CLONING OF A REGION IN THE BRUCELLA ABORTUS CHROMOSOME NECESSARY FOR O-SIDE CHAIN BIOSYNTHESIS

by

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Thesis submitted to the Faculty of Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

MASTERS OF SCIENCE

in

Veterinary Medical Sciences

Approved by:

[Signatures]

November, 1992
Blacksburg, Virginia
Abstract

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As a first step in characterizing the genes involved in O-side chain synthesis in Brucella abortus strain 2308, a portion of the genomic DNA was cloned from a rough mutant created by Tn5 (KnR) mutagenesis. This mutant was rough based on the lack of reactivity by either whole cells or extracted LPS to an O-side chain monoclonal antibody (BRU-38). A 30 kb XbaI genomic fragment (including Tn5) from the rough strain was subcloned into a sequencing vector to create pJM6. When B. abortus 2308 was electroporated with pJM6, KnR clones were unable to react with BRU-38; a Southern analysis of these clones revealed Tn5 in the 30 kb XbaI genomic fragment. Various regions of the 30kb fragment were subcloned and tested for their ability to complement specific rfa and rfb mutants of Escherichia coli and Salmonella typhimurium. One particular DNA fragment complemented an rfbD mutation in E. coli as judged by agglutination with E. coli anti-O (0:85) serum. The same DNA fragment failed to cause E. coli rfbD to react with either BRU-38 or B. abortus anti-O polyclonal antisera. The B. abortus 30 kb XbaI fragment contains a gene which has been identified by complementation as containing the equivalent of the rfbD gene encoding dTDP-rhamnose synthetase in E. coli. Since Brucella is not known to have rhamnose in its' core this enzyme may have a different function in Brucella LPS synthesis.
Acknowledgements

I thank all those involved in helping with the research and writing in this thesis. I am very grateful to Dr. Stephen Boyle for his patience and willingness to help throughout my years at VPI and SU, and also for not giving me a fish, but instead teaching me how to fish, this indeed was a very valuable lesson. I thank Dr. Nammalwar Sriranganathan for his help and understanding, and pulling me up when the pressure of school tried to push me under. I thank Dr. Gerhardt Schurig for his help and knowledge in the immunological aspects of my thesis. I thank Dr. Tom Inzana for his help with the LPS analysis. I am deeply grateful to all the other students, faculty and staff in the Veterinary Microbiological Research Laboratories especially Lynn McGonagle (Jablonski), Bob Moore, and Arden Bond without whom, I doubt I would have made it this far.

I would especially like to thank my family: Mom, Dad, and Colleen, for their continued moral and financial support; they were always there for me when times got rough.

This research was supported by Animal Health and Disease grant 1-37117 to S.M.B. and by a graduate research assistantship to J.R.M. from the Virginia-Maryland Regional College of Veterinary Medicine.
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List of Abbreviations:

u = unit (of enzyme defined by the manufacturer)

kb = kilobase pair

ml = milliliter

ul = microliter

M = molar

mM = milliMolar

g = gram

mg = milligram

ug = microgram

mm = millimeter

V = volts

RT = room temperature (22-25°C)

UV = ultraviolet light

EtBr = ethidium bromide

TE = 10 mM Tris, 1 mM EDTA

TBE = 0.089M Tris, 0.089M Boric acid, 0.002 M EDTA

EtOH = ethanol

SDS = sodium dodecylsulfate

amp = ampicillin

kan = kanamycin

DNA = deoxyribonucleic acid
Literature review

*Brucella abortus* strain 2308 is one of the causative agents of human and animal Brucellosis. Brucellosis was originally called Bang's disease for B. Bang of Denmark. It has also been called Malta fever as it was originally described in humans on the Mediterranean island of Malta; and undulant fever because of the characteristic 12 to 16 hr cyclical fever associated with the clinical symptoms. Early in the twentieth century the name was changed to Brucellosis for the discoverer of the causative agent of the disease, David Bruce. Descriptions of the disease date back to the time of Hippocrates around 500 BC and early Greek history [12]. Currently, the number of human cases of Brucellosis reported in the United States of America is approximately 80 to 90 cases per year [38]. However, this does not reflect the current incidence in the developing nations of the world. For instance, in 1988, in Iran, over 71,000 people were afflicted with this disease [44]; this incidence is more than 1:1,000 per capita which is considered an epidemic. Although the incidence of human brucellosis in Iran is the highest reported, the number of cases in other countries in the Middle East are similarly high. Since the war in the Middle East in 1991, the incidence of Brucellosis has probably increased. Brucellosis is also widespread in Mexico and almost all the South and Central American countries as well as the Far East
including India, China and other Asian countries.

Humans contract this disease from infected animals, primarily from the consumption of unpasteurized milk from cows or goats, or from eating infected meat that hasn't been thoroughly cooked. The clinical symptoms of Brucellosis in humans varies; it is characterized by a severe cyclic fever (101-104°F) reoccurring every 24 hours. Also, anorexia, arthritis, weakness, lethargy, and depression are commonly seen clinical signs. In some severe cases, Brucellosis can become chronic or even fatal [36].

In cattle and other susceptible animals to the disease, the symptoms are less severe with the primary effect being abortion in cattle, goats and camels. The number of cases of infected animals are believed to be very high, especially in the Mediterranean region [43]. Animal infection is the problem that needs to be solved since the primary mode of transmission is from animals to humans.

_E. abortus_ is the causative agent of Brucellosis along with _B. melitensis, B. suis, B. canis_, and _B. ovis_. All of these species are very closely related physiologically, with varying degrees of pathogenicity primarily depending on the host that is infected. The species name comes from the primary host that Brucella infects with the exception of _B. melitensis_ [24], which primarily infects sheep and goats, named for the place of its' original discovery, the
named for the place of its' original discovery, the Mediterranean. The Brucellae are Gram negative coccobacilli and classified as facultative intracellular pathogens. They can survive in the macrophage, a phagocytic cell of the immune system. How the Brucella survive is still a mystery; however, there are a number of mechanisms thought to be operative including protection afforded by lipopolysaccharide (LPS) [5,7,28,29,30]; and possibly enzymes such as superoxide dismutase which protect against oxygen radicals [3,4,9,13,37].

