Mutant Brucella abortus Membrane Fusogenic Protein Induces Protection against Challenge Infection in Mice

Job Alves de Souza Filho, Vicente de Paulo Martins, Priscila Carneiro Campos, Juliana Alves-Silva, Fernanda Souza de Oliveira, Gustavo B. Menezes, Vasco Azevedo, Silvio Lorenzo Cravero, Sergio Costa Oliveira

Brucella species can cause brucellosis, a zoonotic disease that causes serious livestock economic losses and represents a public health threat. The mechanism of virulence of Brucella spp. is not yet fully understood. Therefore, it is crucial to identify new molecules that serve as virulence factors to better understand this host-pathogen interplay. Here, we evaluated the role of the Brucella membrane fusogenic protein (Mfp) and outer membrane protein 19 (Omp19) in bacterial pathogenesis. In this study, we showed that B. abortus Δmfp::kan and Δomp19::kan deletion mutant strains have reduced persistence in vivo in C57BL/6 and interferon regulatory factor 1 (IRF-1) knockout (KO) mice. Additionally, 24 h after macrophage infection with a Δmfp::kan or Δomp19::kan strain expressing green fluorescent protein (GFP) approximately 80% or 65% of Brucella-containing vacuoles (BCVs) retained the late endosomal/lysosomal marker LAMP-1, respectively, whereas around 60% of BCVs containing wild-type S2308 were found in LAMP-1-negative compartments. B. abortus Δomp19::kan was attenuated in vivo but had a residual virulence in C57BL/6 and IRF-1 KO mice, whereas the Δmfp::kan strain had a lower virulence in these same mouse models. Furthermore, Δmfp::kan and Δomp19::kan strains were used as live vaccines. Challenge experiments revealed that in C57BL/6 and IRF-1 KO mice, the Δmfp::kan strain induced greater protection than the vaccine RB51 and protection similar that of vaccine S19. However, a Δomp19::kan strain induced protection similar to that of RB51. Thus, these results demonstrate that Brucella Mfp and Omp19 are critical for full bacterial virulence and that the Δmfp::kan mutant may serve as a potential vaccine candidate in future studies.

Brucellosis is a chronic infectious disease caused by bacteria of the genus Brucella. This disease affects many species of animals, resulting in great economic losses, and is therefore an important bacterial zoonotic disease worldwide (1). The genus Brucella replicates inside trophoblasts, macrophages, and dendritic cells and colonizes the reticuloendothelial system and reproductive organs (2). Additionally, brucellosis is not only the major cause of abortion and infertility in animals but also a debilitating disease in humans (2–7).

To overcome the immune system and establish a chronic infection, B. abortus utilizes diverse evasion mechanisms. This pathogen can penetrate host cells through lipid rafts (8). Once inside cells, the establishment of a persistent infection relies on the ability of the bacterium to form a Brucella-containing vacuole (BCV), which traffics from the endocytic compartment to the endoplasmic reticulum (ER), forming a replicative BCV. It is in this replicative BCV that the bacteria begin to multiply (8, 9).

Extensive vaccination programs have been undertaken to prevent brucellosis in animals. Despite their availability, live vaccine strains have critical disadvantages (10). The main vaccines currently available for brucellosis are S19 and RB51 (derived from B. abortus) and Rev-1 (derived from Brucella melitensis). However, these vaccine strains are virulent for humans and can induce abortion in animals. The strains S19 and Rev-1 do not enable a differential diagnosis between infected and vaccinated animals in serological diagnostic tests. Additionally, the strains RB51 and Rev-1 have variable efficacies, and RB51 is resistant to rifampin, the antibiotic of choice for the treatment of brucellosis in humans (11, 12). Numerous attempts to develop safer and more effective vaccines, including the use of DNA vaccines or recombinant protein vaccines, have had limited success (13–15). Considering the mechanism of infection of Brucella, the use of attenuated strains capable of efficiently inducing cellular immunity offers the best approach.

