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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, *InvA*sion, establishment, and dissemination within the host. Putative *InvA*sion (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 *InvA*ding 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*; *InvA*sion protein; Nudix hydrolase; Persistence; Protection

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Introduction

An intracellular microbial pathogen requires four steps for a successful infection: Adherence, *InvA*sion, establishment, and dissemination within the host [1,2]. Bacteria use a variety of mechanisms to adhere to and *InvA*de host cells. Successful *InvA*sion following adhesion requires the pathogen to evade the humoral immune response and to proliferate in a well-protected niche. Bacteria *InvA*de the non-professional phagocytes by modulating the host cell cytoskeleton dynamics. This includes initiating signaling cascades in the host cell, which leads to the assembly of phagocytotic machinery that induces bacterial uptake [3-5].

Animal brucellosis is a disease affecting various domestic and wildlife species resulting from infection with bacteria belonging to the genus *Brucella* [6,7]. Opsonized or nonopsonized *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 *InvA*sion. 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

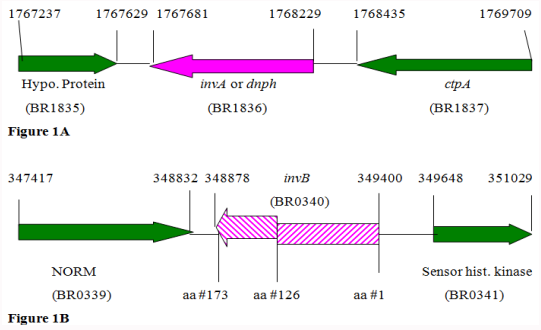

Figure 1: The schematic representations of the organization of *InvA* (also called *dnph*) and adjacent genes (Figure 1A), and *InvB* and adjacent genes (1B) in *B. suis* wild type strain 1330. The arrowheads indicate the direction of transcription of genes. The numbers represent the location of the genes in the chromosome. The GenBank locus tags are given in parentheses under each gene. The regions in the *InvB* sequence identical to (amino acids 1-126) and different from (amino acids 127-173) other *InvB* are shown (Figure 1B).

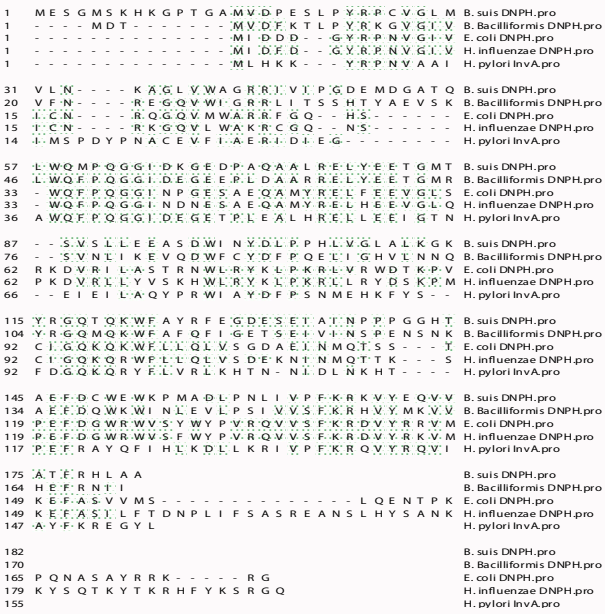

Figure 2: Sequence alignment of *B. suis InvA* with *InvA* or nudix hydrolases (DNPH) of other bacteria. Alignments were performed with ClustalW. The consensus amino acids are shaded with black dots. Numbers to the left of the sequences denote amino acid positions in the individual sequences. The active site of nudix hydrolases is underlined in red.

