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Identification and functional analysis of the cyclopropane fatty acid synthase of *Brucella abortus*

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The brucellae are facultative intracellular pathogens of mammals that are transmitted by contact with infected animals or contaminated materials. Several major lipidic components of the brucella cell envelope are imperfectly recognized by innate immunity, thus contributing to virulence. These components carry large proportions of acyl chains of lactobacillic acid, a long chain cyclopropane fatty acid (CFA). CFAs result from addition of a methylene group to unsaturated acyl chains and contribute to resistance to acidity, dryness and high osmolarity in many bacteria and to virulence in mycobacteria. We examined the role of lactobacillic acid in *Brucella abortus* virulence by creating a mutant in ORF BAB1_0476, the putative CFA synthase gene. The mutant did not incorporate [^{14}C]methyl groups into lipids, lacked CFAs and synthesized the unsaturated precursors, proving that BAB1_0476 actually encodes a CFA synthase. BAB1_0476 promoter–*lux*AB fusion studies showed that CFA synthase expression was promoted by acid pH and high osmolarity. The mutant was not attenuated in macrophages or mice, strongly suggesting that CFAs are not essential for *B. abortus* intracellular life. However, when the mutant was tested under high osmolarity on agar and acid pH, two conditions likely to occur on contaminated materials and fomites, they showed reduced ability to grow or survive. Since CFA synthesis entails high ATP expenses and brucellae produce large proportions of lactobacillic acyl chains, we speculate that the CFA synthase has been conserved because it is useful for survival extracellularly, thus facilitating persistence in contaminated materials and transmission to new hosts.

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INTRODUCTION

Brucellosis is a zoonosis of great importance that has a heavy impact in developing countries. The causative agents belong to the genus *Brucella*, a group of α-2 *Proteobacteria* characterized by their ability to multiply in macrophages, dendritic cells and other cells of a variety of mammals. Although the brucellae do not multiply outside their hosts under natural conditions, they can grow in laboratory axenic cultures and persist in the soil and contaminated materials for weeks or even months depending upon the conditions (Huddleson, 1943; Corbel, 2006). The cell envelope plays an essential role in brucella virulence. This structure displays a number of features that hinder recognition by innate immunity, including high stability when confronted with bactericidal peptides and poorly marked pathogen-associated molecular patterns (PAMPs) in phospholipids, ornithine lipids, lipopolysaccharides and lipoproteins (Barquero-Calvo *et al.*, 2007; Freer *et al.*, 1996; Martínez de Tejada *et al.*, 1995; Martínez de Tejada & Moriyón, 1993; Moriyón & Berman, 1982; Palacios-Chaves *et al.*, 2011). Interestingly, all these lipidic components carry large proportions of lactobacillic acyl chains (Gómez-Miguel & Moriyón, 1986; Thiele & Schwinn, 1973; Velasco *et al.*, 2000). Lactobacillic acid [*cis*-11,12-methylene-octadecanoic (C19cyc)] is a long chain cyclopropane fatty acid (CFA) and, although CFAs are produced by other bacteria under unfavourable conditions, the most common ones [*cis*-9,10-methylene hexadecanoic acid (C17cyc) and other C17 CFAs] have shorter acyl chains.

CFAs result from the modification of pre-existing unsaturated acyl chains by addition of a methylene group to the

Abbreviations: C17cyc, *cis*-9,10-methylene hexadecanoic acid; C19cyc, *cis*-11,12-methylene-octadecanoic; CFA, cyclopropane fatty acid; PAMP, pathogen-associated molecular pattern; SAM, *S*-adenosyl-L-methionine.

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carbon–carbon double bonds (Wang & Cronan, 1994). This reaction is carried out by soluble CFA synthases that use *S*-adenosyl-L-methionine (SAM) as the methylene donor. CFA synthesis typically starts at the onset of the stationary phase of growth and, consistent with this, it has been proposed that CFAs contribute to adaptation of the envelope to unfavourable conditions, including low oxygen tension, nutrient limitation, hydrogen peroxide, acid pH, desiccation and high ionic strength and/or osmolarity. Moreover, several pathogenic *Mycobacterium* species carry CFA synthases and methyltransferases that alter the PAMP of mycolic acids and are thus important in hampering innate immunity recognition (Barkan *et al.*, 2010; Dao *et al.*, 2008; Glickman *et al.*, 2000; Rao *et al.*, 2005, 2006). It is, therefore, conceivable that lactobacillic acid could play a role in brucella virulence by contributing to adapting the envelope to intracellular environments, as proposed by Roop *et al.* (2003) or to reduce the PAMP of envelope molecules. In this work, we report on the identification of the gene coding for the *B. abortus* CFA synthase and on the effects of its dysfunction.

