INFECTION AND IMMUNITY, Oct. 2007, p. 4867–4874 Vol. 75, No. 10
0019-9567/07/$08.00+0 doi:10.1128/IAI.00439-07
Copyright © 2007, American Society for Microbiology. All Rights Reserved.

BvrR/BvrS-Controlled Outer Membrane Proteins Omp3a and Omp3b Are Not Essential for *Brucella abortus* Virulence▽

Lorea Manterola,[1]† Caterina Guzmán-Verri,[2]* Esteban Chaves-Olarte,[2,3] Elías Barquero-Calvo,[2] María-Jesús de Miguel,[4] Ignacio Moriyón,[1] María-Jesús Grilló,[5] Ignacio López-Goñi,[1] and Edgardo Moreno[2]

Departamento de Microbiología y Parasitología, Universidad de Navarra, 31008 Pamplona, Spain[1]; Programa de Investigación en Enfermedades Tropicales (PIET), Escuela de Medicina Veterinaria, Universidad Nacional, 304-3000 Heredia, Costa Rica[2]; Centro de Investigación en Enfermedades Tropicales, Facultad de Microbiología, Universidad de Costa Rica, 1000 San José, Costa Rica[3]; Centro de Investigación y Tecnología Agroalimentaria (CITA), Sanidad Animal, Gobierno de Aragón, Zaragoza, Spain[4]; and Instituto de Agrobiotecnología, CSIC-UPNA-Gobierno de Navarra, Pamplona, Spain[5]

Received 26 March 2007/Returned for modification 6 May 2007/Accepted 17 July 2007

The *Brucella abortus* two-component regulatory system BvrR/BvrS controls the expression of outer membrane proteins (Omp) Omp3a (Omp25) and Omp3b (Omp22). Disruption of *bvrS* or *bvrR* generates avirulent mutants with altered cell permeability, higher sensitivity to microbicidal peptides, and complement. Consequently, the role of Omp3a and Omp3b in virulence was examined. Similar to *bvrS* or *bvrR* mutants, *omp3a* and *omp3b* mutants displayed increased attachment to cells, indicating surface alterations. However, they showed unaltered permeability; normal expression of Omp10, Omp16, Omp19, Omp2b, and Omp1; native hapten polysaccharide; and lipopolysaccharide and were resistant to complement and polymyxin B at ranges similar to those of the wild-type (WT) counterpart. Likewise, *omp3a* and *omp3b* mutants were able to replicate in murine macrophages and in HeLa cells, were resistant to the killing action of human neutrophils, and persisted in mice, like the WT strain. Murine macrophages infected with the *omp3a* mutant generated slightly higher levels of tumor necrosis factor alpha than the WT, whereas the *bvrS* mutant induced lower levels of this cytokine. Since the absence of Omp3a or Omp3b does not result in attenuation, it can be concluded that BvrR/BvrS influences additional *Brucella* properties involved in virulence. Our results are discussed in the light of previous works suggesting that disruption of *omp3a* generates attenuated *Brucella* strains, and we speculate on the role of group 3 Omps.

Members of the genus *Brucella* are intracellular bacterial pathogens of mammals (33). The ability of *Brucella* to invade and replicate in cells has been linked to its outer membrane (OM) properties as well as to structures built within the cell envelope (31, 32, 34). Among these, the lipopolysaccharide (LPS), the β-1,2-cyclic glucans, the type IV secretion system VirB, and the flagellum-like system are the most studied (2, 6, 17, 27). The notion that the *Brucella* OM plays a key role in virulence has been reinforced by the identification of the two-component regulatory system BvrR/BvrS, which controls the expression of at least two OM proteins (Omps), Omp3a and Omp3b, as well as the structure of the LPS (22, 28). Although they do not demonstrate obvious growth defects in vitro, the *bvrS* and *bvrR* mutants are avirulent in mice, displaying reduced invasiveness and replication in professional and nonprofessional phagocytes (7, 42).

Omp3a and Omp3b, also known as Omp25 and Omp22, respectively, belong to group 3 of the *Brucella* Omps (22, 37, 44, 45), a highly conserved family of up to seven members that includes some of the most abundant and immunogenic *Brucella* proteins (10). The function of group 3 Omps is not completely understood. The strong association of some of the members with LPS suggests that they play an important structural role in the OM (19, 37). In *Brucella abortus*, the gene encoding Omp31A is absent and the gene encoding Omp25b is truncated (23, 46), suggesting that neither of these Omps plays a significant role in *Brucella* virulence, although they still may participate in host preferences. Omp31A is a hemin-binding protein, and its expression is induced by iron limitation (11). The virulence of *B. melitensis* Rev1 Omp31A mutants, however, does not differ from the parental Rev1 counterpart (8). These apparent inconsistencies may be accounted for in part by the redundancy of iron uptake systems in *Brucella* (11). There are several reports indicating that *B. abortus* Omp3a is involved in virulence (14–16) and that it acts as a negative regulator of tumor necrosis factor alpha (TNF-α) production in human macrophages (24). Since the levels of Omp3a and Omp3b expression are severely diminished in *B. abortus bvrS* and *bvrR* mutants and these mutants are avirulent (22, 42), we decided to construct *omp3a* and *omp3b* knockout strains and to explore their biological characteristics. Although *omp3a* and *omp3b* mutants displayed some surface properties that distinguished them from the parental strain, we found that they did not reproduce the defects of the *bvrS* or *bvrR* mutants and remained virulent in the systems tested.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The relevant characteristics of the bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* and *Brucella* strains were grown on tryptic soy broth, tryptic soy

* Corresponding author. Mailing address: Programa de Investigación en Enfermedades Tropicales (PIET), Escuela de Medicina Veterinaria, Universidad Nacional, 304-3000 Heredia, Costa Rica. Phone: (506) 238-0761. Fax: (506) 238-1298. E-mail: catguz@medvet.una.ac.cr.

† Present address: Unidad de Proteómica CIC bioGUNE, Parque Tecnológico de Bizkaia, Ed. 801-A, 48160 Derio, Bizkaia, Spain.

▽ Published ahead of print on 30 July 2007.

