The Journal of Microbiology (2010) Vol. 48, No. 5, pp. 668-673
DOI 10.1007/s12275-010-0145-3

Iron Homeostasis in *Brucella abortus*: the Role of Bacterioferritin

Marta A. Almirón* and Rodolfo A. Ugalde

Instituto de Investigaciones Biotecnológicas (IIB, INTECH), Consejo Nacional de Investigaciones Científicas y Técnicas, Universidad Nacional de San Martín (CONICET-UNSAM), Buenos Aires 1650, Argentina

(Received April 15, 2010 / Accepted June 24, 2010)

Brucella abortus **is the etiological agent of bovine brucellosis, an infectious disease of humans and cattle. Its pathogenesis is mainly based on its ability to survive and multiply inside macrophages. It has been demonstrated that if *B. abortus* ferrochelatase cannot incorporate iron into protoporphyrin IX to synthesize heme, the intracellular replication and virulence in mice is highly attenuated. Therefore, it can be hypothesized that the unavailability of iron could lead to the same attenuation in *B. abortus* pathogenicity. Thus, the purpose of this work was to obtain a *B. abortus* derivative unable to keep an internal iron pool and test its ability to replicate under iron limitation. To achieve this, we searched for iron-storage proteins in the genome of brucellae and found bacterioferritin (Bfr) as the sole ferritin encoded. Then, a *B. abortus bfr* mutant was built up and its capacity to store iron and replicate under iron limitation was investigated. Results indicated that *B. abortus* Bfr accounts for 70% of the intracellular iron content. Under iron limitation, the *bfr* mutant suffered from enhanced iron restriction with respect to wild type according to its growth retardation pattern, enhanced sensitivity to oxidative stress, accelerated production of siderophores, and altered expression of membrane proteins. Nonetheless, the *bfr* mutant was able to adapt and replicate even inside eukaryotic cells, indicating that *B. abortus* responds to internal iron starvation before sensing external iron availability. This suggests an active role of Bfr in controlling iron homeostasis through the availability of Bfr-bound iron.**

Keywords**:** bacterioferritin, *Brucella*, siderophores, oxidative stress, iron homeostasis

Brucella abortus (*B. abortus*) is one of the etiological agents of brucellosis, an infectious disease of cattle and humans with a high incidence in developing countries. The intracellular localization of this pathogen, especially inside macrophages, involves an evasion mechanism that allows bacteria to survive, avoiding the immunological host response and action of antibiotics. This strategy usually leads to the development of a chronic disease with a strong impact on public health and economy. However, for *B. abortus* to establish this particular kind of infection, it needs several nutrients difficult to be obtained in the intracellular compartment, particularly iron.

B. abortus is able to acquire iron in different ways. One of them is directly, through two catecholic siderophores, 2,3-dihydroxybenzoic acid and brucebactin, both of them synthesized by the enzymes encoded in the *dhbCEBA* operon (Lopez-Goñi *et al*., 1992; Gonzalez Carrero *et al.*, 2002). Another way is indirectly, by the incorporation of heme (Almirón *et al.*, 2001) and action of hemooxigenase to liberate iron (Puri and O'Brian, 2006). While some reports have shown divergent results about the role of siderophores in the replication or the infectivity of *Brucella* (Bellaire *et al.*, 1999; Parent *et al.*, 2002), the incorporation of external heme is required for the maintenance of chronic *B. abortus* infection in mice (Paulley *et al.*, 2007). To note, heme represents the major iron source available in the host. Furthermore, the biosynthesis of heme in *B. abortus* is essential for the intracellular replication and virulence in mice (Almirón *et al*., 2001). The metabolic pathway for this biosynthesis requires iron.

The excess intracellular iron can be toxic for bacteria and therefore iron is usually stored in ferritin and/or bacterioferritin (Braun, 1997: Andrews, 1998). This mechanism is not only used to protect cells against iron toxicity but also to release iron for cellular metabolism upon demand. Bacterioferritin (Bfr) is only found in bacteria. It is composed of 1 or 2 different subunits and represents a class of ferritin that contains heme (Stiefel *et al*., 1994). Many bacteria express both, ferritin and Bfr. The role of Bfr is so far unpredictable. For example, while in *Escherichia coli* and *Erwinia chrysanthemi*, Bfr is not involved in iron storage (Abdul-Tehrani *et al.*, 1999; Boughammoura *et al.*, 2008) in *Salmonella typhimurium*, it is the main iron reservoir and also participates in hydrogen peroxide resistance (Velayudhan *et al.*, 2007). We observed that the genome of *Brucella* encodes only one homopolymeric Bfr in the *bfr* gene. Bfr has also been detected in *B. mellitensis* and characterized as a T-dominant antigen (Denoel *et al.*, 1997) although its function is far from clear.

