Brucella abortus depends on L-serine biosynthesis for intracellular proliferation

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ABSTRACT

L-serine is a non-essential amino acid and a key intermediate in several relevant metabolic pathways. In bacteria, the major source of L-serine is the phosphorylated pathway, which comprises three enzymes: D-3-phosphoglycerate dehydrogenase (PGDH; SerA), phosphoserine amino transferase (PSAT; SerC), and L-phosphoserine phosphatase (PSP; SerB). *Brucella abortus* genome encodes two PGDHs (SerA-1 and SerA-2), involved the first step in L-serine biosynthesis, one PSAT and one PSP, responsible for the second and third steps, respectively. In this study, we demonstrate that the double mutant *serA-1_serA-2,* and the single mutants *serC* and *serB* are auxotrophic for L-serine. These auxotrophic mutants can be internalized but are unable to replicate in HeLa cells and in J77A.1 macrophage-like cells. Replication defects of auxotrophic mutants can be reverted by cell medium supplementation with L-serine at early times post-infection. Additionally, *serB* mutant is attenuated in the murine intraperitoneal infection model and has an altered lipid composition, since lack of L-serine abrogates phosphatidylethanolamine synthesis in this strain. Taken together, these results reveal that limited availability of L-serine within the host cell impairs proliferation of the auxotrophic strains, highlighting the relevance of this biosynthetic pathway in *Brucella* pathogenicity.

INTRODUCTION

Bacteria of the genus *Brucella* are alpha-2-Proteobacteria that cause brucellosis, a chronic infectious disease affecting diverse species of mammals and humans. *Brucella* infection causes sterility and abortion in animals, and undulating fever and debilitating disorders in humans. Brucellosis remains endemic in many countries, resulting in serious public health problems and economic losses worldwide (1).

The ability to replicate in a wide range of mammalian cell types, including endothelial cells, fibroblasts, epithelial cells and microglia is essential to *Brucella* pathogenesis (2). The pathogen primarily infects and replicates inside phagocytic cells such as macrophages and dendritic cells, before disseminating to placental trophoblasts, reproductive tract and the mononuclear phagocyte system, where they persist to establish a chronic infection in the host (3, 4).

After internalization into host cells, *Brucella* resides in a membrane-bound compartment known as the *Brucella*-containing vacuole (BCV). BCVs traffic along the endocytic and secretory pathways, allowing the bacterium to evade killing in phagolysosomes and to replicate in an endoplasmic-reticulum-derived compartment (5, 6). Afterwards, BCVs mature into compartments with autophagic features which are required for cell-to-cell spreading (7).

To date, efforts to characterize *Brucella* pathogenesis mechanisms have been focused on "classical" virulence determinants such as the Type IV Secretion System and its effectors (8–11), lipopolysaccharide (12), cyclic β-1, 2- glucan (13, 14), two component system BvrS/BvrR (15), autotransporters and adhesins (16), and transcriptional regulators (17, 18). These virulence factors participate in key aspects of *Brucella* pathogenesis, like host cell adhesion and internalization, intracellular replication and innate immune evasion (19).

Recently, bacterial nutrition and metabolism during infection have emerged as new research topics in bacterial pathogenesis (20–22). A recent example is illustrated by *B. abortus* glutamate dehydrogenase (GdhZ), which plays an essential role during intracellular replication, as GdhZ constitutes an entry point into tricarboxylic acid cycle (TCA) for

several amino acids. These results emphasize the importance of amino acids as a main carbon source during *Brucella* intracellular phase (23).

Even though it has been widely accepted that BCVs are nutritionally poor (24, 25), their exact nutrient composition is unknown. About twenty years ago, random scale mutagenesis allowed identification of attenuated mutants in genes coding for transport and catabolism of carbohydrates, as well as genes encoding peptides and amino acids transporters, suggesting that they could be used by the bacterium as sources of carbon and energy during infection (25–31). Later, proteomics studies demonstrated reduced key metabolic pathways early after infection, like TCA, pyruvate and pentose phosphate shunt cycles, as well as sugar uptake systems (32). On the contrary, enzymes involved in catabolism of amino acid and proteins are increased, suggesting that *Brucella* may obtain precursors for TCA cycle from amino acids, like glutamate, during early infection. Altogether, these studies emphasize the ability of *Brucella* to adjust their metabolism to the intracellular conditions encountered at each stage of the infection process.

Contrary to what is described for many intracellular pathogenic bacteria, which have reduced their genomes after long periods of co-evolution with their hosts (33), bacteria of the genus *Brucella* are prototrophic for all amino acids (34, 35). Amino acid biosynthesis has become relevant in *Brucella* virulence after identification of attenuated mutants in genes coding for enzymes involved in these pathways, suggesting that BCVs are poor in amino acids (27, 28, 36–38).

L-Serine biosynthesis is a major anabolic pathway in most organisms. Although commonly classified as non-essential amino acid, L-serine plays essential roles as a precursor for glycine, cysteine, tryptophan, phosphatidyl L-serine, sphingolipids, porphyrins, purines, glyoxalate, and glycine (39). Also, as the precursor to glycine, L-serine is the major source of one-carbon units that serve as the donors in methylation reactions mediated by derivatives of tetrahydrofolate and S-adenosyl methionine (40). Conversion of L-serine to glycine is a reversible reaction catalyzed by serine hydroxy methyl transferase (SHMT E.C. 2.1.2.1) (41). Nonetheless, the major route for L-serine biosynthesis in bacteria is the phosphorylated pathway (Fig. 1A), which comprises three sequential steps catalyzed by 3-phosphoglycerate dehydrogenase (PGDH/SerA; EC 1.1.1.95), phosphoserine

aminotransferase (PSAT/SerC; EC 2.6.1.52), and phosphoserine phosphatase (PSP/SerB; EC 3.1.3.3).

Given its central role in metabolism, we sought to characterize and determine the importance of L-serine biosynthesis pathway in *B. abortus*. In the present study, three deletion mutants auxotrophic for L-serine have been obtained and characterized *in vitro* and *in vivo*. All three auxotrophic mutants failed to replicate intracellularly, and one of them proved to be attenuated in mice in the acute and chronic phases of the infection. These results indicate that during intracellular stages, *B. abortus* depends on the biosynthesis of L-serine via the phosphorylated pathway to sustain its proliferation.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study are listed in Supplementary Table S1. *B. abortus* strains were inoculated in tryptic soy agar (TSA) (Difco/Becton Dickinson, Sparks, MD) or in tryptic soy broth (TSB) at 37°C on a rotary shaker for 16-20 hs. When indicated, media were supplemented with 50 µg/ml kanamycin, 5 µg/ml nalidixic acid, and/or 50µg/ml ampicillin. All work with live *B. abortus* was performed in a biosafety level 3 laboratory facility. *E. coli* strains were grown in Luria Broth (LB) liquid or solid medium, at 37°C overnight. Antibiotics, when required, were added at the following concentrations: 50 µg/ml kanamycin or 100 µg/ml ampicillin.

Construction of *B. abortus* mutants and genetic complementation

To obtain the mutant strains by unmarked gene deletion, the regions flanking each gene (*serA-1: bab1_1697*, *serA-2: bab2_0783*, *serC: bab1_1699* and *serB: bab1_1410*) were amplified and ligated using recombinant PCR technique (42). The primers used for PCR amplification of the 500 bp upstream and downstream regions are listed in Supplementary Table S2. Both PCR fragments obtained were used in an overlapping PCR to obtain a 1000 bp fragment. These fragments were ligated into pK18*mob-sacB* vector (43). The resulting plasmids were transformed in *Escherichia coli* S17λ*pir,* and subsequently conjugated to *B. abortus* 2308 by biparental mating. Single recombinants were selected with kanamycin (Km) and replica plated in TSA supplemented with 10% sucrose (Suc). Colonies Km^r and Suc^s were grown ON in TSB without antibiotics and plated on TSA with 10% Suc to counterselect the double recombinants. Double recombination events (Km^s Suc^r) were selected and gene deletion was confirmed by PCR. To obtain double mutant *serA-1_serA-2*, the plasmid pK18*mob-sacB* containing the 1000 pb-flanking region of *serA-2* was conjugated into *serA1* mutant. Selection of recombination events was performed like in single mutant constructions.

