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Omp16, a conserved peptidoglycan-associated lipoprotein, is involved in *Brucella* virulence *in vitro*

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***Brucella*, the bacterial agent of common zoonotic brucellosis, primarily infects specific animal species. The *Brucella* outer membrane proteins (Omps) are particularly attractive for developing vaccine and improving diagnostic tests and are associated with the virulence of smooth *Brucella* strains. Omp16 is a homologue to peptidoglycan-associated lipoproteins (Pals), and an *omp*16 mutant has not been generated in any *Brucella* strain until now. Very little is known about the functions and pathogenic mechanisms of Omp16 in *Brucella*. Here, we confirmed that Omp16 has a conserved Pal domain and is highly conserved in *Brucella*. We attempted to delete *omp*16 in *Brucella suis* vaccine strain 2 (*B. suis* S2) without success, which shows that Omp16 is vital for *Brucella* survival. We acquired a *B. suis* S2 Omp16 mutant via conditional complementation. Omp16 deficiency impaired *Brucella* outer membrane integrity and activity *in vitro*. Moreover, inactivation of Omp16 decreased bacterial intracellular survival in macrophage RAW 264.7 cells. *B. suis* S2 and its derivatives induced marked expression of IL-1β, IL-6, and TNF-α mRNA in Raw 264.7 cells. Whereas inactivation of Omp16 in *Brucella* enhanced IL-1β and IL-6 expression in Raw 264.7 cells. Altogether, these findings show that the *Brucella* Omp16 mutant was obtained via conditional complementation and confirmed that Omp16 can maintain outer membrane integrity and be involved in bacterial virulence in *Brucella in vitro* and *in vivo*. These results will be important in uncovering the pathogenic mechanisms of *Brucella*.**

Keywords: *B. suis* S2, Omp16, conditional expression, tetracycline, attenuation

Introduction

Brucellosis, one of the most common zoonotic infectious diseases, is caused by *Brucella* spp., which tend to infect specific animal species and humans (von Bargen *et al.*, 2012; de Figueiredo *et al.*, 2015). The characteristics of brucellosis are infertility and reproductive losses with predilection in animals (Olsen and Palmer, 2014). Recurrent fever and debilitating musculoskeletal, cardiac and neurological complications are the main clinical symptoms of chronic brucellosis in humans (Celli, 2015). Economic losses, human morbidity, and poverty have become increasingly serious worldwide with the continuous spread of brucellosis. More than half a million new cases are reported worldwide annually (Elfaki *et al.*, 2015). In addition, the true number of brucellosis cases may be much higher because of the lack of characteristic clinical manifestations. Brucellosis transmission from animals to humans is mainly due to the consumption of unpasteurized dairy products or direct contact with infected animals (von Bargen *et al.*, 2012). Until now, there has been no licensed vaccine to prevent brucellosis in humans (Lalsiamthara and Lee, 2017). In animals, the vaccine of *Brucella abortus* strains 19 and RB51 has been effective in controlling brucellosis all worldwide (Lalsiamthara and Lee, 2017). In 1952, researchers of the China Institute of Veterinary Drug Control isolated *Brucella suis* vaccine strain 2 (*B. suis* S2) from the embryo of an aborted sow. At that time, *B. suis* S2 was the most extensively used vaccine to provide domestic animal immune protection from *Brucella* in China (Mustafa and Abusowa, 1993; Wang *et al.*, 2016). Although these vaccines are used extensively in animals to control brucellosis, they have many drawbacks, such as pathogenicity for humans, interference with diagnostic tests, and the potential to cause abortion in pregnant animals (Dorneles *et al.*, 2015).

Brucella spp. outer membrane proteins (Omps) have been broadly characterized as immunogenic and protective antigens (Cloeckaert *et al.*, 1999; Bowden *et al.*, 2000). Additionally, Omps have been shown to be involved in the virulence of *Brucella* (Caro-Hernandez *et al.*, 2007). As important components of the cell wall and the main virulence-related factors in *Brucella*, Omp22, Omp25, and Omp31 play roles in supporting the intracellular survival of *Brucella*, and their mutants have been attenuated in their natural hosts compared to their parental wild-type (WT) strains (Edmonds *et al.*, 2002; Caro-Hernandez *et al.*, 2007; Seleem *et al.*, 2008; Sancho *et al.*, 2014). Luo *et al.* (2017) indicated that *Brucella* Omp25 can inhibit TNF-α expression via the regulation of different microRNAs in porcine and murine macrophages. Compared to their parental WT strains, however, the *Brucella* Omp10, Omp19, SP41, and BepC mutants are not altered

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in their virulence in macrophage J774.A1 cells (Sidhu-Muñoz *et al.*, 2016).

The Tol-Pal system, a multiprotein complex, plays an important role in the import of certain organic molecules and the maintenance of outer membrane integrity (Lloubes *et al.*, 2001; Walburger *et al.*, 2002; Hirakawa *et al.*, 2019). *Brucella* Omp16 has a conserved pal domain. Pasquevich *et al.* (2010) have shown that Omp16 is a pathogen associated molecular pattern (PAMP) of *Brucella*, can activate dendritic cells (DCs) *in vivo* and induce a Th1 immune response, and is a promising self-adjuvanting vaccine. Moreover, *Brucella* Omp16 is highly conserved in *Brucella* strains, which shows that Omp16 could play an essential role in *Brucella* survival *in vitro*. In addition, Sidhu-Munoz *et al.* (2016) have shown that multiple attempts to delete Omp16 from *Brucella ovis* PA were unsuccessful. Here, we also made several attempts to delete Omp16 from *B. suis* S2 but were not successful, which also indicated that Omp16 is a vital gene for *Brucella*. To date, the precise function of Omp16 remains to be further determined because the Omp16 mutant has not been generated.

The aim of this work was to develop *Brucella* strains in which *omp16* expression was tightly controlled by the inducer tetracycline and could be induced in a controlled manner and to determine whether Omp16 affects *Brucella* virulence *in vitro*. First, we confirmed that Omp16 is homologous to Pal of the Tol-Pal system and is highly conserved in *Brucella* strains. Then, we generated a *B. suis* S2 strain in which Omp16 expression was controlled via the inducer tetracycline. In addition, *Brucella* cells lacking Omp16 presented defects in growth, outer membrane integrity and, intracellular survival. *Brucella* Omp16 mutant strain enhanced IL-1β and IL-6 expression in Raw 264.7 cells. Together, the *Brucella* Omp16 mutant strain was obtained, and Omp16 was involved in *Brucella* virulence; these results lay the foundation for exploring the pathogenic mechanisms of *Brucella*.

Meterials and Methods

Bacterial strains, plasmids, and cultural conditions

Wild-type *B. suis* S2 strains (CVCC Reference number, CVCC70502) were obtained from Shaanxi Provincial Institute for Veterinary Drug Control. A total of 8.5×10^9 CFU live bacteria were contained in each vaccine. The viability and smoothness of the *B. suis* S2 cells were checked before use. To activate bacteria, *B. suis* S2 strains were resuspended in phosphate-buffered saline (PBS) and then plated on tryptic soy agar (TSA; Cat. No. 22091, Sigma-Aldrich). Single bacterial clones were cultured in 20 ml of tryptic soy broth (TSB; Cat. No. 22092, Sigma-Aldrich) with shaking at 37°C. When indicated, tetracycline at different concentrations or 50 μg/ml gentamicin was added to the bacterial culture medium. All experiments performed with *B. suis* S2 strains, including the acquisition of endogenous *omp*16 mutant strains and the use of tetracycline – induced gene expression system, were carried out in a biosafety level 3 facility. *Escherichia coli* strain DH5α was obtained from Takara and was grown on Luria-Bertani (LB) medium with shaking at 37°C. When necessary, 50 μg/ml gentamicin or 50 μg/ml carbenicillin was added to the culture media.