The purpose of this work was to determine the efficiency of Bfr to store iron in *B. abortus* and to investigate its contribution to bacterial replication under iron-restrictive conditions.

Materials and Methods

Bacterial strains and growth conditions

B. abortus 2308 and 2308C strains (Martinez *et al*., 2006), and *E. coli* K-12 DH5αF'IQ (Woodcock *et al.*, 1989) were used. Cultures, prepared

* For correspondence. E-mail: malmiron@iib.unsam.edu.ar; Tel: +5411-4580-7255; Fax: +5411-4752-9639

in Brucella Broth (BB-Gibco, USA) or SOC-B, were incubated at 37°C in a rotary shaker at 250 rpm. Bacto agar was added when necessary to a final concentration of 1.6 g%. When required, the media were supplemented with 100 μg/ml ampicillin, 50 μg/ml kanamycin, 40 μg/ml 5-bromo-4chloro-3indolyl-β-D-galactopyranoside (X-Gal), and/or 100 μg/ml isopropyl-β-D-thiogalactopyranoside (IPTG). For iron-limiting conditions, modified Gherardt medium (MG) was used (Lopez-Goñi *et al*., 1992). For iron enrichment, the media was supplemented with 150 μM ferric chloride (Fe) or 77 μM hemin (Hm). Growth and survival were monitored by measuring the OD at 600 nm and by the determination of colony forming units (CFU)/ml on agar after plating appropriate dilutions. Procedures using live bacteria were performed in a level 3 biosafety laboratory. All the reagents were purchased from Sigma (USA), unless otherwise stated.

Gene cloning and sequencing

The putative *bfr* gene was obtained by colony PCR from *B. abortus* 2308 using primers Ubfr (5′-CGCGGCCGTTGCTGTGG-3′) and Lbfr (5′-GGGGGAGGTAAAGATTGGAAGG-3′). The amplified product (752 bp) was cloned into pGEM-T Easy (Promega, USA), following the instructions given by the manufacturer. The new plasmid was named pTB201. Procedures involving DNA handling and cloning were carried out as described by Sambrook *et al*. (1989) using *E. coli* DH5α F´IQ competent cells. Sequencing reactions were performed with an ABI automated fluorescent sequencing system using plasmid pTB201 and the Ubfr and Lbfr primers. The DNA sequence of the *B. abortus bfr* gene and flanking regions were deposited in GenBank (AY229988). The amplified *bfr* was excised from pTB201 with *Eco*RI and cloned into pBBR1MCS-4 (Kovach *et al.*, 1995). The new plasmid was named pBB23.

Construction of mutants

In order to construct a *B. abortus* 2308 *bfr* mutant, plasmid pTB201 was digested with endonuclease *Bal*I to produce a 210 bp deletion in *bfr*. The linearized plasmid was ligated with a kanamycin resistance cassette (Oka *et al.*, 1981), and this last plasmid was named pTB2K and used to electroporate *B. abortus* 2308, where it is unable to replicate. Electroporation was carried out in a BSTX ECM 600 apparatus, and bacteria were cultured in selective medium. As resultants of a double crossing over recombination, isolates were subjected to colony PCR using Lbfr and Ubfr as primers. The selected strain *B. abortus* 2308 *bfr*::km (2308B) was also confirmed by Southern-blotting. A double mutant, *B. abortus* 2308 *bfr dhbC* (2308BC) was obtained by homologous recombination of pTB2K on 2308C, using the same protocol described above.

β-Galactosidase enzyme assay

Levels of β-galactosidase were measured from *B. abortus* cultured in MG or iron-supplemented MG broths as described by Miller (Miller, 1992).

Quantitation of intracellular iron

Because ferritins accumulate excess iron, cells grown in rich media were used to determine the maximum Bfr capability to store iron. Cells grown in 3 ml of BB or BB supplemented with 150 μM ferric chloride were washed twice with PBS. Dried pellets were weighted and suspended in 2 ml of ultra-pure nitric acid. Suspensions were incubated overnight at 80°C. Then, 8 ml of milliQ water were added to each sample and analyzed by atomic absorption spectrometry (Shimadzu 6501).

