IDENTIFICATION OF A CHROMOSOMAL REGION POSSIBLY INVOLVED IN 0-SIDE CHAIN BIOSYNTHESIS IN *BRUCELLA ABORTUS*

by

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(ABSTRACT)

The gram-negative bacterial pathogen *Brucella abortus* is a zoonotic pathogen causing brucellosis in a variety of animal species including humans. The loss of the O-side chain in the lipopolysaccharide of the outer membrane decreases *Brucella* virulence. To understand the genetics of O-side chain biosynthesis and its relationship to virulence, studies were initiated to characterize specific O-side chain mutants. *B. abortus* rough mutant strain RA2 was derived by transposon (Tn5) mutagenesis of smooth *B. abortus* 2308. The chromosomal region of strain RA2 with the Tn5 and flanking chromosomal region was cloned into the sequencing vector pGEM-3Z to create a suicide plasmid pNW-2. The plasmid pNW-2, or a derivative of it (pNW-3), in which Tn5 was replaced with a KanR gene, were electroporated into wild type smooth *B. abortus* 2308 in order to assess the phenotypic conversion from smooth to rough. The electroporation parameters such as cell growth stage, pulse field strength and pulse length were optimized. It was determined that using late log phase cells (approximately 70-77 Klett units), 10 ms and 13 KV/cm were the

best conditions for achieving transformation by pNW-2 or pNW-3. Kanamycin resistant and ampicillin sensitive *Brucella* were screened for double reciprocal crossovers between the suicide plasmids (pNW-2 and pNW-3) and *Brucella* chromosomal DNA. The recombinants were checked for their O-side chain by crystal violet uptake and immunoblotting with monoclonal antibody specific for the O-side chain. The locations of Tn5 and the flanking region in the genome of these recombinants were characterized by Southern blot using either a Tn5 probe or a flanking region probe. An analysis of KanR colonies showed that none of the recombinants were rough. The *B. abortus* DNA in pNW-2 was sequenced and compared with other genes. No significant homology was found between the *Brucella* DNA in pNW-2 and gene sequences in the gene bank. Analysis of the recombinants suggests no linkage between the Tn5 element in strain RA2 and the rough phenotype.

This thesis is dedicated to

my parents, Hai and Qing,

my sisters, Ting and Jun,

my husband, Jie

with love and gratitude

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List of Abbreviation

Amp= ampicillin

AmpR= ampicillin resistant

bp= base pair

Bru38= monoclonal anti O-side chain antibody

Bru48= monoclonal anti core antibody

Bluo-Gal= halogenated indolyl-β-D-galactoside

CFU= colony forming units

CIP= calf intestinal alkaline phosphatase

CTAB= hexadecyl-trimethylammonium bromide

dH_2O= distilled water

Kan= kanamycin

KanR= kanamycin resistant

KV/cm= kirovolt per centimeter

LB= Luria-Bertani media

LPS= lipopolysaccharide

ms= milisecond

dNTP= deoxynucleotide triphosphate

kb= kilobase pair

PCR= Polymerase Chain Reaction

SDS-PAGE= sodium sodecyl sulphate polyacrylamide gel electrophoresis

STA= standard tube agglutination

TEMED= N,N,N'-N'-tertrametylenediamine

TSB= Trypticase Soy Broth media

U= unit

UV= ultraviolet light

Introduction

Brucella is a gram-negative intracellular bacterial pathogen causing Brucellosis in a wide variety of animal species. The genus *Brucella* includes six species; *B. abortus*, *B. ovis*, *B. melitensis*, *B. canis*, *B. neotomae* and *B. suis*. The first species of *Brucella*, *B. melitensis*, was originally isolated and named *"Micrococcus melitensis"* by Sir David Bruce in 1887. He isolated the bacterium from the spleens of patients who died from "Malta fever" on the mediterranean island Malta. In 1897, Bernard Laurits of Denmark isolated *B. abortus* which at that time was described as "*Bacillus abortus*" from the reproductive tract of a cow having "Bang's disease"; this disease refers to cattle having brucellosis. Alice Evans, an American microbiologist, recognized and proved that both species belong to the same genus and renamed "Malta fever" to "Brucellosis" in order to honor Sir Bruce in 1918. Meyer and Shaw in 1920 suggested the genus name of *Brucella*. Other *Brucella species* were discovered between 1914 and 1974 (28). The name of each species reflect the hosts from which they were isolated.

Under microscopic observation, *Brucella* are either small cocci, coccobacilli or short rods arranged either as single cells, pairs, or groups of cells. The average size of a cell is about 0.5-0.7μm in diameter and 0.6-1.5μm in length (13). They are resistant to weak acid and able to survive in dry and

low temperature for a long time (13). Additionally, they are nonmotile, noncapsulated and possess an outer membrane structure characteristic of gram-negative bacteria. Two of the major surface antigens, A and M, are different arrangements of oligopolysaccharide, the carbohydrate portion of lipopolysaccharide (LPS). The biological properties and biochemical characterization of the *Brucella* species are sufficiently different to permit identification. For instance, lysis by bacteriophage, CO_2 requirement for growth, H_2S production, dye (thionine and basic fuchsin) sensitivity and agglutination with monoclonal A or M antibody, are used to type *Brucella spp.* and strains (36). *Brucella spp.* show very close homology to each other using DNA hybridization techniques (20). The average amount of guanosine and cytosine content in their genome is about 55-59% (36). *B. melitensis* has two separate chromosomes approximately 2.2 and 1.1 megabase pairs in size (31). In the *Brucella* genome, there are repeat insertion sequences (IS) but different species possess different copy numbers of IS. These repeat IS may be responsible for genomic polymorphism of *Brucella* because they have the potential for transposition, recombination and deletion (17, 18). So far, no extrachromosomal DNA, i.e. plasmids, has been found (36). Morphologically, *Brucella* resembles *Bordetella, Haemophilus* and *Pasteurella* based on their gram-negative reaction and size and shape (37).

However, based on 16S rRNA classification, *Brucella* is related to the bacterial species of *Rhizobium* and *Mycoplana* (15). Each *Brucella* species has a fairly broad host range. For example, *B. abortus* can infect cattle, humans and horses, while *B. melitensis* can infect humans, goats and sheep. Brucellosis is characterized by abortion in cattle and by undulant fever in humans. Other common symptoms and signs in humans include myalgia, arthralgia, anorexia, fatigue and weight loss (27). Usually *Brucella* invade their hosts through impaired skin, conjunctival surfaces, oral and respiratory mucosa (27). Although readily engulfed by macrophages and other phagocytic cells involved in host immune system, they survive and replicate in these cells. They are released into lymphoid system and blood as the result of lysis of macrophages and locate in the liver, spleen and reproductive organs (45). Usually antibiotics such as tetracycline, streptomycin, rifampin or a combination of these are used to successfully treat brucellosis in humans.

The mechanisms of how *Brucella* survive and replicate in macrophages are not clear; however, some possible mechanisms have been described. Canning (7) proposed that the release of 5'-guanosine monophosphate and adenine by *B. abortus* can inhibit the degranulation of peroxidase positive granules which blocks the respiratory burst of neutrophils. Latimer et al. (26) tested the hypothesis that cell envelope associated

copper/zinc superoxide dismutase activity was the reason that *Brucella* can resist free oxygen radicals during a respiratory burst. They demonstrated that *Brucella* bearing a nonfunctional Cu/Zn SOD were as virulent as wild type (26). Catalase probably plays some role in the resistance of *Brucella* to peroxides (30). To date, it appears that the lipopolysaccharide (LPS) of the outer membrane is the only major determinant which plays a defined role in the virulence of *Brucella* (3, 32, 40, 43).

LPS is composed of an oligopolysaccharide chain (O-side chain), a sugar core and a lipid A. The major antigenic determinant is associated with the O-side chain probably due to the highly antigenic carbohydrates. The O-side chain's chemical structure is composed of a linear homopolymer of 96-100 α-1,2 linked 4,5-dideoxy-4-formamido-D-mannose (also called perosamine) repeat units (10, 52). Two major carbohydrate antigens in *Brucella* are antigen A and antigen M which is due to the difference in arrangement of O-side chain repeat units. Antigen A (A refers to *abortus*) is made of α-1,2 linked 4,6-dideoxy-4-formamido-D-mannose repeat units. The antigen M (M refers to *melitensis*) is made of one 1,3- and four 1,2-linked 4,6-dideoxy-4-formamido-D-mannose repeat units. Usually antigen A is dominantly expressed in *B. abortus,* and antigen M is dominantly expressed in *B. melitensis* (5). Different strains have been shown to carry

either A or M antigen, both or neither (i.e. rough strain). The variation in O-side chain linkage contributes to the differences or similarities of the serotypes among *Brucella spp.* as well as the cross reactions with other bacteria. For instance, *Yersinia enterocolitica* 0:9 possesses an O-side chain identical to *B. abortus* and can cross-react with anti A monoclonal antibody of *B. abortus* (6,8,9). The sugar core which links O-side chain to lipid A is composed of 7-10 sugar including mannose, glucose, quinivosamine, unknown sugars and 2-keto-3-deoxy-2-octulosonate (KDO) (36, 38). Lipid A attaches the entire core and O-side chain to the outer membrane. Lipid A is the mixture of glucosamine and diaminoglucose bound with long-chain fatty acid including saturated C16:0 to C18:0, and hydroxylated (3-OH-C12:0 to 20-OH-C30:0) fatty acids (34, 36).

The LPS of *Brucella* is very unique not only because of its structure but also because of its properties. The endotoxic effect is less than other enterobacterial endotoxin (16) and is nonpyrogenic. LPS can provoke a humoral mediated immune response in hosts (10). IgG and IgM are the major antibodies induced by *Brucella* LPS (10); and the antibody titer usually persists during adult vaccination for quite a long time even up to several years (10). This persistence can confuse the diagnosis of infected animals versus vaccinated ones using standard agglutination tests (STA) and complement

fixation test. The relationship between *Brucella abortus* LPS and virulence is almost same as found in the enterobacteria, such as *Salmonella typhimurium* and *Escherichia coli*. Usually rough strain without intact O-side chain show less virulence than the smooth strains possessing intact O-side chain (43, 45). *Brucella abortus* RB51, a rough strain derived from *B. abortus 2308,* is a good example (43). It is highly attenuated in cattle (12) and provides good protection against *B. abortus* 2308 challenge in goat, mice and cattle (12, 40, 49).

In 1992, 105 cases of human brucellosis were reported in America; but it is estimated that only 4-10% of case were reported because of lack of recognition of various symptoms and signs of the disease (11). There was an outbreak of human brucellosis reported in 1992 in a meat processing plant in North Carolina; 18 cases of Brucellosis were reported (11). Usually the source of infections to humans is ingestion of *Brucella* contaminated milk or cheese. Animals suffering from brucellosis which are diagnosed by standard diagnostic tests (27) are slaughtered because of a federally mandated Brucellosis eradication program (27). Thus brucellosis really seldom cause health problems in humans and economic loss in livestock in the U.S.A.. The Brucellosis problem outside North America and Western Europe is much worse, especially in the middle east. In 1988, 71,051 cases of Brucellosis were reported in Iran (53). *Brucella abortus* is the most

frequently identified strain for causing brucellosis in cattle. Since 1939 the standard *Brucella* vaccine strain for cattle in the United States of America has been the smooth *B. abortus* strain 19 . Although it is used in Brucellosis eradication programs, it is still not ideal. Strain 19 does not confer 100% protection in vaccinated cattle, and is only effective in avoiding seroconversion when it is inoculated into calves (i.e. calfhood vaccination). As a live vaccine, strain 19 still is virulent and can occasionally induce abortion in pregnant cattle as well as infect humans who mishandle the vaccine strain. Another disadvantage of strain 19 is that vaccinated cattle seroconvert which makes it difficult to distinguish vaccinated cattle from those infected by *B. abortus* field strains. In contrast to strain 19, rough strain RB51 (43) is of very low virulence, apparently does not induce abortion in pregnant cattle, protects cattle vaccinated at any age, and does not induce O-side chain antibodies (i.e., does not seroconvert (12, 43). However, the rough strain RB51 has not been characterized at the genetic level to understand the basis of its O-side chain deficiency.

Although the genetics of LPS biosynthesis of *Brucella* is unknown, the LPS genes of the gram-negative bacterium *Salmonella typhimurium* are well characterized (Figure 1). In *S. typhimurium*, the O-side chain is about 30-32 branched repeat sugar units of which mannose (Man), rhamnose (Rha) and

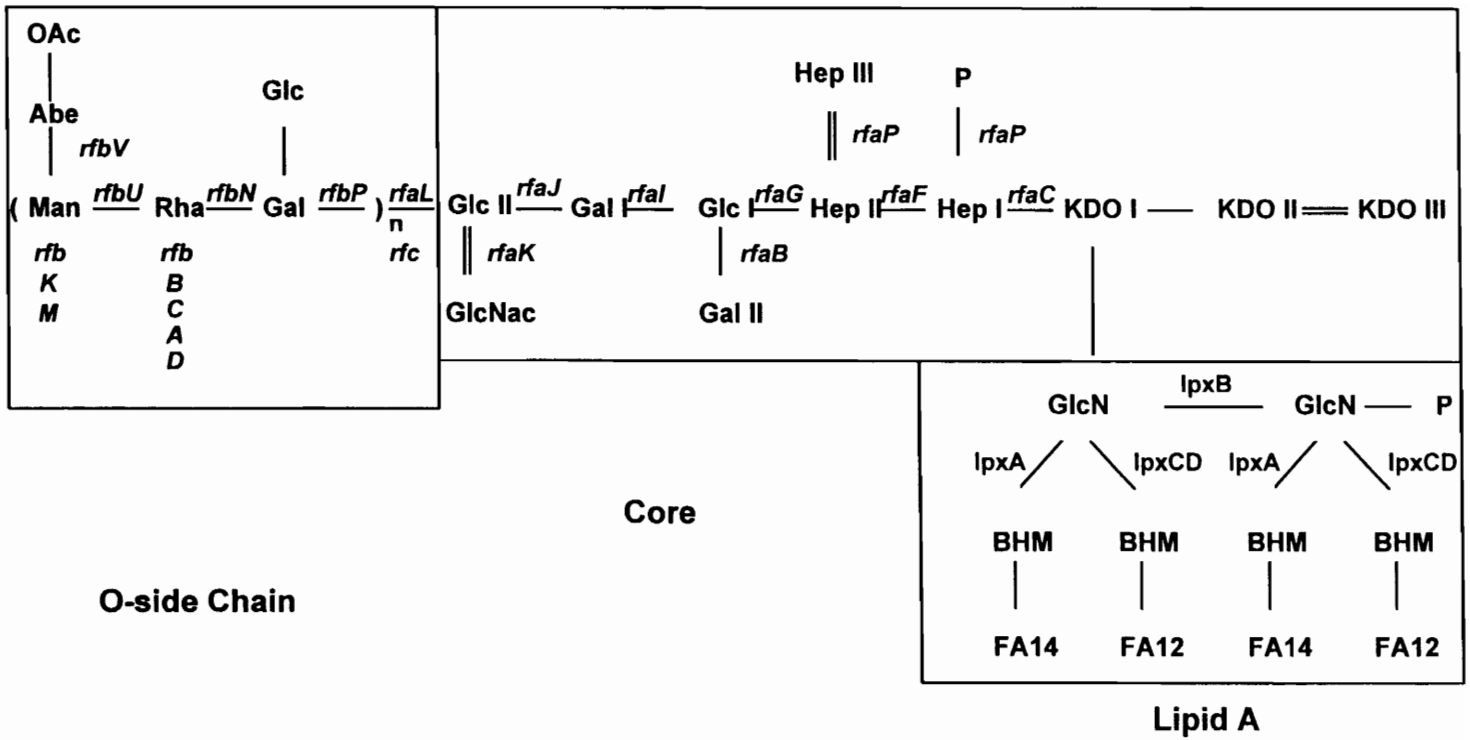

FIG. 1. The structure of the lipopolysaccharide of *S. typhimurium* and the major genes involved in its biosynthesis and assembly. The abbrevations BHM, FA12, FA14, P represent β-hydroxymyristate, laurate, myristate substituents and phosphate respectively. This figure is adapted from Schnaitman's review (42).

