An Atypical Riboflavin Pathway Is Essential for *Brucella abortus* Virulence

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Abstract

Brucellosis is a worldwide zoonosis that affects livestock and humans and is caused by closely related *Brucella* spp., which are adapted to intracellular life within cells of a large variety of mammals. *Brucella* can be considered a furtive pathogen that infects professional and non-professional phagocytes. In these cells *Brucella* survives in a replicative niche, which is characterized for having a very low oxygen tension and being deprived from nutrients such as amino acids and vitamins. Among these vitamins, we have focused on riboflavin (vitamin B2). Flavin metabolism has been barely implicated in bacterial virulence. We have recently described that *Brucella* and other Rhizobiales bear an atypical riboflavin metabolic pathway. In the present work we analyze the role of the flavin metabolism on *Brucella* virulence. Mutants on the two lumazine synthases (LS) isoenzymes RibH1 and RibH2 and a double RibH mutant were generated. These mutants and different complemented strains were tested for viability and virulence in cells and in mice. In this fashion we have established that at least one LS must be present for *B. abortus* survival and that RibH2 and not RibH1 is essential for intracellular survival due to its LS activity in vivo. In summary, we show that riboflavin biosynthesis is essential for *Brucella* survival inside cells or in mice. These results highlight the potential use of flavin biosynthetic pathway enzymes as targets for the chemotherapy of brucellosis.

Introduction

Brucellosis is a worldwide zoonosis that affects livestock and humans and is caused by closely related *Brucella* spp., which are adapted to intracellular life within cells of a large variety of mammals. The most pathogenic species for humans are *B. melitensis*, *B. suis* and *B. abortus* whose preferred host are goats, pigs and cattle respectively. Transmission to humans occurs mainly through the consumption of contaminated unpasteurized dairy products and through direct contact with infected animals. An estimated of 500,000 human infections per year still occur worldwide [1].

*Brucella* can be considered a furtive pathogen that infects professional and non-professional phagocytes. No classical virulence factors, such as exotoxins, cytolyisins, capsules, fimbria, plasmids, lysogenic phages, resistant forms, antigenic variation, endotoxic lipopolysaccharide or apoptotic inducers have been described in *Brucella* spp. so far [2]. Progress in understanding the molecular pathogenesis of the disease, vaccine engineering and postgenomic approaches aimed at the discovery of new pathways used by this pathogen to modify the intracellular environment may lead to new preventive interventions.

Once internalized, *Brucella* resides within a membrane-bound compartment, the *Brucella*-containing vacuole (BCV), which sequentially interacts and fuses with early endosomes, lysosomes and further traffics to the endoplasmic reticulum (ER) to generate an ER-derived replicative niche [3,4,5]. After 12–24 h after cell entry *Brucella* extensively replicates without restricting basic cellular functions or inducing obvious damage to cells [6]. The replicative niche is characterized for having a very low oxygen tension and being deprived in nutrients such as amino acids and vitamins [2]. The true virulence factors of *Brucella* are a complex array of molecular determinants that confer the pathogen the metabolic ability to thrive in the harsh intracellular conditions allowing it to invade, resist intracellular killing, build the replicative niche and replicate.

Although riboflavin (vitamin B2) is the precursor of the essential flavin coenzymes FMN and FAD which participate in a myriad of biochemical reactions, flavin metabolism has been barely implicated in bacterial virulence. Flavoenzymes are involved in dozens of crucial cellular processes such as energy metabolism, RedOx reactions, detoxification and biosynthesis [7]. For example, FMN is required as an electron acceptor for dehydrogenases in the respiratory chain, and its deficiency induces increased levels of...
Cytochrome bd, a terminal oxidase known to work in microaerophilic conditions which is also present in Brucella [8]. Thus, it is likely that flavin metabolism would be related to pathogenesis since Brucella needs to survive to oxidative stress, microaerobic conditions and nutrient starvation and flavoenzymes or flavins themselves may be playing a key role in those processes.

The metabolic pathway of riboflavin in bacteria, plants and fungi has been described in some detail [9,10]. Briefly, the biosynthesis of one riboflavin molecule requires one molecule of GTP and two molecules of ribulose-5-phosphate. The imidazole ring of GTP is hydrolytically opened, yielding a 4,5-diaminopyrimidine that is converted to 5-amino-6-ribitylaminouracil by a sequence of deamination, side chain reduction, and dephosphorylation. Condensation of 5-amino-6-ribitylaminouracil with 3,4-dihydroxy-2-butane-4-phosphate obtained from ribulose-5-phosphate affords 6,7-dimethyl-8-ribityllumazine (lumazine). Dismutation of the lumazine derivative yields riboflavin and 5-amino-6-ribitylaminouracil, which is recycled in the biosynthetic pathway (see Figure S1). Animals incorporate riboflavin as a micronutrient from their diet or throughout saprophyte bacteria to synthesize riboflavin-derived cofactors flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD).

