

Phosphatidylethanolamine Synthesis Is Required for Optimal Virulence of *Brucella abortus*[∇]

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The *Brucella* cell envelope contains the zwitterionic phospholipids phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Synthesis of PC occurs exclusively via the PC synthase pathway, implying that the pathogen depends on the choline synthesized by the host cell to form PC. Notably, PC is necessary to sustain a chronic infection process, which suggests that the membrane lipid content is relevant for *Brucella* virulence. In this study we investigated the first step of PE biosynthesis in *B. abortus*, which is catalyzed by phosphatidylserine synthase (PssA). Disruption of *pssA* abrogated the synthesis of PE without affecting the growth in rich complex medium. In minimal medium, however, the mutant required choline supplementation for growth, suggesting that at least PE or PC is necessary for *Brucella* viability. The absence of PE altered cell surface properties, but most importantly, it impaired several virulence traits of *B. abortus*, such as intracellular survival in both macrophages and HeLa cells, the maturation of the replicative *Brucella*-containing vacuole, and mouse colonization. These results suggest that membrane phospholipid composition is critical for the interaction of *B. abortus* with the host cell.

Besides their role in the formation of membrane bilayers of living cells, phospholipids have a plethora of functions, such as acting as covalent anchors for proteins and glycans, as activators or inhibitors of enzymes, in assembly of supramolecular structures, or as signal transduction mediators (17). In recent years there has been an increasing number of reports recognizing the importance of membrane lipids for the complex symbiotic or pathogenic interaction of pericellular or intracellular microorganism with the host cell. For instance, phosphatidylcholine (PC) is required for the symbiosis of *Bradyrhizobium japonicum* with the plant cell (22), and *Agrobacterium tumefaciens* mutants unable to synthesize PC have reduced transcription of genes encoding the T-DNA conjugative apparatus and are avirulent (33). In addition, the pathogen *Legionella pneumophila* requires PC for the optimal function of virulence determinants such as the flagellum (10). All these findings illustrate that the membrane lipid content is relevant for the function and/or assembly of multiprotein complexes, such as secretion systems or flagella, which are essential for the microbe-host cell interaction.

Members of the genus *Brucella* are the etiological agents of brucellosis, a worldwide-distributed zoonosis affecting both wild and domestic mammals as well as humans (14). They are facultative intracellular pathogens with the ability to invade and proliferate intracellularly in macrophages and nonprofessional phagocytes of the host, establishing long-lasting infec-

tions (5, 25). In animals, brucellosis often causes sterility in males and abortion in pregnant females, whereas in humans the disease causes undulant fever in the acute phase and severe complications in chronicity, such as endocarditis, osteoarticular complications, and neurological symptoms (11).

Once internalized, *Brucella* resides within a modified phagosome called the *Brucella*-containing vacuole (BCV), in which the bacterium survives and eventually replicates (5). The BCV undergoes a maturation process characterized by controlled and limited interaction with the endocytic pathway. Subsequently, the bacterium controls the maturation of the BCV by redirecting its traffic toward the endoplasmic reticulum (ER), where exponential intracellular replication occurs (5, 6). The VirB type IV secretion system of *Brucella* plays a central role in this process, probably by translocating effector proteins that modify the host's vesicular transport. In addition, several factors such as immune modulators, smooth lipopolysaccharide (LPS), and cyclic β -glucans also contribute to the virulence (1, 8, 18).

Although knowledge about the virulence factors that enable *Brucella* to survive inside host cells is increasing, little is known about how membrane lipid composition contributes to the interaction with the host cell. The structure and composition of the *Brucella* cell envelope (CE) are quite peculiar. The *Brucella* membrane is composed of phosphatidylethanolamine (PE), PC, ornithine lipid (OL), cardiolipin (CL), and phosphatidylglycerol (PG) (7, 9, 31). In previous work we demonstrated that in brucellae, PC synthesis occurs exclusively via the novel PC synthase pathway (Pcs) and that the endogenous methylation pathway for PC formation is not functional. Consequently, the pathogen depends on choline synthesized by the host cell to form one of its main phospholipids. The absence of

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PC in the CE is compensated for by increasing the amounts of the other main zwitterionic phospholipid, PE. Although the membrane level of PC does not affect some key virulence traits of *Brucella* such as invasion, intracellular traffic, brucellosome formation, and intracellular replication, its presence is necessary directly or indirectly to sustain a chronic infection in mice (7). In view of the importance of the membrane lipid composition for *Brucella* pathogenesis, in the present work we analyzed PE biosynthesis in *Brucella abortus* and the role it plays in membrane function and virulence by disrupting the phosphatidylserine synthase gene (*psaA*), whose product is responsible for the first committed step in PE synthesis.

