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Role of the Omp25/Omp31 Family in Outer Membrane Properties and Virulence of *Brucella ovis*▽

Paola Caro-Hernández,[1] Luis Fernández-Lago,[1] María-Jesús de Miguel,[2] Ana I. Martín-Martín,[1] Axel Cloeckaert,[3] María-Jesús Grilló,[2,4] and Nieves Vizcaíno[1]*

Departamento de Microbiología y Genética, Edificio Departamental, Universidad de Salamanca, Plaza Doctores de la Reina s/n, 37007 Salamanca, Spain[1]; Centro de Investigación y Tecnología Agroalimentaria del Gobierno de Aragón, Unidad de Sanidad Animal, Carretera de Montañana 930, 50059 Zaragoza, Spain[2]; INRA, UR1282, Infectiologie Animale et Santé Publique, IASP, Nouzilly F-37380, France[3]; and Instituto de Agrobiotecnología y Recursos Naturales, CSIC-UPNA, Ctra. Mutilva Baja, 31192 Pamplona, Spain[4]

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The genes coding for the five outer membrane proteins (OMPs) of the Omp25/Omp31 family expected to be located in the outer membrane (OM) of rough virulent *Brucella ovis* PA were inactivated to evaluate their role in virulence and OM properties. The OM properties of the mutant strains and of the mutants complemented with the corresponding wild-type genes were analyzed, in comparison with the parental strain and rough *B. abortus* RB51, in several tests: (i) binding of anti-Omp25 and anti-Omp31 monoclonal antibodies, (ii) auto-agglutination of bacterial suspensions, and (iii) assessment of susceptibility to polymyxin B, sodium deoxycholate, hydrogen peroxide, and nonimmune ram serum. A tight balance of the members of the Omp25/Omp31 family was seen to be essential for the stability of the *B. ovis* OM, and important differences between the OMs of *B. ovis* PA and *B. abortus* RB51 rough strains were observed. Regarding virulence, the absence of Omp25d and Omp22 from the OM of *B. ovis* PA led to a drastic reduction in spleen colonization in mice. While the greater susceptibility of the Δ*omp22* mutant to nonimmune serum and its difficulty in surviving in the stationary phase might be on the basis of its dramatic attenuation, no defects in the OM able to explain the attenuation of the Δ*omp25d* mutant were found, especially considering that the fully virulent Δ*omp25c* mutant displayed more important OM defects. Accordingly, Omp25d, and perhaps Omp22, could be directly involved in the penetration and/or survival of *B. ovis* inside host cells. This aspect, together with the role of Omp25d and Omp22 in the virulence both of *B. ovis* in rams and of other *Brucella* species, should be thoroughly evaluated in future studies.

The genus *Brucella* comprises six classical species (*Brucella melitensis*, *B. abortus*, *B. suis*, *B. ovis*, *B. canis*, and *B. neotomae*) that infect terrestrial mammals (41), although in recent years an increasing number of *Brucella* strains have been isolated from marine mammals and proposed to be included in two new species, *B. cetaceae* and *B. pinnipediae* (9).

Brucella spp. may have smooth or rough lipopolysaccharide (S-LPS or R-LPS) depending on the presence or absence, respectively, of O-polysaccharide chains. Rough mutants derived from smooth *Brucella* strains show an important reduction in virulence (1, 26, 30, 40, 45). Accordingly, the O-polysaccharide chains of LPS are thought to be necessary for the pathogenicity of *Brucella* strains bearing S-LPS. However, *B. ovis* and *B. canis* lack O chains in the LPS but are pathogenic for rams and dogs, respectively, and induce long-lasting infections with high levels of splenic colonization in laboratory animals (34). Since O chains mask other components of the *Brucella* spp. outer membrane (OM), OM proteins (OMPs) are more exposed at the bacterial surface of rough *B. ovis* and *B. canis* (4, 7) and their involvement in virulence in rough *Brucella* strains may be more relevant than in smooth *Brucella* strains.

The *Brucella* spp. Omp25/Omp31 family comprises seven homologous OMPs. Omp25 and Omp31 are major OMPs, except Omp31 in *B. abortus*, which lacks its coding gene (48, 49, 51), and they have been fairly well characterized in several aspects (11). Regarding virulence, mutant *B. melitensis*, *B. abortus*, and *B. ovis* strains with the *omp25* gene inactivated have been found to be attenuated in mice, goats (*B. melitensis*), and cattle (*B. abortus*) (17–19). Additionally, Omp25 has been shown to inhibit the production of tumor necrosis factor alpha (TNF-α) by human macrophages (35) and to be involved in the permeability of the *Brucella* membrane, allowing the secretion of periplasmic proteins, in acidic medium (3).

In contrast, little is known about the Omp31b, Omp25b, Omp25c, Omp25d, and Omp22 proteins. Although no studies specifically analyzing the presence of these proteins in each *Brucella* species have been reported, the five OMPs have been detected experimentally in at least one *Brucella* species. Thus, Omp31b has been identified in *B. abortus* and *B. suis* (13, 42), Omp25b in *B. melitensis* and *B. suis* (23, 42, 52), Omp25c in *B. melitensis*, *B. abortus*, and *B. suis* (13, 42, 52), and Omp22 in *B. abortus* (13, 32). Evidence for the production of Omp25d in the genus *Brucella* is less conclusive, since the protein has been detected only weakly, by reactivity with an anti-Omp25 monoclonal antibody (MAb), in an *omp25*-defective *B. suis* mutant (42). Studies addressing DNA polymorphism of the Omp25/

* Corresponding author. Mailing address: Departamento de Microbiología y Genética, Edificio Departamental, Universidad de Salamanca, Plaza Doctores de la Reina s/n, 37007 Salamanca, Spain. Phone: 34-923-294 532. Fax: 34-923-224 876. E-mail: vizcaino@usal.es.
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TABLE 1. *Brucella* strains used in this work

Brucella strain	Relevant genotype	Relevant characteristic(s)	Origin
Wild-type strains[a]			
B. abortus RB51		Rough strain obtained under laboratory conditions	BCCN[b]
B. ovis PA	Parental strain	Naturally rough virulent *Brucella* strain	BCCN
Mutant strains			
B. ovis PNV31A	Δ*omp31* Kan^r	*B. ovis* PA with a Kan resistance cassette replacing the XcmI-SalI fragment of *omp31*	This work
B. ovis PNV25A	*omp25*::Kan^r	*B. ovis* PA with a Kan resistance cassette inserted into the AflII site of *omp25*	This work
B. ovis PNV25cA	Δ*omp25c* Kan^r	*B. ovis* PA with a Kan resistance cassette replacing the AspI-HinCII fragment of *omp25c*	This work
B. ovis PNV25dA	Δ*omp25d* Kan^r	*B. ovis* PA with a Kan resistance cassette replacing the NheI-ClaI fragment of *omp25d*	This work
B. ovis PNV22A	Δ*omp22* Kan^r	*B. ovis* PA with a Kan resistance cassette replacing the RsrII-StuI fragment of *omp22*	This work
Complemented mutant strains			
B. ovis PNV31A-com	Δ*omp31 omp31* Kan^r Amp^r	*B. ovis* PNV31A complemented with *omp31* cloned in pNV31300[c]	This work
B. ovis PNV25A-com	*omp25*::Kan^r *omp25* Kan^r Amp^r	*B. ovis* PNV25A complemented with *omp25* cloned in pNV25com[c]	This work
B. ovis PNV25cA-com	Δ*omp25c omp25c* Kan^r Amp^r	*B. ovis* PNV25cA complemented with *omp25c* cloned in pNV25c4[c]	This work
B. ovis PNV25dA-com	Δ*omp25d omp25d* Kan^r Amp^r	*B. ovis* PNV25dA complemented with *omp25d* cloned in pNV25d4[c]	This work
B. ovis PNV22A-com	Δ*omp22 omp22* Kan^r Amp^r	*B. ovis* PNV22A complemented with *omp22* cloned in pNV22D[c]	This work

[a] Reference strains of the *Brucella* species (46) were also used for the bacteriological typing of the *B. ovis* strains obtained in this work.
[b] BCCN, *Brucella* Culture Collection of Nouzilly, INRA Centre de Tours, France. *B. abortus* RB51 and *B. ovis* PA are strains BCCN V5 and BCCN 76-250, respectively.
[c] Plasmids were constructed by cloning the corresponding *B. ovis* PA wild-type *omp* gene in pBBR1MCS-4.

Omp31-encoding gene family in the genus *Brucella* have provided additional information about the occurrence of these five proteins in the OM of the *Brucella* species (46): (i) Omp31b is probably absent from the OMs of *B. melitensis*, *B. ovis*, and *B. canis*; (ii) Omp25b is not expected to be present in the OMs of *B. abortus*, *B. ovis*, *B. canis*, *B. cetaceae*, and *B. pinnipediae*; and (iii) Omp25c, Omp25d, and Omp22 are conserved proteins that would be located in the OMs of the six classical *Brucella* species and of *B. cetaceae* and *B. pinnipediae* (perhaps with the exception of Omp22 in some *B. cetaceae* isolates).

