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Analysis of Outer Membrane Ultrastructure of Pathogenic *Treponema* and *Borrelia* Species by Freeze-Fracture Electron Microscopy

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We analyzed the outer membrane (OM) ultrastructure of four pathogenic members of the family *Spirochaetaceae* by freeze fracture. The OM of *Treponema pallidum* subsp. *pertenue* contained a low intramembranous particle concentration, indicating that it contains few OM transmembrane proteins. The concave OM fracture faces of *Treponema hyodysenteriae* and *Borrelia burgdorferi* contained dense populations of particles, typical of gram-negative organisms. A relatively low concentration of particles which were evenly divided between a small and a large species was present in the concave OM fracture face of *Borrelia hermsii*; the convex OM fracture face contained only small particles. As for gram-negative bacteria, the convex OM fracture face particle concentrations of these pathogens were low. These spirochetes cleaved preferentially within the OM, in contrast to typical gram-negative bacteria, which tend to fracture within the inner membrane. The OM ultrastructure of *T. pallidum* subsp. *pertenue* provides an explanation for the lack of antigenicity of the treponemal surface and may reflect a mechanism by which this pathogen evades the host immune response.

Treponema pallidum subsp. *pertenue*, *Treponema hyodysenteriae*, *Borrelia burgdorferi*, and *Borrelia hermsii* are the etiologic agents of yaws, swine dysentery, Lyme borreliosis, and relapsing fever, respectively. Although the diseases caused by these spirochetes (family *Spirochaetaceae*) have been well described, research on the biology of these pathogens has been hampered by several factors. Rich, serum-containing media are required to cultivate the agents of Lyme borreliosis, relapsing fever, and swine dysentery; none of the treponemes responsible for disease in humans can be cultivated continuously in vitro. No systems for introducing DNA into members of the family *Spirochaetaceae* have been developed. The spirochetal outer membrane (OM) tends to be easily damaged by mild physical and chemical manipulations (3, 12, 14, 15, 27, 28, 32). Work with spirochetes such as *Treponema pallidum* subsp. *pallidum*, the etiologic agent of syphilis, has indicated that this fragility has not been adequately addressed by the techniques used to define the OM constituents of gram-negative bacteria (12, 26–28, 30, 38).

Freeze-fracture electron microscopy has allowed examination of the ultrastructure of the OM of *T. pallidum* subsp. *pallidum* (30, 38). The OM of this spirochete was shown to be unusual in that it contains a low intramembranous particle (IMP) concentration in both the concave and convex fracture faces, indicating that the OM of *T. pallidum* subsp. *pallidum* contains few transmembrane proteins (30, 38). In this study, we used the techniques of freeze-fracture and freeze-etch electron microscopy to examine the OM of *T. pallidum* subsp. *pertenue*, *T. hyodysenteriae*, *B. burgdorferi*, and *B. hermsii*.

Virulent *T. pallidum* subsp. *pertenue*, strain Haiti B, was provided by Paul H. Hardy, Jr., The Johns Hopkins University School of Medicine, Baltimore, Md. The strain was cultivated by passage in New Zealand White male rabbits and harvested as previously described for *T. pallidum* subsp. *pallidum* (23). *T. hyodysenteriae* B204 stocks were provided by Thad B. Stanton, National Animal Disease Center, Ames, Iowa. The organism was cultured for 20 h in brain heart infusion (Difco Laboratories, Detroit, Mich.) supplemented with 10% fetal bovine serum (GIBCO Laboratories, Grand Island, N.Y.) at 39°C in an atmosphere of N_2-O_2 (99:1, vol/vol) as described by Stanton and Cornell (34). A low-passage (fourth in vitro passage), virulent strain of *B. burgdorferi*, B31, was provided by Steven Barthold, Section of Comparative Medicine, Yale University, New Haven, Conn. *B. burgdorferi* B31 (type strain, ATCC 35210) is routinely passaged in our laboratory. *B. hermsii* HS1 (type strain, ATCC 35209), serotype C, is also routinely passaged in our laboratory. Borreliae were grown in Barbour-Stoenner-Kelly II (2) medium for 3 days at 34°C.