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*sion [11-16] and nudix proteins [17-21], in other bacterial species, we hypothesized that the putative *InvA*sion proteins play important roles in intracellular persistence of *Brucella*. We report that functions of both *InvA* and *InvB* are important for growth *in vitro*, *InvA*sion 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 alternative 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 (Difco Laboratories, Sparks, MD). Brucellae were grown in Trypticase Soy Broth (TSB) or on Trypticase Soy Agar (TSA) (Difco) at 37°C in the presence of 5% CO_2 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 DNA kits (QIAGEN Inc., Valencia, CA). Restriction digests, Klenow reactions, and ligations of DNA were performed as described elsewhere [23]. Restriction enzymes, 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 server of the Institute of Bioinformatics of the Tsinghua University. The identity of the *B. suis* putative *InvA* and *InvB* to proteins of the EMBL/GenBank/DDBJ databases was analyzed using the BLAST 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' GGGGTACCAAATGAGCAAGACAAA 3') and a reverse primer (5' GGCTGCGTACGAAAAGAAATACGA 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 pCR*InvA*. 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 *Kpn*I and *Pst*I digestion and cloned into the same sites of plasmid pGEM-3Z (Promega) to produce pGEM*InvA*. *E. coli* Top10 strain carrying the recombinant plasmid were picked from TSA plates containing

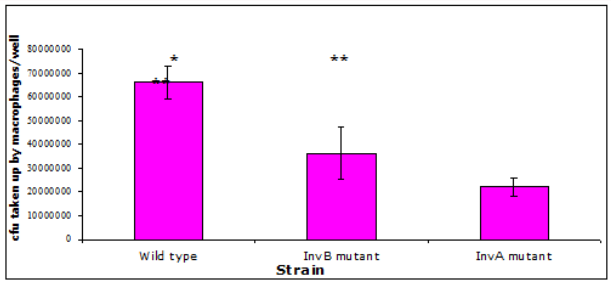

Figure 3A

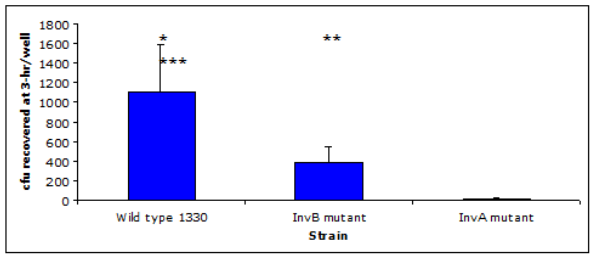

Figure 3B

Figure 3: Uptake into and intracellular persistence of *B. suis* in J774 macrophage cell line. The J774 macrophage cells were seeded at a density of 5 × 10^5/ml into 24-well tissue culture dishes. Nearly 1 × 10^8 cells of the bacterial suspension were added, and the cells were incubated at 37°C for 3 hr. The number of bacteria taken up by or adhered to macrophages (Figure 3A) was calculated by subtracting the number of brucellae remained in cell culture at 3 h post-inoculation from number of brucellae used for inoculation. *P* value for the difference among mean values was <0.005. The brucellae survived intracellular killing was determined by estimating the number of bacteria remained inside macrophages at the end of 3 h incubation (Figure 3B). *P* value for the difference among mean values was <0.005. The mean values that share the same symbol do not differ one another; and the mean values designated by different symbols differ one another significantly.

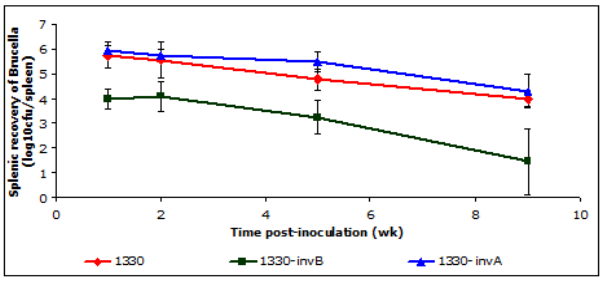

Figure 4A

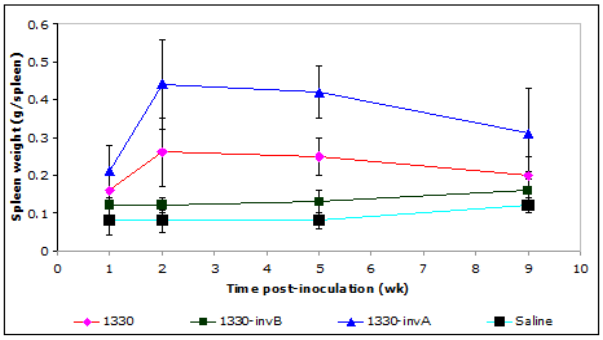

Figure 4B

Figure 4: *In vivo* persistence of *B. suis* in CD1 mice. Mice were intraperitoneally inoculated with 5.0 $\log_{10}$ to 5.2 $\log_{10}$ cfu of *B. suis* strains 1330, 1330Δ*InvA*, and 1330Δ*InvB*. 4A: the splenic cfu counts were determined at 1, 2, 5, or 9 weeks post-inoculation. The standard deviation for the strain 1330Δ*InvB* nine weeks post-inoculation was slightly big since the strain had been totally cleared in two out of five mice. 4B: The average weight of spleens of CD1 mice injected saline, or *B. suis* strains.

