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# The Importance of Haem vs Non-Haem Iron in the Survival and Pathogenesis of *Brucella abortus*

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## 1. Introduction

The genus *Brucella* belongs to the alpha-group of proteobacteria, along with plant pathogens as *Agrobacterium* and symbionts as *Rhizobium* and *Bradyrhizobium*. Several *Brucella* species, highly related at the genetic level, have been identified as the etiological agents of brucellosis. Each one has shown a preferred host although they can infect other animals. Animal brucellosis is usually endemic in developing countries and its main impact is economic. Human brucellosis is difficult to diagnose because symptoms are extremely variable. Few live vaccines are available for animals only, not for humans. The main problems with this pathogen are the lack of virulence factors commonly identified in other bacteria and the ability to establish a niche inside eukaryotic cells, where they replicate and survive evading the immune response and the action of other antibacterial molecules.

In general, the pathogenic mechanisms used by bacteria have been related to iron because this essential cellular nutrient is scarce and not easily available for bacteria either in body fluids or inside eukaryotic cells. Thus, the iron deficient environment that bacteria encounter during infection induces genes which products are needed to support effective survival and thus they became part of virulence factors. Most of them are related to the acquisition of ionic iron or iron-containing molecules (Schaible & Kaufmann, 2004).

In the case of *Brucella*, much effort has been put into the study of this organism under iron limitation. The first reports about the nutritional preferences showed an absolute requirement for iron (Gerhardt, 1958). Further studies, mainly on *Brucella abortus* (the causative agent of brucellosis in cattle) have shown that *Brucella* produce two catecholic compounds, 2,3-dihydroxybenzoic acid (2,3-DHBA) and brucebactin, as siderophores under iron limitation (Gonzalez Carrero et al., 2002; Lopez-Goñi et al., 1992). However, experimental evidence on mutants unable to synthesize those molecules failed to demonstrate that their production is critical for bacterial replication inside macrophages (Bellaire et al., 1999; Jain et al., 2011). In addition, a mutant in the main iron-response regulator gene, with deficient synthesis of siderophores and increased intracellular haem content, showed a better replication pattern inside professional phagocytes than wild type (Martinez et al., 2006). On the other hand, a *B.*

*abortus* mutant that can not ensemble iron into protoporphyrin IX to produce its own haem was unable to survive inside professional phagocytes (Almiron et al., 2001). Another *B. abortus* mutant that can not internalize haem from the medium showed significant attenuation in cultured murine macrophages (Paulley et al., 2007). None of these two mutants could establish a wild-type pattern of infection in the mice model. Therefore, taking into consideration all these results, it seems that under iron limitation, as one of the stress conditions that probably *Brucella* face during infection, the acquisition or biosynthesis of haem renders more benefits to survive than the acquisition of iron. In agreement with this, we found that a mutation in the main iron-reservoir protein of *B. abortus* did not affect its replication inside professional phagocytes (Almiron & Ugalde, 2010).

For a long time, a link has been established between the preference of *Brucella* to use erythritol as a carbon and energy source and the pathogenesis of this organism, based on experimental data (Smith et al., 1962). This molecule is present at high concentrations in placental trophoblast, the preferred niche for *Brucella*. One of the plausible explanations to this link is the bacterial necessity for iron to catabolized erythritol. Without interfering with this theory, it is apparent that placenta is a membranous-vascular organ that receives nutrients from the mother's blood providing in this way a high content of haem. Additionally, haem is known to be the main iron source inside eukaryotic cells.

## 2. Involvement of iron in the replication of *B. abortus*

*B. abortus*, like most pathogenic bacteria, presents an absolute requirement for the micronutrient iron to support growth (Gerardt, 1958). Bacteria utilize iron as a cofactor or as a prosthetic group for essential enzymes that participates in many biological processes, such as metabolism, respiration, oxygen transport, and gene regulation. Iron is one of the most abundant elements in nature but its bioavailability is reduced under physiological conditions. The oxidised  $\text{Fe}^{3+}$  ferric form is predominant in the environment under aerobic atmospheres with very low solubility at neutral pH ( $10^{-18}$  M), and even lower ( $10^{-36}$  M) in solution due to its tendency to hydrolyze and form polymers (Andrews et al., 2003). In animals, iron is bound to proteins in order to keep the metal soluble and to avoid toxic reactions due to its redox potential. Intracellularly, iron is chelated by a porphyrin ring giving rise to haem. However, the chelated metal iron can undergo reversible oxidative changes and thus haem is mainly associated to proteins. In this way, free iron is practically unavailable for bacteria in the host.

*B. abortus* is able to acquire iron in different ways. Directly, through the synthesis and secretion of two catecholic siderophores, 2,3-DHBA and the more complex compound derived from this known as brucebactin. Both of them are synthesized by the enzymes encoded in the *dhbCEBA* operon in response to iron limitation (Gonzalez Carrero et al., 2002; Lopez-Goñi et al., 1992). The indirect way is by the incorporation of haemin as an iron source (Almirón et al., 2001; Paulley et al., 2007).

