Chapter 2

In Vitro Mutagenesis of *Brucella* Species

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Abstract

Three major techniques have been employed for broad-range in vitro mutagenesis of *Brucella* species. Shotgun approaches capable of generating large libraries of randomly inserted transposon mutants include Tn5, mariner (Himar1), and mini-Tn5 signature-tagged mutagenesis. Allelic exchange has also been extensively employed for targeted gene deletion. In general, plasmid and transposon delivery into *Brucella* has relied upon electroporation; however, conjugation has also been successfully employed. Both approaches have been used to identify critical virulence determinants necessary for disease and intracellular survival of the organism. Perhaps more importantly these approaches have provided an opportunity to develop attenuated vaccine candidates of improved safety and efficacy. Future experiments are designed to identify individual functions that govern the interaction between host and agent and control intracellular trafficking and survival. Toward this goal, this chapter describes current approaches used to mutagenize *Brucella* spp.

Key words: *Brucella*, Conjugation, Transposon mutagenesis, Electroporation, Allelic exchange

1. Introduction

Brucella species are a group of Gram-negative, facultative intracellular bacteria that cause brucellosis, a worldwide zoonosis. There are at least six recognized species characterized biochemically, and serologically, but primarily on host preference. Recent identification from marine mammals suggests at least three additional species based upon isolation from porpoises, dolphins, and pinnipeds (1–3). Most work with *Brucella* has been restricted to the three classical species that affect agricultural animals and are readily transmissible to humans: *Brucella melitensis*, *Brucella abortus*, and *Brucella suis*. As a result, prevention of animal disease has been used as the primary approach to reduce human disease.

Jeff Braman (ed.), *In Vitro Mutagenesis Protocols: Third Edition*, Methods in Molecular Biology, vol. 634,
DOI 10.1007/978-1-60761-652-8_2,

These organisms do not express classic virulence factors such as toxins, hemolysins, etc., and express a lipopolysacchraride (LPS) component that is also greatly reduced in toxicity (4–6). The search for genetic factors important for virulence has been explored in many labs worldwide using a variety of mutagenic approaches.

The protocols outlined below describe techniques employed for broad range in vitro mutagenesis of *Brucella* species (7–11), as well as targeted gene deletion (12–14) and delivery methods (8, 14, 15). The absence of naturally occurring plasmids has led to the use of broad-range, low copy number plasmid, RK2-derived delivery vehicles, such as pBBR1mcs, pMR10, and pGL10, capable of replicating in the *Brucella* and providing experimental approaches to restore gene function (complementation) in the second step of the molecular version of Koch's postulates (16–19). Plasmids such as pSUP202-1/Tn5 (ColE1), pUT-mini-Tn5 (oriRK6), pSC189 (oriRK6), and pEX18Ap (oriT) do not replicate in *Brucella* and are used as delivery vehicles for random and site-specific mutagenesis (8, 9, 20–22). Allelic exchange is performed using pUC and pBluescript (ColE1) based replicons due to their inability to replicate in *Brucella* (13, 14). Curiously, despite the use of naturally occurring mechanisms, the *Brucella* spp. lack indigenous plasmids. One explanation for this is that *Brucella* occupies environments that are free of organisms capable of transferring plasmid via conjugation. Although acquired via ingestion, *Brucella* are rapidly taken up and transported to the lymphoreticular system, presumably limiting interaction with other organisms. Finally, experimental evidence indicates that in the absence of selection pressure none of the plasmids described persists in *Brucella*.

It is important to note that the introduction of any antibiotic resistance into class 3 agents such as the *Brucella* species requires the approval of regulatory agencies. Furthermore, antibiotics representing primary treatment regimens should never be considered for introduction. Before considering such experimentation, it is recommended to consult the Johns Hopkins ABX guide (http://prod.hopkins-abxguide.org/) listing of therapies. Select biological agents (SBAT), such as *B. melitensis*, *B. abortus* and *B. suis*, are under the oversight of the Centers for Disease Control or US Department of Agriculture (USDA). Introduction of antibiotic resistance requires the approval by the ISATTAC (Intergovernmental Select Agents and Toxins Technical Advisory Committee). Introduction of recombinant *Brucella* species that are not listed as SBAT requires the approval by the NIH/RAC through the local institutional biosafety committee (IBCs) (Table 1).

Table 1
Antibiotic resistance (Kirby-Bauer technique) expressed by Himar1 transposon mutants of *Brucella melitensis*

	Antibiotic[a]							
Gene	**Am(10)**	**Cm(30)**	**Do(30)**	**Gm(10)**	**Km(30)**	**Nm(30)**	**Rf(5)**	**St(50)**
16M	20	37	37	20	25	25	30	26
bacA	17	36	42	18	0	0	32	20
bacA	18	35	39	19	0	0	31	23
grsT	20	32	39	22	0	0	32	30
grsT	20	34	40	26	0	0	32	30
nifB/elp	20	33	38	20	0	0	30	26
nifB/elp	18	34	44	20	0	0	30	24
hlyD	23	36	45	22	0	0	27	26
aidA-hyp	22	37	43	21	0	0	28	24
dppB	20	35	35	14	0	0	30	26
mbl	19	41	40	22	0	0	30	25
mtrC	19	36	42	26	0	0	30	23
btuB	18	37	40	20	0	0	24	22
uspA	18	34	42	24	0	0	28	25
colV	14	38	40	20	0	0	30	20

Am Ampicillin, *Cm* Chloramphenicol, *Do* Deoxycyclin, *Gm* Gentamycin, *Km* Kanamycin, *Nm* Neomycin, *Rf* Rifampin, *St* Streptomycin

[a]All antibiotic concentrations in (μg/ml); the numbers in the table are the size of the zone (mm) surrounding the antibiotic disks. Mutants are resistant to Km/Nm.

