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Molecular Cloning, Nucleotide Sequence, and Occurrence of a 16.5-Kilodalton Outer Membrane Protein of *Brucella abortus* with Similarity to PAL Lipoproteins

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Recombinant λgt11 phages were selected by screening a genomic library of *Brucella abortus* DNA with monoclonal antibodies specific for a 16.5-kDa *Brucella* outer membrane protein (Omp16). The corresponding gene, named *pal*, was subcloned on a 0.7-kb *Alu*I fragment. Immunoblotting confirmed the expression of a recombinant Omp16 in the transformants. DNA sequence analysis revealed an open reading frame of 168 codons. The deduced amino acid sequence agrees with an internal peptide sequence of native Omp16 and contains a potential lipoprotein signal peptide cleavage site, giving rise to a predicted mature protein of 144 amino acids. The predicted sequence of Omp16 also shows a remarkable degree of similarity to the sequences of three peptidoglycan-associated bacterial lipoproteins. In immunoblotting with a monoclonal antibody specific for Omp16, we demonstrated that Omp16 was expressed in the 34 *Brucella* strains tested, representing all six species and known biovars.

Brucella spp. are facultative, intracellular gram-negative bacteria and the etiological agents of brucellosis in animals and humans. Serological tests are the major tools for brucellosis diagnosis and mainly detect antilipopolysaccharide (anti-LPS) antibodies. This dominant antigen is common to virulent and vaccinal strains. Therefore, the distinction between infection and vaccination is made difficult. Another major problem encountered in bovine brucellosis diagnosis is the increasing occurrence of false-positive reactions due to serological cross-reactivity between *Brucella* spp. and bacterial species which also have smooth LPS structural components (1, 2, 17, 37). Brucellosis research is therefore focused on identification of antigens that are potentially useful for diagnosis as well as for vaccinal prophylaxis and specific for the genus *Brucella*.

In the mouse model, passive protection with monoclonal antibodies (MAbs) directed against smooth-LPS epitopes (7, 31, 32, 39, 42) and protection experiments with the O chain coupled to bovine serum albumin (BSA) (27) demonstrated the importance of anti-smooth LPS antibodies early after the infection (3 weeks). However, the number of *Brucella* colonies in the spleens collected later (8 weeks postchallenge) indicated that the reduction observed in the first weeks was not maintained. The smooth-LPS-specific immunity is a prerequisite but not sufficient for inducing long-term clearance of *Brucella* organisms.

A role for other surface-exposed antigens, the outer membrane proteins (Omps), in immune protection was proposed. Three major and four minor Omps common to *Brucella abortus* and *Brucella melitensis* have been identified by using MAbs; among them is a 16.5-kDa protein (5). Passive immunization with MAbs raised against these seven different Omps did not improve long-term protection in mice (6, 26). Since spleen cells, particularly T cells, were shown to transfer protection to mice, Omps were proposed to play a role in cellular immune protection (44).

In addition, immunoblot analysis and competitive enzyme-linked immunosorbent assay (ELISA) indicated the usefulness of these Omps as diagnostic antigens (8). The availability of MAbs against Omps allowed us to clone the genes encoding these proteins. In this paper, we report the molecular cloning and sequencing of the *Brucella* gene encoding Omp16, named *pal*, as well as its expression in *Escherichia coli*. The *Brucella* species and representative biovars as well as bacteria known to cross-react in brucellosis assays (e.g., *Yersinia enterocolitica*) were analyzed by Western blotting (immunoblotting) to verify the presence of this new Omp.

MATERIALS AND METHODS

Bacterial strains. The *Y. enterocolitica* O:9 strain 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 strains were from the Institut National de Recherche Vétérinaire, Brussels, Belgium. Cell extracts were prepared by sonication as described previously (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 d'Immunologie, Nouzilly, France. Briefly, cell lysis was achieved by boiling in the presence of 1% sodium dodecyl sulfate (SDS).

