Disruption of *Brucella Suis* InvA and InvB Encoding Putative Invasion Proteins Affects Growth in Culture, Uptake and Persistence in Macrophages, and Persistence in CD1 Mice

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Abstract

An intracellular bacterial pathogen requires four steps for a successful infection: Adherence, InvAion, establishment, and dissemination within the host. Putative InvAion (Inv) proteins or nudix hydrolases are believed to contribute in these infection steps. The genome of *Brucella suis* strain 1330 carries (i) an InvA gene that is homologous to InvA or nudix hydrolases; and (ii) and an InvB gene that is homologous to InvB of other bacteria. We investigated the role of *B. suis* InvA and InvB on *in vitro* growth and multiplication in macrophages, and *in vivo* persistence in CD1 mice. The InvA and InvB genes of *B. suis* strain 1330 were disrupted by allelic exchange to construct isogenic InvA and InvB mutant strains, respectively. The wild-type InvA and InvB genes were introduced into the mutant strains to generate isogenic, complemented mutant strains. Compared to the wild type, both mutants grew slower in enriched broth or on agar medium. The mutant strains were less capable of InvAing and persisting in J774.1 macrophage cell line *in vitro*. The InvB mutant cleared from spleens of inoculated CD1 mice faster than the wild type or the InvA mutant. Mice inoculated with the attenuated InvB mutant expressed specific serum antibodies of immunoglobulin 2a isotype and exhibited substantial protection against challenge with virulent *B. suis* strain 1330. Disruption of *B. suis* InvA and InvB impaired the strains’ growth in culture, uptake into and survival in macrophage cell line, and persistence in mice. The InvB mutant strain induced immunity and protection in mice against *B. suis* challenge. The out-bred CD1 mouse model was useful in studying *B. suis* pathogenesis.

Keywords: *Brucella*; InvAion protein; Nudix hydrolase; Persistence; Protection

Introduction

Animal brucellosis is a disease affecting various domestic and wildlife species resulting from infection with bacteria belonging to the genus *Brucella* [6,7]. Opsosized or nonopsosized *Brucella* are able to infect macrophages, suggesting that antibody or complement-mediated phagocytosis as well as the direct *Brucella*-host cell contact allows adherence and InvAion. Following uptake into macrophages, *Brucella* survives and multiplies by inhibiting phagosome-lysosome fusion and transition to a replicative phagosome associated with endoplasmic reticulum [8]. Finally, as a result of destruction of macrophages, the accumulated bacteria are disseminated to other host cells and result in a variety of symptoms including abortion in cattle and swine, and undulant fever in humans [6,7,9].

Little is known about the molecular determinants that mediate the interaction of the brucellae...
with the surface of epithelial and professional phagocytic cells. The genome of B. suis wild type strain 1330 carry two inv homologs, i.e. InvA and InvB [10] [GenBank accession no. NC_004310]. B. suis InvA encodes putative InvA that is homologous to InvA and nudix proteins of other bacteria and higher organisms. Similarly, B. suis InvB encodes putative InvB that is homologous to InvB of other bacteria. Based on the role played by InvA [11-16] and InvB [17-21], in other bacterial species, we hypothesized that the putative InvA and InvB proteins play important roles in intracellular persistence of Brucella. We report that functions of both InvA and InvB are important for growth in vitro, Invasion of phagocytic cells, and in vivo persistence of B. suis. We also report that the attenuated InvB mutant induces protection against virulent B. suis infection, and the outbred mouse strain CD1 provides an advantage to the inbred mouse strains (e.g. BALB/c) to study the pathogenicity of Brucella.

Materials and Methods

Bacterial strains, plasmids and reagents

B. suis strain 1330 was from our culture collection. E. coli strain Top10 (Invitrogen Life Technologies, Carlsbad, CA) was used for producing plasmid constructs. E. coli was grown in Luria-Bertani (LB) broth or on LB agar (Difico Laboratories, Sparks, MD). Brucellae were grown in Tryptic Soy Broth (TSB) or on Tryptic Soy Agar (TSA) (Difico) at 37°C in the presence of 5% CO₂ as previously described [22]. The plasmids used in this study are listed in Table 1. Bacteria containing plasmids were grown in the presence of ampicillin or kanamycin at 100 µg/ml (Table 1).

