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Cloning and Nucleotide Sequence of the Gene Coding for the Major 25-Kilodalton Outer Membrane Protein of *Brucella abortus*

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The cloning and sequencing of the *Brucella abortus* major 25-kDa outer membrane protein (OMP) is reported. The 25-kDa (group 3) OMP has been proposed, on the basis of amino acid composition, to be the counterpart of OmpA (D. R. Verstraete, M. T. Creasy, N. T. Caveney, C. L. Baldwin, M. W. Blab, and A. J. Winter, Infect. Immun. 35:979–989, 1982). However, the amino acid sequence predicted from the cloned *B. abortus* gene did not reveal significant homology with either OmpA sequences from different members of the family *Enterobacteriaceae* or other known protein sequences.

The bacteria of the genus *Brucella* are gram-negative intracellular parasites of both humans and animals. *Brucella abortus* is the major species involved in bovine brucellosis and can cause abortions and infertility in cattle, which result in great economic losses. The *B. abortus* outer membrane contains lipopolysaccharide, proteins, and phospholipids. The major *B. abortus* outer membrane proteins (OMPs) are 25- to 27-kDa proteins (3, 9, 10), also called group 3 proteins (18), and 36- to 38-kDa proteins (3, 9, 10), also called group 2 porin proteins (7, 18). Variation in apparent molecular masses of these proteins has been shown to be probably essentially due to association with peptidoglycan subunits of different sizes (5, 10, 16). Thus, group 2 and group 3 proteins would be in fact peptidoglycan-associated forms of the same gene product. Both the major 25- to 27-kDa and the 36- to 38-kDa OMPs have been shown by immunoelectron microscopy to be surface exposed (3). These major OMPs are also major components of the sodium dodecyl sulfate (SDS)-insoluble cell wall fraction which confers important vaccinal properties (8–10). The role of the two major OMPs in protective immunity against *Brucella* infection is being studied by several research groups (8, 9, 19). The gene encoding the *B. abortus* 36-kDa porin OMP has been recently cloned and functionally expressed in *Escherichia coli* (11, 13).

The major 25-kDa (group 3) OMP (Omp25) has been previously proposed, on the basis of amino acid composition, to be the counterpart of OmpA (18). The present paper reports the cloning and nucleotide sequence of the gene coding for *B. abortus* Omp25 (*omp25* gene). Predicted amino acid sequence homologies with OmpA proteins from different gram-negative bacteria were determined.

Cloning of the *B. abortus omp25* gene. A λgt11 genomic library of *B. abortus* 544 (biovar 1 reference strain) was constructed according to a protocol similar to that described by De Kesel et al. (6). Briefly, the DNA of *B. abortus* 544 was purified as described by Verger et al. (17), partially digested with *Sau*3A (Boehringer GmbH, Mannheim, Germany), and filled in with deoxynucleoside triphosphates (Appligene, Illkirch, France) and Klenow polymerase (Boehringer). *Eco*RI sites of agarose gel-purified DNA fragments of 2 to 8 kb were methylated with *Eco*RI methylase (Promega, Madison, Wis.). *Eco*RI linkers (Boehringer) were then ligated to the DNA fragments. Following *Eco*RI digestion, the *B. abortus* 544 DNA fragments were further ligated into *Eco*RI-digested λgt11 DNA (Promega). The ligated DNA was packaged into phage λ particles by using commercial extract Gigapack II Plus (Stratagene, La Jolla, Calif.) and amplified on *E. coli* Y1090 (Stratagene). Recombinant λgt11 phage was screened following transfer to nitrocellulose filters (Stratagene) with anti-Omp25 monoclonal antibodies (MAbs) (3, 4). Individual plaques were removed from the plates and rescreened several times until all of the plaques recovered reacted positively with the MAbs. Among approximately 4×10^5 plaques tested from the *B. abortus*-constructed λgt11 library, 10 reacted positively with the anti-Omp25 MAbs. One of these recombinant phages was selected on the basis of its strong reactivity with the MAbs. It was further shown that only one MAb (MAb A59/01E11/D11) (immunoglobulin G2a [4]) of a mixture of five anti-Omp25 MAbs reacted with this recombinant phage. The recombinant phage was further propagated in lysogenic *E. coli* Y1089 (Stratagene), and recombinant protein synthesis was induced with IPTG (isopropyl-β-D-thiogalactopyranoside). As shown by immunoblotting after SDS-polyacrylamide gel electrophoresis of the *E. coli* lysate with MAb A59/01E11/D11 and an anti-β-galactosidase MAb (Boehringer), the recombinant phage expressed a β-galactosidase fusion protein with an apparent molecular mass of 116 kDa (data not shown). The size of the *B. abortus* DNA insert in the recombinant phage was estimated at 1,150 bp. To clone the entire *omp25* gene, a λEMBL3 genomic library of *B. abortus* 544 was constructed as described by Grimont et al. (12). Briefly, 9- to 23-kb *Sau*3A-digested fragments of *B. abortus* 544 DNA were ligated into *Bam*HI-digested and phosphatase-treated λEMBL3 vector arms (Promega). The ligated DNA was packaged into phage λ particles by using commercial extract Gigapack II Plus (Stratagene) and amplified on *E. coli* LE 392 (Promega). Immunological screening of plaques was performed with the anti-Omp25 MAbs. Among approximately 10^4 plaques tested from the *B. abortus*-constructed λEMBL3 library, one was positive with the anti-Omp25 MAb mixture. Southern blot hybridization, performed as described by Grimont et al. (12) and Verger et al. (17),

