Identification of Brucella melitensis 16M genes required for bacterial survival in the caprine host

Michel S. Zygmunt a,*, Sue D. Hagius b, Joel V. Walker b, Philip H. Elzer b,c

a UR 1282, Unité de Recherche Infectiologie Animale et Santé Publique (IASP), Institut National de la Recherche Agronomique, 3738 Nouzilly, France
b Department of Veterinary Science, LSU AgCenter, Baton Rouge, LA 70803, USA
c Department of Pathobiological Sciences, LSU, School of Veterinary Medicine, Baton Rouge, LA 70803, USA

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Abstract

Brucella species are Gram-negative bacteria which belong to α-Proteobacteria family. These organisms are zoonotic pathogens that induce abortion and sterility in domestic mammals and chronic infections in humans known as Malta fever. The virulence of Brucella is dependent upon its ability to enter and colonize the cells in which it multiplies. The genetic basis of this aspect is poorly understood. Signature-tagged mutagenesis (STM) was used to identify potential Brucella virulence factors. PCR amplification has been used in place of DNA hybridization to identify the STM-generated attenuated mutants. A library of 288 Brucella melitensis 16M tagged mini-Tn5 Km2 mutants, in 24 pools, was screened for its ability to colonize spleen, lymph nodes and liver of goats at three weeks post-i.v. infection. This comparative screening identified 7 mutants (approximately 5%) which were not recovered from the output pool in goats. Some genes were known virulence genes involved in biosynthesis of LPS (lpsA gene) or in intracellular survival (the virB operon). Other mutants included ones which had a disrupted gene homologous to flgF, a gene coding for the basal-body rod of the flagellar apparatus, and another with a disruption in a gene homologous to ppk which is involved in the biosynthesis of inorganic polyphosphate (PolyP) from ATP. Other genes identified encoded factors involved in DNA metabolism and oxidoreduction metabolism. Using STM and the caprine host for screening, potential virulence determinants in B. melitensis have been identified.

Keywords: Brucella; Signature-tagged mutagenesis; Virulence; In vivo; Caprine host

1. Introduction

Brucella spp. are Gram-negative bacteria belonging to the α-Proteobacteria family which cause brucellosis, an infectious disease affecting animals and humans. Brucella melitensis is the most important species involved in ovine and caprine brucellosis which is characterized by abortion, low production, and infertility in infected animals. B. melitensis is also the most pathogenic species for humans. The capacity of this facultative intracellular bacterium to induce disease is dependent on its ability to survive and to multiply within both professional host and non-professional host phagocytes [1,2]. Identification of virulence determinants of Brucella spp. has been based mainly on in vitro or mouse model approaches. These studies suggest that several stress proteins play a key role in the adaptation of Brucella to the intracellular environment [3–5]. Stress response proteins [6] and smooth lipopolysaccharide (LPS) [7] have been shown to be required for virulence in vitro and in the mouse model. The understanding of bacterial pathogenicity requires the identification of genes involved which are essential for the survival of bacteria in vivo resulting in disease.

A negative screening technique called signature-tagged mutagenesis (STM) has been developed in Salmonella typhimurium which allows the identification of mutant strains that are eliminated from a population of recovered mutants after an animal challenge [8]. The STM approach has been adopted to...
identify essential genes in several bacterial species including *Vibrio cholerae* [9], *Yersinia enterocolitica* [10], and *Legionella pneumophila* [11]. Classical STM methodology involves the use of hybridizing techniques which may result in high background and false positives. In this study, a PCR-based STM was used, avoiding the problems inherent to hybridization and increasing the specificity during the PCR screening [12].

In *B. melitensis* and *Brucella suis*, STM has been applied to identify the gene’s encoding factors required for the survival and multiplication and persistence of *Brucella* in the mouse model and human macrophages [13–16]. A limitation of these studies was the models used to screen the mutants. To gain a better understanding of the *in vivo* pathogenicity of *B. melitensis*, a PCR-based STM was used for screening and identification of seven potential virulence genes in the caprine host.