Recombinant DNA methods

All experiments with live brucellae were performed in a Biosafety Level 3 facility at the Infectious Disease Unit of the Virginia-Maryland College of Veterinary Medicine per Centers for Disease Control and Prevention-approved standard operating procedures.

Genomic DNA and plasmid DNA were isolated by using of Genomic DNA and plasmid DNA kits (QIAGEN Inc., Valencia, CA). Restriction digests, Klenow fragment, and T4 DNA ligase enzyme were purchased from Promega Corporation (Madison, WI). Ligated plasmid DNA was transferred to E. coli Top10 cells by heat shock transformation per the guidelines of the manufacturer (Invitrogen Life Technologies, Carlsbad, CA). Purified plasmid DNA was electroporated into B. suis with a BTX ECM-600 electroporator (BTX, San Diego, CA), as described previously [24].

DNA and protein sequence analyses

The nucleotide sequences of the InvA and InvB genes were analyzed with DNASTAR software (DNASTAR, Inc., Madison, WI). The presence of any signal sequences of genes was predicted by using the SignalP 3.0 server of the Technical University of Denmark [25]. The destination of the putative InvA and InvB proteins upon translation and processing was predicted using the Subloc v1.0 software [26], at the National Center for Biotechnology Information (Bethesda, MD).

Construction of InvA B. suis strain

A 915-bp region including the whole sequence of the InvA gene was amplified via PCR using the genomic DNA of B. suis. A primer pair consisting of a forward primer (5' GGTGTCACCAATGACGACGACA 3') and a reverse primer (5' GGCTGCTACGAAAAGATACCA 3') (Ransom Hill Bioscience, Inc., Ramona, CA) was designed based on the nucleotide sequence (GenBank accession no. NC_004310). PCR amplification was performed in an Omni Gene thermocycler (Hybaid, Franklin, MA) as per standard protocols [23]. The amplified gene fragment was cloned into the pCR2.1 vector of the TA cloning system (Invitrogen) to produce plasmid pCRInvA. Competent E. coli Top10 cells (invitrogen) were transformed with the ligation mixture, and the colonies carrying the recombinant plasmid were picked from TSA plates containing ampicillin (100 µg/ml) per the manufacturer’s guidelines. From this plasmid, the InvA gene was isolated by Kpn1 and PstI digestion and cloned into the same sites of plasmid pGEM-3Z (Promega) to produce pGEMInvA. E. coli Top10 strain carrying the recombinant plasmid were picked from TSA plates containing
ampicillin (100 µg/ml). The plasmid pGEMInvB was digested with Clal, ends were filled in by reaction with Klenow enzyme and ligated to the 1.3-kb SalI fragment of pUC4K (also blunt ended) containing the Tn903 npt gene [27], which confers kanamycin resistance (Kanr) to B. suis. The resulting suicide vector was designated pGEMInvAK. The E. coli Top10 cells carrying the recombinant plasmid were picked from TSA plates containing kanamycin (100 µg/ml).

One microgram of pGEMInvBK was used to electroporate B. suis strain 1330; several colonies of strain 1330 were obtained from TSA plates and incubated at 37°C at 5% CO₂ for four days to visually confirm that a double-crossover event had taken place in all three transformants. One microgram of pBBInv and designated 1330 ΔInvAΔInvB. The 915-bp DNA fragment containing the InvA gene with its native promoter was isolated by digesting plasmid pCRInvA and was cloned into same sites of pBBInvA. One microgram of pBBInv was used to electroporate B. suis strain 1330 ΔInvA; several colonies of strain 1330 ΔInvA were picked from a TSA plate containing ampicillin (100 µg/ml). One microgram of pBBInv was used to electroporate B. suis strain 1330 ΔInvA, and designated 1330 ΔInvAΔInvB.

Construction of InvB B. suis strain

A 832-bp region including the full length of the InvB gene was amplified via PCR using the genomic DNA of B. suis and a primer pair (Forward 5’ GGGGTACCAGTGCAAAAGAAGA 3’; and Reverse 5’ GGCCTGACGCTGTGATGA 3’) (Ransom Hill Bioscience). PCR amplification, restriction digestion, and cloning were performed exactly as described above. The recombinant pCR2.1 vector containing 832-bp InvB gene was designated pCRInvB. From this plasmid, the InvB gene was isolated by Kpnl and PstI digestion and cloned into the same sites of plasmid pGEM-3Z (Promega) to produce pGEMInvB. The plasmid pGEMInvB was digested with EcoNI, ends were filled in and ligated to the Kanr, to produce the suicide vector designated pGEMInvBK that was used to electroporate B. suis strain 1330. One of the recombinant B. suis colonies with double-crossover event was chosen for further analyses and designated 1330 ΔInvB.

