CONSTRUCTION OF *BRUCELLA Abortus* STRAINS REQUIRING AROMATIC AMINO ACIDS AND MUTANT IN *PGI* GENE

BY

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CONSTRUCTION OF *BRUCELLA Abortus* STRAINS

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To my family, I'm grateful for your patience and endurance during the course of my graduate work.

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Chapter I

Background

The genus *Brucella:*

Brucella is a gram-negative coccobacillus and a facultative intracellular organism. *Brucella* was named after Sir David Bruce who, in 1887, first discovered the organism from the spleen of infected British soldiers residing in Malta. The genus *Brucella* is divided according to cultural, metabolic, and antigenic characteristics into six species: *B. melitensis, B. abortus, B.canis, B. ovis, B. suis,* and *B. neotomae.* Three of the six *Brucella* species include several biovars. *B. melitensis* has biovars 1, 2, and 3, *B. abortus* includes biovars 1, 2, 3, 4, 5, 6, and 9, and *B. suis* includes biovars 1, 2, 3, 4, and 5. The major difference among the *Brucella* species is their host preference. *B. melitensis* infects sheep and goats and it is the most common pathogen causing brucellosis in humans. *B. abortus* primarily infects cattle but it can infect buffaloes, camels, deer, dogs, horses, sheep, and man. Different biovars of *B. suis* infect different animal hosts. Biovars 1 and 3 infect swine, biovar 2 infects European wild hares, biovar 4 is responsible for infection in reindeer and wild caribou, and biovar 5 infects rodents. Except for biovar 2, all *B. suis* biovars can be transmitted to humans. *B.canis* is responsible for epididymo-orchitis in the

B.neotomae is only known to infect the desert wood rat [1]. Recently, new uncharacterized strains of *Brucella* were isolated from many marine mammals such as seals, porpoises, dolphins, and a minke whale [2]. These strains were identified as *Brucella* by conventional typing tests, but their overall characteristics, however, are not similar to those of any of the six known *Brucella* species. Therefore it was suggested that these strains be grouped under a seventh species to be named as "*Brucella maris*" [2]. Meyer and Shaw, in 1920, described some basic characteristics of the genus *Brucella*. All the species in the genus are non-motile and non-sporing occurring singly, in pairs, or in short chains. They are aerobic and do not grow under strictly anaerobic conditions. Most of the strains in the genus are carboxyphilic. Their metabolism is mainly oxidative but shows a little fermentative action on carbohydrates in conventional media. Thiamine, biotin, and nicotinamide are required, and the growth of many strains is improved by calcium pantothenate and meso-erthritol. All the *Brucella* species are catalase positive, usually oxidase positive, reduce nitrates, produce H_2S, and do not produce indole or acetyl-methyl carbinol. They do not utilize citrate and their methyl red reaction is negative. The optimum growth temperature for the bacteria in this genus is 37°C and it ranges from 20-40 °C. The optimum pH for growth is between 6.6 and 7.4. The species and the biovars within each species are differentiated on the basis of specific combinations of characteristics. These include phage lysis, CO_2 requirement, H_2S production, serological specificity, tolerance to dyes and oxidative metabolic profiles with standard substrates.

The genus *Brucella* is responsible for a serious infection, causing abortions in animals and a febrile disease known as brucellosis or Malta fever in man. The incubation

period of brucellosis is usually one to three weeks, but sometimes may be several months [28]. The illness may be mild and self-limiting or severe. It may have either a sudden or insidious onset and is accompanied by continued, intermittent, or irregular fever. The symptomatology of brucellosis is like that of many other febrile diseases, but with a marked effect on the musculoskeletal system evidenced by generalized aches and pains and associated with fatigue, prostration and mental depression [28]. Urogenital symptoms may dominate the clinical presentation in some patients. The duration of the disease can vary from a few weeks to several months and laboratory tests are needed to confirm the clinical diagnosis. Brucellosis in humans and animals is increasing in certain parts of the world, especially in developing areas of the Mediterranean Region, Middle East, western Asia and parts of Africa and Latin America. Brucellosis is transmitted to humans through direct contact with infected animals or through the consumption of unpasteurized dairy products. Cases in which *Brucella* was transmitted through inhalation were also reported and it is very common in laboratory-acquired infections. Brucellosis is treated by a combination of antibiotics for several weeks [28].

***Brucella* genome:**

The study of the *Brucella* genome started back in late 60s when Hoyer and Mc Cullough used DNA homology to discover the relationship among different *Brucella* strains [3]. The first attempt to map the *Brucella* genome was done by Altenbern who used chemical mutagenesis to induce auxotrophic mutants of *B. abortus* S19, and then used marker frequency analysis to map certain genes [4]. DNA hybridization assays showed that the *Brucella* strains share over 90% of homology [5]. This fact lead Verger et al in 1985 to

suggest that the genus *Brucella* is a monospecific genus with *B. melitensis* being the main species and the other species just being biovars [5].

The complete genome sequences of *B. suis* and *B. melitensis,* and recently *B. abortus* are now available. The genome sequence of *B. suis* strain 1330, a standard reference strain for *B. suis* biovar 1, was sequenced by the whole genome sequencing method [6]. The genome of *B. suis* 1330 consists of two circular chromosomes. Chromosome I is composed of 2,107,792 bp with 2185 ORFs, while chromosome II is composed of 1,207,381 bp with 1203 ORFs. Chromosome I resembles other bacterial circular chromosomes with the origin of replication adjacent to a gene cluster composed of *dnaA*, *dnaN*, and *recF* [7]. Chromosome II, on the other hand, contains a cluster of plasmid-like replication genes such as initiation protein *RepC* and partitioning proteins *RepA* and *RepB* [7]. Chromosome I encodes the majority of the core metabolic machinery for processes such as transcription, translation, and protein synthesis [7]. In addition to that, chromosome I encodes many phage related proteins due to inserted phage remnants. Chromosome II, on the other hand, encodes proteins responsible for membrane transport, central intermediary and energy metabolism and regulation [7]. Chromosome II possesses genes responsible for cellular processes and plasmid functions primarily because of the presence of three clusters of flagellar biosynthesis and secretion genes and the cluster of conjugation-associated and plasmid-like replication genes on Chromosome II, respectively [7]. Chromosome II also contains essential genes such as the solitary tRNA-Cys and three aminoacyl-tRNA synthetases.

When compared, the genomes of *B. suis* and *B. melitensis* show a very high level of similarity. More than 90% of *B. suis* and *B. melitensis* genes share 98-100% identity at

the nucleotide level [7]. The more variable genes share less than 95% identity and they include hypothetical genes, *UreE* urease component, and probable surface-exposed genes such as outer membrane proteins, membrane transporters, a putative invasin, and Shd-like adhesion protein [7]. These more variable genes may contribute to the differences in pathogenicity and/or host preference between the two species. Whole genome alignments revealed 42 *B. suis* and 32 *B. melitensis* genes that are completely absent in the other's genome. Thirty-three regions of greater than 100 bp unique to either *B. suis* (22 regions) or *B. melitensis* (11 regions) were identified [7]. The presence of two replicons was confirmed in all the *Brucella* species and their biovars with the exception of *B. suis* biovar 3, which has only one replicon of about 3100 kb [8]. At the proteomic level, the annotated protein data sets of *B. melitensis* and *B. suis* also contain additional differences because of frame-shifted or truncated genes and different predictions of hypothetical genes [7].

Like other *Brucella* species, *B. suis* is a member of the alpha-proteobacteria, related to other human/animal pathogens such as Bartonella spp. and more distantly to plant pathogens such as *Agrobacterium tumefaciens* and to plant symbionts such as *Sinorhizobium meliloti* [9]. Complete genome analysis of *B. suis* showed that the predicted *B. suis* proteins are, in fact, very similar to those of species in the rhizobium/agrobacterium group. [7]. A total of 1902 *B. suis* ORFs were conserved in all the three: *Mesorhizobium loti*, *S.meliloti*, and *A. tumefaciens* and 2408 *B. suis* ORFS were conserved in at least one of these three genomes [7]. Like *B. suis*, *M.loti*, *S.meliloti* and *A.tumefaciens* have multiple replicons [7]. *B. suis* Chromosome I shares regions of gene

synteny with *M.loti*. Chromosome II shares limited regions of gene synteny with *M.loti* and the linear plasmid of *A.tumefaciens* and the Sym megaplasmid of *S.meliloti*.

At the genome level, the *Brucella* species and biovars are differentiated from each other by the restriction patterns of certain genes especially genes coding for outer membrane proteins (OMPs). The major porin OMP of 36 KD is encoded in the *omp2* locus, first sequenced in *B. abortus*. [11]. The locus is composed of two genes *omp2a* and *omp2b*, separated by 900 bp and oriented in opposite directions. [11]. Only *omp2b* seems to be expressed at least in *B. abortus*. [11]. The first evidence of polymorphism at the *omp2* locus between *Brucella* species was obtained by Ficht et al [12] in 1990. He showed that all the *Brucella* species reference strains have one copy of both *omp2a* and *omp2b* with the exception of *B.ovis* which has two copies of *omp2a* and lacks the *omp2b*. [12]. Recently, it has been shown that a *Brucella* strain isolated from a marine mammal bears two copies of *omp2b* gene and lacks the *omp2a*, which is a third type of arrangement at the *omp2* locus in the genus *Brucella*. [13]. Specific oligonucleotide probes were designed to differentiate the six *Brucella* strains and their biovars by hybridization to PCR amplified *omp2* fragments [14]. *Omp2* locus can also be used to differentiate *Brucella* strains and biovars by analyzing the restriction profile, which is unique for each strain with some exceptions. Other omp genes like *omp25*, *omp31*, *omp10*, *omp16*, and *omp19* are also used to type *Brucella* strains and their biovars.

