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The *Brucella abortus* Phosphoglycerate Kinase Mutant Is Highly Attenuated and Induces Protection Superior to That of Vaccine Strain 19 in Immunocompromised and Immunocompetent Mice▽

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***Brucella abortus* is a facultative intracellular bacterial pathogen that causes abortion in domestic animals and undulant fever in humans. The mechanism of virulence of *Brucella* spp. is not yet fully understood. Therefore, it is crucial to identify new molecules that can function as virulence factors to better understand the host-pathogen interplay. Herein, we identified the gene encoding the phosphoglycerate kinase (PGK) of *B. abortus* strain 2308. To test the role of PGK in *Brucella* pathogenesis, a *pgk* deletion mutant was constructed. Replacement of the wild-type *pgk* by recombination was demonstrated by Southern and Western blot analyses. The *B. abortus* Δ*pgk* mutant strain exhibited extreme attenuation in bone marrow-derived macrophages and *in vivo* in BALB/c, C57BL/6, 129/Sv, and interferon regulatory factor-1 knockout (IRF-1 KO) mice. Additionally, at 24 h postinfection the Δ*pgk* mutant was not found within the same endoplasmic reticulum-derived compartment as the wild-type bacteria, but, instead, over 60% of *Brucella*-containing vacuoles (BCVs) retained the late endosomal/lysosomal marker LAMP1. Furthermore, the *B. abortus* Δ*pgk* deletion mutant was used as a live vaccine. Challenge experiments revealed that the Δ*pgk* mutant strain induced protective immunity in 129/Sv or IRF-1 KO mice that was superior to the protection conferred by commercial strain 19 or RB51. Finally, the results shown here demonstrated that *Brucella* PGK is critical for full bacterial virulence and that a Δ*pgk* mutant may serve as a potential vaccine candidate in future studies.**

Brucella spp. are responsible for a zoonosis that causes a serious economic impact worldwide, especially in developing countries, and a human disease that is difficult to treat (6). In animals, brucellosis is a major cause of abortions and infertility (25). In humans, infection can cause a serious debilitating disease manifested as undulant fever, endocarditis, arthritis, and osteomyelitis (30). Due to serious economic losses and public health risks, extensive efforts have been conducted to prevent the disease in animals through vaccination programs (28).

Brucella enters the host via the nasal, oral, and pharyngeal cavities; it proliferates within macrophages and prevents fusion of the phagosome with the lysosome by altering the intracellular traffic of the early phagosome vesicle (32, 41), and it is located in structures that resemble the endoplasmic reticulum (ER) (31). Therefore, *Brucella* is capable of establishing chronic infections due to its ability to avoid the killing mechanisms within macrophages and to escape the immune response, persisting in the host during its life span (10).

Despite the availability of live vaccine strains for cattle (S19 and RB51) and small ruminants (Rev-1), the vaccines have several drawbacks, including interference with diagnosis, resistance to antibiotics, and residual virulence, that prevent their use in humans (4, 5). Numerous attempts to develop safe and more effective vaccines, including the use of live vectors, DNA vaccine, or recombinant proteins, has had limited success (23, 29, 40). In the absence of defined protective immunogens, the use of attenuated vaccine strains offers the best approach.

Previous research in our laboratory has identified *Brucella* genes coding for metabolic enzymes (27, 36). Among them is *pgk*, which encodes the phosphoglycerate kinase (PGK). This enzyme catalyzes the reversible transfer of a phosphate group from 1,3-bisphosphoglycerate to ADP, resulting in phosphorylation of ATP and the formation of 3-phosphoglycerate. PGK is required both for glycolysis and gluconeogenesis (26). In the present study, a mutant of *pgk* was generated by gene replacement, and the effects of this mutation on intracellular bacterial survival were evaluated *in vitro* and *in vivo*.

MATERIALS AND METHODS

Mice. A pair of interferon (IFN) regulatory factor-1 knockout (IRF-1$^{-/-}$ or IRF-1 KO) mice in a 129/Sv background was kindly donated by Luis F. Lima Reis from the Ludwig Institute for Cancer Research, São Paulo, Brazil, and the mice were bred and maintained in our animal facility at the Federal University of Minas Gerais (UFMG). BALB/c, C57BL/6, and 129/Sv mice were purchased

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TABLE 1. Bacterial strains and vectors used in this study

Strain or plasmid	Characteristics	Source or reference
Strains		
E. coli TOP 10F	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 recA1 ara*Δ*139* Δ(*ara-leu*)*7697 galU galK rpsL* (Str^r) *endA1 nupG*	Invitrogen
B. abortus strains		
S2308	Wild type; smooth; virulent	Laboratory stock
S19	Vaccine strain; smooth	Laboratory stock
RB51	Rif^r; rough mutant of S2308	Laboratory stock
Δ*pgk* strain	Kan^r; Δ*pgk* mutant of S2308	This study
Plasmids		
pUC4K	ColE1; Amp^r Kan^r	GE Healthcare
pBluescript KS	ColE1; *bla*	Stratagene
pBBR1MCS	Broad-host-range cloning vector; Cm^r	21
pBBR1-*pgk*	1,345-kb KpnI and BamHI fragment containing the complete *B. abortus* S2308 *pgk* gene cloned into pBBR1MCS	This study

from the UFMG and maintained at the Department of Biochemistry and Immunology animal care facility, and 6- to 9-week-old mice were used for experimental infection.

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Brucella abortus* virulent strain S2308 and *B. abortus* vaccine strains RB51 and S19 were obtained from our own laboratory stock. They were grown in brucella broth medium (Becton Dickinson, Sparks, MD) for 3 days at 37°C on a rotary shaker (200 rpm), divided in aliquots, and frozen in 10% glycerol. Then, the number of CFU/ml was enumerated in one aliquot to determine the number of viable bacteria that we were inoculating in the animals. If necessary, the medium was supplemented with ampicillin or kanamycin (25 μg/ml) or chloramphenicol (20 μg/ml) and with 0.1% erythritol. *Escherichia coli* TOP 10F was cultured at 37°C in Luria-Bertani medium containing kanamycin (50 μg/ml) or ampicillin (100 μg/ml) as needed.

