledon, and allantoic fluid and their growth-stimulating activities on *B. abortus*, erythritol has been suspected to play a role in tissue specificity during the infection of cattle by *B. abortus*. The growth-stimulating effect of erythritol was also demonstrated by the observation that when guinea pigs were injected with erythritol, they had increased infection rates of spleen by *B. melitensis* and *B. suis* (25). While the acute placental form of brucellosis was observed in animals like cow, ewe, and goat with easily demonstrable amounts of erythritol in their placentas, the acute form wasn't shown in the placentas of man, rat, and guinea pig that have no detectable amount of erythritol in their placentas, suggesting a role of erythritol in the tissue specificity of brucellosis (25). The correlation between erythritol utilization by *Brucella* and its role in the pathogenicity of *Brucella* are still controversial, so further studies are required to elucidate the relationship and the mechanisms of the growth stimulating effect by erythritol regardless of its involvement in the virulence of *Brucella* (58).

B. abortus used erythritol as the main carbon and energy source in a medium containing high concentrations of glucose (1). The stimulatory effect of erythritol was specific because it could not be imitated with several of three to six carbon homologues

(1). As shown in Figure 1.1, the erythritol utilization of *B. abortus* starts with the phosphorylation of erythritol into D-erythritol-1-phosphate by erythritol kinase, an ATP-dependent kinase. Then the D-erythritol-1-phosphate is oxidized to D-erythrulose-1-phosphate by an NAD-dependent dehydrogenase. The 3-keto-L-erythrose-4-phosphate is the oxidation product of D-erythrulose-1-phosphate by the next NAD-dependent dehydrogenase, which is probably membrane-bound. Finally, the 3-keto-L-erythrose-4-phosphate is oxidized by a membrane-bound dehydrogenase, which is tightly coupled to the electron transport system or a part of the system, to 3-keto-L-erythronate-4-phosphate that is decarboxylated to dihydroxyacetonephosphate by a soluble decarboxylase (64). *B. abortus* US-19 is the only strain of *B. abortus* not growth-stimulated by erythritol and furthermore its growth is inhibited by erythritol in a growth medium containing glucose. The insensitivity of US 19 to growth stimulation by erythritol is due to its missing D-erythrulose-1-phosphate dehydrogenase in the erythritol utilization pathway. The erythritol kinase had eight times higher phosphorylation activity than the hexokinase, so the erythritol kinase uses up the intercellular ATP, inhibiting *Brucella* growth (65). The electron transport system in *B. abortus* is complex and branched (51). Two intermediates of the erythritol pathway could reduce the *Brucella* electron transport system, suggesting that their dehydrogenases are tightly coupled to the electron transport system (51). The erythritol operon has been cloned and characterized by Sangari et al. (59). The operon has four open reading frames identified by sequence homologies with gene products in the databases, as shown in Figure 1.1. As suggested in the above metabolic studies, the operon of *B. abortus* US-19 has a deletion in the region of *eryC* and *eryD* that possibly codes for the missing dehydrogenase and a transcriptional repressor.

Fitzgeorge et al. demonstrated that *B. abortus* grown on basal medium supplemented with bovine allantoic fluid had an increased survival ability inside bovine phagocytes similar to that of organisms grown *in vivo* (17). *B. abortus* could be killed by the classical pathway of complement in the absence of either natural or acquired antibody, as shown by the observation that the killing activity of serum was removed by the treatment of serum with EGTA-$MgCl_2$. The complement-mediated killing activity was blocked by IgG-blocking antibodies that prevent the host from being exposed to excessive amounts of endotoxin released from the killed bacteria, so the complement system of serum can kill *Brucella* only before it enters the host cells (10). *B. abortus* seems to inhibit the degranulation of both primary and secondary PMN granules after ingestion by guinea pig and human polymorphonuclear leukocytes (PMNs) (27). Canning et al. (6) isolated two nucleotide-like substances from *B. abortus*, which inhibit the ability of bovine PMNs to iodinate proteins in a concentration dependent way. Although they inhibited the iodination of proteins by a myeloperoxidase system, the ingestion of bovine PMNs and the production of H_2O_2 by the oxidase on the plasma membrane weren't inhibited by those substances, suggesting that *B. abortus* may survive inside the PMNs by inhibiting the myeloperoxidase system. Rafie-Kolpin et al. (49) demonstrated that some of the protein components of *B. abortus* induced within bovine macrophages were not expressed by *B. abortus* exposed to stress conditions that mimicked the intracellular environments, suggesting a unique protein expression pattern of *B. abortus* inside bovine macrophages. Besides the interaction with phagocytes, the interactions of *B. abortus* and nonphagocytic cells such as Vero cells and HeLa cells were examined. In Vero cells, the intracellular replication of smooth strains occurred in a larger percentage of the host cells

compared to rough strains, although smooth strains adhered to and invaded the host cells less efficiently (12). While the attenuated strain 19 hardly replicates inside HeLa cells and the phagosome containing the strain19 fused with lysosomes, the virulent strain 2308 prevented the formation of phagolysosomes and was distributed within autophagosome-like compartments (47).

Resistance to brucellosis seems to depend on a multigenic trait expressed by bone marrow cells, as shown in the transfer of the resistance by the transfer of bone marrow cells from a resistant mouse strain to a susceptible strain. Smooth virulent strains of *Brucella* grow more rapidly inside macrophages in spite of the slow ingestion by macrophages, suggesting that brucellosis is correlated with the ability to survive inside macrophages (3).

Lifestyles of *Brucella* and Other Intracellular Pathogens in Their Hosts

Although there are some common pathways to cause infections and diseases in their hosts, different pathogens have developed their own distinct mechanisms that enable them to survive inside hosts against the immune systems. These different mechanisms include the toxins that they make, the ways to adhere to and invade the host cells, their intracellular lifestyles in case of intracellular parasites, etc. For example, compared to the absence of any known exotoxins of *B. abortus*, some other pathogens such as *Vibrio cholerae* and *Bordetella pertussis* make cholera toxin with an ADP-ribosylating activity and a pore-forming toxin belonging to the RTX family, respectively (16, 42).

The intracellular or extracellular signals induce an adaptive response including cell motility and gene expression in bacteria. These signal transduction mechanisms play roles in the nitrogen regulation, chemotaxis, osmoregulation, and phosphorus regulation systems in *Escherichia coli* and *Salmonella typhimurium*. The molecular mechanisms of these signal transduction mechanisms involve two component regulatory systems, which are generally a histidine kinase and its cognate response regulator. The histidine kinase is located in the cytoplasmic membrane and has an autokinase activity transferring phosphoryl groups from ATP to a histidine residue. Responding to various signals, the histidine kinase transfers the phosphoryl group from its phosphohistidine to aspartic acid residues in the response regulator, whose phosphorylation state regulates its activity (45, 66).

The expression of virulence genes that enable bacterial pathogens to survive in the hostile environments of the hosts is coordinately regulated, responding to various environmental signals. As with the bacterial adaptive response, coordinate regulation often involves a two component regulatory system to sense the hostile environments and control virulence gene expression. The environmental signals sensed by the two component regulatory systems include temperature, CO_2, osmolarity, carbon source, etc. (36, 39).

One of the best-known two component regulatory systems that control virulence gene expression is the ToxR and ToxS system in *Vibrio cholerae*. ToxR is a transmembrane protein, sensing environmental signals such as temperature, pH, osmolarity, and amino acids and activating the expression of cholera toxin, pilus, and outer-membrane protein in *V. cholerae*. Through the interaction with another

transmembrane regulatory protein, ToxS, ToxR forms a stable dimer capable of transcriptional activation of the various virulence genes (13, 40). Sola-Landa et al. (63) identified a two component regulatory system, BvrR and BvrS in *B. abortus* by isolating two mutants with reduced virulence in the studies using transposon mutagenesis. The BvrR and BvrS have a sequence similarity to *Rhizobium meliloti* ChvI-ExoS and *Agrobacterium tumefaciens* ChvI-ChvG systems previously shown to be critical for endosymbiosis and pathogenicity in plants.

