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Ferrochelatase Is Present in *Brucella abortus* and Is Critical for Its Intracellular Survival and Virulence

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***Brucella* spp. are pathogenic bacteria that cause brucellosis, an animal disease which can also affect humans. Although understanding the pathogenesis is important for the health of animals and humans, little is known about virulence factors associated with it. In order for chronic disease to be established, *Brucella* spp. have developed the ability to survive inside phagocytes by evading cell defenses. It hides inside vacuoles, where it then replicates, indicating that it has an active metabolism. The purpose of this work was to obtain better insight into the intracellular metabolism of *Brucella abortus*. During a *B. abortus* genomic sequencing project, a clone coding a putative gene homologous to *hemH* was identified and sequenced. The amino acid sequence revealed high homology to members of the ferrochelatase family. A knockout mutant displayed auxotrophy for hemin, defective intracellular survival inside J774 and HeLa cells, and lack of virulence in BALB/c mice. This phenotype was overcome by complementing the mutant strain with a plasmid harboring wild-type *hemH*. These data demonstrate that *B. abortus* synthesizes its own heme and also has the ability to use an external source of heme; however, inside cells, there is not enough available heme to support its intracellular metabolism. It is concluded that ferrochelatase is essential for the multiplication and intracellular survival of *B. abortus* and thus for the establishment of chronic disease as well.**

Brucella spp. are gram-negative rods classified in the alpha-2 subgroup of *Proteobacteria*. Six species have been identified (37). Cattle are the reservoir of *Brucella abortus*; humans can also be infected, but the disease, named brucellosis, is not transmitted between humans. After infection, *B. abortus* initially replicates in macrophages; then it reaches the reticuloendothelial system, the mammary glands, and the genital organs, causing infertility in males and abortions in pregnant females and thus disrupting animal reproduction (25).

The mechanisms used by *B. abortus* to cause disease are not yet clear. One of the major challenges to understanding the pathogenesis of *Brucella* is the absence of virulence factors, such as toxins, cytolytic enzymes, and fimbriae. It has been demonstrated that even though these bacteria are phagocytosed, they are able to replicate and survive inside host cells. This fact implies that *Brucella* can overcome intracellular bacteriolytic mechanisms and leads to the hypothesis that the main virulence factors are related to its ability to survive inside eukaryotic cells (25, 36). Recently, *B. abortus* was described as having a colinear arrangement of 13 open reading frames (ORFs) forming an operon which is highly homologous to *Agrobacterium tumefaciens virB* and which is required for intracellular multiplication and virulence (31). The role of this type IV secretion apparatus is not yet clear, but it is probably required for the secretion of proteins that control intracellular traffic in epithelial cells, since VirB mutants are unable to reach the rough endoplasmic reticulum, where *B. abortus* usually multiplies (5).

It is known that one of the conditions needed for intracellular bacteria to survive is their capability to obtain iron inside host cells (11, 26). Most of the iron in mammals is intracellular, mainly as part of the heme molecule. Many pathogenic bacteria express receptors for heme or hemoproteins in their membranes under iron-restricted conditions (6). The evidence for these mechanisms being activated during infection is related to the detection of antibodies against iron-regulated outer membrane proteins in patients infected with *Salmonella enterica* serovar Typhi (8).

In addition to providing a source of iron for bacterial growth, heme is synthesized by bacterial cells and participates as a cofactor of enzymes involved in oxygen transport, energy generation, oxidative reactions, and signal transduction (6, 17, 20).

Starting from δ-aminolevulinic acid, the heme metabolic pathways of eukaryotes and prokaryotes are similar (22). Ferrochelatase is the last enzyme in either pathway, and it introduces one iron molecule into the porphyrin ring. Mutations in the gene that encodes ferrochelatase produce different phenotypes in bacteria. For example, it has been reported that ferrochelatase is not essential in *Escherichia coli* (22) and does not affect the virulence of *Haemophilus influenzae* (30). However, its absence impairs the intracellular survival of *Neisseria gonorrhoeae* inside epithelial cells (35) and that of *Bradyrhizobium japonicum* in soybean nodules (10).