Construction of the conditional expression vector pBB-PZT1-Omp16

To generate a *B. suis* S2 strain in which the expression of *Brucella* Omp16 was controlled by the inducer tetracycline, we constructed a tetracycline inducible gene expression plasmid, pBB-PZT-Omp16. First, the 3,563-bp plasmid backbone was PCR amplified from pBBR1MCS5 (Kovach *et al.*, 1994) using primers F1 (5′-TTGACATAAGCCTGTTCGGTTCGTA-3′) and R1 (5′-CCTCCCAGAGCCTGATAAAAACG-3′), and purified by gel extraction. Afterwards, using the pG-KJE8 plasmid (Tian *et al.*, 2016) as a template, a 1,082-bp fragment containing the tetracycline inducible promoter region with a multiple cloning site (MCS) was amplified using primers F2 (5′-GCTCTGGGAGGCTGCAGCGGCCGTTTCCATTTAGGTGGGTAC-3′) and R2 (5′-CTTATGTCAACTCGAGTTTTAAGCTTGCATGCGGATCCCCGGGTACCGAGCTCTTTCTCCTCTTTAATGAATTC-3′). After purification by gel extraction, the 1,082-bp fragment containing the tetracycline inducible promoter region with an MCS and the 3,563-bp plasmid backbone were fused with the ClonExpress II One Step Cloning Kit (Code No. C112-02, Vazyme). The fused plasmid was named pBB-PZT1 and amplified in *E. coli* DH5α cells. Finally, flag-tagged *omp*16 of *Brucella* was PCR-amplified from *B. suis* S2 using primers F3 (5′-GGTACCATGCGCCGTATCCAGTCGATTGCAC-3′) and R3 (5′-AAGCTTTTACTTATCGTCGTCATCCTTGTAATCCCGTCCGGCCCCGTTGAGAA-3′). After purification by gel extraction, the 543-bp *omp*16 fragment was cloned into the pMD19-T simple vector (Code No. 3271, Takara), digested with *Hin*dIII and *Bam*HI and subcloned into the pBB-PZT1 plasmid, which was digested with *Hin*dIII and *Bam*HI. The recombinant plasmid was named pBB-PZT1-Omp16 and could induce Omp16 expression by tetracycline.

Competent cell preparation and transformation by electroporation

A single bacteria clone was cultured in 20 ml of TSB medium with shaking at 37°C to an optical density at 600 nm (OD_{600}) of ~0.6. Then, bacteria were collected by centrifugation for 5 min at 5,000 × *g* at 4°C and were washed three times with ice-cold sterile water and washed twice times with ice-cold 10% glycerol in deionized water. Finally, *B. suis* S2 cells were suspended in ice-cold 10% glycerol in deionized water. Electroporation-competent cells were successfully prepared. The pBB-PZT1-Omp16 plasmid was transformed into *B. suis* S2 competent cells by electroporation with Gene Pulser II transfection apparatus (Bio-Rad) at 6 msec and 1.8 kV. One milliliter of TSB was added to *B. suis* S2 competent cells that were incubated at 37°C for 12 h. The diluted medium was plated on TSA plates containing 50 μg/ml gentamicin. After incubating for 72 h, the resulting gentamicin-resistant colonies were grown in TSB with shaking at 37°C. The *B. suis* S2 conditional expression strain (*B. suis* S2 <Omp16>) was obtained in the presence of tetracycline and verified by western blotting and immunofluorescence.

Construction of the endogenous *omp*16 deletion strain

The endogenous *omp*16 deletion strain was constructed by allelic replacement as described previously (Zhi *et al.*, 2019). Briefly, primers for constructing a suicide plasmid were designed by using the *B. suis* S2 genome and the sequence of pUC19 (Code No. 3219, Takara). Three fragments, a 654-bp upstream fragment, 651-bp downstream fragment and 978-bp ampicillin resistance gene fragment were generated in independent PCR using primers UF (5′-CTGCAGTTCGCAGATTGTCTTCACATC-3′)/UR (5′-TCTAGATCATATTGAGGTTAAAACAGGCT-3′), DF (5′-GGTACCATGCGCCGTATCCAGTCGATTG-3′)/DR (5′- GAATTCTACCCGATAGCCGACCGACCCT-3′) and AF (5′-TCTAGACGCGGAACCCCTATTTGTTTATTTT-3′)/AR (5′-GGTACCTTACCAATGCTTAATCAGTGAGGC-3′), respectively. After purification by gel extraction, the 3 fragments were cloned into the pMD19T simple vector, and digested with *Pst*I and *Xba*I, *Kpn*I and *Eco*RI, and *Xba*I and *Kpn*I, respectively. Then, the three fragments were subcloned into pUC19 vector, which was digested with *Pst*I and *Eco*RI, and the resulting plasmid was named pUC19-Omp16. The plasmid pUC19-Omp16 was introduced into *B. suis* S2 <Omp16> competent cells by electroporation with a Gene Pulser II transfection apparatus (Bio-Rad) at 6 msec and 1.8 kV. One millilitre of TSB was added to *B. suis* S2 <Omp16> competent cells that were incubated at 37°C for 24 h. The diluted medium was plated on TSA plates containing 50 μg/ml gentamicin and 50 μg/ml carbenicillin. After incubating for 72 h, the resulting gentamicin- and carbenicillin-resistant colonies were grown in TSB at 37°C. The *B. suis* S2 endogenous *omp*16 mutant strain (ΔOmp16 <Omp16>) was verified by PCR using primers IUF (5′-TGCTGCAACTTGAAACCGG-3′)/AR and AF/IRR (5′-CTGGGTTTTTTGCAACTGGTT-3′).

Field emission scanning electron microscopy (FE-SEM) sample preparation and imaging

All bacteria derived from *B. suis* S2 were examined by FE-SEM. The bacteria were washed three times with PBS, and fixed at room temperature for 24 h with 2.5% glutaraldehyde. Then, the bacteria were serially treated with ethanol (once with 10% ethanol for 20 min, 30% ethanol for 20 min, 50% ethanol for 20 min, 70% ethanol for 20 min, 80% ethanol for 20 min and 90% ethanol for 20 min and three times with 100% ethanol for 30 min each time). The bacteria were treated by spray-gold. Then, all samples were examined by FE-SEM (Nova Nano SEM-450).

RNA isolation and quantitative real time PCR

To measure the mRNA expression of exogenous Omp16, the ΔOmp16<Omp16> strain, *B. suis* S2, ΔOmp16<Omp16> and ΔOmp16<Omp16> + T^+ were cultured in 20 ml of TSB medium to the exponential phase (OD_{600} ~0.6) with shaking at 37°C. Then, bacteria were collected by centrifugation for 10 min at 6,000 × *g* at 4°C, and 1 ml of TRIzol reagent (Invitrogen) was added to the collected bacteria. In addition, the cells of infecting *B. suis* S2 and its derivatives were collected at different times, and this samples were used for total RNA extraction using TRIzol (Invitrogen). Total RNA was isolated using the recommended TRIzol extraction procedures. DNA contaminants were removed from the RNA samples. Then, RNA samples were subjected to reverse transcription using a Vazyme RT Reagent Kit according to the manufacturer's protocol. Quantitative real-time PCR (qRT-PCR) was performed using an ABI 7500 Sequencing Detection System and SYBR Premix Ex Taq^{TM}. The primers used for qRT-PCR of Omp16 mRNA were Omp16F (5′-TCGATCTCGATTCGTCGCTG-3′) and Omp16R (5′-CAAGGGCGAGGTTGTACTCA-3′). The relative transcription levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

Western blotting

B. suis S2, ΔOmp16<Omp16> and ΔOmp16<Omp16> (10 ng/ml) were cultured in 20 ml of TSB medium with shaking at 37°C to the exponential phase (OD_{600} ~0.6). Then, bacteria were collected in a tube by centrifugation for 10 min at 6,000 × *g* at 4°C, and the number of bacterial cells was counted by plating on TSA. Approximately 1×10^9 bacteria were prepared in 5 × SDS-PAGE loading buffer and then boiled for 10 min in a water bath. Bacterial samples were run on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels. Then, the gels were transferred to polyvinylidene fluoride (PVDF) membranes for immunoblotting. The membranes were blocked in 5–10% skimmed milk prepared in Tris-buffered saline containing 0.5% tween-20 (TBST) for 2 h at room temperature. The primary mouse monoclonal antibody anti-DYKDDDDK (1:1,000; L10822, Transgen Biotech) was incubated overnight at 4°C in blocking solution. After washing three times with TBST for 5 min, the membranes were incubated for 1 h with secondary antibody. In this study, HRP-conjugated goat anti-mouse (1:5,000; Zhongshan Golden Bridge Biotechnology) was utilized. Finally, the membranes were visualized using the Gel Image System (Tannon, Biotech).