2. Materials

2.1. Isolation of Bacterial Genomic DNA

1. Phenol saline: 0.5% (v/v) phenol, 0.15% (w/v) NaCl.
2. Tris–NaCl–EDTA Buffer (TNE): 10 mM Tris–HCl, pH 8.0, 10 mM NaCl, and 10 mM EDTA.
3. Triton X-100.
4. Lysozyme: 5 mg/ml in water.
5. Proteinase K: 20 mg/ml in water.
6. RNase: 20 mg/ml in water.

2.2. Mariner (Himar1) Transposon Mutagenesis

1. *B. melitensis* 16M American Type Culture Collection (ATCC) 23444. *B. abortus* S2308 (NADC) or *B. suis* 1330T ATCC 23444 as recipient (see Note 1).
2. *E. coli* β2155 [*thr*B1004 *pro thi str*A *hsd*S *lac*ZDM15 (F9 *lac*ZDM15 *lac*I^q *tra*D36 *pro*A1 *pro*B1) Δ*dap*A::*erm* (Erm^r)] *pir*::RP4 [::Km (Km^r) from SM10] as donor control.
3. *E. coli* β2155 bearing plasmid pSC189 as donor strain.
4. Tryptic soy broth (TSB) from Difco™.
5. Tryptic soy agar (TSA), TSB containing 1.5% (w/v) Bacto-Agar (Difco™).
6. Gentamicin (20 mg/ml) in water.
7. Kanamycin (Km) (100 mg/ml) in water.
8. Diaminopimelic acid (DAP) (50 mg/ml) in water.
9. Petri plates for bacterial growth on solid media.
10. TSA-Km (100 μg/ml).
11. TSA-DAP (50 μg/ml).
12. TSA-Km-DAP (100 μg/ml Km, 50 μg/ml DAP).
13. TSB-Km (100 μg/ml).
14. TSB-gentamicin (100 μg/ml).
15. Peptone saline: 1% (w/v) Bacto-peptone™ (Difco™) and 0.5% (w/v) NaCl.
16. 50% (v/v) glycerol in TSB.
17. J774.A1 macrophage (ATCC TIB-67).
18. Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) fetal bovine serum, 1 mM L-glutamine, and 1 mM non-essential amino acids.
19. 3.7% (w/v) formaldehyde.
20. Goat anti-*Brucella* serum.
21. Donkey anti-goat IgG Alexa Fluor 488 (Molecular Probes).
22. Phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic pH 7.4) (PBS).
23. 0.5% Tween-20 in distilled water, filter sterilized.
24. Triton X-100.
25. Restriction enzyme: *Hae*III, *Rsa*I.
26. T4 DNA ligase.
27. Wizard Genomic DNA Purification Kit (Promega®).
28. Inverse PCR primers: forward primer 5′-CAACACTCAACCC TATCTCG-3′; reverse primer 5′-CACTCAACCCTATCTCG GTC-3′ to amplify the region containing the interrupted loci.

29. QIAquick Gel Extraction Kit (Qiagen®).
30. PRISM™ Cycle Sequencing Kit (Applied Biosystems Inc, ABI).

2.3. Signature-Tagged Mutagenesis (STM)

1. Recipients as described in item 1 of Subheading 2.1.
2. Tryptic soy agar (TSA).
3. Tryptic soy broth (TSB).
4. Kanamycin (100 mg/ml) in water.
5. TSA-Km (100 µg/ml).
6. TSB-Km (100 µg/ml).
7. Suicide plasmid pool, pUT carrying signature-tagged mini-Tn5Km2 was obtained from Dr. D.W. Holden (Imperial College, London) and is described in detail in Subheading 3.5 below (10, 23).
8. Primer P2: 5′-TACCTACAACCTCAAGCT-3′.
9. Primer P3: 5′-CATGGTACCCATTCTAAC-3′.
10. Primer P4: 5′-TACCCATTCTAACCAAGC-3′.
11. Primer P5: 5′-CTAGGTACCTACAACCTC-3′.
12. QIAprep Spin Miniprep Kit (Qiagen®).
13. Balb/c mice from commercial vendor.
14. Nitrocellulose membrane circles.
15. 20× SSC: 3 M NaCl, 0.3 M sodium citrate, pH 7.0.
16. Prewash solution: 50 mM Tris–HCl (pH 8.0), 1 M NaCl, 1 mM EDTA, 0.1% (w/v) SDS.
17. Prehybridization/hybridization solution: 5× SSC, 0.5% (w/v) nonfat dried milk, 2.5% (w/v) denatured salmon sperm DNA, 1% (w/v) SDS.
18. ^{32}P-labeled STM probe prepared by PCR amplification of genomic DNA extracted from input and output pools (described in Subheading 3.8 below) using ^{32}P-dATP in the PCR.
19. QIAquick PCR Purification Kit (Qiagen).

2.4. Targeted Gene Deletion

1. Recipients as described in item 1 of Subheading 2.2.
2. *E. coli* Top10 [F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*Δ139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G] from Invitrogen.
3. pBluescriptKSII$^+$ from Stratagene (f1+ origin, Ap^R, β-galactosidase α-fragment, ColE1 origin, *lac* promoter).
4. pKD4 (FLP/FRT, Km^R) from Dr. H.P. Schweizer (24).
5. pEX18Ap (*sacB*, Ap^R) from Dr. H.P. Schweizer (25).

