The invA gene of Brucella melitensis is involved in intracellular invasion and is required to establish infection in a mouse model

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Abbreviations: NUDIX, (di)nucleoside oligophosphate molecules linked to other “X” molecules; pi, post-infection; BCV, Brucella-containing-vacuole; Bm, Brucella melitensis; CFU, colony-forming units

Introduction

Brucellosis is a worldwide zoonotic disease that is mainly distributed in undeveloped countries. In animals, infection with the causative bacterium leads to reproductive problems, abortion and lesions in a variety of tissues, resulting in chronic illness.1 Brucellosis caused by B. melitensis is controlled in animals by the Rev1 vaccine.2 The Brucella genus lacks classical virulence mechanisms, and its metabolism is closely linked to its intracellular lifestyle.3 B. melitensis combats the harsh intracellular environment by transcribing several genetic factors in order to inhibit intracellular destruction and promote bacterial multiplication.4-6

It has been demonstrated that Brucella is capable of modulating the transcription of metabolic genes (for processes such as carbon, nitrogen and lipid metabolism) depending on the environmental conditions.7 A crucial step for intracellular survival is the invasion process, during which Brucella spp. must deal with oxidative and nutrient stress.7,8 A metabolic shift known as the stringent response occurs under nutrient starvation during the invasion process and is characterized by inhibition of RNA and ribosomal protein synthesis.9 These conditions raise the intracellular concentration of recognized molecules known as alarmones (oligophosphate nucleotides). This increase in alarmone concentration is interpreted by the cell as a signal alerting the cell to prepare for a stress adaptation with a transcriptional change.10-12 Although initially beneficial, the accumulation of alarmones can also be detrimental to cells.12,13 To inhibit the toxic effects of alarmone accumulation, a special subfamily of NUDIX enzymes can hydrolyze these oligophosphates. The NUDIX enzymes are a diverse family of enzymes that act on (di)nucleoside oligophosphate molecules linked to other “X” molecules.14 These enzymes have been described in R. prowazekii,15 E. coli,16 L. pneumophila,17 S. Typhimurium,18 and B. bacilliformis.19 The exact link between the transcription of these enzymes and their role in virulence is not fully understood; however, they may act to reduce stress-induced alarmone levels, promoting invasion and intracellular survival.15,19

In the Brucella melitensis genome, the BMEI0215 (invA) gene has phosphate nucleoside hydrolase characteristics. In an amino acid analysis, the Brucella melitensis invA gene had 52% similarity to...
NUDIX enzymes described in other pathogenic bacteria (Fig. 1). This gene exhibited the closest similarity to the invA gene of Rickettsia prowazekii (GenBank access no. RP236) and the ialAB locus of Bartonella bacilliformis (nudH-ialB locus), with an average of 76%.

Given the importance of stress adaptation, the role of the invA gene could be crucial in the virulence of B. melitensis, as has been demonstrated for other pathogenic bacteria. However, to our knowledge, no studies of the BMEI0215 (invA) gene have been performed in B. melitensis.

In the present work, we have shown that expression of the invA gene could be important for intracellular invasion and virulence in a murine model of infection.

**Results**

**HeLa cell invasion**

Invasion was analyzed by infecting epithelial-like HeLa cells with different B. melitensis strains at an MOI of 500 and sampling the cultures at different times pi (Fig. 2). At the onset of infection (incubation time zero), all the strains demonstrated an invasion average of $4.72 \times 10^6$ CFU/mL, except for B. melitensis 16M, for which there were 2.2-fold more bacteria present than compared with the other strains ($P < 0.05$). At 30 min pi, B. melitensis 16M (Bm 16M, reference strain) exhibited 1.6-fold greater survival than B. melitensis 133 ($P < 0.05$, Bm 133, parental) and 4-fold greater survival than B. melitensis 133 invA-km (Bm A, mutant strain). B. melitensis 133 invA-kmC (Bm AC, complemented strain) and B. melitensis Rev1 (Bm Rev1, vaccine strain) ($P < 0.001$). At the same time pi, Bm 133 survival was 2.5-fold greater than that of Bm A, Bm AC, and Bm Rev1 ($P < 0.05$). Later, at 60 min pi, no significant differences were observed for the survival of the strains. An increase in bacterial concentration for Bm 16M, Bm 133, and Bm AC was evident at 120 min pi; in contrast, Bm Rev1 and Bm A retained the same levels of intracellular bacteria compared with 60 min pi. This bacterial increase might be attributable to the Brucella duplication time. It is known that virulent strains of Brucella in log-phase have a duplication time of 2 to 2.5 h in culture media. 20,21 It is probable that virulent strains could be duplicating during the invasion period. The survival of the Bm A and Rev1 strains at 120 min pi was similar ($P > 0.05$) and was 5-fold less than the bacterial concentrations of the parental, reference, and complemented strains ($P < 0.001$, Fig. 2).

The decreased bacterial concentration observed at 120 min pi for the mutant and vaccine strains could be related to their attenuated phenotypes. Although the complemented mutant strain was similar to the parental strain at the final time point, there was no effect before 120 min pi, suggesting partial complementation of the strain (Fig. 2).