galactose (Gal) are the backbone; in addition the acylated abequose (Abe) and glucose (Glu) are the branch sugars. The core structure is composed of heptose (Hep), glu, gal and KDO. Lipid A has glucose disaccharide as its backbone, and bears C_{14} and C_{12} fatty acids as well as the phospholipid (28). Although the detailed chemical structure of LPS of *S. typhimurium* is different from *Brucella*, the overall structures are the same. Both LPS contains lipid A, a sugar core and an O-side chain formed by oligopolysaccharide repeat units as found in other gram-negative bacteria such as *E. coli* and *Yersinia enterocolitica*. The *rfb* gene cluster of *Yersinia enterocolitica*, which is involved in O-side chain biosynthesis and assembly, can be expressed in O-side chain deficient *Escherichia coli* (1). Thus *S. typhimurium* and *E. coli* LPS genes can be used as a model for identifying genes involved in LPS synthesis in *Brucella*.

In *S. typhimurium*, the genes involved in lipid A biosynthesis are distributed in different positions along the genome. The biosynthesis and modification of lipid A genes include *lpxA, B, C, D* (Figure 1). The sugar core biosynthesis genes are located in *rfa* gene cluster at 81-85 minute position (41). The *rfa* gene cluster includes *rfaA, B, C, D, F, G, P, Q, Y, Z* (Figure 1). The *kdsA* and *rfaE* genes are also involved in sugar core synthesis and are located outside of the *rfa* cluster. Whether or not genes in the *rfa* cluster are part of

one operon is unknown. Any mutation of *rfa* gene will induce a deep rough phenotype. The *rfb* gene cluster, responsible for O-side chain synthesis and assembly, is located at 44-48 minute position in the genome. The *rfb* cluster shows more polymorphism (19, 28) than the *rfa* cluster which is consistent with the fact that Lipid A and sugar core have more conserved structures than O-side chain. The *rfb* genes encoding proteins related to sugar biosynthesis, transfer and assembly include *rfbA, B, C, D, F, G, J, X, U, V, W, M, K, P* (21, 42) (Figure 1). Rhamnose biosynthesis is catalyzed by *rfbB, C, A, D* gene products. Mannose biosynthesis is catalyzed by *rfbM, K* gene products (42). The transferases of Rha, Man, and Abe are encoded by the *rfbV, U and N* genes respectively. The attachment of O-side chain is due to the functions of the *rfbP* and *rfaL* genes (4, 42). The assembly of oligopolysaccharide repeat units is dependent on *rfc* product (Figure 1) (42). The current model for the O-side chain synthesis and assembly is that sugars are first synthesized in the periplasmic compartment. The first repeat sugar unit is attached to the core, while the rest of repeat units are assembled to form an O-side chain unit and then are transferred out of the membrane and linked to the first repeat unit which is attached to the core. Basically, *rfb* mutants will exhibit a rough phenotype (28, 42).

This thesis research was initiated to characterize rough

mutants created by Tn5 mutagenesis mediated via bacteriophage P1 transduction and similar to the strain RB51. Strain RA2, the strain used in this study, is a rough mutant of *B. abortus* 2308 (35) created by Tn5 mutagenesis (Fang Lai, unpublished data, VPI&SU). The objective of this research was to identify whether the gene(s) disrupted by Tn5 insertion are responsible for the rough phenotype of strain RA2. The Tn5 element and surrounding *B. abortus* 2308 flanking region were cloned from *B. abortus* RA2 into the vector pGEM-3Z. The cloned region was electroporated into wildtype *B. abortus* strain 2308. The recombinants were selected and assessed for rough phenotype by crystal violet uptake and immunoblot analysis using specific anti O-side chain antibody. The results suggest that the Tn5 insertion may not play a role for inducing the rough phenotype of *B. abortus* strain RA2.

Materials and Methods

Bacterial strains, media and growth conditions:

The strains of bacteria used in this study are described in Table 1. *B. abortus* 2308 and its mutant derivative strains RA1, RA2, RB51 were grown in Trypticase Soy Broth (Becton Dickinson, Microbiology Systems, Cockeysville, MD) or SOB-C media (6% trypticase soy broth, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose) (25) at 37°C with shaking. *E. coli* DH5α were grown in Terrific Broth media (TB, 1.4% tryptone, 2.7% yeast extract, 0.4% glycerol, 17 mM KH_2PO_4, 72 mM K2HPO$_4$) [50] or Luria-Bertani media (LB, 1% tryptone, 0.5% yeast extract, 0.5% NaCl) (29). Tryptone, yeast extract and Bacto-Agar were purchased from Difco Laboratories (Detroit, MI.). The cells replicating plasmids were grown in selective media which contain antibiotics: Ampicillin (Amp) 100μg/ml, Kanamycin(Kan) 25-100μg/ml, or Streptomycin (Strep) 25μg/ml.

Reagents and enzymes:

All the reagents were purchased from Sigma Chemical Corporation (St. Louis, MO) unless otherwise indicated. Restriction endonucleases, calf intestinal alkaline phosphatase (CIP), T4 DNA ligase, halogenated indolyl-β-D-galactoside (Bluo-Gal), Taq DNA polymerase, Wizard PCR Prep, Wizard Clean-up System and Wizard Minipreps were purchased from Promega (Madison, WI). Agarose was obtained from GIBCO-

Table 1. Bacterial strains and plasmids

	Description	Source
Strains		
B. abortus		
2308	Wild type virulent field strain which is pathogenic to cattle.	G.G. Schurig[a]
RA1	Rough mutant of *B. abortus* 2308 by Tn5 insertion, KanR and StrR.	J. McQuiston[a] (MS thesis)
RA2	Rough mutant of strain 2308 by Tn5 insertion, KanR and StrS.	F. Lai[a]
RB51	Rough mutant of *B. abortus* 2308, rifampin R.	G.G. Schurig
E. coli		
DH5-α	F-, *end*A1, *hsd*R17, *sup*E44, *thi*-1, *rec*A1, *gyr*A96, *rel*A1 *lbd*[-], *phi*80d*lacZ*[d]M15.	BRL[b]
Plasmids		
pGEM-3Z	High copy number sequencing plasmid, *lacZ*+, AmpR.	Promega[c]
pUC4-Kixx	KanR gene from Tn5 in pUC4, AmpR.	Promega
pNW-1	7.3Kb *Eco*R I fragment bearing Tn5 with flanking region from RA2 into pGEM-3Z.	This study
pNW2	Subclone of 7.0kb *Eco*R I fragment bearing Tn5 with flanking region from pNW-1; KanR, AmpR.	This study
pNW3	*Xho* I fragment of Tn5 in pNW-2 replaced by 1.6Kb KanR gene from Tn5 in pUC4-Kixx, KanR, AmpR.	This study

[a] VPI& SU, Blacksburg, VA.
[b] GIBCO-BRL Life Technologies, Inc., Gaithersburg, MD.
[c] Promega Corporation, Madison, WI.

Bethesda Research Laboratory (GIBCO-BRL, Gaithersburg, MD). Incert™ agarose and NuSieve low melting point agarose were obtained from FMC BioProducts (Rockland, ME). The Genius Kit was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN).

Restriction digestion:

All restriction digestions were processed in a final volume of 20 μl. This 20 μl mixture contains 500 ng-1000 ng of DNA, 2-12 U of restriction enzyme and 2 μl 10X restriction enzyme buffer; the reaction mixture was incubated at 37°C overnight.

Electrophoresis:

Both mini Sub™ cell and wide Mini Sub™ DNA Electrophoresis cell (Bio-Rad Laboratories, Richmond, CA.) and the power supply EC-103 (E-C Apparatus Corporation, Petersburg, Florida) were used to separate DNA fragments in 1% agarose in 1X TBE buffer (17.8 mM Tris, 17.8 mM Boric acid, 0.4 mM EDTA). The electrophoresis was conducted at 5 V/cm unless specifically mentioned.

Plasmid DNA isolation:

The extraction of plasmids was according to the alkaline-SDS procedure of Ish-Horowicz and Burke (21). Sometimes a Wizard PCR Miniprep (Promega Corporation, Madison, WI) was used to isolate plasmid according to the protocol described by manufacturer.

Chromosomal DNA extraction:

The chromosomal DNA of *B. abortus* strains was isolated by CTAB (Hexadecyl-trimethylammonium bromide) extraction (2) with the following modifications: (1) After adding 600 μl of proteinase K digestion mixture, 200 ul of 2.5 M NaCl was added and mixed thoroughly to remove cell wall debris, denatured protein and polysaccharide complex to CTAB. (2) The chloroform and phenol extraction procedure was repeated twice and the mixture spun at 10,000× *g* for 30 minutes; (3) One third volume of 7.5 M ammonium acetate and three volume of 95% ethanol were added to aqueous phase for 1 hour at -20°C to precipitate DNA .

$CaCl_2$ transformation:

The transformation procedure was the $CaCl_2$ procedure described in Ausubel et al.(2). Hepes (N'-2-Ethanesulfonic acid) was used as the buffer for $CaCl_2$ solution to make *E. coli* DH5α competent.

Construction of pNW plasmid series:

pNW-1 construction:

A 7.3 kb of *Eco*R I fragment was cloned from *B. abortus* RA2 into the *Eco*R I site of high copy number vector pGEM-3Z. This fragment contains Tn5 transposon element (23) and a 1.5 kb of *B. abortus* 2308 chromosome.

Approximately 8 ug of vector pGEM-3Z was digested 18 hours with 20 U of *Eco*R I in 40 ul of buffer at 37°C.

Dephosphorylation of 6.5 μg of this digested vector was conducted in a final volume of 51 μl mixture containing 0.9 U of CIP and 5 μl of 10X CIP buffer at 37°C overnight. Then 51 μl of phenol was added into the mixture and vortexed 30 seconds, spun at 15,000× *g* for 5 minutes. The aqueous phase was removed and added to an equal volume of chloroform: isoamyl ethanol(24:1) and mixed by vortex 30 for seconds; the mixture was spun at 15,000x *g* for 5 min. The aqueous phase was saved and 7.5 M ammonium acetate was added to approximately one fifth volume of the mixture volume. Three volumes of 95% ethanol was added into the mixture for 30 minutes at -20°C to precipitate DNA . The mixture was spun at 10,000× *g* for 10 min. After washing in 75% ethanol, the DNA pellet was dried in speed vacuum (Savant Instruments, Inc, Hicksville, NY) and resuspended in 20 ul of sterile distilled water. For genomic DNA preparation, about 1 μg of *B. abortus* RA2 DNA was digested by 1 U of *Eco*R I in a final volume of 20 ul at 37°C overnight. The ligation reaction was in a 20 ul final volume containing 600 ng of genomic DNA, 30 ng of pGEM-3Z, 0.5 mM of ATP, 1 U of T4 ligase and 4 μl of 5X T4 ligase buffer. After incubation at room temperature overnight, the ligation mixture was used to transform competent *E. coli* DH5α by $CaCl_2$ transformation procedure.

After transformation, 100 μl of culture was spread onto LB plates containing either ampicillin (100 μg/ml), or

kanamycin (25 μg/ml)or both with Bluo-Gal (70 μg/ml) and (6 μg/ml) IPTG. Each of the kanamycin resistant white colonies was patched onto three LB agar plate containing either kan (25 ug/ml), or amp (100 ug/ml), or no antibiotic respectively. The kan and amp resistant colonies were grown at 37°C overnight in TB. The plasmids of the colonies were extracted by Ish procedure (21) and digested with *EcoR* I. The inserts were characterized by restriction enzyme mapping as well as Southern blot analysis (46) using a *B. abortus* 2308 chromosomal probe and a Tn5 probe.

pNW-2 construction:

To delete 300 bp *Eco*R I fragment from pNW-1 (Figure 2), 2 μg of pNW-1 was digested by 20 U of *Eco*R I and separated in 1% NuSieve low melting point agarose gel at 5 V/cm. The agarose containing the 7.0 kb band was removed and purified by Wizard PCR Prep (Promega Corporation, Madison, WI). Ligation of 7.0 kb fragment and vector, transformation, colony selection, plasmid screening and restriction mapping methods were same as described as before.

pNW-3 construction:

The *Xho* I fragment in Tn5 in pNW-2 was replaced by Kan gene of Tn5 from pUC4-Kixx (Pharmacia Biotech Molecular and Cell Biology products, Piscataway, NJ) (Figure 2) .