*B. abortus* stores iron mainly in a bacterioferritin. This molecule represents a ferritin that contains haem. In *Brucella*, the bacterioferritin is a homopolymer that accumulates approximately 70% of the intracellular iron (Almirón & Ugalde, 2010). Another protein considered as a miniferritin, Dps, is expressed in *Brucella* and could be contributing with iron storage in this pathogen during the stationary phase of growth (Almiron et al., 1992;

Lamontagne et al., 2009). According to the phenotype of a *B. abortus bfr* mutant it was suggested that this microorganism senses the Bfr-bound iron available for metabolism, by an unknown mechanism, and thus regulates iron homeostasis independently of the external iron concentration (Almiron & Ugalde, 2010).

## 2.1 Extracellular survival

The acquisition of iron from the environment is a process tightly regulated by bacteria in order to keep iron homeostasis. An excess of free intracellular iron can be devastating for bacterial survival. The iron-uptake system by siderophores, low-molecular chelators with high-affinity towards ferric iron, means the production of the compounds plus the proteins needed for transportation and reception of ferric-siderophores complexes. The transport is usually mediated by the energy-transducing TonB-ExbB-ExbD system in Gram-negative bacteria (Andrews et al., 2003).

In this regard, the TonB system, an ABC transporter, and a putative GTPase were shown to be involved in iron acquisition of *B. melitensis*. The *exbB*, *dstC* and *dugA* genes are needed for the assimilation of DHBA and /or ferric citrate (Danese et al., 2004). Is it probably that they have the same effect in *B. abortus*.

### 2.1.1 The complex regulation of siderophore biosynthesis

The *B. abortus dhbCEBA* operon is repressed during growth under iron-sufficient conditions while it is highly induced under iron limitation (Lopez-Goñi et al., 1992). The transcriptional regulators acting on the promoter region of the *dhbCEBA* operon are Irr and DhbR. The iron-response regulator, Irr, is a member of the Fur family, and DhbR of the AraC family. Both proteins have demonstrated to interact with the DNA *in vitro* by the electrophoretic mobility shift assay (Anderson et al., 2008; Martinez et al., 2006). The *B. abortus irr* gene is constitutively transcribed regardless of the external iron concentration, but no protein could be detected under iron-sufficient conditions (Martinez et al., 2005). The transcription of *dhbR* is iron repressible and seems to be under the transcriptional control of Irr. A putative binding site for Irr was found between the -35 and -10 regions related to the transcriptional start of the *dhbR* gene (Anderson et al., 2008). The induction of *dhbC* transcription by Irr and DhbR has been correlated with the reduced production of siderophores in the culture media of the *irr* and *dhbR* mutants, respectively, and in comparison with the wild type. In both studies, the transcription was analyzed by constructing *dhbC-lacZ* fusions. To study the Irr regulation, the fusion was carried out to the *B. abortus* chromosome causing a mutation in the gene that enabled the strain to produce siderophores. In this case, a two-fold induction was observed in the wild type in comparison with the *irr* mutant (Martinez et al., 2006). In the other work, the authors assayed the  $\beta$ -galactosidase activity of a plasmid-borne *lacZ* fusion expressed in different genetic backgrounds. Interestingly, they found equivalent reduction in the enzyme activity expressed either in *B. abortus dhbR* or in *B. abortus dhbC* mutants. In this way, the authors corroborated that DhbR needs siderophores as a coinducer to repress transcription. This mode of action was described for other AraC-like regulators (Anderson et al., 2008). If DhbR acts in this way, the cause of the remaining levels of the enzyme activity detected in the *B. abortus irr dhbC* mutant under iron limitation should be investigated.

Neither Irr nor DhbR are involved in the transcriptional repression of the *dhbCEBA* operon. In order to have an accurate control of iron metabolism, the Irr protein is unstable under iron-sufficient conditions. The *Brucella* genome encodes two *irr* paralogues as other members of the alpha-proteobacteria group (Martinez et al., 2005). It has not been studied yet if the *B. abortus irr* orthologue is expressed and plays a role in iron-dependent regulation.

Other regulators that could be involved in iron homeostasis have been described by genomic sequence analysis, like RirA (Johnston et al., 2007; Rodionov et al., 2006). But, the lack of experimental results limits our understanding of this subject in *Brucella*.

Beyond this, the fact that more than two regulators modulate the transcription of the genes involved in the biosynthesis of both siderophores is an indication of the importance of the non-haem iron acquisition in the survival of *Brucella* under iron limitation. The idea that siderophores contribute to the virulence of several pathogens –such as *Escherichia coli* and *Vibrio cholerae*– is based on the capacity they give to the bacteria to acquire an essential nutrient under iron limitation. However, this role has not been well defined in *Brucella* yet, as we will discuss below.

### 2.1.2 Could erythritol be related to siderophore production due to its osmotic property?

The attenuation of a *B. abortus dhbC* mutant in pregnant cattle settled the bases for the relationship between iron acquisition and erythritol catabolism (Bellaire et al., 2003a). Bellaire and colleagues found that the production of siderophores was stimulated by the presence of erythritol in the media, and this effect was also observed at transcriptional level. The wild-type *B. abortus* harboring a plasmid-borne *dhbC-lacZ* fusion showed an increment in the  $\beta$ -galactosidase levels when grown in the presence of erythritol than that observed when this polyalcohol was not added. In order to explain this observation, they hypothesized that wild-type *B. abortus* experience an increased demand for iron in order to catabolize erythritol, since one of the enzymes involved in this metabolic pathway needs iron as a cofactor.

