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Release of Outer Membrane Fragments by Exponentially Growing *Brucella melitensis* Cells

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Rough and smooth strains of *Brucella melitensis* released a membranous material that was devoid of detectable NADH oxidase and succinic dehydrogenase activity (cytoplasmic membrane markers) but that contained lipopolysaccharide, proteins, and phospholipids. This material was composed of two fractions that had similar chemical compositions but that were of different sizes which were separated by differential ultracentrifugation. Electron microscopy showed that both fractions are made of unit membrane structures. The membrane fragments were released during the exponential phase of growth, and no leakage of malic dehydrogenase activity (cytosol marker) was detected. Thus, the fragments were unlikely a result of cell lysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis showed that, although group 2 *Brucella* outer membrane proteins and lipoprotein were not detected, the proteins in the membranous material were outer membrane proteins. Gas-liquid chromatography analysis showed a similar fatty acid profile for the cell envelope and the outer membrane fragments of the smooth strain *B. melitensis* 16M. In contrast, the outer membrane fragments from the rough 115 strain were enriched in palmitic and stearic acids. With respect to the unfractionated cell envelope, outer membrane fragments were enriched in phosphatidylcholine, a phospholipid that is unusual in bacterial membranes.

The members of the genus *Brucella* are gram-negative bacteria with a cell envelope (CE) consisting of an inner membrane (IM) and an outer membrane (OM) that are separated by a periplasmic space containing the peptidoglycan layer (13, 14). Up to now, studies on purified OM have not been performed in *Brucella* spp. because of the lack of methods to achieve its separation from the IM. In most gram-negative bacteria such methods are based on the fact that the OM can be rendered permeable to lysozyme by the combined action of Tris and EDTA. The ensuing digestion of the underlying peptidoglycan layer brings about the formation of spheroplasts from which OM and IM can be separated (48). *Brucella* OM, however, is not affected by EDTA (44), and preparation of lysozyme spheroplasts is precluded (14, 44). Therefore, the OM of *Brucella* has been studied by sequential detergent extractions which yield preparations enriched in OM proteins (44, 59) or by using modifications of the methods originally described for *Escherichia coli* by Rosenbusch (49) or by Nakamura and Mizushima (45). Such studies have shown that *Brucella* OM contains three groups of major proteins (59): group 2 proteins (apparent molecular weight, 35,000 to 40,000) are peptidoglycan-associated proteins (45) with porin activity (12). Even though a temperature-dependent molecular weight shift has not been shown for them, group 3 proteins (molecular weight, 25,000 to 30,000) show an amino acid composition resembling that of *E. coli* OmpA (59). Nothing is known about the role or properties of group 1 proteins (molecular weight, 88,000 to 94,000). In addition, a lipoprotein similar to *E. coli* Braun lipoprotein has been characterized recently (21).

However useful, methods relying on the sequential action of some detergents (Sarkosyl [CIBA-GEIGY Corp., Summit, N.J.] and Zwittergent [Calbiochem-Behring, La Jolla, Calif.]) have several intrinsic limitations. First, it has been shown in *E. coli* that Sarkosyl extraction is not quite IM selective (5); and in *Brucella* spp. we have reported (44) that, as judged by the release of 2-keto-3-deoxyoctulosonic acid (KDO), this detergent has also some effect on the OM. Second, exposure to ionic or dipolar ionic detergents is likely to alter some of the properties of the proteins. Finally, the use of detergents does not allow us to study the phospholipid distribution in the CE. Therefore, we have endeavored to search for alternative methods to obtain *Brucella* OM fractions. Here we report that smooth (S) and rough (R) variants of *B. melitensis* release spontaneously a free membranous material (FMM) containing OM components but lacking IM markers.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *B. melitensis* 16M (S, virulent), *B. melitensis* 115 (R, avirulent), *B. abortus* 19 (S), and *B. abortus* 45/20 (R, avirulent) have been described elsewhere (10, 30, 52, 59). All these strains were grown in 3-liter flasks containing 800 ml of Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) on a rotary shaker at 36 to 37°C.

CE preparation and detergent extractions. To obtain the CE, logarithmic-phase bacteria were sedimented by centrifugation (7,500 × *g*, 15 min at 4°C), washed twice with saline, and suspended in a small amount of 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.5)–5 mM $MgCl_2$. The cells were then disintegrated with an MSK cell homogenizer (B. Braun Mesulgen AG, Mesulgen, Federal Republic of Germany); and after removal of glass beads and unbroken cells by low-speed centrifugation, the CE fraction was collected by ultracentrifugation (80,000 × *g*, 2 h), suspended in 10 mM Tris hydrochloride (pH 7.5), and stored at −20°C (43). When *B. melitensis* 16M was used, the bacteria were killed before harvesting by adding phenol (final concentration, 0.5%) to the flasks and overnight incubation at room temperature.