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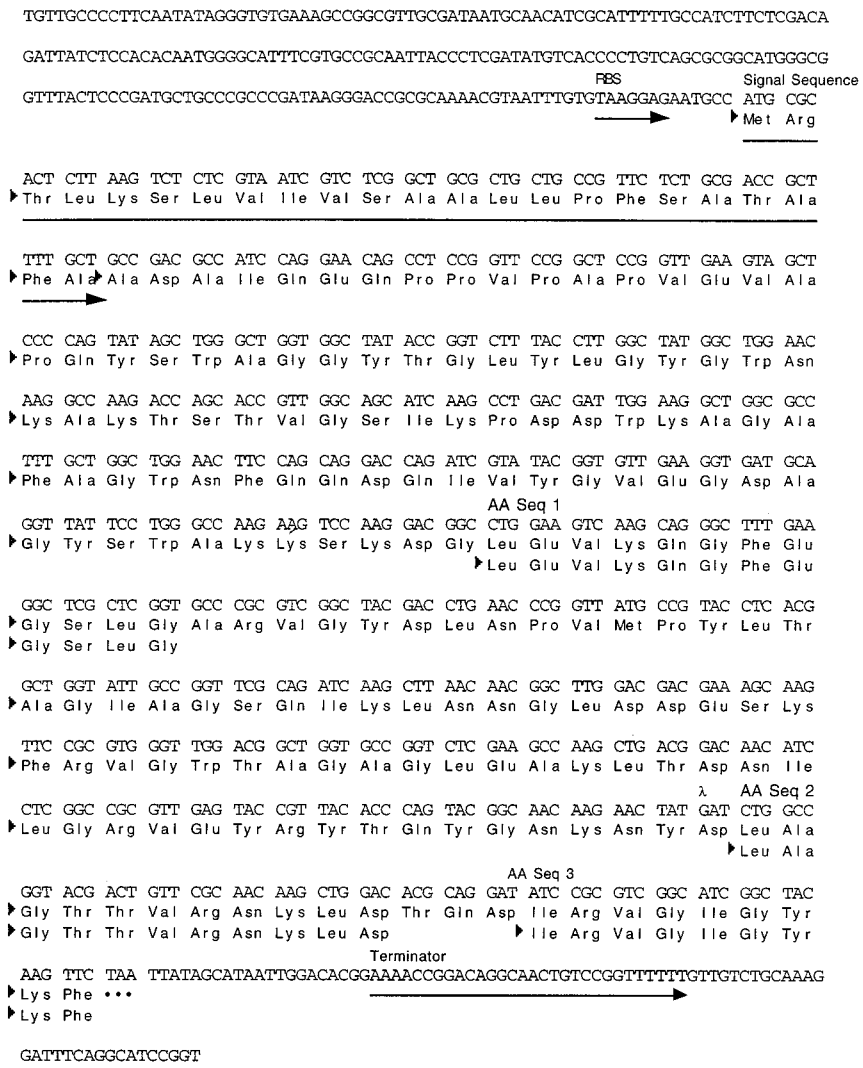

FIG. 1. DNA sequence and predicted amino acid sequence of the *omp25* gene of *B. abortus*. A ribosome binding site (RBS), putative signal peptide sequence, and terminator sequence (underlined with arrows) are shown. The beginning of the λgt11 recombinant phage DNA insert (λ) and amino acid sequences determined from *B. abortus* cell wall-isolated Omp25 (AA Seq 1 through 3) are indicated.

between the DNA insert of the λgt11 recombinant phage and that of the λEMBL3 recombinant phage showed that a 5-kb *Eco*RI-*Sal*I fragment from the λEMBL3 recombinant phage insert was homologous to the λgt11 recombinant phage insert (data not shown).