Isolation and DNA sequencing analysis of the *B. abortus pgk* gene. The *pgk* gene was isolated through screening of the *B. abortus* S2308 genomic library, using the *gap* (glyceraldehyde-3-phosphate-dehydrogenase) gene fragment as a DNA probe (36). In this screening process a clone of approximately 22 kb was obtained and partially sequenced using primer walking to obtain the open reading frame (ORF) of the *B. abortus pgk* gene. Double-stranded DNA was sequenced by the dideoxy chain termination method (38) by using a MegaBACE 1000 system (GE Healthcare, São Paulo, Brazil). The clone was sequenced with a DYEnamic ET Dye Terminator kit (GE Healthcare); the primers used were M13 reverse sequence and M13 universal sequence from GE Healthcare, and specific primers were purchased commercially. The sequences of the specific primers used were as follows: F1, 5′-CGTGGTACGACAATGAATGG-3′; F2, 5′-CATTTTGCCGAAGACTGC-3′; F3, 5′-GGTCTTGATGTCGGCAAA-3′; F4, 5′-GACTTCACCTATATCTCA-3′; R1, 5′-ACCGGGCTTATGTGGATG-3′; R2, 5′-CTGGTGTGGCGTATCGGC-3′; and R3, 5′-CTGCACGAATTCA GGATC-3′. The sequence data were compiled and analyzed by using the sequence analysis program DNASIS, version 5.00 (Hitachi Software). Subsequent homology searches were performed by using BLAST programs.

Generation of *B. abortus pgk* deletion mutant. The *B. abortus pgk* gene was amplified by PCR from the 22-kb genomic clone and subcloned into pBluescript II KS$^+$ (Stratagene, La Jolla, CA). Primers containing one artificial restriction site at each end were constructed according to the *pgk* nucleotide sequence. The primer sequences were (forward) 5′-GTAGGATCCATGATGTTCCGCACCC TT-3′ (containing a BamHI site) and (reverse) 5′-GGGGGTACCTCACTTCT TCAATACATC-3′ (containing a KpnI site). PCR was performed with a 10-μl volume containing assay buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl, 500 mM $MgCl_2$), a mixture of the four deoxynucleoside triphosphates at 10 mM each, a 5 pmol/μl concentration of each primer, 10 ng of template DNA, and 2.5 U of *Taq* DNA polymerase (Promega). Amplification was performed with a PTC-100 Programmable Thermal Controller (MJ Research) at 95°C for 3 min, followed by 30 cycles of denaturation for 30 s at 95°C, annealing for 45 s at 68°C, and extension for 1 min at 72°C. The amplified gene fragment was digested with appropriate restriction endonucleases and cloned into pBluescript II SK$^+$ (pBlue-*pgk*). To generate a *pgk* deletion mutant by homologous recombination, the recombinant plasmid (pBlue-*pgk*) was used to construct the target vector, pBlue-*pgk-kan*. pBlue-*pgk* was digested with EcoRI and ligated with an EcoRI 1.2-kb DNA fragment encoding the kanamycin resistance gene from plasmid pUC4K (GE Healthcare). Five micrograms of pBlue-*pgk-kan* plasmid DNA was added to 50 μl of *B. abortus* S2308 competent cells in sterile electroporation cuvettes with 0.2-cm electrode gaps (Bio-Rad Laboratories, Richmond, CA), and then electroporation was performed with a Gene Pulser II transfection apparatus (Bio-Rad Laboratories) at 25 μF, 2.5 kV, and 400 Ω. Then, colonies that were Kan^r Amp^s and Kan^r Amp^r were selected as colonies in which double- or single-crossover events had occurred, respectively.

Characterization of the *B. abortus pgk* deletion mutant by Southern blotting. To provide genetic evidence that the wild-type *pgk* gene was replaced by a *pgk* gene interrupted by the Kan^r cassette, 10 μg of genomic DNA isolated from both the mutant strain and the wild-type strain (S2308) was digested with EcoRV and then loaded onto a 0.8% agarose gel for Southern blotting, performed as previously described (35).

Western blot analysis. For analysis of *pgk* expression in *B. abortus* S2308, the Δ*pgk* mutant strain and Δ*pgk* strain complemented with pBBR1-*pgk* were grown in 10 ml of brucella broth (BD) overnight at 37°C with agitation (200 rpm). Then, 1 ml of each culture was pelleted and resuspended in SDS sample buffer at an optical density at 600 nm (OD_{600}) of 1. The samples were boiled for 5 min, and 10 μl was loaded on a 12% SDS-PAGE gel. After the gel was run, the samples were transferred onto a nitrocellulose membrane (Hybond-ECL; GE Healthcare) for 1 h at 350 mA using a dry transfer system (Bio-Rad); the membrane was blocked overnight with 10% dry milk in TBST buffer (100 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.2). After the membranes were blocked, they were washed tree times with TBST buffer and probed with anti-PGK antibodies or naive mice serum diluted 1:100 in TBST buffer for 4 h at room temperature. The polyclonal anti-PGK antibodies were produced in our lab by immunizing mice with recombinant PGK (rPGK) protein. After reacting with the primary antibody, the blots were washed six times with TBST buffer and incubated for 1 h at room temperature with anti-mouse IgG conjugated to alkaline phosphatase (Promega) and diluted 1:2,000 in TBST buffer. After three washes with TBST buffer, the reaction was developed after the mixture was incubated at room temperature with NBT (nitroblue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3-indolyl-1-phosphate).

Infection of bone-marrow derived macrophages (BMDM) with the *Brucella* mutant Δ*pgk*. Macrophages were derived from C57BL/6 mouse bone marrow as follows. Each femur and tibia was flushed with 5 ml of Hank's 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) and 10% L929 cell-conditioned medium (LCCM) as a source of macrophage colony-stimulating factor (M-CSF) (24). The cells were distributed in 24-well plates and incubated at 37°C in a 5% CO_2 atmosphere. Three days after seeding, another 0.1 ml of LCCM was added. On the seventh day, the medium was renewed. On the 10th day of culture, when cells were completely differentiated into macrophages, they were infected with *B. abortus* S2308 or Δ*pgk* corresponding to a multiplicity of infection (MOI) of 50:1.

The plates were then centrifuged at 600 × *g* for 10 min at 4°C in order to synchronize the infections. Phagocytosis was allowed to proceed for 30 min at 37°C. At this point, the culture medium was removed, and the monolayer was washed three times with phosphate-buffered saline (PBS). Cultures were incubated for 90 min at 37°C with medium containing 50 μg/ml gentamicin (Sigma) to kill extracellular bacteria. At each time point studied, the infected cells were washed three times with PBS and lysed in 1 ml of 0.1% Triton X-100 in double-distilled H_2O (ddH_2O). The number of viable intracellular *Brucella* bacteria recovered from lysed macrophages was determined at 2, 24, 48, 72, 120, and 168 h following infection. Tenfold serial dilutions of bacterial suspensions in PBS were plated in duplicate on brucella agar with or without kanamycin (50 μg/ml). The number of CFU was determined after 3 days of incubation at 37°C with 5% CO_2. The experiments were performed in triplicate and repeated twice.

