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Molecular Characterization, Occurrence, and Immunogenicity in Infected Sheep and Cattle of Two Minor Outer Membrane Proteins of *Brucella abortus*

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Screening of a *Brucella abortus* genomic library with two sets of monoclonal antibodies allowed the isolation of the genes corresponding to two minor outer membrane proteins (OMP10 and OMP19) found in this bacterial species. Sequence analysis of the *omp10* gene revealed an open reading frame capable of encoding a protein of 126 amino acids. The nucleotide sequence of the insert producing the OMP19 protein contains two overlapping open reading frames, the largest of which (177 codons) was shown to encode the protein of interest. Analysis of the N-terminal sequences of both putative proteins revealed features of a bacterial signal peptide, and homology to the bacterial lipoprotein processing sequence was also observed. Immunoblotting with monoclonal antibodies specific for OMP10 or OMP19 showed that both proteins are present in the 34 *Brucella* strains tested, representing all six *Brucella* species and all their biovars. The OMP19 detected in the five *Brucella ovis* strains examined migrated at an apparent molecular weight that is slightly higher than those of the other *Brucella* species, confirming the divergence of *B. ovis* from these species. OMP10 and OMP19 were produced in recombinant *Escherichia coli* and purified to homogeneity for serological analysis. A large fraction of sera from sheep naturally infected with *Brucella melitensis* were reactive with these proteins in an enzyme-linked immunosorbent assay, whereas sera from *B. abortus*-infected cattle were almost completely unreactive in this assay.

Brucellae are facultative intracellular gram-negative bacteria that produce infectious diseases in humans and domestic animals. Ruminants (sheep, goats, and cows) are predominantly infected by *Brucella abortus* and *Brucella melitensis.*

The *Brucella* cell wall consists of a peptidoglycan layer strongly associated with the outer membrane (12). The cell wall of *B. abortus* prepared by differential centrifugation has been described as "a complex structure populated by at least 75 proteins" (40). These include the major outer membrane proteins (OMPs) of groups 2 (porin, 34 to 40 kDa) and 3 (25 to 30 kDa) first reported by Dubray and Bezard (13) and Verstreate et al. (45), the lipoprotein covalently bound to PG (Braun's lipoprotein equivalent) (18, 19), and the minor OMPs of group 1 (88 to 94 kDa).

Monoclonal antibodies (MAbs) against group 1, 2, and 3 OMPs as well as MAbs to minor surface-exposed OMPs with molecular masses (MMs) of 10, 16, 19, and 31 to 34 kDa (the latter is a major OMP in *B. melitensis* strains but is less abundant in *B. abortus*) have been produced (5). Immunoblotting experiments and competitive enzyme-linked immunosorbent assay (ELISA) with *Brucella*-infected sheep and bovine sera demonstrated an antibody response to the OMPs listed above (6, 47, 48).

Very strong, possibly covalent, interaction of the group 2 and 3 proteins with peptidoglycan has been suggested (8, 14, 40) and would explain the multiple-band profile of these groups. Each group could correspond to different PG-associated forms of only one gene product. The gene encoding the *B. abortus* 36-kDa porin OMP has been cloned and functionally expressed in *Escherichia coli* (16, 30). The availability of MAbs against OMPs allowed us to clone the genes encoding these proteins. The cloning of the *B. abortus* genes *omp25* and *pal*, encoding the major 25-kDa OMP and the minor 16-kDa OMP, respectively, has been reported (11, 41). The predicted sequence of OMP16 revealed significant similarity to the peptidoglycan-associated bacterial lipoproteins. In this paper, we report the cloning and sequencing of the *Brucella* genes encoding the minor OMPs of 10 and 19 kDa referred to as OMP10 and OMP19, respectively, as well as their expression in *E. coli* and the subsequent purification of the recombinant products. The occurrence of both proteins in the classical *Brucella* species and representative biovars, as well as the serological response in naturally infected sheep and cattle, has been investigated.

MATERIALS AND METHODS

Bacterial strains. *Yersinia enterocolytica* O:9 was isolated at the Centre de Dépistage des Maladies Animales, Erpent, Belgium. The *Pasteurella multocida* strain was isolated by S. Bercovich at the Central Diergeneeskundig Instituut, Lelystad, The Netherlands. *B. abortus* B19 and 45/20, *B. melitensis* B115, *Pseudomonas maltophilia*, *Salmonella urbana*, and *E. coli* O:157 were from the Institut National de Recherche Vétérinaire, Brussels, Belgium. Cell extracts were prepared by sonication as previously described (5). All other whole-cell extracts of *Brucella* strains were prepared by J. M. Verger and M. Grayon, Institut National de la Recherche Agronomique, Laboratoire de Pathologie Infectieuse et Immunologie, Nouzilly, France. Briefly, cell lysis was achieved by boiling in the presence of 1% sodium dodecyl sulfate (SDS).

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MAbs and antiserum. Anti-OMP10 MAbs were defined as reacting with a 10-kDa band on immunoblots of *B. melitensis* B115 cell extracts; anti-OMP19 MAbs react with a 19-kDa band under the same conditions. MAb production has been described previously (5, 6). Supernatants of hybridoma cultures of the anti-OMP10 MAbs A68/08E07/B11 (immunoglobulin G2a [IgG2a]) and A68/07G11/C10 (IgG2a) and of the anti-OMP19 MAbs A68/25H10/A05 (IgG2a), A76/02A04/A07 (IgG2b), A76/05C10/A08 (IgG2a), A76/12F02/D06 (IgG2b), and A76/18B02/D06 (IgG2a) were used. Rabbit anti-mouse immunoglobulin antiserum and anti-β-galactosidase goat sera were produced as described previously (5, 41). MAbs reactive with the murine tumor necrosis factor (mTNF) or the Cro-LacI leader peptides were produced by Innogenetics. The rabbit antiserum to *E. coli* proteins was obtained from Dako (Glostrup, Denmark).