Immunofluorescence assay

B. suis S2 and its derivatives were cultured in TSB medium with shaking at 37°C to the exponential phase (OD_{600} ~0.6). Then, bacteria were collected in a tube by centrifugation for 5 min at 6,000 × *g* at room temperature. In addition, RAW 264.7 cells were grown on 24-well plates containing glass coverslips then infected with *B. suis* S2, *B. suis* S2 <Omp16>, *B. suis* S2 <Omp16> (10 ng/ml), Omp16<Omp16> and Omp16 <Omp16> (10 ng/ml) strains at an MOI of 100. Immunofluorescence staining was performed. Bacteria or cells were fixed for 20 min in 4% paraformaldehyde at room temperature, and then permeabilized for 30 min in PBS containing 0.25% Triton X-100 at room temperature. Primary antibodies, anti-DYKDDDDK mouse monoclonal antibody (1:200; L10822, Transgen Biotech) or goat anti-*Brucella* polyclonal antibody (1:100; Noncommercial antibody), were incubated in PBS for 12 h at 4°C. The secondary antibody was incubated in PBS for 1 h at room temperature. In this study, the following secondary antibodies were used: donkey anti-mouse Alexa Fluor 555(1:500; A31570, Invitrogen, Inc). Then, bacteria and cells were visualized under laser scanning confocal microscope. Assays were performed in triplicate.

RAW 264.7 cell culture and infection

Brucella suis S2 and its derivatives were analysed by infection assays, which were carried out as described previously (Wang *et al.*, 2016). Briefly, RAW 264.7 cells were seeded onto 6-well plates (1×10^6 cells/well) or 24-well plates (2×10^5 cells/well). After 12 h, the cells were infected with *B. suis* S2 or its derivatives at an MOI of 100. RAW 264.7 cells were incubated for 4 h and then washed three times with PBS to remove unattached bacteria. Then 50 μg/ml kanamycin was added to kill residual extracellular bacteria (Utaisincharoen *et al.*, 2003). After 1 h, the cells were washed three times with PBS, and 25 μg/ml kanamycin was added to prevent secondary infection. This time was considered the 0 h time point. RAW 264.7 cells were collected, and relevant experiments were conducted. For intracellular survival or adherence assays, the cells that were infected with *B. suis* S2 or its derivatives were washed three times with PBS. PBS containing 0.5% Triton X-100 was added to the wells of infected cells to lyse the cells. Then, the lysates were serially diluted in PBS and plated on TSA for 72 h at 37°C to determine the number

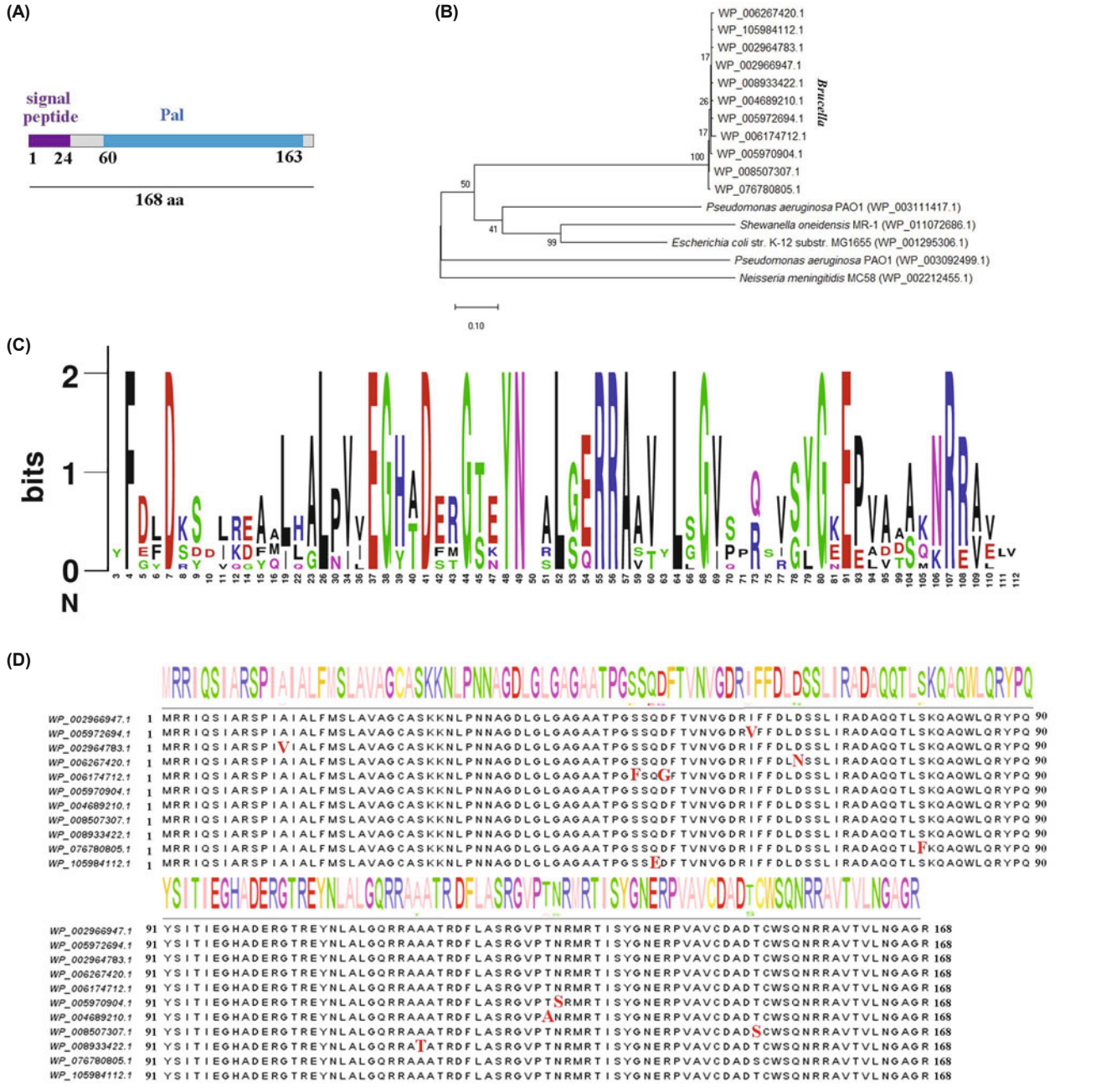

Fig. 1. Omp16 has a conservative Pal domain. (A) Schematic of the Pal domain of Omp16 in *Brucella*. (B) Maximum likelihood phylogenetic tree of 16 proteins from different bacteria. The scale bar represents the number of amino acid substitutions per site. The name of each isolate is followed by its protein_ID. (C) Logo assays for the Pal domain. The logos were generated by the WebLogo service. (D) Alignment of Omp16 amino acid sequences. Amino acid differences are shaded red.

of bacteria.