Genetic complementation of the mutant strains was achieved by expression of C-terminal 3xFlag-tagged versions of the proteins SerB, SerC and SerA-2 from plasmid pBBR1-MCS4-3xFlag (pLF) (44). All genes were amplified by PCR from *B. abortus* 2308 genomic

142 DNA using primers listed in Supplementary Table S3. PCR products were digested with
143 BamHI and SpeI, and the resulting fragments cloned into the same sites of pLF to generate
144 in-frame fusions to the 3xFLAG epitope under *lac* promoter control. The resulting
145 constructions were introduced in the corresponding *B. abortus* mutant strains by biparental
146 mating. To complement *serA-1_serA-2* double mutant, the promoter regions and genes
147 coding for SerA-1 or SerA-2 were amplified by PCR using primers listed in Table 3
148 (Fw_SerA-1_pBBR2_SpeI/Rv_SerA-1_pBBR2_BamHI and Fw_SerA-
149 2_pBBR2_SpeI/Rv_SerA-2_pBBR2_BamHI) . The PCR products were digested with
150 BamHI and SpeI, and the fragments were cloned into pBBR1-MCS2. The resulting
151 plasmids were introduced in *B. abortus* double mutant strain by biparental mating. For
152 genetic complementation with both genes, pLF_*serA-2* and pBBR1 MCS2_*serA-1* were
153 introduced simultaneously in the double mutant.

154 **Growth curve in TSB and minimal medium**

155 Starter cultures were grown in TSB in a rotary shaker (200 rpm) overnight at 37°C, and
156 then diluted with the same medium to OD_{600}= 0.1 (10 ml cultures in 50 ml flasks). Culture
157 growth was monitored by measuring the absorbance at 600nm every 4 hours. When
158 indicated, the medium was supplemented with 10 mM L-serine (Sigma-Aldrich). Bacterial
159 growth in minimal medium Gerhardt-Wilson (GW) (45) was measured as described above,
160 except minimal medium was used as the diluting solution of starters cultures grown until
161 exponential phase (0.6-0.8 OD_{600}). Bacterial growth was monitored by measuring OD_{600}
162 every 24 hours. When indicated, 10mM L-serine (Sigma) was added to the growth medium.
163 Four independent experiments were performed in duplicates.

164

165 **Cell culture infection and replication assays**

166 Log-phase bacteria grown in TSB were used to infect J774A.1 macrophage-like cells at a
167 multiplicity of infection (MOI) of 50:1, or HeLa cells at MOI 1000:1. Bacteria were
168 centrifuged onto cells at 400 x *g* for 10 min to promote bacterium-cell contact. After 60
169 min, wells were gently washed three times with PBS and incubated for 120 min with fresh
170 medium containing 50 µg ml-1gentamicin and 100 µg ml-1 streptomycin to kill non-

internalized bacteria. Thereafter, antibiotics concentrations were decreased to 10 µg ml-1 gentamicin and 20 µg ml-1 streptomycin. At the indicated times, infected cells were either washed three times with PBS and lysed with 500 µl of 0.1% Triton X-100 in PBS (Sigma-Aldrich) for CFU counts or processed for immunofluorescence staining as described below. Intracellular CFU counts were determined by plating serial dilutions on TSA with the appropriated antibiotic.

Immunofluorescence microscopy and antibodies

HeLa cells were seeded on 12-mm coverslips in 24-well plates at $5x10^4$ cells per well. After 24 hs, cells were infected with the indicated *B. abortus* strains and at different times post-infection, cells were washed three times with PBS and fixed with 4% paraformaldehyde (pH 7.4) for 15 min at 37°C. Fixed cells were washed again twice and coverslips were incubated for 30 min in blocking buffer (PBS with 10% horse serum and 0.1% saponin). Afterwards, coverslips were incubated for 60 min in blocking buffer containing primary antibodies. After two washes in 0.1% saponin PBS, the coverslips were incubated for 60 min in blocking buffer containing secondary antibodies. Finally, the coverslips were washed three times in PBS and once in milli Q water and mounted on glass slides using Fluorsave (Calbiochem). The primary antibodies used were rabbit anti-*Brucella*, monoclonal mouse anti-*Brucella* (M84), monoclonal mouse anti-human LAMP-1 antibody (H4A3), monoclonal rat anti-mouse LAMP-1 antibody (1D4B) (Developmental Studies Hybridoma Bank, National Institute of Child Health and Human Development, University of Iowa) and anti-human calnexin antibody (Abcam). The secondary antibodies used were Alexa Fluor goat anti-mouse IgG, goat anti-rat IgG and goat anti-rabbit IgG (Molecular Probes, Invitrogen). Confocal images were acquired using an IX-81 microscope attached to a FV-1000 confocal module, with a PLAN APO 60X NA 1.42 oil immersion objective (Olympus, Japan). The acquisition software used was FV 10-ASW 3.1. Images were treated using ImageJ 1.45s Software (NIH, USA), and. images of 1024 x 1024 pixels were then assembled using Adobe Photoshop CS. Bacterial enumeration in HeLa cells and quantification of LAMP-1 and calnexin colocalization with BCVs were performed on a Nikon microscope (Eclipse TE 2000) at a magnification of X60 with a lens with a

numerical aperture of 1.42. At least 300 bacteria in random fields were analyzed per sample.

Microsomal and acidic pH susceptibility assay

Microsomes derived from J774A.1 murine macrophages were obtained as described in (46). Protease activity in this microsomal fractions was determined using a casein-BODIPY-FL, whose fluorescence is quenched. Protease-catalyzed hydrolysis relieves this quenching, yielding bright green fluorescent peptides. The increase in fluorescence emission is proportional to casein digestion and protease activity. Fluorescence was measured with a fluorescence plate reader (FilterMaxF5 Molecular devices). To determine the sensitivity of *Brucella* strains to lysosomal killing, $2.5x10^5$ CFU were incubated with 20 µg of the purified microsomal fractions or buffer (negative control) for eight hours at 37°C. After incubation, serial dilutions were plated in TSA to determine the number of viable bacteria. Susceptibility to low pH was assessed by incubating log-phase bacteria grown in TSB in phosphate buffer saline (PBS) pH 4 or pH 7 at 37°C for four hours. Serial dilutions were plated after incubation to determine the number of viable bacteria after the treatments.

Mice infection

All experimental protocols of this study were approved by the Committee on the Ethics of Animal Experiments of the University of San Martín (CICUAE UNSAM) and were conducted in agreement with international ethical standards for animal experimentation (Guide for the Care and Use of Laboratory Animals of the National Institutes of Health). Eight-week-old female BALB/c mice were intraperitoneally inoculated with $5 x10^4$ CFU of *B. abortus* strains in PBS (200 µl). At 7, 15 and 30 days post inoculation, spleens from infected mice were removed, weighed, and homogenized in 3 ml of PBS. Spleen homogenates were serially diluted and plated in TSA for CFU enumeration. During the experimental protocol, mice were housed in an appropriate biosafety level 3 facility and handled according to international guidelines required for animal experiments.

Thin-layer chromatography lipid analysis

228 Log-phase bacteria were used to inoculate fresh Gerhardt Wilson medium supplemented
229 with
230 100 µM of choline dihydrogen citrate (Sigma- Aldrich Co.) at an initial OD_{600} of 0.2.
231 Cultures were grown for 48 hs until they reached an OD_{600} of ~ 1 and bacteria were
232 harvested by centrifugation. Lipids were extracted according to the method of Bligh and
233 Dyer (47) and separated on silica gel plates (Kieselgel 60; Merck) by using chloroform-
234 methanol-water (14:6:1 [vol/vol/vol]) as a running solvent. Aminolipids were revealed by
235 spraying the plate with 0.2% ninhydrin in ethanol and heating at 100 °C for three minutes
236 (48). Phospholipids were visualized by destructive treatment with a solution 8% H_3PO_4:
237 10% $CuSO_4$ and charring (49).
238
239 **Statistical Analyses**

240 Statistical analyses were performed with Prism 6 software (GraphPad) with one or two-way
241 ANOVA and Bonferroni post-test for multiple comparisons to assess statistical differences
242 between two experimental data sets. *P*-values: ns, not significant; *, $P<0.05$; **, $P<0.01$;
243 ***, $P<0.001$.

244

RESULTS

Generation and characterization of L-serine auxotrophic mutants

In the phosphorylated biosynthetic pathway, L-serine is derived from the glycolytic intermediate 3-phospho-D-glycerate (PGA), in a three-step reaction (Fig. 1A). The first enzyme is D-3- phosphoglycerate dehydrogenase (henceforth PGDH) that converts PGA to phosphohydroxypyruvate (PHP) with the concomitant reduction of NAD+ to NADH. *B. abortus* genome encodes two isoforms of PGDH/SerA: SerA-1 (BAB1_1697) and SerA-2 (BAB2_0783). Both PGDHs are 38% identical and comprise the catalytic domain and a C-terminal allosteric substrate binding domain (ASB) in SerA-1, or an aspartate kinase–chorismate mutase–TyrA domain (ACT) at the C-terminus of SerA-2.