Confocal microscopy. Infected C57BL/6 mouse BMDM grown on 12-mm glass coverslips in 24-well plates were fixed in 3% paraformaldehyde, pH 7.4, at 37°C for 15 min. Cells were labeled by inverting coverslips onto drops of primary antibodies diluted in 10% horse serum and 0.1% saponin in PBS and incubating the samples for 30 min at room temperature. The primary antibodies used for immunofluorescence microscopy were the following: cow anti-*B. abortus* polyclonal antibody, rat anti-mouse LAMP1 ID4B (Developmental Studies Hybridoma Bank, National Institute of Child Health and Human Development, University of Iowa), and rabbit anticalnexin polyclonal antibody (Stressgene Bioreagent Corp., British Columbia, Canada). Bound antibodies were detected by incubation with a 1:1,000 dilution of Alexa Fluor 488 goat anti-rat, a 1:100 dilution of Texas Red goat anti-cow, or a 1:500 dilution of donkey anti-rabbit Cyanin 3 antibody (Jackson ImmunoResearch Laboratories, Suffolk, United Kingdom) for 30 min at room temperature. Cells were washed twice with 0.1% saponin in PBS, once in PBS, and once in H_2O and then mounted in Mowiol 4-88 mounting medium (Calbiochem, Darmstadt, Germany). Samples were examined on a Zeiss LSM 510 laser scanning confocal microscope for image acquisition. Images of 1,024 by 1,024 pixels were then assembled using Adobe Photoshop, version 7.0. Quantification was always done by counting intracellular bacteria in at least 50 cells in three independent experiments, as previously reported (37).

Persistence of *Brucella* Δ*pgk* mutant in BALB/c, C57BL/6, and 129/Sv mice. To assess the persistence of the *Brucella* Δ*pgk* mutant in different mouse strains, eight animals from each group were examined at each sampling period. Female C57BL/6, 129/Sv, and BALB/c mice 6 to 8 weeks old were injected intraperitoneally (i.p.) with 1×10^6 CFU of either the *B. abortus* S2308 or Δ*pgk* mutant strain in 0.1 ml of PBS. At 1, 2, 3, 4, and 6 weeks postinoculation, all mice in each group were killed, and bacterial counts were determined. Tenfold serial dilutions of the homogenized spleens were plated on brucella agar containing kanamycin to determine the number of *Brucella* Δ*pgk* CFU per spleen compared to the number of the wild-type S2308 strain. *Brucella* colonies were counted after 3 days of incubation at 37°C with 5% CO_2. Data are presented as $\log_{10}$ values of CFU per spleen. The experiment was repeated twice.

Virulence of *Brucella* Δ*pgk* mutant in IRF-1 KO mice. Four groups of eight IRF-1 KO mice were injected i.p. with 1×10^6 CFU of either *B. abortus* S2308, the Δ*pgk* mutant, or vaccine strain S19 or RB51 in 0.1 ml. Mouse survival was observed during 30 days postinfection as previously demonstrated (18). The experiment was repeated twice.

Immunization of mice with the *B. abortus* Δ*pgk* mutant. Female BALB/c, 129/Sv, C57BL/6, and IRF-1 KO mice that were 6 to 9 weeks old were immunized i.p. with brucellae in 0.1 ml of PBS. Groups containing eight mice each were immunized with either *B. abortus* S19 or Δ*pgk* at 1×10^5 CFU or with *B. abortus* RB51 at 1×10^7 CFU. Nonimmunized, control mice were injected i.p. with 0.1 ml of PBS. Twelve weeks after immunization, all mice in each group were challenged by i.p. injection of 1×10^6 CFU of *B. abortus* S2308. Experimentally infected BALB/c, 129/Sv, and C57BL/6 mice were killed 2 weeks later by cervical dislocation, and the spleens were collected and disrupted in 10 ml of PBS. A tenfold serial dilution was plated on brucella agar containing kanamycin or 0.1% erythritol for differentiation of *B. abortus* Δ*pgk*, S19, and S2308. Additionally, we used the crystal violet method to differentiate between the RB51 and S2308 strains. After 3 days of incubation at 37°C, colonies were visualized, and the number of CFU of *B. abortus* S2308 per spleen was determined after the number of *B. abortus* S19 or *B. abortus* Δ*pgk* CFU found by replica plating was subtracted. The degrees of protection in immunized animals and controls were expressed as the mean number of CFU of *B. abortus* S2308 for each mouse group obtained after challenge and $\log_{10}$ conversion. Log_{10} units of protection were obtained by subtracting the mean $\log_{10}$ CFU for the experimental group from the mean $\log_{10}$ CFU for the control group, as previously described (35). For IRF-1 KO mice, survival was observed during 30 days postinfection. The experiment was repeated twice.

Cytokine detection. Six weeks after immunization, IRF-1 KO mice were sacrificed, and their spleens were removed under aseptic conditions. Splenocytes from naive or infected mice were obtained by mechanically disrupting the spleen and collecting the resulting single-cell suspension. Cells were suspended in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 2 mM L-glutamine, 25 mM HEPES, 10% heat-inactivated fetal bovine serum (Sigma), penicillin G sodium (100 U/ml), and streptomycin sulfate (100 μg/ml) (supplemented RPMI medium). Erythrocytes were eliminated with ACK lysis solution (150 mM NH_4Cl, 1 mM Na_2-EDTA [pH 7.3]). Splenocytes were cultured in 96-well microtiter plates with 1×10^6 cells/well in a volume of 0.2 ml to assess cytokine production. The culture was stimulated by the addition of 10^2 heat-killed bacteria (heat inactivation was performed at 80°C for 2 h) per cell or 2 μg/ml of concanavalin A. These cells were incubated at 37°C in a humidified chamber with 5% CO_2. Aliquots of the supernatant were collected after 72 h of culture for IFN-γ measurements. Levels of IFN-γ in the supernatants were measured by a commercially available Duoset ELISA Development System kit (R&D Systems, Minneapolis, MN).

Statistical analysis. Statistical analysis was performed with a Student's *t* test using the computer software package MINITAB (Minitab Inc., State College, PA).

Nucleotide sequence accession number. The nucleotide sequence of the *pgk* gene of *B. abortus* was deposited in GenBank under accession number AF256214.