In the present work, we evaluated the role of the five members of the Omp25/Omp31 family expected to be located in the OM of *B. ovis* (46) in terms of the OM properties and virulence of the bacterium. To achieve these objectives, single-mutant strains with the genes *omp31*, *omp25*, *omp25c*, *omp25d*, or *omp22* inactivated were obtained from virulent *B. ovis* PA and were subsequently complemented with the corresponding wild-type genes.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and cloning vectors. The *Brucella* strains used in this work are listed in Table 1 and were cultured at 37°C in a 10% CO_2 atmosphere. They were typically propagated in tryptic soy broth (TSB; Difco Laboratories, Detroit, MI) or tryptic soy agar (TSA; Difco Laboratories), both supplemented with 0.3% yeast extract (YE; Difco Laboratories) and 5% horse serum (HS; GIBCO-BRL Life Technologies, Germany) (TSB-YE-HS and TSA-YE-HS). For bacteriological typing and virulence experiments, blood agar base no. 2 (Biolife, Italy) supplemented with 5% calf serum (Seromed; Biochrom, Austria) was used. Kanamycin (Kan; 50 μg/ml) or Kan plus ampicillin (Amp) (50 μg/ml each) was added to the culture medium used for mutant or complemented mutant strains of *B. ovis* PA, respectively. When necessary for the selection of mutant strains, the medium was supplemented with 5% sucrose or Amp (50 μg/ml). Bacteriological typing of the *B. ovis* mutant and complemented strains obtained in this work was performed using standard procedures (i.e., Gram staining; catalase, oxidase, urease, and acriflavine agglutination testing; lysis by Tb, Wb, Iz, and R/C phages; agglutination with anti-A and anti-M monospecific sera; CO_2 and serum dependence testing; and testing of susceptibility to thionine, fuchsine, and safranine) (2).

Plasmids pGEM-T Easy (Promega, Madison, WI), pGEM7-Zf (Promega), pUC19 (Fermentas), pCVD442 (16), and pBBR1MCS-4 (36) were used as cloning vectors. Plasmid pCVD442 does not replicate in *Brucella* spp., confers resistance to Amp, and contains *sacB* gene, which renders bacteria susceptible to sucrose, while pBBR1MCS-4 replicates in *Brucella* spp. and confers Amp resistance. The plasmids were propagated either in *Escherichia coli* JM109 (Promega) or, in the case of pCVD442-derived plasmids, in *E. coli* CC118 (λ*pir*). Recombinant *E. coli* cells were grown in Luria-Bertani medium supplemented with 50 μg/ml of the required antibiotic(s) depending on the plasmid.

Primers and DNA techniques. Primers (Roche, Germany) (Table 2) were chosen according to the published *B. melitensis* 16 M and *B. suis* 1330 nucleotide

TABLE 2. DNA primers used in this study

Primer use and name	Nucleotide sequence
Construction of mutant *B. ovis* PA strains	
31MUT-F	5′-AGA ATA AAA CAC ATG CCC-3′
31MUT-R	5′-GCT GAA TGC GGA GAT GGT-3′
25MUTZ-F	5′-CGA CCT TAT CCT CCT GAA-3′
25MUTZ-R	5′-CCA GCA AAA CGT CGC AAA-3′
25cdMUT-F	5′-TGC GTG GTT CAG ATT TCG-3′
25cdMUT-R	5′-TTG CCG CTT CCA TCA GGT-3′
25cMUT-R	5′-AGC CGT AAC CAA CCT GAC-3′
22MUT-F	5′-GGC AAA GAA GAA GAA TAC-3′
22MUT-R	5′-CTG CTG GAA TGC CCT GAA-3′
Complementation of mutant *B. ovis* PA strains	
31sd	5′-TGA CAG ACT TTT TCG CCG AA-3′
31ter	5′-CAT TCA GGA CAA TTC CCG CC-3′
25A-F	5′-GGA CCG CGC AAA ACG TAA-3′
25-R	5′-ACC GGA TGC CTG AAA TCC-3′
25C-F	5′-CTG TGT CCT GTT TGC TAC-3′
25C-R	5′-TAT TGG GTG AGG ATT GAC-3′
25D-F	5′-CTA CAT ACA TTC GAC CAG-3′
25D-R	5′-CTG AAG GGT AAA TGC GGC-3′
22-F	5′-TCA AGC ATG TTT CCC GCC-3′
22-R	5′-GTT TGA ATC CCG GCT GTT-3′

sequences for chromosomes I and II (GenBank accession numbers AE008917, AE008918, AE014291, and AE014292). PCR was performed with the Expand Long Template PCR system (Roche) following the instructions of the manufacturer with 100 ng of *B. ovis* strain DNA template extracted with a High Pure PCR template preparation kit (Roche) and 2 μM each primer. DNA was sequenced by primer-directed dideoxy sequencing with an ABI PRISM 377 DNA sequencer (Perkin-Elmer, Foster City, CA).

Southern blot hybridization was performed as described previously (46) using digoxigenin (DIG)-labeled probes and *B. ovis* strain chromosomal DNA digested with HindIII. Probes were labeled with a DIG DNA labeling kit (Roche), and hybridization was detected with a DIG nucleic acid detection kit (Roche) as instructed by the manufacturer. DNA markers III and VI (Roche) were used as molecular size standards and were hybridized with probes constituted by the same markers labeled with DIG.

MAbs and immunological techniques. MAbs A59/05F01/C09 (C09) and A19/12B10/F04 (F04) (ascitic fluid), specific for *Brucella* sp. Omp25, and A59/10F09/G10 (G10) and A01/08H06/G02 (G02) (hybridoma culture medium supernatant), specific for *Brucella* spp. Omp31 (7, 50), were used. Western blotting with *Brucella* whole-cell lysates was performed as described previously (50). For indirect enzyme-linked immunosorbent assay (iELISA) (46), parental or mutant *B. ovis* PA strains cultured for 24 h in the corresponding TSB-YE-HS media and washed with phosphate-buffered saline (PBS; pH 7.2) were used as coating antigens (100 μl/well of a bacterial suspension adjusted to an optical density at 600 nm [OD_{600}] of 1). Then, a saturation step with 5% skim milk in PBS was included (300 μl/well; 30 min at 37°C). After subsequent incubation steps with MAbs and goat anti-mouse immunoglobulin G (Fc-specific) peroxidase conjugate (Sigma, St. Louis, Mo.; 1:4,000), OD_{405} values were recorded after 30 min of incubation with the substrate solution, and the results were expressed as the mean ± standard deviation (SD) of the OD readings for three wells.

Inactivation of the genes encoding the Omp25/Omp31 family in *B. ovis* PA by homologous recombination. In this work, we obtained five mutant strains (Table 1) derived from parental *B. ovis* PA by individually inactivating the genes coding for Omp31, Omp25, Omp25c, Omp25d, and Omp22 (genes BRA0423, BR0701, BR0119, BR0118, and BR1284, respectively, according to the *B. suis* 1330 genome sequence). For all the genes except *omp25*, inactivation was performed by replacing most of the gene by a Kan resistance cassette, which was placed in the direction opposite to that of the gene. Inactivation of *omp25* was achieved by insertion of the Kan resistance cassette, also in the direction opposite to that of the gene, at the AflII site located just 10 nucleotides downstream from the *omp25* ATG start codon.

Briefly, deletion of *omp31* from parental *B. ovis* PA was performed as follows. Primers 31MUT-F and 31MUT-R (Table 2) were used to amplify, according to the *B. melitensis* 16 M sequence (49), a 1,406-bp fragment comprising *omp31* and DNA flanking both sides of the gene. The amplified fragment was cloned into plasmid pGEM-T Easy, excised by EcoRI digestion from the recombinant plasmid obtained, and then cloned into the EcoRI site of pGEM7-Zf. Then, the XcmI-SalI fragment of *omp31* was replaced from the resulting pNV31201 recombinant plasmid by the Kan^r gene extracted from pUC4K (Amersham Biosciences) to give pNV31211. The DNA insert of pNV31211 was isolated by digestion with SphI and SacI and cloned into pCVD442, resulting in pNV31211-1, which was introduced into *B. ovis* PA by electroporation as described previously (46). To replace wild-type *omp31* by the inactivated *omp31* gene of pNV31211-1 from the *B. ovis* chromosome by double recombination through the homologous regions flanking both sides of *omp31*, a previously described procedure was followed (21). Mutant *B. ovis* PNV31A (Δ*omp31*) was selected by both its resistance to Kan and sucrose and by its susceptibility to Amp.