In order to obtain L-serine auxotrophic mutants, genes coding for PGDHs were deleted to produce single (*serA-1* and *serA-2*) and double (*serA-1_serA-2*) mutant strains. Unmarked gene deletion of *serA-1* or *serA-2* had no significant effect on vegetative growth in TSB (not shown) or in the gluconeogenic medium Gerhardt-Wilson (GW) without L-serine supplementation (Fig. 1B), demonstrating that the effect of the single mutations can be compensated by the presence of the second gene that encodes a functional PGDH. On the contrary, the double mutant *serA-1_serA-2* required L-serine supplementation for growth in minimal medium (Fig. 1B), indicating that L-serine biosynthesis is abrogated in this strain. At this point, it is worth mentioning that 10mM L-serine supplementation to the growth media TSB or GW does not confer any advantage in vegetative growth to *B. abortus* wild type 2308 (Fig. S1 A and B). Genetic complementation of the double mutant with plasmids encoding SerA-1 and/or SerA-2 partially restored wild type growth in minimal medium without L-serine (Fig. 1C), probably due to plasmids instabilities in these strains. These results suggest that SerA-1 and SerA-2 are involved in the first step of L-serine biosynthesis in *B. abortus.*

In the following steps, phosphoserine amino transferase (henceforth SerC; BAB1_1699) converts PHP to L-phosphoserine (PS) with the concomitant conversion of glutamate to α-ketoglutarate, followed by the conversion of PS to L-serine by phosphoserine phosphatase (henceforth SerB; BAB1_1410) (Fig. 1A). Unmarked gene deletion of *serC* or *serB* had no

significant effect on vegetative growth in TSB (not shown). As expected, these mutants required exogenous L-serine (10mM) for growth in GW medium (Fig. 1D). The growth defect of these auxotrophic mutants could be fully rescued by complementation with plasmids coding for the corresponding enzymes (Fig. 1E).

These results indicate that when grown in a gluconeogenic medium such as GW, where the only carbon and nitrogen sources are glycerol and glutamate, *B. abortus* depends on the biosynthesis of L-serine for growth. In addition, the ability to rescue the growth defect of the auxotrophs upon addition of L-serine to the media, suggests that *B. abortus* can incorporate this amino acid through a transport system not identified yet.

L-serine biosynthesis is necessary for *B. abortus* intracellular replication

Brucella can adhere, invade and proliferate inside different cell types including both professional and no-professional phagocytic cells. To assess the impact of L-serine auxotrophy in the intracellular lifestyle of *B. abortus*, we evaluated the intracellular replication of the wild type S2308, *serA-1_serA-2*, *serC* or *serB* mutants and the corresponding complemented strains in HeLa and J774A.1 cell lines, two widely used infection models of non-professional an professional phagocytic cells. Genetic complementation of the double mutant with only one of the genes did not fully restore intracellular replication levels in HeLa and J774A.1 cells (not shown). For this reason, *serA-1_serA-2* mutant was complemented with both genes in these experiments. As can be shown in Fig. 2 (A-F), all mutant strains showed no differences with wild type at 4 hs p.i., but failed to replicate in both cell types, with 1 to 4 log-units decrease in CFU counts when compared to S2308 at 24 hs or 48 hs p.i. Genetic complementation of the mutants with plasmids encoding the corresponding enzymes restored intracellular replication of the mutants with the exception of *serC* at 24 hs p.i. in both cell types (Fig. 2 A-F, green bars). Likewise, addition of 10 mM L-serine to the cell medium RPMI 1640 at the beginning of the infection process rescued the intracellular growth defect of L-serine auxotrophic mutants (Fig. 2 A-F, blue bars), but had no effect on intracellular replication of wild type *B. abortus* (Fig S1 C and D). These results demonstrate that *B. abortus* requires biosynthesis of L-serine to replicate intracellularly, and that exogenous L-serine can be incorporated into

the BCVs to rescue the replication defect of the auxotrophs. Since the three auxotrophic mutants exhibited a similar phenotype in cells, we decided to continue the characterization only with *serB* mutant in the following experiments.

In order to further characterize L-serine requirements of intracellular *B. abortus,* host cell medium was supplemented with the amino acid at 6 hs, 10 hs and 24 hs p.i. Interestingly, supplementation of cell medium with L-serine can only restore intracellular proliferation of *serB* mutant in HeLa and J774A.1 macrophage-like cells if added before 24 hs p.i. (Fig. 3 A and B). These results are in agreement with our previous results demonstrating that *B. abortus* cannot use host cell L-serine to sustain the intracellular replication phase, and therefore depends on *de novo* biosynthesis. The results also highlight the importance of L-serine availability during the first stages of infection in order to successfully proliferate within cells. The fact that supplementation of the cell culture medium with L-serine resumes the intracellular growth of the mutant, indicates that *B. abortus* is capable of transporting the amino acid through both the BCV and the bacterium membranes.

Since intracellular levels of L-serine cannot support proliferation of auxotrophic mutants, we sought to determine the minimal concentration required to rescue the replication defect of *serB* by infecting HeLa cells and adding increasing concentrations of L-serine at the beginning of the *in vitro* infection assay. As can be seen in Fig. 4, there is a minimum threshold of L-serine concentration capable of rescuing the intracellular replication defect of *serB*. At least 1.6 mM L-serine needs to be added to the cell medium RPMI 1640 (containing 0.28-0.4 mM L-serine) to bypass *serB* proliferation defect. These results confirm that *Brucella* can incorporate L-serine through both the bacterial cell envelope and the BCV membrane to support intracellular replication.

Abrogation of L-serine biosynthesis impairs biogenesis of replicative vacuoles in phagocytic cells

Once internalized, *Brucella* resides within a membrane-bound vacuole, the *Brucella*-containing vacuole (BCV), which undergoes remodeling from a compartment with endosomal/lysosomal features into an organelle derived from the host endoplasmic

reticulum (ER) that supports bacterial replication (rBCV) (50–53). Given that *B. abortus* L-serine auxotrophic mutants displayed intracellular replication defects, we investigated the biogenesis of the rBCV in HeLa cells infected with *serB* mutant. We first quantified acquisition and exclusion of the endosomal/lysosomal marker LAMP-1 at 4 hs and 24 hs post-infection, respectively. At 4 hours p.i. 64.59± 6.06% of wild type BCVs were positive for LAMP-1, compared to 67.23 ±4.57% for *serB* BCVs. At 24 hs p.i., *serB* BCVs excluded the lysosomal marker and only 31.20±3.65% remained positive for LAMP-1, compared to 26.95±5.16% of wild type BCVs (Suppl Fig. 2A). Addition of 10 mM L-serine to the cell medium had no effect on LAMP-1 labeling of BCVs at 4 and 24 hours p.i. To further characterize *serB* intracellular traffic, we quantified acquisition of calnexin, an ER resident protein. By 24 hs p.i., 71.37±5.13% of wild type BCVs and 69.95±4.73% of *serB* BCVs were positive for calnexin (Suppl. Fig. 2B).

Detailed inspection and enumeration of intracellular bacteria by immunofluorescence microscopy of infected HeLa cells at 24 hs p.i., revealed that cells infected with wild type *B. abortus* show *foci* of intracellular replication containing more than seven bacteria per cell, whereas no signs of intracellular replication were observed in cells infected with *serB*, with 98.53% of the cells with none or less than four bacteria (Fig. 5A). At 48 hs p.i., the cells infected with *serB* remained free of intracellular replication *foci*, with 88.43±1.31% of cells without bacteria and only 11.25 ±1.47% with less than four bacteria (Fig. 5B). As expected, supplementation of the cell culture medium with 10 mM L-serine restored the replication defect of the auxotrophic mutant, since 2.34±0.04% of cells presented intracellular *foci* with more than seven bacteria. These results are consistent with the intracellular replication curves (Fig. 2E) and are illustrated by representative confocal images of infected HeLa cells at 48 hs p.i. (Fig. 5C). These images show replicative *foci* found in cells infected with the wild type but barely detectable in those infected with *serB*. As expected, supplementation with 10 mM L-serine restored the intracellular proliferation of the mutant as judged by the higher number of replicative *foci* (Fig. 5C). The images also illustrate calnexin association to BCVs in both strains. Together, these results indicate that *B. abortus* L-serine auxotrophs are competent to promote the biogenesis of rBCVs in HeLa cells but are unable to replicate within them.