Virulence:

The genome analysis of three *Brucella* species confirmed the absence of most of the genes that code for the classical virulence factors and pathogenic islands common in other bacteria. In addition to that, the *Brucella* genome lacks a complete set of genes such

as genes encoding types I and II secretion systems, and part of the type III secretion system. Adhesion, invasion, inhibition of phagolysosomal fusion and granule release, and its ability to replicate inside the cell, are essential factors in *Brucella* virulence.

Brucella can invade a wide variety of mammalian cells including professional and nonprofessional phagocytes. Ackermann showed that M cells, macrophages, and neutrophils ingest *Brucella* by zipper-like phagocytosis [15]. Opsonized *Brucella* are internalized through complement and Fc receptors in macrophages and monocytes while non-opsonized *Brucella* penetrate by lectin or fibronectin receptors in addition to some uncharacterized receptors [16]. It was shown that the trafficking of *Brucella* inside the professional phagocytes depends on the mechanism by which the bacteria were internalized into the cell. The opsonized *Brucella* phagocytized by activated macrophages is more prone to be destroyed than the bacteria internalized by other mechanisms [17]. *Brucella* invades the non-professional phagocytes through the recruitment of microtubules and actin cytoskeleton. Treating the target cells with Cytochalasin D or nocodazole impairs the internalization of *Brucella* into the cells, indicating the need for these elements in the cell invasion process [18].

In the first five minutes after invasion, the *Brucella*-containing compartments (BCC) of both *B. abortus* and the attenuated strain 19 were found to contain the transferrin receptor, the small GTP-binding protein rab5, or the early endosomal antigen (EEA1). [19]. The presence of these markers in the BCC indicates an interaction of the *Brucella* with an intracellular compartment related to the early endosomal network. After 10 min. of internalization the number of BCCs labeled with either rab5 or EEA1 decreases significantly and no labeling is detected with these markers after 30 min. [19]. The

integrity of the early endosomal system is relevant to the subsequent normal trafficking of *B. abortus* in host cell [18]. Neither *B. abortus* strain 2308 nor the attenuated strain 19 interacts with the late endosomal network 30 minutes post-invasion [19]. The BCC is transformed gradually to a multimembranous compartment containing the lysosomal-associated membrane protein (LAMP) 1, and the endoplasmic reticulum marker sec61β, but devoid of the luminal lysosomal hydrolase cathepsin D, indicating that the *B. abortus* inhibits the fusion of its phagosome with lysosomal compartments [18]. The late BCC was identified as an autophagosome. The association of *Brucella* with the autophagic pathway is still a mystery. An interaction between the early endocytic compartments and autophagic vacuoles was detected indicating that a physical connection might exist between early BCC and autophagosomes [18].

About 24 hours postinoculation, *B. abortus* and the attenuated strain 19 take two different ways inside the cell. The strain 19 containing phagosomes retain LAMP-1 and acquire the lysosomal marker cathepsin D, indicating that the bacteria are delivered to the lysosomes [19]. On the other hand, phagosomes containing the virulent strain 2308 lose the LAMP-1 marker and never acquire any lysosomal markers [19]. The final BCCs, however, retain the sec61β labeling and acquire other markers of the endoplasmic reticulum [19]. These data as well as other experimental data suggest that the virulent *Brucella* transits from autophagosomes to the endoplasmic reticulum of the host cell where the actual bacterial multiplication occurs, and that the integrity of the host endoplasmic reticulum is essential for *Brucella* multiplication and survival inside the host cell [18].

The benefits that *Brucella* and other intracellular pathogens acquire through association with the host endoplasmic reticulum are still unknown. The intracellular trafficking of *Brucella* is almost the same inside the professional and nonprofessional phagocytes except that fewer bacteria finally reach their replication niche in the macrophages. Certain factors were found essential for the survival of the *Brucella* inside the target cells. *Brucella* outer membrane contains certain lipopolysaccharides that protect the bacteria from the bactericidal activity of the cationic peptides. Another important factor for *Brucella* virulence is that which inhibits the phagosome-lysosome fusion. This factor is not identified yet, but *B. abortus* extracts containing 5′-guanosine monophosphate and adenine were found to inhibit neutrophil degranulation [18].

Hong et al generated a pool of *B. abortus* mutants by signature tagged mutagenesis and identified the genes necessary for establishing chronic infection and those required to sustain the infection [20]. Mutants with defects in establishing an infection include those with an insertion in the *wbkA* gene, which encodes mannosyltransferase, a necessary enzyme in the O-antigen biosynthesis pathway. Mutants with insertions in either *virB1* or *virB10* regions also failed to establish an infection. *VirB* is an operon composed of twelve genes and encodes the type IV secretion system. The operon is induced by phagosome acidification in cells after phagocytosis. Type IV secretion system is essential in *Brucella* virulence since it secretes molecules that allow *Brucella* to pervert the host cell endosomal pathways and to create the intracellular compartment in which it can replicate [21]. Among mutants that could not sustain a chronic infection are those that have insertions in genes involved in metabolic activities. *B. abortus* with mutations in *gluP* (encoding glucose and galactose transporter), and *gltD* (the small subunit of glutamate

synthase) were defective in sustaining a chronic infection, indicating that glucose, galactose, or glutamate may serve as carbon and/or nitrogen source during the growth of *B. abortus* in the target cell [20].

Another important factor in *Brucella* virulence is the (BvrR/BvrS) two component regulatory system, which is essential in the invasion process. *BvrR/BvrS Brucella* mutants poorly invade target cells, fail to reach the autophagoctyic compartments, and are rapidly destroyed in lysosomes[18]. However, the external factors that activate this system as well as the downstream factors that are regulated by the *BvrR/BvrS* system are still to be identified. Identifying genes that are essential for *Brucella* virulence will allow creating null mutants that can be used as live attenuated vaccines.

Figure 1. "Schematic model of the intracellular trafficking of *B. abortus* in host cells. Virulent *B. abortus* strain 2308 as well as attenuated strain 19 interact during the first minutes after invasion with the early endocytic network of host cells, characterized by the presence of markers such as rab5 and EEA1. Brucellae are then located in a multimembranous autophagosome presenting the molecules LAMP-1 and sec61β. During the late stages of infection, attenuated strain 19 is degraded in a mature autophagosome after fusion of the bacterial-containing compartment with lysosomes (rich in cathepsin D), while virulent strain 2308 proliferates in the endoplasmic reticulum of target cells."

Reference: Pizarro-Cerda J, Moreno E, Gorvel JP. 2000. Invasion and intracellular trafficking of Brucella abortus in nonphagocytic cells. *Microbes Infect*. 2000.2:829-35.

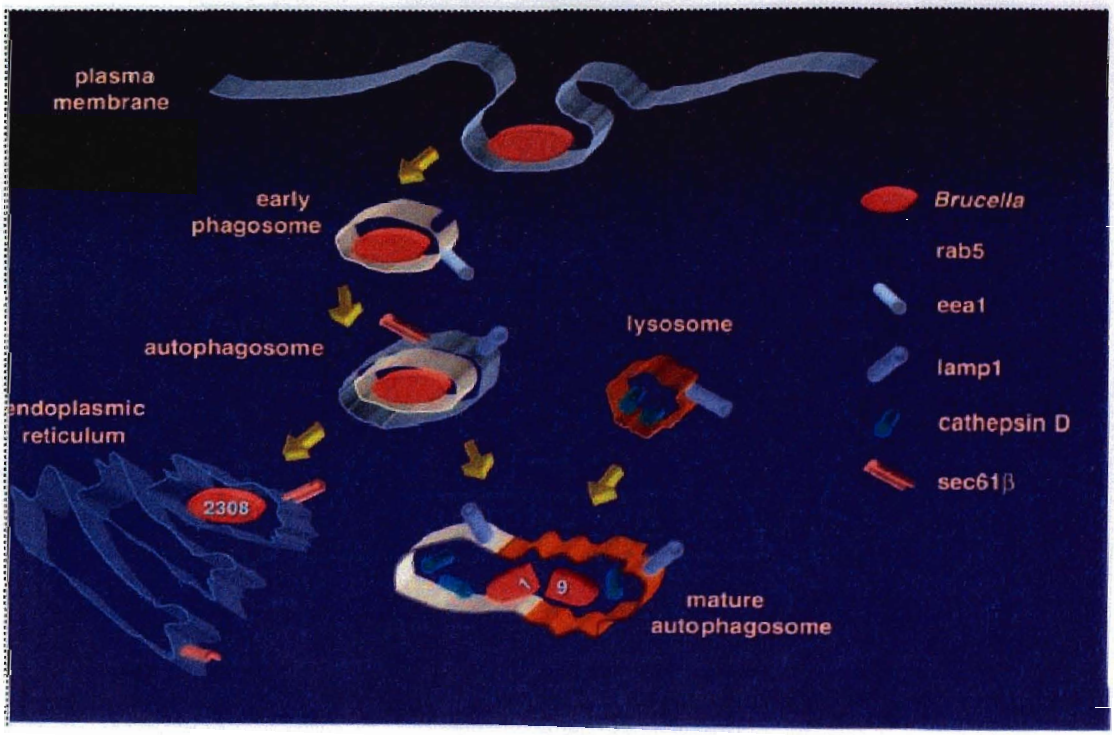
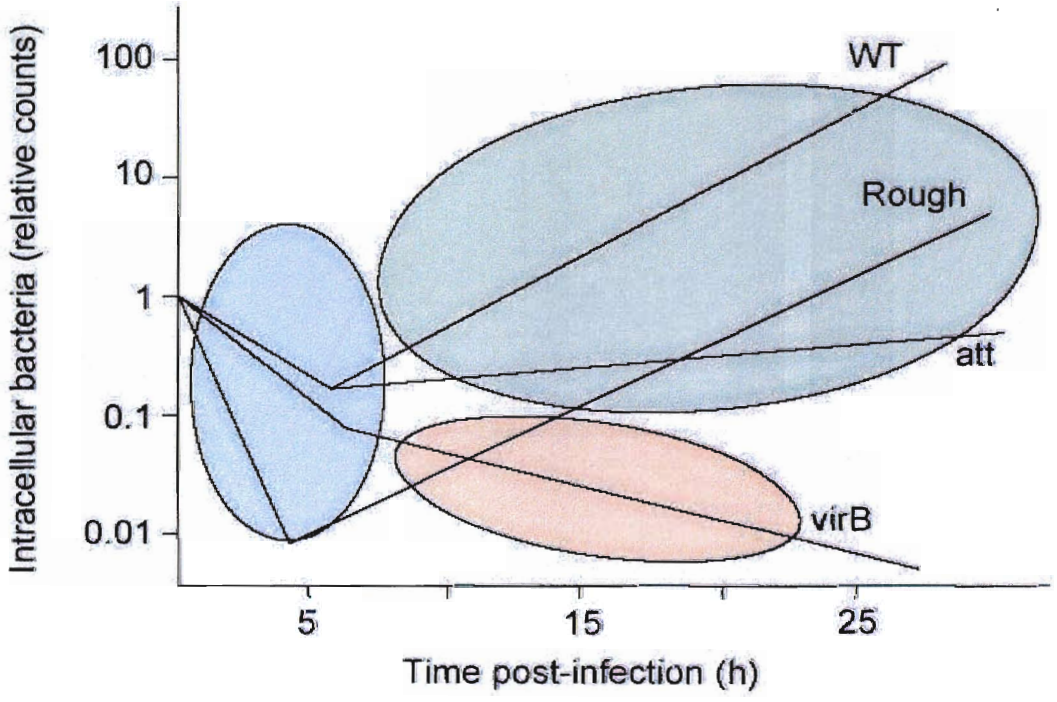

