Dimeric *Brucella abortus* Irr protein controls its own expression and binds haem

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Brucella abortus needs to synthesize haem in order to replicate intracellularly and to produce virulence in mice. Thus, to gain insight into the pathogenesis of the bacterium, regulatory proteins of the haem biosynthetic pathway were sought. An iron response regulator (Irr) from *Bradyrhizobium japonicum*, which is a close relative of *Brucella*, was previously described as being involved in the coordination of haem biosynthesis and iron availability. The *Bru. abortus* genome was searched for an *irr* orthologue gene, and the *Bru. abortus irr* gene was cloned, sequenced and disrupted. A null mutant was constructed that accumulated protoporphyrin IX under conditions of iron deprivation. This phenotype was overcome by a complementing plasmid carrying the wild-type *irr*. Purified recombinant *Bru. abortus* Irr behaved as a stable dimer and bound haem. Interestingly, *in vivo*, Irr was only detected in cells obtained from iron-limited cultures and the protein downregulated its own transcription. Through *lacZ* fusion, it was demonstrated that iron did not regulate *irr* transcription. The data reported show that *Bru. abortus* Irr is a homodimeric protein that is accumulated in iron-limited cells, controls its own transcription and downregulates the biosynthesis of haem precursors.

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INTRODUCTION

Brucellae are Gram-negative facultative intracellular bacteria that are responsible for brucellosis, a chronic zoonotic disease. *Brucella abortus* is the aetiological agent of bovine brucellosis, which induces abortion and sterility in cattle, and undulant fever in humans. The pathogenesis of this organism resides in its ability to invade and replicate within professional and non-professional phagocytes (Smith & Ficht, 1990).

The genus *Brucella* belongs to the α -2 group of proteobacteria, along with plant pathogens and symbionts. Recent analysis of the *Brucella* and *Rhizobium* genomes revealed extensive sequence homology and synteny (Paulsen *et al.*, 2002). This genomic conservation is reflected in common strategies for intracellular survival (Lestrade *et al.*, 2000; Paulsen *et al.*, 2002; Ugalde, 1999). For example, the absence of the haem biosynthetic enzyme HemH affects the virulence of *Bru. abortus* (Almiron *et al.*, 2001) and the development of nodules in *Bradyrhizobium japonicum* (Frustaci & O'Brian, 1992). Haem is required as a prosthetic group in many physiological processes, such as electron transfer,

hydroxylation and the sensing of diatomic gases (O'Brian & Thony-Meyer, 2002). It is also involved in the transcriptional and post-transcriptional regulation of different genes (Ogawa *et al.*, 2001; Pfeifer *et al.*, 1989). Haem is synthesized from the precursor δ -aminolaevulinic acid (ALA) through six enzymic reactions that lead to protoporphyrin IX. In the last step, ferrochelatase (HemH) catalyses the incorporation of ferrous iron into the protoporphyrin IX ring to yield haem. The formation of haem through this pathway could also be compromised by iron limitation, which is a condition faced by the bacteria during infection. Even though *Bru. abortus* has the ability to synthesize haem, as well as to incorporate it from the medium, we showed previously that the absence of *hemH* abolished *Bru. abortus* virulence and intracellular survival (Almiron *et al.*, 2001). Therefore, it is of interest to study the regulation of haem biosynthesis under conditions of iron deprivation.

In *Brad. japonicum*, which is a member of the family *Rhizobiaceae*, the iron response regulator (Irr) has been described as a repressor of haem biosynthesis when iron is scarce, thus avoiding the accumulation of protoporphyrin (Hamza *et al.*, 1998). Irr belongs to the ferric-uptake regulator (Fur) family, the members of which are involved in metal-dependent regulation in bacteria (Escobar *et al.*, 1999).

Bru. abortus genome mining revealed the presence of two putative *irr* genes. Here we describe the characterization and

Abbreviations: ALA, δ -aminolaevulinic acid; DIP, 2,2'-dipyridyl; HRM, haem regulatory motif.

