Pakistan J. Zool., vol. 49(5), pp 1809-1816, 2017. **DOI**: http://dx.doi.org/10.17582/journal.pjz/2017.49.5.1809.1816

Construction and Characteristic Analysis of Omp10 Deletion Mutant of *Brucella abortus*

Tiansen Li[1], Meiling Huang[2], Zhen Wang[1], Fei Guo[3], Hui Zhang[1,*] and Chuangfu Chen[1,*]

[1]*College of Animal Science and Technology, Shihezi University, 832000, Shihezi, Xinjiang, China*
[2]*College of Life Science, Shihezi University, 832000, Shihezi, Xinjiang, China*
[3]*College of Medicine, Shihezi University, 832000, Shihezi, Xinjiang, China*

Article Information
Received 23 July 2016
Revised 15 December 2016
Accepted 24 February 2017
Available online 14 September 2017

Authors' Contribution
TL, MH and FG performed experiments. ZW designed and conducted analysis and HZ performed research. CC did supervision of the work and provision of funds.

Key words
Brucella, Outer membrane protein, Brucella-containing vacuole, Lysosome, Apoptosis.

ABSTRACT

Brucella is pathogenic bacteria that cause animal and human brucellosis. Currently, the mechanism behind the pathogenesis of *Brucella* remains unclear. For this reason *omp10* mutant was constructed to examine the impacts of the outer membrane protein 10 (Omp10) on bacterial survival, virulence, phagosome-lysosome fusion, and apoptosis induction, as assessed in appropriate *in vitro* (cell culture) and animal models. Results showed the *omp10* mutant was dramatically attenuated for survival in macrophages and mice than the parent strain 2308. With decrease in the spleen/body weight ratios of mice infected with *omp10* mutants were noted, inhibition of phagosome-Lysosome fusion was dramaticly impaired, and profound increase of apoptosis rate after exposure of RAW264.7 to *omp10* mutants at 12 h, 24 h and 48 h compared to RAW264.7 exposed to 2308. Results indicate that Δ*omp10* mutant would affect its survival and pathogenicity.

INTRODUCTION

Brucella is gram-negative bacteria and host specific intracellular pathogens that cause human disease and infection of livestock, leading to significant worldwide economic losses. *Brucella abortus* is the causative agent of brucellosis in cattle which results in abortions or birth of weak calves. In humans, chronic *Brucella* infection can lead to reproductive defects as well as undulant fever. It is also wide-spread zoonotic infection in the developing as well as many developed countries (Boschiroli *et al*., 2001; Seleem *et al*., 2010). The mechanism of entry into macrophage and intracellular trafficking varies based on the opsonization status of the *Brucella* (Gorvel and Moreno, 2002). *Brucella* is able to multiply within professional and nonprofessional phagocytes, but the exact mechanisms whereby *B. abortus* intracellularly parasitize the hosts are still unclear (Kim *et al*., 2012).

Outer membrane proteins (Omps) are important immunogens in most of the gram negative bacteria (Moriyón and López-Goñi, 2010). The molecular characterization of several Omps has been reported over the past years (Caro-Hernández *et al*., 2007). The gene *omp25*, *omp31* and *omp2b* encoding the major 25-, 31-, and 36-kDa *Brucella* Omps, respectively, have been cloned and sequenced. Omp2b functions as a porin. The cloning and sequencing of the gene encode three less abundant (minor) Omps has been previously reported. These three minor Omps are expressed in all six species and all of their biovars (Caro-Hernández *et al*., 2007; Estein *et al*., 2003; Jubier-Maurin *et al*., 2001; Mobasheri *et al*., 1997; Tibor *et al*., 1999). Omp10 and Omp19 are among the most abundant proteins in the outer membrane of *Brucella*, having similar antigenic determinant with rhizobia bacteria. Decreased virulence was shown by Δ*omp19* mutant strains (Pasquevich *et al*., 2009; Tibor *et al*., 2002; Verstreate *et al*., 1982).

Brucella has the ability to enter phagocytic and non-phagocytic cells by endocytosis, among which macrophages are major targets in the process of infection, and survive in *Brucella*-containing vacuole (BCV) (Pizarro-Cerdá *et al*., 1998a). Typically, survival of *Brucella* relies upon avoiding fusion of the intermediate BCVs with lysosomes. Cyclic glucans have been proven to modulate maturation of BCVs to avoid fusion with lysosomes (Arellano-Reynoso *et al*., 2005). It also has been proven that the rough LPS mutants of *B. suis* lacking the LPS O-chain entered cells by lipid raft-independent pathway and BCV fuse rapidly with lysosomes (Porte *et al*., 2003).

In recent years, chronic *Brucella* infection have trended upward, which is seriously harmful to human

* Corresponding author: 604118228@qq.com; chuangfu_chen@163.com
0030-9923/2017/0005-1809 $ 9.00/0

health and economic development (Olsen and Stoffregen, 2005). However, the mechanisms underlying the pathogenesis of *Brucella* remains unclear. Omp10 is the major outer membrane protein of *Brucella* and also key virulence factor closely related to intracellular survival of *Brucella* (Tibor *et al*., 2002). Here we focused on the role of the Omp10 in intracellular survival and pathogenicity in infected mice. This study might reveal further pathogenic mechanisms of *Brucella*.