A similar process was followed to inactivate the other genes, and only the significant differences are mentioned below. The primers used for the inactivation of the other genes (Table 2) were as follows: 25MUTZ-F and 25MUTZ-R (*omp25*), 25cdMUT-F and 25cMUT-R (*omp25c*), 25cdMUT-F and 25cdMUT-R (*omp25d*), and 22MUT-F and 22MUT-R (*omp22*). Plasmids pNV25mut-1, pNV25c-1, pNV25d-2, and pNV22-1 were the pCVD442-derived plasmids used to electroporate *B. ovis* PA to obtain the mutant strains described in Table 1. These plasmids respectively contained the *omp25*, *omp25c*, *omp25d*, and *omp22* genes inactivated by the Kan resistance cassette, as shown in Table 1, column 3.

To check the mutant strains, a chromosomal region including the entire fragment used to inactivate the *omp* gene and adjacent DNA on both sides was PCR amplified and sequenced. Southern blot hybridization of HindIII-digested chromosomal DNA with pCVD442 and either pNV31211-1, pNV25mut-1, pNV25c-1, pNV25d-2, or pNV22-1 DIG-labeled probes was also performed. The absence of Omp31 and Omp25 from *B. ovis* PNV31A and *B. ovis* PNV25A (Table 1), respectively, was checked by Western blotting with MAbs G02 and C09. CFU in medium with and without Kan were determined for each mutant strain in order to evaluate the stability of the mutations in vitro.

Complementation of mutant strains. Mutant *B. ovis* PA strains were complemented with the corresponding wild-type genes. Each gene, including the ribosome binding site and the transcription terminator, was PCR amplified with primers 31sd and 31ter (*omp31*), 25A-F and 25-R (*omp25*), 25C-F and 25C-R (*omp25c*), 25D-F and 25D-R (*omp25d*), or 22-F and 22-R (*omp22*) (Table 2). PCR products were cloned in pGEM-T Easy, and the DNA inserts of the selected plasmids were subsequently cloned in pBBR1MCS-4 to leave the *omp* genes under the control of the *lacZ* promoter. Recombinant pBBR1MCS-4 derivatives pNV31300, pNV25com, pNV25c4, pNV25d4, and pNV22D were introduced in the corresponding mutant *B. ovis* PA strain by electroporation, and colonies resistant to both Amp and Kan were selected (Table 1). Complementation was verified by extraction of the plasmid DNA and sequencing of the DNA insert. Synthesis of Omp31 in *B. ovis* PNV31A-com and of Omp25 in *B. ovis* PNV25A-com (Table 1) was also confirmed by reactivity with the MAbs G02 and C09 in Western blotting assays. Plasmid stability of the complemented mutant strains was tested by plating in culture medium without antibiotic or with Kan and Amp.

Susceptibility assays. For susceptibility assays, *B. ovis* strains and *B. abortus* RB51 were cultured for 44 and 24 h, respectively, on TSA-YE-HS supplemented with antibiotic(s) when required. Then, a bacterial suspension was prepared in PBS as detailed for each test.

The susceptibilities of *Brucella* strains to polymyxin B (Sigma) and sodium deoxycholate (Sigma) were determined following a protocol described previously (38) with some modifications. A bacterial suspension containing approximately 1×10^4 CFU/ml was prepared in PBS, and 100 μl was mixed in wells of a 96-well sterile plate with 100 μl of 2 mg/ml polymyxin B or 0.2 mg/ml sodium deoxycholate (final concentrations in the wells, 1 mg/ml and 0.1 mg/ml, respectively). After a 1-h incubation at 37°C in a 10% CO_2 atmosphere, the content of each well was mixed, and 50 μl was spread in triplicate on TSA-YE-HS plates supplemented with antibiotic(s) when required. The CFU obtained after treatment with polymyxin B or sodium deoxycholate were counted, and the percentages of survival were established with respect to the CFU obtained with bacteria incubated in PBS (100% survival). The results were expressed as the mean ± SD of three assays.

Susceptibility to hydrogen peroxide (Sigma) was evaluated by a modification of the disk sensitivity assay described by Elzer et al. in 1994 (22). A bacterial suspension with approximately 10^9 CFU/ml (OD_{600} of 0.2) was prepared in PBS, and 100 μl was spread by triplicate on TSA-YE-HS plates supplemented with antibiotic(s) when required. A Whatman 3MM Chr disk (9-mm diameter) was placed at the center of the plate, and 10 μl of 30% H_2O_2 was deposited on the disk. After 4 days of growth, the diameter of the zone of clearance around the disk was measured in quadruplicate for each plate and the mean diameter was calculated. The results were expressed as the mean ± SD of the diameter (in cm) obtained for the three plates.

The protocol designed to determine the susceptibility of the *Brucella* strains to nonimmune ram serum was based on that described by Corbeil et al. in 1988 (14). Serum was obtained from a 5-month-old ram born in the brucellosis-free flock of the Centro de Investigación y Tecnología Alimentaria (CITA) del Gobierno de Aragón, Spain. Half of the serum was heated at 56°C for 30 min to remove complement (control serum) and the other half was used fresh. Briefly, 50-μl bacterial suspensions with approximately 2×10^4 CFU/ml in PBS were mixed in wells of a 96-well sterile plate with 150 μl of either fresh serum or heated serum. After a 4-h incubation at 37°C in a 10% CO_2 atmosphere, the content of each well was gently mixed by pipetting and 50 μl of each bacterial suspension was spread in triplicate on TSA-YE-HS plates supplemented with antibiotic(s) when required. The CFU obtained after exposure to fresh serum were counted, and the percentages of survival were established with respect to the CFU obtained with the heated serum (100% survival). The results were expressed as the mean ± SD of three assays performed with the same serum.

Autoagglutination test. Bacterial suspensions with an OD_{600} of 0.8 were prepared in TSB-YE-HS from *B. ovis* strains previously cultured for 44 h (or from *B. abortus* RB51 cultured for 24 h) in TSA-YE-HS supplemented with the corresponding antibiotic(s). One ml of each suspension was left in a spectrophotometer microcuvette without agitation, and the OD_{600} readings were scored for 45 h at selected intervals. One microcuvette with TSB-YE-HS was used as a blank. The results were represented as the mean ± SD of the values obtained for three assays at each time point.

Virulence studies in mice. Female 8-week-old BALB/c mice (Charles River Laboratories, Spain) were accommodated in the animal building of the CITA of Aragón (registration number ES-502970012005) under biosafety conditions. The animals were kept in cages with water and food ad libitum for 1 week before the

start of the experiment. The mouse experimental procedures and the facilities used to hold the mice were in accordance with current European legislation (directive 86/609/EEC).

After a preliminary assay to determine the most adequate dose of infection, mice were inoculated intraperitoneally with approximately 5×10^6 CFU of the corresponding *B. ovis* strain in 0.1 ml of PBS, and splenic bacterial counts were determined for five mice per strain at 1, 2, 3, 5, 7, 9, and 11 weeks postinoculation (p.i.). Experimental procedures (i.e., preparation and administration of inocula, retrospective assessment of the exact inoculating doses, and determination of the number of CFU/spleen) were performed as described previously (12). The numbers of CFU/spleen were transformed logarithmically to normalize the distribution of individual counts. Results were expressed as the mean ± SD ($n = 5$) of the log CFU/spleen for each strain at each selected p.i. time point.

To check the stability of mutation after in vivo activity, the strains recovered from the spleens of infected mice were rechecked by PCR amplification, Southern blot hybridization, plating in culture medium with or without the required antibiotic, bacteriological typing, and Western blot assays as described above.

Statistical analysis. Statistical comparisons between means were performed using analysis of variance, and the levels of significance of the differences were determined with the post hoc Fisher's protected least significant differences test.

RESULTS

Inactivation of the genes encoding the Omp25/Omp31 family in *B. ovis* PA and complementation of the mutant strains. DNA sequencing and Southern blot hybridization with chromosomal DNA extracted from the five *B. ovis* PA mutant strains afforded the expected results for inactivation of the corresponding genes, with no modification of adjacent genes. Plasmid DNA could be isolated from the mutant strains complemented with the corresponding wild-type genes, and the insert DNA had the expected nucleotide sequence. After both in vitro subculturing and in vivo activity in mice, all the genetically modified *B. ovis* PA strains behaved like the *B. ovis* PA parental strain in the standard tests for *Brucella* spp. typing and gave similar CFU counts in TSA-YE-HS with and without antibiotic, thus showing the stability of the mutation or complementation. By transmission electron microscopy, no defects were found in the cell wall structures of any of the mutant strains (data not shown).

In vitro growth on solid (TSA-YE-HS) and in liquid (TSB-YE-HS) media was not, in general, dramatically affected for the genetically modified *B. ovis* PA strains. The number of CFU/ml was determined for bacterial suspensions of an OD_{600} of 0.2 prepared from bacteria cultured for 44 h on TSA-YE-HS plates. *B. ovis* Δ*omp25d* and Δ*omp22* mutants and their derived complemented strains had counts similar to that obtained with parental *B. ovis* PA, while Δ*omp31* and Δ*omp25c* mutant strains and their respective complemented strains had CFU/ml values about half as high (the Δ*omp31* and Δ*omp31*-com strains also required one more day than the other strains to develop visible colonies on solid medium) (data not shown). The Δ*omp25* mutant strain also afforded CFU/ml values about half as high as those observed with the parental strain, but complementation of this mutant with wild-type *omp25* recovered the counts obtained with *B. ovis* PA (data not shown).