Study of Erythritol Utilization of *Brucella abortus*

Now that the sequencing projects of a variety of model organisms such as *Escherichia coli*, *Saccharomyces cerevisiae*, *Arabidopsis thaliana*, *Caenorhabditis elegans*, *Drosophila melanogaster*, *Mus musculus*, and *Homo sapiens* have been completed or are in progress, many sequences are available in sequence databases. Thus elucidating the functions of the genes from sequencing projects started to show up as one of the next research goals in the area of functional genomics. One of the possible ways to do so is gene expression study using arrayed open reading frames or a cDNA library to identify the genes expressed in certain nutritional or environmental conditions (9, 29, 46). Besides the model organisms, many microorganisms including a variety of pathogens also had their genomes sequenced or sequencing is in progress. These sequencing projects are expected to help us understand microbial pathogenesis (67).

Arrayed cDNA libraries and open reading frames of *A. thaliana* and *E. coli* have been used in the differential gene expression studies to understand the functions of the

genes (60,68). In the absence of a *B. abortus* genome sequencing project, the identification of erythritol-induced genes will help us elucidate the functions of *B. abortus* genes and increase the understanding of *B. abortus* physiology. Although the erythritol operon of *B. abortus* is characterized, the growth-stimulating effect of erythritol can't be completely explained by the erythritol operon. Thus this growth-stimulating effect might be attributed to the regulatory network beyond the erythritol operon such as a regulon, modulon, or stimulon (43).

The synthesis of enzymes metabolizing other carbon sources is inhibited in a microorganism, when a rapidly metabolized carbon source exists in the medium. This phenomenon, called catabolite repression, is also involved in other cellular processes such as sporulation, antibiotic biosynthesis, and extracellular macromolecular degradation (56). Catabolite repression is generally divided into cAMP-dependent and cAMP-independent pathways. The cAMP-independent catabolite repression is mediated by several distinct mechanisms. The cAMP-dependent pathway includes the phosphoenolpyruvate (PEP)-dependent sugar phosphotransferase system (PTS). In the proposed model (54), when the effector protein is phosphorylated by the PTS system, it allosterically activates adenylate cyclase, resulting in the CRP (cAMP receptor protein)-cAMP-dependent transcription. The dephosphorylation of the effector protein responding to the presence of a sugar substrate of the PTS such as glucose deactivates the adenylate cyclase and inhibits the other permeases and catabolic enzymes, mediating the catabolite repression of other carbon sources (54, 56). The cAMP-independent catabolite repression mechanisms include the fructose repressor (FruR)-mediated control of the central enzymes of carbon metabolism, the synthesis of alternative RNA polymerase sigma

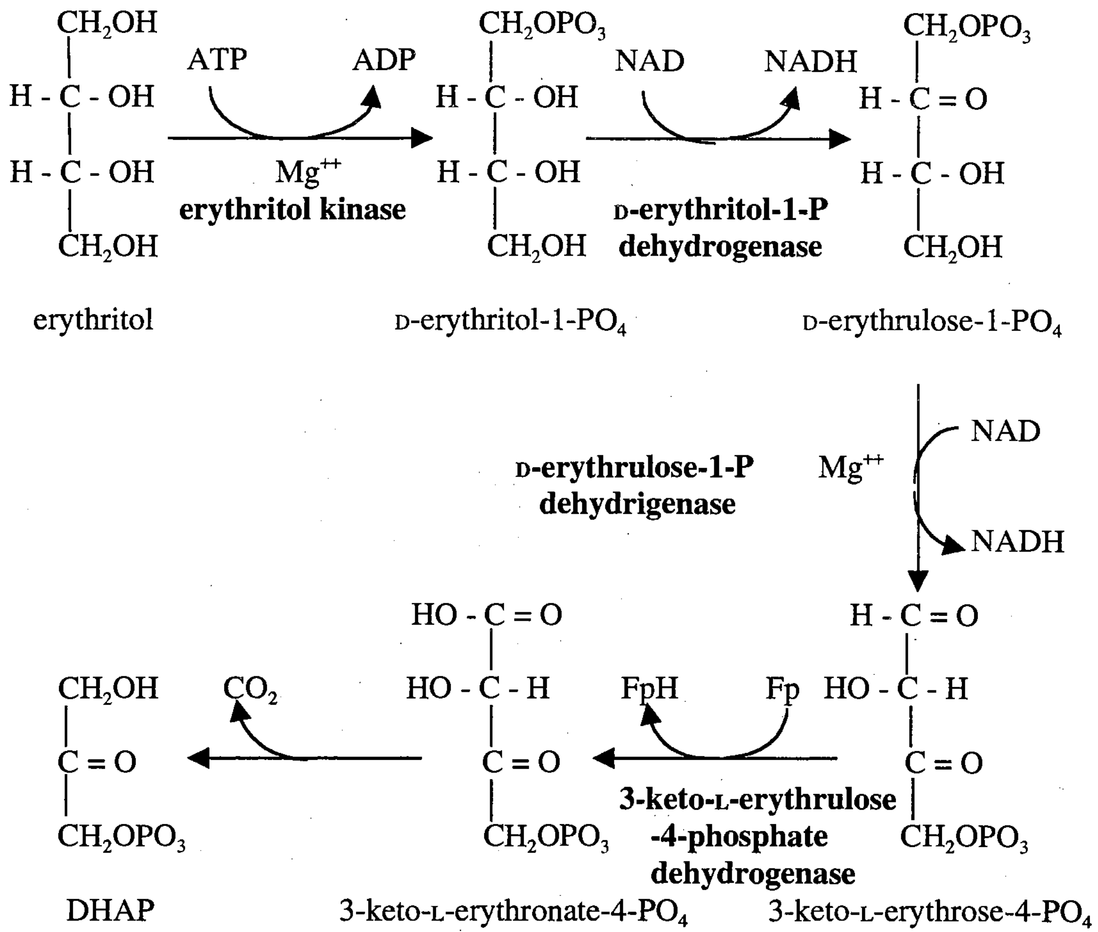
factors in response to starvation or stress, and the control of gene expression by the sensor kinase-response regulator in the two component regulatory system (55, 56). Although bacteria have developed various carbohydrate transport systems such as the PTS system, H symporter, and substrate specific porin, etc. (30), the erythritol transport system of *B. abortus* has not yet been identified. Thus, the study of erythritol-induced gene expression in *B. abortus* might help us get some idea about the transport system.

A rational strategy that a chemotherapeutic agent should kill the parasite without harming the host is based on the fundamental biological and biochemical differences between parasites and hosts (8). One of the biggest problems in the chemotherapy of bacterial pathogens is the occurrence of bacteria resistant to antibiotics. The bacterial pathogens inactivate the antibiotics by three major mechanisms: the destruction or modification of the antibiotics, the prevention of access to the target, and the alteration of the antibiotic target site. Furthermore bacterial resistance is horizontally transferred to other species by plasmids or conjugative transposons located in chromosome (44). As the genome sequences of many pathogens are available now, sequence information can be utilized to find new drug targets. The ideal antimicrobial targets should be essential to the survival of pathogen, highly conserved in a range of pathogens, and absent in the hosts. While the highly conserved targets can be used to develop broad-spectrum antibiotics, the targets unique to a particular organism can also be useful because they provide the opportunity to develop antibiotics highly specific to an organism or a species (53). Therefore, study of erythritol catabolism in *B. abortus* as its unique physiological property will increase the possibility that a new possible drug target is identified, especially considering the absence of *B. abortus* genome sequence. One of the best

vaccine candidates is a live attenuated one that expresses most of the immunogens from pathogens during natural disease and the antigens of which are processed and presented in more natural way. A live vaccine strain is generally constructed by inserting one or multiple mutations in the virulent wild type strain. The mutated genes often encode the virulence factors involved in the pathogenesis and the metabolic enzymes essential to the bacterial survival (37, 48). The study of the metabolism and physiology of *B. abortus* such as erythritol utilization also contributes to the development of a new live *Brucella* vaccine strain.

Figure 1.1. A. Erythritol pathway of *B. abortus* suggested by Sperry et al. (64). The 3-keto-erythrose-4-phosphate was oxidized by incubating with the membrane fraction of 100,000 x g centrifugation without purifying the dehydrogenase or the coenzyme. Enzyme activity of D-erythrulose-1-P dehydrogenase is missing in *B. abortus* US-19. **B.** Erythritol operon of *B. abortus* characterized by Sangari et al. (59). The deletion in US-19 includes 3' region of *eryC* and 5' region of *eryD*.