TABLE 1. Bacterial strains and plasmid constructs used in this study

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
B. abortus		
2308 Nal^r	Virulent WT strain, biotype 1, LPS-S, spontaneous Nal^r mutant	39
bvrS mutant strain 2.13	2308 Nal^r *bvrS*::Tn*5*	42
bvrR mutant strain 65.21	2308 Nal^r *bvrR*::Tn*5*	42
bvrR$^+$ strain 65.21p	65.21 carrying plasmid pBBR2.13	42
omp3a mutant 2308	Nal^r *omp3a*::Km	This work
omp3b mutant 2308	Nal^r *omp3b*::Km	This work
E. coli		
SM10 (λ pir)	*thi-1 thr leu tonA lacY supE recA*::RP4-2-Tet::Mu Km^r; λ pir	40
XL1-Blue	Tet^r *supE44 hsdR17 recA1 endA1 gyrA96 thi relA1 lac* F′ [*proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15* Tn*10* (Tet^r)]	38
TOP 10 F′	F′ [*lacI*^{q} Tn*10* (Tet^r)] *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*Δ*M15* Δ*lacX74* *recA1 araD139* Δ(*ara-leu*) *7697 galU galK rpsL* (Str^r) *endA1 nupG*	Invitrogen
Plasmids		
pCR2.1	High-copy-number cloning vector; Amp^r Km^r	Invitrogen
pUC4K	Plasmid containing kanamycin resistance cassette	Stratagene
pSK-oriT	pBluescript II SK(-) *oriT* RK2 Amp^r	43
pBBR1MCS-4	Intermediate-copy-number cloning vector; Amp^r	25
pAC2553	pUC19 containing Δ*omp3a*::Km	A. Cloeckaert
pSK3aKm	pSK-oriT containing Δ*omp3a*::Km (1.8 kb)	This work
pTA3a	pCR2.1 containing an internal fragment of *B. abortus* 2308 *omp3a* amplified by PCR (0.55 kb)	This work
pTA3b	pCR2.1 containing complete *B. abortus omp3b* gene amplified by PCR (1.1 kb)	This work
pTA3b$^-$	pCR2.1 containing deleted *omp3b* gene (0.6 kb)	This work
pSK3b-Km	pSK-oriT containing Δ*omp3b*::Km (1.9 kb)	This work
pBBR3b	pBBR1MCS-4 containing *B. abortus omp3b* gene (1.1 kb)	This work

agar, or blood agar base (BAB). When needed, nalidixic acid (Nal; 25 μg/ml), kanamycin (Km; 50 μg/ml), or ampicillin (Amp; 100 μg/ml) was added to the cultures. Growth ability was tested in tryptic soy broth, brain heart infusion broth, and Gerhardt's modified minimal medium using an automatic microbiology analyzer (Bioscreen C; Labsystems, Vantaa, Finland).

DNA and RNA manipulations. Plasmid and chromosomal DNA were extracted with QIAprep spin miniprep (QIAGEN GmbH, Hilden, Germany) and Ultraclean microbial DNA isolation (MO BIO Laboratories, Inc.) kits, respectively. Primers were synthesized by Sigma-Genosys (Haverhill, United Kingdom). For RNA manipulation, bacterial cultures adjusted to 10^9 CFU/ml were disrupted with 0.5% Zwittergent 3-16 at 37°C for 1 h. Then, total RNA was extracted using an RNeasy minikit (QIAGEN) according to the manufacturer's instructions. RNA (0.2 μg) was used as a template for the synthesis of cDNA with SuperScript III reverse transcriptase (RT) (Invitrogen) using the random hexamers from the same kit.

Construction and characterization of *omp3a* and *omp3b* mutants. The *B. abortus* 2308 *omp3a*::Km mutant was constructed by homologous recombination between the chromosomal *omp3a* gene and plasmid pSK3aKm carrying an *omp3a*::Km construct from pAC2553 (Table 1). This *omp3a*::Km construct was obtained after cleavage of the *B. abortus* 2308 *omp3a* gene with StyI and the insertion of a kanamycin resistance cassette. In order to facilitate plasmid mobilization by conjugation, *omp3a*::Km was subcloned from pAC2553 into the mobilizable plasmid pSK-*oriT* (43) as an XbaI-SacI 2.1-kb fragment, generating plasmid pSK3aKm. This new construct was confirmed by PCR with primers Omp25U1 (5′-TGCGCTGCT GCCGTTCTCTG-3′) and Omp25L1 (5′-GGATCCGGCCAGATCATAGTTCTT GT-3′), which amplify a specific 547-bp fragment of the *omp3a* gene, and by double digestion with EcoRI and HindIII. Plasmid pSK3aKm was introduced into *B. abortus* 2308 by conjugation with *E. coli* SM10 (λ pir) (39). The first recombination event (vector integration in the *Brucella* chromosome) was selected by Nal and Km resistance, and the second recombination event (excision of the mutator plasmid) was selected by Amp sensitivity. To confirm the mutation, different colonies were screened by PCR with primers Omp25U1 and Omp25L1. EcoRV-digested genomic DNA from selected clones was analyzed by Southern blotting using pSK-*oriT*, pSK3aKm, and pTA3a (Table 1) as probes and by RT-PCR as previously described (28) with primers Omp25U1 and Omp25L1. Failure to express Omp3a was confirmed by Western blotting using OM fragments (18) or Sarkosyl-resistant fractions with anti-Omp3a monoclonal antibodies (MAbs) A70/06B05/A07 and A76/02C12/C11 (9, 42). The *B. abortus omp3b*::Km mutant was constructed as follows. First, *B. abortus* 2308 *omp3b* was amplified by PCR with specific primers BAF-1 (5′-CCCG GCTGTTACATATGCTG-3′) and BAF-2 (5′-CGCGCTGATATCGACATGAC-3′) and cloned into vector pCR2.1 (Invitrogen). The resulting plasmid pTA3b was used as a template for inverse PCR mutagenesis to delete *omp3b*. This plasmid was first denatured with 1 M NaOH and 1 mM EDTA for 15 min at 37°C, neutralized with 3 M sodium acetate (pH 4.8), purified with ethanol, and finally resuspended in water (13). The sample was then inverse amplified with primers IM3B-1 (5′-ACG CGTCGACGCCGGCCTGAACTACAA-3′) and IM3B-2 (5′-ACGCGTCGACG CGGCGACAGGGTCGTTAT-3′) carrying a restriction site for SalI (underlined in the primer sequences). After 5 min at 95°C, amplification was carried out for 30 cycles of 1 min at 95°C, 45 s at 63°C, and 45 s at 68°C, and a final extension at 68°C for 10 min. The 4.5-kb amplified fragment was purified, digested with SalI, and religated to generate plasmid pTA3b$^-$. To facilitate the counterselection of the mutant, a Km resistance cassette from plasmid pUC4K (Amersham Pharmacia Biotech, NJ) was cloned in the SalI site. The mutated *omp3b* gene was subcloned into pSK-*oriT* as an EcoRI 1.9-kb fragment generating plasmid pSK3bKm. This construct was verified by PCR with primers BAF-1 and BAF-2 and by digestion with EcoRI and SalI. Plasmid pSK3bKm was introduced into *B. abortus* 2308 by conjugation with *E. coli* SM10 (λ pir) and Nal^r Km^r Amp^s transconjugants were selected. The resulting colonies were screened by PCR with primers BAF-1 and BAF-2 and primers C3BK-2 (5′-CCGCGCGGACACCAAGCCTA-3′) and C3BK-1 (5′-CGG CGGCGTGACGGATGAAG-3′), which amplify a 1.9-kb fragment of the *omp3b* gene. Mutation was confirmed by Southern blotting using plasmids pSK-*oriT*, pSK3bKm, and pTA3b as probes and EcoRI-digested genomic DNA, as well as by RT-PCR with specific primers 3bZ-1 (5′-GCGCGCAGGTTGGTGGTT-3′) and 3bZ-2 (5′-GGATCCGCCGGCCTTGATCGAATG-3′), which amplify a specific 473-bp fragment of the *omp3b* gene. The absence of Omp3b was corroborated by two-dimensional (2D) gel analysis of the OM fragments of the *omp3b* mutant and the wild-type (WT) strain as previously described (22).

To determine the stability of the *omp3a* and *omp3b* mutants in vitro, bacteria were grown on BAB for 24 h and serial dilutions were plated on BAB, BAB-Nal, BAB-Km, and BAB-Amp. In vivo stability was determined in the mouse model.