As a part of a *B. abortus* genomic sequencing project being performed in our laboratory (28), a putative ORF coding for ferrochelatase was detected. This discovery led us to identify the complete sequence of the *hemH* gene in *B. abortus* and to

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TABLE 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Genotypic and phenotypic description	Reference or source
Strains		
E. col K-12 DH5α-F′IQ	F′ Φ80 d*lacZ* ΔM15(*lacZYA-argF*)*U169 deoR recAI endAI hsdRI7* (r_k^- $m_k^{\pm}$) *phoA supE44* λ^- *thi-I gyrA96 relAI*/F′ *proAB*$^+$ *lacI*^{q}Z ΔM15*zzf*::Tn*5* (Km^r)	38
B. abortus 2308	Wild type, smooth, virulent, Nar^r	Laboratory stock
B. abortus 2308HM	2308 smooth, Nar^r, Km^r, mutant of *hemH*	This work
B. abortus 2308HMC	2308HM harboring plasmid pBBR*hemH*	This work
Plasmids		
pBBR1MCS-4	Broad-host-range cloning vector, Amp^r	16
pBIK9	1.7-kbp fragment with high homology to the *hemH* gene of *B. japonicum* cloned into pBluescript SK II	This work
pT*hemH*	1.3-kbp PCR product containing the *B. abortus* 2308 *hemH* gene cloned into pGEM-T	This work
pBBR*hemH*	1.3-kbp *Eco*RI fragment containing the *B. abortus* 2308 *hemH* gene cloned into pBBR1MCS-4	This work
pSbKm	pB1K9 with a 0.46-kbp *Bal*I deletion and containing a kanamycin resistance cassette	This work

study its implications during the intracellular life cycle of the bacteria. For these goals to be achieved, a knockout mutation in the *hemH* gene was produced in virulent *B. abortus* strain 2308. We characterized the phenotype of the mutant and tested its intracellular survival and multiplication in J774 and HeLa cells and its virulence in BALB/c mice. Our results indicate that ferrochelatase plays an important role in the intracellular survival and virulence of *B. abortus*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. All strains and plasmids used are listed in Table 1. Bacteria were grown in brucella broth (BB; Gibco, Paisley, Scotland) at 37°C in a rotary shaker at 200 rpm. When required, the medium was supplemented with hemin (40 μg/ml), hemoglobin (1 mg/ml), iron chloride (50 μM), iron citrate (50 μM), and fetal bovine serum (10%; Gibco). Hemin and hemoglobin stock solutions were freshly prepared by dissolving hemin in 0.1 N NaOH–50% ethanol and hemoglobin in phosphate-buffered saline (PBS) (pH 7.4). All the reagents were purchased from Sigma Chemical Co. unless otherwise stated.

E. coli DH5α competent cells were prepared using the calcium chloride method and were used for plasmid manipulation. Cells were incubated in Luria broth at 37°C with aeration. Transformants were selected on Luria broth plates supplemented with 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal)/ml and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). When necessary, ampicillin (100 μg/ml) and kanamycin (50 μg/ml) were added to the media.

Preparation of *Brucella* electrocompetent cells and DNA manipulation were performed according to described protocols (27).

Nucleotide sequencing. An insert of 1.7 kbp from plasmid pB1K9 was sequenced by using a BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems) and analyzed with an ABI Prism 377 sequencer (Applied Biosystems). T7 and T3 were used as primers for PCRs with pB1K9. An internal *Bal*I fragment (458 bp) was subcloned in pBluescript SK II (Stratagene, La Jolla, Calif.) and sequenced as described above. Additionally, primers UFQ (5′CAA CTTAATGCACCTCCTC3′) and LFQ (5′TACTGCCCTACTGCCTTTCATC T3′) were used to obtain the complete *hemH* gene from laboratory strain 2308 using the colony PCR method (27). The amplification products were cloned in the pGEM-T Easy vector (Promega) following manufacturer's instructions. The final plasmid was named pT*hemH* and was sequenced as described above using T7 and SP6 as primers. The following primers were needed to complete the sequencing in both directions: FbalU (5′TATCACAATCCGCTCGCA3′) and FbalD (5′TGCTCAATCCTGGTTTCGTGGCCGATT3′).