Statistical analysis

SPSS software was used for all statistical analyses (version 23; SPSS, Inc.). All results were repeated at least three times, and are presented as the means ± standard deviations (SD). An unpaired, two-tailed Student's t test, or one-way analysis with group comparisons was used. A probability (*P*) value of ≤ 0.05 was considered significant.

Results

Omp16 has a Pal domain and is highly conserved in *Brucella* strains

Brucella Omp16 is a homologue to Pal because it has a Pal domain (Fig. 1A). *Brucella omp*16 contains 507 nucleotides, coding 168 amino acids. The Pal domain of Omp16 exists in 60–163 *Brucella* strains. Pal is present in Gram-negative bacteria and is involved in both the maintenance of outer membrane integrity and the import of certain organic molecules as nutrients. We found that the Pal domains of *Pseudomonas aeruginosa* PAO1 (WP 003111417.1), *Escherichia coli* str. K-12 substr. MG1655 (WP 001295306.1), *Shewanella oneidensis* MR-1 (WP 011072686.1), *Shewanella oneidensis* MR-1 (WP 003092499.1), and *Neisseria meningitidis* MC58 (WP 0022-12455.1) were highly conserved with *Brucella* Omp16 by multiple sequence alignment (Fig. 2B). Moreover, in the maximum likelihood (ML) analysis based on amino acid sequences, Pal was classified into three classical genetic lineages (Fig. 1B). In these bacteria, the Pal domain was highly conserved (Fig. 1C). These results suggested that Omp16 has a conserved Pal domain that similar to the above mentioned proteins. In addition, Omp16 is present and highly conserved in all strains, including *B. suis* S2, *B. melitensis* 16M, *B. abotus* 2308, *B. canis* ATCC 23365 and *B. ovis* ATCC 25840 (WP_006267420.1). However, a total of eleven changes were observed in *Brucella* Omp16 (Fig. 1D). Altogether, these results show that *Brucella* Omp16 has a Pal domain and is highly

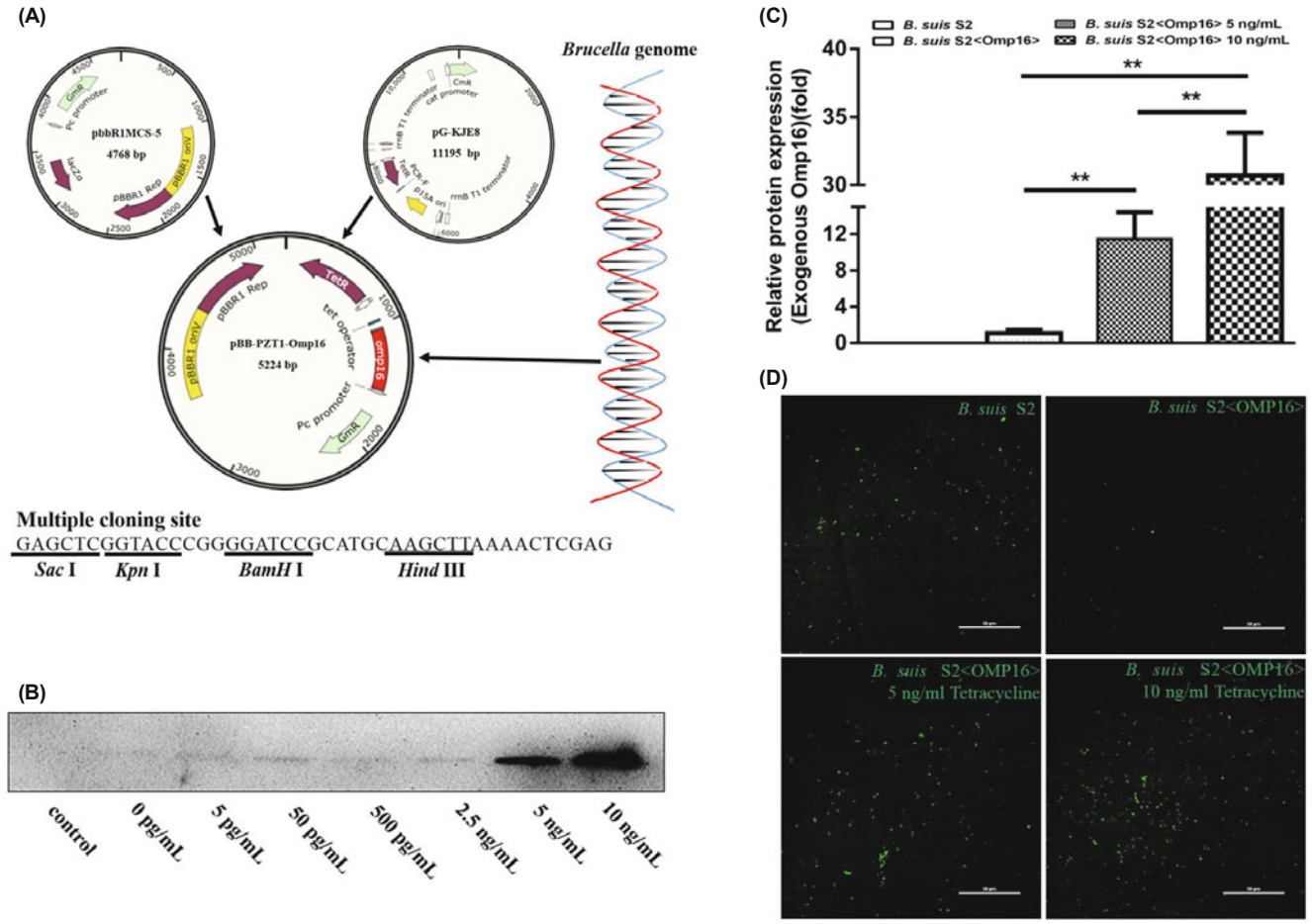

Fig. 2. Inducible expression of flag-tagged Omp16 in *B. suis* S2. (A) Maps of the pBB-PZT1-Omp16 plasmid and the distribution of restriction enzyme sites in the MCS. (B) The inducible expression of flag-tagged Omp16 was detected by western blotting. *B. suis* S2 strain without the pBB-PZT1-Omp16 plasmid served as a negative control in *B. suis* S2. (C) Quantification of band intensities from 3 independent results was determined by densitometric analysis. Data represent the mean ± standard deviation from 3 independent experiments at each time point. ** $P < 0.01$. (D) The inducible expression of flag-tagged Omp16 was confirmed by immunofluorescence. The *B. suis* S2 strain without the pBB-PZT1-Omp16 plasmid was stained with goat anti-*Brucella* serum and donkey anti-mouse Alexa Fluor 488, which served as a control.

conserved in different strains.

Omp16 expression was successfully induced by different concentrations of tetracycline

To induce Omp16 expression by tetracycline, we first constructed the conditional expression plasmid pBB-PZT1-Omp16 (Fig. 2A). The 3,563-bp plasmid backbone (pBBR1 Rep/oriV and gentamicin resistance gene) was obtained by PCR using F1/R1; the plasmid backbone was replicated and used as a selective marker gene. Then, we obtained a 1,082-bp fragment containing a tetracycline inducible promoter region with an MCS using F2/R2; the expression levels of the tetracycline resistance gene and target gene were regulated by the dual promoter of the plasmid. After fusion with the ClonExpress II One Step Cloning Kit, the conditional expression plasmid pBB-PZT1 with an MCS was successfully constructed. In addition, *omp*16 with a flag tag at the C-terminus was obtained from *B. suis* S2 by PCR using F3/R3. Findly, the DNA fragment containing *omp*16 with a flag tag was cloned into the pBB-PZT1 plasmid, which was named pBB-PZT1-Omp16. Based on the conditional expression of the pBB-PZT1-Omp16 plasmid, we next sought to determine whether this system was able to induce exogenous Omp16 expression in *B. suis* S2. The pBB-PZT1-Omp16 plasmid was transformed into *B. suis* S2 competent cells by electroporation, and the expression of flag-tagged Omp16 was tested in the presence of different tetracycline concentrations. The tetracycline controlled expression of flag-tagged Omp16 in *B. suis* S2 was first tested in TSB. The results showed that no production of flag-tagged Omp16 was detected in *B. suis* S2 (Fig. 2B and C), because Omp16 of *B. suis* S2 was not flag-tagged. As the tetracycline concentration increased, the expression of flag-tagged Omp16 gradually increased in *B. suis* S2<Omp16>. However, flag-tagged Omp16 was weakly produced in *B. suis* S2<Omp16> in the absence of tetracycline (Fig. 2B and C). In addition, immunofluorescence was used to detect flag-tagged Omp16 in *B. suis* S2<Omp16> again, and goat anti-*Brucella* serum was used to determine the presence of *Brucella*. The results indicated that the expression of flag-tagged Omp16 in *B. suis* S2<Omp16> gradually increased as the tetracycline concentration increased (Fig. 2D). These results showed that tetracycline can induce Omp16 expression in a dose-dependent manner. Taken together, these results established that we generated a *B. suis* S2 strain in which flag-tagged Omp16 can be controlled *in vitro* using tetracycline, which allowed