RESULTS

Characterization of the *B. abortus* Δ*pgk* deletion mutant. A defined Kan^r Amp^s Δ*pgk* deletion mutant of *B. abortus* S2308 was constructed by chromosomal gene replacement. Chromosomal DNA was isolated from these clones and from the parental strain for Southern blot analysis. The same hybridization profile was observed for all transformants selected from each different phenotype group, as shown in Fig. 1. DNA hybridization of EcoRV-digested chromosomal DNA by using the *pgk* probe produced one fragment at approximately 5.8 kb for wild-type *B. abortus* S2308 (Fig. 1A, lane 3) and a 7-kb band for the Kan^r Amp^s Δ*pgk* mutant (lane 1). A single recombination was confirmed when the Amp^r probe hybridized to only one fragment corresponding to integration of the deletion plasmid (pBlue-*pgk-kan*) in the chromosome (Fig. 1B). When the kanamycin cassette was used as a probe, it hybridized to genomic DNA from the Kan^r Amp^s or Kan^r Amp^r clone but not to genomic DNA of wild-type *B. abortus* S2308, which was used as a negative control (Fig. 1C). To determine whether *pgk* expression was taking place in Δ*pgk* and if we would be able to complement the mutant strain with pBBR1-*pgk* plasmid, Western blot analysis was carried out using anti-PGK polyclonal antibodies. The wild-type and complemented Δ*pgk* strains showed the presence of PGK protein of approximately 42 kDa, whereas the mutant strain lacked *pgk* expression (Fig. 2).

***B. abortus* Δ*pgk* mutant is attenuated in macrophages.** To investigate the role of the *pgk* gene in intracellular *B. abortus* survival, we evaluated the multiplication of *B. abortus* S2308 wild type and the *B. abortus* Δ*pgk* mutant in bone-marrow derived macrophages. The number of viable bacteria was counted at 2, 24, 48, 120, and 168 h postinfection. To inhibit extracellular bacterial growth, gentamicin was added to the medium 30 min after infection at a concentration of 50 μg/ml. Shortly after infection (time zero) there was no difference ($P > 0.05$) between the wild type and the mutant in the number of bacteria infecting the macrophages (Fig. 3). In contrast, by 24 h postinfection there was a 2-log difference ($P < 0.05$) in the number of organisms surviving inside the macrophages. The *Brucella* Δ*pgk* mutant displayed a lower rate

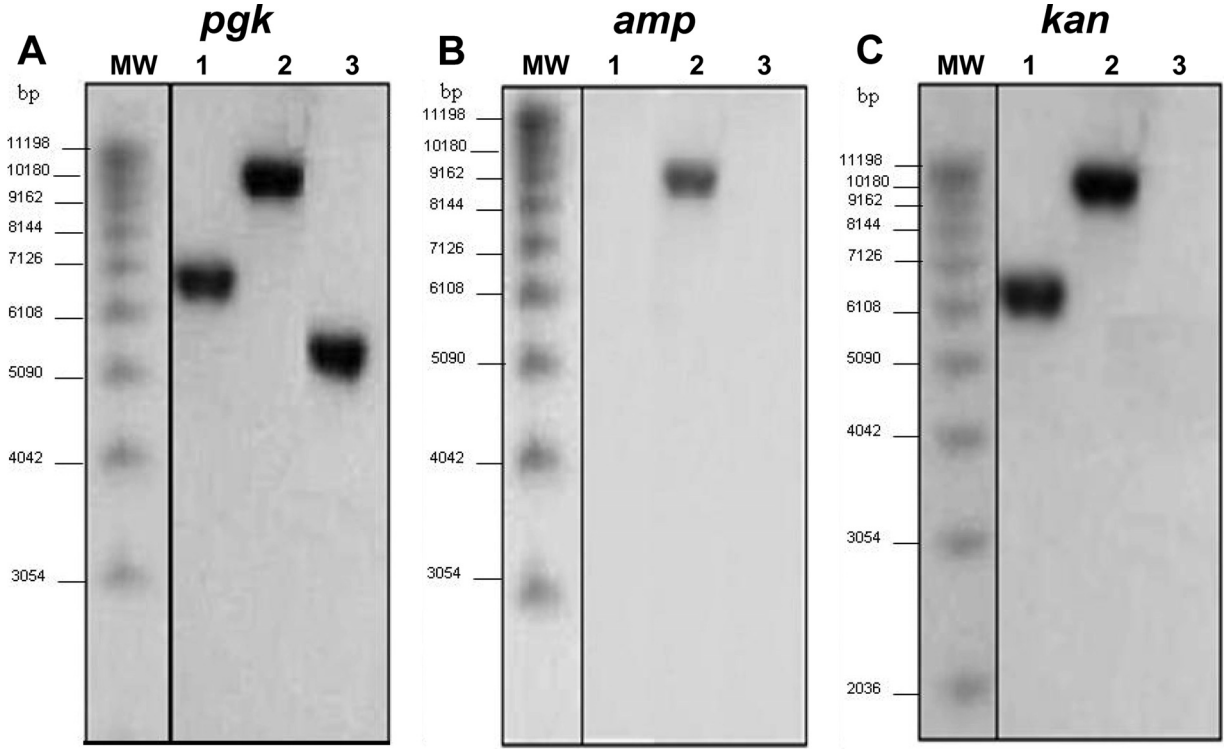

FIG. 1. Characterization of the *B. abortus* Δ*pgk* mutant by Southern blot analysis. EcoRV-digested genomic DNA was probed with the *pgk* (A), *amp* (B), or *kan* (C) DNA fragments. Lanes 1, *B. abortus* Δ*pgk* mutant; lanes 2, clones in which a single recombination event took place; lanes 3, *B. abortus* S2308; lanes MW, molecular weight markers.

of intracellular replication in macrophages than the wild-type strain S2308 at all times studied. These results demonstrate that the Δ*pgk* mutant has a limited ability to replicate within macrophages.

***Brucella* Δ*pgk* mutant does not recruit ER markers in BMDM.** It has been well demonstrated that wild-type *B. abortus* establishes a replicative niche in macrophages, acquiring endoplasmic reticulum markers (9). At 24 h postinfection, wild-type bacteria is found in calnexin (endoplasmic reticulum marker)-positive and LAMP1 (late endosomal/lysosomal marker)-negative compartments. In contrast, immunofluorescence analysis of infected BMDM showed that, unlike wild-type bacteria, at 24 h postinfection the Δ*pgk* mutant was not found in the replication vacuole containing ER markers (Fig. 4C), and instead over 60% of *Brucella*-containing vacuoles (BCVs) retained the late endosomal/lysosomal marker LAMP1 (Fig. 4A and B). Furthermore, at 48 h following infection, around 80% of BCVs containing Δ*pgk* mutant bacteria retained LAMP1. These results are consistent with the attenuation of the Δ*pgk* mutant compared to wild-type strain 2308.