Construction of the mutant. A *Bal*I fragment (458 bp) located 385 bp downstream from the ATG start codon of *hemH* was digested from pB1K9. The deletion plasmid was ligated in the presence of a 1.3-kb *Hin*cII fragment containing a kanamycin resistance cassette (23). The recombinant plasmid was electroporated into 2308 cells. Since the plasmid cannot replicate in *Brucella*, we selected *Brucella* Km^r as a result of a double homologous recombination process. The mutants were recovered when plated on BB-kanamycin medium supplemented with hemin. Determination of sensitivity to ampicillin, PCR, and Southern blotting were done with the wild type and mutants to confirm that chromosomal *hemH* was replaced by *hemH* Δ*Bal*I::Km. The selected *hemH* mutant was named 2308HM.

Construction of plasmid pBBR*hemH* for complementation studies. As mentioned previously, wild-type *hemH* was amplified by colony PCR and cloned, giving rise to pT*hemH* (using primers UFQ and LFQ). In order to have the insert was in a stable plasmid for *Brucella*, it was liberated from pT*hemH* with endonuclease *Eco*RI and ligated into pBBR1MCS4 (16) linearized with the same enzyme, generating plasmid pBBR*hemH*. This plasmid was electroporated into *B. abortus* 2308HM. Km^r Amp^r bacteria were selected on BB-hemin plates and tested for growth without the addition of hemin.

In vitro infection assays. (i) Nonphagocytic cells. Infections were performed with 24-well plates (Falcon; Becton Dickinson, Meylan, France) as previously described (24, 31). Bacteria from overnight cultures grown on BB medium supplemented with hemin and the appropriate antibiotic were suspended in PBS. Cells were centrifuged, washed two times with PBS, and resuspended in culture medium (minimal essential medium supplemented with 5% fetal bovine serum and 2 mM glutamine) (Gibco) to a standardized optical density of about 10^7 bacteria/ml. One milliliter of this suspension was used to infect HeLa cells (10^5/well) at a multiplicity of infection (MOI) of 100. To accelerate the contact between bacteria and cells, the plates were centrifuged for 10 min at $180 \times g$ and room temperature and then incubated at 37°C in a 5% CO_2 atmosphere. After 1 h, nonadherent bacteria were eliminated by washing five times with PBS and then adding medium supplemented with 100 μg of gentamicin/ml and 50 μg of streptomycin/ml. At different times, the infected cells were washed three times with PBS and treated for 5 min with 1 ml of 0.1% Triton X-100 in deionized sterile water. Serial dilutions of these lysates were made in PBS and then plated on BB-hemin medium to determine the number of viable intracellular bacteria.

(ii) Phagocytic cells. The murine macrophage J774 cell line was used to test phagocytic cells. Cells at 10^5/well were infected with a bacterial suspension prepared as described above for HeLa cells except for the following changes: the culture medium was RPMI 1640 supplemented with 5% fetal bovine serum (Gibco), and the MOI was 50.

In vivo experimental infections. Eight-week-old female BALB/c mice were injected intraperitoneally (i.p.) with 0.1 ml of a bacterial suspension prepared in PBS (about 10^4 CFU). At 2 and 4 weeks postinfection (p.i.), mice were bled to death by cardiac puncture after receiving an excess of ether. The spleen and the left lobe of the liver were aseptically dissected. Samples of each organ were immediately fixed in 10% formaldehyde in PBS and then routinely processed for histologic analysis. The rest of the tissues were homogenized in PBS and weighed to determine the number of viable bacteria per gram of tissue using serial dilutions and plating on BB-hemin agar.

Nucleotide sequence accession number. The DNA sequences of the *B. abortus* *hemH* gene and those of the flanking regions were deposited in GenBank under accession number AY027659.