Interestingly, we found that if instead of the minimal media MG (usually employed to test siderophore production in *B. abortus* strains) we used the minimal media MM, no siderophores detection was possible after bacterial growth although iron was not present. The composition of this defined media is:  $(\text{NH}_4)_2\text{SO}_4$  2 g,  $\text{KH}_2\text{PO}_4$  7 g, glucose 4 g, casaminoacids 10 g,  $\text{MnCl}_2$  100  $\mu\text{g}$ ,  $\text{MgSO}_4$  100 mg, biotin 2  $\mu\text{g}$ , nicotinic acid 0.4 mg, piridoxal 0.4 mg, thiamin 0.4 mg and pantoteic acid 0.4 mg per liter; pH 7. A comparative look at the composition of both media led us to find a remarkable difference related to NaCl and glycerol, present in 7.5 g/l (128mM) and 37.5 g/l respectively in MG media. Thus, we decided to supplement MM with NaCl and glycerol and test for catechole production.

The results presented in Table 1 indicate that the *B. abortus* siderophore production was induced by increasing amounts of NaCl or glycerol. Moreover, both osmolytes act in a synergistic mode when added together to the MM media. An increment in siderophore production was also obtained when manitol was added to MM, suggesting that the phenotype was not restricted to ionic osmolytes. To test if this induction was exerted at transcriptional level, the  $\beta$ -galactosidase of the *B. abortus* chromosomal *dhbC-lacZ* fusion was



Medium	MM	MM	MM	MM	MM	MG
NaCl (g/l)	-	5	10	-	10	-
Glicerol (g/l)	-	-	-	37,5	37,5	-
[SD]/OD <sub>600</sub>	1.9	13.0	34.4	21.3	122.6	51.4

Table 1. Effects of NaCl and glycerol on *B. abortus* siderophore production. *B. abortus* siderophore secretion expressed as nmol/ml related to cell density from the wild-type 2308 strain grown in MG or MM media supplemented with NaCl and glycerol at the indicated concentrations. Data are from one experiment made in duplicates and representative of more than four independent experiments.

measured in MM media supplemented with different amounts of NaCl. The results, as shown in Table 2, demonstrate that osmolality plays a role in *dhbC* transcription and consequently in siderophore secretion.

mM NaCl in MM	0	50	100	200
β-galactosidase (Miller units)	21.8	23.2	93.3	439.1

Table 2. Effects of NaCl on the *B. abortus dhbC* transcription. β-Galactosidase activity from *B. abortus dhbC-lacZ* grown in MM supplemented with NaCl at the indicated concentrations. Data are representative of at least three independent experiments.

Even though *Bacillus subtilis* is a Gram-positive bacterium, it produces a similar catechol molecule, 2,3-dihydroxybenzoate, that is finally modified to render the siderophore bacillibactin with stronger chelation capacity than the precursor. The enzymes that participate in their biosynthetic pathway are encoded in the *dhbACEBF* operon. It was reported that high salinity causes iron limitation in *B. subtilis* and triggers the derepression of iron-controlled genes present in the operon mentioned before (Hoffmann et al., 2002). Iron limitation as well as high salinity led to the accumulation of comparable amounts of siderophores in the bacterial culture. This phenotype as well as the growth deficiency observed under high salinity could be reduced by an excess of iron. In this microorganism, the ferric uptake regulator Fur is repressing the genes when sufficient iron is present (Hoffmann et al., 2002). In this relation, it should be noted that erythritol is an osmolyte and curiously, it has been shown that the growth of the *B. abortus dhbC* mutant in iron-limited media supplemented with erythritol is enhanced by the addition of iron (Bellaire et al., 2003a).

Similarly, a *B. abortus entF* mutant presented a growth deficiency under iron limitation in comparison with wild type, which was reverted by the addition of iron (Jain et al., 2011). The product of *entF* is involved in the production of brucebactin (Gonzalez Carrero et al., 2002). It was shown that the *B. abortus entF* mutant was not able to increase the number of viable cells when incubated under iron limitation. Interestingly, the mutant culture had not lost viability before a period of 192 h of incubation as an indication that the internal-iron content was enough to support metabolism in the experimental conditions used. Instead, a decrease in the number of viable cells was observed when the medium was supplemented with erythritol, but this phenotype was reverted by the addition of iron salts. These data

indicate, besides the role that the product of *entF* could have in the erythritol metabolism as suggested, the relationship between siderophore production and erythritol. Is it possible that *Brucella* needs to acquire iron in order to survive under osmotic pressure?

No data about the growth phenotype of iron-depleted cells in media containing erythritol has been reported. This information may be important if it is considered that the host imposes iron limitation as a defense mechanism against microbial infection (Schaible & Kaufmann, 2004). Under this circumstance, it is likely to find iron-depleted bacteria from host's samples. A similar situation could be considered if the bacterial samples came from environment. Taking into account that *Brucella* modulates iron-dependent gene regulation according to their iron content and independently of the external iron concentration, it should be appropriate to test the survival efficiency of iron-depleted *brucella* in media that contain erythritol, such as the modified *Brucella* selective medium that has been developed to be used for diagnostic purposes (Her et al., 2010).