One of the principle differences between smooth and rough Gram negative bacteria has been known for decades to correspond to differences in virulence; smooth bacteria are generally more virulent than rough [30]. This smoothness is due to the O-side chain on a large macromolecule in the outer cell membrane known as LPS (Fig. 1). B. abortus RB51, a rough mutant, exhibits a reduction in virulence when assessed in a mouse spleen clearance model [33]. This dramatic reduction in virulence has also been shown in the Gram negative Salmonella as a result of a mutation in any of the rfa or rfb genes, the two principle loci involved in LPS biosynthesis [5]. Studies with B. abortus RB51 suggests that LPS is an important virulence factor [33].

To study the biosynthesis of Brucella LPS and the genes responsible, it is useful to examine the Salmonella in which the structure, function and genetics of LPS has been
thoroughly documented and can serve as a model. The LPS is anchored in the outer cell membrane by a large lipid molecule, called lipid A. The lipid A is generally the most conserved region of the LPS molecule whose structure is very similar within the family of Enterobacteriaceae. The lipid A is thought to be primarily made up of a β-1,6-D-glucosamine disaccharide backbone with 1,4' phosphate linkages [34]. Attached to the 2,2' amino groups are 3-acyloxyacyl type fatty acids which are very hydrophobic and anchor this large structure in the cell membrane (Fig. 2). On the terminal glucosamine residue is a phosphate molecule to which the core sugars are attached. In the LPS molecule of Salmonella the core consisting primarily of D-glucose, D-galactose, N-acetyl-D-glucosamine, L-glycero-D-manno-heptose and 3-deoxy-D-manno-2-octulosonic acid (KDO) [18], is a slightly more variable region; its' primary purpose appears to serve as the attachment site for the O-side chain. Mutants devoid of O-side chain and core sugars are viable whereas mutations affecting the KDO region produce nonviable cells [27]. The O-side chain is the highly variable region of the LPS molecule responsible for the different O-serotypes of a species. The loss of the O-side chain is responsible for conversion from a smooth to a rough phenotype and consequently a decrease in virulence of the strain. Made up of repeating oligosaccharide units, the O-side chain varies between one oligosaccharide to
number of units being determined by the recently identified rol gene [7]. The three regions of LPS are primarily synthesized and assembled by the products of genes clustered at three different loci in the Salmonella chromosome. The rfa cluster contains the rfa B, C, D, E, F, G, H, I, J, K and L genes responsible for the synthesis and attachment of the core region to lipid A [8]. After the intact core is synthesized, and an enzyme (believed to be the rfaK gene product) has attached the N-acetyl-glucosamine (N- Ac- Glc) branch to the core, the rfaL gene product attaches the first O-polysaccharide subunit to the core [22]. Now the rfb and rfc genes are induced to produce and polymerize the O-side chain. The rfb cluster contains eleven genes, rfb A, B, D, F, G, H, K, L, M, N and two monosaccharide transferases that have yet to be definitively identified. All of these components are involved in the synthesis of the O-polysaccharide subunits [22] (Fig. 3). Although it is not yet known how these rfb genes are transcriptionally regulated, they are believed to be regulated much like the rfa genes. Some of the rfa genes are organized in an operon and are induced by an activator protein, for example; the rfaH gene encodes a protein which positively regulates the rfa genes [22]. To assemble the oligosaccharide subunits, the monosaccharides are attached individually to a undecaprenol carrier. This whole process takes place in the inner membrane where the core sugars are
carrier. This whole process takes place in the inner membrane where the core sugars are also attached to the lipid A molecule. Adding the first O-side chain subunit is believed to be a very complex reaction because of interaction of an intact core containing an intact N-Ac-Glc branch to an intact O-side chain subunit; this is mediated by two different enzymes, the glycosyl transferases encoded by *rfaL* and *rfbT*. These two enzymes bring together the two O-side chain subunits and, in what is believed to be a dephosphorylation reaction, joins them together [22]. After this assembly occurs the *rfc* gene product polymerizes the rest of the O-side chain subunits; the number of subunits is controlled by the *rol* gene mentioned above.

*Brucella* LPS has not been studied at the genetic level nearly as extensively as *Salmonella* LPS. However, the chemical composition of the lipid A region is known while its fine structure is not. The lipid A is composed of 2-amino-2-deoxy-D-glucose (10.1%), n-tetradecanoic acid (12%), n-hexadecanoic acid (33%), 3-hydroxytetradecanoic acid (27%), and 3-hydroxyhexadecanoic acid (4%) [8]. Assuming that most of the mannose found in the LPS analysis is part of the O-side chain subunits, the core appears to be primarily made up of glucose [9]. This analysis determined the percentage of glucose to be equal to mannose in the total LPS with quinovosamine at about half the percentage of either of the
carbohydrates. The amount of glucosamine and KDO have not been determined in the core region of *B. abortus* [25]. Extensive work has been done on the composition and structure of the O-side chain of both *B. abortus* and *B. melitensis*. It was reported by Wu et. al.[43] that the O-side chain represents 99.5% of the *Brucella* antigens recognized by a host infected with smooth strains, as determined by absorption of infected host antibodies by protein free LPS. However, this figure appears high if one considers the reactivity of polyvalent sera to *B. abortus* 2308 with non-LPS associated antigens [33]. It is thought however, that the O-side chain is a major component of the cell's surface molecules that is seen by a host and therefore the immunodominant molecule of *Brucella* [42]. For many years it was assumed that the two predominant antigens termed A(abortus) and M(melitensis) were very different structures on the surfaces of these two *Brucella* species (Fig. 4a&amp;b). After biochemical and nuclear magnetic resonance spectroscopic analysis it was discovered that both the A and M antigens were LPS structures varying only slightly between species. The O-side chain of *B. abortus* is a homopolymer of a 1,2 linked 4,6-dideoxy-4-formido-α-D-mannopyranosyl subunits usually averaging between 96-100 subunits in length [8]. In comparison, this may appear to be much longer than the O-side chains of *E. coli* or *Salmonella* which average about 30 subunits per chain. However, since the
*Brucella* oligosaccharide subunit is a monosaccharide and those in *E. coli* and *Salmonella* are trisaccharides, the 0-side chains average approximately the same length. The difference between the A and M antigens is the addition of a 1,3 linkage for every four 1,2 linkages in the 0-side chain of the M antigen (Fig. 4a) [6]. The M antigen is the predominant form of the 0-side chain in *B. melitensis* and the 1,2 linked homopolymer A antigen is the predominant form in *B. abortus* (Fig. 4b). However, both the A and M form are found in each of the species. The reason for this additional 1,3 linkage in the M antigen is still unknown but one possibility is that it is involved in virulence. For example, Chang et. al. [10] reported that intracellularly grown *Brucella* appeared to produce an M antigen preferentially over the A form.

