Arch Microbiol (2017) 199:971–978
DOI 10.1007/s00203-017-1360-7

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ORIGINAL PAPER

Omp31 plays an important role on outer membrane properties and intracellular survival of *Brucella melitensis* in murine macrophages and HeLa cells

L. Verdiguel-Fernández[1] · **R. Oropeza-Navarro**[2] · **Francisco J. Basurto-Alcántara**[3] · **A. Castañeda-Ramírez**[4] · **Antonio Verdugo-Rodríguez**[1]

Received: 27 September 2016 / Revised: 21 February 2017 / Accepted: 7 March 2017 / Published online: 5 April 2017
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Abstract Brucellosis is an infectious disease that affects practically all species of mammals, including human, and is a major zoonosis worldwide. *Brucella* spp. are facultative intracellular pathogens that have the ability to survive and multiply in phagocytic and nonphagocytic cells such as trophoblast and epithelial cells. Among the six recognized species of the genus *Brucella, Brucella melitensis* is the main etiological agent involved in goat brucellosis and is also the most pathogenic for human. It causes significant losses in livestock production as a result of abortions, metritis, infertility, and birth of weak animals. Outer membrane proteins (OMPs) are exposed on the bacterial surface and are in contact with cells and effectors of the host immune response, whereby they could be important virulence factors of Brucella species. To evaluate this hypothesis, the gene encoding for the major outer membrane protein Omp31 was amplified, cloned into pUC18 plasmid, and inactivated by inserting a kanamycin cassette, rendering pLVM31 plasmid which was transformed into *B. melitensis* wild-type strain to obtain LVM31 mutant strain. The Outer membrane (OM) properties of the mutant strain were compared with *B. melitensis* Bm133 wild-type and *B. melitensis* Rev1 vaccine strains, in assessing its susceptibility to polymyxin B, sodium deoxycholate, and nonimmune serum. The mutant strain was assessed in vitro with survival assays in murine macrophages J774.A1 and HeLa cells. Our results demonstrate that LVM31 mutant is more susceptible to polymyxin B, sodium deoxycholate, and nonimmune serum than control strains; moreover, Omp31 mutation caused a decrease in the internalization and a significant decrease in the intracellular survival compared with the reference strains in both cell lines. These results allow us to conclude that Omp31 is important for maintaining OM integrity, but also it is necessary for bacterial internalization, establishment and development of an optimal replication niche, and essential for survival and intracellular multiplication.

Keywords *Brucella melitensis* · *Omp*31 · Intracellular replication

Communicated by Erko Stackebrandt.

✉ Antonio Verdugo-Rodríguez
antoverduro@hotmail.com

1 Laboratorio de Microbiología Molecular, Departamento de Microbiología e Inmunología, Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autónoma de México, Avenida Universidad 3000, colonia UNAM CU, Coyoacán C.P 04510, CdMx, Mexico

2 Departamento de Microbiología Molecular, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernvaca, Morelos, Mexico

3 Laboratorio de Vacunología, Departamento de Microbiología e Inmunología, Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autónoma de México, Coyoacán, CdMx, Mexico

4 Departamento de Zootecnia, Universidad Autónoma de Chapingo, Texcoco, Mexico

Introduction

Microorganisms belonging to the genus *Brucella* are Gram-negative, facultative intracellular bacteria causing infections in many animal species and humans (Boschiroli et al. 2001). Ten species, classified on the basis of differences in pathogenicity and host preference, are recognized within the genus *Brucella: B. melitensis, B. abortus, B. suis, B. ovis, B. canis* and *B. neotomae, B. pinnipedialis, B. ceti, B. microti, and B. inopinata* (Moreno et al. 2002; Scholz et al.