### 2.1.3 Fur regulation

Bfr is a homopolymeric hemoprotein that functions as the main iron-reservoir of *Brucella*. As other members of the alpha-proteobacteria group, the *Brucella* iron-dependent regulation responds to the internal iron concentration (Almirón & Ugalde, 2010). Hence, a mutation in the *B. abortus bfr* induces siderophore biosynthesis and secretion earlier than in the wild-type strain under iron limitation (Almirón & Ugalde, 2010). It has been described that the promoter region of the *dhbCEBA* operon is subjected to the regulation of Irr and DhbR proteins under iron limitation. Both were able to bind the promoter region *in vitro*. The same region has also two putative Fur boxes. Fur is the main iron-dependent regulator in many bacteria. It usually represses genes when bound to iron. Therefore, if iron is not available there should be a derepression of fur-regulated genes. In order to understand whether Fur participates in the *dhbCEBA* regulation we constructed mutants by inserting a kanamycin-resistant cassette in the amplified *fur* gene and then, by carrying this mutation to the chromosome of the wild-type *B. abortus* 2308 and the isogenic *bfr* or *dhbC-lacZ* mutants by homologous recombination as described previously (Martinez, 2004). As shown in Figure 1, we determined the  $\beta$ -galactosidase activity of the chromosomal *dhbC-lacZ* fusion in the background of the parental and *fur* mutant strains. We included the *bfr dhbC* mutant strain for comparative purpose (Almirón & Ugalde, 2010).

The experiments were done when cells were grown in MG or iron-supplemented MG. Although the absence of Fur did not produce the same effect on the *dhbC* transcription, the small induction observed among cultures at  $OD_{600} \leq 1$  led us to look more precisely at siderophore production in the wild-type, *bfr* and *fur* isogenic mutants.

It is interesting to note that when the cells have the possibility to acquire external iron by means of siderophores, the results can be different. While the wild type did not sense internal iron limitation during the first hours of incubation in MG, the consequence of Fur absence is equivalent to the internal-iron deficiency produced by the mutation in *bfr*, in terms of siderophore secretion (Figure 2A). In contrast, in the presence of external iron the *bfr* mutant represses the biosynthesis as wild type, while *fur* did not (Figure 2B). To determine if the repression observed in *bfr* mutant was due to Fur, a *B. abortus bfr fur* double mutant was constructed. The results suggest that Fur is regulating siderophore production

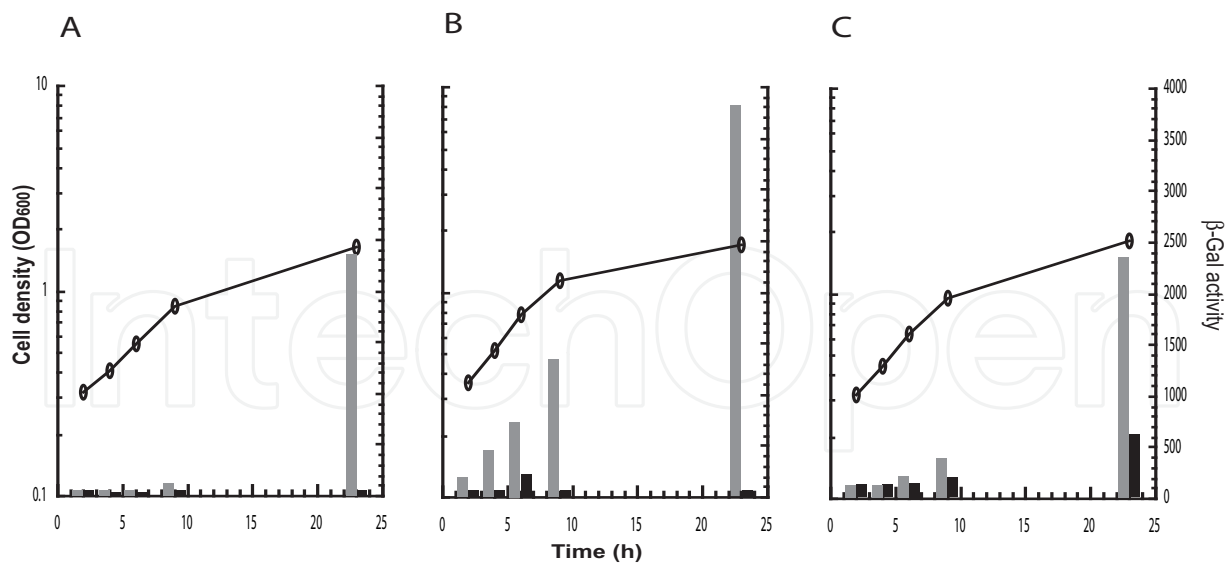


Fig. 1. Effect of iron concentration on *dhbCEBA* transcription. β-Galactosidase activity (Miller units) was determined from *B. abortus* 2308 derivatives growing in MG (gray bars) or in MG supplemented with 50 μM iron citrate (black bars). (A) *B. abortus* 2308 *dhbC*, (B) 2308 *bfr dhbC*, and (C) 2308 *fur dhbC*. Growth curves in MG were determined by measurement of OD<sub>600</sub> (lines). Data are average of duplicates, standard deviation were less than 5%.

