Irr regulates brucebactin and 2,3-dihydroxybenzoic acid biosynthesis, and is implicated in the oxidative stress resistance and intracellular survival of *Brucella abortus*

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Brucella abortus faces iron deprivation in both nature and the host. To overcome this limitation, *Brucella* secretes the siderophores 2,3-dihydroxybenzoic acid and brucebactin. A Fur-like protein named Irr has previously been characterized in *B. abortus*; this protein is present in the α -2 group of *Proteobacteria* only, where it negatively regulates haem biosynthesis when iron is scarce. Additional evidence that Irr also regulates the synthesis of both siderophores is presented here. Transcriptional *lacZ* fusion and chemical determinations revealed that Irr induced the transcription of the operon involved in the synthesis of the catecholic siderophores, which were consequently secreted under conditions of iron limitation. Irr was able to bind the upstream region of the operon, as shown by electrophoretic mobility shift assay. A *B. abortus* *irr* mutant showed higher intracellular haem content, catalase activity and resistance to hydrogen peroxide than the wild-type strain. The mutation also improved the replication and survival of iron-depleted bacteria within cultured mammalian cells. Although the pathogenesis of *Brucella* correlates with its ability to replicate intracellularly, pathogenicity was not attenuated when assayed in a murine model.

INTRODUCTION

Iron is an essential micronutrient for almost all living organisms. In nature, iron is mainly present in a ferric insoluble state, with reduced biological availability. One of the strategies developed by bacteria to acquire iron under restrictive conditions is the synthesis of low-molecular-mass iron chelators, known as siderophores, together with the receptors for the internalization of ferrisiderophores (Wandersman & Delepelaire, 2004). This iron acquisition mechanism is frequently linked to the virulence of bacteria because it contributes to the establishment of a successful infection (Litwin & Calderwood, 1993). In contrast, a high concentration of iron is toxic, due to its participation in reactions that generate reactive oxygen species, which can lead to cellular damage. In these conditions, bacteria usually induce the synthesis of storage proteins and oxidative stress enzymes that remove toxic molecules. According to this scenario, the tight regulation of iron homeostasis is fundamental for bacterial life.

Brucella abortus is the aetiological agent of bovine brucellosis, which is an infection of humans and cattle.

The organism lives mainly intracellularly, and its pathogenesis correlates with the ability to invade and replicate within professional and non-professional phagocytes (Smith & Ficht, 1990). During infection, iron availability is reduced as part of the host defence against micro-organisms, implying that *B. abortus* faces iron limitation in this process (Bullen & Griffiths, 1999). Under conditions of iron depletion, *B. abortus* secretes the two catecholic siderophores brucebactin and 2,3-dihydroxybenzoic acid (2,3-DHBA), which have been associated with virulence of the species in ruminants, suggesting the importance of this mechanism for iron acquisition in the natural host (Bellaire *et al.*, 2003). Brucebactin, whose structure remains unknown, has recently been reported to be the most active siderophore of *B. abortus* (Gonzalez Carrero *et al.*, 2002); it is produced from 2,3-DHBA through a poorly understood pathway. Biosynthesis of 2,3-DHBA requires genes organized in the *dhbCEBA* operon, whose expression is regulated by iron (Bellaire *et al.*, 2003). Even though two Fur boxes have been identified in the promoter region, the ferric-uptake regulator Fur does not repress transcription of this operon (Roop *et al.*, 2004). No additional data on this regulation have been reported heretofore.

We have recently characterized the iron response regulator Irr from *B. abortus*, and it belongs to the Fur family

Abbreviations: CAS, chrome azurol S; 2,3-DHBA, 2,3-dihydroxybenzoic acid; DIP, 2,2'-dipyridyl; EMSA, electrophoretic mobility shift assay; p.i., post-infection.