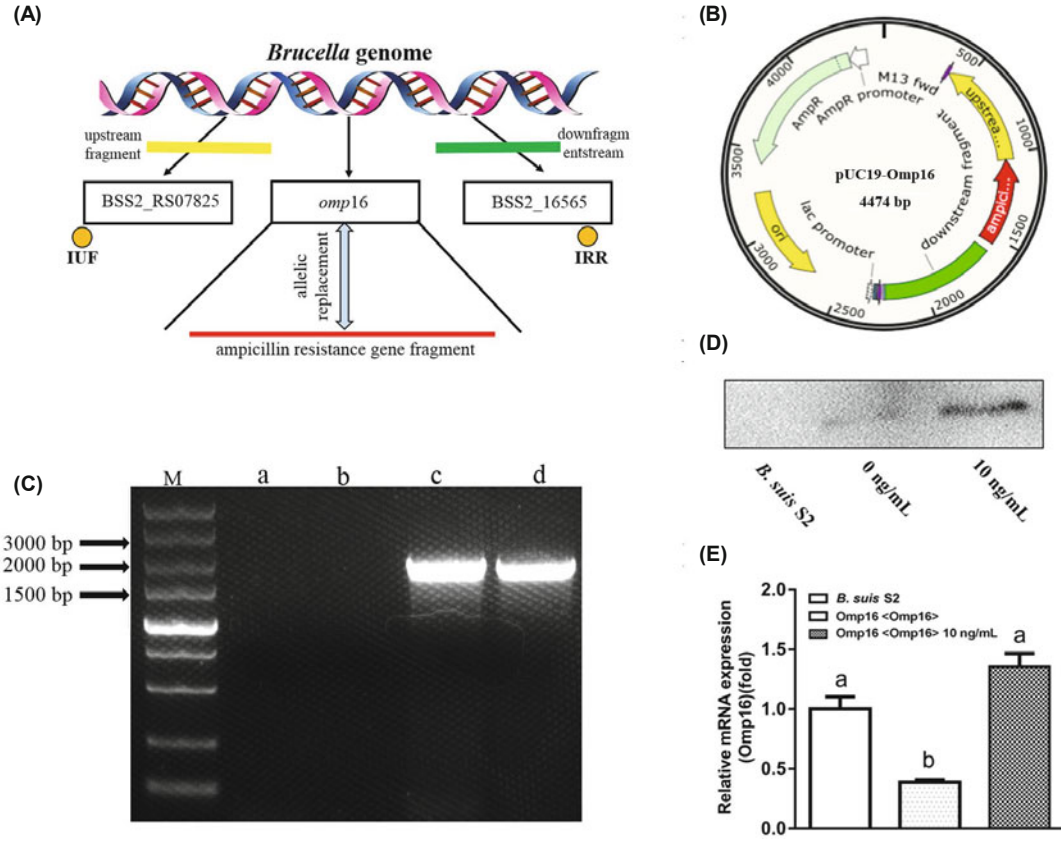

Fig. 3. Identification of the Omp16 mutant strain. (A) Schematic of the construction of the Omp16 mutant strain. (B) Maps of the recombinant suicide plasmid pUC19-Omp16. The upstream fragment, downstream fragment and ampicillin resistance gene fragment were obtained in three independent PCRs. Three fragments were subcloned into pUC19 plasmids. (C) Identification of the Omp16 mutant by PCR amplification. Lanes: a, the gene upstream of Omp16 and the ampicillin resistance gene in *B. suis* S2; b, the gene downstream of Omp16 and the ampicillin resistance gene in *B. suis* S2; c, the gene upstream of Omp16 and the ampicillin resistance gene in the Omp16 mutant strain; d, the gene downstream of Omp16 and ampicillin resistance gene in the Omp16 mutant strain. (D) The inducible expression of flag-tagged Omp16 was detected by western blotting. *B. suis* S2 without the pBB-PZT1-Omp16 plasmid served as a negative control. (E) qRT-PCR confirmed the mRNA expression of Omp16 in the *B.suis* S2 and Omp16 mutants. *Brucella* was collected at the exponential phase.

us to further delete endogenous *omp*16 to acquire ΔOmp16 <Omp16>.

Deletion of endogenous *omp*16 by allelic exchange

To acquire the ΔOmp16<Omp16> strain, we attempted to delete endogenous *omp*16 from *B. suis* S2<Omp16>. The *omp*16 genes and their flanking genes are shown in Fig. 3A. The upstream fragment, downstream fragment and ampicillin resistance gene fragment were obtained in three independent PCRs. Then, the three fragments were subcloned into the pUC19 plasmids, which were digested with *Pst* I and *Eco*RI, and the recombinant suicide plasmid was named pUC19-Omp16 (Fig. 3B). The *omp*16 mutant was verified by PCR amplification using primers IUF/AR and AF/IRR (Fig. 3C). The results indicated that a 1,885-bp fragment containing the gene upstream of Omp16 and the ampicillin resistance gene and a 1,848-bp fragment containing the gene downstream of Omp16 and the ampicillin resistance gene were not amplified from *B. suis* S2, but were amplified from the Omp16 mutant (Fig. 3C). The size of the fragments was as expected. Altogether, this demonstrates that the endogenous *omp*16 gene was successfully deleted from *B. suis* S2 <Omp16>, and that we acquired the ΔOmp16<Omp16> strain.

To confirm whether the tetracycline control system can work well in the ΔOmp16<Omp16> strain, we detected the expression of flag-tagged Omp16 after induction with tetracycline by western blotting and qRT-PCR. The results showed that the expression of flag-tagged Omp16 was also not detected in *B. suis* S2 (Fig. 3D). However, when 10 ng/ml tetracycline was added to the bacterial culture medium, the expression of flag-tagged Omp16 was increased in ΔOmp16<Omp16> (Fig. 3D). In addition, the expression of flag-tagged Omp16 in ΔOmp16<Omp16> was reconfirmed by qRT-PCR. Compared to that in *B. suis* S2 group, the expression of Omp16 decreased in the ΔOmp16<Omp16> group when tetracycline

