CONCERNING *Brucella* LPS: GENETIC ANALYSIS AND ROLE IN HOST-AGENT INTERACTION

A Dissertation

by

JOSHUA EDWARD TURSE

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2005

Major Subject: Genetics

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ABSTRACT

Concerning *Brucella* LPS: Genetic Analysis and Role in Host-Agent Interaction. (August 2005)

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Brucella lipopolysaccharide is an important component of virulence in brucellosis. Recent research in macrophage models has shown that *Brucella* LPS does not behave like classical LPS by stimulating potent inflammatory responses. The central hypothesis of this work is that O-antigen is dynamic signaling molecular and participates in complex interactions with the host to promote productive infection. A corollary to this is that the host environment is dynamic, and *Brucella* has evolved mechanisms to cope with changing environments. In an effort to understand the contribution of *Brucella* LPS to virulence and pathogenesis, the function of a metabolic locus important in the synthesis of LPS has been demonstrated and complemented. The spontaneous loss of LPS expression has been characterized. Contribution of LPS to acquisition of the host environment in tissue culture and mouse models has been explored. This work demonstrated that genes outside the O-antigen biosynthesis (*manBA)* cluster contribute to LPS biosynthesis. Further high

frequency mutation involving manBA is partly responsible for observed dissociation of *Brucella* strains. Finally, work herein attempts to look at the role of LPS in acquisition of the host environment and shows that LPS is important for recruiting particular cell populations within a host model of brucellosis.

DEDICATION

…to my biggest fans, my family,

…my wife, Carol

…Mom & Dad, Jim & Cindy

…my brother, Jason

…my in-laws, Walter & Vivian

…my brother in-law, Gene

…and yes, even my dog, Shawna

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Finally, I thank my wife for her endless understanding of my consuming insanity.

TABLE OF CONTENTS

Page

Page

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

Page

INTRODUCTION AND LITERATURE REVIEW

BACKGROUND

Taxonomy. Members of the genus *Brucellae* are small, non-motile, gram-negative, non-encapsulated, intracellular zoonotic pathogens that opportunistically infect humans. Phylogenetically, *Brucella* is grouped with the α-Proteobacteria, order *Rhizobiales* (56). Related organisms include *Agrobacterium*, *Rhizobium*, and *Bartonella*. Classically, the genus is divided into six species, *B. abortus*, *B. suis*, *B. melitensis*, *B. neotomae*, *B. canis*, and *B. ovis*. *Brucella abortus* and *Brucella melitensis* are the two most studied of these species. *Brucella abortus* is the causative agent of infectious bovine abortion, Bang's disease. *Brucella melitensis* is an important goat and human pathogen, first identified during the Crimean War as causing "Mediterranean gastric remittent fever" or Malta fever (19). There is discussion of the addition of a seventh specie to the genus, and whether it should be added as *Brucella maris*, a specie affecting whales or pinnipeds, *B. pinnipediae*, affecting pinnipeds or *B. cetaceae*, affecting whales (27). The *Brucellae* are further discriminated into biovars, based upon dye uptake, metabolic processes, such as the ability to grow in the absence of carbon dioxide, and susceptibility to phage. Biovar 1

This dissertation follows the style of Infection and Immunity.

strains are typically used for laboratory research and include the strains focused on in this work, *Brucella melitensis* 16M and *Brucella abortus* S2308.

Molecular biology. Characteristic of *Brucellae* are two chromosomes of 1.17 and 2.1 Mb, (102) with the same G + C content and similar proportion of potential coding regions (1,138 and 2,059, respectively) (35, 69). From the sequence of *Brucella melitensis*, several inferences about virulence determinants could be made. Type I, II, and III secretion systems are not expressed (2), though genes encoding sec-dependent, sec-independent, and flagella-specific type III, type IV, and type V secretion systems as well as adhesins, invasins, and hemolysins were identified. Identification of loci encoding essential metabolic and replicative functions is suggestive that both genetic elements are chromosomes rather than mega-plasmids (35, 69).

The disease. The host range of the genus is broad, comprising dogs, swine, sheep, goats, cattle, deer, and humans; producing chronic infection punctuated by periods of recurring acute infection. In humans, the symptoms of brucellosis include undulant fever, arthritis and dementia. Rarely, human cases of Brucellosis will progress to meningitis or endocarditis (85, 136). If left untreated, Brucellosis can develop into debilitating chronic infection, resulting in death. In agriculturally important animals, *Brucella* infection can lead to sterility.

Pathogenesis. Common routes of infection include via the conjunctivae, the alimentary tract, inhalation and broken skin (122), from contact with

contaminated animals and animal products. The organism is then spread throughout the host by way of the lymphatic system: bacteria are phagocytosed by macrophages at the primary site of infection and, transported to regional lymph nodes where *Brucellae* have been shown to persist and multiply within the phagolysosomal compartment (8, 24). The phylogenetic relationship between *Brucella* and the α-subdivision of the Proteobacteriaceae suggests an organism capable of quickly adopting changes in response to changing environments. Most proteobacteria live in soil; as such must adapt to changing conditions present in this environment including reduced nutrient availability. The ability to adapt to nutrient poor conditions is a major contributor to intracellular survival of *Brucella*.

Threat assessment. Brucellosis continues to be a major health threat world wide among humans and domestic animals. Human brucellosis has always been associated with animal disease (60). The principal threats to humans are *B. melitensis*, *B. suis*, and *B. abortus*. Incidence and prevalence vary from country to country, with small-ruminant brucellosis being the most widespread infection. This is remarkable since eradication programs for bovine brucellosis have been largely successful in developed countries (100). Areas considered to have high brucellosis prevalence include the Middle East, countries surrounding the Mediterreanean Sea, Southeastern Europe, Sub-Saharan Africa, and South

and Central America (including Mexico) (60). Brucellosis in these areas is typically small-ruminant brucellosis, caused by *B. melitensis*.

Brucella [suis], weaponized in 1954, was the first biological agent in the United States' arsenal. Several other countries have been suspected of studying the agent as a biological weapon, but to date, no use of *Brucella* in a bioterrorist attack has been reported (67), though there was suspicion of such an undertaking in 1999(1). Since *Brucella* can be weaponized and delivered by aerosol via commonly available equipment (81), there is potential for this organism to be deployed as a tactical [battlefield] weapon (60, 67). In a theoretical attack on a population of 100,000 people, 82,500 cases of brucellosis requiring extending supportive therapy are predicted, with 413 deaths (82). In 1997, the projected economic loss in the case of such an attack was $477.7 million.

***Brucella* virulence factors.** To date, there are two only confirmed virulence determinants, the lipopolysaccharide (LPS), which exhibits antigenic and dissociation (7), and Type IV secretion system (*virB*). *Brucellae* demonstrate antigenic variation in two distinct ways: A versus M epitopes of the O-polysaccharide and rough versus smooth LPS (12, 41, 42). Antigenic variation may account for the appearance of some *Brucella* biovars in which A-antigen predominates over M-antigen and vice versa. For example *B. abortus* biovar 1 produce predominately A-antigen, while *B. abortus* biovar 2 produce M-antigen

and *B. abortus* biovar 3 produce roughly equal amounts of both. One component of the LPS that has not been definitively demonstrated is the presence of an exopolysaccharide. Rosinha et al. (125) demonstrated that *B. abortus* strains deficient in the polysaccharide transporter, ExsA, are attenuated in mice. In *Rhizobium meliloti*, *exsA* genes are involved in the transport of the exopolysaccharide succinoglycan. Speculation is that *exsA* mutants in *Brucella* may have altered polysaccharide architecture.

There is a third antigen that deserves some mention since much literature has been devoted to it; native hapten and polysaccharide B were thought to be antigenic determinants until Moriyón et al., performed a meticulous study comparing outer membranes of *B. abortus* and *B. melitensis* (106). Native hapten and polysaccharide B were found to be byproducts of the chemical reactions used to analyze *Brucella* LPS.

Rough *Brucellae* are characterized by the lack of O-antigen. The term "rough" is a historical term and is based on observations of colony morphology under obliquely reflected light (74). Henry found that rough colonies were yellow-white in color and have a dry, granular appearance, whereas smooth colonies are small, round, glistening and blue to blue-green in color. Some basic phenotypic tests include agglutination of roughs by acriflavine and staining of rough colonies with crystal violet. Rough *Brucellae* are immune to lysis by Berkeley phage BK-2. Rough *Brucella abortus*, but not *Brucella melitensis* are

susceptible to killing by serum complement (4, 50). In the host, infection by rough *Brucella* is seemingly inconsequential, since it is cleared at early time points. Yet interest in the use of rough mutants as vaccines persists due to their lack of interference with standard diagnostic tests (4, 5, 21, 114, 128, 134, 138).

Genetics of *Brucella* O-antigen synthesis. *Brucella* O-antigen is required for persistence in the host and based on studies with rough derivatives of classical smooth strains this survival was thought to originate with increased resistance to intracellular killing mechanisms. However, loss of O-antigen from transposon-derived rough mutants revealed little effect on intracellular survival in macrophages in several separate studies and in at least one study reportedly replicate intracellularly (4, 5, 59, 78, 112, 115). These data suggest that O-antigen may play an important role in survival, but one that is different than originally predicted. In order to understand the role of *Brucella* LPS in survival and virulence it is important to know something about its synthesis and structure.

The nature of the steps leading to the production of *Brucella* O-antigen are not known with certainty, but the following enzymatic functions have been shown to be important. GDP-D-mannose is required for the mannosylation of many bacterial cell surface repeat unit polysaccharides and acts as the precursor for other nucleotide sugars (GDP-L-fucose, GDP-colitose, GDP-perosamine and GDP-D-rhamnose) involved in polysaccharide biosynthesis. Fructose 6-phosphate feeds a biosynthetic pathway consisting of the following steps: *manA*,

converts fructose 6-phosphate to mannose 6-phosphate; *manB*, mannose 6-phosphate to mannose 1-phosphate; *manC*, mannose 1-phosphate to GDP-D-mannose. Catabolism is not the only method *Brucella* can use to acquire mannose-6-phosphate. Studies have demonstrated the presence of hexose permeases, as well as the ability to grow on a number of carbon sources (47, 48).

Phosphomannose isomerase, *manA*, is responsible for the enzymatic conversion of fructose 6-phosphate to mannose 6-phosphate. *ManA* is required in both the glyocolytic pathway (116) and the pathway leading to O-antigen biosynthesis. *ManA* has been identified in several enteric bacteria as being important to the production of O-antigen, but is not often mapped to polysaccharide gene clusters (77), as in *Brucella*.

Phosphomannomutase, *manB,* is responsible for the inter-conversion of mannose 6-phosphate to mannose 1-phosphate in the production of precursors for LPS. Much literature from studies of the opportunistic pathogen *Pseudomonas aeruginosa* and the economically important bacterium *Xanthomonas campestris* focus on *manB*. In *Pseudomonas*, the gene encoding phosphomannomutase, *AlgC* (61) is associated with exopolysaccharide and lipopolysaccharide virulence determinants. *AlgC* deficient Pseudomonads do not produce LPS or exopolysaccharide (30, 62). Knockouts of *algC* have been

shown to be less virulent in mouse models of infection (61, 133), demonstrating the importance of phosphomannomutase to LPS biosynthesis and virulence.

GDP-mannose pyrophosphorylase (GMP), *manC* (BMEI0344), and transfers a guananyl residue to the mannose 1-phosphate synthesized by *manB*, completing synthesis of GDP-D-mannose. Phosphomannomutase and GDP-mannose pyrophosphorylase are thought to participate solely in the production of GDP-D-mannose (77) (FIG. 1). Other genes implicated in O-antigen biosynthesis are identified in Table 1.

O-antigen dissociation. The phenomenon of *Brucella* spontaneous smooth to rough transition (dissociation) is well documented (74). However, the genetic basis for these changes (if any) remains undefined. Alternatively, the spontaneous appearance of rough variants could result from a response to changing environment and preferential growth in the absence of *in vivo* selection pressure. As a result, reversion to smooth phenotype may also be explained by recognition of changing environmental conditions or reversion of mutants

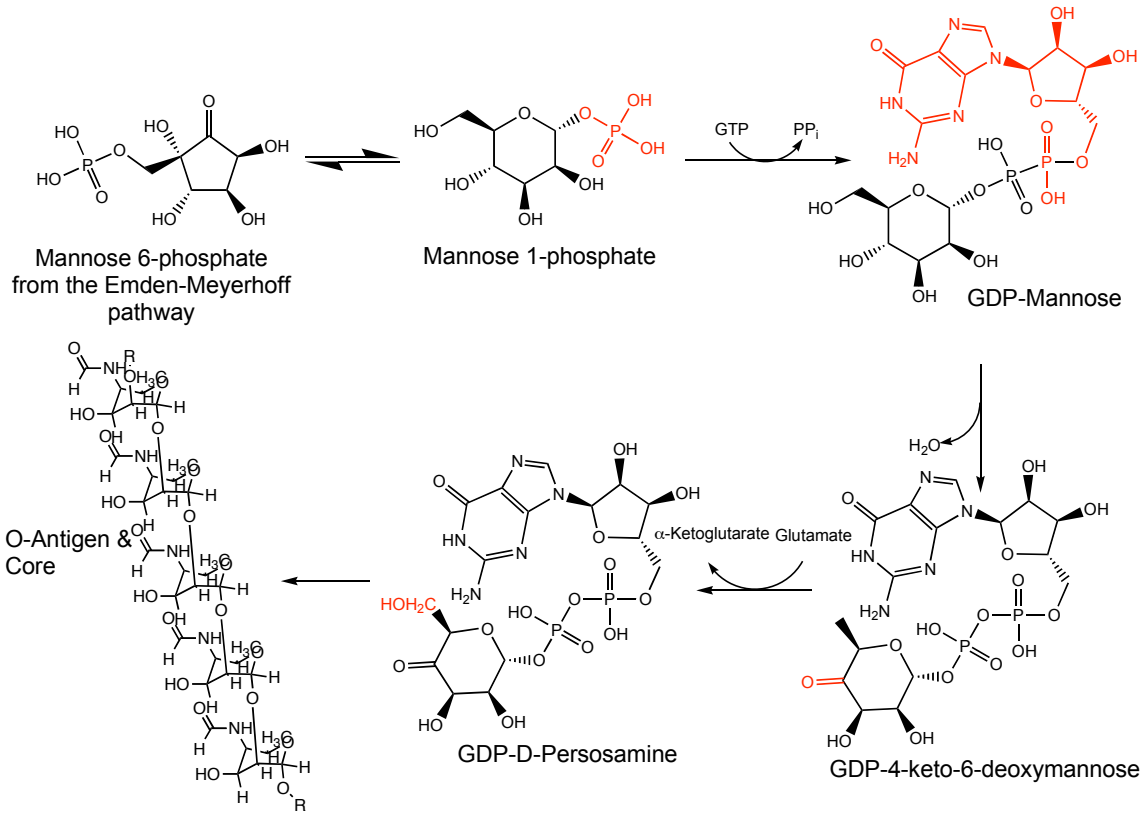


FIG. 1. Schematic showing O-antigen biosynthesis steps in *Brucella* spp. Phosphomannose isomerase, *manA,* is responsible for conversion of fructose 6-phosphate to mannose 6-phosphate in the Embden-Meyerhof pathway (a). Phosphomannomutase, *manB,* is responsible for the inter-conversion of mannose 6-phosphate to mannose 1-phosphate in the production of precursors for LPS (b). GDP-mannose pyrophosphorylase (GMP), *manC* (BMEI0344), transfers a guananyl residue to the mannose 1-phosphate from *manB*, completing synthesis of GDP-D-mannose (c). Phosphomannomutase and GDP-mannose pyrophosphorylase are thought to participate solely in the production of GDP-D-mannose. The pathway is completed in subsequent steps, including elongation of the O-side chain. Allen et al. demonstrated interruption of the phosphomannomutase BMEII0899 (*manB*) locus by the transposon Tn5 causes attenuation and shift from smooth LPS (sLPS) to rough LPS (rLPS) (5), (4). Using similar transposon based techniques or allelic exchange with antibiotic resistance markers, additional loci (Table 1) have been shown to cause defects in O-antigen synthesis.

Table 1. Summary of genes implicated in rough phenotype. This table was adapted from Delrue et al. (33).

Gene	Function	*B. melitensis* ORF
lpsB, lpcC	Mannosyltransferase, Core Biosynthesis	BMEI0509
wbdA	Mannosyltransferase, conversion of GDP-D-mannose to mannan, O-chain biosynthesis	BMEI0997
lpsA	Putative glycosyltransferase, O-Antigen polymerase (13), O-chain biosynthesis	BMEI1326
wbpZ	O-chain biosynthesis, mannosyltransferase C	BMEI1393
manB	Phosphomannomutase, uses GTP and mannose-6-phosphate to produce mannose-1-phosphate, O-chain biosynthesis	BMEI1396
wbkA	Mannosyltransferase, O-chain biosynthesis	BMEI1404
rfbD	GDP-mannose 4,6-dehydratase, converts GDP-mannose to GDP-4-dehydro-6-deoxy-D-mannose + H2O, O-chain biosynthesis	BMEI1413
perA	Perosamine synthetase, converts GDR-4-keto-6-D-deoxymannose to 4-amino-4,6,dideoxymannose (GDP perosamine), O-chain biosynthesis	BMEI1414
wbpL	Putative undecaprenyl-phosphate α-N-acetylglucosaminyltransferase, catalyzes the attachment of the N-actyleglucosamine to the undecaprenol carrier for O-chain biosynthesis	BMEI1426
pgm	Phosphoglucomutase converts α-D-glucose 1-phosphate to α-D-glucose 6-phosphate, O-chain biosynthesis	BMEI1886
manB, pmm	Phosphomannomutase converts α-D-mannose-6-phosphate to α-D-mannose-1-phosphate, O-chain biosynthesis.	BMEII0899
manA, wbpW, pmi	Phosphomannose isomerase converts α-D-mannose-1-phosphate to GDP-mannose O-chain biosynthesis	BMEII0900

(primary or secondary sites) to smooth character. For example, the former vaccine strain 45/20 is a spontaneously appearing rough strain, derived from *B. abortus* 45 after 20 *in vitro* passages. The cause of the rough phenotype of 45/20 is unknown, however, its reversion to virulent smooth form resulted in its discontinued use as a vaccine strain shortly after introduction (129). In contrast, RB51, a current vaccine strain, is also a natural attenuated rough mutant of *B. abortus* S2308 that contains a transposon (IS711) insertion in the *wboA* gene, encoding glycosyltransferase (139). Reversion to smooth phenotype has not been reported for RB51 and the insertion in *wboA* appears to be one of several defects resulting in the rough phenotype (138, 139).

Mechanisms responsible for dissociation may include the activity of transposases, recombinases, resolvases, and integrases to facilitate illegitimate recombination. Analysis of the *B. melitensis* genome indicates 41 transposases (35). Transposases are typically encoded by the transposon they facilitate. The transposase recognizes the cognate element (particular sequences are recognized by different transposon families), breaks the DNA and inserting the transposon (116). To date, only one transposon-like element has been demonstrated in *Brucella* (71); tn2020 is 815bp. Recently, with the completion of the *B. abortus* genomic sequence, deletion events that may be important for speciation have become apparent (69). Similar events could be responsible for the loss of O-antigen expression.

Recombinases catalyze strand exchanges between specific DNA sequences, and can be broken into two categories, resolvases and invertases. No recombinases have been demonstrated in *Brucella*, but a BLAST search with the *B. melitensis* genome suggests several may be present. For our purposes, the most interesting may be the invertases, since they are well characterized in the *Salmonellae* (Hin, Gin, Pin, Cin) (94). *Brucella* possesses several palindromic repeat elements, which form stem loop structures within themselves (70). One could postulate that two of these repeat elements in close proximity could form a larger stem loop structure, essentially deleting the intervening structure by "looping out" (tandem repeat deletions) (17). Authors T. Ficht and C. Allen appear to suggest this possibility in their 1998 publication on transposon derived rough mutants, wherein they identify a Bru-RS1-like repeat near the *manBA* locus in *B. abortus* (5). The situation at *manBA* almost directly reflects that in *Salmonella* at the H2 locus. The H2 locus possesses an upstream element, the H segment that is flanked by two inverted repeats. Inversion of the H segment between the inverted repeats controls the expression at H2. Resolvases are another class of recombinase that shows homology to the Hin, Gin, Pin, and Cin invertases (14). They are mainly associated with transposons.

Other possibilities for genomic modifications exist; for example, *Brucellae* also possess the insertion elements IS711 (71), or IS6501 (110), about 900bp in length. IS6501/711 has been speculated to encode a transposase, similar to a

transposases found in *Mycobacteria.* A genomic island containing several insertion sequences was identified as containing the O-antigen coding genes of *Brucella* (26, 58). Most attention around *Brucella* insertion sequences has focused on IS6501/711, since it may contribute to the attenuation of the current vaccine strain, RB51 (137, 139).

