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Intracellularly Induced Cyclophilins Play an Important Role in Stress Adaptation and Virulence of *Brucella abortus*

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Brucella **is an intracellular bacterial pathogen that causes the worldwide zoonotic disease brucellosis.** ***Brucella*** **virulence relies on its ability to transition to an intracellular lifestyle within host cells. Thus, this pathogen must sense its intracellular localization and then reprogram gene expression for survival within the host cell. A comparative proteomic investigation was performed to identify differentially expressed proteins potentially relevant for** ***Brucella*** **intracellular adaptation. Two proteins identified as cyclophilins (CypA and CypB) were overexpressed in the intracellular environment of the host cell in comparison to laboratory-grown** ***Brucella*****. To define the potential role of cyclophilins in** ***Brucella*** **virulence, a double-deletion mutant was constructed and its resulting phenotype was characterized. The** ***Brucella abortus*** **Δ*cypAB* mutant displayed increased sensitivity to environmental stressors, such as oxidative stress, pH, and detergents. In addition, the** ***B. abortus*** **Δ*cypAB* mutant strain had a reduced growth rate at lower temperature, a phenotype associated with defective expression of cyclophilins in other microorganisms. The** ***B. abortus*** **Δ*cypAB* mutant also displays reduced virulence in BALB/c mice and defective intracellular survival in HeLa cells. These findings suggest that cyclophilins are important for** ***Brucella*** **virulence and survival in the host cells.**

Cyclophilins (Cyps) are folding helper enzymes that belong to the enzyme class of peptidyl prolyl *cis/trans* isomerases (PPIases; EC 5.2.1.8). In addition to cyclophilins, PPIases also includes FK506-binding proteins (FKBPs) and parvulins. These three families of proteins that have no sequence or structural homology can be distinguished by being inhibited by the immunosuppressive compounds cyclosporine, FK506, and rapamycin, respectively (1, 2). PPIases catalyze the *cis*/*trans* isomerization of peptidyl prolyl bonds. This reaction requires free energy and as a consequence is a slow process at lower temperatures, being the rate-limiting step in protein folding (3). PPIases are thought to be important for the correct folding of nascent proteins as well as their refolding (4–6). It is postulated that conformational isomerization by PPIases controls the activity of target proteins, regulating the interaction with other partner proteins to form complexes (3, 7).

Cyclophilins are evolutionary conserved and have been found in all organisms analyzed to date, with the exception of *Mycoplasma genitalium* and some members of the *Archaea* (8). They are ubiquitously distributed proteins and like the other PPIases are critical for cell adaptation under stress conditions (9). Cyclophilins have been reported to be involved in several processes, such as adaptation to environmental stress, cell cycle control, signal transduction, and transcriptional regulation (8, 10–12). In addition, they have been implicated in the virulence of fungal and parasitic pathogens (13–17). Recent reports have shown the involvement of PPIases in stress tolerance and pathogenesis of bacteria, such as *Listeria monocytogenes* (18), *Streptococcus mutans* (19), *Campylobacter jejuni* (20), *Legionella pneumophila* (21), *Burkholderia pseudomallei* (22), *Enterococcus faecalis* (23), *Streptococcus pneumoniae* (24), *Xanthomonas campestris* (25), and *Yersinia pseudotuberculosis* (26).

Brucellosis is an endemic zoonosis in many areas of the world (27). Manifestations of the disease are different in ruminant (cow, sheep, and goat) and human hosts. In animal hosts, *Brucella* spp. target organs and tissues of the reproductive tract, resulting in reproductive failures and abortions (28). In humans, brucellosis may advance from an acute phase to a chronic phase. The acute phase of brucellosis is characterized by debilitating symptoms, along with undulant fever. The chronic phase has several clinical manifestations that include endocarditic and neurological disorders (29). *Brucella* is a Gram-negative facultative intracellular pathogen that comprises several species. It does not produce classical virulence factors, such as exotoxins, cytolytic enzymes, capsules, fimbriae, plasmids, lysogenic phages, or drug-resistant forms (30, 31). Thus, the pathogenicity of brucellae involves adaptation to environmental stressors, such as low levels of oxygen, low levels of nutrients, acidic pH, and reactive oxygen intermediates—conditions encountered by *Brucella* in search of its intracellular replicative niche (32). *Brucella* has evolved strategies to avoid the host's innate immune system, interfere with intracellular trafficking, resist respiratory burst, adapt to oxygen-limiting conditions, and inhibit host cell apoptosis (33). Thus, in order to adapt to the hostile environment of the host, *Brucella* requires temporal and coordinated gene expression. The identification of proteins expressed during its intracellular life will shed light on the mechanisms utilized to establish a bacterium-host cell association. With this purpose in mind, a comparative proteomic analysis of laboratory-grown and intracellularly adapted *Brucella* was performed which resulted in the identification of two *Brucella abortus* cyclophilins (CypA and CypB) that were overexpressed during *B.*

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Phenotype or genotype[a]	Reference or source
Strains		
E. coli		
DH5α F′IQ	F′ ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17*(r_K^+ m_K^+) *phoA supE44* λ$^-$ *thi-1 gyrA96 relA1*/F′ *proAB*$^+$ *lacI*^{q}*Z*ΔM15 *zzf*::Tn*5* (Km^r)	57
XL1-Blue MRF′	Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173* endA1 supE44 *thi-1* recA1 *gyrA96* relA1 lac [F′ proAB lacI^{q}ZΔM15 Tn*10* (Tc^r)]^c	58
S17.1(λpir)	λ lysogenic S17-1 derivative producing π protein for replication of plasmids carrying oriR6K, Nal^s	59
B. abortus		
Wild-type 2308	Virulent, field isolated, wild type, Nal^r, erythritol resistant	Laboratory stock
Δ*cypAB* mutant	*B. abortus* 2308 unmarked double-deletion mutant in *cypA* and *cypB* genes	This study
Δ*cypAB*(pDK51) mutant	*B. abortus* 2308 Δ*cypAB* mutant with plasmid pDK51, Amp^r	This study
Δ*cypAB*(p*cypA*) mutant	*B. abortus* 2308 Δ*cypAB* mutant with plasmid p*cypB*, Amp^r	This study
Δ*cypAB*(p*cypB*) mutant	*B. abortus* 2308 Δ*cypAB* mutant with plasmid p*cypA*, Amp^r	This study
Δ*cypAB*(p*cypAB*) mutant	*B. abortus* 2308 Δ*cypAB* mutant with plasmid p*cypAB*, Amp^r	This study
Δ*cypAB*(p*cypB*$^{R55A/F60A}$) mutant	*B. abortus* 2308 Δ*cypAB* mutant with plasmid p*cypB*$^{R55A/F60A}$, Amp^r	This study
Plasmids		
pK18mobSacB	Mobilizable cloning vector, Km^r Suc^s	36
pK18Δ*cypAB*	Δ*cypAB* deletion cloned into pK18mobSacB	This study
pDK51	pBBR1MCS-4 broad-host-range cloning vector (Amp^r), pLac	52
p*cypA*	1.1-kb ApaI/PstI fragment containing the *B. abortus* 2308 *cypA* gene cloned into pDK51, Amp^r	This study
p*cypB*	0.96-kb ApaI/PstI fragment containing the *B. abortus* 2308 *cypB* gene cloned into pDK51, Amp^r	This study
p*cypAB*	1.7-kb ApaI/PstI fragment containing the *B. abortus* 2308 *cypA* and *cypB* genes cloned into pDK51, Amp^r	This study
p*cypB*$^{R55A/F60A}$	*B. abortus cypB*$^{K55A/F60A}$ gene cloned into pDK51, Amp^r	This study