Catechols determination

The production of *B. abortus* siderophores in MG was determined in triplicate as previously described (Lopez-Goñi *et al.*, 1992). Inocula were prepared as follows: cells from 9 ml overnight culture were centrifuged at 4500×g for 10 min, washed twice with PBS and suspended in 30 ml of MG medium. This suspension was distributed into 3 tubes (10 ml each) and incubated at 37°C. Starting at 4 h of incubation, 1 ml samples from each tube were taken, and the OD at 600 nm was determined. Cells were centrifuged for 5 min, and catechols were determined in the supernatants by the Arnow assay (Arnow, 1937). Different concentrations of commercial 2,3-dihydroxybenzoic acid (2,3-DHBA) were used as standards to correlate OD values with μM concentrations.

Hydrogen peroxide sensitivity assay

Cells were grown overnight in BB, then washed with PBS and diluted 1 in 100 in MG, MG supplemented with Fe or MG supplemented with Hm. Cells were allowed to grow for an additional 17 h, washed and diluted 1 in 10 in PBS. Aliquots of this suspension were challenged with 0.5 mM or 2.5 mM hydrogen peroxide. Assays were carried out at room temperature without shaking. At 0 and 10 min, samples were taken, then serially diluted and plated on BB plates. Colonies were counted after 48 h at 37°C. Survival rates at 10 min were determined as percentages of the number of colonies obtained at 0 min.

Intracellular survival

Infection of HeLa or J774 cell lines was performed in 24-well plates as previously described (Almirón *et al*., 2001), but the addition of fetal bovine serum was avoided during infection. Briefly, at 4, 24, and 48 h p.i., eukaryotic cells were lysed, and the number of viable intracellular bacteria was determined. Bacterial suspensions from overnight cultures grown in MG were prepared in PBS and used as inoculum. As internal controls, bacterial culture supernatants were tested by the Arnow assay to confirm the production of siderophores. Experiments were done in duplicates and repeated at least three times.

Membrane proteins preparation and analysis

Cells grown in BB were washed and diluted 1/10 in MG or MG supplemented with iron. After 24 h of incubation at 37°C by agitation, cells were harvested by centrifugation and resuspended in a buffer solution composed of 50 mM Tris-HCl (pH 8), 3 mM EDTA, and 2 mM PMSF. Cells were disrupted by ballistic disintegration in an MSK cell homogenizer. Unbroken cells and debris were eliminated by centrifugation and membrane proteins from the soluble fraction were sedimented by ultracentrifugation at 100,000×g for 2 h at 4°C. Proteins were quantified by using the Bradford assay (Bradford, 1976), and 25 μg of proteins from each sample were analyzed by sodium dodecyl sulfate-polyacrylymide gel electrophoresis (SDS-PAGE).

Statistical analysis

All statistical analysis was performed using the Student's two-tailed *t* test. p values < 0.05 were considered significant. Results were expressed as means ± SD.

Results

Bfr is the main iron storage protein in *Brucella* spp.

In silico searches among brucellae revealed that a homopolymeric bacterioferritin is present as the sole ferritin. Appropriate primers were designed to clone the complete *bfr* gene by PCR

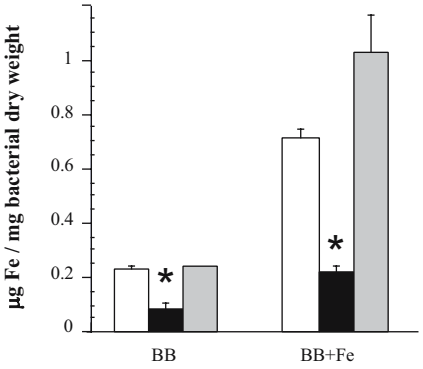

Fig. 1. Intracellular iron content of *B. abortus* 2308 (white bars), 2308B (black bars) and 2308B (pBB23) (gray bars) determined by spectrometry. Data are represented from three independent experiments. * $P<0.05$.