2. Materials and methods

2.1. Bacterial strains and plasmids

*B. melitensis* 16M obtained from laboratory stocks of PHE was used for STM. All *Brucella* strains were grown on Schae
dler blood agar plates (SBA) or liquid Schaedler broth (SB) media (Difco Laboratories, Detroit, MI, USA) at 37 °C in a 5% CO₂ atmosphere with appropriate antibiotics when needed. *Escherichia coli* strains used in this study were S17 λ pir (recA thi pro hsd (r– m+)) RP4::2-Tc::Mu::Km Tn7 lyso
genized with pir phage) [17], JM109 (GE Healthcare), and TOP10 (F⁻ mcrA (mrr-hsdRMS-merBC) 80lacZM15 lacX14 recA1 deoR araD139 (ara–leu)7697 galU galK rpsL (Strr) endA1 nudG) (Invitrogen). *E. coli* strains were grown on *Luria–Bertani* (LB) medium with appropriate antibiotics. Antibi
tics were used at the following concentrations for *E. coli* and *B. melitensis*: ampicillin (Amp), 50 μg/ml; kanamy
cin (Kan), 50 μg/ml. The plasmids used in this study were pUT
tini-Tn5Km2 [12], pCR TOPO 2.1 (Invitrogen), and pUC18 (GE Healthcare).

Inoculation doses of each strain were prepared as previously described [6]. Immediately prior to infection, frozen inocula
tions were thawed and diluted to the appropriate concentration with sterile physiological saline. All doses were verified as
correct on the day of use by serial dilution and plating on the appropriate media.

2.2. DNA manipulation

DNA manipulation was performed following standard tech
niques [18]. Restriction enzymes and oligonucleotide primers were purchased from Eurogentec. *Taq* DNA polymerase and deoxynucleoside triphosphates were purchased from Roche. Southern blotting was performed as described previously [19].

2.3. Construction of 12 mini-Tn5 *B. melitensis* mutant libraries

A collection of 12 *E. coli* S17 λ pir, each containing a uniquely tagged pUT mini-Tn5 Km2 transposon, was a
generous gift from Dario Lehoux and François Sanachagrin (Faculté de médecine, Université Laval, Ste Foy, QC, Canada) [12]. These plasmids were transferred to *B. melitensis* 16M by electroporation as described previously [19]. After 3 days of incubation at 37 °C, the exconjugates were replicated on SB agar containing either Kan or Amp. The Amp-resistant clones were discarded and the Amp-sensitive clones were transferred to 2 ml 96-well plates. In a defined library, each mutant had the same tag but was assumed to be inserted at a different location in the bacterial chromosome. As an STM working scheme, one mutant from each library was picked to form 24 pools of 12 uniquely tagged mutants [12].

2.4. Screening of the STM library

Mutants were grown at 37 °C in 1 ml of SB medium in 96-
well plates with 2 ml square wells (Qiagen) with appropriate antibiotics for 48 h. The bacteria were then pooled, centrifu
gated at 5000 rpm for 10 min in a Jouan centrifuge, and resusp
dended in 2 ml of phosphate-buffered saline (PBS). The bacterial suspension was then diluted to a final concentration of 1 × 10⁸ CFU in 1 ml of *Brucella* broth (Difco Laboratories, Detroit, MI). The number of bacteria was confirmed by plating dilutions on SBA plates.

2.5. Infection of goats

Twenty-four sexually mature male or female Angora goats were obtained from a private herd. All goats were negative for *Brucella*-specific antibodies based on the card test [20]. Throughout the study, the animals were housed in a restricted-access, large animal isolation facility operated under guidelines approved by the United States Department of Agriculture/ Animal and Plant Health Inspection Service (USDA/APHIS).

At the conclusion of each study, the animals were euthanized by the captive bolt. The goats were inoculated with 1 × 10⁹ CFU of the *B. melitensis* STM mutant pools.

The bacterial suspension (100 μl) was injected into the jugular vein. The remaining part of the suspension was plated on medium for DNA isolation. Twenty-one days after the infection, animals were sacrificed; and the spleen, liver and lymph nodes were ho
genized in PBS and dilutions were plated on SBA. The number of bacteria was determined by plating dilutions on SBA plates.

2.6. In vivo competitive assay

In competition experiments, mutant (Kan+) and wild-type bacteria (*B. melitensis* 16M) were grown for 48 h in broth, and then equal amounts of bacteria (about 5 × 10⁸ each in 100 μl of 0.9% NaCl) were mixed and injected intravenously into each goat. Dilutions of the infecting doses were plated on media with and without Kan to estimate the ratio of mutant-
to-wild-type bacteria in the inoculum. Goats were sacrificed after 21 days, and the spleens were removed and homogenized.
To determine the proportion of mutant-to-wild-type bacteria, dilutions of spleen homogenates were plated on media with and without Kan. The competitive index (CI) was calculated as the proportion of mutant-to-wild-type bacteria recovered from the animals divided by the proportion of mutant-to-wild-type bacteria in the inoculum.