To prepare extracts enriched in OM major proteins, CEs of strain 115 were first extracted with Sarkosyl, and the Sarkoysl-resistant fraction was extracted with Zwittergent

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TABLE 1. Chemical characterization of CEs and materials obtained by precipitation [$(NH_4)SO_4$] or ultracentrifugation (FMM) of culture supernatants of *B. melitensis*[a]

Strain	% of dry weight of:			Activity[b]		Yield[d]
	LPS	Protein	FAE-FAA[c]	NADH-ox	SDH	
B. melitensis 115						
CE	21.4	73.0	ND	0.106	0.100	ND
$(NH_4)SO_4$	21.5	26.3	27.1	ND	ND	2.8
FMM	45.0	20.8	30.0	0.030	0.016	2.6
FMM-40,000 × *g*	40.8	23.1	43.7	ND	ND	1.8
FMM-100,000 × *g*	47.8	24.4	34.8	ND	ND	3.4
B. melitensis 16M						
CE	30.0	55.0	27.5	0.273	0.262	ND
$(NH_4)SO_4$	30.3	27.9	42.0	ND	ND	2.4
FMM	37.9	16.9	20.7	0.000	0.000	1.7
FMM-40,000 × *g*	27.0	22.3	40.0	0.004	ND	1.1
FMM-100,000 × *g*	40.9	17.0	22.5	0.001	ND	2.2

[a] Abbreviations: FAE-FAA, fatty acid ester linked and fatty acid amide linked; NADH-ox, NADH-oxidase; SDH, succinate dehydrogenase; ND, not determined.
[b] Reported as the absolute value of the ratio of the optical density change: (10 min × milligrams of protein).
[c] Calculated by substracting the amount of LPS fatty acid from the total fatty acid ester linked and fatty acid amide linked.
[d] Yields are expressed as percentages of the dry weight of the cells obtained from the same culture.

316 (44, 59). When the CEs of the phenol-inactivated 16M cells were extracted, lysozyme digestion of the Sarkosyl-resistant fraction was necessary for the extraction of some of the OM major proteins (59). For sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), Zwittergent extracts were precipitated with 5 volumes of acetone, and the precipitate was collected by centrifugation and extracted with chloroform-methanol (1:2).

Preparation of the FMM. The supernatants of exponentially growing *B. melitensis* cultures were first concentrated 100-fold by dialysis under vacuum at 4°C and then clarified by centrifugation (15,000 × *g*, 30 min at 4°C). The clarified supernatant fluid was precipitated by adding solid ammonium sulfate to a concentration of 28% and incubating for 12 h at room temperature with continuous stirring. The precipitate was collected by centrifugation (15 min at 5,000 × *g* for *B. melitensis* 115 or at 40,000 × *g* for *B. melitensis* 16M), dialyzed against several changes of distilled water, and lyophilized.

Alternatively, the material present in the clarified fluid was obtained directly by ultracentrifugation (6 h at 100,000 × *g*, 4°C), suspended in a small amount of distilled water, and lyophilized.

Phospholipid and fatty acid analysis. Phospholipids were extracted by the method described by Bligh and Dyer (2) and analyzed by thin-layer chromatography on Silica gel H (E. Merck, Darmstadt, Federal Republic of Germany). The plates were developed first with chloroform-methanol-ammonium hydroxide-water (140:50:7:3) and then with chloroform-methanol-acetic acid-water (160:20:4:1.5); both solvent mixtures were run in the same direction. Phospholipid spots were detected with iodine vapors and identified by comparison with standards of pure phosphatidylcholine and *E. coli* K-12 phospholipid extracts (phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin) (40). The identification was confirmed by spraying the plates with one of the following specific mixtures: 3% ninhydrin in acetone, 10% phosphomolybdic acid in ethanol, or 0.0012% Rhodamine 6G in deionized water (31). To estimate the relative amounts of phospholipid species, the corresponding spots were scraped off the plates; the powder was extracted with chloroform; and after drying under nitrogen, total phosphorus was determined by the method described by Bartlett (1).

The fatty acids of total phospholipid extracts were analyzed by gas-liquid chromatography after the corresponding methyl esters were prepared by the method recommended by Moss et al. (46). The analysis was performed in an apparatus with a flame ionization detector (Sigma 300; Perkin-Elmer Ltd., Beaconsfield, England) equipped with a stainless steel Supelcoport (100/120 mesh) 3% SP-2,100 column (Supelco Inc., Bellefonte, Pa.) and a chromatograph data station (Sigma-15; Perkin-Elmer). A 150 to 230°C temperature gradient was used in the analysis. Fatty acids were tentatively identified by comparison of the retention times of the corresponding methyl esters with those of commercial standards (Qualmix M, and bacterial fatty acid methyl esters mixture; Supelco). Equivalent chain lengths (ECLs) were calculated as described by Miyagawa and Suto (43).

Enzyme assays. Succinate dehydrogenase and NADH oxidase were used as IM markers and assayed as described by Johnston and Gotschlich (29) and Osborn et al. (48), respectively. The cytosol marker malic dehydrogenase was assayed by the method described by Smith and Winkler (53).

Electron microscopy. For electron microscopy, the FMMs were sedimented by ultracentrifugation as described above, and the pellet was taken up in a small amount of deionized distilled water. To obtain thin sections, a sample of this suspension was mixed thoroughly with an equal volume of 3% Noble agar (Difco Laboratories, Detroit, Mich.) that had been previously melted and equilibrated at 45°C. After gelling the resulting block of agar was cut into fragments of about 1 mm^3 which were then fixed for 1 h at 0°C with fresh 0.6% $NaMnO_4$ (39) in 340 mM Veronal (pH 7.4). The fixed material was included in Epon 812, and thin sections (20 to 40 nm) were obtained and stained with uranyl acetate and lead hydroxide by standard procedures. A microscope (EM 10C; C. Zeiss, Oberkochen, Federal Republic of Germany) was used to examine the specimens.