ampicillin (100 µg/ml). The plasmid pGEM*InvB* was digested with *Cla*I, ends were filled in by reaction with Klenow enzyme and ligated to the 1.3-kb *Sal*I fragment of pUC4K (also blunt ended) containing the Tn903 *npt* gene [27], which confers kanamycin resistance (Kan^r) to *B. suis*. The resulting suicide vector was designated pGEM*InvA*K. The *E. coli* Top10 cells carrying the recombinant plasmid were picked from TSA plates containing kanamycin (100 µg/ml).

One microgram of pGEM*InvB*K was used to electroporate *B. suis* strain 1330; several colonies of strain 1330 were obtained from a TSA plate containing kanamycin (100 µg/ml). These colonies were streaked on TSA plates containing ampicillin (100 µg/ml) to determine whether a single- or double-crossover event had occurred. Three of the colonies did not grow on ampicillin-containing plates, suggesting that a double-crossover event had occurred. PCR with the primers used for amplifying the *InvA* gene (as described above) confirmed that a double-crossover event had taken place in all three transformants. One of these strains was chosen for further analyses and designated 1330 Δ*InvA*.

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' GGCTGCAGCGTGCTGATGA 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 pCR*InvB*. From this plasmid, the *InvB* gene was isolated by *Kpn*I and *Pst*I digestion and cloned into the same sites of plasmid pGEM-3Z (Promega) to produce pGEM*InvB*. The plasmid pGEM*InvB* was digested with *Eco*NI, ends were filled in and ligated to the Kan^r, to produce the suicide vector designated pGEM*InvB*K 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 Δ*InvB*.

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 *Kpn*I and *Xba*I digestion of plasmid pCR*InvA* and was cloned into same sites of broad-host-range vector pBBR4MCS [28]. The resulting plasmid was designated pBB*InvA*. One microgram of pBB*InvA* was used to electroporate *B. suis* strain 1330 Δ*InvA*; several colonies of strain 1330 Δ*InvA* were picked from a TSA plate containing ampicillin (100 µg/ml). One of these colonies was chosen for further analyses and designated 1330 Δ*InvA* [pBB*InvA*]. The 832-bp DNA fragment containing the *B. suis InvB* gene with its native promoter was introduced to the mutant 1330 Δ*InvB* using a procedure similar to the above. One of the generated colonies was chosen for further analyses and designated 1330 Δ*InvB* [pBB*InvB*].

Growth rates of *B. suis* strains

Single colonies of *B. suis* wild type 1330, mutant strains 1330 Δ*InvA* and 1330 Δ*InvB*, and complemented-mutant strains 1330 Δ*InvA* [pBB*InvA*] and 1330 Δ*InvB* [pBB*InvB*] were streaked on TSA plates and incubated at 37°C at 5% CO_2 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

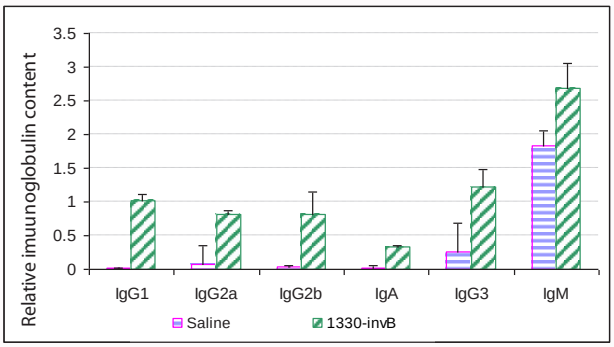

Figure 5: The serum immunoglobulin levels of CD1 mice injected saline or *B. suis* strain 1330Δ*InvB* nine weeks post-inoculation. The sera were collected nine weeks post-injection with saline or strain 1330Δ*InvB*, and serum immunoglobulin levels were determined by ELISA.