(Martínez *et al.*, 2005). Irr downregulates haem biosynthesis when iron is insufficient. This biosynthesis involves six sequential enzymic reactions leading to protoporphyrin IX. In the last step of the pathway, ferrochelatase inserts ferrous iron into the porphyrin ring to yield haem. The interruption of this step is detrimental for *B. abortus* virulence (Almirón *et al.*, 2001). Hence, considering the role of Irr in the regulation of an iron-demanding pathway implicated in *B. abortus* virulence, we decided to investigate whether Irr controls iron-uptake systems, and whether it plays any role during infection. Here, we present evidence that Irr directly induces the biosynthesis of 2,3-DHBA and brucebactin, but that it is not required for virulence in mice. Interestingly, under conditions of iron limitation, a mutant lacking Irr displayed increased resistance to hydrogen peroxide due to higher levels of haem and catalase activity than the wild-type strain. Compared with the wild-type, the mutant showed improved intracellular replication and survival inside HeLa and J774 cells, thus implicating Irr in the pathogenesis of *B. abortus*.

METHODS

Bacterial strains and growth conditions. *B. abortus* strains 2308, 2308IK (2308irr::km), 2308IK(pBBRirr) (the mutant complemented with the wild-type *irr* gene cloned into pBBR1MCS-4) and 2308C (2308dhbC::lacZ) were obtained from the laboratory stock (Martínez *et al.*, 2005). A double mutant 2308IC (2308irr::km, dhbC::lacZ), which carries the chromosomal mutations of 2308IK and 2308C, was constructed for this study by gene replacement (Martínez *et al.*, 2005). All bacterial liquid cultures were incubated at 37 °C in a rotary shaker at 250 r.p.m. Luria–Bertani medium containing the iron chelator 2,2'-dipyridyl (LB-DIP; 0.45 mM), and modified Gerhardt's (MG) medium, were used as iron-deficient media (Lopez-Goni *et al.*, 1992). When required, the media were supplemented with 100 µg ampicillin ml⁻¹ or 50 µg kanamycin ml⁻¹. Procedures using live brucellae were performed in a biosafety level 3 laboratory. All the reagents were purchased from Sigma, unless otherwise stated.

β-Galactosidase enzyme assay. Levels of β-galactosidase were measured from liquid iron-depleted cultures as described by Miller (1992).

Siderophore detection

The chrome azurol S (CAS) assay. This assay was used to determine brucebactin secretion only, since different amounts of commercial 2,3-DHBA had no effect on the absorbance of the CAS reagent, either in plate or in liquid cultures. CAS agar plates were prepared according to the method of Schwyn & Neilands (1987), with the following modifications: (i) MG was used instead of MM9 medium, (ii) the K₂HPO₄ concentration was reduced to 0.3 g l⁻¹, and (iii) glucose was added in place of glycerol and lactic acid to avoid interference of these compounds with CAS (Gonzalez Carrero *et al.*, 2002). Cells grown for 24 h in 1 ml MG were washed and resuspended in 10 µl PBS, which was applied to a sterile filter disk on the CAS plate. The halo produced around the spot was observed after 72 h incubation at 37 °C. For detection in liquid cultures, the supernatants were mixed with an equal volume of CAS reagent, and the absorbance was read at 630 nm. Percentage siderophore activity was calculated by using the formula [(X–Y)/X] × 100, where X and Y are the absorbance values of the growth medium and the sample supernatant, respectively. In order to set up the experimental

conditions for catechol detection, measurements were taken at different time points during the incubation of cells in MG medium. Maximal differences were obtained when the cultures reached OD₆₀₀ ~1. No catechol was detected when determinations were made from supernatants of MG cultures supplemented with 200 µM ferric citrate.

HPLC analysis. This was performed to detect 2,3-DHBA. Supernatants (10 ml) from the bacterial cultures assayed for brucebactin were acidified to pH 2.0 with 6 M HCl. Acidified samples were extracted with ethyl acetate [20 ml (100 ml supernatant)⁻¹]. The catechol-containing extracts were concentrated in a vacuum system, and 0.2 ml of each extract was fractionated on a C18 Sephasil peptide reverse-phase column (12 µM, 4.6 × 250 mm; Pharmacia Biotech). The procedure was performed using a gradient of 10–50 % acetonitrile in water, with 0.1 % trifluoroacetic acid at a constant flow rate of 1 ml min⁻¹. The column profile was monitored by following the absorbance at 254 nm. Commercial 2,3-DHBA dissolved in ethyl acetate was employed as a standard.