**Rationale for thesis:**

Lipopolysaccharide plays an extremely important role in Gram negative bacterial virulence. As LPS is the primary component of the outer membrane, much vaccine research has focused on LPS as an immunoprotective antigen. It has been shown that a loss of virulence is correlated with the loss of the 0-side chain in the LPS [30]. *Brucella* is no exception as the loss of an intact 0-side chain results in a dramatic decrease in the virulence of *B. abortus* [33]. Since the LPS is a virulence factor in *B. abortus* any information on the genetic basis for the synthesis and regulation of LPS would be
possible to extrapolate information concerning the genetics of B. abortus LPS to other species of Brucella ie. B. ovis, B. melitensis, B. suis and B. canis. For example, would rough mutant (O-side chain deficient) strains of other Brucella species be as avirulent as B. abortus RB51? The objective of this thesis was to identify regions of the B. abortus genome containing the genes responsible for LPS biosynthesis, particularly the O-side chain, in order to further our understanding of Brucella's virulence.

Materials and methods

Strains:

Strains used in this research are listed in Table 1. E. coli and S. typhimurium strains were grown at 37°C in either Luria-Bertani (LB; 1% tryptone, 0.5% yeast extract, 0.5% NaCl) media [23], Terrific Broth (TB; 1.4% tryptone, 2.7% yeast extract, 0.4% glycerol, 17mM KH₂PO₄, 72mM K₂HPO₄) [40] or SOB (2.0% tryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM MgCl₂, 10mM MgSO₄) [14].

Strains containing plasmids were grown on selective media containing antibiotics at one of the following concentrations: ampicillin (amp) 100ug/ml, kanamycin (kan) 25ug/ml.
Enzymes and Reagents:

Restriction endonucleases, calf intestinal alkaline phosphatase (CIAP), T4 DNA Ligase, Bluo-Gal (halogenated indolyl-β-D-galactopyranoside) and agarose were obtained from Bethesda Research Laboratories (Gaithersburg, MD). All other chemicals were obtained from Sigma Chemical Corporation (St. Louis, MO) unless otherwise stated. Incert and NuSieve low-melting point agarose were purchased from FMC Bioproducts (Rockland, ME). The Genius Kit was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN) and was used for labeling all DNA probes used in all Southern hybridizations.

Plasmids:

All plasmids in this research were isolated by the procedure described by D. Ish-Horowitz and Burke [17] unless otherwise stated.

Construction of pJM6:

The plasmid pJM6 was constructed by cloning an XbaI digest of the B. abortus RA1 (3A5) chromosomal DNA into the XbaI site in the cloning vector pGEM-3Z (Promega Corp. Madison, WI). The specific 30kb insert was identified by restriction enzyme analysis and Southern analysis.

Nucleic acid digests:

Chromosomal: One 3mm wide Incert agarose (FMC Bioproducts) plug of B. abortus RA1 DNA (see Genomic DNA protocol for DNA preparation) was digested and washed once in
100μl of TE or sterile dH2O for 30 min. and once in 100μl of 1x reaction buffer (10x REact 2 buffer, Bethesda Research Laboratories, Gaithersburg, MD) for 30 min. The buffer was replaced with fresh 1x reaction buffer and the 40 units of XbaI were added (see PFGE protocol). The digest was incubated overnight or at least 16hr at 37°C to ensure total digestion of the DNA.

Vector: 2μg of pGEM-3Z was digested with 10 units of XbaI in a 20 ul reaction including: 2ul of 10x REact 2, 4ul pGEM 250ng/ul and 1ul XbaI (10u/ul) and H2O; the reaction was incubated overnight at 37°C. Three ul of the reaction was electrophoresed on a 1% agarose gel (30 min. at 80V) to assess the extent of digestion. Two units of CIAP was added to the reaction to decrease the probability of vector religation by removal of the terminal 5' phosphate. Two 0.5ul aliquots (20u/ul) of CIAP were added to the vector at 0 and 30 min and incubated at 37°C for 1 hr to ensure total dephosphorylation. To remove any protein, salts or enzyme, a phenol/chloroform extraction [17] was performed; the DNA was reprecipitated with 3 volumes of 95% EtOH and resuspended in 15 ul of dH2O.

**Chromosomal DNA Ligation:**

The digested chromosomal DNA plug (see below) was melted at 65°C for 10 min and slowly cooled to 40°C. One unit of Agarase (Boehringer-Mannheim Biochemicals, Indianapolis, IN) was added and the incubation was continued for 4hr at 40°C.
B. abortus RA1 chromosomal DNA was ligated into pGEM-3Z in a 50ul reaction containing: 10ul of 5x T4 DNA Ligase Buffer, 10ul of chromosomal DNA (approximately 1ug), 5ul of vector DNA (approximately 500ng DNA) and 3ul of T4 DNA Ligase (1 unit/ul) and 22ul of dH₂O. The reaction was incubated at RT for at least 16 hrs.

**Transformation:**

Electroporation was used to introduce plasmids into E. coli and Salmonella. The procedure [1] used the BTX transfector (model 100 BTX Biotechnologies and Experimental Research, Inc., San Diego, Ca.) for electroporation using the following conditions: amplitude = 625V, pulse time = 5ms, and electrode gap = 0.5mm. The electroporation protocol to introduce pJM6 into B. abortus was as described by Lai et.al. [21].

**CaCl₂ Transformation:**

All transformations specified as CaCl₂ were transformed using the procedure described in Ausebel et. al. [1] substituting Hepes (N-2-Hydroxyethylpiperazine, N'-2-ethanesulfonic acid) for Pipes (Piperazine-N,N'-bis[2-ethane sulfonic acid]).

**Ligations:**

All ligations except those involving chromosomal ligations were carried out in a 20ul reaction volume containing: 4ul of T4 DNA Ligase 5x buffer, 300ng of vector
DNA, 500ng of insert DNA, and sterile dH₂O. The reaction was incubated at RT overnight and used to transform competent cells by the CaCl₂ procedure.

In Gel ligations:

Approximately 500ng each of the vector and insert were cut with the appropriate restriction enzymes. If dephosphorylation was required, 1 unit of CIAP and 2ul of CIAP buffer were added to 17ul of the reaction and incubated at 37°C for 2hrs.