We have recently described that Brucella and other Rhizobiales bear an atypical riboflavin metabolic pathway [10,11]. The enzyme 6,7-dimethyl-8-ribityllumazine synthase (lumazine synthase, LS) catalyzes the penultimate step in the biosynthesis of riboflavin (see Figure 1). A phylogenetic analysis on eubacterial, fungal and plant LSs allowed us to classify them into two categories: Type-I LSs (pentameric or icosahedral) and Type-II LSs (decameric). Brucella codes both a Type-I and a Type-II LS called RibH1 and RibH2 respectively. Both enzymes show low catalytic activity in vitro, suggesting that natural selection operating over the riboflavin pathway favored the evolution of catalysts with low reaction rates. The rationale behind this is that the excess of flavins in the intracellular pool in Brucella could act as a negative factor when these bacteria are exposed to oxidative or nitrosative stress [12].

In the present work we analyze the role of flavin metabolism on B. abortus intracellular survival, focusing at its two LS isoenzymes. Mutants on the ribH1, ribH2 genes and a double ribH mutant were generated. These mutants and different complemented strains were tested for viability on cells and mice. In this fashion we have established that at least one LS must be present for B. abortus viability and that RibH2 and not RibH1 is necessary for Brucella intracellular replication due to its LS activity in vivo. In summary, we show that flavin biosynthesis is essential for Brucella survival inside cells and mice.

Results

Brucella Needs at Least One ribH Gene to Survive

In order to determine the role of the LS genes, simple and double mutants for the ribH genes were generated. The ribH1 (BAB1_0791) mutant was made by clean non-polar deletion because this gene is believed to be located in an operon (rib operon). On the other hand, ribH2 (BAB2_0545) is an isolated ORF, thus the ribH2 mutant was generated by disrupting the ribH2 gene with an antibiotic resistance cassette (see Material and Methods). Both simple mutants were obtained without any major difficulty and were confirmed by Western blot analysis (Figure 2A, lanes 1–3). They are not auxotrophic for riboflavin and grow at wild-type rates in both rich (TSB) and minimal media (Gerhardt-Wilson) (data not shown). We then tried to obtain the double ribH1 mutant by disrupting ribH2 in a ribH1 mutant genetic context. All attempts were unsuccessful, suggesting that the double mutation might be lethal. To overcome this difficulty we changed our strategy and the ribH1 mutant was complemented with a plasmid harboring ribH1 under a constitutive promoter before ribH2 mutagenesis (see materials and methods for details). In this way a chromosomal double ribH mutant (ribH1-ribH2) was obtained but at the expense of keeping a rescue copy of ribH1 in an expression plasmid (Figure 2A, lanes 4 and 6). These results indicate that the double mutant ribH1-ribH2 is not viable probably because it lacks a complete flavin biosynthesis pathway and that both genes encode LS activity but only one ribH gene would be sufficient for the bacteria to survive.

RibH1 and RibH2 Have LS Enzymatic Activity In Vivo

Given the above described mutagenesis studies and the fact that both RibH1 and RibH2 exhibit poor in vitro activity as LSs [11,13], we decided to assess if they exhibit this enzymatic activity in vivo. To address this issue we designed a plasmid swap experiment assuming that the pribH1Km plasmid that is harbored by the ribH1-ribH2 strain is essential for the growth in TSB medium (poor in riboflavin) (Figure 2A). Thus we tested if the pribH1Km plasmid could be cured in the presence of another plasmid from the same incompatibility group harboring different inserts but with an ampicillin resistance plasmid (pB8R4) (see Figure 3A for details). Theoretically, only plasmids that encode active LSs would be able to replace the original pribH1Km plasmid.
after several generations. This is because there is a metabolic selective pressure for keeping LS enzymatic activity when bacteria are grown in riboflavin poor media. Figure 3Bi shows that RibH1 and RibH2 share the same enzymatic activity since the ribH1 gene could be exchanged with ribH2. RibH1 also could be replaced by the heterologous and characterized LS from Saccharomyces cerevisiae (LS_sce) [14] confirming that RibH1 and RibH2 are both active LSs in vivo (Figure 3Bi). As expected, pribH1_Km cannot be cured in the presence of an empty plasmid or with pribH2_W22A (Figure 3Bi). RibH2_W22A contains a single amino acid mutation located in the active site of the endogenous RibH2. This mutant lacks enzymatic activity but its stability and structure are unaltered (unpublished results). Thus, these results confirm that Brucella needs at least one active LS to survive in riboflavin poor media. In agreement with this conclusion, the metabolic pressure to keep RibH1 can be overcome when very high levels of riboflavin (500 μM) are added to the media. Under these conditions, both the empty plasmid and the ribH2_W22A construct were able to replace RibH1 in the ribH1-ribH2 mutant strain (Figure 3Bi). The same clones did not survive when plated in TSBl-Amp media without the addition of riboflavin (Figure S2). Overall, these experiments demonstrate that the two ribH genes of B. abortus code for LS isoenzymes.