Complementation of InvA and InvB in mutant strains

The 915-bp DNA fragment containing the B. suis InvA gene together with its native promoter was isolated by Kpnl and XbaI digestion of plasmid pCRInvA and was cloned into same sites of broad-host-range vector pBBR4MCS [28]. The resulting plasmid was designated pBBInvA. One microgram of pBBInvA was used to electroporate B. suis strain 1330 ΔInvA; several colonies of strain 1330 ΔInvA were picked from a TSA plate containing ampicillin (100 µg/ml). One of these colonies was chosen for further analyses and designated 1330 ΔInvAΔInvB.

Growth rates of B. suis strains

Single colonies of B. suis wild type 1330, mutant strains 1330 ΔInvA and 1330 ΔInvB, and complemented-mutant strains 1330 ΔInvAΔInvB and 1330 ΔInvBΔInvA were streaked on TSA plates and incubated at 37°C at 5% CO₂ for four days to visually estimate the approximate colony sizes. The single colonies of strains were grown at 37°C for 72 h to stationary phase in 10 ml of TSB. These cultures were used to inoculate 25 ml of LB or salt-free LB in
Klett side-arm flasks to 25 to 35 Klett units. The cultures were grown at 37°C at 180 rpm; Klett readings were recorded every three hours in a Klett-Summerson colorimeter (New York, NY).

**Preparation of B. suis incula**

TSA plates were inoculated to confluency with B. suis strains. After 96 h of incubation at 37°C in the presence of 5% CO₂, the cells were harvested from plates, washed with Phosphate-Buffered Saline (PBS), resuspended in 20% glycerol, and frozen at -80°C. The number of viable cells (colony forming units-cfu) was determined after dilutions of the cell suspensions were placed on TSA and incubated for 96 h at 37°C.

**Brucella uptake into and survival in J774 macrophages**

This was performed as described elsewhere [29]. Briefly, the mouse macrophage-like cell line J774 (the American Type Culture Collection, Manassas, VA) were seeded at a density of 5 × 10⁵/ml in Dulbecco's Modified Essential Medium (DMEM) (Sigma-Aldrich) Collection, Manassas, VA) were seeded at a density of 5 × 10⁵/ml in mouse macrophage-like cell line J774 (the American Type Culture Collection, Manassas, VA) were allowed one week of acclimatization. Groups of 20 mice each were intraperitoneally injected with PBS or 5.2 log₅ cfu of mutant 1330 invB mutant. Nine weeks after inoculation, mice were challenged 5.0 log₅ cfu of wild-type 1330. Two weeks post-challenge, spleen weights (SB) and splenic cfu counts (6A) were determined. P value for the difference among mean values was <0.005.

**Data analyses**

The data were analyzed by performing analysis of variance, and the mean cfu counts among treatments were compared using the least-significance pair-wise comparison using the standard procedures [30].

**Results**

**Nucleotide and protein sequences of InvA and InvB**

The goal of this study was to elucidate the influence of the InvA gene encoding putative InvA protein (also called dinucleoside polyphosphate hydrolase or nudix hydrolase), and the InvB gene encoding putative InvB protein on pathogenicity of B. suis. The InvA that is also designated as dph (locus tag BR1836) is 548-bp long and located on chromosome 1 of B. suis strain 1330 (Figure 1A). Immediately upstream of InvA is ctpA (locus tag BR1837) encoding carboxyl-terminal protease that is transcribed in the same direction as InvA [29]. Downstream of InvA is a gene (locus tag BR1835) encoding a hypothetical protein that is transcribed in opposite
direction as InvA. The DNA sequence analyses predicted that the putative InvA is localized in the cytoplasm (Reliability Index=2; Expected Accuracy=85%), and does not carry an N-terminal signal sequence (signal peptide probability: 0.00). At the amino acid level, *B. suis* InvA shared up to 77% identity with InvAsian or dinucleoside polyphosphate hydrolases (nudix hydrolases) of other bacterial genera including *Mesorhizobium*, *Rhizobium*, *Sinorhizobium*, *Agrobacterium*, *Vibrio*, *Shigella*, *Escherichia*, *Haemophilus*, *Yersinia*, *Salmonella*, *Burkholderia* and *Francisella* (Table 2.1). This protein also showed some identity with the nudix hydrolases of plant species including *Arabidopsis thaliana* and *Oryza sativa* (data not shown). The nudix diphosphatase linked to some other moiety X (nudix) protein family, consists of about 800 proteins in more than 200 species from all kingdoms [31,32]. The active site of nudix hydrolases corresponds to the consensus sequence GX,EX,REUXEXGU (where X represents any amino acid and U represents Ile, Leu, or Val) [20]. The InvA amino acid sequence carried the exact GX,EX,REUXEXGU sequence (Figure 2). The InvA gene was found conserved among all the sequenced *Brucella* species. These include *B. abortus* strains 9-941 and 2,308, *B. melitensis* 16M, *B. ovis* ATCC 25840, and *B. suis* 1330 (data not shown).

