A Phospholipase A1 modulates cell envelope phospholipid content of Brucella melitensis, contributing to polymyxin resistance and pathogenicity

Running title: B. melitensis Phospholipase A1 alters cell envelope phospholipids

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ABSTRACT

A subset of bacterial pathogens, including the zoonotic Brucella species, are highly resistant against polymyxin antibiotics. Bacterial polymyxin resistance has been attributed primarily to modification of lipopolysaccharide, however it is unknown what additional mechanisms mediate high-level resistance against this class of drugs. This work identified a role for the B. melitensis gene bveA (BMEII0681), encoding a predicted esterase, in the resistance of B. melitensis to polymyxin B. Characterization of the enzymatic activity of BveA demonstrated that it is a Phospholipase A1 with specificity for phosphatidylethanolamine (PE). Further, lipidomic analysis of B. melitensis revealed an excess of PE lipids in bacterial membranes isolated from the bveA mutant. These results suggest that by lowering the PE content of the cell envelope, BveA increases resistance of B. melitensis to polymyxin B. BveA was required for survival and replication of B. melitensis in macrophages, as well as for persistent infection in mice. BveA-family esterases are encoded in the genomes of alphaproteobacterial species that co-exist with polymyxin-producing bacteria in the rhizosphere, suggesting that maintaining a low PE content of the bacterial cell envelope may be a shared persistence strategy for association with plant and mammalian hosts.

INTRODUCTION

The polymyxin class of antibiotics, produced by soil bacteria such as Paenibacillus polymyxa (formerly Bacillus polymyxa) was abandoned for therapy in the 1970’s in favor of newer drugs. However, with emerging multi-drug resistance bacteria such as Acinetobacter baumanii and Klebsiella pneumoniae (1), rehabilitation of these drugs, most notably Polymyxin B (PmB) and Colistin (Polymyxin E) has been proposed as a last line treatment for infections with antibiotic-resistant Gram-negative bacteria especially in critical care settings (2-4).
Polymyxins act at the cell envelope: their initial association with outer membrane is dependent on displacing divalent cations (Mg$^{2+}$ and Ca$^{2+}$) from lipopolysaccharide (LPS). Subsequent association with the cytoplasmic membrane results in insertion of PmB, leading to formation of pore-like structures and membrane permeabilization. The ensuing disruption of the cytoplasmic membrane leads to inhibition of bacterial respiration via loss of proton-motive force and, consequently, to growth inhibition (5).

Several bacterial species are inherently resistant to polymyxins, including *Burkholderia*, *Proteus*, *Neisseria*, and *Brucella* spp., and this characteristic is actually utilized for primary isolation of *Brucella* spp. from clinical samples (6, 7). Understanding the basis for this resistance is important, as this knowledge may inform the design of novel therapies against drug-resistant organisms. Here, we identify a new mechanism for increased resistance to this class of drugs in the bacterial pathogen *Brucella melitensis* and show that this represents part of its adaptation to causing infection in mammalian hosts.

**MATERIALS AND METHODS**

**Bacterial growth conditions:** *E. coli* was grown in LB and *B. melitensis* in tryptic soy broth. For mouse infections *B. melitensis* was cultured on tryptic soy agar plus 5% blood for 3 days (8). *S. Typhimurium* strain IR715 was cultured under low magnesium conditions (10 µM MgCl$_2$) in chemically defined M9 minimal medium supplemented with 1% glucose following Groisman *et al.* (9). When needed, nalidixic acid at 25 µg/ml, ampicillin at 250 µg/ml, kanamycin at 100 µg/ml, or gentamicin at 50 µg/ml was added to media. Work with *B. melitensis* wild type and mutant strains was performed at biosafety level 3 and was approved by the Institutional Biosafety Committee at the University of California, Davis. For expression of proteins an overnight culture was grown to an optical density of 0.4 in LB with glucose and the expression
was induced with 0.5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 6 h at 37ºC with continuous shaking.

