RESEARCH ARTICLES

Ignacio Moriyón
Ignacio López-Goñi

Departamento de Microbiología,
Universidad de Navarra, Pamplona, Spain

Structure and properties of the outer membranes of *Brucella abortus* and *Brucella melitensis*

Summary The brucellae are Gram-negative bacteria characteristically able to multiply facultatively within phagocytic cells and which cause a zoonosis of world-wide importance. This article reviews the structure and topology of the main components (lipopolysaccharide, native hapten polysaccharide, free lipids and proteins) of the outer membranes of *Brucella abortus* and *B. melitensis*, as well as some distinctive properties (permeability and interactions with cationic peptides) of these membranes. On these data, an outer membrane model is proposed in which, as compared to other Gram-negatives, there is a stronger hydrophobic anchorage for the lipopolysaccharide, free lipids, porin proteins and lipoproteins, and a reduced surface density of anionic groups, which could be partially or totally neutralized by ornithine lipids. This model accounts for the permeability of *Brucella* to hydrophobic permeants and for its resistance to the bactericidal oxygen-independent systems of phagocytes.

Correspondence to:
Ignacio Moriyón.
Departamento de Microbiología.
Universidad de Navarra.
Apartado 177. 31080 Pamplona. Spain.
Tel.: +34-948425600. Fax: +34-948425649.
E-mail: imoriyon@unav.es

Introduction

Brucellosis is still one of the most common bacterial zoonosis in the world and an important cause of human suffering and economical losses. This disease is caused by several species of the genus *Brucella*, an homogeneous group of facultative intracellular pathogens belonging to the α-2 subdivision of the Proteobacteria [30]. Accordingly, the brucellae are Gram-negative eubacteria endowed with cell envelopes (CE) composed of inner and outer membranes (OM) enclosing a periplasm with a peptidoglycan (PG) mesh and soluble components [15]. However, the brucellae do not grow in several selective media in which classical Gram-negative bacteria grow, show acid-fastness by the Stamp's modification of the Ziehl-Neelsen method, and are resistant to classical procedures of periplasmic extraction. These simple observations hint at differences between the CE (and OM) of brucellae and those of other Proteobacteria usually taken as models of the Gram-negative cell. Since the OM and adjacent layers are in interplay with the environment, the bases for those differences should be relevant in the relationship between the brucellae and their hosts and could relate, at least in part, to their peculiar intracellular parasitism. This review is focused on the OM features of *B. melitensis* and *B. abortus*, two of the nominal species distinguished within the genus by their surface features and preferential host range, and which have been studied in more detail because of their greater importance in animal and human health.

The smooth lipopolysaccharide

The characterization of phenol-water and ether-water extracts combined with immunoabsorption experiments [11, 36] and electron microscopy observations [15] soon established the presence of an endotoxic smooth (S)-type lipopolysaccharide (LPS) in the surface of S brucellae. Moreover, these early studies also suggested the presence in the CE of a second polysaccharide [the native hapten (NH)] antigenically related to the S-LPS [11]. Although it has been known since then that these molecules carry the serologically dominant antigenic determinants of the cell surface, their characterization is not complete. For the S-LPS, this would mean the elucidation of its main sections: lipid A, oligosaccharide core, and O-chain polysaccharide.
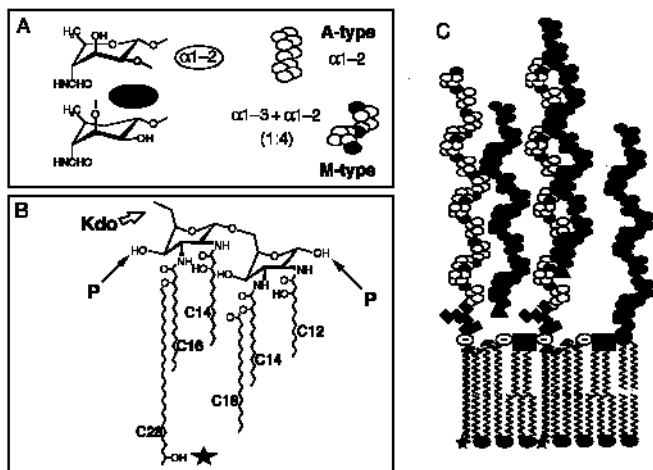
Fig. 1 Non proteic components of the OM of *Brucella melitensis* and *Brucella abortus*. (A) N-formyl-perosamine showing the positions of the inter sugar linkages and oligosaccharide models showing the effect of absence (*B. abortus* biotype 1, A-type O-chain) or presence (*B. melitensis* biotype 1, M-type O-chain) of the α-1,3 linkage. (B) Tentative structure of the *B. abortus* lipid A with the position of possible ester linked phosphates and of the linkage to the Kdo; microheterogeneity could arise from variations in phosphate and acyl-chain substitution. (C) Model showing the packing of LPS, NH, and free lipids in the OM; the O-chain represented would be of the M type (see panel A) and the core is represented as branched to illustrate a hypothetical hindrance to polycations; symbols with – and + in the outer leaflet denote the negatively charged groups (Kdo and phosphates) and cationic heads of ornithine lipids, respectively; for clarity, not all acyl chains of the LPS are represented; the ☆ symbol marks the likely position of the iso hydroxyl group of C28 acyl chains (thicker lines); although the O-chain and NH have the same overall chemical structure, the latter is represented in gray tones, with a s marking the hypothetical sugar of the reducing end or with a hypothetical linkage to an acyl chain carrying molecule (●) (the relative proportions of LPS and NH are unknown); phospholipids (mostly phosphatidylcholine) are depicted only in the inner leaflet of the OM but their topology has not been studied