A



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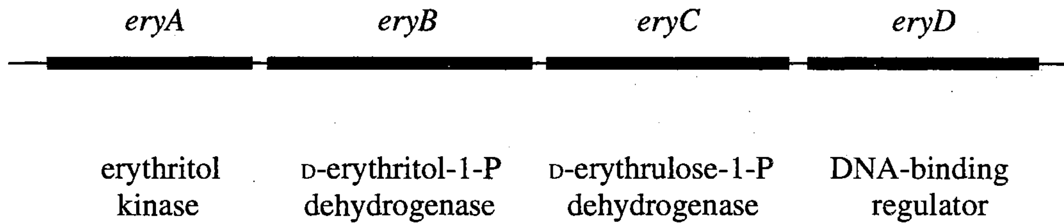


CHAPTER II

IDENTIFICATION OF GENES INDUCED IN *BRUCELLA ABORTUS* INSIDE BOVINE MACROPHAGES BY ARBITRARILY PRIMED PCR OF RNA

Introduction

As a facultative intracellular pathogen, *Brucella abortus* is capable of surviving in professional phagocytes such as macrophages, causing brucellosis in cattle and humans (11). In comparisons of 16 S rRNA sequence and lipid A composition with other bacteria, Moreno et al. (41) indicated that *B. abortus* belongs to the α-2 subdivision of the class *Proteobacteria*, along with *Rochalimaea quintana*, *Agrobacterium tumefaciens*, and *Rhizobium leguminosarum*, which also live in close association with eukaryotic cells. Although there are numerous small insertions or deletions and *B. abortus* has a 640-kb inversion in the small chromosome, the two chromosomes of each of the six *Brucella* species are highly conserved in their restriction and genetic maps (38).

Bovine allantoic fluid increased the survival ability of *B. abortus* inside bovine phagocytes (17). Corbeil et al. showed that the classical pathway of complement was responsible for the killing activity of serum against *B. abortus*, although it was effective only before *B. abortus* enters the host cells due to the IgG-blocking antibodies (10). *B. abortus* seemed to inhibit degranulation after ingestion by guinea pig and human polymorphonuclear leukocytes (PMNs) (27). Canning et al. (6) isolated two nucleotide-like substances that inhibited the ability of bovine PMNs to iodinate proteins in a

concentration dependent way by inhibiting the myeloperoxidase system. In experiments using two-dimensional gel electrophoresis, Rafie-Kolpin et al. (49) demonstrated that some of the protein components induced during survival of *B. abortus* within bovine macrophages were not expressed by *B. abortus* treated with stress conditions that mimicked the intracellular environments, suggesting the unique protein expression pattern of *B. abortus* inside bovine macrophages. In Vero cells, smooth virulent strains had intracellular replication in a larger percentage of cells compared to rough strains, although they adhered to and invaded the host cells less efficiently (12). While the attenuated strain 19 hardly replicated inside HeLa cells and the phagosome containing the strain 19 fused with lysosomes, the virulent strain 2308 prevented the formation of phagolysosomes and was distributed within autophagosome-like compartments (47).

Several methods are used to isolate the genes induced in bacterial pathogens inside host macrophages. Transposon mutagenesis of pathogenic bacteria leading to attenuated virulence in the hosts has been used to identify the genes involved in the virulence of pathogen (4). Finlay et al. (15) identified the *Salmonella* proteins required to adhere and invade the epithelial cells using two-dimensional gel electrophoresis. Penicillin selection method was used to isolate the genes essential in the intracellular replication of bacterial pathogens (5, 13). In this selection method, a pool of transposon mutants of the bacterial pathogen were ingested by host cells and then treated with a β-lactam to isolate mutants defective for intracellular replication, since the β-lactam treatment kills only actively growing mutants of pathogen within host cells. In the *in vivo* expression technology, Mahan et al. (33, 35) isolated the genes induced in *S. typhimurium* when injected into mouse spleen by utilizing a transcriptional fusion vector, which used an antibiotic

resistance (pIVET8) or an auxotrophy as the selective marker. Using a *S. typhimurium* library of random DNA fragments inserted upstream of a promoterless *gfp* gene, fluorescence-based selection for the intracellularly induced genes of *S. typhimurium* inside host cells was used by Valdivia et al. (69). Differential display was developed to isolate the genes differentially expressed by eukaryotes in specific conditions such as various cells or altered conditions by means of PCR using a pair of primers that consist of a poly (dT) primer and one of arbitrary primers (32). This method was modified to isolate the stress-inducible genes of *S. typhimurium* by using pairs of arbitrary primers (70). In this report, we attempted to use the arbitrarily primed PCR of RNA to isolate the intracellularly induced genes of *B. abortus* inside bovine macrophages.

Methods and Materials

Bacterial Strains and Media

B. abortus strain 2308 used in the experiments was obtained from the Oklahoma Animal Disease Laboratory and stored as tryptose-glycerol stocks at –80°C. *B. abortus* cells were grown on tryptose (Difco) agar plates right before the usage in the experiments. *Escherichia coli* strain XL-1 Blue (Stratagene) was transformed as described by Hanahan (14).

RNA Extraction from Extracellularly and Intracellularly grown *B. abortus*

RNA extraction from the intracellularly and extracellularly grown *B. abortus* was carried out by Maryam Rafie-Kolpin as described by Rafie-Kolpin. (50).

Preparation, Isolation, and cloning of Arbitrarily Primed PCR Products from *B. abortus* RNA

RNA fingerprinting by arbitrarily primed PCR (RAP-PCR) was performed by Maryam Rafie-Kolpin by using the RAP-PCR kit (Stratagene) according to the manufacturer's instructions.

Southern Blot Analysis

Total *B. abortus* genomic DNA was isolated as described (2) after a brief treatment of *Brucella* cells grown on tryptose plates with methanol to kill the cells. After restriction digestions with *Eco*RI and *Hin*dIII enzymes and agarose gel electrophoresis, the genomic DNA fragments were transferred to a nylon membrane by capillary action and immobilized by UV crosslinking after treatment as described by Sambrook et al. (57). The probes were photobiotinylated as described by Forster et al. (18) using photobiotin (Sigma). The Southern blots were hybridized to a PCR-amplified and biotin-labelled gel eluent, or a biotin-labelled plasmid DNA cloned from the gel eluent in a solution containing 7% SDS and 0.25 M Na_2PO_4, pH 7.2 at 65°C overnight. Then, the blots were washed twice with a solution containing 0.1% SDS, 150 mM NaCl, and 15 mM Na-citrate, pH 7.0 at 65°C, 20 min each. The bound probes were detected by using the chemiluminescent detection system (Tropix) according to the manufacturer's instructions.

Southern Dot Blot Analysis

Total *B. abortus* genomic DNA was digested with *Eco*RI, phenol-chloroform extracted, and ethanol precipitated. After denaturation with NaOH, 1 µg of the genomic DNA fragments was dotted on a nylon membrane and immobilized by UV crosslinking. The membranes were hybridized and washed, and the hybridized probes were detected as described in the previous section.

Northern Blot Analysis

Total RNA from extracellularly and intracellularly grown *B. abortus* was transferred on a nylon membrane by capillary action and UV-crosslinked after electrophoresis on formaldehyde agarose gel by Maryam Rafie-Kolpin as described by Sambrook et al. (57). The membranes were hybridized at 65°C and the hybridized probes were detected as described in the Southern blot analysis.

Results

Isolation and Cloning of RAP-PCR Products

This experiment was performed by Maryam Rafie-Kolpin as described by Rafie-Kolpin (50). Four primers were used in the RAP-PCR of the RNA from the extracellularly and intracellularly grown *B. abortus*. On the polyacrylamide gel, 22 bands were shown to have the RAP-PCR products from intracellularly induced RNA species and were cloned into pBluescript SKII by Rafie-Kolpin. After the TA-cloning, the cell lysates from the clones were used in PCR amplification and the inserts were determined to have the same sizes as on the polyacrylamide gel, as shown in Figure 2.1. Out of the 22 RAP-PCR products, 13 fragments were sequenced and searched against several sequence databases as described by Rafie-Kolpin (49). Although some of the sequences had some similarities with known proteins in bacterial species, others didn't show any significant homologies against the databases searched.

Southern Blot Analysis

Several out of the gel eluents from the 22 bands, which included the RAP-PCR products from intracellularly induced RNA species, were biotinylated in PCR amplification using biotin-dUTP (Sigma) and hybridized against the *B. abortus* genomic

DNA on nylon membrane. Surprisingly, the probe from each gel eluent gave signals from more bands than a plasmid probe from the same gel eluent, indicating that the gel eluent from a single band on the polyacrylamide gel had several different RAP-PCR products. Figure 2.2 shows one of several Southern blots analyzed. .

