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Outer membrane ultrastructure explains the limited antigenicity of virulent *Treponema pallidum*

(syphilis/freeze-fracture electron microscopy)

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ABSTRACT **Freeze fracture and deep etching were used to investigate the ultrastructural basis for the observation that anti-treponemal antibodies bind poorly to the surface of virulent *Treponema pallidum*. Fractures of *T. pallidum* outer membranes contained scarce, uniformly sized intramembranous particles (IMPs). IMPs on the convex faces often appeared to form linear arrays that wound in spirals about the organism. In contrast to the outer membrane, IMPs of the cytoplasmic membrane were randomly distributed, numerous, and heterogeneous in size. In *Escherichia coli* and *T. pallidum* cofractures, IMPs of the *E. coli* outer membranes were densely packed within the concave fracture faces, while the *T. pallidum* fractures were identical to the experiments lacking the *E. coli* internal controls. Outer membranes of two representative nonpathogenic treponemes, *Treponema phagedenis* biotype Reiter and *Treponema denticola*, contained numerous IMPs, which segregated preferentially with the concave halves. Examination of apposed replicas and deep-etched specimens indicated that at least some of the IMPs extend through the *T. pallidum* outer membrane and are exposed on the surface of the organism. The outer membrane of intact *T. pallidum* appears to contain a paucity of integral membrane proteins that can serve as targets for specific antibodies. These findings appear to represent an unusual parasitic strategy for evasion of host humoral defenses.**

Syphilis, a sexually transmitted disease caused by the bacterium *Treponema pallidum* subsp. *pallidum*, continues to be a major global public health problem. In untreated individuals, syphilis is a chronic infection that progresses through stages with characteristic clinical manifestations. The mechanisms that enable virulent treponemes to survive for years in the face of vigorous humoral and cellular immune responses by the host are among the most poorly understood aspects of syphilis pathogenesis.

Like all spirochetes, *T. pallidum* morphologically consists of an outer membrane that surrounds the periplasmic endoflagella, the cytoplasmic membrane, and the protoplasmic cylinder (1). Investigators have presumed that, as with other bacterial pathogens, surface-exposed molecules mediate the interactions between treponemes and their human hosts (2). For this reason, characterization of the outer membrane of *T. pallidum* and analysis of the structure's protein constituents have become major goals of treponemal research. Complicating such investigations is the fact that *T. pallidum* remains one of the few important pathogens of humans that cannot be maintained by continuous *in vitro* cultivation. Recombinant DNA and monoclonal antibody methodologies have facilitated analyses of a number of treponemal immunogens, although the precise cellular locations of most of them remain uncertain (3–7).

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Despite these limitations, recent investigations have demonstrated that the *T. pallidum* outer membrane differs significantly from the analogous structure of conventional Gram-negative bacteria. It does not contain lipopolysaccharide (8, 9), and it can be disrupted under certain conditions (e.g., low concentrations of detergents) and by physical manipulations (e.g., centrifugation and resuspension) that cause little discernible damage to the outer membranes of conventional Gram-negative bacteria (8, 10–12). Perhaps of profound significance with respect to the ability of the organism to evade host immune defenses, virulent organisms *in vitro* bind only small amounts of the specific antibodies present in human or rabbit syphilitic sera (11–14). This remarkable phenomenon traditionally has been attributed to a layer of host proteins and mucopolysaccharides external to the outer membrane (15, 16). However, cell fractionation experiments by several groups of investigators suggest that the poor antigenicity of the outer membrane results from a paucity of integral membrane proteins and that the large majority of the organism's integral membrane proteins are associated with the cytoplasmic membrane (8, 10, 11).

Freeze-fracture and freeze-etch electron microscopy can visualize integral membrane proteins as discrete intramembranous particles (IMPs) (17). We reasoned, therefore, that these methods would be ideally suited for estimation of the overall protein content of the *T. pallidum* outer membrane relative to that of other microorganisms. These techniques have provided conclusive evidence that the outer membrane contains only a small number of transmembrane proteins and that its surface is not covered by an outer coat. We believe that these findings explain the unusual *in vitro* interactions between the organism and anti-treponemal antibodies and that they reveal a previously undescribed mechanism of bacterial pathogenesis.