Growth in liquid medium of bacterial cultures starting at an OD_{600} of 0.05 and incubated with shaking is shown in Fig. 1. The Δ*omp31* and Δ*omp25* mutant strains, in spite of reaching higher OD values (Fig. 1A), provided CFU/ml numbers in the stationary phase similar to that observed for parental *B. ovis* PA (data not shown). The Δ*omp25c* strain seemed to grow slower than the parental strain, reaching OD_{600} and CFU/ml

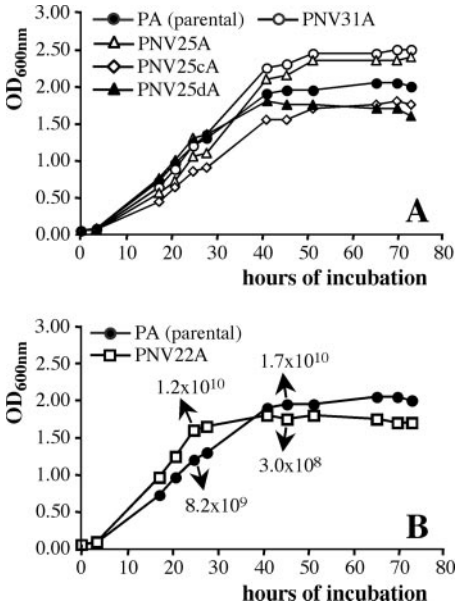

FIG. 1. Growth curves in liquid TSB-YE-HS, as determined by the evolution of OD_{600} with time, of parental *B. ovis* PA (A and B) and the mutant strains with the genes *omp31*, *omp25*, *omp25c*, *omp25d* (A), and *omp22* (B) inactivated. CFU/ml counts are shown at time points 25 and 46 h for parental and Δ*omp22* mutant strains (B). Cultures were started at an OD_{600} of 0.05 and were incubated at 37°C with shaking under a 10% CO_2 atmosphere.

values in the stationary phase that were slightly lower than those corresponding to *B. ovis* PA. As determined by OD_{600} readings and CFU/ml counts, the Δ*omp25d* mutant grew as well as the parental strain did (Fig. 1A and data not shown). The Δ*omp22* mutant grew faster than *B. ovis* PA, reaching the stationary phase earlier (Fig. 1B). However, although the bacterial counts for the Δ*omp22* mutant were in accordance with the OD_{600} scores and both parameters were higher than those observed with *B. ovis* PA up to 25 h of incubation, the number of CFU/ml for this mutant underwent a drastic decrease after 46 h of culture; at this time point, it was almost 2 logs lower than that obtained for this mutant at 25 h and that obtained for the parental strain after 46 h of incubation (Fig. 1B).

The absence of Omp31 and of Omp25 in the respective mutant strains and the recovery of their synthesis in the corresponding complemented mutant strains were verified by reactivity with specific MAbs (Fig. 2). Moreover, by reactivity with rabbit polyclonal antibodies raised against the recombinant proteins of the Omp25/Omp31 family, we obtained additional information about the synthesis of the other members of the Omp25/Omp31 family in the *B. ovis* strains obtained in this work (unpublished results). Thus, we were able to detect the presence of Omp25c and Omp22 in *B. ovis* PA and their absence in the respective mutant strains. Moreover, Omp25c was detected at important levels—higher than those detected for the parental strain—for the complemented mutant strain *B. ovis* PNV25cA-com. In contrast, the synthesis of Omp22 was strongly decreased in the complemented mutant strain *B. ovis* PNV22A-com compared with parental *B. ovis* PA. Unfortunately, attempts to detect Omp25d have been unsuccessful so far, and hence we were not able to identify this protein either

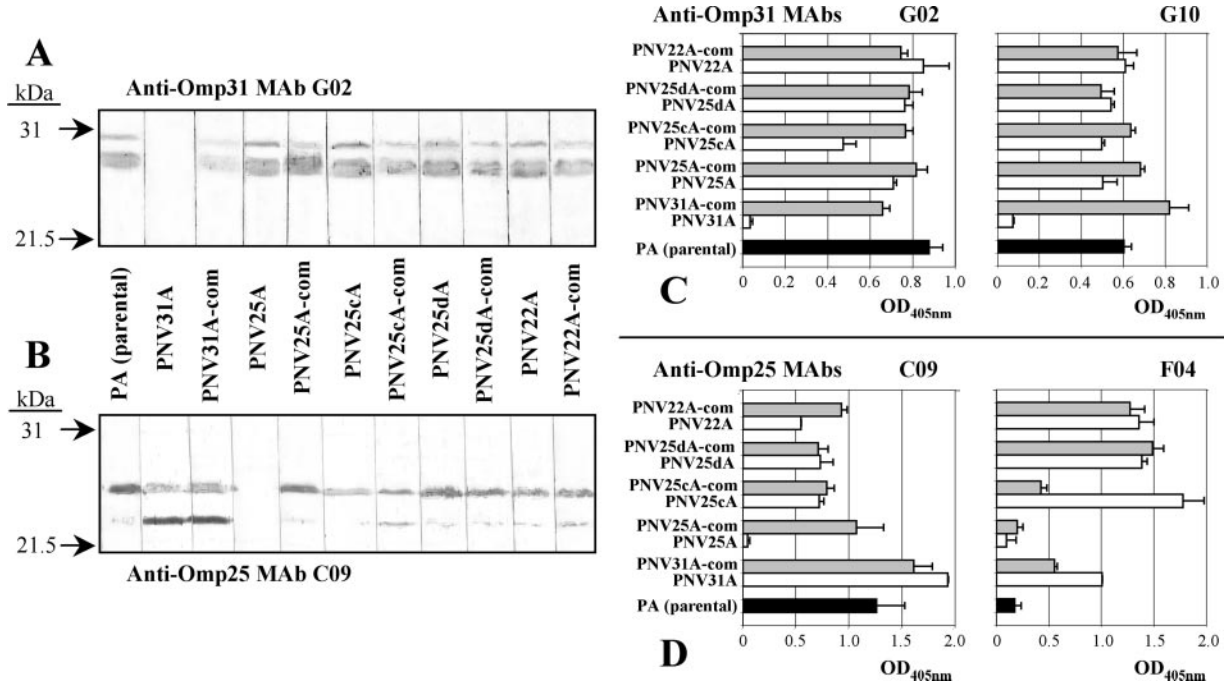

FIG. 2. Western blot (A and B) and iELISA (C and D) analysis of the *B. ovis* strains obtained in this work. Western blotting was performed with MAb G02, specific for the *B. ovis* Omp31 protein, diluted 1/5 (A) or with MAb C09, specific for the *Brucella* spp. Omp25 protein, diluted 1/6,000 (B). The positions of the molecular mass protein standards are shown by arrows on the left. For iELISA, MAbs specific for Omp31 (G02 and G10, diluted 1/50) (C) or specific for Omp25 (C09, diluted 1/6,400, and F04, diluted 1/800) (D) were used. Black, white, and shaded columns correspond to parental *B. ovis* PA, mutant strains, and complemented mutant strains, respectively.

in the parental strain or in the complemented mutant strain PNV25dA-com.

Effect of the inactivation of each gene encoding the Omp25/Omp31 family on the levels of Omp25 and Omp31 in *B. ovis* PA. The five mutant strains derived from *B. ovis* PA and the mutant strains complemented with the corresponding wild-type genes (Table 1) were analyzed by iELISA and Western blotting with MAbs specific for the Omp31 and Omp25 proteins (the only proteins of the Omp25/Omp31 family for which MAbs are available) in order to determine both the absence of Omp31 and Omp25 in *B. ovis* PA mutant strains PNV31A and PNV25A, respectively, and how the inactivation of each gene coding for the Omp25/Omp31 family might affect the amount of and/or accessibility to antibodies of these two major OMPs. The Omp31 protein afforded the characteristic multiple-band pattern in Western blotting (47, 50) with the specific MAb (Fig. 2A). As expected, Omp31 was not detected in the Δ*omp31* mutant strain by Western blotting (Fig. 2A) or by iELISA (Fig. 2C), and complementation of this strain with wild-type *omp31* restored the synthesis of Omp31 (Fig. 2A and C). Upon comparing *B. ovis* PNV31A-com to parental *B. ovis* PA, however, slight differences regarding the reactivity of the anti-Omp31 MAbs were found as follows. (i) MAb G02 showed, in both Western blotting and iELISA, a weaker reactivity with the complemented mutant strain PNV31-com (Fig. 2A and C) than with the parental strain (Fig. 2A and C). (ii) Nevertheless, strain PNV31A-com reacted slightly better than *B. ovis* PA with MAb G10 in iELISA (Fig. 2C). This MAb, raised against *B. melitensis* Omp31, reacts strongly with Omp31 from *B. ovis* in iELISA but very weakly in Western blotting (50). The other four mutant strains and the four respective complemented strains behaved in manners similar to that of the parental strain regarding reactivity with the anti-Omp31 MAbs in Western blotting (Fig. 2A) and iELISA (Fig. 2C), with the exception of *B. ovis* PNV25cA, which reacted more weakly in iELISA with MAb G02 (Fig. 2C).