Freeze-fracture electron microscopy and particle density quantification were performed as described previously (38). In some experiments, the organisms were fixed at 35 or 4°C rather than at room temperature. For freeze-etching, the fixed organisms were prepared in distilled water without cryoprotection and were fractured at −105°C, etched at the same temperature for 90 s, and replicated as described previously (38).

Representative freeze-fracture profiles are shown in Fig. 1A to D, and the concave and convex OM fracture face (OMF) IMP densities are presented in Table 1. No difference in IMP concentration was noted between the virulent and avirulent variants of *B. burgdorferi* B31 (data not shown). Occasional short linear arrays of IMPs were observed in the concave and convex OMFs of *T. hyodysenteriae* and *B. burgdorferi* (data not shown) at all fixation temperatures (indicating that they were not the result of lipid-phase

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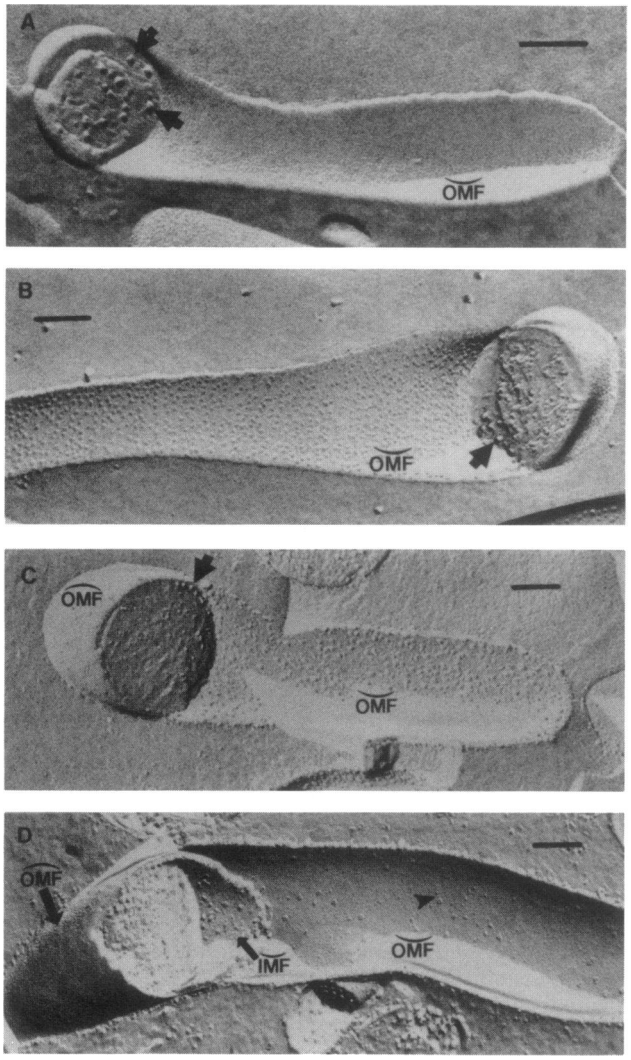

FIG. 1. (A) Freeze-fracture electron micrograph of *T. pallidum* subsp. *pertenue*. A transverse fracture through the cytoplasm and a longitudinal fracture through the concave (⌣) OMF are visible. Endoflagella are visible in the periplasmic space revealed by the transverse fracture (arrows), and cytoplasmic fibrils are exposed in the cytoplasm below the endoflagella. (B) Freeze-fracture electron micrograph of *T. hyodysenteriae*. A transverse fracture through the cytoplasm and a longitudinal fracture through the concave OMF are shown. Endoflagella are visible in the transverse fracture (arrow). (C) Freeze-fracture electron micrograph of *B. burgdorferi*. A transverse fracture through the cytoplasm and a longitudinal fracture through the concave OMF are visible, as is a small portion of the convex (⌢) OMF. Endoflagella are visible in the cross fracture (arrow). (D) Freeze-fracture electron micrograph of *B. hermsii*. Longitudinal fractures of the convex and concave OMFs are visible, as is a portion of the concave inner membrane fracture face (IMF). Note juxtaposed large and small IMPs (arrowhead). Bars = 0.1 μm.

transition); otherwise, there was no discernible order to the arrangement of the IMPs in either OMF of any of the organisms studied. The concave OMF of *B. hermsii* contained approximately equal numbers of large and small IMPs (Fig. 1D); the convex OMF contained only small IMPs. The convex OMFs of the spirochetes contained low IMP concentrations, typical of the OM of gram-negative organisms (5, 19).