Southern Dot Blot Analysis

When one of the gel eluents A3-4 was TA-cloned and 12 different plasmids from its clones were photobiotinylated and used as probes in the hybridization against the *B. abortus* genomic DNA dotted on nylon membrane, only two of them gave hybridization signals as demonstrated in Figure 2.3, suggesting that 10 out of 12 RAP-PCR products from a single band aren't from the *B. abortus* RNA but presumably from the bovine macrophage RNA.

Northern Blot Analysis

Figure 2.4 shows the northern blot analysis using one of the probes that gave a hybridization signal in the Southern dot blot analysis as a probe.

Discussion

As demonstrated in the figures, in this study using RAP-PCR technique to isolate intracellularly induced genes of *B. abortus*, two problems were revealed. Among the gel eluents from the 22 bands, each of the examined ones seemed to include several RAP-PCR products of the same size. Secondly, the RNA from the intracellularly grown *B. abortus* seemed to be contaminated with bovine macrophage RNA. The problem of multiple RAP-PCR products in a single band might be solved by increasing the stringencies of the arbitrary priming during the first and second strands' synthesis of RAP-PCR and thus decreasing the total number of RAP-PCR products from each primer.

The contamination with bovine macrophage RNA was confirmed by the fact that some of the clones showed high sequence similarities to eukaryotic genes or proteins such as a bovine protein p97. The contamination of the RNA from intracellularly grown *B. abortus* with bovine macrophage RNA made many of the bands on the polyacrylamide gel look induced because the macrophage RNA doesn't exist in the RNA from extracellularly grown *B. abortus*. Some of the techniques used to isolate the genes induced in bacterial pathogens in intracellular conditions are based on the isolation of RNA from intracellularly grown bacteria, so a new technique to overcome the host RNA contamination is required to continue this kind of approach. Currently, there are some ways to get over the host RNA contamination. One of the ways is to use the hybridization of RNA or cDNA from intracellularly grown pathogens with bacterial genomic DNA to get rid of the host RNA or cDNA. After the hybridization, the bacterial RNA or cDNA can be isolated from the hybrid (22). Another possible way to overcome the host RNA contamination is to array bacterial open reading frames on a solid support such as nylon membrane or glass plate (60, 68), providing the gene expression profile of intracellularly grown bacteria. The fraction of probe from the host RNA will not hybridize with the arrayed bacterial genes, solving the host RNA contamination problem.

Figure 2.1. PCR amplification of clones after TA-cloning of one of RAP-PCR products, A3-4. The gel eluent from the band on the polyacrylamide gel was PCR-amplified, ligated to pBluescript SKII, and transformed into XL-1 Blue. The cell lysates from several white colonies were used in PCR amplification using the same primer as in the RAP-PCR. The PCR amplified inserts from colony lysates were electrophoresed in 1.0% agarose gel with 0.5 μg/ml ethidium bromide. The vectors of the colonies were shown to have the right inserts.

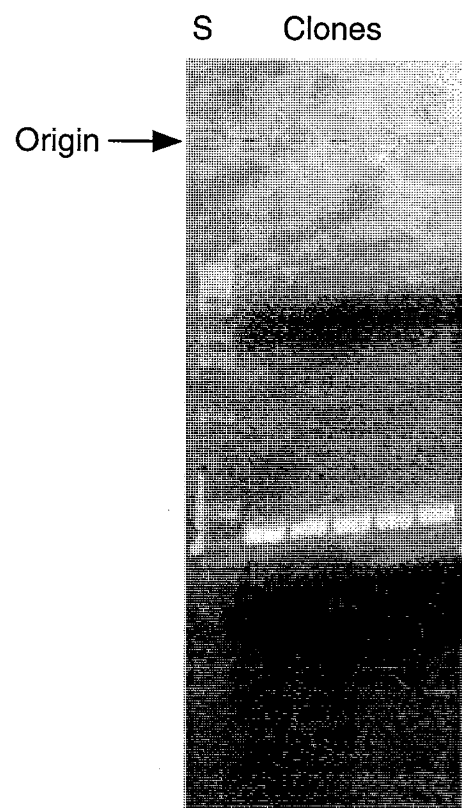

S
Clones
Origin

Figure 2.2. Southern blot analysis. ***A.*** The plasmid from a single colony after the TA-cloning was biotinylated and used as a probe against a Southern blot of *B. abortus* 2308 genomic DNA digested with *Eco*RI or *Hin*dIII. ***B.*** The gel eluent from a single band on the polyacrylamide gel was biotinylated by PCR-amplification using a biotin-dUTP and used as a probe against a Southern blot of *B. abortus* 2308 genomic DNA digested with *Eco*RI or *Hin*dIII.

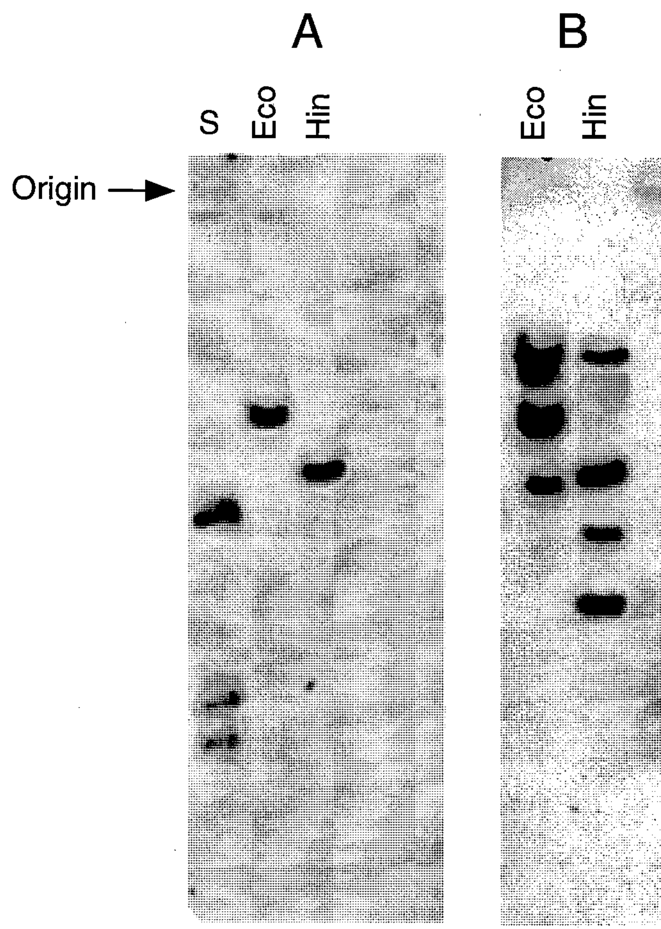

A
B
S
Eco
Hin
Eco
Hin
Origin

Figure 2.3. Southern dot blot analysis. Total *B. abortus* genomic DNA was dotted on 12 different pieces of nylon membrane. After the gel eluent A3-4 was TA-cloned, the plasmids were isolated from 12 different white colonies, photobiotinylated and used as probes separately against 12 Southern dot blots.

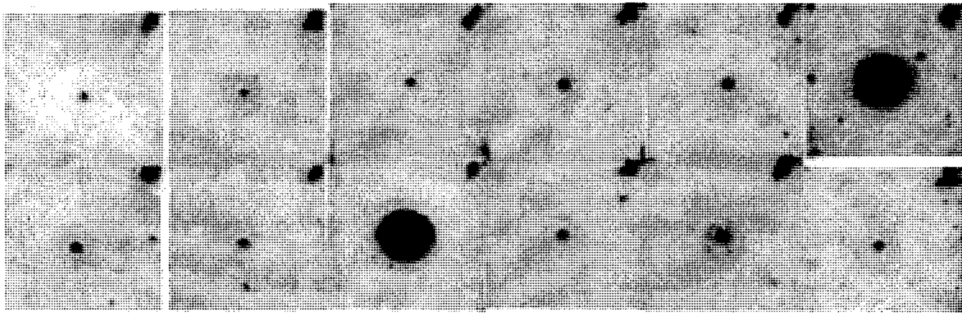
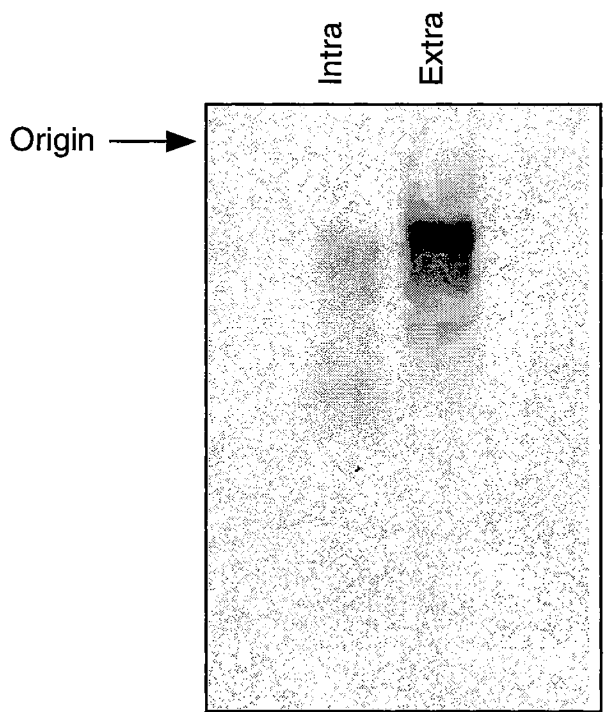