6. Primers:
   (a) Forward primer (F_{Km}) to amplify Km cassette from pKD4: 5′-CGGGATCCCGCACGTCTTGAGCGATTGTGTAGG-3′ (with BamHI linker)
   (b) Reverse primer (R_{Km}) to amplify Km cassette from pKD4: 5′-CGGGATCCCGGGACAACAAGCCAGGGATGTAAC-3′ (with BamHI linker)
   (c) Forward ($F_{5'}$ and $F_{3'}$) and reverse ($R_{5'}$ and $R_{3'}$) primers engineered to amplify flanking regions of the gene(s) to be deleted (sequences are specific for the gene(s) to be deleted) and to contain restriction sites for cloning $F_{5'}$ (site 1), $R_{3'}$ (site 2), $R_{5'}$, and $F_{3'}$ (site 3).
7. QIAquick Gel Extraction Kit (Qiagen®).
8. SOC: 6% trypticase soy broth (w/v), 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, and 20 mM glucose.
9. SOC-B: 6% trypticase soy broth (w/v), 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose.
10. Sucrose broth: TSB supplemented with 6% (w/v) sucrose, lacking salt and antibiotics.
11. Sucrose agar: TSA supplemented with 6% (w/v) sucrose, lacking salt and antibiotic.
12. Luria–Bertani broth (LB).
13. LB agar [LB broth containing 1.5% (w/v) Bacto-Agar (Difco™)].
14. TSB.
15. TSA [TSB containing 1.5% (w/v) Bacto-Agar].
16. Ampicillin (Am, 100 mg/ml) in water.
17. Kanamycin (Km, 100 mg/ml) in water.
18. Carbenicillin (Cb, 100 mg/ml) in water.
19. 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal, 20 mg/ml) in water.
20. TSA-Am (100 μg/ml).
21. TSA-Km (100 μg/ml).
22. TSA-Cb (100 μg/ml).
23. Sigma Miniprep Kit.

2.5. Intracellular Survival Assay

1. J774.A1 macrophage (ATCC TIB-67).
2. DMEM with 10% (v/v) fetal bovine serum, 1 mM L-glutamine, and 1 mM nonessential amino acids.
3. Gentamicin (Gm, 20 mg/ml) in water.
4. 0.5% (v/v) Tween-20 in distilled water, filter sterilized.

5. Peptone saline.
6. TSA.
7. TSA-Km (100 μg/ml).

3. Methods

3.1. Isolation of Genomic DNA

There are many approaches to isolate genomic DNA from *Brucella*. The method described provides sufficient amounts of genomic DNA for Southern blotting and PCR amplification and requires little manipulation of the bacteria.

1. Bacteria are grown overnight with agitation at 37°C in TSB and pelleted by centrifugation (10,000 × *g* for 5 min).
2. Pelleted bacteria are resuspended in the same volume of phenol saline and heated for 1 h at 60°C to kill the organism.
3. DNA sufficient for Southern blotting or PCR amplification may be obtained from a cell culture volume as small as 0.3 ml, as follows.
4. The bacteria are pelleted as described above and the supernatant removed.
5. The cell pellet is washed in 1.0 ml TNE by resuspension using a pipette.
6. Pelleting and washing is repeated at least twice.
7. The cells are then resuspended in 135 μl of TNE.
8. The cell suspension is diluted with 135 μl TNE containing 2% (v/v) Triton X-100.
9. Thirty microliters of freshly prepared lysozyme solution (5 mg/ml) is added and mixed by tapping the tube.
10. The suspension is incubated at 37°C in a water bath for 30 min.
11. Fifteen microliters of proteinase K solution (20 mg/ml) is added and the suspension mixed by inversion.
12. The mixture is incubated at 65°C in a water bath for at least 2 h.
13. Heat-treated RNase is added to a final concentration of 10 μg/ml.
14. These DNA preparations are best stored at –20°C until used.

3.2. Mariner Transposon Mutagenesis

Transposons of the Mariner family integrate nonspecifically at T/A base pairs, and do not require species-specific host factors for efficient transposition. In this protocol, plasmid vector pSC189, containing both the hyperactive transposase C9 and

transposon terminal inverted repeats flanking a kanamycin resistance gene, is used to deliver Himar1 transposable element into the *B. melitensis* 16M genome by conjugation. Conjugation is performed efficiently and rapidly in less than one generation in order to minimize the formation of siblings while assuring the highest level of genome coverage [8].

1. *Brucella* are removed from frozen stock and streaked for growth on TSA plates for 48–72 h (see Note 2).
2. Stock cultures should be checked to make sure that kanamycin resistance is either undetectable or significantly below the frequency observed for transposition. Briefly, frozen stocks are removed from the freezer and evaluated for growth on TSA and TSA-Km plates. Following incubation, the appearance of spontaneous kanamycin resistant (Km^R) colonies is assessed.
3. *E. coli* β2155 with pSC189 is grown for 24 h on TSA supplemented with DAP (50 μg/ml) and Km (100 μg/ml).
4. The bacteria are harvested from plates prepared in steps 1 and 3 above into 5 ml of peptone saline supplemented with DAP (50 μg/ml).
5. Equal volumes of *Brucella* and *E. coli* β2155 bearing pSC189 are mixed together to provide a donor to recipient ratio of approximately 1:100.
6. Nitrocellulose filters (25 mm diameter) are placed on the surface of TSA-DAP plates that have been dried by incubation overnight at 37°C.
7. Two hundred microliters of bacterial conjugation mixtures are pipetted onto individual nitrocellulose filters.
8. The liquid is rapidly absorbed by the dried plates that are then incubated at 37°C for 2 h.
9. The bacterial conjugation mixtures on the nitrocellulose filters are removed by resuspension into 5 ml of peptone saline.
10. Serial tenfold dilutions of the conjugation mixtures are prepared in peptone saline.
11. One hundred microliters of each dilution are plated on TSA-Km and incubated at 37°C for 3 days.
12. The remaining bacterial conjugation mixture is stored at –80°C in peptone saline supplemented with 15% (v/v) glycerol.
13. Colony forming units (CFU) are determined and used to calculate conjugation efficiency and the number of plates necessary to amplify the mutant bank.
14. There should be no Km-resistant growth from control conjugation mixtures, which include *E. coli* β2155 alone or *B. melitensis* 16M alone.