**Survival and intracellular multiplication in goat macrophages**

The intracellular survival of different Brucella strains was measured at different time points in goat macrophages infected at an MOI of 100. At 0 and 4 h pi, no significant differences were evident between the strains in terms of invasion and intracellular
Infection of the murine macrophage J774A.1 cell line at a bacterial MOI of 100 was performed to study intracellular traffic via confocal microscopy using selected protein markers (Fig. 4A–D). Confocal microscopy analysis revealed a similar entry pattern for all strains tested, with some differences at 10 min pi (Fig. 4A), indicating that the reference strain had the highest percentage of EEA1 (early endosomal antigen 1) colocalization (60.6% ± 2%, P < 0.05). EEA1 is a protein that characterizes early endosomes and is involved in the phagosome to early-endosome transition.22 Brucella-containing vacuoles (BCVs) of the parental strain retained the EEA1 protein (40% ± 2%, Fig. 4A) at 15 min pi, resulting in the highest colocalization percentage compared with other bacterial strains (P < 0.001), suggesting sustained invasion by the parental strain. The association of BCVs with EEA1 is transient and occurs immediately after internalization.23 During endosome maturation, BCVs next acquire the LAMP1 glycoprotein (lysosome associated-membrane protein-1), which is characteristic of late endosomes and lysosomes.24 During the adaptation period (4 h pi) BCVs of the different tested strains exhibited a similar pattern of positive LAMP1 colocalization (Fig. 4B). At 12 h pi, the strains demonstrated an average of 52% positive LAMP1 vacuoles, and at 24 h pi, 17.8% positive LAMP1 colocalization was observed, with the exception of the Bm Rev1 strain. The percentage of colocalization of the vaccine strain with LAMP1 at 24 h pi was 45.7% ± 2.08% (P < 0.05). Later during BCVs intracellular trafficking, replicative vacuoles are evident when phagosomes progressively acquire endoplasmic reticulum markers, such as calregulin, and lose late endosomal markers, such as LAMP1.25 As expected, in the parental and reference strains, calregulin-positive BCVs progressively increased during infection (Fig. 4C) and were higher at 24 h pi. This infection time point (24 h pi) positively correlated with the onset of macrophage intracellular replication (Fig. 3). The colocalization of calregulin in the Bm 133 and Bm 16M strains was similar (71% ± 3.6% and 64.3% ± 2%, respectively) at 24 h pi. These percentages were significantly higher than the calregulin-positive BCVs of the mutant strain (49.3% ± 7%, P < 0.05, Fig. 4C). As demonstrated by the macrophage infection results (Fig. 3), complementation of the mutant strain was only partial, as colocalization with calregulin at 24 h pi in the complemented mutant strain was significantly lower than in the parental strain (32.2% ± 2.5% vs. survival, with the exception of the Bm Rev1 strain, which exhibited 2.5-fold lower survival than the others at 4 h pi (P < 0.05, Fig. 3). At 24 h pi, the parental and reference strains survived and replicated uniformly, at higher concentrations, i.e., 11-fold greater than the rest of the strains tested (P < 0.001). At 48 h pi, the parental, reference, and mutant strains demonstrated 16-fold greater replication than the Rev1 and complemented strains (P < 0.001). During the phagocytosis (0 h) and adaptation (4 h) periods, there were no significant differences between the mutant strain and the parental strain (P > 0.05). At 24 h pi, the mutant strain demonstrated 8-fold less replication than the parental strain, which was the most pronounced difference in terms of the intracellular concentration of both strains (P < 0.001). In contrast, no significant differences (P > 0.05) were observed between the parental strain and the mutant strain at 48 h pi, indicating that the invA mutation results in delayed multiplication. As seen in Figure 3, complementation did not restore the survival and replication ability of the mutant strain at 24 h and 48 h pi. In fact, the Bm AC strain demonstrated 4.2-fold lower levels of survival than the Bm A strain at 24 h pi and 6.2-fold lower levels at 48 h pi, indicating less survival and intracellular replication of the complemented mutant strain than of the mutant strain (P < 0.05).

Intracellular traffic in murine macrophages

Infection of the murine macrophage J774A.1 cell line at a bacterial MOI of 100 was performed to study intracellular traffic via confocal microscopy using selected protein markers (Fig. 4A–D). Confocal microscopy analysis revealed a similar entry pattern for all strains tested, with some differences at 10 min pi (Fig. 4A), indicating that the reference strain had the highest percentage of EEA1 (early endosomal antigen 1) colocalization (60.6% ± 2%, P < 0.05). EEA1 is a protein that characterizes early endosomes and is involved in the phagosome to early-endosome transition.22 Brucella-containing vacuoles (BCVs) of the parental strain retained the EEA1 protein (40% ± 2%, Fig. 4A) at 15 min pi, resulting in the highest colocalization percentage compared with other bacterial strains (P < 0.001), suggesting sustained invasion by the parental strain. The association of BCVs with EEA1 is transient and occurs immediately after internalization.23 During endosome maturation, BCVs next acquire the LAMP1 glycoprotein (lysosome associated-membrane protein-1), which is characteristic of late endosomes and lysosomes.24 During the adaptation period (4 h pi) BCVs of the different tested strains exhibited a similar pattern of positive LAMP1 colocalization (Fig. 4B). At 12 h pi, the strains demonstrated an average of 52% positive LAMP1 vacuoles, and at 24 h pi, 17.8% positive LAMP1 colocalization was observed, with the exception of the Bm Rev1 strain. The percentage of colocalization of the vaccine strain with LAMP1 at 24 h pi was 45.7% ± 2.08% (P < 0.05). Later during BCVs intracellular trafficking, replicative vacuoles are evident when phagosomes progressively acquire endoplasmic reticulum markers, such as calregulin, and lose late endosomal markers, such as LAMP1.25 As expected, in the parental and reference strains, calregulin-positive BCVs progressively increased during infection (Fig. 4C) and were higher at 24 h pi. This infection time point (24 h pi) positively correlated with the onset of macrophage intracellular replication (Fig. 3). The colocalization of calregulin in the Bm 133 and Bm 16M strains was similar (71% ± 3.6% and 64.3% ± 2%, respectively) at 24 h pi. These percentages were significantly higher than the calregulin-positive BCVs of the mutant strain (49.3% ± 7%, P < 0.05, Fig. 4C). As demonstrated by the macrophage infection results (Fig. 3), complementation of the mutant strain was only partial, as colocalization with calregulin at 24 h pi in the complemented mutant strain was significantly lower than in the parental strain (32.2% ± 2.5% vs.
Figure 4. Percentage of colocalization ± standard deviation of the *B. melitensis* strains with different endosomal antibodies (left) and representative confocal micrographs of the *B. melitensis* 133 *invA*-km mutant strain (right) at different times pi in J774A.1 macrophages. (A, C, and D): LPS colocalization (Alexa 488) with EEA1, calregulin, and cathepsin D (Alexa 594), respectively. (B) LPS colocalization (Alexa 594) with LAMP1 (Alexa 488). Bottom bar in micrographs corresponds to 5 μm; white arrows show colocalization. Significant differences are shown in letters and stars (black star represents a difference of *P* < 0.05, and red star represents a difference of *P* < 0.001). Lines and symbols represent: solid line and closed circle, *B. melitensis* 133; dashed line and open triangle, *B. melitensis* 133 *invA*-km; dashed line and open inverted triangle, *B. melitensis* 133 *invA*-km²; dashed line and closed diamond, *B. melitensis* Rev1; solid line and open circle, *B. melitensis* 16M.
To measure intracellular destruction, a cathepsin D antibody was utilized (lysosomal protein). Replicative Brucella never colocalizes with cathepsin D, in contrast with attenuated bacteria.28 Cathepsin D colocalization progressively decreased over the course of infection for all strains (Fig. 4D), with the exception of Bm Rev1 at 12 h pi in comparison with the rest of the strains tested (47.3% ± 2.08 versus 12.2% ± 0.6%, respectively; P < 0.001) and at 24 h pi (36.7% ± 1.5% vs. 4.7% ± 1.3%, respectively; P < 0.001). In agreement with the results for LAMP1-negative and calregulin-positive BCVs, for which the onset of intracellular replication was observed (Fig. 3, 24 h pi), it was expected that these strains would have a low percentage of cathepsin D-positive BCVs at 24 h pi. With the exception of the complemented mutant strain (11% ± 3.5%, P < 0.05), the parental, reference and mutant strains yielded similar cathepsin D colocalization results (an average of 75% ± 1.6%). These results indicated that the intracellular destruction of the strains at 24 h pi was not as great as that of the Bm Rev1 strain (Fig. 4D).