MATERIALS AND METHODS

Media and growth conditions. *B. abortus* strains were grown at 37°C on tryptic soy broth (TSB), tryptic soy agar (TSA), or Gerhardt-Wilson (GW) minimal medium (13) supplemented with 100 µM of choline dihydrogen citrate (Sigma-Aldrich Co.) when needed. Media were supplemented with the appropriate antibiotics at the following concentrations: kanamycin (Km), 50 µg ml⁻¹; gentamicin (Gm), 5 µg ml⁻¹; chloramphenicol (Cm), 20 µg ml⁻¹; and ampicillin (Ap), 50 µg ml⁻¹. For *Escherichia coli* strains, antibiotics were used at the following concentrations: Km, 50 µg ml⁻¹; Gm, 20 µg ml⁻¹; Cm, 20 µg ml⁻¹; and Ap, 100 µg ml⁻¹.

Cloning, gene disruption, and generation of mutant strains. A DNA fragment of 847 bp containing the *psaA* gene of *B. abortus* 2308 (BAB1_0470) was amplified from genomic DNA using primers *psaUP* (5'-GCATAAGCCGATGGAAA CGCCT-3') and *psaDOWN* (5'-TCAATGTGTCACCGGACGAGGCT-3') and Platinum *Taq* DNA polymerase (Invitrogen). The amplicon was ligated into pGemTeasy (Promega Corp.) to generate the intermediate vector pGemT-*psaA*. This vector was subsequently digested with *NarI* (New England Biolabs), blunted with T4 DNA polymerase (New England Biolabs), and ligated to a Km resistance cassette from pUC4K (24) to generate the plasmid pGemT-*psaA::kan*. This vector was introduced into *B. abortus* 2308 by electroporation to obtain the corresponding knockout mutant. Double recombination events (Km^r Ap^r) were selected, and the corresponding gene knockout was confirmed by genomic PCR. The strain was the *B. abortus* *psaA* mutant.

To generate the pBBR4-*psaA* complementing plasmid, the *psaA* gene was excised from pGEMT-*psaA* by digestion with *ApaI* and *PstI* enzymes and subcloned into the pBBR4 replicative vector under control of the *lac* promoter. To perform the knock-in strategy, the *psaA* gene was excised from pGEMT-*psaA* by digestion with *NotI* and subcloned into a modified pBlueScriptKS-*sacB/R* vector (27). The resulting plasmid was introduced into the *B. abortus* *psaA* mutant by electroporation, and double recombination events were selected (Ap^r and sucrose resistance). Restoration of the *psaA* locus was confirmed by PCR and sequence analysis. The resulting strain was called the *B. abortus* *psaA* KI mutant.

In vivo labeling of *B. abortus* with [¹⁴C]acetate and quantitative analysis of lipid extracts. The lipid compositions of *B. abortus* 2308, the mutant, and the complemented strains were determined by labeling with sodium [¹⁴C]acetate. The strains were grown overnight in GW medium, and the cultures were washed with GW minimal medium and used to inoculate 12 ml of GW minimal medium (with or without choline) at an optical density at 600 nm (OD₆₀₀) of 0.1. After the addition of 2 µCi of sodium [¹⁴C]acetate (56.50 mCi/mmol; New England Nuclear), cultures were incubated to an OD₆₀₀ of 0.8. The cells were harvested by centrifugation. Lipids were extracted according to the method described previously by Bligh and Dyer (2) 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 exposure to Biomax Kodak films, lipids were visualized by iodine staining. Spots corresponding to labeled lipids were scraped from the plate, and the radioactivity was quantified in a scintillation counter with 500 µl Eco-Lite scintillation liquid (ICN Biomedicals).

Growth inhibition assay. Two Whatman filter discs (6-mm diameter) containing 15 µl of 1% (vol/vol) Sarkosyl (Sigma-Aldrich Co.), 1% (wt/vol) sodium dodecyl sulfate (Invitrogen Co.), or 1% (vol/vol) Zwittergent 3-16 (Sigma-Aldrich Co.) were placed on TSA plates seeded with the different *Brucella* strains. Plates were incubated for at least 48 h, and the diameter of the inhibition halo was quantified.

PmB assay. The bactericidal effect of polymyxin B (PmB) was tested as follows. A total of 2.5 × 10⁵ CFU of each strain were 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 Co.). Afterwards, serial dilutions were plated on TSA. The percent survival was calculated according to the CFU recovered from the control treatment.

