**Brucella melitensis** invA gene (BME_RS01060) transcription is promoted under acidic stress conditions

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**Abstract**

The invA gene of Brucella melitensis codes for a NUDIX (nucleoside diphosphate linked to moiety X) hydrolase related to invasiveness. The objective of this work was to evaluate invA transcription under acidic conditions. The invA gene transcription was up regulated at pH 3 and pH 5 observed with semiquantitative real-time PCR in B. melitensis 133 strain. Results indicated that invA gene transcription at pH 3 showed a basal and decreased transcription compared to that of pH 5 incubation. Transcription levels of the dnaK gene were similar to those obtained with invA gene. The survival rates of wild type and invA mutant strains at pH 5 were above 90% in all post-incubation times. In contrast, at pH 3 there was a time-dependent reduction on both strains at 15 min (P < 0.05). These results suggest that invA gene transcription is promoted under acidic conditions in Brucella melitensis.

**Keywords** Brucella melitensis · Transcription · Acidic stress · NUDIX · Bacterial adaptation

**Introduction**

Members of the genus Brucella are intracellular pathogens that infect animals and humans, producing reproductive disorders in domestic animals and flu-like symptoms with possible human complications (Olsen et al. 2010). In order to have a successful infection, bacterial pathogens have to overcome iron sequestration, oxidative stress, hyperosmotic conditions, among other stressors (Guan et al. 2017). Acid stress is originated as a defense mechanism against pathogens. Before reaching the intracellular niche, Brucella sp. encounters acid conditions in the host stomach (pH 3.0) if the infection is acquired through the oral mucosa (San¬gari et al. 2007). During cell infection, Brucella bacteria are enclosed in a phagosome (called Brucella containing vacuole, BCV) that matures to phagolysosome, with a pH conditions in the range of 4.5–5.0 (Porte et al. 1999). Phago¬some-lysosome fusion increases the vacuole proton concentra¬tion rendering low pH, thereby counteracting intracellular bacteria (Duclos and Desjardins 2000). Not only decreased pH is encountered inside of BCV, but also nutrient deprivation, oxidative environment, and lytic enzymes. This harsh environment worsens until bacteria reach the endoplasmic reticulum (ER), where they became replicative. It is specu¬lated that 90% of Brucella bacterial cells die in this phago¬some maturation process, and only 10% of them survive to become replicative (Celli 2019). Mechanisms of low-pH resistance in Brucella are necessary for full virulence and replication. Different elements have been reported that are associated with the response to acid stress conditions. DnaK protein (Teixeira-Gomes et al. 2000), the response regulator OtpR (Liu et al. 2015), the glutamic acid decarboxylase sys¬tem (gad operon) in B. microti (Occhialini et al. 2012), the HdeA acid-chaperone (Valderas et al. 2005) among others plays a decisive role in controlling acid stress. Of particular interest is the activation of the Type IV Secretion System.
(TIVSS), a principal virulence mechanism that is promoted under intracellular acidic conditions (Boschirol et al. 2002; Celli et al. 2003; Sieira et al. 2010).

Under these considerations’ possible unknown mechanisms of stress resistance or adaptation could interact for Brucella survival. In Brucella melitensis genetic sequence, a possible NUDIX hydrolase gene has been identified, named invA (BME_RS01060). B. melitensis invA mutant strain showed a lower invasion phenotype in non-professional phagocytes and attenuation in the murine model of infection (Alva-Pérez et al. 2014). In the present work, we demonstrate that the invA gene transcription is promoted under acidic conditions in vitro.

Materials and methods

Bacterial strains

Brucella melitensis 133 (Bm 133) is a biotype 1 Mexican field strain, nalidixic acid-resistant (Hernández-Castro et al. 2000), and B. melitensis invA-km (Bm 133 invA-km) is invA gene mutant strain, kanamycin-resistant (Alva-Pérez et al. 2014). Both strains were maintained on Trypticase Soy Broth or Trypticase Soy Agar (TSA, BD Bacto™, Sparks Maryland, USA), at 37 °C. For mutant strain, kanamycin (200 µg/ml, Bio Basic Ontario, Canada) was added to TSB or TSA when needed. All bacterial manipulations were done on biosafety level III facilities.