The Lipid A There is only partial agreement among the lipid A structures that can be derived from recent chemical analyses. Two studies have reported a backbone disaccharide of 2,3-diamino-2,3 dideoxy-D-glucose in β-1,6 linkages [35, 47]. In this diaminoglucose disaccharide, the β-hydroxy fatty acids (C12, C14 and C16) are amide bound, and ester-linked fatty acids are found only in acyl-oxyacyl groups and have acyl chains equal to or longer than C16. According to two studies, they include 27OH-C28:0 and traces of 29OH-C30:0 [30, 47], and preliminary analysis of materials obtained by mild hydrolysis show also 27keto-C28:0 and traces of 29keto-C30:0 fatty acids. Lipid A disaccharides are most often substituted with phosphate, and 1 or 2 orthophosphate molecules in the *B. abortus* GlcN3N-GlcN3N disaccharide has been reported [35, 47]. Although the composition of the sugar backbone seems constant, there is lipid A heterogeneity which possibly relates to the degree of phosphorylation and acyl substitution [17, 47]. Ethanolamine and arabinosamine, often present in the lipid A of enteric bacteria, have not been found. A tentative structure of the *B. abortus* lipid A is presented in Fig. 1B.

The Core oligosaccharide The structure of the LPS core oligosaccharide has not been elucidated in any *Brucella* species. Mannose and 2-amino-2,6-dideoxy-D-glucose (quinovosamine) are components of the core [17]. In addition, there is 3-deoxy-D-manno-2-octulosonate (Kdo), possibly in two types of substitution but with no phosphate [47]. L-glycero-D-manno-heptose, the sugar usually linked to Kdo, is absent. There is also evidence for 2-amino, 2-deoxy-D-glucose (not detected in the lipid A). In all likelihood, Kdo is bound to the nonreducing sugar of the lipid A disaccharide (Fig. 1B), and quinovosamine could bridge the core to the O-chain. Core heterogeneity in some spontaneous rough (R) mutants has also been reported [17]. The absence of phosphate is remarkable because Kdo often carries phosphate groups which contribute to the overall negative charge of the lipid A-core region.

The O-chain polysaccharide Controlled acid hydrolysis of S-LPS yields a polysaccharide which, in *B. abortus* biotype 1, is constituted by an unbranched homopolymer (the O-chain polysaccharide) of α-1,2-linked 4-formamido-4,6-dideoxy-D-mannose [N-formyl-perosamine (Fig. 1A)] [34] plus residual core sugars. In *B. melitensis* biotype 1, the O-chain polysaccharide consists of repeating blocks of five N-formyl-perosamine residues, four α-1,2-linked and one α-1,3-linked (Fig. 1A). Like in other S Gram-negative bacteria, it shows molecular weight heterogeneity within a given strain, and heterogeneity in average length among strains has been shown [2]. From ^{13}C-NMR (nuclear magnetic resonance) and sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) analyses, the average number of monosaccharides has been estimated in 96 for *B. abortus* 1119-3 [34] From data obtained by high performance gel permeation [1], 130 to 35 and 92 to 25 sugar residues can be calculated for *B. melitensis* 16M and *B. abortus* 2308, respectively.

The relative frequency of α-1,2 and α-1,3 linkages explains the epitopic structure of the O-chain. Most authors accept the Douglas and Palmer [13] modification of the A + M antigenic structure of Wilson and Miles, according to which O-chains of biotype 1 *B. abortus* and *B. melitensis* carry exclusively A or M epitopes, respectively, plus a common C epitope(s) accounting for the cross-reactivity. Like *B. melitensis* biotype 1, serologically intermediate biotypes (AM) also have the α-1,3 linkage but in reduced proportions. Thus, in common with *B. abortus* biotype 1, the O-chain of these biotypes have stretches of more than five sugars continuously linked in α-1,2. Because the α-1,3 linkages kink the otherwise homogeneous (α-1,2 N-formyl-perosamine polymer (Fig. 1), the M and AM O-chain polysaccharides can be predicted to establish looser interchain linkages (hydrogen and, perhaps, hydrophobic bonds) in the outer polysaccharide layer of the OM. This explains the greater diffusion of the aggregates of the M-type LPS in gel precipitation tests [37] and could also cause subtle physical differences in the surface of the A, AM, and M serotypes.

The native hapten polysaccharides

These haptenic polysaccharides were first defined by immunoprecipitation of extracts from S brucellae [11], and are found in the literature under a variety of names (fast diffusing component, first component, second polysaccharide and native hapten). A serologically related component present in the R mutant *B. melitensis* 115 was originally named polysaccharide B [1].

NH and polysaccharide B have been the subject of controversy. Using *B. melitensis* 16M extracts some authors have equated the polysaccharide B with some serologically inactive cyclic 1,2-β-glucans [4, 34] similar to the periplasmic glucans of several Rhizobiaceae [3, 30]. Moreover, NH have been considered as mere O-chain-core polysaccharide fragments resulting from a hypothetical hydrolysis occurring during LPS extraction [34]. However, the name polysaccharide B cannot be applied to those cyclic glucans without disregard of the original serological and strain definition, and there is strong and widespread evidence showing that NH do not result from LPS hydrolysis [1]. The existence of O-chain like polysaccharides not linked to core-lipid A in the OM of several Gram-negative bacteria is currently accepted, and it occurs as a result of variations in the standard polymer: lipid A-core ligase reaction (49). Indeed, part of the confusion around NH and polysaccharide B has arisen from the several names used, but also from the oversight of the immunoprecipitation conditions necessary to show their presence [1].