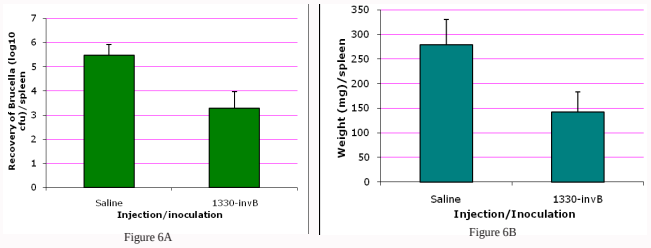

Figure 6: Protection induced in CD1 by the *InvB* mutant *B. suis* strain. Mice were intraperitoneally injected with saline or 5.2 $\log_{10}$ cfu of *InvB* mutant 1330Δ*InvB.* Nine weeks later, mice were challenged 5.0 $\log_{10}$ cfu of wild-type 1330. Two weeks post-challenge, spleen weights (6B) and splenic cfu counts (6A) were determined. *P* value for the difference among mean values was <0.005.

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* inocula

TSA plates were inoculated to confluency with *B. suis* strains. After 96 h of incubation at 37°C in the presence of 5% CO_2, 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 5×10^5/ml in Dulbecco's Modified Essential Medium (DMEM) (Sigma-Aldrich) into 24-well tissue culture dishes and cultured at 37°C with 5% CO_2 until confluent. The tissue culture medium was removed, 108 cells (200 µl) of the bacterial suspension in PBS were added, and the mixture was incubated at 37°C for 3 hr. The unattached bacteria were removed from the wells, and the cfu not adsorbed or taken up by macrophages was determined by plating the serial dilutions on TSA. The number of brucellae taken up/adsorbed to macrophages was calculated by substracting the cfu not adsorbed by total cfu used for inoculating wells. Furthermore, at the end of 3 h incubation, the macrophages remaining in the wells were washed with PBS, and 500 µl of 0.25% sodium deoxycholate was added to lyse them. After 5 min the lysate was diluted in PBS, and the number of viable cells was determined after growth at 37°C for 96 hr on TSA plates. Triplicate samples were taken at all time points, and the assay was repeated two times.

Survival of *B. suis* strains in mice

Six-week-old female CD1 mice (Charles River Laboratories, Wilmington, MA) were allowed one week of acclimatization. Groups of 20 mice each were intraperitoneally injected with 5.0 $\log_{10}$ to 5.2 $\log_{10}$ cfu of *B. suis* wild type 1330, *InvA* mutant 1330 Δ*InvA* or *InvB* mutant 1330 *InvB*. Groups of five mice injected with each strain were sacrificed at 1, 2, 5 and 9 weeks after inoculation, and the *Brucella* cfu count per spleen was determined as described previously [22]. Briefly, spleens were collected and homogenized in TSB. Serial dilutions of each spleen's homogenates were placed on TSA plates. The number of cfu that appeared on plates was determined after 4 days of incubation at 37°C.

Enzyme-Linked Immunosorbant Assay (ELISA)

Mouse antibody isotyping ELISA kit was purchased from Sigma (St. Louis, MO). The antigen-mediated ELISA was performed as per the manufacturer's instructions. Heat-killed cells of *B. suis* wild-type 1330 were suspended in carbonate buffer (pH 9.6) and used to coat the wells of polystyrene plates (100 µl/well; Nunc-Immuno plate with a MaxiSorp surface). The mouse serum samples (1:100 dilution), isotype-specific goat anti-mouse reagents (1:1000 dilution), and rabbit anti-goat IgG (1:5000 dilution) were used. TMB (TMB Microwell peroxidase substrate; Kirkegaard & Perry Laboratories, Gaithersburg, MD) was used as the substrate and the reaction was stopped by adding stop solution (0.185 M sulfuric acid). The A_{450} was recorded with a microplate reader (Molecular Devices, Sunnyvale, CA).