RibH2 Activity Is Essential for B. abortus Intracellular Replication

Once established that both RibH1 and RibH2 can function as active LSs in vivo, we decided to evaluate whether they play a role in virulence. For this purpose, we analyzed the intracellular replication of single and double RibH mutants in a macrophagic cell line and the persistence of these mutants in mice.

In J774 cells the wild-type strain showed a classical infection profile, with a marked decrease in viability between 0 and 4 h post-infection (p.i.) (Figure 4). This reflects cell entry and death by oxidative burst and fusion of the BCVs to lysosomes followed by a recovery phase where replication takes place after approximately 12 h p.i. (Figure 4). The ribH1 strain showed a similar behavior to that of the wild-type strain. In contrast, ribH2 showed a marked decrease in CFU counts at all time-points, suggesting that RibH2 is an important factor for intracellular life style. Complementation of ribH2 by ectopic expression of RibH2 in a plasmid recovers this attenuated phenotype: this strain exhibited an increased survival at early times (1 and 4 h p.i.) and a similar curve to that of the wild-type strain at later times. These results are indicative that RibH2 is more important in the intracellular phase than RibH1. In spite of the fact that the double ribH mutant is unable to synthesize flavins, its intracellular flavin pool content is enriched when it is grown in the presence of 500 μM riboflavin (as analyzed by thin layer chromatography, results not shown). In clear agreement, the double ribH mutant exhibited an increased survival at early time points, but is unable to replicate at later times p.i. (Figure 4). This is indicative that flavin biosynthesis is essential for metabolic adaptation to the replicative niche.

To further characterize the mechanisms by which RibH2 acts in intracellular survival, we analyzed the biogenesis of ribH2 BCVs in HeLa cells by scoring the recruitment kinetics of the late endosome/lysosome glycoprotein LAMP-1 and the lysosomal luminal hydrolase cathepsin D. As previously described, LAMP-1 is rapidly acquired and then gradually excluded from wild-type BCVs [3]. Accordingly, at 24 h p.i. the wild-type BCVs were able to promote the maturation of replicative organelles and proliferated in LAMP-1 negative compartments. In contrast, the ribH2 BCVs acquired this glycoprotein with similar kinetics but 40,3%±5,0 of BCVs retained LAMP-1 at 24 h, suggesting that this mutant fails to control vacuole maturation in HeLa cells (Figure 5A, 5C). As for cathepsin D, the percentage of ribH2 positive BCVs vacuoles increased from 2 to 6 h p.i. Additionally, at 24 h p.i. almost 50% of the phagosomes were positive for cathepsin D, whereas most of the wild-type BCVs excluded this molecule at the time points analyzed (Figure 5B). These results are consistent with bacterial survival at 4 and 48 h p.i. in HeLa cells as assessed by CFU counting (Figure S3). However, the number of ribH2 CFUs retrieved at 48 h p.i. indicates that although the ribH2 mutant is less efficient to promote the BCV maturation in comparison to the wild-type strain, it is capable of further survival and replication.

The ribH mutants were also tested in mice infection. In agreement with cell experiments, ribH1 behaved exactly as the wild-type strain (Figure 6A) showing the expected peak in CFU per spleen count at 14 days p.i. corresponding to the acute phase.
Although we have not tested the effect at longer times, even at 60 days p.i. ribH1 had no differences compared to the wild-type strain. In contrast, the ribH2 strain showed a significant attenuation at all times tested with a spleen bacterial count 100-fold lower compared to the wild-type at 10 days p.i. and an even larger drop at 40 days p.i. (1000-fold, Figure 6B). The complemented ribH2 + pribH2_W22A strain evidenced a larger bacterial count than the wild-type strain at 14 days p.i, demonstrating that RibH2 is required for survival in mice. The double mutant lacking both ribH genes was completely cleared by infected mice by day 14 p.i., indicating again that the biosynthesis of flavins is essential for survival (Figure 6B).

Discussion

It is very difficult to characterize the main activities that support Brucella virulence by means of classical biochemical or microbiological studies. The main reason for this difficulty is the fact that Brucella behaves very differently in culture than inside cells or animal hosts. For these reasons, several genomic or proteomic approaches have been used to detect the genes that are important for virulence and pathogenesis. In some of these studies, ribH2 has been identified as a differentially expressed gene. Al Dahouk et al. have detected that RibH2, among other proteins, is present in higher concentrations in intramacrophagic Brucellae than in culture [15]. Also, Lamontagne et al. made a comparative protein expression analysis between the attenuated S19 and the infectious Brucella 2308 strains where RibH2 was again identified to be augmented in the virulent strain [16]. In fact, RibH2 was originally characterized as a serological marker of active infection of both human and bovine brucellosis, implying that this protein is highly expressed during the infectious process [17]. Remarkably, the LS function is encoded by two different genes in Brucella, with ribH1 located within the rib operon in chromosome I, and ribH2 located in chromosome II (Table 1). Chromosome I is thought to code mainly housekeeping functions and chromosome II is regarded to harbor virulence-related functions that are differentially expressed during adaptation to the replicative niche inside cells [18]. Also, RibH2 has a very unusual regulatory element, the RFN element, which is supposedly a riboswitch found by...