Immunological methods. Anti-lipopolysaccharide (LPS) serum samples were obtained from rabbits that had been inoculated 1 month previously with either *B. melitensis* 16M or *B. melitensis* 115. By immunoelectrophoresis with soluble protein extracts and LPS, these serum samples mostly contained antibodies to the corresponding R or S LPS. Antibodies to *B. melitensis* 115 FMM were raised in rabbits by intramuscular injection of five doses of 600 μg in Freund

incomplete adjuvant which were administered at weekly intervals. A serum sample to *B. abortus* 45/20 OM lipoprotein was generously provided by M. J. Gómez-Miguel; this serum cross-reacts with OM lipoproteins from *B. melitensis* and *B. ovis* (22).

For enzyme-linked immunosorbent assays (ELISAs) the FMM from *B. melitensis* was coupled to polystyrene plates in carbonate buffer, and the assay was performed as described elsewhere (60) with peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG; Nordic Laboratories, Tilburg, The Netherlands). Western blots were performed by the method described by Burnette (3) with the same conjugate and with 4-chloro-1-napthtol as the substrate (24). Coagglutination with protein A-containing staphylococci was performed as described by Kronwall (33).

Other analytic methods. Total protein was determined by the method described by Lowry et al. (38), with bovine serum albumin as the standard. KDO was determined by the thiobarbituric acid method described by Warren (61) and the interference due to 2-deoxysugars corrected as described before (44) by including both pure KDO (Sigma Chemical Co., St. Louis, Mo.) and deoxyrribose as standards; when LPS was estimated by measuring KDO, the corresponding LPS extracted as described elsewhere (17, 35) was used as the standard. Total fatty acid content was determined colorimetrically (23) with tripalmitin as the standard.

SDS-PAGE was performed in 12.5% acrylamide slabs with the discontinuous buffer system described by Laemmli (34) and gels stained either with Coomassie blue (15) or by the alkaline silver method described by Merrill et al. (42). Molecular weight markers were phosphorylase *b* (94,000), BSA (67,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (21,000), and lysozyme (14,000). In addition, OmpF (molecular weight, 38,000) extracted from the CE of *E. coli* B by the method described by Rosenbusch (49) was used as the standard.

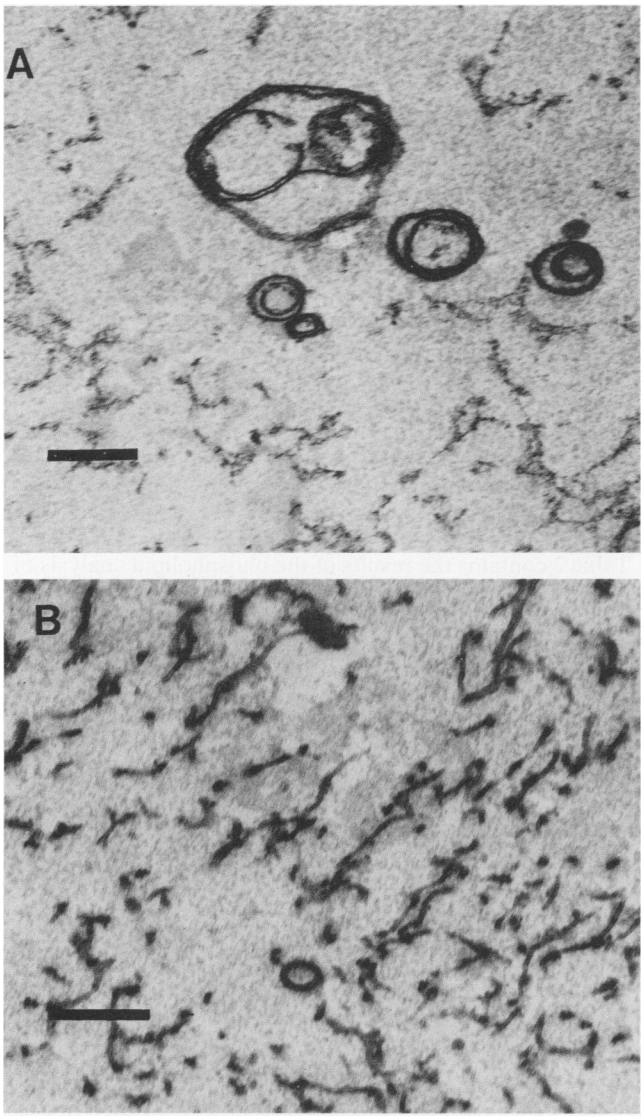

FIG. 1. Electron micrographs of the materials present in the supernatants of exponentially growing cultures of *B. melitensis* 16M. (A) Thin section of the material sedimenting at 40,000 × *g*; (B) thin section of the material remaining in the supernatant of the 40,000 × *g* centrifugation and sedimented at 100,000 × *g*. Bars, 100 nm.

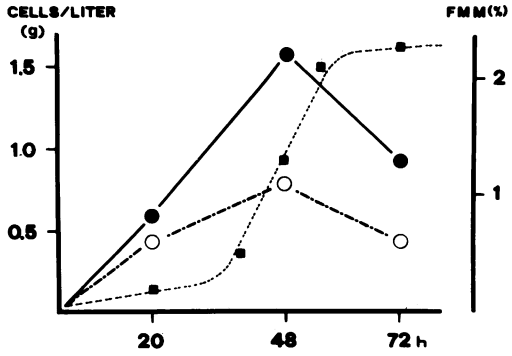

FIG. 2. Growth curve and release of FMM. *B. melitensis* 16M was grown in Trypticase soy broth. After the indicated times of incubation, the cells (■) were harvested, FMM-40,000 × *g* (○) and FMM-100,000 × *g* (●) were collected by ultracentrifugation, and dry weights were determined after lyophilization. The FMM yield is expressed as the ratio (FMM dry weight/cell dry weight) × 100.

