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Nucleotide Sequence and Expression of the Gene Encoding the Major 25-Kilodalton Outer Membrane Protein of *Brucella ovis*: Evidence for Antigenic Shift, Compared with Other *Brucella* Species, due to a Deletion in the Gene

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The nucleotide sequences encoding the major 25-kDa outer membrane protein (OMP) (*omp25* genes) of *Brucella ovis* 63/290, *Brucella melitensis* 16M, *Brucella suis* 1330, *Brucella canis* RM6/66, and *Brucella neotomae* 5K33 (all reference strains) were determined and compared with that of *Brucella abortus* 544 (P. de Wergifosse, P. Lintermans, J. N. Limet, and A. Cloeckaert, J. Bacteriol. 177:1911–1914, 1995). The major difference found was between the *omp25* gene of *B. ovis* and those of the other *Brucella* species; the *B. ovis* gene had a 36-bp deletion located at the 3′ end of the gene. The corresponding regions of other *Brucella* species contain two 8-bp direct repeats and two 4-bp inverted repeats, which could have been involved in the genesis of the deletion. The mechanism responsible for the genesis of the deletion appears to be related to the "slipped mispairing" mechanism described in the literature. Expression of the 25-kDa outer membrane protein (Omp25) in *Brucella* spp. or expression from the cloned *omp25* gene in *Escherichia coli* cells was studied with a panel of anti-Omp25 monoclonal antibodies (MAbs). As shown by enzyme-linked immunosorbent assay (ELISA) and immunoelectron microscopy, Omp25 was exported to the outer membrane in *E. coli* expressing either the truncated *omp25* gene of *B. ovis* or the entire *omp25* genes of the other *Brucella* species. Size and antigenic shifts due to the 36-bp deletion were demonstrated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting and by the differences in binding patterns in ELISA of the anti-Omp25 MAbs at the cell surface of *E. coli* cells harboring the appropriate gene and of cells of *B. ovis* and other *Brucella* species. In particular, MAbs directed against discontinuous epitopes of the entire Omp25 showed the absence of, or a significant reduction in, antibody reactivity with the *B. ovis* truncated Omp25. The results indicated that, as defined by the MAbs, exported Omp25 probably presents similar topologies in the outer membranes of *E. coli* and *Brucella* spp. and that the short deletion found in the *omp25* gene of *B. ovis* has important consequences for the expression of surface B-cell epitopes which should be considered for the development of vaccines against *B. ovis* infection.

Bacteria of the genus *Brucella* are gram-negative intracellular parasites of both humans and animals. Six species are recognized within the genus *Brucella*: *B. abortus*, *B. melitensis*, *B. suis*, *B. ovis*, *B. canis*, and *B. neotomae* (14). This classification is based mainly on differences in pathogenicity and host preference (14). The main pathogenic species worldwide are *B. abortus* and *B. melitensis*, which are involved in bovine and ovine brucellosis, respectively, and can cause abortions and infertility in cattle and sheep, with resulting high economic losses. In countries where sheep are raised, *B. ovis* is an important cause of epididymitis and subsequent infertility in rams and is less frequently associated with abortions (4).

The *Brucella* outer membrane contains lipopolysaccharide (LPS), proteins, and phospholipids. The major *Brucella* outer membrane proteins (OMPs) are 25- to 27-kDa proteins (7, 18, 19) (also called group 3 proteins [37]) and 36- to 38-kDa proteins (7, 18, 19) (also called group 2 porin proteins [16, 37]). Most of the variations in the apparent molecular sizes of these proteins are probably due to association with peptidoglycan (PG) subunits of different sizes (12, 19, 35). Thus, group 2 and group 3 proteins are different PG-associated forms of the same gene product. Both the major 25- to 27-kDa and 36- to 38-kDa OMPs have been shown by use of monoclonal antibodies (MAbs) and immunoelectron microscopy to be surface exposed (7). These major OMPs are also major components of the sodium dodecyl sulfate (SDS)-insoluble cell wall fraction, which has important vaccine properties (17–19). The role of the two major groups of OMPs in protective immunity against *Brucella* infection is currently under investigation by several research groups (17, 18, 38). One MAb to the 25- to 27-kDa OMPs was shown to protect against experimental *B. abortus* infection in CD-1 mice (8). Antibody responses to the major OMPs have been observed in *B. abortus*-infected cattle (9) and *B. melitensis*- and *B. ovis*-infected sheep (27, 29, 39).

The major 25-kDa (group 3) OMP (Omp25) has been previously proposed, on the basis of amino acid composition and antigenic relationship, to be the counterpart of the *Escherichia coli* protein OmpA (21, 37). However, cloning and sequencing of the gene coding for *B. abortus* Omp25 (the *omp25* gene) and determination of its predicted amino acid sequence revealed no significant homologies with OmpA proteins from different gram-negative bacteria (15). Polymorphism of the *omp25* gene among *Brucella* species was further studied by PCR and restriction fragment length polymorphism analysis (10). Two species-specific markers were detected, i.e., (i) the absence of the

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TABLE 1. Strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
E. coli		
INVαF′	Host for pCRII derivatives	Invitrogen
TG1	*supE hsd*Δ*5 thi* Δ(*lac-proAB*) F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	30
Brucella		
B. abortus 544	*B. abortus* reference strain biovar 1	3
B. melitensis 16M	*B. melitensis* reference strain biovar 1	3
B. melitensis B115	*B. melitensis* rough strain isolate	3
B. suis 1330	*B. suis* reference strain biovar 1	3
B. ovis 63/290	*B. ovis* reference strain	3
B. ovis Reo198	*B. ovis* CO_2-independent strain	3
B. neotomae 5K33	*B. neotomae* reference strain	3
Plasmids		
pCRII	Amp^r Kan^r *lacZ′*	Invitrogen
pUC19	Amp^r *lacZ′*	
pAC2501	PCR-amplified *omp25* gene of *B. abortus* 544 cloned in pCRII (coding orientation vs P_{lac})	This work
pAC2503	PCR-amplified *omp25* gene of *B. melitensis* 16M cloned in pCRII (coding orientation vs P_{lac})	This work
pAC2504	PCR-amplified *omp25* gene of *B. melitensis* 16M cloned in pCRII (noncoding orientation vs P_{lac})	This work
pAC2505	PCR-amplified *omp25* gene of *B. ovis* 63/290 cloned in pCRII (coding orientation vs P_{lac})	This work
pAC2506	PCR-amplified *omp25* gene of *B. ovis* 63/290 cloned in pCRII (noncoding orientation vs P_{lac})	This work
pAC2507	PCR-amplified *omp25* gene of *B. suis* 1330 cloned in pCRII (coding orientation vs P_{lac})	This work
pAC2509	PCR-amplified *omp25* gene of *B. canis* RM6/66 cloned in pCRII (coding orientation vs P_{lac})	This work
pAC2521	PCR-amplified *omp25* gene of *B. neotomae* 5K33 cloned in pCRII (coding orientation vs P_{lac})	This work
pAC2533	*Xba*I-*Sac*I insert of pAC2504 cloned into *Xba*I-*Sac*I sites of pUC19 (coding orientation vs P_{lac})	This work
pAC2535	*Xba*I-*Sac*I insert of pAC2506 cloned into *Xba*I-*Sac*I sites of pUC19 (coding orientation vs P_{lac})	This work
pAC2562	Last third of PCR-amplified (primers 25D and 25B) *omp25* gene of *B. melitensis* 16M cloned in pCRII (fusion protein in coding orientation vs P_{lac})	This work

