EXPRESSION OF *BRUCELLA* GENES REQUIRED FOR LIPOPOLYSACCARIDE PRODUCTION UNDER THE CONTROL OF *ARA*C AND P*TAC* PROMOTERS

A Senior Honors Thesis

by

SHUO HAN

Submitted to the Office of Honors Programs
& Academic Scholarships
Texas A&M University
In partial fulfillment of the requirements of the

UNIVERSITY UNDERGRADUATE
RESEARCH FELLOWS

April 2006

Major: Biochemistry/Genetics

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Approved as to style and content by:

Thomas A. Ficht	Edward A. Funkhouser
(Fellows Advisor)	(Executive Director)

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ABSTRACT

Expression of *Brucella* Genes Required for Lipopolysaccaride Production Under the Control of *ara*C and p*Tac* Promoters (April 2006)

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Department of Biochemistry & Biophysics
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Brucella spp. are gram-negative, facultative intracellular pathogen that causes an infectious and contagious bacterial disease, brucellosis, in humans and animals. Previous research showed that lipopolysaccaride (LPS) is a classically demonstrated virulence mechanism in *Brucella*. The main objective of this project was to design an inducible system that would allow or prevent the expression of LPS using the *man*BA genes which encode components of LPS through the regulation of different promoters, *pTac* and *ara*C. This will allow us to better understand the significance of LPS expression and its

link to virulence. If we are able to regulate the expression of LPS in smooth and rough strains, we will be able to further understand these differences and eventually help to create attenuated vaccines against brucellosis.

Construction of *man*BA expression vectors using *pTac* and *ara*C promoters was carried out in the *E. coli* strain, DH10B as well as the conditions required for induction of *man*BA expression. The constructs were transferred into a *Brucella melitensis* (16M) *man*BA deletion mutant to determine optimal induction conditions. During the induction, specific time points were monitored for variations in *man*BA expression. Methods used to detect *man*BA expression include: acriflavine agglutination, SDS-PAGE followed by staining and Western Blots. Results showed that LPS expression could be regulated by p*Tac* and *ara*C promoters under specified induction conditions. Although the *pTac* promoter was found to be leaky, we were able to induce LPS expression in *Brucella melitensis* with the addition of glucose to the growth medium. The *ara*C promoter construct was more tightly regulated than the p*Tac* promoter and required DMEM, a defined media for expression. Therefore, we have developed two inducible systems which would aid in the study of LPS virulence mechanism in *Brucella*. Future work will include infecting murine macrophages using the inducible

Brucella constructs to study intracellular trafficking and survival of the transformed bacteria.

DEDICATION

I would like to dedicate this work to my parents for their support, love, guidance and encouragement. I am eternally grateful to my Mom and Dad for all that they have done for me. I hope this thesis will be able to reflect the amount of love that they have invested in me throughout the years.

ACKNOWLEDGEMENTS

I would like to thank all who have helped me in one way or another throughout the past year. Without you guys, I would not be able to produce this piece of work.

First of all, I would like to thank Dr. Thomas Ficht for agreeing to serve as my mentor. He allowed me to carry out this project at my own pace without any pressure and trusted in me. He was available to help whenever the need arose. Not only was he a mentor for my project, he had also given me valuable advice about my future. Also to Dr. Allison Ficht who has hosted many of the after-lab parties which fostered a friendly, social environment among lab mates.

I would not have been able to carry out this project if not for Josh and Carol Turse. They have become my teachers, my friends and brother, sister throughout my stay in the lab. Especially to Josh, who had rescued me from many embarrassing situations, and had taught me many lessons in lab and in life. To Carol, who coped with my incessant questions wherever she went. You two are the best!

I must extend my gratitude to Melissa Kahl who had helped me immensely ever since Josh and Carol left. She's also a great sushi company! And not forgetting Jenni Weeks who had given me many tips that made my life in the lab a lot easier. Jenni is

also a great friend to talk to. Lastly, I would like to thank my lab mates: Dr. Pei, Dr. Wu, Navina, Troy and Midhat for their patience, consideration, advice and help.

I would also like to thank Dr. Edward Funkhouser, Mrs Cindy Raisor, Jennifer Veracruz and Donna O'Connor for their work in running the Fellows program. Especially to Mrs Cindy Raisor who always replied promptly whenever I had a question, she was also a great Technical Writing teacher who helped to make thesis-writing less stressful. I would like to acknowledge the Honors Department at Texas A&M University for providing part of the funding towards this project.

Of course, to my instructors throughout college who had taught and guided me in one way or another. Finally, I would like to thank my family and friends for accommodating my madness throughout this period. Especially to Nicky who always helped in whenever I was in need.

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I. INTRODUCTION[1]

Brucellosis: Disease & Virulence Mechanism

Brucella spp. are gram-negative, facultative intracellular pathogen that causes an infectious and contagious bacterial disease, brucellosis, in humans and animals. This disease leads to chronic infection and abortion in animals, while undulant fever occurs in infected humans. There are three major species of *Brucella* categorized according to host specificity (15).

Brucella abortus affects cattle, and is the livestock pathogen with the greatest economic impact to the United States. Economic loss in the cattle industry is mainly a result of involuntary abortions in infected female cattle. These cattle are no longer profitable due to their inability to carry fetuses to full term once infected. Beef and dairy industries were severely affected during the 1950s due to this disease. Bovine brucellosis once affected more the 10 percent of the cattle population and about 30 percent of the cattle herds in the United States and dairy producers lost an estimated $499 million annually before an eradication program began. As a result of eradication the number of

[1] This thesis follows the style and format of *Infection & Immunity*.

infected herds has dropped from thousands in 1957 to only 40 in 1996, preventing huge losses that would have otherwise been incurred due to this disease (14).

Brucella suis causes disease in swine while *Brucella melitensis* affects caprine, ovine or bovine species. Human consumption of contaminated, unpasteurized goat milk products originating from infected animals is one important cause of brucellosis in humans. Improper handling of discharges from infected animals (e.g. milk, afterbirth, and fluids of parturition) is another cause of most human clinical cases. There are millions of people at risk to brucellosis worldwide, especially in developing countries where improper treatment procedures of milk and poor hygienic conditions are of main concern. Furthermore, *Brucella spp.*are potential bioweapons since they can be transmitted via aerosol.

Brucella spp. tend to undergo variation during growth. Changes in the colony morphology are related to variations in virulence, and are traditionally defined as smooth vs. rough using the oblique light method first described by Henry in 1933 (2). Smooth colonies are usually small, round, glistening and blue or blue-green in color. Rough colonies are granular and are yellow-white in color. The smooth appearance of the bacterial colony is due to the presence of lipopolysaccharide (LPS).

LPS is found on the outer membrane of gram-negative bacteria. It is composed of three principal parts: Lipid A, core oligosaccharide, and an O-specfic chain (sugar polymer). Smooth colonies tend to be pathogenic while rough colonies are less infectious and do not have the antigenic character found in that of the smooth cultures. In *Brucella*, the core oligosaccharide incorporates mannose and quinovosamine in addition to KDO. The O-specific side chain is N-formyl perosamine. LPS is one of the classically demonstrated virulence mechanism of *Brucella*. Studies have shown that rough bacterial strains are often more susceptible to host defenses including complement and cationic peptides (i.e defensins) (1, 11). Complement is a complex of proteins carried in the serum of mammals that punches holes in the cell membrane of invading microorganisms (5). Defensins are low molecular weight, cationic amphipathic peptides thought to function within phagocytic cells by permeablizing the cytopolasmic cell membranes of gram-negative organisms. Ionic interactions between defensins and LPS and were shown to have the greatest bactericidal effect against *B. abortus* (1). We are

Figure 1. Model of LPS. NH and free lipids in the outer membranes of *Brucella spp*.(11)

able to take advantage of the attenuated characteristics of rough *Brucellae* to create live attenuated vaccines that could combat brucellosis.

Rough *Brucellae* arise spontaneously in cultures as a result of smooth-to-rough transition, otherwise known as phase variation. Several environmental factors have been shown to influence the rate of appearance of rough *Brucella* organisms in culture. Since LPS is a classically demonstrated virulence factor, our understanding of how LPS functions as a virulence mechanism within brucellosis would allow insight for disease prevention in humans and animals to greater heights.

***man*BA Gene**

Previous research in our laboratory identified two genes involved in LPS expression are: *manB* and *manA*. The genes *ManB* and *manA* encode functions for phosphomannomutase and phosphomannose isomerase respectively, both of which are required for the movement of mannose through the LPS biosynthesis pathway.