Both vector and insert were separated by electrophoresis on a 1% NuSieve Agarose Gel in 1x TBE buffer with 0.10 ug/ml EtBr at 80V until the fragments were resolved. The DNA fragments were visualized under UV light and photographed if necessary. An agarose slice containing the DNA fragment was cut out of the gel and placed in a microfuge tube and heated to 68°C for 10 min. The molten gel was slowly cooled to 40°C into which 1 unit of Agarase per 100 ul of agarose was mixed; the mixture was incubated for 3 to 18hrs at 40°C. Judging from the concentration of the DNA in the band and the approximate volume of the gel slice, the amount of DNA/ul was estimated to be a particular concentration for the ligation.

In an 80ul reaction volume: 16ul 5x T4 DNA ligase buffer, 20ul (approximately 300ng DNA) of vector, and 20ul of insert DNA were added to 3-5 units of T4 DNA ligase (1 unit/ul) and 17-19ul of sterile H₂O. The reaction volume was brought up to
80ul with sterile dH$_2$O. The entire reaction volume was incubated at RT for 16 hr and used to transform CaCl$_2$ competent E. coli cells which were then screened for the appropriate marker.

**Genomic DNA Preparations:**

Agarose plug preparation: This procedure was used to avoid shearing of the intact chromosome during physical and chemical manipulation of the DNA. *B. abortus* or *B. melitensis* was grown to a density of 70-80 Klett units from an overnight culture in Trypticase Soy Broth (TSB) (Difco Laboratories Detroit, MI). In the case of rough Brucella strains, such as RA1, cultures were grown to 60 Klett units as this strain had 25% more cells/Klett unit than a smooth strain. The cells were harvested by centrifugation at 3400xg for 15 min, resuspended and washed in a volume of sterile TE equal to the original culture volume, harvested by centrifugation, and resuspended in 1/10 the original culture volume of TE or sterile H$_2$O. The suspension was mixed with an equal volume of 1% Incert agarose boiled previously, to give a final concentration of 0.5% agarose and kept molten at 65°C until ready for use. The mixture of cells and agarose was pipetted into a plug mold (BioRad Laboratories, Melville, NY) and allowed to solidify at 4°C for 10 min. The plugs were placed into 2x volume of solution I, (handle carefully, as these are fragile) (500ul of solution I for every plug), and incubated
for 24 hr at 37°C with rotary shaking at no more than 100rpm. Solution I was removed carefully and replaced with the same volume of Solution II and incubated at 37°C for 48 hrs. Solution II was replaced with Solution III and incubated for 3 hrs; this step is repeated. Finally, Solution IV (which is also the storage solution) was used to replace solution III. To ensure that all of the Brucella have been killed, one plug from each vial was placed into 3 ml of TSB media and shaken at 180 rpm for 72 hrs at 37°C and checked for growth. Solution I: 10 mM Tris-HCl, pH 7.5, 1M NaCl, 100 mM EDTA, pH 7.5, 0.2% deoxycholate, 0.5% Sarkosyl, 20 µg/ml DNase free RNase, 1 mg/ml Lysozyme. Solution II: 100 mM EDTA, pH 8.0, 1% Na-lauryl sarcosine, 1 mg/ml Proteinase K. Solution III: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0, 1 mM PMSF (phenyl methyl sulfonyl fluoride). Solution IV: 10 mM Tris-HCl, pH 8.0, 100 mM EDTA, pH 8.0

CTAB Genomic DNA extraction:

This protocol was used when shearing of the chromosome was not a concern (ex. in a Southern analysis). The CTAB (Hexadecyl-trimethylammonium bromide) protocol was adapted from Ausebel et. al. [1] with the following modifications: 1) Instead of saturated broth cultures, 6 Trypticase Soy Broth (Difco Laboratories, Detroit MI) agar plates were spread with bacteria to cover the agar surface. These cultures were incubated at 37°C for 4-8 days. The bacteria were harvested by lightly dragging a glass coverslip over the agar surface.
These bacteria were suspended in 6ml of TE and collected by centrifugation. 2) During the phenol extraction it was necessary to centrifuge the sample for up to 3 hr at 15,000 rpm to achieve a tight interface and a clean aqueous layer. 3) 95% ethanol was used instead of isopropanol for DNA precipitation. 4.) The DNA pellet was air dried overnight in a hood to make resuspension easier as vacuum drying caused the pellet to become too compact and slowed resuspension. 5) The DNA pellet was resuspended in 500ul of TE at 60°C for 2hrs.

**Pulse Field Gel Electrophoresis (PFGE):**

Preparation of the DNA for PFGE is described above in the section entitled agarose plug preparations. To prepare the DNA for enzyme digestion, one 3mm wide agarose plug (approximately 500ng of DNA) was washed for 30 min at 37°C in 100ul of appropriate 1x reaction buffer (10ul buffer (10x)+ 90ul of dH₂O). The buffer was drawn out and replaced with 10ul of 10x reaction buffer, 85ul of dH₂O. The enzymes were added in the following amounts for complete digestion of the chromosomal DNA: *Xho I* - 60 units, *Xba I* - 50 units, *Nhe I* - 75 units (New England Biolabs Inc., Beverly, MA), *Spe I* - 70 units. The amount of DNA in the plugs was titrated with respect to the units of each restriction enzyme in order to ensure complete digestion of the DNA. The optimum amount of DNA per digest was found to be extremely low; approximately 500 ng for each 100ul digestion described above. For example, the amount of DNA in
the digestion, and consequently in each lane on a pulse field gel, was barely visible when visualized under UV light after EtBr staining.

The DNA was digested for a minimum of 16 hrs at 37°C or at room temperature; either temperature gave equally digested DNA. The enzyme and buffer were removed and the plug was melted at 68°C for 5-10 min. The molten agarose was carefully pipetted into the dry wells of a 1 to 1.5% agarose gel, depending on desired size resolution. Concatamerized lambda DNA (High molecular weight standards (FMC Bioproducts) were cut and placed carefully into the appropriate well(s). The gel was then set into the pulse field chamber (CHEF DRII Pulse Field Electrophoresis System, BioRad Laboratories, Melville, NY) and covered to a depth of 2 to 4 mm with 0.5x TBE buffer. The following parameters were used to resolve the various sizes of DNA fragments.

Resolution:

50-150 kb

200V 1.5% gel

14°C 8-4 sec. pulse.