plasma membrane
early phagosome
autophagosome
lysosome
endoplasmic reticulum
2308
mature autophagosome
Brucella
rab5
eea1
lamp1
cathepsin D
sec61β

Fig. 2. "The typical intracellular growth curves of *Brucella suis* in macrophages. The different types of compartments in which the bacteria might reside during the course of infection are symbolized by coloured ovals as follows: blue, subversion of the macrophage; green, brucellosome; red, phagosome–lysosome fusion. Rough mutants decrease rapidly in number during the first hours and only a few bacteria reach a safe replicative niche, enabling initiation of intracellular multiplication. Abbreviations: att, attenuated mutant with low-level replication; Rough, rough mutants; virB, strongly attenuated *virB* mutant that is rapidly eliminated; WT, wild-type."

Reference: Kohler S, Michaux-Charachon S, Porte F, Ramuz M, Liautard JP. 2003. What is the nature of the replicative niche of a stealthy bug named Brucella? *Trends Microbiol.* 11:215-9.


Intracellular bacteria (relative counts)
100
10
1
0.1
0.01
WT
Rough
att
virB
5
15
25
Time post-infection (h)

Brucellosis Vaccines:

Extensive research has been done to find a vaccine that can limit the economic and medical losses of Brucellosis.

***B. abortus* strain 19:**

B. abortus strain 19 was the first vaccine used for Brucellosis. Strain 19 was first described in 1930. The strain was isolated in 1923 as a virulent strain from Jersey cow milk, and after being kept in the lab at room temperature for over a year, had become attenuated [22]. Strain 19 was able to induce protective immunity in cattle, but, however, vaccination of pregnant cattle can cause abortion. *B. abortus* strain 19 can't grow on media that contain erythritol. Being a smooth strain (i.e. it contains the LPS with an O-chain in its outer membrane) created a technical problem. Antibodies to these outer membrane polysaccharides appear in the serum of vaccinated animals. The production of these antibodies makes the continued use of the vaccine incompatible with the simultaneous application of test and slaughter procedures for the control of Brucellosis.

***B. melitensis Rev.1*:**

B. melitensis Rev.1 is a live attenuated vaccine derived from a *B. melitensis* strain that became dependent on streptomycin for its growth. The strain lost the need for streptomycin after further subculture but didn't lose the streptomycin resistance. It protects sheep and goats against *B. melitensis* infection, and rams against infection with *B.ovis* [22]. Rev.1 is a smooth organism; therefore it induces positive serology which interferes with the diagnosis.

B .abortus strain 45/20:

B. abortus strain 45/20 is a live attenuated rough strain derived from the smooth *B. abortus* strain 45/0 after 20 passages in guinea pigs. This strain can protect guinea pigs and cattle from *Brucella* infection [22]. It is, however, an unstable strain that can revert to a smooth phenotype, therefore it was used as a bacterin incorporated in adjuvants usually based on water and oil emulsions.

B. abortus strainRB51:

B. abortus strain RB51 is a live attenuated rough *B. abortus* strain. Strain RB51 is attenuated in mice, goats, guinea pigs, and cattle. It almost has no abortifacient characteristics, and it induces the same or better protection than the attenuated strain 19 [22].

Other vaccines:

Vaccine preparations, which include killed Brucella, were tested. With the exception of *B. abortus* strain 25/20, the application of these preparations is not practical. Production cost, poor protection, and serological interference are reasons which make using these vaccine preparations not feasible [22]. Antigenic fractions extracted from *Brucella* have been used also in a variety of adjuvants, and protection was achieved with some of these preparations. Preparations used include whole killed cells, cell envelopes, outer membrane proteins, periplasmic proteins, salt extractable proteins, modified *Brucella* proteins, and smooth and rough LPS [22].

DNA vaccines:

The basic principle of DNA vaccines involves the introduction of genes encoding protein antigens responsible for stimulating a protective immune response. The genes are cloned

in a plasmid vector that can just replicate in prokaryotes and can replicate and be expressed in eukaryotic cells [22]. There are a limited number of studies on *Brucella* DNA vaccines, and most of them were done on small animal models. *Brucella* DNA vaccines which have been tried on mice did not achieve the same level of protection that the live attenuated strains did.

Chapter II

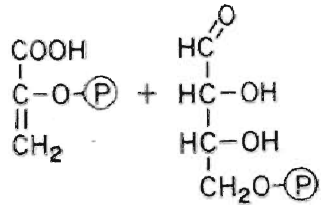
CONSTRUCTION OF *BRUCELLA Abortus* STRAINS REQUIRING AROMATIC AMINO ACIDS AND MUTANT IN *PGI* GENE

Introduction:

The aromatic amino acids phenylalanine, tyrosine, and tryptophan are synthesized in prokaryotes, yeasts and filamentous fungi, apicomplexan parasites, and the plastids of plants and algae. As essential amino acids, phenylalanine and tryptophan are required ingredients in the diet of animals, who can synthesize tyrosine by hydroxylation of phenylalanine. Chorismic acid is a branching point in the aro pathway from which separate pathways lead to individual aromatic amino acids, and to *para*-aminobenzoic acid and hence folic acid. Bacteria aro mutants are auxotrophic for the aromatic amino acids, *para*-aminobenzoic acid, and 2,3 dihydroxybenzoic acid. Some of these compounds are not available at sufficient levels in the host mammalian cell to sustain growth of the aro mutated intracellular bacteria leading to attenuation. In 1981 Hoiseth and Stocker showed that *Salmonella enterica* serovar Typhimirium *aroA* (EPSP synthase) mutant was both attenuated and an excellent live vaccine in the mouse typhoid model [23]. Hong et al. defined a *Brucella* mutant that was auxotrophic for aromatic amino acids and at the same time could not establish a chronic infection. The major aim behind

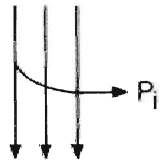
[23]. Hong et al. defined a *Brucella* mutant that was auxotrophic for aromatic amino acids and at the same time could not establish a chronic infection. The major aim behind my research was to construct *Brucella* mutants that are deficient in *aroA* and *aroBE* genes, which are involved in the aromatic amino acids pathway. Fig. 3 shows the role of these genes in the aromatic amino acid biosynthesis pathway. Another goal of this research was to construct *pgi Brucella* mutant. *Pgi* codes for a phosphoglucoisomerase, an enzyme that catalyzes the reaction: fructose-6-phosphate to glucose-6-phosphate. *B. suis* that carries a mutated *Pgi* gene was found to be attenuated [29]. The importance of the *Pgi* gene for the viability of *Brucella* is not yet known. However, *pgi* gene plays an important role in glucose metabolism in *Brucella*. *Brucella* metabolizes glucose through the pentose phosphate pathway. Since *Brucella* lacks the enzyme phosphofructokinase, it cannot utilize fructose-6-phosphate. The *Pgi* gene product therefore is important for *Brucella* since it enables the organism to recycle fructose-6-phophate to glucose-6-phosphate [30].

The ultimate goal is to study the survival of these *Brucella* constructs in macrophage cell culture and in animal model.