Immunoscreening of the λgt11 gene bank. Construction of a λgt11 genomic library prepared from DNA of *B. abortus* 544 was described previously (41). This library was screened with a mixture of culture supernatants of the two anti-OMP10 MAbs (A68/08E07/B11 and A68/07G11/C10) or of five anti-OMP19 MAbs (A68/25H10/A05, A76/02A04/A07, A76/05C10/A08, A76/12F02/D06, and A76/18B02/D06), according to a previously described protocol (41).

SDS-PAGE and immunoblotting. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were performed as previously described (41).

Lysogen production and preparation of whole-cell extracts. To study the putative recombinant phages, lysogens were generated in *E. coli* Y1089 as described by Huynh et al. (25). Lysogenic strains were induced with IPTG (isopropyl-β-D-thiogalactopyranoside) (10 mM) according to published procedures (41). Whole-cell extracts were subsequently analyzed by SDS-PAGE and immunoblotted with anti-OMP10 MAb A68/08E07/B11 or anti-OMP19 MAb A76/02A04/A07 as well as with anti-β-galactosidase serum.

Recombinant DNA techniques. The plate lysate method (39) was used for large-scale preparation of recombinant phage particles, and isolation of phage DNA was done with the LambdaSorb phage adsorbent (Promega Corp., Madison, Wis.) as recommended by the manufacturer. Large-scale isolation of plasmid DNA from *E. coli* was performed by the Qiagen, Inc. (Chatsworth, Calif.), protocol. Restriction endonucleases, Klenow enzyme, T4 DNA ligase, and RNase A (Boehringer, Mannheim, Germany) were used as described by the manufacturer. Ligation of insert DNA into phagemids pTZ18-R and pUC19 (Pharmacia P-L Biochemicals, Uppsala, Sweden) and pBluescriptSK (pSK; Stratagene, La Jolla, Calif.) and subsequent transformation into *E. coli* XL1-Blue (Stratagene) cells were performed by standard techniques (39). DNA fragments were extracted from agarose with the GeneClean II kit (Bio 101, Inc., La Jolla, Calif.).

DNA sequencing. DNA sequence information was obtained from both strands of the inserts by primer-directed dideoxy sequencing of double-stranded templates with Sequenase version 2.0 (U.S. Biochemicals, Cleveland, Ohio) or a T7 sequencing kit (Pharmacia P-L Biochemicals). Single-stranded templates prepared from recombinant phagemids (by using the Qiagen M13 protocol) or 7-deaza-dGTP-containing reaction mixtures (Boehringer Mannheim) were used to avoid compression. The M13 reverse-sequencing primer was purchased from Pharmacia, and the M13 sequencing primer (−40) was from U.S. Biochemicals. All the other oligonucleotides used in sequencing were synthesized at Innogenetics.

DNA and protein sequence analysis. DNA sequence data obtained from sequencing gels were compiled and analyzed by the DNA Strider 1.1 program (29). Other sequence analyses were performed on a Dec/Vax 6220 or on a SUN Sparcserver 10/41. FastA, TFastA, Terminator, PepPlot, and Motifs programs were used with the Genetics Computer Group sequence analysis software package, version 7.3-UNIX (GCG, Inc., Madison, Wis.). DNA and amino acid sequences were used to search the DNA and protein databases at the National Center for Biotechnology Information (National Library of Medicine, Washington, D.C.) by using the BLAST algorithm (2).

Expression of the *B. abortus* recombinant OMP10 (rOMP10) and rOMP19 in *E. coli*. (i) Plasmid construction. The expression vector pIGFH10 is a derivative of the vector pmTNFMPH (17) and allows expression of cloned genes as fusion proteins with a polypeptide comprising the first 25 amino acids of mTNF and a hexahistidine peptide. A 437-bp *Brucella* DNA fragment encoding OMP10 devoid of the putative signal peptide (*Sma*I-*Hin*fI fragment [see Fig. 3]) was subcloned into pIGFH10, to obtain an in-frame fusion with the mTNF fusion partner. The resulting plasmid was termed pBru10.

The vector pIGALMPH used for expression of rOMP19 has been described by Van Gelder et al. (42). The fusion proteins synthesized contain 48 amino acids of Cro-LacI information linked with a stretch of six histidyl residues at the amino terminus. A 588-bp *Xma*I-*Eco*RI *Brucella* DNA fragment containing the *omp19* coding sequence (open reading frame 2 [ORF2]) was subcloned into the *Xma*I-*Stu*I-opened pIGALMPH vector. Subsequently the *Xma*I site was filled in to restore the reading frame between the vector-encoded leader peptide and OMP19, to create pBru19-1. To remove the OMP19 putative signal peptide, a 120-bp *Bam*HI-*Sac*I fragment was deleted from pBru19-1 to create pBru19-2, again fusing the *Brucella* ORF in frame to the Cro-LacI ORF.

Fusion proteins produced by these vectors can be detected with the MAbs targeted to the mTNF and to the Cro-LacI sequences, respectively.

