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**Characterization of B. Melitensis Rev 1
Attenuated Mutants**

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biov., biovar; CFU, colony forming unit; i.p., intraperitoneally; ,STM, signature-tagged mutagenesis.

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Visits/Meetings			1	1
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A. Cooperation, briefly explain whether synergistic, complementary or supportive.

The cooperation between investigators was complementary to start with and became synergistic. The U.S. research efforts were designed to be supportive of the Israeli effort to eliminate brucellosis. This will be accomplished with the construction of the knockouts.

A. ABSTRACT

ORIGINAL OBJECTIVES

TAMU	1. To develop and characterize attenuated <i>B. melitensis</i> Rev1 (Elberg) and Israeli field strain mutants incapable of persistent survival in macrophages.
	2. To characterize attenuated <i>B. melitensis</i> Rev1 (Elberg) mutants in mice
UWISC	1. Produce recombinant proteins from genes associated with <i>B. melitensis</i> virulence and test T lymphocyte response to subunit vaccines
	2. Compare the phenotype of lymphocytes responding to virulence-associated proteins, <i>B. melitensis</i> Rev 1 and field strain mutants
	3. Define the cytokine profile of lymphocytes specific for virulence associated proteins <i>B. melitensis</i> Rev 1 and field strain mutants
ISRAEL	1. To test <i>B. melitensis</i> Rev1 vaccines possessing selected deletions of virulence genes as vaccine candidates in sheep compared to the original Rev 1 strain (Israel).
	2. To test the possibility of fortifying the immunity of adult animals by revaccination using recombinant subunit/DNA vaccines based on brucella immunodominant/virulence gene products.

A.1 *Brucella* Mutagenesis (TAMU)

The working hypothesis for this study was that survival of *Brucella* vaccines was directly related to their persistence in the host. This premise is based on previously published work detailing the survival of the currently employed vaccine strains S19 and Rev 1. The approach employed signature-tagged mutagenesis to construct mutants interrupted in individual genes, and the mouse model to identify mutants with attenuated virulence/survival. Intracellular survival in macrophages is the key to both reproductive disease in ruminants and reticuloendothelial disease observed in most other species. Therefore, the mouse model permitted selection of mutants of reduced intracellular survival that would limit their ability to cause reproductive disease in ruminants. Several classes of mutants were expected. Colonization/invasion requires gene products that enhance host-agent interaction or increase resistance to antibacterial activity in macrophages. The establishment of chronic infection requires gene products necessary for intracellular bacterial growth. Maintenance of chronic infection requires gene products that sustain a low-level metabolism during periods characterized by little or no growth (1, 2). Of these mutants, the latter group was of greatest interest with regard to our originally stated premise. However, the results obtained do not necessarily support a simplistic model of vaccine efficacy, i.e., long-survival of vaccine strains provides better immunity. Our conclusion can only be that optimal vaccines will only be developed with a thorough understanding of host agent interaction, and will be preferable to the use of fortuitous isolates of unknown genetic background.

Each mutant could be distinguished from among a group of mutants by PCR amplification of the signature tag (5). This approach permitted infection of mice with pools of 50 different mutants (including the parental wild-type as a control) and identified 40 mutants with apparently defective survival characteristics that were tentatively assigned to three distinct classes or groups. Group I (n=13) contained organisms that exhibited reduced survival at two weeks post-infection. Organisms in this group were recovered at normal levels by eight weeks and were not studied further, since they may persist in the host. Group II (n=11) contained organisms that were reduced by 2 weeks post infection and remained at reduced levels at eight weeks post-infection. Group III (n=16) contained mutants that were normal at two weeks, but recovered at reduced levels at eight weeks. A subset of these mutants (n=15) was confirmed to be attenuated in mixed infections (1:1) with the parental wild-type. One of these mutants was

eliminated from consideration due to a reduced growth rate in vitro that may account for its apparent growth defect in the mouse model.

Although the original plan involved construction of the mutant bank in *B. melitensis* Rev 1 the low transformability of this strain, prevented accumulation of the necessary number of mutants. In addition, the probability that Rev 1 already carries one genetic defect increases the likelihood that a second defect will severely compromise the survival of this organism. Once key genes have been identified, it is relatively easy to prepare the appropriate genetic constructs (knockouts) lacking these genes in *B. melitensis* Rev 1 or any other genetic background. The construction of "designer" vaccines is expected to improve immune protection resulting from minor sequence variation corresponding to geographically distinct isolates or to design vaccines for use in specific hosts.