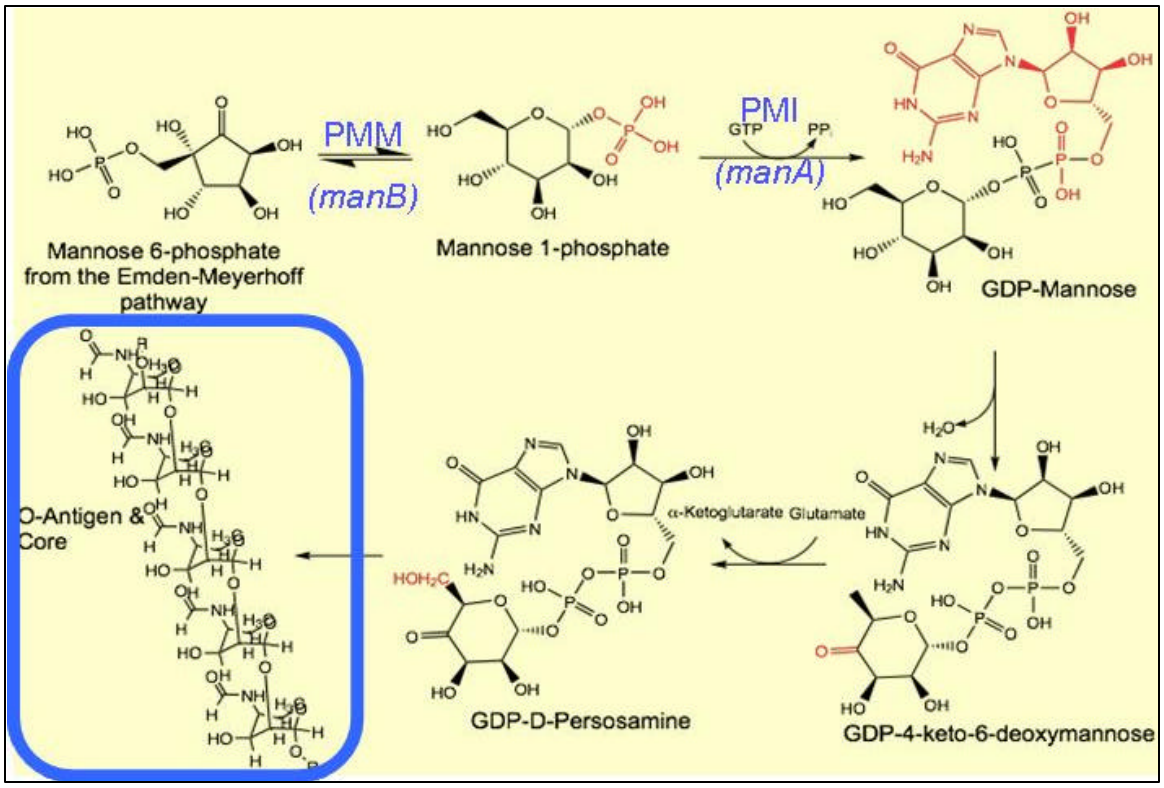


Figure 2. Lipopolysaccharide biosynthesis pathway.

Continuing research in our laboratory has shown that defects in the *manBA* operon are responsible for 30% of smooth-to-rough transition (16). This suggests a central role for *man*BA in phase variation.

The main objective of this project was to design an inducible system that would allow or prevent the expression of LPS using the *manBA* genes and two promoters capable of being regulated in different ways. To date, there have not been any known published papers demonstrating inducible control of LPS expression in *Brucella*. This is a crucial step in helping us to define the pathogenic properties of *Brucella spp*, which preferentially target macrophages in host cells as the replicative niche. From unpublished data in our laboratory, there is a difference in the uptake and trafficking of rough vs. smooth *Brucella* in macrophages which may partially be due to the level of LPS expression Regulation of LPS express in smooth and rough strains will further understanding of these differences, and eventually help to create attenuated vaccines against brucellosis.

p*Tac* and *ara*C Promoters

The plasmid vector, pBAD18Kan (6) contains the promoter of the *ara*BAD (arabinose) operon, *araC*, which encodes important regulatory functions. The arabinose operon is induced in the following way: when glucose is absent and arabinose is present, the inducible promoter is activated. When glucose is present, the inducible promoter is repressed (6). Therefore, the environment necessary for regulation is the presence of glucose and arabinose. The plasmid pBAD18Kan confers kanamycin (kan) resistance and therefore acts as a selective marker for cloning. We propose to link *ara*C to *manBA* to regulate expression of LPS.

The plasmid vector, pMMB207 (10), contains the p*Tac* promoter which is a strong hybrid promoter composed of the -35 region of the *trp* promoter and the -10 region of the lacUV5 promoter/operator (3). Expression of p*Tac* activity is repressed by the *LacI* protein. Isopropylthio-D-galactoside (IPTG) inactivates the *LacI* repressor and turns on the p*Tac* promoter. Allowing the expression of the cloned gene. The amount of expression from p*Tac* is proportionate to the concentration of IPTG added. Therefore, p*Tac* is a candidate promoter for the cloning of *manBA*. pMMB207 confers chloramphenicol (Cm) resistance, acting as a selective marker for cloning.

The two expression constructs, *ara*C-*man*BA and p*Tac*-*man*BA, were transformed into *E. coli* strain DH10B in the BL-2 facility for confirmation before attempting to transform the constructs into a *Brucella melitensis* 16M *manBA* knockout strain in the BL-3 facility.

II. Construction of *manBA* Expression Vectors

Introduction

In order to induce the *man*BA genes in *Brucella* under the control of the respective promoters, the gene must be inserted at suitable cloning sites in the vectors, pMMB207 and pBAD18Kan. These expression vectors will then be transformed into the *E.coli* DH10B strain so as to confirm the presence of the cloned *man*BA genes. Once the vectors have been successfully constructed, they can be transformed into *B.melintensis* 16M strains for induction.

The plasmid pMMB207 is a broad host-range plasmid that contains moblizable elements (MOB) that would allow the expression vector to be readily transformed into *Brucella* 16M strains. Unfortunately, pBAD18Kan does not contain these suitable mobilizable elements. Therefore, we are unable to directly transform the *ara*C-*manBA* expression vector into *Brucella*. One method was to transform the expression vector into *E.coli* strain ß2155, (DAP requiring strain for selection) allowing conjugation of the plasmid transfer to take place with *E.coli* ß2155 as the donor and *B. melitensis* 16M as the recipient (4). An alternative method was to PCR amplify the *ara*C-*manBA* insert into another broad host-range plasmid that contains mobilizable elements suitable for

transformation into *Brucella.* The plasmid chosen was pMR10 (17), which has multiple MOBs and was used effectively in previous research.

Materials and Methods

Plasmid Preparation. The plasmids used were pBAD18Kan, pMMB207, pManBA and pMR10. They were isolated from *E. coli* DH10B strains using the alkaline lysis maxi-prep method (7). The plasmids used in the analysis are summarized in Table 1.

Plasmid Name	Features	Source/ Reference	Intended Use
pMMB207	plasmid containing p*Tac* promoter, capable of replication within *Brucella*, chloramphenicol (Cm) resistant	R. Tsolis/(10)	Expression of *manBA* in *Brucella*
pBAD18Kan	plasmid containing *ara*C promoter, kanamycin (Kan) resistant	P. Hong/(6)	Expression of *manBA* in *E.coli*
pJET1445	plasmind containing *manBA* gene	J. Turse/(16)	*manBA* insert for expression vectors
pMR10	plasmid containing *mob* elements, capable of replication within *Brucella*, kanamycin resistant	J. Turse/(16)	Expression of *manBA* in *Brucella*

Table 1. Plasmids obtained for use in constructing *manBA* expression vectors.

PCR

Primers. The DNA sequences used to generate polymerase chain reaction (PCR) primers were analyzed with MacVector®, (Genetics Computer Group, Campbell, CA) and EnzymeX. The restriction enzyme sites required for cloning were incorporated in the primer sequences and can be found in Table 2. Primers were purchased from Integrated DNA Technologies, Inc. (Coralville, IA).