[a] Abbreviations: Amp^r, ampicillin resistance; Nal^r, nalidixic acid resistance; Tc^r, tetracycline resistance; Km^r, kanamycin resistance.

abortus intracellular life. *Brucella* Cyps were required for stress adaptation, intracellular survival, and virulence in BALB/c mice.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All bacterial strains and plasmids used are listed in Table 1. *Escherichia coli* strains were grown on Luria broth (LB) (34). *B. abortus* strains were grown on tryptic soy broth (TSB), tryptic soy agar (TSA), or Gerhardt-Wilson (GW) medium (35) at 37, 42, or 23°C on a rotary shaker (250 rpm). If necessary, media were supplemented with the appropriate antibiotics at the following concentrations: kanamycin (Km), 50 μg/ml; ampicillin (Amp), 100 μg/ml; and nalidixic acid (Nal), 5 μg/ml. All experiments involving live *B. abortus* cells were conducted in a biosafety level 3 (BSL3) facility at the University of San Martín, Buenos Aires, Argentina.

Cell culture and infection assay. HeLa cells or J774 macrophages were maintained in Dulbecco modified Eagle medium (DMEM) or RPMI 1640, respectively, supplemented with 5% fetal bovine serum (FBS) and streptomycin (50 μg ml^{-1})-penicillin (50 U ml^{-1}) in a 5% CO_2 atmosphere at 37°C. (All of these solutions and media were purchased from Gibco Life Technologies.) Cells (5×10^4 per well) were seeded on 24-well plates in antibiotic-free DMEM and were kept for 24 h. *B. abortus* infections were carried out at a multiplicity of infection (MOI) of 100:1 for gentamicin (gentamicin) protection assay or an MOI of 500:1 for immunofluorescence microscopy. After a 60-min incubation with the bacteria, wells were washed three times with phosphate-buffered saline (PBS) and incubated with fresh medium containing 50 μg ml^{-1} Gm and 100 μg ml^{-1} streptomycin to kill noninternalized bacteria. At the indicated times, infected cells were washed three times with PBS and lysed with 500 μl 0.1% Triton X-100 (Sigma-Aldrich). The intracellular CFU was determined by plating serial dilutions on TSA with the appropriate antibiotic. For proteomic studies, flasks (175-cm^2 culture area) were seeded with J774 cells at a concentration of 1×10^7 cells/flask in antibiotic-free RPMI medium supplemented with 10% (vol/vol) heat-inactivated FBS plus 2 mM L-glutamine and inoculated with an MOI of 500:1 of log-phase-growing cultures of *B. abortus* 2308 for 4 h. At 48 h postinfection (p.i.), infected cells were washed with PBS and lysed with 5 ml 0.1% Triton X-100.

Isolation of *Brucella* from infected J774 macrophages. Infected cell lysates were centrifuged at 210 × *g* for 10 min at 4°C to remove host cell debris. The culture supernatant was collected and centrifuged at 20,000 × *g* for 30 min at 4°C, and the resulting pellet was resuspended in 3 ml Tris-sucrose (TS) buffer (33 mM Tris-hydrochloride containing 0.25 M sucrose [pH 7.4]). Three milliliters of the bacterial suspension was loaded onto 27 ml of Percoll (GE Healthcare Life Sciences) prepared at 30% (vol/vol) in polycarbonate centrifuge tubes. Tubes were centrifuged at 25,000 × *g* for 60 min at 4°C to allow the development of a self-forming-gradient by isopycnic centrifugation leading to development of two gradient bands. The lower band of the gradient, containing more than 85% of *Brucella* cells, was collected, and diluted 10-fold in ice-cold PBS (pH 7.4) and then centrifuged at 20,000 × *g* for 30 min at 4°C. Differential pelleting and density gradient centrifugation were performed in a Sorvall centrifuge. The pellet was resuspended in PBS and recentrifuged to eliminate residual Percoll. The final pellet from each gradient was resuspended in PBS, and protein content and *Brucella* viability were determined. Bacteria from *in vitro* growth were subjected to the same purification steps.

Protein extraction. Protein extraction of *Brucella* from either the intracellular or *in vitro* growth was performed in the same manner. *Brucella* cells (75 μl) were aliquoted, and acetonitrile (ACN) (37.5 μl for a total of 7.5%) and 8 M urea (387.5 μl) were added for a total volume of 500 μl. The cells were sonicated in a tissue culture hood on ice using 5 pulses of 5-s duration each with a 30-s rest between each pulse. After sonication, the cells were centrifuged (12,000 rpm, 10 min, 4°C) and the supernatant was removed and kept. The supernatant was then applied to a Pall 10K Nano-

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sep column and concentrated to approximately 75 μl. A series of buffer exchange and protein cleaning steps were performed as follows, with reconcentration to 75 μl after each step: step 1, addition of 4% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate} buffer (500 μl); step 2, addition of 7.5% ACN plus 4% CHAPS (500 μl); step 3, addition of 4% CHAPS (500 μl) followed by a repeat of step 3; step 4, addition of 0.05% CHAPS (500 μl) followed by a repeat of step 4. After each extraction, the total protein concentration was determined by Bradford analysis.

2-DE. Two-dimensional electrophoresis (2-DE) experiments were carried out with the ElectrophoretIQ3 system (Proteome Systems). All supplies and reagents for 2-DE, except for immobilized-pH-gradient (IPG) strips, were purchased from Proteome Systems and used according to the manufacturer's instructions. *B. abortus*-extracted proteins (50 μg) were separated by isoelectric focusing (IEF) on pH 3.9-to-5.1, 4-to-7, and 7-to-10 linear IPG strips (Bio-Rad). After 12 h of rehydration, the following focusing parameters were applied: 50 mA per strip, a linear voltage increase over 8 h from 100 to 10,000 V, and finally 10,000 V for 8 h. After IEF, IPG strips were equilibrated in equilibration buffer and applied to a 6 to 15% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were electrophoresed for 1.5 h at 500 V and stained with SYPRO ruby (Sigma-Aldrich).

Gel analysis. Each sample was run in triplicate and an average gel was generated using the 2D Phoretix software (Nonlinear Dynamics Limited). Spots present in at least two of the three subgels were included in the average gel.

In-gel trypsin digestion and MALDI-TOF MS. Protein spots were excised from the 2-DE gels using the Xcise robotic workstation (Proteome Systems). Gel plugs were washed with 50 mM ammonium bicarbonate–50% ACN, dried, and treated with 1.6 mg/ml of trypsin in 50 mM ammonium bicarbonate at 37°C overnight. Tryptic peptides were applied to a matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) target plate in a solution of 10 mg/ml α-cyano-4-hydroxycinnamic acid (CHCA) in 0.1% trifluoroacetic acid (TFA) and 50% ACN. MS spectra (100 profiles per spectrum) were obtained using an Axima-CFR plus (Shimadzu Biotech) in a positive-ion reflection mode with a source voltage of 25,000 V and a laser intensity of 55%. Peptide mass fingerprints were analyzed and searched against the theoretical spectra of *B. abortus* 2308 using the MASCOT Daemon software package (Matrix Science).