Residual virulence in the murine model

To analyze residual virulence, different groups of mice were inoculated with B. melitensis strains (1 × 10⁴ CFU) intraperitoneally. Spleen colonization was evident at the beginning of murine infection (Fig. 5). At 7 d pi, mice inoculated with the parental strain achieved the highest levels of spleen colonization, with a tissue concentration of 498.6 ± 44.5 CFU/mg, which was 1.7-fold greater than that of the other strains (P < 0.001). The highest levels of spleen colonization were achieved at 15 d pi: 648.6 ± 153.3 CFU/mg for the parental group vs. the group infected with the mutant strain (480.3 ± 155.5 CFU/mg, P < 0.05), for which spleen colonization was 0.74-fold less than that of the parental group. Conversely, the Bm Rev1 and Bm AC groups demonstrated 6.2-fold less spleen colonization than the Bm A group (P < 0.001) and 8.5-fold less spleen colonization than the Bm 133 group (P < 0.001). Infection control began at 30 d pi; this was evident in the low bacterial recovery from mouse spleens infected with the Rev1, mutant and complemented strains (55.5 ± 19.5 CFU/mg, P > 0.05). At 30 d pi, the bacterial concentrations in spleens from the parental group were 6-fold greater than for the mutant, complemented mutant and Rev1-inoculated groups (P < 0.001); later, at 60 d pi, the recovery of the parental strain was 9.51-fold greater than for the mutant and complemented mutant groups (P < 0.001). Finally, at 90 d pi, the bacterial concentration for the parental group was 5.64-fold greater than that of the mutant group (P < 0.001). There was no bacterial recovery from spleens of either from mice inoculated with the Rev1 strain starting at 30 d pi through to the end of the experiment or mice in the complemented mutant group at 90 d pi.

The spleen weight of the different inoculated groups indicated active infection (Table 1). The most prominent splenomegaly was detected in all bacteria-inoculated groups during the first 15 d of infection. For the complemented mutant group, there was a constant spleen weight from day 30 pi to the end of the experiment (an average of 195.86 ± 41.36 mg), and constant splenomegaly was observed at 60 d and 90 d pi for the mutant group (220.97 ± 22.05 mg and 214.03 ± 40.93 mg, respectively). As seen in Table 1, the parental group had the highest spleen weights throughout the experiment until 30 d pi. At 30 d pi, the spleen weights for the parental and mutant groups were similar (446.1 ± 36.57 mg and 396.53 ± 54.5 mg, respectively; P > 0.05), and were 2.16-fold greater than the spleen weights of the Rev1 and complemented mutant groups (P < 0.001). Interestingly, after 60 d of infection, the spleen weights of the mutant and complemented groups had similar values (P > 0.05), likely indicating similar patterns of inflammation during late in vivo infection. Meanwhile, splenomegaly caused by the parental strain was 1.96-fold greater than that elicited by the mutant and complemented strains and 2.74-fold greater than that elicited by the Rev1 strain. Finally, at 90 d pi, splenomegaly caused by the parental strain was 1.96-fold greater than caused by the mutant, complemented and Rev1 strains. Taken together, the BALB/c mouse infection results demonstrated that, in vivo, the invA mutant strain was less virulent than the parental strain, although spleen colonization and splenomegaly indicated that the attenuation was not as evident as the Rev1 vaccine strain attenuation. Related to the in vitro results, the complemented mutant strain and the mutant strain exhibited similar virulence behaviors, indicating that complementation was partial.