Primer Name	Direction	Primer Sequences (5'-3')	No.of Nucleotides
TAF552	Forward	G**GG GTA CC**C CAG GCA CAT ACA GG *Kpn* I	23
TAF552a	Forward	G**GG GTA CC**C CAT GAG CAG CAA TTC CCT CAA *Kpn* I	30
TAF553	Reverse	GC**T CTA GA**G CAT CCC AAT AGG CCG AAT GCC AA *Xba* I	32
TAF593	Reverse	TCC C**CC CGG G**GG GAT TAT GAC AAC TTG *Xma* I	27
TAF594	Forward	TCC C**CC CGG G**GG GAT CAA ACG CGT CCG *Xma* I	27
TAF594a	Forward	TCC **CCC GGG** GGA TTA TGA CCA CTT GAC GGC *Sma* I	30

Table 2. List of primers used for PCR. The respective engineered restriction endonuclease sites are underlined and listed. Primers used were 5 µM working stocks.

Amplification Protocol. The protocol was performed following the manufacturer's instructions using AccuTaq LA DNA Polymerase Mix (Sigma, St. Louis, MO). Each reaction tube contained the following: 3 µl of DNA template (2.5 ng/µl); 3 µl of each 5 µM forward and reverse primer; 2.5 µl of 200 µM dNTP, 0.5 unit of AccuTaq LA DNA, 5 µl of 10X AccuTaq Buffer and 33 µl of DNAse-Free water in a total of 50 µl per reaction. The reactions were mixed gently and briefly centrifuged to ensure even distributions of the components. The amplification protocol is shown in Table 3.

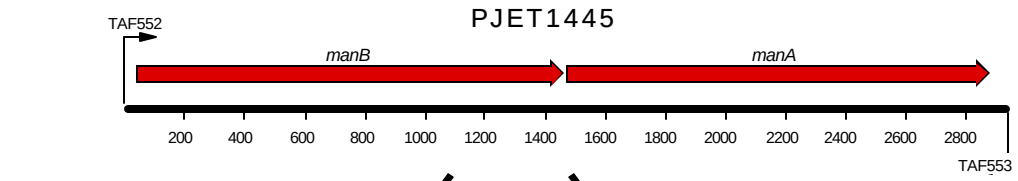
Initial Denaturation	98°C	30 sec
For Cycles 1-30		
Denaturation	95°C	30 sec
Annealing	53-65°C	30 sec
Extension	68°C	3 min
Final Extension	68°C	10 min

Table 3. PCR protocol using AccuTaq DNA LA Polymerase Mix. A temperature gradient was set up to determine the optimum temperature for each reaction. After optimization, the optimum temperature was used for annealing.

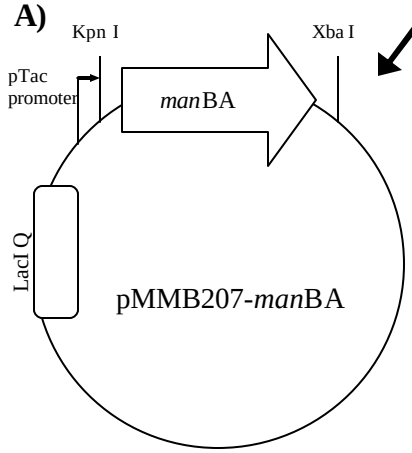
A touchdown PCR was carried out in order to amplify the *ara*C-*manBA* construct (Figure 3) from pBAD-*man*BA. Touchdown PCR uses varying annealing temperatures to increase the specificity of PCR. The initial primer-template duplex formation is enhanced by this method during which constant decreasing annealing temperatures will allow more specific binding of the primer-template duplex. This will ensure more gene products are amplified without trying to optimize the annealing temperatures often.

Initial Denaturation	98°C	30 sec
For Cycles 1-15		
Denaturation	94°C	10 sec
Annealing	65°C	20 sec
Decrease temperature after Cycle 1 by 0.5°C after every 1 cycle		
Extension	68°C	4 min
For Cycles 16-36		
Denaturation	94°C	10 sec
Annealing	50-68°C	20 sec
Extension	68°C	10 min
Final Extension	68°C	4 min

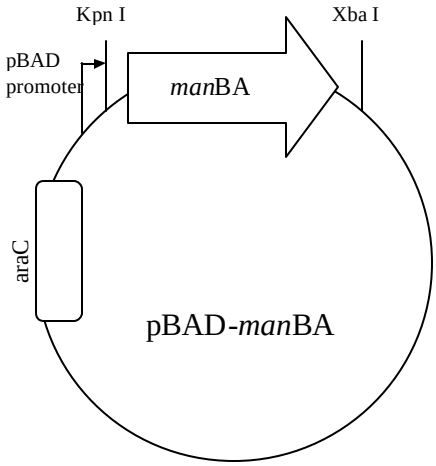
Table 4. Touchdown PCR protocol.

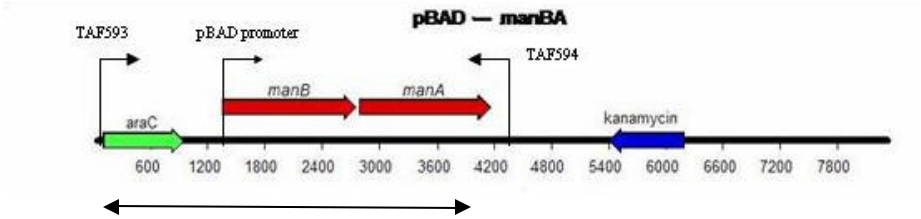


A)



B)

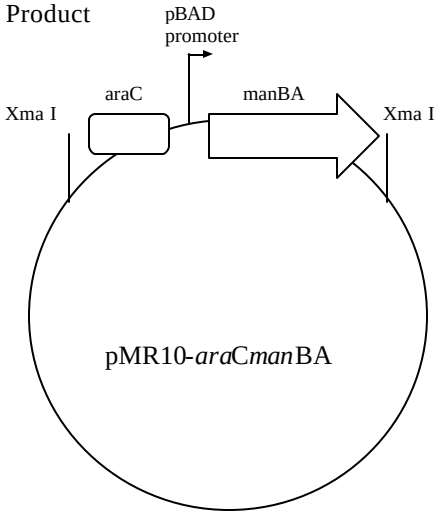




PCR Product

Transformed into *Brucella melintensis* 16M? *man*BA by:

1. Electroporation
2. Conjugation (*E.coli* ß2155 as donor)



Induction under suitable conditions for p*Tac* promoter

Work in progress

Figure 3. Overview of plasmid constructs used in this study. *man*BA was amplified using primers TAF552/TAF553 from pJET1445. The *man*BA PCR product was then cloned into pMMB207 and pBAD18Kan respectively using Kpn I and Xba restriction enzyme sites, creating the inducible constructs: A) p*Tac*-*man*BA and pBAD(*ara*C)-*man*BA. p*Tac*-*man*BA was then transformed into *Brucella* for induction. B) *ara*C-*man*BA was amplified using TAF593/TAF594 from pBAD-*man*BA. The *ara*C-*man*BA PCR product is used to clone into pMR10, creating the plasmid pMR10-*ara*C*man*BA.

Cloning of PCR Products

Restriction Enzyme Digestion. The plasmids, pBAD18kan, pMMB207 and the PCR *manBA* product were cut with restriction enzymes *Kpn*I and *Xba*I (Roche, Molecular Chemicals, Indianapolis, IL) for cloning of the p*Tac*-*man*BA and pBAD(*ara*C)-*man*BA constructs. A further restriction enzyme digestion reaction on the pBAD-*man*BA and the PCR product was carried out using *Sma* I for cloning of the pMR10-*ara*C*man*BA product.

Purification and Ligation. Each of the restriction enzyme digest products was separated by electrophoresis on a 0.8% agarose gel at 90V. Linearized DNA of the cloning vectors (pMMB207, pBAD18kan, pMR10) and the *man*BA amplicon were then extracted from the agarose gels using the QIAquick® Gel Extraction Kit (Qiagen, Valencia, CA) and stored in 30µL elution buffer aliquots at -20°C. The concentration of the purified DNA was determined using NanoDrop (NanoDrop, Wilmington, Delaware). The ligation mixture was prepared using a molar ratio of either 1:3 (vector: insert) or 1:1, depending on the restriction endonuclease sites. The total concentration of vector: insert used was 200ng in 12 µl; 2 µl of 10X T4 Ligation Buffer and DNase-free water to a final reaction volume of 20 µl. The ligation mixture should always be maintained on ice during sample preparation and then incubated overnight at 14°C in a microcooler.