RESULTS

When culture supernatants of *B. melitensis* 16M were tested by coagglutination, a strong reaction was observed with staphylococci sensitized with sera from infected rabbits (mostly anti-S LPS) but not with staphylococci sensitized with nonimmune sera. Such coagglutination persisted after the supernatants were filtered (pore size, 0.22 μm; Millipore Corp., Bedford, Mass.) but not after ultracentrifugation, and similar results were obtained with *B. melitensis* 115 supernatants and sera from rabbits infected with the homologous strain. In contrast, negative or very weak coagglutination reactions were obtained with the supernatants of the two *B. abortus* strains that were tested.

Ammonium sulfate precipitation of the supernatants of *B. melitensis* yielded a material which, on chemical analysis (Table 1), showed the presence of LPS (estimated by KDO), protein, and amounts of fatty acids that could not be accounted for by the fatty acid content of the LPS. A material containing these same components could also be obtained by ultracentrifugation of the culture supernatants (FMM; Table 1). By differential ultracentrifugation, this same FMM was separated into two different fractions. The first one sedimented at 40,000 × *g* (FMM-40,000 × *g*; 2 h),

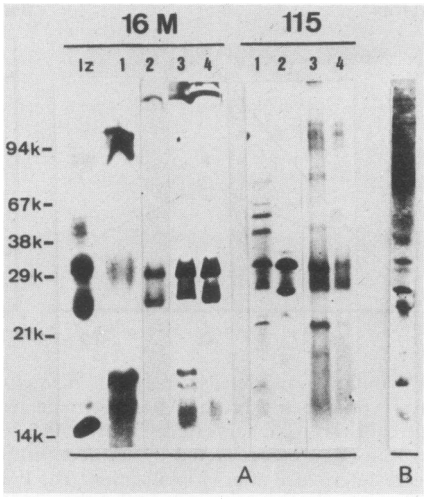

FIG. 3. (A) SDS-PAGE analysis of lysozyme digests of the Sarkosyl-Zwittergent-resistant fraction of the CE of *B. melitensis* 16M (lz), Sarkosyl-Zwittergent extracts of cell envelopes (lanes 1), ammonium sulfate precipitates of culture supernatants (lanes 2), FMM-40,000 × *g* (lane 3), and FMM-100,000 × *g* (lanes 4), of *B. melitensis* 16M and *B. melitensis* 115. (B) Western blot analysis of *B. melitensis* 115 CE Sarkosyl-Zwittergent extracts with serum to homologous FMM.

and the second fraction required for sedimentation at least 100,000 × *g* for 6 h. The overall chemical composition of these two fractions, however, was not significantly different (Table 1); both contained LPS, fatty acids, and protein. With respect to the presence of IM enzymatic markers, neither NADH oxidase activity nor succinic dehydrogenase activity was found in the FMM (Table 1). Furthermore, negative results were also obtained when the assays were performed immediately after brief sonication of the FMM, thus ruling out the presence of inverted vesicles in which the enzymes would be cryptic. In contrast, CE controls that were obtained from the same cultures and processed simultaneously contained both NADH oxidase and succinic dehydrogenase (Table 1).

Electron microscopy of thin-sectioned FMMs showed that it consisted of unit membrane structures. Results of electron microscopy also confirmed the different size of the fragments obtained by differential ultracentrifugation (Fig. 1). When the rate of appearance of FMM in the medium during growth was studied, it was found that whereas the ratio FMM dry weight:dry weight of cells increased steadily during the logarithmic phase of growth, it began to decrease by the stationary phase (Fig. 2). Analysis of culture supernatants for cytosol leakage (malic dehydrogenase activity) was negative throughout the experiment.

SDS-PAGE analysis of the FMM and Sarkosyl-Zwittergent CE extracts revealed that both materials contained two major proteins (molecular weights, 30,000 and 25,000) and several minor proteins (molecular weights, 84,000, 22,000, and 18,000) with similar apparent molecular weights (Fig. 3A; note that the OM proteins of strain 16M are represented by the combined profiles of the fractions obtained before and after lysozyme treatment). Group 2 (molecular weight, 35,000 to 40,000) OM major proteins was not found in the FMM of either *B. melitensis* strain by SDS-PAGE, even when the more sensitive silver staining method was used (data not shown). In addition, the anti-lipoprotein serum failed to react with the FMM of *B. melitensis* 115 in ELISA, despite the fact that the CE of this strain reacted strongly with the same serum sample (Fig. 4). Similar negative results were obtained by Western blot analysis with anti-lipoprotein serum (data not shown). Finally, the identity between the major proteins in the FMM and their molecular weight counterparts in Zwittergent extracts was confirmed by Western blot analysis with serum samples from rabbits that were hyperimmunized with the FMM of the R strain (Fig. 3B).