Acid stress survival assay

Fresh frozen aliquots of Bm 133 and Bm 133 invA-km were thawed at room temperature and seeded in TSA. After 48 h of incubation at 37 °C, five colony forming units (CFU) were added in 100 ml of TSB and incubated in a shaker at 37 °C at 200 rpm for 48 h. Bacterial cell concentration (one milliliter of each bacterial strain, 10⁹–10¹⁰, late log phase) were harvested by 2,000 × g centrifugation for 10 min at 4 °C. Bacterial cells were re-suspended in 1 ml of acidified medium (pH 5 or pH 3, TSB) and incubated 5, 10 or 15 min at room temperature (time post-incubation, i.e. pi). HCl 2 N (Sigma-Aldrich, Missouri, USA) achieved the acidified medium (pH 5 or pH 3, TSB) and incubated 5, 10 or 15 min at room temperature (time post-incubation, i.e. pi). HCl 2 N (Sigma-Aldrich, Missouri, USA) achieved the acidified medium added to the required pH. The control group was designed as 1 ml of each strain centrifuged and incubated at the same conditions, but TSB medium was at pH 7.2. After incubations in acidified medium or neutral pH medium bacterial numbers were calculated by diluting and plating on TSA. Bacterial concentration in neutral pH medium was determined as 100% per cent of survival. The experiment was carried out in triplicate, and every experiment had three replicates.

invA gene transcription under acid condition

The invA gene transcription assay was determined for Bm 133 incubated in acid conditions as described previously. After incubations, bacterial cells were harvested at 2000×g for 1 min at 4 °C. RNA extraction was done with TRI Reagent (MRC Ohio, USA) following the manufacturer’s instructions. Isolated bacterial RNA was re-suspended in 30 µl of nuclease-free water (Sigma-Aldrich, Missouri, USA). DNase I (Invitrogen California, USA) incubation was done following the manufacturer’s instructions for DNA elimination. The RNA concentration and purity were determined by spectrophotometer readings (JENWAY Genova, Staffordshire UK). Samples were kept at −20 °C.

The invA transcription analysis was carried out using a semiquantitative real-time PCR (qPCR) assay. Primers and probes (Table 1) were designed for qPCR with on-line software PrimeQuest of Integrated DNA Technologies (https://www.idtdna.com/Primerquest/Home/Index). 16srRNA gene was used as a housekeeping gene and dnaK gene as acid stress positive control (Teixeira-Gomes et al. 2000). We use the single tube step format, where RNA is transformed into complementary DNA (cDNA), and then PCR is achieved in the reaction. Super Mix RT-PCR 2X (Biocremol, Mexico City, Mexico) was used. The reaction was integrated of 5 µl of retrotranscription PCR buffer (2x), 0.5 µM of reverse and forward primer (1 µl each, Table 1), 0.5 µM of Taq-man probe (1 µl, Table 1), 100 ng of RNA sample, and nuclease-free water (up to 10 µl). qPCR protocol was as follow: reverse transcription step at 42 °C for 30 min, denaturation step at 95 °C for 10 min, followed for 40 cycles of 95 °C for 5 s and 60 °C for 30 s. qPCR was achieved in Step One thermocycler (Applied Biosystems, California USA). invA gene transcription (relative expression) was calculated by the 2−ΔΔCt method (Livak and Schmittgen 2001). Experiments were done in triplicate and for every time evaluated, two independent experiments were done. In order to evaluate primer efficiency, different Bm 133 cDNA concentrations