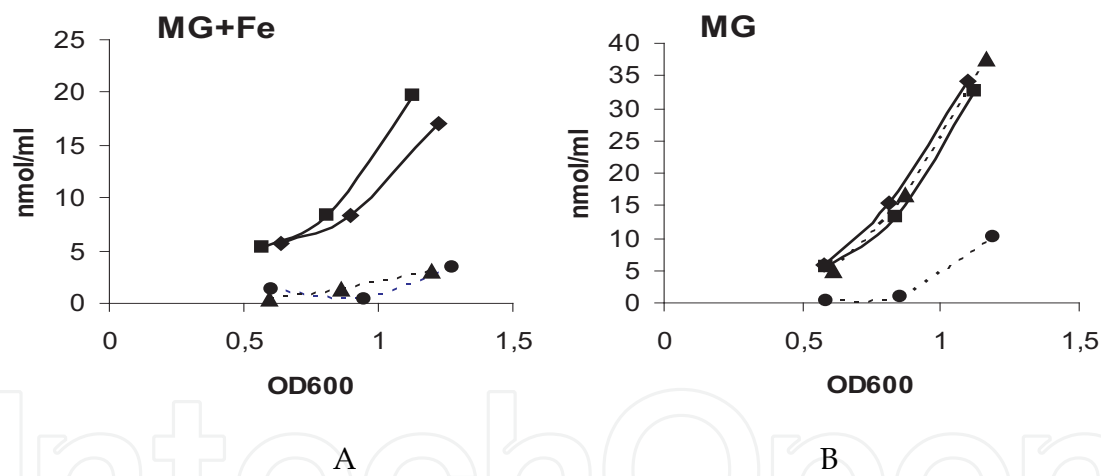


Fig. 2. *B. abortus* siderophore secretion in response to external iron concentration. Concentration of siderophores related to cell density from *B. abortus* 2308 (●), 2308 *fur* (■), 2308 *fur bfr* (◆), and 2308 *bfr* (▲). Values of catechol concentration (determined by the Arnow assay) are expressed from duplicates at three different time points during incubation. Results are representative of three independent experiments.

in *Brucella abortus* under iron sufficient conditions. Further studies are needed to determine the promoter region that interacts with Fur-Fe complexes.

There is a previous report showing that a *fur* mutant was producing less β-galactosidase activities than the wild type in MG. No differences were observed in the presence of iron. It should be noted that the data were obtained from a plasmid-borne *dhbC-lacZ* fusion expressed either in the wild-type *B. abortus* or in the isogenic *fur* mutant (Roop, et al., 2004).



Because the extracellular iron concentration does not represent the *Brucella* iron content, the experimental conditions used to test the *dhbC*-lacZ fusion, sometimes in a plasmid and others in the chromosome causing a mutation in the *dhbC* gene, do not result trivial, especially with Fur that needs intracellular free-iron to bind DNA near the promoter region of a gene. Thus, it should be important to determine the transcriptional level of the *dhbCEBA* operon relative to the internal iron content in *Brucella* strains in order to get comparable results and to better understand iron homeostasis in this pathogen.

#### 2.1.4 Production of outer-membrane vesicles under iron limitation

The dogma that bacteria induce virulence factors under iron limitation and the possibility to find them concentrated in outer-membrane vesicles, as described for other pathogens, led us to isolate *B. abortus* membrane vesicles from cells grown in iron rich (2xYT) and iron-depleted (2xYT treated with 150  $\mu$ M DIP) media at 37 °C for 1 week in a CO<sub>2</sub> incubator. Cells were collected in saline solution and heated at 60 °C for 30 minutes. Cells were discarded by centrifugation at 13,000 x g. Supernatants were centrifuged at 18,500 rpm for 30 min in the 70Ti Beckman-rotor. A second centrifugation of the supernatant was done at 43,500 rpm for 3 h in the same rotor for further purification. The pellet was suspended in PBS and tested under transmission-electron microscopy for the presence of pure vesicles. Samples of equal volume were subjected to electrophoresis in polyacrylamide gels and stained with Coomassie blue for protein detection. Proteins were detected only from cultures grown with iron limitation.

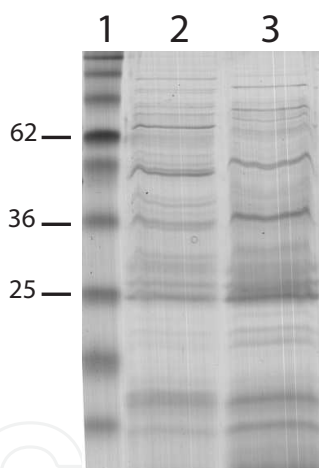


Fig. 3. Outer-membrane vesicles from *B. abortus* in response to iron limitation. 15% SDS-PAGE analysis of proteins obtained from outer-membrane vesicles preparations of *B. abortus* grown under iron limitation at 5 (line 2) and 15 days (line 3). Molecular mass standards (line1); kilodaltons are indicated on the left.