Haem determination. The intracellular concentration of haem in *B. abortus* strains was determined as described by Frustaci *et al.* (1991). Commercial haemin was used as a standard in the range 10–200 nM, and it was assayed at the same time as the *B. abortus* samples.

Catalase assay. *B. abortus* strains were grown to OD₆₀₀ ~1.0 in LB-DIP. Cells were centrifuged, washed, and resuspended in PBS. Suspensions were sonicated, and further centrifuged (20 000 g for 10 min at 4 °C). Aliquots from the supernatants were assayed for catalase activity, which was determined by following the decomposition of 18 mM hydrogen peroxide at 240 nm (Beers & Sizer, 1952). One unit of activity was defined as the amount of enzyme that catalysed the decomposition of one micromole of hydrogen peroxide per minute. Protein concentration was determined as described by Bradford (1976).

Hydrogen peroxide sensitivity assay. Cells were grown in either LB or LB-DIP to the exponential or stationary phase of growth. Cells were centrifuged (20 000 g for 10 min), washed, and diluted 1 in 10 in PBS. Aliquots from the logarithmic- and stationary-phase suspensions were challenged with 33 and 100 mM hydrogen peroxide, respectively. Assays were carried out at room temperature, without shaking. At different time points, samples were taken, and these were serially diluted, and plated on LB plates. Colonies were counted after 48 h at 37 °C. Survival rates at different time points were determined as percentages of the number of colonies in the original inoculum.

DNA-binding assay. The ability of the recombinant Irr protein to bind to the upstream *dhbCEBA* region was determined by an electrophoretic mobility shift assay (EMSA). A 0.62 kb DNA fragment was PCR-amplified with the sense primer (5'-GCTCTAGACCTG-TCCCGGCGCAG-3') and the antisense primer (5'-TTCTGCAGTT-GCCGCTGGCCGCCA-3'). The PCR product was digested with *Hind*III, and the 0.24 kb fragment containing the two promoter regions was purified from an agarose gel. Samples containing 0.7 µg of this DNA were mixed with 0, 0.6 or 0.6 nmol of the recombinant Irr in 1 × binding buffer (20 mM Tris, pH 7.8, 5 % glycerol, v/v, 1 mM DTT, 50 µg BSA ml⁻¹, and 5 µg salmon sperm DNA ml⁻¹). The samples were then incubated at room temperature for 15 min. When needed, 3 µl polyclonal mouse anti-Irr serum was added to the reaction mix, which was incubated for another 15 min. As a control, a 0.76 kb chromosomal DNA fragment was used; this was amplified by PCR with the sense primer (5'-CGGGATCCTCGT-GTTCGAGGCTCA-3') and the antisense primer (5'-TGCACTGC-AGTTCCGGGAGGACGAAT-3'). EMSA reactions were analysed on

1.5% agarose gel in $0.5 \times$ Tris/borate/EDTA buffer. After the electrophoresis, the gel was stained with $0.5 \mu\text{g}$ ethidium bromide ml^{-1} , and visualized under UV light.

Intracellular *Brucella* survival experiment. Infection of HeLa and murine-macrophage-like J774 cell lines was performed as previously described (Almirón *et al.*, 2001), with the following modifications: (i) bacterial strains were grown in either LB or LB-DIP; (ii) during infection, no fetal bovine serum was added to the cell medium in order to avoid any possible iron acquisition; and (iii) at 1 h post-infection (p.i.), when non-adherent bacteria had been eliminated, infected eukaryotic cells were incubated with the appropriate medium supplemented with fetal bovine serum.