**DNA manipulation, construction of mutants and complementation**

Strains and plasmids used in this study are listed in Table 1. The *bveA* gene (BMEII0681) was identified in a bioinformatic screen for cell envelope and Type IV secretion system-related functions (published in part in(10)). It originally came to our attention, because of its similarity to two proteins in *Agrobacterium tumefaciens*, AcvB and VirJ, which are required for transfer of the Ti plasmid (11). However, closer analysis showed it to have a lipase domain, therefore, we hypothesized a role for this protein related to membrane biogenesis. The *bveA* mutant was generated by allelic exchange. Regions up and downstream of BMEII0681 (*bveA*) were amplified using the primer sets H1-For (5'-CTGCAGATAGCTGCGCTCCTGA-3'), H1-Rv (5'-TCTAGAGCTGCGCACTGTCTTCTATGG-3'), H2-Fw (5'-GTCGACCTTCCTGATCAGTGC-3') and H2-Rv (5'-CTGCAGGCAAATCACATGCCGT-3'), and cloning of the amplicons into pCR2.1-TOPO (Invitrogen). The Tn903 kanamycin resistance cassette (KSAC from pUC4-KSAC) was introduced between the up- and downstream fragments to generate plasmid pCR105 (Table 1). This generated a plasmid that was transferred to *B. melitensis* 16M wild type by electroporation and allelic exchange mutants of *bveA* were screened for resistance to kanamycin and loss of the plasmid-encoded ampicillin resistance. The correct position of the insertion was verified by PCR and Southern blotting of a *Hin*dIII digested chromosomal preparation of *bveA* using a *bveA*-specific probe (data not shown). The correct strain was designated CMR27 (Table 1). To complement the *bveA* mutant (CMR27), plasmid pCR108 was constructed as follows: primers CR-forward (5'-TCAGCGCGCAGGGCGCGGCGG-3') and CR-reverse (5'-CGTATTCTTTATCGTCCTGGGGTTGCG-3') were used to amplify *bveA* together with its promoter from the *B. melitensis* 16M genome. This PCR product was introduced into pCR2.1 by TA-cloning (Invitrogen) and was sub-cloned into the pBBR1MCS4 vector (12) using EcoRI to
Influence of Polymyxin B on bacterial growth

obtain the plasmid pCR108. Plasmid pCR108 was introduced into CMR27 to yield strain CMR28. However the expression of bveA in CMR28 was unstable during exposure to polymyxin B. We therefore utilized a previously described strategy for stable, single-copy chromosomal gene expression from the promoter of the secE gene encoding the preprotein translocase (13). To this end, we constructed a suicide plasmid (pTK19) by replacing the mCherry gene of pKSoriT- bla-PsecE-mCherry (kindly provided by X. DeBolle)(13) with an amplicon containing the bveA coding sequence (secE-bveA-Fr 5'-CCTGATCAGACAGAGTATGAAGAAAGAACGCGTATTCTTTATCGTCCTGGGGTTGG-3' and secE-bveA-Rv 5'-CCCTGCAGGTCGAGGTCAGCGCGCAGGGCGCGG-3') from B. melitensis 16 via Gibson Assembly cloning (NEB). All cloning steps were verified by sequencing and following electroporation the subsequent insertion of the fusion gene was confirmed by PCR.

For overexpression and purification of His-tagged BveA in E. coli we amplified the gene using the primer combination His-681-SalI-For (5'-ATAGTCGACTCAGTGGTGGTGGTGGTGGCGCGCAGGGCGCGGCGGC-3') and 0681-BamHI-Rev (5'ATAAGGATCCGAAGAAAGAACGCGTATTCTTTATCG-3'). This amplified the bveA gene with an additional in frame 6xHis-tag and two restriction sites (SalI and BamHI) that were used to clone the fragment into the pET25b(+) vector (EMD Millipore) generating the plasmid pTK07. This step fused the BveA-His6 construct to the PelB leader that enables periplasmic localization of the final fusion protein. The construct was verified by sequencing. To express MBP-BveA, we amplified bveA with the primers pMAL-Fr and pMAL-Rv (5'-GGGATCGAGGGAAGGAAGAAAGAACGCGTATTCTTTATCGTCCTGGGGTTGGC-3' and 5'-catggacatatgtgaaatTCAGCGCGCAGGGCGCGG-3') and cloned the resulting amplicon into pMALp5x (NEB) using the Gibson cloning method, to yield plasmid pTK25. Subsequent sequencing verified the correct sequence of the fusion construct.
The bactericidal effect of polymyxin B (Difco) against *B. melitensis* strains and *S. Typhimurium* IR715 was tested by incubating 1x10^6 colony-forming units (CFU) of each strain for 48 h at 37°C with 0 (control), 5, 10, 15, 50 and 150 µg/ml of PmB in TSB (pH 7.4), and subsequent enumeration of CFU on TSA.