MATERIALS AND METHODS

Bacterial strains, cell lines, mice

B. abortus strain 2308 was obtained from the Center of Chinese Disease Prevention and Control (Beijing, China) and *B. abortus* 2308 Δ*omp10* deletion mutant was engineered for this study. The *B. abortus* strains were cultured in tryptone soya agar (TSA) or tryptone soya broth (TSB) (OXOID, England). Following two days of incubation, pure culture of each strain was harvested in normal saline and pelleted by centrifugation (1700 xg). The pellet of each strain was washed twice with phosphate-buffered saline (PBS) (1M, PH=7.4). *Escherichia coli* strain DH5α was grown on Luria-Bertani (Difco, Becton Dickinson) plates or in broth overnight at 37°C with or without ampicillin (50 mg/liter). The RAW264.7 murine macrophage was purchased from Cell Resource Center, IBMS, CAMS/PUMC (Beijing, China), which was maintained at 37°C in 5% CO_2 atmosphere in DMEM (Gibco, USA) containing 10% fetal bovine serum (FBS) (Gibco, USA). Cells were plated in 6-well tissue culture plates (Nalge Nunc International, Naperville, and III). Six-week-old BALB/c female mice were obtained from Experimental Animal Center of Academy Military Medical Science (Beijing, China). All experimental procedures and animal care were performed in compliance with institutional animal care regulations

Construction of the 2308 Δomp10 deletion mutant

The 2308 Δ*omp10* deletion mutant was constructed in 2308 as previously described with some modifications. The sequence upstream of the *omp10* gene was amplified from *B. abortus* 2308 with the primer pair 5'-*GGA TCC* TAT AGG GCT GGA GCC ATT CT-3'and 5'-CTA ATG GAG AGC ATG GAC TCC CTT TTT GGA AAA CAG AA -3' the sequence downstream of *omp10* was amplified with the primer pair 5'-GTC CAT GCT CTC CAT TAG AAA-3' and 5'-*GAG CTC* GCG TGA AGG GTA TCC ATA TC-3'. These two products were ligated to one another via overlapping PCR with *BamH* I and *Sac* I sites (underlined) engineered between the two sequences. And a *SacB* gene (1477bp), which is a selectable marker gene from *B. subtilis*. Primer sequences are the following: *SacB*-F, 5'-*GAG CTC* GGG CTG GAA GAA GCA GAC CGC TA-3', *SacB*-R, 5'-*GAG CTC* GCT TAT TGT TAA CTG TTA ATT GTC *C-3'*, and inserted within the vector at the unique *Sac* I site (underlined). Those products were then ligated to pGEM-7zf+ to generate suicide plasmid pGEM-omp10-SacB (pOS). Competent *B. abortus* 2308 was electroporated with pOS and potential 2308 Δomp10 deletion mutant was isolated by its Ampr and sucrose phenotype. The mutant was further confirmed by PCR amplification with primers *omp10*-I-F (5'-GTG CCG AGA TTG ACC AGC-3') and *omp10*-I-R (5'-GCC ACT ATG CCT ACC ACC C-3'), which are located in upstream and downstream of homologous arm of omp10, respectively. PCR products were sequenced to confirm the product.

Determination of intracellular viability

Macrophages were seeded in six-well plates and filled with DMEM with 10% FBS, infected at a multiplicity of infection (MOI) of 1:100 at 37 °C with *B. abortus* 2308 and *B. abortus* 2308 Δ*omp10*. 45 min incubation, the cells were washed 3 times with medium without antibiotics and then incubated with 50 μg/ml of gentamicin for 1 h to kill extracellular bacteria. After 1, 4, 12, 24, 48 h post-infection and bacteria were collected. And the live bacteria were enumerated by plating on TSA plates. All assays were performed in triplicate and repeated at least three times.

Virulence determination in mice

To test the virulence of *B. abortus* 2308 Δ*omp10* deletion mutant, groups of six-week-old female BALB/c mice were inoculated intraperitoneally (i.p.) with 1×10^5 CFU of *B. abortus* 2308 Δ*omp10* deletion mutant and *B. abortus* 2308. At 3, 5, 7, 14 and 28 days post-inoculation, five mice from each treatment group were euthanized by CO_2, spleens were weighed, and bacterial survival was determined following homogenization of the mouse spleens in 1 ml of saline. Serial dilutions of the spleen homogenates were placed on TSA plates for bacterial enumeration and to assess the virulence of each strain.

For pathological studies, spleens and livers from infected and PBS-treated mice were fixed in 10% neutral buffered formalin, processed and stained with hematoxylin and eosin or Giemsa stain.

