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Demonstration of Rare Protein in the Outer Membrane of *Treponema pallidum* subsp. *pallidum* by Freeze-Fracture Analysis

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The surface of *Treponema pallidum* subsp. *pallidum* (*T. pallidum*), the etiologic agent of syphilis, appears antigenically inert and lacks detectable protein, as judged by immunocytochemical and biochemical techniques commonly used to identify the outer membrane (OM) constituents of gram-negative bacteria. We examined *T. pallidum* by freeze-fracture electron microscopy to visualize the architecture of its OM. *Treponema phagedenis* biotype Reiter (*T. phagedenis* Reiter), a nonpathogenic host-associated treponeme, and *Spirochaeta aurantia*, a free-living spirochete, were studied similarly. Few intramembranous particles interrupted the smooth convex and concave fracture faces of the OM of *T. pallidum*, demonstrating that the OM of this organism is an unusual, nearly naked lipid bilayer. In contrast, the concave fracture face of the OM of *S. aurantia* was densely covered with particles, indicating the presence of abundant integral membrane proteins, a feature shared by typical gram-negative organisms. The concentration of particles in the OM concave fracture face of *T. phagedenis* Reiter was intermediate between those of *T. pallidum* and *S. aurantia*. Similar to typical gram-negative bacteria, the OM convex fracture faces of the three spirochetes contained relatively few particles. The unique molecular architecture of the OM of *T. pallidum* can explain the puzzling in vitro properties of the surface of the organism and may reflect a specific adaptation by which treponemes evade the host immune response.

Treponema pallidum subsp. *pallidum* (*T. pallidum*), is a noncultivatable pathogenic spirochete with surface characteristics that appear to be unique. The binding of antibody to the surface antigens of the virulent organism has not been demonstrable by standard immunocytochemical techniques (7, 11, 15, 20, 25). The surface inertness suggested by these studies is paralleled by the prolonged kinetics of killing of the organism by specific antibody and complement in vitro. A minimum of 4 h of incubation with high-titer antiserum is required before killing of treponemes can be demonstrated; typically 16 h is required to achieve 100% killing (4, 17, 19). Extrinsic radioiodination, a method commonly used to identify surface-exposed proteins of bacteria, has failed to label *T. pallidum* surface proteins (21). These unique surface properties may reflect a mechanism that allows *T. pallidum* to evade the host immune response.

The fact that *T. pallidum* is propagated in the rabbit testis provides a basis for explaining the unusual surface characteristics of the organism. It has been reported that treponemes harvested from testes have fibronectin, albumin, and other host molecules bound to their surface (1, 8, 9, 22). Uniform binding of host molecules by the surface of *T. pallidum* could contribute to its antigenic inertness. An alternative view has been that the lack of antigenicity of the surface of the organism may represent a unique molecular organization that is an innate property of the *T. pallidum* outer membrane (OM).

In this report we use the technique of freeze fracture, which cleaves within the hydrophobic interior of a lipid bilayer, effectively splitting a membrane in half, to provide evidence that the OM of *T. pallidum* is an unusual, nearly naked lipid bilayer with few integral membrane proteins. This clear demonstration obviates the need to hypothesize a coat of host material to explain the antigenic inertness of the intact surface of the organism. We also show that the OM of *Treponema phagedenis* biotype Reiter (*T. phagedenis* Reiter), a nonpathogenic host-associated treponeme, resembles the OM of *T. pallidum* more closely than it resembles the OM of typical gram-negative organisms. In contrast to *T. pallidum* and *T. phagedenis* Reiter, we found that the OM of *Spirochaeta aurantia*, a free-living spirochete, is densely populated with integral membrane proteins, a property *S. aurantia* shares with typical gram-negative organisms.

MATERIALS AND METHODS

Bacteria. Virulent *Treponema pallidum* subsp. *pallidum* was cultivated by passage in New Zealand White male rabbits and harvested as described previously (18). Treponemes were extracted in phosphate-buffered saline (pH 7.4) from testes infected 10 days before harvest; the organisms were separated from gross host tissue material by centrifugation for 10 min at 400 × *g*. *Treponema phagedenis* biotype Reiter was grown at 34°C in Spirolate broth (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 10% heat-inactivated normal rabbit serum. *Spirochaeta aurantia* J1 was grown at room temperature in a medium described by Leschine and Canale-Parola (13). *T. phagedenis* Reiter and *S. aurantia* were harvested from logarithmic-phase cultures.