METHODS

Bacterial strains and growth conditions. *Brucella abortus* 2308 Nal^R (BAB-parental) is a smooth virulent strain used in previous studies (Conde-Álvarez *et al.*, 2006; Palacios-Chaves *et al.*, 2011). This strain and the mutant, revertant and complemented strains resulting from the genetic manipulations described below were characterized following standard *Brucella* typing procedures (colony morphology, crystal violet exclusion, catalase, oxidase, urease and acriflavine agglutination tests, sensitivity to Tb, Wb, Iz and R/C phages, agglutination with anti-A and anti-M monospecific sera, CO_2 and serum dependence, and susceptibility to thionine blue, fuchsine and safranin) (Alton *et al.*, 1988). Bacteria were routinely grown in tryptic soy broth (TSB) (bioMérieux) or on tryptic soy agar (TSA) either plain or supplemented with 25 μg nalidixic acid (Nal) ml^{-1} and/or 50 μg kanamycin (Km) ml^{-1} and/or 20 μg chloramphenicol (Cm) ml^{-1} (Sigma Aldrich) and/or 5 % (w/v) sucrose.

DNA manipulation, construction of mutants and complementation. Plasmid and chromosomal DNA were extracted with QIAprep spin miniprep (Qiagen) and ultraclean microbial DNA isolation kit (Mo Bio Laboratories), respectively. When necessary, DNA was purified from agarose gels using a QIAquick gel extraction kit (Qiagen). DNA sequencing was performed at the Servicio de Secuenciación de DNA of the Center for Applied Medical Research (University of Navarra, Pamplona, Spain). Primers were synthesized by Sigma Aldrich. DNA and protein homology searches were carried out using the NCBI (http://www.ncbi.nlm.nih.gov) and the EMBL-European Bioinformatics Institute (http://www.ebi.ac.UK/ebi_home.html) servers. Sequences were obtained from the KEGG (http://www.genome.jp/kegg/) and NCBI (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi?view=1) databases.

To construct an in-frame mutant in ORF BAB1_0476 [putative *B. abortus cfa* (cyclopropane fatty acid synthase) gene], oligonucleotides BAB1_0476-F1 (5′-CGGTTGATTGACGGAAAACT-3′) and BAB1_0476-R2 (5′-GTTAAACGCAACGGCTCTTT-3′) were used to amplify a 286 bp fragment including codons 1–54 plus 124 nt upstream of the start codon, and oligonucleotides BAB1_0476-F3 (5′-AAAGAGCCGTTGCGTTTAACTATATCGAGGCGGAGGAAAA-3′) and BAB1_0476-R4 (5′-TCCGCCATGATTCTCTTTTC-3′) were used to amplify a 305 bp fragment including codons 395–426 of the ORF and 206 bp downstream of the stop codon. These fragments were ligated by overlapping PCR using oligonucleotides BAB1_0476-F1 and BAB1_0476-R4 for amplification, and the complementary regions of BAB1_0476-R2 and BAB1_0476-F3 for overlapping. The resulting fragment, containing the BAB1_0476 deletion allele, was cloned into pCR2.1 (Invitrogen) to generate plasmid pLPI-3, sequenced to ensure maintenance of the reading frame, and subcloned into the *Bam*HI and *Xba*I sites of the suicide plasmid pJQK (Scupham & Triplett, 1997). The resulting suicide mutator plasmid (pLPI-4) was introduced into BAB-parental by conjugation (Conde-Álvarez *et al.*, 2006; Palacios-Chaves *et al.*, 2011); integration of this suicide vector was selected by Nal and Km resistance and the excisions [generating both *cfa* mutant (BABΔ*cfa*) and revertant (BAB-revertant) strains recovering the intact gene] were selected by Nal and sucrose resistance and Km sensitivity. The resulting colonies were screened by PCR with primers BAB1_0476-F1 and BAB1_0476-R4 which amplify fragments of 591 or 1611 bp from the deletion mutants and the revertant strains, respectively. The mutation resulted in the loss of both the SAM-methyltransferase consensus motif (VLE/DXGXGXG) and the amino acids equivalent to those responsible for the enzymic activity of the *Escherichia coli* CFA synthase (C139, E239, H266, I268 and Y317) (Courtois *et al.*, 2004; Ingrosso *et al.*, 1989).

For complementation, a plasmid carrying ORF BAB1_0476 was constructed using the Gateway cloning Technology (Invitrogen). Since the sequences of BAB1_0476 and of its *Brucella melitensis* homologue are 99 % identical, the clone carrying BMEI_1484 was taken from the *B. melitensis* ORFeome (Dricot *et al.*, 2004) and subcloned into plasmid pRH001 (Hallez *et al.*, 2007) to produce plasmid pLPI-15. This plasmid was introduced into the BABΔ*cfa* mutant by mating with *E. coli* S17 λpir and the conjugants (BABΔ*cfa* pLPI-15) were selected by plating onto TSA-Nal-Cm plates.