Transformation

Chemical Transformation. Transformation of *E.coli* DH10B and ß2155 follows the protocol found in Molecular Cloning (7). The competent cells were thawed on ice after removal from storage -80°C. Two µl of the ligation reaction was added to 50 µl of competent cells and the samples were stirred gently with a pipette tip. The

transformation mixture was incubated on ice for 30 minutes and then in a water bath at 42°C for 60 seconds followed by a quick-chill in an ice bath lasting 1-2 minutes. Following the heat shock procedure, 250 µL of SOC [6% (w/v) trypticase soy broth (w/v), 10mM NaCl, 2.5mM KCl, 10mM MgCl2 and 20mM glucose] or SOC supplemented with 50 µg/ml DAP (SOCD) was added to the *E.coli* DH10B and ß2155[2] transformation reactions respectively. The reaction mixture was incubated at 37°C for 1 hour with shaking at 275 rpm and 50 and 200 µl portions were plated onto LB plates containing 30 µg/ml chloramphenicol or 100 µg/ml kanamycin for selective growth of *E.coli* containing either p*Tac*-*man*BA pBAD-*man*BA, respectively. Controls used were *E.coli* cells with only the restriction digested cloning vectors (pMMB207, pBAD18Kan and pMR10) with and without the T4 ligase.

Plasmid DNA Isolation

Positive transformants were identified either by antibiotic selection (Cm^R and Kan^R) or using blue-white screening in which plasmids containing the insert have

[2] *E.col* ß 2155 requires 50 µg/ml DAP (diaminopimelic acid) to grow.

disrupted ß-galactosidase activity. Followup analysis includes PCR amplification, restriction enzyme digestion to confirm the presence of the insert fragment and DNA sequence analysis to confirm proper fusion of the regulatable promoters to the structural genes. Positive colonies of *E. coli* culture were then grown up in Luria-Bertani (LB, Difco co Laboratories) broth with appropriate antibiotics.

PCR Screening. Colonies grown on the appropriate media and antibiotics were picked using a pipette tip and resuspended in 50 µl DNase-free water and mixed well. This was used as DNA template in PCR screenings. The conditions used followed the amplification protocol using primers TAF552 and TAF553 (Table 2) to probe for the *man*BA insert.

Restriction Endonuclease Analysis. Each colony picked for screening was placed in 5 ml of LB with either 30 µg/ml chloramphenicol or 100 µg/ml kanamycin and 50 µg/ml of DAP when appropriate. The culture was incubated overnight at 37°C. A miniprep was performed the next day using the PureLink™ Quick Plasmid Miniprep Kit (Invitrogen, San Diego, CA). The purified plasmids containing the *man*BA genes were then analyzed

via restriction digestion using appropriate enzymes (Roche). Each reaction contained the following: 1µg/ml of purified plasmid DNA; 5 µl of appropriate 10X restriction enzyme buffer; 2 µL of each restriction enzyme (depending on single or double digests); 0.5 µL BSA where appropriate (needed for *Kpn* I) and DNase-free water made up to a final reaction volume of 50 µL. The mixture was gently tapped to ensure even mixing of the reagents and was then incubated at 37°C waterbath (25°C for *Sma* I) for at least 4 hours. 25 µL of each sample was electrophoresed on 0.8% (w/v) agarose gels containing 0.5mg/ml of Ethidium Bromide in 1X TAE buffer at 90V and viewed under UV light. The size of the insert (*man*BA) and vector (pMMB207, pBAD18kan and pMR10) in each plasmid preparation was confirmed using Hyperladder I (Bioline, Boston, MA). Cultures of the true positive colonies were grown up and 2 mL of each culture was kept as a frozen stock in 50% glycerol-50% LB solution at -80°C. Plasmid maxipreps was then carried out using High-Speed Plasmid Maxiprep Kit (Qiagen) and stored at -20°C.

Blue/White Screening. For the pMR10-*ara*C*man*BA construct, blue/white screening was a viable approach since *ara*C*man*BA was cloned into the LacZ gene of the pMR10

vector. White colonies were isolated followed by PCR and restriction endonuclease analysis as described above.

DNA Sequencing

pMMB207-*man*BA construct was sent for sequence analysis at Gene Technologies Lab, Department of Biology at Texas A&M University to verify that *man*BA was indeed cloned into the pMMB207 vector.

Results

Plasmid DNA Isolation

*man*BA was isolated from pJET1445 using TAF552/553 primers which contain Kpn I and Xba I restriction endonuclease sites respectively (Fig. 4).

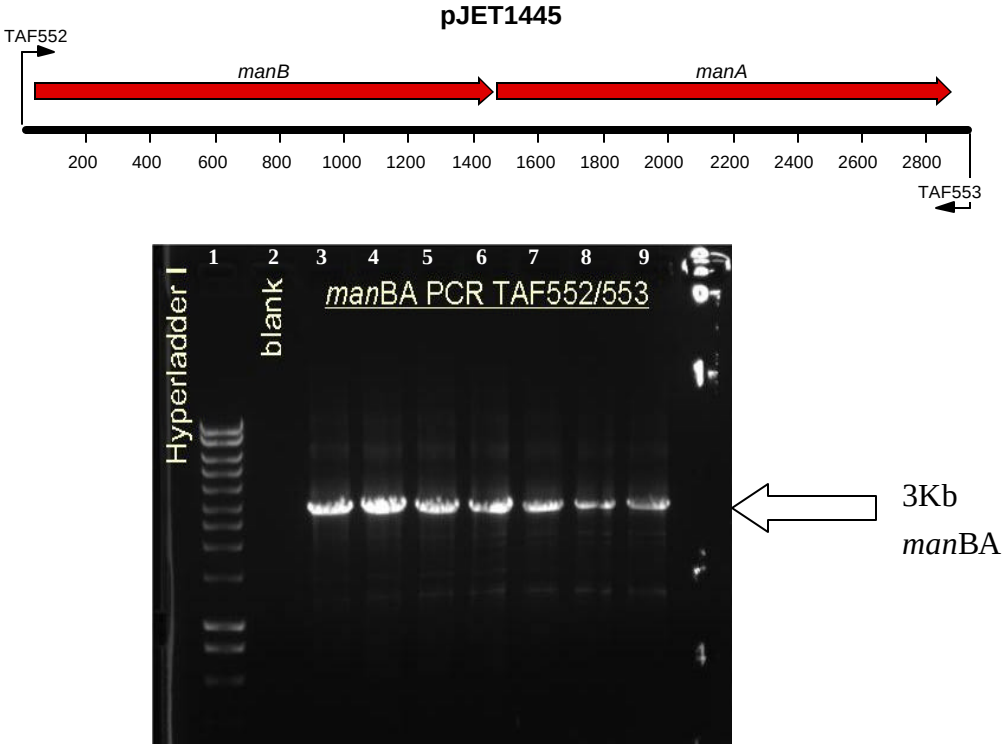


Figure 4. PCR for *man*BA amplicon from pJET1445 using the amplification protocol. The product is about 3Kb as shown in the plasmid map. Lane 1: Hyperladder I molecular marker; lane 2: negative control, no template DNA added; lanes 3-9: *man*BA PCR product.

Ligation and transformation were performed in *E.coli* DH10B cells to obtain p*Tac*-*man*BA and pBAD(*ara*C)-*man*BA constructs. Positive colonies were selected according to their respective antibiotic resistance markers (Cm^R and Kan^R respectively). Several positive transformants were chosen and were verified using restriction endonuclease digestions. A miniprep was carried out on each of the selected colonies and digested with Kpn I and Xba I enzymes to check for the presence of *man*BA inserts. Figure 5 shows p*Tac*-*man*BA construct while figure 6 shows pBAD-*man*BA construct.

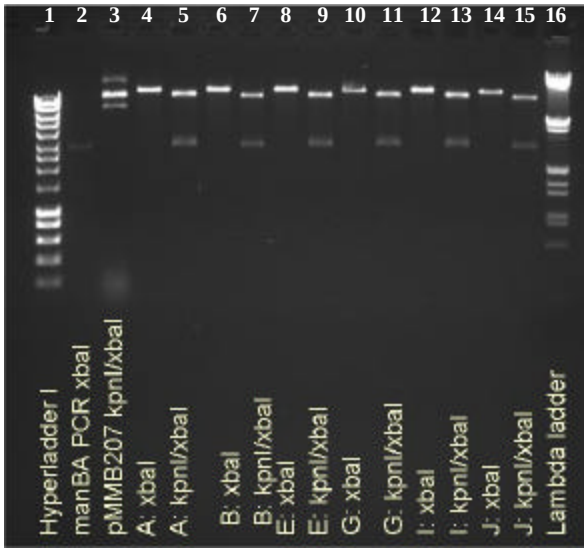


Figure 5. Restriction endonuclease analysis of pMMB207(p*Tac*)-*man*BA positive colonies. Lane 1: Hyperladder I molecular marker; lane 2: positive control of *man*BA PCR product; lane 3: positive control *Kpn* I and *Xba* I of plasmid vector pMMB207; lanes 4, 6, 8, 10, 12, 14: *Xba* I restriction endonuclease digest of pMMB207-*man*BA clones; lanes 5, 7, 9, 11, 13, 15 *Xba* I and *Kpn* I restriction endonuclease double digests of pMMB207-*man*BA clones; lane 16: Lambda DNA/*Eco*R I + *Hin*d III molecular marker.