Freeze-fracture electron microscopy. Suspensions containing approximately 4×10^8 viable spirochetes (100% motility), as determined by enumeration of 20 fields by dark-field microscopy (at least 200 total organisms), were centrifuged at 30,000 × *g* to pellet the organisms. The pellets were suspended in 2 ml of 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). After 15 min of fixation, 1 ml of the suspension was transferred to each of two 1.5-ml microfuge centrifuge tubes and the treponemes were pelleted by centrifugation at 14,000 × *g*. The pellets were suspended in 50 μl of 20% glycerol in 0.1 M sodium cacodylate buffer

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(pH 7.4). These manipulations were performed at ambient temperature (22 to 24°C). From this suspension, 2-μl portions were placed on standard Balzars specimen holders (Balzars Co., Nashua, N.H.). The samples were frozen by immersion in liquid propane (−190°C), using a guillotine-type device. The frozen samples were transferred under liquid nitrogen to the specimen stage of a Balzars 400K freeze-fracture apparatus precooled to −150°C. The frozen suspension of bacteria was fractured at −120°C, using a knife cooled at the temperature of liquid nitrogen. The fracture surface was immediately replicated with platinum-carbon at 45′ and carbon at 90′. The replicas were floated in 3 to 4% sodium hypochlorite to bleach the organic material, washed three times in double distilled water, and placed on Formvar-coated freeze-fracture grids (Ted Pella Inc., Redding, Calif.). The grids were observed in a JEOL 100 CX II electron microscope operated at 80 kV. For each organism studied, a minimum of 50 fields was photographed and printed at a final magnification of ×100,000; typical fractured cells were chosen from these fields for determination of intramembranous particle density.

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RESULTS

We have studied the structural organization of the OM of *T. pallidum* using freeze-fracture techniques. The morphology of the organism is similar in cross section to the gram-negative bacteria in that an OM delimits the outer cellular surface of the organism and the protoplasmic cylinder is bounded by the inner membrane (IM). Unlike *Escherichia coli* and other typical gram-negative bacteria, the organelles of motility of *T. pallidum* lie entirely within the periplasmic space between the IM and the OM. These organelles, the endoflagella, attach subpolarly near either end of the organism and wrap around the helical protoplasmic cylinder. The cytological organization of *T. pallidum* is characteristic of the spirochetes in general, although details, such as the number of endoflagella and the wavelength of the helix, may differ among members of this diverse group of organisms. Figure 1A shows a low-magnification view of a field containing longitudinally and transversely fractured *T. pallidum* and numerous membrane vesicles from host tissue that coisolated with the organisms. The organism appeared as a round profile of 0.2 μm in diameter when fractured perpendicularly to the major axis. One of the treponemes was fractured parallel to the major axis for a distance of 4.7 μm and demonstrates the spiral morphology of the organism with a wavelength of 1.1 μm. Even at this low magnification, the low particle density of this convex OM fracture face (OMF) is evident. The low particle densities of the convex (inner leaflet) and concave (outer leaflet) OMF of *T. pallidum* are clearly visible in the higher-magnification micrograph (Fig. 1B); a few distinct particles are discernible in largely smooth convex and concave fracture faces. This treponeme was fractured along the convex OMF, through the periplasmic space and the cytoplasmic cylinder, to the concave OMF (Fig. 1B). The fracture faces in Fig. 1 are clearly from the OM because there is only a small fracture step between the cleaved membrane and the surrounding ice; the endoflagella in Fig. 1B provide a morphological marker that confirms this judgment. A total of 1.9 μm^2 of convex OMF and 0.9 μm^2 of concave OMF was quantified from 25 typical organisms (fracture profiles from different fields) and demonstrated that there are approximately 100 particles per μm^2 of convex OMF and 70 particles per μm^2 of concave OMF.

The low particle density observed in the OM of *T. pallidum* does not appear to be an artifact of preparation because the IM of the organism was cleaved following a conventional fracture pattern. Figure 1C shows an organism in which the fracture plane passed from the convex OMF across the periplasmic space to the convex IM fracture face (IMF). The contrast in particle density between the convex faces of the two membranes is evident; the convex IMF contains numerous well-defined particles, while the convex OMF has only a few.

Figure 2A shows *T. phagedenis* Reiter fractured along the major axis for a distance of 1.4 μm. The convex and concave fracture faces of the OM and their constituent particles are visible, as is a fragment of the concave IMF. The particles were not densely packed in either OM fracture face and appeared to be randomly distributed. Quantification of a total of 4.33 μm^2 of convex OMF and 2.7 μm^2 of concave OMF from 25 typical organisms demonstrated particle densities of approximately 350 and 800 particles per μm^2, respectively.

Figure 2B shows 0.95 μm of *S. aurantia* in which the convex and concave OMF, and concave IMF can be distinguished. The dense, regular population of particles in the concave OMF of *S. aurantia* is a striking feature of this organism compared with the corresponding fracture faces of *T. pallidum* and *T. phagedenis* Reiter. Like *T. pallidum* and *T. phagedenis* Reiter, the intramembranous particles in the OM convex fracture face of *S. aurantia* are present in a low concentration and are randomly scattered. Quantification of a total of 0.623 μm^2 of convex OMF and 0.143 μm^2 of concave OMF from 10 typical *S. aurantia* yielded particle densities of 340 and 5,250 particles per μm^2, respectively.