MATERIALS AND METHODS

Bacterial Strains. *T. pallidum* (Nichols) was propagated in New Zealand White rabbits and extracted at room temperature in phosphate-buffered saline (PBS; pH 7.2) as described (10). Thioglycolate stock cultures of the two nonpathogenic treponemes, *Treponema phagedenis* biotype Reiter and *Treponema denticola*, were subcultured into 10-ml portions of spirolate medium (BBL Microbiology Systems) supplemented with 10% heat-inactivated normal rabbit serum and were grown at 34°C for 3 days. Ten-milliliter cultures of *Escherichia coli* RR1 in standard LB broth were grown to midlogarithmic phase (OD_{600}, ≈0.6) by shaking with aeration in a water bath at 37°C.

Abbreviation: IMPs, intramembranous particles.

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Fixation of Cells. *T. pallidum* cells were fixed without any manipulations, other than extraction from the rabbit testes, to avoid disruption of the outer membrane and to preserve host material that might be associated with the surface of the organism (15, 16). Five-milliliter portions containing freshly extracted *T. pallidum* were placed in a circulating water bath at 34°C or on ice for 30 min. They then were mixed gently with equal volumes of 4% glutaraldehyde in 100 mM sodium phosphate/100 mM sucrose, pH 7.4, preequilibrated to the same temperatures. Five-milliliter portions from cultures of the nonpathogenic treponemes were fixed at 34°C by the same method. To produce approximate 1:1 suspensions of *T. pallidum* and *E. coli* RRI, midlogarithmic-phase cultures of *E. coli* were washed three times in PBS at room temperature and enumerated by measurement of optical density at 600 nm; 2×10^9 *E. coli* were added with gentle mixing to an equal number of freshly extracted virulent *T. pallidum*. The mixed suspensions were equilibrated at either 34°C or 0°C and fixed as described above. All specimens were fixed for at least 1 hr before the cells were pelleted by centrifugation at 20,000 × *g* (20 min at 4°C) and stored at the same temperature.

Freeze-Fracture and Freeze-Etch Electron Microscopy. For routine freeze fracture, fixed organisms were infused with 30% (vol/vol) glycerol as a cryoprotectant. The cells were washed in distilled water prior to freezing, when deep etching was performed. Blank apposed gold specimen holders for the Denton DFE-3 freeze-etch module were modified by scratching grooves into the smooth apposed surface. With this modification, only 0.2 μl of cell pellet is required and fracture occurs very close to the gold surface where the freezing rate is high and freezing artifacts are minimal. Loaded gold holders were dropped into liquid Freon 22 (DuPont) at its melting point. Specimens were fractured in the Denton DFE-3 freeze-etch module at −115°C to −120°C and immediately platinum shadowed from an electron beam gun of our own design (18). Carbon was deposited by standard resistance evaporation. In a few experiments, the specimens were fractured at −100°C and etched for up to 4 min. Replicas were floated and cleaned for at least 1 hr in 1.2% sodium hypochlorite (from commercial bleach) in the wells of porcelain spot plates. After three rinses in deionized water, the replicas were picked up onto uncoated 300-mesh grids. Breaking of replicas due to surface tension during transfers with a platinum loop was essentially eliminated by adding a 2- to 3-μl drop of 0.5% Tween 20 to cleaning and rinse solutions in each well. Replicas were examined and photographed with a JEOL 100C electron microscope at 80 kV. The electron micrograph film was reversal processed to obtain black shadows on prints (19). The size of membrane particles was measured on prints with a final magnification of ×100,000.

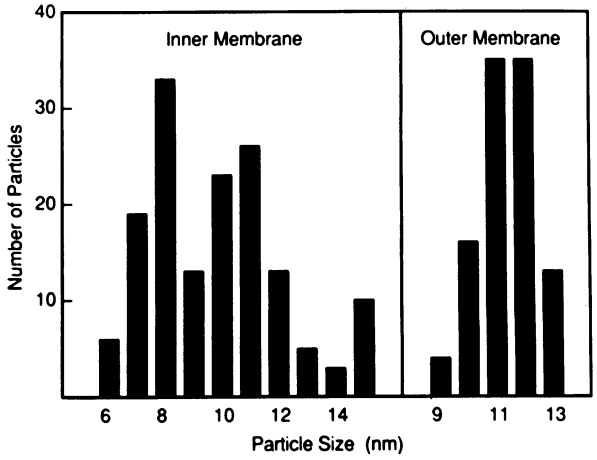

FIG. 2. Histogram plots of IMP sizes for the convex halves of the inner and outer membranes of *T. pallidum*.