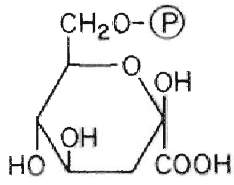


Phosphoenol pyruvate + erythrose 4-phosphate

aroF
aroG
aroH



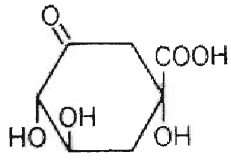
3-Deoxy-D-*arabino*-heptulosonate 7-phosphate synthase (EC 4.1.2.15)



3-Deoxy-D-*arabino*-heptulosonate 7-phosphate

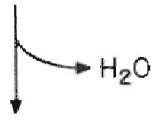
aroB

3-Dehydroquinate synthase (EC 4.6.1.3)

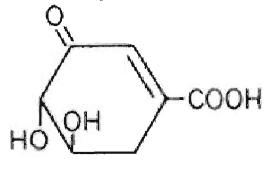


3-Dehydroquinate

aroD

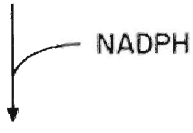


3-Dehydroquinate dehydratase (EC 4.2.1.10)



3-Dehydroshikimate

aroE



Shikimate dehydrogenase (EC 1.1.1.25)

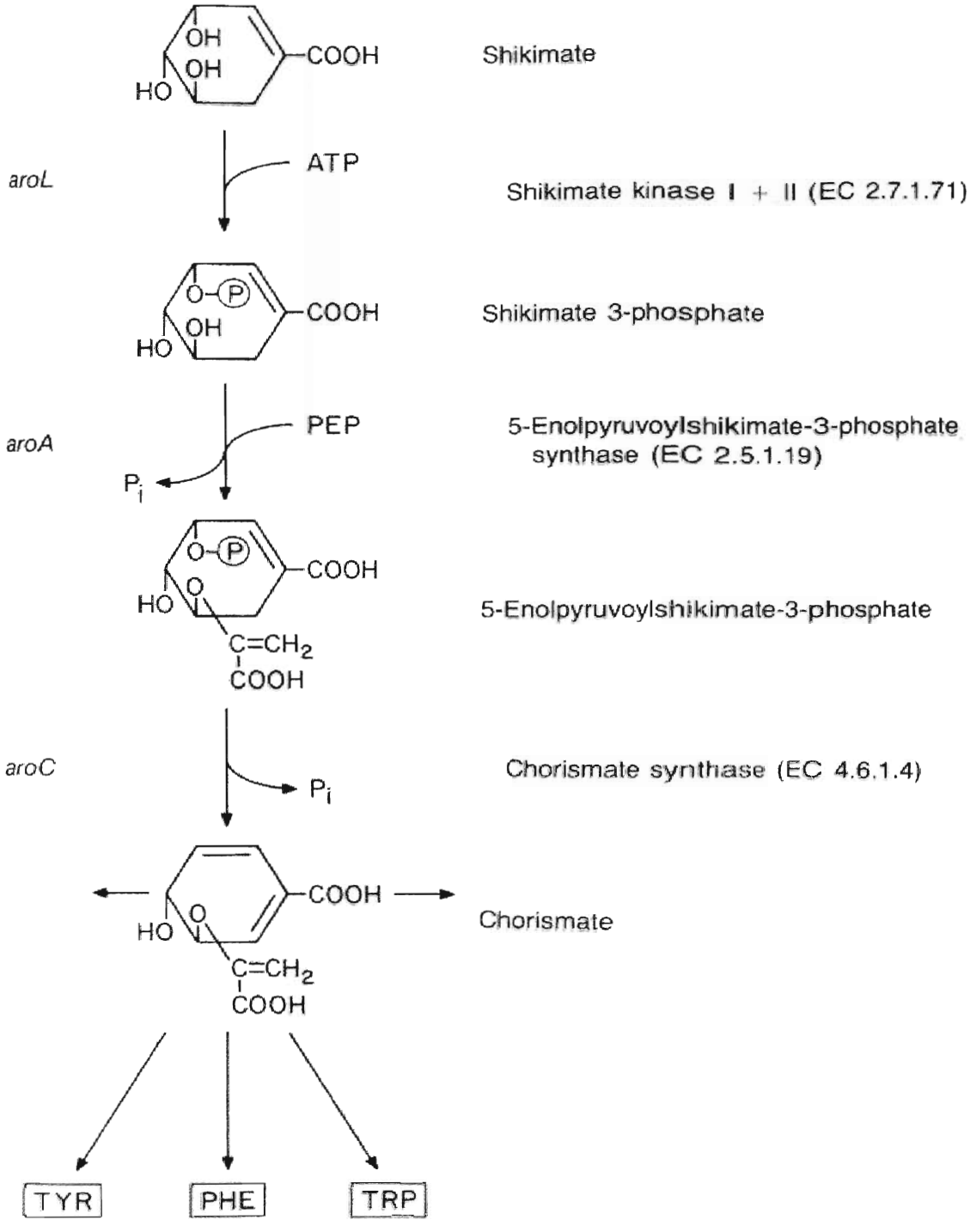


Fig.3.The shikimate pathway

Chapter III

Materials and Methods

Bacterial strains, plasmids, media, and growth conditions:

Virulent *B.abortus* strain 2308 was obtained from the Oklahoma Animal Disease Diagnostic Laboratory. *E.coli* strains were obtained from Dr. Richard Essenberg's lab stocks and they are listed in Table 1.

Plasmids were obtained from *B.abortus* genomic library that was prepared by S. Kay Nida and they are listed in Table 2. The media used to grow *E.coli* and *B.abortus* are listed in Table 3. *E. coli* strains were routinely cultured at 37°C overnight. *Brucella* cells are grown in a BL3 laboratory at 37°C under an atmosphere containing 5% CO_2 for 4 to 6 days. For *E. coli* strains, 50 μg/ml ampicillin, 30 μg/ml kanamycin and 30 μg/ml chloramphenicol were added as required, while *Brucella* strains required 100 μg/ml ampicillin, 60 μg/ml kanamycin and 30 μg/ml chloramphenicol.

Preparation and manipulation of DNA

Plasmid DNA was purified by the QIAprep Spin Miniprep Kit Protocol (Qiagen). Restriction endonucleases were purchased from Gibco BRL. *T4* DNA ligase was purchased from Promega, and ELONGASE Enzyme Mix were purchased from

unless noted otherwise. Digested plasmids were separated by electrophoresis on 0.8% low melting agarose gels and extracted by the QIAquick Gel Extraction Kit Protocol (Qiagen). Competent *E. coli* cells were made and transformed (Heat-Shock) by the method of Inoue et al., 1990. Standard techniques were used for electrophoresis and molecular cloning.

Crossover PCR:

The protocol for the PCR was taken from ref. [25]. The primers used are listed in table 4. Platinum pfx DNA polymerase (Invitrogen) was used. The PCR cycles were as follows: denature: 94°C for 15 seconds, anneal: 55°C for 30 s, extend: 68°C for 1 min per Kb. 1X of the supplied enhancer was used in the reaction mixture.

Preparation of competent *Brucella* cells

Wild type *Brucella* cells (2308) were grown in 5ml YENB (Yeast Extract and Nutrient Broth) media for 33 h at 37°C with shaking. Then 2.5 ml of the overnight culture was added to 50 ml of fresh YENB medium and was placed in a 37°C shaker until the Klett reading was between 70-80. The cells were harvested by chilling the flask on ice for 5 min and spinning the cells at 4000 x g for 10 min at 4°C. The medium was removed and discarded, and the pellet was washed twice by resuspending in 5 ml of cold water and centrifuging at 4000 x g for 10 min at 4°C. The supernatant was again removed and discarded. The pellet was resuspended in 1 ml of cold 10% glycerol and centrifuged, and the supernatant removed and discarded as before. The cells were resuspended to a final volume 200-300 µl of cold 10% glycerol (Sharma and Schimke, 1996).

Electroporation of suicide plasmids into *B. abortus*

50 µl of competent *Brucella* cells were placed in a microfuge tube and 1.5-2.1 µg of DNA was added. This was mixed and placed on ice for 1 min. The cells and DNA were transferred to a 0.1 cm electroporation cuvette. The cuvette was put in its holder and was attached to the power unit. The voltage was set to 1250 V and the resistance to 200 Ω, and then it was pulsed. The cuvette was immediately removed and the cells washed out with 1 ml SOC-B and placed in a sterile microfuge tube. The microfuge tubes were open and incubated at 37°C, 5% CO_2 for 5 minutes, then closed and incubated for another one hour. Then the cells were sedimented for 5 min in a Millipore microfuge. The supernatant was discarded in a disinfectant. The pellet was resuspended in 0.2 ml SOC-B and plated on 2 SOC-B plates (0. 1ml each) with no antibiotic. The plates were incubated overnight at 37°C, 5% CO_2. The next day the growth was removed and resuspended in 0.2 ml tryptose. Cells were spread on 2-4 tryptose plates with selective antibiotics and incubated at 37°C, 5% CO_2 for 7-10 days (Lai et al., 1990).

Killing *B. abortus* Cells with Methanol

Before *B. abortus* cells can be removed from the BL3 lab for DNA extraction, the cells must be killed. First the cells were grown in 3 ml of tryptose broth overnight at 37°C. Next the cells are transferred to a sterile microfuge tube and are centrifuged for 5 min, the supernatant was removed and discarded into a disinfectant. The pellet was resuspended in 0.1-0.2 ml tryptose broth, and 1-2 ml of methanol was added. This was incubated at room temperature for 5 min and then the cells were centrifuged for 5 min, the supernatant was removed and decontaminated. Again, the cells were resuspended in

0.1-0.2 ml tryptose broth and 1-2 ml of methanol was added. Then the cells were again incubated at room temperature for 5 min and were centrifuged for 5 min, and the supernatant was removed and decontaminated. The cells were resuspended in 0.1-0.2 ml tryptose broth and the viability of killed cells was checked by spreading 10 µl on tryptose plates and growing for 2 days at 37°C, 5% CO_2.