Phagosome-lysosome fusion measurement

Cells were seeded on glass coverslips and infected by all of the strains as described above. After 4, 12, 24, 48 h post-infection, cells were washed three times with PBS (1M, PH=7.4), 5 min each time. Green fluorescence from Cell Navigator Lysosome Staining Kit was added to cells in 6-well plate and incubated for 2 h. Then cells

were washed three times before and fixed for 30 min in 4% paraformaldehyde. Then the cells were washed three times again and permeabilized for 10 min in 0.3% Triton at room temperature. Then the cells were incubated for 20 min by 1% Bovine Serum Albumin (BSA). Coverslips were washed three times with PBS (1M, PH=7.4). Then coverslips were incubated with the primary antibodies in PBS sheep anti-*B.abortus* IgG antibodies for 2 h at 37°C or overnight at 4°C, washed in PBS, and then incubated with the secondary antibodies Rhodamine (TRITC) conjugated AffiniPure donkey anti-sheep IgG antibodies for 1 h at 37°C. The coverslips were mounted onto glass slide using 50% glycerinum. Cells were observed by confocal laser scanning microscope (Zeiss LSM710) used 64×oil immersion objectives. To determine the percentage of bacteria that colocalized with the lysosome marker 100 intracellular bacteria were counted. The assays were performed three times.

Flow cytometry

Apoptosis of RAW264.7 was studied by flow cytometric analysis. After the infection of RAW264.7 by Δ*omp10* and 2308, about 1-5×10^5 cells of each sample were collected, suspended with Binding Buffer and treated by Annexin V-FITC and PI at room temperature. After 5-15 min reaction in the dark we detected the apoptosis of RAW264.7 using flow cytometer. The assays were performed in three times.

Statistical analysis

To determine the significance of differences observed in our experiments, pairwise comparisons were performed by Scheffe tests, after a two-way analysis of variance providing the residual mean square estimate with the highest available degree of freedom number.

RESULTS

B. abortus *2308 Δomp10 mutant is attenuated compared with* B. abortus *2308 for survival in RAW264.7 murine macrophages*

RAW264.7 macrophages were infected with the *B. abortus* 2308 and Δ*omp10* mutant at a MOI of 1:100 and the bacteria the survived were enumerated. At 0 h, there were no significant difference in the number of survived bacteria between the Δ*omp10* mutants and *B. abortus* 2308 strains. 4 h post-infection, the number of bacteria had an initial decrease in both group. By 12 h post-infection, there was a 1.12-log (P <0.05) decrease in the bacteria number of *B. abortus* 2308 Δ*omp10* mutant compared with that of *B. abortus* 2308. At 24 h post-infection, there was a 2.89-log (P <0.01) decrease in the bacteria number of *B. abortus* 2308 Δ*omp10* mutant compared with that of *B. abortus* 2308. And at 48 h post-infection, the decrease in the number of *B. abortus* 2308 Δ*omp10* mutant was more significant (3.86-log fold; P <0.01) compared with *B. abortus* 2308 (Fig. 1).

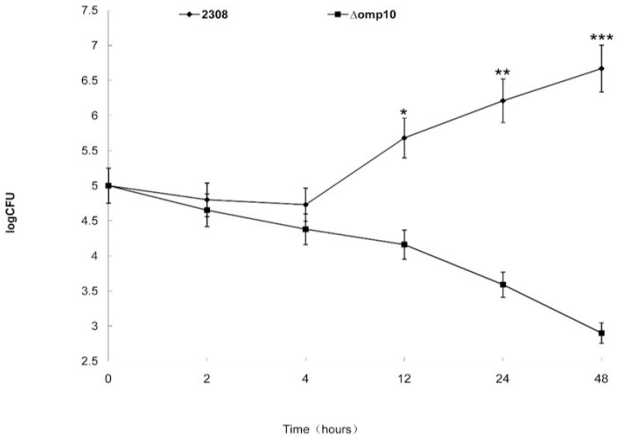

Fig. 1. Intracellular survival of *B. abortus* 2308(♦), Δomp10 (■) mutants in murine RAW264.7 cells. Macrophages were infected with the strains, and incubation at 37 °C for the indicated time. Bacteria intracellular growth in RAW264.7 cells was determined at different incubation time points by counting the viable intracellular bacteria. Each point represents the mean±standard deviation of three experiments. Statistically significant differences between the bacterial growth of the parent strain and the mutants are indicated by asterisks (***, P<0.001).

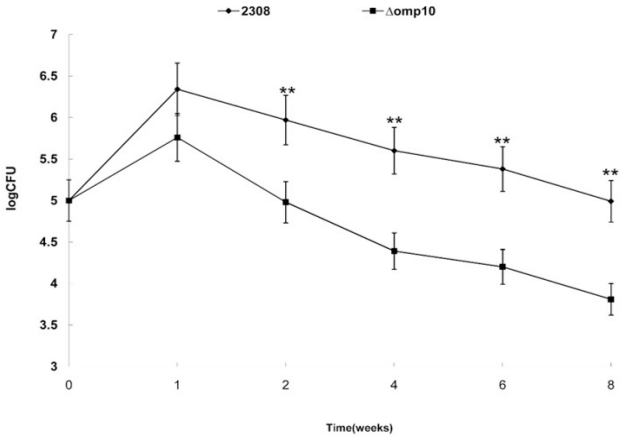

Fig. 2. Survival of *B. abortus* 2308 Δomp10 in the mouse model. Each group of mice was infected intraperitoneally with *B. abortus* 2308, Δomp10 mutant strains. Mice were infected by intraperitoneal injection with 1×10^5 *Brucella*. The values are shown as means ± SEM of samples from 10 mice. Each point represents the mean±standard deviation of three experiments. Statistically significant differences between the bacterial growth of the parent strain and the mutants are indicated by asterisks (***, P<0.001).