**Sensitivity of *B. melitensis* to antimicrobials**

The killing ability of several antimicrobial substances, peptides and proteins against *B. melitensis* was investigated as described by Martinez de Tejada et al. (14) with the exception of using 25 µg of polymyxin B sulfate (Difco), magainin 2 and cecropin P (Sigma-Aldrich).

**Protein expression, periplasmic preparation and purification of BveA**

C-terminal 6xHis-tagged PelB-BveA protein was expressed in *E. coli* Origami™ 2 (DE3) pLysS (EMD Millipore) and a periplasmic fraction was obtained following the recommendations of Novagen’s *pET System Manual 11th edition*. Periplasmic fractions were concentrated using spin concentrator units with a cut off of 3 kDa (Millipore). The presence of the BveA fusion protein was verified by Western blot detection using an anti-His antibody (BioRad). An MBP-BveA fusion protein was purified from the periplasm of *E. coli* Origami™ B (DE3) pLysS (EMD Millipore) using amylose resin (NEB). The eluted protein was concentrated using a 50 kDa concentrator unit (Thermo Fisher Scientific) and the purity of the MBP-BveA protein sample was confirmed by SDS-PAGE.

**Esterase activity Assay**

Esterase activity of BveA was determined as published by Plou et al. (15) using A₂₈₀ adjusted periplasmic samples and the turbidity of the mixture was measured after 16 h incubation in standard clear bottom 96 well plates at 37°C using the FilterMax F3 (Molecular Devices) at 450 nm. To test the specificity of BveA for PE, phosphatidylcholine (PC), and phosphatidylglycerol
(PG; Sigma-Aldrich), we adopted the assay conditions but exchanged Tween80 with 1mM of purified phospholipids. To investigate the specificity of BveA against crude Brucella envelope lipids, Tween80 was substituted with 1% of a bacterial lipid extract from *B. melitensis* 16M. To this end, freshly extracted membrane lipids were re-suspended by vortexing in 20 mM Tris (pH 7.4) and the reaction mixture was incubated at 37°C for 16h and 120 hours. Phospholipase A1-specificity was detected using the substrate PED-1 (EnzChek® Phospholipase A1 Assay Kit, Life Technologies).

**Phospholipid extraction and Thin-layer Chromatography**

To obtain glycerophospholipids from *B. melitensis* wild type and *bveA*-mutant we extracted membrane lipid from OD600 adjusted liquid cultures using MTBE (Sigma-Aldrich) according to Matyash et al. (16). 2D-TLC was performed on silica gel plates (Kieselgel 60, Merck) loaded with equal amounts of lipid preparations using the solvents chloroform/methanol/water (14:6:1) followed by chloroform/methanol/acetic acid (13:5:2). Phospholipids were identified with molybdatophosphoric acid spray solution (Merck) and commercial standards (Avanti) (17). Relative amounts of the known membrane lipids were estimated by densitometry of stained 2D-TLC spots using a gray scale-, 8-bit transformed picture of the stained TLC plates with the BioSpectrumAC imaging system (UVP) and the densitometry tool of the Labworks software suite. The gray intensities for all known lipids spots were combined to calculate the percentage of the contribution of each single to the sum of all known lipid species.

**Lipidomic analysis of the *B. melitensis* cell envelope**

The contribution of PE and PC to the total phospholipid content of *B. melitensis* cell envelope was analyzed by liquid chromatography-electrospray-tandem mass spectrometry (LC-ESI-MS/MS) on a 6530 Q-TOF Mass Spectrometer (Agilent Technologies) coupled with an automated HPLC (1200 Agilent Technologies) using a 2.1x100 mm C18 column from Waters.
The MTBE-extracts were re-dissolved in methanol and analyzed in positive ionization mode. MS/MS spectra were analyzed manually using an in-house developed MS/MS database (LipidBlast) that was used to identify single lipid species (18). To correct for machine drift and to normalize the data each individual value (peak height) was divided against the sum of all peak intensities obtained in the corresponding chromatogram and multiplied by the average of all total intensities.