368 To assess if the same is true in professional phagocytic cells, LAMP-1 acquisition and
369 exclusion was evaluated in infected J774A.1 macrophages. At 4 hours p.i., 71.31±4.84% of
370 wild type BCVs were decorated with LAMP-1, compared to 62.468 ±3.49% for *serB*
371 BCVs. At 24 and 48 hs p.i, wild type BCVs were able to progressively exclude this
372 lysosomal marker with 30.27±0.39% and 19.5±3.5% of LAMP-1 positive vacuoles,
373 respectively. On the contrary, *serB* BCVs remained positive for this marker at 24 and 48 hs
374 p.i. with 51.35±5.63% and 55.50±6.36% of LAMP-1 positive BCVs, respectively (Fig.
375 6A). Confocal microscopy images illustrate *B. abortus* wild type and *serB* mutant inside
376 J77A.1 macrophages at 48 hs p.i. (Fig. 6B). In agreement with the intracellular replication
377 curves (Fig. 2F), *serB* mutant fails to replicate inside these cells and is decorated with
378 LAMP-1. Additionally, *serB* lysosomal degradation inside these cells is evidenced by small
379 *puncta* labeled with total anti-*Brucella* antibodies (Fig. 6 B smaller arrowheads). In contrast
380 to what was observed in non-phagocytic Hela cells, inside a competent phagocytic cell such
381 as J774.A1 macrophages, *serB* serine auxotroph could not complete the biogenesis of the
382 rBCV, remaining mostly in LAMP-1 positive and calnexin negative compartments (not
383 shown) where it was eventually degraded.

384

385 **L-serine auxotrophy alters *B. abortus* membrane lipid composition**

386 The *Brucella* cell envelope is composed of phosphatidylethanolamine (PE),
387 phosphatidylcholine (PC), ornithine lipid (OL), cardiolipin (CL), and phosphatidylglycerol
388 (PG) (54, 55). In *B. abortus*, PE is synthesized by the phosphatidylserine synthase pathway
389 (Fig. 7A). The first reaction requires the condensation of L-serine with CDP-diacylglycerol
390 catalyzed by the Phosphatidylserine synthase (PssA) to produce phosphatidylserine, which
391 is quickly decarboxylated by the Phosphatidylserine decarboxylase (Psd) to produce PE
392 (54). In order to assess whether L-serine auxotrophy affects the membrane lipid
393 composition, *B. abortus* wild type and the *serB* mutant were grown in GW medium
394 supplemented with choline, and the total lipids were extracted and analyzed by thin layer
395 chromatography and revealed by acid charring (total lipids) and ninhydrin staining for
396 aminolipids. As expected, the spot corresponding to PE was absent when the mutant was
397 grown in a defined medium without L-serine. This defect can be circumvented by

supplementing the medium with 10mM L-serine (Fig. 7B). Lack of PE in the cell envelope was compensated by increasing the amount of OL. These findings indicate that impairment of L-serine biosynthesis abrogates PE formation, which impacts the lipid membrane composition suggesting that *B. abortus* L-serine auxotrophs depend on exogenous L-serine to form PE, one of the major membrane phospholipids.

Since phospholipid composition in bacterial cell envelope is critical for the interaction with the host, we analyzed if the lack of PE in *serB* increased its susceptibility to acidic pH conditions or lysosome killing. First, we evaluated the sensitivity of the bacteria to low pH conditions by incubating S2308, *serB* and *pssA* mutant strains in PBS pH 4 for four hours. No differences were detected in the number of viable bacteria recovered after four hours of incubation in this acidic pH (Suppl Fig. 3A). *pssA* mutant strain was included as a control, since it cannot synthesize PE, but L-serine biosynthetic pathway remains intact (54). Additionally, we evaluated if lack of PE synthesis increases *serB* sensitivity to lysosomal proteolytic activity. To achieve this, microsomal fractions obtained from JJ74A.1 macrophages were incubated with S2308, *serB* and *pssA* mutant strains, and *serB* complemented mutant. After eight hours of incubation with the microsomal fractions, no differences in sensitivity to lysosomal proteolytic activity were detected among the strains (Suppl Fig. 3B). Therefore, the inability of *serB* mutant to proliferate intracellularly, whether in professional or non-professional phagocytes, is not related to the impaired PE biosynthesis, but to its failure to produce serine *de novo* and/or to extract it from the host cell.

L-serine biosynthesis is required for full virulence in BALB/c mice

Since auxotrophic mutant strains fail to replicate intracellularly and to produce PE, we sought to evaluate the virulence of *serB* mutant in the murine infection model. As shown in Fig. 8, *serB* auxotrophic mutant exhibited significantly reduced levels of splenic colonization. At 7 days p.i., *serB* mutant showed 2 log units reduction in CFU in comparison to the wild type. This difference became larger at 15 days p.i., with 2.867 CFU log unit reduction (Fig. 8). Although statistically significant, the difference in CFU between wild type and *serB* diminished to 1.641 log CFU at 30 days p.i., indicating that this

auxotrophic mutant was able not only to persist in spleen, but also to replicate. Genetic complementation with a plasmid encoding the fusion protein SerB_3xFLAG partly restored virulence in mice. These results can be explained by the instability of certain plasmids in the mice infection model, where antibiotic selection cannot be achieved. The colonization defect of *serB* mutant was concomitant with reduced splenomegaly and hepatomegaly, hallmarks of *Brucella* infection (data not shown). These results indicate that *B. abortus* requires L-serine biosynthesis to achieve an efficient infection in the mammalian host.

DISCUSSION

L-serine is a non-essential amino acid produced by a biosynthetic pathway in nearly all organisms (56). In this study, we show that disruption of the genes coding for the enzymes involved in the anabolic pathway causes L-serine auxotrophy, which impairs the ability of *B. abortus* to proliferate inside the host cell and affects virulence in the mice infection model of brucellosis.

The crucial role of L-serine biosynthesis in pathogenicity is supported by a previous study describing a transposon-insertion mutant in *B. suis* gene coding for SerB, which displays an attenuated phenotype after 48 hs of infection in THP-1 macrophages (25). To our knowledge, attenuated mutants in the genes coding for SerC or SerA have not been reported nor identified in mutagenesis analyses. In the case of SerA, this can be explained by the presence of two PGDH isoforms encoded in *B. abortus* genome. Despite the little identity and different domain architectures of SerA-1 and SerA-2, both enzymes proved to be functionally redundant. Single mutants were prototrophs for L-serine and only simultaneous deletion of *serA-1* and *serA-2* produced a growth defect in minimal medium without L-serine. *B. melitensis* SerA-2 crystallographic structure has been resolved (PDB entry 3k5p) and it closely resembles *E. coli* PGDH structure (Edwards, T.E. et al., unpublished). ACT domain in *E. coli* PGDH is allosterically and cooperatively inhibited by L-serine to achieve negative feedback regulation of the biosynthetic pathway (57). Further studies are required in order to characterize the function of ACT and ASB domains of PGDH isoforms in regulation of the biosynthetic pathway in *B. abortus*.

Apart from the phosphorylated pathway, L-serine can also be produced from glycine by SHMT (56). However, as this reaction depletes glycine levels, this is not a major route to produce L-serine. In fact, this pathway cannot compensate the effect of the mutations responsible for L-serine auxotrophy. For those reasons, we postulate that *B. abortus* SHMT encoded by gene locus *bab1_0787* functions *in vivo* not for serine biosynthesis but for glycine production.

469 Several amino acid auxotroph strains of bacterial pathogens are often attenuated for
470 intracellular growth and infection. *Mycobacterium tuberculosis* proline and lysine
471 auxotrophs (58–60), and *Salmonella* auxotrophs for histidine and methionine (61–63) are
472 examples of attenuated mutants *in vivo*. In *B. abortus*, L-serine depletion in the mutants
473 *serA-1_serA-2*, *serC* and *serB* was likely the cause of intracellular replication defects,
474 which could be rescued by amino acid supplementation of the host cell medium. The fact
475 that the mutants were able to transport and use extracellular L-serine from RPMI-
476 supplemented medium, suggests that wild-type *B. abortus* does not obtain enough L-serine
477 from the host during infection and must synthesize its own. Delivery of extracellular L-
478 serine into *B. abortus* cytoplasm requires transport across three membranes: the host cell
479 membrane, the phagosomal membrane and ultimately the bacterial membranes. Amino acid
480 supplementation and uptake by intracellular pathogens has already been described in
481 *Francisella* (64, 65) and *Legionella* (66). These bacteria are auxotrophic for some amino
482 acids and must obtain them from the host in order to proliferate.
483
484 According to our results, L-serine availability contributes during the early infection phases
485 to bacterial proliferation. A recent transposon mutagenesis highlights and supports the
486 importance of *B. abortus* amino acid biosynthetic pathways during infection (67). Mutant
487 strains in the genes coding for the enzymes involved in the anabolism of histidine and
488 isoleucine, leucine and valine are attenuated at 24 hs p.i. in RAW 264.7 macrophages. At 2
489 hs and 5 hs p.i., the mutants resemble L-serine auxotrophs, showing no differences with
490 wild type in intracellular bacterial counts. Noticeably, although *serB* auxotrophic mutant is
491 attenuated at 24 hs and 48 hs p.i., it is contained inside BCVs with replicative
492 characteristics in HeLa cells, resembling wild type *B. abortus*. In HeLa cells, similar
493 kinetics of LAMP-1 exclusion and calnexin recruitment to the BCVs suggests a
494 dissociation between traffic events and replication capability for *serB*. However, in cells
495 with proficient phagocytic activity such as J774A.1 macrophages, the biogenesis of the
496 rBCV is impaired and the *serB* mutant resides in intracellular compartments positive for the
497 late endosome/lysosome marker LAMP-1 and negative for the ER chaperone calnexin,
498 where it is subjected to lysosomal degradation. Further studies are needed to address and
499 characterize these observations.