The NH of *B. melitensis* 16M and *B. abortus* 2308 are closely similar to their respective O-chains, both in size and chemical composition (but in the linkage to Kdo carrying oligosaccharides); nevertheless, some aspects remain to be elucidated for their complete characterization. ^{13}C-NMR spectra of serologically and chromatographically pure NH show small signals not corresponding to the N-formyl-perosamine sugars [1]. According to current views, OM homopolymers are synthesized linked to an undecaprenol-PP-N-glucosamine primer by a Wzy-independent pathway [49]. Thus, those signals could correspond to a glucosamine unit linked to the reducing end of NH. Structurally, we have provided evidence supporting a model in which free NH is intertwined with the LPS O-chain and anchored in the OM by polysaccharide to polysaccharide interactions, and there is also evidence for a lipid-linked NH [41, Aragón et al., unpublished] (Fig. 1C). The ratio LPS/NH has not been determined and there could be strain and species differences.

Free lipids

Because of the difficulties in achieving a good physical separation of the OM of *Brucella* [33, 44], there are no specific studies on the free lipids (i.e., those than can be extracted with ethanol-diethyl ether or chloroform-methanol mixtures) of the *Brucella* OM. The free lipid composition of whole *Brucella* cells has been previously reviewed [4], but we discuss here the more relevant aspects. Although the lipid composition depends on medium and age of the culture, it is well known that the major phospholipid in *B. abortus* and *B. melitensis* is phosphatidylcholine [37% of the total phospholipid; others are phosphatidylethanolamine (33%), cardiolipine (20%) and phosphatidylglycerol (10%)]. There is evidence that phosphatidylcholine is enriched in the OM. This is interesting because phosphatidylcholine has structural properties different from those of phosphatidylethanolamine (the main phospholipid in many Gram-negative bacteria) and tends to form more stable bilayers. In addition, *B. abortus* and *B. melitensis* contain neutral lipids, of which the ornithine lipids are remarkable. In those lipids, the ornithine residue is ester-linked to ethylen glycol, and there are two fatty acids, one ester-linked (to the glycol) and other amide-linked. This means that there is one amino group free and, therefore, that the ornithine lipids carry positive charge. The location of these ornithine lipids in the CE is not known, but they are often coextracted with the LPS and a location on the surface of the OM seems possible. An interesting possibility is that they shield the negative charge of the phosphates of the lipid A [26] (Fig. 1C). The dominant fatty acids in both phospholipids and ornithine lipids are C:16 and 19:Ocyclopropane (lactobacillic acid). Although the precise packing is not known, their comparatively longer acyl chains suggest an average acyl-chain membrane span greater in *Brucella* than in classical Gram-negative bacteria.

Outer membrane proteins

General characteristics The major outer membrane proteins (Omp) of *Brucella* were first identified in the 1980s by detergent extraction of CE, and classified according to their apparent molecular mass in SDS-PAGE as group 1 (94 or 88 kDa), group 2 (36–38 kDa), and group 3 (31–34 and 25–27 kDa) (50). Resistance to nonionic detergents and EDTA was noticed in these studies, and a tight interaction of several Omp with the LPS and with the PG was observed. Moreover, it was also shown that group 2 were porins and a lipoprotein similar to *Escherichia coli* Braun's lipoprotein was partially characterized (for a detailed review of these early studies see [44]). In later works, the genes of group 2 and 3 Omp and of several minor Omp have been characterized. There is diversity among the genes of the major Omp at species, biovar and strain level that are of taxonomical and epidemiological interest and that have brought new insights into the evolution of brucellae (for a review see [5]). Although the corresponding genes have been sequenced, the nature and precise role of group 1 proteins remains to be clarified, and we summarize here only those aspects of structural and functional interest of the better known Omp.

Group 2 Two closely linked genes (*omp2a* and *omp2b*) encode porin proteins with a high degree (> 85%) of homology [16]. Interestingly, only Omp2b is expressed in laboratory grown *B. abortus* [25] but, although its expression in *Brucella* has not been observed, Omp2a increases the permeability to maltodextrins when cloned in *E. coli* [25]. As judged by detergent extraction, the ration porin/group 3 Omp is diminished in *B. melitensis* with respect to *B. abortus*, and a further reduction is observed in R mutants of the former species [50]. Association in a trimeric SDS-resistant state (a hallmark of porins) has been shown for group 2 (Omp2b in all likelihood) [50]. Several analytical algorithms coincide in suggesting a more hydrophobic overall structure (Hopp-Woods and Kyte-Doolittle) and a comparatively reduced surface exposure (Karplus-Schulz and Parker) for Omp2b than for the OmpC and OmpF porins of enteric bacteria. Since OmpF and Omp2b are membrane spanning proteins with similar internal pore diameters (see Permeability) and OmpF is larger than Omp2b (362 vs. 308 amino acids), this higher hydrophobicity would be in keeping with the lipid composition and overall OM properties (see Hydrophobicity).

Group 3 Two proteins of 25 and 31 kDa with only 34% of identity are included in group 3 [50] and they are coded for by the *omp25* and *omp31* genes [5]. Based on algorithmic analyses of the predicted amino acid sequences, none of these two proteins would show the hydrophobicity of Omp2b and both would have significantly more surface exposed epitopes. Omp25 shows small identity with *E. coli* OmpA, previously suggested to be the equivalent of group 3, and there is also a large difference in size (113 amino acids). However, although not homologous, it could be that both proteins play a similar or overlapping role in the OM. OmpA interacts with the PG, other Omp and the LPS, and mutants lacking OmpA show considerable membrane instability. At least in part, these properties are related to β-sheet structures which both favor the binding of amphiphiles (like detergents and LPS) and cause the characteristic heat-modifiable electrophoretic migration of OmpA. The same set of features is also present in Omp25. Although *Brucella* porins also carry tightly bound LPS and lipids [32], greater amounts of LPS are observed in the 25 kDa protein of group 3 (Omp25 in all likelihood) after SDS-PAGE [19] and, as previously observed for OmpA, addition of LPS to SDS-denatured *B. abortus* Omp25 restores native conformational epitopes [6]. Moreover, the characteristic heat-modifiable SDS-PAGE migration of OmpA has also been shown for the 25 kDa protein [19] and, although the interactions among *Brucella* Omp have not been studied, an SDS-resistant dimeric state has been observed for group 3 Omp of *B. melitensis* [50]. Finally, a more stringent structural constrain in Omp25 than in porins is suggested by the fact that, as compared to *omp2* genes, *omp25* is highly conserved in *Brucella* species, biovars and strains [5]. Analysis of the OM properties of Omp25 deficient mutants would help to clarify its role.