Genomic DNA Extraction of *B. abortus*

Once the cells were killed (no growth on the tryptose plates after the second day) the genomic DNA from *B. abortus* was extracted as described except that the proteinase K digestion was carried out overnight at 37° instead of 1 hr (Wilson, 1994).

Southern blot

A Southern Blot was performed on the genomic DNA of *B. abortus* to determine if the samples actually had the *aroD* or *aroBE* genes present and to detect if the *sac*B-kanamycin cassette was also present. The transfer of DNA to the membrane was followed as described in the S&S TurboBlotter and Blotting Assembly for Alkaline and Neutral Transfer. The probe was labeled with photobiotin (Forester et al., 1985) and detected by the Southern-Light & Southern-Star Chemiluminescent Detection System (Tropix).

Table 1
Bacterial strains used in the study

Strain	Genotype
B.abortus 2308	Wild type
E.coli XL1-Blue	*recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17*, *supE44*, *relA1*, *lac*
E.coli strain RE 729	*Tsx-356*, *supE42*, λ-, *aroD352*, *gyrA*.

Table 2
Plasmids used in the study

Plasmid	Description
PRE523	*Brucella aroD*
pRE525	*Brucella aroBE*
pRE167 A10	*Brucella aroA*
pRE171	*Brucella pgi*
pUM24	Kan-sac cassette

Table 3
Media used in the study

Medium	Contents
LM	Tryptone, yeast extract, NaCl, $MgSO_4.7H_2O$
5xA	K_2HPO_4, KH_2PO_4, $(NH_4)_2SO_4$, $Na_3Citrate.2H_2O$
E.coli minimal medium	5xA, glucose, $MgSO_4.7H_2O$
Brucella SOC	Tryptic soy broth, NaCl, $MgCl_2$, $MgSO_4.7H_2O$, glucose
5x *Brucella*	NaCl, K_2HPO_4, sodium thiosulfate, $(NH_4)_2SO_4$
500x *Brucella*	Vitamin B1, nicotinic acid, Pantothenic acid, biotin.
Brucell minimal medium	5x, 500x, Erythritol, $MgSO_4.7H_2O$

Table 4. Primers used in crossover PCR. Bases written in lower case are the tail and the ones with the upper case match sequences in the target genes.

Primer	Sequence
AroA Co	cgcacgcatgtcgacGCATGGGCGGAAAGCAC
AroA Ci	gttctgcagcggccgcgaattccgCTCGAAGCTGAAAGGCGTTGT
AroA No	aaaaactgcagGAAATGCCGGACAAGTGGTTA
AroA Ni	cggaattcgcggccgctgcagaacGCCCGTAAGTGCCTGCGAATG
GluP Co	cgcacgcatgtcgacGCGCACACAGACCTCCTC
GluP Ci	gttctgcagcggccgcgaattccgGTACATTATGCCCGTCAACCA
GluP No	aaaaactgcagGAAGCGGGAAAGCACGGTTGA
GluP Ni	cggaattcgcggccgctgcagaacGCCCAGTCCGAAGCCCTGAT

Chapter IV

Results

1-Mutating the cloned Brucella *aroD* by inserting Kan-Sac cassette:

Plasmid pRE523 which contains the *aroD* gene, contains also another gene which encodes acetyl CoA carboxylase subunit. The two genes overlap with each other. *ClaI* cuts in two sites in the plasmid pRE523, one of them in the *aroD* gene (fig4). pRE523 was partially digested with *ClaI*. The ends were blunted, and were ligated to a blunt ended Kan-Sac cassette. The ligation mixture was electroporated to *E.coli* and the bacteria were cultured on kanamycin LM. The plasmid DNA was extracted from bacterial colonies by Qiagen protocol. In order to choose the desired plasmid (which has the kan-sac cassette only in the *aroD* gene), a digestion with *EcoRV* was carried out, and the DNA fragments were analyzed (fig 5). If *ClaI* cuts only once and in the *aroD* gene, the *EcoRV* restriction pattern should show a piece of about 300 bp, and another one of about 2800 (Table 5). Only one colony showed this pattern. (Lane 10 in fig 5). The plasmid was then electroporated into *E.coli* strain RE729 which is a Δ*aroD*, and the cells were cultured on a minimal media with and without aromatic amino acids. The bacteria

acids but could grow on aromatic amino acids containing minimal media, suggesting that the *aroD* gene was mutated. The wild type plasmid pRE523 was used as a control (it could complement *E.coli* RE729). Table 5 shows the different possible *EcoRV* predicted restriction fragments of different clones that might result from kan-sac insertion in different *ClaI* sites. The orientation of the kan-sac cassette was also considered. There are four positions of kan-sac insertion that can produce fragments of about 2.77 Kb and 0.33 Kb with *EcoRV* digestion (listed in Table 5). Among those clones, only the one that got the insertion in the 836 position (*aroD*) will fail to complement the *E.coli* RE729 strain. Further characterization was made using *PstI* (fig.6). Kan-sac cassette has two *PstI* sites, one at each end. The distance from *PstI* site in the multiple cloning site (MCS) to the *PstI* site at either end of the kan-sac cassette can locate the cassette insertion regardless of its orientation. If the cassette was inserted in coding region of *aroD* (*ClaI* site 836), a band of about 800 bp should appear. Rather, a band of about 1300 bp was obtained indicating that the insertion site was actually outside the coding region of *aroD* (at *ClaI* site 401) contradicting the combined *EcoRV* and complementation results.

2-Construction of *aroA*, and *pgi* deletion mutants by crossover PCR:

The deletion by crossover PCR is a two-step process and involves the use of four primers (fig.7). The tails attached to the primers No, and Co carry *PstI* and *SalI* sites respectively. The tails of the primers Ni and Ci are complementary to each other in a way that allows them to anneal to each other, and they carry a *NotI* site. In the first step, a PCR for both N terminal and C terminal of the gene are carried out separately using the No-Ni, and Co-Ci primer pairs respectively.

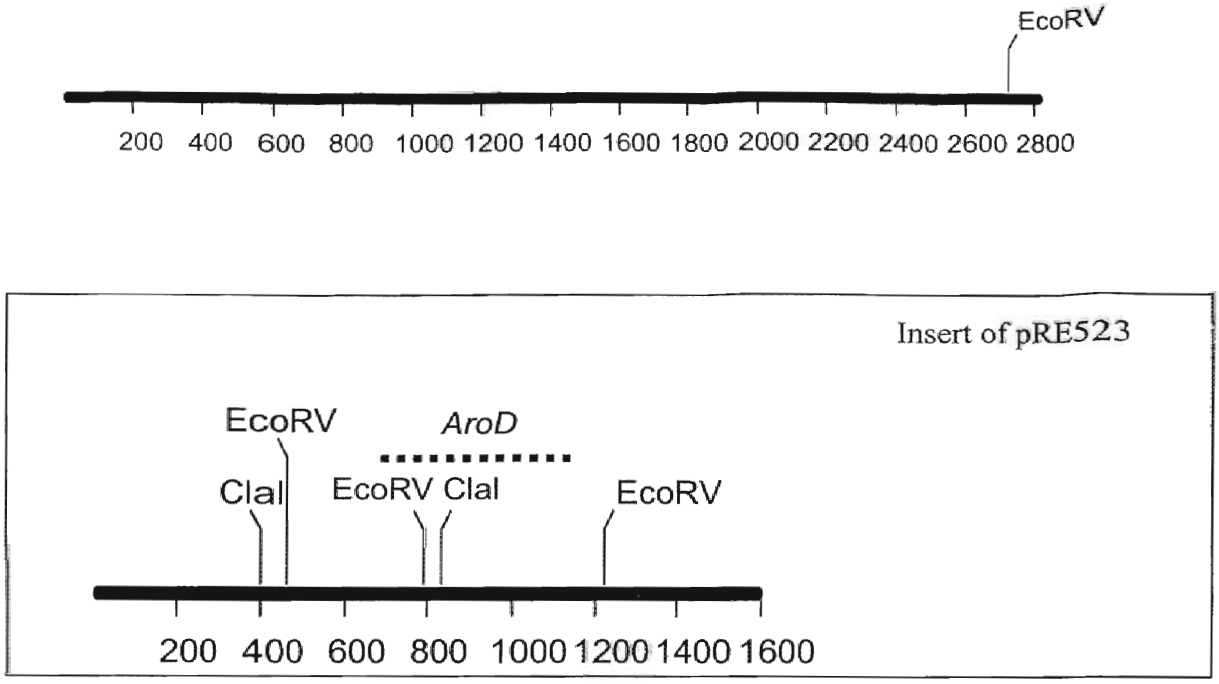


Fig. 4. Restriction maps of the Kan-sac cassette and the *Brucella* insert of plasmid pRE523 containing *aroD*

Table.5. The *EcoRV* predicted restriction patterns for different *aroD* and kan-sac clones. The kan-sac cassette was inserted into plasmid pRE523 after partial digestion with *ClaI*. The *ClaI* cuts twice in the *aroD*: once in 401 and the other is in 836 (within the *aroD* coding region fig.4). *ClaI* also cuts in the multiple cloning site of the plasmid (pSK). The *aroD* is oriented in a way that makes the beginning of the gene close to the T7 promotor. The orientation of the kan-sac cassette is expressed by (+) if the beginning of the cassette was from the T3 side and by (–) if the insertion was in the opposite direction. *EcoRV* has three sites in *aroD*: 457, 789, 1225, one site in the Kan-sac: 2723, and one site in the MCS (between the *ClaI* site and the *aroD* gene).