Integrases are typically associated with phage, though horizontal transfer from phage to *Brucella* could conceivably introduce integrases to the genome. Integrases recognizes a specific site in DNA, and integrate viral DNA into the genome of the viral host. In their analysis of the *B. melitensis* genome, Del Vechhio et al., identified 16 ORFs related to phage on chromosome I.

Detailing modes of chromosomal rearrangement would not be complete without mentioning two other organisms. Prokaryotes are not known to exhibit programmed changes to their DNA. Two notable exceptions are the cyanobacteria *Anabaena* 7120, which excises two nitrogen fixation elements during heterocyst formation (63) and *Bacillus subtilis*, which excises 42kb from the *sigK* gene during sporulation. (88). Rearrangement of the nitrogen fixation gene in Anabaena is mediated by transposase-like elements, *xisA* and *xisC* as part of the programmed rearrangement cyanobacteria undergo in heterocyst formation. *XisA* was characterized by transposon and site directed mutagenesis, while *xisC* has only been characterized by its homology to *xisA*.

Mutation of Gram-negative bacteria from smooth to rough phenotype may be the result of random events or a response to changing environments. *Brucella* may need to be rough in order to adapt the host as a suitable environment. In the paradigm learned from enteric bacteria, an antigenic switch from smooth LPS to rough LPS would cause the host to switch from an inflammatory response, TH1, to TH2 response, during which IL-10 would be released. IL-10 functions to down-regulate TH1 responses and inhibits cytokine release, increasing the chances for colonization of the host. These rough organisms would almost certainly be susceptible to killing, but having served the purpose of modulating the host immune response, chronic infection with the surviving smooth *Brucellae* could be established.

SPECIFIC AIMS

This research focuses on the initial interactions between *Brucella* and the target cells that control the host's innate immune response. O-antigen produces the majority of antibody response *Brucella,* highlighting intimate processing of LPS by host cells. Recent observations demonstrate significant differences in the host response to smooth and rough *Brucella* (21, 112, 121), suggesting an important signaling role for O-antigen during these initial interactions. Ultimately, these tentative exchanges may control survival of *Brucella*, but the exact mechanism(s) remains undefined. The work proposed explores the contribution of O-antigen in establishing a productive infection. The central hypothesis of this work is that O-antigen restricts macrophage activation and promotes productive infection. A corollary to this is that the host environment is dynamic, and *Brucella* has evolved mechanisms to cope with changing environments. To explore this hypothesis, these aims are proposed:

A.1 Demonstrate, definitively, the link between *manBA* expression and LPS biosynthesis.

A.2 Determine the rate of appearance of spontaneous, rough isolates and identify genetic changes associated with their appearance.

A.3 Evaluate the role of *Brucella* LPS in uptake and survival of the organism through comparison of survival of smooth and rough variants *in vitro* and *in vivo*.

ManBA (BMEII0899-0900) IS ESSENTIAL FOR LPS BIOSYNTHESIS

INTRODUCTION

The lipopolysaccharide of *Brucella* is a classical gram negative LPS in form only: it is a tripartite structure consisting of lipid A, core oligosaccharide and O-antigen. At the molecular level and with respect to activity it is clearly non-classical. Compared to enterobacterial LPS, *Brucella* LPS contains only amide bonds as opposed to the typical amide and ester bonds, *Brucella* Lipid-A is a diaminoglucose rather than glucosamine, and the acylation of the Lipid A is much greater (C18-19, C28, rather than C12 and 14)(91). The activity of *Brucella* LPS is much lower than enterobacterial LPS' (23, 104, 119). O-antigen produces the primary antibody response in the host. In *Brucella abortus*, the O-antigen consists of a homopolymer of 4,6-dideoxy-4-formamido-D-mannopyranose (N-formyl perosamine) linked in an α-1,2 fashion (A antigen), while the N-formyl perosamine homopolymer found in *Brucella melitensis* consists of 4 residues linked in an α-1,2 fashion with a fifth in an α-1,3 fashion (91, 101, 104, 106) (FIG. 2).

The decreased activity of *Brucella* LPS can be correlated with an increased acylation state of the lipid A moiety (46, 66, 80). Lipid A interacts with host cells primarily through TLR-4. Altering the pattern of lipid A on the surface of the bacterium changes the dynamic between the TLR-4 receptor and the pathogen (83).

The phosphomannomutase/phosphomannose isomerase locus (*manBA*) has been implicated as being important for the production of LPS in models that use a transposon to interrupt the gene. Allen et al. and Monreal et al. studied transposon mutants of *B. abortus* (4, 5, 103) while Foulonge et al. characterized *manBA* transposon mutants in *B. suis* (53). The goal of this study was to knock out *manBA*, complement the loss of activity. This will definitively demonstrate the role of *manBA* in the production of *Brucella* LPS in *B. abortus* and *B. melitensis*. In these models, LPS has been shown to play an important role in resistance to complement-mediated killing, survival in macrophage models and animal models. Based on these studies, our hypothesis is that *manBA* (BMEII0899-900) is important for LPS biosynthesis.

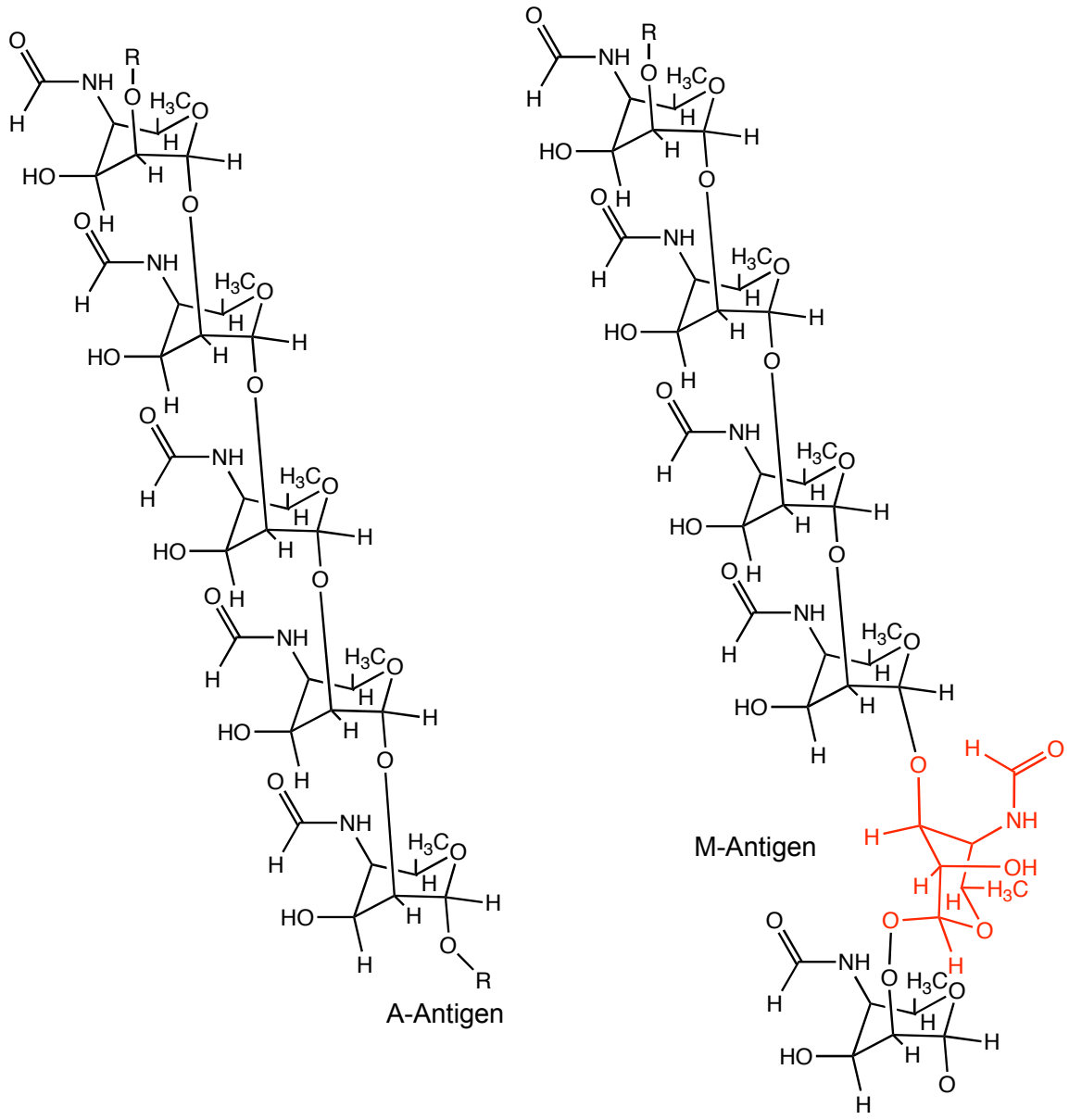


FIG. 2. Molecular schematic of A- and M-antigen. Found in the lipopolysaccharide of Brucella abortus and Brucella melitensis, respectively. Brucella O-antigen is a homopolymer of 4-formamido-4,6-dideoxymannose (N-formyl perosamine), repeating approximately 100 residues. In B. abortus, the homopolymer is linked predominantly in an -1,2 fashion, while B. melitensis N-formyl perosamine is four units linked in -1,2 with a fifth unit linked -1,3. This heterologous linkage in M-Antigen is displayed in red.

MATERIALS AND METHODS

Bacteria, plasmids, growth conditions and restriction endonucleases.

All bacteria and plasmids used during the course of this investigation are listed in Table 2. All of the restriction endonucleases used in this study were purchased from Roche Biochemicals, with the exception of Asc I, which was purchased from New England Biolabs. FastStart Taq was purchased from Roche Biochemicals. AccuTaq LA was purchased from Sigma Chemicals. T4 DNA Ligase was purchased from Promega.

Virulent *B. abortus* S2308 (B. Deyoe, NADC) and *B. melitensis* 16M (ATCC), were re-isolated from aborted fetuses of cattle or goats, respectively. *Brucella* strains used in this study, or their derivatives were grown on tryptic soy agar (TSA, Difco Laboratories, Detroit, MI) medium with appropriate antibiotics, except where noted. Liquid cultures of *Brucella* were grown in tryptic soy broth (TSB, Difco Laboratories, Detroit, MI) or SOC-B [6% (w/v) trypticase soy broth, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose](89). *E. coli* DH10B, DH5a, β2155, bacterial cultures were routinely diluted in either peptone saline [1% Bacto peptone (w/v), 0.5% NaCl (w/v/)], or phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4). All *Brucella* cultures were inactivated by autoclaving or heat killing in phenol saline [0.05% (v/v) phenol/0.85% (w/v) NaCl] at 65°C for a minimum of 2 hours. *E. coli* cultures were incubated on Luria-Bertani (LB, Difco Laboratories)

Table 2. List of bacterial strains and plasmids used in this study.

Strain/Plasmid	Relevant Markers	Source
B. abortus		
S2308	*B. abortus* wild-type isolate, type strain (biovar 1)	Lab stock, bovine isolate
S2308Δ*manBA*	S2308 Δ*manBA*::*Km*^{R}	This study
B. melitensis		
16M	*B. melitensis* wild-type isolate, type strain (biovar 1)	Lab stock, goat isolate
16MΔ*manBA*	16M Δ*manBA*::*Km*^{R}	This study
E. coli		
DH10B	F- *mcrA Δ(mrr-hsdRMS-mcrBC) 80lacZΔM15 ΔlacX74 recA1 endA1 araΔ139 Δ(ara-leu)7697 galU galK rpsL nupG*	Invitrogen
TOP10	F- *mcrA Δ(mrr-hsdRMS-mcrBC) 80lacZΔM15 ΔlacX74 recA1 ara139 Δ(ara-leu)7697 galU galK rpsL endA1 nupG*	Invitrogen
DH5α	F- *80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17(rk-, mk+) phoA supE44 - thi-1 gyrA96 relA1*	Invitrogen
β2155	*ThrB1004 pro thi strA hsdS lacZDM15 (F' lacZΔM15 lacI*^{q} *trajD36 proA1 proB1) ΔdapA::erm (Erm*^{R}*) pir::RP4 (::kan (Km*^{R}*) from SM10)*	Dehio et al.(32)
pBBR1MCS6-y	Cloning vector, Cm^R, GFP	P. Elzer (107)
pBluescript KS II(+)	Cloning vector	Stratagene
pKD4	Source of *nptII* cassette and FRT sites	B. Wanner (31)
pJET1445	*B. melitensis manBA* operon in pBBR1MCS6-y, Cm^R	This study
pJET931MA	pBluescript containing *manBA* 3' and 5' fragments, used to construct pJET940MA, Amp^R	This study
pJET940MA	Suicide vector for deletion of *manBA,* Kan^R, Amp^R	This study

plates for 18 hours with appropriate antibiotics. When appropriate, antibiotics were added to the following final concentrations: kanamycin, 100 μg/ml; chloramphenicol, 30μg/ml and ampicillin, 100 μg/ml. Unless otherwise noted, all bacterial strains were grown at 37°C, 5% (v/v) CO_2.

Molecular biology, polymerase chain reaction, and primers. Genetic manipulations were carried out using standard techniques (127). Primers used in this study can be found in Table 3. All primer design was performed using Accelrys MacVector™.

DNA was digested with selected restriction endonucleases using 1U of enzyme per microgram DNA. Ligations were performed in a total volume of 20μl, containing 2μl 10X ligase buffer, 6U of T4 ligase and 3:1 molar ratios of insert:vector DNA. Total DNA was between 150-500ng. Ligation mixtures were incubated at 14°C for 18 hours before transformation. Typical PCR reactions contained reaction mixture contained 50-200ng target DNA, 0.5μM of each primer, 200μM of each deoxyribonucleoside triphosphate, 2U of FastStart Taq polymerase, 1x PCR buffer, 2mM $MgCl_2$. As necessary, PCR products and digested vectors were gel purified and extracted using the QIAGEN QIAEXII gel purification kit.

Southern blotting is a routine technique and was used for examining the presence or absence of genes in total genomic DNA.

Table 3. List of primers used in this study

Primer	Sequence	Purpose
TAF204	GGCGCGCCACGTCTTGAGCGATTGTGTAGG	*nptII* amplification
TAF205	GGCGCGCCGGACAACAAGCCAGGGATGTAAC	
TAF234	GCTCTCCATGATTTCGGGTA	Amplification of *nylC*
TAF235	CGCTTCGCAAAATTGAAAAT	
TAF251	CGGGATCCCGGCAAAGGGTTCCGCAATAC	5' *manBA* fragment
TAF254	GGCAAAATACCGGCGCGCCGCAAATCCCTGCCGACAAAC	
TAF252	CGGGATCCCGGGTCAGTCATCAGTTGCGGATTC	3' *manBA* fragment
TAF253	GCAGGGATTTGCGGCGCGCCGGTATTTTGCCCCTCGTCCTG	
TAF419	TTCCAGGCAGATACAGG	Amplification of *manBA*
TAF420	ATCCCAATAGGCCGAATGCCAA	
TAF433	GAAGATCTTCCAGGCAGATACAGG	*manBA* expression construct
TAF434	GAGATCTATCCCAATAGGCCGAATGCCAA	

Production of hybrid PCR products. Hybrid PCR products were produced in order to construct an allelic exchange knockout vector. Primers were designed to amplify regions of DNA flanking the 5' and 3' ends of *manBA*. Specifically, the reverse primer of the 5' region and forward primer of the 3' region have been designed to contain complementary sequences, with an engineered restriction site. Products from this PCR reaction were diluted 1:100 and used as template in a subsequent reaction to create a hybrid PCR product, consisting of the 5' product and 3' product, joined. This hybrid product contains a restriction site in the center that was exploited to clone in a selectable marker (FIG. 3).

Transformation and selection of recombinant plasmids. Following ligations, 20 μl of cells (TOP10, DH5α, or DH10B) were mixed with a 1μl portion of the ligation mixture and kept on ice for 30 minutes. Samples were heat shocked at 42°C for 1 minute, then immediately placed on ice for 2 minutes. Two hundred and fifty microliters of SOC [6% (w/v) trypticase soy broth (w/v), 10mM NaCl, 2.5mM KCl, 10mM MgCl2 and 20mM glucose] were added to the samples, which were then incubated at 37°C with agitation for one hour. Samples were plated on plates containing appropriate antibiotics and screened the following day by preparing mini-preparations of plasmid DNA followed by digestion with restriction endonucleases to map the product. Recombinant plasmids in *E. coli* hosts are frozen in 50% peptone saline/glycerol and stored at -80°C.

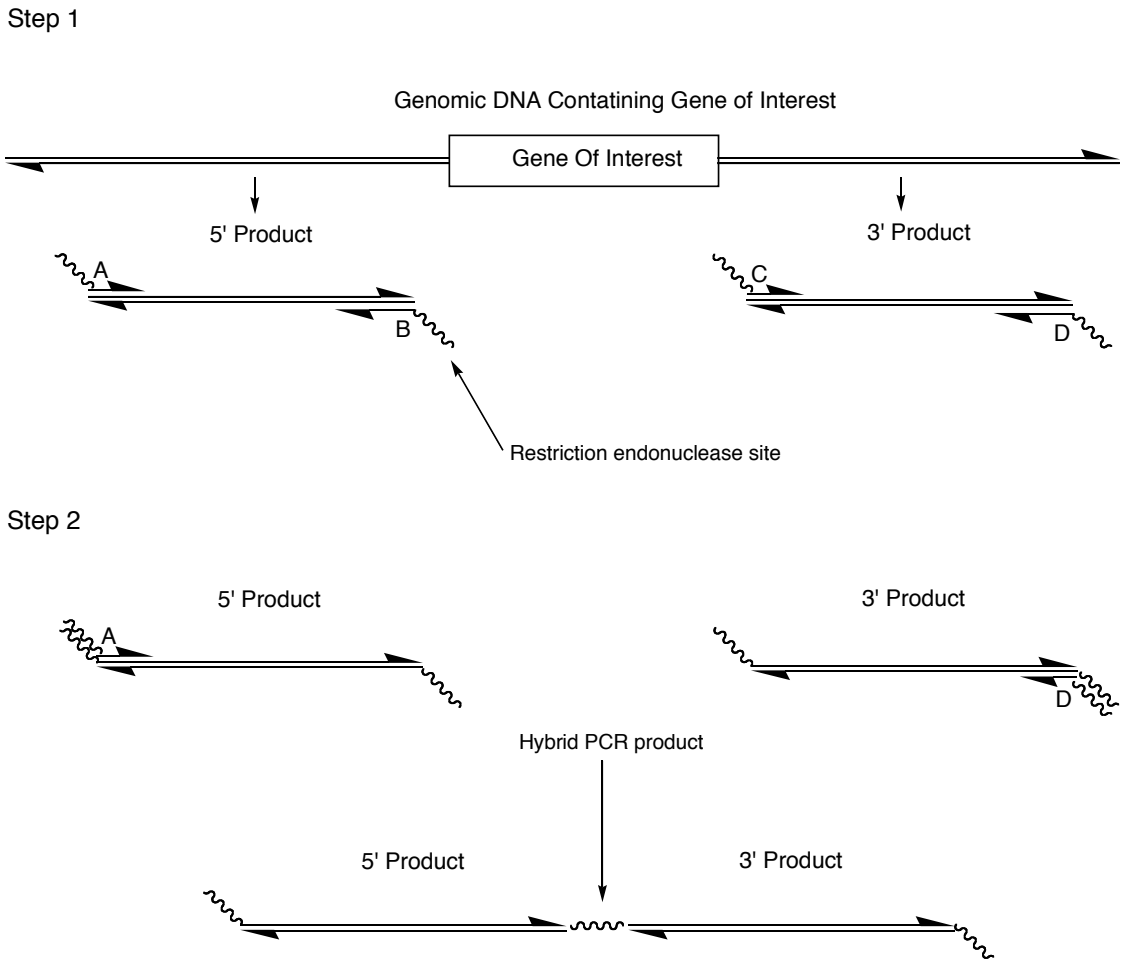


FIG. 3. Production of hybrid PCR products. In the first round of amplification, regions flanking the gene of interest are amplified. In this cartoon, the 5' product is AB and the 3' product is CD. In the second round of amplification, product AB and product CD are used as the template, with the forward primer for product AB and the reverse primer for product CD. The result is a hybrid PCR product that joins AB and CD. Wavy lines signify restriction endonulcease recognition sites that were introduced during primer design.

Transformation and selection of *Brucella* mutants. The knockout vector was delivered to virulent *B. abortus* S2308 and *B. melitensis*16M via electroporation. Strains were grown for electroporation by one of two methods. In the first approach, *Brucella* was grown from the frozen stocks on tryptic soy agar to confluence for 72 hours at 37°C. Bacteria were re-suspended by scraping off the plate into 5 ml of PBS. The resulting suspensions, containing approximately 5 x 10^{11} CFU/ml, were transferred from the plate into a 50ml conical tube.

Alternatively, isolated colonies were selected following 72 hours of growth and used to inoculate 5 ml of fresh TSB. These cultures were grown for 48 hours to achieve saturation. Fifty microliters of the saturated culture was used to inoculate 10 ml of fresh TSB. Cultures were incubated at 37°C for 18 hours, achieving a density of approximately 2x10^9 CFU/ml.