The InvB (locus tag BR0340) is 522-bp long and located on the same chromosome but far apart from InvA (Figure 1B). The gene upstream of InvB (locus tag BR0341) is transcribed in opposite direction and encodes a sensor histidine kinase. The gene downstream of InvB (locus tag BR0339) is also transcribed in the opposite direction and encodes the multidrug resistance protein NorM. The putative InvB sequence was predicted to contain an N-terminal signal sequence (probability: 1.00), and the likely peptidase cleavage site is located between the 23rd and 24th amino acids (maximum cleavage site probability: 0.999). The predicted subcellular localization of InvB was periplasmic space (Reliability Index=4; Expected Accuracy=92%). At the amino acid level, the InvB shared 80% identity with the InvA protein B of *Ochrobactrum anthropi* and up to 49% identity with InvAsian associated proteins of a number of bacterial genera, including *Nitrobroch*, *Mesorhizobium*, *Magnetospirillum*, and *Bartonella* (Table 2.2). The *B. suis* InvB did not share considerable identity with nudix hydrolases. Unlike the InvB genes of other sequenced *Brucella* species (*B. abortus* strains 9-941 and 2308, *B. melitensis* 16M, *B. ovis* ATCC 25840), the *B. suis* InvB possesses an extra T base at the bp #379 position. The presence of this extra base generates an authentic frame-shift in *B. suis* InvB sequence, but it does not introduce any premature stop codons. As such, *B. suis* InvB makes an intact full-length Open-Reading-Frame (ORF). The deduced *B. suis* InvB amino acid sequence between the 1st and the 126th amino acids is highly identical to the InvB of other *Brucella* and other bacteria. However, due to the authentic frame-shift, *B. suis* InvB sequence between the 127th and the 173rd amino acids differ entirely from other InvB sequences.

**Construction of recombinant *B. suis* strains**

The InvA gene of *B. suis* wild type 1330 was disrupted by allelic exchange, and the resulting strain was designated as 1330 ΔInvA or InvA mutant. A strain generated by disrupting the InvB gene of strain 1330 was designated as 1330 ΔInvB or InvB mutant. The PCR assays with the primer pairs used to amplify the InvA and InvB genes (see Materials and Methods) produced predicted amplicons of 0.9-kb and 0.8-kb, respectively from the wild type *B. suis* strain 1330. These primer pairs yielded approximately 2.2-kb and 2.1-kb products from
InvA mutant and InvB mutant respectively, indicating that in mutant strains, due to a double-crossover events, the 1.3-kb Kan’ gene was inserted into each of InvA or InvB (data not shown). PCR assays using the primers specific to B. suis revealed that both InvA mutant and InvB mutant strains are true brucellae. The InvA was cloned into broad-host-range plasmid pBBR4MCS [28] and introduced into the InvB mutant strain to generate the complemented InvB mutant 1330 ΔInvA [InvA‘]. In the same way, the InvB gene was introduced into the InvB mutant strain to generate the complemented InvB mutant 1330 ΔInvB [InvB‘].