Gas chromatography-MS analysis. For fatty acid analysis, dry bacteria (150 mg) were treated with 15 % NaOH (3.75 ml) in 50 % (v/v) methanol for 30 min at 100 °C. The saponified material was cooled and acidified with HCl, extracted three times with hexane-methyl *tert*-butyl ether (1 : 1), and the pooled extracts were evaporated to dryness and dissolved in chloroform. The analyses were performed with a Varian 3800 chromatograph coupled to an ion-trap detector (Varian 4000) using a fused-silica DB-5HT capillary column (12 m × 0.25 mm internal diameter, 0.1 μm film thickness) from J&W Scientific. The oven was heated from 50 to 90 °C at 30 °C min^{-1}, and from 90 to 250 °C at 8 °C min^{-1}. The transfer line was kept at 300 °C and the injector temperature was set at 250 °C. Helium was used as carrier gas at a rate of 2 ml min^{-1} and the injection was performed in the splitless mode. Trimethylsilyl-diazomethane methylation and *bis* (trimethylsilyl)trifluoroacetamide silylation in the presence of pyridine were used to produce the appropriate derivatives. Compounds were identified by mass fragmentography and by comparing their mass spectra with those of the Wiley and NIST libraries.

[^{14}C]SAM labelling of free lipids. Saline-washed stationary phase bacteria grown in TSB were adjusted to OD_{600} 1.0, and 1 ml of this suspension was inoculated into 10 ml minimal Gerhardt's medium (Gerhardt, 1958). Immediately, 1 ml of the culture was supplemented with 0.5 μCi (1.85×10^4 Bq) [^{14}C]SAM (56 mCi $mmol^{-1}$) (Hacker *et al.*, 2008). After incubation for 24 h, bacteria were inactivated with phenol (0.5 %, v/v), harvested by centrifugation, washed with 500 μl water and resuspended in 100 μl water. Lipids were then extracted with chloroform : methanol (Bligh & Dyer, 1959), resolved on silica gel 60 high-performance TLC plates (Merck Chemicals) using n-propanol/propionic acid/chloroform/water (3 : 2 : 2 : 1) and developed by autoradiography and charring with 15 % (v/v) sulfuric acid in ethanol.

CFA synthase gene expression studies. To construct a BAB1_0476 promoter–*luxAB* transcriptional fusion, a fragment

containing the ATG of BAB1_0476 plus 200 bp upstream of this start codon was amplified using primers BAB1_0476-F11 (5′-GGGATCCA-AATTTCGGGCGGTCGCTATTGGA-3′; *Bam*HI site underlined) and BAB1_0476-R12 (5′-TTCTAGACATCGGCCCCATCTCCTCTTTGG-GAA-3′; *Xba*I site underlined), cloned into pGEM-T Easy (Promega) and subcloned (using the *Bam*HI and *Xba*I sites) into pSKori-TKmLuxAB. The resulting plasmid was then transferred to *B. abortus* (Conde-Álvarez *et al.*, 2006; Palacios-Chaves *et al.*, 2011) and the clones carrying the transcriptional fusion [integrated into chromosome I (*B. abortus* pBAB*1_0476-luxAB*)] selected by Nal and Km resistance and by PCR using oligonucleotides BAB1_0476-F11 and *luxAB*-Rv (5′-AGCAACCAAACGGTGTCGAA-3′). To measure luciferase activity, fresh *B. abortus* pBAB1_0476-*luxAB* cells were adjusted to OD_{600} 0.4 in saline and 50 µl was inoculated into 10 ml TSB, TSB adjusted to pH 6 with McIlvaine's buffer (0.2 M Na_2HPO_4; 0.1 M citric acid) or TSB-340 mM sucrose (in this case, a 200 µl inoculum was used to correct for the longer lag phase). Growth was followed by measuring OD_{600} and 1 ml aliquots were taken periodically to measure the luminescence in relative units of luminescence (RLU) after addition of 100 µl ethanol/decanal (1 : 1).

Effect of acid pH on bacterial survival. Bacteria were grown in TSB up to the stationary phase, harvested by centrifugation, washed twice and adjusted to OD_{600} 0.9 with saline. Then, 500 µl was suspended in 10 ml McIlvaine's buffer adjusted to the appropriate pH and incubated at 37 °C with shaking (130 r.p.m.). After 48, 72, 96 and 120 h, 100 µl aliquots were serially diluted and plated onto TSB agar plates that were incubated at 37 °C for up to 6 days.