1.5 ug of pNW-2 was digested by *Xho* I and dephosphorylated as described before. 1.5 μg of pUC4-Kixx was

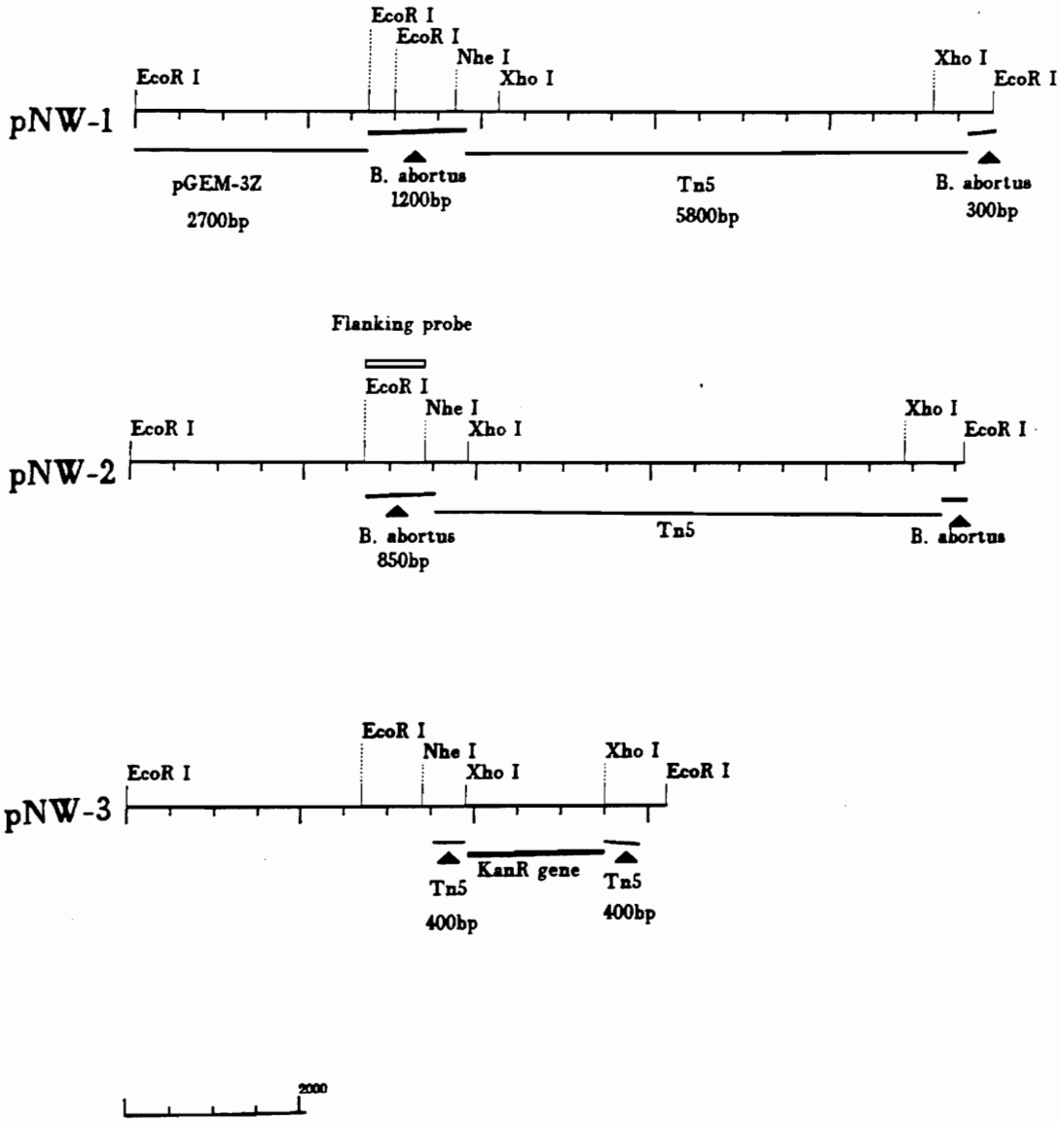

Figure 2. pNW plasmid series.

digested by *Xho* I at 37°C for 3 hours. Both DNA samples were separated by electrophoresis at 5 V/cm on 1.0% NuSieve low melting point agarose. The 5.4 kb band of pNW-2 as well as 1.6 kb band of pUC4-Kixx encoding KanR were cut from the agarose gel and purified by Wizard PCR Prep (Promega Corporation, Madison, WI). These purified fragments were ligated together and used to transform *E. coli*. The kanamycin and ampicillin resistant colonies were selected and the plasmids from these colonies were screened and mapped as described before.

Southern Blot:

This procedure was based on the protocol described by Southern with modifications (47). The TurboBlotter™ Rapid Downward Transfer system (Schleicher & Schuell Inc., Keene, NH) was used to transfer depurinated DNA to the Nylon transfer membrane (Micron Separations Inc, Westboro, MA) as described below. 20X SSC (3 M NaCl, 0.3 M Na_2citrate, pH 7.0) was the transfer buffer system. All paper towels, Whatman paper and nylon membrane used in this experiment were cut to the same size as the gel. Paper towels were placed in the stacking tray and covered with three pieces of Whatman Chromatograph paper (International Ltd, Maidstone, England). Two pieces of presoaked 20X SSC Whatman paper were layered on the paper towel stack, followed by nylon membrane (presoaked in distilled water) on the top of the wet paper. The gel

containing DNA fragments was placed over the membrane, and the air bubbles were removed from between two layers. The gel was covered with two pieces of Whatman paper presoaked in transfer buffer. The assembled materials were placed in a tray which was filled at least 150 ml of 20X SSC. Usually, the time for completing a transfer was about 3 hours to overnight depending on the size of DNA. After completing the transfer, the membrane was allow to air dry and then crosslinked at 120,000 microjoules for 30 seconds in a UV Stratalinker™ (Stratagene Cloning System, Lajolla, CA).

The Genius Kit (Boehringer Mannheim, Indianapolis, IN), a non-radioactive DNA labeling and detection system, was used for probe construction and visualization.

Growth curve of *B. abortus* 2308:

A single colony of *B. abortus* 2308 grown on a TSB agar was inoculated into 25 ml TSB media, incubated at 37°C at 150 rpm. 100 μl of culture (350-450 Klett units) was inoculated into 30 ml of TSB. 100 ul of culture was removed at different time points and serially diluted in TSB; five 10 μl aliquots of these dilutions were dotted on a TSB plate. After three days of incubation at 37°C, the cells were counted and averaged to calculate Colony Forming Units (CFU/ml).

Transformation by electroporation:

B. abortus 2308 cells were made competent by following procedure: A 660 ul aliquot of a culture (400 Klett units) was

inoculated into 200 ml TSB and incubated at 37°C and 150 rpm. At the desired growth status, 200 ml of culture was transferred into eight chilled 50 ml Corning centrifuge tubes (Fisher Scientific, Norcross, GA.) and incubated on ice for 30 min. The cells were centrifuged at 2190× *g* for 25 minutes and were resuspended in 50 ml sterile ice cold water. The water wash was repeated four times. The cells were resuspended in dH_2O in a final volume of 500 ul, aliquoted into 0.5 ml microcentrifuge tube, and incubated on ice until ready to use.

The BTX electroporation system 600 (BTX Inc., San Diego, CA) was used to transform *B. abortus* 2308 with pNW-2 or pNW-3 plasmids. The following settings were used: the resultant pulse length is 6 ms, 11 ms and 17 ms and the resultant field strength was 10-15 KV/cm. Disposable electrode cuvettes (BTX) with a 1 mm gap were used. To initiate electroporation, 3 μg of DNA was added into the 0.5 ml microcentrifuge tube containing 55 ul of cells and mixed well by swirling and incubating on ice for 20 min. This mixture was loaded into the cuvette and the electroporator was discharged; immediately, 1 ml of SOB-C was added into the cuvette, and then transferred to a 15 ml Corning centrifuge tube. The cells were incubated at 37°C with 150 rpm for 16 hours. After recovery in SOC-B medium (25), 100 μl of each recovery mixture was spread on a TSB plate, and the remainder of the mixture

was spread on a TSB/Kan(100 μg/ml) plate. These plates were incubated at 37°C and observed for up to 14 days for colony growth. The kanamycin resistant colonies were selected for further study.

Probe labeling:

A 700 bp chromosomal flanking probe was generated by *Eco*R I and *Xho* I double digestion of pNW-2. Following electrophoresis the fragment was isolated from the gel by Wizard PCR Prep (Promega Corporation, Madison, WI), and labeled using the Genius Kit protocol. 1 kb ladder, Tn5 and *B. abortus* 2308 whole chromosomal probes were prepared using a Polymerase Chain Reaction (PCR). A typical PCR reaction mixture contained 1 μl of 10X concentrated dNTP (Boehringer Mannheim), 0.5 μl of 10X concentrated random primer (Boehringer Mannheim), 0.5 μl (2.5 U) of Taq™ DNA polymerase, 4 μl of 10X polymerase buffer, 25-1000 ng of template DNA, 1.0-2.5 mM of $MgCl_2$ concentration. Sterile dH_2O was added to bring the final volume to 40 μl. The PCR cycle was set at 1.3 minutes at 95°C for denaturation, 1.3 minutes at 42°C for annealing, 2 minutes at 72°C for extension for 30 such cycles.

Colony immunoblot by monoclonal antibody Bru38:

After growing cells on a TSB agar, a piece of MagnaGraph nylon transfer membrane (Micron Separations Inc, Westboro, MA) was placed on the plate for 5 min. The membrane was removed and soaked in 25 ml of chloroform for 15 minutes. The

membrane was air dried, and incubated in a 25 ml mixture containing 2 μg/ml DNase, 80 μg/ml lysozyme and 3% of bovine serum albumin (Fisher) in blocking-digestion buffer (0.15 M NaCl, 0.02 M Tris, 0.005 M $MgCl_2$, pH 7.5) for 1 hour at room temperature. The membrane was washed 5 times with 25 ml of TBST (0.05% Tween-20 in TBS which is made of 0.5 M NaCl, 0.02 M Tris, pH 7.5). The membrane was incubated in 15 ml of Bru38, a rat IgG monoclonal antibody (44) diluted 1:10 in TBST, for 1 hour with agitating at room temperature. The membrane was washed 5 times with 25 ml of TBST and incubated in goat anti-rat IgG conjugated with horseradish peroxidase(1:500 dilution in TSB) with agitating for 1 hour. After washing 5 times in TBST, the membrane was incubated with freshly made developing mixture (combination of 60 mg 4-chloro-1-naphthol in 10 ml methanol and 0.6 ml 3% H_2O_2 in 100 ml of TBS) until the positive control exhibited a purple signal. The visualization reaction was stopped by soaking the membrane in large amount of dH_2O.

Preparation of LPS:

The procedure used was that developed by Hitchcock and Brown (19). Briefly, 1.5 ml of bacterial culture was harvested at the density of 50-60 Klett units by centrifugation at 10000× *g* for 5 minutes. 1.5 ml of acetone was added to pelleted cells for 1 hour to 18 hours to kill *Brucella*. Cells were harvested by centrifugation at 10,000×

g for 5 minutes, acetone removed and the pelleted cells air dried. After washing one time in phosphate-buffered saline, the pellet was resuspended in 0.05 ml of SDS-PAGE sample buffer (2% SDS, 4% 2-mercaptoethanol, 10% glycerol, 0.002% bromophenol blue in 1 M Tris-HCl buffer, pH 6.8). The sample was heated at 100°C for 10 minutes, and incubated at 60°C for 1 hour. 2.5 mg/ml proteinase K was added and the mixture incubated at 60°C for at least 1 hour. 0.01 ml of this treated sample was resolved on a 14% acrylamide gel (24).

SDS-PAGE of LPS:

The procedure is described by Laemmli (24) with the following modifications. A separate running gel: 14% acrylamide (Pierce, Rockford, IL), 0.37% N,N'-Methylenebisacrylamide(bis), 2.83 M urea (Pierce, Rockford, IL), 0.1% SDS(BDH, Biochemicals Ltd, Poole, England), 0.5 M Tris-HCl, pH8.8, 0.03% TEMED, and 0.07% ammonium persulfate. A stacking gel: 4.05% acrylamide, 0.1% Bis, 0.125 M Tris-HCL, ph 6.8, 0.1% SDS, 0.05% TEMED, and 0.05% ammonium persulfate. HPLC H_2O was used to make both stacking gel and separating gel.

Silver stain of LPS:

This procedure is based on Tsai & Frasch (50) and modified as following: (1) The gel containing the LPS was fixed overnight in 200 ml of 40% ethanol, 5% glacial acetic acid, and 55% dH_2O. (2) The gel was removed and incubated for

5 minutes in 200 ml of 0.7% periodic acid solution containing 1.4 mg of periodic acid, 80 ml of 95% of ETOH, 10 ml of glacial acetic acid and 110 of ml dH2O with mild agitation. (3) The gel was washed three times in 1 liter of dH_2O for 15 min with mild agitation on a rotary shaker. (4) The gel was soaked in 150 ml of ammoniacal silver solution: 20% silver nitrate, 28 ml of 0.1 M NaOH, 2 ml of concentrated ammonium hydroxide. (5) Step (3) was repeated . (6) LPS was visualized by adding 6 mM formaldehyde-0.25 mM citric acid solution to the treated gel until a brown signal develops. (7) Colorization was stopped by washing the gel in a large amount of cold water.

Result

Overall experimental approach:

The overall experimental approach for this thesis work is described in Figure 3.