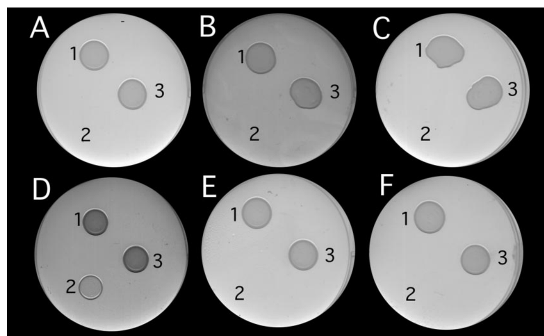

FIG. 1. Hemin auxotrophy of *B. abortus* 2308HM and genetic complementation of the phenotype by *hemH*. Five microliters from an overnight culture of 2308 (1), 2308HM (2), or 2308HMC (3) was spotted onto BB agar (A) or BB agar supplemented with hemoglobin (B), fetal bovine serum (C), hemin (D), iron citrate (E), or iron chloride (F). The six-well plate was incubated for 5 days at 37°C. The *hemH* mutant (spot 2) grew only in the presence of hemin and showed prototrophy when complemented with pBBR*hemH* (spot 3).

RESULTS

Homology of *B. abortus* HemH to ferrochelatases from plant and human pathogens. The nucleotide sequence of the putative ORF (1,059 bp) present in plasmid pB1K9 was obtained. It was located just downstream from the gene that codes for Omp10 (33). The predicted amino acid sequence (352 amino acids, 40 kDa) showed significant homology with those of previously described ferrochelatases of prokaryotes and eukaryotes when the Blast program was used (3). The best scores were obtained in comparisons with (database numbers in parentheses) *Mesorhizobium loti* ferrochelatase (AP003001) (70% identity), *Bradyrhizobium japonicum* ferrochelatase (P28602) (58% identity), and *Rhodobacter capsulatus* ferrochelatase (Q59735) (54% identity); these microorganisms belong to the same alpha subgroup of *Proteobacteria*. Significant identity (35 to 45%) was also observed with ferrochelatases from *Yersinia enterocolitica* (P43413), *Vibrio cholerae* (D82255), *E. coli* (P23871), *H. influenzae* (P43868), and *Neisseria meningitidis* (CAB84199). All of the proteins of the ferrochelatase family conserve residues needed for iron and the protoporphyrin IX ligand. The *B. abortus* putative *hemH* gene has these conserved ferrochelatase signature sequences (1, 12).

A *B. abortus hemH* Δ*Bal*I::Km strain displays hemin auxotrophy. A mutant was constructed by deleting a *Bal*I fragment (458 bp) in the intragenic region of putative *hemH* and replacing it with a kanamycin resistance cassette (1.3 kb) as described in Materials and Methods. The resulting Km^r and hemin auxotrophic mutant strain was named *B. abortus* 2308HM. Mapping of the chromosomal *hemH* Δ*Bal*I::Km mutation was confirmed by Southern blot and PCR techniques (data not shown).

B. abortus 2308HM (spot 2) was unable to grow on BB medium (Fig. 1A) unless exogenous hemin was added (Fig. 1D). We decided to test its ability to grow on medium supplemented with hemoglobin or bovine serum. The mutant was unable to utilize those potential sources of heme (Fig. 1B or C, respectively). Additionally, we tried incubating the mutant strain in BB medium supplemented with hemoglobin at 10 μg/ml to 5 mg/ml; after 5 days, we did not detect any bacterial growth, thus confirming our previous results. In order to rule out a lack of growth caused by insufficient iron concentrations, we repeated the experiment but included iron citrate (Fig. 1E) and iron chloride (Fig. 1F). While the growth of the wild-type parental strain was unaffected by these substrates, the mutant did not grow at all. The mutant could grow only on BB medium supplemented with hemin, a result which indicates that the only source of iron capable of reverting the auxotrophy is hemin, suggesting the presence of a hemin receptor.

Colonies of the mutant showed the characteristic brownish red color due to the accumulation of protoporphyrin IX, as described elsewhere (19). The colonies were smaller than those of the wild type, and this characteristic was not improved by incubation under less aerobic conditions (5% CO_2 atmosphere).

The mutant was also sensitive to phage Tsibili (2), indicating a smooth phenotype.

pBBR*hemH* confers hemin prototrophy to mutant cells. We cloned the wild-type *hemH* gene from *B. abortus* 2308 in plasmid pBBR1MCS4 by colony PCR and used it to transform mutant 2308HM. The resulting complemented strain, named *B. abortus* 2308HMC, grew well on BB plates without the addition of hemin (Fig. 1A to F, spot 3). Colony morphology was similar to that of the wild type, confirming that *hemH* is responsible for the auxotrophic phenotype.