2.7. Analysis of transposon insertion sites

Chromosomal DNA was prepared from the attenuated mutants, digested with EcoRV (no site in mini-Tn5 Km2), and ligated into pUC18-SmaI-BAP (GE Healthcare). The resulting plasmids were used to transform E. coli JM109, selecting for ampicillin and kanamycin resistance. Plasmid DNA was extracted, and the chromosomal DNA flanking the transposon were sequenced by Dye Terminator method (Big Dye Terminator Kit, Perkin Elmer) with an ABI 377 sequencer. GenBank sequence searching was performed with BLASTX program against the non-redundant peptide database and against the B. melitensis database available at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

3. Results

3.1. Construction of signature-tagged mutants of B. melitensis

A collection of unique libraries of B. melitensis 16M signature-tagged mini-Tn5 Km2 mutants was constructed by using 12 differently tagged pUT mini-Tn5 Km2 [12]. To confirm that mutants obtained from the same electroporation arose from independent transposition events, Southern hybridization was performed using a 492-bp PCR probe internal to the kanamycin resistance gene. With EcoRV-digested chromosomal DNA, it was demonstrated that each mutant contained a single copy of the transposon (data not shown).

3.2. Screening of the B. melitensis 16M mutant library in goats

To identify mutants defective in establishing infection, the natural host of B. melitensis 16M, i.e. goat, was used. After 21 days of infection, the spleen, liver and lymph nodes were removed [21]. After incubation for 5 days, the harvested bacteria were resuspended using plates which contained approximately $10^3$–$10^4$ colonies. Chromosomal DNA was prepared from the recovered pool of mutants. To identify mutants not surviving the passage in the goat, PCR screening was performed using chromosomal DNA. PCR products obtained from the in vitro pool were compared with the in vivo pool. Mutants were selected on the basis of the lack of 500-bp amplicon separated by agarose gel electrophoresis. Twenty-four pools of 11 mutants were screened in goats. Of the 288 mutants of the input pool, 11 potentially attenuated mutants were identified which were not recovered from the goats.

3.3. Molecular characterization of attenuated strains

Chromosomal DNA was prepared from the 11 mutants and digested by EcoRV. The EcoRV genomic fragment of each mutant was cloned in pUC18 and the DNA sequence of the region flanking the transposon was determined against the genomic sequence of B. melitensis [22]. The function of each disrupted gene was predicted according to the annotation used in the B. melitensis genomic database allowing each to be assigned to different functional families (Table 1).

The first group corresponded to virulence determinants that were previously identified in Brucella. Two mutations were found in the virB operon (virB6 and virB8) (BMEI10030 and BMEI10032). The polysaccharide group contains one mutant which had an insertion in the gene lpsA coding for a putative glycosyltransferase involved in LPS biosynthesis [23] (BMEI1326). The third group of genes was composed of a gene involved in DNA metabolism. One insertion was found in the ATP-dependent nuclelease subunit A gene (BMEI2023). A mutant was found (10B2) in the flgF gene coding for the basal-body rod of the flagellar apparatus (BMEI1107). It is known that brucellae contain flagellar genes which have been confirmed in a murine model of infection [16]. This is the first observation that the flagellar genes may be involved in Brucella pathogenesis in the natural host. One attenuated mutant had an insertion in a gene coding for a polyphosphate kinase (PPK), an enzyme that catalyzes the polymerisation of the phosphate of ATP into a polyP chain [24] (BMEI1205). Another mutant was identified which was defective in a gene coding for a bacterioferritin co-migratory protein (bcp) (BMEI1049).

<table>
<thead>
<tr>
<th>Functional family</th>
<th>Strain</th>
<th>Gene</th>
<th>Putative function</th>
<th>ORF no.</th>
<th>Goat competitive growth assay(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS synthesis</td>
<td>7A8</td>
<td>lpsA</td>
<td>Putative glycosyltransferase</td>
<td>BMEI1326</td>
<td>NR(^b)</td>
</tr>
<tr>
<td>Flagella</td>
<td>10B2</td>
<td>flgF</td>
<td>Flagellar basal-body rod protein</td>
<td>BMEI1107</td>
<td>0.78</td>
</tr>
<tr>
<td>Type IV secretion</td>
<td>5B2</td>
<td>virB6</td>
<td>Secretion system</td>
<td>BMEI10029</td>
<td>NR(^b)</td>
</tr>
<tr>
<td>Type IV secretion</td>
<td>4B3</td>
<td>virB8</td>
<td>Secretion system</td>
<td>BMEI10032</td>
<td>NR(^b)</td>
</tr>
<tr>
<td>DNA/RNA metabolism</td>
<td>4A4</td>
<td>wvdD</td>
<td>AT-dependent nuclease subunit A</td>
<td>BMEI2023</td>
<td>0.08</td>
</tr>
<tr>
<td>Oxidoreduction</td>
<td>12A3</td>
<td>bcp</td>
<td>Bacterioferritin co-migratory protein</td>
<td>BMEI1049</td>
<td>0.24</td>
</tr>
<tr>
<td>Oxidative metabolism</td>
<td>8A4</td>
<td>ppk</td>
<td>Polyphosphate kinase</td>
<td>BMEI1204</td>
<td>NR(^b)</td>
</tr>
</tbody>
</table>