DISCUSSION

Freeze-fracture analysis of *T. pallidum* clearly demonstrates that the concave and convex fracture faces of the OM contain a low density of intramembranous particles, 70 and 100/μm^2, respectively. *T. pallidum* has a sparse distribution of particles on largely smooth OM fracture faces (Fig. 1). In contrast, gram-negative organisms, typified by *E. coli* and *Salmonella typhimurium*, contain from 6,000 to 10,000 particles per μm^2 of OM concave fracture face (14); essentially the entire concave OMF is covered by intramembranous particles (confirmed for *E. coli* in our laboratory [data not shown]). The OM convex fracture face of typical gram-negative organisms contains few intramembranous particles relative to the concave face; *E. coli* contains from 500 to 700 particles per μm^2 of convex OMF (14), while the concentration of particles in the convex OMF of *S. typhimurium* may approach zero (14). The reason for the preferential partitioning of intramembranous particles to one of the two fracture faces is not known (27).

In general, intramembranous particles in biomembranes represent intramembrane (integral) proteins or intramembrane protein-lipid aggregates (3, 14, 23, 29). The correspondence between particles and integral membrane proteins has been well established in the erythrocyte model (23) and also has been demonstrated in artificial biomembranes (31). The low intramembranous particle density of myelin correlates

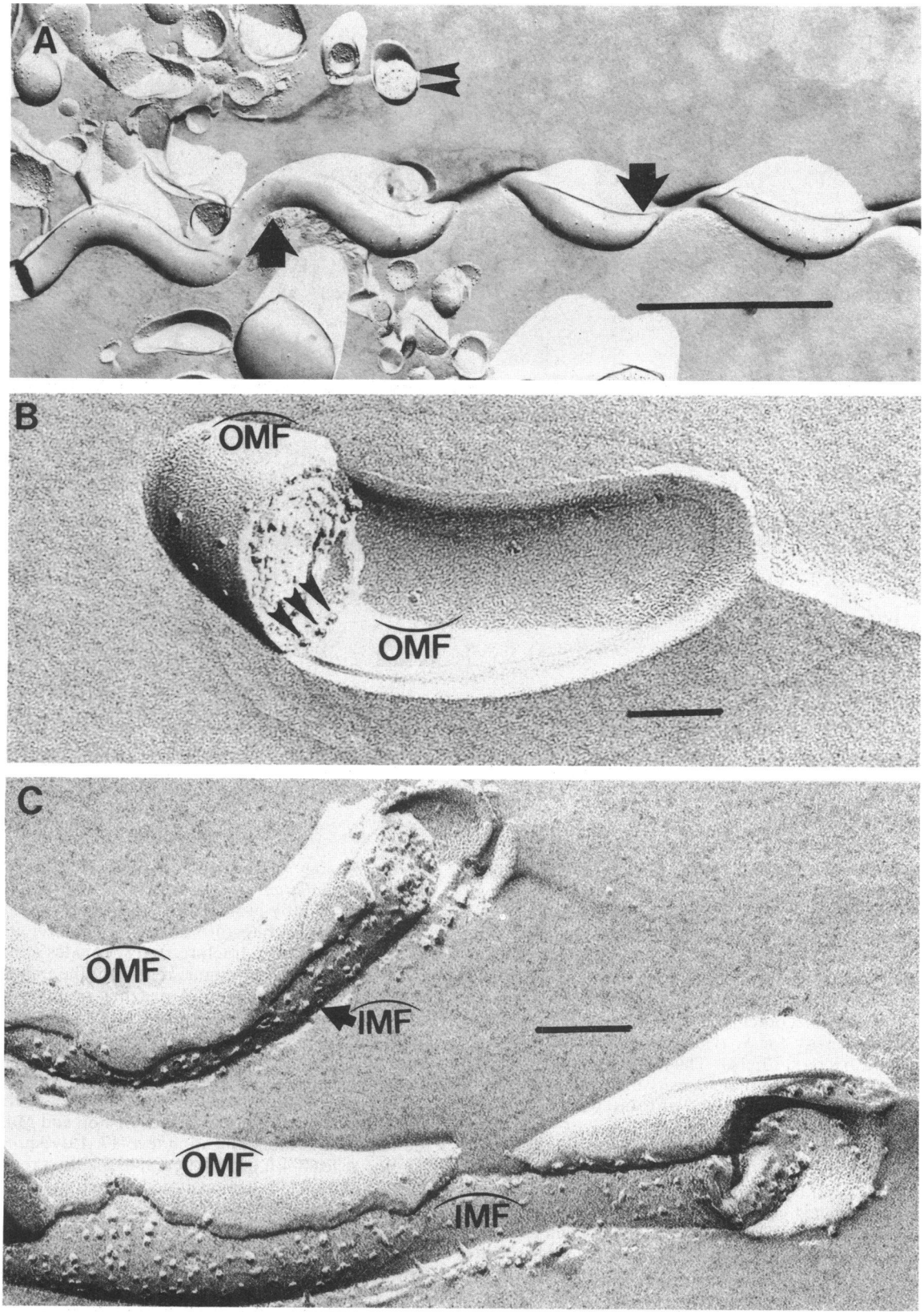

FIG. 1. Freeze-fracture electron micrographs of *T. pallidum*. (A) Low-magnification view showing a treponeme fractured over a 4.7-μm length of the OM convex face (indicated by arrows); a transverse fracture is indicated by arrowheads. Bar = 1.0 μm. (B) High-magnification view of the convex (⌒) OMF and the concave (⌣) OMF of *T. pallidum*; endoflagella are visible in the transverse fracture (indicated by arrowheads). Bar = 0.1 μm. (C) High-magnification view of the convex (⌒) OMF and IMF of *T. pallidum*. Bar = 0.1 μm.

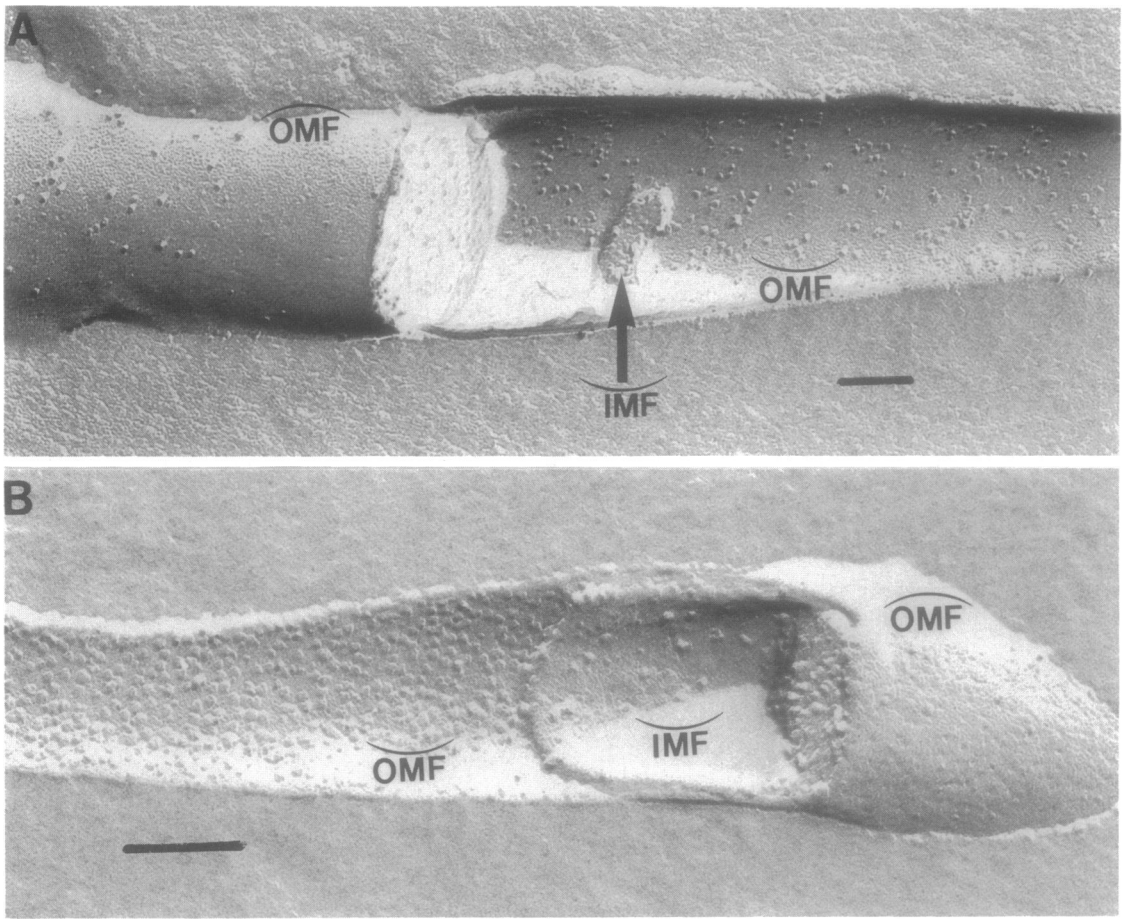

FIG. 2. Freeze-fracture electron micrographs of *T. phagedenis* Reiter (A) and *S. aurantia* (B). (A) High-magnification freeze-fracture electron micrograph of *T. phagedenis* Reiter revealing the concave (⌣) and convex (⌢) OMF and a fragment of the concave (⌣) IMF. Bar = 0.1 μm. (B) High-magnification view of *S. aurantia* in which the convex (⌢) and concave (⌣) OMF and the concave (⌣) IMF are exposed. Bar = 0.1 μm.