Construction of plasmid pK18Δ*cypAB*. In order to delete the DNA region containing the BAB1_1118 and BAB1_1117 genes, two PCR fragments were generated from regions flanking these genes. Oligonucleotides CypF1 (5′-CCGGATCCGTCCTTAAAGGGGCCTTGCG-3′) and CypR1 (5′-TCCAGACTGCTACGTATCGCTTTTCAGGATCGGCAGC CGA-3′) were used to amplify 500 bp of the upstream region, and CypF2 (5′-GCGATACGTAGCAGTCTGGAGTATCGCCGCCGACATCTGA-3′) and CypR2 (5′-GGACTAGTCGTGCCGAGAAAGCAGCTTG-3′) were used to amplify 500 bp of the downstream region. Both fragments, containing complementary regions, were ligated by overlapping PCR using oligonucleotides CypF1 and CypR2. The resulting fragment was digested with SpeI and BamHI and cloned into the pK18mobSacB plasmid (36).

Construction of the *B. abortus* Δ*cypAB* mutant strain. Plasmid pK18Δ*cypAB* was introduced into *B. abortus* strain 2308 by biparental mating, and kanamycin-resistant colonies were selected. These clones are the result of a single homologous recombination and thus harbor the *sacB* gene. Selection with sucrose, excision of plasmids, and generation of deletion mutants were performed as described previously (37). Double-recombination events were confirmed by kanamycin sensitivity and PCR using oligonucleotides CypF1 and CypR2.

Site-directed mutagenesis of the *B. abortus cypB* gene. A synthetic DNA fragment corresponding to an internal DNA region of *cypB* (from SacI to SalI) that included the cyclophilin's active site was purchased from Integrated DNA Technologies, Inc. (IDT). Two critical amino acids for the enzymatic activity (Arg 55 and Phe 60) were replaced with alanine, and the synthetic DNA fragment (362 bp) digested with SacI and SalI was cloned into the p*cypB* plasmid digested with the same restriction enzymes. Plasmid p$cypB^{R55A/F60A}$ was obtained, and nucleotide changes were verified by sequencing.

Complementation of the *B. abortus* Δ*cypAB* mutant. Genetic complementation of the *B. abortus* Δ*cypAB* mutant was carried out with plasmids p*cypB*, p*cypA*, p*cypAB*, and p$cypB^{R55A/F60A}$ (Table 1). Plasmids were introduced into the *B. abortus* Δ*cypAB* mutant by biparental mating using *E. coli* S17.1 as the donor strain (38).

Osmotic stress. *Brucella* cells were harvested and washed with PBS, suspended to an optical density at 600 nm (OD_{600}) of 0.9, and serially diluted in PBS, and 10 μl of each dilution was spotted on LB agar or LB agar with 170 mM NaCl, 250 mM NaCl, or 500 mM NaCl. The plates were incubated at 37°C for 5 days prior to the recording of the number of CFU.

Detergent sensitivity. *B. abortus* cultures were adjusted to a standardized optical density and immediately serially diluted in PBS. Dilutions were plated in TSA plates containing 125 μg/ml Sarkosyl (Sigma-Aldrich), 25 μg/ml Zwittergent 3-16 (Sigma-Aldrich), 1,000 μg/ml deoxycholate (DOC), or 10% Triton X-100. After 3 days of incubation at 37°C, the numbers of viable cells were determined.

Acidic stress. *B. abortus* cultures were adjusted to a standardized optical density and suspended in 1 ml of PBS, adjusted to pH 7.0, 5.0, 4.5, 4.0, 3.5, and 3.0. After 1 h of incubation at 37°C, cells were serially diluted and plated on TSA in order to determine cell viability. Cell viability was also analyzed with phosphate-citrate buffer solution between pH 4.5 and 3.0.

Oxidative stress. Bacterial resistance to oxidative stress was measured using a disk diffusion assay. Overnight cultures of *Brucella* in TSB medium were diluted in PBS and spread on TSB medium plates. A 5-mm-diameter Whatmann 3M paper disk containing H_2O_2 (5 μl of a 10% solution per disk) was placed in the center of each plate. After 3 days of incubation at 37°C with 5% CO_2, the diameter of the bacterium-free zone was determined as a measure of resistance.

PmB assay. The bactericidal effect of polymyxin B (PmB) was tested as follows. A total of 2.5×10^5 CFU of each strain was incubated for 60 min at 37°C with 0 (control), 10, or 30 μg ml^{-1} of PmB in 500 μl of 1 mM HEPES (pH 8) (Sigma-Aldrich). Afterwards, serial dilutions were plated on TSA. The percentage of survival was calculated according to the CFU recovered from the control treatment.

Western blot analysis. Whole-cell lysates of the *B. abortus* Δ*cypAB* mutant and the virulent parental strain 2308 were subjected to 12% SDS-PAGE and transferred onto nitrocellulose membranes using a semidry transfer procedure. Immunoblotting was performed using mouse anti-*Brucella* O-polysaccharide-specific monoclonal antibody M84 (kindly provided by Klauss Nielsen) and mouse monoclonal antibodies against *Brucella* outer membrane proteins (Omp1, Omp2b, Omp10, Omp16, Omp19, and Omp25) (kindly provided by Axel Cloeckaert). Detection was performed using peroxidase-conjugated goat anti-mouse immunoglobulin (Dako) and developed with SuperSignal West Pico chemiluminescent substrate (Pierce).

***In vivo* labeling of *B. abortus* with [^{14}C]acetate and quantitative analysis of lipid extracts.** The lipid compositions of *B. abortus* 2308 and the Δ*cypAB* mutant were determined by labeling with sodium [^{14}C]acetate. *Brucella* strains were grown overnight in GW medium, washed with the same medium, and then used to inoculate 12 ml of fresh GW medium at an optical density at 600 nm (OD_{600}) of 0.1. After the addition of 2 μCi of 56.50 mCi/mmol sodium [^{14}C]acetate (New England Nuclear), cultures were incubated to an OD_{600} of 0.8. The cells were then harvested by centrifugation. Lipids were extracted according to the method described previously (39) and separated by two-dimensional thin-layer chromatography (2D-TLC) on silica gel plates (Kieselgel 60; Merck) using chloroform-methanol-water (14:6:1) in the first phase followed by chloroform-methanol-acetic acid (13:5:2) in the second phase. After 10 days of exposure to Biomax Kodak films, lipids were visualized.

Mouse infection assays. Groups of 5 9-week-old female BALB/c mice were injected intraperitoneally or orally with 5×10^4 or 10^9 CFU, respec-

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tively, of cells of the *B. abortus* wild-type or Δ*cypAB* mutant strain in 0.2 ml of sterile PBS. (In the case of the oral infection, prior to the inoculation, mice were administered 0.1 ml of 10% sodium bicarbonate.) At different times postinfection, animals were euthanized, and spleens were removed and homogenized in 2 ml of PBS. Tissue homogenates were serially diluted with phosphate-buffered saline and plated on TSA with the appropriate antibiotics to determine the number of CFU per spleen.

Immunofluorescence microscopy. HeLa cells were plated on glass coverslips and infected as described above. Coverslips were washed with PBS, and cells were fixed for 15 min in 4% paraformaldehyde (pH 7.4) at room temperature for different periods. Processing for immunofluorescence labeling was accomplished as previously described (40). The primary antibodies used for immunofluorescence microscopy were rabbit anti-*Brucella* polyclonal antibody (1:1,500), M84 mouse anti-*Brucella* OPS monoclonal antibody (1:1,000), and mouse anti-human LAMP-1 H4A3 monoclonal antibody (1:400) (Developmental Studies Hybridoma Bank, Department of Biological Sciences, University of Iowa). The secondary antibodies used were Alexa Fluor 488 goat anti-rabbit or anti-mouse and Alexa Fluor 568 goat anti-rabbit or anti-mouse (Molecular Probes, Invitrogen Co.). Hoechst dye at 2 μg ml^{-1} was used for DNA staining. After immunofluorescence staining, the coverslips were mounted onto slides with FluorSave (Calbiochem). Samples were examined on a Nikon microscope (Eclipse E600). Images were then assembled using Adobe Photoshop CS.