Cellular infection assays

J774.A1 (ATCC® TIB-67™) macrophage-like cells were propagated in DMEM (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies) and 1% nonessential amino acids (ATCC). For macrophage-survival assay, J774.A1 cells were infected with *B. melitensis* strains with a multiplicity of infection of 100 bacteria per cell and the survival of intracellular bacteria was determined after 0, 4, 24 and 48 h as reported previously (19).

Mouse infections

For mice inoculated intranasally (*i.n.*), groups (n=4-5) of 8- to 10-week old female C57BL/6J wild type mice (The Jackson Laboratory) were inoculated with 1x10⁷ CFU containing a 1:1 ratio of *B. melitensis* wild type and bveA mutant in a total volume of 10 μl sterile phosphate-buffered saline. After 1 and 4 weeks post-infection (*p.i.*), cervical lymph nodes from infected mice were removed, weighed, and homogenized in 1 ml of PBS. For intraperitoneal (*i.p.*) infections, groups of 5 C57BL/6 mice were inoculated with 0.1 ml of PBS containing 1x10⁵ CFU of either *B. melitensis* 16M or the bveA mutant (CMR27). For enumeration of bacterial tissue load, serial dilutions of tissue homogenates were spread on TSA with appropriate antibiotics. Mice were maintained in micro-isolator cages with sterile bedding and irradiated feed in a biosafety level 3 laboratory. All animal experiments were approved by the University of California Laboratory.
Animal Care and Use Committee and were conducted in accordance with institutional guidelines.

**Statistical Analysis**

All data in this work are presented as the mean and the standard error of the mean. To determine statistical significance between experimental groups at a given time point, we used either a Student’s t test on log- or arc-sin transformed data as well as Mann-Whitney test on non-parametric values. A p-value of <0.05 was considered significant.

**RESULTS**

**B. melitensis BveA confers resistance against polymyxin B (PmB)**

The remarkable resistance of *Brucella* spp. to cationic compounds such as polymyxins and host-derived antimicrobial peptides has been attributed to features of its cell envelope, including its unique lipopolysaccharide, and these properties contribute to the ability of *Brucella* spp. to infect their mammalian hosts (14, 20-22). However, since *Brucella* species with rough LPS, such as *B. canis* and *B. ovis* are also resistant to cationic antimicrobials (23), it is possible that features of *Brucella* spp. in addition to LPS contribute to this phenotype. A gene with a predicted cell envelope-associated function, *BMEII0681* (designated *bveA* for *Brucella*), was originally identified in a bioinformatic screen for *Brucella* genes with functions related to the cell envelope (partially published in (10)). *In silico* analysis revealed that *BMEII0681* was broadly conserved among *Brucella* spp., with paralogs in the soil-associated Alphaproteobacteria, including *Ochrobactrum* spp., *Rhizobium* spp., and *Agrobacterium tumefaciens*, as well as the more distantly related *Burkholderia* species associated with plant rhizospheres. To determine whether BveA functions in resistance against cationic antimicrobials, we constructed and characterized a *B. melitensis* strain deleted for *bveA*. The *bveA* mutant was indistinguishable from its wild type parent with regard to growth in
Tryptic Soy Broth (Fig. S1A), and its ability to withstand short-term treatment (20 min) with cell envelope disrupting agents including the cationic lipopeptide Polymyxin B (PmB), the antimicrobial peptides magainin 1 and cecropin P1, EDTA, lysozyme and Tween 20 at concentrations that are bactericidal for smooth E. coli K12 ATCC W1485 (14) (Fig. S1B). Additionally, the mutant revealed no obvious defect in its lipopolysaccharide composition (Fig. S1C). Considering that the antimicrobial action of PmB also induces bacteriostasis, we determined whether bveA contributes to resisting the bacteriostatic effect of PmB. To this end, we incubated 1x10⁶ cfu/ml of wild type B. melitensis 16M, and an isogenic bveA mutant (CMR27) in TSB (pH = 7.4) for 48 h with increasing concentrations of PmB to monitor the growth of the strain in the presence or absence of PmB (Fig. 1). As a reference, we used Salmonella enterica serotype Typhimurium (S. Typhimurium) strain IR715 grown under conditions that enhance its PmB resistance (9). As described previously (24), B. melitensis 16M exhibited a high level of PmB resistance compared to S. Typhimurium, with significant growth occurring at concentrations of PmB up to 150 μg/ml. In contrast, while S. Typhimurium grew in low (5 μg/ml) concentrations of PmB, at concentrations that exceeded 15 μg/ml PmB, a bactericidal effect of PmB was observed. The growth of the B. melitensis bveA mutant was inhibited at 5 μg/ml and a bactericidal effect was observed at 10 μg/ml, suggesting that BveA mediates resistance to polymyxin antibiotics. Partial restoration of PmB resistance was achieved by chromosomal expression of the bveA gene under the control of the secE promoter (TOK09; bveA<sup>restored</sup>).