| Gene | Primer | Sequence |
|------|--------|----------|
| invA | Foward | 5’-GAGGCGGATGAAAGTGAAATG-3’ |
| Probe | 5’-ACTCCTGAGATGCTGTGGGAA-3’ |
| Reverse | 5’-CGCAAACCCCTGATCATAGA-3’ |
| 16sr RNA | Foward | 5’-ACCTCCTCTGGTTTTAT-3’ |
| Probe | 5’-CCTGTAGGCGCAAGCTCATCT-3’ |
| Reverse | 5’-GTCGTGAGAGTCTGGTTTAAAG-3’ |
| dnaK | Foward | 5’-GACGGGCTGCTGTTGGAA-3’ |
| Probe | 5’-TGAAGTGAAGCTCCAAATGTGGA-3’ |
| Reverse | 5’-CTTGAACCTCGGAACACATG-3’ |
were tested for dnaK or invA qPCR amplifications (Livak and Schmittgen 2001). The dnaK gene specific primers resulted in efficient amplification and was similar to that of the amplification of endogenous gene (equation of dnaK gene: $-3.993x + 14.556; R^2: 0.9899$; equation of 16sr RNA gene: $-4.0748x + 12.677; R^2: 0.9897$). Similarly, the invA gene specific primers resulted in efficient amplification and was similar to that of the amplification of endogenous gene (equation of invA gene: $-3.4247x + 11.405; R^2: 0.9995$; equation of 16sr RNA gene: $-3.384 + 9.6809; R^2: 0.9993$).

invA and dnaK promoter sequences analysis

A comparison of the promoter sequences of invA and dnaK genes was done. Complete dnaK (BME_RS09910) and invA (BME_RS01060) gene sequences were obtained from GenBank database (https://www.ncbi.nlm.nih.gov/nucleotide/). The B. melitensis dnaK and B. ovis dnaK were obtained from Teixeira-Gomes et al. 2000. For the determination of invA promoter sequence The Berkeley Drosophila Genome Project promoter prediction program was used to predict promoter sites (http://www.fruitfly.org/seq_tools/promoter.html). Promoter sequence score of 1.0 was determined as the more representative prediction of the invA promoter sequence. Comparison of the promoter regions (B. ovis dnaK as consensus sequence) was done with ClustalW software (Larkin et al. 2007).

Statistical analysis

Survival bacterial results and qPCR results were statistical analyzed with two-way ANOVA and mean multiple comparisons were made with Tukey’s test. The significance level was set at $P < 0.05$ or $P < 0.001$. The GraphPad Prism 8 software (GraphPad Software, Inc., La Jolla, CA) was used for analysis and graphics construction.

Results

Bacterial survival rate under acid stress conditions

Survival assay results at pH 5 demonstrate no significant differences, except for mutant survival strain comparison, where Bm 133 invA-km survived almost 10% more at 10 min compared to that of 15 min ($P < 0.05$, Fig. 1a). At 5- and 10-min pi, mutant and wild-type strains had more than 90% of survival rate, and at 15 min pi, a slight decrease in the invA mutant strain survival rate was observed, compared to that of wild type strain (84.6 ± 4.8 and 92.7 ± 4.6, respectively). Nevertheless, no significant difference was observed. In contrast, survival assay results at pH 3 demonstrate a reduced bacterial survival at 15 min pi (Fig. 1b). Bm 133 invA-km survival at 15 min was less compared to that of the same strain at 5 min pi (62.9 ± 6.5 vs 93.8 ± 3.9, $P = 0.009$), in the same way, the mutant strain at 15 min pi. survived less than that of the parental strain at 10 min (62.9 ± 6.5 vs 87.4 ± 4.9, $P = 0.005$). Also, wild-type strain at 10 min survived less than that of the mutant strain at 5 min (87.4 ± 4.9 vs 93.8 ± 3.9, $P = 0.029$). Overall tendencies to better bacterial survival were observed at pH 5 than that of pH 3. However, bacterial survival did not decrease to 50% for both strains, indicating a good resistance to low pH. All the bacterial survival data are given in the Additional File 1.xls.