To better understand the pathogenesis associated with this disease, significant efforts have focused on defining the relevant Brucella virulence genes. Carrica et al. have characterized a highly conserved protein in Brucella, termed Brucella membrane fusogenic protein (Mfp) (16). Although the function of Brucella Mfp is unknown, this protein adopts an amphiphatic alpha-helical structure in the presence of phospholipid vesicles, high ionic strength, or acidic pH, and the interaction of Brucella Mfp with phospholipid vesicles promotes in vitro membrane fusion (16). The structural similarity between Brucella Mfp and fusogenic proteins com-

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were aliquoted, frozen, and quantified (CFU/ml).

kanamycin (50

sary, the medium was supplemented with ampicillin (100

were grown on Luria-Bertani (LB) medium (Invitrogen). When neces-

pared with the plated macrophages at 600 µ/ml and ampicillin (100 µ/ml) (BAkan/amp) to select the Brucella bacteria that expressed GFP, resulting in a Δmfp(pBBR4-gfp) or Δomp19(pBBR4-gfp) strain. The growing CFU were seeded again on BAkan to ensure purification, and the typical B. abortus morphology was assessed by Gram staining. The strain B. abortus S2308(pBBR4-gfp) generated by conjugation was obtained from our laboratory stock. The complementation experiments were performed using the entire mfp or omp19 gene inserted into the plasmid pBBR1MCS (with chloramphenicol resistance) that replicates in Brucella (20). In the present study, the gene replacement Δmfp:kan and Δomp19:kan mutant strains were tested in comparison to the wild-type strain for intracellular localization in vitro and persistence in vivo. Additionally, the potential use of these mutant strains as vaccines was tested in immunocompromised and immunocompetent mice.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. All B. abortus strains were grown on brucella broth (BB) medium (Becton Dickinson) or on plates of BB medium containing 1.5% agar (BA). Escherichia coli strains were grown on Luria-Bertani (LB) medium (Invitrogen). When necessary, the medium was supplemented with ampicillin (100 µg/ml) and/or kanamycin (50 µg/ml) and/or chloramphenicol (5 µg/ml). The bacteria were grown at 37°C with shaking at 180 rpm. After growth, the bacteria were aliquoted, frozen, and quantified (CFU/ml).

Mice. C57BL/6 mice and interferon regulatory factor 1 (IRF-1) knockout (KO) mice were obtained from the Federal University of Minas Gerais (UFMG) animal facility. They were used at 6 to 8 weeks of age and in groups of six to eight animals.

BMDM culture. Bone marrow-derived macrophages (BMDM) were obtained from C57BL/6 mice as follows. Each femur and tibia was flushed with 5 ml of Hanks’ balanced salt solution (HBSS). The resulting cell suspension was centrifuged, and the cells were resuspended in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) containing 10% fetal bovine serum (FBS; Gibco), penicillin-streptomycin (10 µg/ml), and 1% L929 cell-conditioned medium (LCCM) as a source of macrophage colony-stimulating factor (M-CSF). Cells were distributed into 24-well plates and incubated at 37°C in a 5% CO₂ atmosphere. Three days after seeding, another 0.1 ml of LCCM was added. On the 7th day, the medium was renewed, and on the 10th day, the cells were completely differentiated into macrophages (21).

Generation of GFP-expressing B. abortus mutant strains. The B. abortus Δmfp:kan and Δomp19:kan defined deletion mutants were constructed by gene replacement as described previously (21). The mfp or omp19 wild-type gene was replaced by double homologous recombination with the mfp or omp19 allele containing the kanamycin resistance gene, resulting in the Δmfp:kan or Δomp19:kan mutant strain (Table 1). To generate B. abortus wild-type (S2308) or mutant strains expressing green fluorescent protein (GFP), a plasmid containing the GFP sequence (pBBR4-gfp) was transformed into chemically competent E. coli S17, a conjugative donor strain (22). E. coli S17-GFP cells were then cultured with a B. abortus Δmfp:kan or Δomp19:kan strain in brucella agar without antibiotics to allow conjugation. After 48 h at 37°C, the colonies were seeded in brucella agar containing kanamycin (50 µg/ml) and ampicillin (100 µg/ml) (BAkan/amp) to select the Brucella bacteria that expressed GFP, resulting in a Δmfp(pBBR4-gfp) or Δomp19(pBBR4-gfp) strain. The growing CFU were seeded again on BAkan to ensure purification, and the typical B. abortus morphology was assessed by Gram staining. The strain B. abortus S2308(pBBR4-gfp) generated by conjugation was obtained from our laboratory stock. The complementation experiments were performed using the entire mfp or omp19 gene inserted into the plasmid pBBR1MCS (with chloramphenicol resistance) that replicates in Brucella, resulting in the complemented Δmfp(pBBR1-mfp) or Δomp19(pBBR1-omp19) strain as previously described by our group (21). GFP was also expressed in complemented mutant strains by conjugation using the plasmid pBBR4-gfp as described above. The complemented strains expressing GFP were termed the Δmfp(pBBR1-mfp, pBBR4-gfp) and Δomp19(pBBR1-omp19, pBBR4-gfp) strains.