Once the bacterial suspensions were prepared, they were pelleted by centrifugation at 3800g x 15 minutes in a Jouan CR 4.12 preparative centrifuge equipped with a M4 swinging bucket rotor. From this point forward, all reagents were kept at 4°C. The supernatant was removed and the pellet was washed three times with 50ml ice-cold molecular biology grade water (Mediatech, Herndon, VA). After the final wash, the pellet was re-suspended in 1 ml of ice-cold molecular biology grade water. Two microliters of RNA-free plasmid (~0.1-0.3 μg) was added to 70 μl washed cells in a sterile microfuge tube. This

mixture was stirred with a pipet tip and placed in a chilled electroporation cuvette with a 1 mm gap (VWR, West Chester, PA). Current was applied from a BTX 600 electroporation generator set to 265 ohms, 25 μF, 2.5 kV (BTX, Holliston, MA) in a BL3 biological safety cabinet. One milliliter of SOC-B was used to remove cells from the cuvette to a sterile microfuge tube. Cells were allowed to recover at 37°C, with shaking, 6 to 18 hours. After the recovery period, electroporants were plated on TSA with appropriate antibiotics. Colonies arose between 3-8 days and were screened by various techniques. Knockouts were confirmed by PCR analysis and Southern blotting.

Transformation of *Brucella* strains by conjugation. *Brucellae* were incubated on TSA for 72 hours to achieve confluence. The donor *E. coli* strain β2155, was incubated on plates containing appropriate antibiotics and 50μg/ml diaminopimelic acid (DAP) for 24 hours. To prepare the conjugations, both strains, donor and recipient, were harvested from plates in approximately 2 ml peptone saline with DAP. The bacterial suspensions were pelleted for 2 minutes x 12,000G in a microfuge. The pellets were re-suspended in 100μl of peptone saline containing 50μg/ml DAP. The bacterial suspensions were combined and applied to NC20 nitrocellulose filters (Schleicher and Schuell, Dassel, Germany) on a TSA plates containing 50μg/ml DAP. Mating was allowed to occur over a 2-hour period, after which the filter was harvested, placed in a microfuge tube, and the bacteria washed off with 1 ml peptone saline without DAP. Conjugants

were recovered by plating the entire suspension TSA with appropriate antibiotics. Screening of conjugants was performed by crystal violet staining (6).

Molecular detection of LPS. Five milliliters of TSB with appropriate antibiotics were inoculated with *Brucella*. After the culture reached saturation, 48-72 hours, the bacteria were killed by the combination of heat and exposure to phenol. Cultures were pelleted at 3800g x 15 minutes in a Jouan CR 4.12 preparative centrifuge equipped with a M4 swinging bucket rotor. The pellets were re-suspended in phenol saline and incubated in a 65° water bath for 18 hours. The heat killed *Brucella* were pelleted and washed with PBS to remove residual phenol saline as previously described. After the final wash, pellets were re-suspended in PBS to achieve an optical density of 0.8 at a wavelength of 420nm. One and one-half milliliter of this suspension was pelleted at 13,000g x 2 minutes in a microfuge using a fixed angle rotor. The supernatant was aspirated. Fifty microliters of PBS, pH 7.2, was used to re-suspend the pellet; 50 μl of 2x Laemmli sample buffer was added to the suspension. Samples were heated at 100 °C for 15 min, allowed to cool, spun briefly in a microfuge. A volume of 15μl of each sample was loaded onto an SDS-PAGE gel. The gel was electrophoresed at 200V until the dye front reached the bottom edge of the gel. Characterization of LPS occurred using one of the two following methods:

In the first method, fluorescent labeling of LPS was performed directly in polyacrylamide gels, utilizing the fluorescent hydrazide, Pro-Q Emerald 300 dye.

This dye can be conjugated to glycoproteins. The reaction generates a highly fluorescent conjugate that can be visualized easily (132), using a standard gel documentation setup for agarose gels, with UV transilluminator, as described by the manufacturer, Molecular Probes.

For the second method, *Brucella* LPS was visualized by Western blotting utilizing antibody to *Brucella* LPS. The electrophoresed lysates were transferred to a PVDF membrane (Immobilon-P, Millipore) using a semi-dry procedure. The stacking gel was removed from the polyacrylamide gel containing the electrophoresed samples. The gel was equilibrated in cathode buffer [25 mM Tris, 20% (v/v) methanol, 40 mM 6-amino-N-hexanoic acid, pH not adjusted] for 10 minutes. The transfer membrane was soaked in absolute methanol for 10 seconds, and then moved to anode buffer 2 [25 mM Tris, 20% (v/v) methanol, pH 10.4] for 5 minutes. Simultaneously, wicks were prepared by soaking two pieces of filter paper (3mm Whatman Chromatography Paper) in anode buffer 1 [300 mM Tris, 20% (v/v) methanol, pH 10.4], one piece of filter paper in anode buffer 2, and three pieces of filter paper in cathode buffer. The proportions of each component of the transfer stack were the same as the polyacrylamide gel. The transfer stack was assembled on the anode plate of a semi-dry transfer cell (Owl Separation Systems, Portsmouth, NH), placing the two pieces of filter paper soaked in anode buffer 1, followed by the piece of filter paper soaked in anode buffer 2, the transfer membrane, the gel, the three pieces of filter paper

soaked in cathode buffer, and finally the cathode plate. Protein was transferred to the transfer membrane using constant current of 2 mA/cm^2 of gel area 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, cat# A7030). The membrane was washed in PBS containing 0.05% (v/v) Tween-20 (PBS-T). Depending on the species, the whole blot was incubated 2 hours to overnight in either sensitized anti-*Brucella melitensis* goat sera (diluted 1:200 in PBS-T) or anti-*Brucella abortus* LPS mouse monoclonal antibody 39 (diluted 1:200 in PBS-T, kindly provided by Dr. L.G. Adams). After being washed in 3 times in PBS-T, the blot was incubated 2 hours to overnight with a secondary antibody, either 1:5000 rabbit anti-goat or 1:2000 goat anti-mouse, conjugated to alkaline phosphatase. The secondary antibody was removed and the blots were washed 2x in PBS, 1x in molecular biology grade water. Signal was visualized by reaction with NBT-BCIP solution prepared according to manufacturer instructions (Roche Biochemicals). The alkaline phosphatase reaction was stopped by the addition of TE (10mM Tris, 1mM EDTA, pH 8.0).

Phenotypic confirmation of rough mutants. The rough phenotype of selected mutants was confirmed by acriflavine agglutination (6). Ten microliters 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 solution of acriflavine. Agglutination is consistent with a rough phenotype (6).

Complement mediated killing. Fresh serum was collected from goat, cow and human donors for use in these experiments. Complement mediated killing was measured as per Corbeil et al. (29) Bacteria were incubated for 24 hours on solid media as appropriate for the strains being tested. The bacteria were harvested tryptone-phosphate buffer, pH 8.0 [25mM Na_2HPO_4, 25mM NaH_2PO_4, 0.1% tryptone (w/v)]. The cell concentration was adjusted to a reading of 0.125 at OD_{610} (2 x 10^9 cfu/ml). Bacteria were diluted 1:10 with tryptose-phosphate buffer. 50μl of dilute bacteria were added to 50μl of fresh, undiluted serum in the wells of a microdilution plate. Control assays included heat inactivated sera. Mixtures were incubated for 4 hours at 37°.

RESULTS

Targeting of *manBA* for deletion by allelic exchange. Primers suitable for this study were designed with the aid of Accelrys MacVector™. Amplification of *nptII* from pKD4 was performed using primers TAF204 and TAF205, each of which contains an AscI restriction endonuclease recognition site to facilitate cloning. The 5' and 3' *manBA* knockout products were amplified using primer pairs TAF251/TAF254 and TAF252/TAF253 respectively. Primers TAF251 and TAF253 contain a BamHI restriction endonuclease recognition sequence to facilitate cloning. Primers TAF254 and TAF252 contain complementary regions

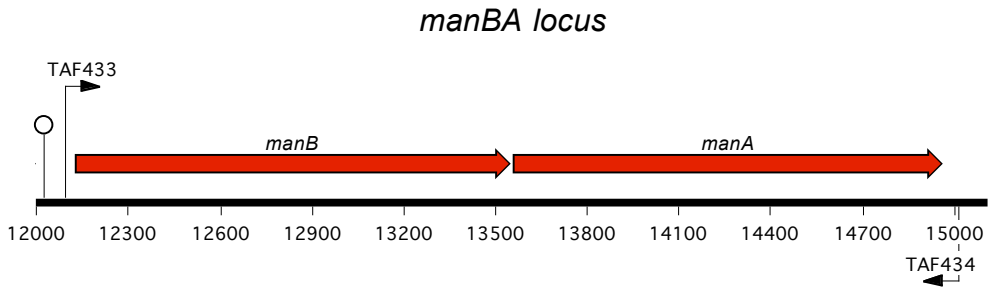
consisting of an AscI restriction endonuclease recognition site to both facilitate cloning of the *nptII* cassette and cross-priming of PCR products to produce a hybrid product. The reaction mixture contained 1μl *B. melitensis* crude extract DNA from a lyzozyme/proteinase K digestion, 0.5μM of each primer, 200μM of each deoxyribonucleoside triphosphate, 2U of FastStart Taq polymerase, 1x PCR buffer, 2mM $MgCl_2$. Amplification of the hybrid product was achieved using a reaction mixture containing 1μl of 1:100 fold diluted PCR product for the 5' and 3' regions.

Construction of *manBA* knockout vector. A vector that is non-replicative in *Brucella* was constructed to knockout *manBA* (FIG. 4). A hybrid PCR product consisting of the 5' and 3' regions flanking *manBA* was cloned into the BamHI restriction endonuclease site of pBluescript II KS(+), and transformed into *E. coli*. Thermal cycler conditions for amplification of the 5' and 3' flanking regions from genomic DNA included initial activation of FastStart Taq, 4 minutes at 95°C, 30 cycles of amplification that included a 30 second denaturing step at 95°, 30 seconds of annealing at 63°C, 2 minutes of elongation at 72°C. Final extension was carried out for 1 cycle at 72°C for 7 minutes. In a second round of PCR the 5' and 3' regions amplified during the first round PCR are used as template, with the forward primer from the 5' region and the reverse primer from the 3' region. Clones were verified for the presence of the hybrid PCR product, and the

resultant plasmid, pJET931MA, was prepared for subsequent cloning steps (FIG. 5)

The second step consisted of amplifying the *nptII* cassette from pKD4 (31) with primers that contain restriction sites matching the restriction site in the middle of the hybrid product from step 1. The primers for amplifying the Km^R cassette from pKD4 amplify a 1600bp fragment that contains two FRT sites in addition to the *nptII* cassette. Thermal cycler conditions for amplification of *nptII* from pKD4 included initial activation of FastStart Taq, 4 minutes at 95°C, 30 cycles of amplification that included a 30 second denaturing step at 95°, 30 seconds of annealing at 65°C, 1.5 minutes of elongation at 72°C. Final extension was carried out for 1 cycle at 72°C for 10 minutes. Though not used in this study, FRT sites can be used to excise the Km^R cassette by use of the flippase recombinase. After the Km^R cassette was amplified, it was cloned into the vector containing the hybrid product from first step. This marked plasmid, pJET940MA (FIG. 5), was isolated from the appropriate *E. coli* hosts for electroporation into both *B. abortus* and *B. melitensis*.

A



B

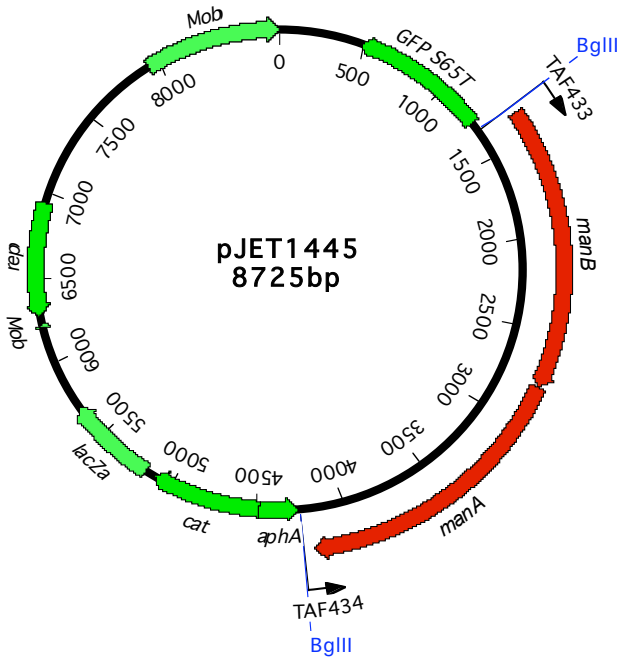
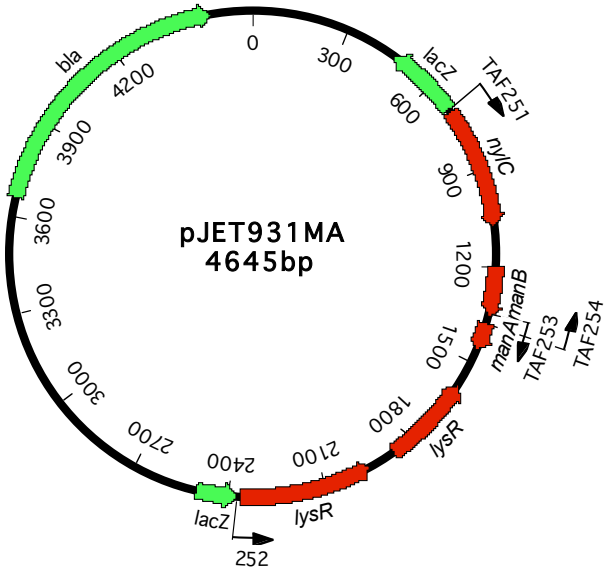


FIG. 4. The *manBA* locus. Panel A, the *manBA* locus was amplified using primers TAF433 and TAF434. The promoter predicted by the Baylor College of Medicine Neural Network Promoter Prediction tool is displayed as an open circle on a stem. Panel B, the complete vector for complementation of *manBA* knockouts.



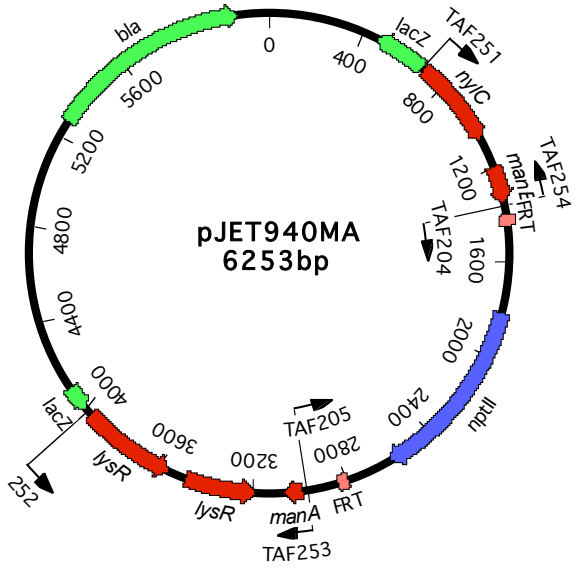


FIG. 5. Vectors constructed for knocking out *manBA*. pJET931MA was a stepping-stone to pJET940MA.

The *manBA* expression vector, pJET1445, was constructed in the broad-host-range plasmid, pBBR1MCS6-y (107). The vector and *manBA* PCR products were digested with Bgl II, and ligated. Primers (TAF433/434) for amplifying *manBA* exclude the putative promoter site predicted by the Neural Network Promoter Prediction tool, with a threshold of 0.80, hosted by the Baylor College of Medicine (131). A schematic is displayed in FIG. 4 and the exact sequence amplified is in Appendix A-1. The reaction mixture was optimized for production of a long-template product and contained 1μl *B. melitensis* crude extract DNA from a lysozyme/proteinase K digestion, 0.5μM of each primer, 500μM of each deoxyribonucleoside triphosphate, 2U of AccuTaq polymerase, 1x PCR buffer, 2mM $MgCl_2$. Thermal cycler conditions for amplification of *manBA* included initial activation of AccuTaq LA, 4 minutes at 95°C, 30 cycles of amplification that included a 30 second denaturing step at 95°, 30 seconds of annealing at 63.9°C, 4 minutes of elongation at 68°C. Final extension was carried out for 1 cycle at 68°C for 10 minutes. All samples were held at 20°C until being removed from the thermal cycler.

Brucella knockout mutants were screened for ampicillin sensitivity and kanamycin resistance. Southern blotting, using *nylC* as a probe, and PCR using primers TAF419/TAF420 were used to screen suspected knockouts (FIG. 6). Knockouts were screened phenotypically by use of acriflavine agglutination.

Results for the PCR screen (FIG. 6, Panel I) showed the presence of the anticipated 2.9kb fragment from the wild-type strain and the presence of a 1.9kb PCR product, indicative of the knockout. Results from the Southern blot (FIG. 6, Panel II) with the *nylC* probe on Hind III digested genomic DNA demonstrated the anticipated 10.2kb fragment from the wild-type strain the 14.8kb from the knockout strains.

Complementation test confirms restoration of LPS expression in rough *Brucella*. Knockout strains of B, *abortus* and *B. melitensis* were transformed by conjugation with *E. coli* strains carrying pJET1445. Complementation of rough defect was confirmed by several phenotypic tests, including acriflavine agglutination, Western Blotting and direct fluorescent labeling of *Brucella* LPS (FIG. 7).

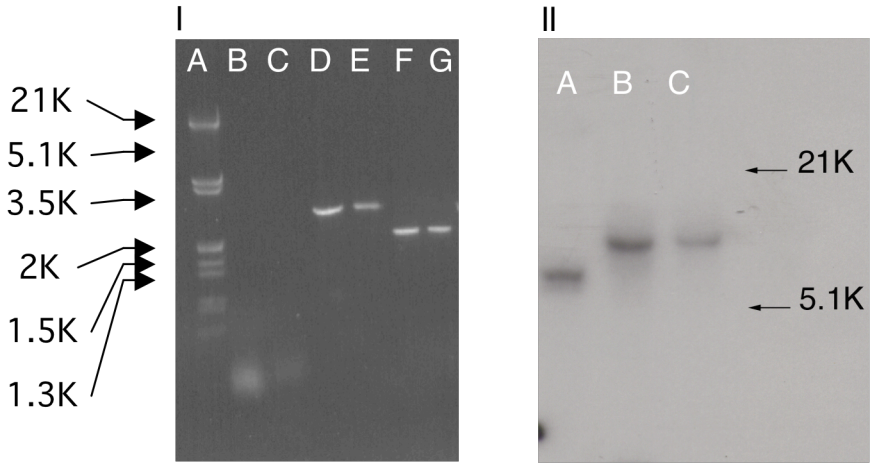


FIG. 6. Screening *manBA* knockouts. *ManBA* knockouts were screened by PCR and Southern Blotting. Panel I, agarose gel with PCR products A) Lambda HindIII/EcoRI, B) Distilled water control, C) TSB control, D) *Brucella melitensis* 16M wild-type product, E) *Brucella abortus* S2308 wild type product, F) *Brucella melitensis* Δ*manBA*, G) *Brucella abortus* Δ*manBA*. These results show the presence of the anticipated 2.9kb fragment from the wild-type strain and the presence of a 1.9kb PCR product, indicative of the knockout. In Panel II, results from the Southern blot (FIG. 6, Panel II) with the *nylC* probe on Hind III digested genomic DNA demonstrated the anticipated 10.2kb fragment from the wild-type strain the 14.8kb from the knockout strains.