Figure 2.4. Northern blot analysis. One of the two probes hybridized to the *B. abortus* genomic DNA in the Southern dot blot analysis of **Figure 2.3**. was used again as a probe against a northern blot of the RNA from extracellularly (Extra) and intracellularly (Intra) grown *B. abortus*.


Intra
Extra
Origin

CHAPTER III

IDENTIFICATION OF GENES INVOLVED IN ERYTHRITOL UTILIZATION OF *BRUCELLA ABORTUS*

Introduction

Brucella abortus is a facultative intracellular pathogen that is able to survive in professional phagocytic cells such as macrophages, causing brucellosis in cattle and humans (11). Based on comparisons of 16 S rRNA sequence and lipid A composition, *B. abortus* belongs to the α-2 subdivision of the class *Proteobacteria*, along with *Rochalimaea quintana*, *Agrobacterium tumefaciens*, and *Rhizobium leguminosarum*, which also live in close association with eukaryotic cells (41). Like other species in the genus *Brucella*, *B. abortus* has two chromosomes as its genome, a feature which is conserved in the other five species, except for a 640-kb inversion in its small chromosome (38).

Erythritol was fractionated from a mixture of infected allantoic fluids collected from a cow which was about to abort after infection with *B. abortus* and shown to promote the growth of *B. abortus* within bovine phagocytes as well as stimulate the extracellular growth of *B. abortus* (62). So, it was suggested that the presence or absence of erythritol in foetal tissues might determine the nature of brucellosis in animals other than cattle. Keppie et al. (25) demonstrated the growth-stimulating effect of erythritol by showing increased infection rates of spleen by *B. melintensis* and *B. suis* in guinea pigs

injected with erythritol. The tissue specificity of brucellosis due to erythritol was suggested from the observation that animals such as ewe, goat, and sow with erythritol in their placentas suffered from the acute placental form of brucellosis, while man, rat, and guinea pig, which have no detectable amount of erythritol in their placentas, didn't suffer from the acute form of brucellosis (25). The correlation between erythritol utilization of *B. abortus* and its role in the pathogenicity of *B. abortus* is still controversial, so further studies are required to elucidate the relationship and the mechanisms of the growth-stimulating effect by erythritol regardless of its involvement in the virulence of *B. abortus* (58).

Methods and Materials

Bacterial strains, media, and growth conditions

B. abortus strain 2308 used in the experiments was obtained from the Oklahoma Animal Disease Laboratory and stored as tryptose-glycerol stocks at –80°C. *B. abortus* cells were grown on tryptose (Difco or Fisher) agar plates right before the usage in the experiments. *Escherichia coli* strain XL-1 Blue (Stratagene) was transformed as described by Hanahan (23).

RNA extraction from *Brucella abortus* grown in glucose and erythritol media

After *B. abortus* 2308 was grown to log phase in 20 ml tryptose media supplemented with 1% glucose or erythritol, the *B. abortus* cells were harvested by centrifuging at 7000 rpm. Total RNA from *B. abortus* cells grown in glucose or erythritol media was prepared as described by Garrido et al. (20). Briefly, the centrifuged cells were resuspended in 0.5 ml of an ice-cold PBS, then 50 µl of an ice-cold solution of 10 mg/ml lysozyme in 20 mM EDTA, pH 8.0 was added. The cells were frozen in a dry ice-

ethanol bath and then thawed in a 37°C water bath for 2 min. After one more cycle of the freezing and thawing, the cells were frozen again for 2 min in dry ice-ethanol bath. To the frozen samples, 10 µl of 1 µg/µl yeast tRNA, 50 µl of 10% SDS, 20 µl of 10% acetic acid, 70 µl of 2 M sodium acetate, pH 4.0, and 0.5 ml of water-saturated phenol were added. After thawing for 2 min in a 37°C water bath, the samples were vortexed at high speed for 1 min. Then the samples were incubated on ice for 10 min and centrifuged at high speed for 15 min. 350 µl- 500 µl of the supernatant was removed to a new tube and then two volumes of ethanol were added. After overnight incubation at -20°C, the total RNA was centrifuged at 14000g for 30 min, washed with 70% ethanol, dried in vacuum, and resuspended in 50 µl –100 µl ddH_2O.

Preparation of arrayed *B. abortus* genomic DNA library on nylon membranes

Total *B. abortus* genomic DNA was prepared as described (2), after briefly treating *B. abortus* cells grown on tryptose plates with methanol to kill cells. The genomic DNA was partially digested with *Sau*3AI, size-fractionated in low-melting temperature agarose gel (Sigma) to isolate 2-6 kb genomic DNA fragments, ligated to pBluescript SKII previously digested with *Bam*HI and then dephosphorylated with alkaline phosphatase. The ligation mixtures were cleaned with PCR purification Kit (Qiagen) before electroporation. The preparation of electroporation-competent cells from XL1-Blue (Stratagene) and electroporation were carried out as described by Sharma et al. (61). After electroporation, the cells were incubated at 37°C with shaking for 1 hr and plated on Luria Broth (LB) (57) plates containing ampicillin (100 µg/ml), IPTG (112µM), and X-gal (27 µg/ml). The cells were incubated at 37°C overnight. 3456 white colonies were inoculated into 96 well blocks (EdgeBioSystem), each well of which had 1.2 ml TB

media (57) containing ampicillin (100 μg/ml), followed by incubation with shaking at 350 rpm for 24 hr at 37°C. The cells were harvested by centrifugation at 549 g for 10 min, resuspended in 200 μl of TE-RNase (50 mM Tris-HCl, pH 7.6, 10 mM EDTA, 42 μg/ml RNaseA, 42 U/ ml RNaseT1), and incubated on a shaker at 37°C for 15 min. To the resuspended cells, 200 μl of 3 M potassium acetate, pH 4.8 was added after lysis with 200 μl of 0.1 N NaOH, 1% SDS, then they were incubated with shaking at 37°C for 30 min, and stored at –20°C overnight. The cell lysates were precipitated by centrifugation at 1336 g for 1 hr at 4°C. The top 300 μl of supernatant was removed into a clean 96 well block, 900 μl 95% ethanol was added, and it was incubated at -20°C for 1 hr to overnight. The plasmid was precipitated by centrifugation at 1336 g for 30 min at 4°C, washed with 300 μl 70% ethanol, dried under vacuum, and resuspended in 100 μl TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) buffer containing RNaseA (20 μg/ml). 20 μl of each of the plasmid solutions was removed from 96 well blocks into 96 well microtiter plates and denatured by addition of 20 μl of 0.5 N NaOH/20 mM EDTA solution followed by incubation at 65°C for 30 min to 1 hr. Using a 96 pin replicator, 864 plasmids were replicated onto a pair of nylon membranes, making 4 pairs of nylon membranes blotted with plasmids from 3456 colonies.

Preparation of random-primed and PCR amplified probes from total RNA

The random-primed and PCR-amplified cDNA was prepared as described by Froussard (19) with minor modifications. Briefly, the total RNA was converted to the first strand cDNA by reverse transcription using random primers (CATTTGGGATCCT GACACCGNNNNNN and CAGCAAGAATTCCCTCACGCNNNNNN), as shown in Table 3.1, which have a defined 5' sequence and a 3' random hexamer, and Superscript II

(GIBCO), followed by the second strand synthesis with Klenow fragment (Promega) according to the manufacturer's instructions. The second strand mixtures were cleaned with PCR purification Kit (Qiagen) and PCR amplified for 40 cycles (94°C for 1 min, 60°C for 2 min, 72°C for 3 min) with the PCR primers that have a defined 5' sequence (CATTTGGGATCCTGACACCG and CAGCAAGAATTCCCTC ACGC), as shown in Table 3.1. 1 μg of the PCR amplified cDNA was photobiotinylated as described by Foster et al. (18) using photobiotin (Sigma) and used in the hybridization experiments.