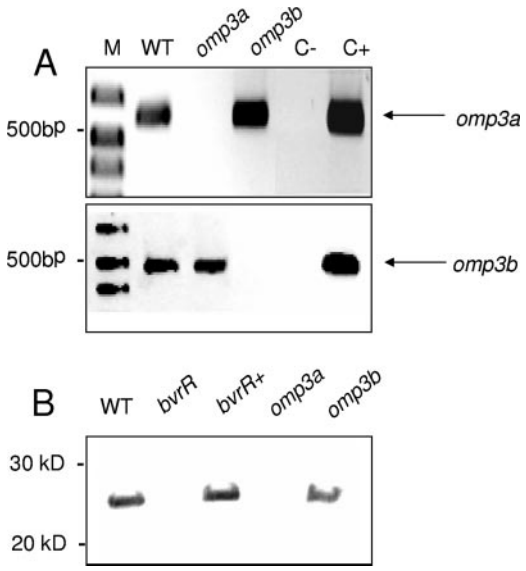

FIG. 1. Construction of *B. abortus omp3a* and *omp3b* mutants. (A) RT-PCR using specific primers for *omp3a* (top) and *omp3b* (bottom). Total RNA was extracted and retrotranscribed, and cDNA was amplified by PCR with primers Omp25U1/Omp25L1 and 3bZ-1/3bZ-2. Lanes: M, molecular size markers; WT, *B. abortus* 2308; *omp3a*, *B. abortus omp3a* mutant; *omp3b*, *B. abortus omp3b* mutant; C-, PCR-negative control with water; C+, PCR-positive control with *Brucella* genomic DNA. (B) Detection of Omp3a by Western blotting in cell envelope Sarkosyl-resistant fractions using anti-Omp3a MAbs. Lanes: WT, *B. abortus* 2308; *bvrR*, *B. abortus bvrR::*Tn*5* mutant; *bvrR*$^+$, reconstituted *B. abortus bvrR*$^+$; *omp3a*, *B. abortus omp3a* deletion mutant; *omp3b*, *B. abortus omp3b* deletion mutant.

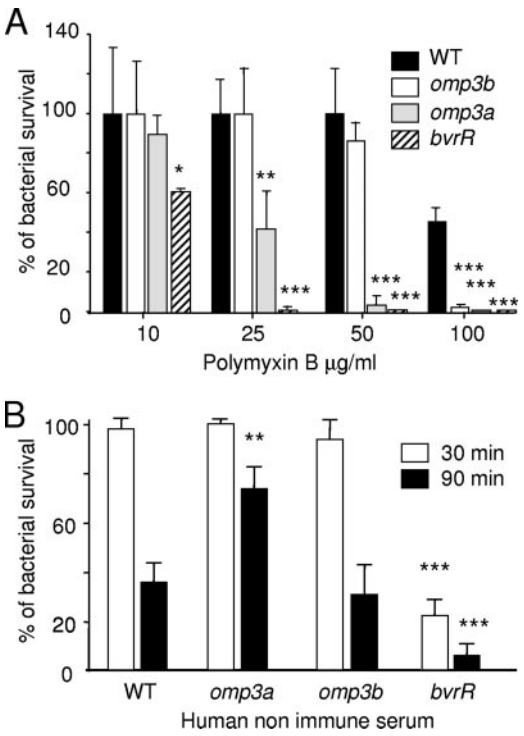

FIG. 2. Sensitivity to polymixin B and human complement. (A) Bacterial survival in the presence of polymyxin B. The graph shows the percent survival after 1 h at 37°C with different peptide concentrations. (B) Bacterial survival after 30- or 90-min incubation with human nonimmune serum. Data represent the means ± standard deviations of percentages of viable bacteria in relation to a bacterial control without polymyxin B or a bacterial control with heat-inactivated human serum. Samples were compared using the Mann-Whitney U test. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$ (with respect to the WT strain).

Groups of five BALB/c mice (see below) were inoculated intraperitoneally with 0.1 ml of a suspension containing 10^5 CFU of each bacterial strain, and 2 weeks later they were sacrificed and the spleens were removed. Each spleen was homogenized, and decimal dilutions were plated in triplicate samples on BAB, BAB-Nal, BAB-Km, and BAB-Amp. Mutants were considered stable in vivo and in vitro when viable counts were the same in all media. LPS stability (smooth LPS versus rough-type LPS) and the presence of native hapten (NH) polysaccharide was also verified by crystal violet staining, immunodiffusion, immunofluorescence, and Western blotting (1, 28). Regular typing and sensitivity to colorants and phages were determined as described elsewhere (1).

Immunological methods. Cell envelope components were analyzed and immunodetected as described previously (22). Direct, indirect, and double immunofluorescence assays for the determination of extracellular and intracellular bacteria were performed as described elsewhere (21, 35). The levels of TNF-α in a supernatant medium of *B. abortus*-infected murine RAW 264.7 macrophages were measured by enzyme-linked immunosorbent assay (BD Biosciences, San Diego, CA) at different time intervals according to the manufacturer's instructions.

Sensitivity to polymyxin B, antibiotics, and nonimmune serum. Sensitivity to polymyxin B was tested as described elsewhere (42). The sensitivity to several hydrophilic and hydrophobic antibiotics was tested as described previously (29). The sensitivity to the bactericidal action of human and bovine serum was estimated by the method described by Skurnik et al. (41).

Internalization, survival, and replication assays in cells and mice. Ex vivo infection assays were performed with HeLa cells (ATCC CCL-2), murine RAW 264.7 macrophages (ATCC TIB-71), and human polymorphonuclear neutrophils (PMN). Cell cultures and gentamicin survival assays were performed as described previously (6, 7, 21, 35, 47). For double immunofluorescence analysis of *Brucella*-infected HeLa or RAW 264.7 cells, procedures described previously were followed (6, 35). Counts of intracellular and extracellular bacteria were performed for at least 50 infected cells and were expressed as the mean and standard deviation of the percentage of intracellular bacteria and the number of bacteria per infected cell. The percentage of cells with associated bacteria (intra- and extracellular) was expressed as the mean and standard deviation of numbers of cells with bound bacteria in five different ×400 magnification fields. Statistical analysis was performed using Student's *t* test. PMN were purified from defibrinated blood extracted from human donors with no history of brucellosis. One part of blood diluted with 1 part minimal essential medium containing 5% inactivated fetal calf serum, with 25 mM HEPES and 2 mM glutamine (Sigma-Aldrich, Co). Eight milliliters of diluted blood was layered on the top of a biphasic gradient of Ficoll-Histopaque (3 ml with a density of 1.077 ± 0.001 on the top of 3 ml with a density of 1.119 ± 0.001; Sigma) on a conic tube and centrifuged at 4°C for 30 min at 700 × *g*. Granulocytes were extracted from the interphase between the two Ficoll layers, washed in supplemented medium without antibiotics, and counted. Infections were performed with 2-ml plastic tubes by mixing 5×10^5 PMN with 5×10^6 *Brucella* cells in a total volume of 250 μl of medium without antibiotics. The tubes were incubated at 37°C for 1 h with mild agitation. Finally, the mixture was centrifuged, the supernatants were removed, and the cells were lysed with 100 μl 0.1% Triton X-100. Dilutions of cell lysates were plated on tryptic soy agar, and the bacterial CFUs were counted after 3 days of incubation. The number of internalized *Brucella* cells in the PMN was recorded by immunofluorescence, as described previously (35).