MAbs and antiserum. Anti-16.5-kDa protein (anti-16.5) MAbs, defined as reacting with a 16.5-kDa band on immunoblots of *B. melitensis* B115 cell extracts, were produced as described previously (5, 6). Supernatants of hybridoma cultures of the anti-16.5 MAbs A68/04G01/C06 (immunoglobulin G2a [IgG2a]) and A66/04E03/B07 (IgG2b) were used. Rabbit anti-mouse immunoglobulin antiserum was produced as de-

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scribed previously (5). Anti-*E. coli* antibodies were removed from the rabbit anti-mouse immunoglobulin serum by incubating the antiserum for 16 h at 4°C with 1 volume of whole-cell *E. coli* Y1090 extracts (with a bacterial optical density at 600 nm of 20). The absorbed antiserum was centrifuged to remove bacterial debris prior to being used. Anti-β-galactosidase goat serum was produced by injection of commercial β-galactosidase (Boehringer, Mannheim, Germany) at 100 μg in complete Freund's adjuvant the first time and in incomplete Freund's adjuvant two times, each at 15-day intervals. The goat was bled the week after the last injection.

Construction of a *B. abortus* genomic library in λgt11. This library was constructed in collaboration with J.-N. Octave, Laboratoire de Neurochimie, Université Catholique de Louvain, Brussels, Belgium. *B. abortus* 544 genomic DNA was extracted by J.-M. Verger and M. Grayon. The genomic DNA was subjected to partial digestion with *Sau*3AI to obtain sizes ranging from 2 to 7 kb. Restriction of the DNA was monitored by agarose gel electrophoresis. DNA fragments were labeled with [α-^{32}P]dATP as the ends were made flush with Klenow enzyme. Endogenous *Eco*RI sites were protected from *Eco*RI digestion by using *Eco*RI methylase. After ligation to *Eco*RI linkers followed by *Eco*RI digestion, the reaction mixture was fractionated by size exclusion chromatography on Biogel A50 (Bio-Rad Laboratories, Richmond, Calif.). Eluted fractions were analyzed by agarose gel electrophoresis and autoradiography. DNA fragments (2 to 7 kb) were then ligated into dephosphorylated λgt11 arms (Promega Corp., Madison, Wis.) and packaged by using the Gigapack system (Stratagene, La Jolla, Calif.). The titer of the library was evaluated after infection of *E. coli* Y1090, and about 60% of the phages contained inserts.

Immunoscreening of the λgt11 gene bank. For screening of the library, approximately 3×10^4 PFU were plated per 150-mm plate on *E. coli* Y1090 (25). Phages were allowed to grow at 42°C for approximately 4 h; at that time, they were overlaid with a 0.45-μm nitrocellulose disk previously saturated with 10 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The plates were incubated at 37°C overnight. The filters were removed, washed briefly in TBS (10 mM Tris-HCl [pH 7.5], 150 mM NaCl), and then saturated for 1 h in TBS containing 3% BSA. The filters were incubated for 3 h at room temperature with a mixture of culture supernatants of the two anti-16.5 MAbs (A68/04G01/C06 and A66/04E03/B07), each at a 1:5 dilution, and then washed three times for 10 min each in TBST (TBS containing 0.05% Tween 20). The filters were incubated at room temperature for 1 h with a 1:100 dilution of rabbit anti-mouse immunoglobulin antiserum in TBST containing 1% BSA (TBST–1% BSA) and washed three times again in TBST. To identify immunoreactive plaques, the filters were incubated for 1 h with a 1:2,000 dilution of *Staphylococcus aureus* protein A-peroxidase conjugate (5) in TBST–1% BSA. After two washes in TBST and two in TBS, the signals were revealed by incubation at room temperature in TBS containing 0.06% (wt/vol) 4-chloro-1-naphthol (Bio-Rad) and 5 mM H_2O_2. The reaction was stopped by washing in distilled water. Positive plaques were picked, eluted into phage suspension medium (20 mM Tris-HCl [pH 7.5], 100 mM NaCl, 10 mM $MgSO_4$, 2% [wt/vol] gelatin), replated, and screened until all plaques tested positive.

SDS-PAGE and immunoblotting. *E. coli* recombinant clones were grown in L broth supplemented when indicated with 1 mM IPTG or 2% glucose. Whole-cell extracts were prepared by boiling the harvested *E. coli* cells for 3 min in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer, containing 60 mM Tris-HCl (pH 6.8), 2% SDS, 2% β-mercaptoethanol, 10% glycerol, and 0.001% bromophenol blue. These extracts were subjected to SDS-PAGE through 3% stacking and 15% separating slab gels as described by Laemmli (30). Proteins were then either stained with Coomassie brilliant blue or transferred electrophoretically to 0.2-μm-pore-size nitrocellulose for immunoblotting (the library immunoscreening protocol is used from the saturation step on) (47).