Confocal microscopy. BMDM were differentiated on 12-mm glass coverslips in 24-well plates. Infection with the S2308(pBBR4-gfp), Δmfp(pBBR4-gfp), Δomp19(pBBR4-gfp), Δmfp(pBBR1-mfp, pBBR4-gfp), or Δomp19(pBBR1-omp19, pBBR4-gfp) strain was performed at a multiplicity of infection (MOI) of 100:1. Bacteria were centrifuged with the plated macrophages at 600 × g for 10 min at 4°C. After 30 min of incubation at 37°C with 5% CO₂, each well was washed four times with HBSS (500 µl) and incubated for 90 min in DMEM supplemented with

TABLE 1 | Bacterial strains and plasmid used in this study

<table>
<thead>
<tr>
<th>Bacterial strain or plasmid</th>
<th>Characteristics</th>
<th>Source or reference</th>
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<tr>
<td>B. abortus strains</td>
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<tr>
<td>B. abortus S2308</td>
<td>Wild type, smooth</td>
<td>LIDI</td>
</tr>
<tr>
<td>B. abortus S2308(pBBR4-gfp)</td>
<td>Wild type expressing GFP, smooth</td>
<td>LIDI</td>
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<tr>
<td>B. abortus S19</td>
<td>Vaccine strain, smooth</td>
<td>LIDI</td>
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<tr>
<td>B. abortus RB51</td>
<td>Vaccine strain, rough</td>
<td>LIDI</td>
</tr>
<tr>
<td>B. abortus Δmfp:kan</td>
<td>Kan', mutant of S2308, smooth</td>
<td>CICVyA-INTA</td>
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<tr>
<td>B. abortus Δmfp(pBBR4-gfp)</td>
<td>Kan' Amp', mutant of S2308, smooth</td>
<td>This study</td>
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<tr>
<td>B. abortus Δomp19:kan</td>
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<td>Kan' Amp', mutant of S2308, smooth</td>
<td>This study</td>
</tr>
<tr>
<td>B. abortus Δomp19(pBBR1-omp19)</td>
<td>Kan' Cm', mutant of S2308, smooth</td>
<td>CICVyA-INTA</td>
</tr>
<tr>
<td>B. abortus Δomp19(pBBR4-gfp)</td>
<td>Kan' Cm', mutant of S2308, smooth</td>
<td>This study</td>
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<td>B. abortus Δomp19(pBBR1-omp19)</td>
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<tr>
<td>E. coli strains</td>
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<tr>
<td>E. coli BL21</td>
<td>Competent strain for protein expression</td>
<td>LIDI</td>
</tr>
<tr>
<td>E. coli S17</td>
<td>Competent conjugative donor strain</td>
<td>LIDI</td>
</tr>
<tr>
<td>Plasmid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GFP</td>
<td>pBBR4-gfp Amp'</td>
<td>DNA 2.0</td>
</tr>
</tbody>
</table>

a Kan', kanamycin resistance; Amp', ampicillin resistance; Cm', chloramphenicol resistance.  
b LIDI, Laboratório de Imunologia de Doenças Infecciosas, UFMG, Belo Horizonte, Brazil; CICVyA-INTA, Instituto de Biotecnología, CICVyA-INTA, Buenos Aires, Argentina (Silvio L. C. Zaveri).

promly observed in viruses, bacteria, and eukaryotes is a strong indicator of their biological relevance (16–19). The distinctive properties of the Brucella outer membrane are critical to Brucella virulence. Omp19 is an immunoreactive outer membrane lipoprotein that is one of many proteins considered critical for Brucella virulence (20). In the present study, the gene replacement Δmfp:kan and Δomp19:kan mutant strains were tested in comparison to the wild-type strain for intracellular localization in vitro and persistence in vivo. Additionally, the potential use of these mutant strains as vaccines was tested in immunocompromised and immunocompetent mice.