*Eco*RV site in *B. melitensis* strains and (ii) an approximately 50-bp deletion, located between the unique *Eco*RV and *Mae*III sites, in *B. ovis* strains.

In the present work, we studied other polymorphisms of the *omp25* gene and precisely located the deletion in the *B. ovis* *omp25* gene by gene sequence determination and analysis. The consequence of the deletion for expression of surface-exposed B-cell epitopes was analyzed by use of MAbs with both *E. coli* and *Brucella* strains expressing Omp25.

MATERIALS AND METHODS

Bacterial strains and cultivation. The *Brucella* strains used in this study are listed in Table 1. All strains were kept freeze-dried in the *Brucella* Culture Collection, INRA, Nouzilly, France. Cultures were grown on Trypcase soy agar (BioMérieux, Marcy l'Etoile, France) supplemented with 0.1% (wt/vol) yeast extract (Difco Laboratories, Detroit, Mich.) (TSAYE medium). For fastidious strains (*B. ovis*), sterile equine serum (BioMérieux) was added to TSAYE medium to a final concentration of 5% (vol/vol) (TSAYES medium). The strains were checked for purity and species and biovar characterization by using standard procedures (3).

B. melitensis B115 and *B. ovis* Reo198 cells, used for Omp25 expression analysis, were grown on liquid LB medium at 37°C for several days until an A_{600} of around 2.0 was obtained. *E. coli* strains (Table 1) were cultured at 37°C overnight on liquid selective LB medium with ampicillin at a concentration of 50 μg/ml (for *E. coli* TG1 carrying plasmid pUC19 or derivatives) or with both ampicillin and kanamycin at a concentration of 50 μg/ml each (for *E. coli* INVαF′ carrying plasmid pCRII or derivatives). For induction of expression (pUC19-derived plasmids), overnight cultures of *E. coli* TG1 grown in liquid selective LB medium were adjusted to an A_{600} of 0.1 in 50 ml of fresh liquid selective LB medium. After 2 h of growth at 37°C, isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma) was added at a final concentration of 1 mM. The cultures were allowed to grow for several hours longer, and aliquots were taken at defined intervals to control expression.

DNA preparation. For each strain, a 1-day 37°C culture on a TSAYE or TSAYES medium slope was harvested with 3 ml of sterile distilled water and centrifuged at 4,500 rpm for 10 min. The pellet was suspended in 567 μl of TE-Na buffer (50 mM Tris, 50 mM EDTA, 100 mM NaCl, pH 8.0). Thirty microliters of a 10% (wt/vol) SDS solution and 3 μl of a 2% (wt/vol) proteinase K solution were then added, and the mixture was held at 37°C for 1 h. The lysed cell suspension was extracted twice with phenol-chloroform. Nucleic acids were precipitated by gently mixing the resultant aqueous phase with 2 volumes of cold ethanol. The precipitate was dissolved in 100 μl of TE (10 mM Tris, 1 mM EDTA, pH 8.0). The amount of DNA was measured by electrophoresis of an aliquot of each sample through 0.8% agarose gels and comparison with standard DNA solutions.

PCR. 20-mer primers for amplification of portions of the *omp25* gene or the entire *omp25* gene (Table 2) were obtained from Appligene (Illkirch, France). For *omp25* gene cloning and sequencing, primers (25A and 25B) were chosen to amplify the entire gene with its Shine-Dalgarno and putative transcription terminator sequences according to the previously determined *B. abortus* gene sequence (15). For gene sequencing and production of fusion proteins, the primers used for amplifications of portions of the *omp25* gene are indicated in Table 2. Amplification reaction mixtures were prepared in volumes of 100 μl containing 10 mM Tris-HCl (pH 9.0)–50 mM KCl–1.5 mM $MgCl_2$–0.1% Triton X-100–0.2 mg of gelatin per ml (1× PCR buffer; Appligene), 200 μM each deoxynucleoside triphosphate, 1 μM primer, 100 ng of genomic DNA, and 2.5 U of *Taq* DNA Polymerase (Appligene). The temperature cycling for the amplification was performed in a Techne PHC-2 thermocycler as follows: cycle 1 was 95°C for 5 min (denaturation), 58°C for 2 min (annealing), and 70°C for 3 min (extension); the next 30 cycles were 95°C for 1 min (denaturation), 58°C for 2 min (annealing), and 70°C for 3 min (extension); and the last cycle was 95°C for 1 min (denaturation), 58°C for 2 min (annealing), and 70°C for 10 min (extension).

The PCR-amplified *omp25* genes were confirmed by restriction digestion with a number of restriction enzymes as described previously (10).

Plasmid construction. Plasmid isolation and subcloning procedures were performed as described by Sambrook et al. (30). The PCR-amplified *omp25* genes of different *Brucella* species and strains were first cloned in plasmid pCRII (TA Cloning Kit) (Invitrogen, San Diego, Calif.), according to the manufacturer's instructions, resulting in the plasmids listed in Table 1. Insert orientations were determined by the sizes of fragments produced after digestion with *Hin*dIII. Digested plasmids were analyzed on a 1% agarose gel. The pCRII plasmid carries both ampicillin and kanamycin resistance genes, and therefore transfor-

TABLE 2. Primers used

Primer	Sequence
25A	5′-GGACCGCGCAAAACGTAATT-3′
25B	5′-ACCGGATGCCTGAAATCCTT-3′
25C	5′-TCCAATCGTCAGGCTTGATG-3′
25D	5′-CTTGGACGACGAAAGCAAGT-3′
25E	5′-CAAGCCTGACGATTGGAAGG-3′
25F	5′-AACCCACGCGGAACTTGCTT-3′

TABLE 3. Characteristics of the MAbs used[a]

