Veterinary Microbiology 172 (2014) 279–284

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Roles of genomic island 3 (GI-3) BAB1_0267 and BAB1_0270 open reading frames (ORFs) in the virulence of *Brucella abortus* 2308

Luisa Ortiz-Román [a,b], Roberto Riquelme-Neira [a], RobertoVidal [c], Angel Oñate [a,*]

[a] *Laboratory of Molecular Immunology, Department of Microbiology, Faculty of Biological Sciences, Universidad de Concepción, Concepción, Chile*
[b] *Biogenesis Research Group, Faculty of Agricultural Sciences, Universidad de Antioquia, Medellín, Colombia*
[c] *Microbiology and Mycology Program, Institute of Biomedical Sciences, Faculty of Medicine, University of Chile, Santiago, Chile*

ARTICLE INFO

Article history:
Received 8 January 2014
Received in revised form 29 April 2014
Accepted 4 May 2014

Keywords:
B. abortus
Genomic island 3 (GI-3)
B. abortus mutant strain
Virulence factors

ABSTRACT

One of the properties of bacteria is their capacity to acquire large fragments of genomic DNA from other bacteria or to loose important parts of their own genome. Such fragments include genomic islands (GIs); nine GIs are present in *Brucella*, including genomic island 3 (GI-3), present in *B. abortus*, *B. melitensis* and *B. ovis*. The GI-3 have 29 open reading frames (ORFs) most of them with unknown function. Within the GI-3, the ORFs BAB1_0267 encodes a hypothetical protein sharing a SH3 domain and BAB1_270 a zinc-dependent metallopeptidase. We have obtained deletion mutants for BAB1_0267 and BAB1_0270 ORFs present within GI-3, which have been named the Δ0267 and Δ0270, respectively; in both cases the mutation did not affect the growth of bacteria. Both mutants were evaluated with respect to their growth rates, their ability to invade and replicate in the non-professional and professional phagocytes, HeLa and J774.A1 cells, respectively. Their persistence in the spleens of mice was also evaluated. The mutants efficiently invaded HeLa and J774.A1 cells but both mutants showed a decreased intracellular survival in macrophages and HeLa cells 72 and 96 h post-infection, respectively, and were non-detected in J774.A1 cells 120 h post infection. With respect to *in vivo* persistence Δ0267 was detected through the fourth week while Δ0270 decreased at 7 days disappearing the second week. Our results indicated that deletion of BAB1_0267 and BAB1_270 are necessary to establish an optimal infectious process in *B. abortus* 2308, having more effect the deletion of ORF BAB1_0270. Therefore these ORFs, principally BAB1_0270 are important virulent of *B. abortus*.

© 2014 Elsevier B.V. All rights reserved.

1. Introduction

Brucella abortus is an intracellular pathogen causing brucellosis, a world-wide zoonotic disease (Corbel, 1997). *Brucella* is a Gram-negative bacteria and facultative intracellular pathogen (Kim et al., 2013). Its virulence depends on survival and replication properties in different cell types avoid innate immune responses and to reach its replicative niche (Atluri et al., 2011; Gorvel and Moreno, 2002). This bacterium causes inhibition of the bactericidal functions of phagocytes, including phagolysosomal fusion and neutrophil degranulation (Skendros et al., 2011).

Studies on *Brucella* pathogenicity have been primarily focused in identifying virulence factors. Two sets of genes have been shown to be essential in this process: those of

* Corresponding author. Tel.: +56 41 204118/+56 41 2204118; fax: +56 41 245975.
E-mail address: aonate@udec.cl (A. Oñate).

http://dx.doi.org/10.1016/j.vetmic.2014.05.005
0378-1135/© 2014 Elsevier B.V. All rights reserved.

the type IV secretion system, essential for the intracellular trafficking of the bacteria in host cells (Gomes et al., 2013; Sieira et al., 2000) and those of the two-component regulator BvrR–BvrS involved in cell invasion and intracellular replication (Gorvel and Moreno, 2002; Guzmán-Verri et al., 2002).

In *Brucella*, comparative analysis of genomes of three species (*B. suis*, *B. melitensis* and *B. abortus*), revealed the absence of classical virulence factors such as exotoxins, flagella, capsules and type III secretion systems (Lamontagne et al., 2010). However, 184 genes important for survival and virulence have been found in all of these three species, some of them present in genomic islands (GIs) (Delrue et al., 2004). The region containing the island 3 (GI-3) is shared by *B. melitensis*, *B. abortus* and *B. ovis* (Mancilla, 2011) and it presents 29 genes encoding mostly proteins with unknown functions (Ratushna et al., 2006).