Relative expression of invA gene

Relative transcription results demonstrate different patterns of invA mRNA concentration depending on pH and time
incubation (Fig. 2). Under pH 5 conditions maximum invA gene transcription was observed at 15 min pi and compared at 5- and 10-min pi, invA gene transcription was 25.4 and 15.4 times more up-regulated, respectively (P < 0.0001, Fig. 2a). In addition, dnaK gene was significantly up-regulated since 10 min pi (above 2 Relative Transcription Units, RTU). In contrast, at pH 3, levels of invA mRNA transcripts were below 1 RTU (maximum level observed at 5 min pi, i.e. 0.79 ± 0.26, Fig. 2b). Likewise, dnaK gene transcription was below 1 RTU and it was significantly lower than that of invA gene transcription at 5 min pi (0.46 ± 0.1 compared to 0.79 ± 0.26, P < 0.05). Levels of mRNA transcripts for both genes beyond 10 min pi were below 0.5 RTU, indicating a constant decrease in transcription. These results demonstrate that a pH 5 invA gene transcription is promoted and is time dependent. In contrast at pH 3 invA gene transcripts remained low at basal levels. All the transcription data and analysis are shown in the Additional File 2.xls.

Promoter sequence analysis

The observation that both genes (invA and dnaK) are up-regulated in pH 5 and pH 3 drive us to the question if both genes are co-regulated by similar transcription factor. It is known that molecular chaperone DnaK is up-regulated in heat and acid stress conditions, and σ^32 transcription factor binds to dnaK promoter sequence in E. coli (Jishage et al. 2002). The B. melitensis dnaK promoter region is homologous to −35 and −10 regions of the E. coli σ^32 specific promoter at nucleotide position 110–116 and 127–134, respectively (Teixeira-Gomes et al. 2000). Figure 3 shows the comparison of invA and dnaK promoter regions and compare them with putative homologous σ^32 specific promoter sequence derived from B. ovis dnaK promoter (consensus sequence). As it can be seen, no homologous sequence is observed between consensus promoter sequence and invA promoter, suggesting different mechanisms of invA gene regulation.

Discussion

Low pH, reduced nutrient availability, and oxidative environment are present during the invasion, at the initial stages of Brucella infection. There are multiple systems to sense and respond to environmental factors that could be detrimental to bacterial cells to survive and multiply. Acid stress is detrimental to bacterial protein structure and function, and cellular bioenergetics (Krulwich et al. 2011). Therefore, Brucella melitensis survival to acid stress is crucial for a successful intracellular infection (Roop et al. 2009). In the present work we demonstrated survival to two different low pH conditions. The oral route of Brucella infection is the most common route of infection. Live Brucella counteract the proteolytic and acid environment of the stomach of mammals (pH 1.0–3.0). Passage to this acid milieu is fast and transitional (in a calf-ligated ileal loop, Brucella bacteremia is detected in 30 min) (Rossetti et al. 2013). Urease activity of Brucella have been related as the principal system for stomach low-pH resistance (Sangari et al. 2007). Subsequently, Brucella bacterial cells can disseminate systemically through transepithelial migration or invasion on M cells (Rossetti et al. 2013). Final intracellular infection in epithelial and monocyte-macrophage cells is determinant for the establishment of successful
infection. In the initial steps of infection (5–15 min), traffic through endosome-phagosome with a pH to 4.0–5.0 determines the intracellular fate of Brucella (Celli 2019). Brucella transition to low-pH environment could be fast (no more than 30 min). Considering these two pH scenarios, we hypothesized that invA gene transcription could be necessary for the Brucella melitensis acid resistance mechanism. In pH 5, survival of both strains was higher up to 80%, still at pH 3 bacterial survival was decreased according to time evaluated, with less survival at 15 min. No differences at the same time evaluated were observed between Bm 133 (wild-type) and Bm 133 invA-km (mutant) strains. Similar results were observed by Cui et al. (2013) at pH 3, where B. melitensis 16 M strain survived less than 40%, and hfq mutant survived 20%. Furthermore, a pH 4 bacterial survival assay with Bm 133 and Bm 133 invA-km (with the same experimental conditions of pH 5 and pH 3) demonstrated similar results compared to that of pH 5 bacterial survival results. Bacterial survival for both strains (Bm 133 and Bm 133 invA-km) were above 80% in pH 4 experiments (Supplementary Fig. 1), with no statistical differences. On the other hand, in our model at pH 3.0, the B. melitensis acid-stress response was not enough to ensure the bacterial survival of both strains at similar levels of the pH 5 results. Brucella urease system is necessary for survival in extreme acidic conditions (pH 2.0–3.0), this mechanism is used through passage to the stomach (Bandara et al. 2007; Sangari et al. 2007). Nevertheless, passage through this anatomic compartment is transient, and probably an important bacterial concentration dies in the stomach. In vitro, B. melitensis do not survive beyond 4 h at pH 3.0 (El-Daher et al. 1990). Since no statistical differences are observed between Bm 133 and Bm 133 invA km strains at pH 3.0 at 15 min. post-infection, it is possible that invA gene transcription is not essential in the survival of Brucella melitensis in this acidic condition.