The virulence attenuation of *B. abortus* caused by L-serine auxotrophy might be merely based on the requirement of this amino acid for protein biosynthesis. In addition, the virulence attenuation might be due to the role of L-serine as a precursor in phosphatidylethanolamine biosynthesis. Previous studies demonstrated that absence of PE in a *pssA* mutant altered cell surface properties, impaired intracellular survival and maturation of the replicative BCVs, and spleen colonization in mice (54). In the *pssA* mutant, the absence of PE was compensated by increasing the relative amount of the other lipids, in particular the ornithine lipid, as described here for *serB* auxotrophic mutant. However, absence of PE in *serB* cell envelope does not affect its resistance to acidic pH or to lysosome killing, suggesting that lack of L-serine is mainly responsible for the defect in intracellular replication.

In the mouse infection model, although attenuated in acute and chronic stages, *serB* mutant manages to persist in the spleen. This finding suggests that host tissues contain enough L-serine to support growth of this auxotrophic mutant. It is well established that macrophages can adopt two different immulogical states: classical activated macrophages (CAM) with inflammatory cytokine production and bactericidal activity, and alternative activated macrophages (AAMs), which are less inflammatory and play relevant roles in homeostasis, allergic inflammation, wound healing and tissue repair (68, 69). During *in vivo* infection, *Brucella* is found within CAMs at early infection times, but survive and replicate preferentially in AAMs (70). In these cells, a metabolic shift to beta-oxidation of fatty acids induced by peroxisome proliferator-activated receptor γ (PPARγ) increases intracellular glucose availability, which promotes intracellular survival and persistence of *Brucella* in AAMs (70). The fact that *serB* mutant is less attenuated in the late stages than in the early stages of mouse infection, could be related in part to the availability of amino acids (in particular L-serine) in each subtype of infected macrophages. Therefore, a better nutritional characterization of the microenvironment surrounding *B. abortus* in infected tissues would be useful to understand how bacteria adapt their metabolism to the different conditions found during the course of infection.

531

532 In the intracellular pathogens *Legionella pneumophila* and *Coxiella burnetti*, the host-cell
533 L-serine is transported into the phagosome where the bacterial cell converts it into pyruvate
534 to feed the TCA cycle, thus serving as the main source of carbon and energy (71).
535 However, it seems not to be the case of *Brucella spp.*, which also require the activity of the
536 TCA cycle for the intracellular stages, but they mainly depend on glutamic acid as an entry
537 point into TCA (23, 72). In agreement with this, preliminary results from our group indicate
538 that the mutant in the gene coding for the L-serine deaminase, involved in L-serine
539 deamination to yield pyruvate, is not attenuated intracellularly like L-serine auxotrophic
540 mutants.

541

542 In conclusion, although it is capable of incorporating L-serine from the extracellular milieu
543 by a yet unidentified transporter, *B. abortus* depends on the biosynthesis of L-serine to
544 sustain intracellular proliferation inside phagocytic and non-phagocytic cells. These
545 findings highlight the L-serine biosynthetic pathway as an interesting target for the
546 development of new drugs and/or strategies to combat brucellosis.

AUTHOR AND CONTRIBUTIONS

VR: data acquisition, data analysis, data interpretation, writing of the manuscript; MIM: data acquisition, data analysis, data interpretation, writing of the manuscript, revising of the manuscript; DC: data analysis, data interpretation, writing of the manuscript, revising of the manuscript, principle investigator.

CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any financial or commercial relationships that would be construed as a potential conflict of interest.

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780

781

782 **FIGURE LEGENDS**

783 **Figure 1. L-serine auxotrophy in *B. abortus*. (A)** Schematic representation of L-serine
784 biosynthetic pathway in *B. abortus*. SerA-1/SerA-2: 3-phosphoglycerate dehydrogenase
785 (EC 1.1.1.95), SerC: 3-phosphoserine aminotransferase (EC 2.6.1.52), SerB: phosphoserine
786 phosphatase (EC 3.1.3.3). **(B, D)** Growth kinetics of *B. abortus* wild type (2308) and the
787 indicated mutant strains in GW medium. Overnight cultures of bacteria grown in TSB were
788 pelleted, washed, and resuspended in fresh GW medium in the presence or absence of 10
789 mM L-serine. Growth was monitored by measuring the turbidity (OD at 600 nm) at
790 different times. **(C, E)** Growth kinetics of *B. abortus* wild type (2308), auxotrophic mutants
791 for L-serine and the complemented strains in GW medium without L-serine. Four
792 independent experiments were performed in duplicates for each growth curve. Data are
793 means ±SD (error bars are within the size of the symbols) of a representative experiment
794 performed in duplicate.
795
796 **Figure 2. L-serine biosynthesis is required for *B. abortus* intracellular replication.**
797 Intracellular replication of the indicated strains in HeLa cells **(A, C, E)** and J774A.1
798 macrophagic cells **(B, D, F)**. CFU were enumerated at 4, 24 and 48 hours post infection
799 (p.i.). When indicated, cell medium was supplemented with 10mM L-serine at the
800 beginning of the infection. Data are means ±SD of a representative experiment performed
801 in triplicate. **P* <0.05; ***P* <0.01; ****P* <0.001.
802
803 **Figure 3. L-serine supplementation rescues *B. abortus* serB intracellular replication**
804 **during early infection phase.** Intracellular replication of the indicated strains in HeLa cells
805 **(A)** and in JJ74A.1 macrophages **(B)**. When indicated, medium was supplemented with 10
806 mM L-serine at the indicated time p.i. Data are means ±SD of a representative experiment
807 performed in triplicate. ***P <0.001.
808
809 **Figure 4. Determination of the minimal concentration of L-serine necessary to rescue**
810 **the *B. abortus serB* intracellular replication defect.** Intracellular replication of the
811 indicated strains in HeLa cells. Cell medium was supplemented with the indicated

812 concentrations of L-serine at the beginning of the infection. Data are means ±SD of a
813 representative experiment performed in triplicate. ***P <0.001.
814
815 **Figure 5. *B. abortus serB* mutant fails to establish intracellular replication *foci.***
816 Enumeration of intracellular bacteria in HeLa cells infected with the indicated strains at 24
817 hours **(A)** and 48 hours post infection **(B)**. When indicated, cell medium was supplemented
818 with 10mM L-serine at the beginning of the infection. Data are means ±SD of a
819 representative experiment performed in duplicate. *P <0.05; **P <0.01; ***P <0.001. **(C)**
820 Representative confocal micrographs of HeLa cells infected with the indicated strains at 48
821 h p.i. HeLa cells were labeled for calnexin (green) or *Brucella* (red) as described in
822 Materials and Methods. Arrowheads indicate replication *foci* and insets show BCVs
823 colocalizing with calnexin (arrows).
824
825 **Figure 6. *serB* is necessary for LAMP-1 exclusion from BCVs in phagocytic cells. (A)**
826 Quantification of LAMP-1 acquisition and exclusion by BCVs of wild type and *serB*
827 mutant at 4, 24 and 48 hs post-infection in J774A.1 macrophages. Bars represent means
828 ±SD of a representative experiment performed in duplicate. **P <0.01; ***P <0.001. **(B)**
829 Representative confocal micrographs of J774A.1 macrophages infected with the indicated
830 strains at 48 h p.i. Cells were labeled for LAMP-1 (red) and *Brucella* (green) as described
831 in Materials and Methods. Arrowheads indicate LAMP-1 and *Brucella* colocalization for
832 *serB* mutant and smaller arrowheads show puncta/dots suggestive of bacterial lysosomal
833 degradation.
834
835 **Figure 7. Phosphatidylethanolamine (PE) synthesis is abrogated in *B. abortus serB***
836 **mutant. (A)** Schematic representation of PE biosynthetic pathway in *B. abortus*. PssA:
837 Phosphatidylserine synthase; Psd: Phosphatidylserine decarboxylase. *B. abortus* strains
838 were grown in minimum medium with choline, and 10mM L-serine was added to the
839 medium when indicated. Total lipids were isolated and separated by thin layer
840 chromatography analysis. Lipid spots corresponding to phosphatidylglycerol (PG),
841 ornithine lipid (OL), phosphatidylethanolamine (PE), cardiolipin (CL) and

phosphatidylcholine (PC) are indicated. Total lipids were visualized by sulfuric acid charring **(B)** and aminolipids were visualized by ninhydrin staining **(C).**

Figure 8. *B. abortus serB* mutant is defective for mice spleen colonization. BALB/c mice were inoculated intraperitoneally with *B. abortus* wild type, *serB* mutant, or the complemented strain, as indicated in Materials and Methods. Bacteria were recovered from spleens at 7, 15 and 30 days post infection. Individual CFU values are plotted and horizontal dashed lines represent the median bacterial load for each treatment group. Statistical significance was determined by one-way ANOVA **P <0.01; *** P <0.001.