RESULTS

General Appearance of Freeze-Fractured *T. pallidum*. *T. pallidum* fractured predominantly through the outer membrane. In many cases, the level of the fracture was confirmed by identifying the periplasmic endoflagella below the level of cleavage (Fig. 1*A*). Sparsely distributed IMPs were found on both the convex and the concave fracture faces of the outer membrane. The IMPs on the convex faces were more prominent and often appeared to form periodic linear arrays that wound in spirals about the organism (Fig. 1*A*). Fractures through the cytoplasmic (inner) membrane were rare and usually exposed only small areas. A single fracture was found that revealed a large portion of the cytoplasmic membrane (Fig. 1*B*). The convex face of the cytoplasmic membrane contained an obviously greater density of IMPs than the corresponding face of the outer membrane. Size measurements for the IMPs of the outer and inner membrane convex faces were compared in histogram plots (Fig. 2). The outer membrane particles had a narrow unimodal size distribution centered about 11–12 nm. In contrast, the size range of the inner membrane particles was much broader and had a bimodal distribution.

Comparison with *E. coli* and Nonpathogenic Treponemes. It has been well established in *E. coli* that IMPs are abundant

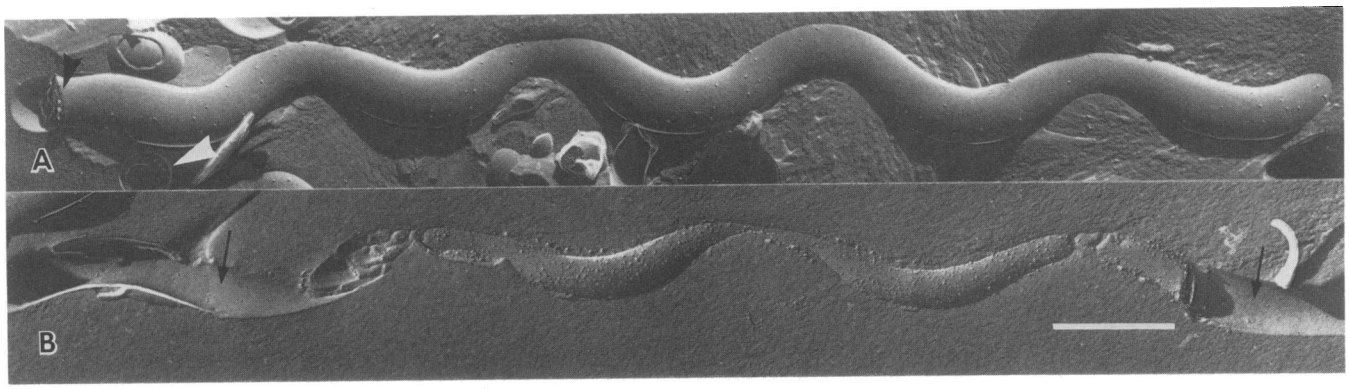

FIG. 1. *T. pallidum* fixed at 34°C, cryoprotected in 30% (vol/vol) glycerol, and fractured at −120°C. (*A*) Convex fracture face of the outer membrane showing prominent but sparse IMPs, which, in some regions, appear to form linear arrays. The two sets of cross-fractured endoflagella (black and white arrowheads at the left of the figure) serve as points of reference for the identification of inner (cytoplasmic) and outer membranes. (*B*) The convex fracture face of the inner membrane (center of micrograph) exhibits numerous IMPs that are randomly distributed and heterogeneous in size. The concave fracture face of the outer membrane (arrows) has sparse particles that are less prominent than, but similar in distribution to, those on the convex outer membrane. (Bar = 0.5 μm.)

in the outer membrane and that they segregate primarily with the concave fracture face (20). We therefore cofractured *T. pallidum* and *E. coli* to confirm that the paucity of IMPs in the *T. pallidum* outer membrane and their apparent tendency to segregate with the convex membrane half were not artifacts. *E. coli* cells displayed their typical freeze fracture behavior in that most of the cleavages occurred through the cytoplasmic membranes. The concave faces of the *E. coli* outer membranes were densely covered with IMPs, as previously observed (20), whereas the distribution of IMPs in the *T. pallidum* outer membranes was unchanged from the previous experiment (Fig. 3). For further comparison, we fractured two nonpathogenic treponemes, *T. phagedenis* biotype Reiter and *T. denticola*. Unlike *T. pallidum*, the outer and cytoplasmic membranes of the nonpathogens fractured in approximately equal proportions. The outer membranes of both nonpathogens displayed an obviously greater (10- to 15-fold) particle density and a preference for the IMPs to segregate with the concave outer membrane halves (Fig. 4). Particle densities in the inner membranes of all three treponemal species were similar.