Protective efficacy of *InvB* mutant against virulent *B. suis* 1330 challenge

Six-week-old female CD1 mice (Charles River Laboratories) were allowed one week of acclimatization. Groups of five mice each were intraperitoneally injected with PBS or 5.2 $\log_{10}$ cfu of *InvB* mutant. Nine weeks post-inoculation, mice were intraperitoneally challenged with 5.0 $\log_{10}$ cfu of wild-type, virulent *B. suis* strain 1330. Two weeks post-challenge, mice were sacrificed by CO_2 asphixiation, and the *Brucella* cfu count per spleen was determined as described above.

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 *dnph* (locus tag BR1836) is 548-bp long and located on chromosome I 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

Table 1: Description of the plasmids and bacterial strains used in this study.

Plasmid or strain	Description	Source or reference
Plasmids		
pCR2.1	TA cloning vector, 3.9-kb, Amp^r	Invitrogen
pCR*invA*	pCR2.1 with 832-bp insert containing the *B. suis invA* gene; Amp^r	This study
PCR*invB*	pCR2.1 with 915-bp insert containing the *B. suis invB* gene; Amp^r	This study
pGEM-3Z	Cloning vector, 2.74-kb, Amp^r	Promega
pGEM*invA*	pGEM-3Z with 832-bp insert containing the *B. suis invA* gene; Amp^r	This study
PGEM*invB*	pGEM-3Z with 915-bp insert containing the *B. suis invB* gene gene; Amp^r	This study
pUC4K	Cloning vector, 3.9-kb, Kan^r, Amp^r	Pharmacia
pGEM*invA*K	pGEM*invA* with 1.3-kb *sal*I-cut and blunt-ended Kan^r cassette from pUC4K inserted at *Cla*I site (that is also blunt-ended) Kan^r, Amp^r	This study
pGEM*invB*K	pGEM*invB* with 1.3-kb *sal*I-cut and blunt-ended Kan^r cassette from pUC4K inserted at *EcoN*I site (that is also blunt-ended) Kan^r, Amp^r	This study
pBBR4MCS	Broad-host-range vector; Cm^r	(28)
pBB4*invA*	pBBR4MCS with 832-bp *invA* from pCR*invA* Amp^r	This study
pBB4*invB*	pBBR4MCS with 915-bp *invB* from pCR*invB* Amp^r	This study
***Escherichia coli* strains**		
Top10	F$^-$*mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*Δ*M15* Δ*lacX74 deoR recA1 araD139* Δ(*ara-leu*) *7697 galU galK rpsL* (StrR) *endA1 nupG*	Invitrogen
***Brucella suis* strains**		
1330	Parent-type, smooth strain	G.G. Schurig
1330 Δ*invA*	*invA* disrupted mutant of 1330, Kan^r	This study
1330 Δ*invB*	*invB* disrupted mutant of 1330, Kan^r	This study
1330 Δ*invA*[*invA*]	1330 Δ*invA*[*invA*] containing pBB4*invA*; Kan^r; Amp^r	This study
1330 Δ*invB*[*invB*]	1330 Δ*invB*[*invB*] containing pBB4*invB*; Kan^r; Amp^r	This study

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 *InvA*sion 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 nucleoside diphosphate 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 $GX_5EX_7REUXEEXGU$ (where X represents any amino acid and U represents Ile, Leu, or Val) [20]. The *InvA* amino acid sequence carried the exact $GX_5EX_7REUXEEXGU$ 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*sion protein B of *Ochrobactrum anthropi* and up to 49% identity with *InvA*sion associated proteins of a number of bacterial genera, including *Nitrobacter*, *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 Δ*InvA* or *InvA* mutant. A strain generated by disrupting the *InvB* gene of strain 1330 was designated as 1330 Δ*InvB* 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

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

GenBank accession	Bacterial species	Identity (%)
NP_104977.1|	*Mesorhizobium loti*	77
YP_471550.1|	*Rhizobium etli*	75
ZP_01413754.1|	*Sinorhizobium medicae*	74
YP_770256.1|	*Rhizobium leguminosarum*	73
AAL43753.1|	*Agrobacterium tumefaciens*	71
AF140364_1	*Bartonella clarridgeiae*	55
NP_230320.1|	*Vibrio cholerae*	38
NP_223867.1|	*Helicobacter pylori*	36
YP_311817.1|	*Shigella sonnei*	36
NP_289382.1|	*Escherichia coli*	36
YP_248600.1|	*Haemophilus influenzae*	36
NP_404411.1|	*Yersinia pestis*	36
NP_457398.1|	*Salmonella enterica*	36
ZP_00931552.1|	*Burkholderia mallei*	35
NP_274687.1|	*Neisseria meningitides*	34
YP_169226.1|	*Francisella tularensis*	33

Table 2.2: Identity of *B. suis* putative invasin protein B with the Invasin protein B of other organisms.