A.2 Mouse Model of *Brucella* Infection (UWISC)

Interferon regulatory factor-1-deficient (IRF-1^{-/-}) mice have diverse immunodeficient phenotypes that are necessary for conferring proper immune protection to intracellular bacterial infection, such as a 90% reduction of CD8⁺ T cells, functionally impaired NK cells, as well as a deficiency in iNOS and IL-12p40 induction. Interestingly, IRF-1^{-/-} mice infected with diverse *Brucella abortus* strains reacted differently in a death and survival manner depending on the dose of injection and the level of virulence. Notably, 50% of IRF-1^{-/-} mice intraperitoneally infected with a sublethal dose in C57BL/6 mice, i.e., 5 x 10⁵ CFU of virulent S2308 or the attenuated vaccine S19, died at 10 and 20 days post-infection, respectively. Interestingly, the same dose of RB51, an attenuated new vaccine strain, did not induce the death of IRF-1^{-/-} mice for the 4 weeks of infection. IRF-1^{-/-} mice infected with four more other genetically manipulated S2308 mutants at 5 x 10⁵ CFU also reacted in a death or survival manner depending on the level of virulence. Splenic CFU from C57BL/6 mice infected with 5 x 10⁵ CFU of S2308, S19, or RB51, as well as four different S2308 mutants supports the finding that reduced virulence correlates with survival of IRF-1^{-/-} mice. Therefore, these results suggest that IRF-1 regulation of multi-gene transcription plays a crucial role in controlling *B. abortus* infection, and IRF-1^{-/-} mice could be used as an animal model to determine the degree of *B. abortus* virulence by examining death or survival.

A.3 Diagnostic Tests for Detection of *B. melitensis* Rev 1 (Kimron)

In this project we developed an effective PCR tool that can distinguish between Rev 1 field isolates and *B. melitensis* virulent field strains. This has allowed, for the first time, to monitor epidemiological outbreaks of Rev 1 infection in vaccinated flocks and to clearly demonstrate horizontal transfer of the strain from vaccinated ewes to unvaccinated ones. Moreover, two human isolates were characterized as Rev 1 isolates implying the risk of use of improperly controlled lots of the vaccine in the national campaign. Since atypical *B. melitensis* biotype 1 strains have been characterized in Israel, the PCR technique has unequivocally demonstrated that strain Rev 1 has not diverted into a virulent mutant. In addition, we could demonstrate that very likely a new prototype biotype 1 strain has evolved in the Middle East compared to the classical strain 16M. All the Israeli field strains have been shown to differ from strain 16M in the PstI digestion profile of the *omp2a* gene sequence suggesting that the local strains were possibly developed as a separate branch of *B. melitensis*. Should this be confirmed these data suggest that the Rev 1 vaccine may not be an optimal vaccine strain for the Israeli flocks as it shares the same *omp2* PstI digestion profile as strain 16M.

complex deficient mutant was similar during the course of infection. These results suggest that *B. abortus* contains no essential terminal oxidase utilized at low oxygen tension in phagosomes requiring the cytochrome *bc₁* complex. Alternatively, other branched cytochrome *bc₁* complex independent respiratory mechanisms that contain the high oxygen affinity terminal oxidases likely exist to facilitate *Brucella* survival in phagosomes. This investigation regarding the *Brucella* respiratory system illustrates the multiple survival strategies used by this bacterium for intracellular survival.

Brucella abortus actively secretes materials and uptakes nutrients to maintain the survival and multiplication of the bacteria in host cells. ATP-binding cassette (ABC) transporters can uptake or secrete diverse materials across the bacterial membrane, and, thus, ABC transporters may be important for survival of the pathogen in the host. In the present study, the *B. abortus* genes encoding tandem repeated *Brucella* ATP-binding proteins, BapA and BapB, were identified. The deduced amino acid sequences of these two genes place BapA and BapB into group 6 containing RTX toxin transporters and cyclic -1,2-glucan transporters, one of twenty-five ABC transporter ortholog groups. One of the ortholog group 6 proteins, *Haemophilus influenzae* LktB, shows the highest similarity and identity with these two *Brucella* proteins. To test the role of these putative tandem repeated ABC transporters in *Brucella* pathogenesis, a *bap* deletion mutant was constructed and used to infect murine RAW 264.7 macrophages and mice. The number of CFU from RAW 264.7 cells and spleens of BALB/c mice infected with wild type or the *bap* deletion mutant was similar during the course of infection, suggesting the *bap* genes are not necessary to maintain the pathogenesis of *B. abortus*, or alternative compensatory mechanisms may exist to permit the intracellular survival of *B. abortus* *in vitro* and *in vivo*. This is the first molecular approach to investigate the role of putative ABC transporters classified into ortholog group 6 in *Brucella* pathogenesis.