The role of Omp31 should also be studied. This protein is able to form oligomers resistant to SDS denaturation, a property also presented by porins. This Omp is absent from *B. abortus* (due to a large deletion) but not from other *Brucella* species [5]. Taken together, both observations have lead to hypothesize that Omp31 could be a porin present in those *Brucella* species which have reduced expression of *omp2* genes [5, 48]. This would be an interesting situation because, whereas in bacteria expressing several types of porins (such as *E. coli*) the different porin genes (*ompC, ompF* and even *lamB*) show considerable homology, *omp2b* and *omp31* are quite different. Functional assays are necessary to elucidate this point.

Lipoproteins By a modification of Braun's protocol based on extensive SDS extraction of CE from exponentially growing *Brucella*, the trypsin fragment of a lipoprotein akin to the PG-linked *E. coli* lipoprotein has been partially characterized and shown to carry the long fatty acids characteristic of the brucellae [20]. In a different study [7], the presence of this lipoprotein was not confirmed but differences in extraction procedures could account for the discrepancy. Genetic studies are necessary for a better understanding of the characteristics of this protein. In addition, several low molecular weight proteins have been identified as minor Omp in *B. abortus* (Omp19, Omp16.5 and Omp10) [45, 46]. According to the predicted amino acid sequence, these proteins are largely hydrophilic and similar to the PG-associated lipoproteins of *E. coli* and other Gram-negative bacteria [the peptidoglycan associated lipoproteins (PAL), different from Braun's lipoprotein]. Based on this similarity, they can be predicted to carry three fatty acids linked to a N-terminal glycerylcysteine (two ester and one amide linked, like Braun's lipoprotein).

Topology of the outer membrane proteins

As expected, hydropathy plots of the major Omp predict both hydrophobic and hydrophilic domains. The former allow for a hydrophobic interaction with other OM elements, and indirect evidence suggests that such interactions are tighter than those described for classical Gram-negative bacteria. The latter make possible interactions with the PG and a surface exposure which, according to sequence analyses (Karplus-Schulz and Parker algorithms), is considerably reduced in Omp2b with respect to Omp25 and Omp31.

Peptidoglycan association Comparative electron microscopy studies show that the *Brucella* PG presents an interaction with the OM much tighter than that observed in *E. coli* [15]. In addition, it has been noted that, whereas heat inactivation of cells causes the *B. abortus* cytoplasmic membrane to collapse, the OM keeps its morphological appearance despite extensive release of OM materials [42]. This suggests that the OM-PG interaction results in an OM stiffness greater in *Brucella* than in other Gram-negative bacteria. Because of the role that its counterpart plays in *E.*

coli, the Braun's type lipoprotein should play an important role in such interaction. Moreover, the PAL features of Omp10, 16.5 and 19 indicate their association to the PG. This, and their predicted hydrophilic and lipoproteic nature, strongly suggests that they are periplasmatically associated to the OM through their lipid moieties. If the fatty acids they carry are of long acyl chains as in other lipidic components of the brucellae, their anchorage to the OM should be comparatively stronger than those of other PAL (see Hydrophobicity).

In some extraction procedures, *Brucella* groups 2 and 3 Omp often appear tightly associated with the PG, and there are conflicting views on the structural meaning of these observations. On one side, the association is thought to be partially caused by inactivation of the bacteria before extraction, since similar observations can be made with *E. coli* [50], and by conditions of extraction not harsh enough to eliminate the stronger hydrophobic effects in the *Brucella* CE (33) (see Hydrophobicity). On the other side, the need for lysozyme digestion to release those Omp from a SDS-resistant fraction of CE obtained from heat-inactivated cells, and the reactivity of Omp prepared in this way with monoclonal antibodies reacting with *B. melitensis* PG (obtained by SDS, NaOH and pepsin treatment), are taken as evidence for a covalent linkage [5, 7]. This interpretation is apparently supported by observations made with *Rhizobium leguminosarum*, another member of the α-2 subdivision of the Proteobacteria. Based on the resistance to conventional SDS extraction and the ³H labeling of SDS-PAGE resolved Omp obtained from cells grown with N-[³H]acetyl-glucosamine, several *R. leguminosarum* Omp have been described as covalently bound to the PG [9] and, noteworthy, these Omp show 35 to 40% identity with several *Brucella* Omp [40, 48]. However, such *R. leguminosarum* Omp are also in a fraction extracted without lysozyme digestion [9], and indirect labeling by a tightly bound LPS (exogenous acetylglucosamine can act as a lipid A precursor) similar to that carried by *Brucella* Omp after SDS-PAGE was not evaluated.