2010). *Brucella melitensis*, besides its important zoonotic aspect, is the most relevant etiologic agent of ovine and caprine brucellosis, a disease that causes abortion in ewes and goats resulting in huge economic losses, particularly in Mediterranean countries (Blasco 1997). Vaccination is the most suitable way to control both infections in sheep in endemic situations. The live attenuated *B. melitensis* Rev.1 strain is considered the best vaccine available for the prophylaxis of brucellosis in sheep and goats (Garin-Bastuji et al. 1997). However, this vaccine may cause abortion if used in animals in late pregnancy and its use is known to induce antibody responses indistinguishable by the current conventional serological tests from those observed in *B. melitensis* infected animals, this fact limits the extended use of Rev.1 in countries applying eradication programs based on serological testing and slaughtering of seropositive animals (de Bagüés et al. 1992; Schurig et al. 2002). Finally, this vaccine strain is fully virulent for humans and many accidental injection infections have been documented. To solve these problems, several strategies have been used to improve current vaccines. For safety improvement, the deletion of virulence-related genes, reduced-dose vaccination, and vaccination via the oral and conjunctival routes have widely been used (Barrio et al. 2009; Wang and Wu 2013; Yang et al. 2013). Outer membrane proteins (OMPs) are exposed on the bacterial surface and are in direct contact with cells effectors of the host immune response, whereby they could be important virulence factors of Brucella species (Martín-Martín et al. 2011). The *Brucella* spp. Omp25/Omp31 family is formed by seven homologous OMPs (Salhi et al. 2003). Regarding virulence, mutant strains of *B. melitensis, B. abortus*, and *B. ovis* by *omp25* inactivation have been found to be attenuated in mice, goats (*B. melitensis*) (Edmonds et al. 2002a, b), and cattle (*B. abortus*) (Edmonds et al. 2001, 2002). In addition, Omp25 has been shown to inhibit the production of tumor necrosis factor alpha (TNF-α) by human macrophages (Jubier-Maurin et al. 2001) and to be involved in the permeability of *Brucella* membrane, allowing for secretion of periplasmic proteins, in acidic medium (Boigegrain et al. 2004). Several reports showed that the inactivation of the genes coding for the five OMPs of this family in virulent *B. ovis* PA, and demonstrated that the absence of Omp25d or Omp22 proteins leads to a striking decrease on virulence of *B. ovis* PA in mice (Caro-Hernández et al. 2007; Vizcaíno et al. 2004). Furthermore, it was demonstrated that Omp25d and Omp22 are essential for invasion and survival of *B. ovis* inside host cells, justifying the strong attenuation of the Δ*omp25d* and Δ*omp22* mutants. In contrast, little is known about the role of the Omp31 on virulence in smooth strains (Martín-Martín et al. 2008). The aim of this work was to generate an *omp31* mutant of *B. melitensis* Bm 133 strain and evaluate its effect on OM properties and intracellular survival of bacteria in murine macrophages J774.A1 and HeLa cells.

Materials and methods

Bacterial strains, growth conditions, and cloning vectors

The strains used in this work are listed in Table 1. *Brucella* strains were cultured at 37 °C in a 5% CO_2 atmosphere for 72 h. They were typically propagated in *Brucella* broth (BB; Difco Laboratories, Detroit, MI) or *Brucella* agar (BA; Difco Laboratories), both supplemented with 0.3% yeast extract (YE; Difco Laboratories) and 5% fetal bovine serum (FBS; GIBCO-BRL Life Technologies, Germany) (BB-YE-FBS and BA-YE-BFS).

Table 1 Bacterial strains and plasmids

	Relevant genotype	Relevant characteristic	Origin
Bacterial strain			
Escherichia coli DH5α	*ΔlacZ* M15, endA1 and recA1 mutations	This strain of *E. coli* is not pathogenic, and was developed for laboratory cloning use	LMM
Brucella melitensis biotype 1 Bm133	Mexican reference strain (wild-type)	Smooth virulent *Brucella* strain	LMM
Brucella melitensis LVM31	*omp*31::Kan^r	*Brucella melitensis* with a Kanamycin resistance cassette inserted into the *Sal*I site of *omp*31	This work
Plasmids			
pLV	Amp^r Kan^r	*Brucella melitensis omp31* gene cloned into pCR2.1 TOPO vector	This work
pLVM	Amp^r	*Brucella melitensis omp31* gene inserted into the *Hin*dIII-*Xba*I sites of pUC18	This work
pLVM31	*omp*31::Kan^r	pLVM with a Kanamycin resistance cassette inserted into the *Sal*I site of *omp*31	This work