Survival of B. abortus *2308 Δomp10 in the mouse model*

To evaluate the survival capability of mutants, the mice were inoculated i.p 1×10^5 CFU of *B. abortus* 2308 and *B. abortus* 2308 Δ*omp10*. Compared to *B. abortus* 2308, splenic CFU in *B. abortus* 2308 Δ*omp10* infected mice were significantly reduced (P <0.01) at 7, 14, 21, 28, 35, 42 days. The counts of bacteria in spleens from mice infected with *B. abortus* 2308 Δ*omp10* mutants were always significantly lower than *B. abortus* 2308-infected mice during the 2-8 weeks, which indicates that survival capability of *B. abortus* 2308 Δ*omp10* mutant was attenuated (Fig. 2).

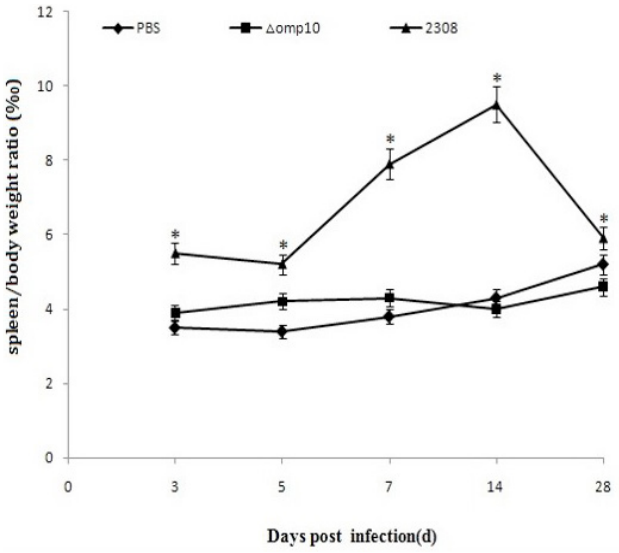

Fig. 3. The changes of spleen weight/body weight at different time points post-infection. At different time points (3d, 5d, 7d, 14d, 28d) after infection, five mice were sacrificed, quickly removed the spleen and weighed. Statistically significant differences between the bacterial growth of the parent strain and the mutants are indicated by asterisks (***, P<0.001).

Virulence of B. abortus *2308 Δomp10 in the mouse model*

Decreases in the spleen/body weight ratios of mice infected with *omp10* mutants were noted, with no significant difference compared to the PBS control. However, enlarged spleens were observed in 2308-infected mice (Fig. 3). The typical tissue response to *Brucella* infection is granulomatous inflammation. However, in the spleen and liver of mice *B. abortus* 2308 Δ*omp10* infection resulted in development of characterized by slight vasculitis and thrombosis, seldom influx of neutrophils (Fig. 4). Collectively, these data demonstrate a critical role for *omp10* in modulating the initial inflammation and pathology in response to *B. abortus* infection, which in turn benefits the pathogen due to enhanced bacterial survival.

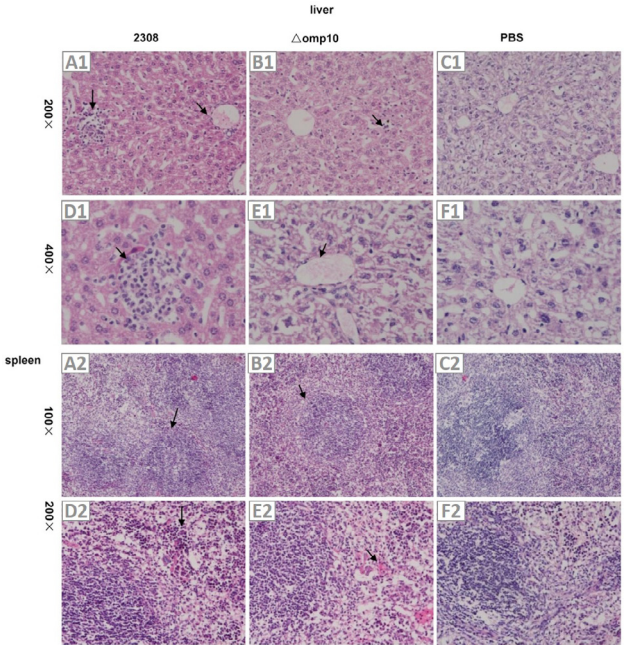

Fig. 4. Virulence of *B. abortus* 2308 Δomp10 in the mouse model. Liver pathological section (A1-F1), the effect of 2308 on liver (A1, D1), the effect of Δomp10 on liver (B1, E1), the PBS control (C1, F1); Spleen pathological section (A2-F2), the effect of 2308 on spleen (A2,D2), the effect of Δomp10 on spleen (B2, E2), the PBS control (C2, F2).