Plasmid swap. To demonstrate the essentiality of either *pcs* or *psaA*, we developed a plasmid swap experiment based on the instability of two vectors from the same incompatibility group in the same bacterial population. To this end, we first generated the strain *B. abortus* *pcs::Gm psaA::kan*(pBBR4-*pcs*) by disrupting *psaA* from a *pcs* mutant strain bearing a plasmid expressing *pcs* (pBBR4-*pcs*) (7). The resulting strain was able to synthesize PC but not PE and was resistant to Gm, Km, and Ap. We then introduced by conjugation the plasmid pBBR1 or pBBR1-*pcs* (encoding resistance to Cm) into this strain. Exconjugants were selected in TSA supplemented with Gm, Km, Ap, and Cm. Three independent clones from each conjugation were selected and cultivated for 20 generations in TSA with Km, Gm, and Cm. Two hundred colonies from each clone were replica plated in TSA with Km, Gm, and Ap and in TSA with Km, Gm, and Cm, and the percentage of chasing plasmid (Ap^r/Cm^r clones × 100) was calculated.

Cell culture infections. The HeLa and J774.A1 macrophage-like cell lines were maintained in Dulbecco modified Eagle medium and RPMI 1640, respectively, supplemented with 5% fetal bovine serum and streptomycin (50 µg ml⁻¹)-penicillin (50 U ml⁻¹) in a 5% CO₂ atmosphere at 37°C (all of those solutions and media were purchased from Gibco). Cells (5 × 10⁴ per well) were seeded on 24-well plates in media without antibiotics and were kept for 24 h. *B. abortus* infections were carried out at the indicated multiplicity of infection (MOI). After 30 or 120 min (for J774.A1 or HeLa cells, respectively) wells were washed three times with phosphate-buffered saline (PBS) and incubated for 60 min with fresh medium containing 50 µg ml⁻¹ Gm and 100 µg ml⁻¹ streptomycin to kill noninternalized bacteria (afterwards, antibiotics were diluted to final concentrations of 10 µg ml⁻¹ and 20 µg ml⁻¹, respectively). At the indicated times, infected cells were washed three times with PBS and lysed with 500 µl 0.1% Triton X-100 (Sigma-Aldrich Co.). The intracellular CFU counts were determined by plating serial dilutions on TSA with the appropriated antibiotic.

Antibodies and reagents. All the antibodies were diluted in 5% horse serum (Gibco) and 0.1% saponin (Sigma). The primary antibodies used were rabbit anti-*Brucella* polyclonal antibody (1:1,500), mouse immunoglobulin G anti-O-antigen monoclonal antibody (23), and mouse anti-human LAMP-1 H4A3 (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.). For DNA staining, Hoechst dye at 2 µg ml⁻¹ (final concentration) was used.

Fluorescence microscopy. Eukaryotic cells were plated on glass coverslips and infected as described above. At the indicated times the coverslips were washed with PBS, and the cells were fixed for 15 min with 3% paraformaldehyde and then blocked for 24 h with 1% horse serum–20 mM NH₄Cl solution. After immunofluorescence staining, the coverslips were mounted onto slides with FluorSave (Calbiochem). Images were made and quantitation performed in a Nikon microscope (Eclipse E600) at a magnification of ×1,000, examining at least 100 cells containing bacteria from aleatory fields. Adobe Photoshop was used to merge the microscopic images taken with SPOT software (Diagnostic Instruments).

Mouse infections. Eight-week-old female BALB/c mice were intraperitoneally inoculated with 1 × 10⁵ CFU of *B. abortus* strains in PBS. At 2 and 8 weeks postinfection (p.i.), spleens from infected mice were removed, weighed, and homogenized in 2 ml of PBS. Serial dilutions from individualized spleen were plated on TSA to quantify recovered CFU.

RESULTS

***B. abortus* synthesizes PE via the phosphatidylserine synthase pathway.** In previous work, we demonstrated that synthesis of PC in *Brucella* is independent of the precursor PE because it occurs through direct condensation of choline with CDP-diacylglycerol in a one-step reaction catalyzed by the novel enzyme PC synthase (7). Since the absence of PC impairs virulence of the pathogen, we examined the role of the other