In vivo experimental infection. Eight-week-old female BALB/C mice were injected intraperitoneally with 0.1 ml of a bacterial suspension prepared in PBS (about 10^4 c.f.u. grown in LB-DIP). At 1 and 3 weeks p.i., an excess of ether anaesthesia was administered, and mice were bled to death by cardiac puncture. The spleen was aseptically dissected, weighed, and then homogenized in PBS. The number of viable bacteria was determined by plating serial dilutions on LB agar.

Statistical analysis. All statistical analysis was performed using Student's two-tailed *t* test. *P* values ≤ 0.05 were considered significant. Results are expressed as means $\pm$ SD.

RESULTS

Analysis of secreted siderophores

Each siderophore released by *Brucella* was detected individually, as described in Methods. Brucebactin was assayed directly with CAS. As shown in Fig. 1(a), after 3 days incubation, the mutant *B. abortus* 2308IK secreted less brucebactin than the wild-type 2308, as judged by the size of the halo. Brucebactin production of the mutant was restored when complemented with the wild-type *irr*, thus confirming that Irr was responsible for the observed phenotype.

To detect secreted 2,3-DHBA, strains 2308, 2308IK and 2308IK(pBBR*irr*) were assayed together with *B. abortus* 2308C, a strain that does not produce siderophores. Supernatants of cultures were analysed by reverse-phase HPLC, and the area of the compound corresponding to the 2,3-DHBA standard was used to determine the amount of 2,3-DHBA secretion. As shown in Fig. 1(b), the *B. abortus* wild-type and the complemented strain secreted three- to fivefold more 2,3-DHBA than the mutant *B. abortus* 2308IK. To compare secretion of the catecholic compounds in the same medium, the relative activity of brucebactin was determined in liquid samples, as described in Methods. In agreement with the above result, the parental strain secreted over twofold more brucebactin than the *irr* mutant. The relative siderophore activities determined in the supernatants of 2308 and 2308IK from three independent experiments performed in duplicate were 87 ± 1 and $29 \pm 6\%$, respectively ($P < 0.05$). The mutant phenotype was also reverted by complementation with the wild-type *irr* gene to give $76 \pm 11\%$ activity, against 1% obtained in the

Fig. 1. Siderophore analysis from iron-restricted *B. abortus* cultures. (a) Detection of brucebactin secretion on CAS plates. *B. abortus* cultures were applied to filter disks on CAS plates, and incubated for 48–72 h at 37°C . Brucebactin was detected as an orange halo around 2308 (1), 2308IK (2), and 2308IK(pBBR*irr*) (3). (b) Detection of 2,3-DHBA (arrow) in ethyl acetate extracts of supernatants assessed by reverse-phase HPLC. The column was eluted with a linear gradient of 10–50% acetonitrile (%B), and absorbance (Abs) was recorded at a wavelength of 254 nm.

supernatant of 2308C. Therefore, active Irr increased the amount of catecholic siderophores secreted by *B. abortus*.

Effect of Irr on the expression of the *dhbCEBA* operon

In order to determine whether the higher amounts of the secreted siderophores were the consequence of induced transcription of the genes involved in their synthesis, a chromosomal *dhbC-lacZ* fusion was analysed in the

background of the parental and *irr* mutant strains, 2308C and 2308IC, respectively. The β -galactosidase activity of the 2308C strain (1252 ± 140 Miller units) was about twofold higher than that of the strain 2308IC carrying the *irr* mutation (609 ± 72 Miller units) ($P < 0.05$). These results were obtained from three independent experiments performed in duplicate. No β -galactosidase activity was obtained when the experiments were done either with cultures at $OD_{600} < 0.4$, or with MG cultures supplemented with ferric citrate. Maximal induction of transcription was observed during the stationary growth phase of iron-limited cultures. These data corresponded to the decreased secretion of siderophores in the absence of Irr, suggesting that Irr contributes to positive transcriptional regulation of the operon when cells are growing under conditions of iron limitation.