There is agreement on the fact that at least a fraction of Omp does not require lysozyme to be released from the *Brucella* CE [7, 33, 44]. Thus, it seems that only an undetermined proportion of each Omp could be covalently linked to PG and what could remain to be studied is the free/bound ratio for each Omp and, perhaps, the influence of the growth phase. It has also be taken into account that, although both *Brucella* Omp31 and *Rhizobium* RopB can be expressed in *E. coli* OM in an apparently native state, their reported covalent association with the PG is not observed [10, 48]. This suggests that either the hypothetical covalent binding is due to peculiarities of the *Brucella* (and *R. leguminosarum*) PG and periplasm, or the lipid environment (similar in *Brucella* and in *R. leguminosarum*) in which the Omp are inserted, accounts for the discrepancies. Thus, further research would have to be performed directly with the *Brucella* CE and for this purpose two points remain critical: the use of CE obtained from bacteria not inactivated by methods introducing CE artifacts; and the use of PG extraction procedures modified to take into account the *Brucella* CE peculiarities, including a follow up of appropriate markers during purification. Unfortunately, the reports on the chemical composition of *Brucella* PG are somewhat contradictory [22, 24] and its structure should be reexamined by modern methods of analysis (and for amino acid determinations the contamination with tightly bound proteins evaluated). An additional point to be considered is the possibility of an oxidative deamination of Omp lysine residues to yield aldehides which could form aldimine crosslinks by Schiff-base formation with available amino groups of PG [12]. Although they could be artifacts, such allysine residues have been proposed to explain the observation that in *E. coli* stationary phase cells several Omp appear bound to the PG in a SDS-resistant manner. Like *E. coli* porins and OmpA, Omp25 and Omp31 have lysine residues (15 and 14, respectively) in hydrophilic domains but their number is reduced to only 2 in Omp2b.

Outer surface exposure Two different probes have been used to examine exposure of proteins on the outer surface of *Brucella*: ¹²⁵I-lactoperoxidase and antibodies. The first method [31] showed an OM protein profile more complex in *Brucella* than in the *E. coli* used as control. Although this method revealed all major Omp plus additional proteins which should correspond to the minor Omp, it failed to reveal differences between S and R brucellae. Differences in the accessibility to antibodies of immunogenically dominant Omp of S *B. melitensis* and both R *B. melitensis* and *B. ovis* cells were noticed in absorption experiments performed with sera of *B. ovis* infected rams [38]. In a refined research, Cloeckaert et al. [8] showed by immunogold labeling with monoclonal antibodies and cells killed with 0.1% peracetic acid that most *Brucella* Omp have epitopes exposed on the surface of S cells, and that exposure is increased in R cells. Moreover, these authors also noticed that Omp 89 kDa and 31–34 kDa were not accessible in S *B. abortus*, whereas they were exposed on R *B. abortus* and also on S *B. melitensis*. Thus, these findings showed not only the differences between S and R cells that had been observed in other Gram-negative bacteria, but also some degree of Omp exposure on S cells and suggestive species differences. Although obtained with heat-inactivated cells, these findings were extended in a later work [2]. Such antibody accessibility also include the three PAL-like Omp [2, 8] and the Braun's type lipoprotein [21] and, according to this evidence, all these lipoproteins could be on both sides of the OM. This is an intriguing feature which should be the subject of further research. For the S strains, all these observations can be interpreted as indicating that the LPS molecules with shorter O-chains are preferentially distributed in the periphery of some Omp, or another key surface element such as the NH polysaccharide is unevenly distributed in the cell surface, or both possibilities. A hypothetical topology of the *B. melitensis* Omp is presented in Fig. 2.
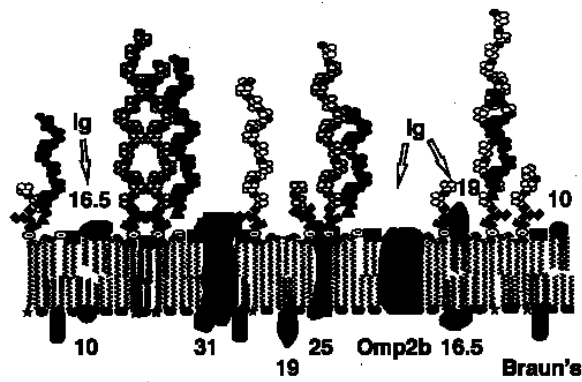
Fig. 2 Topology of the Omp of *Brucella melitensis*. Omp identity is indicated by its molecular weight except for the Omp2b porin. Periplasmic domains of the major proteins [25, 31 (missing in *Brucella abortus*) and Omp2b] would allow for peptidoglycan interactions, and variations in the degree of polymerization of the LPS O-chain and NH density could explain different degrees of antibody (Ig) accessibility among Omp and among strains. Strong association with LPS and lipids should be a relatively common feature, but it could be more marked for Omp25. Omp2b and Omp31 are represented in a trimeric state but protein interactions among different Omp should also exist. For the lipoproteins, a peripheral location and lipid anchorage to either the outer or periplasmic face of the OM is suggested based on their accessibility to antibodies and overall similarity with *Escherichia coli* PAL and Braun's lipoproteins, respectively; lipoprotein acyl chains are indicated as thicker lines. The model does not take into account the relative proportions of the Omp

Properties of functional significance

Permeability The penetration of small hydrophilic molecules through *Brucella* group 2 Omp was shown by Douglas et al. [14] using proteoliposomes. In this functional assay, *E. coli* OmpF and *Brucella* porins had a similar apparent internal diameter (1.2 nm), with slight species and strain differences. Since thionine (used in *Brucella* biotyping) has an exceptionally low hydrophobicity among dyes, it was also suggested that species differences in thionine sensitivity relate to different functional properties of their porins (an exception would be *B. canis*). At least for *B. abortus*, those results can be attributed to Omp2b because Omp2a is not expressed in vitro [16].