RESULTS

***Brucella* cyclophilins BAB1_1118 (CypA) and BAB1_1117 (CypB) are induced within the host cell.** To understand how *Brucella* is able to adapt to an intracellular lifestyle, proteomic studies were performed to compare the intracellular proteomes of *Brucella* cells isolated from macrophages against those from *Brucella* cells cultured in liquid media. A set of proteins that consistently were differentially expressed within the host cell were identified (unpublished data). Two of these proteins, BAB1_1118 and BAB1_1117, which were overexpressed 18 and 1.2 times, respectively, were identified as peptidyl prolyl *cis/trans* isomerases (PPIases) belonging to the cyclophilin family (COG0652) and consequently have been referred to as CypA and CypB (Fig. 1A). *Brucella* genome analysis revealed that *cypA* and *cypB* genes are adjacent in chromosome I and code for two predicted proteins that share 63% protein sequence identity. CypA contains 196 amino acids, whereas CypB has 168 amino acids (Fig. 1B and C). The gene arrangement of the DNA region surrounding the *cypA* and *cypB* genes is conserved in the *Alphaproteobacteria* (Fig. 1B). A typical proisomerase domain present in both proteins ranging from amino acids 9 to 164 (CypB) and 32 to 191 (CypA) containing residues involved in CsA binding and PPIase activity was revealed with SMART program analysis (41) (Fig. 1C). A signal sequence with a probable cleavage site between amino acids 1 and 26 of the CypA protein was predicted by the SignalP algorithm (Fig. 1C) (42), suggesting a periplasmic localization. In CypB, the absence of a predicted signal peptide indicated a potential cytoplasmic localization. Interestingly, in *Escherichia coli* and *Azotobacter vinelandii*, cytoplasmic and periplasmic cyclophilin isoforms were also reported (43, 44).

The observation that cyclophilins CypA and CypB were overexpressed during *B. abortus* intracellular life and the knowledge that PPIases have been involved in stress adaptation and pathogenesis in other bacteria prompted us to speculate on a potential role during *B. abortus* infection. To understand the role of cyclophilin proteins in *B. abortus*, a double-deletion (Δ*cypAB*) mutant was constructed in the *B. abortus* wild-type strain, 2308.

The *B. abortus* Δ*cypAB* mutant grows normally in liquid media and has no altered membrane composition. An evaluation of the growth of the *B. abortus* Δ*cypAB* mutant in TSB and GW media revealed no modification in generation time compared with the wild-type strain (data not shown). No changes in lipopolysaccharide (LPS) composition were detected by Western blotting, crystal violet staining, Tb phage sensitivity, and polymyxin B sensitivity (data not shown). No differences in the expression of outer membrane proteins (Omp10, Omp16, Omp19, Omp25, Omp2b, and Omp1) was observed by Western blotting, and no membrane lipid composition (phosphatidylethanolamine, phosphatidylcholine, ornithine lipid, cardiolipin, and phosphatidylglycerol) was detected by 2D-TLC in the *B. abortus* Δ*cypAB* mutant compared with its parental strain (data not shown).

The *B. abortus* Δ*cypAB* mutant has a reduced ability to survive environmental stressors. To characterize the role of cyclophilins in *Brucella* stress adaptation, the *B. abortus* Δ*cypAB* mutant was exposed to a range of environmental stresses, including osmotic, oxidative, acidic, and detergent sensitivity stresses (Table 2). No difference in the survival rates of the *B. abortus* Δ*cypAB* mutant under osmotic stress was observed. However, the lack of cyclophilins affected survivability caused by hydrogen peroxide and acidic conditions, environmental "stressors" that mimic conditions that *Brucella* must overcome in order to survive within the host cell (32, 45). In addition, the *B. abortus* Δ*cypAB* mutant showed an increased sensitivity to anionic (DOC and Sarkosyl) and zwitterionic (Zwittergent 3-16) detergents compared with the wild-type strain. No difference in sensitivities to nonionic detergent (Triton X-100) was observed (Table 2). Complementation of the *B. abortus* Δ*cypAB* mutant with the medium-copy plasmid p*cypA*, p*cypB*, or p*cypAB* fully restores its abilities to survive environmental stressors.

The *B. abortus* Δ*cypAB* mutant is impaired in the ability to grow at low temperature. It has been reported that the rate of *cis/trans* proline isomerization at low temperature is much slower than at higher temperatures, and consequently, folding of *cis* proline-containing proteins is the rate-limiting step for bacterial growth in cold environments (46). Thus, it was interesting to determine if the *Brucella* Δ*cypAB* mutant has a reduced ability to grow at lower temperatures. As shown in Fig. 2, growth of the *B. abortus* Δ*cypAB* mutant and its parental wild-type strain was evaluated on TSA plates at 23, 37, and 42°C (Fig. 2A) or TSB at 23°C (Fig. 2B). Although there was no difference at 42 and 37°C, the *B. abortus* Δ*cypAB* mutant has a reduced ability to grow at 23°C (Fig. 2A and B). As expected, genetic complementation of the *B. abortus* Δ*cypAB* mutant with the p*cypA*, p*cypB*, and p*cypAB* plasmids restored the growth at 23°C (Fig. 2A and B). These results suggested that *Brucella* cyclophilins may be involved in protein folding at low temperature.

The *B. abortus* Δ*cypAB* mutant is internalized normally but cannot efficiently acquire its replicative niche within host cells because it is less able to survive intracellular killing. After internalization within host cells, *Brucella* migrates in a membrane-bound compartment known as the *Brucella*-containing vacuole (BCV). Early in the internalization process, BCVs interact with vesicles derived from the early endosome, as shown by the presence of lysosomal/endosomal markers on the BCV membrane. As the BCV matures, it becomes more acidic (pH 4 to 5) and interacts with late endosomes and lysosomes, although not extensively. Finally, the BCVs interact with the endoplasmic reticulum, becom-

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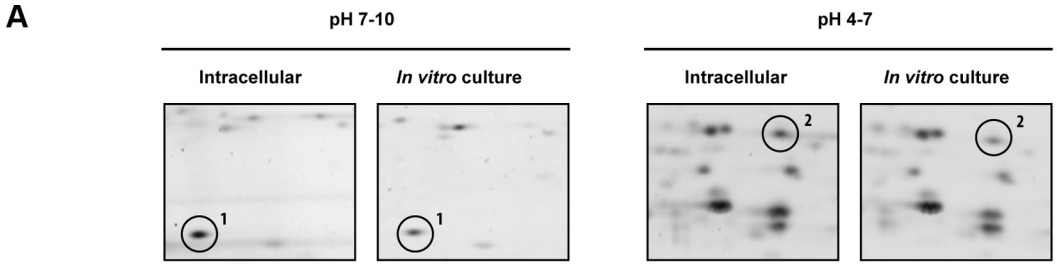
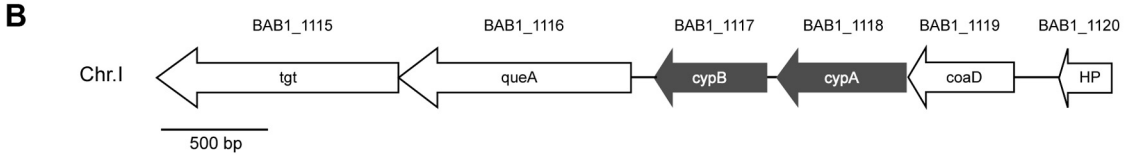