Specificity	MAb no.	Isotype	Binding[b] on rough *B. melitensis*:	
			Cells (in ELISA)	Cell lysates (in immunoblotting)
Omp25	A59/05F01/C09	IgG2a	+++	+++
	A68/04B10/F05[c]	IgG2a	+++	+
	A76/02C12/C11[c]	IgG2a	+++	+
	A68/07D11/B03[c]	IgG2a	+++	+/−
	A68/28G06/C07[c]	IgG2a	+++	+/−
	A76/02F04/F01	IgG2a	++	++
	A19/12B10/F04	IgG1	+++	+++
	A18/13D02/F05	IgG1	+	+++
	A70/06B05/A07[c]	IgG3	++	+
	A76/08H09/A02	IgG3	+	++
	A76/09F04/B11	IgG3	+	++
	A76/12H09/A05	IgG3	+	++
	A76/15H07/E07	IgG3	+	++
	A59/01E11/D11	IgG2a	−	++
PG	3D6	IgG3	−	+++
S-LPS	12G12	IgG1	−	− or +++[d]
36-kDa OMP	A68/25G05/A05	IgG2a	+++	+/−

[a] Characteristics are according to references 7, 8, 9, 11, 12, and 13 and unpublished data.

[b] +++, strong binding; ++, medium binding; +, little binding; +/−, very little binding; −, no binding.

[c] The MAb most probably recognizes discontinuous epitopes of Omp25, since it shows high-level reactivity in ELISA on native Omp25 at the cell surface but weak or very weak reactivity on the denatured Omp25 in immunoblotting.

[d] Binding depends on intracellular expression of O polysaccharide.

mants were selected on LB plates containing ampicillin and kanamycin, both at 100 μg/ml. The pCRII plasmid lacks the *lacI* gene, and therefore expression was controlled without IPTG induction. The *Xba*I-*Sac*I fragments of plasmids pAC2504 and pAC2506 (Table 1), gel purified with the GeneClean kit (Bio 101, La Jolla, Calif.), were further ligated by the standard T4 ligase procedure at 15°C into plasmid pUC19 cut by *Xba*I and *Sac*I and gel purified with the GeneClean kit, resulting in plasmids pAC2533 and pAC2535, respectively (insert coding orientation relative to P_{lac} promoter) (Table 1). The ligation product was transformed into *E. coli* TG1, which was made competent by the method of Sambrook et al. (30). Transformants were selected on LB plates containing ampicillin (100 μg/ml). The pUC19 plasmid carries the *lacI* gene, and consequently expression was tested after IPTG induction. For production of a fusion protein comprising the last third (carboxy-terminal end) of the Omp25 protein of *B. melitensis* 16M fused to β-galactosidase, a portion of the *omp25* gene of *B. melitensis* 16M was amplified with primers 25D and 25B (Table 2) and cloned into the pCRII plasmid as described above.

DNA sequencing. The dideoxy chain termination method of Sanger et al. (32) was employed with the SequiTherm Cycle Sequencing Kit (Epicentre Technologies, Madison, Wis.) according to the manufacturer's protocol. PCR-amplified *omp25* genes from the six *Brucella* species (Table 1) were purified from an agarose gel with the GeneClean kit (Bio 101) and used as templates, and the primers listed in Table 2 were used for annealing in the sequencing reaction. The temperature cycling for the amplification was performed in a Techne PHC-2 thermocycler as follows: cycle 1 was 95°C for 5 min (denaturation), 50°C for 30 s (annealing), and 70°C for 1 min (extension), and the next 30 cycles were 95°C for 30 s (denaturation), 50°C for 30 s (annealing), and 70°C for 1 min (extension).

MAbs. The anti-Omp25, anti-PG, anti-36- to 38-kDa protein (anti-36-38 kDa), and anti-smooth LPS (anti-S-LPS) MAbs were produced as described previously (7, 12). Their characteristics are summarized in Table 3. MAbs were used as hybridoma supernatants throughout the study.

Protein expression and characterization. Protein expression in *E. coli* or *Brucella* strains was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with the anti-Omp25 MAbs as previously described (7). Binding of MAbs (one-third-diluted hybridoma supernatants) in immunoblotting was detected by using rabbit anti-mouse immunoglobulin antiserum (Nordic Immunology, Tilburg, The Netherlands) and peroxidase-conjugated protein A (Sigma, St. Louis, Mo.). Peroxidase activity was revealed with the development kit from Bio-Rad S.A., Paris, France, containing 4-chloro-1-naphthol, according to the manufacturer's instructions. Enzyme-linked immunosorbent assay (ELISA) with *E. coli* and *Brucella* cells was performed as described previously (7, 11). *E. coli* and *Brucella* cells were adsorbed on the microtiter plates at a cell suspension A_{600} of 1.0. Binding of MAbs (hybridoma supernatants diluted one-third) was detected by using peroxidase-conjugated goat anti-mouse immunoglobulins (Bio-Rad). The substrate solution for detecting peroxidase activity was 4 mM H_2O_2 and 1 mM ABTS [2,2-azino-di(3-ethylbenzthiazolinesulfonic acid)] in 50 mM sodium citrate, pH 4.2. A_{414} values were recorded with an automatic ELISA reader (Bio-Tek EL 312; Packard Instruments, Rungis, France).

Immunoelectron microscopy. Whole-mount immunogold labeling was performed as described previously (7). Binding of MAbs (hybridoma supernatants diluted one-third) was detected by using sheep anti-mouse biotinylated immunoglobulins (Amersham) and gold-labeled streptavidin (15-nm-diameter particles) (Amersham). Grids were observed in a transmission electron microscope (Philips CM10).

Nucleotide sequence accession number. The DNA sequences of the *B. melitensis* 16M, *B. suis* 1330, *B. ovis* 63/290, *B. canis* RM6/66, and *B. neotomae* 5K33 *omp25* genes have been submitted to GenBank and assigned accession numbers U33003, U39397, U33004, U39358, and U39359, respectively.

RESULTS

***omp25* gene sequence diversity among *Brucella* species.** The *omp25* gene sequences of *B. melitensis* 16M, *B. suis* 1330, *B. canis* RM6/66, *B. ovis* 63/290, and *B. neotomae* 5K33 (reference strains) were determined and compared with that of *B. abortus* 544 (15) (Fig. 1). The *omp25* gene of *B. ovis* 63/290 consisted of a 603-bp open reading frame encoding 201 amino acids, and those of the other *Brucella* species consisted of a 639-bp open reading frame encoding 213 amino acids. The predicted molecular masses of Omp25 were 21,816 Da for *B. ovis* 63/290 and around 23,185 Da for the other *Brucella* species.