Comparison of phagosome-lysosome interaction between the parent strain and mutant

Phagosome-lysosome fusion was evaluated by the colocalization of both markers, and the markers were described in materials and methods (Fig. 5A). *B. abortus* 2308 Δ*omp10* is decreased compared with *B. abortus* 2308 for phagosome-lysosome fusion in RAW264.7 murine macrophages, 9% of Green Fluorescence-labelled phagosomes colocalized with lysosome at 4 h post-infection. Patterns were similar at 24 and 48 h post-infection (31% and 76%) (Fig. 5B). In contrast, only <10.0% of labelled phagosomes colocalized with these markers at 4 h and 24 h post-infection (Fig. 5B). All results showed that compared with *B. abortus* 2308, *B. abortus* 2308 Δ*omp10* couldn't inhibit the fusion of the phagosome and lysosomal.

The effect of Δomp10 on the apoptosis of RAW264.7

About $1\text{-}5\times10^5$ RAW264.7 cells infected with *B. abortus* 2308 and *B. abortus* 2308 Δ*omp10* were collected and suspended with Binding Buffer and treated by Annexin V-FITC and PI at room temperature. After 5-15 min reaction in the dark we detected the apoptosis of RAW264.7 using flow cytometer (Fig. 6A). The results

showed that *B. abortus* 2308 Δ*omp10* compared with *B. abortus* 2308 couldn't inhibit cell apoptosis (Fig. 6B), indicating that the cells apoptosis was increased in *B. abortus* 2308 Δ*omp10* groups.

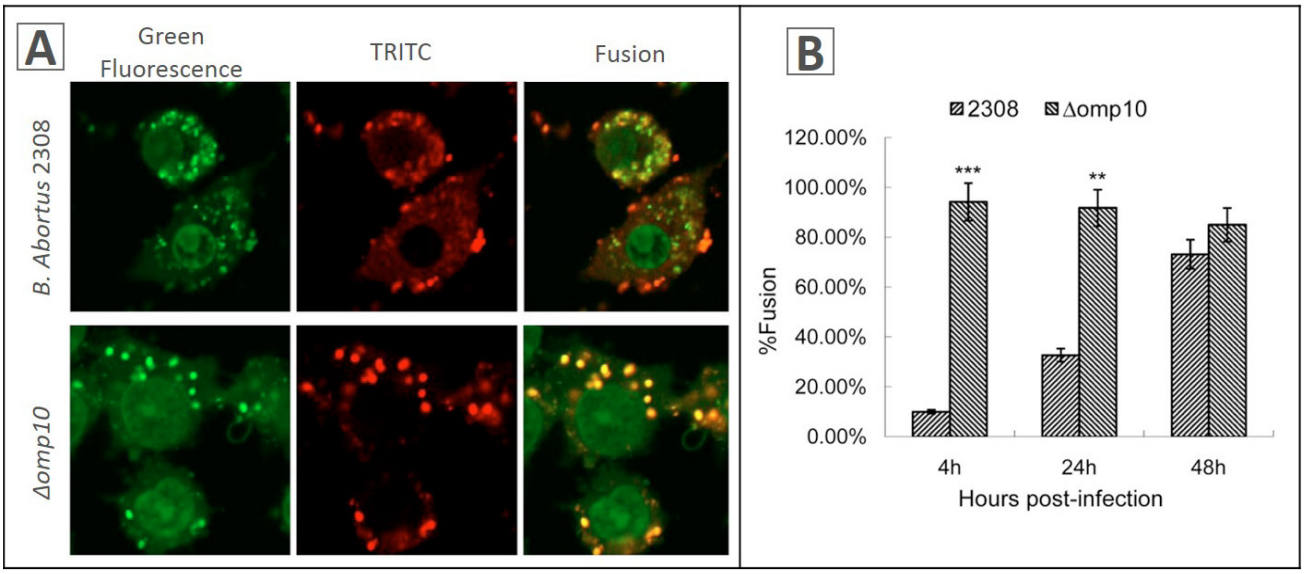

Fig. 5. Interaction of *B. abortus* 2308- and mutants-containing phagosomes with lysosomes in RAW264.7 macrophages. *B. abortus* were labeled with sheep anti-*B. abortus* IgG antibodies and Rhodamine (TRITC) conjugated AffiniPure donkey anti-sheep IgG antibody, lysosomes were labeled with Cell Navigator™ Lysosomes Staining Kit Green Fluorescence. A, cells were fixed at different time points after infection. Confocal images of cells containing *B. abortus* were obtained at 4 h post-infection; B percentage of phagosome-lysosome fusion at different time points after infection. Fusion was evaluated by the colocalization of markers, Green fluorescein and TRD. To determine the percentage of fusion, bacteria were analyzed at each time point.