***B. abortus* Δ*pgk* is highly attenuated in BALB/c, 129/Sv, C57BL/6, and IRF-1 KO mice.** The ability of *B. abortus* to persist within BALB/c mice has been shown to correlate with virulence in the natural host (11). Groups of BALB/c, C57BL/6, and 129/Sv mice with different backgrounds were

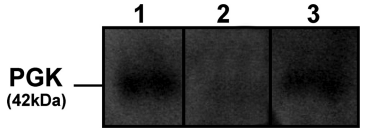

FIG. 2. Western blot analysis of *pgk* expression in different *B. abortus* strains. *B. abortus* S2308 (lane 1), Δ*pgk* mutant (lane 2), or Δ*pgk* mutant complemented with pBBR1-*pgk* plasmid (lane 3) were subjected to SDS-PAGE, and Western blot analysis using polyclonal anti-PGK antibodies demonstrated the presence of a band of approximately 42 kDa in lanes 1 and 3, which indicated *pgk* expression.

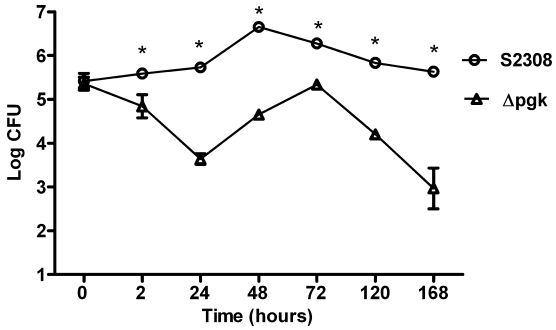

FIG. 3. Intracellular replication of *B. abortus* S2308 and Δ*pgk* in BMDM. Adherent cells were infected at an MOI of 50 with *B. abortus* S2308 or the Δ*pgk* mutant as described in Materials and Methods. At 2, 24, 48, 72, 120, and 168 h postinfection, macrophages were lysed and enumerated by serial dilutions plated in duplicate. The data points, presented as the $\log_{10}$ CFU per well, are the mean with standard error of the mean (SEM) of two independent experiments performed in triplicate. Statistically significant differences ($P \leq 0.05$) between the Δ*pgk* mutant value and that of the parental strain S2308 are indicated by an asterisk. This result is representative of two independent experiments.

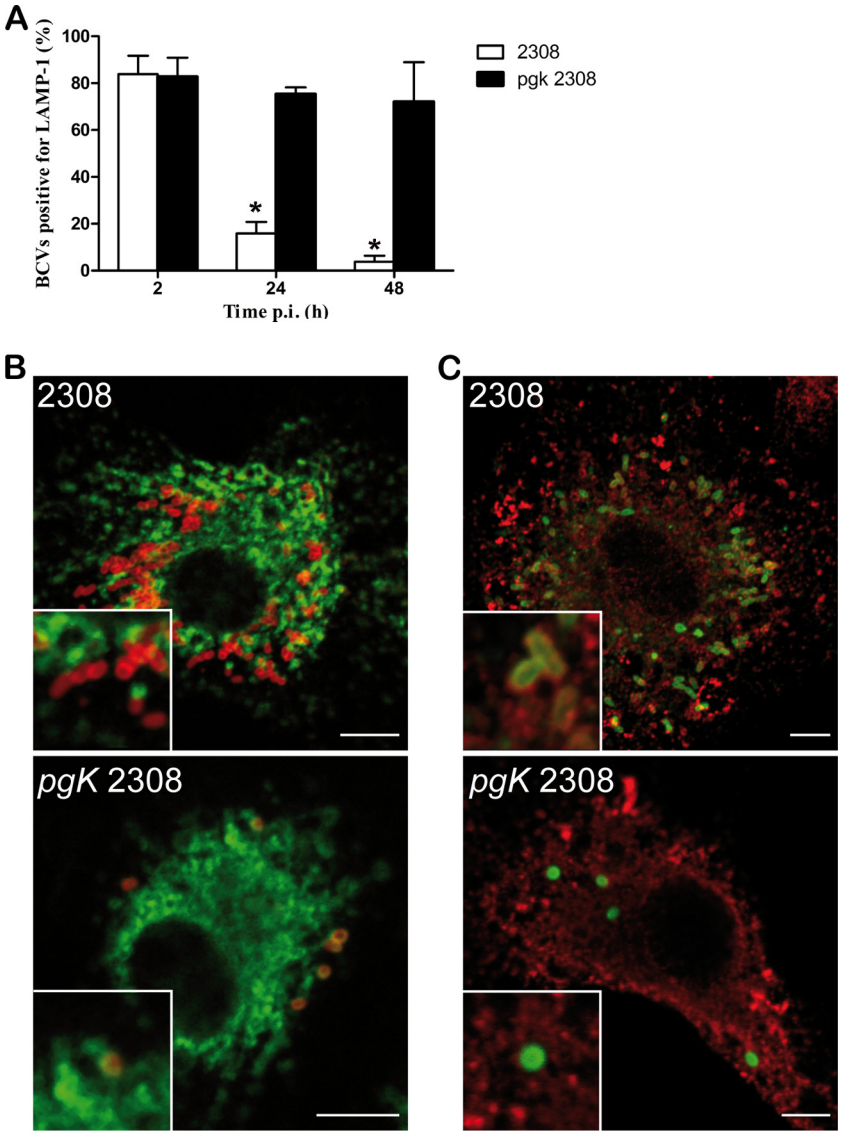

FIG. 4. Multiplication and intracellular localization of *B. abortus* Δ*pgk* mutant and wild-type strain in BMDM. (A) Quantification of the percentage of wild-type or Δ*pgk* mutant BCVs that contain LAMP1 by confocal immunofluorescence microscopy. The difference between the wild type and mutant was statistically significant at 24 and 48 h ($P < 0.001$) postinfection (p.i.). Data are means from three different experiments. (B) Representative confocal images of BMDM at 24 h postinfection with wild-type *B. abortus* or the Δ*pgk* mutant. *Brucella* lipopolysaccharide (LPS) is labeled in red, and LAMP1 is in green. (C) Confocal images of BMDM at 24 h postinfection with wild-type *B. abortus* or the Δ*pgk* mutant. *Brucella* LPS is labeled in green, and calnexin is shown in red. Scale bar, 5 μm.