Effect of osmolarity on bacterial growth. Luria–Bertani broth (LB) [1 % (w/v) tryptic casein–0.5 % (w/v) yeast extract; Becton Dickinson) was used, either plain or supplemented with 340 mM sucrose (LB-340 mM sucrose). Bacteria were grown in LB up to stationary phase, harvested by centrifugation, washed twice and adjusted to OD_{600} 0.17 with saline. An aliquot (10 µl) of this suspension was inoculated into 190 µl LB or LB-340 mM sucrose and incubated in a Bioscreen C (Bioscreen Instruments) at 37 °C with continuous stirring. For studies on solid media, bacteria were suspended to OD_{600} 0.9, serially diluted, and 10 µl was spotted on LB agar or LB-agar-340 mM sucrose; the plates were incubated at 37 °C for 6 days.

Multiplication in murine macrophages. Murine RAW 264.7 macrophages (ATCC TIB-71) were cultured in Dulbecco's modified Eagle's medium [DMEM, (Gibco; Invitrogen)] supplemented with 10 % (v/v) fetal bovine serum, 1 % (v/v) non-essential amino acids 100 × (Gibco; Invitrogen) and 1 % (v/v) L-glutamine 200 mM (Sigma Aldrich) under a 5 % CO_2 atmosphere. Infections were performed using an m.o.i. of 50 : 1 by centrifuging bacteria onto macrophages (400 ***g*** for 10 min at 4 °C), followed by incubation at 37 °C for 30 min under 5 % CO_2. To remove extracellular bacteria, macrophages were extensively washed with DMEM and incubated in medium with 100 µg gentamicin ml^{-1} for 60 min. Thereafter, the antibiotic concentration was decreased to 25 µg ml^{-1}. After 2, 24 and 48 h, infected macrophages were washed three times with 100 mM PBS (pH 7.2), lysed with 0.1 % (v/v) Triton X-100 in PBS and serial dilutions plated onto TSA to enumerate the c.f.u. The attenuated *B. abortus virB* mutant was used as a control (Sieira *et al.*, 2000).

Virulence in mice. Groups of 7-week-old female BALB/c mice (Charles River) were inoculated intraperitoneally with 1.87×10^4 c.f.u. BABΔ*cfa* per mouse or 6.83×10^4 c.f.u. BAB-parental per mouse in 0.1 ml PBS. Spleen weights (mean ± SD, g per spleen) and c.f.u. (mean ± SD, $\log_{10}$ c.f.u. per spleen) were determined in five mice 2 and 8 weeks after inoculation and the identity of the bacterial isolates was confirmed by PCR. The experimental procedures (i.e. preparation and administration of inocula, assessment of the inoculating doses and determination of the number of c.f.u. per spleen) have been described previously (Grilló *et al.*, 2006). Statistical comparisons between means were performed by ANOVA and Fisher's protected least significant differences tests (Grilló *et al.*, 2006). Animal handling and related procedures were in accordance with the current European legislation (directive 86/609/EEC) and approved by the Animal Welfare Committee of the institution (protocol no. R102/2007).

RESULTS

ORF BAB1_0476 encodes a CFA synthase

The genome of *B. abortus* 2308 (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi) contains an ORF (BAB1_0476) annotated as encoding a methyltransferase or a CFA synthase. Since some bacteria have more than one of these enzymes, we searched for orthologues of the three CFA synthases annotated in *Mesorhizobium loti* (Kaneko *et al.*, 2000) and of the *cfa-1* and *cfa-2* CFA synthase genes described in *Sinorhizobium meliloti* (Saborido Basconcillo *et al.*, 2009) as well as for homologues of the methyltransferases that modify *Mycobacterium tuberculosis* mycolic acids (Dao *et al.*, 2008; Rao *et al.*, 2005, 2006). Although we identified several methyltransferase homologues in *B. abortus*, only ORF BAB1_0476 had a significant homology with CFA synthases. Moreover, the predicted protein conserves both the SAM-binding consensus motif (VLE/DXGXGXG) and the amino acids equivalent to the C139, E239, H266, I268 and Y317 residues that are essential for the activity of the *E. coli* phospholipid CFA synthase (Courtois *et al.*, 2004; Ingrosso *et al.*, 1989). ORF BAB1_0476 has identical orthologues in the reference strains of all *B. abortus* and *Brucella suis* biovars as well as in vaccine *B. abortus* S19, *B. ovis* and *Brucella ceti*. A deletion removing an amino acid outside the predicted functional sections exists in the reference strains of all *B. melitensis* biovars, vaccine *B. melitensis* Rev 1 and *B. neotomae*. Also, there is a 2 aa deletion in *Brucella pinnipedialis* (http://www.broadinstitute.org/annotation/genome/brucella_group/).