\(^a\) Competitive index (CI) is calculated relative to the WT parent strain as described in Section 2.

\(^b\) NR: no recovery of mutant organism.
Additionally, four mutants generated by STM were not attenuated in the competitive assay: one had a transposon insertion in an Na\(^+\) driven multidrug efflux (NorM) (BMEI1584) [25]; another had a transposon insertion in a gene coding for a fosmidicine resistance protein (RosA) 5BMEI1291); one had a transposon insertion in a gene coding for a homolog to DnaJ (BMEI0047), a universally conserved heat-shock protein involved in protein folding; and the last one had a transposon insertion in a gene coding for a diaminohydroxyporphirobilinoylaminopyrimidine deaminase (BMEI1189).

4. Discussion

STM is a method that generates a large number of mutants for screening in various model systems. It was initially used to identify new virulence genes in S. typhimurium [26]. STM has previously been used to identify virulence factors in Brucella [13–15] where the screening was performed in an in vitro macrophage model or in an in vivo mice model. A natural infection model is preferable for screening as it may discern factors involved in the establishment of infection as well as those factors involved in resistance to host defense. The purpose of this study was to assess the goat model of Brucella pathogenesis coupled with STM to generate and identify potential virulence genes in B. melitensis. This is the first application of STM with Brucella in a natural host.

The reason we chose i.v. and not conjunctival exposure is because we were not concerned about invasion factors. There are three facets to the ability to cause disease: invasion, colonization and persistence. We needed to know that the bacteria got into the goat so we could test colonization alone. This is also why we killed animals at 21 days, before persistence factors would be called into play [21].

Seven putative virulence genes of B. melitensis were identified. This study revealed some of the same genes identified in an STM screen performed with B. melitensis in the mouse model [16]. These genes included virulence determinants like virB, genes belonging to the VirB type IV secretion system (T4SS), a gene coding for an enzyme involved in LPS biosynthesis, and a gene coding for the rod of the flagellar apparatus. Two mutants had insertions in different genes of the VirB type IV secretion system operon (T4SS) of Brucella. The T4SS is involved in DNA transfer of the Ti plasmid in Agrobacterium tumefaciens [27]. The T4SS of Brucella is required for Brucella to reach its proper niche and to replicate within host cells [28,29].

With smooth LPS, the type IV secretion system (T4SS) of Brucella encoded by the virB operon is a major virulence factor. The T4SS mobilizes the transfer of macromolecules such as monomeric proteins, multimeric toxins, and DNA–protein complexes across the cell envelope. Such secretion systems function in pathogenic bacteria, such as A. tumefaciens, Borde-tella pertussis, Helicobacter pylori, or the intracellular pathogen Legionella pneumophila [30]. The T4SS of Brucella is involved in controlling the maturation of the Brucella-containing vacuole (BCV) into a replication-permissive organelle [28]. In A. tumefaciens, VirB6, a polytopic inner membrane protein, contributes to the formation of outer membrane VirB7 lipoprotein and VirB9 protein multimers required for T4SS. It is proposed that VirB6 coordinates its activities with VirB8 to direct formation and positioning of VirB7 and VirB9 complexes required for biogenesis of a type IV secretion channel and T pilus [31]. The identification of VirB6 and VirB8 with a screening at 21 days after infection confirms that VirB is involved in the early phase of infection [32].