Lysogen production and preparation of whole-cell extracts. To determine whether the putative recombinant phages produced β-galactosidase fusion proteins, lysogens of each of these phages were generated in *E. coli* Y1089 as described by Huynh et al. (25). Lysogenic strains were grown at 32°C to an optical density at 600 nm of 0.5 and then shifted to 42°C for 20 min. IPTG was then added to a final concentration of 10 mM, and the cultures were incubated at 37°C for 1 h. Cells were collected by centrifugation and resuspended in SDS-PAGE sample buffer, and whole-cell extracts were analyzed by SDS-PAGE and immunoblotting with anti-16.5 MAb A68/04G01/C06 and anti-β-galactosidase serum used separately.

Amino acid sequencing. The cell wall fraction of *B. melitensis* B115 was separated by SDS-PAGE on a 16% gel. The gel fragment containing the region where the 16.5-kDa Omp is localized was used for microsequencing of the N-terminal end of internal peptides obtained by formic acid hydrolysis as previously described (48).

Recombinant DNA techniques. The plate lysate method (46) was used for large-scale preparation of recombinant phage particles, and isolation of phage DNA was done with the LambdaSorb phage adsorbent (Promega) 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) were used as described by the manufacturer. Ligation of insert DNA into phagemids pTZ18-R and pTZ19-R (Pharmacia P-L Biochemicals, Uppsala, Sweden) and pBluescriptSK (Stratagene) and subsequent transformation into *E. coli* XL1-Blue (Stratagene) cells were performed by standard techniques (46). DNA fragments were analyzed by electrophoresis on 0.8 to 1% agarose gels. DNA fragments were extracted from agarose with the GeneClean II kit (Bio 101 Inc., La Jolla, Calif.).

Oligonucleotides. M13 reverse sequencing primer was purchased from Pharmacia, and M13 sequencing primer (−40) was from United States Biochemical Corp. (Cleveland, Ohio). All the other oligonucleotides used in sequencing were synthesized at Innogenetics.

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) or a T7 sequencing kit (Pharmacia P-L Biochemicals). Single-stranded templates prepared from recombinant phagemids (following the Qiagen M13 protocol) or 7-deaza-dGTP-containing reaction mixtures (Boehringer) were used to avoid compression.

DNA and protein sequence analysis. DNA sequence data obtained from sequencing gels were compiled and analyzed by the DNA Strider 1.2 program (35). 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.). The following nucleic acid and protein databases were used for sequence homology searches: GenBank (modified) (release 78.0 [updated daily]), EMBL (release 36.0 [updated daily]), and SWISS-PROT (release 27.0 [updated weekly]). The bacterial lipoproteins peptidoglycan-associated

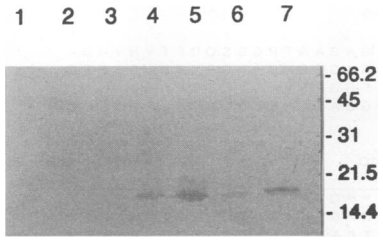

FIG. 1. Immunoblot analysis of native and recombinant *Brucella* Omp16. Whole-cell lysates of *B. abortus* 544 (lane 7) and of *E. coli* XL1-Blue harboring plasmid pSK (lane 1), p161 (lane 6), p162 (lane 5), p163 (grown in the presence of 1 mM IPTG [lane 4] or in the presence of 2% glucose [lane 3]), or p164 (lane 2) were immunoblotted with MAb A68/04G01/C06. Molecular mass standards (in kilodaltons) are indicated on the right.

lipoprotein (PAL) from *E. coli* K-12 (3), PAL from *Haemophilus influenzae* (11), and PplA from *Legionella pneumophila* (34) and the *Brucella* Omp16 sequences were analyzed with the software Match-Box for the simultaneous alignment of several protein sequences (13, 14).

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 number L27996.