Female BALB/c mice were infected by the intraperitoneal route with 10^5 CFU of *B. abortus omp3a*, *omp3b*, or WT strains as described previously (42). For the *bvrS* or *bvrR* mutant, doses of 10^8 CFU were used. For each strain, 30 mice were inoculated and the numbers of CFU in spleens were determined at various times postinfection. Statistical comparisons were performed by the Fisher's protected least significant differences test.

RESULTS

***B. abortus omp3a* and *omp3b* mutants do not display significant phenotypic deviations from the WT.** Independent disruption of *omp3a* and *omp3b* in *B. abortus* by the introduction of a kanamycin cassette was corroborated by RT-PCR and Western

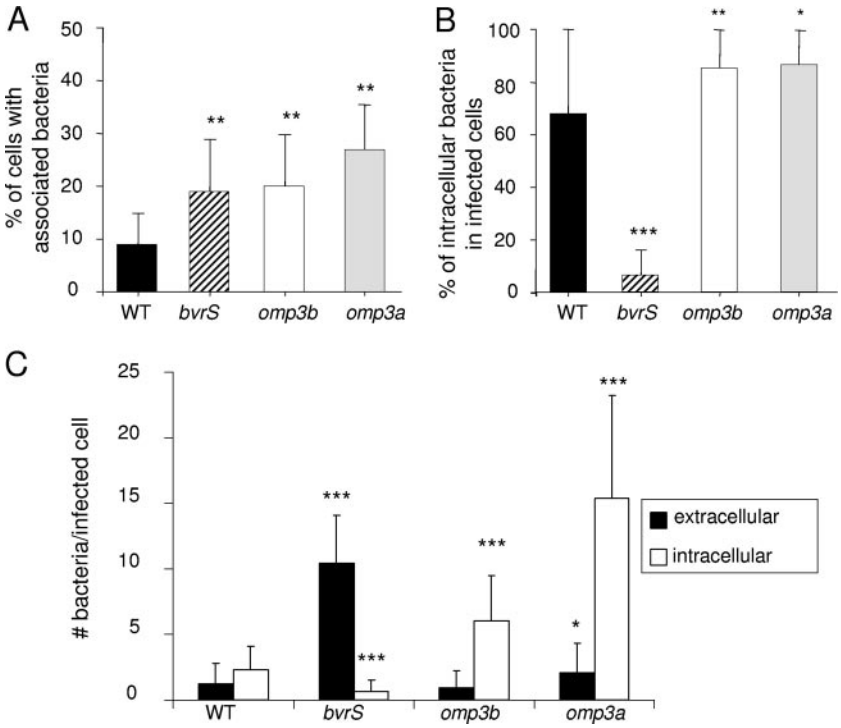

FIG. 3. Gentamicin survival assay by double immunofluorescence 30 min postinfection in HeLa cells. (A) Proportion of cells with associated (intra- and extracellular) bacteria. (B) Proportion of intracellular bacteria with respect to the total of intra- and extracellular bacteria. (C) Absolute number of intra- and extracellular bacteria per infected cell. Data represent means ± standard deviations. Samples were compared using the Student *t* test. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$ (with respect to the WT strain).

blotting. As expected, no transcription of specific mRNAs for *omp3a* or *omp3b* was detected by RT-PCR (Fig. 1A), and the absence of Omp3a in the *omp3a* mutant was demonstrated with MAbs against Omp3a (Fig. 1B). Antibodies against Omp3b are not currently available. However, 2D gel analysis of OM fragments of the *omp3b* mutant, the WT, and the *omp3b* mutant transformed with a plasmid encoding Omp3b demonstrated that protein spots similar to those previously shown to correspond to Omp3b isoforms (22) were absent in the *omp3b* mutant preparations and present in those of the WT and the *omp3b* mutant reconstituted strain (data not shown). In regular bacteriological media, both mutants and the WT showed similar growth patterns. These mutations did not affect the conventional phenotypic or metabolic properties described for *B. abortus* biotype 1 (1) or the ability of the mutants to grow in complex or defined media. The sensitivity to antibiotics such as doxycycline, gentamicin, streptomycin, chloramphenicol, penicillin, rifampin, and ciprofloxacin was similar to that of the WT but different from that of the *bvrS* or *bvrR* mutants, which displayed higher sensitivity (not shown). The LPS from the *omp3a* and *omp3b* mutants was smooth according to crystal violet staining, immunofluorescence, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis. Antibodies against Omp10, Omp16, Omp19, Omp2b, Omp1, and NH polysaccharide revealed quantities of these molecules in the *omp3a* and *omp3b* mutants similar to those in the WT strain (not shown).

***B. abortus omp3a* and *omp3b* mutant resistance to polymixin B and complement.** The avirulent phenotype of the *B. abortus bvrS* and *bvrR* mutants correlates with their higher sensitivity to bactericidal cationic peptides and complement (28, 42). Although these features have been linked to structural alterations of the LPS molecule (28), the role of Omp3a and Omp3b, whose transcription is regulated by the BvrR/BvrS system, has not been explored. At 10 μg/ml of polymyxin B, the *omp3a* mutant displayed levels of polymixin B sensitivity between those of the WT and the *bvrR* mutant, whereas the *omp3b* mutant was as resistant as the WT (Fig. 2A). At 25 μg/ml of polymyxin B, the percent survival for the *omp3a* mutant was less than 50% and the percent survival for the *omp3b* mutant was similar to that of the WT. As expected, the percent survival of the *bvrR* mutant was close to zero. At 50 μg/ml, the *omp3a* mutant displayed very low levels of bacterial survival, but the level of survival of the *omp3b* mutant was similar to that of the WT strain. At concentrations as high as 100 μg/ml, both mutants showed practically no survival. Consistent with previous reports (28), the *bvrR* mutant was highly sensitive to the action of complement in normal serum after a 30-min incubation; however, both *omp* mutants were resistant (Fig. 2B). The bactericidal effect was more pronounced after 90 min of incubation in most of the strains. There were no significant differences between the *omp3b* mutant and the WT strain, but in contrast, the *omp3a* mutant displayed higher resistance than the WT strain. Similar results were obtained with bovine nonimmune serum (data not shown).