(ii) Expression and purification of both rOMPs in *E. coli*. *E. coli* SG4044 (20) containing plasmid pAc1 was transformed with either plasmid pBru10 or plasmid pBru19.2. A 15-liter fermentor was seeded with an overnight saturated culture of the transformants in a 1/50 dilution. Incubation was performed at 28°C until the optical density (OD) at 600 nm reached 0.2. The temperature of the vessel was then shifted to 42°C, and incubation was then continued for 3 to 4 h at 42°C. During the fermentation, the pH, oxygen pressure, and temperature were monitored and adjusted. Bacteria were then concentrated by microfiltration and centrifugation and kept frozen at −70°C until use. After thawing, the cells were resuspended in 150 ml (five-pellet volumes) of lysis buffer (150 mM KCl, 10 mM Tris [pH 6.8], 5 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 25 mM ε-amino-caproic acid) and lysed in a French press (two cycles at 16,000 lb/in^2). The resulting lysate was centrifuged at 30,000 × *g* for 20 min at 4°C. The pellet was solubilized in 80 ml of 6 M guanidine-HCl–0.1 M phosphate (pH 6.5) buffer containing 0.05% Triton X-100, and the extract was centrifuged at 30,000 × *g* for 30 min at 4°C. The supernatant containing the recombinant antigen (50 to 100 mg of total proteins) was then loaded onto a 20-ml IMAC column (Pharmacia P-L Biochemicals) preactivated with $NiCl_2$ and equilibrated with 6 M guanidine-HCl–0.1 M phosphate (pH 6.5) buffer. The column was washed with 6 M guanidine-HCl–0.1 M phosphate (pH 6.5) buffer containing 0.05% Triton X-100 and eluted with a linear pH gradient in the same buffer (pH 6.5 to 3.7). The contaminating bacterial lipopolysaccharide (LPS) was removed from the rOMP19 preparation by passing the eluted fractions through a Q-Sepharose fast-flow column (Pharmacia P-L Biochemicals), equilibrated in 7 M urea at pH 10.2. The purified proteins were aliquoted and stored at −70°C until used. The protein concentration was determined by the bicinchoninic acid method (Pierce, Rockford, Ill.) using bovine serum albumin as the standard. Expression and purification of the recombinant antigens were monitored by SDS-PAGE followed by Coomassie brilliant blue staining or Western blot (immunoblot) analyses. The anti-*E. coli* rabbit serum was used to estimate the level of *E. coli* proteins contaminating the purified recombinant antigens. The reactivity with anti-OMP10 or anti-OMP19 MAbs and with the anti-Cro-LacI or anti-mTNF MAb was also analyzed.

Sera. Sheep sera were obtained from J. M. Blasco (Servicio de Investigacion Agraria, Zaragoza, Spain) and have been collected from 129 sheep from flocks in which *B. melitensis* had been previously isolated and no vaccination had been performed (3). The positive-control sera for the ELISA were produced by intramuscular injection of the recombinant OMP10 or OMP19 at 150 μg in QuilA adjuvant (1 mg in phosphate-buffered saline in a final volume of 2 ml) three times each at 3-week intervals. The sheep were bled before the first injection to obtain the negative-control serum, and the positive-control serum was collected 1 week after the last injection. In addition, sera from 88 unvaccinated sheep from a *Brucella*-free experimental flock were provided by G. Dubray (INRA-Tours, Nouzilly, France) and used as negative controls. Serum samples from 36 naturally *B. abortus*-infected cattle with positive smooth-LPS (S-LPS) ELISA and classical serology for *Brucella* infection (28) were used. Ninety-six serum samples from healthy cattle were used as controls.

ELISA. Sheep and bovine sera were assayed by indirect ELISA for antibody reactivity against rOMPs as follows. (i) Microtiter plates (Maxisorp; Nunc A/S, Roskilde, Denmark) were coated (100 μl per well) by incubation for 1 h at 37°C with rOMP10 or rOMP19, each at 1 μg/ml in 0.1 M $NaHCO_3$ (pH 9.5). Plates were saturated for 1 h at 37°C in blocking buffer (phosphate-buffered saline containing 0.1% caseine and 0.5 mg of Kathon per ml). Sera were diluted 50-fold in blocking buffer containing 0.3% Triton X-705 and *E. coli* lysate to a final protein concentration of 0.5 mg/ml (to reduce interference of anti-*E. coli* antibodies present in the sample sera) and tested in duplicate wells. Binding of the antibodies to rOMPs was visualized by using polyclonal peroxidase-conjugated rabbit anti-sheep or anti-cow immunoglobulin (Dako) diluted 5,000-fold in blocking buffer. Washings between the different incubations and development of the peroxidase activity were performed as described before (7). The difference in ODs at 490 nm and at 630 nm was read in Bio Kinetics reader EL340 (Bio-tek Instruments). The positive- and negative-control sheep sera were included in all plates.

(ii) *Brucella* S-LPS was coated on microplates (69620; Nunc) at 1 μg/ml in carbonate buffer, pH 9.5. The subsequent steps were the same as those for the protein ELISA. The sera from all 129 sheep in the infected flocks tested positive for brucellosis by S-LPS ELISA.

Nucleotide sequence accession number. The *B. abortus* sequence data reported in this paper will appear in the EMBL, GenBank, and DDBJ nucleotide sequence databases under accession numbers L27995 and L27997 for the *omp10* and *omp19* genes, respectively.

RESULTS

Cloning of *omp10* and *omp19* genes. A *B. abortus* 544 genomic library constructed in phage λgt11 was screened with a mixture of MAbs, two anti-OMP10 and five anti-OMP19. Two plaques reacting with anti-OMP10 MAbs (L101 and L102 phages) and one reacting with anti-OMP19 MAbs (L19 phage) were selected for further work. To determine whether the positive phages expressed a *lacZ-B. abortus omp* gene fusion, *E. coli* Y1089 lysogens of these three phages were generated.

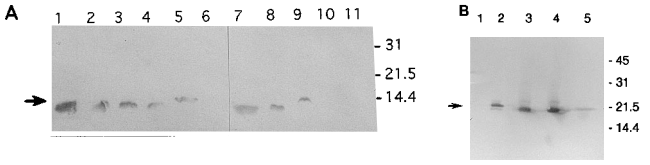


FIG. 1. Immunoblot analysis of native and recombinant *Brucella* OMP10 (A) and OMP19 (B). (A) Whole-cell lysates of *B. abortus* 544 (lanes 1 and 7) and of *E. coli* XL1-Blue harboring plasmid p101.2 (lane 2), p102 (lane 3), p103 (lanes 4 and 8), p104 (grown in the presence of 1 mM IPTG [lane 5] or in the presence of 2% glucose [lane 6]), p105 (grown in the presence of 1 mM IPTG [lane 9] or in the presence of 2% glucose [lane 10]), or p106 (lane 11) were immunoblotted with MAbs A68/07G11/C10 and A68/08E07/B11. (B) Whole-cell lysates of *B. abortus* 544 (lane 5) and of *E. coli* XL1-Blue harboring plasmid p191 (lane 4), p192 (lane 3), or p193 (grown in the presence of 1 mM IPTG [lane 2] or in the presence of 2% glucose [lane 1]) were immunoblotted with MAb A68/25H10/A05. Arrows, rOMP10 (A) and rOMP19 (B), which have slightly higher MMs than the corresponding native OMP. MM standards (in kilodaltons) are indicated on the right.