To analyse the activity of the protein encoded by BAB1_0476, we constructed a *B. abortus* 2308 Nal^R (BAB-parental) BAB1_0476-deleted mutant by making an in-frame deletion encompassing the SAM-binding consensus motif and the C139, E239, H266, I268 and Y317 equivalents. The mutant was identical in colony morphology and growth, as well as in all standard tests used to type smooth brucellae, to the parental strain (Alton *et al.*, 1988). Then, we analysed the fatty acids of stationary phase cells of BAB-parental, BABΔ*cfa* and BABΔ*cfa*-pLPI-15 (BABΔ*cfa* complemented strain). As can be seen in Fig. 1, the major fatty acid in BAB-parental was lactobacillic acid (C19cyc). Palmitic (C16 : 0), *cis*-9,10-methylene hexadecanoic (C17cyc), stearic (C18 : 0), vaccenic [C18 : 1(*n-7*), the C19cyc precursor] acid and trace amounts of palmitoleic [C16 : 1(*n-7*), the C17cyc precursor] acid were also present. In contrast, mutant BABΔ*cfa* lacked CFAs and showed increased amounts of cyclopropane acid precursors, vaccenic in particular (Fig. 1). Complementation restored

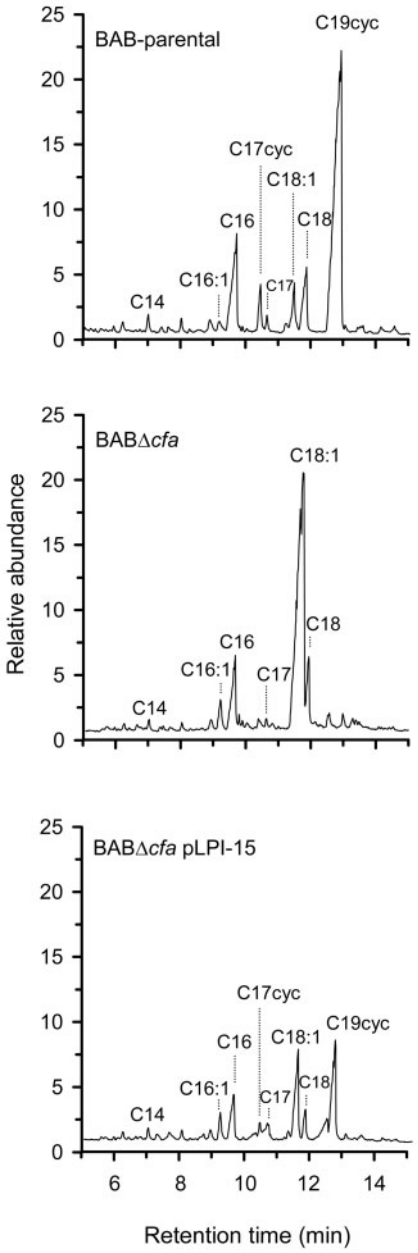

Fig. 1. Gas chromatography-MS analysis of fatty acids (as methyl esters) from BAB-parental, BABΔ*cfa* mutant and BABΔ*cfa* mutant carrying plasmid pLPI-15 encoding the complete ORF BAB1_0476.

the ability to generate CFAs, although not to the level of the parental strain (Fig. 1).

To extend these results, we tested the ability of BAB-parental and BABΔ*cfa* to incorporate methylene groups into lipids using SAM as the donor. To this end, we used a minimal medium supplemented with [^{14}C]SAM and extracted the free lipids of stationary phase bacteria. TLC followed by autoradiography showed that whereas the ornithine lipids

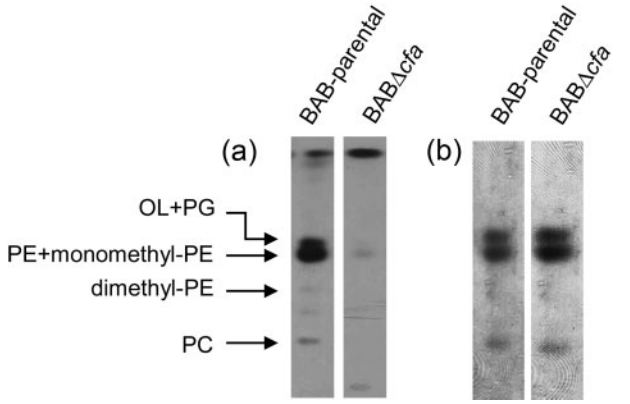

Fig. 2. *B. abortus* ORF BAB1_0476 encodes a protein with methyltransferase activity. TLC analysis of the free lipids of BAB-parental and BABΔ*cfa* grown in Gerhardt's minimal medium supplemented with [^{14}C]SAM. (a) Autoradiography and (b) charring. OL, Ornithine lipids; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PC, phosphatidylcholine.

and major phospholipids of BAB-parental were labelled, those of BABΔ*cfa* were not (Fig. 2). Altogether, the results of the fatty acid analysis and [^{14}C]SAM experiments clearly indicate that BAB1_0476 encodes a CFA synthase and that no other ORF is involved in CFA synthesis under these growth conditions. Accordingly, the name *cfa* was used for BAB1_0476.