RESULTS

Cloning of *pal* gene. A *B. abortus* 544 genomic library constructed in λgt11 was screened with a mixture of two MAbs raised against the 16.5-kDa Omp (Omp16). Initially, six plaques were found to be positive. To determine whether the recombinant bacteriophages expressed a *lacZ-B. abortus pal* gene fusion, *E. coli* Y1089 lysogens of these phages were generated. In whole-cell extracts derived from IPTG-induced and noninduced lysogens, no β-galactosidase fusion protein could be detected. Independently of the addition of IPTG, a specific band recognized by the mixture of anti-16.5 MAbs with an apparent molecular mass identical to that of the native Omp16 present in *B. abortus* extracts appeared (data not shown), suggesting that the entire *pal* gene was present on the phage insert and fully expressed in the lysogens.

DNA isolated from two of these recombinant phages was subsequently cleaved with *Eco*RI, generating inserts of 1.8 and 2.1 kb, respectively. These inserts were cloned into the *Eco*RI site of vector pTZ19-R, resulting in constructs p161 and p162, respectively. Both constructs allowed production of a 16.5-kDa protein that reacted with the two anti-16.5 MAbs on Western blots (Fig. 1). This result confirms that each of these inserts contains the entire *pal* gene. Since synthesis of Omp16 could be detected independently of the insert cloning orientation in pTZ19-R (data not shown), the *Brucella pal* gene seems to be transcribed from its own promoter.

The restriction map of the inserts showed that they largely overlapped (Fig. 2). To localize the *pal* gene more precisely, the 0.75-kb *Eco*RI-*Sph*I and the 0.85-kb *Sph*I-*Sph*I fragments were subcloned into the vector pTZ18-R. By immunoblotting, these two subclones showed no expression of Omp16, suggesting that the *Sph*I recognition site was located within the *pal* gene. Sequencing was therefore performed upstream and downstream from this *Sph*I site.

Sequence analysis of the *pal* gene. The nucleotide sequence and deduced amino acid sequence of the *pal* gene and the corresponding Omp16 protein of *B. abortus* are presented in

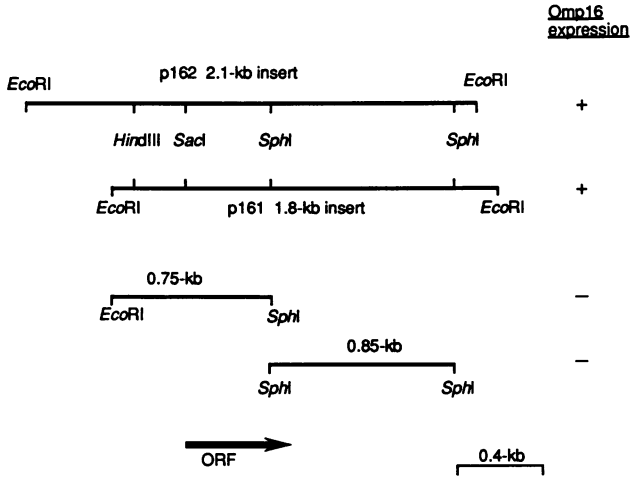

FIG. 2. Restriction endonuclease map of the cloned 1.8- and 2.1-kb DNA fragments of *B. abortus*. Subclones were constructed as shown. Expression of Omp16, detected by immunoblotting, is indicated to the right of each clone. The thick arrow indicates the position of the ORF.

Fig. 3. In agreement with the high G+C content of *Brucella* DNA (57 to 59%) (11), the 845-bp sequence determined contains 57.4% guanosine and cytosine. An open reading frame (ORF) of 507 nucleotides crossing the *Sph*I site (positions 162 to 668) was identified (Fig. 3). There is a potential Shine-Dalgarno ribosome-binding site (TAAGGAG) from

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   1  CGGGAGAATTGCGGTCGGCGCCCCGGCTTGCAGATGAGGTTTGCATCGGGGCATATATCT
  61  GGTCGCAAGAACGGTTAACTATAATTTTTGAGCCTGTTTTAACCTCAATATGATTATCGG

   1                                                  M  R  R  I  Q  S  I
 121  ATGGTTACGCAGGAACATCAAATCTTAAGGAGCTCTGGCCCATGCGCCGTATCCAGTCGA
                                    AluI
   8   A  R  S  P  I  A  I  A  L  F  M  S  L  A  V  A  G  C  A  S
 181  TTGCACGTAGCCCGATCGCTATTGCGCTTTTCATGTCGCTCGCCGTTGCCGGCTGTGCGT