TABLE 1. IMP concentration of the concave and convex OMFs of *T. pallidum* subsp. *pertenue*, *T. hyodysenteriae*, *B. burgdorferi*, and *B. hermsii*

Organism	IMPs/μm² of concave OMF	IMPs/μm² of convex OMF
T. pallidum subsp. *pertenue*	93 ± 57	86 ± 43
T. hyodysenteriae	2,601 ± 242	60 ± 21
B. burgdorferi[a]	1,860 ± 132	80 ± 30
B. hermsii	382 ± 84	189 ± 86

[a] Virulent variant strain B31.

Each spirochete fractured almost exclusively within the OM; inner membrane fracture faces were rarely observed. Similar results have been reported for *T. pallidum* subsp. *pallidum* (30). In contrast, typical gram-negative bacteria fracture preferentially within the inner membrane (5, 19). Extensive OM cleavage occurs in *Escherichia coli* K-12 mutants deficient in OmpA and in mutants with a rough lipopolysaccharide chemotype (19). No associations have been made between specific spirochetal components and preferred fracture plane; it is interesting that these spirochetes appear to lack a typical gram-negative lipopolysaccharide (1, 3, 13, 27, 36).

A representative freeze-etched profile is shown in Fig. 2. The etched membranes of all the spirochetes examined in this study were generally smooth and lacked observable features such as ordered arrays. This result must be interpreted carefully; externally exposed molecules may lack sufficient relief above the surface of the organism to be shadowed (24, 33, 35). The OMFs of the fractured and etched spirochetes resembled the cryoprotected fracture faces, although without cryoprotection, membrane damage such as swelling and blebbing was more commonly observed, especially for *T. hyodysenteriae* (data not shown).

The freeze-fractured OM of *T. pallidum* subsp. *pertenue* is indistinguishable from that of *T. pallidum* subsp. *pallidum* (30, 38); the OM of *T. pallidum* subsp. *pertenue* evidently also contains few transmembrane proteins. This unusual ultrastructure suggests that the treponemal OM may contain few antigenic targets (30, 38), which correlates with the lack of antigenicity of the surface of *T. pallidum* subspecies that has been inferred from immunocytochemical studies (16, 20, 29) and the prolonged kinetics of killing of the treponemes by specific antibody and complement in vitro (6, 17, 23, 25, 37). Work in our laboratory with a modified in vitro killing assay and freeze fracture indicates that antibody-dependent com-

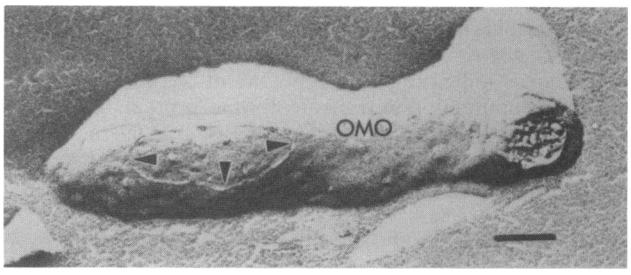

FIG. 2. Freeze-etch electron micrograph of *T. pallidum* subsp. *pertenue*. Arrowheads point from the convex OMF to the step between the convex OMF and the outer surface of the OM (OMO). Bar = 0.1 μm.

plement fixation on the treponemal OM is inefficient because of the scarcity of antigenic targets (7). The yaws and syphilis treponemes are very closely related; they are identical in DNA base sequence at the level of resolution of DNA-DNA hybridization analysis (21). Yaws and syphilis are similar in pathogenesis, although they differ in some details of natural and experimental infection (22, 37). As has been hypothesized for *T. pallidum* subsp. *pallidum*, the OM ultrastructure of *T. pallidum* subsp. *pertenue* may reflect a mechanism that allows evasion of host immune mechanisms and establishment of chronic infection (7, 30, 38).