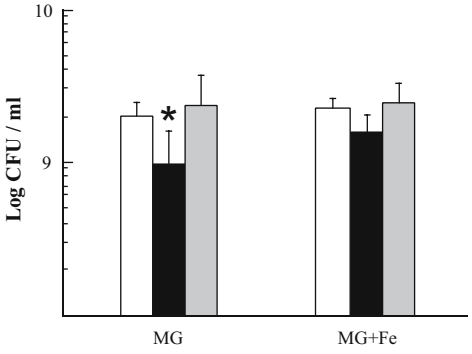

Fig. 2. The growth increment of *B. abortus* 2308 (□), 2308B (■), and 2308B (pBB23) (■) in iron-limited MG and iron-enriched MG media after an overnight incubation in the indicated media. Data are from three independent experiments performed in duplicate. * $P<0.05$.

from *B. abortus* 2308 genome. DNA sequence analysis revealed four changes: $asp_{13}xglu$, $asn_{60}xasp$, $phe_{65}xleu$, and $glu_{146}xgly$, compared with that reported in *B. melitensis* (Denoel *et al.*, 1995). These changes did not include amino acids taking part either in the ferroxidase center or in the heme-binding site. The first three changes were also encoded in the *B. suis bfr* sequence.

To determine whether Bfr behaves as a real ferritin regarding iron storage, a *bfr* null mutant was made as described in Material and Methods and atomic absorption spectrometry was used to analyze the iron content of wild-type and mutant cells grown in rich medium. Results shown in Fig. 1 demonstrate that the lack of Bfr caused a deficiency in the total amount of accumulated iron. When the mutant was complemented with the medium-copy plasmid pBB23 carrying the wild-type *bfr* gene, the amount of iron increased to wild-type levels. However, we were expecting a higher value for the complemented mutant than for the wild type due to an increase in the copy number of *bfr* gene. Thus, to discard a limitation in the external iron source, the experiment was repeated with cells grown in the same medium but supplemented with a concentration of iron that did not affect the cell viability. In this iron-enriched condition, we observed that the intracellular iron levels increased in all the strains, reaching a maximum level in the complemented strain. The ratio of iron concentration between 2308B and 2308 remained constant in both conditions, indicating that Bfr contributes to about 70% of the total iron content.

Inactivation of *bfr* causes a deficient growth phenotype in iron-restricted media

Considering that Bfr is the main iron-storage protein in *B. abortus*, we decided to test if iron from this pool was available for metabolic requirements. First, the *B. abortus bfr* mutant and wild-type cells were grown under normal iron conditions to let Bfr to be loaded with iron. Then, washed cells were diluted into iron-depleted medium, and growth curves were obtained by determining the number of viable cells at different time points. A delay was observed in the mutant growth that usually comprises the lag and logarithmic phases, but either the extension of the first or growth rate of the second phase were variable according to the number of iron-deprived cells, the batch media, and the incubation time. Thus, the overall increment in cell number obtained after an overnight incubation was determined. The results shown in Fig. 2 indicate that 2308B presented a significant growth disadvantage under iron limitation after an overnight when compared to 2308. The complemented 2308B was able to grow as the wild type. To discard an iron-independent growth deficiency of the mutant, iron was added to culture medium in simultaneous experiments. The results showed no difference among strains.

The Bfr-bound iron is not needed for intracellular replication

Since *B. abortus* is able to replicate inside eukaryotic cells and it is accepted that in this milieu free iron is not accessible for bacterial replication, we decided to test if the absence of Bfr also affected *B. abortus* replication inside professional and non-professional phagocytes. The gentamicin protection assay was done as described in Materials and Methods. The values of intracellular replication of *B. abortus* in HeLa cells, expressed in log units as the fraction between the CFU/ml at 24 or 48 h p.i. and CFU/ml at 4 h p.i., were 1.5±0.04 and 1.9±0.02 for 2308, and 1.5±0.02 and 1.9±0.03 for 2308B. Inside J774, a murine macrophage-derived cell line, the percentage survival at 24 and 48 h p.i. related to that detected at 4 h p.i (set as 100%) were 3.3±0.4% and 23.2±1.7% for 2308, against 2.4±0.4% and 24.6±1.5% for 2308B.

Absence of Bfr increases the sensitivity to hydrogen peroxide under iron limitation

The main defense mechanism of professional phagocytes is based on oxidative stress produced by hydrogen peroxide. Since the results obtained for the survival of 2308B inside J774 at 24 h p.i. were consistently lower than those of 2308 ($p=0.05$), we decided to test the sensitivity to that oxidant *in vitro*. Thus, bacterial cells were challenged to sublethal concentrations of hydrogen peroxide. Data from three independent experiments showed that the survival of 2308B was highly reduced in relation to 2308 under iron deficient conditions when exposed for 10 min to 0.5 mM (24±0.4% vs.