  28   K  K  N  L  P  N  N  A  G  D  L  G  L  G  A  G  A  A  T  P
 241  CAAAGAAGAACCTTCCGAATAATGCCGGTGATCTGGGTCTCGGTGCAGGCGCTGCAACGC

  48   G  S  S  Q  D  F  T  V  N  V  G  D  R  I  F  F  D  L  D  S
 301  CGGGCTCCTCGCAGGACTTCACCGTTAATGTCGGCGACCGCATCTTCTTCGATCTCGATT

  68   S  L  I  R  A  D  A  Q  Q  T  L  S  K  Q  A  Q  W  L  Q  R
 361  CGTCGCTGATCCGCGCCGATGCGCAGCAGACGCTTTCCAAGCAGGCCCAGTGGTTGCAGC

  88   Y  P  Q  Y  S  I  T  I  E  G  H  A  D  E  R  G  T  R  E  Y
 421  GTTATCCGCAGTATTCGATCACGATCGAAGGCCATGCCGACGAGCGCGGCACGCGTGAGT

 108   N  L  A  L  G  Q  R  R  A  A  A  T  R  D  F  L  A  S  R  G
 481  ACAACCTCGCCCTTGGCCAGCGCCGTGCTGCCGCCACCCGCGACTTCCTCGCTTCGCGCG

 128   V  P  T  N  R  M  R  T  I  S  Y  G  N  E  R  P  V  A  V  C
 541  GTGTGCCGACCAACCGCATGCGCACCATTTCCTACGGTAATGAGCGCCCGGTTGCCGTCT
                    SphI
 148   D  A  D  T  C  W  S  Q  N  R  R  A  V  T  V  L  N  G  A  G
 601  GCGATGCCGACACATGCTGGTCGCAGAACCGTCGCGCCGTCACCGTTCTCAACGGGGCCG