Properties of *B. abortus* RA2:

The bacteriophage P1 was used to introduce the transposon Tn5 (KanR) into *B. abortus* (F. Lai, VPI&SU, unpublished data). All Kanamycin resistant (KanR) *B. abortus* clones were selected for further phenotypic characterization of their LPS. Strain RA2 is one of the Tn5 mutants deficient in LPS; the presence of Tn5 was confirmed by a Southern blot of RA2 chromosomal DNA using a Tn5 probe (Figure 4). The fact that strain RA2 can be stained by crystal violet (data not shown) indicates a rough phenotype; smooth strains repel the dye (51). Macroscopically, it is very hard to distinguish between smooth strain 2308 and rough strain RA2. However, strain RA2 has the tendency autoagglutinate. It was reported that strain RA2 is not as rough as strain RB51 because it still expresses some minor amount of high molecular weight O-side chain (John McQuiston, unpublished data, VPI&SU). An immunoblot of strain RA2 LPS reveals that strain RA2 LPS can bind Bru-38, a rat monoclonal IgG antibody specific to O-side chain, although very weakly compare to *B. abortus* 2308. In this study, however, the silver stain of LPS revealed no any high molecular weight O-side chain was detectable in strain RA2

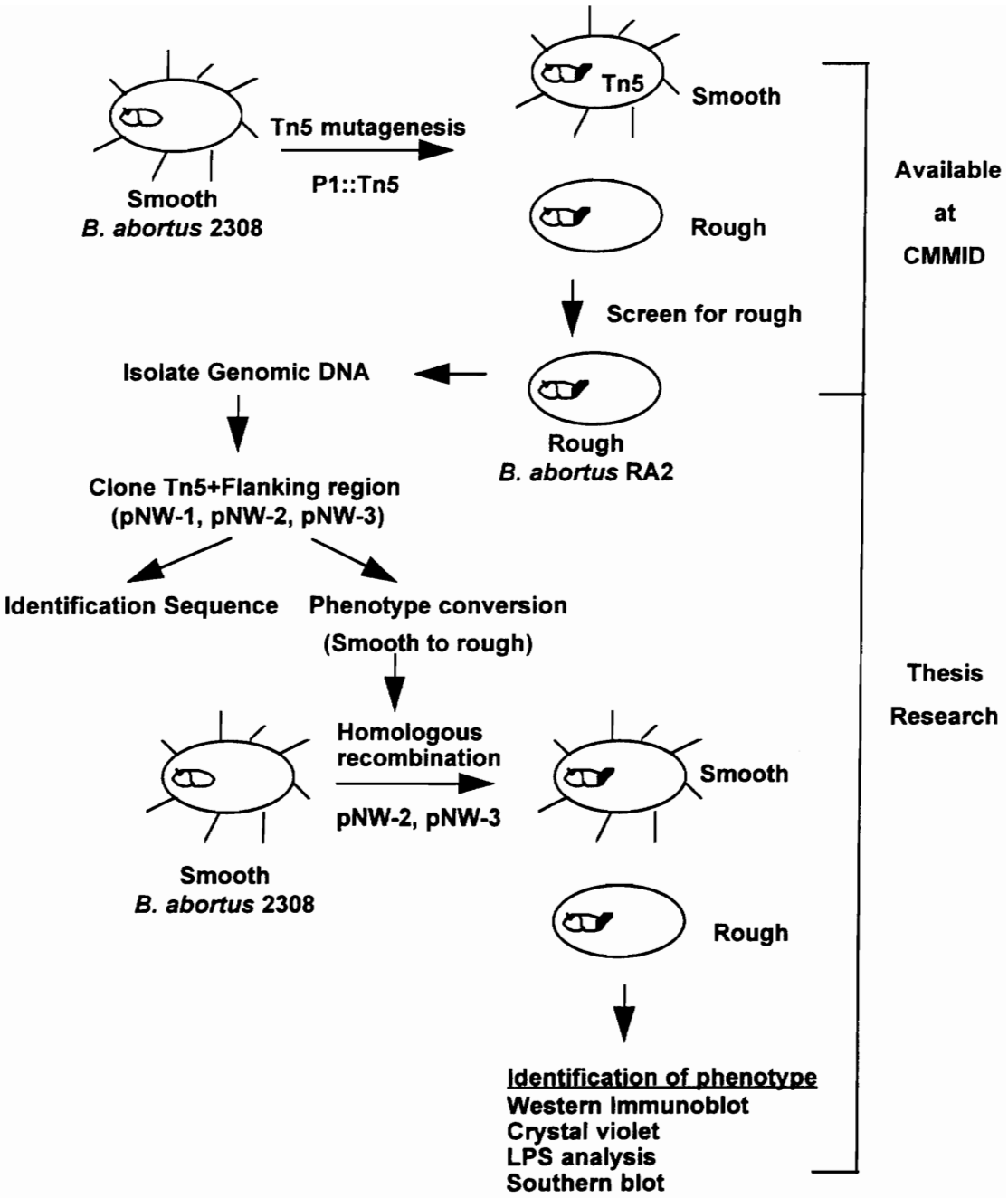

Figure 3. Overall experimental approach.

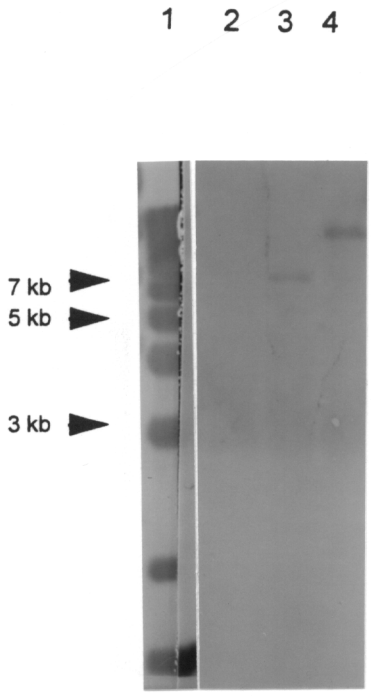

Figure 4. Southern blot of genomic DNA digested by *EcoR* I. Lane 1: 1 kb ladder; lane 2: *B. abortus* 2308; lane 3: strain RA2; lane 4: strain RA1.

(Figure 5). Both LPS from strain RA2 and strain RB51 did not show any Bru-38 binding activity (Figure 6). The immunoblot of LPS by specific Bru48 anti core reveals that RA2 had strong binding activity as did strains RB51 and 2308 (Figure 7).

Cloning of the Tn5 element and flanking region of *B. abortus* RA2:

Because *Eco*R I does not have any recognition sites in Tn5 (23), this enzyme was used to digest strain RA2 genomic DNA. The intact *Eco*R I fragments, including the one containing the Tn5 element with chromosomal flanking region, were ligated into the vector pGEM-3Z to create an *Eco*R I bank. Because the vector contains the AmpR gene and Tn5 element carries the KanR gene, clones were screened for kanamycin and ampicillin resistant; plasmids from several AmpR and KanR clones of *E. coli* DH5α were extracted and digested by *Eco*R I. In one clone, the restriction pattern revealed that there were two *Eco*R I fragments ligated into pGEM-3Z; this plasmid was designated pNW-1. These two *Eco*R I fragments were approximately 7.0 kb and 300 bp (Figure 8) and hybridized with *B. abortus* 2308 chromosomal DNA (Figure 9). These hybridization results indicate that the cloned genomic DNA originated from *B. abortus*. To confirm that the kanamycin resistant property is due to Kan resistant gene on the Tn5 element in pNW-1 as opposed to spontaneous mutation, a restriction analysis was performed. The restriction fragments

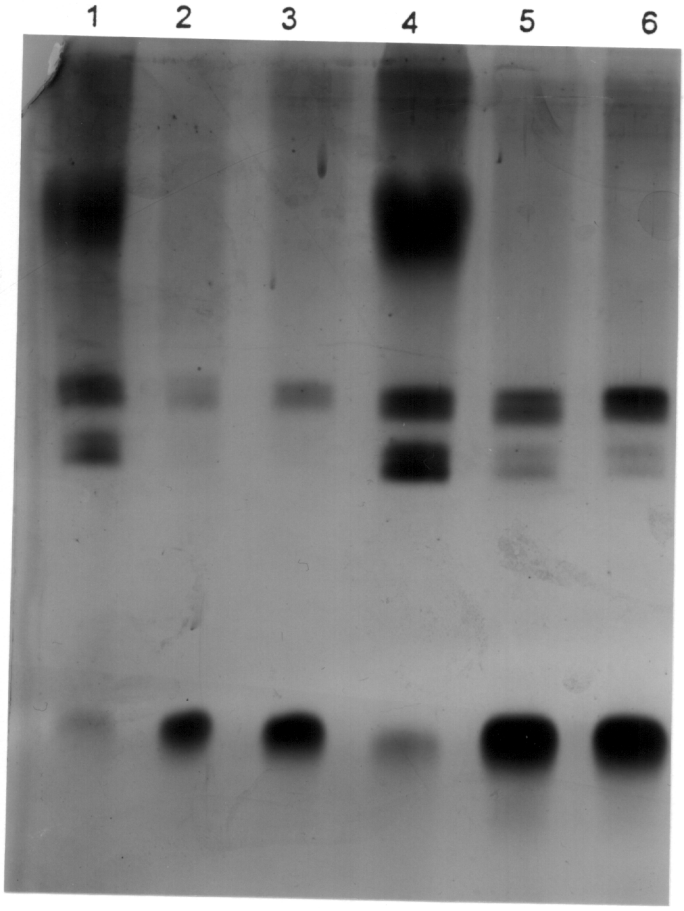

Figure 5. Silver stain of crude LPS from *B. abortus*. Lanes 1, 4 are *B. abortus* 2308 with 0.5X, 1X LPS respectively; lanes 2, 5 are strain RB51 with 0.5X, 1X LPS respectively; lanes 3, 6 are strain RA2 with 0.5X, 1X LPS respectively.

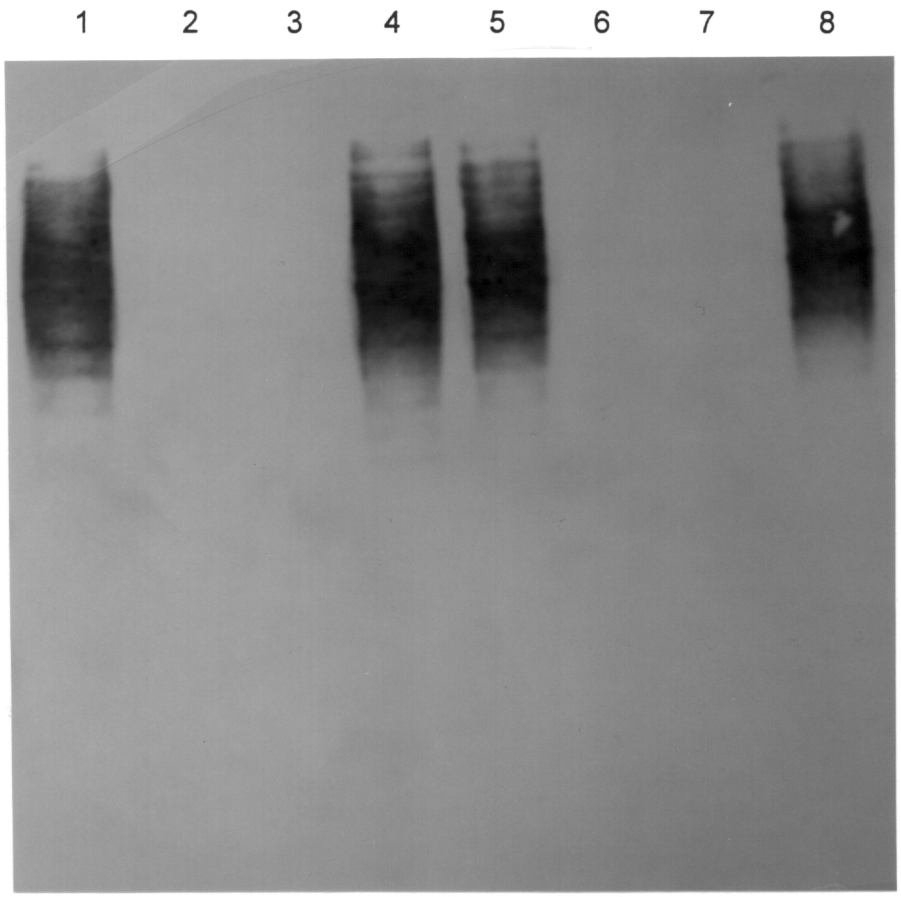

Figure 6. Western immunoblot of *Brucella* LPS by monoclonal antibody Bru38. Lanes 1, 5 are *Brucella abortus* 2308 with 0.5X, 1X LPS respectively; lanes 2, 6 are strain RB51 with 0.5X, 1X LPS respectively; lanes 3, 7 are strain RA2 with 0.5X, 1X LPS respectively; lanes 4, 8 are recombinant #25 with 0.5X, 1X LPS respectively.

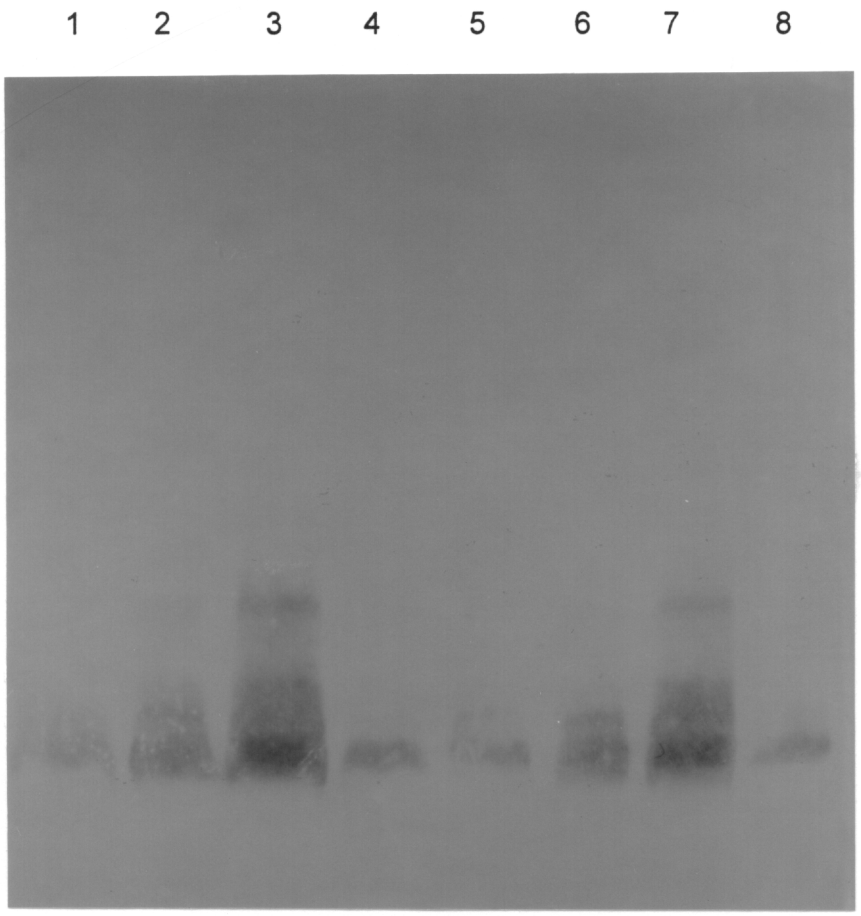

Figure 7. Western immunoblot of *Brucella* LPS by monoclonal antibody Bru48. lanes 1, 5 are recombinant #25 with 0.5X, 1X LPS respectively; lanes 2, 6 are strain RA2 with 0.5X, 1X LPS respectively; lanes 3, 7 are strain RB51 with 0.5X, 1X LPS respectively; Lanes 4, 8 are *Brucella abortus* 2308 with 0.5X, 1X LPS respectively.