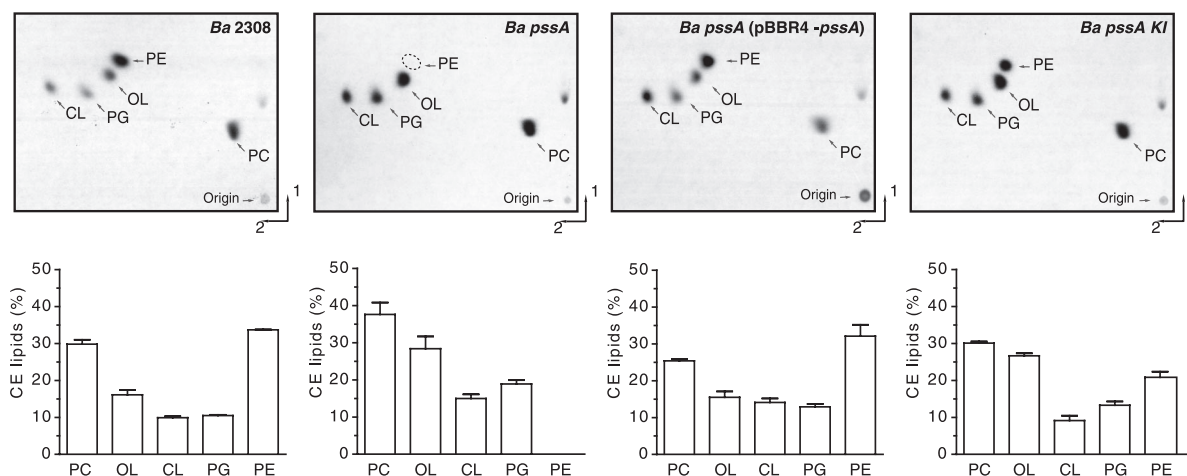


FIG. 1. The *B. abortus pssA* mutant is not able to synthesize PE. Total [^{14}C]acetate-labeled lipids were extracted from GW cultures in the presence of choline and analyzed by 2D-TLC. Lipids spots corresponding to CL, PG, OL, PE, and PC are indicated. A dashed circle highlights the absence of a PE spot in the *B. abortus pssA* mutant. The relative percentage of lipids for each strain is represented in the graph. Data shown are means and standard deviations for triplicate samples.

main zwitterionic phospholipid, PE, in the interaction with the host.

In bacteria, the first step of PE synthesis is catalyzed by phosphatidylserine synthase (PssA) (15). Analysis of the *Brucella* genome sequences revealed the presence of orthologs of *pssA* from *Sinorhizobium meliloti*, a gene whose product is a CDP-diacylglycerol serine *O*-phosphatidyltransferase responsible for PE synthesis (29).

The *B. abortus* 2308 gene BAB1_0470 (gi. 3788867) encodes a 278-amino-acid protein that shares 70% identity with the PssA from the related endosymbiont *S. meliloti*. The protein has eight putative transmembrane regions and belongs to subclass II of phosphatidylserine synthases (20).

To determine whether the product of BAB1_0470 is implicated in PE biosynthesis, an insertion mutant in the corresponding locus was generated and named the *B. abortus pssA* mutant. The membrane lipid compositions of *B. abortus* 2308 (wild type), the *B. abortus pssA* mutant, and the complemented *B. abortus pssA*(pBBR4-*pssA*) and *B. abortus pssA* KI strains, grown in a defined medium in the presence of sodium [^{14}C]acetate, were analyzed by 2D-TLC (Fig. 1, upper panel). It was observed that disruption of *pssA* abrogated PE synthesis, indicating that the phospholipid is formed via synthesis of the precursor phosphatidylserine by the action of PssA. Besides the absence of the spot corresponding to PE in the *B. abortus pssA* mutant, no other difference was observed between the wild type and the mutant strain. Synthesis of PE in the *pssA* mutant was recovered by knock-in or by ectopic expression of the wild-type copy of the gene, confirming that the product of BAB1_0470 is the phosphatidylserine synthase. The absence of PE was compensated for by increasing the amounts of the other zwitterionic lipid PC (37.6% versus 29.8%), OL (28.4% versus 18.1%), and the anionic CL/PG (34% versus 20%) (Fig. 1, lower panel).

The *B. abortus pssA* mutant requires choline supplementation for growth. To analyze whether the absence of PE affects growth of *B. abortus*, the generation time in rich complex medium (TSB) or in defined medium (GW) was determined.

No differences in the growth constants were observed between the mutant and the wild-type strains grown in TSB (data not shown). However, in the minimal defined medium GW, the growth of the *B. abortus pssA* mutant was severely impaired (Fig. 2). Given that in media without choline supplementation, *B. abortus* is unable to synthesize PC (7), a possible explanation for the drastic growth phenotype of the *B. abortus pssA* mutant might be the lack of the two major zwitterionic lipids in the membrane. Alternatively, as was observed for a PE-deficient *E. coli* strain, the *B. abortus pssA* mutant could require bivalent cation supplementation to grow (12). The growth defect was not rescued by supplementation of GW with 50 mM MgCl_2 (Fig. 2) or CaCl_2 (not shown). When 10 μM or 100 μM choline was added, the growth defect of the *B. abortus pssA* mutant was rescued, but the growth rate was lower than that of the wild type (30 h for the mutant versus 17 h for the wild type) (Fig. 2). These results indicate that synthesis of PC is required for