 168   R  *
 661  GACGGTAAAACCGGCAACGTCCCGAGGGTCGGTCGGCTATCGGGTAAACCGGCGATAACA

 721  CAACAGTGTTACGGAAAAAGCGGCCTTCAGGCCGTTTTTTTCGTCATTGTTGCTCAAGAT
                <<<<< <<<<<    >>>>>>>>>>
 781  ACAGGCTTTTGACAAACTTTAGCCGAAGTCCCCCATGTCATAGAGGATGGTATGGCGAAG
                                                              AluI
 841  CTCTT  845
```

FIG. 3. Nucleotide sequence and deduced amino acid sequence of the *pal* gene of *B. abortus* and of the corresponding protein. *Sph*I and *Alu*I restriction sites are indicated. The putative ribosome-binding site is underlined twice. The possible initiator methionine is underlined, as is the dyad symmetry of the putative terminator stem-loop structure (<<<>>>). The asterisk denotes the termination codon. The putative signal peptide is in italics. The proposed site of lipidation appears in boldface and is doubly underlined. The sequence of the internal peptide is underlined.

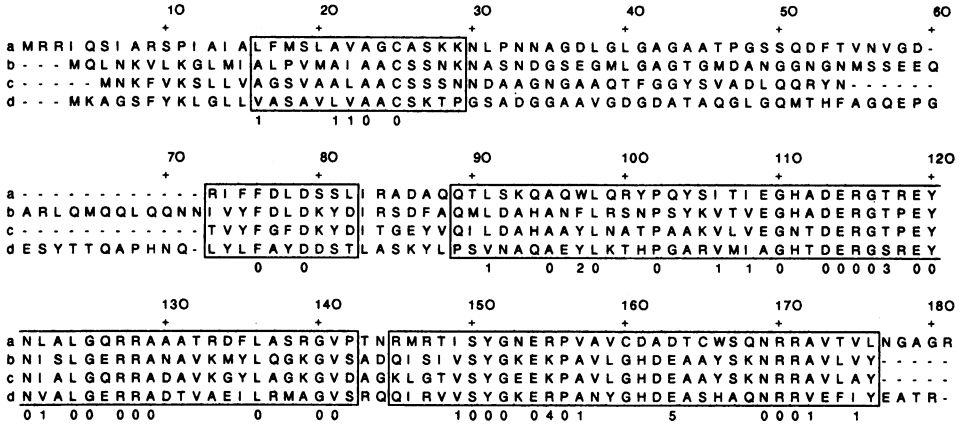

FIG. 4. Simultaneous multiple alignment of protein sequences from (a) *B. abortus* Omp16, (b) *E. coli* PAL, (c) *H. influenzae* PAL, and (d) *L. pneumophila* PplA. Boxes outline matching regions among the four sequences. Amino acids are numbered above the sequence. Residue identities (0) or similarities (1, A, V, L, I, P; 2, W, Y, F; 3, S, T; 4, R, K, H; 5, D, E) between the four sequences are indicated under the alignment.

−16 to −10 (bases 146 to 152), and a region of dyad symmetry (bases 735 to 759) was also identified 66 bases downstream from the termination codon. Such GC-rich regions capable of forming a stable hairpin upstream of a poly(T) stretch have already been found beyond other *Brucella* coding sequences (15, 33, 36) and were suggested to function as rho-independent transcription terminators. When a poly(A) stretch precedes the GC dyad symmetry, this site can serve (at least in *E. coli*) as a termination site for two genes, the upstream gene and a downstream gene located on the other strand (43). No data are available concerning the gene located downstream of the *pal* gene, since this region has not been sequenced.

The putative ORF was subcloned in both orientations as an *Alu*I-*Alu*I fragment spanning bases 151 to 839 into the *Sma*I site of pBluescriptSK− (Fig. 3). This subcloning deleted the *pal* gene of its putative promoter and Shine-Dalgarno sequences. The resulting plasmids presented the Omp16 ORF either on the same DNA strand as the β-galactosidase α-peptide ORF (p163) or on the opposite strand (p164). Expression assays showed that synthesis of *Brucella* Omp16 by *E. coli* cells transformed with p163 is induced by IPTG and repressed by 2% glucose (Fig. 1). In contrast, we were not able to detect expression of *Brucella* Omp16 from p164 *E. coli* transformants. These results indicate that in p163, *Brucella pal* gene transcription is under the control of the *lac* promoter.

Predicted Omp16 sequence analysis. The ORF encodes a putative polypeptide of 168 amino acids, with a calculated molecular mass of 18,221 Da. The deduced N-terminal amino acid sequence of Omp16 exhibits features of a signal peptide (45). Positively charged residues are present at positions 2, 3, and 5 and are followed by a core of hydrophobic amino acids. Moreover, residues 22 to 25 (Val-Ala-Gly-Cys) show similarity to the consensus sequence of bacterial lipoprotein precursors, Cys being the proposed site of lipidation [Leu-(Ala, Ser)-(Gly, Ala)-Cys from −3 to +1 accounting for about 75% of the cases (23)]. The predicted *B. abortus* Omp16 sequence has a Val at position −3, which is favored in 8% of the cases. Ala is present at +2 (favored in 12% of the cases), and Ser is present at +3 (favored in 38%). This consensus sequence defines a signal peptidase II cleavage site between residues Gly and Cys; cleavage would yield a polypeptide of 144 amino acids with a predicted molecular mass of 15,667 Da. The theoretical size of the cleaved, fully acylated (mature) Omp16 lipoprotein would be 16.4 kDa (assuming that acylation results in covalent linkage with 3 palmitic acid molecules). This is consistent with the molecular mass of the native Omp16 estimated by SDS-PAGE followed by Western blotting.

The lipoprotein precursors are predicted to have a β-turn secondary structure immediately following the cleavage site at the +2 or +3 position (28). Hydrophilic residues from positions +3 to +6 could be part of a β-turn; however, the highest propensity to form a β-turn secondary structure, as predicted by the Chou-Fasman algorithms (4), is obtained for the Pro-Asn pair that follows these residues.