LMM Molecular Microbiology Laboratory, Immunology and Microbiology Department, FMVZ-UNAM

Plasmids pCR2.1 TOPO (Invitrogen, USA), pUC4K, and pUC18 were used as cloning vectors. pUC18 does not replicate in *Brucella* spp., so it works as a suicide plasmid required for homologous recombination. Plasmids were propagated in *Escherichia coli* DH5α. Recombinant *E. coli* cells were grown in Luria–Bertani (LB; Difco Laboratories) medium supplemented with 50 µg/ml of the required antibiotic(s) depending on plasmid resistance. *Brucella* and *E. coli* strains were provided by LMM (Molecular Microbiology Laboratory, Immunology and Microbiology Department, FMVZ-UNAM, Mexico).

Primers and DNA techniques

Chromosomal DNA of *Brucella melitensis* Bm133 was obtained by the guanidine thiocyanate method, as directed by the protocol described by Sambrook and Russell 2001. Prior to DNA extraction, biomass of bacteria was suspended in 5 ml of sterile distilled water and incubated in a water bath at 80 °C for 45 min for inactivation.

The *omp*31 nucleotide sequence was retrieved from the genome sequence of *B. melitensis* reference strain 16 M (GenBank accession no. JF918757.1). Primers were designed to amplify the entire *omp*31 gene (723 bp). The primers were omp31Fw (forward primer 5′ATGAAATCC GTAATTTTGGCG 3′) and omp31Rv (reverse primer 5′ TTAGAACTTGTAGTTCAGACC 3′). Primers were purchased from Sigma Genosys (United States). PCR was performed on extracted DNAs as follows: 35 cycles of PCR, with 1 cycle consisting of 1 min at 95 °C for DNA denaturation, 1 min at 50 °C for DNA annealing, and 1 min at 72 °C for polymerase-mediated primer extension. The last cycle included incubation of the sample at 72 °C for 5 min. Five microliters of the amplified product were analyzed by electrophoresis in 1.5% agarose gels in TAE buffer (20-mM Tris base, acetate acid, 2-mM EDTA [pH 8.0]) (Sigma–Aldrich).

Inactivation of *omp*31 gene in *B. melitensis* Bm 133 by homologous recombination

Construction of recombinant pLV plasmid was carried out using the amplification product of *omp31* and cloned into plasmid pCR2.1-TOPO (Invitrogen, USA). The ligation mixture was transformed into electrocompetent *E. coli* DH5 α cells, plated on LB agar with kanamycin (100 µg/ml), and incubated at 37 °C overnight. Subsequently, plasmid extraction was performed. pLV plasmid was digested with *Hin*d III (Thermo-SCIENTIFIC) and *Xba* I (Thermo-SCIENTIFIC) enzymes and *omp*31 was subcloned into pUC18 plasmid previously digested with both enzymes, rendering pLVM. The pLVM plasmid was digested with *Sal*I (Thermo-SCIENTIFIC) and *omp31* inactivation was performed by inserting a kanamycin resistance cassette obtained from pUC4K to render pLVM31. This plasmid was transformed by electroporation into the *B. melitensis* strain, obtaining the mutant strain *B. melitensis* LVM31 (Fig. 1).

Growth kinetics of bacterial strains

To determine *B. melitensis* 133 biovar 1, *Brucella melitensis* Rev1, and Brucella melitensis LVM31 growth rates, five colonies of fresh cultures were taken and inoculated into a tube with 5 ml of Brucella broth, which were incubated at 37 °C to 150 rpm for 22 h. Thus, a 1/50 dilution of each culture in 200 ml of Brucella broth was made and cultures were incubated at 37 °C at 150 rpm for 120 h. To determine