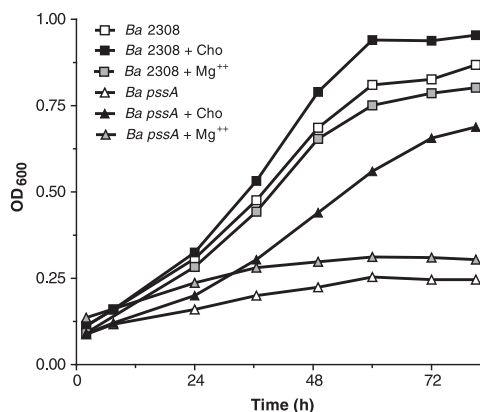


FIG. 2. The *B. abortus pssA* mutant needs exogenous choline for growth. *B. abortus* 2308 and the *B. abortus pssA* mutant were grown in GW, in GW with 100 μM choline (Cho), or in GW with 50 mM MgCl_2 . At the indicated times, aliquots were taken to measure OD_{600} . The *pssA* mutant was able to grow only when choline was added.

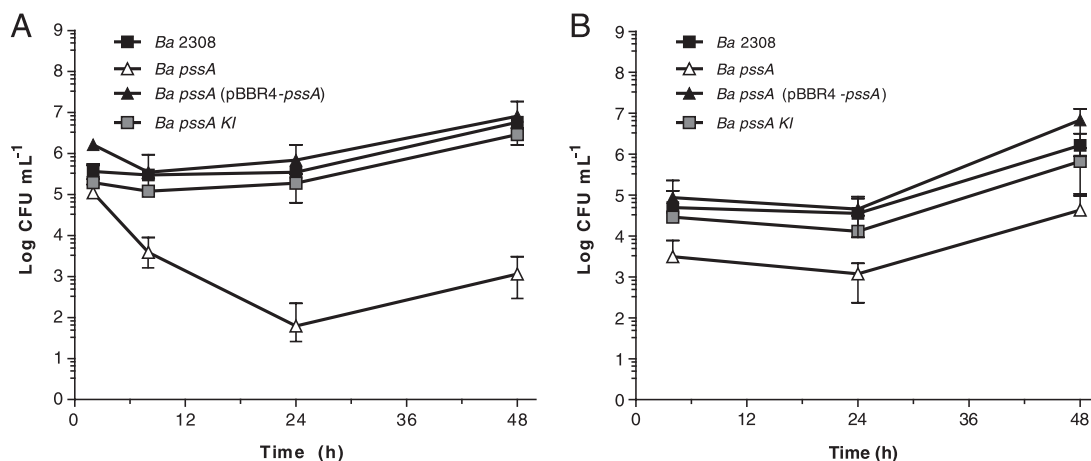


FIG. 3. The *B. abortus pssA* mutant has defective intracellular survival. Kinetics of intracellular survival of the wild type, the *B. abortus pssA* mutant, and the complemented *B. abortus pssA*(pBBR4-*pssA*) and *B. abortus pssA* KI strains in J774A.1 macrophage-like cells (MOI, 50:1) (A) and HeLa cells (MOI, 500:1) (B) are shown. Intracellular bacterial CFU numbers were determined after lysis of infected cells as described in Materials and Methods. In both professional and nonprofessional phagocytic cells, the absence of PE severely affected the survival during the first 24 h p.i. Each determination was performed in duplicate, and values are the means \pm standard deviations from three independent experiments.

growth of the *B. abortus pssA* mutant, and they suggest that either PE or PC is essential for viability. Indeed, in spite of several efforts, we were unable to construct a *B. abortus pcs pssA* double mutant. To assess whether *Brucella* viability depends on the two zwitterionic phospholipids, we first generated the *B. abortus pcs::Gm pssA::kan*(pBBR4-*pcs*) strain by disrupting *pssA* from a *pcs* mutant strain bearing a plasmid expressing *pcs* (pBBR4-*pcs*) (7). Using this strain, we attempted to swap pBBR4-*pcs* with an identical vector encoding a different selectable marker expressing or not expressing *pcs*. The pBBR4-*pcs* vector was easily replaced when pBBR1-*pcs* was used (90% of the colonies obtained were Ap^s Cm^r), whereas no colonies were recovered using pBBR1 without the insert. Thus, the requirement of a plasmid bearing a copy of *pcs* in the double mutant indicates that synthesis of either PC or PE is essential for *Brucella* viability.