Figure 1

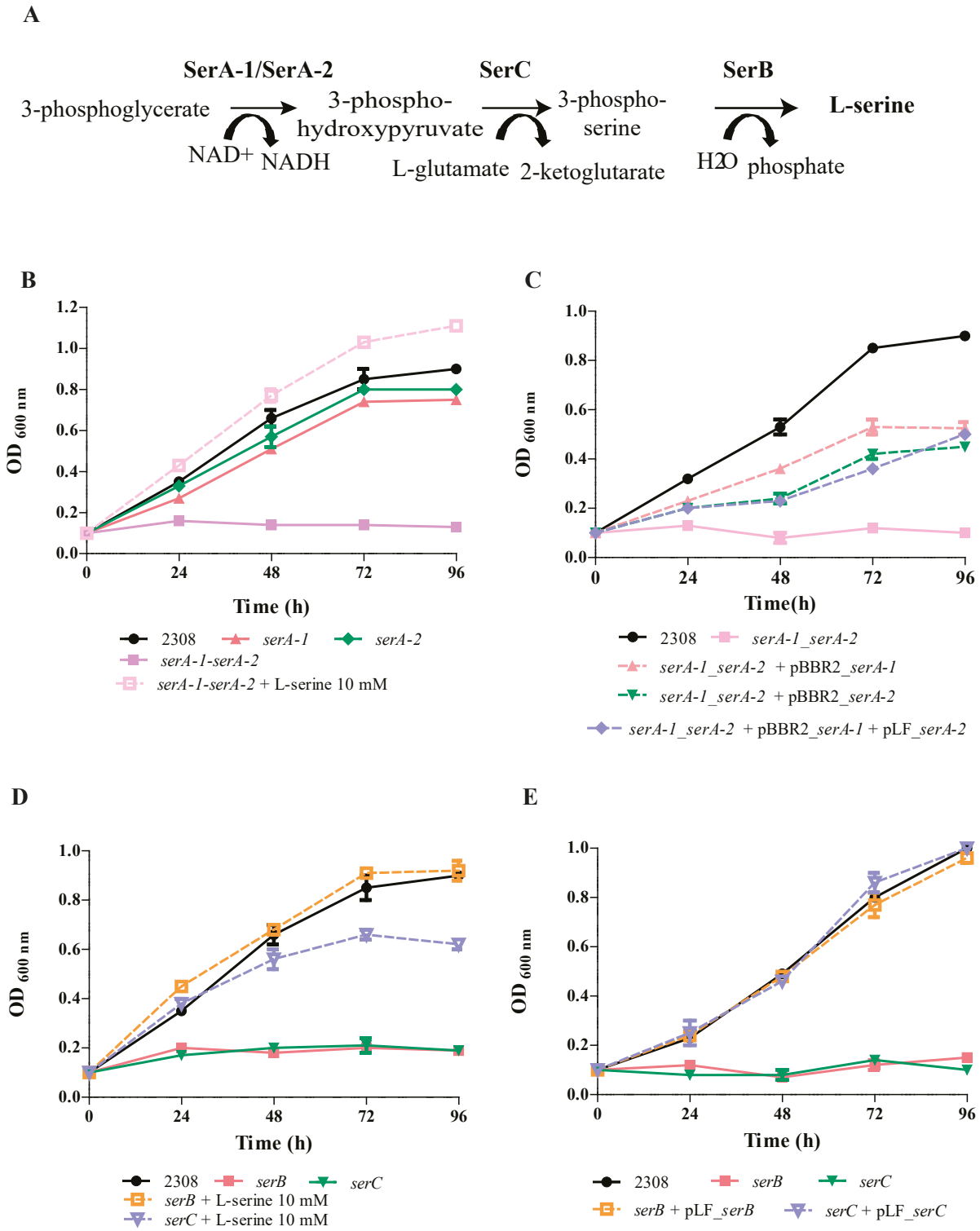
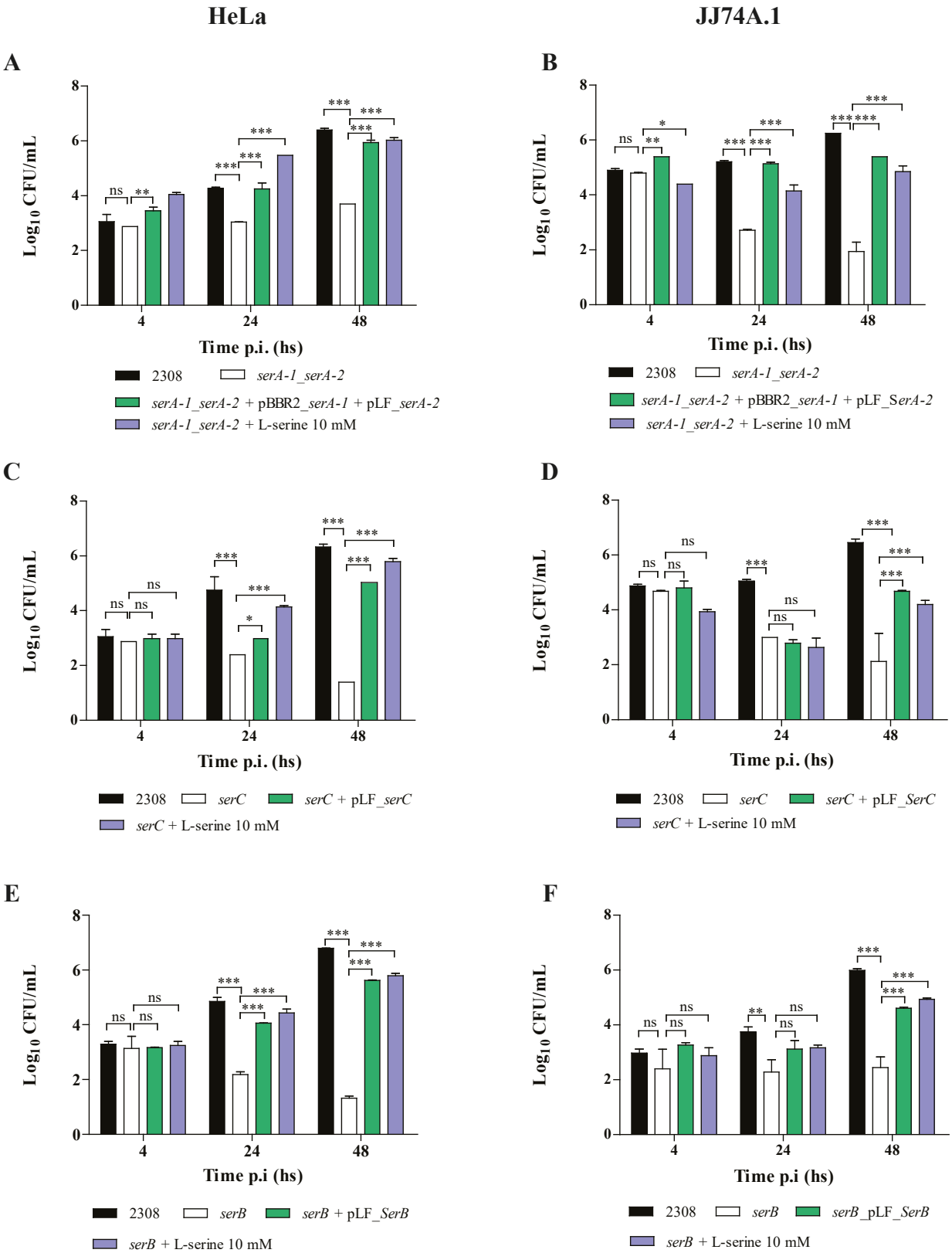