Humoral immune responses in the murine model

Sera from infected mice during the residual virulence assay were collected to determine isotype antibody switching at different time points throughout in vivo infection (Fig. 6A–E). There was a general increase in antibody levels during the first 30 d pi. From 60 d to 90 d pi, there was a plateau for all antibodies tested, except for the IgM antibody titers. IgM antibodies were predominant over the course of infection (Fig. 6E), but the IgG3 antibody titers were also high, indicating that they played a key role during infection (Fig. 6D). During the first 7 d of infection for the group inoculated with the parental strain, the IgM antibody titers were predominant over the other IgG subclass antibodies (0.159 ± 0.082, Fig. 6E); however, negligible IgG2b
(Fig. 6C) and IgG3 antibody titers (Fig. 6D) were detected during the same time period. In the group challenged with the parental strain, the IgM antibody titers increased at a constant rate throughout the experiment. However, in the mutant group, the IgM antibody titers were significantly higher than those elicited by the parental strain at 30 d pi (0.436 ± 0.03 vs. 0.270 ± 0.024, respectively; $P < 0.001$). IgG3 antibodies, as expected, were evident starting at 7 d pi in the parental group. These IgG antibodies predominated over the other IgG subclasses in all groups. Differences in the IgG3 titers between the parental and mutant groups were perceptible at 30 d pi; the titers of the parental group were 1.3-fold greater than mutant titers ($P < 0.001$). Interestingly, the Rev1 group had similar IgG3 and IgG2b titers compared with the mutant group at 30 d pi, indicating similar humoral responses. IgG2b antibodies were more pronounced than IgG2a subclass antibodies, and were elicited earlier for the parental group (Fig. 6C). At 30 ($P < 0.001$) and 90 ($P < 0.05$) days pi, the parental group exhibited significantly higher IgG2b antibody titers than the mutant group. As seen in Figure 6C, the parental group IgG2b titers always demonstrated values above the IgG2b titers of the mutant group, thus indicating that the humoral response was elicited to a considerably greater extent.
by the parental strain than by the mutant strain. However, the mutant IgG2a antibody titer demonstrated constant humoral stimulation beyond 15 d pi and were considerably higher at 30 d pi compared with those of the parental group (P < 0.001, Fig. 6B). In contrast to IgG2 antibodies, the IgG1 titers were markedly lower than the IgG2a and IgG2b titers in all inoculated groups. From 30 to 60 d pi, constant and similar IgG1 antibody levels were evident for the parental and mutant groups and at 90 d pi, a slight increase in parental IgG1 titers was significantly different from mutant IgG1 titer (P < 0.05, Fig. 6A). IgG2a and IgG2b antibodies are markers of the Th1 immune response; they are elicited to promote macrophage phagocytosis and IFN-γ production. In contrast, the IgG1 antibody is a typical marker of the Th2 immune response, which is characterized as the regulatory and humoral response. In general, in the parental and mutant groups, the IgG2b or IgG2a antibody titers were dominant over the IgG1 antibody levels, indicating a Th1 immune response in vivo infection. Conversely, in the complemented mutant group, antibody titers revealed a pattern that was similar to that elicited by the mutant strain, indicating in vivo plasmid instability as previously described.

Compared with the Rev1 group, the mutant group exhibited comparable antibody titers. There was no significant difference between IgM elicited by the Rev1 strain and that elicited by the mutant strain during the first 30 d pi. In relation to IgG antibodies, only the Rev1 IgG2b antibody titers were higher than those elicited by the mutant strain at 7 (0.084 ± 0.031 vs. 0.008 ± 0.005, P < 0.05) and 15 (0.145 ± 0.010 vs. 0.073 ± 0.02, P < 0.001) days pi.

IFN-γ expression in the murine model

mRNA samples were obtained from the blood of infected mice throughout the residual virulence assay to determine IFN-γ expression levels by real-time PCR (Fig. 6F). As observed, IFN-γ transcription was upregulated during the first week pi for all inoculated groups. There was no difference in IFN-γ transcription between the parental, mutant and Rev1 inoculated mice, indicating that the Th1 polarization response was elicited similarly by these strains. After the first week pi IFN-γ transcription was inhibited, likely indicating that other cytokines were readily taking its place during in vivo infection.

Discussion

NUDIX hydrolases have been described in both prokaryotes, eukaryotes and even in viruses. They are implicated in several functions, including RNA processing, metabolic processes, genetic detoxification, and bacterial pathogenesis. Their involvement in bacterial invasion was demonstrated with the E. coli YgdP protein, 16 IaA of B. bacilliformis, 19 and InvA of R. prowazekii. 15 The principal objective of the present work was to elucidate the role of a potential NUDIX (BMEI0215) gene in Brucella virulence. The epithelial-like cell invasion results demonstrated that survival of the B. melitensis invA-km strain was significantly compromised at 2 h pi, indicating the inability of this mutant strain to survive during the adaptation period of infection. Additionally, intracellular survival assay results with the caprine macrophage infection model revealed a considerable decrease in the intracellular concentration of the mutant strain at 24 h pi, which was 81% lower than the parental and reference strain concentrations. However, at 48 h pi, no significant concentration differences were observed between the mutant strain and the virulent strains, which indicate that the mutant strain maintains some degree of intracellular replication ability. In agreement with the caprine macrophage results, the colocalization results for cathepsin D revealed that there was no significant difference at 24 h pi. Conversely, the calregulin-positive BCV percentage for the mutant strain was less than the calregulin colocalization in the parental strain at the same time point (24 h pi), indicating lower overall intracellular multiplication of the mutant strain, which was not correlated with intracellular destruction (cathepsin D results). These findings show that the invA mutation restricts intracellular multiplication at 24 h pi. However, at 48 h pi, the intracellular concentration was not affected; multiplication of the mutant strain was similar to that of the Bm 133 and Bm 16M virulent strains. This observation could indicate a delayed intracellular multiplication of the mutant strain at 24 h pi but attainment of the same intracellular multiplication at 48 h as that of the other virulent strains, as has been observed for other Brucella virulence factors. These findings are in agreement with previous observations of mutated NUDIX enzymes in bacterial strains. Edelstein et al. showed that a L. pneumophila nudA mutant exhibited a significant decrease in guinea pig macrophage multiplication after 2 d pi; however, virulence restoration was evident on day 3 pi. The invA gene likely acts as a factor that limits the accumulation of alarmones during the first stage of intracellular stress. It is known that the accumulation of alarmones (such as diadenosine 5′,5″′-P4,P4-tetraphosphate, Ap4A, or diadenosine 5′,5″′-P4,P4-pentaphosphate, Ap5A) can be detrimental to intracellular bacteria; however, NUDIX hydrolases counteract the excess of alarmones to detoxify the intracellular niche. It is probable that the invA gene of B. melitensis could restore homeostatic levels of these alarmones, indirectly promoting invasion. However, biochemical characterization of the InvA enzyme is crucial to determine the substrate specificity.