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10% FBS, streptomycin (100 µg/ml), and gentamicin (100 µg/ml) to kill extracellular bacteria. Thereafter, the antibiotic concentration was decreased to 10 µg/ml. At 2 and 24 h postinfection, the BMDM were washed twice with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde, pH 7.4, at room temperature for 15 min. Once the cells were fixed, the coverslips were washed again and maintained in PBS at 4°C overnight. The cells were incubated with the primary antibody rabbit anti-LAMP-1 (Sigma) diluted in 10% FBS and 0.1% saponin in PBS for 1 h at room temperature. After samples were washed three times with PBS, the coverslips were incubated for 30 min at room temperature with an anti-rabbit secondary antibody conjugated to Alexa 546 (Jackson ImmunoResearch). The cells were washed once with 0.1% saponin in PBS, once in PBS, and once in water and then mounted in the mounting medium Prolong Gold with 4',6’-diamidino-2-phenylindole (DAPI; Invitrogen). The samples were examined by confocal microscopy (Nikon C2 confocal microscope).

Persistence of *B. abortus Δmfp and Δomp19 strains in C57BL/6 mice*. C57BL/6 mice were intraperitoneally (i.p.) injected with 10^6 CFU of a *B. abortus Δmfp::kan or Δomp19::kan* strain or with *B. abortus* S2308 as a control. Eight mice from each group were sacrificed at 1, 3, 6, and 12 weeks postinfection, and spleens were removed, macerated, and plated on BA medium after serial dilutions. CFU were counted after 3 days of incubation at 37°C with 5% CO_2.

**Virulence of *B. abortus Δmfp and Δomp19 strains in IRF-1 KO mice**. IRF-1 KO mice were i.p. injected with 10^6 CFU of the wild-type strain S2308, the Δmfp::kan or Δomp19::kan mutant strain, and the Δmfp(pBBR1-omp19) or Δomp19(pBBR1-omp19) complemented strain. Mouse survival was monitored daily for 90 days postinfection.

**Effectiveness of immunization with Δmfp and Δomp19 strains in C57BL/6 and IRF-1 KO mice**. C57BL/6 and IRF-1 KO mice were i.p. injected with 10^6 CFU of the Δmfp::kan or Δomp19::kan strain or with the vaccine strain S19 (10^5 CFU) or RB51 (10^6 CFU) as a control. Control immunized mice were injected with 0.1 ml of PBS. At 6 weeks postimmunization, all C57BL/6 and IRF-1 KO mice were challenged i.p. with *B. abortus* S2308 at 10^6 CFU. Two weeks later, the CFU counts from the spleens of C57BL/6 mice were taken as described above. For IRF-1 KO mice, survival was monitored for 30 days after infection with *B. abortus* S2308. We used a higher dose for the rough vaccine strain because the Δomp19 strain demonstrated reduced persistence during all time periods analyzed compared to that of the wild-type strain *B. abortus* S2308 (Fig. 2).

To determine whether *B. abortus* Mfp and Omp19 are involved in the persistence and confer immunoprotection to C57BL/6 mice. To determine whether the *B. abortus* Δmfp::kan or Δomp19::kan strain demonstrated reduced persistence during all time periods analyzed compared to that of the wild-type strain *B. abortus* S2308 (Fig. 2). The persistence of the Δomp19::kan strain was significantly reduced at 6 weeks postinfection. At 12 weeks postinoculation, both mutant strains were completely eliminated from the mouse spleens.