Likewise, Omp25 was not detected in the *B. ovis* PNV25A mutant strain by Western blotting or by iELISA with a specific MAb (Fig. 2B and D), and the recovery of the synthesis of the protein was detected in the complemented strain *B. ovis* PNV25A-com (Fig. 2B and D). Anti-Omp25 MAb C09 developed in Western blotting two protein bands in parental *B. ovis* PA that correspond to Omp25, as previously shown (10, 29). These Omp25 protein bands were more intensely marked both in mutant *B. ovis* PNV31A and in its corresponding strain complemented with wild-type *omp31* (Fig. 2B) than in the other mutant or complemented strains (Fig. 2B), which displayed the same band profile as the parental strain (Fig. 2B). With regard to iELISA, further differences were found between strains in the reactivity with the two anti-Omp25 MAbs (Fig. 2D). Thus, while the Δ*omp31* strain and its complemented strain showed slightly higher reactivity with MAb C09, inactivation of *omp25c*, *omp25d*, and *omp22* led to a lower reactivity with this MAb, which persisted after complementation with the respective wild-type genes (Fig. 2D). The opposite situation was observed with MAb F04. This MAb did not react with parental *B. ovis* PA or with the derived PNV25A or PNV25A-com strains. The absence of reactivity with parental and PNV25A-com complemented strains was not surprising, since MAb F04 was previously shown to react in ELISA much

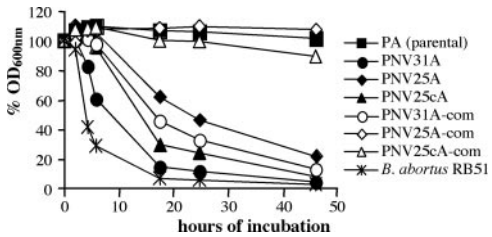

FIG. 3. Autoagglutination capacity of parental *B. ovis* PA, its derived strains obtained in this work, and *B. abortus* RB51. The OD_{600} values for bacterial suspensions adjusted to an initial OD_{600} of 0.8 were scored at several time points of static incubation, and the percent OD_{600} was determined with respect to the OD_{600} of the initial suspension (100% of OD_{600}). *B. ovis* PA mutants PNV25dA and PNV22A and the corresponding strains complemented with wild-type *omp25d* and *omp22*, respectively, behaved as parental *B. ovis* PA and are not represented. The SD, which was always lower than 5% of the mean, is not shown.

more weakly with the *B. ovis* Omp25 protein than with the *B. melitensis* protein and not to react at all with *B. ovis* Omp25 located in the OM of *E. coli* (10). Surprisingly, however, the mutant Δ*omp31* strain, and especially the Δ*omp25c*, Δ*omp25d*, and Δ*omp22* strains, reacted intensely with MAb F04 (Fig. 2D). This was also the case for the corresponding complemented mutant strains, with the exception of strains PNV31A-com and especially PNV25cA-com, which, although showing reactivities higher than that of the parental strain, afforded reactions less intense than those observed for their corresponding mutant strains (Fig. 2D).

Role of the proteins of the Omp25/Omp31 family in the OM properties of *B. ovis* PA. Several tests related to the OM properties of *Brucella* spp. (i.e., autoagglutination and resistance to polymyxin B, sodium deoxycholate, hydrogen peroxide, and nonimmune ram serum) that might be linked to virulence were performed with all the *B. ovis* strains obtained in this work, and the results were compared with those given by parental *B. ovis* PA and *B. abortus* RB51.

When bacterial suspensions were incubated statically at room temperature, the parental rough strain *B. ovis* PA remained in suspension throughout the experiment, while the rough strain *B. abortus* RB51 settled quickly, showing a reduction of about 60% in the OD_{600} after 4 h of incubation and an almost complete clearance after 18 h (Fig. 3). *B. ovis* PA Δ*omp31* and Δ*omp25c* mutant strains, and to lesser extent mutant PNV25A, also showed a strong capacity to autoagglutinate (Fig. 3). Complementation of mutant strains PNV25A and PNV25cA with wild-type *omp25* and *omp25c*, respectively, restored the phenotype of the *B. ovis* PA parental strain. However, complementation of strain PNV31A with wild-type *omp31*, although reducing its autoagglutination properties, did not completely recover the characteristics of the parental strain (Fig. 3). Finally, both the PNV25dA and PNV22A mutant strains and the corresponding complemented strains behaved like the parental strain, remaining in suspension throughout the experiment (data not shown).

Parental *B. ovis* PA was relatively resistant to 1 h of exposure to 1 mg ml^{-1} polymyxin B (survival of 69.59% ± 5.26%) while *B. abortus* RB51 was completely killed after this treatment (Table 3). *B. ovis* PNV25cA was the only mutant strain that was significantly less resistant ($P < 0.0005$) to polymyxin B than parental *B. ovis* PA (survival of 27.70% ± 11.65% versus 69.59% ± 5.26%) (Table 3). The corresponding mutant strain complemented with wild-type *omp25c* (survival of 7.88% ± 5.18%) did not recover the resistance of parental *B. ovis* PA and was even more susceptible ($P < 0.05$) than mutant strain PNV25cA (Table 3). *B. ovis* PNV22A-com was also more susceptible to polymyxin B than *B. ovis* PA ($P < 0.005$) but was not different from its corresponding mutant strain, PNV22A,

TABLE 3. Susceptibility to polymyxin B, sodium deoxycholate, hydrogen peroxide, and nonimmune ram serum of *B. abortus* RB51, parental *B. ovis* PA, and its derived strains obtained in this work[a]

Brucella strain	% Survival after 1-h exposure to:		Inhibition zone diam (cm) with hydrogen peroxide	% Survival after 4-h exposure to nonimmune ram serum
	Polymyxin B (1 mg/ml)	Na deoxycholate (0.1 mg/ml)		
B. abortus RB51	0.00 ± 0.00B	88.69 ± 5.45	4.28 ± 0.11C	25.73 ± 4.84B
B. ovis PA (parental)	69.59 ± 5.26	89.34 ± 9.30	5.04 ± 0.05	85.52 ± 5.20
B. ovis PNV31A	70.07 ± 13.83	75.51 ± 20.97	6.23 ± 0.11B	64.43 ± 6.97C
B. ovis PNV31A-com	69.83 ± 8.63	62.99 ± 22.90D	6.64 ± 0.37B(D)	69.44 ± 3.62D
B. ovis PNV25A	75.73 ± 22.13	63.81 ± 3.98D	5.88 ± 0.02B	71.67 ± 2.89D
B. ovis PNV25A-com	75.42 ± 4.41	64.74 ± 15.67D	6.08 ± 0.43B	87.21 ± 2.27(D)
B. ovis PNV25cA	27.70 ± 11.65B	39.21 ± 7.92B	5.88 ± 0.15B	55.68 ± 7.32B
B. ovis PNV25cA-com	7.88 ± 5.18B(D)	32.54 ± 18.15B	7.08 ± 0.04B(B)	17.51 ± 14.17B(B)
B. ovis PNV25dA	61.16 ± 8.93	55.44 ± 21.69C	6.05 ± 0.07B	54.05 ± 7.42B
B. ovis PNV25dA-com	58.02 ± 12.87	48.70 ± 15.19C	6.29 ± 0.27B	56.13 ± 2.98B
B. ovis PNV22A	52.74 ± 3.97	30.68 ± 3.60B	5.90 ± 0.25B	25.38 ± 3.98B
B. ovis PNV22A-com	38.52 ± 1.91C	23.31 ± 8.23B	6.09 ± 0.02B	17.41 ± 5.47B

[a] Assays were performed as detailed in Materials and Methods. Values are means ± SD ($n = 3$). Statistical comparisons were performed with Fisher's protected least significant differences test. Significant differences between the mutant or complemented strains and parental *B. ovis* PA or between the complemented strains and the corresponding mutant strain (in parenthesis) are as follows: B, $P < 0.0005$; C, $P < 0.005$; D, $P < 0.05$.