Spot N°	pH range	Protein identification	ORF	Name	Change fold	pI		MM(kDa)	
						Exp.	Theor.	Exp.	Theor.
1	7-10	Peptidyl-prolyl cis-trans isomerase	BAB1_1118	CypA	+ 18	7.75	8.30	17.0	20.8
2	4-7	Peptidyl-prolyl cis-trans isomerase	BAB1_1117	CypB	+ 1.2	6.10	5.61	17.0	18.2



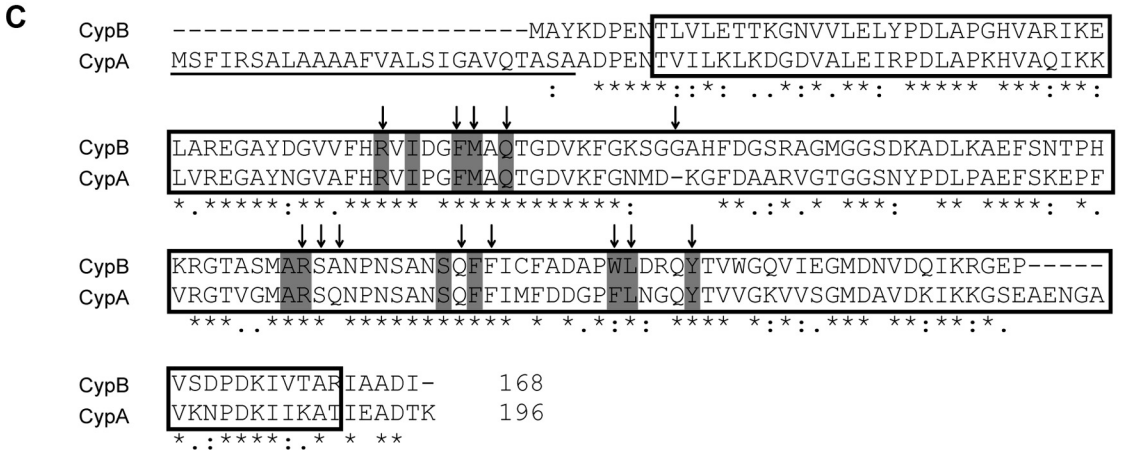


FIG 1 *B. abortus* cyclophilins are induced within the host. (A) 2DE analysis of intracellular *B. abortus* isolated from J774.A1 cells. Total bacterial protein extracts from *in vitro* culture or intracellular *B. abortus* (50 μg) were focused with IPG strips and electrophoresed by 6 to 15% SDS-PAGE. Gels were stained with SYPRO ruby and imaged at 470 nm. The panels show zoom views of gels with pH ranges of 4 to 7 and 7 to 10. Spot numbers 1 and 2, identified by MALDI-TOF MS, correspond to overexpressed CypA and CypB proteins, respectively. The table summarizes the information obtained from 2-DE MALDI-TOF MS. MM, molecular mass. (B) Physical map of the *B. abortus* cyclophilins. Arrows indicate the direction of transcription. The scale bar provides a reference for the approximate sizes of cyclophilins and surrounding genes. (C) Sequence alignment of *B. abortus* cyclophilins. Identical amino acids are marked with asterisks, highly conserved amino acids with two dots, and conserved amino acids with one dot; gaps are depicted with horizontal lines. The proisomerase domain is indicated by a box. Amino acid residues involved in binding of cyclosporine (CsA) are indicated in black arrows, and those involved in peptidyl prolyl *cis*/*trans* isomerase (PPIase) activity are shaded gray. Signal sequence determined by SignalP 4.0 is underlined. The alignment was performed with ClustalW.

ing a permissive vesicle for replication (47). These observations suggest that *Brucella* has to be able to cope with important stress challenges in order to reach its intracellular replicative niche. As shown above, the *Brucella* mutant lacking cyclophilins is more sensitive to environmental stressors. A gentamicin protection assay in HeLa cells was performed to determine the relevance of cyclophilins in intracellular adaptation of *Brucella*. As shown in Fig. 3A, the *B. abortus* Δ*cypAB* mutant presents a reduction of about 50 times in the number of intracellular bacteria at 4, 24, and 48 h postinfection compared with its wild-type parental strain. Complementation of the *B. abortus* Δ*cypAB* mutant with the medium-copy plasmid p*cypA* or p*cypB* fully restored the abilities to survive within the host cells (Fig. 3A).

Further efforts were focused on understanding if the reduced intracellular fitness of *B. abortus* Δ*cypAB* mutant was due to (i) diminished bacterial-host cell association, (ii) less-efficient internalization within host cells, or (iii) a defect in intracellular survival. As shown in Fig. 3B, the numbers of HeLa cells associated with either the *B. abortus* Δ*cypAB* mutant or its parental strain were similar and represented about 9% of the total cells. Inside-out staining studies (Fig. 3C) showed that 50% of total host-cell-associated *Brucella* cells (either the Δ*cypAB* mutant or its parental

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TABLE 2 Sensitivity of *B. abortus* strains to different stresses[a]

Strain	Sensitivity to stress			Sensitivity to detergent (log CFU)[e]			
	Osmotic (log CFU)[b]	Acidic (log CFU)[c]	Oxidative (mm)[d]	DOC	Zwittergent 3-16	Sarkosyl	Triton
Wild-type 2308	6.03 ± 0.11	5.47 ± 0.06	8.0 ± 1.4	6.47 ± 0.01	4.65 ± 0.21	4.79 ± 0.01	5.63 ± 0.21
Δ*cypAB* mutant	6.07 ± 0.05	2.91 ± 0.13	13.5 ± 0.7	4.54 ± 0.09	1.85 ± 0.07	2.81 ± 0.04	5.59 ± 0.08
Δ*cypAB*(pDK51) mutant	ND	2.67 ± 0.21	12.5 ± 0.7	4.54 ± 0.09	1.90 ± 0.14	2.84 ± 0.08	ND
Δ*cypAB*(p*cypA*) mutant	ND	5.38 ± 0.05	7.0 ± 0.0	6.50 ± 0.01	4.40 ± 0.14	4.72 ± 0.04	ND
Δ*cypAB*(p*cypB*) mutant	ND	5.42 ± 0.01	7.5 ± 0.7	7.13 ± 0.25	4.50 ± 0.28	5.10 ± 0.04	ND
Δ*cypAB*(p*cypAB*) mutant	ND	5.20 ± 0.02	7.5 ± 0.7	7.09 ± 0.12	4.75 ± 0.07	4.89 ± 0.08	ND
Δ*cypAB*(p*cypB*$^{R55A/F60A}$) mutant	ND	ND	10.5 ± 0.7	5.12 ± 0.09	2.65 ± 0.21	3.25 ± 0.01	ND