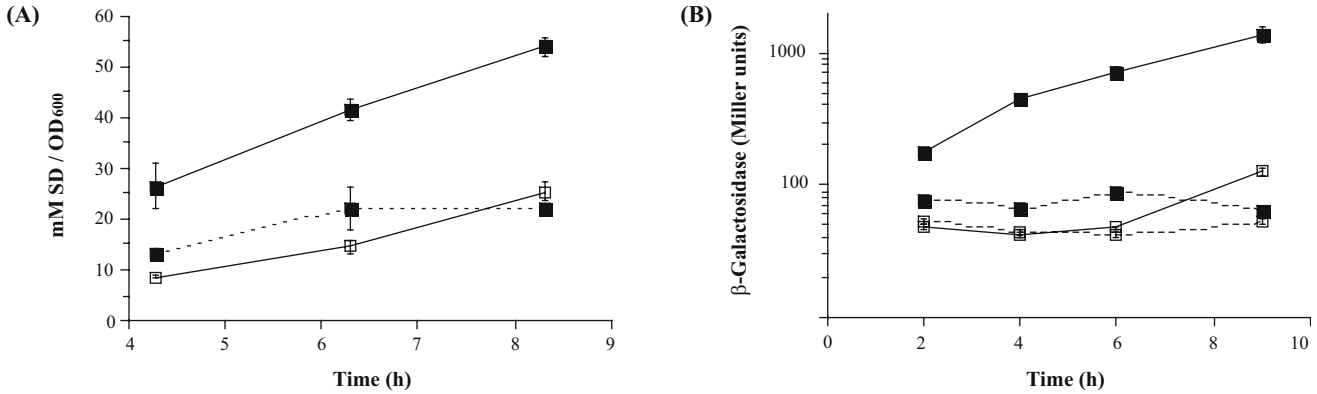

Fig. 3. *B. abortus* siderophores secretion and synthesis in response to internal iron content. (A) Concentration of siderophores related to cell density from *B. abortus* 2308 (-□-), 2308B (-■-), and 2308B (pBB23) (··■··). (B) β-Galactosidase activity (Miller units) from *B. abortus* 2308C (□) and 2308BC (■) grown in MG (-) or in MG supplemented with 50 μM iron citrate (---). Data are from one experiment made in triplicate and representative of more than three independent experiments; standard deviation was less than 5%.

62±27%) or 2.5 mM (17±2% vs. 37±17%) hydrogen peroxide.

The *bfr* complementation exacerbated hydrogen peroxide susceptibility unless heme was added to the media. So, we tried to chemical complement the phenotype by adding extra iron or heme to the culture media. In both conditions, the survival of 2308B was similar to wild type when cells were treated with the same concentrations of hydrogen peroxide (data not shown).

Absence of Bfr activates siderophores production and *dhbC*-lacZ expression

B. abortus 2308B was able to overcome its deficiency to survive inside phagocytes and could replicate under iron limitation. So, it is conceivable that this mutant had triggered a genetic regulation faster than the wild type, in order to adapt to this nutritional starvation. Among the genes known to be induced under iron limitation are those located in the *dhbCEBA* operon. To assess if the internal iron pool related to Bfr participates in this response, the amount of secreted catechols in *B. abortus* cultures grown under iron limitation was measured for 10 h. As shown in Fig. 3A, 2308B signify-cantly secreted more siderophores than did 2308. Values obtained from 2308 at 8.3 h of incubation were similar to those obtained earlier from 2308B. As it is shown in the same figure, the levels of catechols detected from the complemented strain were similar to those of 2308.

This induced secretion of siderophores caused by the absence of Bfr could be the consequence of iron-dependent gene activation of the *dhbCEBA* operon. To test the transcriptional induction of this operon, the β-galactosidase activity of a *dhbC-lacZ* fusion carried out in the chromosome of *B. abortus* wild type and *bfr* mutant (2308C and 2308BC) was assayed. Experiments were done in minimal medium as well as in iron-sufficient conditions as control. As shown in Fig. 3B, 2308BC displayed an immediate activation that was gradually increasing under iron-limited conditions, while 2308C had started transcription at 9 h when compared to cells that kept transcription under repression in iron-sufficient conditions. After 23 h of incubation, 3,900 and 2,400 Miller units were detected for 2308BC and 2308C, respectively, under iron-deficient conditions, clearly indicating an induction of transcription at stationary phase for both strains.

bfr mutant expresses an altered pattern of membrane proteins under iron limitation

Considering that the bacterial mechanisms for the uptake of iron or iron-containing molecules involve the expression of membrane proteins, we decided to determine the pattern of membrane proteins expressed by *B. abortus* 2308 and 2308B when grown in iron-deficient media. As shown in Fig. 4, the protein profile of 2308B was different to that of 2308 when cells were grown under iron limitation for 24 h. The effects due to the absence of Bfr were reversed by the addition of extra iron in the culture media.