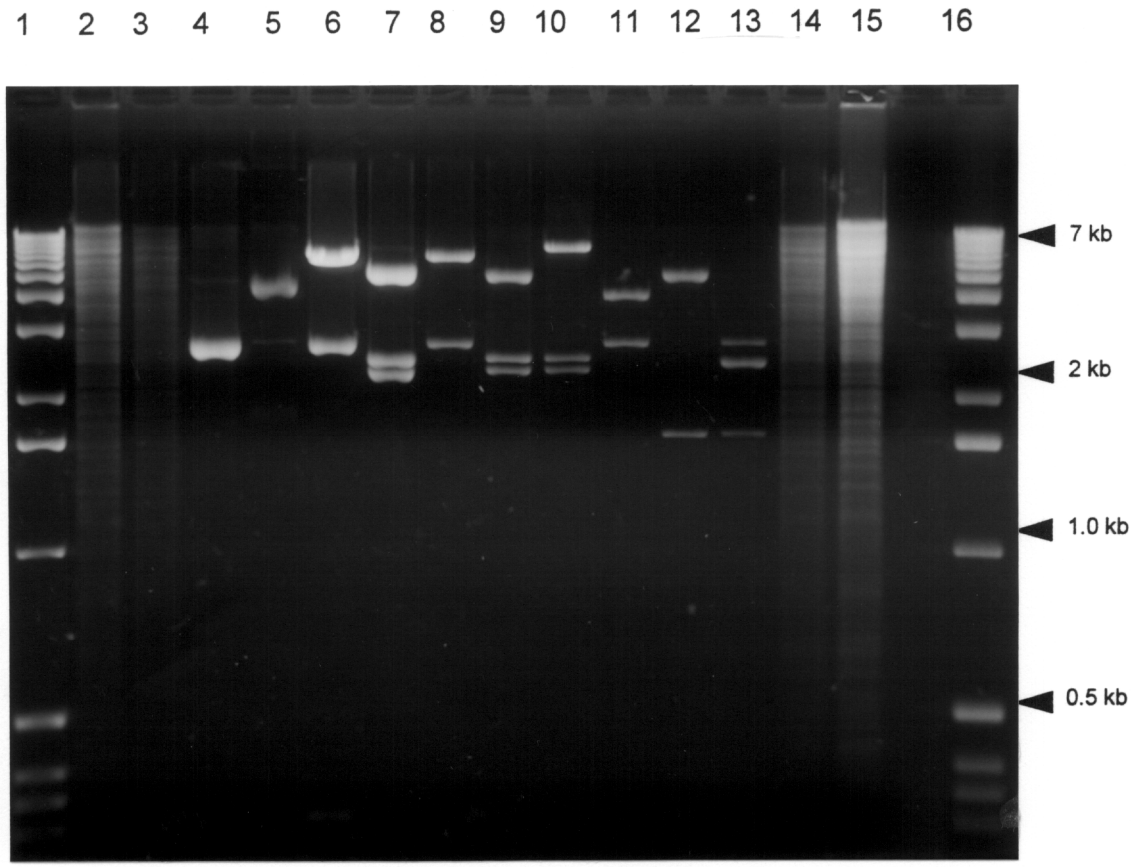

Figure 8. Restriction digestion pattern of pNW plasmids. Lanes 1 and 16: 1 kb ladder; lanes 2 and 15: *B. abortus* 2308 *Eco*R I digestion; Lanes 3 and 14: strain RA2 *Eco*R I digestion; lanes 4 and 5: pGEM-3Z *Eco*R I and *Xho* I digestion respectively; lanes 6 and 7: pNW-1 *Eco*R I and *Xho* I digestion respectively; lanes 8 and 9: pNW-2 *Eco*R I and *Xho* I digestion respectively; lane 10: pSUP-2021 *Xho* I digestion; lanes 11 and 12: pNW-3 *Eco*R I and *Xho* I digestion respectively; lane 13: pUC4-KIXX *Xho* I digestion.

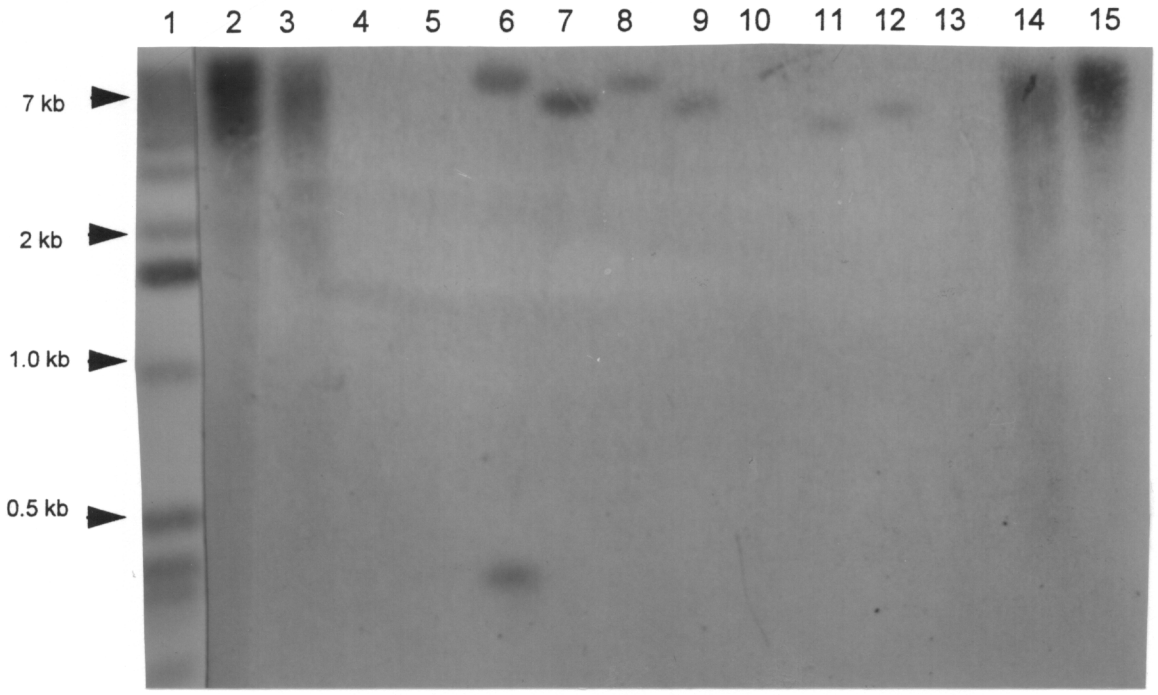

Figure 9. Southern blot of pNW plasmids using *B. abortus* 2308 genomic DNA as a probe. Lane 1: 1 kb ladder; lanes 2 and 15: *B. abortus* 2308 *Eco*R I digestion; Lanes 3 and 14: strain RA2 *Eco*R I digestion; lanes 4 and 5: pGEM-3Z *Eco*R I and *Xho* I digestion respectively; lanes 6 and 7: pNW-1 *Eco*R I and *Xho* I digestion respectively; lanes 8 and 9: pNW-2 *Eco*R I and *Xho* I digestion respectively; lane 10: pSUP-2021 *Xho* I digestion; lanes 11 and 12: pNW-3 *Eco*R I and *Xho* I digestion respectively; lane 13: pUC4-KIXX *Xho* I digestion.

generated by *Xho* I digestion of Tn5 and pNW-1 were compared using Tn5 as probe. The digestion of pNW-1 or Tn5 by *Xho* I generated two fragments approximately 2.3 kb and 2.4 kb (Figure 8). The digestion of pNW-1 using a Tn5 probe showed that only 7.0 kb fragment of pNW-1 is hybridizing with Tn5 probe (Figure 10). Thus the 7.0 kb fragment of pNW-1 plasmid contains Tn5 element and the surrounding flanking region of *B. abortus* 2308 genome. However, the 300 bp fragment in pNW-1 may or may not originate in the flanking region next to the Tn5 element; it may originate from another position in the genome. In order to avoid confounding results, the 7.0 kb fragment which contains Tn5 and flanking region was cloned into pGEM-3Z to create the plasmid designated pNW-2 (Figure 2). To confirm that the cloned fragment in pNW-2 was from pNW-1, restriction enzyme analysis of both plasmids revealed that they share the same restriction pattern except for the 300 bp fragment in pNW-1 (Figure 8, 9).

The transposon Tn5 belongs to the "composite element" class of transposon families. It contains two 1.2 kb inverted insertion sequence (IS) on two sides and a central region bearing antibiotic resistant markers. It integrates into chromosome by recognizing different "hot spots" (39). The inverted repeat sequence residing outside of the IS and the transposase encoded by the insertion sequence are directly involved in transposon recognition and transposition. Because

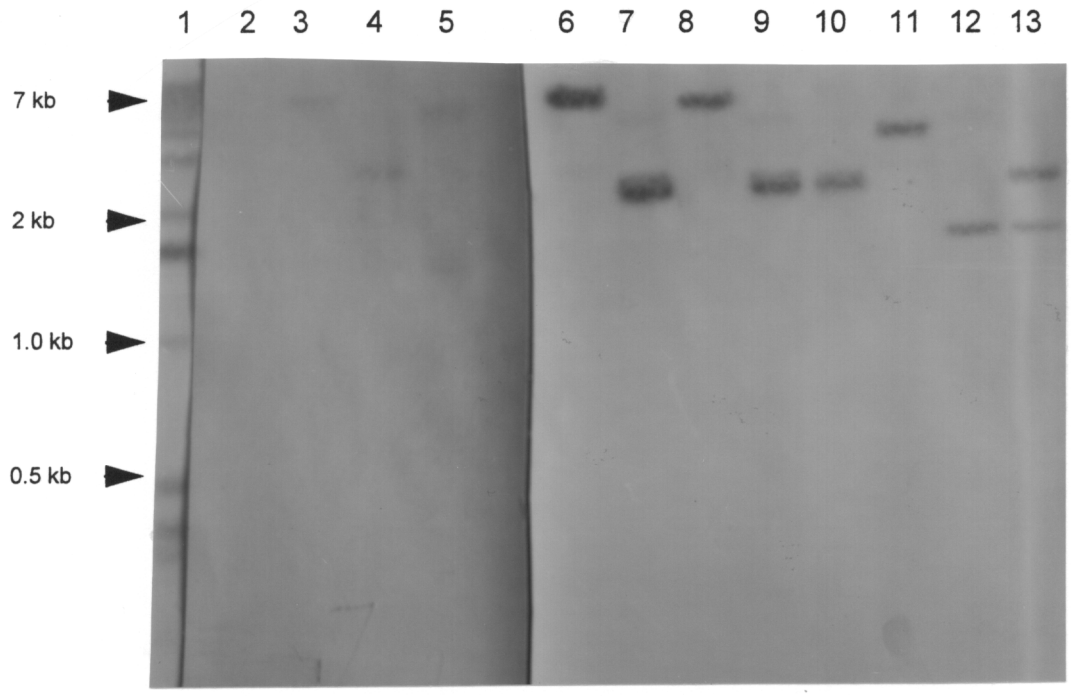

Figure 10. Southern blot of pNW plasmids using Tn5 as a probe. Lane 1: 1 kb ladder; lanes 2: *B. abortus* 2308 *Eco*R I digestion; Lane 3: strain RA2 *Eco*R I digestion; lanes 4 and 5: pGEM-3Z *Eco*R I and *Xho* I digestion respectively; lanes 6 and 7: pNW-1 *Eco*R I and *Xho* I digestion respectively; lanes 8 and 9: pNW-2 *Eco*R I and *Xho* I digestion respectively; lane 10: pSUP-2021 *Xho* I digestion; lanes 11 and 12: pNW-3 *Eco*R I and *Xho* I digestion respectively; lane 13: pUC4-KIXX *Xho* I digestion.

the plasmid pNW-2 contains an intact Tn5, it may be able to move and integrate into the genome randomly. If this occurred, it would be difficult to determine if the Tn5 primary integration event into *B. abortus* 2308 caused the rough phenotype. Therefore, the *Xho* I fragments of Tn5 which includes most part of DNA sequences encoding transposase were replaced by 1.6 kb Kanamycin resistant gene from Tn5 to eliminate the transposition capacity of Tn5 but retain the kanamycin resistant marker. This plasmid was designated as pNW-3. Both pNW-2 and pNW-3 were used to transform wildtype *B. abortus* 2308 to determine if rough phenotypes were induced by homologous recombination of either plasmid into the *B. abortus* 2308 genome.

Optimization of electroporation condition:

Brucella is difficult to transform by the traditional $CaCl_2$ protocol used for *E. coli* (25). Fang et al. (25) reported that the transformation by electroporation was not only dependent on electroporation parameters but also on cell growth status. The BTX electroporation system 600 has many advantages compared with BTX Transfector 100™. For example, one can use disposable electrode cuvettes to decrease the chances of contamination and they are safer for the person handling the system as one does not have to decontaminate the reusable electrodes.

The effect of cell growth status on the efficiency of

electroporation by the BTX system 600 was assessed. *B. abortus* 2308 exhibited the same typical growth pattern as other bacteria: lag, exponential (log), stationary and death phases (Figure 11). The generation time of *B. abortus* 2308 in TSB at 37°C, 150 rpm is approximately four hours. Cells were harvested at early, middle, late log phases (35, 50, 77 Klett Units respectively) and subjected to electroporation using pNW-2. Cells in late log phase were more competent than those in early and middle log phases (Figure 12) and validates the findings of Lai et al. (25). A second set of electroporation parameters evaluated were those of field strength and pulse length. Field strengths of 11, 12, 13, 14, 15 KV/cm were employed as a function of a fixed pulse length, and conversely, set constant when pulse length was fixed; additionally 6, 11, 17 ms of pulse length were employed as a function of a fixed field strength. This experiment was done once. The results revealed that a field strength of 13 KV/cm and a pulse length of 11 ms gave the best conditions to transform the suicide plasmids into *B. abortus* 2308 (Table 2). This is very similar to 5 ms pulse length, 12.5 KV/cm field strength which reported by Lai et al. (25). Overall, these two electroporation units can achieve the same efficiency of transformation of *B. abortus*.