The *B. abortus pssA* mutant has altered membrane properties. To evaluate whether the absence of PE alters membrane properties, we analyzed the sensitivity of the *pssA* mutant to several detergents and polycationic peptides. The *B. abortus pssA* mutant showed an increased sensitivity to the anionic detergents sodium dodecyl sulfate and Sarkosyl, whereas it was more resistant to Zwittergent 3-16 than the wild type (data not shown). Differences in sensitivity to nonionic detergent such as Triton X-100 were not significant. In addition, we did not detect any difference in the sensitivity to low pH, low temperature, or microaerobic growth conditions between the wild type and the *pssA* mutant. The *Brucella* CE is normally resistant to the bactericidal action of polycationic peptides such as PmB. However, the survival of the *B. abortus pssA* mutant was severely affected by PmB in a dose-dependent manner. This defect was not related to major changes in the LPS structure of the *pssA* mutant, since it was indistinguishable from that of the wild type or the *pcs* mutant as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis (data not shown). Together, these results indi-

cate that the absence of PE affects the CE stability of *Brucella*.

Absence of PE affects the ability of *Brucella abortus* to sustain a productive interaction with the host cell. To assess whether the absence of PE affects some traits of *Brucella* virulence, such as adhesion, internalization, trafficking, and replication within both professional and nonprofessional phagocytes, a Gm protection assay was performed by infecting J774A.1 macrophages with the *B. abortus pssA* mutant, the wild type, or both complemented strains. As shown in Fig. 3A, the *B. abortus pssA* mutant showed a marked defect in intracellular survival characterized by an exponential drop of the intracellular CFU counts (3 log₁₀ units) during the first 24 h of culture in comparison with the wild type. Afterwards, the CFU increased exponentially with growth rates comparable to that of the wild type, thus indicating that a surviving fraction of the intracellular *B. abortus pssA* mutant cells were able to replicate. As expected, the defect was completely reversed in both complemented strains. When a cell line with less bactericidal activity, such as HeLa, was used, a similar result was observed (Fig. 3B). Taken together, these results strongly suggest that the absence of PE does not influence intracellular growth but may affect the internalization of *Brucella* and/or the biogenesis of the intracellular replicative vacuole. Deficiency in the internalization of the *B. abortus pssA* mutant was excluded, since no differences in the distribution of bacteria per infected cell at 1 hour of infection were observed (around 50% of intracellular bacteria for both the *B. abortus pssA* mutant and the wild type) (data not shown).

Next we examined the biogenesis of the BCV in HeLa cells by scoring the recruitment kinetics of the late endosome/lysosome glycoprotein LAMP-1 (5, 25). As was widely reported, the recruitment kinetics of LAMP-1 were biphasic, with LAMP-1 being rapidly acquired and then gradually excluded from the wild-type BCVs (Fig. 4E). At 24 h p.i., the wild type was able to promote the maturation of the replicative BCV and proliferated in LAMP-1-negative compartments (Fig. 4B). In