Hybridization and Data Analysis

Four pairs of nylon membranes were hybridized with the photobiotinylated cDNA probes from *B. abortus* grown in glucose- or erythritol-containing medium at 65°C overnight and washed as described in the Chapter 2. The bound probes were detected with the chemiluminescent detection system (Tropix) according to the manufacturer's instructions. The exposed films (Kodak) were scanned on the GS-700 Densitometer (BioRad). The resulting TIFF image files were saved electronically and analyzed by using Molecular Analyst (version 5.0). A grid of rectangles was overlaid on the array image to calculate the intensities for each spot. The signal intensities for each spot were exported from Molecular Analyst into a Microsoft Excel spreadsheet. The total intensity of each spot was expressed as a percentage of the total of intensities of all the spots on each blot. These percentage values of each spot were used to calculate the ratio of the corresponding spots on the two blots, which are used to identify the spots induced or repressed in erythritol medium. We chose only those log expression ratios with values greater than 2 standard deviations from the mean and having a 95% confidence interval

as determined by the t-test in at least one of triplicate hybridizations, and positive in the others, as the induced or repressed spots.

Results and discussion

Growth of *B. abortus* in tryptose media containing glucose or tryptose as a carbon source

As shown in Figure 3.1, when *B. abortus* 2308 was grown in tryptose medium containing 1% erythritol as a carbon source, the growth rate was several times higher than when it was grown in glucose-containing medium in exponential phase. This growth-stimulating effect of erythritol may play a role in *Brucella* pathogenicity (25, 58, 62). Although the role of erythritol in the tissue specificity of *B. abortus* is still unclear, its growth stimulating effect was confirmed in this study.

Preparation of random-primed and PCR amplified cDNA, and *B. abortus* genomic DNA library

Total RNA was extracted from *B. abortus* grown in tryptose media containing two different carbon sources, glucose or erythritol, In order to use the same amounts of total RNA in probe preparation, besides measurement of absorbance, more accurate quantitation was achieved by comparing both of the total RNA in formaldehyde agarose gel after 3 µl of 1 mg/ml ethedium bromide was added to each of RNA samples before loading. The 23S, 16S, and 5S rRNAs appeared on the agarose gel with the same intensities in both lanes, as shown in Figure 3.2. In the probe preparations, the synthesis of the first and second strands was random-primed and the double stranded cDNAs were PCR amplified. The size of random-primed and PCR amplified cDNAs ranged from 0.5 kb to 3.0 kb, as indicated in the agarose gel electrophoresis of Figure 3.3. In the *B. abortus* genomic DNA library construction, the electroporated cells were plated on LB

plates containing ampicillin (100 μg/ml), X-gal (27 μg/ml), and IPTG (112 μM) and 24 out of the examined 28 white colonies had expected inserts.

Gene expression profiles and data analysis

The data from separate triplicate hybridization experiments was used to increase the significance and reproducibility in the identification of *B. abortus* genes induced and repressed in erythritol-containing medium. The log expression ratio of the percent intensities of corresponding spots on a pair of blots were used as criteria to identify the spots containing induced or repressed genes in erythritol-containing tryptose medium. As shown in Table 3.2, 32 spots contained the genes induced in erythritol-containing medium, while 52 spots includes the repressed genes in the erythritol medium. As described by Sangari et al. (57), the erythritol operon doesn't have a full set of components involved in erythritol utilization. As shown in Figure 1.1, the erythritol operon doesn't encode the enzymes after D-erythrulose-1-P-dehydrogenase of the pathway, thus the erythritol utilization by *B. abortus* requires the involvement of other gene(s) or operon(s). The mechanism of erythritol transport is also unknown and thus it is possible that a gene or operon for erythritol transport is involved in the erythritol utilization by *B. abortus*. Thus, far from the growth-stimulating effect of erythritol, erythritol utilization itself needs the involvement of gene(s) or operon(s) other than the erythritol operon. In the hybridization experiments, we identified 32 spots containing erythritol-induced genes and 52 spots with genes repressed in erythritol-containing medium. Since the average size of inserts is about 4 kb, the *B. abortus* genomic library is 3.65 times bigger than *B. abortus* genome size. Based on a simple calculation, the 5.37 kb erythritol operon may be included in about 5 induced spots. Thus, the rest of the induced

spots and the repressed spots include genes or operons other than the erythritol operon. Besides the erythritol operon, this identification of multiple genes and/or operons involved in the erythritol utilization by *B. abortus* suggests that the growth-stimulating effect requires the induction of genes or operons other than the erythritol operon, thus possibly the involvement of multiple cellular processes. As described in Chapter I, besides carbon catabolic enzymes, many proteins involved in a variety of cellular processes are subject to catabolite repression (56). Thus there might be a common regulator involved in the coordinate regulation of induced and repressed genes responding to erythritol, like CRP (cAMP receptor protein) and cAMP playing a central role in response to glucose in catabolite repression. The existence of repressed spots, thus ones including genes possibly induced by *B. abortus* in glucose-containing media, is also interesting, since some of the genes expressed by *B. abortus* growing in glucose-containing media might inhibit the expression of genes involved in the erythritol utilization by *B. abortus* and the growth-stimulating effect of erythritol. Erythritol might be involved in the *B. abortus* pathogenicity (25, 62). The growth stimulating effect by erythritol involves the induction and repression of multiple genes or operons, some of those genes might be connected to the virulence mechanism of *B. abortus*, although this possibility should be examined through the characterization of the erythritol response genes.

The 5' and 3' ends of 10 inserts from the induced and repressed spots were sequenced and examined for the homology between the end sequences and the gene products in the databases. Although 6 end sequences shows no significant similarities, the rest of them have significant similarities with the gene products in the databases. As

expected, most of the inserts except for one have similarities to different gene products in the databases. The insert, which has similarities to the same gene product in the databases, is from the *B. abortus* cyclic β(1-2) glucan synthetase that is a known virulence factor in *Brucella* infection, suggesting a possible connection between the growth-stimulating effect and the *Brucella* pathogenicity in the host (33).

Figure 3.1. Growth curve of *B. abortus* in tryptose media with 1% glucose or 1% erythritol as a carbon source. *B. abortus* grown in tryptose medium with erythritol has several times higher growth rate in exponential phase than *B. abortus* grown in glucose medium.

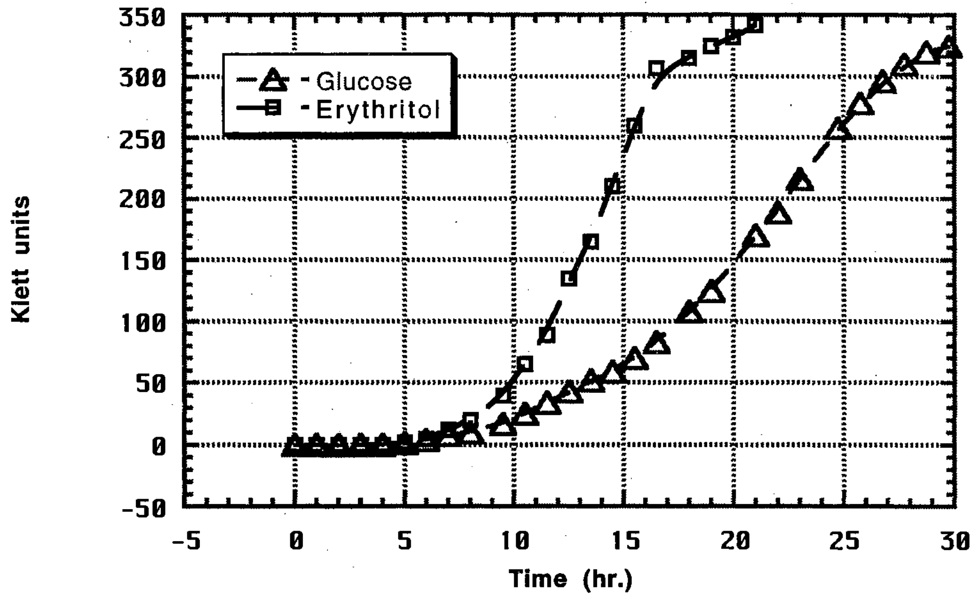

Growth Curve of B. abortus
Glucose
Erythritol
Klett units
350
300
250
200
150
100
50
0
-50
-5
0
5
10
15
20
25
30
Time (hr.)