Homologous recombination between suicide plasmid and *B. abortus* 2308 genome:

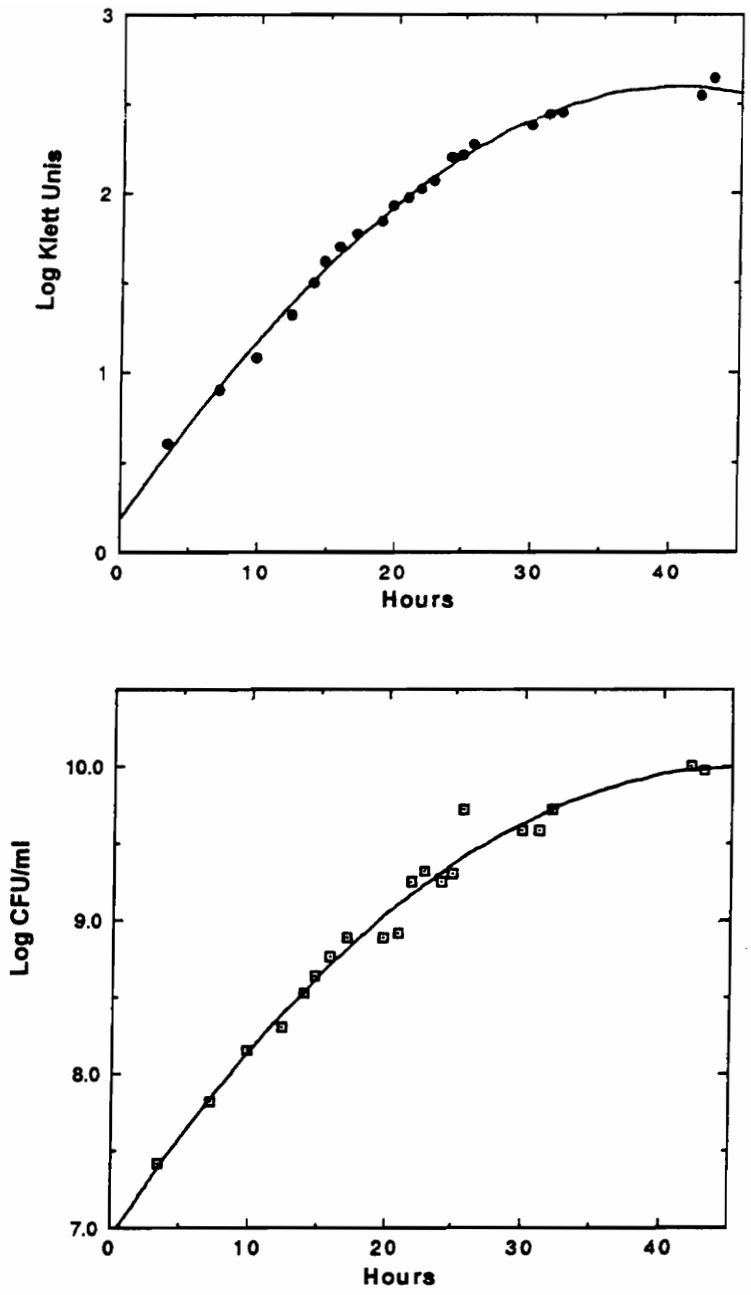

Figure 11. Growth of *B. abortus* 2308 at 150 rpm and at 37°C in TSB. A: klett units; B: colony forming units (CFU/ml).

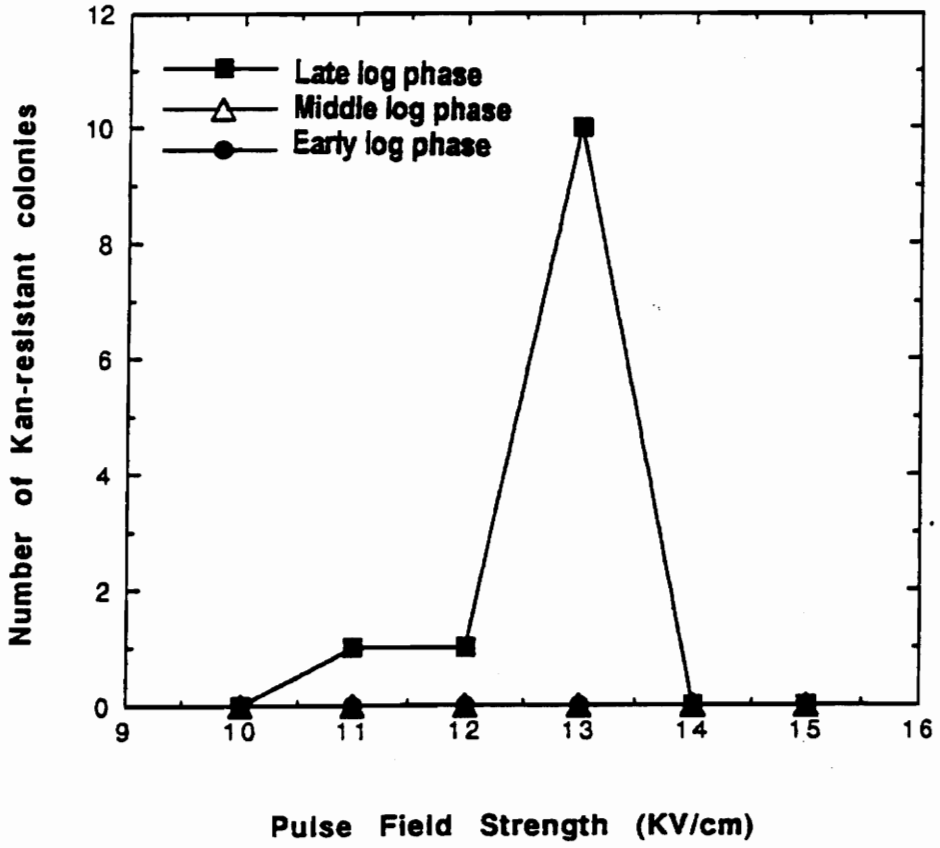

Figure 12. Electroporation induced KanR clones as a function of pulse field strength. The pulse length used was 11 ms; the pulse field strengths used were 10, 11, 12, 13, 14, 15 KV/cm respectively.

Table 2. Optimization of electroporation parameters for *B. abortus* 2308 at late log phase growth (77 Klett Units).

Pulse length (ms)	field strength (KV/cm)	# of KanR clones
6	12	3
	13	0
11	12	1
	13	9
16	12	0
	13	2

The nonreplicating plasmids pNW-2 and pNW-3 were electroporated into wildtype *B. abortus* 2308. The KanR clones were patched onto TSB plates containing 100 ug/ml ampicillin or 100 ug/ml kanamycin to select for double reciprocal crossovers (i.e. KanR and AmpS). Those colonies were further analyzed by crystal violet uptake test (51). All of these KanR, AmpS colonies were not stained by crystal violet and suggested they possessed a smooth phenotype. Colony immunoblot with monoclonal antibody Bru-38 showed that these colonies carried the same amount of O-Side chain as wildtype strain 2308 (Figure 13). The immunoblotting of one of these recombinants, #25 recombinant, with Bru38 and Bru48, also show the smooth phenotype (Lane 4 and 8 in Fig. 6, lane 1 and 5 in Fig. 7). The chromosomes of a number of the recombinants were extracted and hybridized with a Tn5 probe in order to distinguish those resulting from Tn5 insertion versus those resulting from spontaneous mutation. When pNW-2 was electroporated into *B. abortus* 2308, the location of the fragment of Tn5 in the recombinants exhibited polymorphism because Tn5 transposed into different size fragments (Figure 14). The results demonstrated that Tn5 was integrated into the genome but in a different position in each of the recombinants. Furthermore, the location of Tn5 in these recombinants was not in same place as the Tn5 in strain RA2 (Figure 14). The chromosomal DNA of these recombinants were

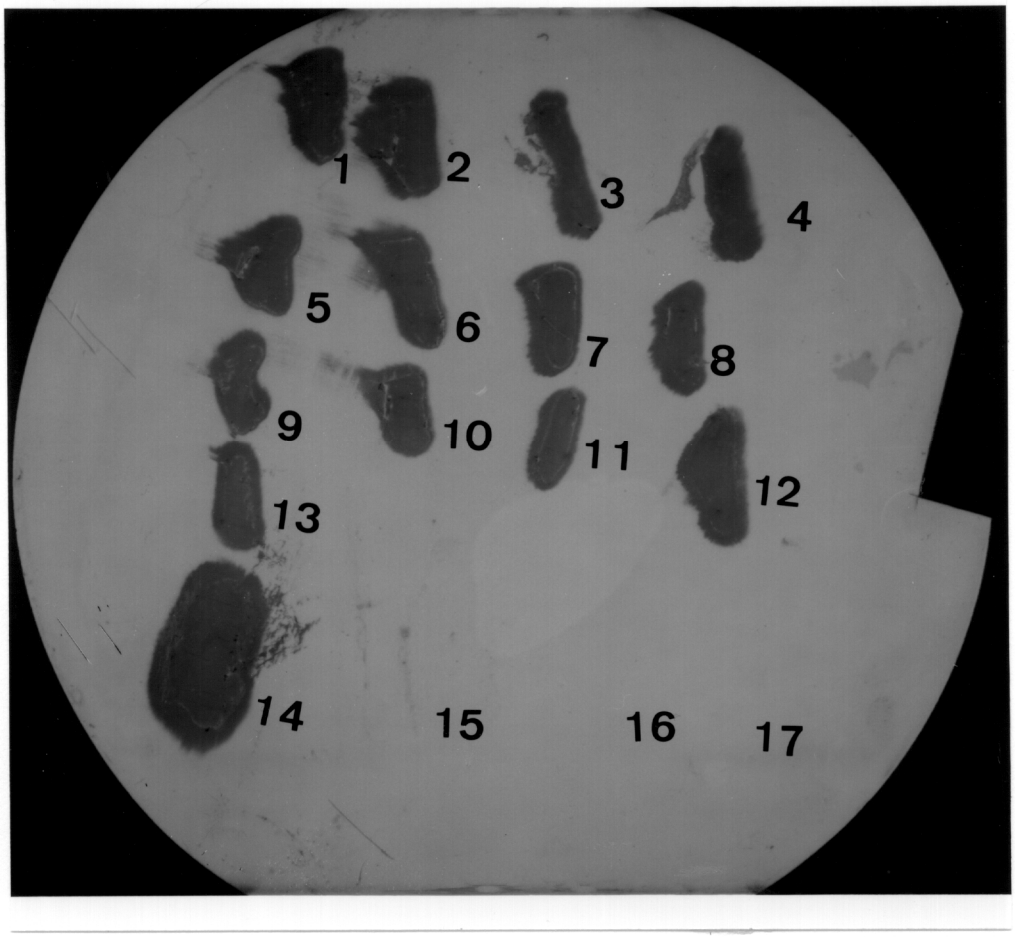

Figure 13. The colony blot of recombinants by monoclonal antibody Bru38. 1-13: recombinants; 14: *B. abortus* 2308; 15: strain RA2; 16: strain RA1; 17: strain RB51.

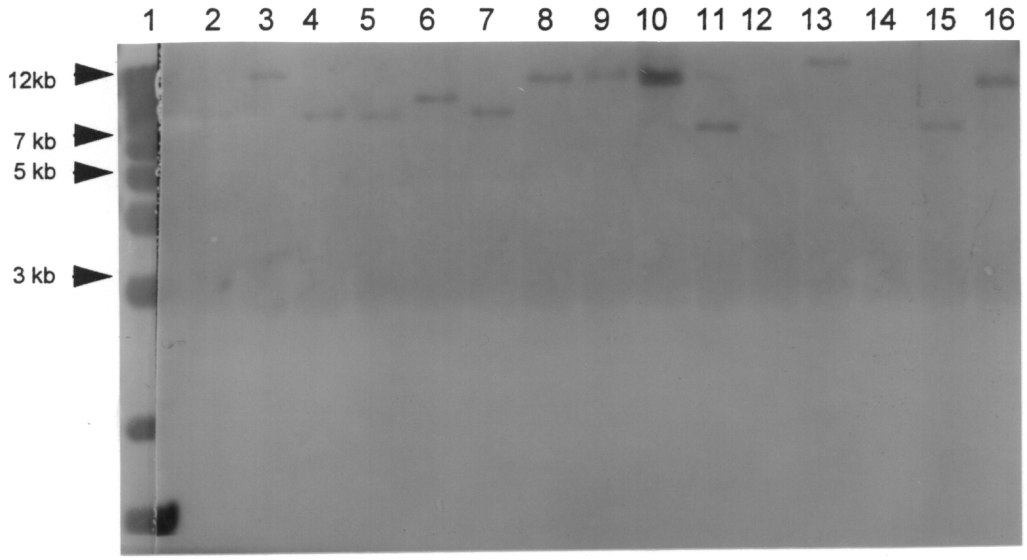

Figure 14. Southern blot of genomic DNA digested with *Eco*R I and hybridized with Tn5 probe. Lane 1: 1kb ladder; lanes 2-13: the mutants created by electroporating pNW-2 into *B. abortus* 2308; lane 14: B. *abortus* 2308; lane 15: strain RA2; lane 16: strain RA1.

hybridized to a 700 bp *Nhe* I/*Eco*R I fragment containing the flanking region of *B. abortus* in pNW-2 (Figure 2). In this Southern blot, all the fragments hybridizing with the flanking region probe were about 1.3 kb, the same as the genomic fragment of strain 2308 hybridized with same probe; however, this probe is hybridizing to the fragment located at 7.0 kb in strain RA2, no other fragments were detected which hybridized with the flanking region probe in strain RA2 (Figure 15).