Figure 2



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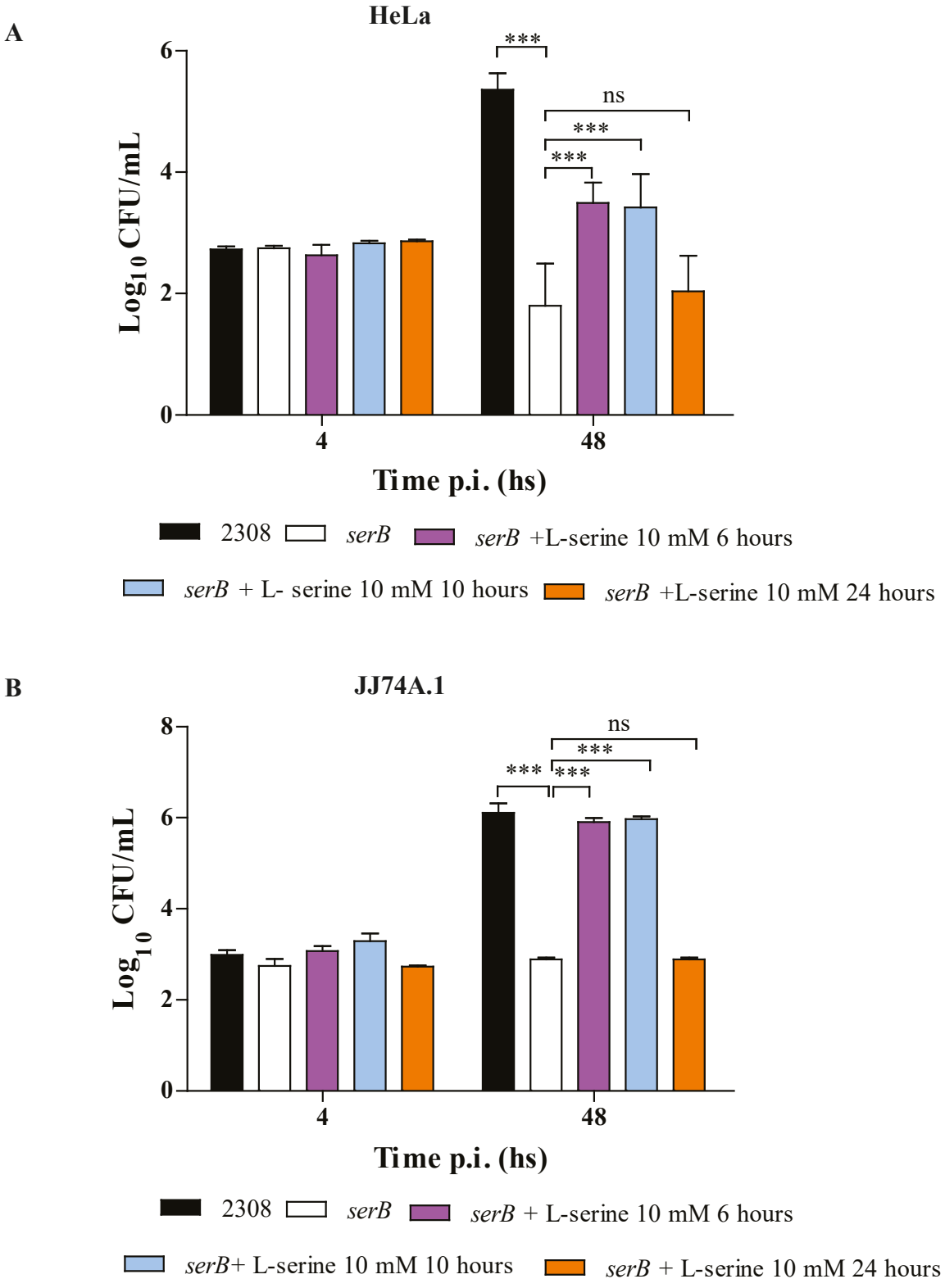

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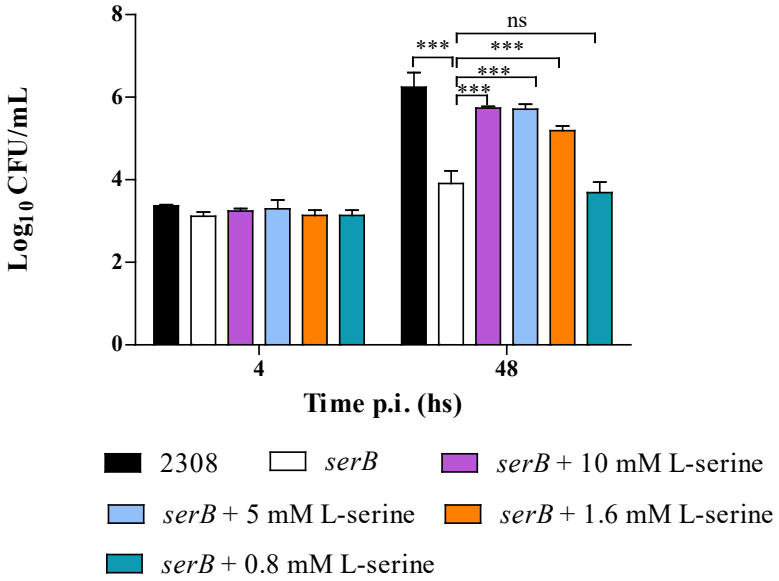


Infection and Immunity

Figure 5

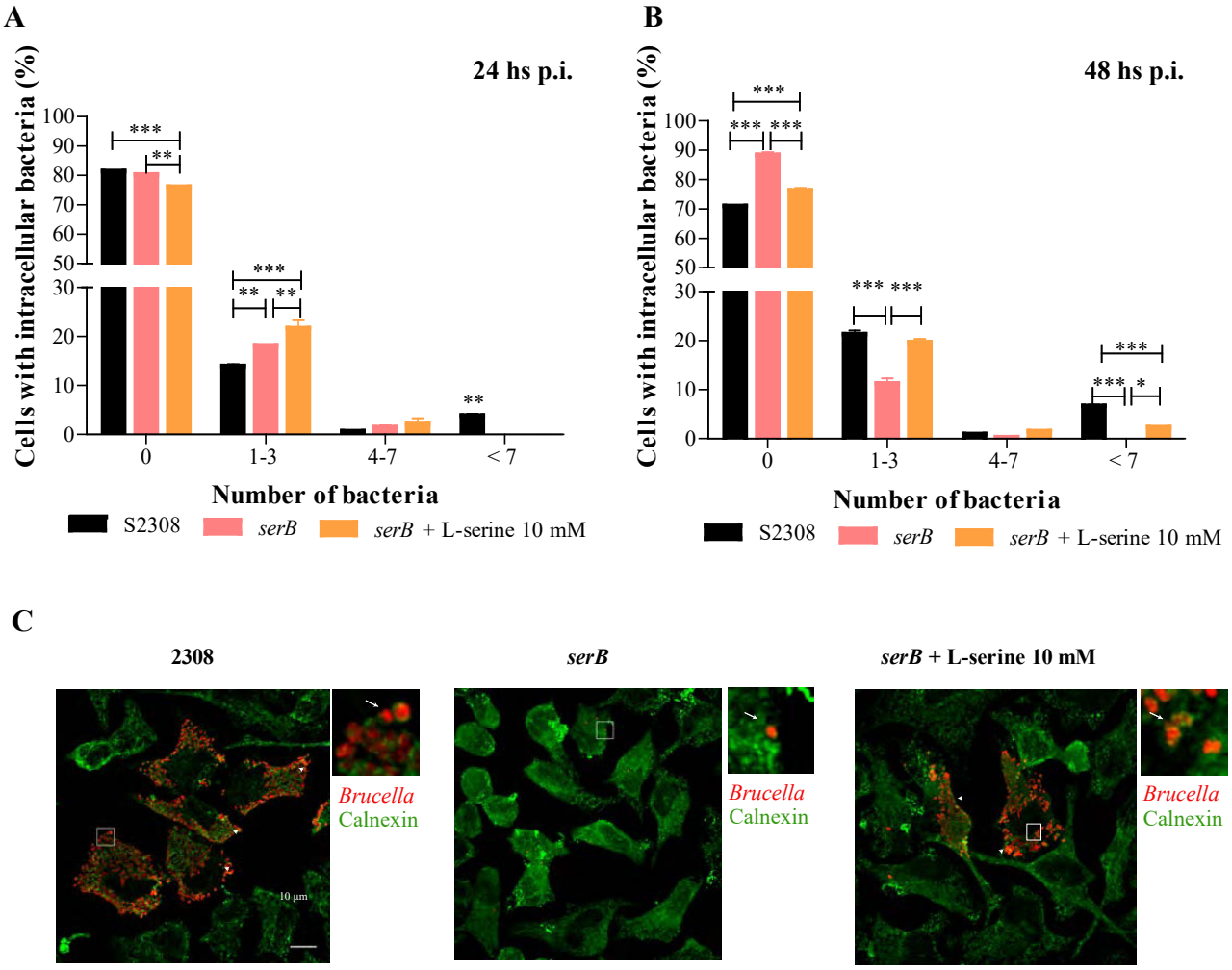
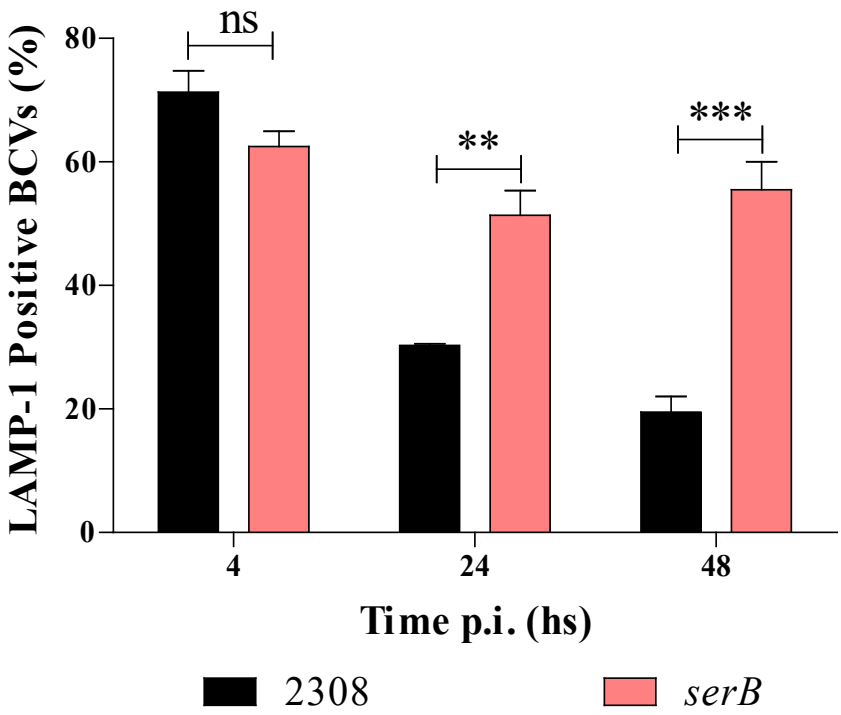


