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Outer Membrane Proteins Omp10, Omp16, and Omp19 of *Brucella* spp. Are Lipoproteins

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The deduced sequences of the Omp10, Omp16, and Omp19 outer membrane proteins of *Brucella* spp. contain a potential bacterial lipoprotein processing sequence. After extraction with Triton X-114, these three proteins partitioned into the detergent phase. Processing of the three proteins is inhibited by globomycin, a specific inhibitor of lipoprotein signal peptidase. The three proteins were radioimmunoprecipitated from [^{3}H]palmitic acid-labeled *Brucella abortus* lysates with monoclonal antibodies. These results demonstrate that Omp10, Omp16, and Omp19 are lipoproteins.

Brucellae are facultative intracellular gram-negative bacteria that cause human disease and significant worldwide economic loss due to infection of livestock.

The *Brucella* cell wall consists of a peptidoglycan layer strongly associated with the outer membrane (9). The cell wall of *Brucella abortus* has been described as "a complex structure populated by at least 75 proteins" (21). The molecular characterization of several of these outer membrane proteins (OMPs) has been reported over the past years. The genes *omp25*, *omp31*, and *omp2b*, encoding the major 25-, 31-, and 36-kDa *Brucella* OMPs, respectively, have been cloned and sequenced (5, 8, 10, 25). Omp2b functions as a porin (18). The cloning and sequencing of the gene encoding three less abundant (minor) OMPs has been previously reported. These three minor Omps are expressed in all six *Brucella* species and all of their biovars (23, 24). The 16-kDa OMP, named Omp16, shows significant similarity to the peptidoglycan-associated lipoproteins (PALs) of many gram-negative bacteria (24). The 10- and 19-kDa OMPs (Omp10 and Omp19, respectively) have still no homologs in the sequence databases (16, 23). However, these three minor OMPs share antigenic determinants with bacteria of the family *Rhizobiaceae* (4). Antibody is elicited to the three OMPs. By using purified recombinant Omp10, Omp16, and Omp19, a significant antibody response specific for these OMPs could be detected in a large fraction of sera from sheep naturally infected by *Brucella melitensis*. However, there was almost no serologic response to these recombinant OMPs in cattle naturally infected by *B. abortus* (17, 23).

A fourth minor OMP of 89 kDa has been identified by use of monoclonal antibodies (MAbs), and its gene sequence is available in the GenBank database (accession no. U51683) (1, 3).

The NH_2-terminal signal peptides of the predicted Omp10, Omp16, and Omp19 contain a tetrapeptide showing a high degree of similarity to the consensus sequence required for the modification and processing of bacterial lipoprotein precursors: the lipobox Leu-(Ala or Ser)-(Gly or Ala)-Cys at the -3 to $+1$ positions (13). The first lipoprotein described for *Brucella* spp. is the equivalent to the peptidoglycan-linked Braun lipoprotein, also called murein lipoprotein. This polypeptide contains fatty acids, both ester and amide linked (11). It is partially exposed on the surface of smooth *B. abortus* and *B. melitensis* (12).

The sequence information suggests that Omp10, Omp16, and Omp19 are lipoproteins, and this report describes the experimental demonstration that this is indeed so.

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Behavior of the three OMPs during Triton X-114 extraction and phase partitioning. To study biochemically the lipoprotein nature of the three OMPs, their partitioning was analyzed after Triton X-114 extraction of *Brucella* and *Escherichia coli* p102, p104, p161, p192, and p193 transformants (23, 24). The plasmids p102, p161, and p192 encode the entire coding sequence of the *omp10*, *pal*, and *omp19* genes, respectively, and express the corresponding recombinant OMP with an apparent molecular mass identical to that of native *Brucella* OMP. The subclones p104 and p193 express a fusion protein between β-galactosidase α-peptide and Omp10 or Omp19, respectively, under control of the *lac* promoter. These fusion proteins lack the signal peptide and the N-terminal cysteine. Membrane-associated proteins such as integral membrane proteins or lipoproteins segregate with the detergent phase upon phase separation (2). *B. melitensis* B115 and *E. coli* extracts were prepared by sonication and extracted with a mixture of 20 mM Tris HCl (pH 8.0), 10 mM EDTA, and 2% Triton X-114. After incubation for 4 h at 4°C with gentle agitation, cellular debris was removed by centrifugation. The supernatant was warmed to 37°C to allow phase separation to occur. After centrifugation for 10 min at 13,000 × *g*, the upper aqueous phase was separated from the detergent phase. The aqueous phase was cleaned by addition of 20% Triton X-114 to a final concentration of 2%, and the detergent phase was diluted to the original volume by addition of 20 mM Tris HCl (pH 8.0)–10 mM EDTA at 0°C. The washing procedure was repeated two times to clean up both phases. The cleaned-up detergent phase was precipitated with acetone and suspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. Whole-cell extracts and materials of the cleaned-up aqueous and detergent phases were analyzed by immunoblotting with anti-Omp10, anti-Omp16, or anti-Omp19 monoclonal antibodies (MAbs). The soluble p39 gene product was used as a marker for the aqueous phase (6). The proteins Omp25 and Omp31 as well as the rough lipopolysaccharide were used