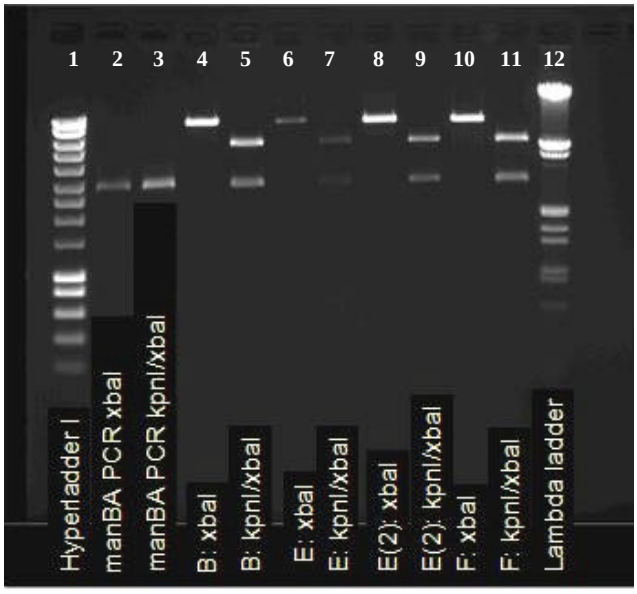


Figure 6. Restriction endonuclease analysis of pBAD(*ara*C)-*man*BA positive colonies. Lane 1: Hyperladder I molecular marker; lane 2: positive control *Xba* I digest of *man*BA PCR product; lane 3: positive control of *man*BA PCR product *Kpn* I and *Xba* I digest of *man*BA; lanes 4, 6, 8, 10: *Xba* I restriction endonuclease digest of pBAD-*man*BA clones; lanes 5, 7, 9, 11 *Xba* I and *Kpn* I restriction endonuclease double digests of pBAD-*man*BA clones; lane 12: Lambda DNA/*Eco*R I + *Hin*d III molecular marker.

DNA Sequencing

The pMMB207-*man*BA sequence overlaps both upstream and downstream of the *man*BA and pMMB207 expected gene sequences.

Construction of pMR10-*ara*C*man*BA Vector

As mentioned in the introduction of Chapter II, *ara*C*man*BA must be amplified from pBAD-*man*BA to be cloned into pMR10 in order to replicate in *Brucella.* Figure 3B illustrates the process of the vector construction. The amplification of *ara*C*man*BA made use of the Touchdown PCR protocol and results are shown in Figure 7. Figure 8 illustrates in Sma I digestion of the vector pMR10 and the amplicon *ara*C*man*BA.

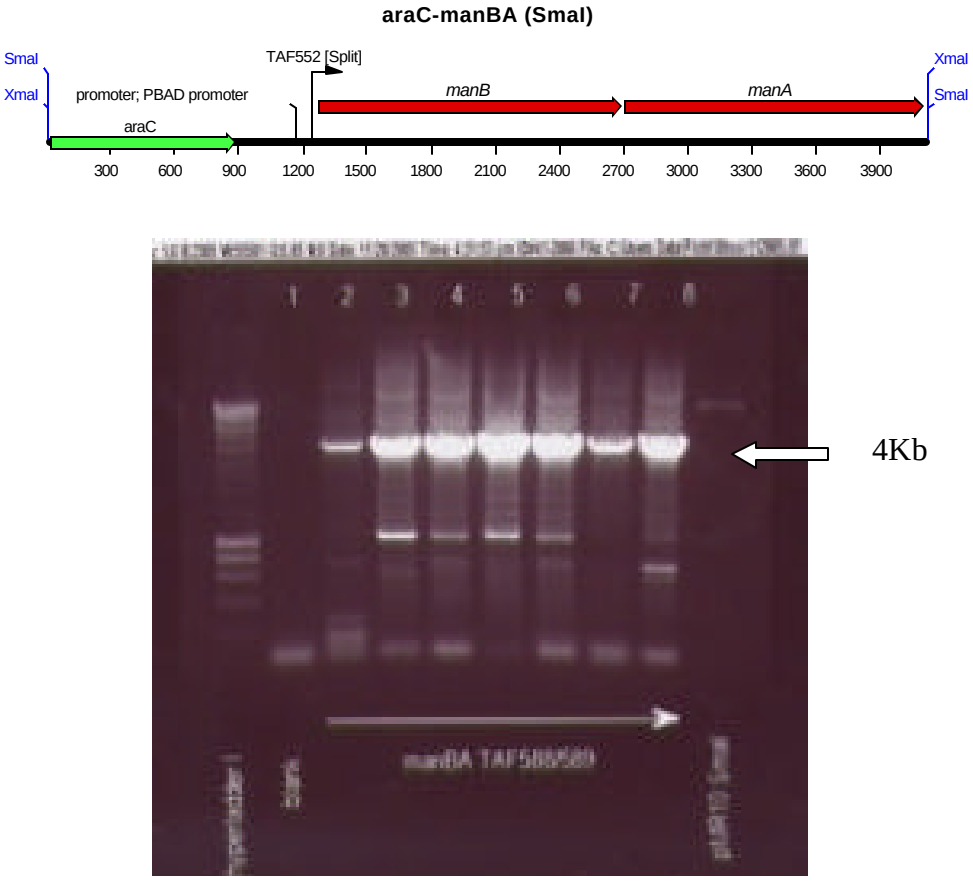


Figure 7. Amplification of *ara*C*man*BA using Touchdown PCR protocol. Lane 1: Hyperladder I molecular marker; lane 2: negative control, no DNA template added; lanes 3-9: *ara*C*man*BA amplicon using TAF593/TAF594, the product is 4Kb according to the map shown; lane 10: pMR10 *Sma* I restriction endonuclease digestion.

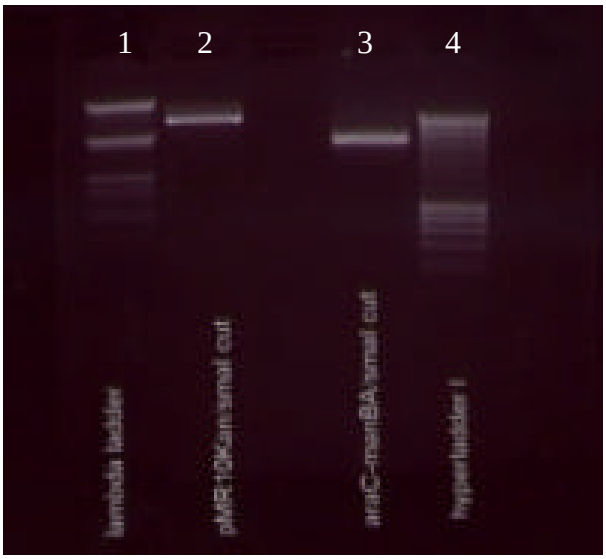


Figure 8. *Sma* I restriction endonuclease digestion. Lane 1: Lambda DNA/*Eco*R I + *Hin*d III molecular marker; lane 2: pMR10 digest; 3: *ara*C*man*BA digest; lane 4: Hyperladder I molecular weight marker.