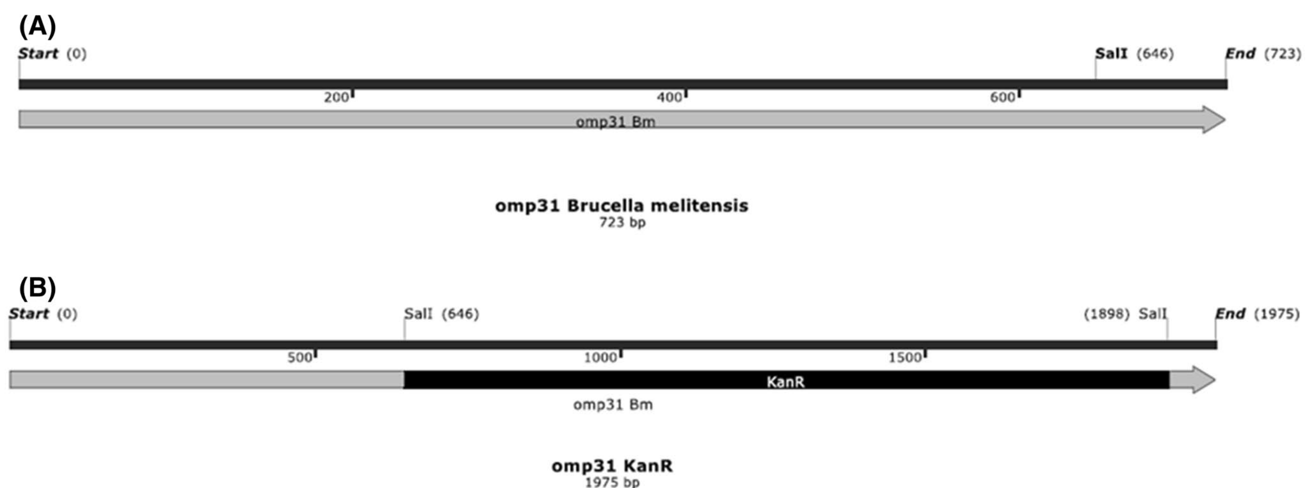

Fig. 1 Schematic view of the gene inactivation performed in *B. melitensis* 133. **a** Fragment of 723 bp of the *omp*31. **b** Inactivation by inserting a kanamycin cassette into the *Sal* I restriction site of *omp*31

the number of CFU, absorbance was measured at 4, 6, 8, 12, 16, 24, 48, 72, 96, and 120 h of culture and 20 ml of a 10^{-6} dilution were platted in triplicate on Brucella agar with or without antibiotic to count colony-forming units (CFUs).

Susceptibility assays

To evaluate the susceptibility of *Brucella* strains to polymyxin B (Sigma–Aldrich) and sodium deoxycholate (Sigma–Aldrich), bacterial suspensions containing 1×10^5 CFU/ml were prepared, and 1 ml of them was mixed in wells of a 24-well sterile plate with 1 ml of 2-mg/ml polymyxin B or 0.2-mg/ml sodium deoxycholate (final concentrations in the wells, 1 and 0.1 mg/ml, respectively). After a 2-h incubation at 37 °C in a 5% CO_2 atmosphere, the content of each well was mixed, and 50 µl were spread by triplicate on BA-YE-FBS plates supplemented with antibiotic(s) when required. The CFU obtained after each treatment were counted, and the percentages of survival were established with respect to a control culture of bacteria incubated in PBS (100% survival) (De Tejada et al. 1995). To determinate the susceptibility to nonimmune serum, 5 ml of serum was obtained from a specific free pathogens (SPF) goat. Half of the serum was heated at 56 °C for 30 min to remove complement (control serum) and the other half was used fresh. Briefly, 1 ml of bacterial suspensions with approximately 1×10^5 CFU/ml in PBS were mixed in wells of a 24-well sterile plate with 200 µl of either fresh serum or heated serum. After a 4-h incubation at 37 °C in a 5% CO_2 atmosphere, the content of each well was gently mixed by pipetting and 50 µl of each bacterial suspension were spread in triplicate on BA-YE-SFB plates supplemented with antibiotic(s) when required. The CFU obtained after exposure to fresh serum were counted, and the percentages of survival were established with respect to the CFU obtained with the heated serum (100% survival) (Corbeil et al. 1988).