Whole-cell extracts derived from IPTG-induced and noninduced lysogens were immunoblotted with anti-OMP10 or anti-OMP19 MAbs. No β-galactosidase fusion protein was detected. Independent of the addition of IPTG, a specific immunoreactive band with an apparent (MM) identical to that of the native OMP present in *Brucella* extracts (data not shown) appeared. This indicated that the phage's insert contained the entire *omp10* gene (L101 and L102 phages) or *omp19* gene (L19).

DNA purified from the three isolated phages was cleaved with *Eco*RI. The inserts of 4.5, 3.2, and 0.96 kb were cloned into plasmid vectors pSK+, pUC19, and pTZ18-R, respectively, and were named p101.1, p101.2, and p191. XL1-Blue p101.1 and p101.2 transformants expressed a recombinant OMP10 with an apparent MM identical to that of native *Brucella* OMP10 (Fig. 1A). In extracts of XL1-Blue cells harboring the plasmid p191, anti-OMP19 MAbs detected a band that comigrated with native *Brucella* OMP19 (Fig. 1B). Both recombinant OMPs were expressed independently of the orientation of the insert.

In order to localize the *omp10* gene precisely within the 3.5- and 4.2-kb inserts, restriction maps were generated. A set of subclones was constructed as described in Fig. 2A. Expression of the recombinant OMP10 by these subclones was tested by immunoblotting. A 1.2-kb *Hin*dIII-*Kpn*I fragment was the smallest insert able to mediate production of a protein which comigrated with *Brucella* OMP10 during SDS-PAGE. The two subclones containing an insert limited by the *Sma*I site (p104 and p105) produced, upon induction by IPTG, an immunoreactive protein which had a slightly higher MM than the native OMP10. Furthermore, no expression could be detected from the 1.6-kb *Sma*I-*Eco*RI fragment cloned in the reverse orientation to *lacZ* (p106). These results indicated that the *Sma*I-limited subclones p104 and p105 express a β-galactosidase α-peptide–OMP10 fusion protein under control of the *lac* promoter. This implies that the *omp10* gene 5′ terminus is located upstream from the *Sma*I site. Sequencing was therefore initiated both upstream and downstream from this restriction site.

In order to characterize the *omp19* gene, the entire 0.96-kb *Eco*RI p191 insert was sequenced (Fig. 2B).

Sequence analysis of the *omp10* and *omp19* genes. The nucleotide sequences of the *omp10* and *omp19* regions of *B. abortus* are presented in Fig. 3 and 4. Nucleotide sequences of *omp10* and *omp19* are GC rich (G+C contents, 55 and 58%, respectively) as expected for *Brucella* genes (10).

The *omp10* gene sequence (Fig. 3) revealed an ORF terminated by a TGA stop codon at position 489. The *Sma*I site lies within the *omp10* ORF, as previously predicted on the basis of the expression pattern of the subclones. Three ATG codons are present in frame at nucleotide positions 94, 103, and 109. A putative Shine-Dalgarno sequence (GGAG) occurs 9 bp upstream from the third ATG; an identical ribosome-binding site has been reported to be present 10 bp 5′ to the *B. abortus ssb* gene starting codon (46). If one considers the third ATG as the start codon, since its location downstream of the putative Shine-Dalgarno site is adequate, the *omp10* ORF codes for a 126-amino-acid polypeptide with a calculated MM of 13,251 Da. A putative stem-loop structure with diad symmetry is present 18 bases downstream from the stop codon and is suggested to function as a rho-independent transcription terminator despite the short length of the poly(T) stretch. Some good hairpin terminators with few consecutive uridines immediately adjacent have been described (36).

Two putative overlapping ORFs were identified in the p191 insert sequence (Fig. 4). ORF1 begins with one of the two ATG codons at position 101 or 113 and ends with a TAA codon at position 494. A potential Shine-Dalgarno sequence (GAGG) is located 6 bases upstream from the second ATG. The second ORF (ORF2) starts with one of the three potential ATG initiator codons at nucleotide 387, 402, or 414 and ends with a TGA stop codon at nucleotide 945. There is a putative ribosome-binding site (GGAG, bases 404 to 407) 7 bases upstream from the third ATG. ORF1 encodes a putative poly-

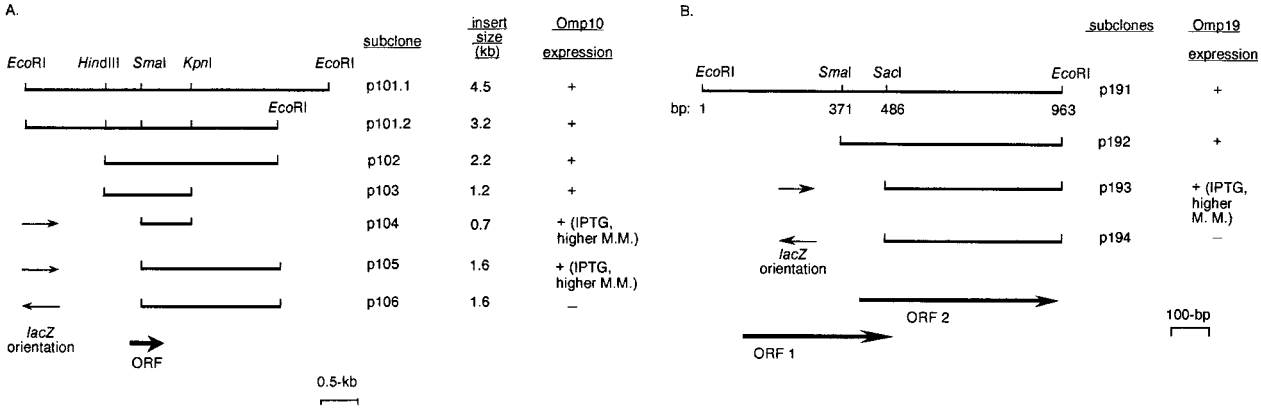


FIG. 2. Restriction map of the cloned 3.5- and 4.2-kb DNA fragments of *B. abortus* expressing OMP10 (A) and of the 0.96-kb insert expressing OMP19 (B). Subclones were constructed as shown. Insert size, expression of OMP10 or OMP19, detected by immunoblotting, and positions of the putative ORFs are indicated.