Based on a comparative bioinformatics analysis of GI-3, we discovered two ORFs, BAB1_0267 and BAB1_0270, which are possibly involved in the virulent capacity of *Brucella abortus*. The ORF BAB1_0267 encodes a potential protein with SH3 domains (SRC homology 3). It has been found that SH3 is involved in pathogen–host interaction influencing cell division (Bakal and Davies, 2000). In *Shigella* it was found that a protein with SH3 domains plays a role in entry of the bacteria to a host cell (Bougnères et al., 2004). In other bacteria such as *Mycobacterium tuberculosis*, it was shown that a Src homology 3-domain interacts with TLR2 and triggers functional maturation of human DCs, that contributes to the Th2 cytokine profile (Bansal et al., 2010). The ORF BAB1_0270 was recently reported as a zinc-dependent metallopeptidase in *B. abortus* (accession AM040264.1). Studies with some bacterial peptidases indicate that they may play a role in the regulation of critical processes in the host, including bacterial catabolism and in the degradation of intracellular peptides generated in response to starvation (Barrett et al., 2004). In *Helicobacter pylori* a peptidoglycan-modifying enzyme belonging to the family of zinc-metallopeptidases was reported to be involved in morphology and virulence of the bacterium. Deletion of the zinc–metallopeptidase gene altered the virulence of the bacteria by decreasing its colonization properties (Bonis et al., 2010).

We have set out to investigate the role of both new ORFs in controlling the virulence of *B. abortus*. Therefore, the aim of this study is to evaluate the two deletion mutants in ORFs BAB1_0267 and BAB1_0270 present within the GI-3 of *B. abortus* with respect to their ability to invade and replicate in professional and non-professional phagocytic cells. Their virulence capacity in mice was also investigated.

2. Materials and methods

2.1. Bacterial strains, plasmids, and growth conditions

All strains and some plasmids used in this study are listed in Table 1. *B. abortus* wild type and *B. abortus* mutants were cultured in Brucella broth (Becton, Dickinson and Company, BD) and incubated at 37 °C. *Escherichia coli* strains were cultured in Luria broth and incubated at 37 °C (Céspedes et al., 2012).

2.2. Construction of mutants of B. abortus by deletion of BAB1_0267 and BAB1_0270 ORFs

To assess whether the BAB1_0267 and BAB1_0270 ORFs affect virulence of *B. abortus* 2308 mutant strains Δ0267 and Δ0270 were generated using the phage λ red system, according to that described previously (Datsenko and Wanner, 2000). Briefly, 10^{10} CFU/ml of *B. abortus* 2308 were mixed with 200 ng of recombinant plasmid pSIM7 (λ red) and pulsed with 2500 kV/5 ms and incubated at 30 °C for 72 h in Brucella agar plates supplemented with 30 μg/ml chloramphenicol. After this time, colonies were cultured in Brucella broth for 72 h followed by incubation at 42 °C for 30 min since the genes, which induce the homologous recombination process are activated at temperatures between 37–42 °C. Later, to accomplish the mutation, kanamicyn resistance cassette (km) was amplified by PCR from plasmid pKD4 employed the primers 0267kan FW/0267kan RV and 0270kan

Table 1
Bacteria and plasmids used in this study.

Strain or plasmid	Characteristics	Reference or source
Brucella abortus 2308	Wild-type, smooth, virulent	Laboratory stock
E. coli DH5α	F-, ompT, hsdS (r^-_B, m^-_B), gal, dcm	Laboratory stock
B. abortus Δ0267	*B. abortus* 2308 mutant to BAB1_0267, km^r	This work
B. abortus Δ0270	*B. abortus* 2308 mutant to BAB1_0270, km^r	This work
B. abortus Δ0267 (pVB1-267)	*B. abortus* Δ267 complemented with vector pVB1-267, amp^r, km^r	This work
B. abortus ΔZ0270 (pVB1-270)	*B. abortus* Δ0270 complemented with vector pVB1-270, amp^r, km^r	
Plasmids		
pSIM7	Plasmid with replication origin wide range and carrying the genes of λ recombinase temperature inducible	Donald L Court NCI. USA
pKD4	Plasmid carrying the kanamycin resistance gene (km^r)	Laboratory stock
pVB1	Plasmid contains oriV and wild type trfA from RK2 which gives the vector broad-host-range and contains the *bla* gene encoding β-lactamase to enable selection with ampicillin.	(DualSystem Biotech AG Switzerland)

km^r, Kanamycin resistance; amp^r, ampicillin resistance.