Cell culture and infection of HeLa and J774.A1 cells

Prior to infection, cell suspensions were prepared in the basal medium Dulbecco's Modified Eagle [Dulbecco's Modified Eagle Medium (DMEM)] (Gibco-BRL), supplemented with fetal bovine serum (FBS); 5% (v/v) for HeLa cells and 10% (v/v) for murine macrophages, and with 4-mM L-glutamine (complete DMEM medium) at a concentration of 1×10^5 cells/ml. Subsequently, cells were distributed in 24-well culture plates (1 ml/well) and incubated for 18 h in the case of HeLa cells, and 24 h for macrophages J774.A1. To perform infection assays, *Brucella melitensis* Bm133 and *Brucella melitensis* LVM31 were grown on *Brucella* agar plates (supplemented or not with antibiotic as required) for 48 h, and thus, five colonies were grown in 5 ml of *Brucella* broth, at 37 °C, 150 rpm for 22 h. A 1/50 dilution of each culture in 200 ml of *Brucella* broth was performed, with incubation at 37 °C, 150 rpm for 26 h. From this culture, serial dilutions 1/2 to 1/10 of the bacteria in *Brucella* broth were made for a multiplicity of infection (MOI) of 100:1 for murine macrophages and 300: 1 for HeLa cells.

Statistical analysis

Analyses were performed using the commercial package for statistical analysis R®, using the Chi-square method with a normal distribution of data by time, achieving greater significance of 99% ($P < 0.01$). Statistical analysis took into account the absolute UFC values obtained in survival tests to determine whether the observed variation in the number of CFU is attributed to the dependent variable; for purposes of our studies, it was the intracellular survival of *B. melitensis* LVM31 mutant strain in HeLa cells and murine macrophages.

Results

Amplification and inactivation of the gene encoding Omp31 in *B. melitensis* Bm 133 by homologous recombination

The *omp31* gene was amplified and interrupted as described in methodology. Thus, to obtain the mutant strain, the pLVM31 plasmid was transformed in *B. melitensis*. Bacteria were spread on Brucella agar plates supplemented with 5% FBS, 3% YE, and 50 µg of kanamycin, which were incubated at 37 °C for 48 h. Kanamycin-resistant and ampicillin sensitive transformants were selected. DNA extraction was performed to verify, by PCR, the integration of the inactivated omp31 gene by homologous recombination into the genome of the wild strain (Fig. 2).

Growth rate of *Brucella* strains

To determine the effect of interrupt *omp31* on the growth rate, growth kinetics using control and mutant strains were performed and CFU/ml were determined by the method of Miles and Misra at different times during 120 h (Slack and Wheldon 1978). A significant decrease ($P < 0.01$) in the growth rate of the mutant strain *B. melitensis* LVM31 was observed at 4, 12, 16, 24, 48, 72, 96, and 120 h as compared to the field *B. melitensis* 133 and *B. melitensis* Rev1 vaccine strains (Fig. 3).

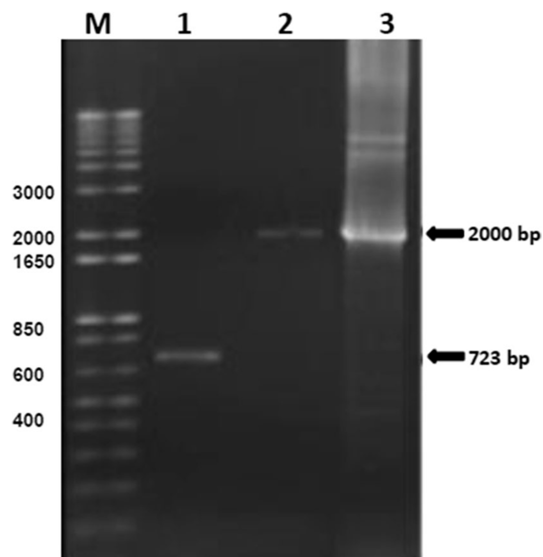

Fig. 2 Agarose gel electrophoresis of PCR-amplified *omp*31 gene fragments from *Brucella* prototype strains. *Lane M* 1 kb "*ladder*" plus. *Lane 1* amplified omp31 fragment (723 bp) from *B. melitensis* Bm133 strain (Wild-Type). *Lane 2* amplified omp31 fragment (2000 bp) from *B. melitensis* LVM31 (mutant strain). *Lane 3* amplified omp31 fragment (2000 bp) from pLVM31 plasmid