Besides a 36-bp deletion found in the *B. ovis* 63/290 *omp25* gene, few differences (not more than 12 single-nucleotide substitutions) were detected between the *omp25* genes of the different *Brucella* species, indicating that the *omp25* gene is highly conserved in the genus *Brucella* (Fig. 1). These nucleotide substitutions altered the predicted amino acid sequence for not more than five amino acids (Fig. 2).

The region of the 36-bp deletion found at the 3′ end of the *omp25* gene of *B. ovis* 63/290 contains two 8-bp direct repeats (5′-GCAACAAG-3′) in the other *Brucella* species (Fig. 1). One of these repeats remains in the *omp25* gene of *B. ovis* 63/290. Two 4-bp inverted repeats (5′-GAAC-3′) located close to these 8-bp repeats are also found in the *omp25* genes of the other *Brucella* species (Fig. 1). The predicted amino acid sequence downstream of the deletion was not altered compared with those of the other *Brucella* species.

Expression of the *omp25* gene in *E. coli* and *Brucella* strains. (i) Size variation of Omp25. The PCR-amplified *omp25* gene of each *Brucella* species was cloned in plasmid pCRII and expressed in *E. coli* INVαF′ (Table 1). Since plasmid instability was observed in *E. coli* INVαF′, the *omp25* genes of *B. melitensis* 16M and *B. ovis* 63/290 were further subcloned in the inducible-expression plasmid vector pUC19, which carries the *lacI* gene, and transformation was done in *E. coli* TG1 (Table 1). Expression of *E. coli*, under the control of the P_{lac} promoter, was tested without (for pCRII-derived plasmids) or with (for pUC19-derived plasmids) induction by IPTG.

Immunoblotting after SDS-PAGE of whole-cell lysates of *B. melitensis*, *B. ovis*, and *E. coli* expressing either the truncated *omp25* gene of *B. ovis* 63/290 or the entire *omp25* gene of the other *Brucella* species, revealed by anti-Omp25 MAb A59/05F01/C09, indicated that the deletion causes a decrease in the apparent molecular mass of Omp25 of *B. ovis* of about 1 kDa. Indeed, in *B. melitensis* and *B. ovis* strains, one major band was revealed by the anti-Omp25 MAb, at 25 and 24 kDa, respectively (Fig. 3). However, additional bands of 23 kDa and between 21 and 22 kDa were detected for *E. coli* expressing the entire or truncated *omp25* gene, respectively. The 23-kDa band was expressed only upon induction by IPTG in *E. coli*(pAC2533) expressing the *omp25* gene of *B. melitensis* 16M cloned in

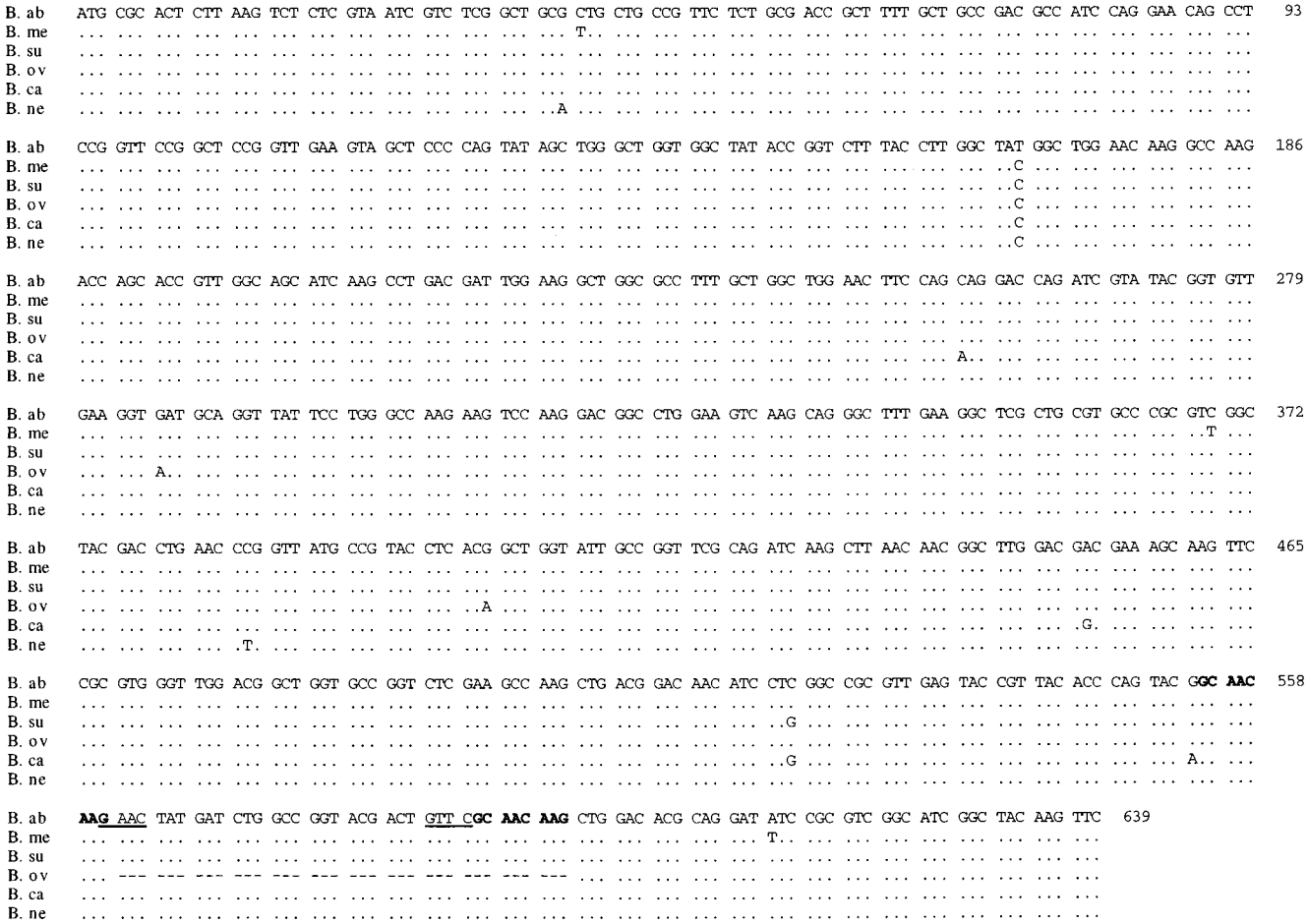

FIG. 1. Nucleotide sequences (open reading frames) of the *omp25* genes of *B. abortus* 544 (B. ab), *B. melitensis* 16M (B. me), *B. suis* 1330 (B. su), *B. ovis* 63/290 (B. ov), *B. canis* RM6/66 (B. ca), and *B. neotomae* 5K33 (B. ne). Differences are indicated by the one-letter code for the nucleotide. Identity is noted by a period. The 36-bp deleted segment of *B. ovis* 63/290 is indicated by dashes. The 8-bp direct repeats are indicated by boldface letters. The 4-bp inverted repeats are underlined.

plasmid pUC19. As shown in Fig. 3, the 23-kDa band was major, whereas the 21- to 22-kDa band of *E. coli*(pAC2505) or *E. coli*(pAC2535) expressing the *omp25* gene of *B. ovis* 63/290 was a minor band.