In contrast with the in vitro infection results, mutation of the invA gene of B. melitensis resulted in in vivo attenuation. At 15 d pi, mice infected with the mutant strain had high levels of bacterial spleen colonization compared with the levels in mice infected with the Rev1 vaccine and complemented strains; however, bacterial concentrations were not the same as they were for mice infected with the parental strain. At 15 d pi the mutant strain CFU concentration decreased to levels similar to those of the Rev1 vaccine strain, and those of the parental strain remained above the mutant and vaccine bacterial concentrations. Splenomegaly as a sign of inflammation can indicate bacterial colonization, as was evident in mice inoculated with the parental strain (Table 1). A constant decrease in bacterial concentration suggests control of the infection (Fig. 5). If we can link low bacterial concentrations with immune control, it is clear that immune control in the mutant-inoculated group was more effective than that observed in the parental strain group. The immune response
in Brucella infection is principally mediated by a Th1 response.\textsuperscript{26} The humoral response observed in the inoculated mice suggests this type of immune response, as IgG2a and IgG2b were predominant over IgG1 antibodies (Fig. 6). The principal differences in antibody titers between the parental and mutant group could be observed at 30 d pi; beyond this point, the differences were minimal. The parental strain elicited an earlier immune response than the mutant strain, as IgM and IgG3 antibodies were evident only in the parental inoculated group (Fig. 6). At 30 d pi, IgG2a was higher in the mutant inoculated group and IgG2b was lower in the mutant inoculated group, compared with the parental group. IgG1 antibody levels were evident during this time period, suggesting that a regulatory response was taking place after 30 d of infection. Similar antibody levels were detected starting at 30 d pi in the Rev1 group and the mutant group. In general, there was an analogous humoral response; however, differences between the parental and mutant inoculated groups could be indicators of specific immune responses. The representative cytokine of the Th1 response is IFN-\(\gamma\), which exerts brucellicidal activity by activating macrophages.\textsuperscript{26} As seen in Figure 6F, IFN-\(\gamma\) transcription was upregulated during the first week of infection for all inoculated groups. After 7 d pi, no IFN-\(\gamma\) transcription profile was evident, suggesting that \textit{B. melitensis} control relies on other immune mechanisms. TNF-\(\alpha\) and IL-2 are pro-inflammatory cytokines that participate in the Th1 immune response of BALB/c mice, in addition to IFN-\(\gamma\).\textsuperscript{31} It is possible that elevated TNF-\(\alpha\) and IL-2 levels, as well as those of other cytokines, could mask IFN-\(\gamma\) transcription beyond 7 d pi. However, it is clear that there is no difference in IFN-\(\gamma\) transcription, suggesting that mutation of the \textit{invA} gene elicits similar cellular immune responses as the parental strain at the beginning of infection. In vivo attenuation of the mutant strain could be related to intracellular destruction during the infection process (as was observed in the HeLa assay) and, consequently, antigen presentation.

Survival, intracellular replication and in vivo virulence results for the complemented mutant strain (Bm 133 \textit{invA}-km\textsuperscript{c}) indicated that complete restoration to wild-type levels was not possible. Genetic \textit{Brucella} complementation in trans with the use of pBBR1MCS, a member of a moderate-copy number plasmid family, was shown to result in incomplete in vivo and in vitro restoration.\textsuperscript{27,28,32} In the goat macrophage model, intracellular survival and replication of the complemented mutant strain were even lower than those of the mutant strain (Fig. 3). Similar effects were observed with \textit{Brucella ovis} outer membrane protein (OMP) complemented mutants by Caro-Hernández et al. who observed that complemented strains were more susceptible to hydrogen peroxide, non-immune ram serum and selected drugs than mutant strains. In this study, the expression of OMPs in the complemented mutant strains was not identical as wild-type levels, suggesting that a certain level of OMP expression is necessary for membrane integrity.\textsuperscript{33} Additionally, it has been reported that genetic complementation of the \textit{nudA} gene of \textit{L. pneumophila} and the \textit{invA} gene of \textit{R. prowazekii} in respective mutant strains does not restore their invasive capacity to wild-type levels; this is mainly attributed to the low production and effectiveness of the enzyme.\textsuperscript{31,37} Detection of InvA protein expression in the Bm 133 \textit{invA}-km\textsuperscript{c} mutant will be important to determine whether its partial complementation is due to low protein expression. However, the mutation in the \textit{invA} gene was created by interrupting the gene via the insertion of a kanamycin cassette from a promoterless pSU411 plasmid; a polar effect in adjacent genes was improbable. The use of the pBBRMC1–4 plasmid for genetic complementation in this work, as well as the low production and effectiveness of plasmid transcription, likely resulted in incomplete phenotypic restoration.

We conclude that the \textit{invA} gene is important for intracellular invasion and adaptation but is not necessary for intracellular replication. In vivo infection results suggest that the \textit{invA} gene is required for full virulence of \textit{B. melitensis} in the mouse model of infection. Indeed, mutation of the \textit{invA} gene elicits a different humoral immune response but similar IFN-\(\gamma\) transcription levels during the first week of infection. The immune response is in agreement with bacterial recovery, as the peak in antibody titers at 30 d pi is related to the decrease in bacterial recovery. Functional gene redundancy in \textit{B. melitensis} detoxification indicates that this bacterial species uses several mechanisms to avoid destruction inside the cell. These mechanisms include the Cu–Zn superoxide dismutase (SOD), AhpC (alkyl hydroperoxide dismutase), and the cysteine oxidoreductase, recently cloned and sequenced from the \textit{B. melitensis} genome. The \textit{invA} gene product is predicted to be a transmembrane protein of approximately 340 amino acids and is localized to the cytoplasmic membrane. The precise function of the \textit{invA} gene product is not yet clear, but it is likely involved in the immune evasion mechanisms of \textit{Brucella} species.