Southern analysis:

To transfer the DNA from the gel to a Nytran membrane (Sleicher and Schuell Inc., Keene, NH), a capillary transfer method [35] was used with 20xSSC (3M NaCl, 0.3M Na₂citrate, pH 7.0) as the buffer medium. The minimum time needed for
complete transfer of the large fragments (> 150kb) of DNA from a pulse field gel was determined to be 48hrs. For horizontal gels with fragment sizes smaller than 20 kb, 24 hr was sufficient time for complete transfer. The DNA was UV crosslinked to the membrane with a Stratalinker (Stratagene Cloning Systems, La Jolla, CA) and allowed to dry. The membrane was stored at 4°C until needed.

**DNA-DNA Hybridization:**

Non-radioactive probes were prepared and hybridized using the Genius kit and accompanying procedures from Boehringer Mannheim Biochemicals (Indianapolis, IN).

**Complementation studies:**

To assay for complementation of the *rfa*, *rfb* or *rfc* genes, the mutant strains of *S. typhimurium* were transformed with the plasmids containing the putative O-side chain genes: pJM6, pJM63, pJM5, and pJM51 (Table 3). The transformed strains were subjected to plasmid isolation to ensure that they contained the plasmid. Each strain was tested by phage analysis (see below) for conversion from rough to smooth or production of O-side chain. Some strains were further tested with monoclonal antibodies Bru 38 and Bru 28 for production of specific *Brucella* O-side chain epitopes.

**Phage analysis:**

*Salmonella* mutants transformed with the plasmids, pJM6, pJM5, pJM63, and pJM51 were cultured overnight in
TB/ampicillin to an approximate cell density of 10⁹ cfu/ml. 100ul of the cell suspension was spread uniformly onto the surface of LB/amp plates and allowed to air dry in a class II containment hood. 5ul of each of the three bacteriophages, F(0), Ffm, and P22c2, (each at a titer of 10⁸ phage/ul) was carefully placed on the cells and allowed to dry. The plates were incubated at 37°C for 16 hrs. Lysis was assessed by determining whether the formation of a clear plaque occurred in the cell lawn.

**Antibody reactions:**

Colonies containing the above plasmids were grown on LE/amp plates. A circular piece of nitrocellulose membrane the size of the petri plate was placed gently on the colonies. After the membrane became completely wet, it was lifted carefully off the plate and allowed to dry. As controls, 5ul each of acetone killed strain 2308 and RB51 were spotted separately on the membrane and allowed to air dry. The membrane was placed in 15ml of chloroform for 15min, removed and allowed to air dry. The cell material excluding the LPS on the membrane was digested with 10ml of blocking/digestion buffer (0.15M NaCl, 0.02M Tris, 0.005M MgCl₂ pH 7.5, 2ug/ml DNase, 80ug/ml lysozyme and 3% BSA) for 1 hr at RT on a rotary shaker. This procedure left the crude LPS and cell material bound to the membrane which was washed 5 times with TBST (0.5M NaCl, 0.02M Tris, pH7.5, 0.05% Tween 20). The washed membrane
was incubated at RT with 20 ml of a 1:10 dilution of the primary antibody (e.g. Bru38) for 1 hr with shaking. Again the membrane was washed 5 times with TBST and incubated with the 20 ml of the secondary antibody (i.e. goat anti-rat IgG conjugated with horse radish peroxidase; Cappell Research Products, Durham, NC) at a 1:500 dilution. The membrane was washed 5 times with TBST and placed in a developing solution containing 6 mg/ml of 4-chloro-1-napthol in methanol, combined with 100 ml of TBS (TBST - Tween) and 0.6 ml $H_2O_2$. A positive reaction was indicated by a purple color change.
Results

Identification of rough *B. abortus* strain RA1:

When surveyed by the colony blot procedure utilizing Bru 38 (a monoclonal antibody to the *Brucella* O-side chain) 10 Kan resistant *Brucella* clones containing the Tn5 element [21] had decreased ability to produce O-side chain. One of these clones 3A5 (later named RA1) had lost the ability to produce O-side chain as judged by the inability of the strain to react with Bru 38 in an immunoblot analysis of crude LPS. This lack of O-side chain was verified by demonstrating the uptake of crystal violet by rough colonies. A smooth colony repels the dye and remains white and glossy [31]. A colony immunoblot was performed with monoclonal antibodies Bru 38 (specific for the O-side chain) and Bru 48 (specific for the core) on the putative rough colonies; smooth strain 2308 was used as a positive control. *B. abortus* RA1 exhibited no reactivity to Bru 38, while it did react with Bru 48. These results were consistent with the loss of the O-side chain in *B. abortus* RA1, since the O-side chain in the smooth strain *B. abortus* 2308 blocks access to the core by Bru 48.

LPS analysis:

In order to assess the degree of the roughness of strain RA1, an LPS analysis was performed. LPS was extracted by a phenol/water extraction method [24], separated by SDS-PAGE and visualized by silver staining (Fig. 5). The silver stained
gel showed no *Brucella* O-side chain associated with RA1 when compared with strains 2308, RB51 and *Salmonella typhimurium*. It is noteworthy that the LPS of strain RA1 shows a configuration much like that associated with the rough strain RB51. The reason for the shift in the LPS near the bottom of the gel is not yet known although one possible explanation is that a change in the core size has occurred. Since the monoclonal antibody Bru38 does not react with either of these bands in a western analysis (data not shown), it is reasonable to conclude that the bands are part of the core and not an incomplete O-side chain subunit.