Expression of *B. abortus cfa* occurs during the exponential phase of growth and increases in acid or hyperosmotic media but it is not involved in virulence

Since brucella temporarily passes through an acidic intracellular vesicle before reaching the intracellular replicative niche, it has been proposed that CFAs play a role in resistance to this unfavourable milieu (Roop *et al.*, 2003). Similarly, the periplasmic cyclic glucans that act as a brucella virulence factor are osmoregulated (Arellano-Reynoso *et al.*, 2005; Iñón de Iannino *et al.*, 2000; Roset *et al.*, 2006), suggesting osmotic changes during intracellular life. Thus, we studied the influence of pH and osmotic tension on *B. abortus cfa* expression *in vitro* using a *cfa* (BAB1_0476) promoter–*luxAB* transcriptional fusion. Fig. 3 shows that there was transient *cfa* promoter activity in TSB at pH 7 and a threefold increase in promoter activity at pH 6 or in 340 mM sucrose during the exponential growth phase. Then, we tested the virulence of BABΔ*cfa* in macrophages and mice. Fig. 4(a) shows that, in contrast with the decreased viability of the *virB* mutant (Sieira *et al.*, 2000) used as a reference of attenuation, BAB-parental and BABΔ*cfa* multiplied similarly in murine RAW 264.7 macrophages. Likewise, we did not find differences in multiplication and persistence in the spleens (Fig. 4b) or in spleen weights (not shown) of intraperitoneally inoculated BALB/c mice,

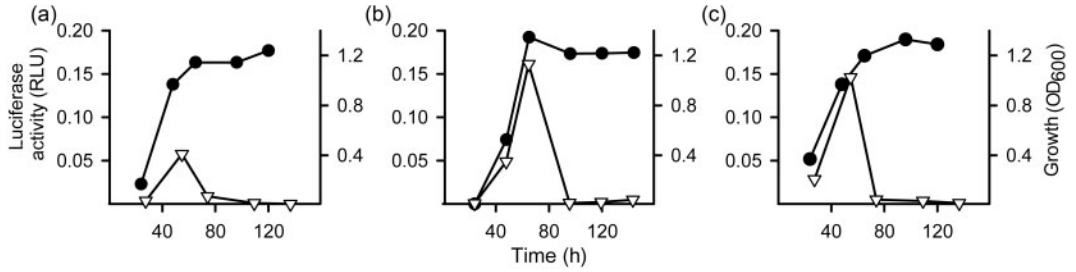

Fig. 3. Activity of the *B. abortus cfa* promoter during growth under standard (a), acidic (b) and hyperosmotic (c) conditions. The results show the luciferase activity [▽; in relative luminiscence units (RLU)] of a *B. abortus* BAB1_0476 promoter–luciferase transcriptional fusion grown to stationary phase in different media. Growth is also shown (●).

proving that the *cfa* deletion and the subsequent inability to synthesize CFA did not affect the ability to establish chronic infections in this model.

Dysfunction of *B. abortus cfa* affects extracellular growth and survival

The brucellae remain infectious for various periods of time on tissues from abortions, related tissues, fomites and soil, where they are exposed to dryness and subsequent osmotic stress and to the acidity that results from the activity of other microbiota. Thus, it is conceivable that, like in other bacteria, CFAs could contribute to persistence under these unfavourable extracellular conditions. To test this possibility, we first inoculated LB or LB-340 mM sucrose and measured growth. These experiments did not reveal significant differences between BAB-parental and BABΔ*cfa* (data not shown). Then, we grew the bacteria to the stationary phase in LB and spotted aliquots of serial dilutions on LB agar with or without 340 mM sucrose. We found that growth of BABΔ*cfa* but not of the revertant strain (generated after the excision of the mutator plasmid, and carrying the wild-type *cfa* gene) was impaired on LB agar-340 mM sucrose (Fig. 5a), and that the parental phenotype was partially restored by complementation of BABΔ*cfa* with plasmid pLPI-15 (not shown). We could not use a similar protocol to test growth on plates adjusted to an acidic pH because of the inability of BAB-parental and BABΔ*cfa* to grow at pH 6 or lower. Instead, we tested the survival after 48, 72, 96 and 120 h in McIlvaine's buffer adjusted to pH 7, 6 or 5 by spotting aliquots of serial dilutions on standard TSA. We observed that BABΔ*cfa* had a reduced ability to survive at pH 6 and 5 and that the differences were most obvious at 120 h (Fig. 5b). This deficiency was partially repaired by complementation with plasmid pLPI-15 (not shown).