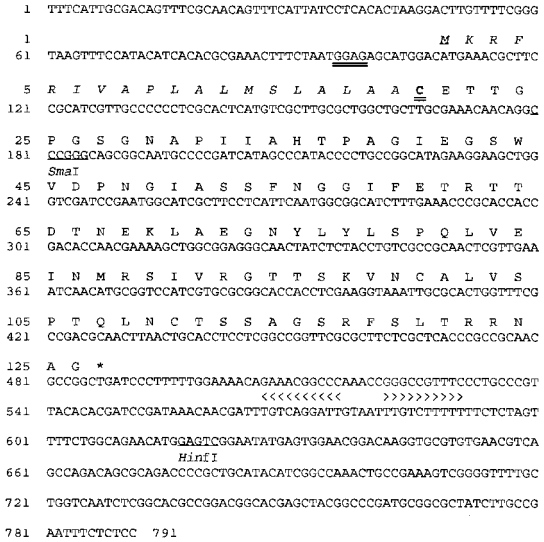


FIG. 3. Nucleotide sequence and deduced amino acid sequence of the *omp10* gene of *B. abortus* and of the corresponding protein. The *Sma*I and *Hin*fI restriction sites used for expression cloning are indicated. The putative ribosome-binding site is underlined twice. The possible initiator methionines are underlined, and the dyad symmetry of the putative terminator stem-loop structure is indicated (<<< >>>). Asterisk, termination codon. The putative signal peptide is italicized. The proposed site of acylation is boldfaced and doubly underlined.

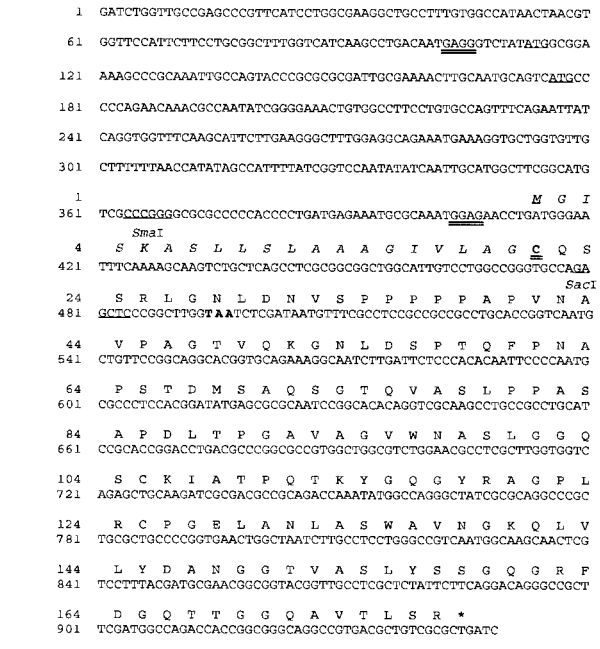


FIG. 4. Nucleotide sequence of the *B. abortus omp19* region. The *Sma*I and *Sac*I restriction sites used in the expression cloning are underlined. Only the amino acid sequence deduced from ORF2 is presented. For both ORFs, the putative ribosome-binding sites are doubly underlined. The possible initiator codons of ORF1 and methionines of ORF2 are underlined. Asterisk, ORF2 termination codon. The ORF1 termination codon is boldfaced. The OMP19 putative signal peptide is italicized. The proposed site of acylation is boldfaced and underlined twice.

peptide of 127 amino acids with a calculated MM of 14,122 Da (assuming that the second ATG is the start codon). The MM of the 177-amino-acid polypeptide deduced from ORF2 is calculated to be 17,592 Da (assuming that the third ATG is the start codon).

In order to identify which ORF codes for OMP19, subclones were constructed and recombinant OMP19 expression was tested by immunoblotting with an anti-OMP19 MAb. ORF2 was subcloned as a *Sma*I-*Eco*RI fragment spanning nucleotides 364 to 949 into the multiple cloning site of pSK (Fig. 2B). XL1-Blue cells harboring the resulting plasmid p192 expressed a protein recognized by the anti-OMP19 MAb and that comigrated with *Brucella* native OMP19, suggesting that the *omp19* gene is located downstream from the *Sma*I site. A *Sac*I (base 479)-*Sac*I (from pTZ18-R) fragment lacking the first 65 bases of ORF2 was subcloned in frame with the *lacZ* gene from pSK− (construction p193). This resulted in expression of a fusion protein between 14 pSK-encoded residues and the last 155 residues of ORF2. This fusion protein reacted with anti-OMP19 MAb and migrated in SDS-PAGE at a slightly higher MM than the *Brucella* native OMP19. Expression of the fusion protein was induced by IPTG and abolished by glucose, confirming that it was under the control of the *lac* promoter. When the *Sac*I-*Sac*I insert was cloned in the opposite direction to *lacZ*, no protein was detected by anti-OMP19 MAbs in the resulting *E. coli* transformants because the ORF2 was separated from its expression signals. Expression results of all three ORF2 subclones demonstrated that ORF2 encodes OMP19.