**DNA analysis:**

In order to clone the genes involved in *Brucella* LPS synthesis, the Tn5 element in strain RA1 was located by physical mapping of the chromosome utilizing pulse field gel electrophoresis. The Tn5 element (identified by Southern analysis) was located within a 30kb *XbaI* band; this *XbaI* band was within a 256kb *XhoI* band (Fig. 6). Since *XbaI* does not cut the Tn5 element [19] this restriction enzyme was used to digest the chromosome of *B. abortus* strain RA1 and produce a fragment containing the Tn5 and the flanking regions. This fragment was ligated into the *XbaI* site of pGEM3-Z to create pJM6 (Fig. 7). A restriction enzyme digestion of pJM6 and analysis using the known *XhoI* and *XbaI* sites verified the successful cloning of this *XbaI* fragment. Digestion of pJM6
with XhoI produced 2.3kb and 2.5kb fragments corresponding to the three enzyme sites within the Tn5. In a Southern analysis of *B. abortus* RA1 DNA using Tn5 as a probe, it was shown that the closest XhoI sites were 64kb and 193kb from either side of the Tn5 element. It was suspected and confirmed by restriction enzyme mapping that there were no XhoI sites in pJM6 other than the sites in the Tn5 element. Therefore, the third fragment generated by an XhoI digestion of pJM6 represented the *Brucella* DNA flanking the Tn5 along with the vector pGEM-3Z; the total size of this fragment was estimated to be 25.2kb. Digestion of pJM6 with XbaI produced a 2.7kb fragment corresponding to pGEM-3Z and the expected 26.3kb *Brucella* insert. Southern hybridization using both the Tn5 element and *Brucella* chromosomal DNA as probes, further confirmed pJM6 contained both the Tn5 and the flanking genomic DNA from *B. abortus* RA1 (data not shown). A restriction enzyme analysis was performed on pJM6 and a restriction map generated (Fig. 7). Expression of the genes on pJM6 apparently has a toxic effect on the host since *E. coli* DH5α transformed with pJM6 has an average life of 6 to 7 days on LB/amp agar and 9 days on SOB/amp agar; compared to *E. coli* DH5α which has a lifespan approximately 3 weeks on LB agar and about 6 weeks on SOB agar. The toxic effect is presumably due to the high copy number of pGEM (40-50 copies per cell) and a product produced by the insert. Because of this toxicity,
genomic fragments from pJMJ6 were subcloned to produce pJMJ61, pJMJ62, and pJMJ63 (Fig. 8).

**Conversion of *B. abortus* 2308 from a smooth to rough phenotype:**

If the fragment of *Brucella* DNA in pJMJ6 or pJMJ63 containing the Tn5 element is responsible for loss of O-side chain production in *B. abortus* strain RA1, then recombination of these plasmids into the wild type strain 2308 chromosome should cause strain 2308 to convert from smooth to rough. To examine whether this was true, *B. abortus* 2308 was transformed with either pJMJ6 or pJMJ63 and screened for homologous recombination of the flanking regions into the genome of *Brucella abortus*. Putative recombinants were identified by the conversion of strain 2308 from Kan and Streptomycin (Str) sensitivity to resistance to these antibiotics i.e. from the corresponding ntr and neo genes encoded on the Tn5 element. The vector of pJMJ6 (pGEM3-Z) encodes β-lactamase (amp resistance). Since a double crossover of the genomic insert in pJMJ6 into the *Brucella* chromosome caused the exclusion of the vector, strain 2308 sensitivity to ampicillin was maintained. Twenty-five Kan/Str resistant, Amp sensitive clones were tested for the presence of rough LPS by staining with crystal violet. All but one of the clones retained the crystal violet stain and indicated conversion to the rough phenotype. The 24 clones reacted with Bru 48 (monoclonal
antibody to the core) and did not react with Bru 38 or Bru 28 (monoclonal antibodies to O-side chain) indicating the loss of the O-side chain. To verify that the Tn5 element had recombined into the chromosome in the same location as in RA1, a Southern analysis was performed on a digest of chromosomal DNA from strain RA1 and from two of the new recombinants. The Southern analysis showed the Tn5 had recombined in the same location (Fig. 9). These results indicate that this region of the Brucella chromosome is necessary for the synthesis of the O-side chain in Brucella abortus.

Complementation studies:

As a first step in identifying either rfa or rfb genes on the cloned Brucella DNA, a series of rfa and rfb mutants of Salmonella typhimurium were transformed with pJM6 and assessed for complementation by phage sensitivity [20]. Salmonella hsd strain LB5000 as well as rfa mutants SL3748, SL3750, SL3769 and SL3749 were transformed with pJM6 and subclones of pJM6. After confirming that the ampicillin resistant Salmonella strains contained pJM6 (i.e. by plasmid isolation), the strains were tested for complementation by phage analysis. Phage Ffm attaches to the core, whether complete or not, and therefore is a good indicator of a rough strain [41]. Lysis by the phage F(0), also called Felix O, is indicative of a complete core; the receptor for this phage is a complete core with the N-Ac-Glc branch attached. Lysis by the phage P22c2
is indicative of complementation as a smooth strain possessing an O-side chain will act as a receptor for the phage. Complementation studies on rfa and rfb mutant Salmonella strains transformed with pJM6, pJM61, pJM62 and pJM63 were negative in the phage analysis and also for reactivity to the monoclonal antibody Bru 38 (Table 3). The Salmonella rfb deletion mutants transformed with pJM6 and its' derivatives also remained rough (no lysis) and exhibited no reactivity to Bru 38. Thus it appears that the cloned Brucella genomic fragments do not express products capable of complementing the rfa of rfb mutants of Salmonella. Alternatively, if analogous products are produced by these plasmids, they are not providing the correct phage receptors (i.e. for F(O), FfM or P22c2) or the epitopes recognized by Bru 38. The possibility exists that the Tn5 element interrupted a gene (e.g. within an operon) in Brucella which blocked the synthesis of the O-side chain. Therefore the 30kb XbaI Brucella genomic fragment without the Tn5 element was cloned from wild type B. abortus 2308. Using an EcoRI fragment of pJM63 as a DNA probe, the corresponding EcoRI chromosomal fragment was identified in a Southern analysis of B. abortus 2308 genomic DNA; it was subcloned into the EcoRI site in pGEM3-Z. Three-hundred and fifty ampicillin resistant, β-galactosidase deficient colonies were screened using the colony lift procedure and pJM63 as a probe. One colony was positive when hybridized to pJM63 (Fig.
10). The plasmid from this clone was isolated and analyzed by restriction enzyme mapping. This plasmid, now designated pJM5, matched the expected pattern produced by restriction digestion of pJM63 without the Tn5 element (Fig. 11). Southern hybridization using *Brucella* genomic DNA and pJM63 as a probe also supported the conclusion that the pJM5 insert contained *Brucella* DNA and which corresponded to the flanking regions of pJM63. This 7.5kb EcoRI fragment in pJM5 does not contain the Tn5 element and therefore should be expressing whatever gene(s) were inactivated by the Tn5 provided that all necessary components of the genes are present (i.e. promoter, etc.). Several unsuccessful attempts were made to clone out the large 25.6kb XbaI fragment from the genome of *B. abortus* strain 2308 corresponding to pJM6. Complementation studies of *Salmonella* and *E. coli* transformed with pJM5 using the phage sensitivity and reactivity to the monoclonal Bru 38 were performed; however pJM5 did not complement any of the strains of *Salmonella* or *E. coli* (Table 2).