Interferon regulatory factor-1-deficient (IRF-1^{-/-}) mice have diverse immunodeficient phenotypes that are necessary for conferring proper immune protection to intracellular bacterial infection, such as a 90% reduction of CD8⁺ T cells, functionally impaired NK cells, as well as a deficiency in iNOS and IL-12p40 induction. Interestingly, IRF-1^{-/-} mice infected with diverse *Brucella abortus* strains reacted differently in a death and survival manner depending on the dose of injection and the level of virulence. Notably, 50% of IRF-1^{-/-} mice intraperitoneally infected with a sublethal dose in C57BL/6 mice, i.e., 5 x 10⁵ CFU of virulent S2308 or the attenuated vaccine S19, died at 10 and 20 days post-infection, respectively. Interestingly, the same dose of RB51, an attenuated new vaccine strain, did not induce the death of IRF-1^{-/-} mice for the 4 weeks of infection. IRF-1^{-/-} mice infected with four more other genetically manipulated S2308 mutants at 5 x 10⁵ CFU also reacted in a death or survival manner depending on the level of virulence. Splenic CFU from C57BL/6 mice infected with 5 x 10⁵ CFU of S2308, S19, or RB51, as well as four different S2308 mutants supports the finding that reduced virulence correlates with survival of IRF-1^{-/-} mice. Therefore, these results suggest that IRF-1 regulation of multi-gene transcription plays a crucial role in controlling *B. abortus* infection, and IRF-1^{-/-} mice could be used as an animal model to determine the degree of *B. abortus* virulence by examining death or survival.

B.3 Development of Molecular Protocol to Distinguish Vaccine and Field Strains

B. melitensis Rev 1 has been used worldwide in the last decades. In a few rare events, the strain has caused adverse effects including strain persistence and secretion in the milk of vaccinated animals. A more frequent occurrence are the abortions due to misuse of the vaccine in pregnant animals, and retained titers after the first gestation hampering surveillance programs.

These problems have been mostly overlooked due to the zoonotic nature of the disease and the reluctance of the farmers to collaborate with the veterinary services once problems were encountered. **The lack of a molecular marker on the strain has hampered epidemiological characterization in cases where field isolates exhibited properties similar to Rev 1.**

Use of Rev1 in national control programs has been strongly proposed by international agencies involved in the improvement of the agricultural and social systems of developing countries. Since eradication by test and slaughter program in endemic countries is difficult to achieve due to heavy budgetary constraints necessary for compensations vaccination of the whole flock has been considered a preferred alternative. In the last decade Israel maintained a conservative vaccination policy in which only young females between the ages of 2 to 6 months were vaccinated, using a full dose, sub-cutaneous route. Nevertheless, our retrospective data have demonstrated that the Rev 1 vaccine caused adverse effects similar to those mentioned above. **Moreover, we were able to demonstrate horizontal transfer of the strain in the field and in two rare cases the vaccine strain was isolated in rough form, further complicating diagnosis.** In addition, there was a single confirmed case of human infection with Rev 1 isolated from a farmer who was in close contact with vaccinated ewes. The fact that the vaccination did not always confer the expected resistance on the animals, including excretion of field strain in the milk of vaccinated animals, had compromised the efficacy of the entire vaccination program.

The vaccination method was altered in November 1997, including the employment of the original Elberg strain, and application in smaller doses via ocular inoculation. In light of the improved results, veterinarians had taken the liberty to vaccinate adult animals. The application of the vaccine in pregnant animals led to abortion storms in several intensively managed flocks followed by the secretion of the organism in the milk. This experience led to vigorous debate among leading scientists in the field of brucellosis concerning the feasibility of whole flock, vaccination scheme. **Although isolates were characterized bacteriologically, the lack of molecular markers to distinguish Rev 1 from field strains made it impossible to determine whether a virulent strain had mutated towards a Rev 1 like phenotype or the reverse had occurred.** This has prevented conclusion of the debate regarding the safety of use of Rev 1 in adult animals. Perhaps most importantly, the problems potentially derived from the use of Rev 1 vaccination was the concern that farmers will refuse to cooperate with veterinarians in the future.