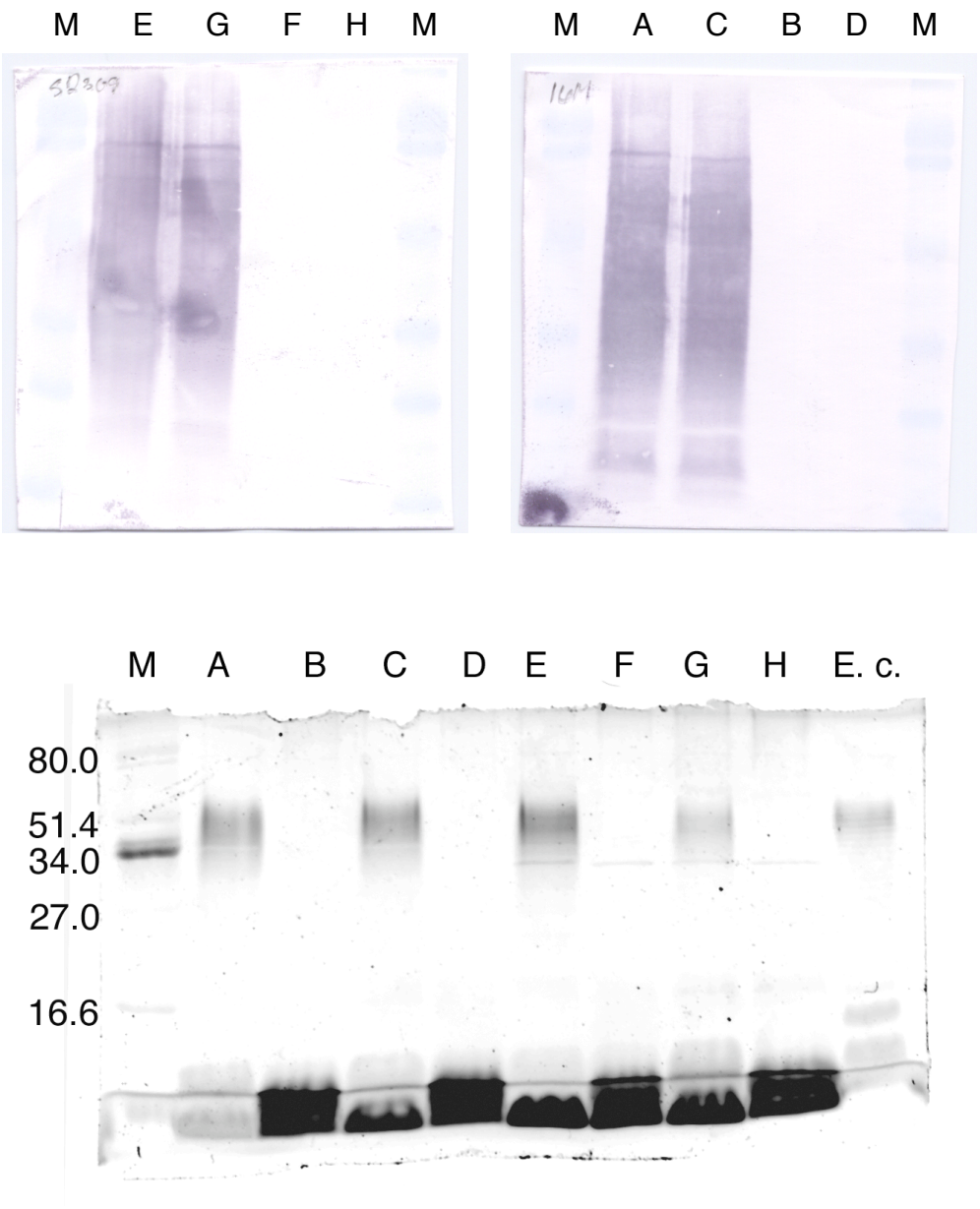


FIG. 7. Western blots of *Brucella*. Top: Western blots of *Brucella melitensis* and *Brucella abortus*. Bottom: ProQ-Emerald 300 glycoprotein staining of *Brucella* run on SDS-PAGE. Lanes are M – Marker, A – *B. melitensis* 16M, B– *B. melitensis* Δ*manBA*, C– *B. melitensis* 16M Δ*manBA*/pJET1445, D – *B. melitensis* 16M Δ*manBA*/pBBR1MCS6–y, E – *B. abortus* S2308, F – *B. abortus* Δ*manBA*, G – *B. abortus* 16M Δ*manBA*/pJET1445 H – *B. abortus* 16M Δ*manBA*/pBBR1MCS6–y, E.c. – *E. coli* O55:B5 smooth strain LPS.

Results from the acriflavine agglutination showed no agglutination with complemented strains. LPS production, as observed by Western Blotting and Pro-Q Emerald 300 staining was indistinguishable in complemented strains versus wild-type smooth strains.

Complement mediated killing of rough *Brucella* strains. Normal cow, human, and goat sera from healthy donors were tested for the ability to kill smooth and rough *B. abortus* and *B. melitensis* strains, as described in the Materials and Methods. As shown in FIG. 8 rough *B. abortus* strains were sensitive to serum complement, whereas *B. melitensis* is generally resistant to serum-mediated killing. Rough *B. abortus* strains were killed approximately 3 logs, while smooth strains were immune to killing. Smooth and rough *B. melitensis* strains were reduced at most by 0.3 log. The result with *B. melitensis* mirrors results with the naturally rough, virulent *B. ovis* and *B. canis* strains (22, 117).

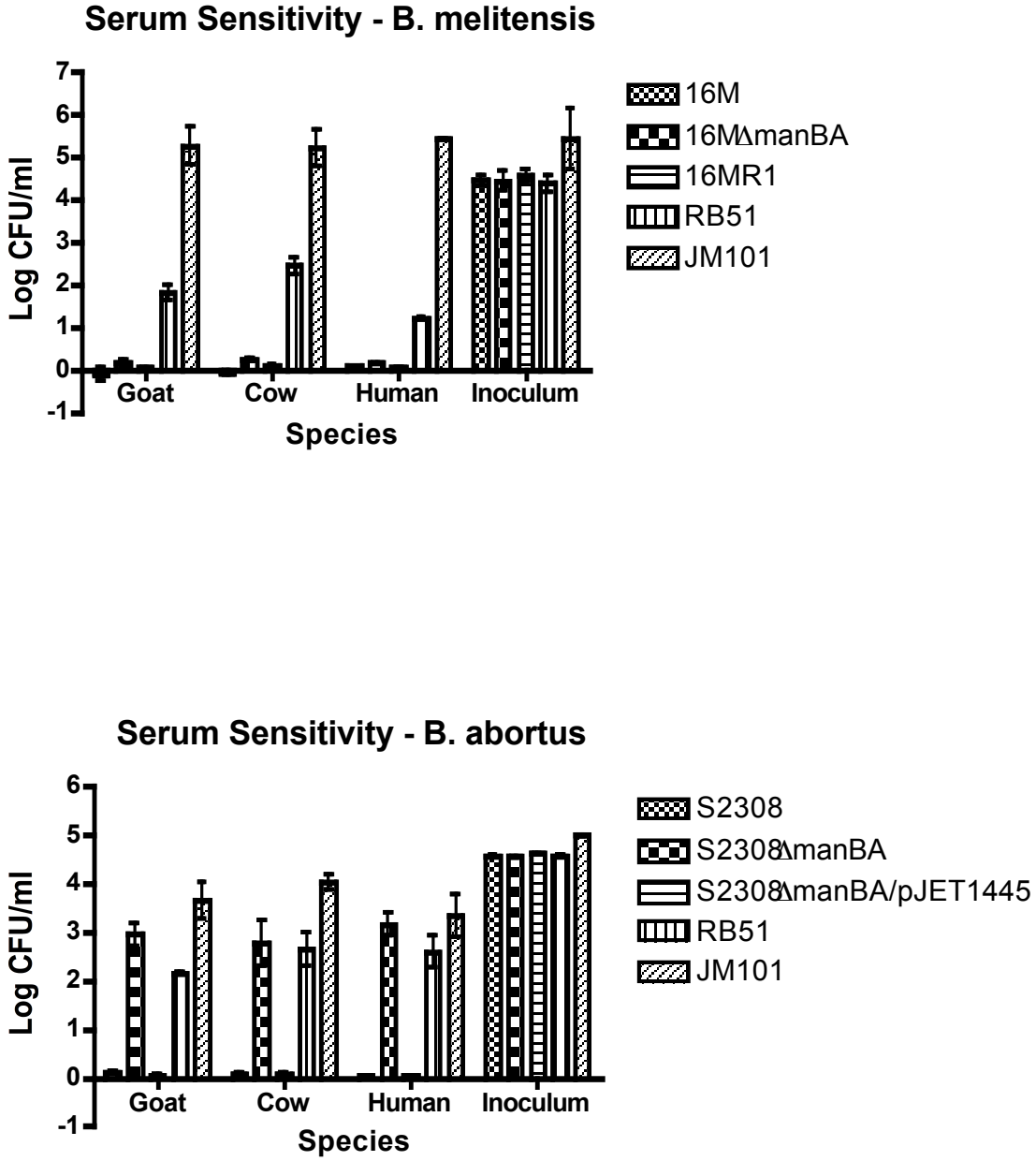


FIG. 8. Serum mediated killing of *Brucella*. Smooth and rough *Brucella* strains were incubated at 37°C with 50% normal cow, normal goat, and normal human serum for 4 hours. Portions of each suspension were plated, and bacterial counts enumerated. Values shown in the graph are the log of the CFU/ml in fresh serum subtracted from the log CFU/ml in heat-inactivated serum. The values shown for the inoculum are the CFU/ml in tryptone phosphate buffer after four hours.

DISCUSSION

In this study, the connection between phosphomannomutase and phosphomannose isomerase and LPS expression was definitively explored by constructing genetically defined mutants in both *Brucella abortus* and *Brucella melitensis*. Previous studies looking at the connection between this locus and LPS biosynthesis have relied on transposon mutants, which may suffer downstream effects as the result of transposon insertion (4, 53, 103). Understanding expression of LPS biosynthesis genes is an important first step to being able to control biosynthesis of LPS for either vaccines or understanding *Brucella* LPS' contribution to virulence.

Rough strains of *B. abortus* and *B. melitensis* were derived by allelic exchange of the *manBA* locus with a kanamycin resistance cassette. Further, trans-complementation of the genetic defect was achieved, restoring full biosynthetic properties. Western blotting and fluorescent labeling of *Brucella* LPS demonstrated restoration of phenotype. A functional assay examining resistance to serum-mediated killing showed that trans-complementation was effective in restoring phenotypic properties characterized for smooth *Brucella* strains (16, 29, 49, 104).

This study opens the door to construct *Brucella* strains with controllable LPS biosynthesis to investigate the role LPS plays in *Brucella* virulence.

GENETIC CHANGES AT *manBA* ARE RESPONSIBLE FOR HIGH FREQUENCY LOSS OF LPS EXPRESSION

INTRODUCTION

The appearance of rough *Brucellae* in culture has been regarded as a random occurrence attributable to changing environmental conditions. Dissociation in *Brucella* has previously been documented for the rough vaccine strain, 45/20. Unpredictable reversion to smooth phenotype was the main reason for discontinued use of strain 45/20 (105, 128, 129). The genetic basis for smooth to rough transition in strain 45/20 remains uncharacterized.

RB51, a spontaneously appearing rough variant of *B. abortus* was found to contain a transposon insertion in the *wbo*A gene (139). Complementation experiments attempting to restore the function of this locus did not restore the organism's normal smooth phenotype. Additional defects may have occurred because of *in vitro* passage. The rate of appearance of rough mutants has never been described in the literature although anecdotal evidence suggests that it is significant. The experiments described below were designed to determine the rate of appearance of rough variants and to determine whether this variation differs under *in vivo* conditions.

In experiments with T-1 phage, Luria and Delbrück established the number of mutations per genome per generation is equivalent to the number of phage resistant mutant bacteria compared to the total number of bacteria in the culture

(97). Further, they found that the phage resistant mutants were already in the bacterial culture rather than being induced by the presence of the phage. Generally, this frequency is 10^{-8} mutant bacteria per generation. Drake has extended this research to find that the range for background mutation in bacteria is 10^{-6} to 10^{-8} mutations per generation (39, 40). Mutation rates higher than 10^{-6} mutations per genome per generation are considered mutators.

Hypermutation in pathogenic bacteria is not unknown, and can be a method to affect dissociation. Oliver et al. have conducted extensive investigations examining the mutation of Pseudomonas in cystic fibrosis patients, wherein they characterized the mutation rate to Rifampin resistant strains as 3.2×10^{-6} (109). One aspect of these hypermutable Pseudomonas strain is changes in the expression of LPS and EPS on the surface of the bacterium (118). In fact, the mutation that allows *Pseudomonas* to adapt in cystic fibrosis patients is often the development of mucoid phenotype resulting from loss of control of *algC*, an ortholog of *manB*. A mutation that has been shown to bring this phenotype about is a C to T transition in the phosphomannomutase gene (142). Similarly, studies focused on *Salmonella* and *E. coli* have demonstrated hypermutable phenotypes in pathogenic strains, that confer an adaptive advantage to the bacterium in the host (15, 20, 57, 93, 108, 135).

The work presented here paralleled the work of Luria and Delbrück, utilizing BK-2 *Brucella* phage isolated from the Isfahan strain of *B. melitensis* (37, 38) to

establish the rough content of smooth colonies. The receptor(s) for BK-2 phage is largely uncharacterized, but one component is smooth LPS. Since the recognition incorporates smooth LPS, only smooth strains are susceptible to phage-mediated killing. This phage also has broad specificity enabling comparison of multiple *Brucella* species.

MATERIALS AND METHODS

Molecular biology, polymerase chain reaction, and primers. Genetic manipulations were carried out as described above. DNA Sequencing was performed on an ABI 3100 Genetic Analyzer at the DNA Technologies Lab, Department of Veterinary Pathobiology, Texas A&M University College of Veterinary Medicine and Biomedical Sciences. Primers used for amplification with were also used for sequencing. Sequence analysis was performed using Accelrys MacVector™ and DNAStar Lasergene™.

Bacteria, plasmids, growth conditions and restriction endonucleases.

In addition to the strains and plasmids listed in Table 2, *E. coli* strain AT2538 (CGSC), *Brucella melitensis* 16MΔ*manBA*, *Brucella abortus* S2308Δ*manBA* were used. The two *manBA* knockout strains are described in the previous chapter. Strain 20D2 is a transposon mutant with a transposon insertion in *pleD* (BMEII0660). All strains were grown as previously described.

The *Brucellaphage*, BK2, was obtained from J. Douglas (37, 38). Propagation of BK-2 phage was carried out in tryptic soy broth inoculated with a

suspension from a 24-hour culture of *B. abortus* S2308, so that the final bacterial concentration was approximately 5 x 10^8 cfu/ml. Phage was added to give a concentration of approximately 10^9 plaque-forming units. The culture was incubated at 37°C, with agitation, for 18 hours. The broth was then centrifuged to remove gross cellular debris and filtered through a 0.2μm polyethersulfone (PES) (Nalgene, Rochester, NY) membrane syringe filter.

Rough mutant isolation from smooth colonies. *Brucella abortus* and *Brucella melitensis* virulent isolates were grown on TSA to isolate individual colonies. Isolation of rough mutants was carried out using independent smooth *Brucella* colonies. Five individual colonies from each plate were suspended in 1ml of peptone saline, each, serially diluted, and plated on TSA in the presence of absence of BK-2 phage. Plates were overlaid with soft agar [7% (w/v) Bacto agar, 15 g/L TSB] containing at least 600,000 pfu of BK2-phage. After 72 hours, the total colony forming units were enumerated.

Determination of the background mutation rate in *Brucella*: Antibiotic resistance. *Brucella melitensis* and *Brucella abortus* were each grown on TSA, verified as antibiotic sensitive, and harvested (individual colonies or whole plates) into peptone saline after 72 hrs at 37°C. These bacterial suspensions were serially diluted and portions plated on TSA with and without antibiotics, including rifampin (100μg/ml), spectinomycin (100μg/ml), or gentamicin (10μg/ml). The number of colonies appearing on each of these substrates was

enumerated. The background mutation rate is equal to the number of colonies appearing on selective media divided by the number of colonies appearing on permissive media.

Determination of the background mutation rate in *Brucella*: Pyrimidine auxotrophy. To measure background mutation rate, pyrimidine auxotrophy was used through selection on 5-fluororotic acid (40μg/ml) in *Brucella* minimal media [BMM, 1.5% Bacto agar (w/v), 100mM NaCl, 60mM K_2HPO_4, 30% glycerol (w/v), 5% lactic acid (w/v), 10mM glutamic acid, 10mM $MgCl_2$, 5mM $FeSO_4$, 10mM $MnCl_2$, 60μM thiamine, 160μM nicotinic acid, 84μM Ca^{2+}-pantothenate, 8μM biotin] containing 50μg/ml uracil.

For determination of pyrimidine auxotrophy as a result of *pyrE* mutation (FIG. 9), independent *Brucella* colonies were re-suspended in 200 μl peptone saline. This suspension was then diluted 1000-fold in fresh peptone saline. A final 10-fold dilution was made into 200 μl aliquots of TSB in the wells of an ELISA plate. The resulting cultures, each inoculated with about 1000 cells, were incubated for 2 days, yielding visible turbidity. Cultures were removed from incubation when

the cell density reached approximately 5 x 10^7/ml. The density of viable cells was determined by plating a subset of these cultures on non-selective media. A 70-μl aliquot of each culture was plated on selective medium, and FOA-resistant colonies were analyzed after 8-10 days of incubation unless otherwise noted. As a positive control, the *E. coli*, uracil auxotroph strain, AT2538 was carried through all steps of the experiment. The *pyrE* mutation rate was calculated from a total of 94 independent *B. abortus* and *B. melitensis* colonies respectively.

Mutants from the *pyrE* study were randomly chosen for sequence analysis by applying the technique of Grogan et al. (65). Mutants were chosen for sequencing by marking a small dot on the back of the Petri plates on the day that cultures were plated. The colony closest to the dot was chosen for sequencing. Portions of each of the clonally pure culture were preserved at -70°C. Total genomic DNA was extracted from the remainder of the culture by use of lysozyme digestion followed by proteinase K digestion.

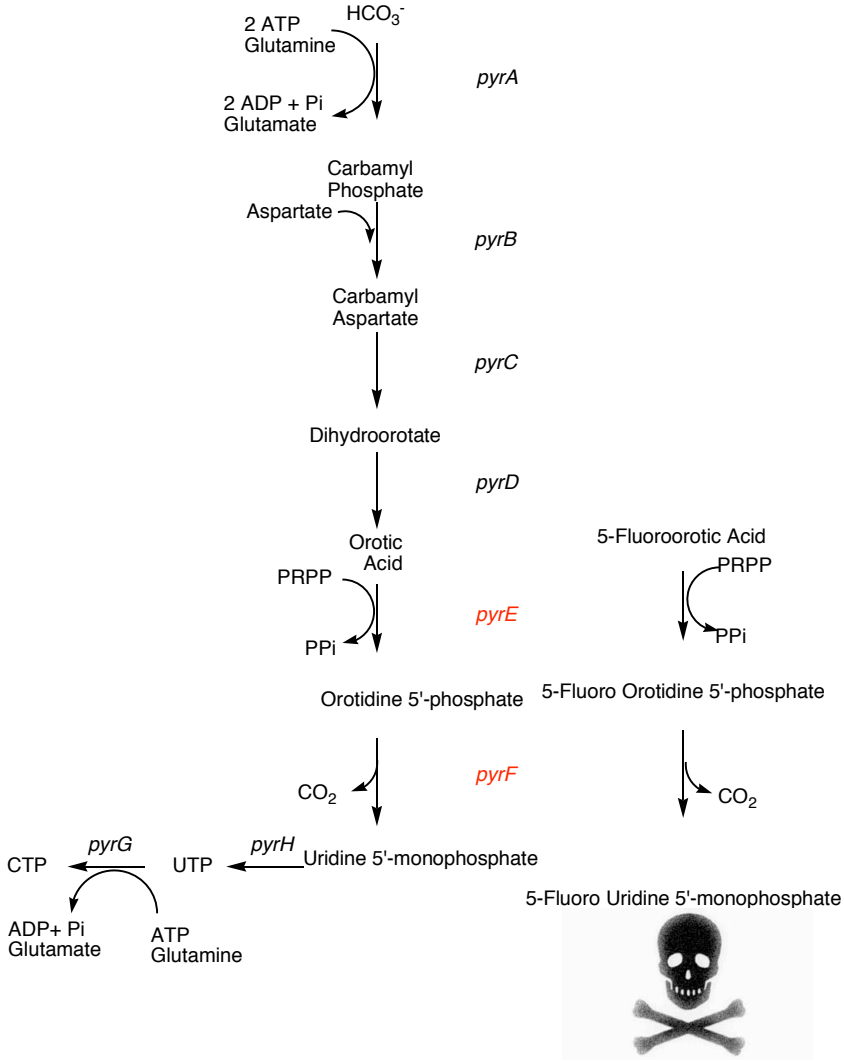


FIG. 9. Co-opting the pyrimidine biosynthetic pathway. The normal pyrimidine biosynthetic pathway in *Brucella melitensis* is shown on the left. The addition of 5-fluoroorotic acid (FOA) co-opts the pathway, resulting in cell death. Cells may avoid death by inactivation of either *pyrE* or *pyrF* (shown in red). The gene designations are as follows: *pyrA*, carbamoyl-phosphate synthase (EC 6.3.5.5, BMEI0522, BMEI0526, BMEI1781); *pyrB* aspartate carbamoyltransferase (EC 2.1.3.2, BMEII0670); *pyrC*, dihydroorotase (EC 3.5.2.3, BMEI1281, BMEII0669); *pyrD*, dihydroorotate dehydrogenase (EC 1.3.3.1, BMEI1611); *pyrE*, orotate phosphoribosyltransferase (EC 2.4.2.10, BMEI1295); *pyrF*, orotidine 5'-phosphate decarboxylase (EC 4.1.1.23, BMEI1999); *pyrH*, uridylate kinase (EC 2.7.4.4, BMEI0825); *pyrG*, CTP synthetase (EC 6.3.4.2, BMEI0850). Adapted from (84, 90).