**RESULTS**

BCVs containing Δmfp and Δomp19 mutants retain lysosomal markers in BMDM. Inside the cell, the maturation process of the *Brucella*-containing vacuoles (BCVs) is characterized by transient interactions and partial fusions with organelles of the endocytic pathway. Such fusion events are required for bacteria to redirect their trafficking to the ER and for further maturation of BCVs into ER-derived replicative organelles. The formation of the replicative BCVs is characterized by the loss of lysosomal markers, such as lysosomal-associated membrane protein 1 (LAMP-1) (8, 9). Therefore, we analyzed whether the BCVs containing a Δmfp (pBBR4-gfp) or Δomp19(pBBR4-gfp) mutant strain lose or retain the LAMP-1 marker after BMDM infection. At 2 h postinfection, approximately 70% of the BCVs of the wild-type strain *B. abortus* S2308, the mutant Δmfp(pBBR4-gfp) or Δomp19(pBBR4-gfp) strain, and the complemented Δmfp(pBBR1-omp19, pBBR4-gfp) or Δomp19(pBBR1-omp19, pBBR4-gfp) strain were found in LAMP-1-positive compartments. However, at 24 h postinfection, approximately 60% of the BCVs containing the *B. abortus* S2308 and the complemented Δmfp(pBBR1-omp19, pBBR4-gfp) or Δomp19(pBBR1-omp19, pBBR4-gfp) strains were found in LAMP-1-negative compartments, while around 80% or 65% of the BCVs containing the Δmfp(pBBR4-gfp) or Δomp19(pBBR4-gfp) strain retained the LAMP-1 marker, respectively. Therefore, we conclude that Δmfp(pBBR4-gfp) and Δomp19(pBBR4-gfp) strains remain in LAMP-1-containing compartments 24 h after infection (Fig. 1). Additionally, we determined whether bacterial growth in *Brucella* broth was modified by the absence of the proteins Mfp or Omp19. Both the Δmfp::kan (193 ± 10 min) and Δomp19::kan (196 ± 6 min) mutant strains showed a generation time (*T_0*) slightly greater than that of the wild-type strain *B. abortus* S2308 (180 ± 2.8 min); however, these differences were not statistically significant (*P* < 0.05), as determined by posttests using the Bonferroni method after ANOVA (data not shown).

*B. abortus Δmfp and Δomp19 strains have reduced persistence and confer immunoprotection to C57BL/6 mice*. To determine whether *Brucella* Mfp and Omp19 are involved in the persistence of *B. abortus*, we compared the bacterial load in spleens of C57BL/6 mice inoculated with *B. abortus* wild-type strain S2308 or the Δmfp::kan and Δomp19::kan mutant strains. The Δmfp::kan strain demonstrated reduced persistence during all time periods analyzed compared to that of the wild-type strain S2308 (Fig. 2). The persistence of the Δomp19::kan strain was significantly reduced at 6 weeks postinfection. At 12 weeks postinoculation, both mutant strains were completely eliminated from the mouse spleens.

To determine whether the Δmfp::kan and Δomp19::kan strains could induce protective immunity against infection, C57BL/6 mice immunized with these mutant strains were challenged with *B. abortus* S2308. Bacterial CFU numbers in the spleens were determined 2 weeks after challenge. The degree of vaccine efficacy in C57BL/6 mice was determined by subtracting the mean number of CFU/spleen recovered from mice after immunization and challenged with *B. abortus* S2308 from the mean number of CFU/spleen recovered from unimmunized but challenged control mice (PBS). All immunized mice had significant reductions in the numbers of *B. abortus* S2308 bacteria in the spleen compared with the amounts in the spleen of unimmunized mice (Table 2). No significant differences were observed between mice immunized...
with the S19 and Δmfp::kan strains as well as with the RB51 and Δomp19::kan strains. However, the S19 and Δmfp::kan strains (1.73 and 1.47 log units, respectively) induced greater protection than the RB51 and Δomp19::kan strains (0.77 and 0.73 log units, respectively).

**B. abortus Δmfp induces IFN-γ production similar to that of the vaccine strains S19 and RB51.** IFN-γ is required for the bactericidal activity of macrophages and is a key cytokine in immunity against *Brucella* (24, 25). Therefore, we investigated the IFN-γ production of splenocytes in mice previously immunized with the Δmfp::kan or Δomp19::kan strain in response to challenge with the *B. abortus* wild-type strain. The Δmfp::kan mutant strain induced levels of IFN-γ similar to the level induced in response to the vaccine strains RB51 and S19 (Fig. 3). In contrast, the Δomp19::kan strain induced less IFN-γ in spleen cells of vaccinated mice than the Δmfp::kan, S19, and RB51 strains in spleen cells of immunized animals.