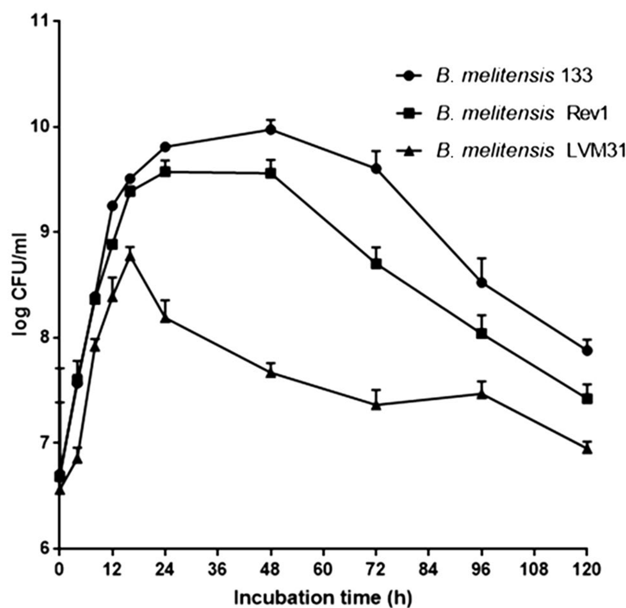

Fig. 3 Growth kinetics of *B. melitensis* 133, *B. melitensis* Rev1, and *B. melitensis* LVM31. The results are expressed as the means ± SD ($n = 3$) of the log CFU/well at each time point

Role of the Omp31 on the OM properties of *Brucella melitensis*

To indirectly evaluate the OM integrity of the *B. melitensis* strains under study, the susceptibility of them to polymyxin B (1 mg/ml), sodium deoxycholate (0.1 mg/ml), and serum was determined. A 100% survival for each strain was assessed, as described in "Materials and methods" section. A statistically significant decrease ($P < 0.001$) in mutant survival exposed to polymyxin B and sodium deoxycholate (42.32 and 50.71% respectively) compared with both *B. melitensis* Bm133 (77.15 and 86.52 respectively) and *B. melitensis* Rev1 (68.57 and 74.28% respectively) strains was observed. In contrast, LVM31 mutant strain was only more susceptible ($P < 0.01$) (59.53%) to serum than *B. melitensis* Bm133 strain (70.59%) (Fig. 4). Therefore, Omp31 could be involved in maintaining outer membrane integrity and bacterial virulence.

Role of the Omp31 protein in intracellular survival of *Brucella melitensis*

To determine the effect of *omp31* mutation, the intracellular survival of *B. melitensis* 133 and *B. melitensis* LVM31 strains in HeLa cells and murine macrophages was evaluated. The number of viable bacteria in the assay was determined at 0-, 4-, 8-, 12-, 24-, 48-, and 72-h postinfection. The number of CFU for *B. melitensis* LVM31 mutant strain invading the macrophage and HeLa cells (time zero) was lower statistically significant ($P < 0.001$) (1.5×10^3 and 4.1×10^3 CFU/ml respectively) as compared with *B. melitensis* 133 strain (3.8×10^4 and 5.4×10^4 CFU/ml respectively). Intracellular survival of *B. melitensis* LVM31 was lower statistically significant ($P < 0.001$) in both HeLa cells and murine macrophages at any sampling time as compared with *B. melitensis* Bm133 wild-type strain (Fig. 5).

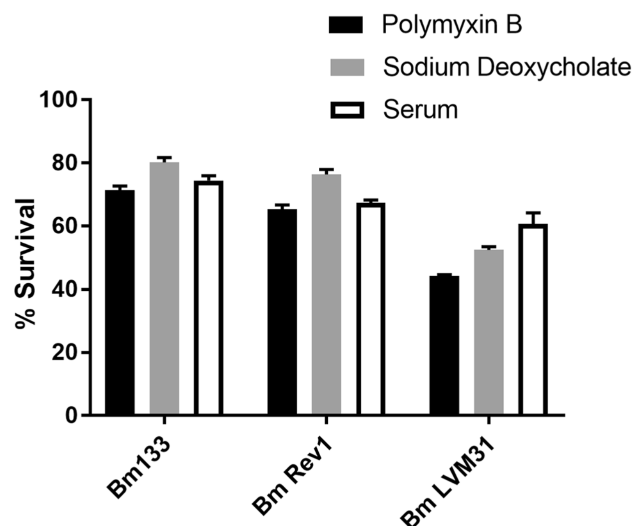

Fig. 4 Susceptibility to polymyxin B, sodium deoxycholate, and non-immune serum of *B. melitensis* Bm133, *B. melitensis* Rev1, and *B. melitensis* LVM31