with its low protein content (29). Although evidence indicates that the particles of the concave OMF of gram-negative bacteria are lipopolysaccharide-protein aggregates (3, 14), *E. coli* mutants deficient in the outer membrane proteins OmpA, OmpF, and OmpC have a concave OMF particle density that is 25% of the concave OMF particle density of wild-type cells (3, 14). A similar relationship appears to exist between the protein content of the OM and the concave OMF particle density for *S. typhimurium* as well as other gram-negative bacteria (3, 14). Thus, the accumulated evidence indicates that the particle density of the gram-negative concave OMF correlates directly with the integral protein content of the membrane.

The low particle densities of both faces of the OM of *T. pallidum* represent a low OM integral membrane protein content that has not been described previously among other spirochetes or typical gram-negative bacteria. The concave OMF of protein-deficient mutants of *E. coli*, which contains 75% fewer intramembranous particles than the corresponding fracture face of wild-type *E. coli* (3, 14), has an intramembranous particle density that is an order of magnitude greater than either fracture face of the OM of *T. pallidum*. Although *T. pallidum* has typical gram-negative cross-sectional morphology, lipopolysaccharide has not been demonstrated as a component of the organism (2, 12, 21). The lack of lipopolysaccharide does not preclude the probability that the morphology of the particles seen on the OM fracture faces of *T. pallidum* is determined by lipid-protein interaction rather than by protein alone.

The unusual architecture of the *T. pallidum* OM demonstrated by this freeze-fracture study provides a likely explanation for many observations regarding the immunobiology of this fastidious pathogen. A puzzling property of *T. pallidum* has been the resistance of freshly extracted, virulent organisms to the detectable binding or action of specific antibodies in serum when the organisms are tested in several immunological and serological reactions. Minimal demonstration of complement-dependent antibody activity in the in vivo-in vitro neutralization test of Bishop and Miller (4) and the *T. pallidum* immobilization test (17, 19) requires incubation for at least 4 h at 34°C; complete killing requires 16 h. Hardy and Nell (10) demonstrated that prolonged incubation at 4°C was required before the treponemes could be agglutinated by syphilitic serum. Freshly extracted treponemes require aging (15) or acetone fixation (20) to exhibit reactivity in indirect immunofluorescence assays. Hovind-Hougen et al. (11) showed that the binding of specific antibodies against *T. pallidum* from human serum could not be detected by immunoelectron microscopy unless the organisms were incubated with the antiserum and active complement for 16 h under the conditions of the *T. pallidum* immobilization test; similar experimental conditions were required to detect

the binding of monospecific antibody against either one of two recombinant treponemal proteins (7, 25). Although a protective coat of host or treponemal material exterior to the OM has been postulated to explain the lack of antigenicity of the surface of *T. pallidum* (1, 8, 9, 22), the very low protein composition of the OM reported here is sufficient to explain the antigenic inertness that has been observed. In order for the binding of antibody to reach the level of detection of agglutination, immunofluorescence, or immunoelectron microscopy, disruption of the OM by physical, chemical, or immunological means may be required to allow antibody to bind to the more abundant subsurface and inner membrane epitopes. The long incubation time necessary for in vitro complement-dependent antibody-mediated killing of *T. pallidum* may represent the kinetics of complement fixation by antibody bound to an OM that apparently contains few target protein molecules. This issue is under investigation in our laboratory.

The observation by freeze-fracture electron microscopy that the OM of *T. pallidum* has a low integral membrane protein content provides an explanation at the molecular level for recent observations that suggest the OM has little or no constituent protein. Penn et al. (21) showed that only a protein corresponding in molecular weight to rabbit serum albumin was radiolabeled extrinsically on intact, unwashed *T. pallidum*, whereas many proteins were labeled when the organisms were treated first with detergent. Stamm et al. (28) isolated putative outer membrane material after treating *T. pallidum* with 0.04% sodium dodecyl sulfate. The sole protein constituents of this material were derived from the periplasmic endoflagella. Radolf et al. (24) reported that the nonionic detergent Triton X-114 appeared to solubilize the OM without the concomitant release of protein; however, conclusions about the integrity of the OM after detergent treatment were based on whole-mount electron microscopy and did not include evaluation of thin sections. Therefore, a quantitative assessment of the degree of OM release was not possible. In our laboratory, Cunningham et al. showed by thin-section electron microscopy that 1% Triton X-114 removed the OM of *T. pallidum* but left the IM morphologically intact (6). After phase partitioning of the Triton-soluble material, a set of eight polypeptides remained in the detergent phase, characteristic of integral membrane proteins. These results suggested that these polypeptides may be constituents of the treponemal OM. However, as Cunningham et al. (6, addendum) reported, the abundance of the individual detergent-phase molecules relative to the scarcity of the integral OM protein demonstrated by our freeze-fracture studies implied that none of the eight hydrophobic polypeptides were likely to be the integral OM proteins seen by freeze fracture; the TX-114-soluble polypeptides may have been extracted from the IM or the periplasmic space of *T. pallidum*.