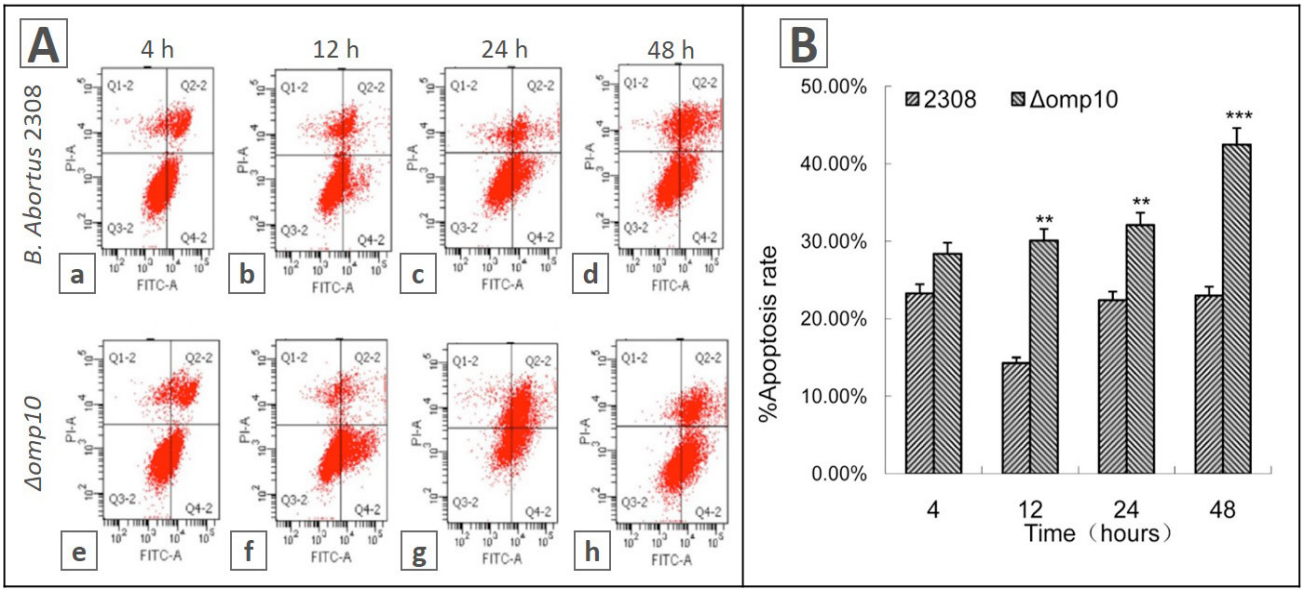

Fig. 6. Flow cytometric analysis of RAW264.7 macrophages exposed to *B. abortus* 2308 and *omp10* mutants. RAW264.7 macrophages were suspended with Binding Buffer and treated by Annexin V-FITC and PI at room temperature. A, cells were collected at different time points after infection, cell apoptosis images containing *B. abortus*. B, percentage of apoptosis at different time points after infection.

DISCUSSION

Omps of Gram-negative bacteria, play an important role in bacterial pathogenesis (Krinos *et al*. 1999). *Brucella* Omps are essential for antibiotic resistance, virulence regulation, and other unknown functions in bacteria (Verstreate *et al*., 1982). *Brucella* Omp10 belongs to lipoprotein (Tibor *et al*., 2002), which is one of the most abundant proteins in the outer membrane of *Brucella*, has been shown to play key roles in nutrient uptake, signal transduction, adhesion and antibiotic resistance (Kim *et al*., 2012).

We constructed the deletion mutant of *omp10*, which showed that the intracellular viability of the *omp10* mutants in RAW264.7 cells were decreased compared to that of the parent strains. Surface properties changed by Omp10 may influence the intracellular viability of bacteria.

B. abortus inhibited phagosome-lysosome fusion after uptake by macrophages, allowing survival and replication (Pizarro-Cerdá *et al*., 1998b; Weber *et al*., 2009). However, inhibition of phagosome-Lysosome fusion was strikingly impaired by *omp10* mutants, suggesting that the lipoprotein contributes to intracellular trafficking of *B. abortus* within phagocytes. *B. abortus* could inhibit apoptosis of macrophages through the dependence of O side chain of polysaccharides after infection (Maria-Pilar *et al*., 2005). However, suppression of apoptosis was attenuated by *omp10* mutants as shown in our study, since deletion of the *omp10* may affect the *Brucella* outer membrane lipid, and influence the surface properties of the *Brucella* lipopolysaccharide (Tibor *et al*., 1999). We also found that survival of *omp10* mutants in mouse model was attenuated, correlated with defective intracellular survival as assessed in RAW264.7 cell model. Indeed, lipoproteins are crucial for the survival of *B. abortus* in mice. Splenomegaly induced by *B. abortus* was also ameliorated by *omp10* mutants at 2 weeks post-inoculation. These results, together with previous reports suggest that lipoprotein is the key mediator of the pro-inflammatory response (Giambartolomei *et al*., 2004), as well as vital factor needed to evade lysosome fusion to multiply inside the phagocytes (Kim *et al*., 2002; Naroeni *et al*., 2001; Naroeni and Porte, 2002; Porte *et al*., 2003; Starr *et al*., 2008). Other pathogenic bacteria are known to avoid fusion with lysosome as has been demonstrated with *Mycobacterium avium* and *Mycobacterium tuberculosis* (Armstrong and Hart, 1971). *Legionella* is another intracellular pathogen that is well adapted to life inside phagosome (Amer *et al*., 2006). Virulent strain 2308 possesses mechanisms to escape lysosome degradation (Pizarro-Cerdá *et al*., 1998b). In contrast, it is clear that the Δ*omp10* mutant can invade cells but multiplies poorly because the bacteria degrad after the *Brucella*-containing phagosomes fuse with lysosome. The results obtained here suggest that Δ*omp10* would be a good vaccine against *B. abortus* (Moreno *et al*., 1981).