Discussion

The purpose of this work was to define the role of Bfr in *B. abortus*, considering that it is the only ferritin present in this pathogen and that iron requirement appears to be critical for

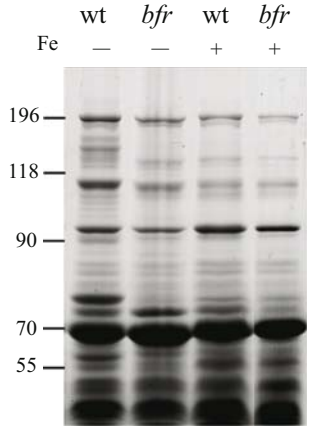

Fig. 4. Membrane protein profiles from *B. abortus* strains in response to iron limitation. 8% SDS-PAGE analysis of membrane proteins of 2308 and 2308B strains grown in (-) low- or (+) high-iron medium. Molecular mass standards in kilodaltons are indicated on the left.

intracellular survival of *Brucella* spp.

Here, we demonstrate that Bfr is the main iron-storage protein in *B. abortus*. The comparison of intracellular-iron levels between the wild type and the *bfr* mutant leads to the conclusion that this protein contributes to approximately 70% of the total intracellular iron content. Although Bfr is the only ferritin in *B. abortus*, it cannot be ruled out that other proteins, such as Dps, could participate in iron storage. Dps was first described as a DNA-binding protein with the ability to protect DNA from oxidative stress (Almirón *et al*., 1992). However, it is now considered a mini-ferritin since it shares structural similarities with ferritins and can also store iron. Recently, it has been reported that *B. abortus* expresses Dps (Lamontagne *et al.*, 2009). Nonetheless, the fact that Bfr stores 70% of the total iron makes the eventual contribution of Dps or other iron-proteins to be minor in this process.

The excess iron stored in Bfr allows *B. abortus* to improve its growth in iron-deficient media when compared with cells that lack Bfr. The growth disadvantage phenotype of the *bfr* mutant when exposed to iron limitation was reversed by *bfr* complementation and also by the addition of iron to the culture media, indicating a direct relation between the absence of Bfr-bound iron and the deficient growth in iron-limited conditions. Interestingly, wild-type bacteria showed the same growth pattern in iron-deficient and iron-sufficient conditions as if they were ignoring the external iron scarcity. These results suggest that wild-type cells employed iron stored in Bfr on metabolic demand. This ferritin function is not shared with its homologue from *E. coli* Bfr.

On the contrary, the excess iron stored in Bfr was not critical for bacteria to replicate inside eukaryotic cells. In this regard, both strains showed a similar pattern of infection in HeLa cells. Moreover, the wild type and the mutant strains were equally efficient to replicate inside the macrophage-cell line at 48 h p.i. However, a slight but constant deficiency for 2308B to survive inside the macrophage-cells during the first hours of infection was detected. One possible explanation is that Bfr confers protection from oxidative stress. When the wild type and *bfr* mutant were exposed to hydrogen peroxide, it was evident that *B. abortus bfr* was more sensitive than the wild type under iron limitation. Previous studies had suggested that iron-storage proteins offer oxidative stress protection by sequestrating and buffering detrimental excess ferrous iron in the cytoplasm (Harrison and Arosio, 1996; Wai *et al*., 1996). However, this mode of protection can be ruled out in our experimental conditions since the sensitivity of *B. abortus bfr* was not increased when extra iron was added to the media, as an indication that no excess ferrous iron was available for the Fenton reaction in the *bfr* mutant. On the other hand, it has been suggested that *Pseudomonas aeruginosa* BfrA protects against hydrogen peroxide because it provides the iron for the heme prosthetic group of catalase (Ma *et al.*, 1999). According to our results, it is likely that *B. abortus* Bfr protects cells in a similar way by providing the iron required to maintain an appropriate level of active iron-containing molecules participating in the bacterial defense against oxidative stress. The paradoxical result that the *bfr* complementation increased the sensitivity to hydrogen peroxide was also observed in *Salmonella* (Velayudhan *et al.*, 2007). One possible explanation is an improper assembly of Bfr, leaving ferrous iron accessible for the Fenton reaction, as a consequence of a misbalance between the heme (prosthetic group for Bfr) and apoprotein biosynthesis when mutant cells are grown under iron limitation. The low ratio holo/apoprotein have been described as a problem for the overexpression of recombinant hemoproteins (Jung *et al*., 2001).