To investigate the interaction of Irr with DNA, gel mobility shift assays were performed, as shown in Fig. 2. The fragment of DNA containing the two promoters of the *dhbCEBA* operon was mixed with the recombinant Irr protein, as indicated in Methods. The DNA mobility was retarded in the

presence of increasing concentrations of Irr (Fig. 2b, lanes 3 and 4), and also when the anti-Irr serum was added to the reaction mix (Fig. 2b, lane 5). Even though the shift observed in the DNA mobility was slight, it was highly reproducible. This binding seems to be specific for two reasons. First, the non-specific binding of Irr was minimized by the addition of salmon-sperm DNA to the binding buffer. Second, the mobility of a DNA fragment without the ICE motif was not retarded in the presence of the maximal amount of Irr protein used in our experimental conditions (Fig. 2b, lanes 6 and 7). The extent of retardation could not be further improved by use of different binding buffers, native PAGE, or manipulation of the agarose concentration.

Oxidative stress response

Bearing in mind the relation between iron and oxidation, the phenotype of the *irr* mutant was investigated under oxidative stress. Preliminary results obtained from LB and LB-DIP plates with disks containing different concentrations of hydrogen peroxide showed a discernible phenotype between the wild-type and the mutant when cells were grown on LB-DIP. Thus, the sensitivity to this oxidative agent was investigated by measuring the percentage survival from cultures growing in liquid iron-restricted medium, using control cultures grown in LB. Since Gram-negative bacteria are more resistant to hydrogen peroxide during the stationary phase of growth than during the exponential phase (Almirón *et al.*, 1992), logarithmic *B. abortus* 2308 and 2308IK cells were challenged with 33 mM hydrogen peroxide, and stationary-phase cells with 100 mM hydrogen peroxide. As shown in Fig. 3, mutant cells were more resistant than the wild-type when grown in iron-deficient medium. Between 40 and 60% of the mutant population survived after 35 min treatment when cells were in exponential phase (Fig. 3a), and after 15 min when they were in stationary phase (Fig. 3b). At similar time points, less than 10% of the wild-type population survived. After 60 min exposure, while both logarithmic and stationary *B. abortus* 2308 cells could not recover from the imposed stress condition, mutant cells remained viable. Non-viable cells were recovered after 15 min exposure to 33 or 100 mM hydrogen peroxide when the experiment was done with logarithmic- or stationary-phase cells grown in LB medium (data from four independent experiments).

Catalase is the enzyme that inactivates hydrogen peroxide, and *Brucella* has one catalase encoded by *katE* (Sha *et al.*, 1994). Thus, we determined the specific activity of catalase in the *B. abortus* strains. The *B. abortus irr* mutant expressed significantly more catalase activity than the wild-type. Values obtained from three independent experiments performed when the cultures reached $OD_{600} \sim 1$ were 562 ± 85 and 47 ± 5 units mg^{-1} for 2308IK and 2308, respectively ($P < 0.05$). These data support the survival phenotype shown in Fig. 3.

As catalase is a haemoprotein, and we have previously shown that the *B. abortus irr* mutant accumulates haem precursors

Fig. 2. DNA binding of Irr. (a) Nucleotide sequence of the *dhbCEBA* promoter region used in EMSA. The -35 and -10 regions of the two promoters are underlined, and the putative ICE-like motifs are indicated in bold. (b) Gel retardation assay. The DNA fragment shown in (a) was run in the absence (lane 2), and in the presence of 0.06 (lane 3) and 0.6 nmol (lane 4) recombinant Irr protein. Anti-Irr serum was added to the complex shown in lane 4 (lane 5). An unrelated DNA fragment was run in the absence (lane 6) and presence of 0.6 nmol Irr (lane 7). Ladders of 100 bp were run in lanes 1 and 8 as molecular mass markers. After electrophoresis, the 1.5% agarose gel was stained with ethidium bromide. A negative image of the gel is shown for clarity.