Figure 3. Both RibH1 and RibH2 have LS activity in vivo. (A) A schematic draft of the plasmid swap experiment is depicted. Different pBBR4 (Amp) constructs were introduced by conjugation into the ribH1-ribH2+pribH1Km strain (X: ribH1, ribH2, LS_sce, ribH2_W22A or an empty plasmid). Exconjugants were selected by Amp and Km resistance. Then, three independent clones from each conjugation were cultured separately in TSB with Amp for more than 15 generations. One hundred Amp’ clones were then replicated in Amp and Km plates and the percentage of Amp’/Km’ was determined. In the case when empty pBBR4 or pribH2_W22A were used, riboflavin (Ribo) was added or not to the TSB and TSA to demonstrate that the presence of the final metabolite of the pathway could lead to the complete loss of LS activity in the cells. (B) The replica plates showed that more than 90% of the clones were Amp’/Km’ after the plasmid swap experiment when ribH1, ribH2 or LS_sce inserts were tested (panel i). In the case of the ribH2_W22A insert or the empty plasmid (panel ii) less than 5% Amp’/Km’ clones were obtained. When riboflavin was added during the plasmid swap experiment more than 90% of the clones tested were Amp’/Km’ when pribH2_W22A or pBBR4 (empty) were used to replace pribH1Km (panel iii). doi:10.1371/journal.pone.0009435.g003
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Figure 4. RibH2 is involved in survival and replication in J774A.1 macrophages. Macrophages infected with wild-type B. abortus, ribH1, ribH2, ribH1-ribH2 mutants and the ribH2 complemented strain (ribH2+ribH2) were lysed and intracellular CFUs per ml quantified at different times after inoculation. Data shown (mean ± standard error of the mean) are representative of three independent experiments performed.

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bioinformatic methods [19] that down-regulates the translation of the protein by sensing the FMN concentration [20,21]. Thus, the presence of this regulatory element added to the experimental evidence shown in this and other works are indicative that RibH2 harbors an important role in Brucella virulence.

Although Brucella RibH1 and RibH2 share only 21% of protein sequence identity, their monomers are structurally very similar, with a very low root mean square deviation (R.M.S.D.) of 1.50 Å when 128 Cα are aligned from a total of 157–158 Cα. We have previously speculated that one plausible explanation for the important role of RibH2 in Brucella virulence is that this new type of decameric LS could harbor a novel yet unknown function [22]. To address this hypothesis we complemented the ribH2 attenuated strain with different constructs (Figure 7). When ribH2 is complemented with an active LS (endogenous RibH2 or the heterologous LS from yeast), the bacteria not only recover their virulence but also acquire an increased capacity to survive inside macrophages. In clear contrast, the complementation with the point-mutated RibH2_W22A does not recover the attenuated phenotype. Thus, it is unlikely that RibH2 is exerting a function different than LS.

Although the expression of two very similar LSs with the same function in Brucella is an intriguing observation, our working hypothesis is that the two ribH genes may be differentially expressed during the Brucella infection cycle. Brucella would use RibH1 for flavin biosynthesis during the extracellular phase and RibH2 during intracellular growth. In any case, the role of RibH2 points out to the importance of the biosynthetic pathway of flavins in virulence. We speculate that the LS activity could be a key point for the regulation of this pathway. In order to corroborate this hypothesis we are currently studying the regulation of ribH genes under different conditions.

As clearly shown in Figure 3, the ribH1-ribH2 mutant is only viable at very high concentrations of exogenous flavins. This result supports previous bioinformatic analyses that indicate that Brucella lacks riboflavin transport activities. We infected cells with the ribH1-ribH2 strain previously grown at high levels of riboflavin (Figure 4). Strikingly, these bacteria show an increased capacity to survive inside macrophages within the first stages of infection (supposedly because of their elevated intracellular flavin pool) but then they show an exponential drop in intracellular CFU counts until they are completely eliminated from the cells. This result may indicate that during the infection there is a high demand for flavins and without endogenous biosynthesis the internal pool is depleted within a few hours. Flavins would be essential for Brucella lifestyle inside the replicative niche where bacteria are isolated and need to survive to starvation and microaerobic respiration. Because there are no LS enzymes present in animals, Brucella LSs constitute very interesting targets for chemotherapy. LS inhibitors could lead to bacterial riboflavin auxotrophy or to attenuated bacterial virulence without endangering the mammalian host.

Two recent discoveries involving flavins in virulence and symbiosis in Rhizobiales have raised particular interest: Taga et al. demonstrated that in the symbiont Sinorhizobium meliloti the last step of biosynthesis of vitamin B12 is based on the fragmentation of FMN through the flavin deconstruct BfuB, and Swartz et al. demonstrated that B. abortus contains a light-activated histidine kinase that binds a flavin chromophore and undergoes photochemistry indicative of cytoeineyl-flavin adduct formation. This latter protein (BA-LOV-HK) appears to function as a photoreceptor that is directly related to Brucella survival and replication within macrophages [23,24]. Thus, the role of flavin metabolism in Brucella pathogenesis is a question of biological relevance and appears not to be limited to the genus Brucella. We have performed bioinformatic and biochemical analyses in other bacteria [11,22].