The iron-pool provided by Bfr prevents the production of siderophores. In the *bfr* mutant, the transcription of *dhbC* was highly induced under iron limitation. The promoter activity of this operon correlates with the increased secretion of siderophores, and this was shown to be a direct consequence of the absence of Bfr. This early induction clearly indicates that the *bfr* mutant suffered in advance from iron starvation when compared to the wild type under the same iron-restrictive conditions. On the other hand, it can be observed that the iron-pool related to Bfr seems to induce the expression of some membrane proteins when cells are exposed to iron limitation. The *bfr* mutant did not have the chance to set a proper response for adaptation. This lack of adaptation is probably the same that accounts for the sensitivity to oxidative stress, as previously discussed. Thus, even though the iron sensor has to be identified, the signal is given by the presence of iron in Bfr.

Here, we present results obtained with *B. abortus* that, to our knowledge, are the first description of a bacterioferritin as the main iron reservoir protein involved in the signaling for iron starvation in this pathogen. This strategy indicates a high control of iron homeostasis developed in *B. abortus* to ensure its replication in different environments.

Acknowledgements

This work was supported by grants from the Agencia Nacional de Promoción Científica y Tecnológica de la República Argentina PICT 6580, given to M A, and PICT 651 given to R U and by Grant PIP 5463 obtained from the Consejo Nacional de Investigaciones Científicas y Técnicas de la Argentina (CONICET).

This paper is dedicated to the memory of R. A. Ugalde, who passed away on august 2009.

References

Abdul-Tehrani, H., A.J. Hudson, Y.S. Chang, A.R. Timms, C. Hawkins, J.M. Williams, P.M. Harrison, J.R. Guest, and S.C. Andrews. 1999. Ferritin mutants of *Escherichia coli* are iron deficient and growth impaired, and fur mutants are iron deficient. *J. Bacteriol*. 181, 1415-1428.

Almirón, M., A.J. Link, D. Furlong, and R. Kolter. 1992. A novel DNA-binding protein with regulatory and protective roles in starved *Escherichia coli*. *Genes Dev.* 6, 2646-2654.

Almirón, M., M. Martinez, N. Sanjuan, and R.A. Ugalde. 2001. Ferrochelatase is present in *Brucella abortus* and is critical for its intracellular survival and virulence. *Infect. Immun.* 69, 6225-6230.

Andrews, S.C. 1998. Iron storage in bacteria. *Adv. Microb. Physiol*. 40, 281-351.

Arnow, L.E. 1937. Colorimetric determination of the components of 3,4-dihydroxyphenylalanine-tyrosine mixtures. *J. Biol. Chem*. 118, 531-537.

Bellaire, B.H., P.H. Elzer, C.L. Baldwin, and R.M. Roop 2nd. 1999. The siderophore 2,3-dihydroxybenzoic acid is not required for

virulence of *Brucella abortus* in BALB/c mice. *Infect. Immun*. 67, 2615-2618.

Boughammoura, A., B.F. Matzanke, L. Böttger, S. Reverchon, E. Lesuisse, D. Expert, and T. Franza. 2008. Differential role of ferritins in iron metabolism and virulence of the plant-pathogenic bacterium *Erwinia chrysanthemi* 3937. *J. Bacteriol.* 190, 1518-1530.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*. 72, 248-254.

Braun, V. 1997. Avoidance of iron toxicity through regulation of bacterial iron transport. *Biol. Chem*. 378, 779-786.

Denoel, P.A., M.S. Zygmunt, V. Weynants, A. Tibor, B. Lichtfouse, P. Briffeuil, J.N. Limet, and J.J. Letesson. 1995. Cloning and sequencing of the bacterioferritin gene of *Brucella melitensis* 16M strain. *FEBS Lett.* 361, 238-242.

Denoel, P.A., T.K. Vo, V.E. Weynants, A. Tibor, D. Gilson, M.S. Zygmunt, J.N. Limet, and J.J. Letesson. 1997. Identification of the major T-cell antigens present in the *Brucella melitensis* B115 protein preparation, Brucellergene OCB. *J. Med. Microbiol*. 46, 801-806.