Figure 3.2. Formaldehyde agarose gel electrophoresis of *B. abortus* total RNA. 10 μg of each of the total RNA from *B. abortus* grown in tryptose media containing 1% glucose or 1% erythritol was loaded on the gel after ethidium bromide was added to each of the RNA samples. The top band is 23S rRNA, the middle band is 16S rRNA, and the lowest band is 5S rRNA.

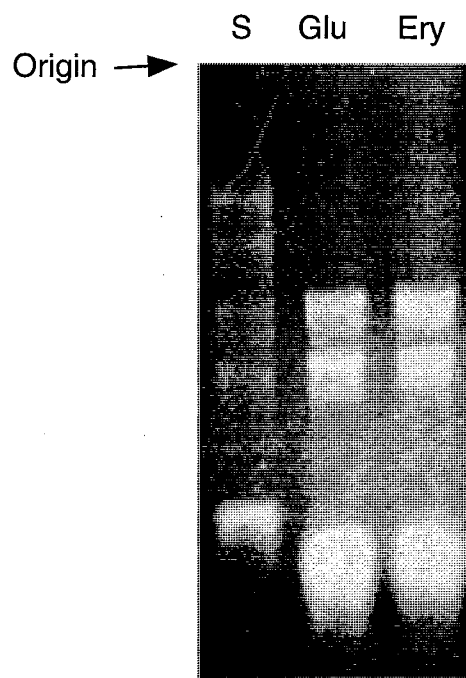

S
Glu
Ery
Origin

Figure 3.3. Random-primed and PCR amplified cDNA from *B. abortus* total RNA. The size range of the cDNA is from 0.5 kb to 3.0 kb. cDNA was synthesized by random-priming and PCR amplifying the total RNA from *B. abortus* grown in glucose (Glu) or erythritol (Ery) medium and electrophoresed in 1% agarose gel with 0.5 μg/ml ethidium bromide.

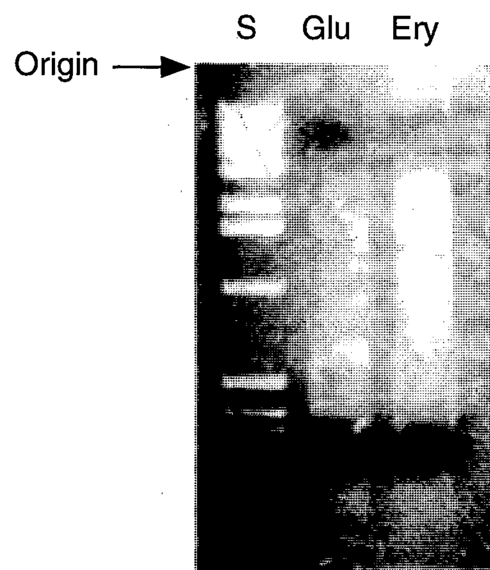
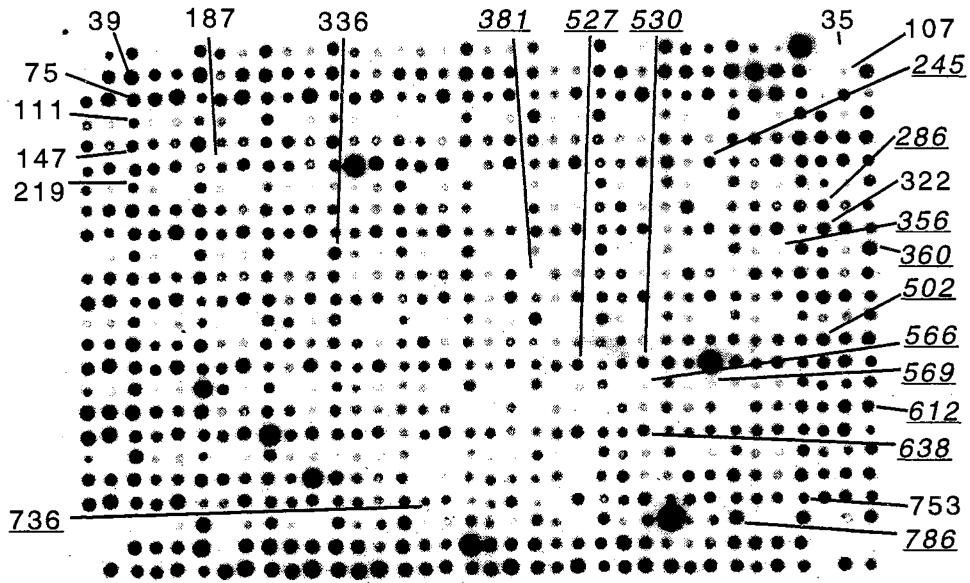

S
Glu
Ery
Origin

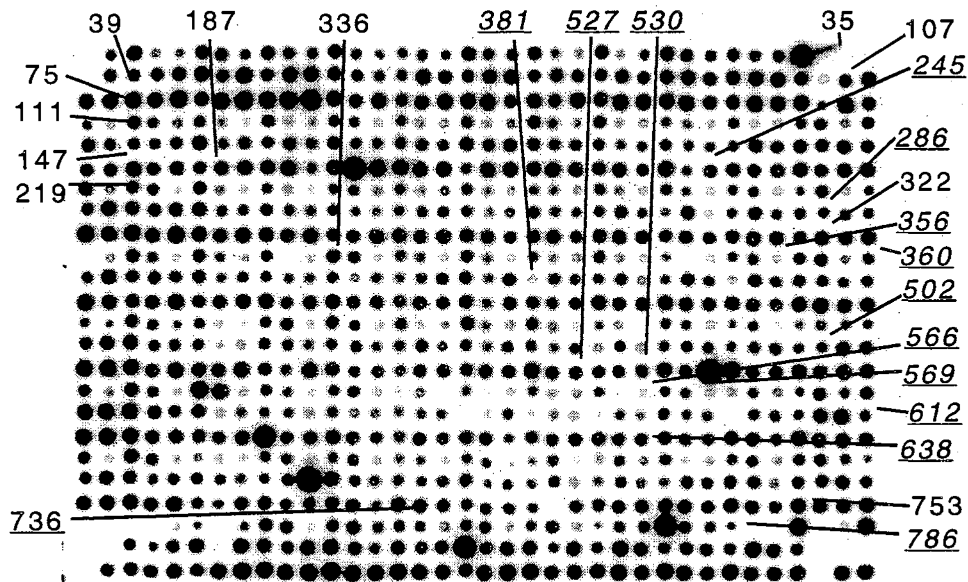
Figure 3.4. Gene expression profiles of *B. abortus* grown in tryptose media containing 1% glucose (Glu) or 1% erythritol (Ery) as a carbon source. *B. abortus* genomic DNA clones were arrayed on 4 pairs of nylon membranes and hybridized with cDNA probes.**A.** Blot 3 **B.** Blot 4. Spots numbered with regular type include the genes induced in erythritol-containing medium, the spots numbered with *italic type underlined* have repressed genes.