Fig. 3. Sensitivity to hydrogen peroxide. *B. abortus* 2308 (○) and 2308IK (●) cells were grown in LB-DIP, and treated with 33 mM during the exponential phase of growth (a), and with 100 mM hydrogen peroxide during stationary phase (b). The number of c.f.u. ml⁻¹ recovered at different time points was related to the initial number of viable cells (about 1×10^8 c.f.u. ml⁻¹), and expressed as a percentage. Data are means $\pm$ SD of three independent experiments. * $P < 0.05$.

under iron limitation (Martínez *et al.*, 2005), we investigated whether there was a higher level of haem in mutant cells that could account for the higher catalase activity observed. Increases of more than twofold in the haemin intracellular concentration of *B. abortus* 2308IK were obtained in comparison with 2308 when the cells were grown in MG. The data obtained from three independent experiments were 15.4 ± 3.6 and 40.2 ± 8.1 nM ($P < 0.05$) for the wild-type and the mutant cells, respectively; data obtained from three independent experiments repeated in LB-DIP medium were in agreement with these results: 48.2 ± 1.1 and 80.8 ± 3.1 nM for wild-type and mutant cells, respectively; $P < 0.05$.

Intracellular survival

The capacity to invade and replicate inside HeLa and J774 cell lines was assayed with 2308 and 2308IK grown in iron-deficient and iron-sufficient media, and 2308IK(pBBRirr) grown in iron-deficient medium. Cell invasion of HeLa cells (Fig. 4a) and the macrophage-like J774 cells (Fig. 4b) was similar among the strains tested. However, at 24 h p.i. of bacteria grown in iron-deficient medium, it was observed that while 2308IK was able to replicate inside HeLa cells, 2308 showed a decrease in the number of viable intracellular bacteria. At 48 h p.i., both strains were replicating. As shown in the inset, when bacteria were grown in iron-sufficient medium, this decline was not observed for the wild-type.

Fig. 4. Intracellular survival of *B. abortus* strains in HeLa (a) and J774 (b) cell lines. (a) Cells were infected with *B. abortus* 2308 (○), 2308IK (●) and 2308(pBBRirr) (▲) at an m.o.i. of 100 after growth under iron limitation. At different times p.i., eukaryotic cells were lysed, and the number of viable intracellular bacteria was determined. The inset shows, using the same units, the infection with *B. abortus* 2308 and 2308IK grown without iron limitation. (b) J774 cells were infected at an m.o.i. of 50 with *B. abortus* 2308 (○) and 2308IK (●) grown under conditions of iron limitation. The inset represents the infection with the same strains grown without iron limitation. Data are expressed as means $\pm$ SD of three independent experiments.

Thus, to investigate whether the enhanced replication of 2308IK at 24 h p.i. was due to the absence of Irr, 2308IK(pBBRirr) was assayed under the same conditions. This complemented strain behaved in the same way as the wild-type, suggesting that the phenotype was a consequence of the mutation in the *irr* gene. When J774 cells were infected with 2308 or 2308IK, an initial reduction in the number of viable intracellular bacteria was detected at 10 h p.i. Whilst 2308IK showed replication at 24 and 48 h p.i., the viability of 2308 cells was seriously affected; replication of intracellular 2308 was detected at 48 h p.i. only. Both 2308 and 2308IK behaved similarly when the cells were infected with bacteria grown in iron-sufficient medium (inset). The complemented 2308IK(pBBRirr) strain was able to invade J774 cells; however, we were not confident of the accuracy of data obtained, since the infected cells were unstable. We are currently unable to explain the reason for this behaviour.

Virulence in mice

As the ability to survive inside eukaryotic cells is essential for the pathogenesis of *B. abortus*, we tested the virulence of the

B. abortus strains in the mouse model. Two groups of 10 BALB/C mice were inoculated, one with 2308 and one with 2308IK, both grown in iron-deficient medium. As described in Methods, the number of viable bacteria recovered from the spleen of each mouse was determined at 7 and 21 days after inoculation, and the values obtained were 5.2 ± 0.4 and $4.9 \pm 0.3 \log(\text{wild-type c.f.u. ml}^{-1})$, and, 5.2 ± 0.4 and $5.0 \pm 0.5 \log(\text{mutant c.f.u. ml}^{-1})$, respectively; thus, both strains showed similar behaviour. It is possible that the phenotype observed *in vitro* for 2308 was in some way compensated *in vivo*, at least in the experimental conditions used.