### Table 1. Spleen weights of infected mice on different days pi (d pi)

| Inoculated groups | d pi | Bm 133 | Bm 133 invA-km | Bm 133 invA-km\textsuperscript{a} | Bm Rev1 | Control\textsuperscript{b} |
|-------------------|-----|--------|----------------|-------------------------------|--------|-------------------|
| 7                 | 126.78 ± 8.87 | 160.38 ± 49.25 | 161.15 ± 14.6 | 108.22 ± 44.33 | 115.93 ± 43.22 |
| 15\textsuperscript{c} | 497 ± 20.35 | 587.37 ± 14.8 | 276 ± 45.34 | 266.23 ± 52.99 | 106.3 ± 3.08 |
| 30\textsuperscript{d} | 446.1 ± 36.57 | 396.53 ± 54.5 | 188.29 ± 33.52 | 210.03 ± 37.77 | 107.55 ± 4.93 |
| 60\textsuperscript{e} | 431.87 ± 64.96 | 220.97 ± 22.05 | 217.63 ± 40.69 | 157.78 ± 16.09 | 111.7 ± 14.24 |
| 90\textsuperscript{f} | 352.2 ± 101.37 | 214.03 ± 40.93 | 181.68 ± 49.88 | 140.9 ± 6.79 | 110.93 ± 8.15 |

Spleen weight was measured on the indicate days pi (d pi). Measurements are milligrams ± standard deviation. \textsuperscript{a}The parental and mutant groups exhibited similar spleen weights at 30 d pi that were significantly higher than those of the Rev1 and complemented groups (\(P < 0.001\)). There was no significant difference between the Rev1 and complemented groups (\(P > 0.05\)). \textsuperscript{b}The control group demonstrated similar spleen weights between sampling periods. \textsuperscript{c}The parental and mutant groups were different when compared (\(P < 0.001\)). \textsuperscript{d}The parental group had the highest spleen weights at 60 d pi (\(P < 0.001\)). The mutant and complemented groups had similar spleen weights (\(P > 0.05\)) that were higher than those of the Rev1 group (\(P < 0.001\)). \textsuperscript{e}The parental group had the highest spleen weights at 90 d pi (\(P < 0.001\)) compared with the other inoculated groups.

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Table 2. Plasmids and bacterial strains used in this study

| Plasmids and strains | Features | References |
|----------------------|----------|------------|
| Brucella melitensis 133 | Biotype 1 Mexican field strain Na<sup>a</sup> | 41 and 42 |
| Brucella melitensis 133 invA-km | 133 mutant strain (BMEI0215 gene interrupted) Km<sup>b</sup> | This work |
| Brucella melitensis 133 invA-km<sup>c</sup> | 133 invA-km complemented mutant strain (pJA4 complemented) | This work |
| Brucella melitensis Rev1 | Biotype 1 vaccine strain; Str<sup>a</sup> | |
| Brucella melitensis 16M | Biotype 1 ATCC 23456 Na<sup>a</sup> | |
| E. coli DH5<sup>a</sup> | fhuA2 Δ(argF-lacZ)U169 phoA glnV44 λ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17 | Invitrogen |
| E. coli S17.1 | recA pro hsdR RP4–2-Tc•Mu•Km•Tn7 | |
| pCR 2.1 | Cloning vector 3.9 kb. Km<sup>a</sup>, Amp<sup>a</sup> | This work |
| pJA1 | pCR 2.1 with B. melitensis 1.6 kb BMEI0215 gene fragment | This work |
| pJA2 | pJA1 with 1.6 kb BMEI0125 gene fragment interrupted by kanamycin resistant cassette | This work |
| pJA3 | pKOK.4 with the 2.9 kb pJA2 fragment | This work |
| pJA4 | pBBR4MCS with the complete 1.6 kb BMEI0215 gene fragment Amp<sup>a</sup> | This work |
| pSU4111 | Kanamycin plasmid source. 9.3 kb. Km<sup>a</sup> | 43 |
| pKOK.4 | Brucella, suicide vector Amp<sup>a</sup>, Tc<sup>c</sup>, Cm<sup>a</sup> | 44 |
| pBBR4MCS | Cloning vector, Amp<sup>a</sup> | 45 |

reductase), Kat E (catalase), transcription of chaperones (dnaK, groEL, clpB), and several others. Consequently, the invA gene may represent another mechanism that promotes invasion, such that mutation does not affect the intracellular replication of B. melitensis. Further research is required to evaluate the InvA enzymatic functionality and specificity.

**Methods**

**Bacterial strains**

The bacterial strains and plasmids used in the present work are shown in Table 2. The 133, Rev1, and 16M strains of B. melitensis were grown in Brucella broth and on Brucella agar (Becton Dickinson and Company, 211088 and 211086). The DH5<sup>a</sup> and S17.1 strains of E. coli were grown in Luria Bertani broth and on Luria Bertani agar (Becton, 241420 and 241320). When necessary, kanamycin (50 μg<sup>c</sup>, Sigma Aldrich, 6015) and ampicillin (100 μg<sup>c</sup>, Sigma Aldrich, A9393) were added. Genetic manipulations were performed according to standard procedures.<sup>34</sup>