BveA is a phospholipase A1 that cleaves PE in the envelope of B. melitensis

To predict the function of BveA in B. melitensis we used the Phyre2.0 engine (25) to characterize orthologs in other organisms based on similarities to solved protein structures. Table S1 shows the five best hits based on similarity at the amino acid sequence and structural levels. All these entries suggest that BveA is a putative hydrolase of the α/β2 superfamily, which
includes lipases and esterases (26). Further, in silico prediction (SignalIP (27)) predicted that BveA contains a signal peptide as well as two intra-molecular disulfide bonds (DiANNA 1.1 (28)), suggesting a periplasmic localization of the mature protein. To test for a potential esterase function for BveA, we expressed a 6xHis-tagged fusion protein of BveA in E. coli (pTK07). To this end, we obtained periplasmic fractions from E. coli carrying pTK07 or the empty vector pET25b, and determined esterase activity in these fractions, using Tween80 as a substrate. In this assay, cleavage of the ester bond linking the sorbitol backbone of Tween 80 to oleate leads to release of free oleate, yielding a precipitate with CaCl₂ that can be quantified spectrophotometrically (15). Cleavage of Tween 20 by periplasmic fractions containing BveA-6xHis, but not control fractions from E. coli carrying the empty vector (Fig. 2A), demonstrated that BveA has esterase activity. To better characterize the specificity of BveA, we determined whether fractions containing BveA-6xHis could cleave the fluorogenic phospholipase A1 selective substrate PED-1 (Fig. 2B). Both the periplasmic fraction containing BveA-6xHis and a purified MBP-BveA fusion protein (Fig. 2C), were able to cleave PED-1, demonstrating that BveA is a phospholipase A1 (Fig. 2D).

Since both in silico analysis and expression in E. coli indicated a periplasmic localization of BveA, we tested whether BveA would act on cell envelope lipids of B. melitensis. To this end, periplasmic fractions containing BveA-6xHis were incubated with phospholipids extracted from B. melitensis 16M (Fig. 2E). We observed a low activity of the enzyme after 16 h incubation, which increased with prolonged incubation times. Assuming a low amount of Brucella lipids in the assay we extended the assay duration to 120 h of incubation, and this gave comparable precipitate accumulations as observed for Tween80. Since the major lipid component of the Brucella envelope are the glycerolipids phosphatidylcholine (PC), phosphatidylethanolamine (PE) or phosphatidylglycerol (PG), we tested the activity of BveA-6xHis containing periplasmic fractions against purified PC, PE and PG (1mM). This assay detected phospholipase activity...
against PE, but when PC or PG were provided as substrates no esterase activity was detected (Fig. 2F).