Many plant symbionts like Rhizobium, Sinorhizobium, Mesorhizobium and Bradyrhizobium spp. and some pathogens like Ochrobactrum anthropi, Pseudomonas syringae pv. tomato and Roseobacter denitrificans also present two ribH genes in their genomes exhibiting one Type-I and one Type-II LS. They are located in the respective genomes in the same fashion as in Brucella, meaning that ribH1 is coded within the rib operon while ribH2 is located as an isolated ORF with the regulatory RFN element upstream. A Type-I LS-ribH gene duplication might have occurred in a bacterial ancestor where one of these genes evolved to a Type-II LS, providing positive fitness for symbiosis or pathogenesis. Vertical and horizontal gene transfer could be responsible for the distribution of the ribH genes through the different taxa where two different types of LS are found.

Two mammal pathogens, Rhodococcus equi and Actinobacillus pleuropneumoniae, become avirulent when their riboflavin operons are disrupted [25,26], and therefore these mutants seem to be good candidates for live attenuated vaccines [27,28]. For these reasons the ribH1-ribH2 strain generated in this work could also be a very good candidate for developing a Brucella vaccine because it can not synthesize riboflavin and it is completely attenuated in cells and in mice. This strain also lacks the serological marker RibH2 which could eventually be used to discriminate between vaccinated and infected animals [29,30]. In summary, in this work we show that flavin biosynthesis is essential for Brucella survival inside cells and that the LSs constitute attractive targets for chemotherapy and vaccine design.

Materials and Methods

Bacterial Strains

All live Brucella strains have been manipulated in a BSL-3 facility at UNSAM University. Bacterial strains, plasmids and oligonucleotides used in this study are listed in Tables 2, 3 and 4. B. abortus strains were grown in Tryptic Soy Broth (TSB, BD), Tryptic Soy Agar (TSA, BD) or Gerhardt-Wilson minimal medium at 37°C on a rotary shaker (250 rpm) or stove for plates [31]. When necessary,
the following antibiotics were added to the indicated final concentrations: kanamycin (Km), 50 \( \mu \)g/ml; gentamicin (Gm), 3 \( \mu \)g/ml; carbenicillin (Cb), 50 \( \mu \)g/ml; nalidixic acid (Nal), 5 \( \mu \)g/ml; and ampicillin (Amp), 50 \( \mu \)g/ml. 

*Escherichia coli* strains were grown at 37\( ^\circ \)C on a rotary shaker (250 rpm) in Luria-Bertani broth. For *E. coli* strains antibiotics were used at the following concentrations: Km, 50 \( \mu \)g/ml; Gm, 20 \( \mu \)g/ml; Amp, 100 \( \mu \)g/ml and streptomycin (Str), 25 \( \mu \)g/ml.

Construction of the *B. abortus* Mutants

**ribH1 mutant.** For the construction of the ORF BAB1_0791 (ribH1) unmarked chromosomal mutant two PCR fragments were generated from regions flanking this ORF. Oligonucleotides ribH1_F1 and ribH1_R1 were used to amplify a 0.3 Kbp fragment including codons 1–7 and oligonucleotides ribH1_F2 and ribH1_R2 were used to amplify a 0.3 Kbp fragment including 150–157 codons of ribH1. Both fragments (containing complementary regions) were ligated by overlapping PCR using oligonucleotides ribH1_F1 and ribH1_R2. The resulting fragment containing the ribH1 deletion allele was cloned into pBluescript-KSII (Stratagene) in which the *Bacillus subtilis* sacB gene encoding levansucrase (that induces lethality upon exposure to 5% (w/v) sucrose in the growth medium) had been cloned in the PsiI site to generate pBlueKSIIsacB\( \Delta \)ribH1. This plasmid was introduced in *B. abortus* 2308 by electroporation. The first recombination event (integration of the suicide vector in the chromosome) was selected by Cb resistance and 10% (w/v) sucrose sensitivity in TSA plates. Cells were grown overnight in TSB in the absence of antibiotic selection to promote recombination. The second recombination event (excision of the plasmid and generation of the mutant strain by allelic exchange) was selected by Cb sensitivity and 10% (w/v) sucrose resistance in TSA plates. Colonies were screened by PCR using oligonucleotides ribH1_F1 and ribH1_R2 that amplify a fragment of 0.6 Kbp in the mutant strain and a fragment of 1.2 Kbp in the wild-type strain. The mutant strain carrying an unmarked deletion of ribH1 was called ribH1.