As shown in Figure 3, similar patterns were observed from cultures incubated between 5 and 15 days under the conditions mentioned before. Interestingly, it was also observed in samples prepared from *B. abortus irr* and *bfr* mutants.

The peptides obtained from the 36 KDa (adaivaxepeaveyv and ntvaednaxggiv) and the 25 KDa bands (enxgyv and adaivaqpateid) identified after trypsin digestion followed by HPLC-MS, led us to identify the porin Omp2b and OmpW as the major proteins expressed in *brucella* vesicles produced under iron limitation.

The bacterial liberation to the culture media of outer-membrane vesicles represents a phenomenon that has been described for many bacteria including *Brucella* (Gamazo et al., 1987; 1989). The origin of these bacterial structures and their function are still under investigation. So far, there is no genetic evidence supporting the idea that bacteria can regulate its production. In terms of function, it has been hypothesized that they can contribute to bacterial virulence by evading the immunological host-defense mechanisms and by redirecting or preserving the virulence factors accumulated in their lumen. In this regard, further studies are needed to understand whether the presence of these proteins in the *brucella* vesicles has a meaning in the virulence of this pathogen.

## 2.2 Intracellular growth

The growth- deficient phenotype of the *B. abortus bfr* observed under iron limitation *in vitro* was not reproduced when cells were growing intracellularly (Almiron & Ugalde, 2010). Nonetheless, we investigated whether siderophores were assisting *B. abortus bfr* for intracellular replication. The gentamicin protection assay was done in HeLa cells with the parental strain and the isogenic *bfr* and *bfr dhhC* mutants. Similar results were obtained for all strains (Figure 4) indicating that siderophores are not involved in *B. abortus bfr* intracellular survival.

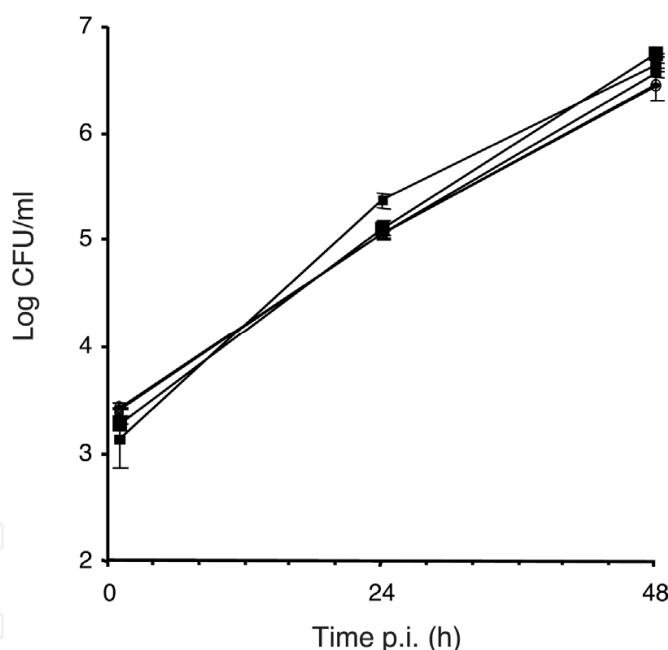


Fig. 4. Intracellular survival of *B. abortus* strains in HeLa cells. Monolayers of HeLa ( $10^5$  cells/well) were infected with wild-type 2308 (■), 2308 *bfr* (●), 2308 *dhhC* (Δ) and 2308 *bfr dhhC* (▲). At different times p.i. cells were lysed to determine the number of viable intracellular bacteria (CFU/ml). Data are expressed as means and standard deviations from one experiment performed in triplicate. They are representative of three independent experiments.

*B. abortus* mutants unable to produce 2,3-DHBA and brucebactin (*B. abortus dhhC* and *entF*) presented a wild-type behavior when infecting murine macrophages (Bellaire et al., 1999; Jain et al., 2011).

The role of *exbB*, *dstC* and *dugA* in the intracellular replication of *Brucella* were tested in bovine macrophages and in HeLa cells. None of these mutants showed attenuation in comparison to the wild type (Danese et al., 2004). These results are in agreement with those obtained with mutants that do not produce 2,3-DHBA.

A proteomic analysis of intracellular *B. abortus* recovered at different times after infection of murine macrophages revealed that proteins related to iron and haem transport were reduced during the first hours post-infection (p.i.) and interestingly, were induced after 20 or 44 h p.i (Lamontagne et al., 2009). These results suggest that once in the endoplasmic reticulum-related vacuole *Brucella* could get access to iron-sources facilitating the intracellular replication. In general, when *Brucella* infects a professional phagocytic cell-line, there is about one log reduction in the intracellular number of colony-forming units after 12 h p.i. Thus, it can not be discarded that the iron source encountered by *Brucella* latter in times have bacterial origin. The ferritins Bfr and Dps were reported to remain uninduced after 3 h of infection.

### 2.3 In animals

*B. abortus dhlbC* was as virulent as wild type in the murine model using Balb/C mice, though this strain showed attenuation in pregnant cattle (Bellaire et al., 1999; 2003b). The author attributed the difference to the presence of erythritol in the bovine placental trophoblast which is absent in mice.