The GenBank/EMBL/DDBJ accession number for the sequence of the *Brucella abortus irr* gene is AY243456.

disruption of the gene with the highest similarity to the *Brad. japonicum irr* gene, including the flanking regions. The haem biosynthesis under iron limitation in the *Bru. abortus irr* mutant was analysed, as well as the effects of iron on *irr* transcription and Irr protein levels. A recombinant *Bru. abortus Irr* was investigated for its ability to bind haem, dimerize and regulate its own transcription.

METHODS

Bacterial strains and growth conditions. *Bru. abortus* strains 2308 and 2308C (2308*dhbC::lacZ*) were obtained from the laboratory stock. *Escherichia coli* DH5 α (Woodcock *et al.*, 1989) competent cells were used for plasmid manipulation. All bacterial liquid cultures were carried out at 37 °C in a rotary shaker at 250 r.p.m., in Luria–Bertani medium (LB) or Brucella Broth (BB) (Difco). LB with 0.45 mM of the iron chelator 2,2'-dipyridyl (DIP) and modified Gerhardt's medium (MG) were used as iron-deficient media. In the latter medium, iron was removed by exhaustive treatment with 8-hydroxyquinoline and the oxime was further extracted with chloroform (Lopez-Goni *et al.*, 1992). For solid media, Bacto agar (1.6%, w/v) was added. When required, the media were supplemented with 100 μ g ampicillin ml⁻¹ or 50 μ g 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.

Gene cloning and sequencing. Classic recombinant DNA techniques were performed according to standard protocols (Sambrook *et al.*, 1989). The putative *irr* gene was obtained by colony PCR from *Bru. abortus* 2308 using the primers Upirr (5'-TGGATTTAG-TGGTTCAGG-3') and Loirr (5'-GACTTCCTTCACCTTTTCCA-3'), both obtained from the putative *Brucella melitensis irr* sequence in GenBank (AE009628). The amplified product (1527 bp) was cloned into pGEM-T Easy (Promega), following the instructions from the manufacturer. The new plasmid was named pGEM-Tirr. Nucleotide sequencing of the amplified fragment was performed using dye-labelled universal terminators on an ABI Prism 377 sequencer (Applied Biosystems) with the primers T7 and Sp6 (Promega). The 1.5 kb *EcoRI* fragment obtained from pGEM-Tirr was ligated to pBBR1MSC-4 (Kovach *et al.*, 1995) digested with *EcoRI*. The resulting plasmid, named pBBRirr, was employed for complementation experiments.

Construction of mutants. (i) *Bru. abortus* 2308IK. In order to disrupt the *Bru. abortus irr* gene, the plasmid pGEM-Tirr was linearized with *NruI* (47 bp downstream of the ATG) and ligated to a 1.3 kb *HincII* fragment containing a kanamycin-resistance cassette (Oka *et al.*, 1981). The new plasmid, named pGEM-TIK, was electroporated into *Bru. abortus* 2308, where it is unable to replicate. Thus, double-homologous recombinants were selected as *Bru. abortus* Km^r Ap^s. PCR and Southern-blot analysis were performed to confirm the mutant.

(ii) *Bru. abortus* 2308IL. To construct a transcriptional *lacZ* fusion to the *irr* gene, the plasmid pGEM-Tirr was digested with *NruI* and ligated to a 4.5 kb *SmaI* fragment containing the *lacZ* cassette (Becker *et al.*, 1995), resulting in plasmid pGEM-TIL. The transcriptional fusion was confirmed by PCR and sequence analysis; β -galactosidase activity was determined as described by Miller (1992). pGEM-TIL was electroporated into *Bru. abortus* 2308. Double recombinants were selected by their antibiotic resistance and confirmed using the methodology indicated above.