DISCUSSION

This research demonstrates that BAB1_0476 encodes the *B. abortus* CFA synthase that accounts for C19cyc (and C17cyc) synthesis in this bacterium. Since C19cyc is also a major component of the lipids of all *Brucella* species analysed thus far (Coloe *et al.*, 1984; De *et al.*, 2008; Dees *et al.*, 1981; Iriarte *et al.*, 2004; Thiele *et al.*, 1969; Thiele & Schwinn, 1974; Vasiurenko *et al.*, 1977) and all *Brucella* species carry an ORF identical or almost identical to BAB1_0476, it is likely that this conclusion also applies to those BAB1_0476 orthologues. Taking into account the presence of *cfa* in all *Brucella* species, the large amounts of C19cyc produced, and the fact that synthesis of each C19cyc molecule requires one SAM and, therefore, three ATP (Cronan, 2002), it seems that C19cyc must confer some adaptive advantage to these pathogens.

As summarized in the Introduction, CFAs have been linked to an adaptation of the cell envelope to stressful environmental conditions, including those prevailing in the stationary phase, and indirect evidence suggests that at least low oxygen tension, nutrient limitation and acidic pH

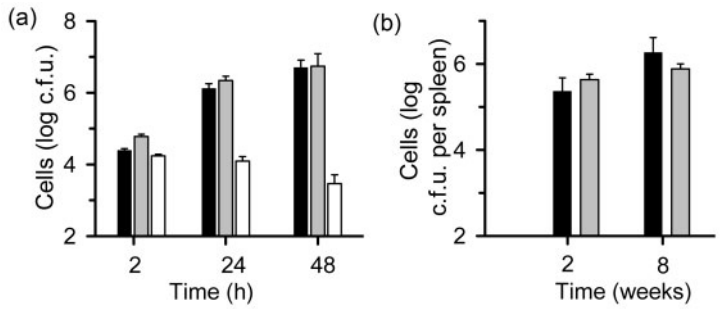

Fig. 4. The CFA deficiency does not alter the ability of *B. abortus* to multiply in RAW 264.7 macrophages (a) and in mice (b). Values are the mean ± SEM of triplicate infections, and the results shown are representative of three independent experiments. Bars: black, BAB-parental; grey, BAB△*cfa*; white, *virB*.

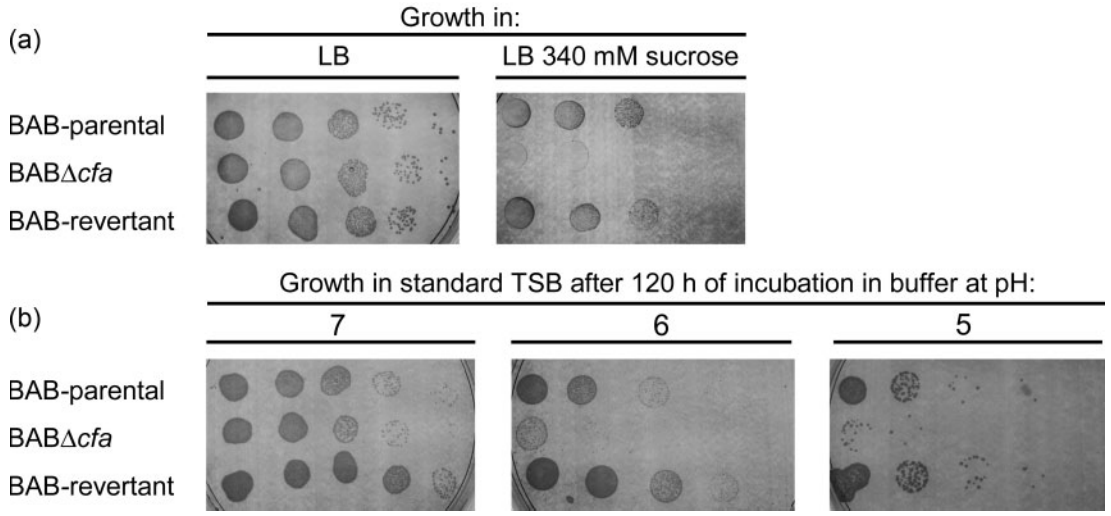

Fig. 5. *B. abortus cfa* is necessary for growth on hyperosmotic agar media (a) and for survival at acid pH (b). Serial dilutions of bacterial suspensions were made in saline and 10 μl of each (from 10^{-4} to 10^{-8}) was spotted on the indicated media and incubated at 37 °C for 7 days.