[a] Statistical significance was evaluated by Student's *t* test. The results are representative of three independent experiments. ND, not determined.
[b] Dilutions of different *B. abortus* strains were plated in duplicate onto LB agar containing 250 mM NaCl. Plates were incubated for 72 h, and the number of CFU was scored.
[c] Sensitivity to acidic stress was determined after exposure of different *B. abortus* strains to citrate buffer (pH 3.5) for 1 h at 37°C as described in Materials and Methods. Cells were serially diluted and plated on TSB agar in order to determine cell viability.
[d] Sensitivity to oxidative stress was studied by the disk diffusion assay using 10 μl of 10% peroxide hydrogen (H_2O_2) for B. abortus strains as described in Materials and Methods. The values shown represent the inhibition zone diameters (mm).
[e] For the detergent sensitivity assay, dilutions of different *B. abortus* strains were plated in duplicate onto TSB agar plates containing Sarkosyl (125 μg/ml), deoxycholate (DOC) (1,000 μg/ml), Zwittergent 3-16 (25 μg/ml), or Triton X-100 (10%). Plates were incubated for 72 h, and the number of CFU was scored.

strain) were inside the host cells at 1 h postinfection. In addition, the number of intracellular replicative bacteria was determined at earlier postinfection times (1, 2, and 4 h) (Fig. 3D). As shown in Fig. 3D, although no difference in intracellular CFU was observed at 1 h postinfection, the CFU of the *B. abortus* Δ*cypAB* mutant significantly dropped at 2 and 4 h postinfection (Fig. 3D). These results taken together indicate that the lack of cyclophilin expression does not affect *Brucella* host cell adhesion or internalization, suggesting that the difference observed in CFU of the *B. abortus* Δ*cypAB* mutant at 4 h postinfection (Fig. 3A and D) is a consequence of a reduced ability to survive intracellular killing. To determine if the *B. abortus* Δ*cypAB* mutant has a reduced ability to

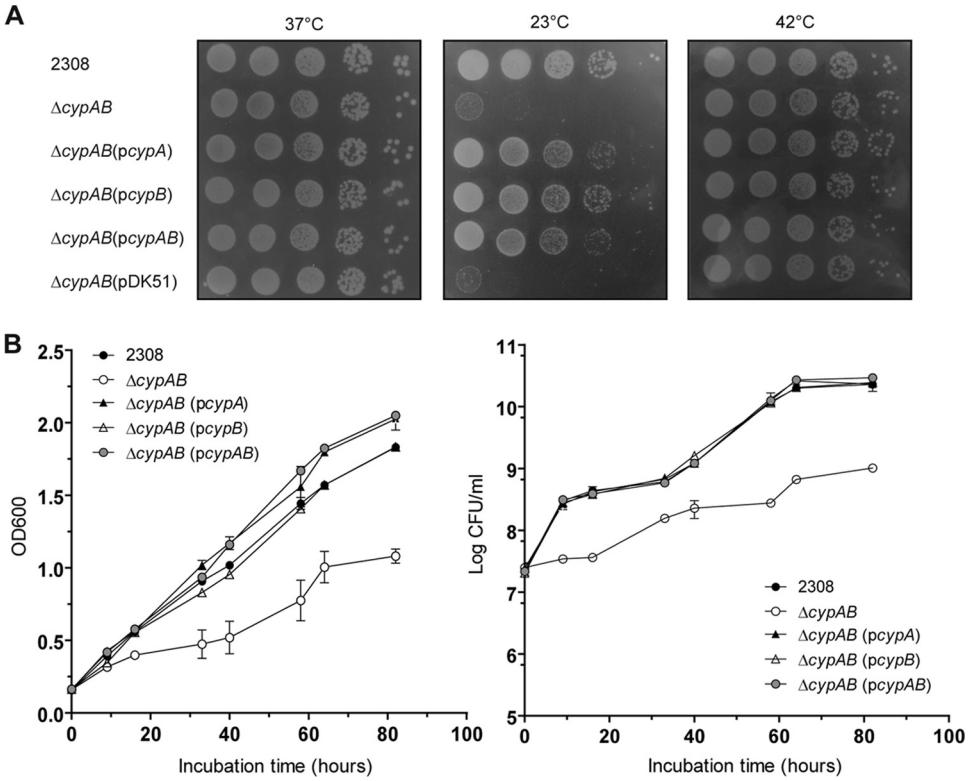

FIG 2 Growth of wild-type, *B. abortus* Δ*cypAB* mutant, and complemented strains of the *B. abortus* Δ*cypAB* mutant at different temperatures on TSA (A) or in TSB (B). (A) Serial dilutions of different strains containing equivalent numbers of CFU (as determined at 37°C) were spotted onto TSA plates and then incubated at either 37, 23, or 42°C. Images of bacterial growth were obtained after incubation during 3 days at 37 and 42°C and after 10 days at 23°C. Results are representative of at least three independent experiments. (B) Log-phase bacteria were inoculated into TSB at 23°C, and bacterial growth of the indicated strains was monitored by recording the optical densities (OD_{600}) or CFU of the cultures at the indicated times. Figures show the means and standard deviations of duplicate cultures and are representative of three independent experiments.

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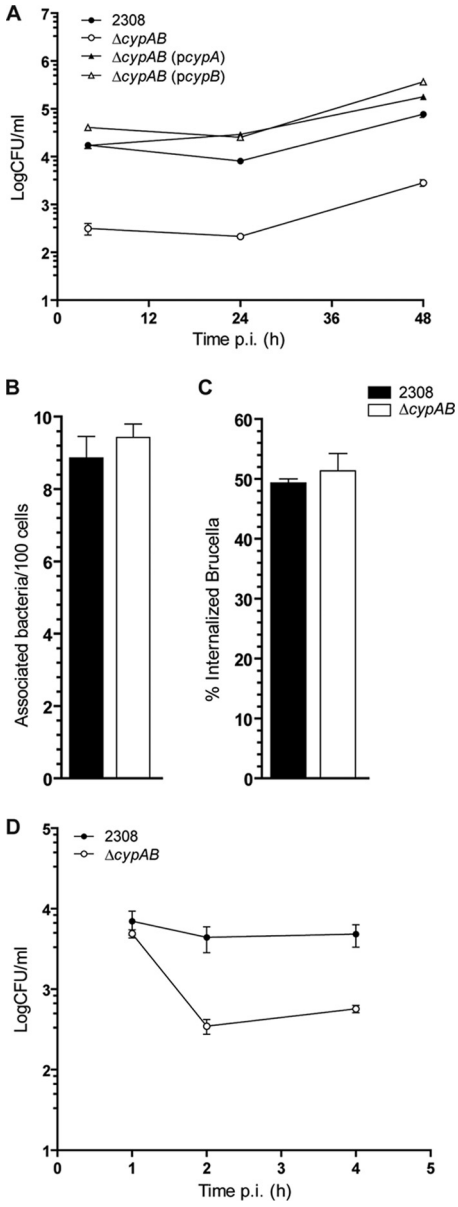

FIG 3 Interaction of the *B. abortus* Δ*cypAB* mutant with HeLa cells. (A and D) Intracellular multiplication of the *B. abortus* strains in HeLa cells. Numbers of CFU of intracellular bacteria were determined after lysis of infected cells at the indicated times postinfection. Each determination was performed in duplicate, and values are shown as the means ± standard deviations and are representative of three independent experiments. (B) Quantification of HeLa cell-associated bacteria after 1 h p.i. Approximately 3,000 cells were counted per strain. (C) Quantification of extracellular and intracellular bacteria was performed by double-immunofluorescence staining after 1 h p.i. with *B. abortus* 2308 or the Δ*cypAB* mutant. Experiments were done in duplicate. Approximately 300 bacteria were counted per strain. Statistical significance was evaluated by Mann-Whitney test.