***B. abortus omp3a* and *omp3b* mutants invade, survive, and replicate within professional and nonprofessional phagocytes.** *B. abortus bvrS* and *bvrR* mutants are poor invaders and fail to survive and replicate within professional and nonprofessional phagocytes (42). To test whether their deficiency in Omp3a

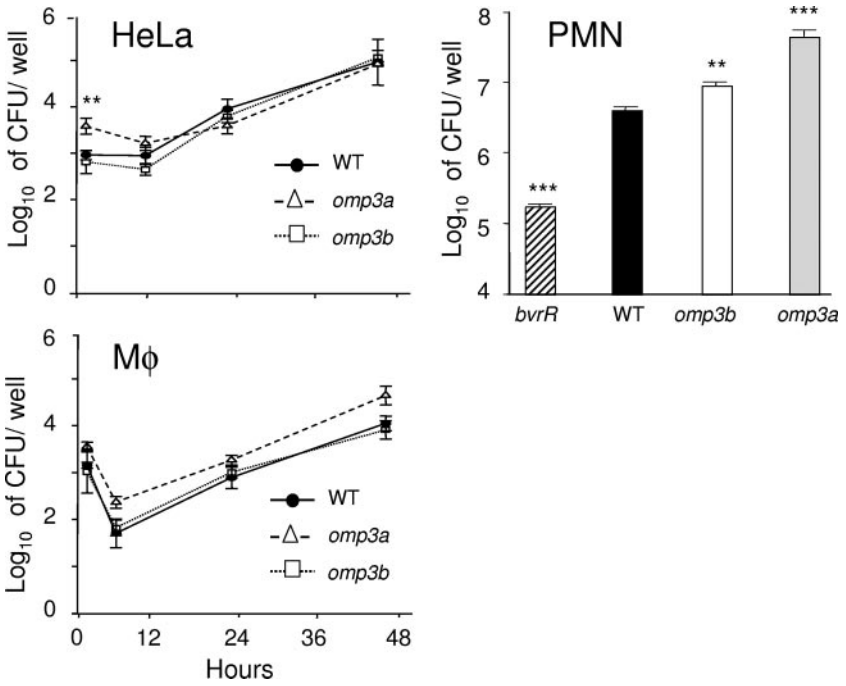

FIG. 4. Intracellular replication of *B. abortus* strains in epithelial HeLa cells, RAW 264.7 macrophages (Mϕ), and PMN. Data represent means ± standard deviations of plate counts. Samples were compared using the Mann-Whitney U test. **, $P < 0.005$; ***, $P < 0.0005$ (with respect to the WT strain).

and Omp3b proteins could explain this phenotype, both *omp* mutants were evaluated with HeLa cells by using the gentamicin survival assay and double immunofluorescence microscopy to distinguish intracellular from extracellular bacteria (Fig. 3). Similar to the *bvrS* mutant, the *omp3a* and *omp3b* mutants attached to more cells than the WT (Fig. 3A). In contrast to the *bvrS* mutant, however, the percentage of intracellular bacteria was higher for these mutants than for the WT strain (Fig. 3B). Moreover, the absolute number of bacteria per cell was higher for all mutants than for the WT. As expected, the number of intracellular bacteria per cell was significantly higher for the *omp3b* and *omp3a* mutants and significantly lower for the *bvrS* mutant than for the WT (Fig. 3C). In order to analyze the sensitivity of the *omp3a* and *omp3b* mutants to the killing action of cells, HeLa cells, PMN, and RAW 264.7 cells were infected and the replication rates were compared with those of the WT strain and the avirulent *bvrR* mutant (Fig. 4). As reported previously (42), the *bvrR* mutant failed to replicate in nonprofessional phagocytic HeLa cells and macrophages (not shown). Although the *omp3a* mutant consistently displayed higher counts at initial times of infection, the replication levels of both *omp* mutants were not considerably different from that of the WT strain at later times (Fig. 4). Similar results were obtained with naïve bone marrow-derived murine macrophages (not shown). As expected, the *bvrR* mutant was readily killed by PMN, while both *omp* mutants displayed a resistance slightly higher than that of the WT strain (Fig. 4).

***B. abortus omp3a* and *omp3b* mutants induce higher levels of TNF-α in murine macrophages than the *bvrS* mutant.** It has been proposed that Omp3a from *B. suis* is involved in the inhibition of TNF-α production during infection of human macrophages (24) but not of murine macrophages (5, 12, 20). We measured the production of TNF-α in murine RAW 264.7 macrophages infected with *omp3a* or *bvrS* mutants (Fig. 5). Consistent with the rates of replication in macrophages, the levels of TNF-α induced by the *omp3a* mutant were in the same range as those of the WT. However, the levels of this cytokine induced by the attenuated *bvrS* mutant were significantly lower.

***B. abortus omp3a* and *omp3b* mutants replicate in BALB/c mice.** As described previously (42), the WT strain maintained high counts in the spleens of BALB/c mice 24 weeks after infection, while *bvrS* and *bvrR* mutants were eliminated within 3 weeks. Similar to the results obtained with cells, the replication of *omp3a* and *omp3b* mutants in mice did not significantly

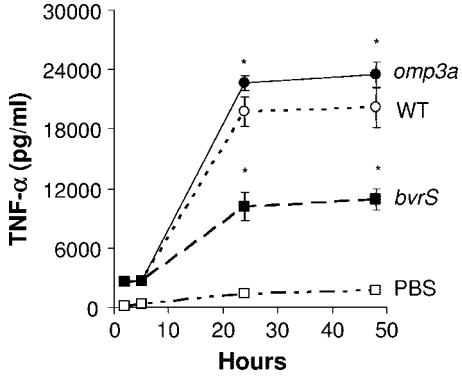

FIG. 5. Induction of TNF-α in murine RAW 264.7 macrophages infected with different *B. abortus* strains. *, $P < 0.05$ (with respect to the WT strain).

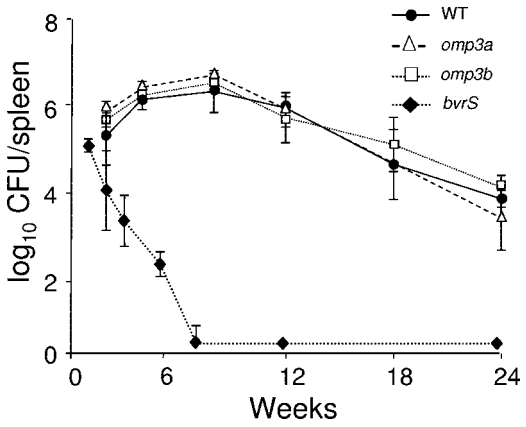

FIG. 6. Infection of the spleens of BALB/c mice with different *B. abortus* strains. Mice were infected intraperitoneally with 10^5 CFU/mouse, except for the *bvrS* mutant, for which the dose was 10^8 CFU/mouse. Values are means ± standard deviations ($n = 5$). The detection limit was 0.6 log CFU/spleen (3 to 4 CFU/spleen).

depart from that of the WT throughout the 24-week period of the experiment (Fig. 6).

DISCUSSION

We have shown that the absence of either Omp3a or Omp3b does not lead to phenotypes resembling *bvrS* or *bvrR* mutants but rather to phenotypes similar to that of the WT strain. Indeed, both *omp* mutants replicated in macrophages and epithelial cells to the same extent as the WT, and more importantly, persisted in mice for up to 24 weeks, exhibiting profiles similar to those of the WT strain. There were, however, some discrete differences between the *omp* mutants and WT *Brucella*. For instance, both *omp* mutants bound more readily to epithelial cells (similar to *bvrS* and *bvrR* mutants) and were slightly more resistant to the killing action of human PMN. The *omp3a* mutant was more sensitive to polymixin B and more resistant to the bactericidal action of complement after 90 min than the WT strain and induced slightly larger amounts of TNF-α in murine macrophages than the WT (see below). These results and those obtained for the *B. abortus omp3b* mutant in the same set of experiments are slightly different from those recently reported for two *Brucella ovis omp3a* and *omp3b* mutants (4). The differences in experimental setups, as well as the fact that *B. ovis* is a rough bacterium, might explain these discrepancies. The reason why the *B. abortus omp3a* mutant is more resistant to the bactericidal action of complement than the WT remains elusive. It is not known why *B. abortus* is more resistant to complement than other bacteria. Although there is some evidence that indicates that this could be attributed to its LPS (30), we did not detect any differences between the mutants' LPS and the parent strain's LPS with the methods used. Nevertheless, the purpose of using these kinds of experiments is to reveal OM properties that might be altered in the mutants compared to the WT. In this sense, we could speculate that the absence of Omp3a but not of Omp3b or both in the mutant strains allows the exposure in the OM of other molecules that are able to activate complement in higher levels than the WT.