***omp25* gene sequence.** Both the *Eco*RI insert of the λgt11 recombinant phage and the 5-kb *Eco*RI-*Sal*I fragment of the λEMBL3 recombinant phage insert were subcloned into both pTZ18R and pTZ19R phagemid vectors (Pharmacia Biotech). The dideoxy chain termination method of Sanger et al. (15) was employed, using a T7 sequencing kit (Pharmacia Biotech) according to the manufacturer's protocol. Figure 1 shows the DNA sequence and predicted amino acid sequence of Omp25 of *B. abortus*. The amino acid sequences of three peptidic fragments of Omp25 isolated from *B. abortus* cell walls were determined (protein sequencer 477A-120A; Applied Biosystems) to confirm that the sequenced gene corresponded to the *omp25* gene. The *omp25* gene sequence revealed an open reading frame that may begin at two different start codons generating proteins with predicted molecular masses of 26,773 and 23,037 Da. However, DNA sequences bordering the open reading frames suggest that the *omp25* gene starts at the second start codon. Indeed, 6 bp before this start codon, seven nucleotides (TAAGGAG) are homologous to the 16S RNA sequence of *E. coli* and most probably constitute the ribosome binding site. In addition, after the start codon, a predicted amino acid sequence characteristic of signal peptides for protein export was found. Localization of the signal peptide cleavage site indicated that the mature Omp25 protein has a predicted molecular mass of 20,649 Da. The recombinant λgt11 insert encoded the last 24 amino acids of the *omp25* gene product. Therefore, an epitope recognized by MAb A59/01E11/D11 is located in the last 24 amino acids of the carboxy-terminal end of Omp25. Downstream of the *omp25* gene, a DNA sequence of 30 bp characteristic of rho-independent transcription termination sequences was found. The 30-bp sequence showed a high degree of DNA homology with the gene *omp2b* encoding the *B. abortus* 36-kDa porin OMP (11) (data not shown).

Amino acid sequence alignment. The amino acid composition of the *B. abortus* Omp25 protein has been reported to be similar to that of *E. coli* OmpA (18). Therefore, by use of the

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E. coli          --mkktaiaiavalagfatvaqaaPKDNTW----------
E. aerogenes     --mkktaiaiavalagfatvaqaaPKDNTW----------
S. typhimurium   --mkktaiaiavalagfatvaqaaPKDNTW----------
S. marcescens    --mkktaialavalagfatvaqaaPKDNTW----------
S. dysenteriae   --mkktaiaitvalagfatvaqaaPKDNTW----------
B. abortus       MRtlkslvivsaallpfsatafaaDAIQEQPPVPAPVDVAPQY

E. coli          -----ytgaklgwSQYHDTGFINNNGPTHEN-------qlgag
E. aerogenes     -----yaggklgwSQFHDTGWYNSNLNNNGPTHES---qlgag
S. typhimurium   -----yagaklgwSQYHDTGFIHNDGPTHEN-------qlgag
S. marcescens    -----ytgaklgwSQYHDTGFYGNGYQNGIGNGPTHKDqlgag
S. dysenteriae   -----ytgaklgwSQYHDTGFIDNNGPTHEN-------qlgag
B. abortus       SWAGGytglylgyGWNKAKTSTVGSIKPD---------dwkag

E. coli          afggyqvnPYVG-----femgydwlgrMPYKGSVENGAYKAQG
E. aerogenes     afggyqvnPYLG-----femgydwlgrMPYKGVKVNGAFSSQA
S. typhimurium   afggyqvnPYVG-----femgydwlgrMPYKGDNINGAYKAQG
S. marcescens    aflgyqanQYLG-----felgydwlgrMPYKGSVNNGAFKAQG
S. dysenteriae   afggyqvnPYVG-----femgydwlgrMPYKGSVENGAYKAQG
B. abortus       afagwnfqQDQIVYGVEgdagyswakkSKDGLEVKQGFEGSLG

E. coli          VQLTAKLGypitddlDIYTRLGGMVWRADTKSNVYGKNHDTGV
E. aerogenes     VQLTAKLGypitddlDIYTRLGGMVWRADSSNSIAGDNHDTGV
S. typhimurium   VQLTAKLGypitddlDFYTRLGGMVWRADTKSNVPGGPSTKDH
S. marcescens    VQLAAKLSypiaddlDIYTRLGGMVWRADSKANYGRTGQRLSD
S. dysenteriae   VQLTAKLGypitddlDVYTRLGGMVWRADTKAHNNVTGESEKN
B. abortus       ARVGYDL-npvmpylTAGIAGSQIKLNNGLDDESKFRVGWTAG

E. coli          SPVFAGGVEYAI-----tpeiatrleyqwtNNIGDAHTIGTRP
E. aerogenes     SPVFAGGVEWAM-----trdiatrleyqwvNNIGDAGTVGVRP
S. typhimurium   DTGVSPVFAGGIEYAI-tpeiatrleyqwtNNIGDANTIGTRP
S. marcescens    HDTGVSPLAAVGVEYALtknwatrldyqfvSNIGDAGTVGARP
S. dysenteriae   HDTGVSPVFAGGVEWAItpeiatrleyqwtNNIGDAHTIGTRP
B. abortus       AGLEAKL----------tdnilgrveyrytQYGNKNYDLAGTT