Following electroporation of pNW-3 into wildtype strain 2308, the genomic DNA of KanR recombinants were extracted. Digestion of genomic DNA from strain RA2 and strain 2308 as well as the recombinants with *Eco*R I or *Eco*R I and *Nhe* I was performed. When the Kan resistant gene from Tn5 element was used as the probe, the Southern blot showed that the location of Tn5 in these recombinant's genome was either in a 4.0 kb *Eco*R I fragment or in a 3.2 kb *Eco*R I/*Nhe* I fragment (Figure 16). This result indicates that the transposition capacity of Tn5 was eliminated by the deletion of DNA sequence encoding transposase in pNW-3. As expected, the Tn5 was located in 7.0 kb *Eco*R I fragment or the 3.7 kb *Eco*R I/*Nhe* I fragment in strain RA2, whereas no Tn5 element was detect in the negative control *B. abortus* 2308 (Figure 16). A 700 bp *Nhe* I/*Eco*R I fragment containing *B. abortus* flanking region from pNW-2 (Figure 2) was used as a probe in order to reveal the interrupted gene in these recombinants compared to strain RA2

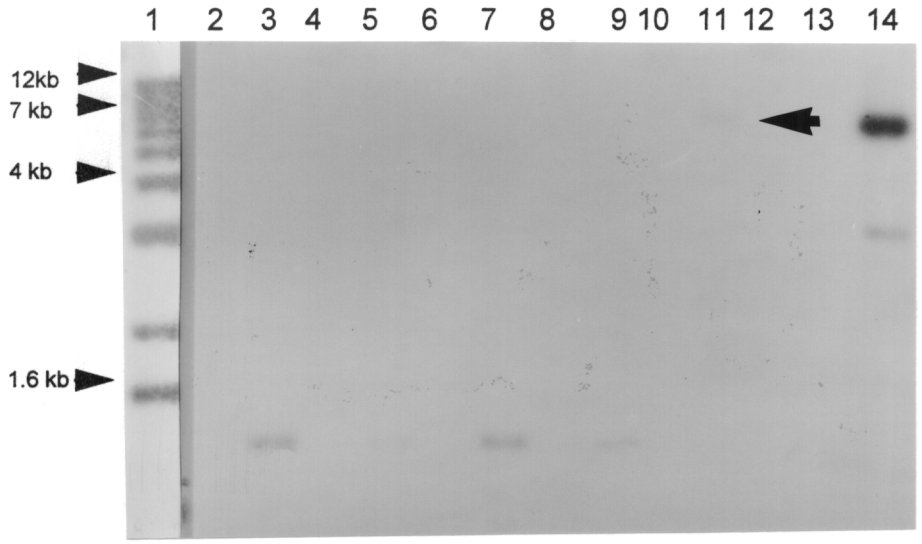

Figure 15. Southern blot of genomic DNA digested with *Eco*R I and using a 700 bp chromosomal flanking region as the probe. Lane 1: 1kb ladder; lanes 2 and 3: #6 recombinant undigested and digested; lanes 4 and 5: #7 recombinant undigested and digested; lanes 6 and 7: #11 recombinant undigested and digested; lanes 8 and 9: #12 recombinant undigested and digested; lanes 10 and 11: strain RA2 undigested and digested; lanes 12 and 13: *B. abortus* 2308 undigested and digested; lane 14: pNW-2 digested.

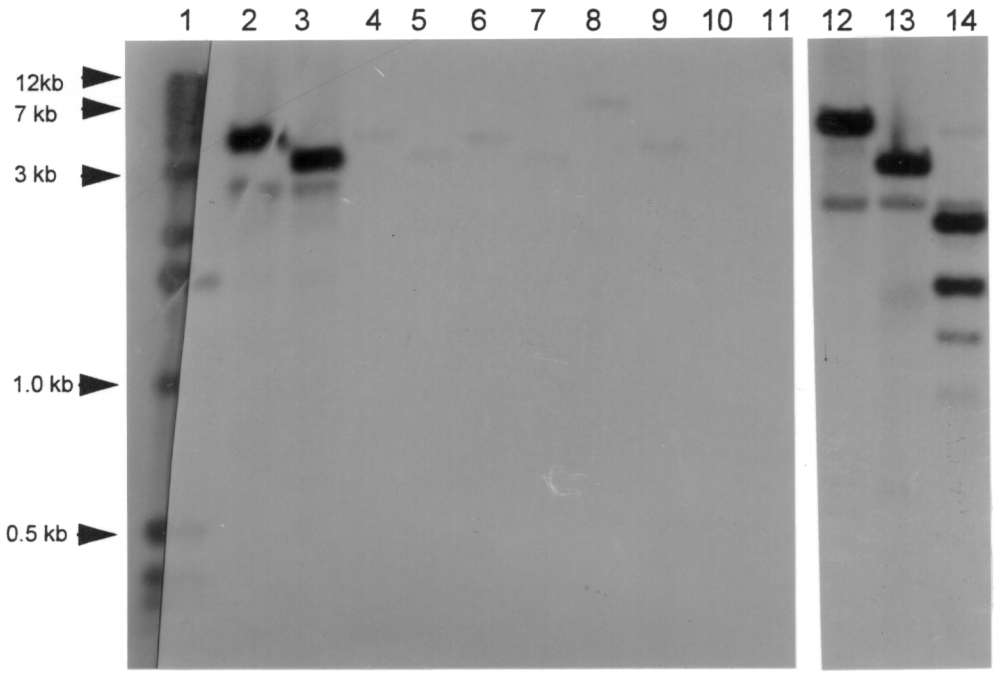

Figure 16. Southern blot of genomic DNA of the recombinants created by electroporating pNW-3 into *B. abortus* 2308 using Kan resistant gene as the probe. Lane 1: 1 kb ladder; lanes 2 and 3: pNW-3 *Eco*R I and *Eco*R I/*Nhe* I digestion respectively; lanes 4-7: #24 and #25 recombinants *Eco*R I and *Eco*R I/*Nhe* I digestion respectively; lanes 8 and 9: strain RA2 *Eco*R I and *Eco*R I/*Nhe* I digestion respectively; lanes 10 and 11: *B. abortus* 2308 *Eco*R I and *Eco*R I/*Nhe* I digestion respectively; lanes 12 and 13: pNW-2 *Eco*R I and *Eco*R I/*Nhe* I digestion respectively; lane 14: pUC4-KIXX *Xho* I digestion.

(Figure 17). The results showed that not only the 4.0 kb *Eco*R I fragment but also 1.3 kb *Eco*R I fragment hybridized to the flanking probe in these recombinants (lane 4 and 6, figure 17). In strain RA2 only the 7.0 kb *Eco*R I fragment and in strain 2308 only the 1.3 kb *Eco*R I fragment hybridized to the probe (Lane 8, 10 respectively, Figure 17). However, when the genomic DNA of the recombinants were digested by both *Eco*R I and *Nhe* I, both the 4.0 kb and 1.3 kb fragments disappeared and only one 700 bp fragment appeared which hybridized with the flanking region probe (Lane 5 and 7, Figure 17). In strain RA2, the 7.0 kb fragment disappeared and 700 bp fragment appeared which hybridized to the probe (Lane 9, Figure 17). In strain 2308, the 1.3 kb fragment disappeared and 700 bp fragment was created which was hybridized to the probe (Lane 11, Figure 17). No other signals could be detected. These results suggest that both 4.0 kb and 1.3 kb *Eco*R I fragment had *Nhe* I sites in the recombinants. These *Eco*R I and *Nhe* I fragments are of the same size as the *Eco*R I/*Nhe* I fragment I in strain RA2 as well as in strain 2308. All these results indicated that a homologous double crossover occurred between chromosome and pNW-3.

To test if the genome of strain RA2 contains one copy of Tn5, the chromosomal DNA was digested by *Xho* I. Southern blot using Tn5 as probe showed that there was no signal on any other fragments other than the 2.3 kb and 2.4 kb fragments

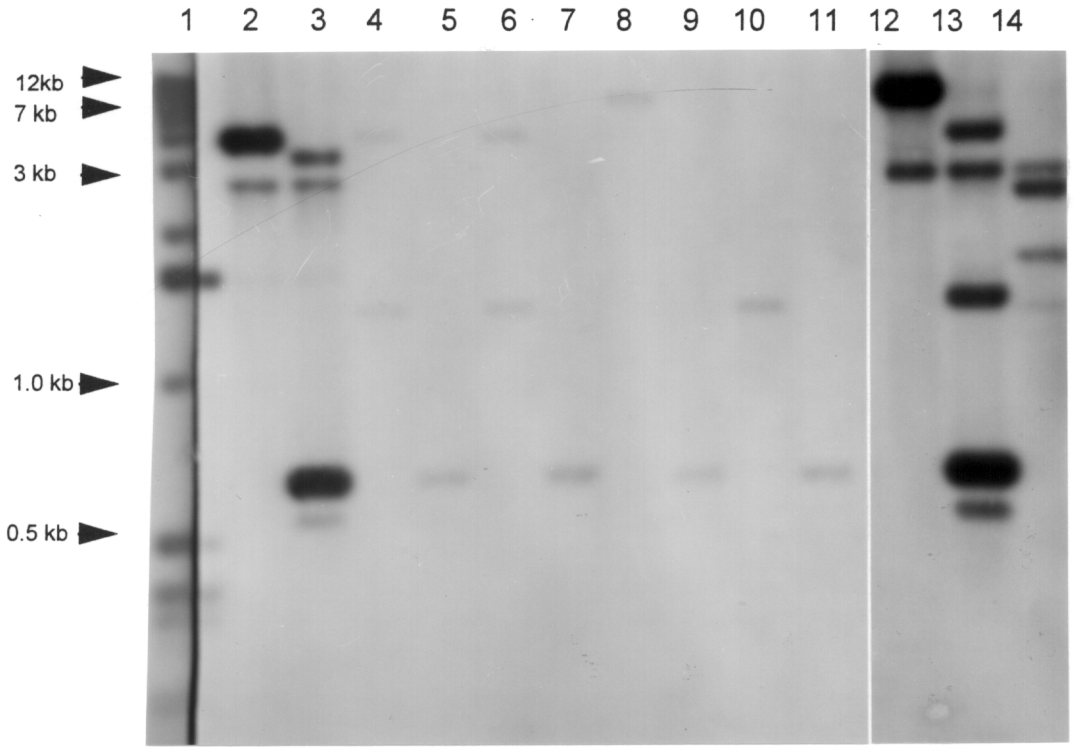

Figure 17. Southern blot of genomic DNA of the recombinants created by electroporating pNW-3 into *B. abortus* 2308 using the flanking region as the probe. Lane 1: 1 kb ladder; lanes 2 and 3: pNW-3 *Eco*R I and *Eco*R I/*Nhe* I digestion respectively; lanes 4-7: #24 and #25 recombinants *Eco*R I and *Eco*R I/*Nhe* I digestion respectively; lanes 8 and 9: strain RA2 *Eco*R I and *Eco*R I/*Nhe* I digestion respectively; lanes 10 and 11: *B. abortus* 2308 *Eco*R I and *Eco*R I/*Nhe* I digestion respectively; lanes 12 and 13: pNW-2 *Eco*R I and *Eco*R I/*Nhe* I digestion respectively; lane 14: pUC4-KIXX *Xho* I digestion.

(Figure 18). This suggests that no Tn5 element can be found in an *Xho* I fragment smaller than 23 kb in strain RA2. Pulse field gel electrophoresis and Southern blot analysis revealed that only one DNA fragment which was bigger than 30 kb is disrupted by Tn5 (John McQuiston, unpublished data, VPI&SU) in strain RA2 genomic DNA.

Sequence analysis of *B. abortus* 2308 flanking region in plasmid pNW-1:

Figure 19 is the sequence of *B. abortus* 2308 flanking region in pNW-1 (David Fritzinger, unpublished data, Walter Reed Army Institute of Research). The sequence was compared with known *Brucella* genes in GenBank, and other genes in the EMBL GenBank Database by DNASTAR. There were no significant homologies found between the *B. abortus* genomic DNA in pNW-1 and published LPS gene sequences. Although there was 21% sequence homology between the *Brucella* DNA in pNW-1 and *Rhizobium trifolii* Sym plasmid nodulation genes *nod*ABCD, the comparison of both ORFs indicated that no sifnificant homology at the amino acid level.

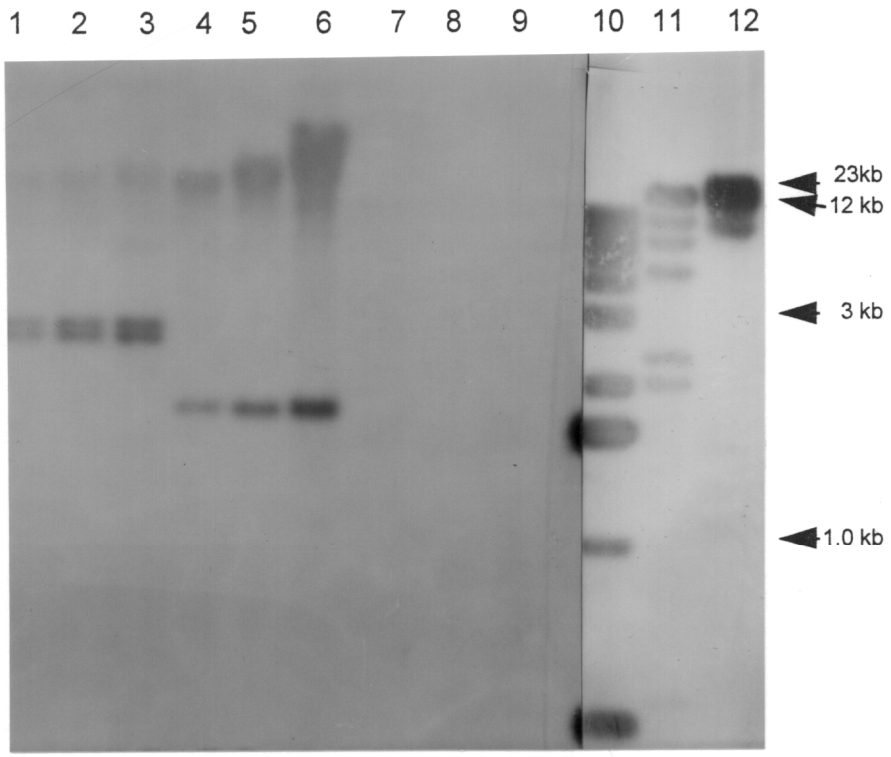

Figure 18. Southern blot of strain RA2 using a Tn5 probe. Lanes 1-3: strain RA2 digested by *Xho* I with 1X, 2X, 4X DNA concentration respectively; lanes 4-6: #25 recombinant digested by *Xho* I with 1X, 2X, 4X DNA concentration respectively; lanes 7-9: *B. abortus* 2308 digested by *Xho* I with 1X, 2X, 4X DNA concentration respectively; lane 10: 1 kb ladder; lane 11: λ/HindIII ladder; lane 12: higher molecular marker.