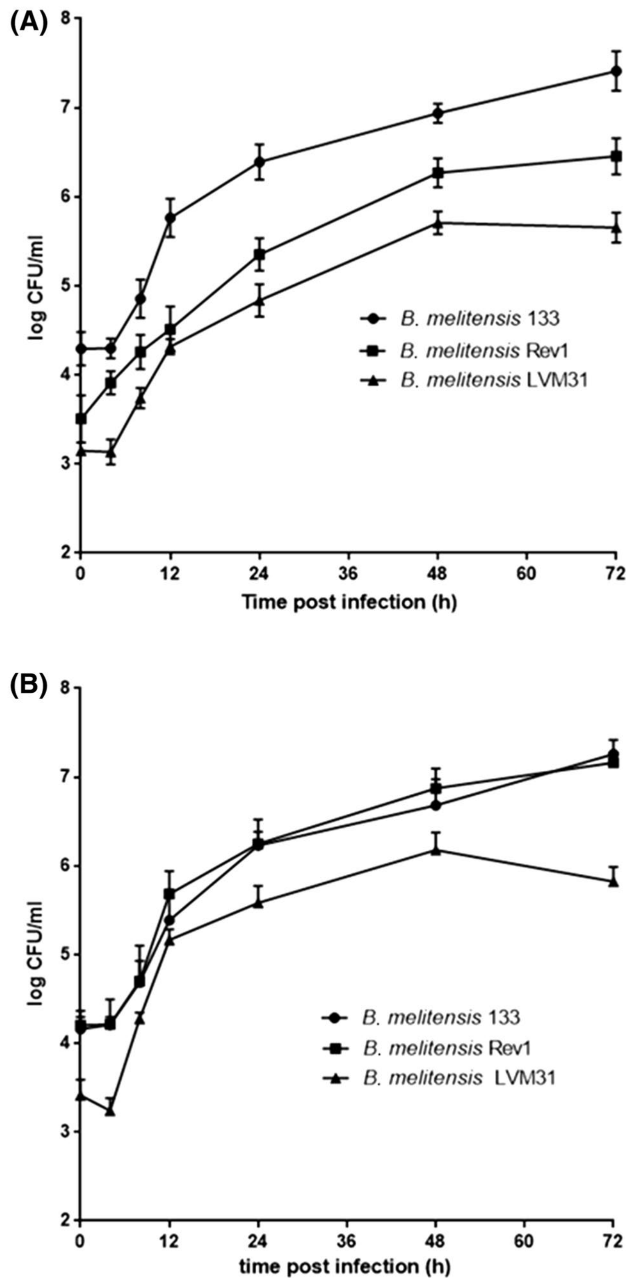

Fig. 5 Evaluation of the intracellular survival in murines macrophages (**a**) and HeLa cells (**b**) of wild-type *B. melitensis* Bm133 and the mutant strain *B. melitensis* LVM31. Results are expressed as means ± SD of the log CFU/ml; the sampling at different times was made by triplicate