**ribH2 mutant.** A PCR product of 0.9 Kbp containing ORF BAB2_0545 (ribH2) was amplified using oligonucleotides ribH2_F1 and ribH2_R1 and ligated to pGEM-T-Easy vector (Promega) to generate pGem-T\( \text{ribH2} \). The plasmid was linearized with EcoRV. Linearized pGem-T\( \text{ribH2} \) was ligated to a 0.7 Kbp Smal fragment containing a acc(3)-I cassette to generate pGem-T\( \text{ribH2-acc(3)-I} \). This plasmid was electroporated into *B. abortus* 2308 where it is incapable of autonomous replication. Homologous recombination

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**Figure 5. RibH2 is required to control BCV maturation in HeLa cells.** HeLa cells were infected with *B. abortus* wild-type (black bars) or ribH2 (white bars) strains expressing GFP and were labeled with (A) anti-cathepsin D or (B) anti-LAMP-1 antibodies at 2, 6 and 24 h p.i. (C) Representative fluorescence microscopy images at 4 and 24 h p.i. labeled with anti-LAMP-1 antibodies. White arrows show BCVs in infected HeLa cells. For (A) and (B) each determination is the average of two independent experiments. Values are expressed as mean ± standard error of the mean.

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events were selected using Gm resistance (3 µg/ml) and Cb sensitivity (50 µg/ml) in TSA plates. PCR and sequencing analyses showed that the ribH2 wild-type gene was replaced by the disrupted one. The mutant strain obtained was called ribH2.

**ribH1-ribH2 mutant.** A 1 Kbp PCR product containing ribH1 gene was first cloned into pGem-T-Easy and then excised and ligated to the EcoRI site of pBBR4 [32] to generate pribH1/Km. Then pGem-T_TribH2::acc3-I plasmid was electroporated into this strain and homologous recombination events were selected as previously described. PCR and sequencing analyses showed that the ribH2 wild-type gene was replaced by the disrupted one. The mutant strain obtained was called ribH1-ribH2+pribH1/Km.

**Conjugation by biparental mating.** E. coli S17-1 [33] donor strain and B. abortus strains were cultured separately, cells were harvested during the exponential phase and 1 ml of each culture was pelleted and washed twice with TSB. Bacteria were then centrifuged, resuspended in residual liquid, mixed and plated together in a single spot in a TSA plate without antibiotics. Plates were incubated at 37°C overnight, afterwards the mix was plated in TSA-Nal plates to select Brucella and exconjugants were selected for the appropriate plasmidic antibiotic resistance.

**Construction of the Complemented ribH2 Strains**

**RibH1.** A 1 Kbp PCR product containing the ribH1 gene was first cloned into pGem-T-Easy and then excised and ligated to the EcoRI site of pBBR4 [32] to generate pribH1 where ribH1 is under the lac promoter. This plasmid was conjugated into the ribH2 strain by biparental mating to generate ribH1+pribH1/Amp.

**RibH2.** A 0.9 Kbp EcoRI fragment containing ribH2 gene was excised from pGem-T_TribH2 and ligated to the EcoRI site of pBBR4. The resulting plasmid, pribH2, was conjugated into the ribH2 strain by biparental mating to generate ribH2+pribH2.

**LS_sce.** ORF YOL143C was amplified using the oligonucleotides LS_sce_F and LS_sce_R from S. cerevisiae genomic DNA. The resulting 0.6 Kbp PCR fragment was purified and digested with XhoI and XbaI and ligated into pBBR4 digested with the same restriction enzymes. The resulting plasmid, pLS_sce, was confirmed by sequencing and conjugated into ribH2 strain by biparental mating to generate ribH2+pLS_sce.

**RibH2_W22A.** The ribH2_W22A mutation was generated using the oligonucleotides W22A_F and ribH2_R2 to amplify the first part of the ribH2 ORF, then the PCR product was purified and used as a “megaprimer” for a second amplification round using the oligonucleotide ribH2_F2. The resultant PCR product was purified, digested with BamH1 and ligated into pBBR4 digested with the same restriction enzymes. The resulting plasmid, pribH2_W22A, was confirmed by sequencing and conjugated into ribH2 strain by biparental mating to generate ribH2+pLS_sce.

**Plasmid swap.** To demonstrate that RibH1 and RibH2 are LSs in vivo, we developed a plasmid swap experiment based the instability of two vectors from the same incompatibility group harbored in the same bacterial population. To this end, a pBBR4 plasmid harboring different inserts (ribH1, ribH2, ribH2_W22A, LS_sce or an empty plasmid) was introduced by biparental mating conjugation into the ribH1-ribH2+pribH1/Km strain. We selected the presence of both vectors with Amp and Km. Three independent Amp+/Km clones from each conjugation were cultured for at least 15 generations in TSB added with Amp only, then dilutions were plated in TSA-Amp plates. One hundred colonies from these plates were replica plated in TSA with Amp or Km. When necessary, riboflavin (500 µM) was added to the TSB or TSA. The percentage of Amp+/Km clones was calculated for each case (n = 100).