(ii) Antigenic shift of Omp25. Figure 4 shows binding activity in ELISA of anti-Omp25, -PG, -S-LPS, and -36-38 kDa OMP MAbs to sonicated and nonsonicated cells of *E. coli* (pAC2503) and *E. coli*(pAC2505), expressing, respectively, the *omp25* genes of *B. melitensis* 16M and *B. ovis* 63/290 cloned in plasmid pCRII. The anti-S-LPS and -36-38 kDa OMP MAbs were used as negative controls. The anti-PG MAb 3D6 was used to test the integrity of the cells applied to the plates. Indeed, this MAb bound only to *E. coli* cells, provided that they were disrupted by sonication (Fig. 4). Rough *B. melitensis* B115 and *B. ovis* Reo198 strains were used as controls for comparative purposes (Fig. 4B and C, respectively). Rough *Brucella* strains were chosen because we have previously demonstrated that O chain expressed on the surface of smooth *Brucella* strains greatly affected the binding of anti-OMP MAbs (5, 7, 8). The binding of anti-S-LPS MAb 12G12 to rough *B. melitensis* B115 cells is due to the presence of intracellularly expressed O chains (13).

A first observation was the binding of anti-Omp25 MAbs to nonsonicated cells of *E. coli* expressing either the entire *omp25* gene of *B. melitensis* 16M or the truncated *omp25* gene of *B. ovis* 63/290, thus indicating that both Omp25 proteins are exported to the *E. coli* outer membrane. On sonicated *E. coli* (pAC2503) or *E. coli*(pAC2505) cells, the binding of the anti-Omp25 MAbs was slightly increased. This could be due to the fact that the Omp25 epitopes are more accessible when cells are sonicated. The anti-Omp25 MAbs did not bind to control *E. coli* cells carrying plasmid pCRII without an insert (data not shown). The patterns of binding of the anti-Omp25 MAbs to *E. coli* cells expressing the entire *omp25* genes of the other *Brucella* species, such as *B. abortus* 544, *B. suis* 1330, *B. canis* RM6/66, and *B. neotomae* 5K33 (Table 1), were similar to that shown in Fig. 4 for *E. coli* expressing the *omp25* gene of *B. melitensis* 16M (data not shown). Another observation was that the patterns of binding of the anti-Omp25 MAbs to *E. coli* (pAC2503) and *E. coli*(pAC2505) cells were similar to those observed with *B. melitensis* B115 and *B. ovis* Reo198 cells, respectively. Thus, the accessibilities of the different Omp25 epitopes seemed to be the same irrespective of whether the protein was produced by *E. coli* or *Brucella* spp., suggesting that both the entire and truncated Omp25 proteins probably adopt similar topologies in the *E. coli* and *Brucella* outer membranes.

The most interesting observation was the antigenic shift caused by the short deletion in the *omp25* gene of *B. ovis*. Compared with results with *E. coli* and *Brucella* strains express-

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B. ab   MRTLKSLVIVSAALLPFSATAFAADAIQEQPPVPAPVEVAPQY   43
B. me   ........................................
B. su   ........................................
B. ov   ........................................
B. ca   ........................................
B. ne   ........................................

B. ab   SWAGGYTGLYLGYGWNKAKTSTVGSIKPDDWKAGAFAGWNFQQ   86
B. me   ........................................
B. su   ........................................
B. ov   ........................................
B. ca   .......................................K
B. ne   ........................................

B. ab   DQIVYGVEGDAGYSWAKKSKDGLEVKQGFEGSLRARVGYDLNP  129
B. me   ........................................
B. su   ........................................
B. ov   .........N..............................
B. ca   ........................................
B. ne   .......................................L

B. ab   VMPYLTAGIAGSQIKLNNGLDDESKFRVGWTAGAGLEAKLTDN  182
B. me   ........................................
B. su   ........................................
B. ov   ........................................
B. ca   ........................................
B. ne   ........................................

B. ab   ILGRVEYRYTQYGNKNYDLAGTTVRNKLDTQDIRVGIGYKF    213
B. me   ..............................F.........
B. su   ........................................
B. ov   ............-----------.................
B. ca   ...........S............................
B. ne   ........................................
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FIG. 2. Predicted amino acid sequences of the Omp25 proteins of *B. abortus* 544 (B. ab), *B. melitensis* 16M (B. me), *B. suis* 1330 (B. su), *B. ovis* 63/290 (B. ov), *B. canis* RM6/66 (B. ca), and *B. neotomae* 5K33 (B. ne). Differences are indicated by the one-letter code for the amino acid. Identity is noted by a period. The 12-amino-acid deleted segment of *B. ovis* 63/290 is indicated by dashes.

ing the entire Omp25, a number of anti-Omp25 MAbs showed a significant reduction in or the absence of binding to truncated Omp25 of *B. ovis*, while other anti-Omp25 MAbs showed increased binding to this Omp25 (Fig. 4). In particular, MAbs probably directed to discontinuous epitopes of the entire Omp25 (Table 3) showed a significant reduction of binding to Omp25 of *B. ovis*. In addition, these MAbs do not recognize the deleted segment of Omp25, since they did not react with *E. coli*(pAC2562) (Table 1) expressing the last third of entire Omp25 fused to β-galactosidase (data not shown), where the deleted segment is located. Only one anti-Omp25 MAb (MAb A59/01E11/D11) reacted in immunoblotting and ELISA with the fusion protein (data not shown). This MAb was previously shown to recognize an epitope located in the last 24 amino acids of Omp25 (15). Interestingly, in contrast, a number of anti-Omp25 MAbs showed increased binding to both *B. ovis* and *E. coli*(pAC2505) cells expressing the *omp25* gene of *B. ovis*. Among these were four immunoglobulin G3 (IgG3) MAbs (A76/08H09/A02, A76/09F04/B11, A76/12H09/A05, and A76/15H07/E07) which could possibly recognize the same epitope. As shown in Fig. 5, this was even more evident on *E. coli* (pAC2535) cells expressing the *omp25* gene of *B. ovis* 63/290 cloned in plasmid pUC19 and when expression was induced by IPTG. However, binding of the anti-PG MAb to nonsonicated *E. coli*(pAC2535) and also to *E. coli*(pAC2533) cells (Fig. 5) indicated that a proportion of the cells applied to the plates were not intact. This was confirmed by electron microscopy (not shown). Thus, overproduction of Omp25 upon induction by IPTG seemed to be toxic for the *E. coli* cells.