Analysis of the predicted OMP10 and OMP19 proteins. The amino termini of the predicted OMP10 and OMP19 strongly resemble a bacterial lipoprotein signal peptide. Both have a positively charged amino-terminal region (OMP10 positions 2, 3, and 5; OMP19 position 5) followed by a hydrophobic core (residues 6 to 16 for OMP10 and residues 6 to 17 for OMP19) and a tetrapeptide showing a high degree of similarity with the consensus sequence of bacterial lipoprotein precursors (Fig. 5) (24). This consensus sequence, Leu-(Ala or Ser)-(Gly or Ala)-Cys, defines the site of prolipoprotein modification and specific cleavage by signal peptidase II. Cleavage of the signal peptide in front of the Cys residue would result in a 107-amino-acid polypeptide for OMP10 and in a polypeptide of 157 amino acids for OMP19. The theoretical sizes of the cleaved, fully acylated (mature) OMP10 and OMP19 lipoproteins would be 12 and 16.6 kDa, respectively (assuming that acylation results in covalent linkage with three palmitic acid molecules).

In addition, the amino acid residues at the +2 and +3 positions of lipoproteins are predicted to be enriched with those having a high propensity to form a β-turn secondary structure (26). This is the case for mature OMP19 Gln (+2) and Ser (+3) residues. However, concerning the mature OMP10 N terminus, the highest propensity to form a β-turn secondary structure, as predicted by the Chou-Fasman algorithms (4), is obtained for the Pro-Gly couple at positions +6 and +7.

Predicted mature OMP10 serine (11.2%) and threonine (11.2%) contents are higher than the predicted frequencies of these residues in proteins as determined by McCaldon and Argos (6.9 and 5.8%, respectively) (32). The deduced mature

FIG. 5. Deduced N-terminal amino acid sequences of OMP10 and OMP19. The consensus sequences of bacterial lipoprotein precursors are aligned under the OMP sequences (the numbers are the frequencies of amino acid residues in hundreds, as measured by Hayashi and Wu [24]). In both OMP sequences, the tetrapeptide showing a high degree of similarity with this consensus is boxed.

OMP19 has a high proline content (10.8%) compared with the 5.1% proline occurrence in proteins in general (32). DNA and protein similarity searches were performed, and no significant similarities of the OMP10 ORF and deduced amino acid sequence to known genes or proteins were identified. The OMP19 predicted sequence shows a high identity (>98%) to a *B. abortus* S19 18-kDa protein that appeared very recently in the EMBL database under the accession numbers L42959 and U29211 (9a). The Cys residue at position 21 of the predicted OMP19 is replaced by a Ser residue in the predicted sequence of the 18-kDa protein. The two sequences also differ from one another in the last residues because of a frameshift occurring at nucleotide 523 of the ORF.

Occurrence of OMP10 and OMP19 in the genus *Brucella*. Thirty-four *Brucella* strains, including all six of the classical species and all their reported biovars, were examined for occurrence of OMP10 and OMP19 (Table 1). SDS-PAGE of whole-cell preparations from each of these strains was followed by Western blotting with anti-OMP10 MAb A68/7G11/C10 and with anti-OMP19 MAb A68/25H10/A05 separately. The anti-OMP10 MAb revealed a 10-kDa band in all *Brucella* strains tested. These data indicate that the apparent OMP10 size is identical among strains. In all these strains anti-OMP19 MAb reacted with a 19-kDa band except for *B. ovis*, whose OMP19 had an apparent MM that was slightly higher (Fig. 6). Western blotting analysis of four additional *B. ovis* strains confirmed that OMP19 migrates more slowly in this species.

In contrast, in extracts of five other bacterial species (Table 1) no protein reacting with either of the MAbs could be detected. These species are not taxonomically closely related to but are known or presumed to induce immunological cross-reactions with *Brucella* spp.

Expression and purification of the recombinant OMP10 and OMP19. Upon expression in *E. coli* transformed with the expression vector containing ORF2 (pBru19-1) and by using anti-19 MAbs for detection on Western blots of whole-cell extracts, two major proteins were detected, one of which was the size of the mature OMP19. The MAb anti-Cro-LacI peptide recognized the highest band as well as a lower band that would correspond to the cleaved peptide (data not shown). This indicates that the OMP19 signal sequence is probably processed in *E. coli*, even when it is not localized at the amino terminus of the protein. To avoid this cleavage and to possibly enhance the expression level, the putative signal peptide was removed by deletion of the *Sma*I-*Sac*I fragment, thus removing the first 70 nucleotides from the *omp19* coding sequence. The recombinant OMP19 expressed by this second construct, pBru19-2, accumulated in *E. coli* in insoluble inclusion bodies (data not shown) that were solubilized in 6 M guanidine-HCl and purified on a nickel chelate affinity chromatography column and by ion-exchange chromatography. This procedure yielded 3.7 mg of a highly purified rOMP19 (purity, >95%) per liter of culture. The rOMP10 expressed by pBru10 was shown to be almost exclusively localized in the insoluble fraction. The yield of rOMP10 purified by the Ni chelate affinity chromatography was 9.3 mg/liter of culture (purity, >95%) (Fig. 7).

Analysis of sheep and bovine sera. To evaluate whether OMP10 and OMP19 are immunogenic in vivo, serum samples from the 129 sheep belonging to infected flocks and 88 normal control sera were tested in ELISA for reactivity with each of the two recombinant OMPs. Figure 8 presents the results of these ELISAs. For both rOMP-based ELISAs, the distribution of the ODs corresponding to the sera from the *Brucella*-free sheep was clearly discriminated from that of the sera from the sheep belonging to the infected flocks. The cutoff was set at the mean plus 3 standard deviations for OD values of the normal control sera, and cutoff values of 0.431 and 0.246 have been calculated for the rOMP10- and rOMP19-specific ELISAs, respectively. On the basis of these values, 66 and 50% of sera from serologically positive sheep contain antibodies to rOMP10 and rOMP19, respectively. In contrast, for both