Growth rates of recombinant B. suis strains

Wild type, mutant and complemented inv strains were cultured on Trypticase Soy Agar (TSA) plates or in Trypticase Soy Broth (TSB) to determine the influence of InvA and InvB genes on in vitro growth. After 96 h of growth on TSA plates, colonies of InvA mutant and InvB mutant appeared approximately 30% and 60%, respectively of the size of the colonies of wild type strain 1330 (data not shown). In TSB, the InvA mutant and the InvB mutant grew slower (approximately 3.7 h and 4.0 h doubling time, respectively) than the wild type 1330 (approximately 2.8 h doubling time) (data not shown). The results suggest that disruption of InvA as well as InvB influences the in vitro growth of B. suis. The complemented InvA mutant grew at a rate similar to that of the wild-type 1330 in TSB (data not shown). However, the complemented InvB mutant was not different from the InvB mutant with regard to the growth rate in TSB (data not shown). These observations suggest that the disruption of InvA had a specific effect, whereas the disruption of InvB had a polar effect.

Uptake into and persistence of B. suis strains in J774 macrophage cell line

The influence of InvA and InvB genes on in vitro adherence to and survival in macrophages was studied by inoculation of J774 macrophages with the wild type and the inv B. suis strains. Almost similar doses of brucellae were used for inoculation, i.e., 8.02 log<sub>10</sub> cfu/well, 7.86 log<sub>10</sub> cfu/well and 8.02 log<sub>10</sub> cfu/well, respectively of wild type 1330, InvA and InvB strains. After 3 hr initial incubation period, the viable brucellae remaining in culture medium (that had not adhered to or was not taken up by macrophages) was estimated. The number of brucellae taken up by or adhered to macrophages was calculated by subtracting the number of viable brucellae remaining in cell culture medium from the total number of brucellae used for inoculation (Figure 3A). Three strains differed significantly in terms of the estimated number of bacteria taken up by or adhering to macrophages. Wild type strain 1330 displayed the least number of brucellae outside macrophages suggesting that a greater number of this strain successfully adhered to or entered into macrophage cells. The relative greater numbers of InvA or InvB mutant brucellae recovered from outside macrophages suggested that these inv mutants have impaired ability to attach to macrophages.

In order to assess the ability of strains to survive intracellularly, the number of viable brucellae present in macrophages at the end of 3 h incubation was determined (Figure 3B). Three strains differed significantly with wild type 1330 having the greatest viable brucellae, the InvB mutant with a moderate number and the InvA mutant having the least viable. The findings suggest that mutations in InvA and InvB genes impaired the ability of brucellae to persist in J774 macrophages.

Survival of the B. suis strains in CD-1 mice

The importance of InvA and InvB genes on in vivo pathogenicity of brucellae was evaluated by determining the splenomegaly and viable Brucella Colony Forming Units (cfu) in spleens of CD1 mice inoculated with the wild type or the inv mutants. Almost similar doses of brucellae were used for inoculation, respectively of 4.95 5.20 log<sub>10</sub> cfu/mouse, 4.95 5.20 log<sub>10</sub> cfu/mouse and 5.20 log<sub>10</sub> cfu/mouse, respectively of wild type 1330, InvA mutant and InvB mutant. The InvA mutant did not differ significantly from the wild type with regard to splenic clearance following intraperitoneal inoculation in CD1 mice (Figure 4A). In contrast, one week after inoculation, the InvB mutant was 1.8 log<sub>10</sub> cfu less than the wild type. Furthermore, the InvB mutant cleared from spleens faster than the wild type or the InvA mutant strains throughout the nine-week trial period. Nine weeks post-inoculation, InvB mutant had been totally cleared in two out of five inoculated mice, whereas, approximately 4.0 log<sub>10</sub> cfu of wild type or InvA mutant were still present (Figure 4A). The colonies of InvA mutant harvested from spleens appeared approximately 30% of the size of colonies of wild-type. Similarly, the majority of colonies of InvB mutant recovered from spleens appeared nearly 20% the size of wild-type (data not shown). The InvA mutant caused greater splenomegaly than the wild type strain 1330, whereas the InvB mutant did not appear to cause significant splenomegaly i.e., relative to saline inoculated mice (Figure 4B). The results suggest that functions of the

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Table 2.1: Identity of B. suis putative invasin protein A with the dinucleoside polyphosphate hydrolase/Invasin protein A of other organisms.

Table 2.2: Identity of B. suis putative invasin protein B with the Invasin protein B of other organisms.
**InvA**, but not **InvB**, gene are dispensable for the *in vivo* persistence of *B. suis*.