The *omp2* gene exists in two homologous copies that differ slightly among *Brucella* spp. and biotypes that has recently been used to establish a molecular typing method for *Brucella* spp. A PstI polymorphism has been shown between *B. abortus* and *B. melitensis* (3). We used this information to study the stability of the *omp2* gene among local *B. melitensis* isolates from small ruminants, cattle and humans, representing the currently prevailing population in Israel. **This study included 37 isolates that were characterized as the vaccine strain Rev 1 according to conventional bacteriological methods. The results of this study are presented in figures 4 through 7 and reveal complete identity of PstI digestion profiles for strain 16M, (prototype strain for virulent *B. melitensis* biovar 1), and the vaccine strain Rev 1.** In contrast, the *omp2a* gene of Israeli field isolates, representing each of three biotypes, revealed a different profile indicating diversion of the local Israeli strains from the predicted pattern. These results have highlighted the potential use of the PstI digest as a molecular method to monitor Rev1 secretion from vaccinated animals, and the risk to the human population.

B. ACHIEVEMENTS

B.1 Identification of mutant *Brucella* (TAMU)

The STM screen in the mouse model identified 27 mutants (out of 178) putatively attenuated for chronic infection. A statistically significant competitive infection defect could be demonstrated for 14 of these mutants when mixed infection (1:1) with the parental wild-type S2308. Thus, statistically significant evidence for attenuation was obtained for 8% (14 of 178) of the mutants screened (7). Assuming that our mutagenesis was random and that the coding density of the 3.2 Mb *B. abortus* genome is similar to that of *E. coli*, our data suggest that an estimated 257 genes may be required for chronic persistence in mice after i.p. inoculation. In contrast, i.p. infection of mice with a signature-tagged mutant bank of *S. typhimurium* revealed only 3% of its 4.4 Mb genome, or an estimated 153 genes are required for the acute infection caused by this intracellular pathogen (5). The greater number of virulence genes required for chronic infection as compared to acute disease may reflect the requirement for additional adaptations to ensure long-term persistence, such as those which prevent clearance of *B. abortus* by the host immune system.

The attenuated mutants could be separated in three groups based on differences in their survival characteristics. Of the twenty four mutants that were reduced at 2 weeks post infection, thirteen recovered to normal levels by eight weeks (group I). Eleven mutants were much reduced by two weeks post infection and did not recover at later times (group II). Sixteen mutants (group III) appeared to establish normal infections at two weeks, but displayed a defect for chronic persistence by eight weeks post infection (Table 1 and Table 2). Typically organisms in group III did not show as drastic a reduction in survival as those observed at 8 weeks for group I organisms (Table 2). The first set of mutants (group I) were not characterized and appear to only be important for early colonization, i.e., greatly reduced recovery at two weeks post-inoculation. However, recovery of these mutants by eight weeks post-inoculation is consistent with normal virulence once they have acquired the appropriate intracellular niche.

The studies described have done much to identify virulence genes in *Brucella* and to evaluate these organisms for their potential efficacy as vaccines. The construction of knockout strains in several genetic backgrounds will help to verify the essential nature of the genes identified as well as producing vaccine candidates. The availability of vaccine candidates for field testing has been delayed by difficulties in obtaining knockout mutants devoid of recombinant DNA. This appears to be a problem with the *sacB* counter-selection. Alternate methods for construction are being explored including the Cre-Lox and other such recombinase based systems (4, 6). Once the candidates are available in Rev 1 they will be tested in goats.

Knockout strains defective in these genes are being constructed to provide candidate vaccines in several genetic backgrounds, i.e., Rev-1, S19, etc. Recovery of the knockout mutants has been the most problematic portion of the work. Sucrose selection requires the use of low osmolarity medium (no salt) and the sucrose tolerant organisms recovered include the knockout mutant, the wildtype and organisms containing point deletions in *sacB*. A significant background of sucrose resistant colonies is caused by point mutations in *sacB*. The resistance of these organisms to ampicillin can be used to distinguish them from other recombination outcomes. Among the sucrose resistant, ampicillin sensitive organisms are the knockout and the wild-type which are formed by excision of the plasmid in one of two different pathways. The wild type is formed when the plasmid is excised using the same sequences as it did to integrate, i.e., 5' and 5', or 3' and 3'. The knockout is formed when the alternate sequence is used to excise

the plasmid, i.e., 5' and 3', or 3' and 5'. The theoretical ratio is predicted to be 1:1, but this has not been the case. Selection of 1000's of sucrose resistant colonies may yield a single mutant. Since PCR is required to distinguish these isolates, it has been decided to prepare pools of 10 isolates each and to look for the shortened product among the PCR products derived from a pool of organisms. Once a pool has been identified individual organisms in the pool will be examined.