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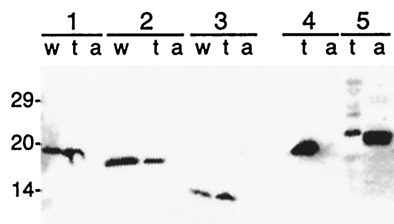

FIG. 1. Behavior of the three OMPs in Triton X-114. Immunoblot analysis of the whole organism (w) and Triton X-114 detergent phase (t) and aqueous phase (a). Lanes 1, 2, and 3 correspond to *B. melitensis* fractions revealed with anti-Omp19, anti-Omp16, and anti-Omp10 MAbs, respectively. Lanes 4 and 5 contain *E. coli* p192 and p193 clone fractions, respectively, and were revealed with anti-Omp19 MAb. The locations of the molecular size standards are shown (in kilodaltons) on the left.

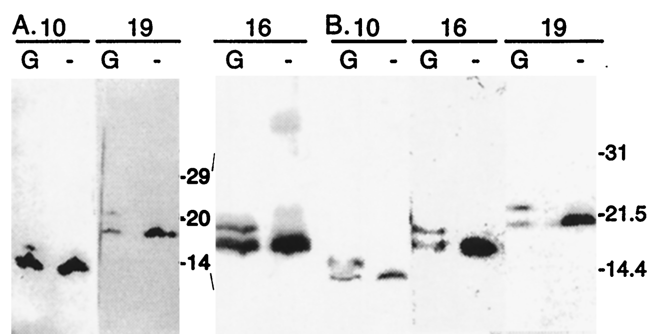

FIG. 2. Inhibition of processing by globomycin. Bacteria were grown in the presence (G) or absence (−) of globomycin. (A and B) Immunoblot analysis of *B. melitensis* (A) and *E. coli* (B) lysates with anti-Omp10 (10), anti-Omp16 (16), and anti-Omp19 (19) MAbs. In panel B, lanes 10, 16, and 19 contain lysates of p102, p161, and p192 clones, respectively. The locations of the molecular size standards are shown (in kilodaltons) on the right.

as markers for the detergent phase, as described for *Brucella ovis* (15).

The three native OMPs selectively partitioned into the detergent phase (Fig. 1). The recombinant OMPs expressed by the p102, p192, and p161 clones partitioned almost exclusively into the Triton X-114 detergent phase. Kyte and Doolittle hydropathy plots of deduced OMPs suggested that the three proteins are hydrophilic, except for the hydrophobic region of their signal peptide. Furthermore, Omp10 and Omp19 fusion proteins devoid of signal peptide were detected almost exclusively in the aqueous phase (Fig. 1). Therefore, the hydrophobic behavior of the three OMPs is consistent with modification by fatty acids. Since the recombinant polypeptides possess hydrophobic properties similar to those of the native proteins, they seem to be recognized by the *E. coli* enzymes which perform the posttranslational lipid modification.

Globomycin inhibits processing of the OMPs. Globomycin (a kind gift from M. Inukai, Sankyo Co., Ltd., Tokyo, Japan) is a specific inhibitor of signal peptidase II and induces accumulation of the lipoprotein precursors (7, 14). To an exponentially growing culture of *B. melitensis* B115 and *E. coli* transformants, globomycin dissolved in methanol was added to a final concentration of 100 μg/ml (final methanol concentration, 2%). Untreated cells were grown in the presence of 2% methanol. After 12 h (for *Brucella*) or 2 h (for *E. coli*) of incubation, bacteria were washed in phosphate-buffered saline (PBS) and suspended in SDS-PAGE sample buffer. These lysates were analyzed by immunoblotting with the corresponding anti-OMP MAbs. As expected, globomycin has no detectable effect on the size of the OMP fusion proteins expressed by p104 and p193 clones (data not shown). In contrast, both in *Brucella* and in *E. coli* p102, p161, and 192 transformants treated with globomycin, each MAb revealed a major band and a fainter band of slightly higher molecular mass corresponding to the mature and precursor forms of the OMP, respectively (Fig. 2). In untreated cells, only the mature OMPs are detected. Inhibition of the maturation by globomycin confirms the lipoprotein nature of the three OMPs. The results imply that *B. melitensis* possesses mechanisms similar to those of *E. coli* for processing of lipoproteins.