**Bacterial infection assays.** Monolayers of J774.A1 cells were seeded in 24-well plates (10^5 cells per well) and inoculated with RPMI 1640 supplemented with 5% fetal calf serum (FCS) containing 5×10^5 bacteria (MOI = 5:1). In order to ensure close contact between cells and bacteria, multilow plates were centrifuged at 400 g for 10 min. After 30 min of incubation at 37°C in a 5% CO2 atmosphere, cells were gently washed with
phosphate buffered saline (PBS, pH 7.4) and then incubated for 1 h in medium supplemented with Str (100 μg/ml) and Gm (50 μg/ml) to kill remaining extracellular bacteria. Thereafter, the antibiotic concentration was decreased to 20 μg/ml of Str and 10 μg/ml of Gm. At the indicated times p.i., the number of intracellular viable bacteria was determined as follows: cells were washed three times with PBS and treated for 5 min with 0.5 ml of 0.1% Triton X-100. Lysates were serially diluted and plated on TSA plates with the appropriate antibiotic to determine colony forming units per ml (CFU/ml). Each determination is the average of two independent experiments. Values are expressed as mean ± standard error of the mean.

HeLa cells were seeded in coverslips (10^5 cells per coverslip) and inoculated with minimal essential medium (MEM; Gibco) supplemented with 5% FCS and 2 mM glutamine containing 5.610^7 CFU of bacteria (MOI = 500:1). Multiwell plates were centrifuged at 400 g for 10 min. After 1 h of incubation at 37°C in a 5% CO₂ atmosphere, cells were gently washed with PBS (pH 7.4) and then incubated for 1 h in medium supplemented with 100 μg/ml of Str and 50 μg/ml of Gm to kill remaining extracellular bacteria. Thereafter, the antibiotic concentration was decreased to 20 μg/ml of Str and 10 μg/ml of Gm. At the indicated times p.i. cells were washed and fixed for immunofluorescence.

### Table 1. Comparison between RibH1 and RibH2 from B. abortus.

|                           | RibH1 | RibH2 | Reference |
|---------------------------|-------|-------|-----------|
| Monomer Length (residues) | 157   | 158   | [22,34]   |
| Classification            | Type-I LS | Type-II LS | [11,22]   |
| Quaternary arrangement    | Pentamer | Decamer | [11,22,33]|
| LS in vitro k cat (s⁻¹)   | 0.005 s⁻¹ (very low) | 0.006 s⁻¹ (very low) | [11]       |
| Chromosome location       | Chromosome I | Chromosome II | [11]       |
| Part of the Rib operon    | Yes    | No    |           |
| Predicted regulatory element | -     | RFN   |           |
| Involved in host immune response | No    | Yes   |           |
| Involved in virulence     | No     | Yes   | This work |

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Figure 7. LS enzymatic activity of RibH2 is essential for Brucella survival. Wild-type B. abortus (black bar), ribH2 mutant (white bar) and complemented ribH2 strains (grey bars) with pBBR4 plasmid harboring different inserts (ribH1, ribH2, LS_sce, ribH2_W22A) were tested for survival at 4 h p.i. in J774A.1 macrophages. Data shown (mean ± standard error of the mean) are representative of three independent experiments performed.

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For immunofluorescence, the primary antibodies used were mouse anti-human LAMP-1 H4A3 (Developmental Studies Hybridoma Bank, Department of Biological Sciences, University of Iowa) or rabbit anti-human cathepsin D (Dako). The secondary antibodies used were goat anti-mouse or goat anti-rabbit Alexa Fluor 568 (Molecular Probes, Invitrogen Co.). For DNA staining, Hoechst dye at 2 μg/ml (final concentration) was used. For Western Blots, the primary antibodies used were monoclonal anti-RibH2 and polyclonal anti-RibH1 mice serum (both generated in our laboratory). The secondary antibody used was goat anti-mouse IgG (Fc Specific) Peroxidase Conjugate (Sigma).

Western blots. Bacteria were grown overnight with the proper antibiotics and extra added riboflavin when necessary. The same amount of protein was loaded into each lane in the gels. Chemiluminiscent ECL Plus Western Blotting Detection System (Amersham Biosciences), Storm Image and Detection system (Molecular Dynamics) and Immobilon-NC Transfer Membranes (Millipore) were used for the process.
# Table 2. *B. abortus* and *E. coli* strains used in this study.

| Strain            | Name               | Characteristics                  | Source or reference |
|-------------------|--------------------|----------------------------------|---------------------|
| *B. abortus* strains | 2308               | wild-type, smooth, virulent, Na⁺  | Laboratory stock    |
|                   | 2308 pGFP          | 2308, containing plasmid pGFP, Km' | This work           |
|                   | ribH1              | 2308, unmarked deletion of ribH1  | This work           |
|                   | ribH1+pribH1       | ribH1, containing plasmid pribH1, Amp' | This work           |
|                   | ribH2              | 2308, Gm', insertion of acc(3)-I cassette in ribH2 | This work           |
|                   | ribH2 pGFP         | ribH2, containing plasmid pGFP, Km' | This work           |
|                   | ribH2+pribH2       | ribH2, containing plasmid pribH2, Amp' | This work           |
|                   | ribH1-ribH2+prbH1Km| ribH1, ribH2, containing plasmid prbH1Km, Km' | This work           |
|                   | ribH1-ribH2        | ribH1, ribH2                      | This work           |
| *E. coli* strains  | S17-1              | recA pro hsdR RP4-2-Tc::Mu-Km::Tn7 | [33]                |