Together, these results suggested that the truncated Omp25 of *B. ovis* probably presents a conformation in the outer membrane different from that of the entire Omp25 proteins of the other *Brucella* species, rendering one or more epitopes more exposed or accessible to MAbs, while others are absent or less accessible.

(iii) Immunoelectron microscopic localization of Omp25. Electron microscopy and immunogold labeling with anti-Omp25 MAbs confirmed the surface exposure of both the entire and truncated Omp25 proteins expressed in *E. coli* (Fig. 6A and B). Immunogold labeling seemed to be as dense as on *B. melitensis* B115 cells (Fig. 6C). The latter cells however, are much smaller than *E. coli* cells. Negative control *E. coli*(pCRII) cells appeared to be almost unlabeled (Fig. 6D).

DISCUSSION

In the present study we analyzed the *omp25* gene sequence diversity among the six *Brucella* species. The *omp25* nucleotide sequence was highly conserved among the six *Brucella* species. The *Brucella* genus has been previously shown by DNA-DNA hybridization (36) to constitute a homogeneous group, and it was proposed that brucellae should be considered biotypes of a single species, *B. melitensis*. The *omp25* nucleotide sequence data strengthened this hypothesis. The 36-bp deletion found in the *omp25* gene of *B. ovis* is unique and distinguishes this species from all others. This conclusion was also drawn from the study of polymorphism of the genes (*omp*2a and *omp*2b) coding for the 36-kDa OMP of brucellae (10, 20). The occurrence of a larger number of insertion sequences in *B. ovis* than in the other *Brucella* species also makes this species phenotypically and genetically distant from the others (25, 28).

The region of the 36-bp deletion found in the *omp25* gene of *B. ovis* contains two 8-bp direct repeats in the other *Brucella* species. One of these direct repeats remains in the *omp25* gene of *B. ovis*. Close to these direct repeats, two 4-bp inverted repeats are found in the other *Brucella* species. Such observations were also made for the 702-bp deletion found in the erythritol catabolic gene of the *B. abortus* vaccine strain B19, which in this case occurred between two short direct repeats of 13 bp (31). A few inverted repeats have also been found inside the deleted region of the erythritol catabolic gene. These naturally occurring deletions have also been reported for other gram-negative bacteria like *E. coli* (1, 2) and for bacteriophages (2, 34). The deletions generally occur between two short direct repeats of 5 to 17 bp and may also occur at sites that flank small repeated sequences in an inverted orientation (2, 23). The latter repeats, called quasipalindromes, could form transient hairpin structures, bringing the deletion endpoints into close proximity at the moment of deletion formation. In the case of *B. ovis*, two short, 4-bp inverted repeats (5′-GAAC-3′) were found within the deleted segment and close to the 8-bp direct repeats and could therefore be responsible for

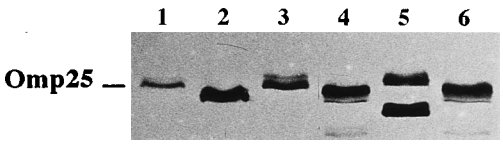

FIG. 3. Expression and size variation of Omp25 in *B. melitensis*, *B. ovis*, and *E. coli* cells expressing the *omp25* gene of *B. melitensis* or *B. ovis*. Immunoblotting after SDS-PAGE of whole-cell lysates of *B. melitensis* B115 (lane 1), *B. ovis* Reo198 (lane 2), *E. coli*(pAC2503) (lane 3), *E. coli*(pAC2505) (lane 4), *E. coli* (pAC2533) (lane 5), and *E. coli*(pAC2535) (lane 6), revealed by anti-Omp25 MAb A59/05F01/C09, is shown. *E. coli*(pAC2503) and *E. coli*(pAC2505) express, respectively, the *omp25* genes of *B. melitensis* 16M and *B. ovis* 63/290 cloned in plasmid pCRII. *E. coli*(pAC2533) and *E. coli*(pAC2535) express, respectively, the *omp25* genes of *B. melitensis* 16M and *B. ovis* 63/290 cloned in plasmid pUC19. Expression from the pUC19-derived plasmids was induced by IPTG as described in Materials and Methods.