Detection of protoporphyrin IX in supernatants of *Bru. abortus* cultures. Accumulation of porphyrins was assayed as described elsewhere (Cox & Charles, 1973). Briefly, *Bru. abortus* cells grown in

BB were centrifuged (4500 g for 15 min), washed twice with phosphate-buffered saline (PBS) and diluted into 50 ml MG or MG supplemented with 50 μ M ferric citrate. Cells from those cultures, grown to saturation, were harvested and the porphyrins from the supernatants were extracted with 12.5 ml ethyl acetate/glacial acetic acid (3:1, v/v) at 18 °C overnight with shaking. Then, the ethyl acetate layer was washed once with MilliQ water and concentrated at low temperature in a vacuum system. The absorption spectrum of this extract was recorded between 700 nm and 325 nm (Spectronic GENEYS 5). Known dilutions of purchased protoporphyrin in a mixture of ethyl acetate and glacial acetic acid were used as standards.

Expression, purification and detection of Irr. To express Irr, the sense primer (5'-ATCGGATCCGATGCATTCTTCACATACCC-AC-3'; *Bam*HI site underlined) and the antisense primer Loirr (see above) were used to amplify a 741 bp fragment from *Bru. abortus* 2308 chromosomal DNA. The PCR product was digested with *Bam*HI and *Hind*III (located 101 bp downstream of the stop codon) and directionally cloned into pTrcHis B (Invitrogen). The new plasmid, pTrcHisirr, was transformed into and expressed in *E. coli* DH5 α . The His₆-tagged Irr was purified from the soluble fractions following the instructions from the manufacturer. It was further used to obtain antibodies against Irr by immunization of mice, following standard procedures.

To detect the levels of the Irr protein in cells that were grown in iron-sufficient or iron-deficient media, *Bru. abortus* 2308IK(pBBRirr) was first grown twice in MG in order to deplete the cells' iron content. Iron-limited cells from late exponential phase were diluted 1/20 either in MG or in MG supplemented with 50 μ M ferric citrate. Exponential-phase cells from each culture were harvested, washed with PBS, and resuspended in lysis buffer (10 mM Tris/HCl, pH 8.0; 10 mM NaCl; 1 mM sodium citrate; 1.5% SDS) for sonication in an ice-water bath (one cycle of 30 s and two cycles of 20 s). Proteins from the soluble extracts were quantified by the Lowry method using BSA as standard. Further, equal amounts of protein from different samples were resolved using 15% SDS-PAGE and transferred onto a nitrocellulose membrane to detect Irr by chemiluminescence (Pierce).

Haem-binding assays. The interaction of Irr with haem was studied through the spectral properties of haem (Zhang & Guarente, 1995). Haemin was dissolved in a 50:48:2 mixture of ethanol/water/5 M NaOH and binding studies were carried out using an appropriate dilution in 50 mM Tris/HCl pH 8.0. The absorption spectrum of 10 μ M haem was recorded in the presence or absence of 10 μ M Irr. Negative and positive controls were carried out using lysozyme and BSA respectively.

Molecular mass determination. The molecular mass of the recombinant Irr was determined by gel filtration using a Superdex 75 10/30 HR column (Amersham Pharmacia Biotech). The molecular mass standards used were BSA (66 kDa), carbonic anhydrase (29 kDa) and cytochrome *c* (12.4 kDa). The samples were resolved at a flow rate of 0.7 ml min⁻¹ using 50 mM Tris/HCl as eluent. The column profile was monitored by following the absorbance at 215 nm. The fractions from the major peaks were pooled, concentrated by precipitation with TCA, dissolved in loading buffer (Sambrook *et al.*, 1989) and resolved using 15% SDS-PAGE.

RESULTS

Search for an Irr orthologue in *Bru. abortus*

To investigate whether an Irr protein was present in *Bru. abortus*, we used the *Brad. japonicum Irr* sequence to carry out a BLAST search (Altschul *et al.*, 1990) against the

Fig. 1. Amino acid alignment of Irr from the α -2 group of proteobacteria. The *Brucella abortus* Irr sequence (AAO89498) is compared with the sequences from its close relatives *Mesorhizobium loti* (BAB51996), *Rhizobium leguminosarum* (CAD37806) and *Agrobacterium tumefaciens* (AAK85974). It is also aligned with sequences from the less related species *Bradyrhizobium japonicum* (AAC32183) and *Rhodopseudomonas palustris* (ZP00010023). Sequences were from GenBank. Conserved amino acids are shaded in grey. The haem-binding domains HRM and HXH are outlined in black. Comparison was performed with the CLUSTAL method using the MegAlign program.

translated genome of *Bru. abortus* (R. A. Ugalde, unpublished). Two homologous sequences were found in chromosome I. This study was performed with the paralogue (Ba12233) that displays the highest similarity (56% identity) and is located, similar to *Brad. japonicum irr*, downstream of genes involved in lipid biosynthesis.