The rare integral membrane protein that we have identified in the OM of *T. pallidum* may be functionally important in the pathogenesis of syphilis. On the basis of the observation that fixation of *T. pallidum* at low temperature before quenching for freeze fracture does not induce aggregation of the OM intramembranous particles, Radolf et al. (26) have reported that the particles may have constrained lateral mobility. However, data from our laboratory has shown that although the particles do not aggregate in a temperature-dependent fashion, significant aggregation occurs after the organisms are incubated for 16 h in heat-inactivated immune rabbit serum and this aggregation precedes the in vitro complement-dependent killing of *T. pallidum* (D. R. Blanco, E. M. Walker, D. H. Haake, C. I. Champion, J. N. Miller, and M. A. Lovett, submitted for publication). This suggests that antibody-mediated cross-linking of OM integral membrane protein, which occurs with slow kinetics as a result of the scarcity of targets, is necessary for efficient complement fixation. Thus, there is reason to believe that the integral OM protein constituents of *T. pallidum*, which we have termed generically the treponemal rare outer membrane protein, represent an important bacterial strategy for evading the host immune response. Because the treponemal rare outer membrane protein represents such a small molar proportion of the organism, the immune response to the treponemal rare outer membrane protein may be delayed, perhaps providing an explanation for the following observations. (i) Complete infection-derived immunity to reinfection with *T. pallidum* takes several months to develop in the rabbit model of experimental syphilis (4). (ii) Successful protection of rabbits against experimental syphilis required vaccination of the animals with a 37-week time course of 3.71×10^9 gamma irradiation-attenuated *T. pallidum* (16).

Although the freeze-fracture appearance of *T. pallidum* appears to show unequivocally that the OM of the organism contains little protein, some caution must be observed in interpreting our results. Freeze fracture does not address the possibility that extrinsic peripheral membrane proteins may exist on the surface of *T. pallidum*. The lack of detectable binding of antibody to the surface of intact *T. pallidum* by immunofluorescence (15, 20) or immunoelectron microscopy (7, 11, 25) suggests that such molecules are either not abundant or not present on the OM of *T. pallidum*. Because *T. pallidum* cannot be passaged in vitro, the organisms are extracted from infected rabbit testes in which an ongoing host response is occurring; the effects of this complex environment on the organism might be questioned. Examination of many freeze-fracture replicas showed that the OM fracture faces were consistent in appearance; no changes in morphology or particle distribution were observed that might indicate the action of host factors such as proteases on the surface of the organism (5). Suspensions obtained with our extraction protocol contain viable (100% motile), virulent (18) treponemes. These organisms are also capable of several biological functions in vitro that correlate with pathogenesis in vivo; they attach to a number of cell types in culture (9) and can penetrate endothelial cell monolayers through tight junctions (30). Our thin-section electron microscopy studies have shown that freshly extracted organisms have intact cross-sectional morphology characteristic of that of the spirochetes (6). Thus, the organisms used in this study represent, by all available biological parameters, virulent, structurally intact *T. pallidum*.

The OM of *T. phagedenis* Reiter, a commensal treponeme that can be grown in vitro in a serum-supplemented medium, is similar to *T. pallidum* in that the intramembranous particles of the concave OMF are not densely packed (Fig. 2A). Although the OM concave fracture face of *T. phagedenis* Reiter contains more particles than the corresponding fracture face of *T. pallidum* (800 and 70 per μm^2, respectively), the concave OMF particle content of *T. phagedenis* Reiter is approximately 1 order of magnitude less than that of *E. coli* and other typical gram-negative organisms. The low particle concentration of the OM convex fracture face of *T. phagedenis* Reiter is slightly less than one-half that of the concave OMF; some preferential partitioning of particles into the concave OMF appears to be occurring.

In contrast to *T. phagedenis* Reiter and *T. pallidum*, the OM of *S. aurantia*, a free-living spirochete that can be grown

in vitro in a defined medium, is densely populated with integral membrane proteins. The intramembranous particle concentration in the concave OMF of *S. aurantia* (5,250/μm^2) and the packing of these particles into an almost continuous two-dimensional array highlight the structural similarity between *S. aurantia* and typical gram-negative organisms. Similar to typical gram-negative bacteria, the OM convex fracture face of *S. aurantia* contains relatively few intramembranous particles; the concentration of particles that partition into the convex OMF of *S. aurantia* is approximately 1 order of magnitude less than the concentration of particles that partition into the concave OMF.