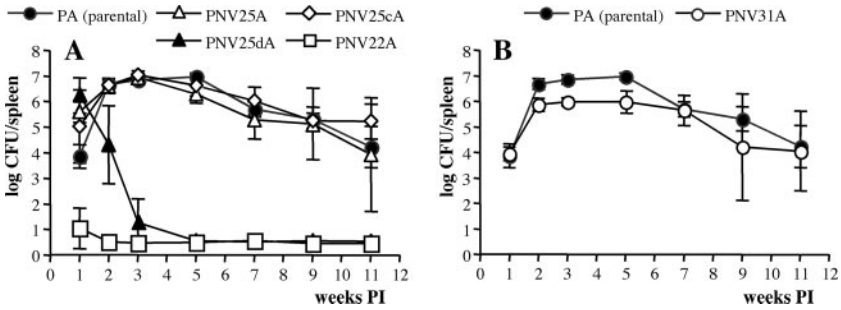

FIG. 4. Kinetics of splenic infection of parental *B. ovis* PA (A and B), and the mutant strains with the genes *omp25*, *omp25c*, *omp25d*, and *omp22* (A) and *omp31* (B) inactivated. Mice were inoculated intraperitoneally with 5×10^6 CFU. Results are expressed as the mean ± SD ($n = 5$) of the log CFU/spleen.

which was not significantly more susceptible than *B. ovis* PA (Table 3).

Both *B. ovis* PA and *B. abortus* RB51 rough strains were highly resistant to 1 h of exposure to 0.1 mg ml^{-1} sodium deoxycholate, while all the *B. ovis* PA Omp25/Omp31 family mutant strains, except the Δ*omp31* strain, showed percentages of survival to this detergent that were significantly lower than that observed for parental *B. ovis* PA (Table 3). The Δ*omp25c* and Δ*omp22* mutant strains were the most susceptible strains ($P < 0.0005$), with percentages of survival of 39.21% ± 7.92% and 30.68% ± 3.60%, respectively, and none of the complemented mutant strains recovered the resistance levels observed for the parental strain, proving to be as susceptible to deoxycholate as the corresponding mutant strains were (Table 3).

Regarding susceptibility to hydrogen peroxide, all the *B. ovis* PA Omp25/Omp31 family mutant and complemented mutant strains were more susceptible ($P < 0.0005$) than the parental strain (Table 3). *B. abortus* RB51 was more resistant ($P < 0.005$) than *B. ovis* PA and therefore more resistant than the *B. ovis* PA mutants (Table 3). Complementation of the mutant strains with the corresponding wild-type genes failed to restore the behavior of the parental strain and, in particular, the complemented mutant strain *B. ovis* PNV25cA-com was even more susceptible ($P < 0.0005$) to hydrogen peroxide than its corresponding mutant strain, PNV25cA.

Exposure for 4 h to nonimmune ram serum strongly reduced the viability of *B. abortus* RB51 (survival of 25.73% ± 4.84%), with no dramatic effect on the survival of parental *B. ovis* PA (survival of 85.52% ± 5.20%) ($P < 0.0005$ for the statistical differences between both strains) being observed (Table 3). All the *B. ovis* PA Omp25/Omp31 family mutant strains were more susceptible than the parental strain to the bactericidal action of serum, with the Δ*omp22* mutant strain displaying more defects in this regard (survival of 25.38% ± 3.98%; $P < 0.0005$). The properties of the complemented strain *B. ovis* PNV25A-com were similar to those of the parental strain. By contrast, the complementation of the Δ*omp31*, Δ*omp25c*, Δ*omp25d*, and Δ*omp22* mutant strains with the corresponding wild-type genes did not restore the survival capacities of the parental strain to nonimmune ram serum, with strain *B. ovis* PN25cA-com proving to be even significantly more susceptible to the bactericidal action of ram serum than the corresponding Δ*omp25c* mutant strain (survival of 17.51% ± 14.17% versus 55.68% ± 7.42%; $P < 0.0005$) (Table 3).

Role of the proteins of the Omp25/Omp31 family in the virulence of *B. ovis* PA in mice. The virulence of the *B. ovis* PA Omp25/Omp31 family mutant strains obtained in this work was evaluated in a BALB/c mouse model by intraperitoneal inoculation of 5×10^6 CFU/mouse and subsequent determination of bacterial counts in spleen at selected intervals p.i. Mice inoculated with the *B. ovis* PA parental strain were used as controls. The exact inoculation doses (CFU/mouse) for each *B. ovis* strain, determined retrospectively, were as follows: for parental PA, 5.40×10^6; for PNV31A, 0.65×10^6; for PNV25A, 4.40×10^6; for PNV25cA, 5.50×10^6; for PNV25dA, 8.50×10^6; and for PNV22A, 7.00×10^6.

The Δ*omp25* and Δ*omp25c* mutant strains showed kinetics of infection in spleen similar to that displayed by the parental strain, with maximum levels of infection between weeks 2 and 5 p.i. declining slowly thereafter until the end of the experiment at week 11 p.i. (Fig. 4A). Mutant strain PNV31A reached a level of splenic infection equivalent to that seen in the parental strain at week 1 p.i. and also between weeks 7 and 11 p.i. However, maximum spleen colonization was about 1 log lower for the Δ*omp31* mutant than for parental *B. ovis* PA ($P < 0.0005$ and $P < 0.005$ at weeks 3 and 5 p.i., respectively) (Fig. 4B).

In contrast, the absence of Omp25d and Omp22 from the OM of *B. ovis* PA dramatically reduced the virulence of the bacterium, as demonstrated by the striking decrease in splenic colonization observed for mutant strains PNV25dA and PNV22A compared to that observed for parental *B. ovis* PA (Fig. 4A). In spite of giving bacterial counts about 2 logs higher than those for the parental strain at week 1 p.i. ($P < 0.0005$), the Δ*omp25d* mutant strain underwent a quick reduction in splenic bacterial counts after week 2 p.i., being 5.5 logs lower than those detected for the parental strain at 3 weeks p.i. ($P < 0.0005$) and undetectable thereafter in any mice (Fig. 4A). Even more dramatic was the attenuation observed for the Δ*omp22* mutant strain, since this strain was isolated from only two out of five mice at week 1 p.i., and it was completely cleared from spleens after week 2 p.i. (Fig. 4A). The complemented mutant strains *B. ovis* PNV25dA-com and PNV22A-com inoculated into mice at doses of 5×10^6 CFU/mice had bacterial counts in spleen at weeks 2 and 9 p.i. that were similar to those observed for the corresponding mutant strains at the same times p.i. (data not shown).

DISCUSSION

In this work, five mutant strains with the *omp31*, *omp25*, *omp25c*, *omp25d*, and *omp22* genes inactivated were obtained from virulent *B. ovis* PA and characterized. Inactivation of *omp25b* and *omp31b* was not attempted in *B. ovis*, since *omp25b* is included in a 15-kb DNA fragment that is absent from this *Brucella* species (46) and *omp31b* has a nucleotide substitution that generates a stop codon that would truncate the encoded protein, probably preventing its localization in the OM (46). All the genetically modified *B. ovis* PA strains afforded the expected results in the genotypic characterization and behaved as the parental strain in conventional bacteriological tests currently used for *Brucella* typing. As reported in Results, the absence of the target OMP in the corresponding mutant strains and the recovery of its synthesis in the respective strain complemented with the wild-type gene were confirmed by reactivity with MAbs (Fig. 2) or antisera (unpublished results). The exceptions were mutant *B. ovis* PNV25dA and the corresponding complemented strain PNV25d-com, since the Omp25d antiserum did not react with either of these two strains, but neither did they react with the parental strain.

The *B. ovis* PA mutant strain with *omp25d* inactivated displayed behavior similar to that of the parental strain as regards in vitro growth, in both solid and liquid culture media (Fig. 1A and data not shown). Accordingly, the marked reduction in virulence observed for this *B. ovis* mutant in mice (Fig. 4A) cannot be justified on this basis. The in vitro growth of the Δ*omp22* strain was also similar to that of the parental strain, with the exception of a marked reduction in viability in the stationary phase (Fig. 1B). This fact could denote difficulties for the bacterium to grow in deprived media and/or at acid pH, conditions that it would find inside the host cells, and this could be related to the attenuation observed for the *B. ovis* PA strain lacking Omp22 (Fig. 4A).

The integrity of the OM is essential for the viability of bacteria. Thus, the removal of one OMP of the Omp25/Omp31 family might lead to a compensatory effect by increasing the synthesis of other members of this family. The possibility of a compensatory effect, analyzed only for the levels of Omp25 and Omp31 (major OMPs for which MAbs are available), was detected only in the Δ*omp31* mutant strain. In this strain, the levels of Omp25 seemed to be increased (Fig. 2B and D), as was also the case for the strain complemented with wild-type *omp31* (Fig. 2B and D), which also seemed to have levels of Omp31 lower than those for parental *B. ovis* PA. However, these results should be considered with caution, since the synthesis of the other members of the Omp25/Omp31 family was not evaluated and the limitations to immunological detection of the protein must be taken into account (i.e., the reactivity of a MAb may be influenced by the particular conformation of the protein in each strain, even under the denaturing conditions of sodium dodecyl sulfate-polyacrylamide gel electrophoresis). The existence of a compensatory effect has been described for the genus *Bartonella* for a group of homologous proteins that are also homologous to the *Brucella* spp. Omp25/Omp31 family (39). Additionally, there is a work reporting that the inactivation of *omp25c*, *omp25d*, and *omp22* in *B. suis* 1330 seems to increase the levels of Omp25b (42), but this effect is not possible in *B. ovis*, since this *Brucella* species lacks Omp25b (46). A *B. suis* strain with the *omp31* gene inactivated was not obtained in this previous study (42), and it is therefore impossible to compare the results obtained in our work with *B. ovis*.