# Table 3. Plasmids used in this study.

| Name               | Characteristics                  | Source of reference |
|--------------------|----------------------------------|---------------------|
| pBBR4              | pBBR1MCS-4 Broad-host-range cloning vector, Amp' | [32]               |
| pBBR2              | pBBR1MCS-2 Broad-host-range cloning vector, Km' | [32]               |
| pGemTribH2         | 0.9 Kbp EcoRI fragment containing ribH2 gene, cloned into pGemT-Easy vector, Amp' | This work           |
| pribH1Amp          | 1 Kbp EcoRI fragment containing ribH1, cloned into pBBR1MCS-4 | This work           |
| pribH1Km           | 1 Kbp EcoRI fragment containing ribH1, cloned into pBBR1MCS-2 | This work           |
| pribH2             | 0.9 Kbp EcoRI fragment containing ribH2 gene, cloned into pBBR1MCS-4 | This work           |
| pribH2_W22A        | point mutant W22A ribH2 gene, cloned into pBBR1MCS-4 | This work           |
| pLS_sce            | RIB4 gene from *Sacharomyces cerevisiae*, cloned into pBBR1MCS-4 | This work           |
| pGFP               | gfp-mut3 cloned into pBBR1MCS-2 | This work           |
| pBluescript-KSII   | Commercial plasmid              | Stratagene          |
| pGem-T-Easy        | Commercial plasmid              | Promega            |
| pBlueKSII sacBΔribH1| Δri fibH1 deletion allele and sacB from *B. subtilis*, cloned into pBluescript-KSII | This work           |
| pGem-TribH2:acc(3)-1| pGem-TribH2:acc(3)-1             | This work           |

# Table 4. Oligonucleotides used in this study.

| Name    | Sequence*                        | Restriction Enzyme |
|---------|----------------------------------|--------------------|
| ribH1_F1| CGGGATCCGGCCGCTTCATCGGGCGAAGA    | BamHI              |
| ribH1_R1| TCCAGACTGCTAGTATCGCTTTGGACATGAAACTCCAT | -                  |
| ribH1_F2| GGATACGTAGCTGTGAGCGAAGAATTTTGAGGCACTGA | -                  |
| ribH1_R2| GGACTAGCTGGCAACGCCGAGGCTTTCCTC | Spel               |
| ribH2_F1| GGAGAATGCGTATAAAATTCAA           | -                  |
| ribH2_R1| TCAGACAGGGCGCGAT                | -                  |
| ribH2_F2| TTAAGGATCCATGCTAGGCAACCTCAAGC | BamHI              |
| ribH2_R2| ATTAGGATCCATGCTAGGCAACCTGAAGGC | BamHI              |
| LS_sce_F| ATTACTCGAGATCGATCATGAGCAACCTGACACTGAGCCACTGACCTG | XhoI               |
| LS_sce_R| CGATTCTAGATCAAAAAGCATTTTTTACGGAAC | XbaI               |
| W22A_F  | ATTACGAGCCCGCGCCACCGCGCACATC | -                  |

*Restriction endonuclease cleavage sites are underlined.
Mice infection assays. 60-day-old female BALB/c mice were inoculated intraperitoneally with 5 × 10^5 CFU of the indicated strains. At 14, 30, 40 or 60 days p.i. mice were sacrificed and spleens were removed and homogenized in 2 ml of PBS. Tissue homogenates were serially diluted and plated on TSA plates with the appropriate antibiotics to determine colony forming units per spleen (CFU/spleen). Values are expressed as mean ± standard errors of the mean (N = 4).

All research involving animals has been conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and all experimental protocols have been approved by the Institutional Animal Care and Use Committee (IACUC) of Leloir Institute.

Statistical analysis. Data are presented as mean ± standard error of the mean. Significance was reported using Student’s unpaired two-tailed T-test (Prism, GraphPad Software). p-values < 0.05 were considered statistically relevant.

Supporting Information

Figure S1 Plants, fungi, and microorganisms share a common flavin biosynthetic pathway. Animals take riboflavin as a vitamin in their diets to synthesize FMN and FAD cofactors (by RK and FS respectively).

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Author Contributions

Conceived and designed the experiments: HRB MIM RAU DJC FAG. Performed the experiments: HRB MIM SK JEU VZ DJC. Analyzed the data: HRB MIM DJC FAG. Contributed reagents/materials/analysis tools: RAU DJC FAG. Wrote the paper: HRB MIM DJC FAG.
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