Besides the nonsignificant variations with respect to the WT at the LPS level, the *B. abortus* Omp mutants did not show important variations in the quantities of other cell envelope components, such as NH or β-cyclic glucans, Omp10, Omp16, Omp19, Omp2b, and Omp1, or display major differences in OM permeability or growth rates. However, this does not rule out the existence of additional changes in other surface molecules, given that group 3 Omps are highly abundant and they strongly associate with the LPS (19, 22, 36). A search of the *B. abortus* genome reveals more than 25 proteins and lipoproteins predicted to be located in the OM, a fact that is sustained by a recent proteomic analysis of *B. abortus* OM fragments (26). Therefore, it is possible that some of these proteins are also affected, as we have seen in 2D gels of OM fragments from these mutants (data not shown).

We observed that RAW 264.7 murine macrophages infected with the *B. abortus omp3a* mutant generated slightly larger amounts of TNF-α than the WT strain. These results are in agreement with those previously reported (5, 12, 20), showing that the production of TNF-α in murine macrophages is not related to Omp3a. However, the *bvrS* mutant, harboring very low quantities of Omp3a, Omp3b, and most likely other membrane defects (28), induced small amounts of TNF-α. This effect is probably due to phenotypic changes in the cell envelope influencing the biological behavior of the mutant strains.

Consistent with the lack of important OM defects, the *omp3a* and *omp3b* mutants and the WT strain displayed almost identical replication rates in cells and similar numbers of colonies in mouse spleens. This is in sharp contrast with the fast clearing of the *bvrS* mutant. The *omp3a* and *omp3b* mutants bind to more cells and are internalized more by the cell population, and in absolute numbers, they show higher counts of total and internalized bacteria per cell than the WT strain. Therefore, and contrary to what is observed with the *bvrS* mutant, the internalization process in these bacteria is not impaired. This observation suggests that the two-component system is affecting the internalization process in a way not directly related to the presence of Omp3a or Omp3b. Although the *omp3a* and *omp3b* mutants are more efficiently internalized than the WT, they show replication and survival rates in cells and mice similar to those shown by the WT. The reason for this remains elusive. One possibility is that, in spite of their higher internalization rates, the actual absolute number of mutant bacteria reaching the final niche, i.e., the endoplasmic reticulum, is the same as the absolute number of WT bacteria. Overall, these results indicate that the absence of one of these two group 3 Omps does not generate attenuated *B. abortus* phenotypes. In previous reports, it has been described that a *B. abortus* 2308 *omp3a* deletion mutant opsonized with hyperimmune murine serum has delayed growth in late gestational bovine chorionic trophoblasts and cultured bovine macrophages. In fact, the percent survival of this mutant does not reach the levels of the WT after 48 h of infection (14). However, the data were normalized so that 100% survival represented the number of bacteria recovered after a 2-h incubation with macrophages. Our data show that both the *omp3a* and *omp3b* mutants were more efficiently internalized than the WT, and therefore their initial CFU counts were higher than those obtained for the WT. This difference is more obvious for HeLa cells than for murine macrophages. Conversely, during

the following hours after infection but before replication occurs, the CFU counts decreased to the WT level. This shows that these mutants have an initially higher destruction rate than the WT strain, as suggested by Edmonds et al. (14). However, the CFU counts after 12 h of infection show that both mutants reached the same absolute numbers as the WT, indicating that the numbers of bacteria that replicate and consequently adapt to the intracellular niche are the same. Indeed, if our results are represented in the same way as they were by Edmonds et al. (14), the data are the same. Therefore, it is clear that increased efficiency of internalization compensates for augmented intracellular destruction.

Caro-Hernández et al. (4) showed that a *B. ovis omp3b* mutant is attenuated in a mouse model compared with the parent strain. However, and in contrast to what we observed with our *B. abortus omp3b* mutant, the *B. ovis omp3b* mutant has greater susceptibility to nonimmune serum and has growth defects, particularly when reaching the stationary phase. These differences might be explained from the OM physiology context; because *B. ovis* is a rough bacterium, it is more likely that the absence of Omp3b might result in drastic modifications of its biology. On the other hand, and consistent with our results, Caro-Hernández et al. also showed that a *B. ovis omp3a* mutant is virulent in the same mouse model (4). Conversely, Edmonds et al. (14, 15) reported that a *B. abortus omp3a* mutant was attenuated in cattle and in mice 18 to 20 weeks postinfection. In our mouse experiments, we did not detect significant variations in a 24-week follow-up period with respect to the WT. At the present time, we do not have an explanation for this discrepancy. However, there is more evidence consistent with our observations. Neither the kinetics of spleen infections nor the residual virulence of *B. melitensis* Rev1 in mice is modified by deletion of group 3 Omp31 (8). Although further research is needed, a hypothesis is that group 3 Omps are an interdependent and coordinated group of proteins that have become redundant to secure the presence of at least one member in the OM and to permit certain cell envelope plasticity in order to live in different environments. This would account for the role of BvrR/BvrS and for the somewhat surprising phenotype of the *omp3a* and *omp3b* mutants since, according to the hypothesis, the absence of one protein of the group will be balanced by one or more of the other members. In fact, it has been observed that knocking out *omp25c*, *omp25d*, or *omp3b* increases Omp25b production in *B. suis*, and on this basis, a compensatory regulation within group 3 Omps is suggested (37). Furthermore, a tight balance of the group 3 Omps seems to be essential for the integrity of the *B. ovis* membrane (4). Virulent WT *B. abortus* naturally lacks functional genes for group 3 Omp31 and Omp25b (23), showing that the absence of one or two members of this family is not decisive for virulence. All this indirect evidence has to be considered with caution because *B. melitensis* and *B. abortus* do not have the same profile of group 3 Omps and there are contradictory reports on the level of group 3 Omps in *B. suis* mutants with mutations in *bvrR* and/or *bvrS* (3, 37). Nevertheless, the compensatory hypothesis would explain why mutations in a single Omp do not cause marked phenotypic changes, while dysfunction in BvrR/BvrS has a profound influence, at least in *B. abortus*. In this regard, it can also be hypothesized that the BvrR/BvrS influence may extend directly or indirectly to other group 3 members, to other OM molecules, or beyond the OM structure. A proteomic analysis of the complete cell envelope of the *B. abortus* WT and avirulent *bvrS* and *bvrR* mutants indicates that this might be the case, since these mutants have small amounts of different group 3 Omps and altered quantities of other Omps and periplasmic components compared with the WT (26).