Discussion

The *man*BA locus was amplified using TAF552/TAF553 (Figure 4), the 3Kb product has restriction endonuclease sites *Kpn* I and *Xba* I. Ligation of the vectors pMMB207 and pBAD18kan using appropriate molar ratios resulted in the recombinant constructs p*Tac*-*man*BA (Figure 5) and pBAD-*man*BA (Figure 6), and were transformed into *E.coli* DH10B (also ß2155 for p*Tac*-*man*BA). DNA sequence revealed that the p*Tac*-*man*BA construct was accurate. Future experiments were carried out based on the

confirmed p*Tac*-*man*BA sequence only. Since pBAD-*man*BA is not able to replicate in *Brucella*, *ara*C*man*BA was amplified from this construct using TAF593/TAF594 (Figure 7) and was engineered into pMR10, a replicable vector in *Brucella*. Although the *ara*C*man*BA was also successfully obtained using Touchdown PCR, ligation and transformation or insertion of *ara*C*man*BA into pMR10 failed after many attempts. The primers used to amplify *ara*C*man*BA was originally designed for the enzyme *Xma* I but as the enzyme was extremely expensive and non-economical, *Sma* I, an isoschizomer of *Xma* I was used instead. *Sma* I recognizes the same sequences (CCC GGG) as *Xma* I but cuts at different positions. *Sma* I is a blunt cutter while *Xma* I is a staggered cutter. Therefore, there might be some problems associated with the ligation procedure as the engineered *Xma* I sites in the *ara*C*man*BA might not be suitable for *Sma* I digestion even though they recognize the same sequences. As seen in Figure 8, the *Sma* I digestion for *ara*C*man*BA (5Kb) was not the right size (4Kb), hence the amplicon might not have been properly digested providing suitable sites for cloning. The work in progress to rectify the problem is to design new primers which contain nucleotides for *Sma* I restriction endonuclease sites before trying to ligate and transform into *E. coli* cells.

DNA sequencing will also be performed to confirm the construct. The p*Tac-man*BA construct alone was therefore used for the induction experiments in *Brucella.*

III. TRANSFER OF CONTRUCTS INTO *BRUCELLA*

Introduction

Brucella are identified as Class B Select Agents by the United States Department of Health and Human Services (HHS) and the United States Department of Agriculture (USDA) as potential threats to public health or welfare. In order to work with *Brucella spp.*, one has to obtain access to Biohazard Level 3 laboratories. Although undergraduate are not cleared to handle BL-3 agents they may observe cleared personnel working with *Brucella*. Therefore, in order to ensure efficiency, the inducible system is first transformed into *E.coli*, after sequence confirmation, and is then transformed into *Brucella*.

To determine the virulence of *Brucella*, it is necessary to transform *Brucella* with replicating plasmids that will be maintained by the bacteria for future experiments. in pMMB207 and pMR10 are vectors which contain MOB elements as mentioned in the previous chapter. Transformation of *Brucella* in this study is carried out by electroporation (9) and conjugation of the *E.*coli ß2155 (see Chapter II). In order to verify the identity of the transformants, the *Brucella* cultures were heat-killed prior to analysis of the *man*BA constructs using PCR.

Materials & Methods

Electroporation

p*Tac-man*BA was delivered to a *B. melitensis* 16M *man*BA knockout strain (16M?*man*BA) via electroporation. 16M?*man*BA was grown for electroporation from frozen stocks on tryptic soy agar (TSA) for 72 hours at 37°C. Bacteria resuspended into 5 mL of PBS containing approximately $5x10^{11}$ CFU/mL were pelleted by centrifugation at 3800xg for 15 minutes in a Jouan CR 4.12 preparative centrifuge with a M4 swinging bucket rotor. Unless stated otherwise all steps of this procedure were performed at 4°C. The supernatant was removed and the pellet was washed three times with 50 mL ice-cold molecular biology grade water (HyClone, Logan, Utah). The pellet was then resuspended in 1 mL of the water. Two microliters of p*Tac-man*BA plasmid was mixed with 70 µl of washed bacteria cells in a sterile microcentrifuge tube, then placed in a cold electroporation cuvette with a 1 mm gap (VWR, West Chester, PA). The parameters for the electroporation are as follows: current was applied from a BTX 600 electroporation generator set at 246 ohms, 25 µF, 2.5 kV (BTX, Holliston, MA) in the BL3 biological safety cabinet. 1 ml of SOC-B [6% (w/v) trypticase soy broth, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 10 mM MgSO4, 20 mM glucose] was added to the

cuvette and the electroporated cells were stored in a sterile microcentrifuge tube. Cells were then allowed to recover at 37°C, with shaking 6 hours to overnight. The samples were then plated on TSA/Cm (30 µg/ml) and incubated at 37°C for 5-8 days. The colonies recovered were screened using PCR.

Conjugation

E.coli ß2155::p*Tac-man*BA donor was incubated on LB plates containing Cm (30 µg/mL) and 50 µg/mL of DAP for 24 hours. The recipient is the *B. melitensis* 16M?*man*BA knockout strain. Both strains were harvested from plates in 2 mL of peptone saline with DAP. The bacterial suspensions were pelleted for 2 minutes at 12,000xg in a microcentrifuge. The pellets were resuspended in 100 µL of peptone saline with 50 µg/ml of DAP. The two suspensions were combined and applied to 0.45 µM NC20 nitrocellulose filters (Schleicher and Schuell, Dassel, Germany) on TSA/DAP plates. After a 2-hour mating period, the filters were harvested, placed in a microcentrifuge tube and the bacteria was resuspended in 1 ml peptone saline without DAP. The suspension was then plated on TSA with chloramphenical (30 µg/ml). The conjugants were confirmed using the acriflavine agglutination test (2).

Acriflavine Agglutination Test

10µl of bacteria from an 18-24 hour liquid culture was spotted onto a glass slide. This sample was mixed with 10µl of a 0.1% (wt/vol) aqueous acriflavine solution. Agglutination is consistent with a rough phenotype (2).

Results

For transformation via electroporation of p*Tac*-*man*BA, 14 chloramphenicol resistant colonies were observed on TSA plates after 5 days of incubation. These colonies were picked and analyzed using PCR to detect *man*BA inserts.

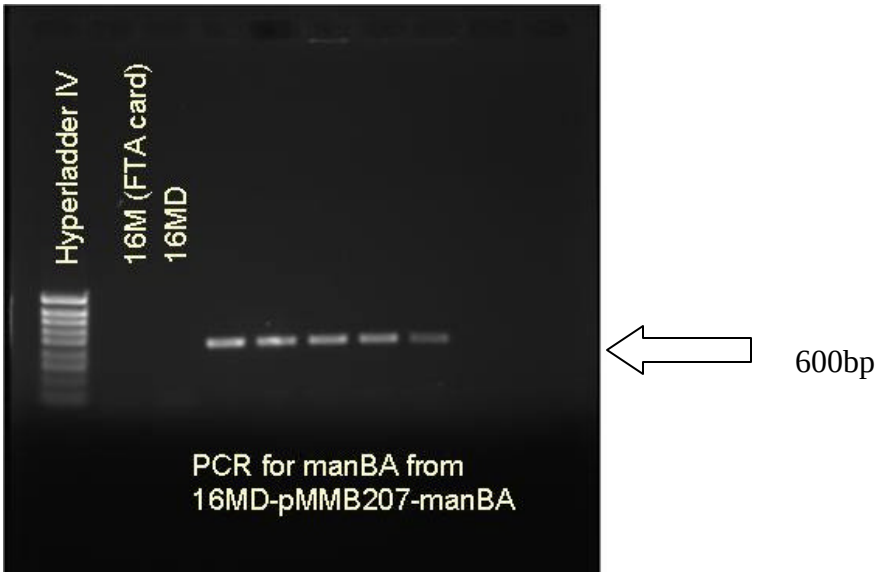


Figure 9. Verification of 16M?*man*BA-pMMB207(p*Tac*)-*man*BA clones using PCR. The primers used were TAF190/191. Lane 1: Hyperladder IV molecular weight marker; lane 2: *B. melitensis* 16M wildtype on FTA card; lane 3: *B.melitensis* 16M?*man*BA; lanes 4-8: positive clones of p*Tac*-*man*BA isolated from *Brucella* culture, 600bp.

Discussion

For transformation using electroporation, the plasmid of p*Tac-man*BA isolated from heat-killed *Brucella* culture. PCR amplification using TAF190/TAF191 revealed that *man*BA was present in the plasmid. In Figure 9, the positive control used was 16M heat-killed culture on FTA card, unfortunately, *man*BA was not detected due to insufficient DNA on the FTA card. Further amplification using liquid culture 16M gave a 600bp product that matched the size of the p*Tac-man*BA amplicon (data not shown). Therefore, p*Tac-man*BA was transformed into *B. melitensis* 16M?*man*BA. Agglutination occurred for the acriflavine test, indicating that the bacteria were still rough and that LPS was not expressed. Using the information for PCR amplification and the acriflavine test, it can be concluded that *man*BA in the *B. melitensis* 16M?*man*BA was not turned on and was probably regulated by the p*Tac* promoter.