The *B. melitensis exbB*, *dstC* and *dugA* mutants were also tested for virulence in Balb/C mice. According to the bacterial counts obtained from mouse spleens at 1 and 4 weeks p.i., no difference was observed between wild type and mutants (Danese et al., 2004).

According to these data, the iron acquisition systems do not seem to be involved in *Brucella* pathogenicity when tested in a mouse model. The significance in the natural host requires further experimentation.

### 3. Involvement of haem in the replication of *B. abortus*

Whilst pathogenic bacteria possess different mechanisms for extracellular haem incorporation, their own biosynthesis is considered to be the main haem source.

The haem biosynthetic pathway in alpha-proteobacteria is similar to that in animal and yeast mitochondria. It starts with the action of the ALA synthase and finishes with the ferrochelatase. The last step, corresponding to the incorporation of a ferrous iron into protoporphyrin IX, can be affected by the availability of intracellular iron, a condition usually faced by bacteria during infection. The oxidative characteristic of the chelated iron allows haem to participate as a cofactor in oxidative reactions. This type of reactions plays an important role in electron transfer for metabolism or energy generation (O'Brian & Thony-Meyer, 2002) and can also be employed in signal transduction systems. It has been described that haem participates in the sensing of diatomic gases and in the transcriptional and post-transcriptional regulation of several genes (Genco & Dixon, 2001).

Bacteria can produce cell lysis to gain access to the intracellular haem by secreting cellular proteases or haemolysins, or via complement. Also, bacteria can get extracellular haem through haemophores that will compete with haem-containing molecules for the haem

group. Once haem is accessible, it is transported across the outer membrane via a TonB-dependent process. Because haem is hydrophobic and tends to aggregate at physiological pH it needs a recognition molecule at the bacteria surface and carrier proteins to be translocated to the cytoplasm. Then, haem can be used as an iron source after the action of haemoxigenases or incorporated to proteins (Lee et al, 1995; Wandersman & Delepelaire, 2004).

The synthesis of haem in *B. abortus* was reported to be altered by mutations in the *hemH* as an indication that *Brucella* is able to synthesize its own haem (Almirón et al, 2001). The protophorphyrin accumulation together with the decreased number of  $\beta$ -galactosidase units obtained from a plasmid-borne *hemB-lacZ* fusion expressed in the *irr* mutant under iron limitation, in contrast to the wild type, suggest that Irr down-regulates the haem biosynthesis. This kind of Irr regulation at the level of *hemB* has been described in *Bradyrhizobium japonicum* and in *Rhizobium leguminosarum* (Hamza et al., 1998; Wexler et al., 2003). Thus, it can be concluded that *B. abortus* has a haem biosynthetic pathway regulated by Irr.

Additionally, *B. abortus* can acquire haem from the environment for metabolic use (Almirón et al, 2001). The BhuA was shown to be involved in the internalization of haem during the stationary phase of *B. abortus* growth, under iron limitation. This outer-membrane protein is an homologue of Ton-B dependent haem transporters already characterized for other Gram-negative bacteria such as *Shigella dysenteriae*, *Yersinia pestis*, and *Bradyrhizobium japonicum* (Paulley et al., 2007).

The genome of *Brucella* possesses sequences coding for putative haemolysins. However no expression of such DNA has been described yet.

### 3.1 Extracellular survival

The auxotrophy of the *B. abortus hemH* mutant was restored only by the addition of haemin indicating the presence of some mechanisms in *Brucella* that allow the internalization and utilization of exogenous hemin as described for other bacteria (Almiron et al., 2001). Iron salts or hemoproteins did not revert the auxothorophy.

As predicted, a *B. abortus bhuA* mutant cannot use haem as an iron source. When this mutant and the parental strain were cultured in the low iron medium MG, no differences were observed during the logarithmic and the stationary phases of growth. However, the death phase of the mutant culture started earlier than the wild type. Since the addition of external iron salts prevented the loss of viability, the authors have suggested that BhuA plays a role in stationary-phase iron acquisition in *B. abortus*. It is hard not to relate iron with haem. In that sense, another data interpretation might suggest that iron allowed the cells to resume haem biosynthesis or instead, no more haem was used as an iron source thus, preserving this molecule. Is it haem or iron what *Brucella* needs to survive extracellularly under iron limitation or stationary phase? The growth capability of *B. abortus hemH* was not restored by the addition of iron salts, even under iron-sufficient conditions. This indicates that haem is essential for *B. abortus* to live as a free microorganism.

The transcription of *bhuA* is under the positive regulation of Irr in *B. abortus* (Anderson et al., 2011). Although this regulation was observed during the stationary phase, it should be

considered that iron limitation occurs in *Brucella* when the internal iron content reaches a threshold, independently of the external iron concentration. This situation could be achieved by cells after several hours of incubation under iron-limited conditions. That is the stationary phase.