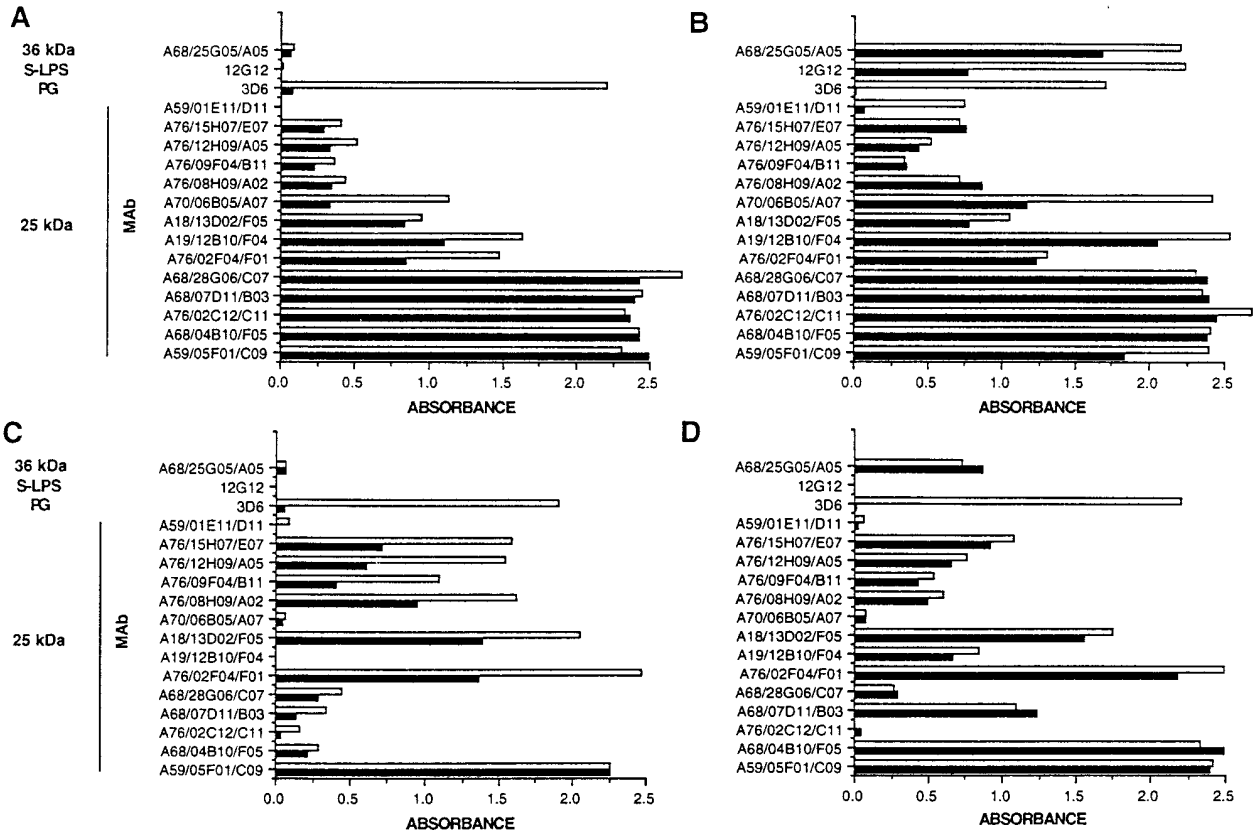

FIG. 4. Expression and antigenic shift of Omp25. Binding, measured by ELISA, of anti-Omp25 (25 kDa), -PG (PG), -36-38 kDa OMP (36 kDa), and -S-LPS MAbs (absorbance values of threefold-diluted hybridoma supernatants) to nonsonicated (■) and sonicated (□) cells of *E. coli*(pAC2503) (A), *B. melitensis* B115 (B), *E. coli*(pAC2505) (C), and *B. ovis* Reo198 (D) is shown. *E. coli*(pAC2503) and *E. coli*(pAC2505) express, respectively, the *omp25* genes of *B. melitensis* 16M and *B. ovis* 63/290 cloned in plasmid pCRII.

hairpin formation and consequently could contribute to deletion formation. The deletion should then arise during DNA synthesis by a mechanism called slipped mispairing (1, 2, 34). The question remains, however, why the 36-bp deletion in the *omp25* gene is specific for *B. ovis* strains (10), since the direct and inverted repeats occur in the *omp25* genes of all other *Brucella* species. Cellular components specific to *B. ovis* might participate in deletion formation. This hypothesis could be studied by constructing a *B. ovis omp25* mutant and complementing it with an entire *omp25* gene. If deletions occurred in the complementing gene, the presence and identification of *B. ovis*-specific cellular components could be further investigated.

Besides the 36-bp deletion found in the *omp25* gene of *B. ovis*, the few single nucleotide substitutions altered the Omp25 predicted amino acid sequence for not more than five amino acids. The 36-bp deletion did not alter the predicted amino acid sequence downstream from the deletion. Thus, the antigenic shift observed for Omp25 of *B. ovis* could essentially be attributed to the deletion found in the *omp25* gene. The deletion caused decreased or loss of binding of a set of MAbs which are probably directed to well-surface-exposed discontinuous epitopes of the entire Omp25 proteins of the other *Brucella* species. Thus, Omp25 of *B. ovis* probably presents a different conformation in the outer membrane, which could possibly expose specific discontinuous epitopes. All anti-Omp25 MAbs described until now were produced by immunization with fractions or bacteria containing the entire Omp25 (7). Immunization with *B. ovis* cells should be useful to produce MAbs specific to discontinuous epitopes of the truncated Omp25 of *B. ovis*. Interestingly, a number of MAbs showed increased binding to *B. ovis* cells or *E. coli* cells expressing Omp25 of *B. ovis*. One or more Omp25 epitopes, which are probably buried

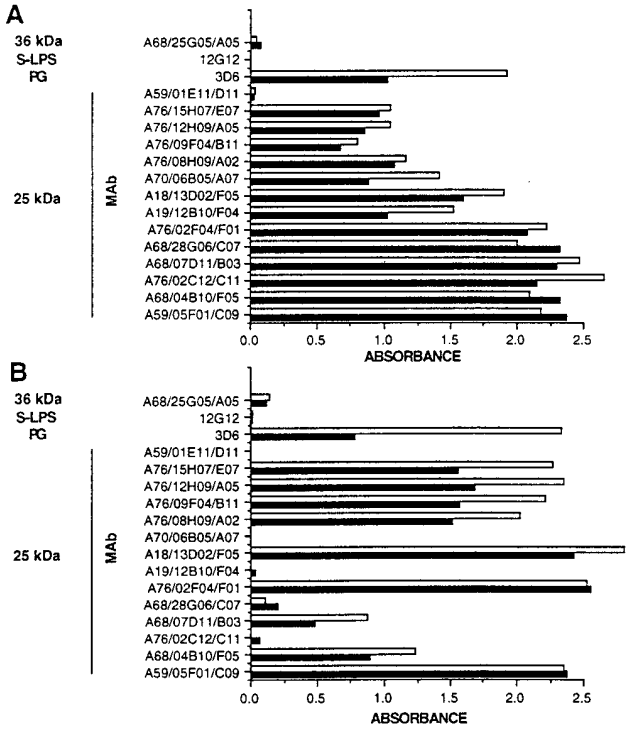

FIG. 5. Binding, measured by ELISA, of anti-Omp25 (25 kDa), -PG (PG), -36-38 kDa OMP (36 kDa), and -S-LPS MAbs (absorbance values of threefold-diluted hybridoma supernatants) to nonsonicated (■) and sonicated (□) cells of *E. coli*(pAC2533) (A) and *E. coli*(pAC2535) (B). *E. coli*(pAC2533) and *E. coli* (pAC2535) express, respectively, the *omp25* genes of *B. melitensis* 16M and *B. ovis* 63/290 cloned in plasmid pUC19. Expression from the pUC19-derived plasmids was induced by IPTG as described in Materials and Methods.