TABLE 1. Species and strains tested

Species and strain (biovar)	OMP10[a]	OMP19[a,b]
B. abortus		
544 (1)	+	+
B19 (1)	+	+
86/8/59 (2)	+	+
Tulya (3)	+	+
292 (4)	+	+
B 3196 (5)	+	+
870 (6)	+	+
80-236 (6)	+	+
89-43 (6)	+	+
87-46 (9)	+	+
90-64 (9)	+	+
91-135 (9)	+	+
76-299 (9)	+	+
75-60 (9)	+	+
80-133 (9)	+	+
77-9 (9)	+	+
C 68 (9)	+	+
91-28 (9)	+	+
45/20 (rough)	+	+
B. melitensis		
16 M (1)	+	+
Rev.1 (1)	+	+
63/9 (2)	+	+
Ether (3)	+	+
B115 (rough)	+	+
H38 (rough[c])	+	+
B. suis		
1330 (1)	+	+
S2 (1)	+	+
Thomsen (2)	+	+
686 (3)	+	+
40 (4)	+	+
513 (5)	+	+
B. canis RM 6/66	+	+
B. ovis		
BOW 63/290	+	+
Reo 198	ND[d]	+
91-70	ND	+
91-266	ND	+
91-268	ND	+
B. neotomae 5 K 33	+	+
E. coli O:157	−	−
Y. enterocolitica O:9	−	−
S. urbana	−	−
P. maltophilia	−	−
P. multocida	−	−

[a] +, detected; −, not detected.
[b] OMP19 detected in extracts of each *B. ovis* strain tested has a larger size than OMP19 present in the other *Brucella* species.
[c] Variant of the smooth H38 strain.
[d] ND, not determined.

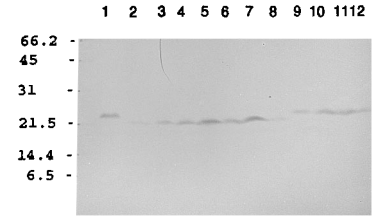


FIG. 6. Immunoblot analysis of *Brucella* native OMP19. Whole-cell lysates of *B. ovis* 91-268 (lane 1); *B. abortus* B19 (lane 2) and 45/20 (lane 3); *B. melitensis* B115 (lane 4), Rev. 1 (lane 5), and H38 (rough variant) (lane 6); *B. suis* S2 (lane 7); *B. abortus* 544 (lane 8); and *B. ovis* BOW 63/290 (lane 9), Reo 198 (lane 10), 91-70 (lane 11), and 91-266 (lane 12) were immunoblotted with anti-OMP19 MAb A68/25H10/A05. MM standards (in kilodaltons) are indicated on the left.

rOMP-based ELISAs, the distribution of the ODs corresponding to the sera from 36 field infected cattle was not different from that of healthy cattle sera (data not shown).

DISCUSSION

We report the cloning from a *B. abortus* 544 library of two genes encoding the minor 10- and 19-kDa OMPs. The gene *omp10* was cloned in *E. coli* on two overlapping inserts (3.2 and 4.5 kb), and it was more accurately mapped within these inserts by subcloning and subsequent expression assays with *E. coli*. Nucleotide sequence analysis of the *omp10* gene region revealed an ORF encoding a protein of the expected size. The gene *omp19* was isolated on a 0.96-kb insert; its sequence contained two short and overlapping potential ORFs. Expression of fusion proteins confirmed the identity of the *omp10* gene ORF and demonstrated that ORF2 encodes OMP19. Despite the presence of a potential ribosome-binding site upstream from ORF1, we have no experimental evidence that this ORF overlapping with the OMP19-encoding ORF is expressed in brucellae. Expression of OMP10 and OMP19 in *E. coli* seems to be driven from their own gene promoter.

The predicted sequences of OMP10 and OMP19 exhibit features of bacterial lipoprotein precursors: an amino-terminal signal peptide ending with a tetrapeptide sequence that conforms to the consensus sequence required for prolipoprotein modification and processing (38). The first *Brucella* sp. lipoprotein that has been isolated is the pepdidoglycan-linked Braun protein's equivalent (18, 19). We described recently the molecular characterization of the *pal* gene encoding another *B.*

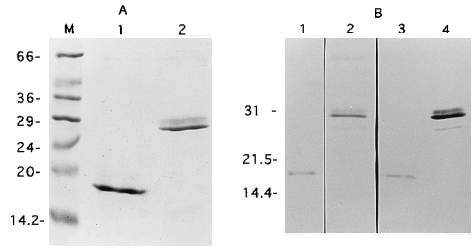
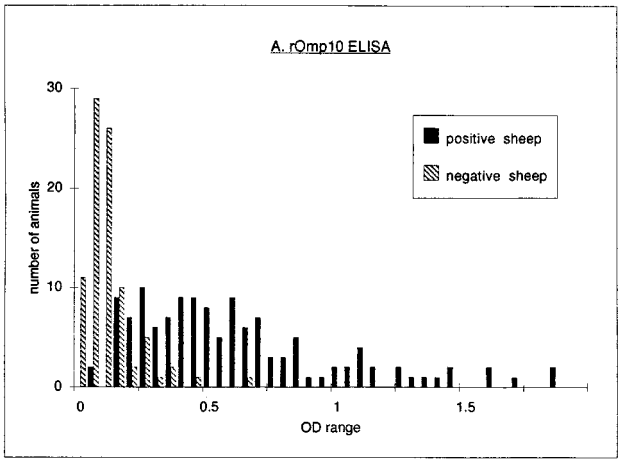


FIG. 7. Analysis of the purified *E. coli*-produced rOMP10 and rOMP19. (A) Coomassie blue staining of purified rOMP10 (2 μg) (lane 1) and purified rOMP19 (2 μg) (lane 2). (B) Purified rOMP10 was immunoblotted with the anti-mTNF MAb (lane 1) and with the anti-OMP10 MAb A68/07G11/C10 (lane 3). Purified rOMP19 was immunoblotted with the anti-Cro-LacI MAb (lane 2) and with the anti-OMP19 MAb A76/02A04/A07 (lane 4). The additional band of a higher size revealed by this MAb probably resulted from an initiation of translation at an AUG codon located upstream, whereas the one of the lower size would correspond to a degradation product. MM standards (lane M) (in kilodaltons) are indicated on the left.