Gene transcription analysis showed that invA is progressively upregulated at pH 5. Conversely, at pH 3 invA transcription is basal; nevertheless, invA mRNA transcripts are present, suggesting a constitutive expression under acidic conditions. These results demonstrate that invA gene transcription is up-regulated under acidic stress. The invA gene promoter sequence demonstrates no homology with dnaK promoter sequence (Fig. 3). It is described that DnaK is a molecular chaperone that is necessary for correct protein folding under heat and acid stress, and it is necessary for intramacrophagic replication (Köhler et al., 2002). Moreover, this protein is virB-dependent translocated substrate (Liu et al. 2016a, b), suggesting its participation on intracellular pathogenesis. The DnaK chaperone protein is related to DnaJ (BME_RS07560) and GrpE (BME_RS08820) molecular chaperones expression. Meanwhile, dnaK and dnaJ are up-regulated under acidic conditions, grpE are down-regulated (Liu et al., 2016a, b). It is probably that DnaK and related molecular chaperones are co-regulated by the same σ52 putative transcription factor. On the other hand, invA gene, whose predicted protein is a NUDIX enzyme, could be regulated by different transcription factor. The prokariotic NUDIX hydrolases are a wide family of proteins related to detoxification, messenger RNA degradation, anti-mutator activities, among others (Srouji et al. 2017; Mildvan et al. 2005). The B. melitensis invA gene preserves the NUDIX box and has 76% similarity with the invA gene of Rickettsia prowazekii and nudH-ialB locus of Bartonella bacilliformis (Alva-Pérez et al. 2014). Furthermore, invA gene is related to E. coli and B. subtilis RppH enzymes (Foley et al. 2015). In E. coli this enzyme triggers 5’-end-dependent RNA degradation by removing orthophosphate from