***B. abortus hemH* mutants fail to survive inside macrophages and HeLa cells.** According to our results, the heme molecule is essential for *B. abortus* to live as a free microorganism. Therefore, we decided to test the role of heme during intracellular multiplication. To accomplish this goal, HeLa cells and J774 murine macrophages were infected as described in Materials and Methods with strain 2308, 2308HM, or 2308HMC. Figure 2A shows the growth curve for *B. abortus* 2308 inside HeLa cells. Even though similar inocula were used for the strains, we found a smaller number of viable 2308HM cells than of wild-type cells at 2 h p.i. At 24 h p.i., we observed an increase in the number of intracellular 2308HM; then the number slowly decreased with time. Complementation (2308HMC) restored a pattern of invasion and intracellular replication similar to that seen for the wild type.

When J774 murine macrophages were infected with similar inocula (Fig. 2B), no viable 2308HM cells were recovered as early as 1 h p.i. The experiment was repeated using 10 times the MOI; no viable 2308HM cells were observed at any time p.i., but 2308 cells showed an increase of 1 $\log_{10}$ unit. Again, complementation restored a pattern similar to that of the wild type.

We determined the viability of the three strains under the same experimental conditions but without eukaryotic cells to discard the chance of 2308HM dying prematurely. We counted the viable cells only during the first 2 h in consideration of the fact that *B. abortus* invades cells within 15 min p.i. (25). The results for all strains indicated no variations in the numbers of viable cells; counts were obtained using the plating method.

***B. abortus hemH* mutants are avirulent for BALB/c mice.** Three groups of 10 mice were inoculated i.p. with strain 2308, 2308HM, or 2308HMC. Results obtained at 2 and 4 weeks p.i. showed neither spleen nor liver colonization by 2308HM. After 2 weeks p.i., the sizes of the spleens obtained from mice infected with strain 2308 were significantly larger ($P < 0.05$) than those of the spleens obtained from animals infected with strain

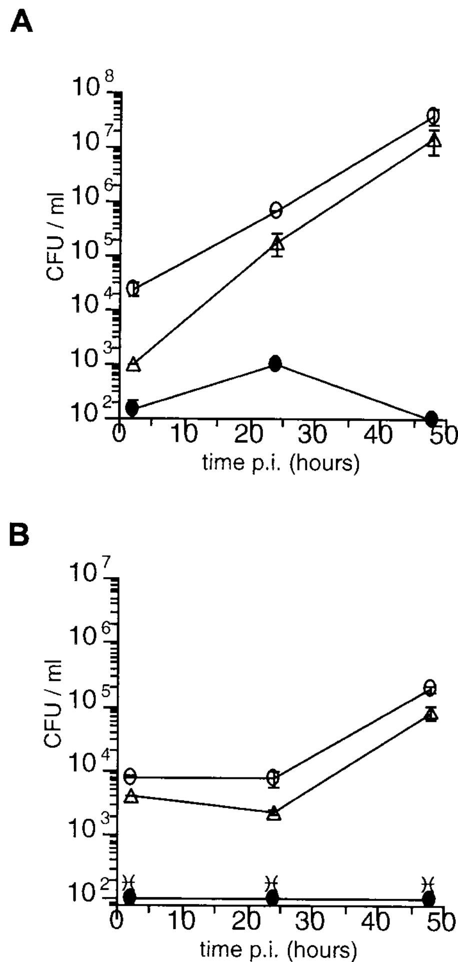

FIG. 2. Intracellular survival of *B. abortus* strains in nonprofessional (A) and professional (B) phagocytes. (A) HeLa cells (10^5 cells/well) were infected with *B. abortus* 2308 (open circles), 2308HM (closed circles), and 2308HMC (open triangles) as described in Materials and Methods. At different times p.i., the cells were lysed, and the numbers of viable intracellular bacteria (CFU/milliliter) were determined. (B) J774 cells were infected at an MOI of 50 with the same strains as those used in panel A as indicated in Materials and Methods.)(, below the experimental threshold of detection. Data represent means and standard deviations from one experiment performed in duplicate and are representative of four independent experiments.