The results of our freeze-fracture analysis of *T. pallidum* and *T. phagedenis* Reiter are similar to results reported recently by Radolf et al. (26). However, the inclusion of *S. aurantia* in this study and our careful quantitation of the particle densities of *T. pallidum*, *T. phagedenis* Reiter, and *S. aurantia* elucidate the OM ultrastructural diversity among the spirochetes. Our freeze-fracture results demonstrate a degree of OM structural relatedness between the two treponemes and clearly distinguish them from *S. aurantia* and typical gram-negative bacteria.

It is apparent from this study that a low OM protein concentration is not a structural requirement of the spirochetes; therefore, the low integral OM protein content of *T. pallidum* may be a specific evolutionary adaptation by which this organism evades the host immune response. The strikingly smooth OM fracture faces of *T. pallidum*, in light of the known lack of antigenicity of the surface of this organism, strongly suggest this. Whether the relatively low concentration of integral proteins in the OM of *T. phagedenis* Reiter may contribute to survival of the organism in the host is unclear. It has been reported that the kinetics of in vitro killing of *T. phagedenis* Reiter are also somewhat prolonged under conditions similar to the *T. pallidum* immobilization test (32); 3 to 4 h was required for complete immobilization of *T. phagedenis* Reiter compared with 16 h for *T. pallidum*. Interpretation of these results is complicated by the following two considerations. (i) *T. phagedenis* Reiter does contain a 10-fold-greater concentration of OM intramembranous particles than *T. pallidum*. (ii) Unlike *T. pallidum*, the OM of *T. phagedenis* Reiter appears to contain lipopolysaccharide (2). Preliminary freeze-fracture results obtained in our laboratory for *Borrelia burgdorferi*, the etiologic agent of Lyme borreliosis, indicate that this organism has an OM integral membrane protein content 20-fold greater than that of *T. pallidum*. Therefore, the OM architecture of *T. pallidum* may represent a parasitic strategy specific to the treponemes and not general to the host-associated spirochetes. We are continuing and extending our studies of *B. burgdorferi* and other pathogenic spirochetes in order to address this question.

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LITERATURE CITED

1. **Alderete, J. F., and J. B. Baseman.** 1979. Surface-associated host proteins on virulent *Treponema pallidum*, Infect. Immun. **26:**1048–1056.
2. **Bailey, M. J., C. W. Penn, and A. Cockayne.** 1985. Evidence for the presence of lipopolysaccharide in *Treponema phagedenis* (biotype Reiterii) but not in *Treponema pallidum* (Nichols). FEMS Microbiol. Lett. **27:**117–121.
3. **Beveridge, T. J.** 1981. Ultrastructure, chemistry and function of the bacterial wall. Int. Rev. Cytol. **72:**229–317.
4. **Bishop, N. H., and J. N. Miller.** 1976. Humoral immunity in experimental syphilis. II. The relationship of neutralizing factors in immune serum to acquired resistance. J. Immunol. **117:**197–207.
5. **Branton, D.** 1971. Freeze-etching studies of membrane structure. Philos. Trans. R. Soc. Lond. B Biol. Sci. **261:**133–138.
6. **Cunningham, T. E., E. M. Walker, J. N. Miller, and M. A. Lovett.** 1988. Selective release of *Treponema pallidum* outer membrane and associated polypeptides with Triton X-114. J. Bacteriol. **170:**5789–5796.
7. **Fehniger, T. E., J. D. Radolf, A. M. Walfield, T. M. Cunningham, J. N. Miller, and M. A. Lovett.** 1986. Native surface association of a recombinant 38-kilodalton *Treponema pallidum* antigen isolated from *Escherichia coli* outer membrane. Infect. Immun. **52:**586–593.
8. **Fitzgerald, T. J., and R. C. Johnson.** 1979. Surface mucopolysaccharides of *Treponema pallidum*. Infect. Immun. **24:** 244–251.
9. **Fitzgerald, T. J., J. N. Miller, and J. A. Sykes.** 1975. *Treponema pallidum* (Nichols strain) in tissue cultures: cellular attachment, entry, and survival. Infect. Immun. **11:**1133–1140.
10. **Hardy, P. H., Jr., and E. E. Nell.** 1957. Study of the antigenic structure of *Treponema pallidum* by specific agglutination. J. Hyg. **66:**160–162.
11. **Hovind-Hougen, K., A. Birch-Andersen, and H. A. Nielsen.** 1979. Electron microscopy of treponemes subjected to the *Treponema pallidum* immobilization (TPI) test. Acta Pathol. Microbiol. Scand. **87:**263–268.
12. **Johnson, R. C.** 1983. Composition, p. 29–37. *In* R. F. Schell and D. M. Musher (ed.), Pathogenesis and immunology of treponemal infection. Marcel Dekker, Inc., New York.
13. **Leschine, S. B., and E. Canale-Parola.** 1986. Rifampin-resistant RNA polymerase in spirochetes. FEMS Microbiol. Lett. **35:** 199–204.
14. **Lugtenberg, B., and L. Van Alphen.** 1983. Molecular architecture and functioning of the outer membrane of *Escherichia coli* and other gram-negative bacteria. Biochim. Biophys. Acta **737:**51–115.
15. **Metzger, M., and J. Ruczkowka.** 1964. Influence of lysozyme upon reactivity of *Treponema pallidum* in the fluorescent antibody reaction. Arch. Immunol. Ther. Exp. **12:**702–708.
16. **Miller, J. N.** 1973. Immunity in experimental syphilis. VI. Successful vaccination of rabbits with *Treponema pallidum*, Nichols strain, attenuated by γ-irradiation. J. Immunol. **110:** 1206–1215.
17. **Miller, J. N., F. P. Fazzan, and S. J. Whang.** 1963. Studies on immunity in experimental syphilis. II. *Treponema pallidum* immobilization (TPI) antibody and the immune response. Br. J. Vener. Dis. **39:**199–203.
18. **Miller, J. N., S. J. Whang, and F. P. Fazzan.** 1963. Studies on immunity in experimental syphilis. I. Immunologic response of rabbits immunized with Reiter protein antigen and challenged with virulent *Treponema pallidum*. Br. J. Vener. Dis. **39:** 195–198.
19. **Nelson, R. A., Jr., and M. M. Mayer.** 1949. Immobilization of *Treponema pallidum* in vitro by antibody produced by syphilitic infection. J. Exp. Med. **89:**369–393.
20. **Penn, C. W.** 1981. Avoidance of host defenses by *Treponema pallidum* in situ and on extraction from infected rabbit testes. J. Gen. Microbiol. **126:**69–75.
21. **Penn, C. W., A. Cockayne, and M. J. Bailey.** 1985. The outer membrane of *Treponema pallidum*: biological significance and biochemical properties. J. Gen. Microbiol. **131:**2349–2357.