15. Conjugation mixtures are diluted with peptone saline based on the conjugation efficiency to give a concentration of conjugant about 100–300 CFU/100 µl.
16. One hundred microliters of diluted conjugation mixture is spread on the surface of TSA-Km.
17. The plates are incubated at 37°C for 3 days.
18. Well-isolated single colonies are selected using sterilized toothpicks and used to inoculate 100 µl of TSB-Km in 96-well microtiter dishes.
19. The dishes are incubated at 37°C for 2 days.
20. Duplicate plates are prepared by inoculating 10 µl from each well of the microtiter dishes into new dishes containing 90 µl TSB-Km.
21. These dishes are incubated at 37°C for 48 h.
22. Fifty microliters of 50% (v/v) glycerol is added to each well and mixed.
23. The plates are sealed with parafilm and stored at −80°C (see Fig. 1).

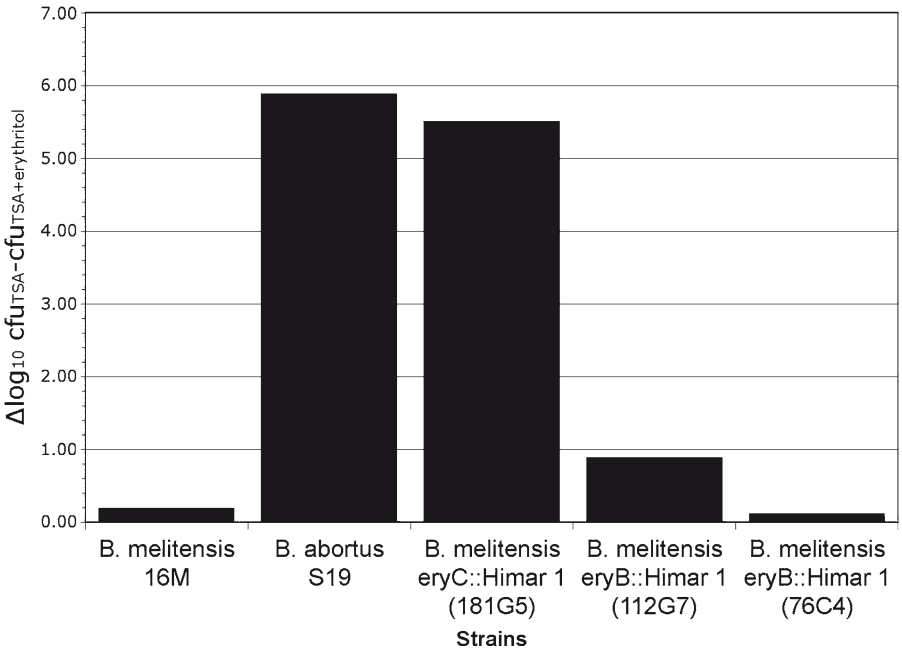

Fig. 1. Erythritol sensitivity was first described in *B. abortus* S19. The cause of the defect is believed to be the buildup of a toxic intermediate (D-erythrulose-1-phosphate, a product of the reaction catalyzed by eryB). In S19, the locus is truncated by a deletion that removes portions of the genes encoding EryC and the repressor protein EryD. The result is uncontrolled expression of the locus and buildup of the toxic product produced in a reaction catalyzed by EryB and the failure to reduce its concentration due to the absence of EryC. Interruption of eryC by Himar1 has an identical effect to the observed deletion. In contrast, Himar1 interruption of eryB has little effect on survival in the presence of erythritol. This may be explained by the failure to produce the toxic product.

3.3. Identification of Attenuated Mutants Using Immunofluorescence

1. J774.A1 macrophage form monolayers on the flat bottom of 96-well dishes when seeded at a density of 5×10^4 cells/well in 0.1 ml DMEM 1 day prior to infection.
2. Bacterial cultures are removed from frozen stock and grown on TSA-Km plates (mutants) or TSA (16M) for 72 h.
3. J774.A1 macrophage monolayers are infected with *B. melitensis* at a multiplicity of infection (MOI) of 100:1 using 10 µl of bacterial culture.
4. Uninfected cells are used as negative control.
5. Cell culture plates are centrifuged for 5 min. at $200 \times g$ to initiate the infection.
6. The macrophages are incubated at 37°C in an atmosphere containing 5% CO_2 for 20 min.
7. The culture supernatant in each well is removed using a sterile pipette and replaced with 100 µl of TSB supplemented with 100 µg/ml of gentamicin to kill extracellular bacteria.
8. The macrophages are incubated at 37°C in atmosphere containing 5% CO_2 for 48 h.
9. The culture media and the monolayers are washed twice with an equal volume of PBS as described in step 7 above.
10. Three hundred microliters of 3.7% (v/v) formaldehyde in PBS is added to each well, and the plates are incubated at room temperature for 1 h to fix the cells and any intracellular bacteria.
11. Each well is washed with 300 µl of PBS three times as described in step 9 above.
12. Fifty microliters of goat anti-*Brucella* serum (1:1000) diluted in PBS-TT is added to each well.
13. The plates are incubated at room temperature for 1 h.
14. Each well is washed three times with 300 µl of PBS-T as described in step 11 of Subheading 3.2.
15. PBS-T is removed and replaced with 50 µl of donkey anti-goat IgG Alexa Flour 488 (1:1000) diluted in PBS-TT.
16. Mutant virulence is determined microscopically by evaluating fluorescence intensity compared with positive and negative controls. Mutants that are unable to replicate within the macrophage are present in reduced number or in fewer cells compared to the control *B. melitensis* wildtype. As such, wildtype fluorescence is stronger than mutant fluorescence, and uninfected macrophages are expected to display no fluorescence.
17. A second round of fluorescence screening is used to provide statistically valid results and to confirm the attenuated phenotype.