inoculated i.p. with the Δ*pgk* mutant or *B. abortus* parental strain S2308 to determine differences in persistence. The number of *Brucella* CFU was evaluated at 1, 2, 3, 4, and 6 weeks postinfection in the spleen of each animal. The *B. abortus* Δ*pgk* mutant strain displayed reduced virulence at all times tested in different mouse models compared to the virulence of the wild-type *Brucella* strain (Fig. 5). Additionally, IRF-1 KO mice were infected with *B. abortus* S2308, S19, RB51, or the Δ*pgk* mutant strain. IRF-1 KO mice can detect different levels of *Brucella* virulence, providing a useful tool to screen *B. abortus* mutants regarding their level of intracellular survival (18). IRF-1 KO mice infected with S19, RB51, or Δ*pgk* survived longer than mice infected with wild-type S2308 ($P \leq 0.005$). All IRF-1 KO mice infected with strain S2308 died within 11 days postinfection. Eighty percent of IRF-1 KO mice injected with *B. abortus* S19 were alive at 30 days postinfection. Additionally, at 30 days postinfection all mice infected with attenuated *B. abortus* strain RB51 or the Δ*pgk* mutant strain were alive, demonstrating that both strains are less virulent than S19 in this mouse model (Fig. 6).

Immunoprotection conferred by vaccination with the *B. abortus* Δ*pgk* mutant strain in BALB/c, C57BL/6, 129/Sv, and IRF-1 KO mice. To determine if the *B. abortus* Δ*pgk* mutant strain is able to induce protective immunity against infection, BALB/c, 129/Sv, C57BL/6, and IRF-1 KO mice immunized with the Δ*pgk* mutant or with the S19 or RB51 vaccine strain were challenged with the *B. abortus* virulent S2308 strain. The numbers of bacterial CFU in the spleens were determined 12

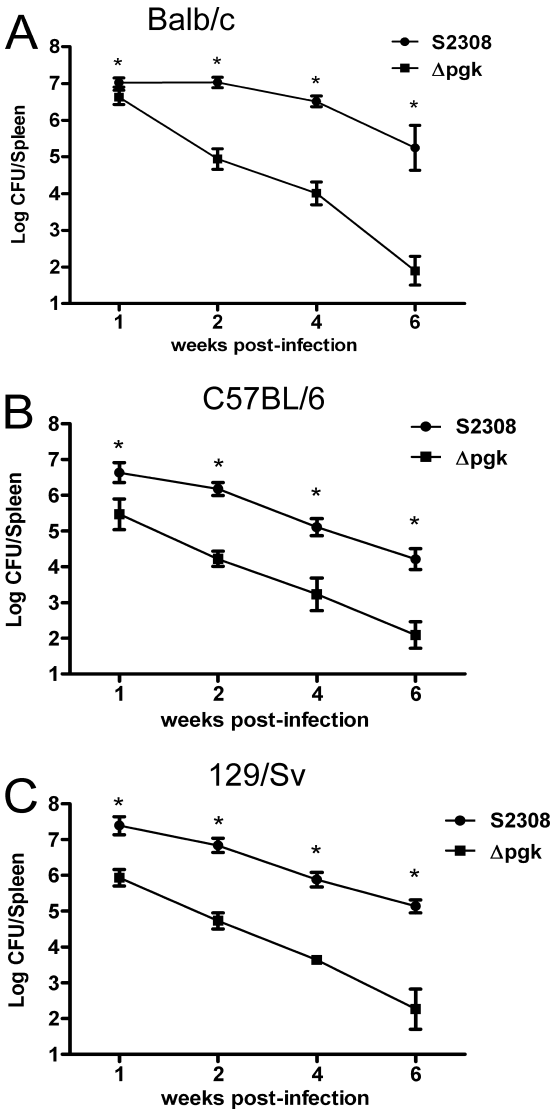

FIG. 5. Persistence of *B. abortus* S2308 or Δ*pgk* in BALB/c, C57BL/6, or 129/Sv mice. Eight mice were infected i.p. with a dose of 10^6 CFU of bacteria. Spleens were harvested at different times, and the number of CFU in disrupted tissue was determined by 10-fold serial dilution and plating. The values are means and standard deviations. The asterisks indicate statistically significant differences between the results obtained for the group that received the *B. abortus* Δ*pgk* mutant compared to the animals injected with the *B. abortus* parental strain S2308 ($P \leq 0.05$).

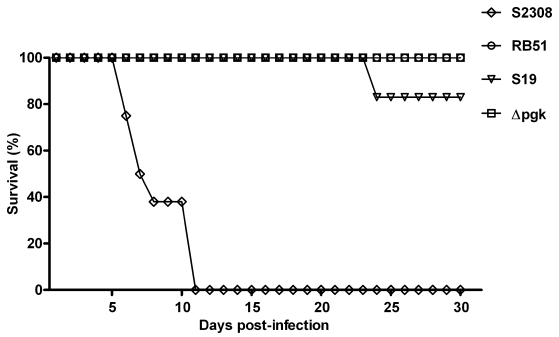

FIG. 6. Virulence of *B. abortus* S2308, S19, RB51, or the Δ*pgk* mutant strain in IRF-1 KO mice. Groups of 10 mice received intraperitoneally 10^6 CFU of each strain and were monitored daily for survival during 30 days.

weeks after challenge since Araya et al. (2) showed that nonspecific resistance to infection with unrelated bacteria is very low 6 weeks after immunization with *Brucella*. BALB/c, C57BL/6, and 129/Sv mice immunized with the *B. abortus* Δ*pgk* mutant had significantly fewer splenic brucellae than nonimmunized animals (Table 2). Additionally, we observed similar log units of protection in BALB/c and C57BL/6 mice immunized with the Δ*pgk* mutant strain (0.96 and 1.36 log units, respectively) compared to mice immunized with the commercial vaccine strain S19 (0.94 and 1.73 log units, respectively) and higher log units of protection in mice immunized with Δ*pgk* than with the commercial vaccine strain RB51 (0.58 and 0.76 log units, respectively) following challenge. In 129/Sv mice, immunization with the *B. abortus* Δ*pgk* mutant strain induced protection (3.28 log units) superior to that in animals immunized with the commercial vaccine strain S19 (2.46 log units) and higher log units of protection than in mice immunized with commercial vaccine strain RB51 (1.97 log units) following challenge. IRF-1 KO mice were immunized with the *Brucella* Δ*pgk* mutant or the S19 or RB51 vaccine strain for 12 weeks and challenged with 1×10^6 CFU of virulent S2308. All IRF-1 KO mice immunized with any *Brucella* vaccine strain survived longer than nonimmunized IRF-1 KO mice, suggesting that immunological memory was activated in these animals and that it could provide protection. However, 90% of IRF-1 KO mice immunized with Δ*pgk* were alive at 30 days postchallenge while 70% or 80% of animals vaccinated with RB51 or S19, respectively, survived during this period (Fig. 7). Therefore, the *B. abortus* Δ*pgk* mutant significantly enhanced resistance to experimental infection compared to the S19 or RB51 commercially available vaccine strain. In order to determine if IFN-γ responses were altered in IRF-1 KO mice immunized