Phenotypic confirmation of rough mutants. Rough mutants from were confirmed for rough phenotype by acriflavine agglutination (6). Ten microliters of bacteria from an 18-24 hour liquid culture, is spotted onto a glass slide. This sample is mixed with 10μl of a 0.1% (wt/vol) aqueous solution of acriflavine. Agglutination indicates a rough phenotype (6). Alternatively, crystal violet staining was used to test the presence of rough colonies on plated media, when harvesting a single colony was undesirable. The dye [2%(wt/vol) crystal violet dye, 20% (vol/vol) ethanol, 0.8% (wt/vol) ammonium oxalate] was used to flood a Petri plate containing the bacteria; colonies formed from rough mutants will absorb the dye readily and turn a dark purple. This dye is excluded by smooth colonies.

Genotypic tests for rough mutants. The presence of *manBA* was determined for all spontaneous rough isolates by PCR amplification. Cultures were grown 16-24 hours in tryptic soy broth. Samples were applied to an FTA CloneSaver^{TM} card, which inactivates the bacteria (Whatman). Two-millimeter punches were taken from each sample and used in a subsequent PCR. Complementation tests with the expression vector pJET1445 were performed for each mutant as previously described

Recovery of rough isolates from mice. Bacterial strains were grown on TSA plates and harvested in 5 ml of PBS. The bacterial concentration of suspension was estimated with an absorbance spectrophotometer, and adjusted

to a concentration of approximately 5 x10^6 CFU/ml. Plating the inocula and enumerating total CFU confirmed the infectious dose. Six to eight week old mice were injected with 100μl, in six parallel groups. The different inocula consisted of pure populations of *Brucella melitensis* 16M, *Brucella melitensis* 16MΔ*manBA*, 1:1 *B. melitensis* 16M wild type and Δ*manBA* and the same strains on a *B. abortus* background. The mice were sacrificed at 1 and 8 weeks post-inoculation by CO_2 asphyxiation. Spleens were collected from each group, spleen weight was determined, and spleens were homogenized with a tissue homogenizer (Omni 2000; Omni International, Inc.) for determining bacterial load. In addition to plating on TSA with appropriate antibiotics, a portion of each homogenate was plated on BK-2 phage as previously described, for enumeration of total rough bacteria. Data are the average $\log_{10}$ value of CFU per spleen over five mice.

***In vitro* competitive growth.** Genetically engineered rough *B. melitensis* was mixed with wild-type *B. melitensis* 1:1 in 5 ml of TSB. After 48 hours, 50μl was removed and passaged to fresh TSB for further culture. This was repeated for three passages. The rough content of each passage was ascertained by differential plating.

RESULTS

Dissociation rate determination. The dissociation rate for 60 *B. melitensis* and 59 *B. abortus* individual smooth colonies was determined. The number of rough colonies derived from each single smooth colony was enumerated, the ratio, rough cfu/smooth cfu from each smooth colony is the dissociation rate. The rate of dissociation for *B. melitensis* is 1.15×10^{-4}, while the frequency of dissociation of *B. abortus* rough variants is 1.27×10^{-5}.

In order to assess the appearance of rough mutants *in vivo*, mice were inoculated with 5×10^{5} CFU of each strain (smooth, rough) or a 1:1 mixture of smooth and rough. The survival and virulence characteristics were compared over eight weeks. The rate of dissociation remained similar to the *in vitro* model. After 1 week, rough mutants were isolated from mice infected with smooth *B. melitensis* at frequency of 5.94×10^{-3} and mice infected with *B. abortus* had a dissociation rate of 9.95×10^{-3}. After 8 weeks, these frequencies changed only slightly to 1.42×10^{-2} and 6.44×10^{-4} respectively. Mice infected with pure rough populations eliminated the bacteria rapidly; both *B. melitensis* and *B. abortus* rough strains were below the limit of detection after 1 week, and *B. abortus* rough mutants recovered slightly towards 8 weeks. In mice infected with 1:1 ratios of smooth and rough bacteria, rough strains were recovered out to 8 weeks, at similar frequencies to animals infected with pure populations of smooth strains. Since the marked mutant was not recovered from these

animals, these roughs represent newly derived mutants from the smooth portion of inoculum (FIG. 10).

Mutation rate estimation. Luria-Delbrück fluctuation calculations were applied to multiple independent cultures to estimate the rates of *pyrE* or *manBA* mutation. The method of the median is generally accepted as an unbiased method to determine the mutation rate in bacterial populations (86, 124). The method consists of calculating the median number of mutants and using that number to find the theoretical number of mutants per culture from the tables of Lea (92). Attempts to establish the background mutation rate of *Brucella* by spontaneous appearance of antibiotic resistant colonies were inconclusive due to the lack of isolation of resistant colonies. Based on the median culture size (approximately 10^8/ml), the rate from this test is less than 10^{-8} mutations per genome per generation.

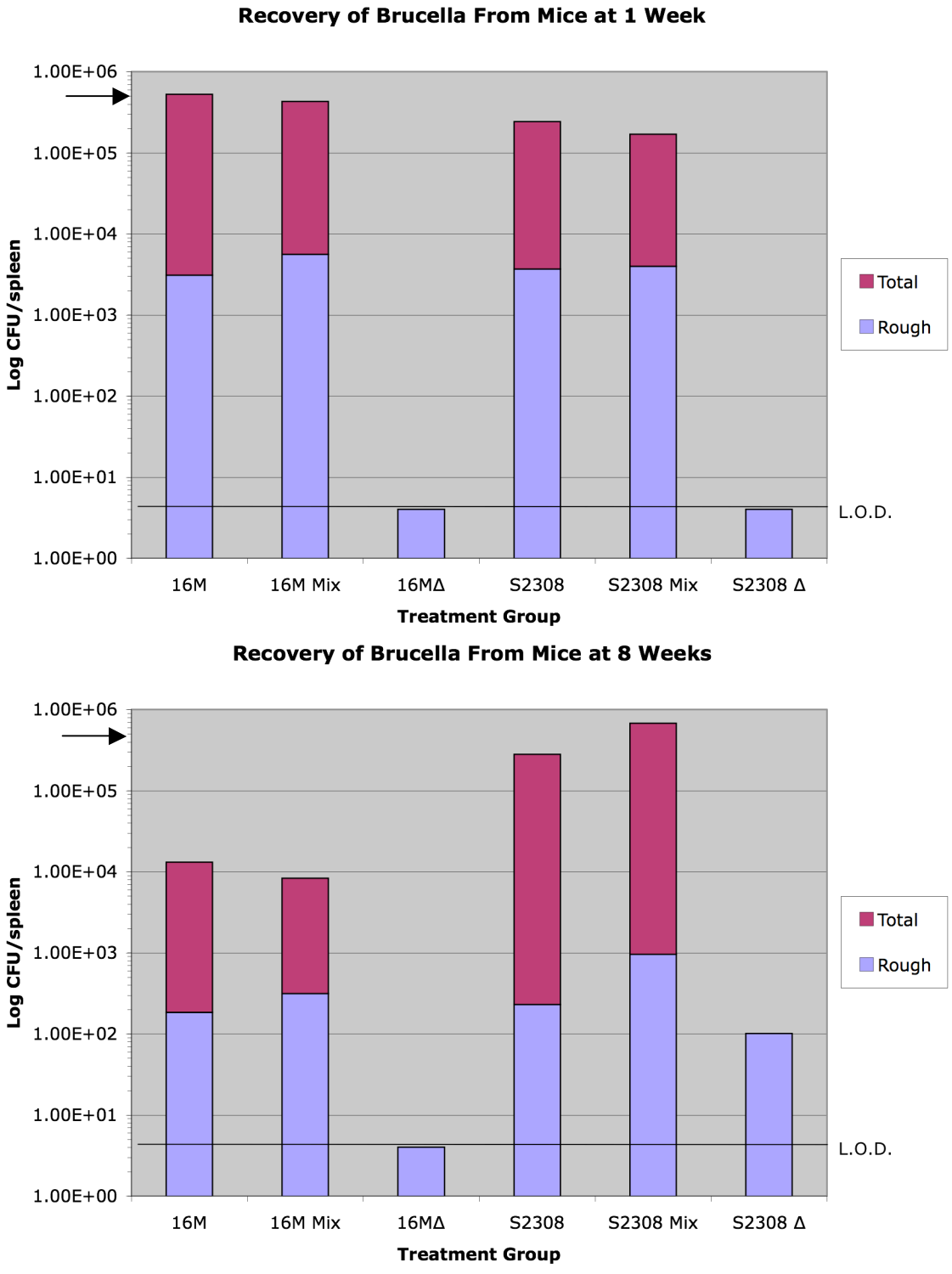


FIG. 10. Recovery of rough *Brucella* in mice at 1 and 8 weeks. Data is presented as log10 value of bacteria present in spleens, averaged over 5 mice. The limit of detection (L.O.D.) is less than 50 organisms. Solid arrows represent the initial inoculum.

An alternative approach utilizing auxonography was utilized. Growth on the toxin 5-fluororotic acid selects mutants deficient in either of two UMP biosynthetic enzymes: orotate phosphoribosyl transferase (OPRTase) and orotidine 5'-monophosphate decarboxylase, encoded by the *pyrE* and *pyrF* genes, respectively (65). FOA is not toxic outright, but the 5-F-UMP made from it by sequential action of the two enzymes kills cells (FIG. 9). Loss of either enzyme thus spares the mutant in the presence of FOA, but also renders it dependent on exogenous uracil for growth. The parameters needed to calculate the background mutation rate were derived from 94 independent *B. abortus* and *B. melitensis* cultures.

With the exception of jackpot cultures (>>3000 colonies/plate), 261 mutant colonies were collected from *B. abortus*, and 930 mutant colonies were collected from *B. melitensis*. Median numbers of mutants for these cultures were 2 and 18.5, respectively. The most probable number of mutational events per culture for the set, *m*, was determined by using the method of the median of Lea and Coulson (92, 97, 124). This leads to an estimation of 1.32 and 6.07 mutational events per culture for *B. abortus* and *B. melitensis* respectively. To calculate the average number of cells per culture, the viable titers were determined by serial dilution and plating on nonselective medium. Values were averaged, deriving the average number of bacteria in a given culture (N_{av}). Mutation rate per cell generation, μ, was calculated by using the relationship $\mu = (\ln 2)(m/N_{av})$. For *B.*

abortus this value was 1.63 x 10^{-8}, and for *B. melitensis* 7.48x 10^{-8} mutations per genome per generation. This indicates an extremely stable genome.Similar experiments were carried out to estimate the mutation rate for *manBA*. The theoretical number of mutations per culture (m) was found in two ways. Using the median of all rough colonies (*B. melitensis* or *B. abortus*) to find m, led to m= 293 mutations per culture, for *B. melitensis* and m=312 mutations per culture for *B. abortus*. Using the median of only those colonies complemented by *manBA in trans*, m=197 mutations per culture for *B. melitensis* and 331 mutations per culture for *B. abortus*. Luria-Delbrück mutation frequencies, μ, were calculated for each method. In general the rate of mutation is about 6 times greater than that observed for reversion of *E. coli* auxotrophy and 2 times greater than the rate observed for *S. typhimurium* (39). Mutation rates for *manBA* are about 100-1000 times greater than the rate of mutation resulting in loss of function of lac^I in *E. coli* (39). These rates, summarized in Table 4, are consistent with a mutator phenotype.

Genotypic tests for rough mutants. Contribution of genetic events at the *manBA* locus was assessed by PCR, Southern blotting, and a complementation test for each mutant. For the complementation test, plasmid pJET1445 (Table 2, FIG. 4) was introduced into each rough isolate to determine if a defect in *manBA* was the reason these strains had lost LPS expression. Indeed, 36.67% of *B. melitensis*, 20.34% of *B. abortus*, and 28.57% overall rough mutants

Table 4. Dissociation frequency, mutation rate, mutation frequency and complementation test results for *B. melitensis* and *B. abortus*.
In the column for mutation frequency, column A represents all rough, while column B represents only those rough complemented by *manBA in trans*.

Species	Dissociation frequency	Mutation Rate	Mutation Frequency		Complementation Test
			A	B	
B. melitensis	1.15×10^{-4}	293	1.27×10^{-3}	1.25×10^{-3}	36.67%
B. abortus	1.27×10^{-5}	312	1.95×10^{-4}	2.06×10^{-4}	20.34%

were complemented by *manBA in trans*. This complementation test represents the first time LPS expression has been fully restored to any O-antigen deficient *Brucella* strain. The ability of this expression vector to restore lipopolysaccharide expression in *manBA* deficient strains was confirmed by both Western blotting (FIG. 7) and staining with Pro-Q Emerald 300, a glycoprotein stain (FIG. 7).

PCR and Southern blots failed to show gross rearrangements at *manBA*, but sequencing did show change that may be related to the loss of function. In sequenced roughs, two transitions, T135C and G335A were found. These transitions cause a coding shift from serine to leucine and glutamic acid to glycine, respectively. Both transitions are found within the putative phosphoglucomutase/phosphomannomutase, alpha/beta/alpha domain I of phosphomannomutase (*manB*, BMEII0899) (98). Presumably, similar polymorphisms exist in spontaneous roughs that still need to be sequenced. Thermal cycler conditions for amplification of *manBA* for screening spontaneous rough isolates, using primers TAF419 and TAF420 were developed. The reaction mixture contained a 2mm punch from a Whatman FTA CloneSaver™ card that had sample applied to it from a liquid *Brucella* culture. Punches were prepared for PCR according to manufacturer instructions. Further, the reaction mixture contained 0.6μM of each primer, 500μM of each deoxyribonucleoside triphosphate, 1U of AccuTaq LA polymerase, 1x PCR buffer, 2mM $MgCl_2$.

Instrument conditions included activation of the polymerase at 98°C for 30 seconds, 30 cycles of amplification with 94°C x 30 seconds melting, 58°C annealing x 30 seconds, and 68°C x 3 minutes elongation. A final elongation was performed one time at 68°C for 10 minutes.

Rough *B. melitensis* grow faster than smooth strains. The ability of rough strains to outgrow smooth strains was test in a competitive assay over five passages. Starting with a ratio of 1:1, the engineered rough mutant was found to outgrow the smooth variant 2:1 by the end of three passages (FIG. 11).

Creation of non-dissociative *Brucella* strains. To see if dissociation could be prevented, smooth *Brucella melitensis* isolates were transformed with pJET1445, containing wild-type copies of the *B. melitensis manBA* genes. BK-2 phage was used to test the rate of appearance of rough isolates from this non-dissociative strain. Dissociation rates of these strains were indistinguishable from wild-type *B. melitensis*.

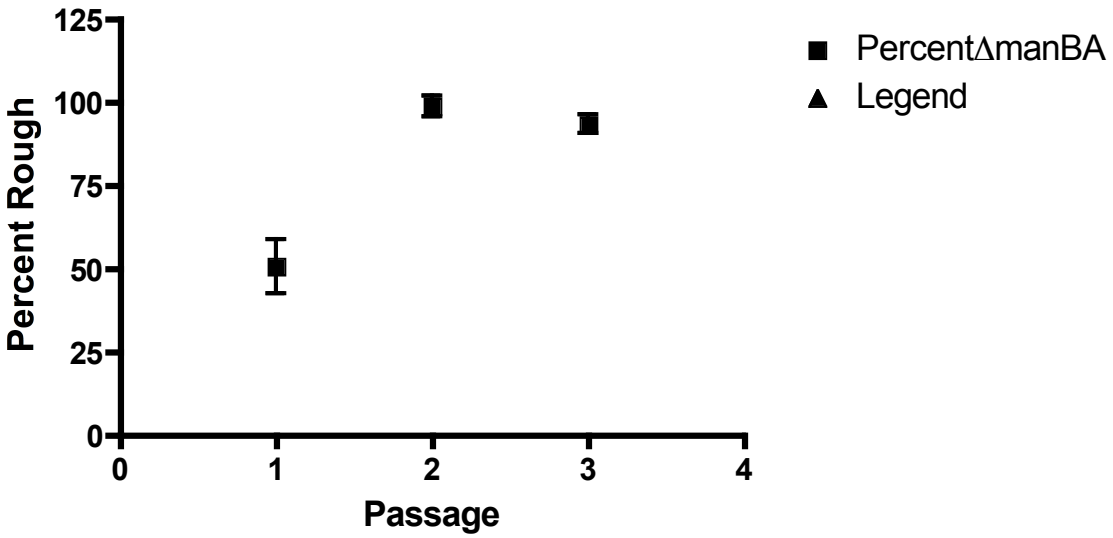
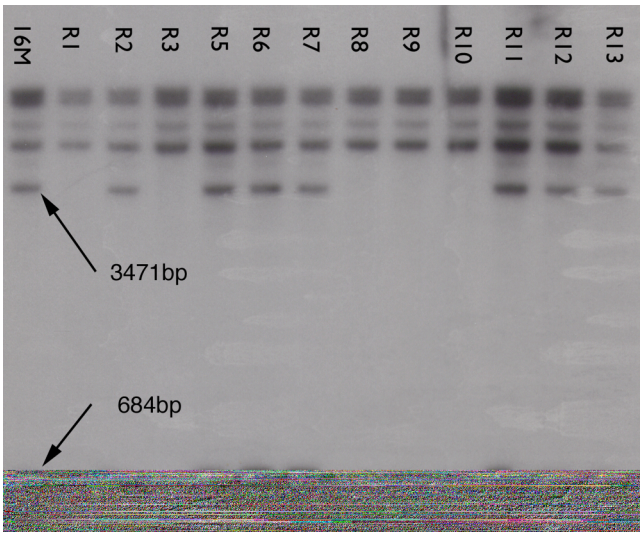


FIG. 11. Rough *Brucella*e outgrow smooth *Brucella*e over time. Smooth and rough strains were mixed 1:1 and grown in 5 ml of TSB. Strains were passaged every 48 hours. Bacterial CFU were enumerated by differential plating. Each data point is the average of two independent trials, ± standard deviation.



manBA locus

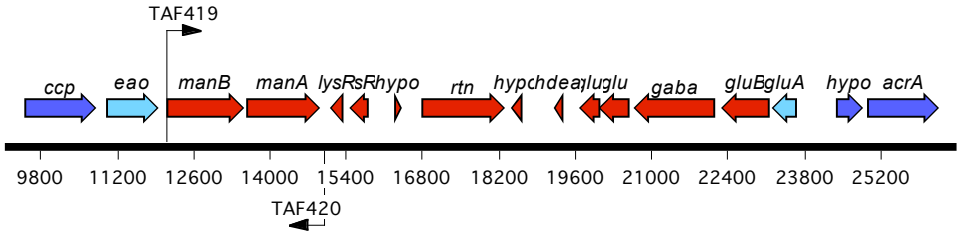


FIG. 12. Preliminary screen of *Brucella* rough mutants. A) Spontaneous rough isolates contain a 12kb deletion. Genomic DNA was prepared and digested from virulent *Brucella* and rough isolates (R1-R13). The digested DNA was electrophoresed and Southern blotted. Detection was performed by using a randomly ^{32}P labeled probe for type-6-aminohexanoate, immediately upstream of *manBA*. The full extent of the deletion was characterized by primer walking. B) the *manBA* locus, showing the full extent of the deletion that was originally characterized. The dark blue genes are undeleted, the light blue genes are partially deleted and red genes are completely removed.

DISCUSSION

Preliminary analysis revealed that there were two genotypes associated with spontaneous rough mutants present in stock cultures obtained from ATCC. One of these genotypes is a deletion of a 12kb region of the genome encompassing a region that includes the *manBA* locus as revealed by Southern blots of genomic DNA, cloning and sequencing (FIG. 12). The second genotype contained no detectable rearrangement when measured at the *manBA* locus. This fits the expectation that more than one locus is involved in the loss of O-antigen, though trans-complementation restored LPS expression in each of these strains (both types). In the process of collecting additional, non-sibling, rough mutants from a virulent isolates of *B. melitensis* and *B. abortus*, we were unable to show a large deletion, but were able to demonstrate trans-complementation of LPS deficient strains in approximately 30% of isolates. Deletion events could represent rare endpoints of smooth to rough transition.

Utilizing a Poisson approximation for the binomial distribution, we calculated the number of rough mutants needed in order to investigate all twelve of the known genes that could cause a rough phenotype. A listing of these genes is in Table 1. Using Poisson's distribution, sample size can be chosen for the probability of showing a defect in at least one individual. This is an extension of the methods used to estimate the number of mutants needed to saturate a genome during signature tagged mutagenesis:

$$n = -\frac{\ln(1-\gamma)}{p} \quad \textit{(i)}$$

where n= sample size, γ=probability of finding at least one defective gene, p=estimated proportion of defective genes in our population (1 out of 12 genes). For 99% confidence:

$$n = -\frac{\ln(1-0.99)}{\frac{1}{12}} = \frac{4.61}{0.08} = 57.63 \approx 58 \text{ mutants} \quad \textit{(ii)}$$

The number of mutants needed to reach saturation increases rapidly with the number of potential targets (Table 5).