During his sabbatical at Texas A&M University, Dr. Banai has standardized an electroporation system for 16M and Rev1. *B. melitensis* 16M requires about 500 nanograms of plasmid DNA compared to *B. melitensis* Rev1 that is best transformed with 5 micogram plasmid DNA. *B. melitensis* 16M appeared to have a greater transformation efficiency producing several hundred colonies in a single transformation under optimal conditions compared to 100 CFU or less for Rev1.

We have tested the relationship between persistence of the vaccine strain and the level of protective immunity in the mouse model. In these trials we used strains with varied attenuation properties in an attempt to establish the correlation between duration of survival of vaccine candidates and protective immunity. Four organisms were examined; S19, a classical vaccine strain used in ruminants since the 1920's, and three attenuated transposon mutants. BA582, deficient in cytochrome d oxidase, BA114, interrupted in *virB10* encoding the type IV secretion system, and BA102, disrupted in the gene encoding glycine dehydrogenase. Three of the strains were rapidly cleared from the mice and were undetectable by eight weeks post-vaccination (Fig. 1). To support our hypothesis these organisms should provide less protection than BA102 that persists in the spleens of mice. **Protection against challenge did not appear to correlate directly with persistence in the spleens of mice among the vaccine strains tested** (Fig. 2). Due to the rapid clearance of three of four vaccine candidates from the spleens of mice, the experiment was repeated using a 100-fold increase in vaccinating dose. In this experiment significant levels of protection were also observed for all candidate vaccines over naïve mice (Fig. 3). However, at this dose one of the vaccine candidates BA582 was less rapidly cleared and persisted out to 8 weeks post inoculation. Naïve mice and mice vaccinated with BA114 exhibited a statistically different level of infection from BA102, BA582 and S19 vaccines that is probably the result of the small number of animals employed. Since the short term vaccine candidate did not behave as expected in this trial we cannot confirm or deny the hypothesis that long-term surviving vaccine candidates are more effective than short-term candidates at providing protection in the mouse model. However, each of the vaccine candidates does induce a significant level of protection.

B.2 Mouse model of infection (UWISC)

To maintain survival in macrophages, *Brucella* must overcome a hostile phagosomal environment defined as low pH, limited nutrition, as well as low oxygen tension. The specific mechanisms utilized by *Brucella* to surmount such unfavorable environmental factors in phagosomes are not well understood. In general, to adapt to a change in environmental oxygen tension, bacteria use different terminal oxidases that have different oxygen affinity. To survive in phagosomes where low oxygen tension exists, *Brucella*, like other bacteria, may require high oxygen affinity terminal oxidases that can accept electrons through a cytochrome *bc₁* complex dependent or independent pathway. Using a *Brucella abortus* cytochrome *bc₁* complex deficient mutant, *fbcF*, the requirement for a high oxygen affinity terminal oxidase governed by the cytochrome *bc₁* complex dependent pathway was tested. The number of CFU from RAW 264.7 macrophage cells and spleens of BALB/c mice infected with wild type or the cytochrome *bc₁*,

C. IMPACT

The significance of the findings reported will impact the development of vaccine strains for use in both animals and humans. Knowledge of the basic mechanisms required for intracellular survival will permit the development of "designer" vaccines for use in specific species and at the appropriate stage of development. In the past vaccination depended on the use of fortuitous isolates of reduced virulence that required careful examination of their properties in host species. S19 and Rev 1 are perfect examples of vaccine strains used in cattle and sheep which retain virulence in humans. The genetic basis for the attenuated virulence of these strains remain undefined. Knowledge of the mechanisms resulting in attenuated virulence will permit application of vaccine strains under different sets of conditions. Perhaps even in pregnant animals.