The smooth LPS has been classified as a major virulence determinant of Brucella while rough mutants are usually attenuated or nonvirulent [7,33]. One mutant was identified which is attenuated in the goat with the transposon in the lpsA gene, a gene required for LPS biosynthesis. This gene encoded a putative glycosyltransferase involved in inner core synthesis and the resulting mutant had a disruption in this region [34]. When probed with anti-O-polysaccharide monoclonal antibodies, the lpsA mutant failed to react and tests with acriflavine showed that this mutant is rough. The B. melitensis lpsA mutant seems promising and it will be interesting to test this potential vaccine candidate against a virulent B. melitensis challenge in goat or sheep. One advantage of any rough vaccine is that they should not cause interference in classical serodiagnostic tests. Rough mutants with disruption in the manB gene which encodes a phosphomannomutase and in the wbkA gene coding for mannosyl transferase have been previously described [34]. The genes inactivated in these mutants are required for O-polysaccharide chain biosynthesis. The gene lpsA is involved in the biosynthesis of the Brucella abortus and B. melitensis LPS core. The genes inactivated in these mutants are predicted to play a role during early infection. An lpsA mutant was highly attenuated and was eliminated early in the infection process.

Although Brucella are described as non-motile bacteria, all the structural genes for the flagellar apparatus are present [22,35]. It has been shown that a mutant in the flfF gene, a gene coding for the M-ring monomer, is attenuated in a mouse model [36]. A mutant in the flgF gene which encodes for the flagellar basal-body rod of the flagellar apparatus was identified in the goat model. Brucella are non-motile and do not appear to express flagellum in vitro. The flagellum might be expressed only in vivo under specific circumstances like starvation or minimal growth conditions and function like a type III secretion system as reported in Yersinia [37].

A mutant in which the ppk gene is disrupted was identified in this study. The inorganic polyphosphate kinase (PPK) is an enzyme responsible for the synthesis of inorganic polyphosphate (polyP) from ATP. The importance of PPK has been established previously for the stationary-phase survival of E. coli [38] and virulence of Pseudomonas aeruginosa [39]. Virulence factors of many pathogens are expressed in the stationary phase [40], and polyP participates in the activation of phase responses and starvation in E. coli [41]. In contrast to the situation in E. coli, a ppk null mutant in P. aeruginosa had no effect on rpoS transcription into stationary phase. Moreover, PPK is essential for various forms of motility or Quorum Sensing (QS) in P. Aeruginosa [39]. In the goat, the ppk gene seems to be playing a role early in infection before the stationary phase, suggesting a new regulation independent of rpoS.
which does not exist in *B. melitensis* as well as the other α-Proteobacteria. A mutation in the ppk gene could render *Brucella* aberrant in QS and affect responses in that production of the QS controlled virulence factors virB and flagellar apparatus.

We can suggest that like in *H. pylori*, PPK may play an essential role during the initial steps of host colonization and may act on the virulence of *Brucella* through an energy dependent mechanism [42].

An insertion in a gene coding for a bacterioferritin co-migratory protein (bcp) which is characterized as a thiol peroxidase, a putative member of TSA/AskPC family [43], gives rise to an attenuated phenotype. This gene product may be necessary for intracellular survival in the host macrophage with regards to reactive oxygen intermediates. Bacterioferritin co-migratory protein was found initially in *E. coli* as a protein with a molecular mass of 18 kDa which co-migrated with bacterioferritin in SDS-PAGE [44]. These peroxidases reduce hydrogen peroxides with electrons derived from NADPH through the thioredoxin (Trx)/Trx reductase pathway or from another reducing agent(s) [45]. These peroxidases are well conserved from prokaryotes to eukaryotes and form a large group now called the peroxiredoxin (Prx) family or the conserved from prokaryotes to eukaryotes and form a large

A strain was mutated in a gene which encodes an ATP-dependent nuclease subunit A. This protein is homologous to UvrD helicase which is known for its involvement in DNA repair and recombination. DNA repair systems seem to play an important role in intracellular persistence, possibly by preventing DNA damage that might be induced by reactive oxygen intermediates. The *uvrD* and *ppk* genes may participate in the resistance of *Brucella* spp. to the harsh conditions of the phagosome. These genes along with previously described ones identified by Kolher et al. [47] could be required for virulence in the intramacrophagic virulome.

These results demonstrate the applicability of screening in the caprine host as seven potential virulence genes involved in colonization were identified. Similar results were obtained by an STM screen performed with *B. melitensis* and *B. abortus* in the mouse model and with *B. suis* in the macrophage model [48]. These mutants included genes involved in the *virB* operon, LPS biosynthesis, and in the flagellar apparatus. We identified genes with a previously unrecognized role in colonization as *uvrD*, *ppk* and a gene encoding a bcp. Although the number of mutants screened in the caprine host was low compared to other systems, this model offers the advantage of using in vivo approaches for understanding the molecular basis of pathogenicity under the natural conditions of infection.

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**References**