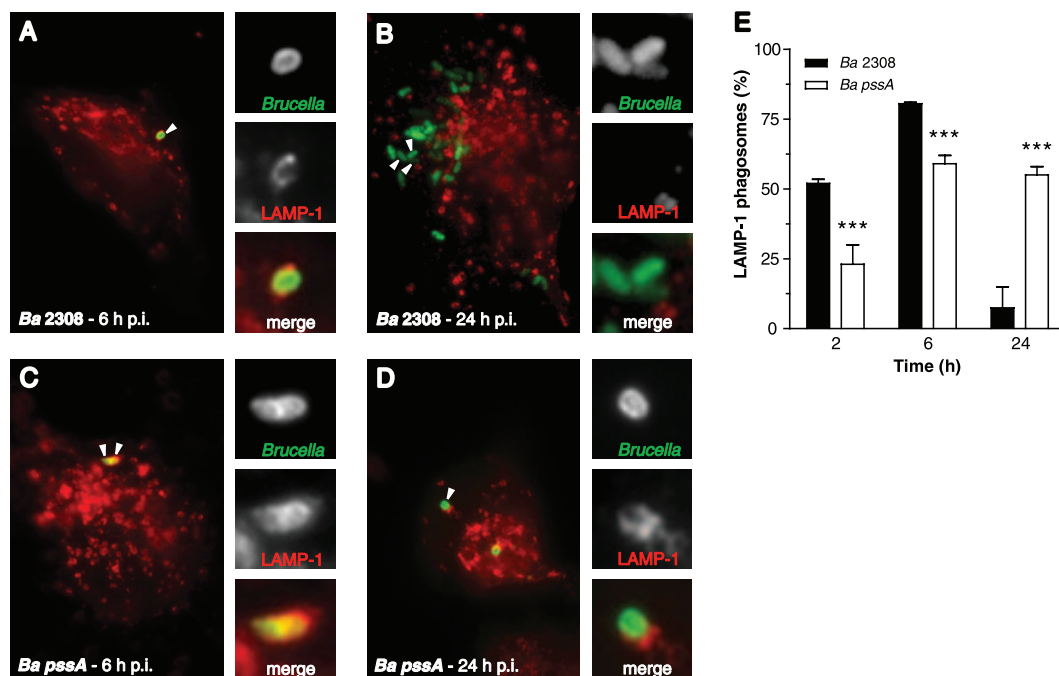


FIG. 4. The absence of PE impairs trafficking in the host cell. (A to D) Representative epifluorescence micrographs of infected HeLa cells at 6 and 24 h p.i. (panels A/C and B/D, respectively). Infections were done with the wild type (A and B) or the *pssA* mutant strain (C and D), and HeLa cells were labeled for LAMP-1 (red) or *Brucella* (green) as described in Materials and Methods. Arrowheads indicate the bacterium and the surrounding area which is magnified ($\times 3$) in the insets. (E) Quantification of the LAMP-1 marker acquired by BCVs. Immunofluorescence stains of HeLa infections were made as described in Materials and Methods. The percentage (mean \pm standard deviation) of LAMP-1-positive BCVs is indicated for the wild type and the *pssA* mutant strain. ***, $P < 0.001$, Mann-Whitney test.

contrast, LAMP-1 was progressively recruited to the *B. abortus pssA* mutant BCVs during the first 6 h p.i. and thereafter reached a plateau (Fig. 4E) ($59.0\% \pm 4.2\%$ at 6 h p.i. and $55.0\% \pm 5.0\%$ at 24 h p.i.), concomitant with the bacterial killing observed during the first 24 h p.i. (Fig. 4). Indeed, replicative LAMP-1-negative BCVs were barely detectable at 24 h p.i. (Fig. 4D). However, from 24 to 48 h p.i., a small fraction of intracellular viable *B. abortus pssA* mutant cells were able to promote maturation of the ER-derived replicative BCV and multiplied in calreticulin-positive organelles similarly to the wild type (data not shown). Hence, the absence of PE leads to a defect in the maturation of the BCV which affects the ability of *Brucella* to efficiently avoid the intracellular killing.

The membrane PE content is critical for virulence in mice.

The increased sensitivity to polycationic peptides and the inability of the mutant to achieve a productive infection in cell cultures led us to evaluate the virulence of the *B. abortus pssA* mutant in the murine infection model. Mice were inoculated intraperitoneally with the wild type, the *B. abortus pssA* mutant, and the complemented *B. abortus pssA* KI strain. The *B. abortus pssA*(pBBR4-*pssA*) strain was excluded from the analysis since we observed plasmid instability without antibiotic selection.

Figure 5 shows the recovered CFU per spleen at 2 and 8 weeks p.i. The *B. abortus pssA* mutant was significantly attenuated in comparison to the wild type or the complemented strain ($P = 0.0159$ at 2 weeks p.i.; $P = 0.0286$ at 8 weeks p.i.), showing 10-fold-lower spleen CFU counts. The colonization

defect of the *B. abortus pssA* mutant was concomitant with reduced splenomegaly, a hallmark of *Brucella* infection (data not shown). These results indicate that *Brucella* requires PE to achieve an efficient infection in the mammal host.

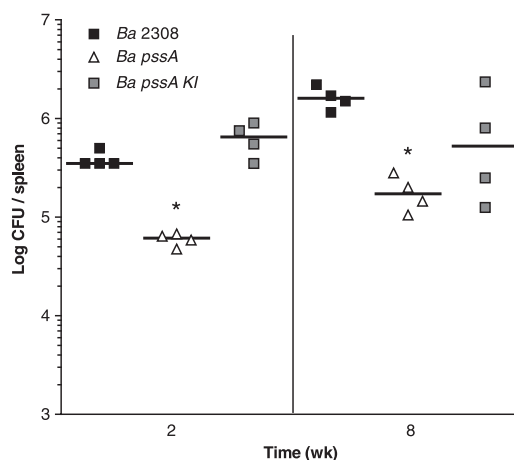


FIG. 5. The *B. abortus pssA* mutant is defective for spleen colonization. BALB/c mice were inoculated intraperitoneally with the wild type, the *pssA* mutant, or the complemented knock-in (KI) strain as indicated in Materials and Methods. Individual spleen CFU values were plotted, and the horizontal bars represent the median CFU for each treatment group. *, $P < 0.05$ (compared to the group that received the wild-type strain), Kruskal-Wallis test.