That some hydrophobic substrates could be entering *Brucella* cells directly through the lipid bilayer was first suggested by Douglas et al. [14]. Later studies proved this insight and showed that, in contrast to many Gram-negative bacteria, the brucellae readily take up hydrophobic permeants [27]. Insertion of *Salmonella montevideo* S-LPS into the OM of R *B. abortus* reduces its permeability to hydrophobic probes without affecting cell viability [18], and insertion of *B. abortus* LPS into the OM of *Ochrobactrum anthropi* (a closely related bacteria with and effective OM barrier) increases its permeability to such probes [Velasco and Moriyón, unpublished]. Although the absence in *Brucella* of efflux pumps for hydrophobic permeants cannot be ruled out, this evidence shows that the increased cell permeability of *Brucella* is mostly

due to the properties of the LPS. In *E. coli*, it is thought that hydrophobic permeants are kept out because of the cooperation between efflux systems and the barrier created by divalent cations bridging the LPS between the anionic groups of lipid A and core. Thus, consistent with other evidence (see Interaction with polycations), the *Brucella* surface should be different at this last level.

The significance of the hydrophobic pathway in *Brucella* is unknown and it could be influenced indirectly by the environment. There should be variations among species and biotypes, as shown by their variable sensitivity to basic fuchsine, a very hydrophobic dye used in *Brucella* biotyping. The hydrophobic pathway of uptake accounts for the suggestive effect of sexual hormones on *Brucella* CE and metabolic rates described by Mayer [29]. Finally, because siderophores of the 2,3-dihydroxybenzoate family are moderately hydrophobic, direct penetration of siderophore chelates through the OM could explain why no iron induced Omp are observed in *B. abortus* under conditions in which they are manifested in *E. coli* [23]. In fact, the moderately hydrophobic iron chelator bipyridyl is readily taken up by *Brucella* [López-Goñi and Moriyón, unpublished].

Interaction with polycations Many polycations, including peptides of lysosomes and body fluids, have a potent bactericidal activity due, in part, to their ability to interact with anionic targets on the bacterial surfaces. Based on earlier observations on the resistance of *Brucella* CE to EDTA, resistance to polycations was suggested by Martin and Hancock [26] and proved experimentally by Martínez de Tejada et al. [28]. These authors showed that the OM of wild type *Brucella* and R mutants were comparatively resistant to a wide range of polycations including defensin NP-2, cecropin PI, lactoferricin B, and active peptides from cationic protein 18 and bactenecin. This explains why the oxygen-independent systems of phagocytes are inefficient in killing S and R brucellae [39].

B. abortus LPS plays a clear role in the resistance to polycations. Involvement of the O-chain is shown by the fact that insertion of the homologous S-LPS into the OM of R *B. abortus* mutants increases their resistance to polycations [18, 28]. Moreover, insertion of *S. montevideo* S-LPS into the OM sensitizes the R mutants and, therefore, the core and lipid A of *B. abortus* LPS must play a significant role [18, 28]. Since *B. abortus* lipid A lacks the arabinosamine and ethanolamine residues implicated in polycation resistance in some enteric bacteria, hypothetical explanations for the *Brucella* resistance are that its LPS core lacks phosphate and acidic sugars (both polycations targets) and that lipid A phosphates could be neutralized by ornithine lipids (see Fig. 1). Moreover, *B. abortus* LPS aggregates saturated with polymyxin B still show residual negative charge and, accordingly, steric hindrance in the access of polycations to lipid A phosphates caused by core peculiarities can be postulated [47]. Elucidation of the core structure is necessary for a definite explanation of those

observations.

Hydrophobicity

We have proposed previously that the lipid composition, conditions of extraction of Omp, and strong LPS-Omp interactions strongly suggest a structural role of the hydrophobic effect more important in *Brucella* that in classical Gram-negative bacteria [31, 32]. Elucidation of the fatty acid composition of the lipid A has added more weight to those arguments with an interesting modification: the 27OH-C28:0 and of 29OH-C30:0 should have the hydroxyl groups located in the periplasmic space and, therefore, the lipid A should act as a membrane spanning toggle bolt (Fig. 1). Obviously, this greater hydrophobicity could explain the acid-fastness of the brucellae and the predicted hydrophobicity of Omp2b. It has to be stressed that the greater hydrophobicity of *Brucella* CE is consistent with the permeability to hydrophobic compounds, the resistance to EDTA and conventional detergent extraction, the failure of classical protocols of periplasm extraction, the absence of phosphate in the LPS core and the resistance to polycations. This is so because a stronger hydrophobic anchorage of the S-LPS means a reduced structural role for divalent cation bridging at lipid A-core level, and this simultaneously makes the OM resistant to EDTA and to polycations, and accessible to nonpolar substrates. We consider this unusual set of properties critical for *Brucella* pathogenicity.

Outer membrane properties and environmental sensing systems

By transposon mutagenesis of *B. abortus* 2308, we have isolated two mutants which, although fully S and not showing obvious in vitro growth defects, are markedly avirulent in mice and essentially unable to promote their uptake by HeLa cells and mouse macrophages. Analysis of the mutated regions shows two adjacent genes corresponding to a two-component regulatory system [*Brucella* virulence related (or Bvr) system] with an inner membrane sensor (a histidine protein kinase) exposed on the periplasmic side and a cytoplasmic response regulator. In addition to their avirulence, both mutants have altered OM properties: they show increased sensitivity to polycations and permeability to hydrophobic probes and surfactants [43]. Thus, it seems that some *B. abortus* OM molecules related to both properties are under the regulation of BvrR-BvrS and that the functional characteristics of this OM vary under different environmental conditions.