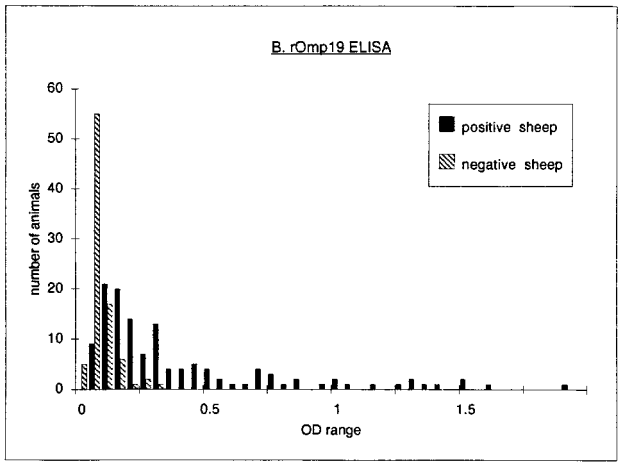


FIG. 8. Distribution of the rOMP10 (A) and rOMP19 (B) ELISA OD values in the sera from 88 *Brucella*-free sheep (negative) and 129 sheep from infected flocks (positive).

abortus putative outer membrane lipoprotein, named OMP16 (41). The apparently correct processing of OMP10, OMP16, and OMP19 in *E. coli* suggests that the pathway for lipoprotein maturation is functionally conserved between Brucella spp. and *E. coli*. Addition of fatty acids is indeed reported as the most abundant posttranslational modification of bacterial secretory proteins, and all bacterial lipoproteins are believed to be modified in the same way as the Braun lipoprotein of *E. coli* (38). Our results suggest that this posttranslational modification is also used in brucellae. It is remarkable that among the four minor OMPs identified by Cloeckaert et al. (5) by use of MAbs (OMP10, -16, -19, and -89) and further characterized, three are putative lipoproteins. To demonstrate unequivocally that these three minor OMPs are lipoproteins, the evaluation of the effect of globomycin (a signal peptidase II-specific inhibitor) on their maturation and the metabolic labeling with palmitic acid are in progress.

The calculated size of the mature OMP10 (12 kDa) is consistent with the MM of the OMP10 expressed by brucellae or by a recombinant *E. coli*, as estimated by SDS-PAGE followed by Western blot. The predicted mature OMP19 has a high proline content that probably accounts for the difference between the observed MM and the calculated MM (16.6 kDa) (37). Western blot analyses indicated, as expected, that the

sizes of the β-galactosidase–OMP fusion proteins produced by the p104, p105, and p193 constructs were slightly higher than the size of the native corresponding OMP. Indeed, this increase in size resulted probably from the addition of 33 (p104 and p105) or 14 (p193) amino acids of the pSK-encoded β-galactosidase α-peptide and from the deletion of the first five and two amino acids (including the modified cysteine) of the mature OMP10 and OMP19, respectively. The anti-OMP10 and anti-OMP19 MAbs reacted with the corresponding β-galactosidase–OMP fusion protein, indicating that the acylation of the cysteine residue is not necessary for the MAb-OMP recognition.

Kyte and Doolittle hydropathy plots of deduced OMP10 and OMP19 sequences suggested that both proteins are hydrophilic except for the hydrophobic region of their signal peptide (27). Furthermore, immunoelectron microscopy and ELISA analysis of whole bacterial cells demonstrated surface exposure of both OMPs in *Brucella* spp. (5). The lipid moiety proposed to be covalently linked to both OMP N termini could be embedded into the outer leaflet of the outer membrane to anchor the OMP molecules in this membrane.

Immunoblot analysis using anti-OMP10 and anti-OMP19 MAbs demonstrated that both OMPs are expressed in all *Brucella* species and biovars tested. In addition, the OMP10 and OMP19 epitopes recognized by the corresponding MAb could be unique to the *Brucella* genus because no cross-reactivity was observed with extracts of the five other bacterial species tested. However, it would be interesting to study the reactivity of the MAbs with extracts of more closely related bacteria, like *Agrobacterium tumefaciens* and *Rhizobium leguminosarum*. The apparent MMs of both OMPs seem to be similar throughout the *Brucella* genus excepted for OMP19, which displays a slightly higher size in the five *B. ovis* strains tested. Despite the overall genomic homogeneity of the genus *Brucella* (21, 43, 44), several studies have suggested that *B. ovis* represents the most widely diverged lineage. This proposal is based on DNA restriction patterns (1, 33), on the copy number of a repetitive DNA element that has the characteristics of an insertion sequence (22, 23, 35), and on the genetic variation of the *omp2* locus (8, 15). In addition, only *B. ovis* could not be shown to express a *B. abortus* 31-kDa protein (31), and Cloeckaert et al. report a deletion of about 50 bp in the *B. ovis omp25* gene (8). We are currently investigating if the different size of the OMP19 expressed by *B. ovis* is due to genetic variability of *omp19*.

Both OMPs devoid of their signal peptide were expressed in *E. coli* as fusion polypeptides. The large-scale production and purification of these recombinant OMP10 and OMP19 provides large quantities of proteins devoid of all other contaminating *Brucella* macromolecules. By use of this material in an indirect ELISA, we demonstrated that a humoral immune response toward OMP10 and OMP19 is mounted in *B. melitensis*-infected sheep. Sera collected in *B. melitensis*-infected sheep flocks were clearly discriminated from sera of *Brucella*-free animals. These data confirm the immunoblot analysis of a cell envelope fraction with infected sheep sera reported by Zygmunt et al. (47). In contrast, no significant antibody response specific for OMP10 or OMP19 could be detected in sera from naturally *B. abortus*-infected cattle. Thus, the host humoral responses appear to differ between the animal species and/or the infecting *Brucella* strains. The availability of both purified recombinant OMPs also allows us to explore the potential of these proteins to specifically induce a T-lymphocyte response. Indeed, recently recombinant protein L7/L12 was used to demonstrate the T-cell reactivity of the protein (34).

The cloning and characterization of these two *omp* genes enable us to project the construction of specific *Brucella* deletion mutants for each of these genes. If viable mutants can be isolated, the contribution of each of these OMPs to bacterial survival in vitro in macrophages and in vivo in mice, as well as to the organization of the *Brucella* outer membrane, will be evaluated.

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