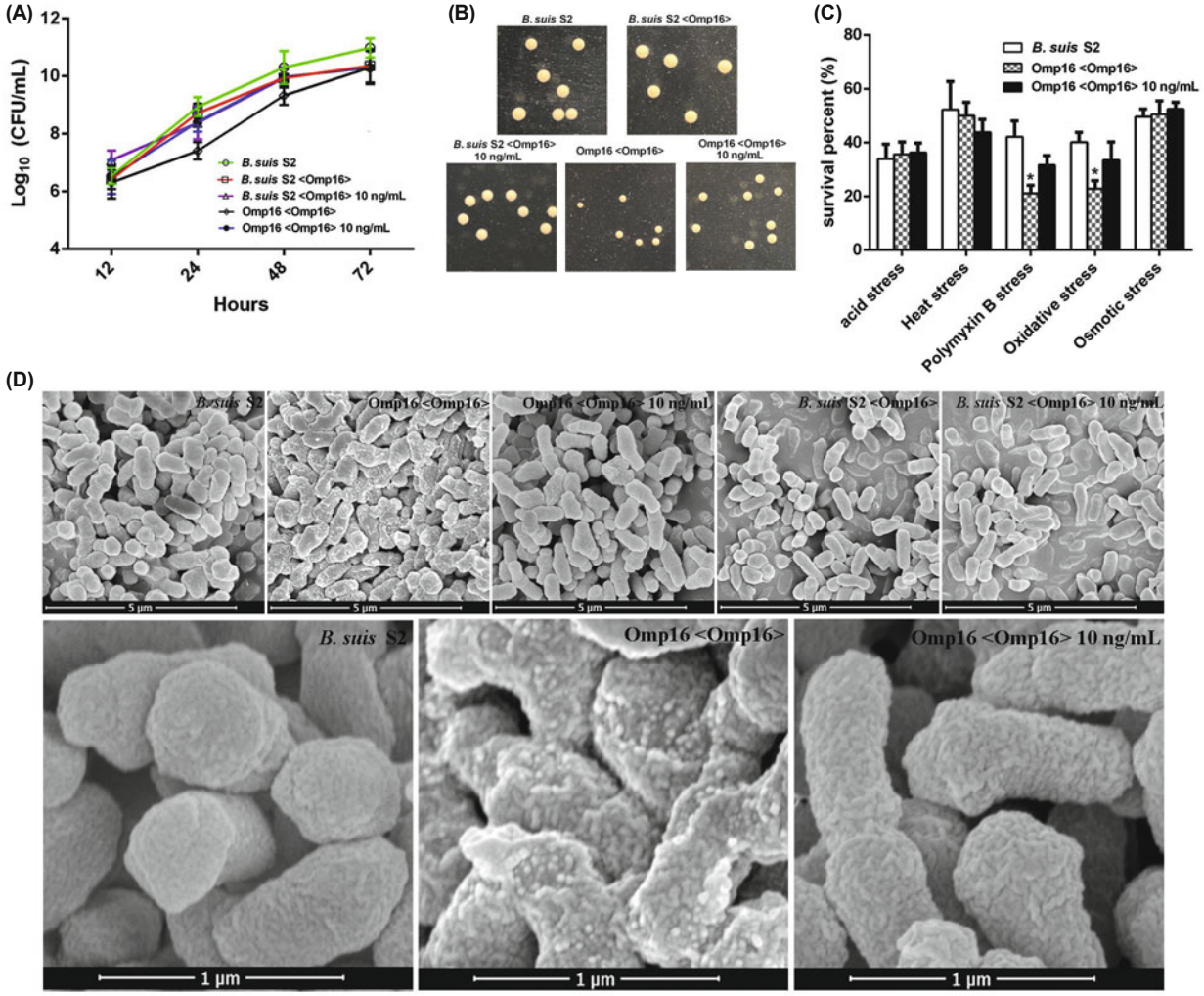

Fig. 4. Effect of Omp16 on the characteristics of *B. suis* S2. (A) Viability of all bacteria derived from *B. suis* S2 in rich medium. CFU of *B. suis* S2, *B. suis* S2 <Omp16> and Omp16<Omp16> cultivated in TSB medium with or without tetracycline. All data represent the means ± standard deviations from 3 independent experiments. (B) Colony size in TSA after 72 h of incubation. (C) Stress resistance assays. *B. suis* S2 and Omp16<Omp16> were cultivated with or without tetracycline at pH 5.5 and 42°C in a water bath and treated with 10 μg/ml polymyxin B, 0.5 mM H_2O_2, or 0.5 M sorbitol. All data represent the means ± standard deviations from 3 independent experiments. *$P \leq 0.05$. (D) Representative FE-SEM images of *B. suis* S2, *B. suis* S2 <Omp16> and Omp16<Omp16> cultivated with or without tetracycline. The image shown is representative of 3 independent experiments.

was not added to the bacterial culture medium but was enhanced in the ΔOmp16<Omp16> group when 10 ng/ml tetracycline was added to the bacterial culture medium (Fig. 3E). Taken together, these experiments demonstrated that the ΔOmp16<Omp16> strain carrying flag-tagged Omp16 under the control of a tetracycline-inducible promoter was successfully acquired.

Omp16 is required for *B. suis* S2 outer membrane integrity and growth *in vitro*

On the basis of the ΔOmp16<Omp16> strain, we suggested that Omp16 could affect *Brucella* outer membrane integrity and growth in vitro. To this end, the viability of the Omp16 <Omp16> strain in TSB medium was assessed by counting the number of CFU. In any group, the number of CFU was not significantly different (Fig. 4A). However, compared to that of the wild strain and *B. suis* S2 <Omp16> strain, the colony size of the Omp16<Omp16> strain was decreased without tetracycline, and this phenotype was partially restored with the addition of tetracycline (Fig. 4B). Therefore, the lack of difference in the viability of Omp16<Omp16> cells in TSB medium was probably due to the reduced sensitivity of the viability assay. In addition, stress resistance assays were performed to detect *Brucella* activity in hostile survival conditions. Acid stress, heat stress, polymyxin B stress, oxidative stress and osmotic stress did not affect *B. suis* S2 <Omp16> strain survival regardless of the presence or absence of tetracycline compared to the wild-type strain. However, in the presence of polymyxin B stress and oxidative stress, the survival rate of the Omp16<Omp16> strain was significantly decreased without tetracycline, and this phenotype was partially restored in the presence of 10 ng/ml tetracycline (Fig. 4C). This result shows that bacterial activity was decreased when the expression of Omp16 was lacking. In addition, by using FE-SEM, we observed that the outer membrane integrity of the Omp16<Omp16> strain was impaired, indicating that Omp16 contributed to maintaining outer membrane integrity in *Brucella* (Fig. 4D). Collectively, these results show that *Brucella* Omp16 can affect bacterial activity and is involved in outer membrane integrity.

Inactivation of Omp16 impairs bacterial intracellular survival in RAW 264.7 cells

Based on the effect of Omp16 on *B. suis* S2 outer membrane integrity and growth, we reasoned that Omp16 may affect the intracellular survival of *Brucella*. To this end, RAW 264.7 cells were infected with *B. suis* S2, *B. suis* S2 <Omp16>, and Omp16<Omp16> with or without tetracycline. Infected cells