Protein sequence analysis was performed to further validate the identified ORF. Attempts to obtain the N-terminal protein sequence were unsuccessful, probably because of chemical blocking of the N-terminal end. Alternatively, the sequence of an internal peptide resulting from acid hydrolysis was determined; this sequence (Phe-Thr-Val-Asn-Val-Gly) perfectly matches amino acids 53 to 58 of the precursor Omp16 protein predicted by the nucleotide sequence (Fig. 3).

Similarities between *Brucella* Omp16 and other bacterial lipoproteins. Similarities between the deduced Omp16 amino acid sequence and the sequences in the databases were searched for with the algorithms FastA and TFastA (41). Omp16 shows significant similarity (optimized score, >200) to the peptidoglycan-associated lipoproteins PAL of *E. coli* K-12 (3), PAL of *H. influenzae* (10), and PplA of *L. pneumophila* (34). To investigate the extent of conserved regions between Omp16 and these three lipoproteins, the four proteins were aligned by using the package Match-Box (14). Boxes indicate the regions of similarity between the four sequences (Fig. 4). The first box encompassed the tetrapeptide defining the site of cleavage and maturation of these lipoproteins. Amino acid identities between the four sequences are focused mainly in three regions: residues 113 to 129, 149 to 157, and 169 to 172, as numbered in Fig. 4. However, no function can be assigned to these segments.

Expression of Omp16 in the genus *Brucella*. Thirty-four *Brucella* strains, including all six of the known species and also all reported biovars, were examined for expression of Omp16 (Table 1). SDS-PAGE and Western blotting with anti-16.5 MAb A68/04G01/C06 of whole-cell preparations from each of these strains were performed. This MAb revealed a 16.5-kDa band in all *Brucella* strains tested. These data indicate that the apparent Omp16 size is identical among strains (within the experimental accuracy of SDS-PAGE). In contrast, in extracts

TABLE 1. *Brucella* species, biovars, and strains and other species tested

Species	Biovar	Strain	Omp16 detected
Brucella			
B. abortus	1	544	+
	1	B19	+
	2	86/8/59	+
	3	Tulya	+
	4	292	+
	5	B 3196	+
	6	870	+
	6	80-236	+
	6	89-43	+
	9	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	+
	Rough	45/20	+
B. melitensis	1	16 M	+
	1	Rev1	+
	2	63/9	+
	3	Ether	+
	Rough	B115	+
	Rough[a]	H38	+
B. suis	1	1330	+
	1	S2	+
	2	Thomsen	+
	3	686	+
	4	40	+
	5	513	+
B. canis		RM 6/66	+
B. ovis		BOW 63/290	+
B. neotomae		5 K 33	+
Escherichia coli O:157			−
Yersinia enterocolitica O:9			−
Salmonella urbana			−
Pseudomonas maltophilia			−
Pasteurella multocida			−

[a] Variant of the smooth H38 strain.

of five bacterial species that are known or presumed to induce immunological cross-reactions with *Brucella* spp. (listed in Table 1), no protein reacting with the anti-16.5 MAb could be detected.

DISCUSSION

We report the cloning and sequencing of a *B. abortus* gene encoding a 16.5-kDa minor Omp. The *pal* gene was cloned in *E. coli* on two largely overlapping inserts (1.8 and 2.1 kb), and expression in *E. coli* was probably driven from its own promoter, as described for other *Brucella* genes cloned in *E. coli* (15, 36). Expression of the protein does not seem to have a detrimental effect on the viability of *E. coli* cells (data not shown). Nucleotide sequence analysis of the cloned fragments revealed an ORF with the capacity to encode a protein of the expected size. The identity of this ORF was confirmed by subcloning and expression in *E. coli* as well as by the identification of an internal peptide sequence also found in the native Omp16.

The first 24 amino acids of the ORF encode a potential leader peptide ending with Val-Ala-Gly-Cys, a sequence that conforms to the consensus sequence required for the modification and processing of prokaryotic prolipoproteins (45). The calculated size of the mature Omp16 is consistent with the 16.5-kDa molecular mass of native and recombinant Omp16 estimated by immunoblotting. As could be expected for a lipoprotein, the N terminus is blocked and prevents Edman degradation (50). Furthermore, the *Brucella* Omp16 sequence has a very high degree of similarity with three other prokaryotic lipoproteins. Hydrophobicity analysis, performed by the algorithm of Kyte and Doolittle (29), failed to identify amino acid stretches, other than that of the leader peptide, likely to encode transmembrane domains. Considering the observations listed above, we suggest the hypothesis that the lipid moiety proposed to be covalently linked to the Omp16 N terminus would anchor this protein molecule in the outer membrane. A pathway for lipoprotein maturation thus seems to be present in *Brucella* spp. and is sufficiently similar to that of *E. coli* to allow an apparently correct processing of Omp16 in *E. coli*. Lipoproteins are indeed the most abundant posttranslationally modified bacterial secretory proteins and are probably all modified in exactly the same way as the Braun lipoprotein of *E. coli* (45). The peptidoglycan-linked Braun lipoprotein's equivalent is the only lipoprotein described so far for *Brucella* spp. (18, 19). This lipoprotein has been shown to be partially exposed on the surface of smooth *B. abortus* and *B. melitensis*. The *pal* gene described here would be the first putative *Brucella* lipoprotein gene to be sequenced. To demonstrate unequivocally that Omp16 is modified by fatty acid acylation, testing of globomycin (a signal peptidase II-specific inhibitor) effect on Omp16 maturation and metabolic labeling with palmitic acid and glycerol are in progress.