In vitro growth characteristics of rough strains were established in competition to smooth strains (FIG. 11). Though the rough mutant outgrows the smooth strain, the rate is linear, as opposed to the logarithmic dissociation frequency. *In vivo*, it is anticipated that the rough would not outgrow the smooth strain since the rough mutant is readily killed.

Table 5. Increase in the number of mutants that needed to be screened in proportion to the suspected contributing genes.

Number of Candidate Genes	Confidence Level		
	99%	95%	90%
12	55	36	28
13	60	39	30
25	115	75	58
50	230	150	115
100	461	300	230

In vivo, the frequency of dissociation was found to be similar to the frequency with which rough isolates were found in smooth colonies. Smooth to rough transition is not simply an *in vitro* artifact, as has been suggested, but represents a real event that may be the result of adaptation to the host environment. The role that LPS plays in acquiring the host is just beginning to be understood, and some modification of the LPS may occur within the host. Dissociation has been demonstrated in many gram-negative bacteria. The major difference between this and other regulatory mechanisms is that it is mediated by changes to the DNA, which can occur during replication, rather than in response to a stimulus (44, 73, 126). There are many mechanisms for dissociation including inversions and transposon movement. The *Brucella* genome is replete with transposons and repeat regions that could be involved. Bacteria have diverse methods for changing the expression of their genes by changing their genomes.

Establishing the background mutation rates for *Brucella* is an important component of the overall project goal of determining the significance of the rate of appearance of rough variants. To accomplish this, a bank of antibiotics was used. This method has been successful in determining the frequency of mutator phenotypes of pathogenic forms of *E. coli*, *Salmonella*, and *Pseudomonas* from 10^8 bacteria (93, 130). The selection of these antibiotics was determined by their mechanism of action, in order to compare as wide a variety of cellular functions (replication, transcription, and translation) as possible. However,

Brucella cultures failed to produce any mutants by this method. Instead, conversion to pyrimidine auxotrophy was used to examine the background mutation rate. As expected, *Brucella* demonstrated a very low rate of mutation.

In order to determine what the rate of appearance of rough variants is under *in vivo* growth conditions, mice were infected with smooth, pure rough and mixed smooth and rough inocula. Spleens of infected mice were harvested at 1 or 8 weeks post inoculation. There was essentially no difference in the rate of smooth to rough transition in the host compared to isolation from smooth colonies.

Attempts undertaken to prevent the appearance of rough mutants by inclusion of *manBA in trans* in smooth strains were unsuccessful. The appearance of rough mutants from transformed smooth strains was essentially the same as from wild-type strains. Since this genetically modified smooth strain contains approximately ten copies of each plasmid, the appearance of rough mutants was expected to be a rare event. Loss of the plasmid is a possibility. In order to assure plasmid retention, chloramphenicol was maintained during *in vitro* studies. Failure to maintain the plasmid will produce results indistinguishable from smooth or rough organisms depending on the genetic background employed. A second mechanism that could limit interpretation is silencing of essential gene functions by trans acting factors including DNA methylation or gene regulation. In such a case the presence of multiple gene

copies may not be sufficient to counteract such a mechanism and rough variants may still appear.

Emergence of spontaneous rough mutants could involve changes at a single locus or at multiple loci. Current evidence suggests major rearrangements are possible, whether this is a rare event or part of a programmed process is not clear. Furthermore, large deletions would appear to limit survival unless the presence of these rough organisms enhances survival of the overall population. In this study, rough mutants were found to be the result of base transitions occurring within the active site of phosphomannomutase. Both base transitions replace a hydrophobic residue within the active site.

Evidence described elsewhere suggests the presence of a population of spontaneous rough organisms that revert to full virulence (128, 129). However, the frequency of such events is not known. It is clear that rough mutants induce greater levels of inflammatory cytokines upon interaction with macrophages indicating presence of O-antigen is important for preventing cellular activation (121). Prevention of activation enhances survival. The fact that rough mutants are observed at the same frequency *in vivo* as *in vitro* argues for a role in survival or dispersion of the bacteria throughout the host. Hypermutation can offer substantial improvement in adaptive potential. Preservation of this hypermutable trait may reflect the ancestry of the strain, as the closely related *Rhizobia* (55) also dissociate from LPS. Loss of LPS in *Rhizobium* has been

shown to cause defects in colonizing the root nodule. The ultimate pathogen would possess transient mutation that confers adaptive advantage in the host environment.

During the course of this work, the signature-tagged mutagenesis project revealed a rough mutant that could be complemented by *manBA in trans*, but was not defective in *manBA*. A two-component response regulatory element, *pleD*, was inactivated by insertion of mini-tn5. Acriflavine agglutination revealed that this strain was rough, and attempts to complement this rough defect with pJET1445 were successful. The role of *pleD* in LPS expression, is unclear, but studies in other organisms have shown that it plays a role in cell division in *Caulobacter crescentus* and cellulose biosynthesis in *Rhizobia* (3, 9, 99). *Rhizobia pleD* null mutants produce long cellulose tendrils from the surface of the bacterium, rather than a uniform capsule. The role of pled needs further investigation. Mutations at second sites in the genome may affect the expression of *manBA* at its native promoter, contributing to dissociation. This may account for lack of gross rearrangements at *manBA*, though pJET1445 was able to complement loss of function. The complementation plasmid, pJET1445 does not carry the putative promoter for *manBA*.

LPS PROMOTES 'PROPER' INNATE IMMUNE RESPONSE

INTRODUCTION

Lipopolysaccharide is commonly accepted as an essential virulence factor for Gram-negative bacteria because it is necessary for survival of classical strains (*B. suis*, *B. abortus*, *B. melitensis*). Contradictory to LPS' classical role as an inflammatory agent, *Brucella* LPS actually limits macrophage activation, which is the hallmark of its traits as a virulence factor (91). In this aim, the main goal of the experiments were to characterize the role of *Brucella* LPS articulating uptake and survival of smooth organisms. Nitric oxide and cytokine production levels were also measured. Finally, the ability of smooth and rough to recruit the macrophage in the host was compared by using a short-term mouse model that looked mainly at invasion.

Though trafficking of *Brucella* is documented in several cell types, macrophages appear to be particularly important to the spread of *Brucella* throughout the host (95). To illustrate the connection between *Brucella* LPS with uptake and survival, LPS knockout mutants with or without complementing plasmid were compared to survival of the parental smooth strain. The murine macrophage cell-line, J774A.1 is well characterized and widely used for screening *Brucella* mutants and was used here for comparative purposes (8, 34, 68, 72, 78, 79, 96, 112). We observed restricted activation of macrophages by smooth *Brucellae* characterized by reduced production of reactive nitrogen

intermediates and reduced cell death (necrosis). Understanding the function of *Brucella* LPS in a productive infection compared to the abortive infection observed with rough *Brucella* will provide greater insight about the role LPS plays in acquiring the host.

The mouse model is well established in *Brucella* research. With previous murine models of brucellosis, it has been shown *Brucella* administered intraperitoneally results in colonization of the spleen and peripheral lymph (4, 5). This study used two approaches to the mouse model, to answer distinctly separate questions. One of the issues approached was to assess the survival of *B. melitensis* smooth and rough mutants at acute (1 week) and chronic (5 weeks) stages of infection in the mouse. Since this study is seeking to define the role of LPS in host cell/bacterial interaction, the more important question asked was about the acquisition or recruitment of the host environment by the pathogen. This experiment was designed to ask which host cells were recruited to the site of infection and what cytokine responses were elicited in the host.

Innate immune response to *Brucella* is mediated by professional phagocytes (8, 11, 18, 87). In macrophages, cytokines typically studied include the proinflammatory cytokines, IL1α, TNFα, IFNγ, and the proinflammatory cytokine antagonist, IL10. Compared to *E. coli*, *Brucella* strains are poor inducers of cytokines in the macrophage model (121). However, work in the mouse model has shown that smooth *Brucella* LPS is an antagonist of proinflammatory

cytokines (21). This inflammatory response appears to be mediated through the toll-like receptor 4 pathways. Herein, data is presented from infection of mice, that corroborates the proinflammatory nature of *Brucella* LPS in the mouse model.

MATERIALS AND METHODS

Bacteria, plasmids, growth conditions and restriction endonucleases. All bacterial strains used in this study are listed in Table 2. Additionally the 16MΔ*manBA*, 16MR1, 16MR6, and S2308Δ*manBA* from the second study were used in this study. 16MR1 and 16MR6 are spontaneous roughs isolates that can be complemented by *manBA in trans*. All strains were grown as previously described.

Macrophage infection. Macrophage infection by *Brucella* is standard practice. Monolayers of cells are cultured in tissue culture plates; bacteria were added to J774A.1 macrophages at multiplicity of infection (MOI) of 100. The infected cell culture plate was centrifuged (room temperature, 200g, 5 minutes) to associate the bacteria with the macrophage monolayer. Cell cultures were placed in a 37°C incubator in the presence of 5% CO_2 for 20 minutes. The infected monolayers were then washed three times with peptone saline. Tissue culture media containing Gentamicin was added back to monolayer, killing bacteria external to the macrophage. At each time point, the cell supernatant was removed to determine cytokine levels, nitric oxide levels or lactate

dehydrogense (LDH) response, as appropriate. Invasion was judged after 1 hour by lysing the macrophage monolayer and plating the lysate on TSA with or without appropriate antibiotics. To measure uptake, the recovered bacterial CFU is compared with the infectious dose. Survival was judged at later time points. Supernatants collected at indicated time points were stored at –80°C for cytokine and nitric oxide detection.

LDH assay to measure cell death. Cells cultured in 24-well plates were infected with *B. abortus* in triplicate wells as described above. Culture supernatants were collected at various time points post-infection, and the lactate dehydrogenase (LDH) release was determined by use of the CytoTox 96 non-radioactive cytotoxicity assay (Promega, Madison, Wisconsin) according to the manufacturer's instructions. Cell death is expressed as a percentage of maximum LDH release, i.e., 100 x (optical density at 490 nm [OD_{490}] of infected cells - OD_{490} of uninfected cells)/(OD_{490} of lysed uninfected cells - OD_{490} of uninfected cells). This assay was performed in triplicate; data shown is the average of three independent experiments, ± the standard deviation.

Assay to measure release of nitric oxide. Nitric oxide in tissue culture supernatants was detected using Griess Reagent (Sigma). Fifty microliters of the culture supernatants were mixed with 50 μl of Griess regent in 96-well assay plate and incubated for 15 min at room temperature. Sodium nitric solutions with 1 to 50 μM concentrations were included as standards. This assay was

performed in triplicate; data shown is the average of three independent experiments, ± the standard deviation.

Murine infection assay (Long-term). Bacterial strains were grown on TSA plates and harvested in 5 ml of PBS. The bacterial concentration of suspension was estimated with an absorbance spectrophotometer, and adjusted to a concentration of approximately 5 x 10^7 cfu/ml of *Brucella melitensis* wild type or 5 x 10^8 cfu/ml *Brucella melitensis* rough mutant. Plating the inocula and enumerating total CFU confirmed the infectious dose. Four groups of 5 mice each, 6 week old BalbC/ByJ mice were injected with 100*µ*l, in parallel groups. The mice were sacrificed at 1 and 5 weeks post-inoculation by CO_2 asphyxiation. Spleens were collected from each group and homogenized with a tissue homogenizer (Omni 2000; Omni International, Inc.) for determining bacterial load. Data are represented as average $\log_{10}$ value of CFU per spleen for each mouse.

Murine infection assay (Short-term). To evaluate uptake in the mouse model, two groups of 8 mice each were infected parenterally via peritoneal injection with either *B. melitensis* wild type or the *manBA* deletion mutant with 1 x 10^8 cfu/mouse. Mice were sacrificed at 1-, 4-, 8-, 24-, and 72-hour time points. Samples from the mice were evaluated for bacterial survival, cellular infiltration of the peritoneum, and cytokine responses. Cell-mediated responses were quantified using differential cell analysis based on morphology, a common

clinical approach used to distinguish different cytological populations. Samples of spleen, liver, and mesenteric lymph node were collected, fixed in 10% formalin. Texas A&M University's College of Veterinary Medicine histology service performed the embedding and sectioning of these tissues for later analysis.

Cytokine analysis. Initial characterization of cytokine response was carried out using the RayBio® Mouse Cytokine Antibody Array II from RayBiotech (Atlanta, GA), as per manufacturer instruction. The sample applied was ascitic fluid from infected mice.

Evaluation of cytokine production was carried out by way of a sandwich ELISA. Whole animal cytokine profiles were developed on recovered ascites and serum (51, 111). All cytokines, unless otherwise stated, were detected using sandwich ELISA development kits purchased from PeproTech, Inc. (Rocky Hill, NJ), following the manufacturer's instructions.

RESULTS

Evaluation of infection by rough and smooth *B. melitensis* in murine macrophages. Both rough and smooth bacteria were internalized by the mouse macrophage cell-line J774A.1. At early time points, 0, 4, and 8 hours, it was apparent that rough bacteria were being internalized approximately 10-fold more than smooth bacteria. After 24 hours, the outcome for rough bacteria was markedly different from smooth bacteria, with a sharp reduction in bacterial

numbers recovered, with the exception of the rough strain 16MR6. Smooth bacteria appear to have begun to replicate within the macrophage environment, as evidenced by an increase of one order of magnitude (FIG. 13).

Infection of macrophages with rough *B. melitensis* causes macrophage death. Inspection of infected macrophages revealed that macrophages infected with rough *B. melitensis* were undergoing cell death. This was quantitated using an assay that measured the release of LDH to the media. High quantities of LDH in the media of macrophages infected with rough strains after 24 hours indicated that these cells were dying, while cells infected with smooth strains showed little cell death (FIG. 14). In fact, observations of cells infected with smooth strains showed that there was no gross morphological difference between these cells and uninfected cells. Cell death may be the endpoint of activation. Consistent with activation mediated death of macrophages was the release of reactive nitrogen intermediates, as measured by Griess reagent. Generally, more reactive nitrogen was observed from macrophages infected with rough *Brucella* (FIG. 15).

Survival of rough *Brucella*e in mice (long-term). The survival characteristics of rough and smooth *Brucella melitensis* were assessed in the mouse model of brucellosis. Since the literature concerning rough mutant survival in mice has already made apparent that rough mutants are attenuated, the rough mutant dose was increased relative to the smooth inoculum. The

result was a significant decrease of spontaneous rough isolates in the mice by 1 week, with all rough mutants being significantly attenuated at 5 weeks (FIG. 16).

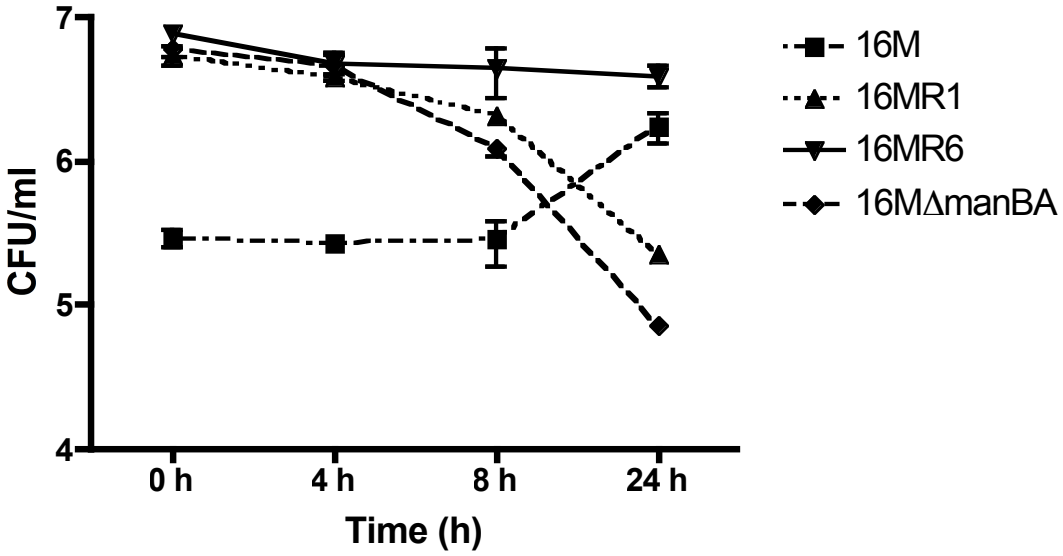


FIG. 13. Infection of murine macrophages by rough and smooth *B. melitensis*. Bacteria were grown overnight at 37°C, then left in contact with macrophages for 1 hour. Monolayers were washed and further incubated with gentamicin containing media for the specified amount of time. At the selected time points, macrophages were lysed and total numbers of bacteria were enumerated by plating serial dilutions on TSA. Data shown are the mean ± standard deviation (error bars).

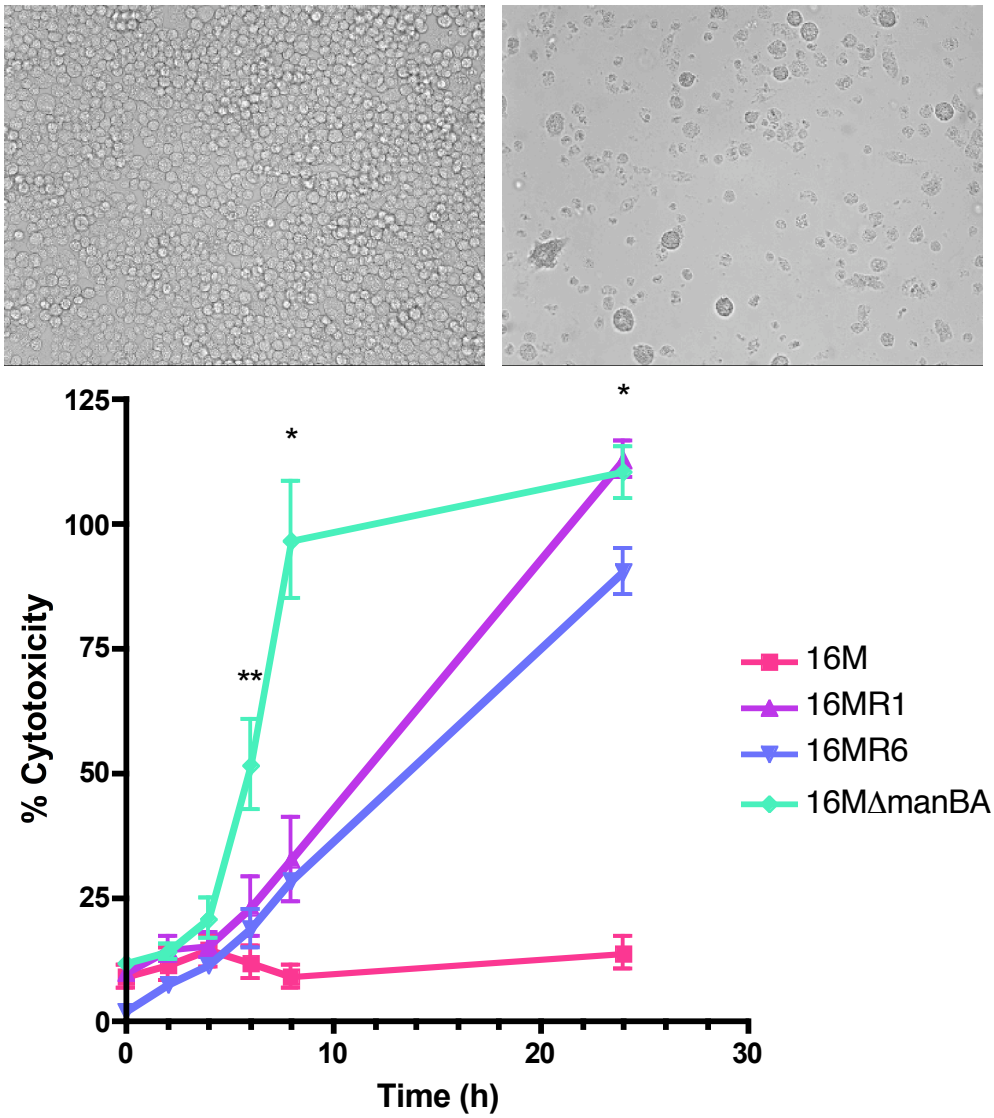


FIG. 14. Rough *Brucella melitensis* are cytotoxic in macrophages. J774A.1 macrophages are infected with *Brucella* at an MOI of 1:100. To synchronize infection, the infected cells were centrifuged 5 minutes, incubated 20 minutes. The tissue culture media is removed and replaced with media containing Gentamicin, which kills all the extracellular bacteria. At each time point, media is removed from each well and assayed for the release of lactate dehydrogenase, an indicator of host cell lysis. The bright field microscopy images are smooth and rough *Brucella* infected macrophages at 24 hours post infection, respectively. In the graph, LDH values are shown as they relate to the percent cell death of the macrophage monolayer. Values with a single asterisk are significant for all rough strains versus the wild-type smooth strain, 16M. Values with a double asterisk are significant for the *manBA* allelic exchange knockout mutant. This assay included *Brucella melitensis* 16M, 16MΔ*manBA*::Km^R, and one each type of the spontaneous rough isolates of *Brucella melitensis*, R1 and R6

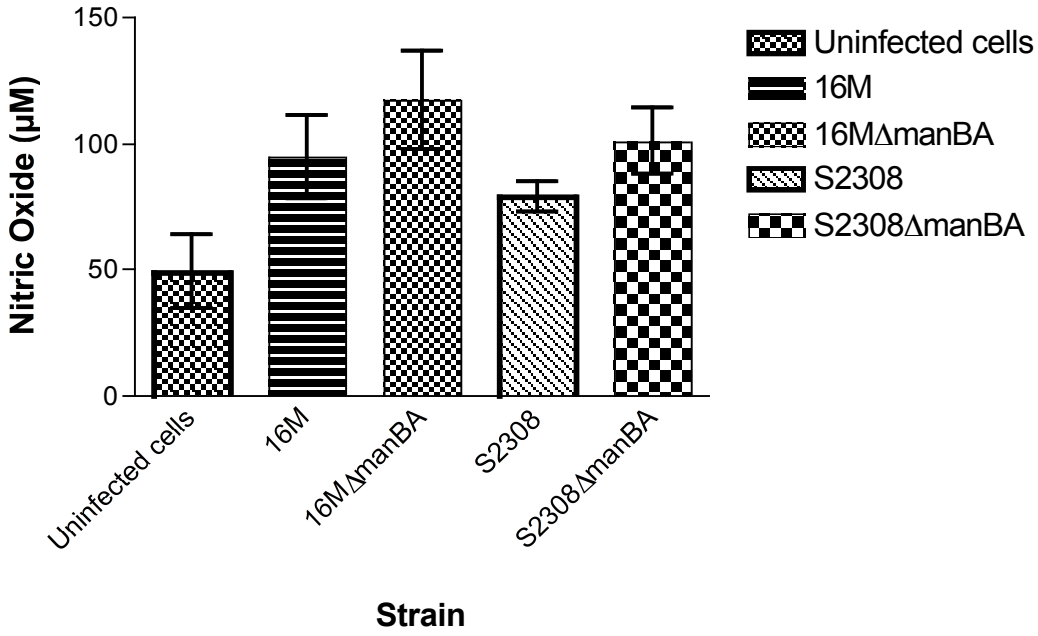


FIG. 15. Release of nitric oxide is greater in macrophages infected with rough *Brucella*. J774A.1 macrophage-like cells were infected as described in the Materials and Methods. Supernatants were assayed for reactive nitrogen 24 hours post-infection. Data is averaged over three independent assays ± standard deviation. Differences between isogenic smooth and rough strains are significant ($p<0.05$) by use of Student's t test. Differences between all strains and uninfected control are significant.