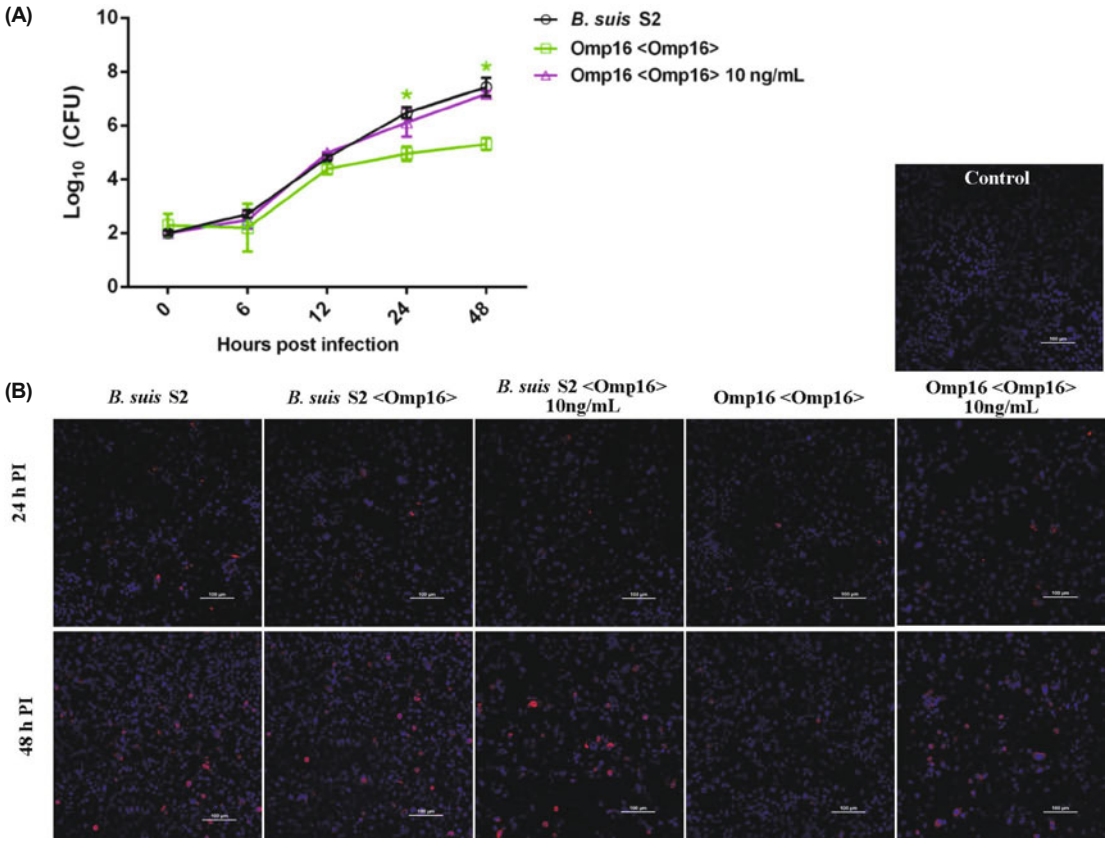

Fig. 5. The intracellular survival of the Omp16<Omp16> strain in RAW 264.7 cells. (A) Intracellular survival of the wild-type and Omp16<Omp16> strains in RAW 264.7 cells with or without tetracycline. The results are expressed as the means ± standard deviation from 3 independent experiments at each time point. *$P < 0.05$. (B) confocal microscopy analysis of the intracellular survival of *B. suis* S2 and its derivatives in RAW 264.7 cells. RAW 264.7 cells were infected with *B. suis* S2 and its derivatives for 24 and 48 h and then immunostained using anti-*Brucella* antibody (red) and DAPI (blue). RAW 264.7 cells with *B. suis* S2 that were not immunostained with anti-*Brucella* antibody served as a control. Red represents *Brucella*. Blue represents the cell nucleus. The image shown is representative of at least 3 independent experiments.

were lysed, and CFU were counted at 0, 6, 12, 24, and 48 h post infection. The bacterial adherence capacity was confirmed via CFU detection. We observed no significant difference in CFU in any groups at -1 h post infection, indicating that there was no defect in entry for any strain (data not shown). Then, the intracellular survival of bacteria was detected by measuring the CFU. We observed that the number of CFU was not significantly different at any time post infection between the wild strain and *B. suis* S2 <Omp16> with or without tetracycline, indicating that the intracellular survival of *B. suis* S2 <Omp16> with or without tetracycline was not affected (data not shown). These results allowed us to further explore the intracellular survival of the Omp16<Omp16> strain. The intracellular survival of the Omp16<Omp16> strain was decreased at 24 and 48 h postinfection compared to the wild-type strain, and this phenotype was partially restored with the addition of tetracycline (Fig. 5A). These results suggested that Omp16<Omp16> drastically decreased intracellular survival. Consistently, confocal microscopy analysis further showed that the intracellular survival of Omp16 <Omp16> cells was reduced at 24 and 48 h PI but restored with the addition of tetracycline (Fig. 5B). Altogether, these experiments indicated that Omp16 was required for efficient intracellular survival in RAW 264.7 cells.

Omp16 inhibits IL-1β and IL-6 expression in RAW 264.7 cells *in vitro*

To assess differences in inflammatory cytokines in RAW 264.7 cells infected with the wild strain and its derivatives with or without tetracycline, we harvested infected cells and detected inflammatory cytokines expression by qRT-PCR. First, we estimated the effects of tetracycline on RAW 264.7. No changes were observed in the expression of mRNA for TNF-α, IL-6, IL-1β, IL-4, and IL-10 at any time in RAW 264.7 with or without tetracycline, indicating that tetracycline did not affect the expression of inflammatory cytokines in RAW 264.7 cells (data not shown). Following *B. suis* S2 and its derivatives challenge, the expression of IL-1β, IL-6, and TNF-α mRNA were upregulated compared with that of control group (Fig. 6); however, the mRNA expression of IL-1β and IL-6 was decreased significantly at 24 h post infection in *B. suis* S2<Omp16> and *B. suis* S2 <Omp16> 10 ng/ml groups compared with that in *B. suis* S2 group (Fig. 6A). Most interesting, the mRNA expression of IL-1β and IL-6 was higher in Omp16<Omp16> group than in *B. suis* S2 group, but restored in adding tetracycline group (Fig. 6C and E). No differences were found in the expression of mRNAs for genes encoding IL-4 or IL-10 among the any groups (Fig. 6D and F). Altogether, these experiments indicated that Omp16 can inhibit IL-1β and IL-6 expression in RAW 264.7 cells.

Discussion

Bacteria of the genus *Brucella* are the causative agents of brucellosis, a worldwide zoonosis threatening animal husbandry and the development of healthy animals and resulting in considerable economic losses (Van der Henst *et al.*, 2013; de