Table 2 contains the results of the phospholipid analysis of the FMM and the CE. Phosphatidylcholine, phosphatidylethanolamine, and cardiolipin were the major phospholipids in both CE and FMM, a result that is consistent with the analysis of total lipid extracts of whole cells (57). The relative proportions of the major phospholipids, however, were different in the CE and FMM, the latter being clearly

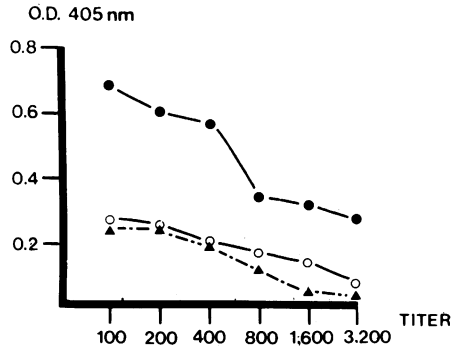

FIG. 4. Analysis by ELISA of the presence of the lipoprotein in the FMM of *B. melitensis* 115 (○) and cell envelopes of the same strain (●). Both antigens were bound to the same plate at the same concentration (10 μg/ml) and under the same conditions. LPS (▲) was used as the negative control. OD, optical density.

TABLE 2. Phospholipids in CEs and FMM of *B. melitensis* 16M[a]

Fraction studied	% Total phosphorus in:				
	CL	PE	PC	Other	(PE-PC)/CL
CE	25.1	25.1	24.5	25.3	1.98
FMM-40,000 × *g*	18.9	20.1	45.9	15.1	3.49
FMM-100,000 × *g*	14.9	19.4	45.9	19.8	4.38

[a] Abbreviations: CL, cardiolipin; PE, phosphatidylethanolamine; PC, phosphatidylcholine.

TABLE 3. Phospholipid fatty acid composition of CEs and FMM obtained from exponentially growing *B. melitensis* 16M and 115[a]

Strain and fraction	% Fatty acid								
	12:0	14:0	16:1	16:0	18:1	18:0	Unknown[b]	19cyc[c]	Unknown[d]
16M									
CE	1.9	7.7	2.1	20.4	24.3	18.6	3.9	7.9	10.2
FMM	4.7	5.6	1.3	20.0	22.8	17.4	4.4	8.4	12.2
115									
CE	0.4	0.9	0.8	8.9	26.0	21.2	5.6	11.5	23.2
FMM	2.7	3.1	1.3	23.8	35.4	18.0	3.1	5.5	6.5

[a] Values were determined by gas-liquid chromatography; unknown fatty acids present in amounts of less than 5% of the total fatty acid are not included.
[b] Unknown with an ECL of 18.3.
[c] 19:0 cyclopropane.
[d] Unknown with an ECL of 19.2.

enriched in phosphatidylcholine. Most of the phosphatidylcholine increase in the FMM correlated with a decrease in cardiolipin content (Table 2).

Gas-liquid chromatography analysis showed that palmitic (16:0), stearic (18:0), oleic (18:1), and lactobacillic (19:0 cyclopropane) acids were the major fatty acids in both CE and FMM *B. melitensis* 16M (Table 3), a result that is in good agreement with those reported by others for *B. melitensis* whole cells (7, 28, 56). These same fatty acids were identified in CE and FMM obtained from the R strain but, in contrast with the results obtained with the S strain, the proportions of palmitic acids, oleic acid, lactobacillic acid, and the unknown fatty acid with an ECL of 19.2 were different in the CE and the FMM.

DISCUSSION

Large amounts of membranous material (FMM) were present in the supernatants of cultures of R and S strains of *B. melitensis*. This material contained LPS, phospholipids, and several OM proteins but lacked the IM markers that were investigated. Thus, although both group 2 proteins and lipoprotein could not be detected, the evidence obtained demonstrates that such FMM is made up of OM fragments. Furthermore, the kinetics of appearance in the growth media showed that FMM is unlikely to result from cell lysis because it accumulated only during the exponential phase of growth, and leakage of malic dehydrogenase activity was not detected. Fragments of CE blebbing off *B. melitensis* 115 cells have been observed by Dubray (13) by electron microscopy of thin sections, and they should correspond to the FMM described here. Indeed, release of OM fragments has been reported for other gram-negative bacteria (4, 9, 18, 19, 25, 26, 32, 36, 37, 41, 47, 50, 55, 64). This phenomenom, however, did not occur in either *B. abortus* 19 or *B. abortus* 45/20, at least to the extent that it could be detected by coagglutination.

Two FMM fractions with different sedimentation properties were separated by sequential ultracentrifugation, and electron microscopy confirmed both their membranous structure and their different sizes. Gankema et al. (18) have shown that the OM blebs released by enteropathogenic strains of *E. coli* can also be fractionated by ultracentrifugation into two fractions that differ both in size and chemical composition. Our results, however, did not demonstrate significant differences in the composition of FMM-40,000 × *g* and FMM-100,000 × *g*. These same data indicate that the smaller FMM fraction cannot be considered free LPS, as it could be suggested by the ultracentrifugation conditions required for its sedimentation. Because the large membrane fragments found in FMM-40,000 × *g* would not be expected to be released without extensive cell damage, it is possible that FMM-40,000 × *g* is the result of the reaggregation of the smaller FMM-100,000 × *g* fragments. This interpretation is consistent with the fact that the ratio FMM-100,000 × *g*/cells increased faster than FMM-40,000 × *g*/cells during the exponential growth phase and that the opposite was true during the stationary phase.