FW/0270kan RV (Table S1). PCR product was purified and electroporated in 10^{10} CFU/ml of *B. abortus* 2308 strains previously transformed with pSIM7. Finally, 100 μl of this suspension was incubated at 37 °C for 72 h in Brucella agar plates supplemented with 50 μg/ml kanamycin for selection of mutants. Colonies were screened by PCR using the primers BAB1_0267 km^r (FW), BAB1_0267 km^r (RW) and BAB1_0270 km^r (FW), BAB1_0270 km^r (RW) (Table S1) (Datta and Costantino, 2006).

2.3. Generation of complemented Δ0267 and Δ0270 strains

In order to ensure that possible changes observed were due to the Δ0267 and Δ0270 mutant strains, two strains complemented with their respective ORFs linked to the vector pVB1 were prepared (DualSystem Biotech AG, Switzerland). A fragment of 366 bp containing the ORF BAB1_0267 and another of 561 bp containing ORF BAB1_0270 were amplified using primers carrying cutting sequences for EcoRI and BamHI enzymes at their ends (Table S1). Later, these fragments were purified and cloned into pVB1 using the enzyme T4 DNA ligase, to generate two plasmids named pVB1-267 and pVB1-270. Then pVB1-267 and pVB1-270 were electroporated within their respective mutant strains and incubated at 37 °C for 72 h in Brucella agar plates supplemented with 100 μg/ml of ampicillin, thereby generating the Δ0267(pVB1-267) and Δ0270(pVB1-270). The pVB1 expression vectors comprise the most versatile and sophisticated system available for production of recombinant proteins. The system combines two unique properties: the highly customizable *Pm/xylS* expression system and a broad-host-range RK2 minimal replicon. The pVB1 vectors are ideal for high-level, controllable recombinant protein production in Gram-negative bacteria.

2.4. Growth curve

To analyze whether the deletion of BAB1_0267 and BAB1_0270 ORFs affected the growth rate of *B. abortus* 2308, growth curves were plotted for *B. abortus* 2308, mutants strains (Δ0267, Δ0270) and complemented strains: Δ0267(pVB1-267) and Δ0270(pVB1-270). For this purpose, 100 μl of each strain (all at an OD_{600} of 0, 5) was cultivated in Brucella broth (Becton, Dickinson and Company, BD) and incubated at 37 °C for 144 h. Growth was determined by measuring OD_{600} after 12, 24, 48, 72, 96, 120 and 144 h.

2.5. Intracellular survival in non-professional and professional phagocytic cell

To assess whether the mutation of the BAB1_0267and BAB1_0270 ORFs affected the ability of *B. abortus* 2308 to replicate within professional and non-professional phagocytic cells, intracellular survival assays using J774 macrophages and HeLa cells were performed as described previously (Céspedes et al., 2012). Briefly, *B. abortus* 2308 suspension was added to HeLa or J774 cells at a 500:1 or 50:1 multiplicity of infection (MOI), respectively (Bonomi et al., 2010; Bukata et al., 2008). After 4, 24, 48, 72, 96, 120 and 144 h post-infection, the cell monolayer was washed, lysated and plated on Brucella agar plates supplemented with 5% fetal bovine serum and 50 μg/ml of kanamycin for the mutant or 100 μg/ml of ampicillin for complemented strains; finally, the number of CFU/ml recovered was determined.

2.6. Persistence in the spleen of BALB/c mice

To test whether the deletion of the BAB1_0267 and BAB1_0270 ORFs affects the ability of *B. abortus* 2308 *in vivo*, assays were performed in the murine model (Silva et al., 2011). For this, BALB/c mice were intraperitoneally injected with 10^5 CFU/ml of the mutant strains, the complemented strains and *B. abortus* 2308 (Ugalde et al., 2000). One, three, seven days, and two, four and six weeks after infection, animals were sacrificed by cervical dislocation to extract their spleens. Subsequently, spleens were homogenized in PBS and serial dilutions were performed which were plated on Brucella agar to determine the number of CFUs recovered per spleen from each strain. Ethical approval to carry out these procedures was obtained from the Universidad de Concepción Ethical Committee.

2.7. Statistical analysis

To analyze whether there is difference between the experimental groups compared with the control group, two-way ANOVA and multiple comparison analysis (Tukey) were performed.

3. Results

3.1. Sequence analyses of BAB1_0267and BAB1_0270

The BAB1_0267 and BAB1_0270 ORFs are located in the genomic island 3 (GI-3) reported in *B. abortus* and *B. melitensis* (Ratushna et al., 2006). Amino acid sequences predicted by PSI-BLAST (Altschul et al., 1997), show that ORF BAB1_0267 encodes a hypothetical protein of 117 amino acids and shares a SH3 domain. ORF BAB1_0270 encodes a protein of 182 amino acids; analysis of the deduced amino acid sequence showed a 100% identity with a Zn-dependent metallopeptidase reported in other species like *Salmonella typhimurium*, *Pseudomonas fluorescens* and *Bacillus subtilis*.