3.4. Identification of Interrupted Loci

1. Genomic DNA isolated from attenuated mutants is digested with restriction enzymes *Hae*III or *Rsa*I.
2. The digested DNA fragments are self-ligated and amplified by inverse PCR (see Subheading 2.2, step 28 above for primers).
3. Inverse PCR is performed by heating to 95°C 4 min., followed by 30 cycles of (95°C 30 s, 57°C 30 s, 72°C 90 s), and 72°C for 7 min.
4. Agarose gel electrophoresis is performed to ensure the production of a unique PCR product, reflecting a single transposon insertion.
5. PCR products are purified from gels using QIAquick Gel Purification Kit.
6. The purified products are sequenced using reverse primer (see Subheading 2.2, step 28 above) with ABI PRISM™ Cycle sequencing kits.
7. The sequence obtained is compared to the *B. melitensis* genome sequence available in GenBank using any of the commercially available software packages.

3.5. Signature-Tagged Mutagenesis (STM)

Signature-tagged mutagenesis was developed for in vivo selection of Tn5 transposon mutants that are defective in colonization of specific tissue in the host. The advantage of this approach is that the signature tags can be used to distinguish individual mutants within a pool of mutants permitting distinction of survival characteristics of multiple mutants in a single host. In practice, recovery of the organism limits the diversity of the input pools to 50–100 mutants per host and multiple hosts are used for statistical evaluation. Tags are arrayed on nitrocellulose for comparison of recovery based on hybridization of the tags amplified from input and output pools of mutants. In this protocol, tagged transposons were obtained from Dr. D.W. Holden (see Subheading 2.3, step 7 above) and individual tags identified that readily amplify and provide a strong hybridization signal without cross hybridization between tags [26]. Plasmids containing these tags are used to generate *B. melitensis* mutants by performing separate conjugations for each tag and selecting 80 mutants or more per tag. Pools of mutants are arrayed in groups of 48 reflecting the 48 unique tags in 96-well microtiter dishes for replica plating. Pools of 48 mutants were washed off the plates and used to infect the host (input pool) and at desired time points after infection, bacteria are recovered from selected tissues (output pools). For *Brucella* infection, the spleen is the preferred tissue, although the liver also has elevated colonization, and the lymph nodes may hold special interest. The signature tags present in the output pool are PCR-amplified and labeled as are the signature tags present

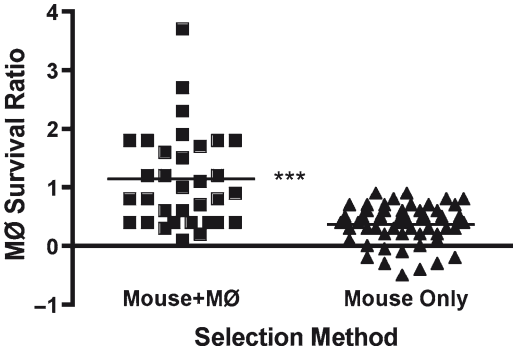

Fig. 2. Comparison of macrophage survival of attenuated *Brucella melitensis* mutants. Mutants were obtained by screening for survival in mice and macrophage (Mϕ) in culture and divided into two groups based on identification in the macrophage screen. The replication ratio (CFU48h/CFU0h) for each mutant was determined relative to the parental strain and presented as the $\log_{10}$ of wild type to mutant. Mutants that were only identified in the mouse model exhibited an average survival ratio that was significantly lower than those mutants identified using the macrophage and confirmed in the mouse model. The enhanced sensitivity of the mouse model may be explained in part by the contribution of extracellular killing present in the mouse model, but missing from macrophage screening. The horizontal line represents the average mutant survival ration from the group.

in the input pool. Following the removal of the flanking arms by *Hind*III digestion, the probes are hybridized to replica arrays of the original signature tags corresponding to the pool. Attenuated mutants are identified based upon differential hybridization signals for input and output pools, tags present in attenuated mutants will not be amplified [10, 26] (see Fig. 2).

3.6. Identification of Useful Signature-Tagged Transposons

1. Signature-tagged miniTn5Km2 transposons or others are prepared as described elsewhere [26]. Briefly, DNA tags are prepared from the variable oligonucleotide pool, RT1 (5′-CTAGGTACCTACAACCTCAAGCTT-$[NK]_{20}$-AAGCTTGGTTAGAATGGGTACCATG-3′) in a 100 µl volume PCR containing 1.5 mM $MgCl_2$, 50 mM KCl, and 10 mM Tris–Cl (pH 8.0) with 200 pg of RT1 as target; 250 µM each of dATP, dCTP, dGTP, dTTP; 100 pM of primers P3 and P5; and 2.5 U Amplitaq (Perkin-Elmer). Cycling conditions are 30 cycles of 95°C for 30 s, 50°C for 45 s, and 72°C for 10 s. The PCR product is gel purified (see Subheading 3.4, step 4 above) and digested with restriction enzyme KpnI prior to ligation into pUT-mini-Tn5Km2.
2. *E. coli* bearing plasmid are grown on TSA-Km plates and individual colonies selected using toothpicks to inoculate fresh TSB-Km in the wells of microtiter dishes.