The apparent molecular weight of the major proteins present in the FMMs were 30,000 and 25,000, and accordingly, they should belong to group 3 *Brucella* OM proteins (59). Santos et al. (51) have shown that, as compared with *B. abortus*, the OM of *B. melitensis* contains more group 3 proteins than groups 1 or 2 when examined by the Sarkosyl-Zwittergent method. Although this observation could explain in part why group 3 proteins were also the most important proteins in *B. melitensis* FMM, the fact that group 2 proteins could not be detected indicates that the FMMs were not representative of the whole of the OM protein. Quantitative differences in the protein profiles of OM blebs and isolated OM have also been described in other gram-negative bacteria (18, 25, 36, 37), and they relate probably to the mechanism underlying their release during exponential growth. It has been suggested that OM blebs represent areas where new material is incorporated into the OM (25), a hypothesis that is supported by the results of pulse-chase experiments (47). A very intense OM synthesis, like the one that takes place during logarithmic growth, could bring about the release of some membrane patches before interactions with other structural elements, such as peptidoglycan-associated proteins or linked lipoprotein, would ensure stability. This hypothesis is consistent with the increased OM blebbing displayed by *E. coli* mutants lacking the Braun lipoprotein (16, 54, 62, 65) and with reports in which it has been shown that *E. coli* OM blebs contain reduced proportions of lipoprotein (free form) with respect to the purified OM (63). The fact that we could not detect the presence of the lipoprotein in the FMMs also supports such a mechanism for the release of OM fragments in *B. melitensis*. Because group 2 (peptidoglycan-associated) *Brucella* OM could not be detected in the FMMs, we must postulate either that they become stabilized more rapidly, perhaps by association with the peptidoglycan, or else that they are not incorporated into the OM at the same points as the proteins detected in OM blebs.

It was interesting that, in comparison with the CE, the FMMs were enriched in phosphatidylcholine. Even though the FMMs were not representative of the OM protein, they could be representative of the OM phospholipid. In *E. coli* newly formed lipid molecules diffuse in the membrane much faster than the protein components of transport systems (58), and there is also evidence that the major proteins diffuse very little once they are incorporated into the OM (8). Likewise, it has been shown that the lateral diffusion of both LPS and phospholipid in reconstituted *Salmonella typhimurium* membranes is over 1,000 times more rapid than that of LamB (52). Thus, the FMM is more likely to represent the average membrane lipid than the average membrane protein. Finally, it has been shown that the OM of several gram-negative bacteria is enriched in phosphatidylethanolamine, the major phospholipid in their CEs (11, 27, 40). The interpretation that the FMM phospholipids represent that of the OM is consistent with those data because the FMM was also enriched in one of the major phospholipids in *B. melitensis* CE (phosphatidylcholine). Phosphatidylcholine occurs very seldom in bacteria (20), and as a membrane constituent it has properties that are different from those of phosphatidylethanolamine (6), the usual major phospholipid in bacterial membranes (20). Although much research is necessary to understand the significance of the presence of phosphatidylcholine, it could relate to some of the unusual characteristics of *Brucella* CE (44).