TABLE 2. Protective immunity induced by Δ*pgk* mutant immunization

Mouse strain and inoculum	Mean (SD) $\log_{10}$ CFU in mouse spleen	Log_{10} U of protection[a]
BALB/c		
PBS	7.07 (0.12)	
B. abortus RB51	6.49 (0.42)	0.58*
B. abortus S19	6.13 (0.59)	0.94*
B. abortus Δ*pgk* strain	6.11 (0.41)	0.96*
C57BL/6		
PBS	6.66 (0.14)	
B. abortus RB51	5.90 (0.41)	0.76*
B. abortus S19	4.93 (0.15)	1.73*#
B. abortus Δ*pgk* strain	5.30 (0.15)	1.36*#
129/Sv		
PBS	7.38 (0.20)	
B. abortus RB51	5.42 (0.19)	1.97*
B. abortus S19	4.93 (0.42)	2.46*#
B. abortus Δ*pgk* strain	4.10 (0.11)	3.28*#†

[a] Significance is indicated as follows: *, $P \leq 0.05$ compared to PBS control group; #, $P \leq 0.05$ compared to the RB51-vaccinated group; †, $P \leq 0.05$ compared to the S19-vaccinated group.

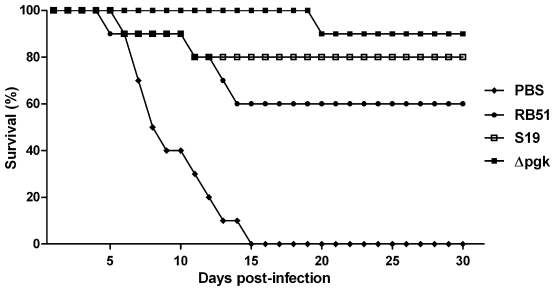

FIG. 7. Protection of IRF-1 KO mice vaccinated with *B. abortus* S19, RB51, or the Δ*pgk* mutant strain. Animals received intraperitoneally 10^5 CFU of *B. abortus* S19, 10^5 CFU of Δ*pgk*, or 10^7 CFU of RB51, and 12 weeks after immunization all mice were challenged by i.p. injection of 10^6 CFU of *B. abortus* S2308. Groups of 10 mice were monitored daily for survival for 30 days. Vaccination of mice with the Δ*pgk* mutant induced protection superior to that of immunization with vaccine strain S19 or RB51.

with different vaccine strains, cytokine production in splenocyte culture was determined. Figure 8 demonstrates that the Δ*pgk* mutant and S19 induced similar levels of IFN-γ in vaccinated IRF-1 KO mice. However, animals immunized with strain RB51 showed reduced production of IFN-γ compared to mice vaccinated with the Δ*pgk* mutant and S19. This difference might be one of the reasons for the increased susceptibility to brucellosis in IRF-1 KO mice immunized with RB51 and challenged with virulent S2308.

DISCUSSION

Intracellular bacteria require special features, such as adhesion, invasion, and replication, for interacting with the host and causing infection (15). *Brucella* behaves as a stealthy organism, disturbing the cell functions as little as possible (20). It is well established that upon entry the bacteria-lipid raft interaction in host cells allows *Brucella* to escape from the degradative endocytic pathway and further favors intracellular replication (22). Therefore, it is crucial to identify new molecules that can function as virulence factors to better understand this host-pathogen interplay.

The development of vaccines to control brucellosis has proven to be a challenge for years. The observation that the highest levels of protection are obtained when the host is immunized with live vaccines indicates that persistence and vaccine viability are key aspects required for an efficient vaccine (17, 43). Previous studies by other investigators and from our laboratory have identified genes related to survival and bacterial virulence (3, 7, 16, 27, 33, 35, 42). Among them, we have identified, sequenced, and disrupted the *pgk* gene of *B. abortus*. Furthermore, the *Brucella pgk* mutant has been evaluated for survival in macrophages and in different mouse strains to confirm attenuation and potential use as a vaccine candidate.

To address the role of *Brucella* PGK in bacterial virulence, a mutant was constructed. To confirm that inactivation of the *Brucella pgk* gene was achieved, Southern and Western blot analyses were performed. As shown in Fig. 2, Western blot analysis using polyclonal anti-PGK antibodies demonstrated the lack of *pgk* gene expression in the Δ*pgk* mutant and showed that PGK production was restored when this mutant strain was complemented with the pBBR1-*pgk* plasmid. Further, the mutant was evaluated for survival and attenuation in bone marrow-derived macrophages. As shown in Fig. 3, Δ*pgk* was defective for survival in macrophages compared to the wild-type strain. Additionally, to determine the fate of the Δ*pgk* mutant intracellularly, confocal microscopy was performed in BMDM. As demonstrated in Fig. 4, at 24 h postinfection, wild-type bacteria was found in calnexin-positive and LAMP1-negative compartments. However, immunofluorescence analysis of infected BMDM showed that, unlike S2308, at 24 h postinfection the Δ*pgk* mutant was not found within the ER-derived compartment and that, instead, over 60% of BCVs retained the late endosomal/lysosomal marker LAMP1.