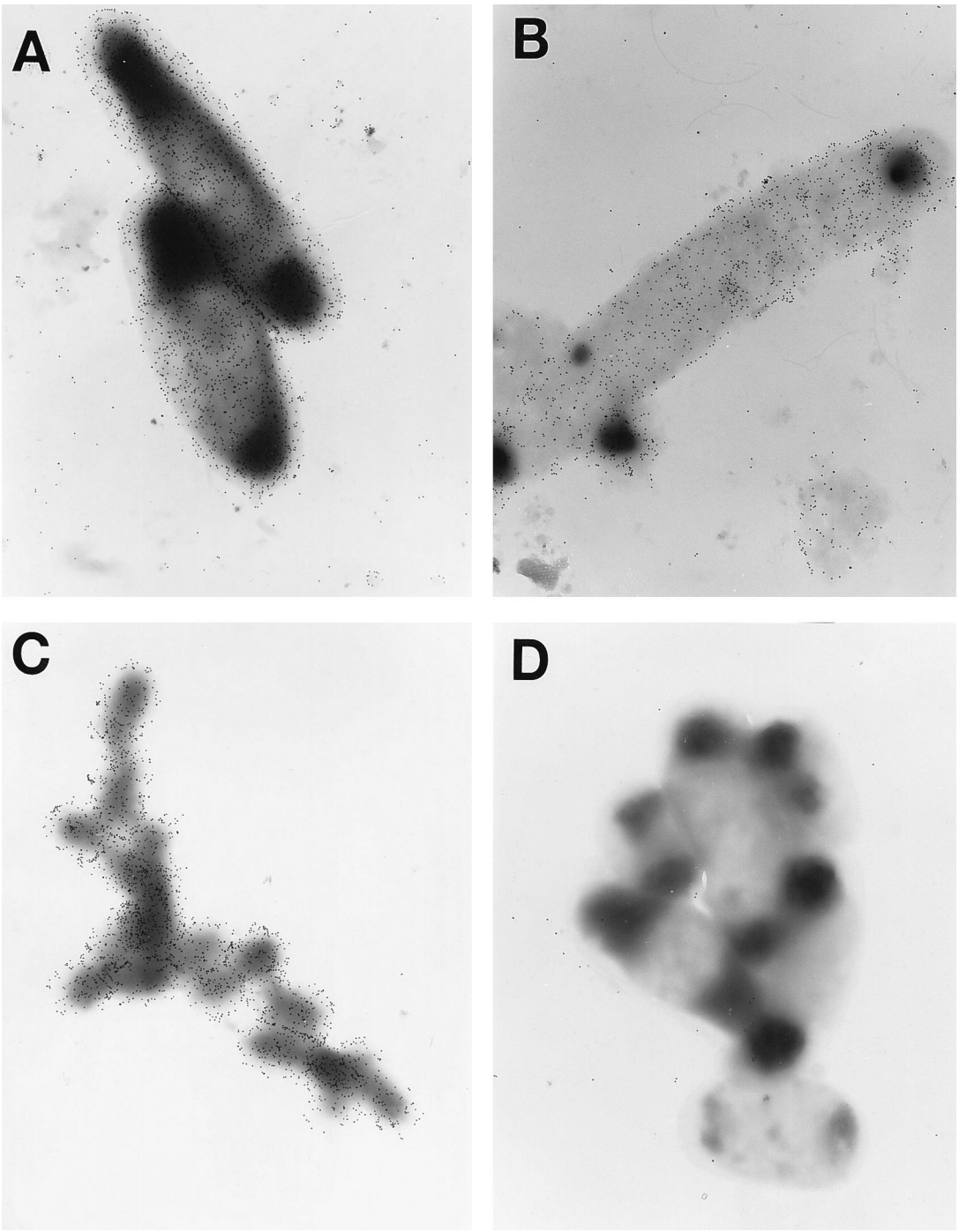

FIG. 6. Electron microscopy and immunogold labeling with anti-Omp25 MAbs A68/04B10/F05 (A, C, and D) and A59/05F01/C09 (B) of whole cells of *E. coli* (pAC2503) (A), *E. coli*(pAC2505) (B), *B. melitensis* B115 (C), and negative control *E. coli*(pCRII) (D). *E. coli*(pAC2503) and *E. coli*(pAC2505) express, respectively, the *omp25* genes of *B. melitensis* 16M and *B. ovis* 63/290 cloned in plasmid pCRII. Magnification, ×14,900.

deeper in the outer membrane of *Brucella* species expressing the entire *omp25* gene, would therefore be more exposed or accessible on the surface of *B. ovis*. In particular, IgG3 anti-Omp25 MAbs showed such improved binding. Omp25 was the only OMP among seven for which we obtained MAbs of the IgG3 isotype (unpublished results). IgG3 MAbs are reported to be preferentially directed against polysaccharide epitopes (24). The major 25-kDa OMP was previously described as possibly being glycosylated, which was demonstrated by its polysaccharide-like staining in SDS-PAGE (19) and by the fact that the IgG3 MAbs were found to recognize epitopes sensitive to periodate oxidation and resistant to pepsin action (12). However, since the epitopes recognized by the IgG3 anti-Omp25 MAbs are well expressed in *E. coli*, the latter hypothesis seems now to be doubtful. Nevertheless, the identification of the epitopes recognized by the IgG3 anti-Omp25 MAbs by

using synthetic peptides will probably give interesting information on the nature of epitopes recognized by the IgG3 antibody. Epitope mapping with the MAbs will also determine the amino acid loops of Omp25 that are surface exposed.

Protective immunity to *B. ovis* infection has been shown to be predominantly antibody mediated (26). A fraction of *B. ovis* called hot-saline extract was shown to be protective against a *B. ovis* challenge in mice either by active immunization or by passive transfer of immune serum. This fraction was shown to be rich in group 3 OMPs (22, 29) or the 25-kDa OMP, as shown by the use of the anti-Omp25 MAbs (unpublished results). Thus, *B. ovis* Omp25 could be an important protective antigen. Therefore, *E. coli* expressing Omp25 of *B. ovis* could be an interesting recombinant vaccine candidate, since Omp25 is highly abundant at the surface of *E. coli*, where it probably retains its topology, including presentation of the same discontinuous epitopes as those present in the *B. ovis* outer membrane. In addition, hot-saline extract afforded protection against challenge infection with *B. ovis* that was as good as or even better than that provided by attenuated *B. melitensis* vaccine strain Rev.1, the vaccine strain currently used for ovine brucellosis (26). The vaccine strain Rev.1 produces an entire *omp25* gene (10). The antigenic shift observed for *B. ovis* Omp25 should therefore be considered in the development of vaccines against *B. ovis* infection.

Antigenic variations of OMPs and their mechanisms, reviewed by Brunham et al. (6) and Seifert and So (33), have been studied for other gram-negative pathogens, such as *Neisseria*, *Borrelia*, and *Chlamydia* spp. The mechanisms at the gene level included recombination, insertion-deletion, and point mutation. Antigenic variation has been proposed as a mechanism to escape from host immune response (reviewed by Brunham et al. [6] and Seifert and So [33]). The origin of *B. ovis* is still unknown, as is the mechanism of its specific pathogenesis and tropism compared with those of the other *Brucella* species. *B. ovis* could possibly represent an escape variant of an ancestor *Brucella* organism. The antigenic shift of Omp25, its particular conformation in the *B. ovis* outer membrane, and other specific features of the outer membrane of *B. ovis*, such as its 36-kDa porin protein (10, 20), might contribute to the specific pathogenesis of *B. ovis* and should be further investigated by studying mutant strains lacking these OMPs.

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