3.2. The growth rate in the mutant strains Δ0267 and Δ0270 is similar to the complemented strains and B abortus 2308

In order to determine whether the deletion of the BAB1_0267 or BAB1_0270 ORFs affect growth of *B. abortus* 2308 in culture media, the growth of each strain under study was analyzed (Fig. 1). The growth curves obtained for the Δ0270 mutant strains were similar to the growth curves for the wild-type 2308 strain and complemented strains Δ0270 (pVB1-270). Moreover, the mutant strain Δ0267 shows a growth curve nearly similar to the growth curves for the wild-type 2308 strain and complemented strains Δ0267(pVB1-267), except for a slight reduction in

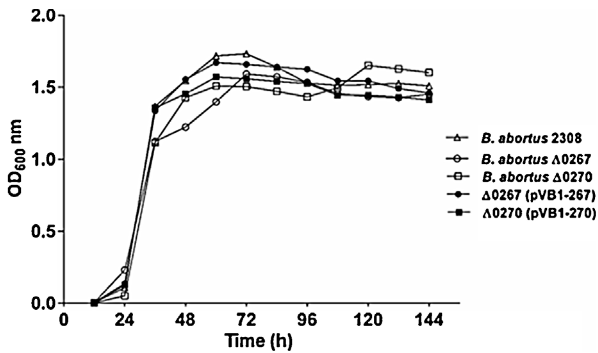

Fig. 1. Growth curves of *B. abortus* 2308 (Wt) (open triangle), *B. abortus* Δ0267 (open circle), *B. abortus* Δ0270 (open square), complemented Δ0267 (pBV1-267) (closed circle) and complemented Δ0270 (pBV1-270) (closed square).

growth at 48 and 60 h post inoculation. These results suggest that the lack of these ORFs is not lethal to the bacterium and it does not affect rate of growth in the culture medium.

3.3. Deletion of BAB1_0267 and BAB1_0270 affects the ability of B. abortus 2308 to proliferate within professional and non-professional phagocytes

As shown in Fig. 2A, the mutant strains were internalized to the same speed as the complemented strains and the 2308 strain, observed that 4 h after infection of macrophages J774.A1 with the different strains studied, no difference in the number of CFU/ml from the cells infected with the mutant strains and was infected with the complemented strains or the wild type *B. abortus* 2308 (*i.e.* approximately 2.2 $\log_{10}$ CFU/ml). However, although there was no significant difference at 24 h post infection, the mutant strains were recovered in lower numbers compared to the wild type strain, indicating that after 4 h of infection, complemented strains and *B. abortus* 2308 began to multiply at a faster rate than the mutated strains Δ0267 and Δ0270. Most evident results are observed at 72 and 96 h, where a significant decrease in the mutant strains compared with *B. abortus* 2308 and complemented strains Δ0267 (pVB1-267) and Δ0270 (pVB1-270), disappearing at 120 h post-infection.

Subsequently the same analysis was performed but, in this case, using HeLa cells infected with the same bacterial strains. As seen in Fig. 2B, 4 h after infection the same behavior observed in professional phagocytes was seen, as the number of CFU/ml recovered from the HeLa cells infected with both mutant strains was similar to the number of CFU/ml recovered from the cells infected with the complemented strains and *B. abortus* 2308 (*i.e.* approximately 2.2 $\log_{10}$ CFU/ml). After 24, 48 and 72 h post-infection, the mutant strains were recovered at a lower number compared to the wild type strain, however, no significant difference was found. Most evident results are observed at 96 h, where a significant reduction of the mutant strains is observed reaching approximately 1.5 $\log_{10}$ CFU/ml lower than *B. abortus* 2308 and complemented strains ($p < 0.001$). These results indicate that deletion of BAB1_0267 and BAB1_0270 ORFs affects the survival of the bacteria within professional and non-professional phagocytes.

3.4. The survival of Δ0267 and Δ0270 is attenuated in mice

After evaluating the effect of the deletion of the BAB1_0267 and BAB_0270 ORFs *in vitro*, the effect of these deletions on *B. abortus* 2308 was determined *in vivo* by determining bacterial loads in the spleen of infected mice.