Discussion

The resistance of *Brucella* spp. to oxygen-independent mechanisms has not been explained on a structural basis. Oxygen-independent mechanisms include the synergistic actions of several cationic proteins and peptides which, in a first step, bind to OM anionic targets and render the cell envelope permeable and susceptible to lytic enzymes, thereby blocking cell functions that depend on membrane integrity (Groisman 1994; Lehrer et al. 1993). The previous studies have shown that *Brucella* envelope has permeability properties and sensitivities to anionic detergents (de Tejada and Moriyon 1993) that are different from those common to Gram-negative bacteria, and on this basis, it has been suggested a relationship between *Brucella* OM properties and its pathogenicity. In this work, *B. melitensis* LVM31 mutant strain was studied for its resistance to polymyxin B, sodium deoxycholate, and nonimmune serum compared to control strains. Polymyxin B and sodium deoxycholate were used to determine OM stability and also as an indicator of *B. melitensis* LVM31 mutant susceptibility to the bactericidal effect mediated by cationic peptides in the host. In this case, *B. melitensis* LVM31 mutant was more susceptible to polymyxin B and sodium deoxycholate ($P < 0.001$) as compared with wild-type and vaccine strains. *Brucella* spp. are also at least partially resistant to killing mediated by the action of host nonimmune serum (Corbeil et al. 1988; Eisenschenk et al. 1999; Estein et al. 2004), which may contribute to virulence. Thus, *B. melitensis* LVM31 mutant survival was evaluated in the presence of nonimmune goat serum. Our results showed that mutant strain is more susceptible to serum action as compared with the wild-type strain ($P < 0.01$). These results suggest that Omp31 might be involved in maintaining the integrity of *B. melitensis* OM. As mentioned, *Brucella* is a facultative intracellular bacterium. Importantly, smooth *Brucella* species are able to enter, survive, and multiply in professional phagocytic cells as macrophages and dendritic cells as well as in non-phagocytic cells such as trophoblastic cells and epithelial cells (HeLa cells) (Billard et al. 2007; Pizarro-Cerdá et al. 2000). Accordingly, we set out to determine the effect of mutate *omp31* in *B. melitensis* by evaluating internalization and intracellular survival of the mutant strain in HeLa cells and murine macrophages. Our results indicated that *omp31* mutation caused a significant decrease in *Brucella* intracellular survival, both in murine macrophages and HeLa cells. These results differ from those obtained in other studies (Martín-Martín et al. 2008), where different mutant strains of *B. ovis* PA on genes belonging to the omp25/omp31 family were evaluated. On that instance, only the absence of Omp25d or Omp22 severely affected bacterial intracellular replication. Thus, while *Δomp25d* mutant strain was able to survive in macrophages, *Δomp22* mutant strain was completely eliminated at 24-h postinfection. Therefore, these contradictory results could be explained considering that the O chains in S-LPS mask other surface antigens such as outer membrane proteins (OMPs), preventing both a specific immune response against them and antibodies accessibility (Bowden et al. 1995; Cloeckaert et al. 1991). Conversely, rough strains of *Brucella*, such as *B. ovis and B. canis*, do not have this problem, since they lack

of O-polysaccharide, and thus, OMPs are exposed on the bacterial surface (Bowden et al. 2000; Vizcaíno et al. 2004; Vizcaíno et al. 2001). Based on these results it is necessary to evaluate the role of the OMP25/31 family in B. melitensis to clarify differences between smooth and rough phase species of *Brucella*.

Achieving a replication niche in macrophages, the main host cells for this pathogen, provides protection to complement and antibodies action, allowing for dissemination into the host, and to remain for longer periods of time in the organism, resulting in subsequent chronic infection. This ability of *Brucella* smooth phase strains involves a complex process in which this pathogen interferes with host cell functions even to control their own intracellular trafficking. Thus, when a smooth *Brucella* strain is phagocytized by the macrophage, it starts an endocytic pathway in which prevents hydrolytic degradation by fusion with lysosomes, favoring in early stages of infection and intracellular survival (Celli 2006). Brucella virulence is associated with survival in phagocytic cells. The results obtained in this study highlight a relationship between a decrease in intracellular survival and replication of *B. melitensis* LVM31 mutant strain in both murine macrophages in HeLa cells and the absence of Omp31. Therefore, Omp31 is involved on maintaining outer membrane integrity, but also could be relevant to virulence of *B. melitensis* 133 by exerting important roles in intracellular survival and multiplication, although studies must be done in mice to test this hypothesis.

Acknowledgements This work was supported by PAPIIT IN212610, PAPIIT IN-221513, and PAPIIT IN-222516, UNAM. The authors acknowledge Beatriz Arellano for her technical support and Mrs. Francisca Muñoz for her administrative support.

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