E. coli          DNGMLS-----lgvsyrfGQGEAAPVVAPAPAPAPEVQTKHFT
E. aerogenes     DNGMLS-----vgvsyrfGQEDNAPVVAPAPAPAPEVTTKTFT
S. typhimurium   DNGLLS-----vgvsyrfGQQEAAPVVAPAPAPAPEVQTKHFT
S. marcescens    DNTMLS-----lgvsyrfGQDDVVAPAPAPAPAPVVETKRFTL
S. dysenteriae   DNGLLS-----lgvsyrfGQGEAAPVVAPAPAPAPEVQTKHFT
B. abortus       VRNKLDTQDIRvglgykf------------------------
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FIG. 2. Predicted amino acid sequence alignment of OmpA proteins with Omp25 of *B. abortus*. Amino acid sequence homologies with *B. abortus* Omp25 (lowercase and boldface) were determined with the Match-box program (Pro-Explore, Biostructure). The first homology box corresponds to the signal peptide sequence.

Match-box program (Pro-Explore, Biostructure), which aligns amino acid sequences with regard to both peptide identity and physicochemical relationship, the amino acid sequence predicted from the *omp25* gene of *B. abortus* was compared with those of OmpA proteins from *E. coli*, *Enterobacter aerogenes*, *Salmonella typhimurium*, *Serratia marcescens*, and *Shigella dysenteriae* (Fig. 2). No significant homology was found, although the predicted amino acid composition of *B. abortus* Omp25 was almost identical to that reported for *B. abortus* group 3 proteins (Table 1) (18). Nevertheless, seven homology boxes were found, including the region coding for the signal peptide (Fig. 2). The homology among these boxes was sometimes poorly significant (especially the third and the sixth). Identity between the five OmpA amino acid sequences was, however, much more important. Therefore, we may conclude that Omp25 of *B. abortus* does not belong to the OmpA family. It must also be noted that none of the anti-Omp25 MAbs we have produced (3) cross-reacted with *E. coli* antigens (2). Moreover, a search for homologies was carried out in the MIPSX database, release 30-2 (Martinsried), and at the Belgian EMBnet Mode (BEM) by using the FASTA method (14). NCBI services were also used to consult the Swiss-PROT (release 26.0), GenBank (release 78.0), and EMBL (release 37.0) databases with the BLAST network service (1). No significant homology between *B. abortus* Omp25 and other known proteins in the databases was revealed. Moreover, none of the OmpA proteins were found in the first homology values.

TABLE 1. Amino acid compositions of OmpA of *E. coli*, group 3 proteins, and Omp25 from *B. abortus*

Amino acid	Mol%			
	E. coli OmpA[a]	*B. abortus* group 3 proteins[a]	Omp25	Mature Omp25[b]
Asx	12.6	11.4 ± 0.7	10.3	11.5
Thr	6.5	5.5 ± 0.4	5.6	5.2
Ser	4.9	4.6 ± 0.4	5.1	4.2
Glx	8.9	9.8 ± 0.7	8.4	9.4
Pro	5.8	4.2 ± 0.2	4.2	4.2
Gly	11.4	14.5 ± 0.4	12.6	14.1
Ala	8.9	10.2 ± 0.8	10.7	9.4
Cys	0.6	ND	0	0
Val	7.7	6.7 ± 0.6	7.0	6.8
Met	1.5	0.8 ± 0.7	0.9	0.5
Ile	4.3	6.7 ± 3.1	4.2	4.2
Leu	6.8	7.2 ± 0.2	7.9	6.8
Tyr	5.2	3.2 ± 2.3	6.1	6.8
Phe	2.5	3.4 ± 0.3	3.3	2.6
His	1.5	2.2 ± 1.6	0	0
Lys	5.2	6.7 ± 0.6	7.0	7.3
Arg	4.0	3.4 ± 0.3	3.3	3.1
Trp	1.5	ND	2.8	3.1

[a] Data from reference 18. ND, not determined.
[b] Amino acid composition of Omp25 without its putative signal peptide.

The possible *Brucella* genus specificity of Omp25 has several implications for both vaccinal and diagnostic purposes. We are currently testing the potential role of *E. coli*-expressed Omp25 in protective immunity against *Brucella* infection and the usefulness of recombinant Omp25 as a diagnostic antigen.

Nucleotide sequence accession number. The DNA sequence of the *B. abortus omp25* gene has been submitted to GenBank and assigned accession no. X79284.

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