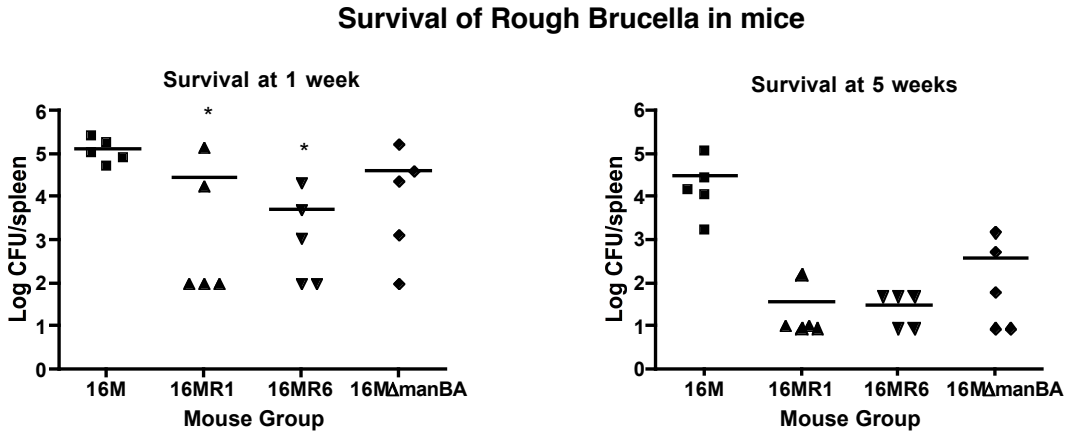


FIG. 16. Rough *B. melitensis* is attenuated in the mouse model of infection at both acute and chronic time points. Mice were infected with smooth and rough *B. melitensis* as indicated in the materials and methods. Spleens were removed at the indicated time points and total bacterial load per spleen was determined. Data on the graph indicate recovery from individual mice, with a solid bar to indicate median infection level. At one week post-infection, significant differences between the smooth and rough are indicated with an asterisk. At five weeks post infection all rough strains a significantly attenuated. Significance was judged using Student's t t-test and $p<0.05$.

Acquisition of the host environment. As a measure of the bacterium's ability to acquire cellular targets in a host model of brucellosis 8 mice were infected parentally with wild-type *B. melitensis* 16M and 8 mice with the engineered rough strain, 16MΔ*manBA*. Within 1 hour of infecting the mice, there was a 1-log difference between smooth and rough strains, reflecting the severe attenuation of rough *Brucella* in an animal model.

Consistent with the observations in the macrophage model, rough bacteria acquired the host cell sooner than smooth bacteria, as measured by the use of gentamicin to treat recovered ascities (FIG. 17). At early time points there was one order of magnitude more extracellular smooth bacteria in the ascities than intracellular bacteria. In the case of rough bacteria, most had acquired a host cell. Both populations decrease in the ascities, presumably as the bacteria are trafficked to peripheral organs (FIG. 17).

Bacterial load in the spleen and liver essentially parallel one another. In the case of both the smooth and rough, bacterial load in the spleen and liver essentially remain the same from 1 hour until about day 7. Rough strains never colonize these organs to the same extent as smooth strains, presumably to killing as the bacteria traffic to these organs. Interestingly, there is an increase in rough bacteria present in the spleen between day 3 and day 7, while there is a decrease in rough bacteria present in the liver (FIG. 17).

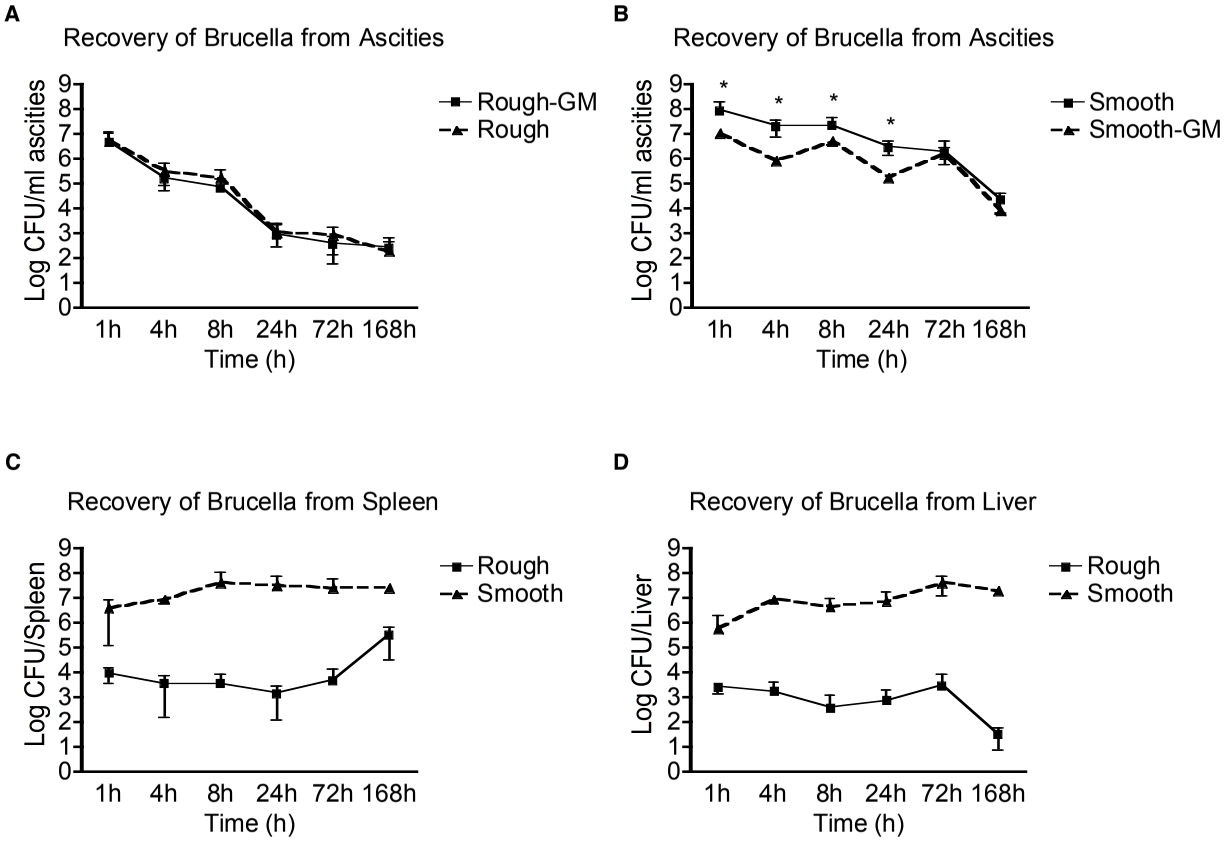


FIG. 17. Acquisition of the host environment. Eight mice were infected with *Brucella melitensis*. Ascities, spleen and liver were collected at 1, 4, 8, 24, 72, and 168h post infection. Total bacterial counts were enumerated per milliliter of recovered tissue. In panel A: *Brucella* recovered from the ascities after infection, treatment with Gentamicin allows differentiation between bacteria in the peritoneal fluid and those that have been internalized by resident phagocytic cells. Panel B more clearly shows the differences between bacteria internalized by phagocytes and those merely present in the peritoneal space. Panel C shows the migration of *Brucella* to the peripheral organs, spleen and liver. In panels A& C, all data points are shown as the average of 5 mice and the standard deviation. Within the dataset for each graph, differences between smooth and rough *Brucella* are significant ($p < 0.05$) at all time points. In panel B, all data points marked with an asterisk (*) are considered significantly different ($p<0.05$).

Total cellularity was assessed in mice infected with both rough and smooth bacteria. There were no significant differences in total cellular numbers elicited by smooth or rough bacteria or from mice injected with a similar volume of PBS. However, differential cell analysis found differences in the cellular populations recruited by smooth and rough bacteria.

In mice infected with smooth bacteria, the major cell populations present between 24 and 72 hours were macrophages, while mice infected with rough bacteria presented more neutrophils in their ascities. By 24 hours, differential cell count in animals infected with smooth bacteria revealed approximately 60% of neutrophils, 25% of macrophages, and 15% of other nucleated cells including mesothelial cells and lymphocytes. By contrast, the cellularity in mice infected with rough *Brucella* revealed approximately 70% of neutrophils, 20% of macrophages, and 10% of other nucleated cells including mesothelial cells and lymphocytes. For both strains at this time point, macrophages often contained large numbers of intracytoplasmic bacteria and clear vacuoles. By 72 hours, the population of cells found in the peritoneum of mice infected with smooth bacteria had not changed, 60% of macrophages, 25% of neutrophils, and 15% of other nucleated cells including mesothelial cells and lymphocytes. Meanwhile, mice infected with rough bacteria showed peritoneal population consisting of 75% of neutrophils, 15% of macrophages, and 10% of other nucleated cells including mesothelial cells and lymphocytes. In uninfected mice, the vast majority

(approximately 90%) of the nucleated cells present were medium-sized round cells with a single, blue, hyperchromatic, round nucleus, moderate amounts of pale to blue cytoplasm containing a peripheral red rim, presumably mesothelial cells. Macrophages and neutrophils were rarely observed (FIG. 18).

Mice release higher amounts of proinflammatory cytokines on challenge with smooth organism. Gram-negative bacteria usually stimulate high levels of pro-inflammatory cytokines, owing to the activity of Lipid A. Difference in cytokine expression, because of infection by rough or smooth bacteria could account for the difference in virulence between bacterial strains. To narrow the scope of pro-inflammatory cytokines screened from the mouse model of infection, a protein based cytokine array was used to determine differential expression of cytokines in mice infected with rough or smooth *Brucella*. Samples applied to these arrays were pooled from mice from each group over all time points. The output from this array (FIG. 19) showed increased induction of the pro-inflammatory cytokines IL6, IL12, IFNγ, MIP2, and TNFα in mice infected with smooth strains. Levels of some key chemokines, KC, MCP1, TIMP1, were greater in rough *Brucella* infected mice.

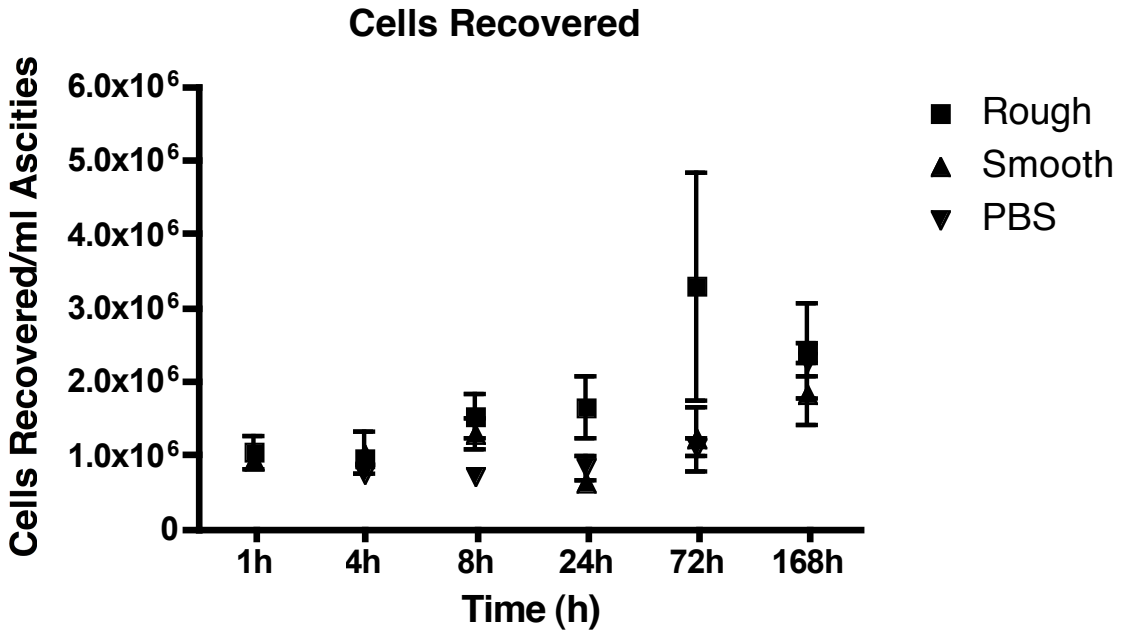


FIG. 18. Total cellularity as the result of infection. The total cells per milliliter PBS were collected to assess whether smooth or rough *Brucella* elicited inflammatory cells to the peritoneum in different quantities. Values shown are the average and the standard deviation.

ELISA was performed on a subset of these cytokines to characterize the temporal expression of pro-inflammatory cytokines and smooth or rough *Brucella* infection (FIG. 20). Proinflammatory cytokines responded as expected for gram-negative bacteria. In general, there was increased expression of pro-inflammatory cytokines in mice infected with smooth *Brucella melitensis*. The difference between parental smooth strains and constructed rough strains was apparent from the outset, particularly in MIP1α. TNFα showed a somewhat delayed increase, but still quite profoundly different between the two strains. TNFα levels peaked early, by four hours, and then began a gradual decline, which is characteristic of this cytokine (121). MIP1α and IL12 show a gradual peak over the 72-hour timeframe, while IL1α seems to show a gradual increase for smooth strains. There is sudden, early peak in IL1α for mice infected with rough strains, which quickly declines at later time points.

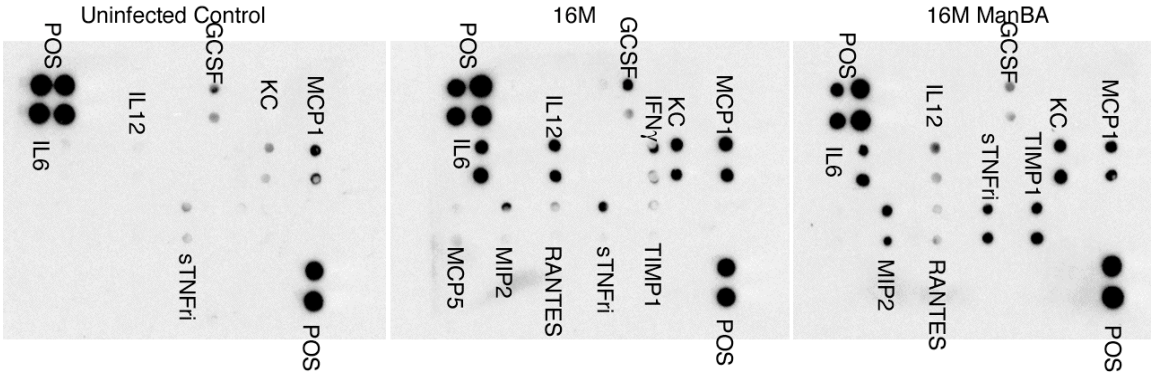


FIG. 19. Induction of different cytokine and chemokine responses by *Brucella melitensis* 16M wild type and the deletion mutant *Brucella melitensis* 16MΔ*manBA*::Km^R.An antibody-based array containing specific antibodies for 32 different cytokines/chemokines was used to determine differences in expression of cytokines/chemokines in mice infected with smooth or rough bacteria. Samples applied to the blots were pooled ascitic fluid of mice over the entire 7-day time course. Key differences were seen between the pro-inflammatory cytokines, IL6, IL12, IFNγ, MIP2, and TNFα as well as differences in levels of the chemokines, KC, MCP1, and TIMP1.

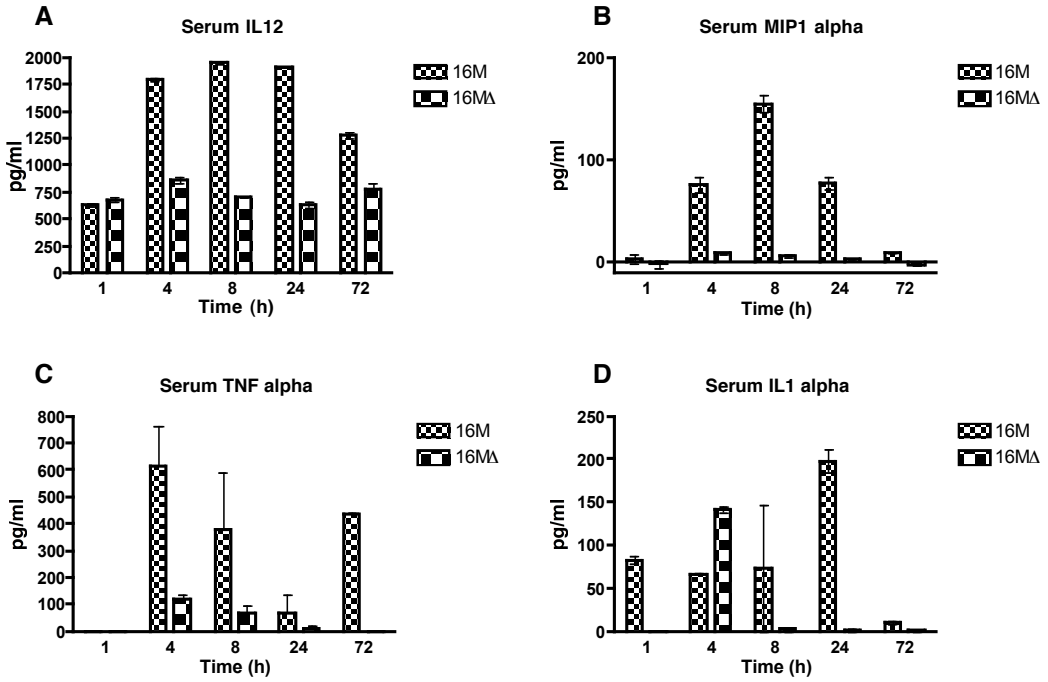


FIG. 20. Increased levels of pro-inflammatory cytokines from mice infected with smooth bacteria. Sera were taken from mice infected with 10^8 smooth or rough bacteria. Cytokine concentration was determined by sandwich ELISA, as described in the Materials & Methods. Smooth *Brucella* consistently induces higher levels of proinflammatory cytokines. The bimodal distribution apparent with levels of TNFα and IL1α is probably due to circulation of resident phagocytic cells from the initial site of infection, to the peripheral blood and recruitment of peripheral phagocytic cells to the peritoneum. Data are the average of pooled samples from duplicate experiments ± standard deviation.