**Immune and protective responses of mice immunized with InvB mutant**

As the **InvB** mutant was found attenuated in CD1 mice, the immunogenicity and protective efficacy against brucellosis of this strain was evaluated. The sera were collected from CD1 mice inoculated intraperitoneally with **InvB** mutant, and serum immunoglobulin levels were determined by the Enzyme Linked Immunosorbent Assay (ELISA). At nine weeks post-inoculation, mice inoculated with **InvB** mutant contained greater levels of **B. suis** specific serum immunoglobulins (IgG1, IgG2a, IgG2b, IgG3, and IgA) relative to those injected with saline (Figure 5). The greatest increment due to inoculation of **InvB** mutant was seen in the IgG1 level and the least in IgA. As the **InvA** mutant was not attenuated in mice, the immunogenicity or the protective efficacy of this strain was not determined.

The CD1 mice inoculated intraperitoneally with the **InvB** mutant were challenged intraperitoneally with the wild type **B. suis** strain 1330 nine weeks post-inoculation. The spleen weights and splenic cfu were determined two weeks post-challenge. Mice immunized with **InvB** mutant exhibited 2.20 log units of protection against a wild-type **B. suis** mutant strain in culture medium, it was completely different from that of any other bacteria. These observations suggest that disruption of **InvA** caused greater intracellular accumulation of toxic substances and cell signaling molecules such as AP-A, that resulted in greater splenomegaly in mice.

**Discussion**

Using isogenic **B. suis** strains, we characterized the importance of **InvA** and **InvB** genes to growth, multiplication and pathogenicity of **B. suis**. The **InvA** gene was found conserved among all the *Brucella* species including **B. suis**. Based on the growth of the **InvA** mutant and the complemented **InvA** mutant strains in culture media, it can be suggested that **InvA** gene regulates *in vitro* growth of **B. suis**. Disruption of **InvA** gene made **B. suis** highly less capable in adhering to and surviving in *J774.1* macrophages *in vitro*. This is particularly significant as no antibody was used to opsonize the brucellae. The **B. suis InvA** shared considerable identity with and carried the same conserved active site of the **InvA** or nudix hydrolase proteins of other organisms [31]. It has been proposed that nudix hydrolases decrease the intracellular level of toxic substances and cell signaling molecules, ex: Dinucleoside polyphosphates [33]. The dinucleoside polyphosphate AP-A (adenosine[5’]-tetraphospho-[5’]-adenosine) is induced upon oxidative stress and heat shock both in prokaryotes and eukaryotes [34,35], and the nudix enzyme subsequently degrades this signaling component to restore the intracellular balance. Nudix enzymes have also been suggested to play a role in bacterial *InvA*ion of eukaryotic cells. The nudix hydrolase encoded by the *iiaA* gene of *Bartonella bacilliformis* was shown to be associated with the ability to InvAide human erythrocytes [21]. An upregulation of the expression of the *Escherichia coli* K1 orthologue ygdP during *InvA*ion of human brain microvascular endothelial cells has also been demonstrated [17]. It was also shown that transcription of the *Rickettsia prowazekii* orthologue **InvA** is temporarily increased during the early stages of infection, similar to the stress-related protein GroEL [19]. The nudix hydrolase MutT of *E. coli* hydrolyzes 8-oxo-dGTP, a compound generating A-T/C-G transversions, and thereby prevents DNA damage caused by oxidative stress [18]. The nudix hydrolase NudA of *Helicobacter pylori* hydrolyzes AP-A and protects cells against hydrogen peroxide stress [20]. Based on these reports, it is highly possible that **B. suis InvA** decreases the intracellular level of toxic substances and cell signaling molecules like AP-A, and thereby regulates InvAion of and survival in macrophages.

In contrast to the relatively greater intracellular killing of the **InvA** mutant in macrophage cell line *in vitro*, this mutant did not differ from the wild type in terms of recovery from the spleens of infected mice *in vivo*. This observation suggests that functions of **InvA** gene are dispensable for the *in vivo* persistence of **B. suis**. It may be possible that functions of **InvA** are important only during the initial few hours of infection. Interestingly, the extent of splenomegaly in mice inoculated with the **InvA** mutant was greater than that in mice inoculated with wild-type. It is possible that disruption of **InvA** caused greater intracellular accumulation of toxic substances and cell signaling molecules such as AP-A, that resulted in greater splenomegaly in mice.