BveA fine-tunes the PE content of the B. melitensis cell envelope

The apparent specificity of BveA for PE suggested that it targets PE in the B. melitensis cell envelope. To test this idea, we compared the relative amounts of the major membrane lipid species in methyl-tert-butyl ether (MTBE) extracts of B. melitensis wild type (16M), bveA mutant (CMR27), and the chromosomally restored bveA mutant (TOK09 bveA restored) by two dimensional thin layer chromatography (2D-TLC), using conditions reported previously (17). The abundance of the main membrane lipids phosphatidylcholine (PC), phosphatidylethanolamine (PE), ornithine-lipids (OL), phosphatidylglycerol (PG) and cardiolipin (CL) was determined. Individual spots were assigned according to the position of corresponding lipid standards from E. coli (Avanti-Polar; data not shown). While PC, PE, OL, PG and CL were detected in the cell envelope of wild type and bveA mutant (CMR27), and bveA restored strain (TOK09), the signal intensity of the PE spot compared to the PC spot was higher in the bveA mutant (CMR27) than in the wild type and the bveA restored strain (TOK09) (Fig. 3A, dashed arrows). Based on three biological replicates for each strain we quantified the contribution of each lipid species to the known lipid content based on densitometry of the spot intensities (Fig. S2) and calculated the ratio of PC to PE for each strain (Fig. 3B). Significantly more PE was observed in the bveA mutant (CMR27; p value = 0.018) than the wild type. When expressed as PC:PE ratio, the values were 4.6 for B. melitensis wild type, 2.1 in the bveA mutant and 3.7 for the complemented mutant (TOK09). No significant differences between strains were observed for the other major cell envelope lipid species (Table S2). These results supported specificity of BveA for PE. Compared to the wild type the lipid profile of the bveA restored strain (TOK09) was only altered in the PG levels. The significance of this result is not clear, but it may be related to expression of bveA from the strong secE promoter at its chromosomal insertion site rather than
from its own promoter. This slight alteration in lipid composition in the restored \textit{bveA} mutant (TOK09) compared to wild type may underlie the partial restoration of resistance against PmB in this strain (Fig. 1).

While densitometric quantification of 2D-TLC signals provided a first clue to changes in the abundance of PE, a more quantitative measure of lipid abundance would require specific measurement of lipid species. We there determined levels of PE and PC species in MTBE extracts of wild type and \textit{bveA} mutant by liquid chromatography-electrospray-tandem mass spectrometry (LC-ESI-MS/MS). Lipid species were identified using the LipidBlast \textit{in silico} tandem mass spectrometry database search (18), with a focus on PE and PC species. All individual identifiable PE and PC species were grouped and analyzed to determine overall changes in these phospholipids. We chose this approach because PE is used by \textit{Brucella} spp. for the synthesis of PC species (29) and thus, a non-PE specific membrane defect might be consequently reflected in changes of the PC pool. As presented in figure 3C, we again detected a significant increase in the total amount of PE species in the mutant over the wild type. In contrast, no significant difference in the amount of PC species between wild type and mutant was observed. Taken together, these results suggest that BveA modulates the phospholipid content of \textit{B. melitensis} by degrading PE, and that loss of BveA results in an increased abundance of PE in the cell envelope.

\textbf{BveA is important for interaction of \textit{B. melitensis} with mammalian hosts}

To determine whether modulation of PE in the cell envelope is important for the ability of \textit{B. melitensis} to interact with host cells, we compared the ability of the \textit{bveA} mutant (CMR27) and wild type to survive intracellularly within macrophages, using a gentamicin protection assay. To this end, we infected the murine macrophage-like cell line J774.A1 with wild type \textit{B. melitensis} strain 16M, the \textit{bveA} mutant (CMR27) and the \textit{bveA} mutant complemented with a
plasmid-encoded copy of bveA (CMR28). As presented in Fig. 4A, the survival of the bveA mutant was reduced beginning at 4 hours post infection compared to the wild type strain 16M, and this defect was restored by complementation.

We next determined whether modulation of PE abundance by BveA was required for colonization and persistence of B. melitensis in a mammalian host. To this end, we used a competitive infection assay, in which C57BL/6J mice were inoculated via the intranasal route with an inoculum containing a 1:1 ratio of B. melitensis 16M and bveA mutant. At 1 and 4 weeks after inoculation, colonization of the draining cervical lymph nodes was assessed (Fig. 4B). At 1 week post infection, threefold higher levels of B. melitensis wild type were recovered compared to the bveA mutant (CMR27). This competitive advantage was increased at 4 weeks, suggesting that modulation of membrane lipid content by BveA contributes to the fitness of B. melitensis during persistent infection.

The intranasal route of inoculation, while mimicking the natural mucosal infection route, is very inefficient at establishing infection, as dissemination to the draining cervical lymph node requires passage through a narrow infection bottleneck. We therefore performed an additional experiment in which we inoculated mice via the i.p. route with individual B. melitensis strains. The results of this experiment, shown in Fig. 4C, revealed that the bveA mutant could disseminate to the spleen initially at wild-type levels, but at 4 weeks, the mutant was recovered at an order of magnitude less than the wild type. Thus, the bveA mutant is deficient for persistence in both draining lymph nodes and systemically in the spleen.