**B. melitensis 133 invA-km mutant construction**

The invA (BMEI0215) 1.6-kb fragment was PCR amplified using the 466 (5′-AGGATGTGAC CGGTTTCGAT-3′) and 467 (3′-CGATGCGCAA AATGATAAGG-5′) oligonucleotides. The amplification product was directly cloned into the pCR2.1-TOPO plasmid (Invitrogen-Life Technologies, K4500-01) to generate the pJA1 plasmid. The pJA1 plasmid was digested at the Clal unique site to interrupt the invA gene and introduce the kanamycin resistance cassette, thereby generating plasmid pJA2. The kanamycin resistance gene (1100 pb) was obtained by PCR from the plasmid pSU4111. The Clal restriction site in the kanamycin gene was inserted by PCR. The generated pJA2 plasmid was digested with the EcoRI enzyme to obtain the 2.9-kb fragment containing the invA gene interrupted by the kanamycin cassette. We cloned the resulting fragment into the suicide plasmid pKOK.4 to generate the plasmid pJA3. The pJA3 plasmid was introduced into B. melitensis 133 from E. coli S17.1 through conjugation assays, and the trans-conjugated colonies were chosen from plates containing kanamycin, tetracycline and ampicillin (all antibiotics from Sigma Aldrich; tetracycline, T7660). Kanamycin-resistant colonies susceptible to tetracycline and ampicillin were chosen. Interruption of the invA gene was confirmed through PCR and sequencing.

**Complementation of the B. melitensis 133 invA-km mutant strain**

To restore the invA gene, the plasmid pBBR4MCS was used. The complete invA gene contained in the 1.6-kb ampiclon was subcloned into the pBBR4MCS-EcoRI-digested plasmid to generate plasmid pJA4. The electrocompetent B. melitensis 133 invA-km mutant was electroporated with the pJA4 plasmid. The electroporation conditions were as follows: 1.6 kV voltage, 50 μF capacitance, and less than 200 Ω resistance with 4 to 8 ms of electroporation time. Trans-conjugating colonies were chosen from plates containing ampicillin and kanamycin. The presence of the plasmid was determined through alkaline lysis, and analysis was performed via PCR.

**HeLa cell invasion**

HeLa cells were used as a model to identify Brucella invasion. These epithelial-like cells were grown at 37 °C in a 5% CO<sub>2</sub> atmosphere in Dulbecco’s minimal essential medium (DMEM, Invitrogen-Life Technologies, 11995-065) supplemented with 10% fetal bovine serum (Invitrogen-Life Technologies, 1.12413-020), 2 mmol/L glutamine (Invitrogen-Life Technologies, 25030-081), and 1.1% MEM non-essential amino acids (Invitrogen-Life Technologies, 11140-50). Cells were seeded (5 × 10<sup>4</sup>) in 24-well cell culture plates one day before each infection assay. HeLa cells were infected as previously described,<sup>35</sup> with different B. melitensis strains at a multiplicity of infection (MOI) of 500 bacteria per cell. The culture plates were centrifuged for 10 min at 400 g at room temperature and placed in an incubator with a 5% CO<sub>2</sub>
were seeded in 75-cm² tissue culture flasks, and monocytes were formed at 1000
1.074
of 30 mL. A Percoll® (GE Healthcare, 17089101) gradient of fraction was re-suspended in PBS-citric acid in a total volume.

Autonomous University of Mexico. The blood was centrifuged Committee for Laboratory Animal Welfare of the National obtained from healthy, two-year-old brucellosis-free goats. The
trose (D9559, all reagents from Sigma Aldrich). The blood was collected by jugular venipuncture (average: 200 mL) and re-sus -
gated in 8 mL of ACD (74.8 mmol/L sodium citrate tribasic; S4641), 38 mmol/L citric acid (251275), and 135.9 mmol/L dex-
ted in 8 mL of ACD (74.8 mmol/L sodium citrate tribasic; S4641), 38 mmol/L citric acid (251275), and 135.9 mmol/L dextrose (D9559, all reagents from Sigma Aldrich). The blood was obtained from healthy, two-year-old brucellosis-free goats. The animal protocols and management were approved by the Internal Committee for Laboratory Animal Welfare of the National Autonomous University of Mexico. The blood was centrifuged at 1000 g for 20 min to obtain the leukocyte-rich fraction. This fraction was re-suspended in PBS-citric acid in a total volume of 30 mL. A Percoll® (GE Healthcare, 17089101) gradient of 1.074 g/mL was layered in a microfuge tube, and the leukocyte fraction was gently poured. Isopycnic centrifugation was performed at 1000 g for 30 min for leukocyte separation. The cells were seeded in 75-cm² tissue culture flasks, and monocytes were separated via their adherent capacity. To verify monocyte to macrophage differentiation (13 d of incubation), nitric oxide (NO₂) production was measured with the Griess reagent (Promega, G2930), according to the manufacturer’s instructions. For macrophage infection, 5 × 10⁴ caprine macrophages were seeded on 24-well cell culture dishes one day before infection. Macrophages were infected as previously described with different B. melitensis strains at an MOI of 100 bacteria per cell. Intracellular bacteria were determined in the same manner as the invasion assay. Bacterial concentrations were determined at the beginning of the infection and at 4 h, 24 h, and 48 h pi. Experiments were performed in triplicate and repeated three times.

Macrophage immunofluorescence
The intracellular trafficking of B. melitensis strains was analyzied by infecting J774A.1 murine macrophage cells in the same manner described for goat macrophages. These cells were chosen because they are an accurate model for intracellular trafficking analysis. For immunofluorescence microscopy, infected J774A.1 cells were seeded on circular glass coverslips. The cells were fixed with 3% paraformaldehyde (Sigma Aldrich, P6148) for 15 min or with cool methanol (Sigma Aldrich, 322415) for 4 min. The cells were PBS-washed three times, and free radicals were neutralized with 50 mmol/L PBS-NH₄Cl (Sigma Aldrich, 254134), only in paraformaldehyde-fixed cells. Incubation in 0.1% PBS-saponin for 1 min and 0.1% PBS-Triton for 4 min was performed before blocking the free receptors with PBS-10% horse serum. Primary antibody incubation was performed with goat polyclonal anti-EEA1 (Santacruz Biotechnology, SC6415), goat polyclonal anti-calcregulin (Santacruz, T-19 sc-7431), mouse monoclonal anti-(H43A) LAMP1 (ABCAM, AB25630), goat polyclonal anti-cathepsin D (Santacruz, G-19 sc-6494), goat polyclonal anti-Brucella and mouse polyclonal anti-Brucella (both antibodies from our laboratory stock, Brucellosis Laboratory, College of Veterinary Medicine, UNAM). All incubations were performed for 40 min. Fluorescent secondary antibodies (Alexa 594, A11005, and Alexa 488, A1100; both reagents from Invitrogen-Life Technologies) were incubated for 40 min after the initial primary antibody incubation. Finally, coverslips were washed and mounted on glass slides with Mowiol® (Sigma Aldrich, 324590). Colocalization analysis was performed with a Leica DM100 immunofluorescence microscope (Leica Microsystems), and microphotographs were taken with an Olympus FV100 confocal microscope (Olympus America Corporate).