ACKNOWLEDGMENTS

This work was partially supported by grant 8-N-2005 from NeTropica; grants from the MICIT/CONICIT, Costa Rica; a grant from Florida Ice & Farm; FIDA from the Universidad Nacional (UNA); and FS from the CONARE UNA/UCR agreement. Other grants were from the IFS (B/3456-1 and B/3456-2), the MEC of Spain (AGL2004-01162 and BIO2005-04985), and RTIC del FIS (objetivo 2, 2000-2006, G03/204). Fellowship support to L. Manterola from the Gobierno Vasco is gratefully acknowledged.

We thank C. Chacón and F. Mena for their assistance in the serologic analysis and A. Cloeckaert for providing some of the MAbs and plasmids used. We thank Samuel Wagner for the critical reading of the manuscript.

REFERENCES

1. **Alton, G. G., L. M. Jones, R. D. Angus, and J. M. Verger.** 1988. Techniques for the brucellosis laboratory. Institut National de la Recherche Agronomique, Paris, France.
2. **Arellano-Reynoso, B., N. Lapaque, S. Salcedo, G. Briones, A. E. Ciocchini, R. Ugalde, E. Moreno, I. Moriyón, and J. P. Gorvel.** 2005. Cyclic beta-1,2-glucan is a *Brucella* virulence factor required for intracellular survival. Nat. Immunol. **6:**618–625.
3. **Boigegrain, R.-A., I. Salhi, M.-T. Alvarez-Martinez, J. Machold, Y. Fedon, M. Arpagaus, C. Weise, M. Rittig, and B. Rouot.** 2004. Release of periplasmic proteins of *Brucella suis* upon acidic shock involves the outer membrane protein Omp25. Infect. Immun. **72:**5693–5703.
4. **Caro-Hernández, P., L. Fernández-Lago, M.-J. de Miguel, A. I. Martín-Martín, A. Cloeckaert, M.-J. Grillo, and N. Vizcaíno.** 2007. Role of the Omp25/Omp31 family in the outer membrane properties and virulence of *Brucella ovis*. Infect. Immun. **75:**4050–4061.
5. **Caron, E., A. Gross, J. P. Liautard, and J. Dornand.** 1996. *Brucella* species release a specific, protease-sensitive, inhibitor of TNF-alpha expression, active on human macrophage-like cells. J. Immunol. **156:**2885–2893.
6. **Celli, J., C. de Chastellier, D. M. Franchini, J. Pizarro-Cerda, E. Moreno, and J. P. Gorvel.** 2003. *Brucella* evades macrophage killing via VirB-dependent sustained interactions with the endoplasmic reticulum. J. Exp. Med. **198:**545–556.
7. **Chaves-Olarte, E., C. Guzmán-Verri, S. Meresse, M. Desjardins, J. Pizarro-Cerda, J. Badilla, J. P. Gorvel, and E. Moreno.** 2002. Activation of Rho and Rab GTPases dissociates *Brucella abortus* internalization from intracellular trafficking. Cell. Microbiol. **4:**663–676.
8. **Cloeckaert, A., I. Jacques, M. J. Grillo, C. M. Marin, M. Grayon, J. M. Blasco, and J. M. Verger.** 2004. Development and evaluation as vaccines in mice of *Brucella melitensis* Rev. 1 single and double deletion mutants of the *bp26* and *omp31* genes coding for antigens of diagnostic significance in ovine brucellosis. Vaccine **22:**2827–2835.
9. **Cloeckaert, A., J.-M. Verger, M. Grayon, M. S. Zygmunt, and O. Grépinet.** 1996. Nucleotide sequence and expression of the gene encoding the major 25-kilodalton outer membrane protein of *Brucella ovis*: evidence for antigenic shift, compared with other *Brucella* species, due to a deletion in the gene. Infect. Immun. **64:**2047–2055.
10. **Cloeckaert, A., N. Vizcaino, J. Y. Paquet, R. A. Bowden, and P. H. Elzer.** 2002. Major outer membrane proteins of *Brucella* spp.: past, present and future. Vet. Microbiol. **90:**229–247.
11. **Delpino, M. V., J. Cassataro, C. A. Fossati, F. A. Goldbaum, and P. C. Baldi.** 2006. *Brucella* outer membrane protein Omp31 is a haemin-binding protein. Microbes Infect. **8:**1203–1208.
12. **Dornand, J., A. Gross, V. Lafont, J. Liautard, J. Oliaro, and J. P. Liautard.** 2002. The innate immune response against *Brucella* in humans. Vet. Microbiol. **90:**383–394.
13. **Dorrell, N., V. G. Gyselman, S. Foynes, S. R. Li, and B. W. Wren.** 1996. Improved efficiency of inverse PCR mutagenesis. BioTechniques **21:**604–608.
14. **Edmonds, M. D., A. Cloeckaert, N. J. Booth, W. T. Fulton, S. D. Hagius, J. V. Walker, and P. H. Elzer.** 2001. Attenuation of a *Brucella abortus* mutant lacking a major 25 kDa outer membrane protein in cattle. Am. J. Vet. Res. **62:**1461–1466.
15. **Edmonds, M. D., A. Cloeckaert, and P. H. Elzer.** 2002. *Brucella* species