Fixation at low temperatures may induce the aggregation of IMPs and the formation of particle-free patches within the inner membrane of *E. coli* (21). This phenomenon was exploited to determine whether the stereotypical pattern of the *T. pallidum* outer membrane IMPs was indicative of constrained mobility in the plane of the membrane. The inner membranes of both *E. coli* and *T. pallidum* exhibited aggregation of IMPs when fixed at 0°C (data not shown). However, fixation at this temperature had no effect on the freeze-fracture behavior (preferred outer membrane fracture) or the sparse distribution of IMPs within the outer membrane (data not shown).

Outer Membrane Architecture in *T. pallidum*. In most freeze-fracture experiments, pits are left in one membrane half when IMPs segregate with the other (17). However, in spite of meticulous examination of many *T. pallidum* replicas, pits were rarely found on either outer membrane fracture face. Instead, we noticed that the distribution of IMPs was similar in both fracture faces but that the IMPs of the concave halves had lower profiles (Fig. 1). This suggested that the IMPs were cleaved along the fracture plane of the outer membrane, a phenomenon sometimes referred to as plastic deformation (22). This was confirmed upon examination of apposed replicas in which matching IMP patterns were found on complementary outer membrane halves (Fig. 5 *A* and *B*). Deep etching revealed that at least some of the IMPs appear to extend entirely through the outer membrane and are exposed on the surface of the organism. Except for the rare protruding particles, the surface of the organism appeared to be entirely smooth (Fig. 5*C*).

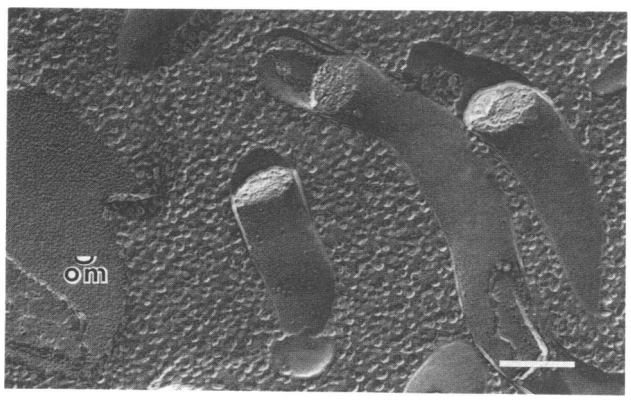

FIG. 3. Outer membrane fractures of *E. coli* and *T. pallidum*. The concave outer membrane (om) of *E. coli* shows a uniformly dense distribution of IMPs in sharp contrast to the scarce particles in the fracture faces of the *T. pallidum* outer membrane. (Bar = 0.2 μm.)

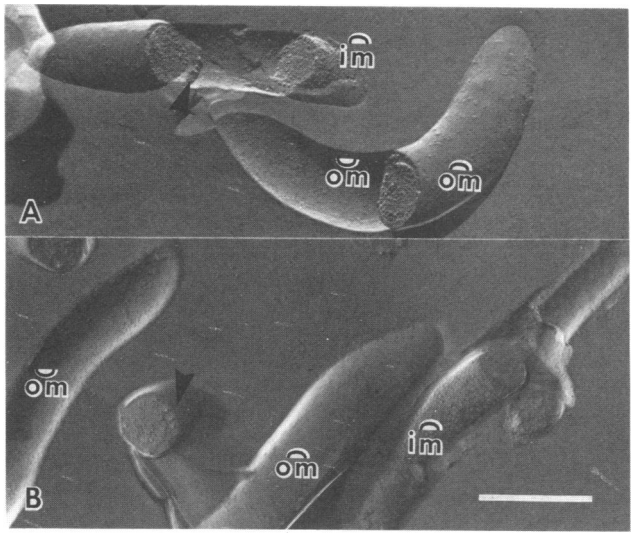

FIG. 4. Nonpathogenic treponemes prepared and fractured identically to *T. pallidum* in Fig. 1. (*A*) *T. phagedenis* biotype Reiter. (*B*) *T. denticola*. Convex (om) and concave (om) outer membrane halves of both organisms are distinctly richer in IMPs than the outer membrane halves of *T. pallidum*. The particle populations on the convex inner membrane (im) halves appear similar in all three treponemal species. Arrowheads, endoflagella. (Bar = 0.5 μm.)