Infection of BALB/c mice
BALB/c female mice that were 6- to 8-wk-old were used and distributed homogeneously among 5 independent groups. Each group had 17 mice, and which were inoculated intraperitoneally with 0.1 mL of PBS containing 10⁴ UFC of each bacterial strain or 0.1 mL of PBS only (control group). Sampling was performed at 7, 15, 30, 60, and 90 d pi by sacrificing 3 mice per bacterial group. All mouse maintenance and procedures were approved by the Internal Committee for Laboratory Animal Welfare of the National Autonomous University of Mexico following ethical international standards.

Residual virulence in the murine model
At each sampling time point, the spleens were aseptically collected. The weight of each spleen was measured and recorded. Later, the spleens were macerated in 1 mL of PBS to determine the bacterial concentration and CFU counts, which were obtained with 10-fold serial dilutions and agar plating.

Humoral immune response
Serum obtained during the different sampling periods were collected for antibody isotype determination by ELISA. The samples were taken from whole blood obtained from the orbital sinus of desensitized mice. IgG1, IgG2a, IgG2b, IgG3, and IgM antibodies were analyzed with Mouse Monoclonal Antibody Isotyping Reagents (Sigma Aldrich, ISO2-1) following the manufacturer’s instructions. Briefly, ELISA plates were prepared with 100 ng of B. melitensis LPS (laboratory stock, Brucellosis Laboratory, College of Veterinary Medicine, UNAM). The plates were maintained at 4 °C before the ELISA procedure. PBS-5% skim milk was used to block unspecific receptors, and plates were incubated for 1 h at 37 °C. Next, the plates were washed three times with 0.05% PBS-Tween 20 (Sigma Aldrich,
P9416). Sera were diluted to a working concentration of 1:200, and samples were analyzed in triplicate; 100 μL of each diluted sample was deposited in a well of a prepared ELISA plate. The ELISA plates were incubated at 37 °C for 1 h and washed three times with 0.05% PBS-Tween 20. Before adding 100 μL of mouse anti-IgG or anti-IgM antibody isotypes and incubating for 30 min at room temperature, every mouse anti-IgG or anti-IgM antibody was diluted 1:1000. The plates were then washed as previously described. The rabbit anti-goat IgG peroxidase-labeled ( Sigma Aldrich, A5420) antibody was diluted 1:5000 before depositing 100 μL in each well and incubating at room temperature for 15 min. The plates were washed as previously described. The enzyme substrate was prepared as follows. A 1 mg/mL solution of 5-aminosalicylic acid ( Sigma Aldrich, A3537) in 0.02 M sodium phosphate ( Sigma Aldrich, 342483) at pH 6.8 was prepared. One hundred microliters of 1% H2O2 ( Sigma Aldrich, 216763) was added for every 10 mL of fresh substrate solution. The substrate solution was added to each well (100 μL) and incubated in the dark for 15 min at room temperature. Then stop solution (50 μL of 3 N NaOH, Sigma Aldrich, S5881) was added to each well. Absorbance measurements at 490 nm were performed with a VECTOR3 multilabel counter (PerkinElmer) with negative controls.

**IFN-γ transcript quantification by RT-PCR**

To provide evidence for the Th1 immune response IFN-γ transcription expression was measured with real-time PCR (RT-PCR). RNA was obtained from whole blood from infected mice at different time points. RNA isolation was performed with Tripure Isolation Reagent (Roche Diagnostics GmbH, 11667157001) following the manufacturer's instructions. Total genomic DNA was eliminated by incubation with DNase I (Invitrogen-Life Technologies,18068-015). The RNA concentration and purity were determined by spectrophotometry. The Omniscript RT Kit (Qiagen, 21012) and Fermentas Oligo-dT (Invitrogen-Life Technologies,18068-015) were used to transform mRNA (mRNA) into cDNA (cDNA). Reverse transcription was performed at 37 °C for 60 min followed by 95 °C for 5 min. The purity and concentration of the cDNA were determined by spectrophotometry. RT-PCR was performed with LightCycler 480 DNA SYBR Green Master Mix I (Roche, 04707516001) using 100 ng of cDNA, 0.5 M of oligonucleotides, and 8 μL of SYBR Green Master Mix I in a final volume of 20 μL of nuclease-free water. The following oligonucleotide sequences were used: 5’T-GGATACCTTT GGCATCATG AAAC-3’ and 5’T-AAACCGCAG CTCAATAAC GTCCG-3’ for the β-actin transcript; 5’-AGCGGTCGAC TGAACTCAGA TTGTAG-3’ and 5’-GTCACTGT TGCACTGTAG AGGG-3’ for the IFN-γ transcript.10 RT-PCR was performed on a LightCycler 480 thermal cycler (Roche Diagnostics GmbH) following the manufacturer's recommendations. The mRNA levels were normalized against mouse β-actin, and the ratio between infected and non-infected mice (control group) was determined at different times pi.

**Statistical analysis**

Statistical significance was calculated using ANOVA two-way analyses, and treatment mean comparisons were evaluated with the Bonferroni post-test analysis. For colocalization results, the geometric mean was used for ANOVA two-way analyses. The software used for these analyses was GraphPad Prism 5. The significance level was set at $P < 0.05$ or $P < 0.001$.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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