DISCUSSION

Bacteria compensate for iron limitation by inducing iron-transport systems, and by reducing the cellular demand for iron. Irr seems to fulfil both functions in *B. abortus*. Previously, it has been shown that Irr down-regulates haem biosynthesis (Martínez *et al.*, 2005). In this work, we demonstrate that *B. abortus* Irr upregulates siderophore production in *B. abortus* under conditions of iron limitation. The *B. abortus irr* secreted significantly less brucebactin than the wild-type strain. Further, detection of 2,3-DHBA from iron-limited bacterial cultures revealed that secretion of this siderophore was also reduced in the mutant. The deficiency in siderophore secretion in the mutant was restored by genetic complementation with a plasmid harbouring wild-type *irr*, indicating the involvement of Irr in the higher amounts of siderophores that accumulated in the extracellular medium of the wild-type cells. This could be the result of an induction in the biosynthesis of the catechols, and/or an induction of unknown secretion machinery. When the transcription of the *B. abortus dhbC* was investigated, gene expression was decreased about twofold in the *irr* mutant compared with the wild-type under conditions of iron limitation. Therefore, Irr positively regulates 2,3-DHBA and brucebactin production; however, these results do not exclude regulation of secretion. Since the factor by which the secretion of the siderophores was affected was higher than that for transcription alone, it can be supposed that secretion was also induced. At this point, it must be considered that transcription and secretion are two different processes that do not necessarily respond with equal intensity to the same regulation.

In general, iron-uptake systems have been reported to be negatively regulated by Fur-like proteins that use ferrous iron as a cofactor. Thus, when iron is scarce, those regulated genes are de-repressed (Wandersman & Delepelaire, 2004). The *dhbCEBA* operon is expressed under conditions of iron limitation from two promoters containing Fur boxes (Bellaire *et al.*, 2003); no evidence of its repression has yet been described. However, the fact that siderophores were secreted in the absence of the positive regulation in the *irr* mutant strongly suggests that the *dhbCEBA* operon is under dual regulation: it is repressed by an unknown regulator in the presence of iron, and induced by Irr in the absence of

this metal. Using a gel retardation assay, we demonstrated that Irr is able to bind the upstream region of the operon. This region contains two putative Irr boxes (ICE motifs) (Rudolph *et al.*, 2006), which are located just overlapping each of the two -10 promoters described in the *dhbCEBA* operon. The DNA–Irr complexes were better resolved from naked DNA in agarose than they were in native polyacrylamide gels, possibly because they were large assembled complexes. In this regard, it must be kept in mind that Irr is able to dimerize *in vitro*, and the target DNA used in our gel mobility retardation assays presents two putative DNA-binding motifs. However, further analyses are needed to identify the DNA sequence that interacts with Irr, and whether it binds DNA as a dimer.

Irr is also implicated in oxidative stress resistance. While almost no *B. abortus* wild-type cells were recovered after exposure to hydrogen peroxide, more than 40 % of *B. abortus irr* cells survived this oxidative stress, regardless of the physiological iron-deficient bacterial state. The *B. abortus irr* showed higher catalase activity than the wild-type. *B. abortus* catalase is transcriptionally regulated by OxyR, increasing its synthesis in response to hydrogen peroxide (Kim & Mayfield, 2000; Kim *et al.*, 2000). Beyond the induction of the gene, its product needs haem as a cofactor to be an active enzyme. Thus, the oxidative stress resistance of the *irr* mutant may be the result of extra haem molecules available to facilitate rapid formation of haemoproteins. It is also possible that Irr regulates the expression of catalase. In this regard, Irr could be a functional homologue of *Bacillus subtilis* Per (peroxide regulon regulator), which is a Fur-like protein that regulates both *katA* and a haem biosynthesis enzyme (Bsai *et al.*, 1998). Similarly, a *Campylobacter jejuni perR* mutant has been described as being hyper-resistant to oxidative stress, with the *katA* and the *ahpC* genes being under derepression (van Vliet *et al.*, 1999; Harvie *et al.*, 2005). Per does not seem to be encoded in the genomes of *B. abortus* (Halling *et al.*, 2005; Chain *et al.*, 2005), *Brucella melitensis* (DelVecchio *et al.*, 2002) and *Brucella suis* (Paulsen *et al.*, 2002). A BLAST search (Altschul *et al.*, 1990) using these translated genomes showed sequence homologies with very low values with a protein that belongs to the Irr family (Martínez *et al.*, 2005), and with Fur. On the other hand, Irr is not present in *Bac. subtilis* or *C. jejuni*. In contrast, *Bac. subtilis* Per does not participate in the regulation of the *Bacillus* catecholic siderophores, while *Bacillus* Fur does (Chen *et al.*, 1995).