Table 2 shows that at 24 h post-infection few bacteria were recovered in the spleen of mice infected for all experimental groups; this is consistent with the notion that *Brucella* must move from the peritoneal cavity to the spleen leading to septicemia. However, at 72 h post

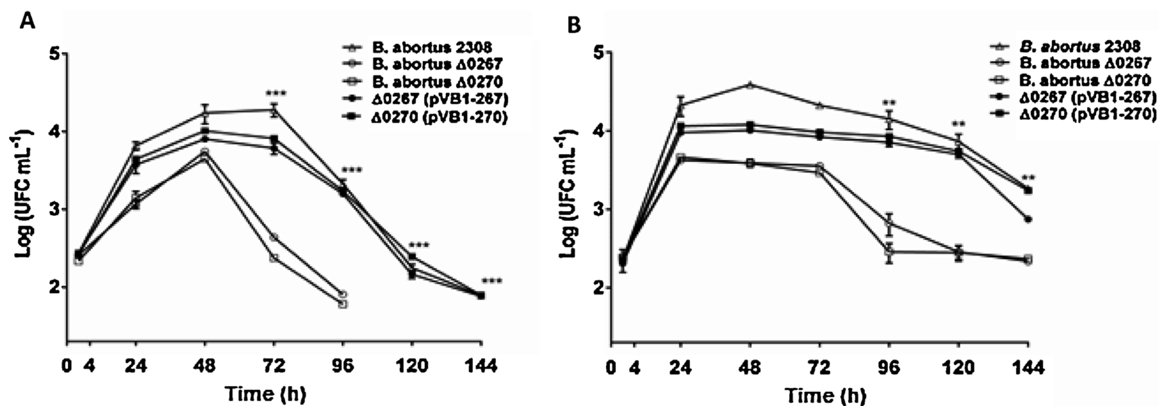

Fig. 2. Intracellular survival of mutant strains Δ0267 and Δ0270 and *B. abortus* strain 2308 in professional and nonprofessional phagocytes. (A) J774.A1 macrophages were infected at a MOI of 50:1. *B. abortus*2308 (Wt) (open triangle), *B. abortus* Δ0267 (open circle), *B. abortus* Δ0270 (open square), complemented Δ0267 (pBV1-267) (closed circle) and complemented Δ0270 (pBV1-270) (closed square). (B) HeLa cells were infected at a MOI of 500:1. At different times after infection, the cells were lysed, and the numbers of viable intracellular bacteria (CFU/ml) were determined. Results correspond to a representative experiment out of two separate experiments. For each time point and each strain results are expressed as means and standard deviations. *** $p < 0.0001$, ** $p < 0.001$ when compared to parental *B. abortus* 2308.

Table 2
BALB/c mice spleen colonization by *B. abortus* Δ0267, *B. abortus* BAB1_0270 mutants, the complemented B. abortus Δ0267 (pVB1-267), Δ0270 (pVB1-270) and the parental *B. abortus* 2308 strain.

Brucella abortus strain	One day post infection (mean ± SD)	Three days post infection (mean ± SD)	Seven days post infection (mean ± SD)	Two weeks post infection (mean ± SD)	Four weeks post infection (mean ± SD)	Six weeks post infection (mean ± SD)
2308	0.952 ± 0.028	2.874 ± 0.017	3.944 ± 0.027	4.345 ± 0.017	1.838 ± 0.023	1.823 ± 0.025
Δ0267	0.881 ± 0.036	2.838 ± 0.013	3.895 ± 0.17	3.771 ± 0.065	0[a]	0[a]
Δ 0270	0.602 ± 0.02[a]	2.098 ± 0.036[a]	1.767 ± 0.077[a]	0[a]	0[a]	0[a]
Δ0267 (pVB1-267)	0.937 ± 0.017	2.790 ± 0.012	3.874 ± 0.017	3.929 ± 0.013	1.568 ± 0.137	1.543 ± 0.082
Δ 0270 (pVB1-270)	0.918 ± 0.036	2.834 ± 0.011	3.883 ± 0.019	4.272 ± 0.013	1.77 ± 0.025	1.67 ± 0.019

$\log_{10}$ CFU of *B. abortus* in spleen of BABL/c mice inoculated intraperitoneally with 10^5 CFU of parental *B. abortus* 2308, both mutants Δ0267 and Δ0270 or complemented Δ0267 (pVB1-267) and Δ0270 (pVB1-270).
[a] $p < 0.05$ (significant) as compared with mean value for control mice inoculated with the parental *B. abortus* 2308 strain.

infection, the persistence of Δ0270 was lower compared to the wild type strain and the complemented strains, this difference was even more striking seven days after infection where 2.2 $\log_{10}$ CFU/spleen least were recovered compared with control strains *B. abortus* 2308. Two weeks after infection, the persistence of Δ0270 strain was down to zero confirming that deletion of the ORF BAB1_0270 generates a significant attenuation in virulence of *B. abortus* 2308. Unlike for Δ0270 strain, until day 14 post-infection no significant differences were observed in the number of colonies recovered from the spleen of the Δ0267 strain compared to *B. abortus* 2308 and complemented strain Δ0267(pVB1-267) and 28 days post-infection a significantly lower number of CFUs when compared to control strains was recovered.