B. abortus **acylates Omp10, Omp16, and Omp19.** A culture of *B. abortus* 45/20 was seeded in tryptic soy broth with an overnight culture in a 1/10 dilution and labeled by addition of [9,10(*n*)-^{3}H]palmitic acid to 25 μCi/ml, followed by further incubation at 37°C under vigorous agitation for 24 h until the optical density at 600 nm reached 0.7. Cells were harvested, inactivated by heat, washed twice, resuspended in Tris-buffered saline (10 mM Tris-HCl [pH 7.2], 150 mM NaCl) containing 1 mM phenylmethylsulfonyl fluoride, and lysed by sonication. Triton X-100 and sodium deoxycholate were added to 1%, and the lysate was centrifuged at 85,000 × *g* for 1 h at 4°C. To the supernatant were added antimouse immunoglobulin G-conjugated magnetic beads (Dynabeads, Dynal, Norway) coated with one anti-OMP MAb. (Two MAbs were tested separately for each OMP.) After gentle agitation for 2 h at 4°C, the bead-antibody-antigen complexes were washed four times in TBS containing 1% Triton X-100 and 1% sodium deoxycholate and boiled in SDS-PAGE sample buffer. The samples were analyzed by SDS-PAGE and prepared for fluorography as described by Thirkell et al. (22). Dried gels were exposed to Amersham hyperfilm MP for several weeks at −70°C. *B. abortus* incorporated [^{3}H]palmitate into a limited number of proteins. The majority of the label was incorporated by a low-molecular-mass compound corresponding to rough lipopolysaccharide and/or the equivalent to murein lipoprotein (data not shown). Immunoprecipitation experiments done in duplicate with two different anti-OMP MAbs (Fig. 3) confirm that Omp10, Omp16, and Omp19 are lipid modified.

Omp10, Omp16, and Omp19 satisfy three criteria used for definition of a lipoprotein (13): (i) their deduced amino acid sequence contains a cysteine residue in the C-terminal portion of a signal sequence as part of a lipobox, (ii) their processing is inhibited by globomycin, and (iii) they are labeled by tritiated palmitic acid. Furthermore, Triton X-114 extraction and phase separation result in their partitioning exclusively into the detergent phase. The presence of the lipobox has successfully predicted the lipoprotein nature of these proteins.

The proposed pathway for the biosynthesis of lipoproteins in

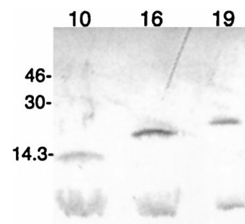

FIG. 3. Proteins from [^{3}H]palmitic acid-labeled *B. abortus* were radioimmunoprecipitated with anti-Omp10 MAb A68/07G11/C10 (10), anti-Omp16 MAb A76/08C03/G03 (16), and anti-Omp19 MAb A76/02A04/A07 (19); separated by SDS-PAGE; and fluorographed. The locations of the molecular size standards are shown (in kilodaltons) on the left. The additional band of very small size present in all lanes is from contamination by rough lipopolysaccharide.

E. coli (for a review, see reference 26) implies that the prolipoprotein is translocated by the Sec machinery and covalently linked at the cysteine residue to diacyl glycerol which contains two esterified fatty acids. Subsequently, signal peptidase II cleaves the molecule and the N-terminal cysteine is linked to another fatty acid by an amide linkage. The localization of the mature lipoprotein to either the inner or outer membrane follows. A periplasmic chaperone, LolA, mediates the release of the outer membrane-directed lipoproteins from the inner membrane (19). When the soluble lipoprotein-LolA complex interacts with an outer membrane receptor, LolB, the lipoprotein is transferred to LolB and then incorporated into the outer membrane (20). *Brucella* could possess a similar pathway for lipoprotein sorting and insertion into the relevant membrane.

Immunoelectron microscopy and enzyme-linked immunosorbent assay of whole bacterial cells demonstrated surface exposure of the three OMPs in *Brucella* spp. (3) and in *E. coli* clones expressing the entire protein (p102, p161, and p192 [data not shown]). As expected, Omp10 and Omp19 fusion proteins devoid of signal peptide were not detected on the surface of *E. coli* p104 and p193 cells.

The topology of the three OMPs in the membrane remains unknown. However, we propose that the lipid moiety covalently linked to the Omp10 and Omp19 N termini would be embedded into the outer leaflet of the outer membrane to place these proteins at the interface of the membrane and the external environment. As mentioned previously, the apparent discrepancy between the surface exposure of Omp16 and other PALs and their tight association with the peptidoglycan layer remains to be resolved (24). Therefore the topology of Omp16 in the cell wall is still an intriguing question.

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