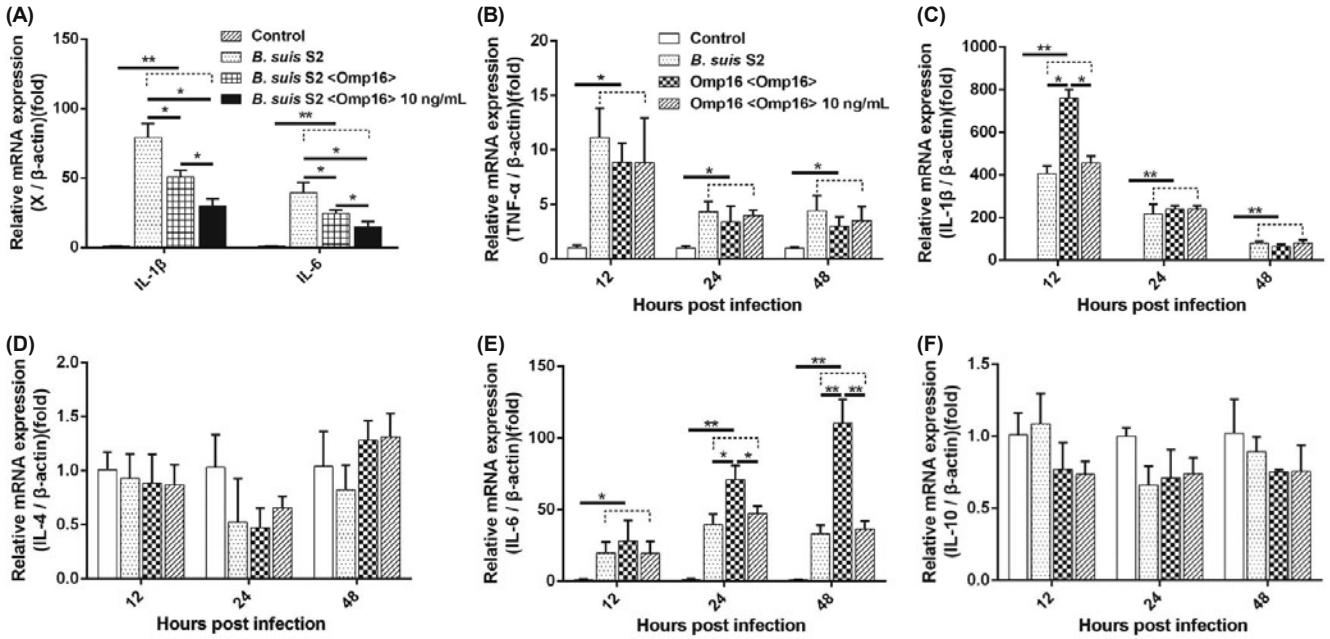

Fig. 6. Effect of the Omp16<Omp16> on the secretion of inflammatory cytokines. (A) The mRNA expression of IL-1β and IL-6 were assayed by qRT-PCR at 24 h phi between wild strain and *B. suis* S2 <Omp16> incubated with or without tetracycline in RAW 264.7. The results are expressed as the means ± standard deviation from 3 independent experiments. Dotted line represents that the mRNA expression of inflammatory factor exhibited significant difference in *B. suis* S2, *B. suis* S2 <Omp16> and *B. suis* S2 <Omp16> 10 ng/ml compared to control. **P* < 0.05; ***P* < 0.01. The mRNA expression of TNF-α (B), IL-1β (C), IL-4 (D), IL-6 (E), and IL-10 (F) were assayed by qRT-PCR at 12, 24, and 48 h phi between wild strain and DnaA <DnaA> incubated with or without tetracycline in RAW 264.7. The results are expressed as the means ± standard deviation from 3 independent experiments. Dotted line represents that the mRNA expression of inflammatory factor exhibited significant difference in *B. suis* S2, Omp16<Omp16> and Omp16<Omp16> 10 ng/ml compared to control. **P* < 0.05; ***P* < 0.01.

Figueiredo *et al.*, 2015). Vaccines, particularly the attenuated vaccine, remain the primary means of prevention and control brucellosis. However, limitations, including potential problems with safety and other side effects, remain a principal concern by using traditional *Brucella* vaccines. *Brucella* spp. outer membrane proteins are immunogenic and protective antigens and serve an important role in interactions with the host. Understanding the role of *Brucella* Omps is the key to develop the vaccine for protecting animals and people from *Brucella* infection. *Brucella* Omps, such as Omp16, Omp19 (Luo *et al.*, 2006; Pasquevich *et al.*, 2009; Tabynov *et al.*, 2014; Sadikaliyeva *et al.*, 2015), Omp25, Omp28, and Omp 31 (Edmonds *et al.*, 2002; Caro-Hernandez *et al.*, 2007; Kaushik *et al.*, 2010; Luo *et al.*, 2017), are particularly attractive for developing vaccines and improving diagnostic tests. *Brucella* Omp16, the homologue to Pal, is associated with PAMP (Pasquevich *et al.*, 2010), but its mutant strain has not been generated in any *Brucella* strains. Hence, a novel method should be established to further explore the precise effects of *Brucella* Omp16. Here, the *Brucella* Omp16 mutant strain was successfully obtained through a tetracycline control system. *Brucella* lacking Omp16 presented defects in growth, outer membrane integrity and intracellular survival, and enhanced IL-1β and IL-6 expression in Raw 264.7 cell.

Up to now, the technologies of gene deletion, site-directed mutation, complementation, over-expression is common method to uncover gene function. Often, allelic replacement is a common method to construct bacterial mutant stains. However, the studies of bacterial vital gene are still limited, because mutant strains cannot be obtained. For example, Sidhu-Muñoz *et al.* (2016) repeatedly attempted to delete Omp16 from *Brucella ovis* PA were unsuccessful. In order to resolve this limitation, a novel way should be proposed. In this study, we obtained the *Brucella* Omp16 mutant via tetracycline-induced conditional complementation. We found that the *Brucella* Omp16 mutant biological characters can be changed, such as outer membrane integrity and activity, but this phenotype was restored with the addition of tetracycline, indicating that this novel method is useful for studying the function of vital gene, such as Omp16. Therefore, we proposed this novel method can overcome the limitation of not being able to obtain mutant strains.

Outer membrane proteins are critical to maintaining the integrity and surviving under stress (Moriyón and López-Goni, 1998). In the present study, the outer membrane integrity of the Omp16<Omp16> strain was impaired. The *Brucella* outer membrane is markedly resistant to bactericidal cationic peptide present in lysosomes and bactericidal polycations like polymyxin B (Sola-Landa *et al.*, 1998). It was found that the *B. melitensis* were more susceptible to polymyxin B due to alteration of the outer membrane integrity by mutated LPS or Omp31 (Feng *et al.*, 2017; Verdiguel-Fernandez *et al.*, 2017). *Brucella abortus* present similar phenotype in Tn5 mutant stain when applied polymyxin B (Sola-Landa *et al.*, 1998). We found that *Brucella* lacking Omp16 lost the typical *Brucella* resistance of polymyxin B. The change in the outer membrane was also shown to affect sensitivity to oxidative stress *in vitro* (Wang *et al.*, 2010). To survive in host cells, intracellular bacteria have developed the capability to adapt to intracellular environments include oxidative burst (Roop *et al.*, 2009). These findings are corroborated our results that inactivation of Omp16 decreased survival under oxidative stress and impaired bacterial intracellular survival in RAW 264.7 cells. In addition, these results indicated that the cell surface characteristics that allow *Brucella* to overcome the stress were Omp16 dependent.

Pro-inflammatory cytokines production by innate immune cells is critical in the process of host control of intracellular pathogens. In the present study, *B. suis* S2 and its derivatives induced marked production of IL-β, IL-6, and TNF-α in macrophage. IL-6 has remarkable functional duality and is strongly engaged both in anti-inflammatory and pro-inflammatory functions (Hunter and Jones, 2015). *Brucella* and its agonists, the outer membranes Omp10, Omp16, Omp19, and Omp28, markedly induced the high level of IL-6 expression in macrophage (Giambartolomei *et al.*, 2004; Pasquevich *et al.*, 2010; Im *et al.*, 2018). In addition, *B. abortus* diminished the expression of MHC-II and CIITA transcripts by IL-6, which is a strategy used by several pathogens to subvert the immune surveillance and to persist inside of the host (Velásquez *et al.*, 2017). However, in this study, our data provide evidence of feedback regulation by tetracycline-induced Omp16 expression in production of IL-6. We found that the mRNA expression of IL-6 was increased during Omp16<Omp16> infection, whereas this increase was attenuated by overexpression Omp16 through added 10 ng/ml tetracycline in macrophage. A recent study revealed that IL-6 contributes to host resistance against *B. abortus* infection by controlling brucellacidal activity in macrophages and priming cellular immune responses (Hop *et al.*, 2019a). These findings are consistent with our results that mutant Omp16 induced a marked IL-6 expression and reduced the survival of *B. suis* S2 within RAW 264.7 cells. Similarly, the expression of IL-1β in *B. suis* S2 and its derivatives-infected macrophages also presented a similar trendency to IL-6. IL-1β expression was marked increase in Omp16 mutant infected macrophages, whereas this tendency was eliminated by treatment with tetracycline. IL-1β contributes to the control of *B. abortus* infection in mice. Previous study indicates that the survival of *B. abortus* was increased in mice lacking IL-1R (Hielpos *et al.*, 2018). In addition, infection of RAW 264.7 cells with *B. abortus* augmented expression and secretion of IL-1β (Hop *et al.*, 2019b). This suggests that the increased production of cytokines may be one of the factors that contribute to the control of *Brucella* infection in macrophages. Further studies will be required to disclose the pathogenesis underlying of Omp16.

In summary, the *Brucella* Omp16 mutant strain was successfully obtained through a tetracycline tightly control system. *Brucella* cells lacking Omp16 presented defects in growth, outer membrane integrity and intracellular survival. Impaired Omp16 expression in *Brucella* enhanced IL-1β and IL-6 expression in Raw 264.7 cells. The present work provides new insight into the pathogenic mechanism of *Brucella* and provides new ideas for exploring vital genes in *Brucella*.

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