DISCUSSION

Certain aspects of human and experimental syphilis suggest that the ability of virulent *T. pallidum* to avoid binding anti-treponemal antibodies plays a fundamental role in syphilis pathogenesis. Patients with early syphilis undergo recurrent episodes of spirochetemia despite high serum titers of specific antibodies (23, 24). In the rabbit model of experimental syphilis, resistance to intradermal challenge correlates poorly with titers of anti-treponemal antibodies (25, 26). The freeze-fracture and deep-etch experiments in this report explain the ultrastructural basis for this phenomenon.

IMPs were scarce in *T. pallidum* outer membrane fractures. Their uniform size and nonrandom distribution suggested that they may represent a single integral membrane protein or oligomer. Examination of apposed replicas and deep-etched specimens indicated that at least some of the IMPs extend through the outer membrane and are exposed on the surface of the organism. The smoothness of the portions of the *T. pallidum* surface revealed by deep-etching did not appear to be consistent with the presence of an outer coat of host proteins and/or mucopolysaccharides. Entirely unexpected was the finding that IMPs on the convex faces often encircle the organism in spiral arrays. This pattern suggested that they might be associated with the periplasmic endoflagella, the organelles of motility that encircle the protoplasmic cylinder in a similar manner (1). In contrast to the outer membrane, the particle density of the cytoplasmic membrane was at least 10-fold greater; the cytoplasmic membrane IMPs also differed by being randomly distributed and heterogeneous in size. The paucity of IMPs in the *T. pallidum* outer membrane was in sharp contrast to the densely covered concave faces of cofractured *E. coli* outer membranes and the significantly greater particle density in the concave halves of outer membranes from *T. phagedenis* biotype Reiter and *T. denticola*, two representative nonpathogens.

The distinctly different freeze-fracture behaviors of *T. pallidum* and *E. coli* are noteworthy. *E. coli* outer membranes fracture poorly in comparison with the cytoplasmic mem-