3. The bacteria are replica-plated onto the surface of TSA-Km plates using a 48 prong replica plater.
4. The plates are incubated at 37°C for 16 h.
5. The plates are used for plasmid isolation and tag amplification, as well as "colony lifts".
6. Each well contains a unique signature-tagged transposon.
7. *E. coli* are washed off the surface of the plates by adding 5–10 ml sterile LB and gently scraping with a sterile plate spreader. The liquid is then removed with a sterile pipette, and pooled plasmids are purified using commercial kits, such as the QIAprep Miniprep kit.
8. Signature tags are labeled by incorporation of P^{32}-dATP during PCR amplification with primers P2: 5′-TACCTACAACCT-CAAGCT-3′ and P4: 5′-TACCCATTCTAACCAAGC-3′ using conditions described in step 1 of Subheading 3.6.
9. The radioactive tags are used as the probe during hybridization with the colony lifts.
10. Colony lifts are obtained by overlaying the plates with 100 mm nitrocellulose circles that are peeled back in order to transfer the colonies to the nitrocellulose.
11. The nitrocellulose circles are laid colony-side up on a stack of filter paper soaked with 0.4 N NaOH/1.5 M NaCl for 5 min to lyse the cells and denature the genomic DNA, and then neutralized with 0.5 M Tris–HCl, pH 7.4/1.5 M NaCl using the same method.
12. The nitrocellulose filters are baked at 80°C for 2 h under vacuum to fix the DNA to the membranes.
13. The filters are wetted with 2× SSC, and then transferred to glass tubes or seal-a-meal bags and prehybridized in excess solution at 68°C for at least 2 h.
14. Hybridization is performed for 16 h at 68°C in a minimal volume of solution containing ^{32}P-labeled probe (100,000 dpm/cm^2 nitrocellulose).
15. The filters are washed in 2× SSC, 0.1% (w/v) SDS twice for 15 min at room temperature, and then in 0.2× SSC, 0.1% (w/v) SDS twice for 15 min at 68°C.
16. The membranes are air-dried on Whatman 3MM paper at room temperature and sealed in plastic bags.
17. The membranes are exposed to X-ray film overnight.
18. Tags that are useful for screening are identified as those producing strong signals on the colony lifts due to stable hybridization, and tag amplification without cross hybridization.

3.7. Transposon Mutagenesis and Mutant Bank Assembly

1. Plasmid pUT containing transposon miniTn5Km2 with signature tags are introduced into *B. melitensis* by conjugation as described in Subheading 3.2 above.
2. Following conjugation, serial dilutions are prepared and the transformation efficiency is determined by plating portions of the serial dilutions on TSA-Km plates.
3. The plates are incubated at 37°C for 3 days. Depending on the number of signature tags employed ($n=48$) and the complexity of the genome, between 80 and 400 mutants are picked from 48 conjugations with plasmid having different tags (see Note 3).

3.8. Mutant Screen and Identification of Attenuated Mutants

1. Pools are assembled from 48 mutants grown in the wells of microtiter dishes and replica-plated as described above (Subheading 3.6, step 3 above).
2. Forty-eight mutants from each plate are pooled by washing the cells from the surface of the replica plate.
3. A 1-ml portion of the mutant pool is removed for genomic DNA extraction using lysozyme and proteinase K treatment (input pool) (see Subheading 3.1 above).
4. The concentration of the bacterial pool is adjusted to approximately 1×10^7 CFU/ml with PBS.
5. Six Balb/c mice are inoculated (i.p.) with 0.1 ml of the pooled bacteria.
6. Three mice are euthanized at 2- and 8-week post-infection.
7. Spleens are removed and homogenized in PBS.
8. Serial tenfold dilutions are prepared in peptone-saline.
9. Homogenates and dilutions (0.1 ml) are plated on TSA-Km.
10. Plates are incubated at 37°C for 4 days.
11. Bacteria are collected from plates containing 1,000–5,000 colonies (output pool) (see Note 4).
12. Genomic DNA is isolated from input pools and output pools as described in Subheading 3.1 above.
13. PCR amplification of signature tags using input pool and output pool genomic DNA as template and primers P2 and P4 is performed as described in step 8 of Subheading 3.6.
14. Signature tags are labeled by incorporation of ^{32}P-dATP during PCR amplification.
15. The PCR tags are digested with *Hind*III (1 U enzyme/μg DNA) to release the shared flanking regions.
16. Labeled tags are hybridized to the corresponding colony blots prepared from 96-well plates generated in Subheading 3.6 above.

17. Mutants that hybridize to the probe from the input pool but weakly or not to the probe from the output pool are attenuated. Failure to amplify signature tags is due to their absence from the output pool.
18. Mutant attenuation is confirmed using intracellular survival assay described in Subheading 3.16 below.

3.9. Identification of Interrupted Loci

1. Genomic DNA isolated from attenuated mutants is digested with *Rsa*I, self-ligated and used as template for inverse PCR.
2. Inverse PCR conditions include an initial 4 min. at 95°C, 30 cycles (95°C 30 s, 57°C 30 s, 72°C 90 s), and a final elongation at 72°C for 7 min with forward 5′-GCCGAACTTGT GTATAAGAGTCAG-3′ and reverse 5′-AAAGGTAGCGTT GCCAATG-3′ primers.
3. PCR products are gel purified using QIAquick PCR Purification Kit and the products sequenced using the reverse primer.
4. DNA sequences are compared to the *B. melitensis* sequence in GenBank to identify the disrupted genes.