22. **Peterson, M., J. B. Baseman, and J. F. Alderete.** 1983. *Treponema pallidum* receptor binding proteins interact with fibronectin. J. Exp. Med. **157**:1958–1970.
23. **Pinto da Silva, P.** 1979. Interpretation of freeze-fracture and freeze etch images: morphology and realism, p. 185–193. *In* J. E. Rash and C. S. Hudson (ed.), Freeze fracture: methods, artifacts, and interpretations. Raven Press, New York.
24. **Radolf, J. D., N. R. Chamberlain, A. Clausell, and M. V. Norgard.** 1988. Identification and localization of integral membrane proteins of virulent *Treponema pallidum* subsp. *pallidum* by phase partitioning with the nonionic detergent Triton X-114. Infect. Immun. **56**:490–498.
25. **Radolf, J. D., T. E. Fehniger, F. J. Siverblatt, J. N. Miller, and M. A. Lovett.** 1986. The surface of virulent *Treponema pallidum*: resistance to antibody binding in the absence of complement and surface association of recombinant antigen 4D. Infect. Immun. **52**:579–585.
26. **Radolf, J. D., M. V. Norgard, and W. W. Shulz.** 1989. Outer membrane ultrastructure explains the limited antigenicity of virulent *Treponema pallidum*. Proc. Natl. Acad. Sci. USA **86**:2051–2055.
27. **Satir, B. H., and P. Satir.** 1979. Partitioning of intramembrane particles during the freeze-fracture procedure, p 43–49. *In* J. E. Rash and C. S. Hudson (ed.), Freeze fracture: methods, artifacts, and interpretations. Raven Press, New York.
28. **Stamm, L. V., R. L. Hodinka, P. B. Wyrick, and P. J. Bassford, Jr.** 1987. Changes in the cell surface properties of *Treponema pallidum* that occur during in vitro incubation of freshly extracted organisms. Infect. Immun. **55**:2255–2261.
29. **Stolinski, C., and A. S. Breathnach.** 1975. Freeze-fracture replication of biological tissues. Academic Press, Inc., San Francisco.
30. **Thomas, D. D., M. Navab, D. A. Haake, A. M. Fogelman, J. N. Miller, and M. A. Lovett.** 1988. *Treponema pallidum* invades intercellular junctions of endothelial cell monolayers. Proc. Natl. Acad. Sci. USA **85**:3608–3612.
31. **Wehrli, E., and P. D. Morse II.** 1974. Recombination of human erthrocyte apoprotein and lipid. II. Visualization of apoprotein-lipid bilayer complex by freeze-etching. J. Supramol. Struct. **2**:71–78.
32. **Zaffiro, P., G. Di Chiara, and G. Tringali.** 1964. Azione immobilizzante e treponemicida dei siereimmuni anti-Reiter. Riv. Ist. Sieroter. Ital. **39**:421–425.