**Construction of pJM51:**

After analyzing the restriction enzyme maps of pJM5 and pJM6 (Fig. 12), it was concluded that a BamHI and XbaI digestion of each plasmid followed by a religation of appropriate fragments would allow a reconstruction of the majority of the large fragment lacking only the 2.5kb fragment from the left side of pJM6. Of the 15 transformants analyzed
by colony lift procedure using rabbit polyclonal anti-sera (against Brucella O-side chain and adsorbed with E. coli, Salmonella, and B. abortus RB51 sonicates) only one transformant reacted strongly. Three of the transformants reacted weakly compared to the negative controls (E. coli/pGEM-3Z, data not shown). The plasmids were isolated from these transformants and digested with EcoRI to determine if they contained the expected fragments of genomic DNA subcloned from the two plasmids (i.e. pJM6 and pJM5). Only one transformant had a plasmid with the correct pattern; this transformant had reacted strongly with the polyclonal antibodies against the Brucella O-side chain in the colony blot procedure described earlier. This new plasmid construct, designated pJM51, was used to transform both the rfa and rfb strains of Salmonella as well as E. coli DH5α which is an rfbD mutant. Attempts to reproduce the seroreactivity of E. coli DH5α/pJM51 in a colony blot with anti-O sera were unsuccessful. Additionally, phage sensitivity using E. coli DH5α and the Salmonella strains transformed with pJM51 were negative.

Agglutination reactions:

In order to assess if any of the cloned Brucella genes were allowing for the production of an intact E. coli O-side chain, E. coli C600 (rfbD) transformed with pJM6, pJM5, and pJM63 were sent to the E. coli Reference Center (Department of
Veterinary Science, Pennsylvania State University) for O-serotyping. The results reported back by the reference center indicate the E. coli C600/pJM63 demonstrated a definitive tube titration reaction of 1:2560 using 0:85 antisera. E. coli with and without pJM6 and pJM5 exhibited titration reactions of 1:640. These results are interpreted to mean that the rfbD gene mutated in E. coli C600 is being complemented by an analogous Brucella gene (i.e. on pJM6) and permitting the synthesis of E. coli O-side chain. Further analysis of extracted LPS from these strains will be necessary to confirm this apparent complementation seen in the agglutination analysis.
Discussion:

In the rough strain, *B. abortus* RB51, the nature of the mutation responsible for the loss of the O-side chain is still unknown [33]. This mutation, if in a global regulatory gene controlling the LPS genes, could mimic a mutation in one of the LPS genes. Therefore, to correlate this loss of the O-side chain directly with a specific LPS gene mutation could be misleading. However, since this correlation between LPS and virulence has been seen in a number of bacteria [5] including *Brucella* [31] it can be assumed that the loss of the O-side chain plays some role in *Brucella*'s virulence. The results provided in this thesis raise the question as to what role the *Brucella* genomic fragments in pJM63, pJM5 and pJM6 play in LPS biosynthesis. In *B. abortus* RA1 the Tn5 element has insertionally inactivated a gene or operon to cause this strain to be completely deficient in O-side chain production. However, it is not known if this deficiency is due to the inactivation of a specific gene, an operon or perhaps a regulatory gene. When *E. coli* C600 (*rfbD*) was transformed with pJM63 (containing a 9kb EcoRI fragment derived from pJM6), the strain produced an agglutination reaction with *E. coli* antisera 0:85. These results suggest that the deficient *rfbD* gene, encoding TDP rhammanose synthetase [39,13], in *E. coli* C600 is being complemented by some analogous *Brucella* gene. However, the parent plasmid pJM6 (or pJM5) does not
seem to complement *E. coli* C600. The reported agglutination results suggest that the genomic fragment in pJM6 and its derivatives contain the region of the *Brucella* chromosome comparable to the *rfb* locus in *E. coli*. However, the agglutination reactions are not supported by the results of the complementation studies in *Salmonella*. The lack of complementation could be explained by a number of possibilities: 1) The cloned *Brucella* genomic fragments lacked the ability to produce a correct phage receptor. Thus a hybrid O-side chain produced by the combination of the *Salmonella* and *Brucella* LPS genes was unrecognizable by P22c2 (O-specific phage) and would therefore be interpreted as a rough phenotype in the phage analysis. Additionally, the O-side chain would not be recognized by O-side chain serum because of the loss or masking of the epitope recognized by Bru38; 2) Since *rfbD* is complemented in *E. coli* C600 by pJM63, *E. coli*’s LPS regulatory system is sufficient to activate *Brucella* LPS genes. However, since *E. coli* C600 is not complemented by pJM5, one can conclude that a gene on pJM5 is repressing the *rfbD* gene; moreover, this gene may have been inactivated by the insertion of the Tn5. This regulatory model explains the complementation of *E. coli* C600 by pJM63 as well as lack of complementation by pJM5. However, when the lack of complementation by pJM6 in *E. coli* C600 is examined, the model does not provide a satisfactory explanation. If the Tn5 had
inactivated a repressor then the \textit{rfbD} gene should also express in pJM6.

To explain these apparently contradictory results, it is proposed that \textit{Brucella}'s LPS genes are regulated by a two component regulatory system in which both components are needed for activation of the genes (Fig. 13). If a protein (X) encoded by a gene located to the far right on pJM6 would normally repress the \textit{rfb} genes unless bound to another protein (Y), it would be possible to explain these results if the gene for protein (Y) had been interrupted by the Tn5 in \textit{B. abortus} RA1. To fit this model both proteins (X) and (Y) would have to repress these \textit{rfb} genes individually, otherwise the \textit{E. coli} mechanism for LPS gene activation would substitute for the missing \textit{Brucella} genes.
References


[38] Summary of Notifiable Diseases, United States, 1990. Morbidity and Mortality Weekly Report, Centers for Disease Control, Atlanta, GA.