Bru. abortus irr encodes a protein of 145 amino acids that, according to a TBLASTX search (Altschul *et al.*, 1990), is confined to the α -2 group of proteobacteria. In this group, most of the proteins lack the haem regulatory motif (HRM) that is associated in *Brad. japonicum* with its iron-conditional stability (Qi *et al.*, 1999), and they form a highly homogeneous group with 68–71% identity among its members (Fig. 1). The amino acid sequences of these proteins display conserved domains that are characteristic of Fur-like proteins, thus suggesting a role in the transcription of metal-ion-responsive genes.

Analysis of protoporphyrin accumulation in *Bru. abortus* cultures

To investigate whether *Bru. abortus* Irr is involved in the regulation of haem biosynthesis, a *Bru. abortus* 2308irr-null mutant was constructed, as described in Methods. The mutant showed the same growth phenotype as wild-type cells in the media used (data not shown). The wild-type *Bru. abortus* 2308, the mutant *Bru. abortus* 2308IK and the complemented *Bru. abortus* 2308IK(pBBRirr) were tested for porphyrin accumulation after being grown in iron-sufficient or iron-deficient media. Only the mutant strain that was grown under conditions of iron deprivation showed a brownish-red colour. This pigmentation appeared in both the cell pellet and the supernatant of the culture.

The pigment was further identified as protoporphyrin IX by spectrophotometry (Fig. 2). No protoporphyrin IX accumulation was detected in the mutant strain grown under iron-sufficient conditions (data not shown). Therefore, the absence of Irr induced the accumulation of protoporphyrin IX under iron-limited growth conditions. These results suggest that, as was described in *Brad. japonicum*, *Bru. abortus* Irr is involved in the down-regulation of haem biosynthesis under conditions of iron deprivation.

Fig. 2. (a) Protoporphyrin accumulation in iron-restricted *Bru. abortus* 2308IK. Strains 2308 (1), 2308IK(pBBRirr) (2) and 2308IK (3) were cultured in iron-deficient and iron-sufficient media. Supernatants from cultures were extracted with a mixture of ethyl acetate and glacial acetic acid and absorption spectra of concentrated extracts were recorded. The figure shows the recorded scans from iron-restricted cultures. (b) Standard protoporphyrin IX. Absorption peak wavelengths are indicated.

Regulation of *irr* expression by iron

As *Irr* is involved in the regulation of haem metabolism when iron is limited, we investigated whether the iron concentration affects *irr* expression. *Bru. abortus* 2308*irr::lacZ* (strain 2308IL) was constructed and β -galactosidase activity was assayed in cells grown in iron-sufficient (LB) or iron-deprived (LB-DIP) media. As shown in Fig. 3(a), the β -galactosidase activities were similar in both media. Moreover, when the assays were repeated with bacteria grown in MG medium or MG supplemented with iron, 1919 ± 28 and 1856 ± 77 Miller units were obtained, respectively. These results indicate that the transcription of *irr* was not affected by the presence of iron or by the composition of the growth media. As a control for the experimental conditions, the β -galactosidase activity of a *dhbC-lacZ* transcriptional fusion strain (2308C) in LB and LB-DIP media was measured (Fig. 3a). *dhbC* is the first gene of the *Bru. abortus* siderophore operon and is known to be strongly derepressed under iron-deficient growth conditions (Bellaire *et al.*, 2003); it can be seen from Fig. 3(a) that *dhbC* transcription is inhibited by the addition of iron to the medium.