4. Discussion

Little is known about the cellular and molecular mechanisms used by *B. abortus* to survive and replicate within host cells. It has been suggested that the protection of the host against this bacterium is mediated by Th1 $CD4^+$ T cells, which secrete interferon gamma (IFN-gamma) to stimulate bactericidal activity and $CD8^+$ T cell cytotoxic activity (Vitry et al., 2012). To date, few specific *B. abortus* antigens involved in the stimulation of protective cell-mediated immunity have been described. It is therefore necessary to identify new proteins in the bacteria, which might be critical for *B. abortus* infection and thus being able to become target for new vaccination. We have recently shown that some of the antigens involved in the virulence of the bacteria are present in GI-3 of the chromosome I of *B. abortus* 2308 (Céspedes et al., 2012). In an effort to identify potential virulence factors in *B. abortus*, we have focused on ORFs BAB1_0267 and BAB1_0270 gene of the GI-3 (Rajashekara et al., 2004).

We have conducted bioinformatic analysis of the ORF BAB1_0267 and revealed the presence of a SH3 domain, which is present in proteins of the signaling pathways regulating cytoskeletal, tyrosine kinases, and the state of activity of adapter proteins (Elena et al., 2011; Stoll and Bosserhoff, 2008). In bacteria, these domains are present in protein that regulates a variety of cellular processes, including chemotaxis, osmoregulation and sporulation of the bacterium (Bilwes et al., 1999). In *Mycobacterium tuberculosis* it was demonstrated that this domain SH3 interacts with Toll Receptor Type-2 (TLR-2) and activated the maturation of dendritic cells. However, a link with the secretion of high amounts of Th2 cytokines was observed, while Th1 cytokines such as IL-12 were not induced. This suggests that SH3 domains in *Mycobacterium tuberculosis* could act as immunomodulators allowing evasion of mycobacteria by activating a Th2 response (Bansal et al., 2010). This is of importance considering that in intracellular microorganisms such as *Brucella* sp., requires a Th1 mediated cellular immune response in order to be eliminated. So in *Brucella* this domain could have a role in their ability to survive within phagocytes since when macrophages were infected with *B. abortus* mutant Δ0267, 72 h post-infection a significant decrease in the ability of bacteria to replicate was observed, this difference was reversed by the complemented strain *B. abortus* pVB1-267 showing a similar number of CFUs to the "Wild-type" strain. Furthermore, it was found that mutant Δ0267 exhibited attenuated growth in HeLa cells. These results in professional and non-professional phagocytes were confirmed in the mouse model, observing a significant reduction two weeks after infection, suggesting that *B. abortus* 2308 requires expression of BAB1_0267 for survival of the bacterium.

The other ORF studied was BAB1_0270 a zinc dependent metallopeptidase. It was studied in *Salmonella enterica* and *Lactococcus lactis*, indicating that these proteases may have a role in the regulation of critical processes in the host in order to survive (Mathew et al., 2000; Uzelac et al., 2013). A virulence factor that is involved in the cleavage of the host extracellular matrix is important in the process of bacterial dissemination and/or invasion (Soares et al., 2008). We by contrast, observed that this protein may not participate in the invasion stage of the HeLa cells and macrophages, because four hours post-infection the mutants and wild-type virulent strain 2308 were recovered in a similar number of CFU/ml, however, 96 h after infection a significant decrease in the count of CFU/ml of the *B. abortus* mutant Δ0270 compared to the wild type, this result could lead us to think that in *B. abortus* 2308 this protein could act as a protease involved in the survival of the bacteria since deletion of this gene affects the ability of intracellular replication of the bacteria. Most marked results, observed *in vivo*, were significant reduction in the number of bacteria observed from 24 h post-infection. Further, 14 days after infection no

bacteria were recovered from the spleen of infected mice. Similar results were found in a study in *Helicobacter pylori* where to infect mice orally with mutated strains for a metallopeptidase, the ability of the mutant to colonize the gastric mucosa of the mouse after 1, 4 and 7 days, was affected compared to the parental strain (Bonis et al., 2010).

In conclusion these results suggest that the deletion of BAB1_0267 and BAB1_0270 ORFs in *B. abortus* 2308 affects the process of cell invasion, the efficiency of bacterial growth within non-professional and professional phagocytes and its virulence in BALB/c mice and therefore affects host–pathogen interaction needed to be established in infection.