CONCLUSION

Collectively, the results from our study showed that Δ*omp10* mutant could be attributed to altered outer membrane properties which would influence its intracellular survival and pathogenicity. Further studies are necessary to establish Omp10 function in exploiting a good vaccine against *B. abortus* infection.

ACKNOWLEDGEMENTS

This work was supported by the National Science and Technology Major Project (2013DFR31110), and the National Natural Science Foundation of China (31572494, 31260596, 31460650, 31360610, 31402166).

Conflict of interest statement

Authors declare no conflict of interest.

REFERENCES

Amer, A., Franchi, L., Kanneganti, T.D., Body-Malapel, M., Özören, N., Brady, G., Meshinchi, S., Jagirdar, R., Gewirtz, A. and Akira, S., 2006. Regulation of legionella phagosome maturation and infection through flagellin and host Ipaf. *J. biol. Chem.,* **281**: 35217-35223. https://doi.org/10.1074/jbc.M604933200

Arellano-Reynoso, B., Lapaque, N., Salcedo, S., Briones, G., Ciocchini, A.E., Ugalde, R., Moreno, E., Moriyón, I. and Gorvel, J.P., 2005. Cyclic β-1, 2-glucan is a Brucella virulence factor required for intracellular survival. *Nature Immunol.,* **6**: 618-625. https://doi.org/10.1038/ni1202

Armstrong, J. and Hart, P.A., 1971. Response of cultured macrophages to Mycobacterium tuberculosis, with observations on fusion of lysosomes with phagosomes. *J. exp. Med.,* **134**: 713-740. https://doi.org/10.1084/jem.134.3.713

Boschiroli, M.L., Foulongne, V. and O'Callaghan, D., 2001. Brucellosis: a worldwide zoonosis. *Curr. Opin. Microbiol.,* **4**: 58-64. https://doi.org/10.1016/S1369-5274(00)00165-X

Caro-Hernández, P., Fernández-Lago, L., de Miguel, M.J., Martín-Martín, A.I., Cloeckaert, A., Grilló, M.J. and Vizcaíno, N., 2007. Role of the Omp25/Omp31 family in outer membrane properties and

virulence of *Brucella ovis*. *Infect. Immun.,* **75**: 4050-4061. https://doi.org/10.1128/IAI.00486-07

Estein, S.M., Cassataro, J., Vizcaíno, N., Zygmunt, M.S., Cloeckaert, A. and Bowden, R.A., 2003. The recombinant Omp31 from *Brucella melitensis* alone or associated with rough lipopolysaccharide induces protection against *Brucella ovis* infection in BALB/c mice. *Microb. Infect.,* **5**: 85-93. https://doi.org/10.1016/S1286-4579(02)00075-8

Giambartolomei, G.H., Zwerdling, A., Cassataro, J., Bruno, L., Fossati, C.A. and Philipp, M.T., 2004. Lipoproteins, not lipopolysaccharide, are the key mediators of the proinflammatory response elicited by heat-killed *Brucella abortus*. *J. Immunol.,* **173**: 4635-4642. https://doi.org/10.4049/jimmunol.173.7.4635

Gorvel, J.P. and Moreno, E., 2002. *Brucella* intracellular life: from invasion to intracellular replication. *Vet. Microbiol.,* **90**: 281-297. https://doi.org/10.1016/S0378-1135(02)00214-6

Jubier-Maurin, V., Boigegrain, R.A., Cloeckaert, A., Gross, A., Alvarez-Martinez, M.T., Terraza, A., Liautard, J., Köhler, S., Rouot, B. and Dornand, J., 2001. Major outer membrane protein Omp25 of *Brucella suis* is involved in inhibition of tumor necrosis factor alpha production during infection of human macrophages. *Infect. Immun*., **69**: 4823-4830. https://doi.org/10.1128/IAI.69.8.4823-4830.2001

Kim, D.H., Lim, J.J., Lee, J.J., Kim, D.G., Lee, H.J., Min, W., Kim, K.D., Chang, H.H., Rhee, M.H. and Watarai, M., 2012. Identification of genes contributing to the intracellular replication of *Brucella abortus* within HeLa and RAW 264.7 cells. *Vet. Microbiol.,* **158**: 322-328. https://doi.org/10.1016/j.vetmic.2012.02.019

Kim, S., Watarai, M., Makino, S.I. and Shirahata, T., 2002. Membrane sorting during swimming internalization of *Brucella* is required for phagosome trafficking decisions. *Microb. Pathog.,* **33**: 225-237. https://doi.org/10.1006/mpat.2002.0531

Krinos, C., High, A. and Rodgers, F., 1999. Role of the 25 kDa major outer membrane protein of Legionella pneumophila in attachment to U-937 cells and its potential as a virulence factor for chick embryos. *J. appl. Microbiol.,* **86**: 237-244. https://doi.org/10.1046/j.1365-2672.1999.00667.x