Position of Kan-Sac	Orientation of Kan-sac	Fragments in bp	Predicted ability to complement *E.coli* RE729
836	+	470, 332, 150, 3112, 3383	–
836	–	470, 332, 2770, 490, 3382	–
401	+	501, 2779, 332, 436, 3382	+
401	–	3124, 150, 332, 436, 3382	+
ClaI in MCS	+	2723, 457, 332, 436, 3483	+
ClaI in MCS	–	101, 457, 332, 436, 6105	+
401-836 fragment removed	+	502, 3112, 3382	–
401-836 fragment removed	–	3124, 490, 3382	–
From MCS- 401 removed	+	2779, 332, 436, 3483	+
From MCS- 401 removed	–	150, 332, 436, 6105	+
From MCS- 836 removed	+	3312, 3483	–
From MCS- 836 removed	–	490, 6105	–

Table 5

Fig.5. *EcoRV* restriction patterns for different *aroD* and Kan-sac clones.
The DNA ladder is 1Kb plus ladder.

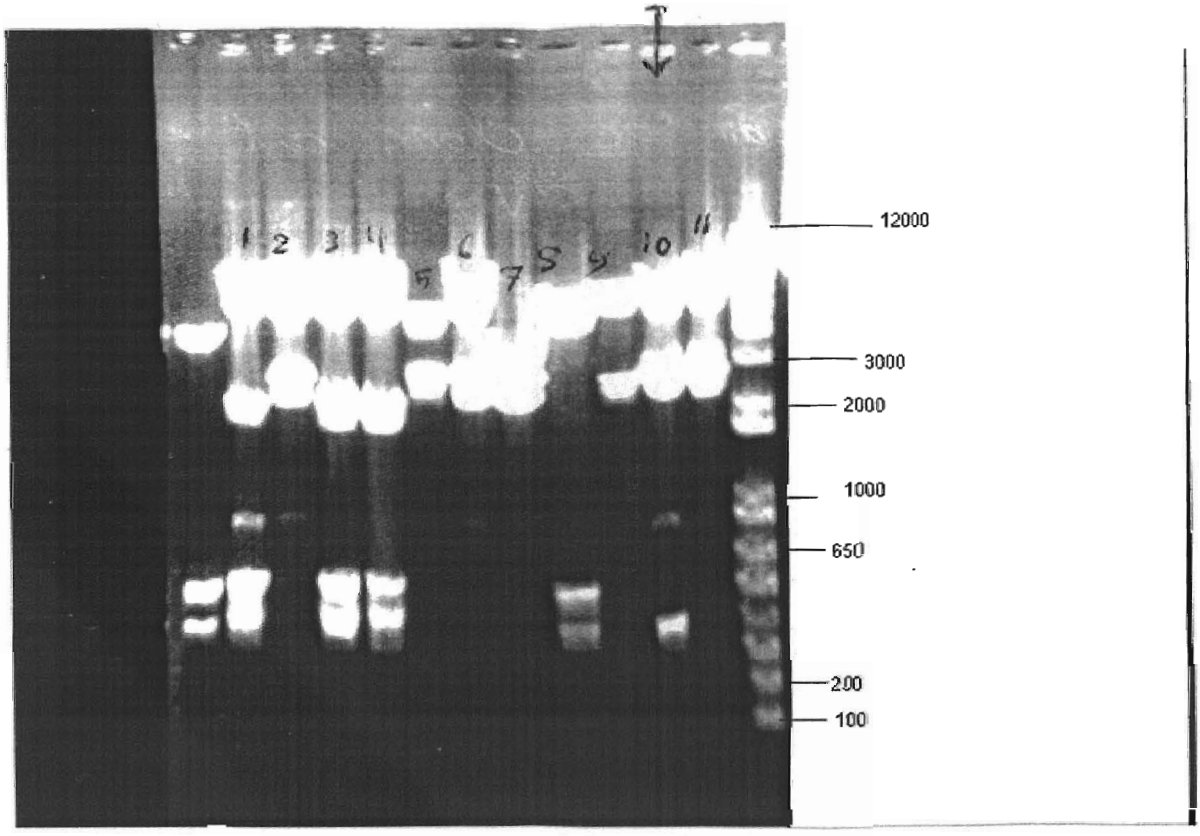


Fig.5.

Fig.6. The *PstI* restriction pattern for the selected *aroD* clone.

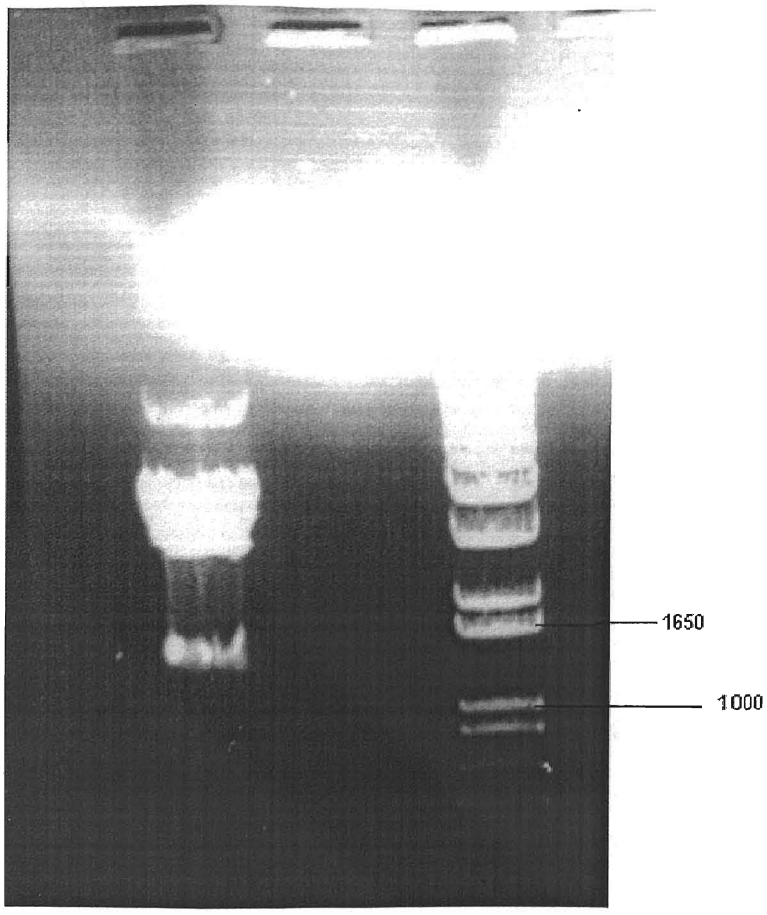


Fig.6.

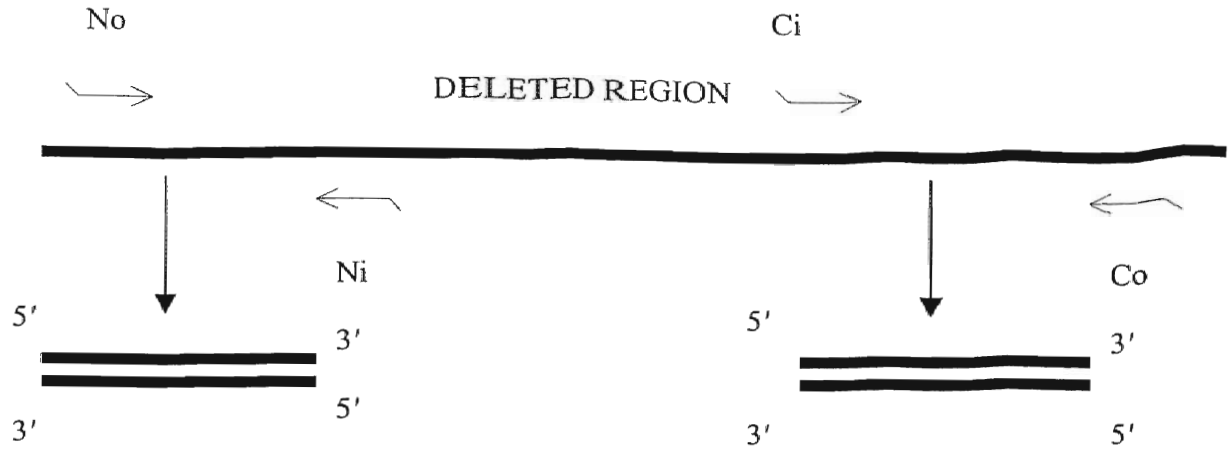
The gel in fig. 8 shows the bands which correspond to the *aroA* N terminal (left lane), and the *pgi* N terminal (right lane). The gel in fig.9 shows the bands that correspond to the C parts of *aroA* (the last three right lane), and *pgi* (the first two left lanes). In this round of PCR bands of the expected sizes were obtained. In the second round of PCR, 1 µl of both the previous PCR mixtures was used as a template, and the primer pair Co-No was used. Fig. 7 shows that the DNA synthesis starts from the complementary tails and proceeds to give the full fragment (N terminal plus C terminal with the deletion). From that point, the primer pair Co-No will amplify this fragment (N terminal plus C terminal). Fig. 10 shows the *pgi* second step PCR. I obtained the bands of the expected size (2.5 Kb) plus a band of about 1 Kb. Changing the reaction conditions did not remove the interfering band. Fig.11 shows the *aroA* second step PCR and the bands are of the expected size (about 1000 bp). The PCR products were then cloned into the PCR vector pCR2.1 TOPO of the TA cloning kit (Invitrogen). Ligation mixtures were transferred into the provided cells and the plasmid DNA was extracted. Plasmids were analyzed using the restriction enzymes *ApaI* and *SacI* and they were found to contain the PCR products.