IV. INDUCTION OF LPS EXPRESSION

Introduction

The induction conditions for p*Tac* and p*Ara*C are found in the literature review. With the 16M?*man*BA-p*Tac*-*man*BA, induction conditions were determined using IPTG, glucose and a defined media, Dulbecco's Modified Eagle's Medium (DMEM) containing 0.45% glucose (4500mg/L). Induced bacteria were heat-killed and analyzed by SDS-PAGE, staining and Western Blots.

Materials & Methods

Induction

p*Tac* promoters are induced when IPTG is added (see Chapter I). The induction experiment consists of several controls so as to confirm the regulation of the *man*BA gene by p*Tac* promoter. The bacterial strains were grown in conditions as defined in Table 5.

	DMEM	IPTG	Cm(30)	Mode of Expression
16M (WT)	10 mL	1mM	-	-
16M?*man*BA-p*Tac-man*BA	10 mL	1mM	10 µL	activated
16M?*man*BA-p*Tac-man*BA	10 mL	-	10 µL	repressed
16M?*man*BA-p*Tac-man*BA	-	-	10 µL	unrepressed

Table 5. Conditions for induction of 16M?*man*BA::p*Tac-man*BA and expected mode of expression.

The strains were allowed to grow under these various conditions for a period of 24 hours at 37°C. Prior to the experiment, Acriflavine test was performed on 16M?*man*BA::p*Tac-man*BA. Acriflavine solution was prepared using a concentration of 1mg/mL in DNase-free water and was then filter sterilized (0.1% wt/vol). Acriflavine tests were performed according to the following procedure: each culture was brought to OD490nm: 0.125 using to ensure that an approximately equal amount of cells were used. 100 µL of each cuture was mixed with RNAprotect Bacterial Agent and vortexed. The suspension was centrifuged for 10 minutes at 5000xg. The supernatant was discarded and the pellet was resuspended in 1 mL of the appropriate induction media. 10 µL of each culture was then added to 10 µL acriflavine solution on a glass slide. Agglutination

was consistent with rough morphology. The acriflavine test was carried out every four hours.

SDS-PAGE Gel

The cultures used in the induction experiment were heat-killed, pelleted at 45000xg for 10 minutes. Pellets were then washed in 1ml of peptone saline and recentrifuged at 12000xg for 2 minutes for a total of two times. The pellets were resuspeneded in 1mL PBS. Molecular biology grade water was then added accordingly to achieve optical density of 0.8 at 420nm. 15 µL of the OD0.8 bacteria solution was mixed with 15 µL of 2X Laemmli sample buffer. The samples were then heated at 100°C for 10 minutes, allowed to cool and spun briefly in a microcentrifuge. 30 µL of each sample was loaded onto a SDS-PAGE gel and electrophoresed at 200V.

Characterization of LPS

The samples run on polyacrylamide gels were stained using the fluorescent hydrazide, Pro-Q Emerald 300 dye (Invitrogen). The reaction of the fluorescent conjugation to glycoproteins can be visualized using a UV transilluminator using the

manufacturer's instructions. Another method used to characterize LPS was to visualize by Western Blotting using the antibody to *Brucella* LPS. Using the semi-dry procedure, the electrophoresed lysates were transferred to a PVDF membrane (Immobilon-P, Millipore). The stacking gel was equilibrated in a cathode buffer [25mM Tris, 20% (v/v) methanol, 40 mM 6-amino-N-hexanoic acid] for 10 minutes. The transfer membrane was soaked in absolute methanol for 10 seconds and transferred to the anode buffer 2 [25mM Tris, 20% (v/v) methanol pH 10.4] for 5 minutes. At the same time, wicks were made by soaking two pieces of filter paper (3mm Whatman Chromatography Paper) in anode buffer 1 [300mM Tris, 20% (v/v) methanol pH 10.4], one piece of paper in anode buffer 2, and three pieces of paper in the cathode buffer. The transfer stack of paper were cut according to the size of the polyacrylamide gel and assembled on the anode plate of a semi-dry transfer cell (Owl Separation Systems, NH). A current of 2mA/cm2 was run through the transfer membrane allowing the protein to transfer for one hour. Unbound sites on the membrane were blocked by incubation for 30 minutes in PBS with 3% (w/v) bovine serum albumin (SIGMA). The membrane was washed in PBS containing 0.05% (v/ v) Tween-20 (PBS-T). The blot was incubated overnight in sensitized anti- *Brucella melitensis* goat sera (diluted 1:200 in PBS-T). After being washed in 3 times in PBS-T,

the blot was incubated overnight with a secondary antibody: 1:5000 rabbit anti-goat, conjugated to alkaline phosphatase. The secondary antibody was removed and the blots were washed twice in PBS, once in molecular biology grade water. Signal was visualized by reaction with NBT-BCIP so solution prepared according to manufacturer instructions (Roche). The alkaline phosphatase reaction was stopped by the addition of TE (10mM Tris, 1mM EDTA, pH 8.0).

Results & Discussion

	DMEM	IPTG	Cm(30)	Mode of Expression	Acriflavine Agglutination Test
16M (WT)	10 mL	1mM	-	-	-
16M? *man*BA-p*Tac-man*BA	10 mL	1mM	10 µL	activated	-
16M? *man*BA-p*Tac-man*BA	10 mL	-	10 µL	repressed	+
16M? *man*BA-p*Tac-man*BA	-	-	10 µL	unrepressed	++

Table 6. Results of the acriflavine test. When IPTG was added, 16M?*man*BA::p*Tac-man*BA, the strain of bacteria containing the inducible *pTac* promoter, was activated as no agglutination appeared for the acriflavine test. No agglutination indicates that the bacteria was still smooth and that LPS was expressed. indicating that the induction was successful (2).

The inducible system is repressed by the $LacI^Q$ protein and activated when IPTG is added as it will inactivate the $LacI^Q$ repressor. It is important to note that DMEM which contains glucose, is used to repress the system more tightly as the p*Tac* promoter is leaky (8). Without glucose, even in the absence of IPTG, p*Tac* was induced above basal levels of expression as the samples agglutinated in acriflavine.

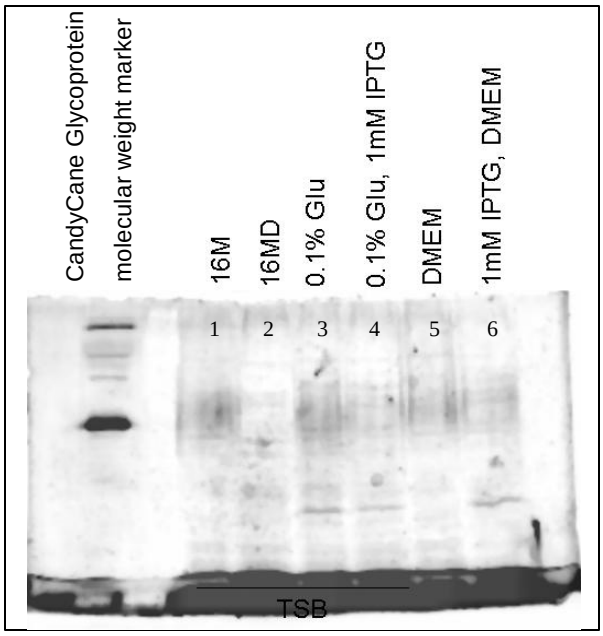


Figure 10. Pro-Q Emerald 300 glycoprotein staining of *Brucella* run on SDS-PAGE. Samples from lanes 1-4 was grown in tryptic soy broth (TSB, Difco Laboratories, Detroit, MI) while lanes 5 and 6 uses DMEM as media. Lane 1: wildtype *B. melitensis* 16M; lane 2: 16M?*man*BA; lane 3-8: 16M?*man*BA-p*Tac*-*man*BA; lane 3: 0.1% glucose added to repress basal level of expression; lane 4: 0.1% glucose and induced with 1mM of IPTG; lane 5: sample grown in DMEM (contains 0.45% glucose); lane 6: induced with 1mM IPTG in DMEM.

Since glycoprotein staining is able to detect proteins other than LPS, it is not clearly defined to which bands or blots belong to LPS from the gel. Lane 5 and 6 were induced under favorable conditions, and the extra band that was detected in lane 6 might indicate that LPS is present as lane 3 and 4 also contain the extra band while the *man*BA knockout strain (lane 2) does not. However, the result is inconclusive when looking at the wildtype 16M strain since the extra band was not detected. If this extra band indicates LPS expression from p*Tac* activity of the *man*BA gene, then the wildtype 16M should also contain it because this strain has the *man*BA gene present.