Simultaneous multiple alignment of Omp16 and three bacterial PALs (peptidoglycan-associated lipoproteins) resulted in identification of amino acid stretches (covering a major part of the protein's length) with highly conserved physicochemical profiles, which generally point to structurally conserved regions (12, 49). Omp16 seems to belong to the class of gram-negative outer membrane lipoproteins known as PALs. Mizuno (38) has shown the widespread existence of these PALs in the cell envelopes of many different genera of gram-negative bacteria. Although the biological function of PALs is not known, the high degree of structural similarity between the four lipoproteins from widely divergent organisms indicates a strong selective pressure on PAL conformation. We have at present no experimental evidence that Omp16 is associated with peptidoglycan. Cloeckaert et al. (9) have shown that the Omp16 immunoblot pattern is not affected by lysozyme treatment of cell extracts and that peptidoglycan-specific MAb 3D6 does not recognize this Omp. According to these authors, there was no evidence for association of Omp16 with peptidoglycan. However, their association detection method applies only to proteins which are covalently linked to peptidoglycan. In contrast, PALs have the unusual property of being strongly although noncovalently linked to the peptidoglycan structure (24, 34, 40).

Immunoelectron microscopy and ELISA analysis of whole bacterial cells demonstrated surface exposure of Omp16 epitopes in *Brucella* spp. (5). The topology of most membrane lipoproteins in gram-negative bacteria is yet to be determined. On the basis of reactivity with antibodies, *H. influenzae* PAL has been described as being surface exposed (22). A recombinant *H. influenzae* PAL without signal peptide was expressed but was not acylated in *E. coli* and was localized in the cytoplasmic fraction (20). A recent paper describes the expression on the *E. coli* surface of epitopes of an immunoglobulin

heavy- and light-chain-variable domain fused to a nonlipoprotein signal peptide (at the N terminus) and with mature *E. coli* PAL (Cys lipidation residue being replaced by Gly) at the C-terminal end (16). The authors proposed that PAL enables the hybrid protein to cross both inner and outer membranes. This implies that acylation is not required for sorting or insertion into the outer membrane. We would like to point out the apparent discrepancy between the surface exposure of PALs and their tight association with the peptidoglycan layer at the inner surface of the outer membrane. PALs should contain putative transmembrane segments forming amphipathic structures, since one of the features of Omps is the absence of regions of high overall hydrophobicity in predicted membrane-spanning segments (45). These complex findings do not allow us to propose a hypothesis for the topology of Omp16 in the cell wall. Further investigations will be conducted to address this intriguing question.

The gene encoding PAL appeared to be essential in *E. coli* and in *Haemophilus* spp., since attempts to create null mutations in these genes have been unsuccessful (3, 21). The cloning and characterization of the *pal* gene enable us to project construction of specific *Brucella* mutants deleted for this gene. If such viable mutants can be isolated, they will be studied to determine the contribution of Omp16 to virulence and protection.

By using an Omp16-specific MAb, an immunoblot analysis of *Brucella* lysates demonstrated that a similar protein with the same electrophoretic mobility occurs in all *Brucella* species and biovars tested. Epitopes shared between *Brucella* Omp16 and an *E. coli* protein could in fact be expected from sequence similarities demonstrated with *E. coli* PAL. Our results indicate that at least the Omp16 determinant recognized by the Mab A68/04G01/C06 is conserved throughout the *Brucella* genus and is specific for this genus.

The cloning and overexpression of the *pal* gene in *E. coli* will provide sufficient Omp16 devoid of all other contaminating *Brucella* macromolecules to permit its evaluation as a diagnostic tool and protective antigen.

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