**B. abortus Δmfp and Δomp19 strains are attenuated and confer immunoprotection in IRF-1 KO mice.** Immunocompromised IRF-1 KO mice can detect different levels of *Brucella* viru-
lence, providing a useful tool for evaluating attenuation of Brucella mutants (23). To test the attenuation of the mutants, IRF-1 KO mice were infected with the S2308, Δmfp:kαn, and Δomp19::kan strains and with the Δmfp(pBBR1-mfp) or Δomp19(pBBR1-omp19) complemented strain at 10^6 CFU, and survival was monitored. All mice inoculated with the wild-type strain S2308 died between the 5th and 11th day after infection. After inoculation with the Δomp19::kan strain, 87.5% (7/8) of mice survived the first 4 weeks, and 25% (2/8) survived the entire period of observation (90 days). All mice inoculated with the Δmfp::kan strain survived the entire period of observation. These results indicate an attenuation of virulence of both mutant strains and especially of the Δmfp::kan strain, which showed complete attenuation (Fig. 4). Additionally, complementation of the Δmfp and Δomp19 mutants restored the wild-type strain 2308 phenotype since all mice inoculated with the Δmfp(pBBR1-mfp) or Δomp19(pBBR1-omp19) strain died within 15 days of infection. Our research group has previously demonstrated the virulence of the vaccine strains S19 and RB51 in IRF-1 KO mice. Only 70 to 80% of IRF-1 KO mice inoculated with S19 at 10^6 CFU survived the first 4 weeks after infection, while all mice inoculated with RB51 at 10^6 or 10^8 CFU survived the first 4 weeks (21, 26). Our data suggest the following order of virulence in IRF-1 KO mice: the S2308 strain > Δomp19::kan strain > Δmfp::kan strain.

To determine the protection efficacy of immunizations with the Δmfp::kan and Δomp19::kan strains, IRF-1 KO mice were vaccinated with the B. abortus mutants or with the vaccine strain S19 or RB51 as a control. Eight weeks after vaccination, all mice were challenged with wild-type S2308, and mouse survival was monitored for 30 days. After the challenge with B. abortus S2308, all mice that received saline died between the 8th and 14th days. All vaccinated mice presented increased survival compared to that of control animals inoculated with PBS, demonstrating that all strains activated the immune system and induced some degree of protection. However, the Δmfp::kan strain was the only one with 100% protection efficacy. At the end of the observation period, the percent survival of immunized mice was as follows: for the Δmfp::kan strain, 100%; S19, 83.3%; RB51, 50%; and the Δomp19::kan strain, 50% (Fig. 5).

### TABLE 2 Protective immunity induced by immunization with a B. abortus S19, RB51, Δmfp, or Δomp19 strain in C57BL/6 mice

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Log_{10} CFU of B. abortus S2308 in spleen (mean ± SD)^a</th>
<th>Protection (log_{10} U)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS control</td>
<td>6.67 ± 0.13</td>
<td></td>
</tr>
<tr>
<td>B. abortus S19</td>
<td>4.94 ± 0.41</td>
<td>1.73^b,c</td>
</tr>
<tr>
<td>B. abortus RB51</td>
<td>5.90 ± 0.42</td>
<td>0.77^a</td>
</tr>
<tr>
<td>B. abortus Δmfp::kan</td>
<td>5.20 ± 0.42</td>
<td>1.47^b,c</td>
</tr>
<tr>
<td>B. abortus Δomp19::kan</td>
<td>5.94 ± 0.35</td>
<td>0.73^a</td>
</tr>
</tbody>
</table>

^a Mice were inoculated with PBS, 10^6 CFU of RB51, or 10^7 CFU of the other strains. Six weeks later, mice were challenged i.p. with 10^6 CFU of S2308, and spleen CFU were enumerated 2 weeks after challenge. Results are representative of two independent experiments.

^b Significantly different compared to the PBS control group (P ≤ 0.0004).

^c Significantly different compared to the Δomp19::kan strain and RB51 (P = 0.003).

FIG 2 Persistence of B. abortus S2308, Δmfp, and Δomp19 strains in C57BL/6 mice. Mice were intraperitoneally injected with 10^6 CFU of B. abortus S2308 and the Δmfp::kan or Δomp19::kan strain. Splenocytes were removed at 1, 3, 6, and 12 weeks after infection, and the CFU were enumerated. The data points are presented as the log_{10} CFU per spleen. Statistically significant differences in relation to B. abortus wild-type S2308 are indicated as follows: *, P < 0.05; **, P < 0.01; ***, P < 0.001. This result is representative of two independent experiments.