DISCUSSION

In this work we established that bacterial phospholipid composition is critical for the interaction of *Brucella abortus* with the host cell. The *Brucella* CE is unusual because it is composed of PC, PE, and OL as the main membrane lipids (32). In previous work we demonstrated that synthesis of PC in *B. abortus* occurs through the PC synthase pathway and that the endogenous methylation pathway from the precursor PE is not functional (7). Interestingly, *Brucella* requires PC for full virulence, since its absence affects the ability of the pathogen to infect mice. Here, we report the isolation of a *Brucella abortus* strain deficient in the synthesis of PE and examine its effect on membrane stability and interaction with the host. As was observed in other *Alphaproteobacteria*, such as *Sinorhizobium spp.* (29) and *Agrobacterium spp.* (16), the lack of PE does not affect the growth of *Brucella* in rich complex medium. However, in minimal defined medium, the *B. abortus pssA* mutant showed a strong dependence on choline for growth, indicating that PC synthesis is essential in a PE-deficient background. Therefore, PE and PC seem to be interchangeable for viability of *Brucella*. Several observations support this conclusion: (i) if the synthesis of PC is blocked in the *pssA* strain by omitting choline from the medium, they fail to grow, and this defect cannot be rescued by Mg^{2+} supplementation (Fig. 2); (ii) despite numerous efforts, we were unable to construct a double *pssA pcs* mutant; and (iii) the plasmid swapping data indicate that either PC or PE is essential. The dependence on choline supplementation for growth when synthesis of PE is abrogated is in line with the observation that *Brucella* needs exogenous choline to synthesize PC (7, 19) and implies that the pathogen is auxotrophic for choline. Recently, it was postulated that *B. abortus* is able to synthesize choline de novo (9). However, this hypothesis does not fit with several observations: (i) the dependence of *Brucella melitensis* on choline but not *S*-adenosylmethionine for PC synthesis (19), (ii) the absolute dependence of *B. abortus* on the *Pcs* pathway for PC formation (7), and (iii) the requirement of choline supplementation for the growth of the *B. abortus pssA* mutant (Fig. 2). All the above data support the hypothesis that *B. abortus* is auxotrophic for choline, although more studies are necessary to draw unambiguous conclusions.

The *B. abortus pssA* mutant showed altered envelope properties characterized by high sensitivity to the disrupting action of anionic detergents as well as elevated resistance to zwitterionic ones. Notably, the mutant was very sensitive to polycationic peptides, and this defect was not related to major changes in the structure of the LPS. However, we cannot rule out minor changes in LPS structure that could be relevant for PmB resistance. All these observations strongly suggest that the absence of PE is responsible for CE perturbations associated with the effects of detergents and membrane-disrupting peptides.

Although it is not required for vegetative growth, PE is critical for *Brucella* virulence. The *B. abortus pssA* mutant is defective in promoting the maturation of the replicative BCV, as illustrated by the presence of the late endosomal/lysosomal LAMP-1 marker decorating more than 50% of the BCV at 24 h p.i. (Fig. 4). This defect explains the deficient intracellular performance of the mutant in both professional and nonprofessional phagocytes (Fig. 3). The *B. abortus pssA* mutant was attenuated in the mouse model of infection, probably as a

consequence of the high sensitivity to polycationic peptides and the traffic defects (Fig. 5). These findings, and our previous report showing that *Brucella* needs PC for full virulence (7), support the concept that membrane lipid composition plays an important role in the *Brucella*-host cell interaction. A likely explanation is that the lipid composition influences the structure or function of certain bacterial surface protein complexes needed to survive inside the cell. There are several reports describing how lipid composition influences the function or assembly of multiprotein complexes such as flagellum or secretion systems. In *E. coli*, PE is essential for the function of the electron transfer chain (21), for motility and chemotaxis (26), and for assembly and function of the lactose permease (3, 4). A PC-defective mutant of *Agrobacterium tumefaciens* has a loss of virulence due to reduced transcription of the VirB apparatus (33). In *Legionella pneumophila*, a mutant unable to form PC is avirulent, and this defect could be attributed to the loss of flagellin (10).

The major virulence factor of *Brucella* is the type IV secretion system encoded by the *virB* operon, which is essential for the maturation of the BCV and the establishment of an ER-derived vacuole where bacterial replication occurs (6, 28, 30). It was postulated that the *Brucella* VirB system delivers effectors to the host cell in order to control the fate of the BCV, but the nature of the putative effector(s) has not yet been identified. Given the reduced efficiency of the *B. abortus pssA* mutant in avoiding degradation in phagolysosomes and the establishment of the intracellular replicative vacuole, it is tempting to speculate that the membrane lipid composition could affect the assembly or function of the VirB machinery. To date, the lack of a model system to check the function of the *Brucella* VirB system precludes us from directly testing whether a link between the membrane lipid composition and the VirB apparatus exists. Independently of the molecular mechanism involved, the altered membrane properties, the defects in intracellular traffic, and the attenuated virulence in mice clearly indicate that the membrane lipid composition is critical for the virulence of *Brucella*. Further work is required to understand how phospholipids influence the *Brucella*-host cell interaction.

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