References

1. Aragón V, Díaz R, Moreno E, Moriyón I (1996) Characterization of *Brucella abortus* and *Brucella melitensis* native haptens as outer membrane O-type polysaccharides independent from the smooth lipopolysaccharide. J Bacteriol 178:1070-1079

2. Bowden RA, Cloeckaert A, Zygmunt MS, Bernard S, Dubray G (1995) Surface exposure of outer membrane protein and lipopolysaccharide epitopes in *Brucella* species studied by enzyme-linked immunosorbent assay and flow cytometry. Infect Immun 63:3845-3852

3. Briones G, de Iannino NI, Steinberg M, Ugalde RA (1997) Periplasmic cyclic 1,2 β-glucan in *Brucella* spp. is not osmoregulated. Microbiology 143:1115-1124

4. Cherwonogrodzky J, Dubray G, Moreno E, Mayer H (1990) Antigens of *Brucella*. In: Nielsen K, Duncan JR (ed) Animal Brucellosis. Boca Raton, FL: CRC Press, pp 19-64

5. Cloeckaert A, Verger JM, Grayon M, Vizcaíno N (1996) Molecular and immunological characterization of the major outer membrane proteins of *Brucella*. FEMS Microbiol Let 145:1-8

6. Cloeckaert A, Zygmunt MS, Bezard G, Dubray G (1996) Purification and antigenic analysis of the major 25-kilodalton outer membrane protein of *Brucella abortus*. Res Microbiol 147:225-235

7. Cloeckaert A, Zygmunt MS, de Wergifosse P, Dubray G, Limet LN (1992) Demonstration of peptidoglycan-associated *Brucella* outer-membrane proteins by use of monoclonal antibodies. J Gen Microbiol 138:1543-1550

8. Cloeckaert A, de Wergifosse P, Dubray G, Limet JN (1990) Identification of seven surface-exposed *Brucella* outer membrane proteins by use of monoclonal antibodies: immunogold labeling for electron microscopy and enzyme-linked immunosorbent assay. Infect Immun 58:3980-3987

9. de Maagd RA, Wientjes FB, Lugtenberg BJ (1989) Evidence for divalent cation (Ca²⁺)-stabilized oligomeric proteins and covalently bound protein-peptidoglycan complexes in the outer membrane of *Rhizobium leguminosarum*. J Bacteriol 171:3989-3995

10. de Maagd RA, Mulders IHM, Canter Cremers HCJ, Lugtenberg BJ (1992) Cloning, nucleotide sequencing, and expression in *Escherichia coli* of a *Rhizobium leguminosarum* gene encoding a simbiotically repressed outer membrane protein. J Bacteriol 174:214-221

11. Díaz R, Jones LM, Leong D, Wilson JB (1968) Surface antigens of smooth brucellae. J Bacteriol 96:893-901

12. Diedrich DL, Schnaitman CA (1978) Lysil-derived aldehides in outer membrane proteins of *Escherichia coli*. Proc Natl Acad Sci USA 75:3708-3712

13. Douglas JT, Palmer DA (1988) Use of monoclonal antibodies to identify the distribution of A and M epitopes on smooth *Brucella* species. J Clin Microbiol 26:1353-1356

14. Douglas JT, Rosenberg EY, Nikaido H, Verstreate DR, Winter AJ (1984) Porins of *Brucella* species. Infect Immun 44:16-21

15. Dubray G (1976) Localisation cellulaire des polyosides des bactéries des genres *Brucella* et *Escherichia* en phase lisse (S) or rugueuse (R). Ann Microbiol (Inst Pasteur) 127B:133-149

16. Ficht TA, Bearden SW, Sowa BA, Adams LG (1989) DNA sequence and expression of the 36-kilodalton outer membrane protein gene of *Brucella abortus*. Infect Immun 57:3281-3291

17. Freer E, Rojas N, Weintraub A, Lindberg AA, Moreno E (1995) Heterogeneity of *Brucella abortus* lipopolysaccharides. Res Microbiol 146: 578-596

18.   Freer E, Moreno E, Moriyón I, Pizarro-Cerdá J, Weintraub A, Gorvel J-P (1996) *Brucella/Salmonella*-lipopolysaccharide chimeras are less permeable to hydrophobic probes and more sensitive to cationic peptides and EDTA than their native *Brucella* spp. counterparts. J Bacteriol 178:5867-5876

19.   Gamazo C, Vitas AI, Moriyón I, López-Goñi I, Díaz R (1993) *Brucella* Group 3 outer membrane proteins contain a heat-modifiable protein. FEMS Microbiol Lett 112:141-146

20.   Gómez-Miguel MJ, Moriyón I (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

21.   Gómez-Miguel MJ, Moriyón I, López J (1987) *Brucella* outer membrane lipoprotein shares antigenic determinants with *Escherichia coli* Braun's lipoprotein and it is exposed on the cell surface. Infect Immun 55:258-262

22.   Lacave C, Roux MJ (1965) Composition chimique des parois de *Brucella*. Glycosamino-peptide de *Brucella abortus* et *Brucella melitensis*. CR Acad Sc Paris 260:1514-1517

23.   López-Goñi I, Moriyón I, Neilands JB (1992) Identification of 2,3-dihydroxybenzoic acid as a *Brucella abortus* siderophore. Infect Immun 60:4496-4503

24.   Mardarowickz C (1966) Isolierung and charakterisierung des murein-sacculus von *Brucella*. Z Naturforschg 21b:1006-1007

25.   Marquis H, Ficht TA (1993) The *omp2* gene locus of *Brucella abortus* encodes two homologous membrane proteins with properties characteristic of porins. Infect Immun 61:3785-3790