2308HM: 188 ± 0.3 mg versus 90 ± 0.3 mg (mean and standard deviation). At 4 weeks p.i., we also observed significant differences in spleen sizes for mice infected with strains 2308 and 2308HM. A similar enlargement of spleens was observed in the group of mice infected with 2308HMC.

Histologic analysis revealed a granulomatous reaction mainly composed of macrophages and mononuclear cells in all of the experimental mice at 2 and 4 weeks p.i. (data not shown). As shown in Table 2, the numbers of viable bacteria recovered from the spleens of 2308- and 2308HMC-infected mice were similar at both times, while the numbers of viable cells recovered from the third group were below the threshold of experimental methods. Data, expressed as the number of viable cells per gram of tissue, were standardized for accuracy.

TABLE 2. Mouse spleen colonization by *B. abortus*[a]

Strain used to infect BALB/c mice	Log CFU/g of spleen at the following wk of infection:	
	2	4
2308	5.8 ± 0.4	5.8 ± 1.0
2308HM	<2[b]	<2[b]
2308HMC	5.5 ± 0.7	5.3 ± 1.0

[a] Three groups of mice were inoculated i.p. with 10^4 CFU of the strains indicated. At 2 and 4 weeks p.i., five mice per group were killed, and spleens were removed aseptically, weighed, and processed for histologic and bacteriologic analyses. Values are means and standard deviations from five spleens homogenized independently in PBS, diluted, and plated in duplicate to determine the CFU per gram of tissue.

[b] Significantly different ($P < 0.05$) from the wild type when Student's t test was applied.

Results similar to those described above were obtained when the left lobes of the livers were processed (data not shown).

DISCUSSION

Ferrochelatase (HemH) is the last enzyme involved in the biosynthetic pathway of heme. Ferrochelatases from eukaryotes and prokaryotes compose a large family of monomeric proteins that have molecular masses ranging from 36 to 40 kDa and that catalyze the incorporation of a ferrous ion into protoporphyrin IX (1, 12). The chelated iron of heme is capable of undergoing oxidative change, thus allowing heme, as a prosthetic group of enzymes, to participate in oxidative reactions.

In this study, we have identified and characterized *B. abortus hemH*, the first gene in this genus described as being involved in the metabolic pathway for heme biosynthesis. The predicted 40-kDa protein encoded by this gene shows high homology to other ferrochelatases, especially those of closely related organisms, such as *M. loti*, *B. japonicum*, and *R. capsulatus* (10, 14), and conserves the 21 residues which are important to the catalytic activity of the enzyme (12).

We constructed a chromosomal mutation in *hemH* by deleting a fragment of the coding region and replacing it with a kanamycin resistance cassette in virulent *B. abortus* strain 2308. The *hemH* mutant (2308HM) displayed hemin auxotrophy. In agreement with other bacterial *hemH* mutants, the colonies were brownish red and comparatively smaller than wild-type colonies (10, 21, 35). The growth capability of 2308HM was restored by the addition of hemin but not by hemoglobin or iron salts. These results suggest the existence of some mechanisms in *B. abortus* that allow the internalization and utilization of exogenous hemin, as described for other bacteria (6, 18, 34). Moreover, these results indicate that, besides the supply of iron that heme carries, heme itself is essential for in vitro growth. The *hemH* gene, which was cloned in a plasmid, was sufficient to complement the auxotrophy, indicating that the mutation in *hemH* did not affect any adjacent genes and indicating that the *B. abortus* wild-type gene codes for an active ferrochelatase.

Auxotrophic mutations in pathogens are explored as candidates for a live vaccine because of their known attenuation. In the genus *Brucella*, two auxotrophic mutants were described previously. One was a *Brucella melitensis purE* mutant (7), and

the second was an *aroC* mutant of *Brucella suis* (9). As far as we know, this is the first reported mutant of this kind in *B. abortus*. The attenuation of the *B. abortus hemH* mutant was confirmed by the infection of professional and nonprofessional phagocytic cell lines. Our results clearly show that *B. abortus* 2308HM is unable to survive inside murine J774 macrophages or in human HeLa cells.