Figure 6

A



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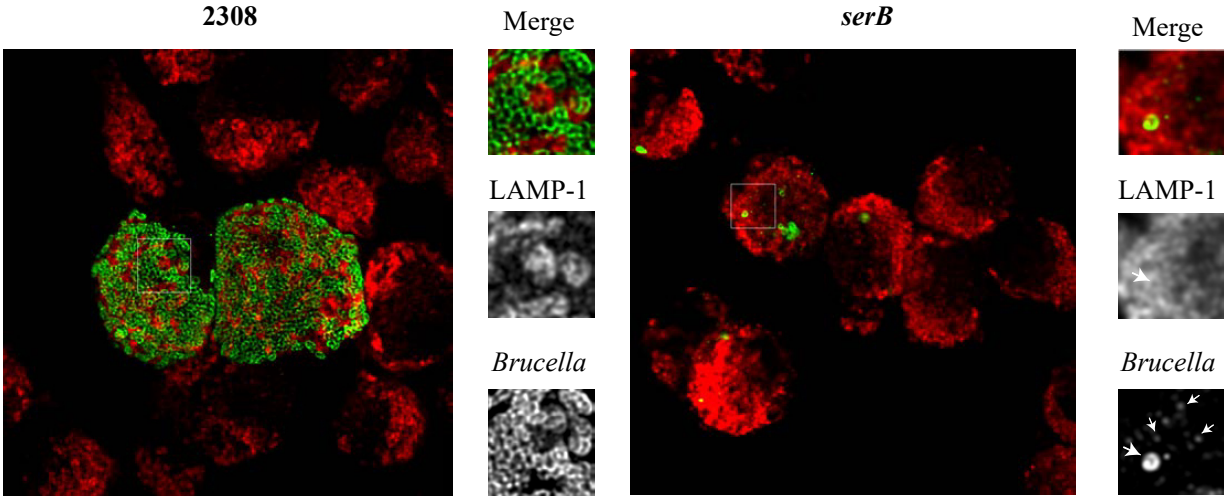

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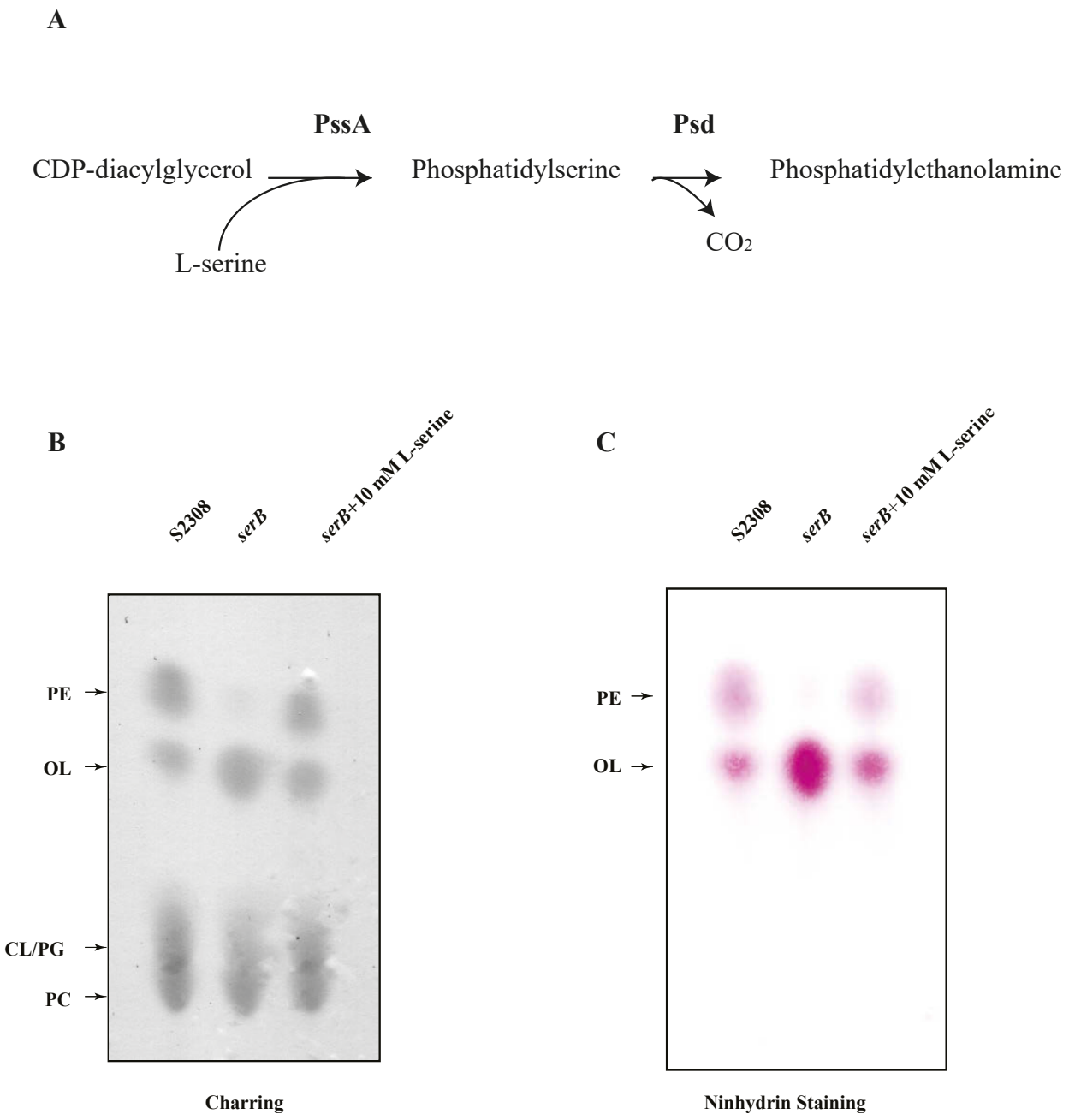

Figure 8