26.   Martin NL, Hancock REW (1990) Function and structure of the major components of the outer membrane of Gram-negative bacteria. In: Adams LG (ed) Advances in Brucellosis Research. College Station, TX: Texas A & M University Press, pp 55-75

27.   Martínez de Tejada G, Moriyón I (1993) The outer membranes of *Brucella* sp. are not a barrier to hydrophobic permeants. J Bacteriol 175:5273-5275

28.   Martínez de Tejada G, Pizarro-Cerdá J, Moreno E, Moriyón I (1995) The outer membranes of *Brucella* spp. are resistant to bactericidal cationic peptides. Infect Immun 63:3054-3061

29.   Mayer ME (1976) Evolution and taxonomy in the genus *Brucella*: Steroid hormone induction of filterable forms with altered characteristics after reversion. Am J Vet Res 37:207-210

30.   Moreno E, Stackebrandt E, Dorsch M, Wolters J, Busch M, Mayer H (1990) *Brucella abortus* 16S rRNA and lipid A reveal a phylogenetic relationship with members of the alpha-2 subdivision of the class Proteobacteria. J Bacteriol 172:3569-3576

31.   Moriyón I, Berman DT (1982) Effects of nonionic, ionic and dipolar ionic detergents and EDTA on *Brucella* cell envelopes. J Bacteriol 152:822-828

32.   Moriyón I, Berman DT (1983) Isolation, purification and partial characterization of *Brucella abortus* matrix protein. Infect Immun 39:394-402

33.   Moriyón I, Gamazo C, Díaz R (1987) Properties of the outer membrane of *Brucella*. Ann Inst Pasteur/Microbiol 138:89-91

34.   Perry MB, Bundle DR (1990) Lipopolysaccharide antigens and carbohydrates of *Brucella*. In: Adams LG (ed) Advances in Brucellosis Research. College Station, TX: Texas A&M University Press, pp 76-88

35.   Qureshi N, Takayama K, Seydel U, Wang R, Cotter RJ, Agrawal PK, Bush CA, Kurtz R, Berman DT (1994) Structural analysis of the lipid A derived from the lipopolysaccharide of *Brucella abortus*. J Endotox Res 1:137-148

36.   Redfearn MS (1960) An immunochemical study of antigens of *Brucella* extracted by the Westphal technique. Ph D Thesis. University of Wisconsin-Madison, WI

37.   Redfearn MS, Berman DT (1960) Application of the gel-diffusion technique for typing brucellae. Bull Wld Hlth Org 23:133-134

38.   Riezu-Boj JI, Moriyón I, Blasco JM, Gamazo C, Díaz R (1990) Analysis by immunoblot of the antibody response of sheep infected by smooth and rough brucellae to outer membrane proteins extracted with hot saline. Infect Immun 58:489-494

39.   Riley LK, Robertson DC (1984) Brucellicidal activity of human and bovine polymorphonuclear leukocyte granule extracts against smooth and rough strains of *Brucella abortus*. Infect Immun 46: 231-236

40.   Roest HP, Bloemendaal C-JP, Wijffelman CA, Lugtenberg BJ (1995) Isolation and characterization of RopA homologous genes from *Rhizobium leguminosarum* biovars *viciae* and *trifolii*. J Bacteriol 177:4985-4991

41.   Rojas N, Freer E, Weintraub A, Ramírez M, Lind S, Moreno E (1994) Immunochemical identification of *Brucella abortus* lipopolysaccharide epitopes. Clin Diag Lab Immunol 1:206-213

42.   Rubbi CP (1991) Antígenos proteicos de *Brucella abortus*: aislamiento y estudio de su asociación al LPS y de factores que afectan a la producción de anticuerpos monoclonales contra los mismos. Ph D Thesis, Universidad Nacional de la Plata, Argentina

43.   Sola-Landa A, Pizarro-Cerdá J, Grilló M-J, Moreno E, Moriyón I, Blasco JM, Gorvel J-P, López-Goñi I (1998) A two component regulatory system playing a critical role in plant pathogens and endosymbionts is present in *Brucella abortus* and controls cell invasion and virulence. Mol Microbiol (in press)

44.   Sowa BA (1990) Membrane proteins of *Brucella* spp. In: Adams LG (ed) Advances in Brucellosis Research. College Station, TX: Texas A&M University Press, pp 89-105

45.   Tibor A, Saman E, de Wergifosse P, Cloeckaert A, Limet J, Letesson J-J (1996) Molecular characterization, occurrence, and immunogenicity in infected sheep and cattle of two minor outer membrane proteins of *Brucella abortus*. Infect Immun 64:100-107

46.   Tibor A, Weynants V, Denoel P, Lichtfhouse B, De Bolle X, Saman E, Limet J, Letesson JJ (1994) Molecular cloning, nucleotide sequence and occurrence of a 16.5-kilodalton outer membrane protein of *Brucella abortus* with similarity to PAL lipoproteins. Infect Immun 62:3633-3639

47.   Velasco J (1997). Relaciones antigénicas y estructurales entre *Ochrobactrum anthropi* y *Brucella* spp. Ph D Thesis, Universidad de Navarra, Spain

48.   Vizcaíno N, Cloeckaert A, Zygmunt M, Dubray G (1996) Cloning, nucleotide sequence, and expression of the *Brucella melitensis omp31* gene coding for an immunogenic major outer membrane protein. Infect Immun 64:3774-3751

49.   Whitfield C, Amor PA, Köplin R (1987) Modulation of the surface architecture of Gram negative bacteria by the action of surface polymer:lipid A-core ligase and by determinants of polymer chain length. Mol Microbiol 23:629-638

50.   Winter AJ (1987) Outer membrane proteins of *Brucella*. Ann Inst Pasteur/Microbiol 138:87-89