The effect of iron on the intracellular level of the *Irr* protein was also investigated. The experiment was carried out with the 2308IK(pBBR*irr*) strain expressing *Irr* from a medium-copy-number plasmid, as we failed to detect *Irr* by immunoblotting analysis in the wild-type strain. The cells were grown under conditions of iron limitation and were diluted into iron-deficient or iron-sufficient media. The

Fig. 3. Expression of *Bru. abortus irr* according to the external iron concentration. (a) Effect of iron on the expression of the *irr* promoter. *Bru. abortus* 2308IL was grown in iron-deficient (–) or iron-sufficient (+) media and assayed according to Miller (1992). β -Galactosidase activity of the iron-responsive *dhbC* gene from strain 2308C was simultaneously assayed as a control. Activities (means $\pm$ SD of four determinations made in duplicate) are shown. The results are representative of at least three independent experiments. (b) Effect of iron on the *Irr* levels. Cells carrying the medium-copy plasmid harbouring wild-type *irr* were grown in low-iron medium (lane 1). Aliquots from this culture were used to inoculate fresh iron-deficient (lane 2) or iron-sufficient (lane 3) media. After growth, cells from each culture were processed as described in Methods. From each culture processed culture, 40 μ g samples of proteins were resolved using 15% SDS-PAGE and analysed by Western blotting using polyclonal anti-*Irr* serum.

cultures were allowed to grow to the late exponential phase and were further processed in order to analyse the *Irr* levels by Western blotting analysis. As shown in Fig. 3(b), *Irr* was only detected in cells grown under our experimental iron-restrictive conditions (lanes 1 and 2). *Irr* was undetectable in cells that were regrown under iron-sufficient conditions, whereas it was increased in cells grown under conditions of iron limitation. Thus, the presence of iron reduced the amount of *Irr* that was present in the cells. As mentioned above, *irr-lacZ* activity was not affected by the iron concentration, so it is possible that iron affects *Irr* protein stability.

Assays of haem binding to *Irr*

Bru. abortus *Irr* lacks the HRM that is associated with binding to haem and the turnover of the protein. However, as haem can interact with proteins lacking this motif, we analysed its binding to *Irr*. The absorption spectrum of haem changes upon interaction with peptides or proteins (Zhang & Guarente, 1995). Thus, the absorption spectrum of haem in the presence or absence of purified recombinant *Irr* was recorded. As shown in Fig. 4, the 382 nm absorption peak of haem shifted to 394 nm in the presence of *Irr*. This shift is characteristic of haemoproteins, in which the Soret bands shift to longer wavelengths due to changes of electron distribution in the porphyrin ring.

Analysis of oligomerization of *Irr*

Irr belongs to the Fur family, and Fur was previously reported to act as a dimer (Stojiljkovic & Hantke, 1995). Therefore gel-filtration chromatography of a purified recombinant protein was used to determine the possible presence of *Irr* oligomers. The molecular mass of the protein estimated by SDS-PAGE was about 20 kDa. The elution profile from the chromatography revealed the presence of a major molecular form of 43 kDa and a less-abundant molecular form of 81.9 kDa (Fig. 5). The fractions were

Fig. 4. Effect of *Irr* on the absorption spectrum of haem. (a) Absorption spectrum of 10 μ M haem was recorded in the absence (1) or in the presence (2) of 10 μ M recombinant *Irr*. A scan of 10 μ M *Irr* alone (*) is also shown. (b) Absorption spectra of 5 μ M haem with 5 μ M lysozyme (3) or 5 μ M BSA (4) were also recorded as negative and positive controls, respectively. Absorption peak wavelengths are indicated.

Fig. 5. Analytical gel filtration chromatography of *Bru. abortus* Irr. (a) Elution profile of 75 mg of the purified recombinant Irr from a Superdex 75 HR 10/30 HPLC column. A major peak at 43 kDa (2) and a minor one at 81.9 kDa (1) were detected. (b) Proteins from both peaks were resolved using 15% SDS-PAGE together with molecular mass markers (M) and stained with Coomassie blue.

pooled and resolved using SDS-PAGE. As shown in Fig. 5(b), the 43 kDa peak yielded only a 20 kDa protein. The minor peak showed high-molecular-mass proteins that co-purified in the affinity chromatography. Therefore, we concluded that Irr was present exclusively as a dimer of 43 kDa. Moreover, this homodimeric complex was highly stable, as it was obtained even when the sample was heat treated for 1 min before the chromatographic analysis.