are encountered by brucellae in macrophages (Jubier-Maurin *et al.*, 2004). On this basis, it has been hypothesized that *Brucella cfa* is a stationary phase gene and that it plays a role in intracellular life (Roop *et al.*, 2003). Concerning the first hypothesis, our results show that expression of *B. abortus cfa* occurs mostly during the exponential phase of growth. Although *Brucella cfa* is expressed during exponential phase and not during the stationary phase, this evidence cannot be interpreted to mean that the C19cyc is synthesized only during exponential phase. In fact, C19cyc is a small proportion of the acyl chains of the free lipids present in the outer membrane blebs released by exponentially growing *B. melitensis*; C18 : 1 is present in much larger amounts (Gamazo & Moriyón, 1987). In *E. coli*, only a low proportion of unsaturated acyl chains are converted into CFAs during exponential growth (Grogan & Cronan, 1997). In this bacterium, *cfa* expression depends on RpoD (σ70; active throughout growth) and RpoS (σ38), which regulates stationary phase genes (Wang & Cronan, 1994). Moreover, there is a proteolytic degradation of CFA synthase at the beginning of stationary phase that makes its activity transitory (Chang *et al.*, 2000). Thus, CFA accumulation in stationary phase cells is the outcome of the action of RpoS and lipid synthesis arrest (Wang & Cronan, 1994). This indicates that CFA accumulation is a complex phenomenon compatible with *cfa* expression in exponentially growing cells. With respect to the possibility that C19cyc could be relevant in intracellular life, the ability of the *cfa* mutant to multiply in murine macrophages and to establish chronic infections in mice seem to disprove this hypothesis, at least in these laboratory models. Indeed, disruption of this ORF completely abolished synthesis of cyclopropane fatty acids *in vitro* and, although the possibility that another CFA synthase could be activated *in vivo* cannot be completely excluded, BAB1_0476 is the only ORF in the *Brucella* genomes that conserves both the SAM binding motif and all the residues necessary for CFA synthase activity. It is known that cyclopropane rings modulate innate immunity recognition of mycolic acids (Dao *et al.*, 2008; Rao *et al.*, 2005, 2006) and that *B. abortus* escapes early detection by innate immunity because of the reduced PAMP of envelope molecules with lipidic moieties (Barquero-Calvo *et al.*, 2007). Although the presence of long acyl chains in these molecules is essential in PAMP reduction in *Brucella* (Lapaque *et al.*, 2006; Palacios-Chaves *et al.*, 2011), our results suggest that C19cyc is not critical and that it can be substituted by C18 : 1. This conclusion is consistent with the observation that *B. abortus* expresses *cfa* at the same level *in vitro* as in baby hamster kidney cells (Viadas *et al.*, 2010).

It has been postulated that the acidic pH of stomach represents a barrier when infection occurs orally in humans. If this is true, CFAs could play a role at this level. However, there is considerable evidence indicating that penetration of brucellae in humans (or ruminants) occurs through the oral mucosae rather than through the small or large intestine (for a discussion, see Gorvel *et al.*, 2009). Like in *B. abortus*, mutation of the CFA synthases of the phylogenetic relative *S. meliloti* does not hamper intracellular life, and it has been suggested that CFAs could be useful during *S. meliloti* extracellular life (Saborido Basconcillo *et al.*, 2009). A similar assumption can be made for *B. abortus*. Under natural conditions, these bacteria do not multiply significantly outside the hosts but can persist on tissues from abortions or related tissues, on materials contaminated with vaginal fluids, in milk and in dairy products. It has been known for a long time that, protected from direct sunlight and at temperatures below 55 °C, *Brucella* is not easily destroyed: *B. abortus* can persist for 6–8 months on fetuses,

for 3–4 months in faeces, for 1–3 months in soil, depending on the humidity, and for up to 4 days in milk at room temperature (Huddleson, 1943; Corbel, 2006). Indeed, the environmental conditions must be very different from those encountered in the endoplasmic reticulum-derived vacuolae where brucellae multiply normally. Undoubtedly, such extracellular conditions include acidity as a result of fermentations carried out by other micro-organisms, and the high tonicity associated with dryness of surfaces exposed to air. In this work, we have presented evidence showing that acidity and high osmolarity negatively affected the survival of a *B. abortus cfa* mutant. The fact that these observations were made on agar but could not be reproduced in broth indicates that additional factors must also be relevant. Whereas stirred broth provides homogeneous growth, micro-organisms are constrained to grow as colonies on structured products like agar media, many foodstuffs or contaminated surfaces. This results in a heterogeneous environment that causes an additional stress and slower growth rates, possibly because of the local depletion of oxygen and nutrients and accumulation of metabolites with altered pH gradients as a consequence (Theys *et al.*, 2008). These conditions on structured media probably reflect more closely those on the materials that become contaminated when *B. abortus* is released in the environment. Thus, a reasonable hypothesis that is consistent with our *in vitro* results is that CFAs play a role in survival outside the hosts, thus favouring the possibility of transmission. This hypothesis could explain why an energetically costly function that does not play a role in intracellular life is conserved in this facultative intracellular parasite.

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