The close packing of the IMPs in the concave OMF of *T. hyodysenteriae* and *B. burgdorferi* resembles that of *E. coli* and other typical gram-negative bacteria (5, 19), although the absolute concave OMF particle concentrations of these spirochetes were somewhat lower than that of *E. coli* (5,000 to 10,000/μm^2 [19]). The dramatic preferential partitioning of the IMPs to the concave OMF of these two spirochetes is also characteristic of gram-negative bacteria (5, 19); the phenomenon of preferential partitioning of IMPs in freeze-fractured membranes has not been definitively explained (31). On the basis of detergent solubilization studies, Boyden et al. (8) proposed that the OM of *T. hyodysenteriae* may contain few OM proteins, like that of *T. pallidum* subsp. *pallidum*; divergent results have been obtained in other studies using selective detergent solubilization (32, 39). Our results clearly do not support the suggestion that the OM of *T. hyodysenteriae* resembles that of *T. pallidum* subspecies. The OM of *B. burgdorferi* has been characterized as containing the abundantly expressed proteins OspA and OspB (3); these molecules have been shown to be lipoproteins (9). Proteins anchored in a membrane solely by lipid domains may not form particles in fracture faces (19). Therefore, the abundance of IMPs in the OM of *B. burgdorferi* suggests that the OM of this organism may contain numerous proteins that are neither OspA nor OspB, although the number of possible protein species is impossible to estimate from our data. The OM architecture of *B. burgdorferi*, with its close packing of potential antigens, correlates well with the observation that this organism is rapidly killed (within 2 h) in vitro by antibody and complement (18). In contrast, the kinetics of antibody-dependent, complement-mediated in vitro killing of *T. pallidum* subsp. *pallidum*, an organism with apparently few OM proteins (30, 38), are prolonged to 16 h (6, 17, 23, 25, 37).

The OM of *B. hermsii* apparently contains relatively few transmembrane proteins, although the particle concentration of the concave OMF of *B. hermsii* is approximately fourfold greater than those of *T. pallidum* subspecies. The pattern of alternating febrile and afebrile periods, characteristic of relapsing fever, is apparently mediated by sequential expression of antigenic variants of the abundant variable major protein (VMP [3, 4]). The low-density, scattered distribution of the IMPs in the OMFs of *B. hermsii*, and the observation that these fell into two distinct size classes, does not seem to account for the amount of VMP expressed by the organism. As the VMPs appear to be lipoproteins (10), and, therefore, may not form IMPs (19), our results suggest that at least two species of transmembrane proteins exist in the OM of *B. hermsii*, possibly as minor constituents relative to the VMP.

Direct comparison of the freeze-fracture results is complicated by the need to obtain *T. pallidum* subsp. *pertenue* from animals, whereas the other spirochetes were grown in rich media in order to obtain the numbers needed for freeze fracture. Virulent strains of *T. pallidum* subsp. *pertenue*, *T. hyodysenteriae*, and *B. burgdorferi* were freeze fractured. Although a culture-adapted, nonswitching strain of *B. hermsii* was examined in this study, the results of sodium dodecyl sulfate-polyacrylamide gel electrophoresis, immunoblot, and radioiodination studies indicate that this strain is representative of the other serotypes in terms of overall protein complexity (4).

In this study, we have shown that the OMs of the pathogenic *Treponema* and *Borrelia* species vary greatly in IMP content and that the OM of these organisms differs from that of typical gram-negative bacteria, as reflected by the preference of the cleavage plane for the OM of these spirochetes. A better understanding of these observations will require the development of methodologies that will allow molecular characterization of the OM constituents of these spirochetes. Such studies will be necessary to address definitively such issues as the nature and complexity of the spirochetal transmembrane proteins, the anchoring and orientation of the Osp and VMP molecules, and the recently described treponemal lipoproteins (11), as well as to understand the molecular basis of the differences between the OMs of typical gram-negative bacteria and these spirochetes.

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