The pathogenicity of *Brucella* spp. is based on their ability to survive and replicate inside macrophages. As one of the main bactericidal mechanisms used by macrophages is to produce hydrogen peroxide (Jiang & Baldwin, 1993), it can be expected that a *B. abortus* strain with high catalase activity will offer more resistance to being killed by the macrophage-like J774 cells. The results presented here support this hypothesis: the *B. abortus irr* showed a better adaptation to the intracellular environment, recovering sooner than the wild-type from the initial loss of viability. The mechanisms

against bacteria used by non-phagocytic cells are different; nonetheless, the *irr* mutant was able to successfully replicate during the first 24 h p.i. Wild-type cells lost viability during the same period; nevertheless, they replicated to the same extent as the *irr* mutant cells. It is noteworthy that the infection of HeLa and J774 cell lines with wild-type iron-starved *B. abortus* in the absence of fetal bovine serum was less efficient than when the cell lines are infected in non-limiting conditions (Almirón *et al.*, 2001); nonetheless, the invasion capability of the bacteria was not affected.

Previous reports have shown that the absence of catalase does not attenuate the virulence of *B. melitensis* in goats (Gee *et al.*, 2004) or mice (Sangari & Aguero, 1996). In a different way, 2,3-DHBA is not required for *B. abortus* virulence in mice (Bellaire *et al.*, 1999), while it is considered a virulence factor in its natural host (Bellaire *et al.*, 2003). Here, we present evidence that the combined effects of an increment in catalase activity and haem content, together with a decline in siderophore synthesis, do not alter the virulence of *B. abortus* in mice. This observation suggests that the observed *in vitro* phenotype of the *irr* mutant is not relevant in this animal model.

When the results are analysed together, it can be observed that differences between the *B. abortus* wild-type and *B. abortus irr* phenotypes are evident after short time periods, as if they are reflecting a delay in the wild-type response to the imposed stress condition. This lag may correspond with the time needed for *B. abortus* to increase its haem level through iron uptake and haem biosynthesis, or through haem-uptake systems. Once the goal of increasing the haem level is achieved, the stability of Irr is affected (Martínez *et al.*, 2005; Qi & O'Brian, 2002; Qi *et al.*, 1999). Thus, the wild-type phenotype in the iron-limited condition will resemble that of the *irr* mutant, resulting in an improved stress response.

To conclude, Irr directly induces iron assimilation to ensure haem biosynthesis in *B. abortus*. Haem biosynthesis is essential for the intracellular survival of this animal pathogen (Almirón *et al.*, 2001). In a similar way, *Bradyrhizobium japonicum* Irr coordinates high-affinity iron-uptake systems with the synthesis of haem, a molecule indispensable to efficiently nodulate soybeans (Hamza *et al.*, 1998). Whilst *Brucella* and *Bradyrhizobium* belong to the α -2 *Proteobacteria*, and Irr is confined to this group, it is possible that the regulation exerted by Irr represents a common strategy for intracellular adaptation among plant symbionts and animal pathogens (LeVier *et al.*, 2000; Ugalde, 1999).

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