Acknowledgments

This work was supported by grant 1130093 from "Fondo Nacional de Investigación Científica y Tecnológica" (FONDECYT), Chile. Luisa Ortiz-Román was supported by a fellowship from Agencia de Cooperación Internacional de Chile (AGCI). The authors thank Donald L. Court, PhD from the National Cancer Institute for providing us the plasmid pSIM7.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetmic.2014.05.005.

References

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.

Atluri, V.L., Xavier, M.N., de Jong, M.F., den Hartigh, A.B., Tsolis, R.M., 2011. Interactions of the human pathogenic *Brucella* species with their hosts. Annu. Rev. Microbiol. 65, 523–541.

Bakal, C.J., Davies, J.E., 2000. No longer an exclusive club: eukaryotic signalling domains in bacteria. Trends Cell Biol. 10, 32–38.

Bansal, K., Sinha, A.Y., Ghorpade, D.S., Togarsimalemath, S.K., Patil, S.A., Kaveri, S.V., Balaji, K.N., Bayry, J., 2010. Src homology 3-interacting domain of Rv1917c of *Mycobacterium tuberculosis* induces selective maturation of human dendritic cells by regulating PI3K-MAPK-NF-kappaB signaling and drives Th2 immune responses. J. Biol. Chem. 285, 36511–36522.

Barrett, A.J., Woessner, J.F., Rawlings, N.D., 2004. Handbook of Proteolytic Enzymes, second ed., Vol. 1Elsevier, New York, NY, , pp. 231–268 (Access online).

Bilwes, A.M., Alex, L.A., Crane, B.R., Simon, M.I., 1999. Structure of CheA, a signal-transducing histidine kinase. Cell 96, 131–141.

Bonis, M., Ecobichon, C., Guadagnini, S., Prévost, M.C., Boneca, I.G., 2010. A M23B family metallopeptidase of *Helicobacter pylori* required for cell shape, pole formation and virulence. Mol. Microbiol. 78, 809–819.

Bonomi, H.R., Marchesini, M.I., Klinke, S., Ugalde, J.E., Zylberman, V., Ugalde, R.A., Comerci, D.J., Goldbaum, F.A., 2010. An atypical riboflavin pathway is essential for *Brucella abortus* virulence. PLoS One 5, e9435.

Bougnères, L., Girardin, S.E., Weed, S.A., Karginov, A.V., Olivo-Marin, J.-C., Parsons, J.T., Sansonetti, P.J., Van Nhieu, G.T., 2004. Cortactin and Crk cooperate to trigger actin polymerization during *Shigella* invasion of epithelial cells. J. Cell Biol. 166, 225–235.

Bukata, L., Altabe, S., de Mendoza, D., Ugalde, R.A., Comerci, D.J., 2008. Phosphatidylethanolamine synthesis is required for optimal virulence of *Brucella abortus*. J. Bacteriol. 190, 8197–8203.

Céspedes, S., Salgado, P., Retamal-Díaz, A., Vidal, R., Oñate, A., 2012. Roles of genomic island 3 (GI-3) BAB1_0278 and BAB1_0263 open reading frames (ORFs) in the virulence of *Brucella abortus* in BALB/c mice. Vet. Microbiol. 156, 1–7.

Corbel, M.J., 1997. Brucellosis: an overview. Emerg. Infect. Dis. 3, 213.

Datsenko, K.A., Wanner, B.L., 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Nat. Acad. Sci. U.S.A. 97, 6640–6645.

Datta, S., Costantino, N., 2006. A set of recombineering plasmids for gram-negative bacteria. Gene 379, 109–115.

Delrue, R.M., Lestrate, P., Tibor, A., Letesson, J.J., Bolle, X., 2004. Brucella pathogenesis, genes identified from random large-scale screens. FEMS Microbiol. Lett. 231, 1–12.

Elena, T., Andrea, V., Francesca, Z., Carlo, B., Elisa, M., Oriano, M., Antonio, T., Luciana, B., Veronica, M., Mario, A.P., 2011. Interaction between the SH3 domain of Src family kinases and the proline-rich motif of HTLV-1 p13: a novel mechanism underlying delivery of Src family kinases to mitochondria. Biochem. J. 439, 505–516.

Gomes, M.T.R., Campos, P.C., Oliveira, F.S., Corsetti, P.P., Bortoluci, K.R., Cunha, L.D., Zamboni, D.S., Oliveira, S.C., 2013. Critical role of ASC inflammasomes and bacterial type IV secretion system in caspase-1 activation and host innate resistance to *Brucella abortus* infection. J. Immunol. 190, 3629–3638.