Effect of Irr on its own expression

We then investigated whether Irr regulated its own expression, as other members of the Fur family do. For this purpose, we analysed the β -galactosidase activity of the 2308IL strain transformed with pBBRirr, which carries a chromosomal *irr-lacZ* fusion and plasmid-borne wild-type *irr* under the control of its own promoter. The strain that was complemented with the vector alone was used as a control. The assay was carried out in LB-DIP medium, as Irr was only detected under conditions of iron deprivation. The β -galactosidase activity of the chromosomal fusion (2425 ± 224 Miller units) was reduced by about one-half in the presence of Irr (1034 ± 72 Miller units). A similar result was obtained with the 2308IL isogenic mutant carrying an additional *irr* copy in the chromosome (data not shown), indicating that the downregulation was not an artefact of the plasmid-copy number.

DISCUSSION

We have demonstrated the existence of a functional protein in *Bru. abortus* that regulates the synthesis of haem. The analysis of the *Bru. abortus* genome showed a strong

homology to *Brad. japonicum* Irr (Hamza *et al.*, 1998), thus revealing that *Bru. abortus* has a putative *irr* gene. A chromosomal mutation in *irr* of the wild-type *Bru. abortus* strain led to protoporphyrin IX accumulation in the cells when grown under iron-limited conditions. This phenotype, which is based on the repression of haem biosynthesis when iron availability is low, was also recently assigned to an Irr protein described in *Rhizobium leguminosarum* (Wexler *et al.*, 2003).

In both *Brad. japonicum* and *R. leguminosarum*, the regulation of haem biosynthesis by the Irr proteins was observed at the transcription level of *hemB*, which encodes the second enzyme of haem biosynthesis from ALA. Irr represses the expression of *hemB*. As this phenotype was observed in all three species, it is possible that *Bru. abortus* Irr also exerts *hemB* repression. We have observed that wild-type *Bru. abortus* transformed with a pBBR1MSC4 derivative encoding a *hemB-lacZ* fusion yielded approximately 10-fold less β -galactosidase activity than an isogenic *irr* mutant strain transformed with the same plasmid (data not shown). Whilst this is consistent with Irr-mediated repression of *hemB* transcription, we observed that growth of the transformed wild-type bacteria was impaired in iron-limited medium. Thus further studies will be needed to confirm that Irr regulates *hemB* transcription and to determine the site of DNA binding by Irr and whether it corresponds to a consensus sequence.

The Irr protein belongs to the Fur family and is confined to the α -2 proteobacteria, according to an analysis of the available bacterial genome databases. Interestingly, the genomes of *Bru. abortus* and *Brad. japonicum* contain more than one putative sequence encoding Irr-like proteins, without homology to *perR* and besides the *fur* and *zur* genes. This might reflect the importance of keeping iron under restrictive control in these organisms. It would be interesting to know whether these putative Irr-like proteins are expressed simultaneously and have similar functions.

Hence, Irr is a regulator protein that responds to iron concentrations in a particular way. In the present work, we present evidence that the rate of *irr* transcription in *Bru. abortus* is high, regardless of the iron level in the growth medium. Nonetheless, the protein was only detected in cells carrying many copies of the wild-type *irr* gene when grown under iron-limited conditions. The fact that Irr could not be detected by Western blotting analysis when expressed from the chromosomal *irr* gene under conditions of iron limitation could be an indication that it is generally poorly expressed. In this regard, the transcription of the homologous *irr* genes in *Brad. japonicum* and *R. leguminosarum* has been reported to be upregulated under low iron concentrations. Although in *Brad. japonicum*, *irr* transcription depends on Fur-Fe (Hamza *et al.*, 2000), it does not involve Fur in *R. leguminosarum* (Wexler *et al.*, 2003).