3.10. Targeted Gene Deletion

In order to eliminate specific genes of interest, primers are designed to amplify sequences flanking the segment or gene to be deleted. The flanking regions referred to are located 5′ and the 3′ to the gene of interest, and are joined to each other using overlap extension PCR, i.e., the reverse primer of the 5′ fragment and the forward primer of the 3′ fragment contain complimentary sequences, as well as unique restriction sites [27]. Deletion is typically constructed to avoid downstream polar effects, where genes downstream in an operon would be affected during transcription. Genes in an operon are typically deleted so as to severely truncate the gene product, but avoid having the ribosome disrupted. The 5′ and 3′ fragments are amplified in separate reactions, gel-purified, and PCR-amplified in the same reaction to produce a joined product. The final products are digested with the restriction enzymes engineered into the primers and the final fragment is gel purified for cloning into pBluescript KSII$^+$. Antibiotic resistance cassettes are inserted between the 5′ and 3′ fragments and the construct is used to generate marked deletion mutants. To create unmarked deletion mutants (free of foreign DNA or selectable markers), the joined PCR product (without the kanamycin cassette) is cloned into the plasmid pEX18Ap, which contains *sacB*, encoding levansucrase. This *sacB* gene product is lethal to the cell, and bacteria possessing the plasmid are eliminated in the presence of sucrose. Thus, the presence of sucrose selects for the loss of the plasmid, and growth of the unmarked knockout.

3.11. Recombinant Plasmid Construction

1. Sequences flanking the gene of interest are amplified via PCR using conditions determined empirically for the gene and primers employed (see Note 5).
2. Amplify the 5′ fragment using *Brucella* genomic DNA as template and upstream primers $F_{5'}$ and $R_{5'}$ (see Subheading 2.4, step 6c above). PCR conditions depend upon the gene size and GC%.
3. Amplify the 3′ fragment using *Brucella* genomic DNA as template and downstream primers $F_{3'}$ and $R_{3'}$ (see Subheading 2.4, step 6c above).
4. The 5′- and 3′-fragments are joined during a second amplification in which only the primers $F_{5'}$ and $R_{3'}$ are used.
5. The amplification product is isolated by gel electrophoresis and purified using the QIAquick Gel Extraction Kit.
6. The PCR product and plasmids are digested with appropriate restriction enzymes to clone the insert into either pBluescript KSII$^+$ (the first step in plasmid construction to develop marked *Brucella* mutants) or pEX18Ap (to develop unmarked *Brucella* mutants).
7. Ligation is performed overnight at 15°C using a 3:1 molar ratio of insert to plasmid DNA and T4 DNA ligase.
8. Antibiotic resistance cassettes such as kanamycin (nptII) are amplified via PCR from plasmid template pKD4 with specific primers: F_{Km} 5′-CGGGATCCCGCACGTCTTGAGCGATTG TGTAGG-3′ (with BamHI linker) and R_{Km}5′-CGGGATCC CGGGACAACAAGCCAGGGATGTAAC-3′ (with BamHI linker) (see Subheading 2.4, step 6).
9. The primers F_{Km} and R_{Km} have been constructed to contain the same unique restriction enzyme sites (in this case $BamH_I$) as the junction of the 5′- and 3′-fragments (5′-Km^R-3′) (see Subheading 2.4, step 6c above).
10. The amplified resistant cassette is isolated by gel electrophoresis and purified using Qiagen's QIAquick Gel Extraction Kit.
11. Ligation of the antibiotic resistance cassette between the upstream and downstream regions is performed as described in step 6 of Subheading 3.11.

3.12. Transformation and Selection of Recombinant Plasmids

1. Competent *E. coli* are transformed with plasmid DNA as described by the manufacturer, and the culture is plated onto solid media supplemented with appropriate antibiotic (depending upon the plasmid backbone) and X-gal (20 μg/ml).
2. Colonies are selected by blue–white screening after overnight growth at 37°C and individual white colonies are used to prepare fresh cultures in (LB) broth supplemented with appropriate antibiotic (depending upon plasmid encoded resistance).

3. Recombinant plasmids are purified (Sigma Miniprep Kit) and verified using restriction enzyme digestion.
4. Bacterial frozen stocks are prepared in LB broth supplemented with 50% glycerol (v/v) and stored at −80°C.

3.13. Creation of Marked Mutants by Electroporation

1. *Brucella* are harvested from the surface of confluent plates after 3–4 days of growth at 37°C.
2. The bacteria are pelleted by centrifugation at 5,000 × *g* for 15 min at 4°C.
3. The cell pellet is washed three times with sterile, ice-cold water by repeating the previous step and is then resuspended in 1-ml ice-cold water.
4. Seventy microliters of the cell suspension is placed into a pre-chilled 1-mm gap electroporation cuvette along with 1 μg plasmid in 1–5 μl water (see Note 6).
5. The mixture is electroporated using a BT× 3000 apparatus set at 2.2–2.5 kV and 246 Ω.
6. The bacterial suspension is immediately diluted with 1 ml of SOC-B in the cuvette, transferred to a microfuge tube and subsequently incubated overnight at 37°C with agitation.
7. One hundred microliters of cell suspension is spread on the surface of TSA-Km plate and incubated at 37°C for 3 days.
8. If necessary (low efficiency), the remaining cell suspension is pelleted by centrifugation at 10,000 × *g* for 1 min, resuspended in TSB-Km and plated on TSA-Km plates.
9. Individual colonies are replica-plated onto TSA-Km and TSA-Cb.
10. Marked deletion mutants are kanamycin resistant (Km^R) and Carbenicillin sensitive (Cb^S) due to loss of the plasmid during allelic exchange.
11. Following verification (Subheading 3.15 below) individual colonies are resuspended in TSB containing 50% (v/v) glycerol and stored frozen at −80°C.