Maria-Pilar, J.D.B., Dudal, S., Dornand, J. and Gross, A., 2005. Cellular bioterrorism: how *Brucella corrupts* macrophage physiology to promote invasion and proliferation. *Clin. Immunol.,* **114**: 227-238. https://doi.org/10.1016/j.clim.2004.07.010

Mobasheri, H., Ficht, T., Marquis, H., Lea, E. and Lakey, J., 1997. *Brucella* Omp2a and Omp2b porins: single channel measurements and topology prediction. *FEMS Microbiol. Lett.,* **155**: 23-30. https://doi.org/10.1111/j.1574-6968.1997.tb12681.x

Moreno, E., Speth, S., Jones, L.M. and Berman, D.T., 1981. Immunochemical characterization of *Brucella* lipopolysaccharides and polysaccharides. *Infect. Immun.,* **31**: 214-222.

Moriyón, I. and López-Goñi, I., 2010. Structure and properties of the outer membranes of *Brucella abortus* and *Brucella melitensis*. *Int. Microbiol.,* **1**: 19-26.

Naroeni, A., Jouy, N., Ouahrani-Bettache, S., Liautard, J.P. and Porte, F., 2001. *Brucella* suis-impaired specific recognition of phagosomes by lysosomes due to phagosomal membrane modifications. *Infect. Immun.,* **69**: 486-493. https://doi.org/10.1128/IAI.69.1.486-493.2001

Naroeni, A. and Porte, F., 2002. Role of cholesterol and the ganglioside GM1 in entry and short-term survival of *Brucella suis* in murine macrophages. *Infect. Immun.,* **70**: 1640-1644. https://doi.org/10.1128/IAI.70.3.1640-1644.2002

Olsen, S.C. and Stoffregen, W., 2005. Essential role of vaccines in brucellosis control and eradication programs for livestock. *Expert Rev. Vaccines,* **4**: 915-928. https://doi.org/10.1586/14760584.4.6.915

Pasquevich, K.A., Estein, S.M., Samartino, C.G., Zwerdling, A., Coria, L.M., Barrionuevo, P., Fossati, C.A., Giambartolomei, G.H. and Cassataro, J., 2009. Immunization with recombinant *Brucella* species outer membrane protein Omp16 or Omp19 in adjuvant induces specific CD4+ and CD8+ T cells as well as systemic and oral protection against *Brucella abortus* infection. *Infect. Immun.,* **77**: 436-445. https://doi.org/10.1128/IAI.00123-09

Pizarro-Cerdá, J., Méresse, S., Parton, R.G., van der Goot, G., Sola-Landa, A., Lopez-Goñi, I., Moreno, E. and Gorvel, J.P., 1998a. *Brucella abortus* transits through the autophagic pathway and replicates in the endoplasmic reticulum of nonprofessional phagocytes. *Infect. Immun.,* **66**: 5711-5724.

Pizarro-Cerdá, J., Moreno, E., Sanguedolce, V., Mege, J.L. and Gorvel, J.P., 1998b. Virulent *Brucella abortus* prevents lysosome fusion and is distributed within autophagosome-like compartments. *Infect. Immun*., **66**: 2387-2392.

Porte, F., Naroeni, A., Ouahrani-Bettache, S. and Liautard, J.P., 2003. Role of the *Brucella suis* lipopolysaccharide O antigen in phagosomal genesis and in inhibition of phagosome-lysosome

fusion in murine macrophages. *Infect. Immun*., **71**: 1481-1490. https://doi.org/10.1128/IAI.71.3.1481-1490.2003

Seleem, M.N., Boyle, S.M. and Sriranganathan, N., 2010. Brucellosis: a re-emerging zoonosis. *Vet. Microbiol.,* **140**: 392-398. https://doi.org/10.1016/j.vetmic.2009.06.021

Starr, T., Ng, T.W., Wehrly, T.D., Knodler, L.A. and Celli, J., 2008. *Brucella* intracellular replication requires trafficking through the late endosomal/lysosomal compartment. *Traffic*, **9**: 678-694. https://doi.org/10.1111/j.1600-0854.2008.00718.x

Tibor, A., Decelle, B. and Letesson, J.J., 1999. Outer membrane proteins Omp10, Omp16, and Omp19 of *Brucella* spp. are lipoproteins. *Infect. Immun.,* **67**: 4960-4962.

Tibor, A., Wansard, V., Bielartz, V., Delrue, R.M., Danese, I., Michel, P., Walravens, K., Godfroid, J. and Letesson, J.J., 2002. Effect of omp10 or omp19 deletion on *Brucella abortus* outer membrane properties and virulence in mice. *Infect. Immun.,* **70**: 5540-5546. https://doi.org/10.1128/IAI.70.10.5540-5546.2002

Verstreate, D., Creasy, M., Caveney, N., Baldwin, C., Blab, M. and Winter, A., 1982. Outer membrane proteins of *Brucella abortus*: isolation and characterization. *Infect. Immun.,* **35**: 979-989.

Weber, S.S., Ragaz, C. and Hilbi, H., 2009. Pathogen trafficking pathways and host phosphoinositide metabolism. *Mol. Microbiol.,* **71**: 1341-1352. https://doi.org/10.1111/j.1365-2958.2009.06608.x