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Original article

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

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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

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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 Schaedler blood agar plates (SBA) or liquid Schaedler broth (SB) media (Difco Laboratories, Detroit, MI, USA) at 37 °C in a 5% CO_2 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 Tn*7* lysogenized with pir phage) [17], JM109 (GE Healthcare), and TOP10 [F^- *mcrA* (*mrr-hsd*RMS-*mcr*BC) 80*lacZM15 lacX14 recA1 deoR araD139* (*ara–leu*)*7697 galU galK rpsL* (Strr) *endA1 nupG*] (Invitrogen). *E. coli* strains were grown on Luria–Bertani (LB) medium with appropriate antibiotics. Antibiotics were used at the following concentrations for *E. coli* and *B. melitensis*: ampicillin (Amp), 50 μg/ml; kanamycin (Kan), 50 μg/ml. The plasmids used in this study were pUT-mini-Tn*5Km*2 [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 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 techniques [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-Tn*5* Km2 transposon, was a generous gift from Dario Lehoux and François Sanchagrin (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, centrifuged at 5000 rpm for 10 min in a Jouan centrifuge, and resuspended in 2 ml of phosphate-buffered saline (PBS). The bacterial suspension was then diluted to a final concentration of 1×10^9 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 1×10^9 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 removed aseptically. For recovery of bacteria, the spleen, liver and the lymph nodes were homogenized in PBS and dilutions were plated on SBA. Plates containing approximately 10^4 clones were used for DNA extraction.

2.6. In vivo competitive assay

In competition experiments, mutant (Kan^r) and wild-type bacteria (*B. melitensis* 16M) were grown for 48 h in broth, and then equal amounts of bacteria (about 5×10^8 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.