Infections were performed with similar inocula of the three strains. The intracellular behavior of mutant 2308HM in HeLa cells showed some unexpected features: a reduced number of viable bacteria at 1 h p.i.; an increase in the number of intracellular bacteria, of almost 1 log unit, at 24 h. p.i.; and the loss of viability at later times. Interestingly, no viable intracellular 2308HM bacteria were recovered from infected macrophages at any time p.i. These results cannot be explained in terms of auxotrophy for the following reasons: 2308HM in eukaryotic-free culture medium, without the addition of hemin, does not show a reduction in viable counts by the first point examined (1 h p.i.), and live or dead *B. abortus* bacteria are phagocytosed by murine macrophages in a few minutes (4). Thus, one possible explanation is that 2308HM has some invasion deficiency due to the lack of the ferrochelatase. It has been reported that the invasive capability of *B. abortus* in macrophages and HeLa cells is affected by mutations that alter the outer membrane, such as those produced in *bvrR* and *bvrS* genes (32). Ferrochelatase does not localize in the outer membrane and is involved only in bacterial metabolism (13). Hence, it is doubtful that 2308HM does not enter into eukaryotic cells due to some invasion deficiency. Furthermore, this mutant has smooth lipopolysaccharide. It is more likely that the invasion of eukaryotic cells was not affected but that the mutant was more sensitive to bactericidal intracellular conditions. In this regard, it should be noted that professional phagocytes are capable of eliminating bacteria more efficiently than nonprofessional phagocytes. This hypothesis is further supported by observations made with *N. gonorrhoeae*, which is also a gram-negative bacterium that replicates inside epithelial cells. It was demonstrated that wild-type and *hemH Neisseria* strains have equal capabilities regarding attachment to and invasion of eukaryotic cells but that the mutant cannot survive in the cells (35).

Many different complex mechanisms that are involved in bacterial pathogenesis in vivo entail both bacteria and hosts. Most of these mechanisms are not clearly understood. In this work, we demonstrate that the lack of ferrochelatase abolished *B. abortus* 2308 virulence in mice. No spleen colonization was observed in 2308HM-infected mice after 2 weeks. It is possible that the bacteria were cleared from the spleens before that time. Splenomegaly occurred only in mice infected with 2308 and 2308HMC and did not occur in the 2308HM group, indicating a good correlation between splenomegaly and bacterial colonization. The same basic lesion was observed with all three strains at histologic examination: a granuloma composed of macrophages surrounded by lymphoid cells with some neutrophils. This lesion was more evident at 2 weeks p.i. At 4 weeks p.i., the granulomatous reaction was diminished in mice infected with 2308HM compared to those infected with 2308. The presence of a granulomatous reaction at 2 weeks p.i., when the spleen does not contain any viable bacteria, can be justified because it is not necessary for bacteria to be alive in order to elicit this type of reaction. A granuloma can appear because of the presence of intramacrophage undigested bacteria and/or the stimulation of macrophages by the activation of T lymphocytes. The different spleen sizes for 2308- and 2308HM-infected mice can be explained not only by the different kinetics of the granulomatous reactions but also by the remarkable hyperplasia of the white pulp observed in 2308-infected animals. The hyperplasia was present to a lesser degree in mice inoculated with 2308HM.

The remarkable attenuation observed for 2308HM may be the result of a failure in one or more mechanisms used by virulent *Brucella* to succeed in intracellular survival. In this regard, many speculations can be made considering the kinds of molecules where heme is present, e.g., catalase and cytochromes *b* and *c*. It has been reported that mutations that rendered *B. abortus* deficient in catalase or in cytochromes did not strongly alter the intracellular survival of the bacteria (15, 29). Thus, it is possible that their attenuation in the *hemH* mutant was synergistic. It has also been reported that heme participates in the sensing domain of a *Rhizobium meliloti* histidine kinase (20) and in a *Rhodospirillum rubrum* transcriptional activator (17). Thus, we can also consider inefficient signal transduction misdirecting the *hemH* mutant into the wrong intracellular pathway. Recently, a type IV secretion system encoded by the *virB* operon in *B. abortus* was found to be required for intracellular trafficking in HeLa cells. Active VirB is essential in preventing the fusion of phagosomes with lysosomes so that bacteria can reach the rough endoplasmic reticulum, where multiplication takes place (5).

In conclusion, we have demonstrated that an inability of *B. abortus* to synthesize heme is detrimental to its intracellular survival and to the establishment of infection in mice.

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