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## Table 1

**Brucella, E. coli and Salmonella strains, plasmids and bacteriophage**

<table>
<thead>
<tr>
<th>Strains (B. abortus)</th>
<th>Description</th>
<th>Source</th>
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<tr>
<td>2308</td>
<td>Wild type strain of <em>Brucella abortus</em></td>
<td>BCC</td>
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<tr>
<td>RA1</td>
<td>Rough Tn5 mutant, Kan(R), Str(R)</td>
<td>This study</td>
</tr>
<tr>
<td>RA2</td>
<td>Semi-rough Tn5 mutant, Kan/Str(R).</td>
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<tr>
<td>RB51</td>
<td>Rough mutant, Rifampin(R)</td>
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<td>E. coli C600</td>
<td>rfbD, thr-1, leuB6, lacY1, supE44, rfbD,F, endA1, hsdR17, supE44, thi-1,</td>
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<tr>
<td>DH5α</td>
<td>rfbD, pyrE(+)</td>
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**Salmonella**

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<th>Description</th>
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**Plasmids**

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<td>pGEM</td>
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<td>pUC4KIX</td>
<td>amp(R), kan(R) from Tn5</td>
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<td>pJM6</td>
<td>amp(R), 30kb insert from RA1 genomic DNA into XbaI site in pGEM.</td>
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<td>20kb right side BamHI frag. of pJM6</td>
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<td>10kb left side of pJM6 BamHI to XbaI</td>
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<td>11.6kb internal EcoRI fragment containing the Tn5 element.</td>
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<td>6kb EcoRI from strain 2308 corresponding to pJM63 from strain RA1</td>
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<td>pJM51</td>
<td>Reconstruction of 2308 chromosomal fragment from pJM6 and pJM5.</td>
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Table 1 cont'd

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<td>P22c2</td>
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Table 1 (R) = Resistant, Pharm. = Pharmacia, BCC = Brucella Culture Collection; National Animal Disease Center, Ames, Iowa, SGSC = Salmonella Genetic Stock Centre, University of Calgary, Calgary, Alberta, Canada. PGMA= Promega Corporation
Table 2

Complementation Study with *Salmonella typhimurium* mutants

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**E. coli**

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Table 2  L = Lysis, NL = No Lysis, NT = Not tested, (-) = negative reaction, (+) = positive reaction, (+/-) Positive initially, however, not reproducible.
Fig. 1

The Cell Membrane of a Gram Negative Bacterium

The primary component of the outer membrane of a Gram negative bacterium is the lipopolysaccharide (LPS) shown here.
Fig. 2

**Lipid A**

The LPS is anchored in the Gram negative outer cell membrane by the strongly hydrophobic lipid A molecule. The lipid A is constructed of a B-1-6-D Glucosamine backbone (in box) with the hydrophobic branches of 14 to 18 carbon atom fatty acid molecules represented here by the "R" group.
**Fig. 3**

**The LPS genes of Salmonella typhimurium.**

The genes in *Salmonella typhimurium* that synthesize the LPS molecule are found primarily at two clusters on the chromosome. The *rfa* genes which construct and attach the core to the lipid A molecule are located at 79 min on the *Salmonella* chromosome [33]. The *rfb* genes, responsible for the O-side chain synthesis are found at approximately 42 min on the *Salmonella* chromosome [33]. A mutation in any of these genes results in a loss of synthesis and/or assembly of the LPS which results in a rough phenotype.
1,3-4,6 dideoxy-4-formido-D-mannopyranosyl linkage

Fig. 4a

The Brucella M antigen

The Brucella M antigen is composed of four 1,2 linked 4,6-dideoxy-4-formido-α-D-mannopyranosyl subunits linked to one 1,3 linked subunits.
1,2-4,6-dideoxy-4-formido-D-mannopyranosyl linkage

Fig. 4b

The Brucella A Antigen

The Brucella A Antigen is a homopolymer of 1,2 linked 4,6-dideoxy-4-formido-α-D-mannopyranosyl subunits.
Fig. 5

Silver stained SDS-PAGE of LPS

Lane 1 *S. typhimurium* LPS, Lane 2 *B. abortus* 2308 LPS, Lane 3 *B. abortus* RA1(3A5) LPS, Lane 4 *B. abortus* RB51 LPS.
Fig. 6

**Pulse field gel of Brucella abortus DNA**

(stained with EtBr); Lane 1 and 10 Lambda MW standards, Lanes 2-5 XbaI digest of *B. abortus* 2308, RB51, RA1(3A5), RA2(3D); Lanes 6-9 XhoI digest of 2308, RB51, RA1(3A5), and RA2(3D).
Fig. 7

Restriction enzyme map of pJM6

pJM6 contains a 30kb insert of B. abortus genomic DNA and the Tn5 element cloned into the Xba I site in pGEM.
Southern analysis of rough *Brucella* recombinant.

Southern analysis of rough *Brucella* recombinant using a probe to the Tn5. Lane 1 and 6, 1kb molecular weight markers (Bethesda Research Laboratories, Gaithersburg MD.); Lane 2 EcoRI digest of *B. melitensis* 16M genomic DNA; Lane 3 EcoRI digest of *B. abortus* 2308 DNA; Lane 4 EcoRI digest of *B. abortus* RA1 DNA; Lane 5 EcoRI digest of *B. abortus* RA3 (recombinant) DNA.
Fig. 9

Partial restriction enzyme maps of pJM6, pJM61, pJM62, pJM63 and pJM5.
DNA blot of *E. coli* DH5α using pJM63 as a probe. Colony number 15 (→) was positive for hybridization to pJM63.
pJM5 is a 7 kb fragment of B. abortus 2308 DNA corresponding to the region of the genome interrupted by the Tn5 in strain RA1. This fragment also corresponds to the insert in pJM63 excluding the Tn5 element. Eco= EcoRI, Nde= NdeI, H= HindIII, B= BamHI.
Fig. 12

Construction of pJM51

Using the 20kb BamHI/XbaI fragment of pJM6 ligated into a BamHI/ XbaI site in pJM5 as indicated the large intact genomic fragment was reconstructed.
Fig. 13
A proposed model to explain the results of the agglutination tests with *E. coli* C600/pJM63. If both proteins (X) and (Y) are repressors individually but as a complex they are incapable of repressing the *rfbD* gene their interaction could explain the results obtained from the agglutination tests. NT = Not Tested.
Curriculum Vitae

John R. McQuiston, was born on September 30, 1965 in Sacramento, California. After finishing high school at Vestal Senior High School, Vestal, NY in June 1984, he attended Broome Community College, Binghamton, NY, until May of 1986. In August of 1986 he enrolled in the Recombinant Gene Technology Program at State University of New York, College at Fredonia, Fredonia, NY, where he was graduated with a Bachelor of Science degree in May of 1988.

In August of 1989, he enrolled at the Virginia-Maryland Regional College of Veterinary Medicine at Virginia Polytechnic Institute and State University in Blacksburg, VA.

John R. McQuiston