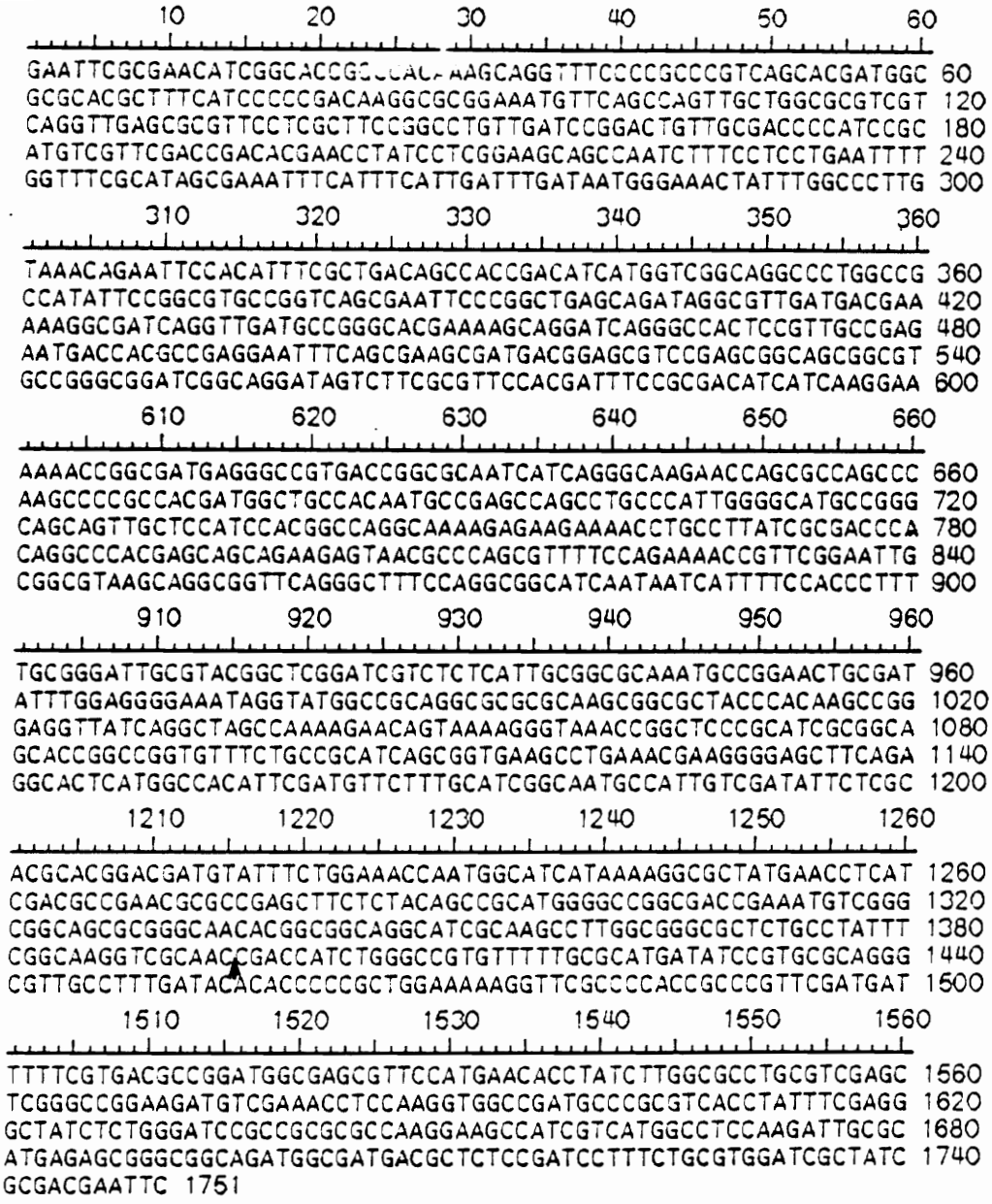

Figure 19. The sequence of pNW-1 flanking region of *B. abortus* 2308. ▲ represents the position of Tn5 insertion.

Discussion

Previous studies revealed that LPS is the major antigenic determinant related to the virulence in *Brucella* infections (10, 12, 26, 40, 43). The identification of the genes involved in LPS synthesis, especially O-side chain biosynthesis will be useful for further study of the role of LPS antigenicity and virulence at the genetic level. Mutagenesis of *Brucella abortus* 2308 by Tn5 is a practical tool to identify those genes. A rough strain which lacks its intact O-side chain was selected. In this thesis study, strain RA2, one of the Tn5 mutants of *B. abortus* 2308 created by P1 transduction, was characterized according to its LPS properties. The experiments included crystal violet uptake, LPS extraction, Southern blot, immunoblot by specific anti O-side chain, and anti core antibodies; all results indicated that strain RA2 is a rough Tn5 mutant (Figure 5, 6, 7). Preliminary LPS analysis on strain RA2 showed that some Bru38 binding capacity from previous work (John McQuiston, unpublished data, VPI&SU). The absense of similar binding capacity in this study may be because strain RA2 has been sufficiently subcultured so that it lost its minor high molecular weight O-side chain.

If it is assumed that the rough phenotype was caused by Tn5 insertion, it is possible that the gene interrupted by Tn5 encodes a product involved in O-side chain biosynthesis and/or

assembly. To identify the role of the putative gene, studies were initiated by cloning the Tn5 element and the chromosomal flanking region from strain RA2. When the suicide plasmid carrying the intact Tn5 and flanking region (pNW-2) was electroporated into wildtype *B. abortus* 2308 in order to assess the phenotype conversion from smooth to rough by homologous recombination, none of the recombinants showed a rough phenotype. The Southern blot of the genomic DNA of these recombinants (Figure 14, 15) indicate that Tn5 had integrated into the genome randomly rather than at a specific site. Thus the Tn5 element in these recombinants did not disrupt the same gene as that in strain RA2. In this case, two possible events could have happened: Tn5 transposition occurred preferentially at a specific-site (eg. as in RA2); or transposition occurred but subsequently the Tn5 element transposed to another position in the genome. In other studies of Tn5 induced rough *Brucella* (McQuiston, M.S. Thesis, VPI&SU), when an intact Tn5 element having longer chromosomal flanking regions (pJM63) was electroporated into smooth *Brucella*, homologous recombination occurred, and no Tn5 random integration was observed. The plasmid pJM63 contains Tn5 with 2.5 kb and 2.1 kb flanking region, while pNW-2 contains only 300 bp and 850 bp outside the Tn5 element. It is possible that reciprocal DNA exchange is favored when longer homologous sequences are used.

To avoid the Tn5 movement, the transposase coding region in Tn5 and middle sequence was replaced by Kanamycin resistant gene from Tn5 in pNW-2. When this nonreplicating plasmid (pNW-3) was electroporated into *B. abortus* 2308, no recombinant exhibited a rough phenotype. The restriction pattern and the Southern analysis of the chromosomal DNA from these recombinants using Tn5 as a probe revealed that homologous exchange happened between the plasmid and the genome (Figure 16). Thus, it is possible to conclude that the gene disrupted by the KanR gene in the recombinants is the same one as in strain RA2. If one compares the strength of the hybridization signal in the *Eco*RI fragment to that in the *Eco*R I/*Nhe* I fragment using the flanking region probe, the signal in *Eco*R I/*Nhe* I is stronger (Figure 17). One explanation is that the increased signal is the sum of both *Eco*R I fragment signals in these recombinants. However, both in strain RA2 and strain 2308, the signals show the same intensity in either the *Eco*R I fragment or in *Eco*R I/*Nhe* I fragment (Figure 17). The fact that Tn5 was located in same fragment in these recombinants as in strain RA2 and that the sequence data analysis of flanking region did not show any homology with other sequence in the gene bank suggest that the gene Tn5 interrupted may not be related to LPS biosynthesis. However, both the 4.2 kb and 1.3 kb fragments hybridized with flanking region probe (Figure 17). The Southern blot and the

comparison of the hybridizing signal (Figure 17) suggested that these two fragments share homology, however, one interrupted by Tn5 and one kept intact in the recombinants. The repeat insertion sequence in *Brucella* spp. is not unique (17, 18); *E. coli* and *S. typhimurium* also contain different repeat insertion sequence as well as in other bacteria (14). Usually these repeat sequences in the genome are involved in DNA rearrangement and deletion. Interestingly, only one 7.0 kb fragment hybridized with the flanking region probe (Figure 17), no other signal could be detected. Regular agarose gel electrophoresis (Figure 18) and pulse field gel electrophoresis (John, McQuiston, unpublished data, VPI&SU) results show that only one Tn5 element was detected in RA2 genome. It is possible that a second copy of Tn5 is located nearby the region in which the first copy of Tn5 inserted into the genome. Thus the homologous rearrangement of Tn5 fragments could cause the deletion of the sequence between these two copies of Tn5 in strain RA2 (Figure 20)(14). This also explains the presence of one copy of the repeat sequence and one copy of Tn5 element in strain RA2.

It is possible the rough phenotype of strain RA2 may be caused by an indirect event (eg. Tn5 induced polarity) instead of the Tn5 insertion. It has been reported that P1 phage prefer adsorbing to rough strains rather than smooth strain (46). Thus, it is possible that Tn5 transposition occurred in

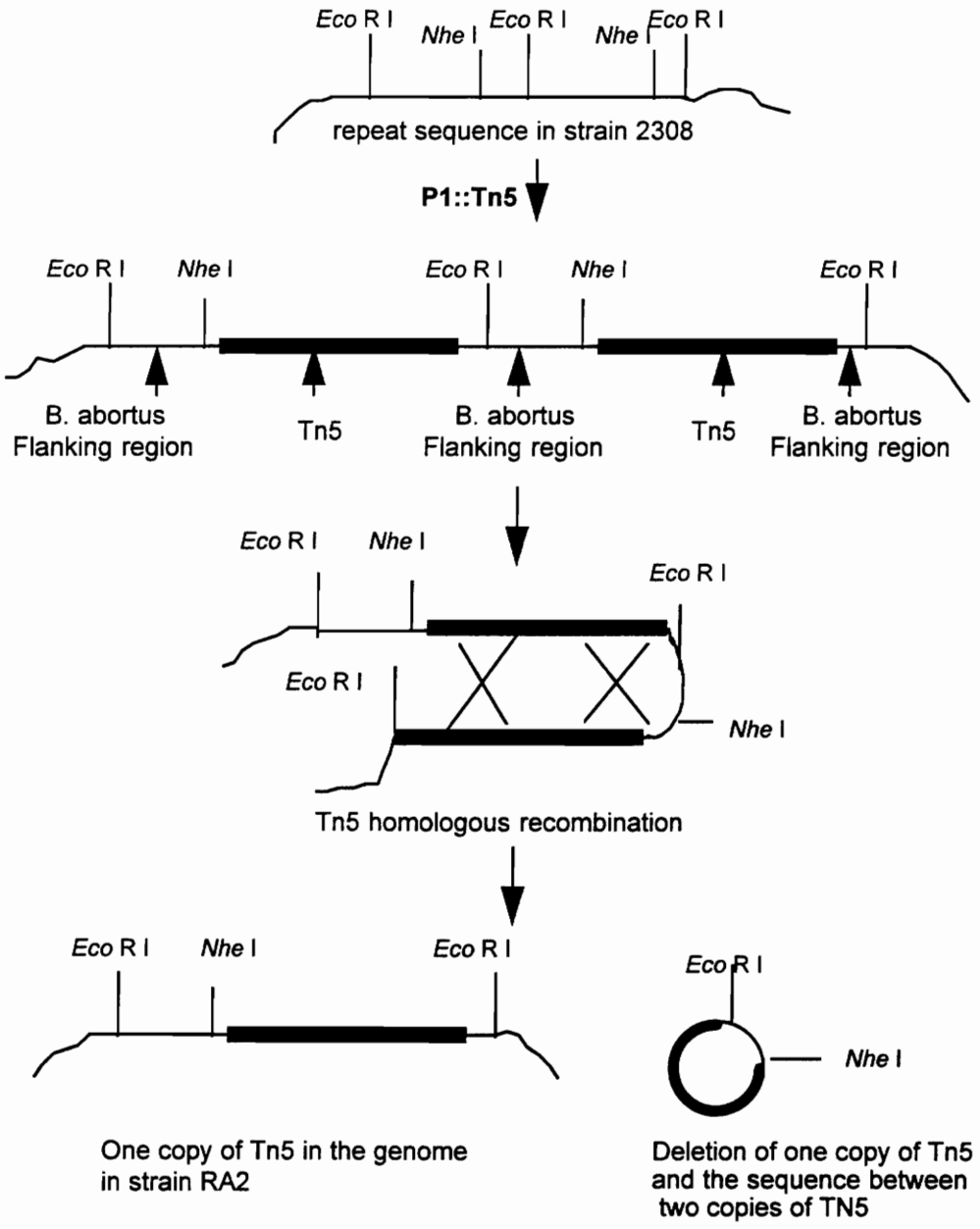

Figure 20. Possible model for only one copy of the repeat insertion sequence existing in strain RA2.

a *B. abortus* 2308 pre-existing rough mutant. This thesis work supports the possibility that an as yet to be defined mutation is responsible for the rough phenotype of strain RA2.

Although the best condition for electroporation in this study is similar as that Lai et al. reported (25), the absolute number of kanamycin resistant colonies in this study was less. The exact mechanism or reason for the lower number of KanR clones obtained with pNW-2 or pNW-3 relative to pSUP2021 (Lai et al., 25) is not known. However it is possible to speculate that the *Brucella* DNA sequences on pNW-2 or pNW-3 limit the sites of integration wherease no such sequences are present on pSUP2021. Thus it is reasonable that the absolute number of kanamycin resistant colonies obtained with pNW-2 or pNW-3 is less than that reported by Lai et al. (25).

It is most likely that the gene interrupted by Tn5 in strain RA2 is not related to LPS biosynthesis. Alternatively, it is possible that one of the repeat sequence in the genome next to the Tn5 element is somehow involved in rough phenotype. To see if candidate genes are present and subject to transposition or their expression is affected by Tn5 (eg. polarity), regions further downstream of Tn5 in RA2 need to be cloned and sequenced.

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Curriculum Vitae

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