FIG 3 Production of IFN-γ in splenocytes of C57BL/6 mice vaccinated with a B. abortus Δmfp or Δomp19 mutant strain. C57BL/6 mice were intraperitoneally injected with the wild-type S2308 (10^6 CFU), with vaccine strain S19 (10^6 CFU) or RB51 (CFU 10^7), or with the Δmfp::kan (10^7 CFU) or Δomp19::kan (10^6 CFU) strain. Unimmunized control mice were inoculated with PBS. After 6 weeks, a splenectomy was performed, and spleen cells were activated in vitro with B. abortus S2308 or ConA. After 72 h, the supernatant was collected, and the amount of IFN-γ was measured by ELISA. Statistically significant differences are indicated as follows: *, P < 0.001, compared to the PBS control group; #, P < 0.001, compared to the Δomp19::kan group. This result is representative of two independent experiments.

FIG 4 Virulence of the B. abortus S2308, Δmfp, Δomp19, Δmfp(pBBR1-mfp), or Δomp19(pBBR1-omp19) strain in IRF-1 KO mice. Mice were intraperitoneally injected with 10^6 CFU of each strain, and mouse survival was monitored for 90 days after infection. This result is representative of three independent experiments.

FIG 5 Persistence of B. abortus S2308, Δmfp, Δomp19 strains in C57BL/6 mice. Mice were intraperitoneally injected with 10^6 CFU of B. abortus S2308 and the Δmfp::kan or Δomp19::kan strain. Splenocytes were removed at 1, 3, 6, and 12 weeks after infection, and the CFU were enumerated. The data points are presented as the log_{10} CFU per spleen. Statistically significant differences in relation to B. abortus wild-type S2308 are indicated as follows: *, P < 0.05; **, P < 0.01; ***, P < 0.001. This result is representative of two independent experiments.
therefore analyzed the endosomal/lysosomal marker LAMP-1 (8, markers on BCVs is a hallmark of virulent establishes a replicative BCV. Consequently, the loss of lysosomal lysosomes, the bacterium redirects its trafficking to the ER and subsequently in the formation of fusogenic membrane fusion activity of Mfp (16) and its similarity to the mouse models studied here, the mutation in these mouse models. Additionally, the Δmfp::kan strain was more efficient in both animal models. Over 30 days of observation, the Δmfp::kan strain conferred 100% protection to the IRF-1 KO mice when they were challenged with the B. abortus virulent strain.

Previous studies with Brucella mutants suggest that some level of persistence is required for protection (12). This residual virulence cannot be so great as to cause disease or so low as to fail to successfully stimulate protective immunity. Our results suggest that the Δmfp::kan strain has a level of persistence that allows it to elicit potent protective immunity without causing disease. Moreover, although it was more attenuated, the Δmfp::kan strain induced greater protection than the Δomp19::kan strain. The low protection induced by the Δomp19::kan strain highlights the antigenic importance of Omp19, which corroborates the fact that immunization with Omp19 is enough to confer partial protection against wild-type B. abortus 2308 infection (31). Additionally, mutation in the mpf and omp19 genes does not grossly alter lipopolysaccharide (LPS) formation since both the Brucella Δmpf::kan and Δomp19::kan strains showed a smooth LPS phenotype, similar to that of the wild-type 2308 strain (data not shown). Further, Brucella Δmpf::kan presented levels of Omp19 expression similar to the level of wild-type bacteria, demonstrating that lack of Mfp does not influence the production of important antigens such as Omp19 (data not shown).

In conclusion, Omp19 and Mfp are critical for the full virulence of B. abortus. Additionally, the Δmfp::kan and Δomp19::kan mutant strains are able to induce protective immunity in C57BL/6 and IRF-1 KO mice at levels similar to those of the commercial strains S19 and RB51, respectively. Due to its protection efficacy in the mouse models studied here, the Δmpf::kan strain shows great potential as a vaccine for brucellosis, and this result justifies further investigation in large animals.

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REFERENCES