Gonzalez Carrero, M.I., F.J. Sangari, J. Agüero, and J.M. García Lobo. 2002. *Brucella abortus* strain 2308 produces brucebactin, a highly efficient catecholic siderophore. *Microbiology* 148, 353-360.

Harrison, P.M. and P. Arosio. 1996. The ferritins: molecular properties, iron storage function and cellular regulation. *Biochim. Biophys. Acta.* 1275, 161-203.

Jung, Y., J. Kwak, and Y. Lee. 2001. High-level production of heme-containing holoproteins in *Escherichia coli*. *Appl. Microbiol. Biotechnol*. 55, 187-191.

Kovach, M.E., P.H. Elzer, D.S. Hill, G.T. Robertson, M.A. Farris, R.M. Roop 2nd, and K.M. Peterson. 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene*. 166, 175-176.

Lamontagne, J., A. Forest, E. Marazzo, F. Denis, H. Butler, J.F. Michaud, L. Boucher, I. Pero, A. Villeneuve, and *et al*. 2009. Intracellular adaptation of *Brucella abortus*. *J. Proteome Res*. 8, 1594-1609.

Lopez-Goñi, I., I. Moriyón, and J.B. Neilands. 1992. Identification of 2,3-dihydroxybenzoic acid as a *Brucella abortus* siderophore. *Infect. Immun*. 60, 4496-4503.

Ma, J.F., U.A. Ochsner, M.G. Klotz, V.K. Nanayakkara, M.L. Howell, Z. Johnson, J.E. Posey, M.L.Vasil, J.J. Monaco, and D.J. Hassett. 1999. Bacterioferritin A modulates catalase A (KatA) activity and resistance to hydrogen peroxide in *Pseudomonas aeruginosa*. *J. Bacteriol.* 181, 3730-3742.

Martinez, M., R.A. Ugalde, and M. Almirón. 2006. Irr regulates brucebactin and 2,3-dihydroxybenzoic acid biosynthesis, and is implicated in the oxidative stress resistance and intracellular survival of *Brucella abortus*. *Microbiology* 152, 2591-2598.

Miller, J.H. 1992. A short course in bacterial genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, N.Y., USA.

Oka, A., H. Sugisaki, and M. Takanami. 1981. Nucleotide sequence of the kanamycin resistance transposon Tn903. *J. Mol. Biol*. 147, 217-226.

Parent, M.A., B.H. Bellaire, E.A. Murphy, R.M. Roop 2nd, P.H. Elzer, and C.L. Baldwin. 2002. *Brucella abortus* siderophore 2,3-dihydroxybenzoic acid (DHBA) facilitates intracellular survival of the bacteria. *Microb. Pathog*. 32, 239-248.

Paulley, J.T., E.S. Anderson, and R.M. Roop 2nd. 2007. *Brucella abortus* requires the heme transporter BhuA for maintenance of chronic infection in BALB/c mice. *Infect. Immun.* 75, 5248-5254.

Puri, S. and M.R. O'Brian. 2006. The *hmuQ* and *hmuD* genes from *Bradyrhizobium japonicum* encode heme-degrading enzymes. *J. Bacteriol.* 188, 6476-6482.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular cloning: A laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, N.Y., USA.

Stiefel, E.I., M.J. Grossman, S.M. Hinton, V. Minak-Bernero, G.N. George, R.C. Prince, R.E. Bare, and G.D. Watt. 1994. Bacterio-ferritin: a hemoprotein member of the ferritin family. *Adv*. *Exp. Med. Biol*. 356, 157-164.

Velayudhan, J., M. Castor, A. Richardson, K.L. Main-Hester, and F.C. Fang. 2007. The role of ferritins in the physiology of *Salmonella enterica* sv. Typhimurium: a unique role for ferritin B in iron-sulphur cluster repair and virulence. *Mol. Microbiol*. 63, 1495-1507.

Wai, S.N., K. Nakayama, K. Umene, T. Moriya, and K. Amako. 1996. Construction of a ferritin-deficient mutant of *Campylobacter jejuni*: contribution of ferritin to iron storage and protection against oxidative stress. *Mol. Microbiol*. 20, 1127-1134.

Woodcock, D.M., P.J. Crowthwe, J. Doherty, S. Jefferson, E. DeCruz, M. Noyer-Weidner, S.S. Smith, M.Z. Michael, and M.W. Graham. 1989. Quantitative evaluation of *Escherichia coli* host strains for tolerance to cytosine methylation in plasmid and phage recombinants. *Nucleic Acids Res*. 17, 3469-3478.