| a | dnaK: ACCTTGAGCGACGCGATTGTGAAACCGATAGGAACTGCCAT |
| b | invA: ATTTGAGTGAAGCGGTGAAAGCTGCTGTGATATCAATCGAGTGAAT |

**Fig. 3** invA and dnaK promoter sequence comparison. Complete promoter sequences and their comparison were obtained as described in materials and methods section. a Complete promoter sequences. dnaK promoter sequence is located 46 nt. upstream the initiation codon, meanwhile invA promoter sequence is located at 46 nt. upstream the gene, respectively. b Alignment of the promoter sequences. Consensus sequence regions in the figure are the −35 nt (left) and −10 nt (right) of the dnaK of B. ovis (σ52 putative promoter sequence). Black boxes represent identical nucleotides between the consensus and dnaK promoter sequences, and gray boxes represent identical nucleotides between the three sequences.
5′-diphosphorylated transcripts (Gao et al. 2020). Multiple related bacterial RppH enzymes have been implicated in invasiveness and virulence (Edelstein et al. 2005; Gaywee et al. 2002; Conyers and Bessman 1999; Ismail et al. 2003) As mentioned before, Brucella melitensis invA mutant strain was less invasive and virulent than parental wild-type strain (Alva-Pérez et al 2014). The transcription regulator protein, named MucR, up-regulates the B. abortus NUDIX-hydrolase (BAB1_1511) (Caswell et al. 2013), and Mirabella et al. (2013) demonstrate that B. melitensis MucR is transcribed under stress conditions. This transcriptional regulator is involved in pathogenesis, metabolism regulation, and bacterial envelope modification. It is tempting to speculate that MucR regulates invA, under acid stress conditions, however further work need to be done to address this question.

The molecular mechanisms of acid-stress response in Brucella sp. are the two-component response regulator BMEI1329 (Liu et al. 2016a, b), the two-component system OtpR (W. Liu et al. 2015) the NtrX response regulator protein (Carrica et al. 2013), the vjbR gene at pH of 5.5 (Arocena et al. 2012), among others. All these molecular factors act in concert to counteract the low-pH stress in Brucella. Furthermore, virB operon expression under low-pH environment indicates that acidification is essential for intracellular survival (Ke et al. 2015). Additionally, it is known that a shift on bacterial metabolic pathways is necessary for adaptation under acidic stress (i.e., differential expression of acetyl-CoA synthase, vitamin B12-dependent methyltransferase on Rev1 vaccine strain, that could explain partially bacterial attenuation) (Salmon-Divon et al. 2019). As a hydrolase of oligophosphate nucleosides and/or RNA transcripts, invA gene transcription could contribute to the overall metabolism shift observed through acid-stress response. We hypothesize that InvA works together with other factors, as well, for adaptation. It would be of interest to specify how InvA protein contributes to the stress response network of Brucella and clarify the specific time InvA protein is contributing to intracellular pathogenesis. Current experiments are underway to decipher these questions.

Through this study we demonstrate that invA gene is up-regulated under low-pH conditions. At pH 5 invA gene up-regulation was observed according to time, nevertheless at pH 3 up-regulation was almost basal, probably indicating minimal invA gene intervention in low-pH adaptation. Bacterial survival results demonstrate that, under pH 5 conditions, acid-stress mechanisms of adaptation are ensuring bacterial survival. This was not observed under pH 3 incubation. Acid stress adaptation for intracellular invasion (pH 4–pH 5) in Brucella spp. is critical in order to have a successful infection. It has been demonstrated that an invA gene mutant strain had a lower invasion phenotype compared to parental strain. This observation indicates that, under in vitro intracellular infection, invA gene aids to Brucella melitensis invasion. In conclusion, low-pH up-regulates invA transcription, suggesting that the InvA protein is contributing to stress adaptation.

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Author contributions RSB: carried out the experiments, interpreted and analyzed data and drafted the manuscript. HBG: carried out the experiments, made substantial contributions to the work and revised the manuscript critically. JMB: made substantial contributions to the work and revised the manuscript critically. BAR: designed the experiment and revised the manuscript critically. ABG: interpreted and analyzed the data and revised the manuscript critically. JAP: funding obtaining, designed and carried out the experiments, interpreted and analyzed the data and drafted the manuscript. All authors read and approved the final manuscript.

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Availability of data and material Data on bacterial survival experiments are in the Additional File 1.xls. Data on invA and dnaK gene transcriptions analysis are in the Additional File 2.xls.

Code availability Not applicable.

Declarations

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

Ethical approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

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