The results for the reactivity in iELISA of MAbs specific to Omp25 and Omp31 with the *B. ovis* PA Omp25/Omp31 family mutant strains suggest that the conformation of Omp31 and especially of Omp25 would be modified when some of the other OMPs of the family are removed from the OM. The change in the conformation, which also indicates alterations in the OM, was particularly evident when the anti-Omp25 MAbs C09 and F04 were used. The specific epitope of MAb C09 seems to be either less exposed at the surface or disposed in a different conformation in some mutant strains compared to what is seen for parental *B. ovis* PA (Fig. 2D). The reactivity of MAb F04 in iELISA has previously been shown to be stronger with Omp25 from *B. melitensis* than with the *B. ovis* protein, and no reactivity was detected between this MAb and recombinant *E. coli* bearing the *B. ovis* Omp25 protein at the surface (10). Surprisingly, although this MAb did not react with parental *B. ovis* PA or with mutant strain *B. ovis* PNV25A, it reacted strongly with the other *B. ovis* PA Omp25/Omp31 family mutants (Fig. 2D). In light of the results obtained from Western blotting and iELISA with the anti-Omp25 MAb C09, this higher reactivity of MAb F04 (except perhaps for mutant PNV31A) does not seem to be related to increased levels of Omp25 but rather seems related to a different topology of Omp25, suggesting modifications of the OM in these *B. ovis* strains.

Accordingly, the OM alterations detected by reactivity with the MAbs specific for Omp25 and Omp31 do not by themselves justify the marked reduction in virulence observed with mice for the Δ*omp25d* and Δ*omp22* mutant strains, since the Δ*omp25c* mutant strain exhibited a similar pattern of reactivity and was not attenuated in mice (Fig. 4A).

The autoagglutination properties could not be related with either virulence or the rough phenotype, since parental rough *B. ovis* PA and the attenuated strains *B. ovis* PNV25dA and PNV22A did not agglutinate, while rough *B. abortus* RB51 settled rapidly (Fig. 3 and data not shown). Taking into account that the Δ*omp31* mutant of *B. ovis* PA displayed an autoagglutination pattern similar to that observed for *B. abortus* RB51 (Fig. 3) and that it has been shown that *B. abortus* lacks Omp31 (49, 51), the autoagglutination phenomenon of *B. abortus* RB51 could be related to the rearrangements of the OM caused by the absence of Omp31. However, the possibility of other differences in the OM, related or not to the Omp25/Omp31 family, that might help to explain the different autoagglutination properties of both strains cannot be ruled out. In this respect, other divergences between *B. abortus* and *B. ovis* with respect to the members of the Omp25/Omp31 family have been described previously (i.e., the absence of Omp31b and the presence of Omp25 of a smaller size in *B. ovis*) (10, 46). It has been also observed that the presence of O-polysaccharide chains in LPS prevents the autoagglutination phenomenon, since other smooth strains, including smooth *B. abortus* 2308, from which rough strain RB51 derives, remain in suspension (data not shown).

The particular properties of the OM are thought to be responsible for the resistance of *Brucella* spp. to the bactericidal action of cationic peptides compared to what is seen for other

gram-negative bacteria, such as enterobacteria (27, 37, 38). This resistance has been associated in part with the presence of O-polysaccharide chains in smooth strains, which would reduce the surface exposure of inner anionic groups (38), although the core-lipid A complex also plays a major role (33, 38). As in previous reports (27, 28, 38, 43), polymyxin B was used in this work to determine the stability of the OM and also as an indicator of the susceptibility of the *B. ovis* PA mutants to the bactericidal effect mediated by cationic peptides in the host. Surprisingly, rough parental *B. ovis* PA was much more resistant to a 1-h exposure to 1 mg/ml polymyxin B than rough *B. abortus* RB51, which did not survive this treatment (Table 3), and it was even slightly more resistant than smooth *B. abortus* 2308 (data not shown). The discrepancies with the results described in other works reporting that *B. ovis* Reo 198 is more susceptible to polymyxin B than smooth *B. abortus* S19 and behaves like rough *B. abortus* 45/20 (28, 38) might be related to the slight differences between the experimental protocols and/or the different *B. ovis* and *B. abortus* strains used. It should be noted that *B. ovis* Reo 198, employed in other works, is an atypical *B. ovis* strain, since it does not require a CO_2 atmosphere or serum supplementation in the culture medium to grow and it is in addition an avirulent strain (28, 37, 38). In any case, results presented in this work regarding polymyxin B susceptibility reveal differences in the OM properties between *B. abortus* RB51 and *B. ovis* PA that do not depend on the presence or absence of O-polysaccharide chains at the surface, since both strains exhibit a rough phenotype.

Regarding the *B. ovis* PA Omp25/Omp31 family mutant strains, a higher susceptibility to polymyxin B cannot explain the attenuation observed in mice for the Δ*omp25d* and Δ*omp22 B. ovis* PA mutant strains, since they behaved like the parental strain (Table 3) and the fully virulent *omp25c* mutant was significantly more susceptible. However, the possibility that the Δ*omp25d* and Δ*omp22* strains might be more susceptible than parental *B. ovis* PA to other bactericidal cationic peptides present in mammals cannot be ruled out. On the other hand, the complemented strain PNV25cA-com, which recovered the synthesis of Omp25c but at levels higher than those observed for the parental strain (unpublished results), was significantly more susceptible to polymyxin B than the corresponding mutant strain, suggesting that balanced levels of Omp25c would be required for the stability of the *B. ovis* PA OM.

Again, no explanation for the attenuation of the Δ*omp25d* and Δ*omp22* strains was found on the basis of susceptibility to sodium deoxycholate, since both strains, although more susceptible than the parental strain (Table 3), did not show any differences with the Δ*omp25c* strain, which is fully virulent in mice (Fig. 4A). Mutant strain PNV25A was also more susceptible to this anionic detergent, and complementation of the four susceptible mutant strains with the respective wild-type genes did not restore the resistance properties of parental *B. ovis* PA in any case. Since recoveries of the synthesis of Omp25, Omp25c, and Omp22 were detected in the corresponding complemented strains (Fig. 2 and unpublished results), these results again suggest that balanced levels of the members of the Omp25/Omp31 family would be important to maintain the stability of the OM.

In the present work, susceptibility to hydrogen peroxide was selected as an indicator both of the stability of the OM and of the ability of the *B. ovis* PA Omp25/Omp31 family mutant strains to resist killing by the oxygen-dependent bactericidal mechanisms of phagocytes. All the *B. ovis* mutants, and also all the complemented mutants, were significantly more susceptible to H_2O_2 than the parental strain, again showing the difficulties involved in restoring the phenotype of parental *B. ovis* PA by complementation with the wild-type gene when the encoded protein is located in the OM. The loss of virulence caused by the absence of Omp25d or Omp22 from the *B. ovis* PA surface cannot be explained in terms of a higher susceptibility to hydrogen peroxide, although the possibility that the Δ*omp25d* and Δ*omp22* mutants might be more susceptible to intracellular killing mediated by other oxygen-dependent mechanisms cannot be excluded. All the *B. ovis* strains, including the parental strain, were also more susceptible than *B. abortus* RB51 (Table 3), again showing up the differences between rough strains from different *Brucella* species.

Brucella spp. are also at least partially resistant to killing mediated by the action of host nonimmune serum (1, 14, 20, 24, 25), which may contribute to virulence. Since OM properties may influence the behavior of *Brucella* spp. towards nonimmune serum to a considerable extent, we have evaluated the role of the members of the Omp25/Omp31 family in the survival of *B. ovis* PA in the presence of nonimmune ram serum. Although the virulence assays were performed in a murine model, ram serum was selected for susceptibility studies because ram is the preferred host for *B. ovis* and because of the difficulty involved in obtaining the required amount of serum from mice.

Exposure for 4 h to nonimmune ram serum dramatically reduced the viability of rough *B. abortus* RB51, while the susceptibility of rough *B. ovis* PA was very low (Table 3). Smooth *B. abortus* 2308 has previously been reported to be more resistant than the rough strains of the same species to the bactericidal effect of bovine and human nonimmune serum (1, 14, 20, 25), and these differences have been attributed to the presence of O-polysaccharide chains in the LPS of smooth *B. abortus* 2308 (1, 14, 20, 25). Nevertheless, while rough *B. abortus* strains are susceptible to exposure to human nonimmune serum, rough *B. melitensis* mutant strains are not (25). Therefore, in addition to the presence or absence of O-polysaccharide chains in the LPS, other differences, probably in the bacterial surface, must exist between the *Brucella* species to account for their different levels of susceptibility to nonimmune serum. This idea is also supported by the differences between *B. ovis* PA and *B. abortus* RB51 found in this work (Table 3).