Discussion

It is known that the cell envelope of Brucella species has a phospholipid composition that differs markedly from that of other Gram-negative mammalian pathogens (30). Two major differences between Brucella spp. and other Gram-negative bacteria such as S. Typhimurium or E. coli are the presence of PC and a remarkably small PE content –the PE content of E. coli is
80% in *E. coli*, whereas it is only 25% in *B. melitensis* (30-33). Interestingly, a link between PE abundance and antimicrobial susceptibility has been noted previously in studies with model bio-membranes. The amphiphilic cationic peptide NK2 binds preferentially to PE-rich model-membranes (34), which was related to a higher affinity of NK2 for PE than other lipids and correlated with the degree of accessibility of the lipid phosphate groups to the cationic side chains of the NK2 peptide (35).

Previous work on *B. abortus* demonstrated that disruption of the individual synthesis pathways for either PC (*pcs* and *pmtA*) (29) or PE (*pssA*) (17) resulted in reduced fitness of *B. abortus* strains *in vitro* and *in vivo* (17), demonstrating that phospholipid composition of the envelope is critical to the biology of this organism. Our results detail a mechanism by which *B. melitensis* maintains a low abundance of PE in the cell envelope via expression of the BveA phospholipase A1 enzyme. This property of the cell envelope contributes to both polymyxin resistance and to persistence in an infected host.

Although inactivation of *bveA* and the consequent increase in PE abundance did not compromise the integrity of the *B. melitensis* cell envelope as evidenced by an unchanged sensitivity to membrane-disrupting agents, it did affect its ability to survive in a mammalian host. BveA could contribute to host-pathogen interactions of *B. melitensis* either directly, by enhancing resistance to host antimicrobials, or indirectly. Since phospholipids are determinants of membrane topology (36), a change in balance between phospholipid species could affect insertion or proper function of membrane proteins involved in interaction with host cells. For example, *Agrobacterium tumefaciens* encodes two proteins of this family, VirJ (encoded on the octopine-type plasmid pTiA6) and AcvB, that play overlapping roles in tumorigenesis, by promoting T-DNA transfer to plant cells (11). Thus it is possible that esterase activity of these proteins modulates phospholipid composition of the cytoplasmic membrane to enable the proper assembly of the Type IV secretion system that mediates transfer of T-DNA and effector proteins.
The best characterized mechanism of resistance to polymyxins in bacteria is modification of the Lipid A moiety of LPS (37). The regulation and biochemistry of these processes have been described in detail for S. Typhimurium and Pseudomonas aeruginosa, which respond to the presence of antimicrobial peptides by modification of Lipid A to reduce its net negative charge, a strategy that prevents PmB binding to the bacterial surface (37-39).

Recently, part of the S. Typhimurium response to cationic peptides was shown to be palmitoylation of PG by the lipid A-modifying enzyme PagP, suggesting that modification of other membrane lipids may also play a role in defense against cationic peptides such as PmB (40).

B. abortus LPS is known to have several distinctive features that mediate its resistance to PmB, including a lack of divalent cations (14), a low degree of acyl chain fluidity (41), and the glycosylation pattern of its LPS core (22, 42). These features of LPS could be considered as a first line of defense against PmB, since B. melitensis mutants with rough LPS are killed rapidly, in contrast to the bveA mutant, which is killed after prolonged incubation with higher concentrations of PmB. Thus, it is likely that LPS of Brucella spp. mediates resistance to PmB, but during prolonged incubation small amounts can enter the cell envelope, and it would be at this point that the reduction of PE composition in the cell envelope by BveA would be important to resist formation of pore-like structures and permeabilization of the cytoplasmic membrane by PmB that result in loss of proton-motive force and inhibition of growth (5). Considering the presence of bveA orthologs (e.g. AtvA from Rhizobium tropici CIAT899 (43)) in phylogenetically related alphaproteobacterial species inhabiting the rhizosphere, reduction of PE abundance may be a conserved mechanism among this group of bacteria with relevance for bacterial-plant interactions as well as interactions with mammalian hosts.