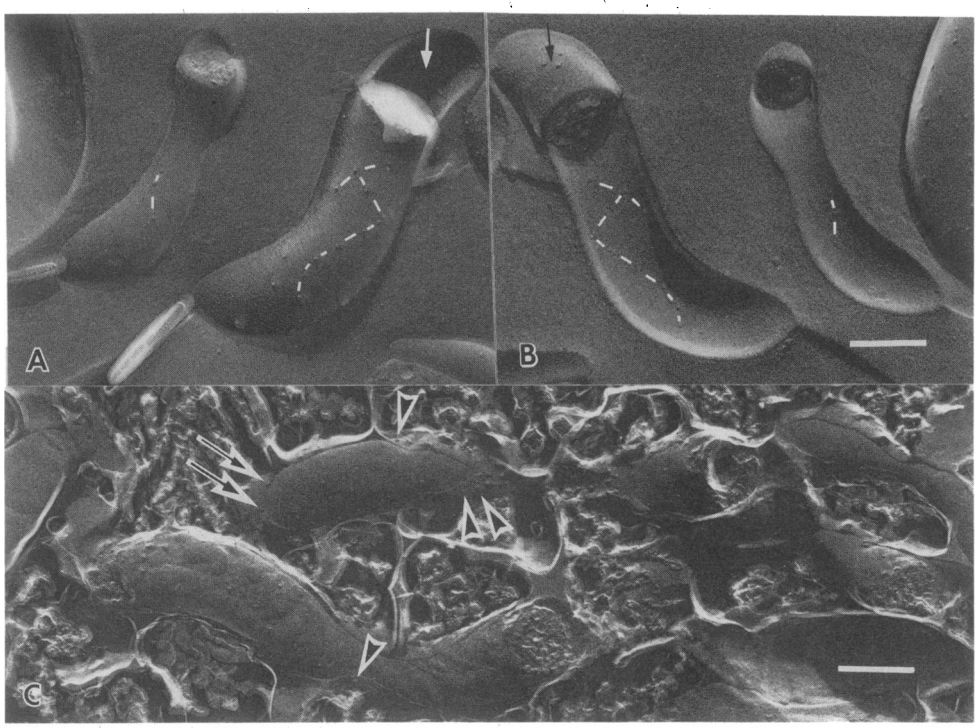

FIG. 5. Apposed and deep-etched fracture faces of *T. pallidum*. (*A* and *B*) Apposed replicas. The IMPs on complementary outer membrane halves are arranged in clearly matching patterns (broken white lines and small arrows). (*C*) Deep-etched replicas. The surface of the organism is smooth and has very few particles (arrowheads), which appear to be similar in size and distribution to the IMPs. Occasional IMPs can be seen extending through the outer membrane half along a fracture line (arrows). (Bar = 0.2 μm.)

brane (20, 27). Mutant cells that are either deficient in major outer membrane proteins or that contain a rough lipopolysaccharide chemotype fracture more readily through the outer membrane, findings attributed to the increased phospholipid/protein ratio in the mutant cell outer membranes (20, 27). The fact that nearly all of the *T. pallidum* fractures included generous portions of outer membrane is consistent with the absence of lipopolysaccharide and an extremely high phospholipid/protein ratio in the outer membrane. The relative rarity of fractures through the cytoplasmic membrane of *T. pallidum* was somewhat surprising, especially since cytoplasmic membrane fractures occurred frequently in the nonpathogenic treponemes. The biochemical basis for this observation remains obscure.

The current studies have provided significant support for the contention that the outer membrane of *T. pallidum* contains a paucity of integral membrane proteins (10). Nevertheless, the freeze-fracture data must be interpreted cautiously because the technique can identify only integral membrane proteins whose polypeptide chains reside at least partly within the phospholipid bilayer (17). Proteins anchored to a membrane solely by one or more hydrophobic moieties presumably would not be identified by this technique. This point is relevant because we have discovered recently that a number of abundant *T. pallidum* membrane immunogens are lipoproteins (N. R. Chamberlain, M. E. Brandt, A. L. Erwin, R. S. Munford, J.D.R., and M.V.N., unpublished data). However, the overall smoothness of the *T. pallidum* surface, as depicted by deep-etching, indicates that lipoproteins would have to be confined primarily to the inner leaflet of the outer membrane. In this regard, experiments based on detergent fractionation of whole *T. pallidum* do not support a predominantly outer membrane location for these protein antigens (10, 11).

We believe that many of the *in vitro* interactions between virulent *T. pallidum* and specific antibodies can be interpreted readily in light of these findings. The intrinsic structure of the outer membrane appears to allow access of antibodies to only a limited number of surface-exposed proteins. In fact, it remains to be determined whether the proteins identified by freeze-etching are antigenic, although this can be inferred by the activity of syphilitic sera in assays of immobilizing activity (28), cytadherence (29, 30), and phagocytosis (31). Such interactions may be below the level of detectability of certain techniques, particularly immunoelectron microscopy, that have been used to identify surface-exposed antigens (12, 14). The limited antigenicity of the entire *T. pallidum* surface by these techniques is also consistent with the absence of nonproteinaceous surface-exposed antigens. The apparently constrained lateral mobility of the IMPs, indicated by their failure to aggregate during fixation at 0°C, helps to explain the extremely slow kinetics of complement activation in assays such as the *T. pallidum* immobilization test (28). Efficient complement activation by IgG antibodies requires contiguous surface-bound IgG molecules and would be retarded when membrane antigens are incapable of freely diffusing within the plane of the membrane (32).

The unique ultrastructure of the *T. pallidum* outer membrane appears to represent an unusual parasitic strategy for evasion of host humoral immune defenses. Current concepts relating to other aspects of treponemal virulence also may require reevaluation as a result of these findings. Finally, although many treponemal antigens have been identified by immunologic and recombinant DNA methodologies (3–7), it is presently uncertain whether the identity of the outer membrane protein(s) identified by freeze fracture is known. Identification and characterization of this protein may be a prerequisite to the development of an effective treponemal vaccine.

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