lacking the major outer membrane protein Omp25 are attenuated in mice and protect against *Brucella melitensis* and *Brucella ovis*. Vet. Microbiol. **88:**205–221.
16. **Edmonds, M. D., A. Cloeckaert, S. D. Hagius, L. E. Samartino, W. T. Fulton, J. V. Walker, F. M. Enright, N. J. Booth, and P. H. Elzer.** 2002. Pathogenicity and protective activity in pregnant goats of a *Brucella melitensis* Δ*omp25* deletion mutant. Res. Vet. Sci. **72:**235–239.
17. **Fretin, D., A. Fauconnier, S. Kohler, S. Halling, S. Leonard, C. Nijskens, J. Ferooz, P. Lestrate, R. M. Delrue, I. Danese, J. Vandenhaute, A. Tibor, X. DeBolle, and J. J. Letesson.** 2005. The sheathed flagellum of *Brucella melitensis* is involved in persistence in a murine model of infection. Cell. Microbiol. **7:**687–698.
18. **Gamazo, C., and I. Moriyón.** 1987. Release of outer membrane fragments by exponentially growing *Brucella melitensis* cells. Infect. Immun. **55:**609–615.
19. **Gamazo, C., A. I. Vitas, I. Moriyón, I. López-Goñi, and R. Diaz.** 1993. *Brucella* group 3 outer membrane proteins contain a heat-modifiable protein. FEMS Microbiol. Lett. **112:**141–146.
20. **Gross, A., S. Spiesser, A. Terraza, B. Rouot, E. Caron, and J. Dornand.** 1998. Expression and bactericidal activity of nitric oxide synthase in *Brucella suis*-infected murine macrophages. Infect. Immun. **66:**1309–1316.
21. **Guzmán-Verri, C., E. Chaves-Olarte, C. von Eichel-Streiber, I. López-Goñi, M. Thelestam, S. Arvidson, J. P. Gorvel, and E. Moreno.** 2001. GTPases of the Rho subfamily are required for *Brucella abortus* internalization in non-professional phagocytes: direct activation of Cdc42. J. Biol. Chem. **276:** 44435–44443.
22. **Guzmán-Verri, C., L. Manterola, A. Sola-Landa, A. Parra, A. Cloeckaert, J. Garin, J. P. Gorvel, I. Moriyón, E. Moreno, and I. López-Goñi.** 2002. The two-component system BvrR/BvrS essential for *Brucella abortus* virulence regulates the expression of outer membrane proteins with counterparts in members of the Rhizobiaceae. Proc. Natl. Acad. Sci. USA **99:**12375–12380.
23. **Halling, S. M., B. D. Peterson-Burch, B. J. Bricker, R. L. Zuerner, Z. Qing, L.-L. Li, V. Kapur, D. P. Alt, and S. C. Olsen.** 2005. Completion of the genome sequence of *Brucella abortus* and comparison to the highly similar genomes of *Brucella melitensis* and *Brucella suis*. J. Bacteriol. **187:**2715–2726.
24. **Jubier-Maurin, V., R.-A. Boigegrain, A. Cloeckaert, A. Gross, M.-T. Alvarez-Martinez, A. Terraza, J. Liautard, S. Köhler, B. Rouot, J. Dornand, and J. P. Liautard.** 2001. Major outer membrane protein Omp25 of *Brucella suis* is involved in inhibition of tumor necrosis factor alpha production during infection of human macrophages. Infect. Immun. **69:**4823–4830.
25. **Kovach, M. E., R. W. Phillips, P. H. Elzer, R. M. Roop II, and K. M. Peterson.** 1994. pBBR1MCS: a broad-host-range cloning vector. BioTechniques **16:**800–802.
26. **Lamontagne, J., H. Butler, E. Chaves-Olarte, M. S. J. Hunter, M. T. C. Paquet, P. Kearney, L. Hamaidi, D. Chelsky, I. Moriyón, E. Moreno, and E. Paramithiotis.** 2007. Extensive cell envelope modulation is associated with virulence in *Brucella abortus*. J. Proteome Res. **6:**1519–1529.
27. **Lapaque, N., I. Moriyón, E. Moreno, and J. P. Gorvel.** 2005. *Brucella* lipopolysaccharide acts as a virulence factor. Curr. Opin. Microbiol. **8:**60–66.
28. **Manterola, L., I. Moriyón, E. Moreno, A. Sola-Landa, D. S. Weiss, M. H. Koch, J. Howe, K. Brandenburg, and I. López-Goñi.** 2005. The lipopolysaccharide of *Brucella abortus* BvrS/BvrR mutants contains lipid A modifications and has higher affinity for bactericidal cationic peptides. J. Bacteriol. **187:**5631–5639.
29. **Martínez de Tejada, G., and I. Moriyón.** 1993. The outer membranes of *Brucella* spp. are not barriers to hydrophobic permeants. J. Bacteriol. **175:** 5273–5275.
30. **Moreno, E., D. T. Berman, and L. A. Boettcher.** 1981. Biological activities of *Brucella abortus* lipopolysaccharides. Infect. Immun. **31:**362–370.
31. **Moreno, E., and J.-P. Gorvel.** 2004. Invasion, intracellular trafficking and replication of *Brucella* organisms in professional and non-professional phagocytes, p. 287–312. *In* I. López-Goñi and I. Moriyón (ed.), *Brucella*: molecular and cellular biology. Horizon Bioscience, Norfolk, United Kingdom.
32. **Moreno, E., and I. Moriyón.** 2002. *Brucella melitensis*: a nasty bug with hidden credentials for virulence. Proc. Natl. Acad. Sci. USA **99:**1–3.
33. **Moreno, E., and I. Moriyón.** 2006. The genus *Brucella*, p. 315–456. *In* M. Dorkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, and E. Stackebrandt (ed.), The prokaryotes, vol. 5. Springer-Verlag, New York, NY.
34. **Moriyón, I., and I. López-Goñi.** 1998. Structure and properties of the outer membranes of *Brucella abortus* and *Brucella melitensis*. Int. Microbiol. **1:**19–26.
35. **Pizarro-Cerdá, J., S. Méresse, R. G. Parton, G. van der Goot, A. Sola-Landa, I. Lopez-Goñi, E. Moreno, and J. P. Gorvel.** 1998. *Brucella abortus* transits through the autophagic pathway and replicates in the endoplasmic reticulum of nonprofessional phagocytes. Infect. Immun. **66:**5711–5724.
36. **Riezu-Boj, J. I., I. Moriyón, J. M. Blasco, C. Gamazo, and R. Díaz.** 1990. Antibody response to *Brucella ovis* outer membrane proteins in ovine brucellosis. Infect. Immun. **58:**489–494.
37. **Salhi, I., R.-A. Boigegrain, J. Machold, C. Weise, A. Cloeckaert, and B. Rouot.** 2003. Characterization of new members of the group 3 outer membrane protein family of *Brucella* spp. Infect. Immun. **71:**4326–4332.
38. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
39. **Sangari, F., and J. Aguero.** 1991. Mutagenesis of *Brucella abortus*: comparative efficiency of three transposon delivery systems. Microb. Pathog. **11:**443–446.
40. **Simon, R., U. Priefer, and A. Puhler.** 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria. Bio/Technology **1:**784–791.
41. **Skurnik, M., R. Venho, J. A. Bengoechea, and I. Moriyón.** 1999. The lipopolysaccharide outer core of *Yersinia enterocolitica* serotype O:3 is required for virulence and plays a role in outer membrane integrity. Mol. Microbiol. **31:**1443–1462.
42. **Sola-Landa, A., J. Pizarro-Cerda, M. J. Grillo, E. Moreno, I. Moriyón, J. M. Blasco, J. P. Gorvel, and I. López-Goñi.** 1998. A two-component regulatory system playing a critical role in plant pathogens and endosymbionts is present in *Brucella abortus* and controls cell invasion and virulence. Mol. Microbiol. **29:**125–138.
43. **Tibor, A., V. Wansard, V. Bielartz, R.-M. Delrue, I. Danese, P. Michel, K. Walravens, J. Godfroid, and J.-J. Letesson.** 2002. Effect of *omp10* or *omp19* deletion on *Brucella abortus* outer membrane properties and virulence in mice. Infect. Immun. **70:**5540–5546.
44. **Verstreate, D. R., M. T. Creasy, N. T. Caveney, C. L. Baldwin, M. W. Blab, and A. J. Winter.** 1982. Outer membrane proteins of *Brucella abortus*: isolation and characterization. Infect. Immun. **35:**979–989.
45. **Vizcaino, N., P. Caro-Hernandez, A. Cloeckaert, and L. Fernandez-Lago.** 2004. DNA polymorphism in the *omp25*/*omp31* family of *Brucella* spp.: identification of a 1.7-kb inversion in *Brucella cetaceae* and of a 15.1-kb genomic island, absent from *Brucella ovis*, related to the synthesis of smooth lipopolysaccharide. Microbes Infect. **6:**821–834.
46. **Vizcaino, N., J. M. Verger, M. Grayon, M. S. Zygmunt, and A. Cloeckaert.** 1997. DNA polymorphism at the *omp-31* locus of *Brucella* spp.: evidence for a large deletion in *Brucella abortus*, and other species-specific markers. Microbiology **143:**2913–2921.
47. **Weiss, D. S., K. Takeda, S. Akira, A. Zychlinsky, and E. Moreno.** 2005. MyD88, but not Toll-like receptors 4 and 2, is required for efficient clearance of *Brucella abortus*. Infect. Immun. **73:**5137–5143.

Editor: J. B. Bliska