Interestingly, a mutation in the *B. abortus irr* has rendered an increase in haem content due to a derepression of the haem biosynthesis (Martinez et al., 2005; 2006). This phenotype assists *brucella* to survive under the oxidative stress produced by exposition to hydrogen peroxide. On the other hand, another mutation in the *B. abortus irr* prevented iron acquisition through siderophores, as previously reported, and haem internalization via the haem transporter BhuA (Anderson et al, 2011). Consequently, if a *Brucella irr* mutant is grown for several hours under iron limitation, it is expected that this mutant will not survive even if haem is added to the media. The iron depletion imposed does not support its own haem biosynthesis and the mutant is unable to internalize haem. But, if iron is added to the media, the *irr* mutant can resume the haem biosynthesis and survive. This hypothesis has been supported by the experimental data reported by Anderson. These data indicate that, besides the supply of iron that haem carries, haem itself is essential for *in vitro Brucella* growth.

### 3.2 Intracellular survival

*Irr* desregulation in *B. abortus* caused by a mutation in *irr* enables the microorganism with a better performance inside eukaryotic cells (Martinez et al., 2006). Thus, while wild type experienced a decrease in the number of intracellular bacteria during the first 24 h p.i. in HeLa or J774 cell lines, the *irr* mutant showed an increment in the bacterial count. Even though the invasion was not affected for the mutation, it is evident that it led *Brucella* to be better equipped than wild type for the survival strategy under iron limitation. Catalase as other haem-containing molecules can be considered as part of that equipment since the *irr* mutant showed higher intracellular haem content, catalase activity and resistance to hydrogen peroxide than the wild type. Paradoxically, the iron-acquisition system through siderophores was deficient in this mutant. This data is in agreement with those that indicate that siderophores are not involved in the intracellular replication of *Brucella*.

The *hemH* mutant was assayed for *in vitro* survival inside HeLa cells and J774 murine macrophages. This mutant was completely attenuated in both cell lines in comparison with the wild type. It was impaired in both invasion capability and intracellular survival. The mechanism that failed in this mutant remains unknown. Considering that haem is present in different kinds of molecules, it is possible to speculate that, with an outer-membrane haemoprotein deficiency, it could be involved in cell invasion. Once inside the cell, it is more likely that a synergistic effect from different haemoproteins leads to the unsuccessful survival.

Furthermore, the *B. abortus bhuA* mutant was attenuated in cultured murine macrophages compared to wild type (Paulley et al., 2007).

### 3.3 In animals

When Balb/C mice were infected with the *Brucella hemH* mutant it was interesting to note that as early as 2 weeks p.i. neither spleen nor liver colonization were observed in comparison with the wild type or the *hemH* mutant complemented with the wild-type *hemH*. In spite of this, the histological examination of the spleens revealed the same



granulomatous reaction in mice infected with all three strains at 2 and 4 weeks p.i. Hyperplasia occurred to a lesser degree in mice inoculated with the *hemH* mutant.

Interestingly, mutations in *B. abortus* genes that affect the haem acquisition system, like *bhuA* or *irr*, were shown to be attenuated in C57BL6 mice at 4 weeks p.i. (Anderson et al., 2011; Paulley et al., 2007). When a different *B. abortus irr* mutant was previously tested in Balb/C mice, at 7 and 21 days after inoculation, neither the increment in catalase activity and haem content, nor the decline in siderophores biosynthesis affected the wild type virulence (Martinez et al., 2006). Although a different mice strain was used, the attenuation in the *brucella* virulence observed after 4 weeks p.i. could be an indication that *Brucella* suffers from iron limitation after one month of infection. Most importantly, it can be a clear indication that cells have access to haem but not to free iron at this stage in the infected animal.

#### 4. Conclusion

It is as much impossible to dissociate free iron from haem as it is difficult to know whether bacteria incorporate haem in response to a real demand for this molecule or it just represents an iron source. Nonetheless, if we analyze the data presented here, there is a line of evidence that suggests the preferred value of haem over non-haem iron in the survival of *B. abortus* during its life cycle as a free organism or as an intracellular pathogen.

First of all, the transcriptional regulation as the protein stability of the major iron responsive regulator in *Brucella abortus* depends on haem. The *irr* gene is transcribed independently of the external iron concentration but it is autoregulated under low iron conditions. The Irr protein is able to bind haem in vitro and this situation probably contributes to the formation of dimers. Intracellularly, Irr is degraded when bacteria do not sense iron limitation. In this condition, bacteria are provided with both non-haem iron and haem.

In general, as it has been proved, the inability to acquire iron through siderophores does not alter the intracellular replication and the capacity to infect mice. On the contrary, the inability to synthesize its own haem or to acquire this molecule from the media affects both the *B. abortus* intracellular replication and the possibility to establish a normal infection in the mouse model.

#### 5. Perspectives

Future research in haem biosynthesis under conditions that result more representative of those faced by *brucellae* during infection might help to increase our knowledge about the survival and pathogenesis of *B. abortus*.

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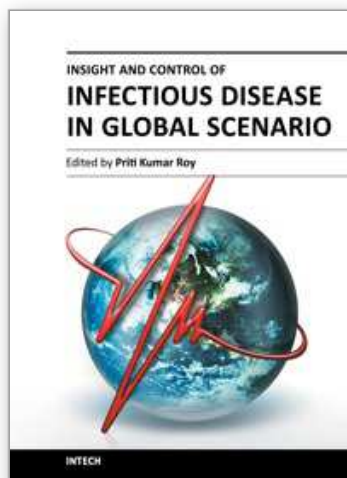
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