Considering that *B. abortus* lacks a DNA fragment of about 25 kb that includes *omp31* (48, 49, 51), the absence of Omp31 or other proteins encoded in this DNA fragment in *B. abortus* has been suggested as a possible explanation for the differences between *B. melitensis* and *B. abortus* as regards their susceptibilities to nonimmune serum (25). In the present work, we observed that the Δ*omp31* mutant strain was more susceptible to killing by nonimmune ram serum than parental *B. ovis* PA, but it was also significantly more resistant than rough *B. abortus* RB51 (Table 3). Accordingly, by itself the absence of Omp31 in *B. abortus* RB51 does not explain its higher susceptibility to the bactericidal effect of nonimmune ram serum, and

hence other differences, known or unknown, between the *B. abortus* and *B. ovis* rough strains used in this work must be involved in their different susceptibilities. In this respect, it should be noted that between the two *Brucella* species, in addition to the absence of Omp31 in *B. abortus*, there are other differences regarding the members of the Omp25/Omp31 family, such as the absence of Omp31b in *B. ovis* (46) and the presence of Omp25 of a smaller size in *B. ovis* (10).

The absence of Omp22 from the OM of *B. ovis* PA elicited an important reduction in its resistance to killing by nonimmune ram serum (Table 3), which could account at least in part for the almost complete loss of virulence observed in the murine model for the Δ*omp22* mutant strain (Fig. 4A). On the other hand, the attenuation of the Δ*omp25d* mutant strain (Fig. 4A) cannot be explained exclusively on the basis of its higher susceptibility to nonimmune serum, since it was as susceptible as the fully virulent Δ*omp25c* strain (Table 3; Fig. 4A). Again, complementation of the mutant strains with the corresponding wild-type genes of the Omp25/Omp31 family did not restore the resistance to nonimmune ram serum observed for parental *B. ovis* PA (Table 3), even in complemented mutant strains where the synthesis of the removed protein was detected. Additionally, strain PNV25cA-com was significantly more susceptible than the corresponding PNV25cA mutant strain (Table 3), which again shows that a tight balance of the members of the Omp25/Omp31 family is essential for maintaining the properties of the OM.

Regarding the role of the members of the Omp25/Omp31 family in the virulence of *B. ovis* PA in mice, removal of Omp31 from the OM had no dramatic effect on the persistence of the bacterium in spleen (Fig. 4B). The only remarkable effect was that in comparison with the parental strain, the mutant strain *B. ovis* PNV31A showed a reduction of about 1 log in the maximum levels of splenic colonization, which were obtained between weeks 2 and 5 p.i. (Fig. 4B). The fact that Omp31 has no relevant effect in the virulence of *B. ovis*, in spite of being a major OMP, is not surprising, considering that this protein is not synthesized in virulent *B. abortus* (49, 51) and that it is not essential for the virulence of *B. melitensis* Rev.1 in either the murine or sheep model (8, 31). It has been shown that Omp31 functions as a hemin-binding protein in *B. melitensis*, *B. suis*, and *B. ovis*, and its synthesis seems to be increased under iron-limiting conditions in the culture medium (15). Despite the relevance that the hemin-binding capacity of Omp31 might have for the virulence of *Brucella* spp., the results obtained in this work regarding the virulence in mice of the *B. ovis* PA Δ*omp31* mutant (Fig. 4B), together with those reporting that Omp31 is not necessary for the virulence of *B. abortus* or *B. melitensis* Rev.1 (8, 31, 49, 51), suggest that Omp31 would not be essential for iron uptake. However, the other members of the Omp25/Omp31 family might also have hemin-binding properties that would compensate the effect of the absence of Omp31 from the OM. In this respect, it should be noted that the hemin-binding protein HbpA from *Bartonella quintana* shares 32% amino acid sequence identity with Omp31 from *B. melitensis* (6) and that other four proteins homologous to HbpA and thought to be able to bind hemin exist in *B. quintana* (39).

The Δ*omp25* and Δ*omp25c* mutant strains displayed kinetics of splenic colonization similar to that observed for the parental strain (Fig. 4A), and therefore Omp25 and Omp25c do not play an important role in the virulence of *B. ovis* PA, at least under our experimental conditions. The full virulence of the Δ*omp25c* mutant strain was surprising, considering that it was the *B. ovis* PA mutant that showed in the tests performed in this work the most evident alterations of the OM (Fig. 2 and 3; Table 3) and that *omp25c* is highly conserved, with almost identical nucleotide sequences, in all *Brucella* species (46). On the other hand, the results obtained with the Δ*omp25* strain differ from those obtained in a previous work with a Δ*omp25* mutant of *B. ovis* LSU99 (18). The differences between the two studies might be related to the different genetic backgrounds of the respective *B. ovis* parental strains or to differences in the doses or routes of infection employed in each case. The first of these options is the most likely, since the infection levels reached in spleen with the parental strains were similar in both studies and it has been demonstrated that *B. ovis* PA splenic growth curves after intravenous or intraperitoneal infection are almost identical (34).

Some indirect evidence has been reported previously about the possible involvement of Omp25 in the virulence of *Brucella* spp., but this evidence might be dependent on the host and/or *Brucella* species. Thus, on one hand it has been shown that Omp25 of *B. suis* inhibits the production of TNF-α in human macrophages (35) and that it is involved in the release of periplasmic proteins in acidic conditions (3) and that *B. abortus* attenuated mutants with the *bvrS-bvrR* two-component regulatory system impaired do not synthesize Omp25 (32). On the other hand, other works have shown that the protein factor responsible for the inhibition of the TNF-α production is not active in mouse macrophages (5) and that attenuated mutants of *B. suis bvrS-bvrR* showing defects in intracellular multiplication in human macrophages do not present lower Omp25 levels (42).

As discussed above for each test, the marked reduction in virulence observed for the Δ*omp25d* mutant strain cannot be explained on the basis of the results obtained in any of the analyses regarding the OM properties performed here. Accordingly, Omp25d could be directly involved in the penetration and/or survival of *B. ovis* inside host cells. The same reasoning could be applied to hypothesize the role of Omp22 in virulence, although as discussed before the higher susceptibility of the Δ*omp22* mutant strain to nonimmune serum and its difficulty in surviving during the stationary phase of growth could be related to the striking degree of attenuation observed for this *B. ovis* PA mutant strain in mice (Fig. 4A). It should also be mentioned that *B. abortus* attenuated mutants with the *bvrS-bvrR* two-component regulatory system impaired do not synthesize Omp22 (32).

Complementation of Δ*omp25d* and Δ*omp22* mutant strains of *B. ovis* PA with the corresponding wild-type genes did not recover the virulence or the OM properties of the parental strain. Although the possibility of a spontaneous mutation being the origin of the defects observed for these mutant strains cannot be completely disregarded, it should be noted that a clear rho-independent transcription terminator is located downstream from all the genes of the Omp25/Omp31 family, and hence the probability of a polar effect on a downstream gene in *B. ovis* PNV25dA and PNV22A seems to be very low. Moreover, the gene downstream from *omp22* is located in the

opposite orientation in the *Brucella* spp. genome. Additionally, by reactivity with specific antisera (unpublished results), Omp25d has not been detected in the complemented strain PNV25dA-com, and very low levels of Omp22 were detected in the complemented strain PNV22A-com in comparison to what was detected for the parental strain. Complementation in *trans* of *Brucella* mutant strains with wild-type genes coding for OMPs has frequently proved unsuccessful (3, 18, 44) due to the difficulty involved in recovering levels of the surface protein exactly the same as those found in the parental strain, which is probably essential for maintaining the integrity of the OM, especially in rough strains. This was clearly seen in complemented strain PNV25cA-com, which recovered the synthesis of Omp25c—although at levels higher than those found for parental *B. ovis* PA (unpublished results)—but had even more OM defects (Table 3) than the corresponding Δ*omp25c* mutant strain.

The results reported here are pioneering in the study of the virulence factors involved in the pathogenicity of *B. ovis*, which unlike rough mutant strains obtained from smooth virulent *Brucella* spp. (1, 26, 30, 40, 45) is a natural rough *Brucella* species that is virulent in its preferred host and in animal models. Taking into account that Omp25d and Omp22 are essential for the virulence of *B. ovis* PA in mice and that both proteins are highly conserved with almost identical amino acid sequences in all *Brucella* species (46), the possibility of a role in the virulence of the other *Brucella* species should be considered. It would be also of great interest to determine the relevance for the virulence of *B. ovis* in rams not only of Omp25d and Omp22 but also of the other members of the Omp25/Omp31 family. These aspects, together with the role of these OMPs in intracellular survival, will be addressed in a forthcoming study to be conducted in our laboratory.

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