The genomes of *B. abortus* 9-941, *B. abortus* 2308, *B. melitensis* 16M, and *B. ovis* ATCC 25840 carry a conserved copy of **InvB** gene. Paulson et al. [10] suggested that **B. suis InvB** is a pseudogene due to an authentic frame-shift in its sequence. However, our analyses contradict the above suggestion and reveal that this frame-shift does not generate any premature stop codons, and **B. suis InvB** produces a full-length ORF. The **B. suis** amino acid sequence between the 1<sup>st</sup> and the 126<sup>th</sup> residues was identical to InvB of other *Brucella* species. However, due to the addition of extra T base at the bp #379 position, between the 127<sup>th</sup> and the 173<sup>rd</sup> amino acids, the **B. suis InvB** was entirely different from InvB of other *Brucella*. Based on this observation it can be speculated that the extra T base incorporated into **B. suis InvB** after this species separated from other species of genus *Brucella*. Furthermore, the amino acid sequence between the 127<sup>th</sup> and the 173<sup>rd</sup> residues of **InvB** of other *Brucella* shared identity with InvB of other bacteria including *O. anthropi*. However, **B. suis InvB** amino acid sequence between the 127<sup>th</sup> and the 173<sup>rd</sup> residues did not share any identity with InvB of any other bacteria. These observations suggest that addition of the extra T base into **B. suis InvB** occurred at a relatively recent time of evolution after *Brucella* species separated from other closely related bacterial species.

Disruption of **InvB** impaired the growth of **B. suis** in media, suggesting that this gene regulates *in vitro* growth of **B. suis** either directly or indirectly. Disruption of **InvB** gene made **B. suis** less capable in adhering to and surviving in *J774.1* macrophages *in vitro*. In CD1 mice, in contrast to the wild type or the **InvA** mutant, the **InvB** mutant induced less splenomegaly and was recovered in less numbers from the spleens. These findings suggest that disruption of **InvB** gene made **B. suis** less persistent both in acute as well as chronic phases of infection. Overall, the slow *in vitro* growth in enriched media, and less persistence in *J774.1* macrophages and CD1 mice of **InvB** mutant suggest that disruption of **InvB** impacted extracellular as well as intracellular growth and multiplication of **B. suis**.

In Gram-positive facultative intracellular bacterium *Listeria monocytogenes*, the *InvA*ion proteins InlA and InlB function as both adhesions and *InvA*ions [11,34]. InlA is sufficient for uptake into gut epithelial cells [13] and is required for crossing the intestinal barrier
al. [39] reported that in BALB/c mice in our previous studies [29]. Petrovska et ctpA comparable to the clearance patterns of strain 1330 and mutant 1330 Brucella. observations confirm that CD1 model could be an alternative to the sion and persistence in tissues in CD-1 and BALB/c mice. Our InvA 1330 and the multiplication of Brucella InvB and need to be identified. establishment and dissemination of bacterium within the host. The

Since InvB mutant was found attenuated in vivo, the immunogenicity and protective efficacy against brucellosis of this strain was evaluated. The CD1 mice inoculated with this strain induced significant levels of all immunoglobulin classes. This may probably be due to its smooth phenotype and uninterrupted expression of lipopolysaccharide O-side chain. Compared to saline injection, inoculation with InvB mutant induced a significant protection of 2.2 log$_{10}$ cfu in CD1 mice, suggesting that this strain can be a potential live vaccine candidate against brucellosis.

The BALB/c mouse model has been extensively used in studies of Brucella in vivo. The splenic clearance patterns of B. suis wild type 1330 and the InvB mutant strain in CD1 mice in this study are quite comparable to the clearance patterns of strain 1330 and mutant 1330 ΔctpA in BALB/c mice in our previous studies [29]. Petrovska et al. [39] reported that B. melitensis strains display similar kinetics of Invision and persistence in tissues in CD-1 and BALB/c mice. Our observations confirm that CD1 model could be an alternative to the BALB/c model in studying the pathogenesis of Brucella.

Conclusion

Mutations in InvA and InvB genes affected in vitro growth and multiplication of B. suis, whereas in InvB affected in vivo survival during acute as well as chronic phases of infection. The InvB mutant induces significant immunity and protection in CD1 mice against challenge with virulent brucellae. The CD1 mouse model can be used as alternative animal model to study the Brucella pathogenesis.

Acknowledgement

We thank Kay Carlson for technical assistance, and Lynn Heffron, Dustin Lucas and the staff of the Virginia-Maryland College of Veterinary Medicine non-client animal facility for the expert handling of the mice.

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