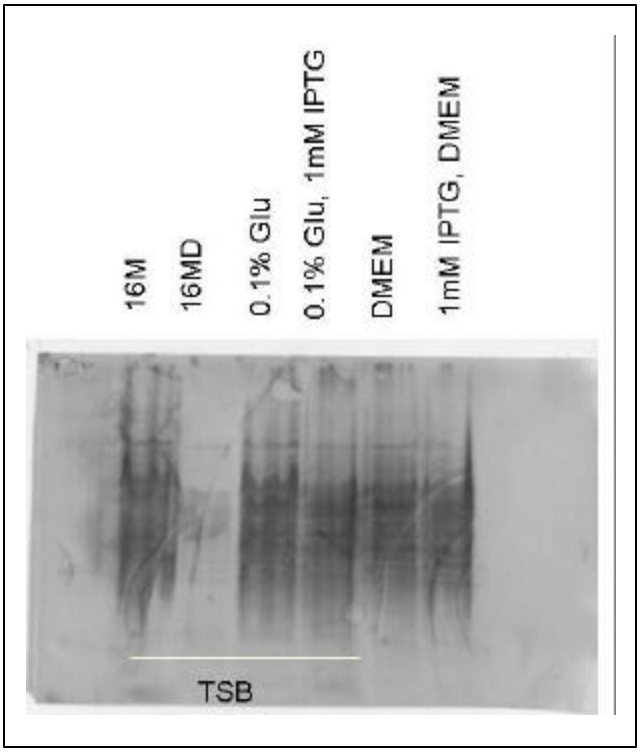


Figure 11. Western blot for *Brucella*. Samples from lanes 1-4 was grown in tryptic soy broth (TSB, Difco Laboratories, Detroit, MI) while lanes 5 and 6 uses DMEM as media. Lane 1: wildtype *B. melitensis* 16M; lane 2: 16M?*man*BA; lane 3-8: 16M?*man*BA-p*Tac*-*man*BA; lane 3: 0.1% glucose added to repress basal level of expression; lane 4: 0.1% glucose and induced with 1mM of IPTG; lane 5: sample grown in DMEM (contains 0.45% glucose); lane 6: induced with 1mM IPTG in DMEM.

LPS was detected in samples which were uninduced (lane 3 and lane 5). This might indicate that p*Tac* was not regulatable in *Brucella*.

We found that p*Tac* was a leaky promoter prior to using DMEM as media. A literature review indicates that glucose is required to repress the basal level of expression from p*Tac*. Since DMEM contains 0.5% glucose and is a defined media, it is a good media to grow the inducible strain 16M?*man*BA::p*Tac*-*man*BA. The smooth morphology of the acriflavine test suggests that the promoter was activated by the addition of 1mM IPTG. The rough morphology of strain indicates that p*Tac* was able to repress the expression of LPS. Therefore, from this set of results, we could conclude that the inducible system is functioning.

However, the Pro-Q Emerad 300 glycoprotein staining and the Western Blot results are not consistent with that of the acriflavine test. Several problems could have caused this inconsistency. Samples may have been contaminated during gel electrophoresis, creating errors in the above results. Alternatively, a new promoter sequence could have been created during cloning due to extra nucleotide sequences used in the primers. This new promoter sequence could require a different set of induction conditions as opposed to the predicted conditions.

V. SUMMARY, CONCLUSIONS & FUTURE WORK

In this study, two inducible systems of LPS expression through *man*BA genes were designed and one was tested in *Brucella*. The construction of these two systems required the use of three plasmid vectors, namely pMMB207, pBAD18kan and pMR10. Using methods such as PCR, Touchdown PCR, ligation and transformation, cloning of the expression vectors has been completed with the exception of the pMR10 construct. The p*Tac*-*man*BA construct was also successfully transformed into *B. melintensis* 16M? *man*BA using electroporation and conjugation. Using this construct, we were able to determine the conditions for induction of the *man*BA genes. The characterization of LPS expression was carried out using SDS-PAGE analysis, acriflavine testing, Pro-Q Emerald staining and Western Blotting. The second inducible system, pMR10-*ara*C*man*BA has been designed and is in the process of optimizing the ligation reaction using appropriate primers designed for

Sma I engineered restriction enzyme sites.

Although we have determined that the p*Tac* system is inducible through the acriflavine test, the contradictory results shown in the LPS staining and Western Blotting raise concerns. Contamination of the samples could be a factor linked to this

inconsistency. However, we are certain that p*Tac* is a leaky promoter and requires glucose to repress activation. Therefore, we found DMEM containing glucose, to be the best medium used to repress such an activity. More work needs to be done to confirm the inducibility of the p*Tac* system through staining and Western Blotting. As for the a*raC* system, a similar induction and characterization approach will be followed to test for inducibility.

Our future goal is to infect J774A.1 murine macrophages and compare the intracellular trafficking and survival of the transformed bacteria. It is believed that rough mutants of *Brucella spp*. are attenuated for survival in animals, however there has been conflicting in vitro evidence concerning the intracellular survival of rough mutants. Recent publications report that rough mutants exhibited increased macrophage uptake relative to smooth strains (12). The reduction in numbers at the end of the assay has been associated with intracellular killing. According to previous work in our lab, there had been a 10- to 20-fold-increased uptake of rough mutants over that of smooth organisms under standard conditions. The number of rough organisms recovered only declined when intracellular replication of the smooth organisms was observed after 8h postinfection. Thus, the decline in the number of rough organisms was the result of the

lysis of macrophages and not from intracellular killing. These findings suggest a role for O antigen which is an important part of LPS during the early stages of host-agent interaction which will result in disease of the host (12). Therefore, with our inducible system, we could study the intracellular trafficking and survival of the transformed bacteria.

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EDUCATION

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Bachelor of Science in Biochemistry and Genetics (Double Major)
Graduation: May 2006 GPA: 3.71

HONORS & AWARDS

- Dean's Honor Roll
- Emerald Honors Conference, Golden Key Honor Society, Sigma Xi
- Senior Honors Thesis (April 2006)
- Student Research Week 2006: Oral Presentation (4th Place)
- Honors Incentive Award, Ben T & Mattie B Little Foundation Scholarship, Summer Undergraduate University Research Scholarship, International Student Education Scholarship, A L Darnell Agricultural Scholarship, Hamrick Harris Scholarship,

TECHNICAL SKILLS

- **Laboratory Processes**
  Competent Cells, Mini Prep, Maxi Prep, Transformation. Gel Electrophoresis, PCR, HPLC, Tissue Culture, Gene Sequencing, Restriction Enzyme Digest, DNA Purifcation, Western Blot
- **Computer Skills**
  Proficient in Microsoft Word, Excel, Publisher, Outlook; familiar with HTML and Adobe Photoshop, Sequencer, Gel electrophoresis software, MacVector.
- **Language**
  Fluent in English, Mandarin and conversational French.

RELEVANT EXPERIENCE

AP Beutel Health Center **Fall 2006**

- Pharmacist Orientation Program; Shadow pharmacists to familiarize with work environment

Dr Tom A. Ficht Lab **Fall 2003 – Present**

(TAMU, Veterinary Medical Research)

Undergraduate Research Fellow

- Carry out independent research on *Brucella* under the most prestigious undergraduate research program coordinated by the Honors Department

Omniprobe Pte Ltd, Dallas, Texas **Aug 2003, Jan 2003**

- Assisted in assembly of nanoprobes sold to various universities and governmental organizations worldwide; helped the budding company set up its own inventory system.

LEADERSHIP

Singapore Student Association

President; Vice President **Fall 2004 – Present**

- Organize activities for members, uphold association motto

Secretary **Summer 2003 – Spring 2004**

- Arrange meetings, record minutes, administrative work

Spring Festival Concert **Spring 2004**

- Invited by Chinese Students and Scholars' Association to be *Erhu* soloist in above concert attended by officials from the China Consulate in Houston.

International Week – Talent Show, Cultural Display **Spring 2004 & 2005**

- Solo performance at the talent show for Mayors of College Station and Bryan, school officials and a strong TAMU audience.
- Organized a team to promote Singapore culture during the annual event, clinched 3rd overall.

ACTIVITIES

Big Event HealthForAll Volunteer TAMU Photography Club

College Station Chinese Church Indonesian Christian Fellowship