DISCUSSION

In this study, invasion and survival phenotypes of rough *Brucella* mutants were investigated in macrophage and mouse models of brucellosis. *Brucella* has two predominant host cell types that it inhabits *in vivo*, macrophages and trophoblasts (123). The macrophage appears to be the primary residence of *Brucella* in experimental hosts (45), while in pregnant animals the trophoblast has been shown to play host to *Brucella* (36). This study shows that lack of LPS does not permit *Brucella* to recruit the correct cell type to establish infection.

First, macrophage-like cell lines were used to characterize the survival phenotype of smooth *Brucella melitensis* and an isogenic *manBA* knockout. Knocking out the *manBA* operon has been shown to ablate LPS biosynthesis, with the resultant immunological consequences being reduced survival in serum mediated killing assays, macrophages, and the host model (4, 5, 50, 53). Rough mutants were found to have impaired survival, but possibly only as result of the death of the host cell. This cytotoxic phenomenon was observed over 40 years ago by Freeman et al. (54), and has only been recently rediscovered (50, 112). Death of the host cell in this experimental system would expose the bacterium to gentamicin in the media, killing the bacteria. *In vitro*, the reduction of rough *Brucella*e by macrophages, although significant, is not very great.

Rough mutants were also found to have increased uptake at early time points in the macrophage and acute mouse model, by an unknown mechanism.

Fernandez-Prada et al. showed that *Brucella* rough mutants have increased affinity for serum complement (50) (49). Enhanced uptake due to complement may play a role in increased uptake *in vivo*. Presence of serum complement is one of the key differences between the *in vivo* mouse model and *in vitro* macrophage model. This phenomena needs to be investigated more carefully, as characterization of uptake differences between smooth and rough bacteria may help elucidate immunological responses to virulent organism.

In an acute model of disease, differences in cellular recruitment were evident by differential cellular analysis. Smooth *Brucella* recruited macrophages to the site of infection, while rough *Brucella* recruited larger numbers of neutrophils. This dichotomy in cellular recruitment is most likely the root of decreased survival of rough *Brucella*. Generally, neutrophils exhibit much greater killing ability compared to macrophages, though *Brucella* has evolved some defense mechanisms to prevent neutrophil degranulation (95). One of these defenses is LPS; increased killing of rough *Brucella* by neutrophils has previously been demonstrated (87). Other aspects of *Brucella*'s defense against neutrophils include the secretion of GMP and adenine (95).

These differences in cellular recruitment were borne out by observations from an antibody based array assessing the chemokines and cytokines present during the course of infection. On the array, pro-inflammatory cytokines were up-regulated in mice infected with smooth strains, while a basal level of

expression was apparent on the array treated with ascitic fluid from mice infected with rough *Brucella*. However, the real difference between these arrays was in the up-regulation of neutrophil specific chemokines.

In rough *Brucella* infected mice, there is increased expression of TIMP-1 and the mouse CXCL1 chemokine, KC. TIMP-1 degrades basement membrane, promoting infiltration of inflammatory cells (25). The presence of KC is indicative of neutrophil influx. In fact, in murine models of anthrax, KC has been shown to cause influx of neutrophils, followed by neutrophil mediated killing of bacteria (113). Differences in cellular recruitment probably account for differences in the cytokine and chemokine profile measured by ELISA, as the proinflammatory cytokines measured in this experiment are primarily secreted by macrophages.

Modifications to the bacterial cell surface would most certainly change the way the host perceives *Brucella*. Current insights into innate immunity suggest that the primary receptor for gram negative LPS is TLR-4 mediated (10, 75). In the past six months, data has shown temporal involvement of TLR-2 then TLR-4 in the stimulation of innate immunity by gram-negative pathogens (141). Indeed, CHO cells transfected with TLR-2 and TLR-4 show a response to the presence of both smooth and rough LPSs from *Brucella*. However, rough LPS alone is insufficient to stimulate TLR-4 (21).

Brucella LPS plays an important role in acquisition of the host environment. LPS is protective against macrophage killing, complement mediated killing and

reactive nitrogen and oxygen intermediates. More importantly, it appears to play a role in recruitment of the 'correct' cellular partner to advance brucellosis from an acute infection to a chronic infection.

CONCLUSIONS AND FUTURE WORK

Lipopolysaccharide is the major component of Gram-negative cell walls and as a result, is an active participant during the early stages of host agent interaction. O-antigen is the immunodominant antigen on the *Brucella* surface and as a result, the serological response is dominated by antibody to O-antigen. This body of work has demonstrated that the metabolic locus, *manBA*, participates in the synthesis of O-antigen. The *manBA* locus is subject to higher than expected mutation rates, and is a substantial participant in the phase-variant nature of pathogenic *Brucella melitensis* and *Brucella abortus* strains. Further, *manBA* may be subject to regulation by a trans-acting factor, *pleD*.

The role of O-antigen in acquisition of the host was tested using macrophage and mouse models. In both mice and macrophages, rough bacteria are more readily taken up. Contradictory to indications from the macrophage model, smooth strains of *Brucella* stimulated more proinflammatory cytokine activity in the mouse. Smooth LPS appears to be important in recruiting macrophages to the site of infection, in contrast to the neutrophils recruited by rough strains, to effectively colonize the host. This data adds credence to the adage that LPS plays an important role in the intracellular trafficking of *Brucella* (52, 64, 121), although not in a traditional role of protecting the bacterium from physical effects

of host killing mechanisms. Rather, the role of *Brucella* LPS as a signaling molecule needs to be explored.

Signaling through toll-like receptors needs to be studied in more detail. The deep rough strains presented in this work are ideal for carrying these signaling studies forward, since unlike RB51, they make no lipopolysaccharide (28). From the literature, there are implications that the toll-like receptors are important for processivity of *Brucella* (21, 43, 76). Toll-like receptors are pattern recognition receptors, and as such have been shown to have affinities for particular Pathogen Associated Molecular Patterns (PAMPs). For gram-negative bacteria, the essential PAMPs are lipids and LPS. Lipids activate macrophages through TLR2 and LPS activates macrophages through TLR4. So far, studies investigating *Brucella* and TLR's are limited in scope and have concentrated on cementing the link between TLR4 and *Brucella* LPS, rather than focusing on TLR mediated signaling in the presence of live bacteria. Campos et al. have taken the first look at live *Brucella* in a mouse model, and present cytokine data is contradictory to the accepted paradigm from macrophage models, though it agrees with the data presented here. The disjoint between macrophage derived data and data from animal models is a dangerous pitfall, and displays the limitations of both systems.

Experimental *Salmonella* infections have been shown to include temporal involvement of both TLR4 and TLR2 (141). Toll-like receptors are expressed at

low level on resting macrophages (140). Upon activation, TLR4 is expression down-regulated early (6 hours), while TLR2 expression comes up gradually (by 24 hours). In this particular line of experimentation, the early involvement of TLR4 is largely responsible for control of the bacterium. TLR4 knockout mice did not produce the same inflammatory response as TLR4 competent mice, though both TLR4 and TLR2 were necessary to clear the infection. The roles of TLR4 and 2 could be investigated for *Brucella* infections using similar techniques. Rough *Brucella*e should be unable to signal through the TLR4 mediated pathway. This may be the source of the decreased cytokine production by the rough mutant in the mouse, despite profound activation *in vitro*.

The dynamics of *Brucella* LPS expression, as a line of investigation, have been opened up by the current study. Regulation of LPS expression/phase is the next logical step. A clear link between the appearance of rough forms and dissociation has yet to be established; fluorescence microscopy appears to be the tool of choice to make these observations with antibody specific to smooth LPS. Already, from the signature-tagged mutagenesis bank, it appears a candidate gene, the transcriptional regulator, *pleD,* has been found. Loss of function at *pleD* resulted in a rough phenotype that was complemented *in trans* by *manBA*. LPS control by a transcriptional network has huge implications for the virulence model. Fluorescence microscopy assays can be put to work

immediately to look at dissociation inside host cells. Currently, transcriptional regulation of LPS biosynthesis in *Brucella* is “black box”. In other species, dissociation is an integral component of virulence.

With the advent of microarray technologies, picking apart the LPS biosynthesis pathway may become more feasible. For instance, *Vibrio cholerae* has both smooth and rugose forms. Rugosity actually has been found to confer an advantage to *Vibrio* in the environment (120). In 2004, Yildiz et al. used microarray analysis and determined that there are significant changes in bacterial transcriptional networks, beyond the scope of the predicted LPS biosynthesis genes.

Lipopolysaccharide plays an important role in the pathogenesis of brucellosis. This study has established the central role of a metabolic locus in the formation of LPS and the importance of LPS for acquisition of the host. Further questions to be asked include the regulation of LPS biosynthesis in *Brucella* as well as the importance of *Brucella* LPS as a signaling molecule.

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APPENDIX A

A-1 Exact sequence of *manBA* amplified from *Brucella melitensis*

```
                          25                          50
TTCCAGGCAG ATACAGGTTC GATGGCAGCA TGAGCAGCAA TTCCCTCAAA
AAGGTCCGTC TATGTCCAAG CTACCGTCGT ACTCGTCGTT AAGGGAGTTT

                          75                         100
TTTGGCACGA GCGGCCTTCG CGGGCTGGCG GTAGAACTGA ACGGCCTGCC
AAACCGTGCT CGCCGGAAGC GCCCGACCGC CATCTTGACT TGCCGGACGG

                         125                         150
CGCCTATGCC TATACGATGG CCTTTGTGCA GATGCTTGCT GCAAAAGGGC
GCGGATACGG ATATGCTACC GGAAACACGT CTACGAACGA CGTTTTCCCG

                         175                         200
AGTTGCAGAA GGGCGACAAG GTGTTTGTCG GCAGGGATTT GCGCCCCTCC
TCAACGTCTT CCCGCTGTTC CACAAACAGC CGTCCCTAAA CGCGGGGAGG

                         225                         250
AGCCCTGATA TTGCAGCCCT TGCCATGGGT GCCATCGAAG ATGCCGGCTT
TCGGGACTAT AACGTCGGGA ACGGTACCCA CGGTAGCTTC TACGGCCGAA

                         275                         300
CACACCGGTC AATTGCGGCG TCCTGCCCAC GCCTGCGCTG AGCTATTATG
GTGTGGCCAG TTAACGCCGC AGGACGGGTG CGGACGCGAC TCGATAATAC

                         325                         350
CGATGGGCGC GAAAGCACCC AGCATCATGG TCACGGGAAG CCATATTCCA
GCTACCCGCG CTTTCGTGGG TCGTAGTACC AGTGCCCTTC GGTATAAGGT

                         375                         400
GATGATCGCA ACGGGCTGAA ATTCTATCGC CGCGACGGTG AAATCGACAA
CTACTAGCGT TGCCCGACTT TAAGATAGCG GCGCTGCCAC TTTAGCTGTT

                         425                         450
GGATGATGAG GCGGCAATCA GTGCAGCCTA TCGCAAGCTG CCTGCCATTC
CCTACTACTC CGCCGTTAGT CACGTCGGAT AGCGTTCGAC GGACGGTAAG

                         475                         500
TCGCTGCCCG CAAACATGTC GGCTCCACCG AAACCGATGC GGCCTTGCAG
AGCGACGGGC GTTTGTACAG CCGAGGTGGC TTTGGCTACG CCGGAACGTC

                         525                         550
GCTTATGCCG ATCGCTATGC AGGTTTTCTT GGGAAAGGGA GCCTGAATGG
CGAATACGGC TAGCGATACG TCCAAAAGAA CCCTTTCCCT CGGACTTACC

                         575                         600
CCTGCGGGTC GGCGTTTATC AACATTCTTC CGTGGCGCGC GATCTTCTGA
GGACGCCCAG CCGCAAATAG TTGTAAGAAG GCACCGCGCG CTAGAAGACT
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                          625                         650
TGTACCTGCT CACGACACTC GGCGTGGAAC CCGTGGCGCT CGGACGATCC
ACATGGACGA GTGCTGTGAG CCGCACCTTG GGCACCGCGA GCCTGCTAGG

                          675                         700
GATATATTCG TGCCGGTCGA TACCGAGGCA TTGCGCCCCG AAGACATTGC
CTATATAAGC ACGGCCAGCT ATGGCTCCGT AACGCGGGGC TTCTGTAACG

                          725                         750
GCTGCTTGCC CAATGGGGCA AAAGCGACAG GCTTGATGCC ATCGTCTCCA
CGACGAACGG GTTACCCCGT TTTCGCTGTC CGAACTACGG TAGCAGAGGT

                          775                         800
CCGACGGAGA CGCGGATCGC CCGCTGATTG CCGATGAGCA TGGACAATTC
GGCTGCCTCT GCGCCTAGCG GGCGACTAAC GGCTACTCGT ACCTGTTAAG

                          825                         850
GTTCGCGGCG ATCTTGCTGG CGCCATCACC GCCACATGGG TGGGGGCGGA
CAAGCGCCGC TAGAACGACC GCGGTAGTGG CGGTGTACCC ACCCCCGCCT

                          875                         900
TACGCTCGTC ACGCCAGTCA CCTCCAACAC CGCATTGGAA AGCCGCTTTC
ATGCGAGCAG TGCGGTCAGT GGAGGTTGTG GCGTAACCTT TCGGCGAAAG

                          925                         950
CCAAGGTTTT GAGAACGCGC GTCGGTTCGC CTTATGTCAT CGCAAGCATG
GGTTCCAAAA CTCTTGCGCG CAGCCAAGCG GAATACAGTA GCGTTCGTAC

                          975                        1000
GCACAGGTAT CCACGGGCAA TTCCGGCCCG GTCATCGGGT TTGAGGCCAA
CGTGTCCATA GGTGCCCGTT AAGGCCGGGC CAGTAGCCCA AACTCCGGTT

                         1025                        1050
TGGCGGCGTT CTGCTTGGCA GCACGGTCGA GAGGAATGGA CGAAGCCTGA
ACCGCCGCAA GACGAACCGT CGTGCCAGCT CTCCTTACCT GCTTCGGACT

                         1075                        1100
CGGCCCTGCC GACGCGCGAC GCCTTGTTGC CCATTCTGGC TTGCCTTGCC
GCCGGGACGG CTGCGCGCTG CGGAACAACG GGTAAGACCG AACGGAACGG

                         1125                        1150
ACGGTTCACG AAAAGAAAAC GCCGCTTTCA ACAATCGCCC GGTCCTATGG
TGCCAAGTGC TTTTCTTTTG CGGCGAAAGT TGTTAGCGGG CCAGGATACC

                         1175                        1200
CTTCCGCGTC GCGCTTAGCG ACCGGCTGCA AAACATTCCG CAGGAGGCGA
GAAGGCGCAG CGCGAATCGC TGGCCGACGT TTTGTAAGGC GTCCTCCGCT

                         1225                        1250
GCACCGCCTT CCTCGCGCTC TTGGAGGATG CGGATAAACG CGCCTCGCTC
CGTGGCGGAA GGAGCGCGAG AACCTCCTAC GCCTATTTGC GCGGAGCGAG

                         1275                        1300
TTTCCTGCTG GCGACGCAAT CGTGCGGGTG GAAACCATCG ACGGCGTGAA
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CGAAAAGAAA GTTAGTCCGT TACGCCAAGT AATAGCCCGT AGCCCGTTAC

                         1375                        1400
CGCCGGAACT GCGCTGCTAT GTGGAATCTT CGGATGACAC ACAAGCCGCC
GCGGCCTTGA CGCGACGATA CACCTTAGAA GCCTACTGTG TGTTCGGCGG

                         1425                        1450
AAGCTTCAGG CGCTTGGCTT GGAAATCGCA CGCAAAGCAC TAAAGGATGC
TTCGAAGTCC GCGAACCGAA CCTTTAGCGT GCGTTTCGTG ATTTCCTACG

                         1475                        1500
GACGAGGCCA TGAGTTTCAT ACCGGTAATT ATCAGCGGGG GATCGGGTTC
CTGCTCCGGT ACTCAAAGTA TGGCCATTAA TAGTCGCCCC CTAGCCCAAG

                         1525                        1550
AAGGCTTTGG CCGCTTTCGC GGGATGCACA TCCAAAACCC TTTATCAAAC
TTCCGAAACC GGCGAAAGCG CCCTACGTGT AGGTTTTGGG AAATAGTTTG

                         1575                        1600
TGCCGGATGG CGAAACACTC ATCGGCAAGA CCTATGCGCG CGCTTCGCGC
ACGGCCTACC GCTTTGTGAG TAGCCGTTCT GGATACGCGC GCGAAGCGCG

                         1625                        1650
CTTGTAAACG CCGAACAGAT CCTTACGGTT ACGAACCGCG ATTTTCTTTT
GAACATTTGC GGCTTGTCTA GGAATGCCAA TGCTTGGCGC TAAAAGAAAA

                         1675                        1700
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GGACTGCGAG CTGCGAATAC GCCGCCGTCC ACGGCGCGTC TACCTTTTGT

                         1725                        1750
CTTTCCTTCT GGAGCCGCTT GGCCGCGACA CCGCGCCCGC AGTGGCGCTT
GAAAGGAAGA CCTCGGCGAA CCGGCGCTGT GGCGCGGGCG TCACCGCGAA

                         1775                        1800
GCCGCCCTTC ATGCCGCTGA AGCCTATGGG CCGGATGCCA CGCTTCTGGT
CGGCGGGAAG TACGGCGACT TCGGATACCC GGCCTACGGT GCGAAGACCA

                         1825                        1850
CATGCCGGCC GATCACCTGA TCGAGGATGA ACAAGCCTTC GCGGAAGCGG
GTACGGCCGG CTAGTGGACT AGCTCCTACT TGTTCGGAAG CGCCTTCGCC

                         1875                        1900
TTGCGAAAGC GCGCGCGCTT GCCGAGGCAG GGCGCATCGT CACCTTCGGC
AACGCTTTCG CGCGCGCGAA CGGCTCCGTC CCGCGTAGCA GTGGAAGCCG

                         1925                        1950
ATTGTGCCGG ATCGGCCCGA GACCGGCTTC GGCTATATCG AAGTGCAAGG
TAACACGGCC TAGCCGGGCT CTGGCCGAAG CCGATATAGC TTCACGTTCC

                         1975                        2000
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ATGCCTACAA GTCGCTAAAC AGCTTTTCGG CCTACTCCGG TGGCGGGTCT

                        2025                        2050
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GGATACACCT TTCGCCTGCG ATAAAGACCT TAAGGCCGTA CAAGACGAAG

                        2075                        2100
AAGGCATTGA GCATGATCGA CGCCATGCAG CGTTACGCAC CGCAGGTACT
TTCCGTAACT CGTACTAGCT GCGGTACGTC GCAATGCGTG GCGTCCATGA

                        2125                        2150
GGCAGGCGCT AGGGCTGCCC TCGCCCAGGC CCGGCGTGGC AATAACGGCG
CCGTCCGCGA TCCCGACGGG AGCGGGTCCG GGCCGCACCG TTATTGCCGC

                        2175                        2200
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TCTGGTTTTG GGAACTTTAG CGGTCCCTGC TCAAACGGCG TTGCGGACGG

                        2225                        2250
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                        2275                        2300
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CGGGCAAAGG ACGCCGACCA GCCTATAGCC GAGTACCCGC CGCTACCGGC

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TAGAGCACTG TGGGCTACTT TTGCCGTTAG CGGACGCGCC TCTTTGACAA

                        2375                        2400
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GACCTTCTAT GCTGGTTGTC AAAGCAGGAA AGACTTTGGG CAGACCACTC

                        2425                        2450
CCTTGTCGGT GTGCATGATC TTCTCGTGGT TGACACGCCG GACGCCCTTC
GGAACAGCCA CACGTACTAG AAGAGCACCA ACTGTGCGGC CTGCGGGAAG

                        2475                        2500
TCGTCGCTCA TCGCGACAAA GCGCAGGAAG TACGCAGCGT TTTCAACAAA
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                        2525                        2550
TTGCGCAAGC AAGGTCATGA AGCTGCAAAG CTGCACCGCA CAGCCCATCG
AACGCGTTCG TTCCAGTACT TCGACGTTTC GACGTGGCGT GTCGGGTAGC

                        2575                        2600
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GGGTACCCCG TGGATATGGC ACGACCTTCT CCCGCTGCCG AAGTTCTAGT

                        2625                        2650
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                          2675                        2700
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                          2725                        2750
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ACCGCTGGCC CTTTAGGACG AGTGGTGGTT AGTCAGTTGG ATATAAGGGA

                          2775                        2800
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                          2825                        2850
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                          2875                        2900
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ACTGCTACAA ATGCCTGCGC AAACTTTGTA TAAGTCGGTT TACGCTTGGC

                          2925
GCTTTGGCAT TCGGCCTATT GGGAT
CGAAACCGTA AGCCGGATAA CCCTA
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VITA

Joshua Edward Turse received his Bachelor of Science degree in biochemistry from Bates College in 1996. He entered the Genetics Program at Texas A&M University in August 1998, and received his Doctor of Philosophy degree in August 2005. His research interests included emerging infectious diseases and development of novel therapeutics.

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