GenBank accession	Bacterial species	Identity (%)
YP_001369001.1|	*Ochrobactrum anthropi*	80
ZP_01046780.1|	*Nitrobacter winogradskyi*	49
NP_104332.1|	*Mesorhizobium loti*	45
ZP_00049991.2|	*Magnetospirillum magnetotacticum*	40
ZP_00947418.1|	*Bartonella bacilliformis*	35
YP_031865.1|	*Bartonella quintana*	35
YP_033020.1|	*Bartonella henselae*	34
ZP_01545090.1|	*Stappia aggregata*	35

InvA mutant and *InvB* mutant respectively, indicating that in mutant strains, due to a double-crossover events, the 1.3-kb Kan^r 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 *InvA* mutant strain to generate the complemented *InvA* mutant 1330 Δ*InvA* [*InvA*$^+$]. In the same way, the *InvB* gene was introduced into the *InvB* mutant strain to generate the complemented *InvB* mutant 1330 Δ*InvB* [*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 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 $\log_{10}$ cfu/well, 7.86 $\log_{10}$ cfu/well and 8.02 $\log_{10}$ 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 relatively 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, i.e., 4.97 5.20 $\log_{10}$ cfu/mouse, 4.95 5.20 $\log_{10}$ cfu/mouse and 5.20 $\log_{10}$ 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 $\log_{10}$ 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 $\log_{10}$ cfu of wild type or *InvA* mutant were still present (Figure 4A). The colonies of *InvA* mutant harvested from spleens appeared approximately 30% 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

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 $\log_{10}$ units of protection against a challenge of strain 1330 (Figure 6A). All brucellae harvested from spleens of challenged mice were sensitive to kanamycin (Kan^s), indicating that they all were from the challenge strain 1330 (Kan^s), as opposed to the vaccine strain (*InvB* mutant; Kan^r). Compared to those inoculated with *InvB* mutant, mice injected with saline and subsequently challenged with strain 1330 exhibited spleens approximately twice as large (Figure 6B). The results suggest that compared to saline injection, inoculation with *InvB* mutant induces a significant protection of 2.2 $\log_{10}$ cfu in CD1 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 medium, 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 consensus 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_4A (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*sion of eukaryotic cells. The nudix hydrolase encoded by the *ialA* gene of *Bartonella bacilliformis* was shown to be associated with the ability to *InvA*de human erythrocytes [21]. An upregulation of the expression of the *Escherichia coli* K1 orthologue *ygdP* during *InvA*sion 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_4A and protects cell 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_4A, and thereby regulates *InvA*sion 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_4A 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 1st and the 126th 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 127th and the 173rd 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 127th and the 173rd 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 127th and the 173rd 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*sion proteins InlA and InlB function as both adhesions and *InvA*sions [11,34]. InlA is sufficient for uptake into gut epithelial cells [13] and is required for crossing the intestinal barrier

[14]. InlB mediates uptake into a variety of cell types, e.g., hepatocytes, endothelial cells, and some epithelial cells [15,36]. The receptor for InlA is human E-cadherin [37]. In enteric bacterium *Yersinia pseudotuberculosis*, the outer membrane *InvA*sin protein regulates the uptake of bacterium into M cells [16]. *InvA*sin binds to members of the beta-1 integrin family [38] and induces formation of pseudopods that envelop the bacteria. Further work is required to determine the specific functions of *Brucella InvA* and *InvB* on adhesion, *InvA*sion, establishment and dissemination of bacterium within the host. The host receptors for *Brucella InvA* and *InvB* need to be identified.

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 Δ*ctpA* in BALB/c mice in our previous studies [29]. Petrovska et al. [39] reported that *B. melitensis* strains display similar kinetics of *InvA*sion 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 that 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.

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