Gorvel, J.P., Moreno, E., 2002. *Brucella* intracellular life: from invasion to intracellular replication. Vet. Microbiol. 90, 281–297.

Guzmán-Verri, C., Manterola, L., Sola-Landa, A., Parra, A., Cloeckaert, A., Garin, J., Gorvel, J.-P., Moriyon, I., Moreno, E., Lopez-Goni, I., 2002. The two-component system BvrR/BvrS essential for *Brucella abortus* virulence regulates the expression of outer membrane proteins with counterparts in members of the *Rhizobiaceae*. Proc. Nat. Acad. Sci. U.S.A. 99, 12375–12380.

Kim, D.H., Son, B.G., Lim, J.J., Lee, J.J., Kim, D.G., Lee, H.J., Min, W., Rhee, M.H., Kim, K.D., Chang, H.H., 2013. The role of a *Brucella abortus* lipoprotein in intracellular replication and pathogenicity in experimentally infected mice. Microb. Pathog. 54, 34–39.

Lamontagne, J., Beland, M., Forest, A., Cote-Martin, A., Nassif, N., Tomaki, F., Moriyon, I., Moreno, E., Paramithiotis, E., 2010. Proteomics-based confirmation of protein expression and correction of annotation errors in the *Brucella abortus* genome. BMC Genomics 11, 300.

Mancilla, M., 2011. The Brucella genomic islands. In: López-Goñi, I., O'Callaghan, D. (Eds.), Brucella: Molecular Microbiology and Genomics. Horizon Scientific Press, Norwich, UK, pp. 36–57.

Mathew, Z., Knox, T.M., Miller, C.G., 2000. *Salmonella enterica* serovar typhimurium peptidase B is a leucyl aminopeptidase with specificity for acidic amino acids. J. Bacteriol. 182, 3383–3393.

Rajashekara, G., Glasner, J.D., Glover, D.A., Splitter, G.A., 2004. Comparative whole-genome hybridization reveals genomic islands in *Brucella* species. J. Bacteriol. 186, 5040–5051.

Ratushna, V.G., Sturgill, D.M., Ramamoorthy, S., Reichow, S.A., He, Y., Lathigra, R., Sriranganathan, N., Halling, S.M., Boyle, S.M., Gibas, C.J., 2006. Molecular targets for rapid identification of *Brucella* spp. BMC Microbiol. 6, 13.

Sieira, R., Comerci, D.J., Sánchez, D.O., Ugalde, R.A., 2000. A homologue of an operon required for DNA transfer in *Agrobacterium* is required in *Brucella abortus* for virulence and intracellular multiplication. J. Bacteriol. 182, 4849–4855.

Silva, T., Costa, E.A., Paixão, T.A., Tsolis, R.M., Santos, R.L., 2011. Laboratory animal models for brucellosis research. J. Biomed. Biotechnol. 2011, 10.

Skendros, P., Pappas, G., Boura, P., 2011. Cell-mediated immunity in human brucellosis. Microbes Infect. 13, 134–142.

Soares, G., Da Silva, B.A., Dos Santos, M.H.B., Da Costa, A.F.E., Dos Santos, A.L.S., Morandi, V., Nagao, P.E., 2008. Metallopeptidases produced by group B *Streptococcus*: influence of proteolytic inhibitors on growth and on interaction with human cell lineages. Int. J. Mol. Med. 22, 119.

Stoll, R., Bosserhoff, A., 2008. Extracellular SH3 domain containing proteins-features of a new protein family. Curr. Protein Pept. Sci. 9, 221–226.

Ugalde, J.E., Czibener, C., Feldman, M.F., Ugalde, R.A., 2000. Identification and characterization of the *Brucella abortus* phosphoglucomutase gene: role of lipopolysaccharide in virulence and intracellular multiplication. Infect. Immun. 68, 5716–5723.

Uzelac, G., Kojic, M., Lozo, J., Aleksandrzak-Piekarczyk, T., Gabrielsen, C., Kristensen, T., Nes, I.F., Diep, D.B., Topisirovic, L., 2013. A Zn-dependent metallopeptidase is responsible for sensitivity to LsbB, a class II leaderless bacteriocin of *Lactococcus lactis* subsp. lactis BGMN1-5. J. Bacteriol. 195, 5614–5621.

Vitry, M.-A., De Trez, C., Goriely, S., Dumoutier, L., Akira, S., Ryffel, B., Carlier, Y., Letesson, J.-J., Muraille, E., 2012. Crucial role of gamma interferon-producing CD4+ Th1 cells but dispensable function of CD8+ T cell, B cell, Th2, and Th17 responses in the control of *Brucella melitensis* infection in mice. Infect. Immun. 80, 4271–4280.