The STM screen employed in this work focused on persistence of the parental strain and mutants in the mouse model. This is a direct result of intracellular survival in macrophages. The ability of *Brucella* to persist in these cells results in its distribution throughout the system and delivery to the reproductive system. The decreased persistence of S19 and other vaccine strains prevents abortion. However, when pregnant animals are inoculated with S19 or Rev 1 a high percentage of inoculated animals abort their fetus. This has also been observed with RB51 in elk and bison. In the future, it may be possible to develop vaccine strains that are unable to target, replicate or survive in reproductive tissue. Such vaccines may be used safely in animals on unknown status.

The financial impact of improved vaccines in the U.S. may not be realized in agriculture, but may have a significant impact on tourism including the protection of wildlife species elk and bison. In Wyoming alone, the financial impact of hunting (\$1 billion per year) exceeds the annual agricultural contribution to the state economy. International impact will be realized by increased agricultural productivity, in Latin American and Middle Eastern countries in which brucellosis is endemic. This decrease will also help to reduce human infection that is a direct result of animal infection.

The resemblance of biological properties between the atypical strain previously described in Israel and the vaccine strain Rev 1 regarding susceptibility to penicillin and dyes has been described on more than one occasion. The unique Pst I pattern described for strain 16M, which also applies to the vaccine strain Rev 1 permitted determination of the origin of the atypical strain. Origination from Rev1 would maintain the Pst I digestion pattern of strain Rev1 and strain 16M. The contrary is true if the strain had emerged from a *B. melitensis* biotype 1 field strain. It may be inferred, therefore, that emergence of atypical strains occurred due to a mutation in the biological properties of field strain that increased susceptibility to penicillin and dyes. This conclusion contributed to solving the dispute on the stability of attenuation of strain Rev1, since it became clear that strain Rev1 had not acquired virulence properties.

D. REFERENCES

1. **Baldwin, C. L., and A. J. Winter.** 1994. Macrophages and *Brucella*. Immunol.Ser. **60**:663-680.
2. **Birmingham, J. R., and E. L. Jeska.** 1981. Characterization of macrophage functions in mice infected with *Brucella abortus*. Infect. Immun. **32**:1079-1083.
3. **Ficht, T. A., S. W. Bearden, B. A. Sowa, and H. Marquis.** 1990. Genetic variation at the *omp2* porin locus of the brucellae: species-specific markers. Mol.Microbiol. **4**:1135-1142.
4. **Hasan, N., M. Koob, and W. Szybalski.** 1994. *Escherichia coli* genome targeting, I. Cre-lox-mediated in vitro generation of ori- plasmids and their in vivo chromosomal integration and retrieval. Gene **150**(1):51-6.
5. **Hensel, M., J. E. Shea, C. Gleeson, M. D. Jones, and E. Dalton.** 1995. Simultaneous identification of bacterial virulence genes by negative selection. Science **269**: 400-403.
6. **Hoang, T. T., R. R. Karkhoff-Schweizer, A. J. Kutchma, and H. P. Schweizer.** 1998. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. Gene **212**(1): 77-86.
7. **Hong, P. C., R. M. Tsolis, and T. A. Ficht.** 2000. Identification of genes required for chronic persistence of *Brucella abortus* in mice. Infect. Immun. **68**(7): 4102-7.

E. APPENDIX I(TABLES)**Table 1 Mutants Identified by STM**

Mutants not recovered at 2 or 8 weeks (group II)	Mutants not recovered at 8 weeks (group III)
BA11	BA18
BA41	BA27
BA73	BA31
BA87	BA38
BA100	BA63
BA102	BA70
BA109	BA99
BA114	BA105
BA157	BA116
BA159	BA119
BA184	BA122
	BA130
	BA138
	BA140
	BA142
	BA152

Table 2 Confirmation of competitive defects of STM mutants by competitive infection of mice with *B. abortus* S2308

Mutant	Competitive Index ^a in vivo		Competitive Index in vitro	Mutant	competitive index in vivo
	2 weeks	8 weeks			
BA11	2.29*b	1.86		BA18	0.064
BA41	4.74*	>29512***	0.97	BA27	2.24
BA73	3.36	147.9*	2.74	BA31	3.63*
BA87	1.08	10.47**	0.79	BA38	7.76*
BA100	20.89***	>758.6***	4.70	BA63	1.86*
BA102	1.47	93.3***	1.80	BA70	3.39
BA109	0.92	3.21		BA99	1.02
BA114	133.07**	>1047.1***	0.91	BA105	2.00
BA157	0.74	1.58		BA119	6.31
BA159	1.50	23.44*	2.20	BA116	2.51
BA184	1306.8***	>2168.2***	0.57	BA122	95.50*
				BA130	3.02
				BA138	3.72
				BA140	1.20
				BA142	70.79***
				BA152	72.44***

^a competitive index was calculated as $(CFU_{S2308}/CFU_{mutant})_{recovered}/(CFU_{S2308}/CFU_{mutant})_{inoculated}$

^b significance of differences between competitive indices of STM mutants and a control, non-attenuated mutant were compared using a Student's t-test.

*, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.005$

Table 3 Characterization of *B. abortus* genes identified by STM

Mutant	Closest homolog (organism)	GenBank Accession Number	Inferred Function
BA41	<i>virB1 (Brucella suis)</i>	AF141604	Type IV secretion
BA114	<i>virB10 (B. suis)</i>	AF141604	Type IV secretion
BA184	<i>wbkA (B. melitensis)</i>	AF047478	O-antigen biosynthesis
BA159	<i>gluP (B. abortus)</i>	U43785	uptake of glucose and galactose
BA102	P-protein (<i>Synechocystis</i> sp.) <i>gcvP (M. tuberculosis)</i>	D90914	glycine dehydrogenase
BA152	<i>gltD (Rhizobium etli)</i>	AF107264	glutamate synthase
BA87	hypothetical protein (<i>Helicobacter pylori</i>)	AE000628	conserved inner membrane protein
BA73	no homology	none	unknown

F. APPENDIX II (FIGURE LEGENDS)

Fig. 1 Persistence of *B. abortus* mutants in the Spleens of Vaccinated Mice. Mice (10 per treatment group) were inoculated i.p with 0.1 ml containing the bacterial doses shown ($2-6 \times 10^4$ cfu). At the times indicated the mice were sacrificed and the bacterial burden in the spleen determined.

Fig. 2 Protection from challenge with virulent *B. abortus* strain 2308. Mice (5 per treatment group) were inoculated i.p with 0.1 ml containing 5×10^4 cfu of each strain. At the times indicated the mice were sacrificed and the bacterial burden in the spleen determined.

Fig. 3 Persistence in the Spleens of Vaccinated Mice. Mice (5 per treatment group) were inoculated i.p with 0.1 ml containing $\approx 5 \times 10^6$ cfu of each strain. Following clearance of the vaccine strain at 4-6 week, the mice were challenged with S2308 at a dose of $\approx 5 \times 10^4$ cfu. At the times indicated the mice were sacrificed and the bacterial burden in the spleen determined. The asterisk (*) indicates a significant difference ($p < 0.05$) with the result obtained using naïve mice. The double asterisk (**) indicates a significant difference ($p < 0.05$) with the result obtained using S19 vaccinates.

Fig. 4 PCR amplification of *omp2* fragments from *Brucella* prototype strains. Marker (lane M); *B. abortus* biov. 1 2308 (lane 1); *B. melitensis*-B115 (INRA) (lane 2); *B. abortus* biov. 3-Tulya (lane 3); *B. abortus* biov. 1 544 (lane 4); *B. abortus* vaccine strain S19 (lane 5); *B. melitensis* biov. 1-16M (INRA) (lane 6); *B. suis* vaccine strain 2 (lane 7); *B. melitensis* vaccine strain Rev 1 (subcutaneous) (lane 8); *B. melitensis* vaccine strain Rev 1 (ocular) (lane 9); *B. melitensis* biov. 3-Ether (lane 10).

Fig. 5 PstI digestion of *omp2* amplified fragments from *Brucella* prototype strains. *B. abortus* biov. 1 2308 (lane 1); *B. melitensis*-B 115 (INRA) (lane 2); *B. abortus* biov. 3-Tulya (lane 3); *B. abortus* biov. 1 544 (lane 4); *B. abortus* vaccine strain S 19 (lane 5); *B. melitensis* biov. 1-16M (INRA) uncut (lane 6); Marker; *B. abortus* biov. 1 544 uncut (lane 7); *B. suis* vaccine strain 2 (lane 8); *B. melitensis* biot. 1-16M (lane 9); *B. melitensis* vaccine strain Rev 1 (subcutaneous) (lane 10); *B. melitensis* vaccine strain Rev 1 (ocular) (lane 11); *B. melitensis* biov. 3 Ether (lane 12).