Even though there is constitutively high transcription of *irr* in *Bru. abortus*, observations with *Brad. japonicum* suggest

that the Irr protein may be degraded when the intracellular iron levels are increased (Qi *et al.*, 1999). *Bru. abortus* cells that were grown in iron-deficient media accumulated Irr at a certain level. This Irr protein was undetectable when the same cells were diluted into iron-containing media, whereas an increased concentration was observed when the cells were diluted into iron-deficient media. These results suggest an efficient iron-dependent mechanism for the protein degradation. Another possibility is that the intracellular concentration of Irr is lowered as a consequence of the cell dilution, and that this protein level is under the limit of the detection system used in the current work. However, two lines of evidence support the former hypothesis: first, the cell mass of the cultures did not increase substantially, as the cells were growing in the minimal medium for only a relatively short time; and second, similar results were obtained when the experiments were repeated with more protein loaded onto the SDS-polyacrylamide gel for Irr detection. On the other hand, it is evident that intracellular iron depletion prompts the synthesis of a stable Irr protein.

Of the two other Irr homologues described in the species mentioned above, *Brad. japonicum* Irr is the best studied. For the stability of this protein, a mechanism based on two haem-binding sites in the Irr sequence has been proposed (Yang *et al.*, 2005). The *Brad. japonicum* Irr has an HRM that binds ferric haem and a second motif of three histidines (H₁₁₇, H₁₁₈ and H₁₁₉) that interacts with ferrous haem. It has been shown that changing one of these histidines to another amino acid reduces the binding efficiency (Yang *et al.*, 2005). The *Bru. abortus* Irr does not contain the HRM and conserves only two histidines (HXH) of the second motif. In spite of this, we report here that the recombinant *Bru. abortus* Irr protein is able to bind haem. There is a possibility that this haem-binding domain tested *in vitro* could be related to the third uncharacterized ferric haem-binding site in the *Brad. japonicum* Irr. The red shift of the haem spectrum displayed by the *Bru. abortus* Irr is characteristic of haemoproteins that retain a methionine/histidine or bis-histidine coordination to the haem iron (Zhang & Guarente, 1995). It has also been reported that direct iron binding to histidine-rich proteins affects the stability of these proteins (Guo *et al.*, 1995; Pohl *et al.*, 2003), and Irr itself is a histidine-rich protein. Even though the haem-binding site of the *Bru. abortus* Irr remains unknown, it is possible that, in this micro-organism, post-transcriptional regulation is being exerted by binding to the haem molecule, as was established in *Brad. japonicum*. Studies involving a ferrochelatase mutant have suggested that the *Brad. japonicum* Irr degradation occurs at the site of haem biosynthesis via an interaction between Irr and ferrochelatase (Qi & O'Brian, 2002). This type of study cannot be easily carried out in *Bru. abortus*, due to the high demand for exogenous haemin of *Bru. abortus* cells lacking ferrochelatase.

An increase in the intracellular Irr concentration down-regulates its own transcription, thereby allowing the cells to

maintain Irr at a suitable level. This kind of regulation was observed when the experiments were performed with a chromosomal copy and was also demonstrated in the strain that carried many copies of the wild-type *irr* *in trans*, in order to confirm the presence of Irr in the cells under our experimental conditions. This autoregulation is characteristic of members of the Fur family, as is the ability to dimerize. Here we present evidence that the *Bru. abortus* Irr is able to form homodimers *in vitro* and that this conformation is highly stable.

As far as we know, this is the first report to describe the presence of an Irr in *Bru. abortus*. Furthermore, we conclude that this protein behaves as a regulator with the ability to dimerize and is present in cells only when the iron concentration is limited, thereby repressing haem biosynthesis. Under iron-sufficient conditions, the protein becomes less stable, probably due to its ability to bind iron from haem. As low iron availability is one of the conditions imposed by a host during bacterial infection, it will be of interest to evaluate whether Irr regulates other genes that are involved in *Bru. abortus* pathogenesis. To this end, studies are currently being developed in our laboratory.

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