3- Electroporation of mutated genes *aroBE* and *aroD* into *B. abortus* 2308:

aroBE was cloned and mutated by insertion of kan-sac cassette within the coding sequence (work done before in the lab). Kan-sac cassette contains the kanamycin resistance gene and the *Bacillus subtilis sacB* gene. The *sacB* gene encodes levansucrase, an enzyme involved in levan synthesis and sucrose hydrolysis [26]. Production of levansucrase in many gram-negative bacteria was found to be lethal in the presence of 5% sucrose.

Homologous recombination was used in an attempt to replace the endogenous chromosomal copy of the *aroD*, and *aroBE* genes with an inactivated copy of these genes. The plasmid pRE525 that carries the mutated genes *aroBE* was electroporated into *Brucella* and wild type strain was use as a control. The cells were plated on tryptose agar for one day and then were plated on selective plates with kanamycin for 4-7 days. The colonies were then tested for the double crossover by plating on sucrose-kanamycin plates, kanamycin plates, and ampicillin plates (all tryptose plates). The cells that had a double crossover should not grow on plates with ampicillin or on plates with sucrose but should grow on plates with kanamycin only. Almost all the cells did not grow on ampicillin plates. Two colonies from each plate were not able to grow with sucrose. These colonies were picked and plated on a minimal medium with and without aromatic amino acids. All the colonies were able to grow on minimal medium lacking the aromatic amino acids mix. It is worthy to note that these colonies were able to grow on sucrose plates when re-plated. The genomic DNA was extracted from these colonies after killing them with methanol. The genomic DNA was digested by *EcoRV* and was hybridized against plasmid pMU24 that contains the kan-sac cassette. No bands were detected from the mutated bacteria.

Step: 1



Step: 2

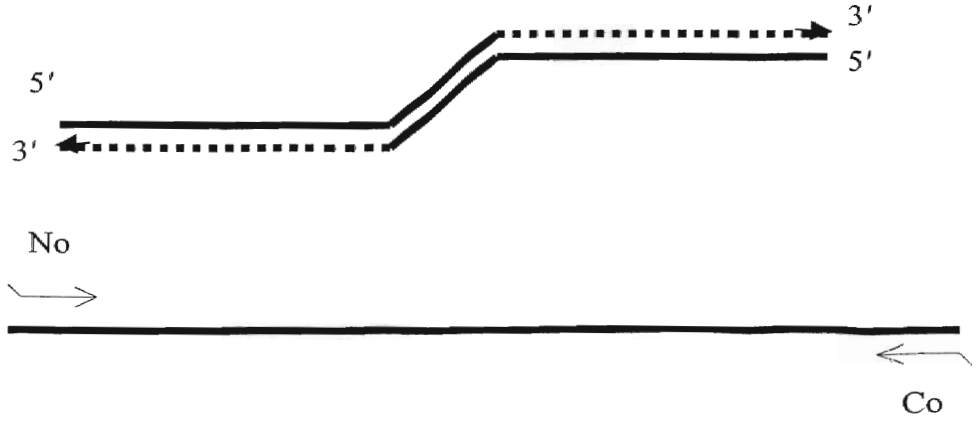


Fig.7. The crossover PCR

Fig.8. The crossover PCR for the N terminal of the *aroA* gene (left lane), and *pgi* gene (right lane). The bands are of the expected sizes. The DNA ladder is 1kb plus ladder

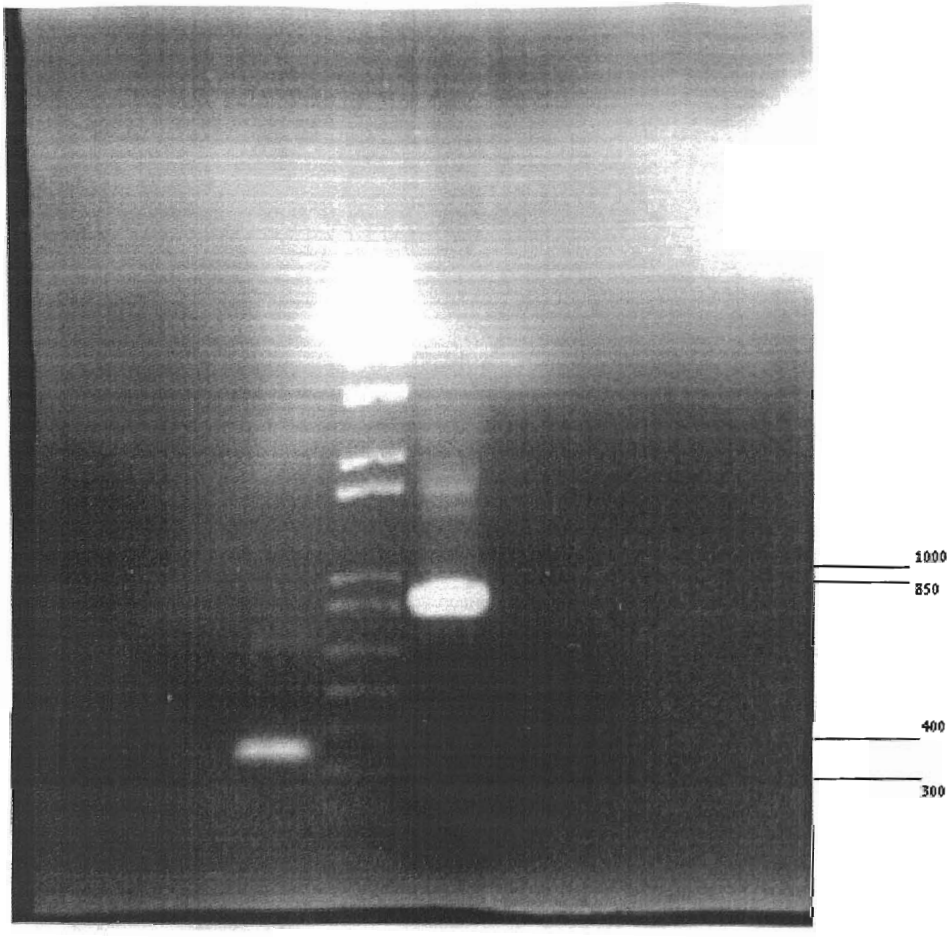


Fig.8.

Fig.9. The crossover PCR for the C terminal of the *aroA* gene (the three lanes to the right of the ladder), and the *pgi* gene (the two lanes to the left of the ladder). The bands are of the expected sizes. The DNA ladder is 1kb plus ladder

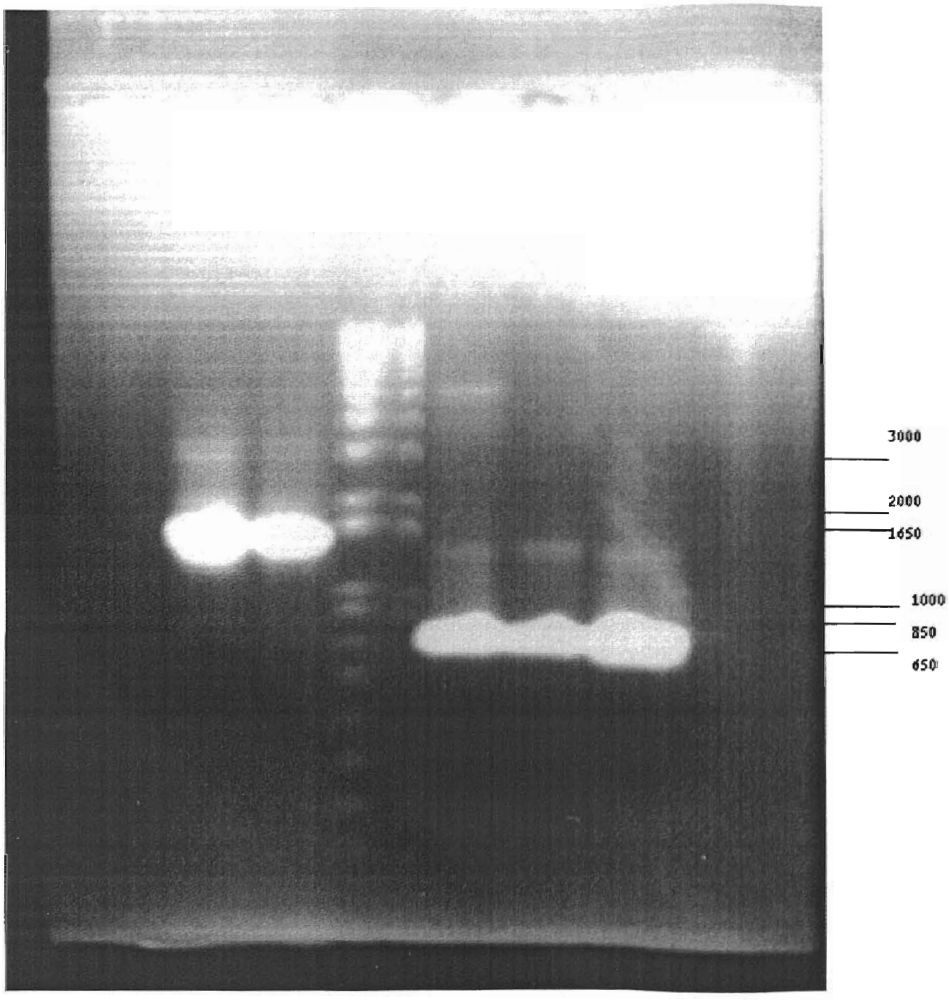


Fig.9.