3.14. Creation of Unmarked Mutants by Electroporation

1. Marked deletion mutants are harvested from the surface of confluent plates after 3–4 days of growth at 37°C.
2. Repeat the procedure described in the previous section to prepare electrocompetent cells.
3. Electroporation is performed using plasmid pEX18Ap-containing the insert composed of 5′ and 3′ fragments (see Note 7) and bacterial suspensions are plated on TSA-Cb.
4. Individual colonies are replica-plated onto three different solid media: TSA-Cb, TSA-Km, and sucrose plates.
5. Co-integrants form due to homologous recombination between genomic and plasmid gene copies, and are Cb^R-, Km^R- and sucrose-sensitive.

6. Sucrose-sensitive colonies are used to inoculate 5 ml of sucrose broth and incubated at 37°C for 24 h with agitation (see Note 8).
7. The culture is diluted 10- to 100-fold with TSB-sucrose and 100 µl of undiluted and diluted cultures are plated onto TSA-sucrose plates.
8. The plates are incubated at 37°C for 3–5 days.
9. Sucrose-tolerant colonies are replica-plated onto TSA-sucrose and TSA-Km plates.
10. Unmarked deletion (and the original parental organism) mutants are sucrose-tolerant and kanamycin-sensitive (see Note 9) and require genetic analysis to distinguish.

3.15. Confirmation of Gene Deletion

1. Genomic DNA is extracted from sucrose-tolerant Kan^S colonies and PCR amplification of the target gene uses the 5′-upstream and 3′ reverse primers described above in step 6c of Subheading 2.4.
2. The choice of primers and size of the amplification product depends on the gene deleted and the sequence flanking the gene of interest.
3. Amplification of a deleted locus produces a smaller PCR product that may be distinguished from either revertants to wildtype and/or the parental strain.

3.16. Confirmation of Mutant Attenuation

1. Stock cultures of mutants or the parental wildtype 16M are inoculated into 5 ml TSB or TSB-Km and incubated at 37°C for 48–72 h with agitation.
2. Fresh cultures are prepared by diluting the stock cultures (1:1000) into fresh TSB or TSB-Km and incubated at 37°C for 24 h with agitation.
3. Macrophages are seeded into 24-well plates (2.5×10^5/well in 0.5 ml DMEM) 1 day prior to infection.
4. The bacteria are pelleted by centrifugation and resuspended in an equal volume of PBS. This step is repeated twice and following the last centrifugation the bacterial suspension is diluted fivefold.
5. Approximately 10 µl of bacterial suspension is added to each well of the microtiter dish reflecting bacteria to macrophage ratio or MOI of 50:1.
6. The microtiter dishes are centrifuged at $200 \times g$ for 5 min at room temperature and then incubated at 37°C for 20 min.
7. The supernatant is removed and the infected cell monolayer is washed with PBS. This step is repeated three times.
8. Fresh DMEM is added to each well containing gentamicin (50 µg/ml) to destroy extracellular bacteria.

9. The dishes are incubated at 37°C up to 48 h. At that time the DMEM is removed and 1.0 ml 0.5% (v/v) Tween-20 is added to lyse macrophages and release intracellular bacteria.
10. Tenfold dilutions of the lysate are prepared using PBS and the dilutions are plated in triplicate on TSA with or without kanamycin.
11. The plates are incubated at 37°C for 3 days, at which time the bacterial recovery is determined, i.e., CFU/well.
12. Recovery of mutants is compared to wildtype organism to observe attenuation.

3.17. Summary

The work described has been developed in or laboratory over the past 10 years and is the culmination of the work of several individuals as well as collaborators who generously provided reagents, cloning and delivery vehicles, and signature-tagged transposons. The methods presented are meant to describe their use in the development of *Brucella* mutants and not to imply their original development that is described in those works provided in the bibliography. The work has resulted in the identification of several important virulence factors and vaccine candidates currently under evaluation.

4. Notes

1. It is best to passage the bacteria through a host organism, including mice or small ruminants. In this way, the parent organism may be expected to exhibit the highest level of virulence.
2. To ensure that the starting organism is fully virulent, it is also best to streak for isolation and to select a smooth colony to inoculate either fresh plates or broth. This will help to minimize the presence of spontaneously appearing rough organisms.
3. These mutants will be screened for susceptibility to ampicillin to eliminate strains (fewer than 2% of mutants) carrying cointegrates of the suicide vector pUT inserted in the chromosome. As *B. melitensis* is not allowed to replicate before plating the transformants, the isolation of siblings with this procedure is unlikely.
4. This number of organisms is sufficient to provide recovery of virulent organism with 95% confidence.
5. Primers used contain restriction sites for subsequent cloning.
6. Plasmids used for electroporation should be eluted into water prior to use in electroporation, since high salt concentrations present in commercial elution buffers negatively affects the procedure.

7. The unmarked plasmid, containing the *sacB* gene, insert, and *bla* gene (same function as Carbenicillin) is used for electroporation into marked deletion strains. Utilizing the newly created marked strain enhances selection, since loss of kanamycin resistance identifies unmarked mutants formed via allelic exchange.
8. After 24 h of growth, the cultures will not look saturated because the sucrose was toxic to the majority of the cells, but there are enough cells for plating.
9. Unmarked deletion mutants are sucrose tolerant, resulting from the loss of the integrating plasmid-containing *sacB*, and kanamycin-sensitive, since the original kanamycin cassette is replaced during plasmid integration.

Acknowledgments

We would like to acknowledge the support of NIH (R01048496) and the WRCE (1U54AI057156-0100) for the construction of *B. melitensis* mutant banks and the USDA (99-35204-7550) for the development of *B. abortus* mutant banks. We gratefully acknowledge the contributions of Chris Allen, Renée Tsolis, Priscilla Hong, and Carol Turse without whose efforts this publication would not be possible.

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