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LITERATURE CITED

1. **Bartlett, G. R.** 1959. Phosphorus assay in column chromatography. J. Biol. Chem. **234**:466–468.
2. **Bligh, E. G., and W. J. Dyer.** 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **43**:911–917.
3. **Burnette, W. N.** 1981. Western blotting: electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. **112**:195–203.
4. **Chatarjee, S. N., and J. Das.** 1967. Electron microscopic observations on the excretion of cell-wall material by *Vibrio cholerae*. J. Gen. Microbiol. **49**:1–11.
5. **Chopra, I., and S. W. Chales.** 1980. Comparison of the polypeptide composition of *Escherichia coli* outer membranes prepared by two methods. J. Bacteriol. **144**:425–427.
6. **Cullis, P. R., and B. de Kruijff.** 1979. Lipid polymorphism and functional roles of lipids in biological membranes. Biochim. Biophys. Acta **559**:399–420.
7. **Dees, S., S. Thanabalasundrum, C. W. Moss, D. G. Hollis, and R. E. Weaver.** 1980. Cellular fatty acid composition of group IVe, a nonsaccharolytic organism from clinical sources. J. Clin. Microbiol. **11**:664–668.
8. **de Leig, L., J. Kingma, and B. Witholt.** 1979. Nature of the regions involved in the insertion of newly synthesized protein into the outer membrane of *Escherichia coli*. Biochim. Biophys. Acta **553**:224–234.
9. **Devoe, I. W., and J. E. Gilchrist.** 1973. Release of endotoxin in the form of cell wall blebs during in vitro growth of *Neisseria meningitidis*. J. Exp. Med. **138**:1156–1167.
10. **Díaz, R., L. M. Jones, and J. B. Wilson.** 1967. Antigenic relationship of *Brucella ovis* and *Brucella melitensis*. J. Bacteriol. **93**:1262–1268.
11. **Diedrich, D. L., and E. H. Cota-Robles.** 1974. Heterogeneity in lipid composition of the outer membrane and cytoplasmic membrane of *Pseudomonas* BAL-31. J. Bacteriol. **119**:1006–1018.
12. **Douglas, J. T., E. Y. Rosenberg, H. Nikaido, D. R. Verstreate, and A. J. Winter.** 1984. Porins of *Burcella* species. Infect. Immun. **44**:37–41.
13. **Dubray, G.** 1972. Etude estructurale des bacteries de colonies lisses (S) et rugueuses (R) du genre *Brucella*. Ann. Inst. Pasteur (Paris) **123**:171–193.
14. **Dubray, G.** 1976. Localisation cellulaire des polyosides des bacteries des genres *Brucella* et *Escherichia* en phase lisse (S) or rugueuse (R). Ann. Inst. Pasteur Microbiol. **127**:133–149.
15. **Fairbanks, G., T. L. Steck, and D. F. Wallach.** 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry **10**:2606–2617.
16. **Fung, J., T. J. MacAlister, and L. I. Rothfield.** 1978. Role of murein lipoprotein in morphogenesis of the bacterial division septum: phenotypic similarity of *lykD* and *lpo* mutants. J. Bacteriol. **133**:1467–1471.
17. **Galanos, C., O. Lüderitz, and O. Westphal.** 1969. A new method for extraction of R-lipopolysaccharides. Eur. J. Biochem. **9**: 245–249.
18. **Gankema, H., J. Wenski, P. A. M. Guinee, W. H. Jansen, and B. Witholt.** 1980. Some characteristics of the outer membrane material released by growing enterotoxigenic *Escherichia coli*. Infect. Immun. **29**:704–713.
19. **Gmeiner, J., and S. Schlecht.** 1979. Molecular organization of the outer membrane of *Salmonella typhimurium*. Eur. J. Biochem. **93**:609–620.
20. **Goldfine, N.** 1984. Bacterial membranes and lipid packing theory. J. Lipid Res. **25**:1501–1507.
21. **Gómez-Miguel, M. J., and I. Moriyón.** 1986. Demonstration of a peptidoglycan-linked lipoprotein and characterization of its trypsin fragment in the outer membrane of *Brucella* spp. Infect. Immun. **53**:678–684.
22. **Gómez-Miguel, M. J., I. Moriyon, and J. Lopez.** 1987. *Brucella* outer membrane lipoprotein shares antigenic determinants with *Escherichia coli* Braun lipoprotein and is exposed on the cell surface. Infect. Immun. **55**:258–262.
23. **Haskins, W. T.** 1961. Spectrophotometric determination of fatty acid amide in lipids. Anal. Chem. **33**:1445–1446.
24. **Hawkes, R., E. Niday, and J. Gordon.** 1982. A dot-immunobinding assay for monoclonal and other antibodies. Anal. Biochem. **119**:142–147.
25. **Hoekstra, D., J. W. Van der Laan, L. de Leij, and B. Witholt.** 1976. Release of outer membrane fragments from normally growing *Escherichia coli*. Biochim. Biophys. Acta **455**:889–899.
26. **Holt, S. C., A. C. R. Tanner, and S. S. Socransky.** 1980. Morphology and ultrastructure of oral strains of *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus*. Infect. Immun. **30**:588–600.
27. **Ishinaga, M., R. Kanamoto, and M. Kito.** 1979. Distribution of phospholipid molecular species in outer and cytoplasmic membranes of *Escherichia coli*. J. Biochem. **86**:161–165.
28. **Jantzen, E., E. Knudsen, and R. Winsnes.** 1982. Fatty acid analysis for differentiation of *Bordetella* and *Brucella* species. Acta Pathol. Microbiol. Immun. Scand. Sect. B **90**:353–359.
29. **Johnston, K. H., and E. Gotschlich.** 1974. Isolation and characterization of the outer membranes of *Neisseria gonorrhoeae*. J. Bacteriol. **119**:250–257.
30. **Jones, L. M., R. Diaz, and D. T. Berman.** 1976. Endotoxic activity of rough organisms of *Brucella* species. Infect. Immun. **13**:1638–1641.
31. **Kates, M.** 1972. Isolation analysis and identification of lipids, p. 435–441. *In* Techniques in lipidology. Elsevier/North-Holland, Amsterdam.
32. **Knox, K. W., J. Cullen, and E. Work.** 1967. An extracellular lipopolysaccharide-phospholipid-protein complex produced by *Escherichia coli* grown under lysine-limiting conditions. Biochem. J. **103**:192–201.
33. **Kronwall, G.** 1973. A rapid slide-agglutination method for typing pneumococci by means of specific antibody adsorbed to protein A-containing staphylococci. J. Med. Microbiol. **6**:187–190.
34. **Laemmli, U. K.** 1970. Cleavage of structural proteins during assembly of bacteriophage T4. Nature (London) **227**:680–685.
35. **Leong, D., R. Díaz, K. Milner, J. Rudbach, and J. B. Wilson.** 1970. Some estructural and biological properties of *Brucella* endotoxin. Infect. Immun. **1**:174–182.
36. **Loeb, M. R., and J. Kilner.** 1978. Release of a special fraction of the outer membrane from both growing and phage T4-infected *Escherichia coli* B. Biochim. Biophys. Acta **514**:117–127.
37. **Logan, S. M., and T. J. Trust.** 1982. Outer membrane of *Campylobacter jejunii*. Infect. Immun. **38**:898–904.
38. **Lowry, O. H., N. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
39. **Luft, J. H.** 1956. Permanganate: a new fixative for electron microscopy. J. Biophys. Biochem. Cytol. **2**:799–801.
40. **Lugtenberg, B., and R. Peters.** 1976. Distribution of lipids in cytoplasmic and outer membranes of *Escherichia coli* K12. Biochim. Biophys. Acta **441**:38–47.
41. **MacIntyre, S., T. J. Trust, and J. T. Buckley.** 1980. Identification and characterization of outer membrane fragments released by *Aeromonas* sp. Can. J. Biochem. **10**:1018–1025.
42. **Merrill, C. R., R. C. Switzer, and M. L. Van Keuren.** 1979. Trace polypeptides in cellular extracts and human body fluids detected by two-dimensional electrophoresis and a highly sensitive silver stain. Proc. Natl. Acad. Sci. USA **76**:4335–4339.
43. **Miyagawa, E., and T. Suto.** 1980. Cellular fatty acid composition in *Bacteroides oralis* and *Bacteroides ruminicola*. J. Gen. Appl. Microbiol. **26**:331–343.
44. **Moriyón, I., and D. T. Berman.** 1982. Effects of nonionic, ionic and dipolar ionic dergents and EDTA on the *Brucella* cell envelope. J. Bacteriol. **152**:822–828.
45. **Moriyón, I., and D. T. Berman.** 1983. Isolation, purification and partial characterization of *Brucella abortus* matrix protein. Infect. Immun. **39**:394–402.
46. **Moss, C. W., M. A. Lambert, and W. H. Merwin.** 1974. Comparison of rapid methods for analysis of bacterial fatty acids. Appl. Microbiol. **28**:80–85.