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REFERENCES:


Figure legends:

**Figure 1.** BveA contributes to antibiotic resistance of *B. melitensis*.
Impact of polymyxin B (PmB) on the growth of *B. melitensis* wild type (Bm, ●), the bveA-mutant CMR27 (bveA, ■), the restored mutant TOK09 (BveA\textsuperscript{restored}, □) as well as *S. Typhimurium* IR715 (STM, ◆) in TSB (pH 7.4) for 48 hours at 37°C. The starting inoculum was 10\(^6\) cfu/ml (dashed line). *S. Typhimurium* IR715 was pre-incubated under conditions that induce the expression of the PhoP/Q regulon (9).

**Figure 2.** BveA is a phospholipase A1 with specificity for PE
Lipase activity against Tween80 (A) and the phospholipase A1 substrate PED-1 (B) using periplasmic fractions containing BveA (black bars) and the empty vector control (white bars). Purification (C) and lipase activity of MBP-BveA fusion protein (97kDa) (D) compared to increasing units of gelatinase, a positive control for phospholipase A1 activity (PLA\(_1\), Life technology). (E) Lipase activity of BveA against lipid extractions of *B. melitensis* cell envelope and (F) against phospholipid standards (Sigma) PC, PE and PG. Asterisks denote significant differences between BveA-containing samples and the empty vector control with a p-value < 0.01 (**), and p < 0.001 (***)

**Figure 3: BveA fine-tunes the PE-content of the *B. melitensis* cell envelope**
Membrane lipid composition of wild type *B. melitensis* (Bm) compared to an isogenic bveA mutant CMR27 (bveA) and the chromosomally restored strain TOK09 (bveA\textsuperscript{restored}). (A) 2D-TLC of lipid extracts of the cell envelope of *B. melitensis* 16M, bveA-mutant (CMR27) and the restored strain TOK09 extracted with MTBE. Phospholipids were detected with molybdatophosphoric acid spray solution (Merck). Dashed line highlight PC : PE ratio.
(B) Ratio of phospholipids PC : PE in the cell envelope of *B. melitensis* 16M (black bar), *bveA*-mutant CMR27 (white bar) and chromosomally restored *bveA*-mutant TOK09 (gray bar). Ratios were calculated based on relative densitometric abundance (percentage) of individual MBTE-extracted membrane lipids from 2D-TLC of PC, PE, OL, PG, and CL ([Fig.S2](#)). (C) Q-ToF analysis to estimate relative quantities of PE and PC in *B. melitensis* 16M (black bar) and the *bveA*-mutant CMR27 (white bar). Asterisks indicate significant differences between wild type 16M and *bveA* mutant CMR27 with a *p*-value < 0.05 (*) and *p*-value < 0.01 (**).

Figure 4. *BveA* contributes to intracellular survival of *B. melitensis* in macrophages and to persistent infection *in vivo*. (A) Intracellular replication of wild type *B. melitensis* (Bm, ○), the *bveA*-mutant CMR27 (bveA, □) and the complemented strain CMR28 (pbveA, ▲) as recovered cfu/ml at specific time points post infection in J774.A1 macrophage-like cells. (B) Colonization levels of wild type and *bveA* mutant CMR27 in cervical lymph nodes of C57BL/6J mice at 1 week (n=5) and 4 weeks (n=4) after inoculation via the i.n. route with a 1:1 mixture of WT and CMR27 (bveA). Competitive index (ratio of WT to CMR27) was calculated based on recovered cfu/g tissue. (C) Colonization levels of *B. melitensis* WT and CMR27 (bveA) after inoculation of individual strains via the i.p. route. Symbols represent values from individual mice and horizontal bars represent the geometric mean of each group. Asterisks indicate significant differences between wild type (Bm) and CMR27 (bveA) with *p*-values < 0.05 (*) and *p*<0.01 (**).
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<tr>
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Figure A: Graph showing the number of cfu/ml over time p.i. [h] for Bm, bveA, and bveA + pbveA.

Figure B: Scatter plots for log cfu/g cervical lymph node at week 1 p.i. and week 4 p.i. for Bm and bveA. Cl_{w/mut} = 3.01 for Bm and Cl_{w/mut} = 6.93 for bveA.

Figure C: Scatter plots for log cfu/g spleen at week 1 and week 4 for Bm and bveA. **P < 0.01, ***P < 0.001.