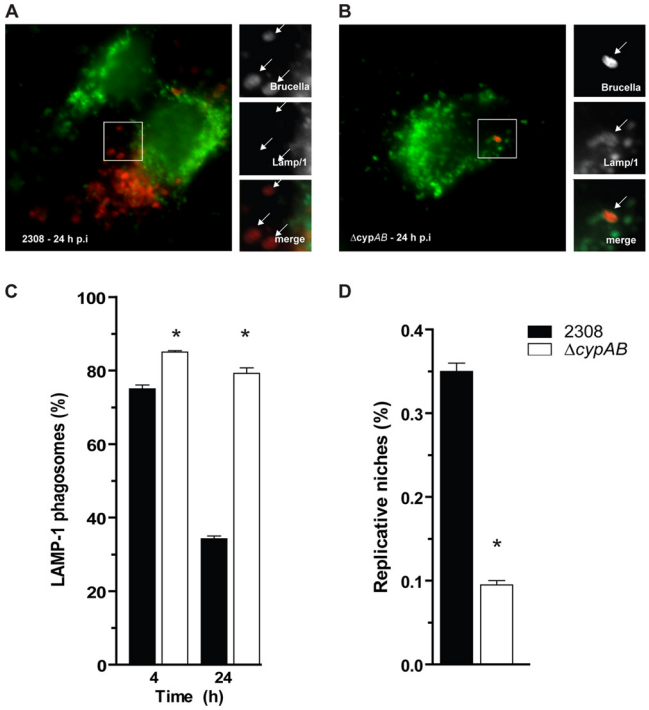

FIG 4 The *B. abortus* Δ*cypAB* mutant presents impaired intracellular trafficking within the host cell. (A and B) Representative fluorescence microscopy images of infected HeLa cells at 24 h p.i. Infections were done with the wild-type *B. abortus* strain (A) or its isogenic Δ*cypAB* mutant (B). HeLa cells were labeled for LAMP-1 (green) or *Brucella* (red) as described in Materials and Methods. Arrowheads indicate a bacterium that is magnified (×3) in the insets. (C) Quantification of LAMP-1 acquisition on BCVs in HeLa cells infected with *B. abortus* 2308 or the *B. abortus* Δ*cypAB* mutant at 4 and 24 h p.i. The data depicted are means of two independent experiments. *, $P < 0.05$, Mann-Whitney test. (D) Quantification of replicative niches at 48 h p.i. *, $P < 0.05$, Mann-Whitney test.

acquire its replicative niche, the recruitment of the late endosomal/lysosomal glycoprotein, LAMP-1, on BCV was scored. As reported, acquisition of LAMP-1 on wild-type BCV resulted in a biphasic kinetics, being rapidly recruited (4 h p.i.) and then gradually excluded (24 h p.i.) from BCV (Fig. 4C). At 4 h postinfection, the recruitment of LAMP-1 on the *B. abortus* Δ*cypAB* mutant-containing vacuole was higher than that observed in the wild-type BCV. After 24 h postinfection, the *Brucella* wild-type strain was able to replicate in a LAMP-1-negative compartment (Fig. 4A and C). In contrast, at 24 h postinfection, the LAMP-1 marker is retained on the *B. abortus* Δ*cypAB* mutant-containing vacuole (Fig. 4B and C), indicating fusion of the bacterial compartment with lysosomes. Interestingly, after 24 h postinfection, the CFU increased exponentially with a growth rate comparable to that of the wild-type strain, indicating that a surviving fraction of the intracellular *B. abortus* Δ*cypAB* mutant was able to replicate (Fig. 3A). In addition, the total numbers of cells carrying more than 25 bacteria/cell were counted by immunofluorescence microscopy at 48 h postinfection (Fig. 4D). As expected, the *B. abortus* Δ*cypAB* mutant showed a reduced number of intracellular replicative niches (Fig. 4D). In conclusion, although the *B. abortus* Δ*cypAB* mutant enters the host cell to the same extent as the wild-type strain, it cannot efficiently reach its replicative niche because it is less able to survive intracellular killing.

Defective stress adaptation of the *B. abortus* Δ*cypAB* mutant is dependent on the PPIase activity. It has been reported that functions assigned to cyclophilins do not always depend on its PPIase activity (44, 48, 49). To determine if the PPIase activity of *B. abortus* CypB is required for its physiological role, the amino acid residues Arg 55 and Phe 60 were replaced by alanine (Fig. 5A).

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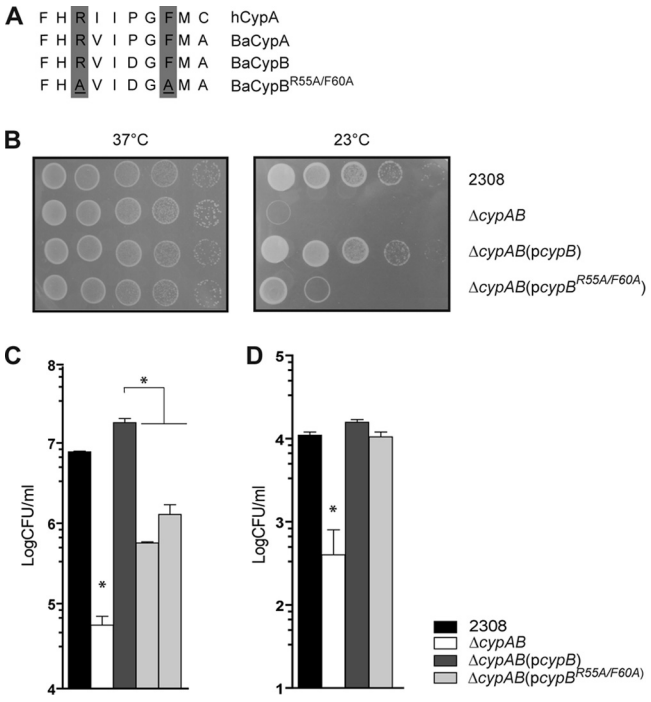

FIG 5 Peptidyl prolyl *cis*/*trans* isomerase activity is required for cyclophilin stress adaptation. Two critical amino acids for the enzymatic activity of Cyps, Arg 55 and Phe 60, were replaced by alanine (A). *B. abortus* Δ*cypAB*(p*cypB*$^{R55A/F60A}$) was assayed for bacterial growth at 23°C (B), detergent sensitivity (DOC) (C), and survival in HeLa cells (D) as described in Materials and Methods. *, $P < 0.05$, Mann-Whitney test.

These point mutations were previously reported to substantially reduce the enzymatic activity of the human CypA (48). As shown in Fig. 5B and C, plasmid p*cypB*$^{R55A/F60A}$ partially rescued the *B. abortus* Δ*cypAB* mutant for DOC sensitivity (Fig. 5C and Table 2) and bacterial growth at 23°C (Fig. 5B). These results demonstrate that all traits associated with *Brucella* stress adaptation are linked to the PPIase activity of cyclophilins. Intermediate results observed with p*cypB*$^{R55A/F60A}$ complementation suggest that the protein CypB$^{R55A/F60A}$ maintains some residual activity. Interestingly, p*cypB*$^{R55A/F60A}$ restored intracellular replication of the *B. abortus* Δ*cypAB* mutant to the wild-type level (Fig. 5D), suggesting that the residual PPIase activity of CypB$^{R55A/F60A}$ is sufficient to complement intracellular survival in HeLa cells.