Fig.10. The second step of the crossover PCR for the *pgi* gene. The size of the bands should be around 2500 bp. Smaller bands can be also seen. The DNA ladder is 1kb plus ladder.

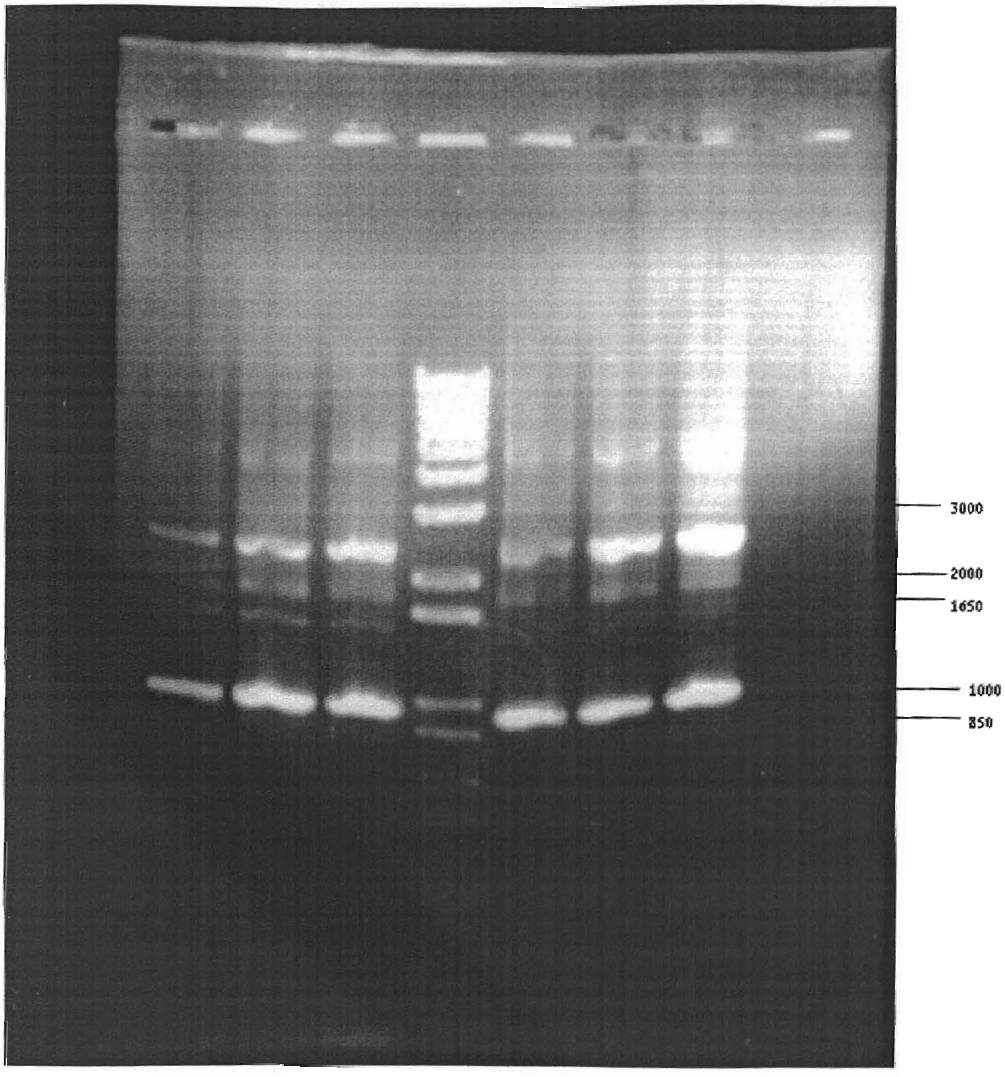


Fig.10.

Fig 11. The second step in the crossover PCR for the *aroA* gene. The bands were of the expected sizes. The DNA ladder is 1kb plus ladder.

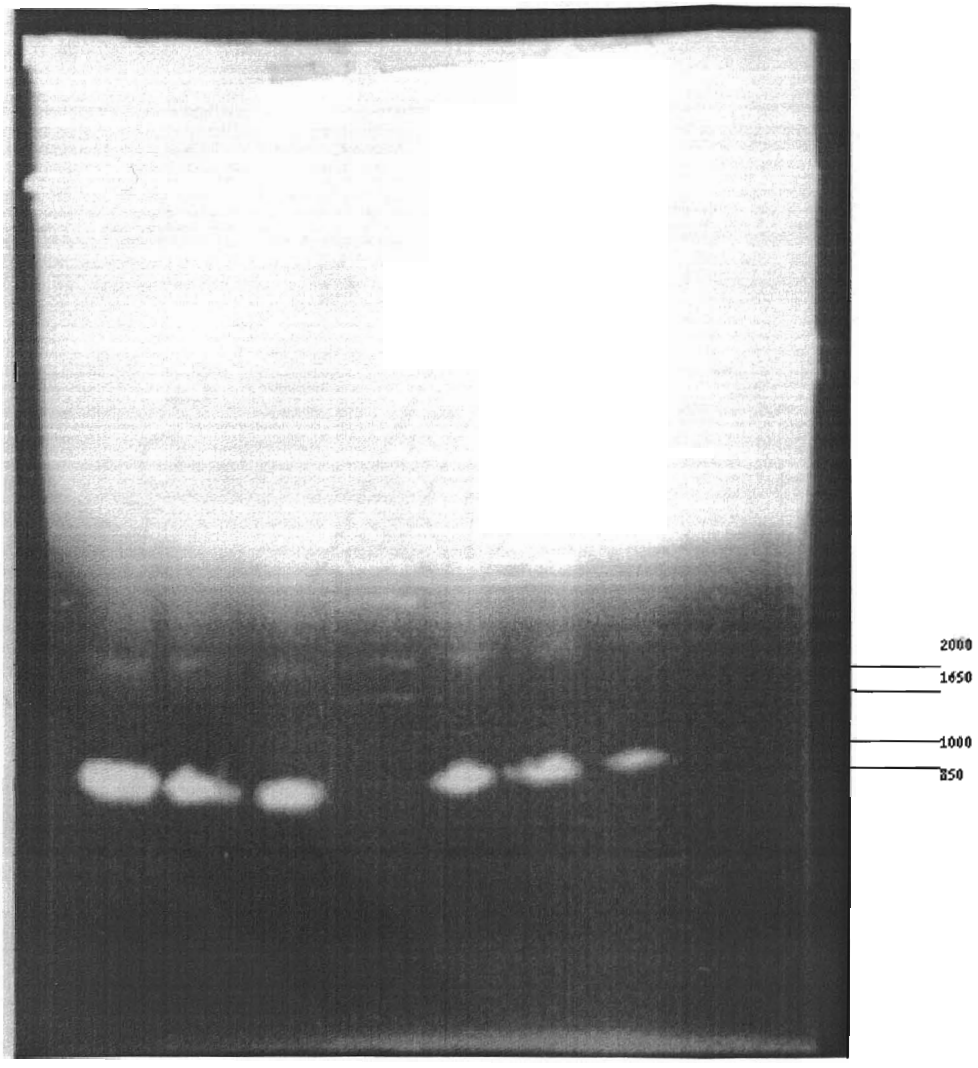


Fig.11.

Chapter V

Discussion

Knocking out the aromatic amino acid pathway attenuated many intracellular bacterial pathogens such as *Salmonella typhimirium* and *Yersinia.* In addition to that, Hong et al reported that a *B.abortus* mutant that couldn't establish an infection was auxotrophic for aromatic amino acids [20]. These findings suggest that the aromatic amino acid pathway is crucial for the survival of the intracellular bacteria inside the host cells. This importance may come from the fact that the aro pathway supplies the bacteria with nutrients that are not available in the animal eukaryotic host cells. These nutrients include phenylalanine, tryptophan, *para*-aminobenzoic acid, and folic acid. Mutant bacteria are constructed by homologous recombination, which replaces the genome copy of the target gene by another mutated copy carried on a suicide vector that cannot replicate in the target cells. Two markers are at least required for the detection of the double crossover bacteria. In the case of *aroD*, and *aroBE* genes, a kan-*sac* cassette was inserted in the coding region of these genes and an ampicillin resistance gene was carried on the plasmid. That will allow the detection of the double crossover (loss of both the wild type genome copy of the gene and the plasmid) by one step. The ability of the cells to grow on minimal media even though they could grow on kanamycin is puzzling since *B.abortus*

Another thing that might be tried is to transfer the plasmid via bacterial mating. To do this, a nalidixic acid *Brucella* strain should be used, and the genes should be cloned in pSK out plasmid, a vector that has the mobilizing and transferring genes [27]. Using this method may help since mating is actually quite a bit more efficient than electroporation. Since the *aroD* gene overlaps with acetyl CoA carboxylase subunit, it would be important to try to mutate *aroD* without affecting the translation of the acetyl CoA carboxylase subunit (polar effect). The same problem was faced before with *aroA* gene, which overlaps with CMP kinase. Mutants in which both *aroA* and CMP kinase are interrupted were not viable. To avoid this I tried to make a deletion of *aroA* by inverse PCR. In inverse PCR mutagenesis, primers are designed so that they go out of the deleted region. The deleted region should be divisible by 3 to avoid the polar effect. Then the PCR products were cloned into a pBluescript vector. The PCR product was then sequenced using T3 and T7 primers. Unfortunately, the sequence obtained did not match the expected sequence. Then the *aroA* gene was deleted by crossover PCR. Like the inverse PCR, the primers were designed in a way that deletes a region that is divisible by 3. The future research on this topic may figure out ways to obtain the required *Brucella* mutants and to study the viability of these mutants inside a macrophage cell culture or a live animal model.

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