47. **Mug-Opstelten, D., and B. Witholt.** 1978. Preferential release of new outer membrane fragments by exponentially growing *Escherichia coli*. Biochim. Biophys. Acta **508**:287–295.
48. **Osborn, M. J., J. E. Gandner, E. Parisi, and J. Carson.** 1972. Mechanism of assembly of the outer membrane of *Salmonella typhimurium*. Isolation and characterization of the cytoplasmic and outer membrane. J. Biol. Chem. **247**:3962–3972.
49. **Rosenbusch, J. P.** 1974. Characterization of the major envelope protein from *Escherichia coli*. J. Biol. Chem. **249**:8019–8029.
50. **Rothfield, L., and M. Pearlman-Kothencz.** 1969. Synthesis and assembly of bacterial membrane components. A lipopolysaccharide-phospholipid-protein complex excreted by living bacteria. J. Mol. Biol. **44**:477–492.
51. **Santos, J. M., D. R. Verstreate, V. Y. Perera, and A. J. Winter.** 1984. Outer membrane proteins from rough strains of four *Brucella* species. Infect. Immun. **46**:188–194.
52. **Schindler, M., M. J. Osborn, and D. E. Koppel.** 1980. Lateral mobility in reconstituted membranes: comparison with diffusion in polymers. Nature (London) **283**:346–350.
53. **Smith, D. K., and H. H. Winkler.** 1979. Separation of inner and outer membranes of *Rickettsia prowazeki* and characterization of their polypeptide composition. J. Bacteriol. **137**:963–971.
54. **Sonntag, I., H. Schwartz, Y. Hirota, and U. Henning.** 1978. Cell envelope and shape of *Escherichia coli*: multiple mutants missing the outer membrane lipoprotein and other major outer membrane proteins. J. Bacteriol. **136**:280–285.
55. **Stirling, P., and S. J. Richmond.** 1980. Production of outer membrane blebs during chlamydial replication. FEMS Microbiol. Lett. **9**:103–105.
56. **Thiele, O. W., C. Lacave, and J. Asselineau.** 1969. On the fatty acids of *Brucella abortus* and *Brucella melitensis*. Eur. J. Biochem. **7**:393–396.
57. **Thiele, O. W., and G. Schwinn.** 1973. The free lipids of *Brucella melitensis* and *Bordetella pertussis*. Eur. J. Biochem. **34**:333–344.
58. **Thilo, L., and P. Overath.** 1976. Randomization of membrane lipids in relation to transport system assembly in *Escherichia coli*. Biochemistry **15**:328–334.
59. **Verstreate, D. R., M. T. Creasy, N. T. Caveney, C. L. Baldwin, M. W. Blab, and A. J. Winter.** 1982. Outer membrane proteins of *Brucella abortus*: isolation and characterization. Infect. Immun. **35**:979–989.
60. **Voller, A.** 1975. A microplate method of enzyme-linked immunosorbent assay and its application to malaria. Bull. W.H.O. **51**: 209–211.
61. **Warren, L.** 1959. The thiobarbituric acid assay of sialic acids. J. Biol. Chem. **245**:1971–1975.
62. **Weigand, R. A., K. D. Vinci, and L. I. Rothfield.** 1976. Morphogenesis of the bacterial division septum: a new class of septation-defective mutants. Proc. Natl. Acad. Sci. USA **73**:1882–1886.
63. **Wensik, J., and B. Witholt.** 1981. Outer-membrane vesicles released by normally growing *Escherichia coli* contain very little lipoprotein. Eur. J. Biochem. **116**:331–335.
64. **Wood, D. D. L., S. C. Holt, and E. R. Leadbetter.** 1979. Ultrastructure of *Bacteroides* species: *Bacteroides asacharolyticus*, *Bacteroides fragilis*, *Bacteroides melaninogenicus*, subspecies *melinogenicus* and *Bacteroides melaninogenicus*, subspecies *intermedius*. J. Infect. Dis. **139**:534–546.
65. **Yem, D. W., and H. C. Wu.** 1978. Physiological characterization of a *Escherichia coli* mutant altered in the structure on murine lipoprotein. J. Bacteriol. **133**:1419–1426.