Fig. 6 PstI digests of *omp2* amplified fragments from *Brucella* atypical human isolates. *B. melitensis* biov. 1#117790 (lane 1); *B. melitensis* biov. 1#118762 (lane 2); *B. melitensis* biov. 2#118808 (lane 3); *B. melitensis* biov. 2#160621 (lane 4); *B. melitensis* vaccine strain Rev 1 (ocular) (lane 5); *B. melitensis* vaccine strain Rev 1 (subcutane) (lane 6); *B. melitensis* rough #119056 (lane 7); *B. melitensis* vaccine strain Rev 1 (subcutaneous): uncut (lane 8); Marker; *B. melitensis* biov. 1 16M (INRA): uncut (lane 9); *B. melitensis* Rev 1-5000 (lane 10); *B. melitensis* biov. 1 16M (INRA) (lane 11); *B. melitensis* Rev 1#204216 (lane 12); *B. melitensis* biov. 3#119917 (lane 13); *B. melitensis* biov. 3#119919 (lane 14); Lane 15, *B. melitensis* biot. 1 atypical #124386 (lane 15); Lane 16, *B. melitensis* biov. 2 atypical #124906 (lane 16).

Fig. 7 Pst I digestion of *omp2* amplified fragments from animal isolates showing vaccine strain Rev 1 characteristics. *B. melitensis* vaccine strain Rev 1 #134172 (lane 1); *B. melitensis* vaccine strain Rev 1#116375 (lane 2); *B. melitensis* vaccine strain Rev 1#124596 (lane 3); Lane 4, *B. melitensis* strain Rev 1 (subcutaneous) (lane 4); *B. melitensis* strain Rev 1 (subcutaneous) uncut (lane 5); Marker; *B. melitensis* vaccine strain Rev 1 (ocular) uncut (lane 6); *B. melitensis* vaccine strain Rev 1 (ocular) (lane 7); *B. melitensis* vaccine strain Rev 1#225877 (lane 8); *B. melitensis* vaccine strain Rev 1#226390 (lane 9); *B. melitensis* vaccine strain Rev 1#226997 (lane 10); *B. melitensis* vaccine strain Rev 1#223713 (lane 11).

G. FIGURES

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7

APPENDIX III PUBLICATIONS AND ACKNOWLEDGMENTS

- Hong, P.C., R.M. Tsolis, and T.A. Ficht. 2000. Identification of genes required for chronic persistence of *Brucella abortus* in mice. *Infect. Immun.* 68:4102-7.
- Endley, S., McMurray, D., and T.A. Ficht. 2000. Interruption of the *cydB* locus in *Brucella abortus* attenuates intracellular survival and virulence in the mouse model of infection. *J. Bacteriol.* Submitted.
- Ficht, T.A. Adaptation and survival of *Brucella abortus* in acidic environments. 2000. *Infect. Immun.* Submitted.
- Hong, P., M. Kahl and T.A. Ficht. Reporter gene fusions to monitor survival and gene expression in *Brucella abortus*.
- Hong, P., M. Kahl and T.A. Ficht. Survival of *Brucella abortus* candidate vaccine strains in the mouse model and relation to protective immunity.
- Kahl, M. and T.A. Ficht. Creation of knockout mutants in *Brucella melitensis* and examination of their virulence in mice.
- Kurar, E. and Splitter, G.A. Nucleic acid vaccination of *Brucella abortus* ribosomal *L7/L12* gene elicits humoral and cellular immunity. *Vaccine*, 15:1858-1862, 1997.
- Oliveira, S.C., Harms, J.S., Rech, E.L., Rodarte, R.S., Bocca, A.L., Goes, A.M., and Splitter, G.A. The role of T cell subsets and cytokines in the regulation of intracellular bacterial infection. *Brazilian J. of Medical and Biological Research* 31:77-84, 1998.
- Ko, J. and Splitter, G.A. Residual virulence of *Brucella abortus* in the absence of the cytochrome *bc₁* complex in a murine model *in vitro* and *in vivo*. *Microbial Pathogenesis*, In Press.
- Ko, J., and Splitter, G.A. *Brucella abortus* tandem repeated ATP-binding proteins encoded by *bapA* and *bapB*: cloning, sequencing and residual virulence *in vitro* and *in vivo* of a deletion mutant. Manuscript submitted.
- Ko, J., Ficht, Thomas A. and Splitter, G.A. The differences in virulence of *Brucella abortus* strains detected in interferon regulatory factor-1-deficient mice. Manuscript submitted.