***B. abortus* cyclophilins are required for maintenance of chronic infection in mice.** As shown above, the lack of expression of cyclophilins impaired the ability of *Brucella* to cope with different environmental stresses and to reach its intracellular replicative niche within the host cell. To determine if these characteristics alter the ability of *Brucella* to infect and chronically colonize mice, a mouse infection assay was performed. As shown in Fig. 6A, after intraperitoneal infection no significant differences in the numbers of recovered bacteria were observed until 4 weeks postinfection in both *Brucella* strains. Interestingly, at 12 weeks postinfection, the number of bacteria recovered from the *B. abortus* Δ*cypAB* mutant-infected mice decreased 10 times, suggesting that cyclophilins are important for maintenance of chronic infection in mice (Fig. 6A). Similar results were observed when the oral infection route for *Brucella* was performed. This route is more challenging because the bacterium must progress through different tissues and organs to reach its replicative niche within the host (Fig. 6B). As shown in Fig. 6B, orally infected mice had a reduced number of *B. abortus* Δ*cypAB* mutant cells at 6 weeks postinfection compared with the mutant's parental strain, confirming that cyclophilins are required for maintenance of a successful *Brucella* chronic infection in mice.

DISCUSSION

Living organisms are constantly confronted with never ending environmental changes that can have tremendous consequences on growth and survival. Thus, organisms must to elicit adaptive responses to external stressors and challenges. One initial manifestation of stress is protein denaturation within the cell. This triggers the induction of specific proteins whose function is to restore the equilibrium by assisting the process of protein folding. To this end, both eukaryotes and prokaryotes require the activity of a highly conserved family of proteins, such as chaperonins, thioredoxin, and PPIases that refold proteins, recovering their functional state. Herein, we have shown that for *Brucella* to adequately respond to acidic, oxidative, and low-temperature stresses

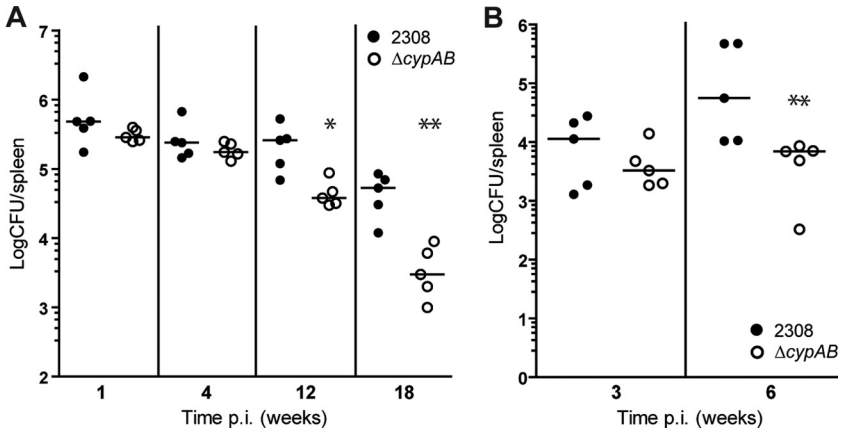

FIG 6 *B. abortus* cyclophilins are required for maintenance of chronic infection in mice. BALB/c mice were infected intraperitoneally (1×10^4 CFU) (A) or orally (1×10^9 CFU) (B) with *B. abortus* 2308 or its isogenic (Δ*cypAB*) mutant. At different weeks postinfection, the numbers of CFU recovered from spleens were determined by serial dilutions and plating onto TSA. Five animals were used for each determination. *, $P < 0.05$; **, $P < 0.01$, Mann-Whitney test.

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and detergents requires the presence and participation of the cyclophilins, members of the PPIase family. Since microbial pathogen-host cell interaction is a stressful for both participants, it is not unexpected that a *Brucella* mutant that lacks cyclophilin expression is also attenuated in a mouse virulence model. It is interesting to note that the *B. abortus* Δ*cypAB* mutant behaves normally in the absence of a stress condition, and its phenotype becomes apparent only when certain environmental stressors are present or when the bacteria are internalized within the host cell. Although the *B. abortus* Δ*cypAB* mutant can enter the host cell, it is less efficient at reaching its replicative niche, presumably by succumbing to the combination of acidic and oxidative stresses and detergent-like compounds encountered in the harsh lysosomal environment.

Intracellular bacterial pathogens that have coevolved in long-standing association with the mammalian host have acquired specific mechanisms to survive and replicate within the host cell. For example, *Brucella* has evolved modified PAMPs (such as LPS and flagellin) that allow it to evade host cell innate immunity responses or have acquired specific mechanisms to inhibit host cell apoptosis. Such traits promote the establishment of a safe haven for *Brucella* replication within the host cell. Survival in the host cell entails *Brucella* sensing its location and consequently coordinating the expression of genes that help to subvert the host cell defenses for its own benefit. The comparative proteomic investigation showed that *Brucella* cyclophilins are upregulated in the intracellular milieu and are involved in stress adaptation and virulence. By *in silico* analysis of the *Brucella* genome, seven putative PPIases were revealed: BAB1_1117 (CypB), BAB1_1118 (CypA), BAB1_1944, BAB1_1162, BAB1_0706 (SurA), BAB1_0917 (trigger factor), and BAB2_0908, with CypA and CypB the only members of cyclophilin family of PPIases. Delpino et al. (50), characterized another *Brucella* PPIase, the protein SurA, as a substrate for the *Brucella* type IV secretion system (T4SS), the most important virulence mechanism in *Brucella*. Others have described PPIases involved in *Brucella* virulence, like the trigger factor, which plays a critical role in the acute phase of *Brucella* infection (51, 52).

Bacterial molecular chaperones have been described as "moonlighting or multitasking proteins," since in addition to their biological function as helpers in protein folding, they also have a distinct role in bacterial virulence. Thus, it has been reported that in *Listeria monocytogenes*, *Neisseria meningitidis*, and *Mycobacterium tuberculosis*, Hsp70 protein (DnaK) is located on the bacterial surface, functioning as a plasminogen receptor (53–55). In *Brucella*, Hsp70 is also secreted to the bacterial supernatant in a T4SS-dependent manner (50). In this context, it is conceivable that in addition to *Brucella* cyclophilin's role in bacterial stress adaptation, it may also function in the pathogen-host cell relationship. Interestingly, in preliminary experiments using the *Bordetella pertussis* adenylate cyclase fusion assay (CyaA), we have determined that *Brucella* CypB is translocated to host cell cytosol in a T4SS-dependent manner in the course of the bacterial infection (unpublished data). However, it remains to be seen if *Brucella* cyclophilins function as a T4SS effector protein. In the class *Alphaproteobacteria*, members of which are known to form close association with eukaryotic cells, the presence of two adjacent cyclophilins along with surrounding genes is highly conserved. Because of the divergence in the amino acid sequence and composition, *Brucella* cyclophilins CypA and CypB appear to be the result of separate evolutionary pathways. CypA shared homologies with the cyclophilins of Gram-negative bacteria. CypB has a primary protein structure characteristic of eukaryotic cyclophilins, including the Trp^{125} residue, which has been described as the critical residue for interaction with the immunosuppressor CsA (56). In addition, CypA and CypB are predicted to be located within the periplasm and the cytoplasm, respectively. Nevertheless, in this study we determined that both cyclophilins CypA and CypB complement the *B. abortus* Δ*cyp*AB mutant, indicating that both proteins function equally and are redundant regardless of their predicted subcellular localization. Here, we conclude that cyclophilins make an important contribution to *Brucella* intracellular adaptation and virulence.

ACKNOWLEDGMENTS

We would like to dedicate this work to the memory of Dr. Rodolfo A. Ugalde.

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