In BALB/c, C57BL/6, and 129/Sv mice, the Δ*pgk* mutant demonstrated different levels of spleen colonization from those of the wild-type S2308, indicating that virulence *in vivo* was altered by the absence of PGK (Fig. 5). The number of *Brucella*

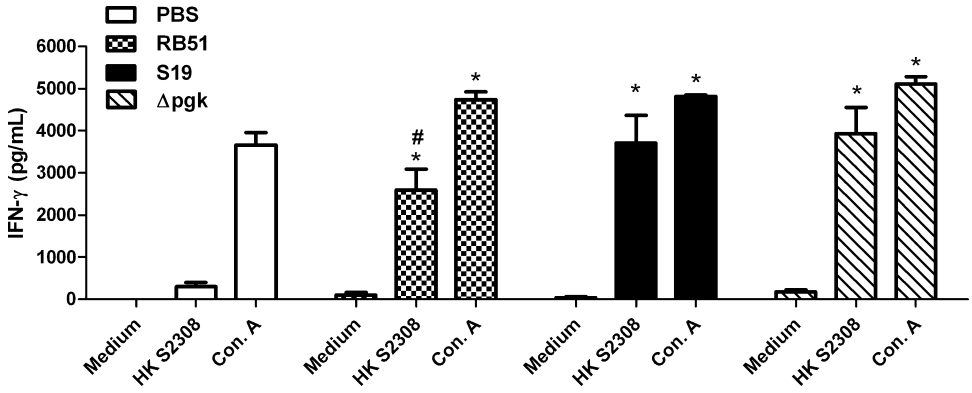

FIG. 8. IFN-γ production by spleen cells of IRF-1 KO mice vaccinated with S19, RB51, or the Δ*pgk* mutant strain. IRF-1 KO mice were inoculated with 10^7 CFU of RB51, 10^5 CFU of S19, or 10^5 CFU of Δ*pgk*. Six weeks after vaccination, splenocytes were recovered and stimulated with heat-inactivated *B. abortus* (HK 2308) or concanavalin A as a positive control. Splenocyte culture supernatants were harvested after 72 h of culture. Bars represent the mean ± standard deviation of quadruplicate sets of cells. Statistically significant differences are indicated by an asterisk ($P \leq 0.05$) for the comparison to medium alone and by a number sign (#) for the comparison to the RB51-vaccinated group stimulated with HK 2308.

CFU was evaluated at 1, 2, 3, 4, and 6 weeks postinfection in the spleen of each animal. The *B. abortus* Δ*pgk* mutant strain displayed reduced persistence at all times tested in different mouse models compared to the virulence of the wild-type *Brucella* strain. Additionally, IRF-1 KO mice were infected with *B. abortus* S2308, S19, RB51, or the Δ*pgk* mutant strain. IRF-1 KO is an interesting animal model to analyze the contribution of an individual *Brucella* gene to bacterial virulence (18). IRF-1 KO mice infected with S19, RB51, or Δ*pgk* survived longer than mice infected with wild-type S2308 ($P \leq 0.005$). Eighty percent of IRF-1 KO mice injected with *B. abortus* S19 were alive at 30 days postinfection, and all mice injected with attenuated *B. abortus* strain RB51 or Δ*pgk* mutant strain were alive.

Pathogenicity of brucellae and chronicity of brucellosis are due to the ability of the pathogen to adapt to the harsh environmental conditions found in host cells. The analysis of the intramacrophagic virulome of *Brucella suis* showed that low levels of nutrients and oxygen are major features of its replicative niche (19). A recent proteomic study has shown that *Brucella* adapts to low-oxygen conditions intracellularly by upregulating glycolysis and denitrification (1). Most of the upregulated proteins were involved in energy metabolism, such as transketolase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and glycerol-3-phosphate ABC transporter. In the past, it was demonstrated that *Brucella* uses the pentose phosphate pathway via glycolysis for sugar degradation (34) and that a functional ribose kinase is essential for intramacrophagic growth (19). In parallel with these studies, we show here that PGK enzyme, involved in the downstream pathway of glycolysis, is critical for intramacrophagic growth and bacterial virulence *in vivo*. Recently, Fugier et al. (14) have shown that inhibition of host cell GAPDH and enolase, both involved in glycolysis, resulted in reduced intracellular replication of *Brucella*. It is possible that *Brucella* is also using the host cell glycolysis to its advantage, as a source of energy.

Although certain subunit vaccines have been demonstrated to be efficacious and useful (8, 12), one of the most promising of the vaccine approaches, based on published results and effectiveness in the field, is use of a live, attenuated agent (13). Vaccines currently in use are derived from spontaneously occurring attenuated forms that arise randomly and that therefore do not provide a means to control the combination of defects that attenuate survival (13). Therefore, to investigate whether the Δ*pgk* mutant strain induces protection, the protective efficacy of this potential vaccine was assessed in BALB/c, 129/Sv, C57BL/6, and IRF-1 KO mice. We observed similar levels of protection in BALB/c and C57BL/6 mice immunized with the Δ*pgk* mutant strain and in mice immunized with commercial vaccine strain S19 and higher log units of protection than with the commercial vaccine strain RB51. In 129/Sv mice, immunization with the *B. abortus* Δ*pgk* mutant strain induced protection that was superior that achieved in animals immunized with commercial vaccine strains S19 and RB51. IRF-1 KO mice are defective in multiple immune components; however, they mount an adaptive immune response sufficient to protect against virulent challenge, and the protection is vaccine strain dependent. IRF-1 KO mice immunized with Δ*pgk* were alive at 30 days postchallenge while 70% or 80% of animals vaccinated with RB51 or S19, respectively, survived during this period. Additionally, the number of Δ*pgk* CFU in these mice at 6 weeks postimmunization decreased rapidly compared to the values for the S19 strain, showing the tendency of low residual vaccine load (data not shown). However, the Δ*pgk* mutant still is more protective than the S19 strain. In conclusion, the *B. abortus* Δ*pgk* mutant significantly enhanced resistance to experimental infection compared to the S19 or RB51 commercially available vaccine strain.

IFN-γ is a critical cytokine in *Brucella* immunity and is required for macrophage bactericidal activity (39). Therefore, we decided to investigate the role of IFN-γ induced by the Δ*pgk* mutant, RB51, or S19 vaccine strains in protection achieved in IRF-1 KO mice. As shown in Fig. 8, RB51-vaccinated IRF-1 KO mice produced less IFN-γ than S19- and Δ*pgk* mutant-vaccinated mice, which paralleled the reduced protection induced by RB51 in this mouse model. Even though the IRF-1 KO mice have a dysregulation of interleukin-12 (IL-12) p40 induction, critical for Th1 cell development, these animals mounted a strong IFN-γ response when immunized with S19 or the Δ*pgk* mutant strain. Additionally, the IRF-1 KO animals serve as an important model to rapidly assess vaccine efficacy of *Brucella* strains. The level of bacterial attenuation requires fine-tuning to avoid undesired effects on survival that may attenuate the organism so that the level of protective immunity provided is insufficient. Finally, the results reported here demonstrate that the Δ*pgk* mutant possesses reduced persistence in macrophages and mice and that it induces protection superior to that of S19 and RB51; therefore, it should be considered a new potential vaccine candidate against brucellosis.

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