A

Glu

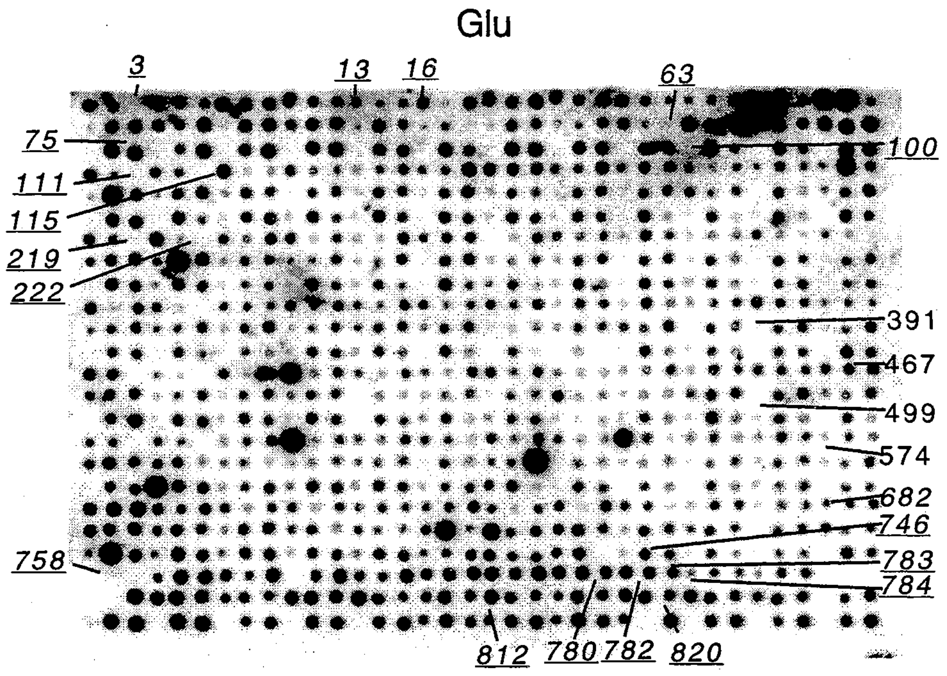


Ery



B

Glu



Ery

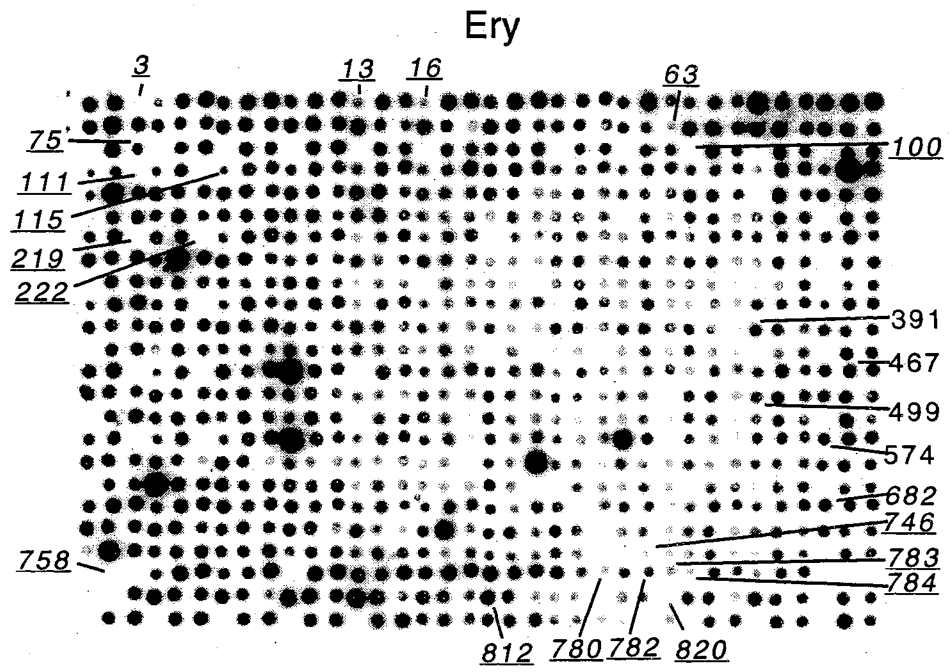


Table 3.1. Synthetic oligonucleotides used in this study

Sequence	Use
CATTTGGGATCCTGACACCGNNNNNN CAGCAAGAATTCCCTCACGCNNNNNN	Random primers used in the first and second strand synthesis of cDNA
CATTTGGGATCCTGACACCG CAGCAAGAATTCCCTCACGC	PCR primers used in PCR amplification of cDNA

Table 3.2. Identification of *B. abortus* genes induced or repressed in erythritol-containing tryptose medium.

B. abortus genomic library blots	Induced	Repressed
Blot 1	7	1
Blot 2	8	21
Blot 3	12	13
Blot 4	5	18
Total	32	53

Table 3.3. Homology of between end sequences of inserts from induced and repressed spots and gene products in the databases

Identification of spots	Expression	Closest homologous gene/gene product (accession number)	aa identity (%)+ /similarity(%)
10-D12-T3	Repressed	nss*	
10-D12-T7	Repressed	nss	
11-C1-T3	Repressed	*Mesorhizobium loti* unknown protein (NP104062.1)	64/70
11-C1-T7	Repressed	*Mesorhizobium loti* unknown protein (NP104065.1)	82/87
11-G1-T3	Repressed	nss	
11-G1-T7	Repressed	nss	
11-H2-T3	Repressed	*Mesorhizobium loti* lysyl tRNA synthetase (NP106258.1)	88/94
11-H2-T7	Repressed	*Mesorhizobium loti* unknown protein (NP106257.1)	44/59
13-B12-T3	Induced	*Brucella abortus* cyclic β 1-2 glucan synthetase (T31419)	77/78
13-B12-T7	Induced	*Brucella abortus* cyclic β 1-2 glucan synthetase (T31419)	86/87
14-B9-T3	Induced	*Rhodobactor capsulatus* hypothetical protein (T03518)	32/50
14-B9-T7	Induced	*Mesorhizobium loti* hypothetical protein (NP103776.1)	63/74
18-C1-T3	Repressed	Flagellar basal-body rod protein (Q52946)	76/85
18-C1-T7	Repressed	*Sinorhizobium meliloti* flagellar basal-body rod protein (CAA11959.1)	50/62
18-E1-T3	Repressed	*Escherichia coli* high affinity ribose transport protein (AAA62102.1)	39/57
18-E1-T7	Repressed	nss	
18-G1-T3	Repressed	nss	
18-G1-T7	Repressed	*Pisum sativum* putative senescence-associated protein (BAB33417.1)	53/58
24-A1-T3	Induced	*Mesorhizobium loti* thiamin biosynthesis ThiG (NP106390.1)	71/84
24-A1-T7	Induced	Phosphomethylpyrimidine kinase (HMP-phosphate kinase) (P56904)	46/55

* No significant similarity (nss) with gene products in the databases.
+ Percentage identity and similarity were determined using the Blastx program from the National Center for Biotechnology Information (NCBI)

CHAPTER IV

CONCLUSION

Differential gene expression of *B. abortus* within bovine macrophages was studied by using RAP-PCR as an effort to understand how *B. abortus* manages to survive inside host phagocytic cells. In the RAP-PCR technique, the synthesis of cDNA is based on arbitrary priming both in the first strand and second strand synthesis using a low stringency condition and then the later amplification steps use a high stringency condition. In the later steps of this study, the bands on the polyacrylamide gel, which were used in cloning and sequencing, have several RAP-PCR products of similar size from different RNA species and furthermore some of them are from contaminating bovine macrophage RNA. Thus a new technique seems to be essential in the approaches depending on the RNA extraction from pathogenic bacteria intracellularly grown inside host cells. Recently, several new ways to avoid the contamination by RNA from host cells have been reported. One of the methods utilizes the hybridization of the bacterial genomic DNA and the cDNA from the bacterial RNA contaminated with host RNA to identify the cDNA from the differentially expressed genes by pathogens inside host cells (22). Another method relies on the arrayed bacterial open reading frames on solid support such as nylon membrane or glass plate. In the later case, out of the probes from the contaminated bacterial RNA, only those from the bacterial RNA will hybridize against the arrayed bacterial genes, eliminating the effect of the contaminating host RNA in the hybridization. These new methods to get over the contamination by host RNA will help

us understand how pathogens survive the hostile environment inside host cells and contribute to the development of more effective live vaccine and the identification of new drug targets.

In the absence of *B. abortus* genome sequence, one of the ways to identify the genes involved in erythritol utilization by *B. abortus* is to use the arrayed genomic DNA library of *B. abortus.* Compared to the study using arrayed open reading frames identified from genome sequences, the arrayed genomic library has a couple of differences. First of all, it is difficult to make the genomic library including all the genes, so we won't be able to identify all the genes involved in erythritol utilization by *B. abortus.* Secondly, each of genomic DNA inserts includes multiple open reading frames, making it difficult to identify induced or repressed open reading frames from arrayed spots. In spite of these difficulties, in case of the absence of genome sequence, an arrayed genomic library is one of the best ways in gene expression studies. Erythritol has been suspected to be involved in the virulence and tissue specificity of *B. abortus.* The erythritol operon is the only gene known to be induced by erythritol in *B. abortus*. Thus the identification of genes induced and repressed in B. abortus responding to erythritol will give some clues to the mechanism of the growth stimulating effect and the possible involvement of erythritol in B. abortus pathogenicity.

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