Spirochete Attachment Ultrastructure: Implications for the Origin and Evolution of Cilia

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Spirochete Attachment Ultrastructure: Implications for the Origin and Evolution of Cilia

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Abstract. The fine structure of spirochete attachments to the plasma membrane of anaerobic protists displays variations here interpreted as legacies of an evolutionary sequence analogous to that from free-living spirochetes to undulipodia (eukaryotic "flagella" and homologous structures). Attached spirochetes form a vestment, a wriggling fringe of motile cells at the edge of the plasma membrane of unidentified cellulolytic protist cells in the hypertrophied hindgut of the digestive system of *Mastotermes darwiniensis*, the large wood-feeding termite from northern Australia. From the membrane extend both undulipodia and a complex of comparably sized (10–12 μm × 0.2–0.3 μm) ectosymbiotic spirochetes that resembles unruly ciliated epithelium. In the intestines are helical (swimming) and round-body morphotypes. Round bodies (RBs) are slow or immotile spirochetes, propagules known to revert to typical swimming helices under culture conditions favorable for growth. The surfaces of both the spirochete gram-negative eubacteria and the parabasalid protists display distinctive attachment structures. The attached hypertrophied structures, some of which resemble ciliate kinetids, are found consistently at sites where the spirochete termini contact the protist plasma membranes.

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Abbreviations: RBs = spirochete round bodies, cysts, L-forms, propagules, resting stages, vesicles, granules, *etc.* See Margulis *et al.* (2009) for explanation.

Introduction

Spirochetes, a cohesive phylum of gram-negative eubacterial organo-chemoheterotrophs (Margulis and Chapman, 2010) tend to swim in synchrony and attach to each other and to other live cells. They penetrate high-viscosity gels, muds, and tissues. Spirochete attachment to the membranes of cellulolytic termite hindgut protists takes many forms—from loose and casual to complex, repeated and tight, permanently ectosymbiotic structures. Heterologous spirochete-membrane attachments to eukaryotic cells in an anoxic, microoxic-to-oxic gradient environment are described here. The termite hindgut is anoxic only in its center, while the edges of the gut are microoxic (Brune *et al.,* 1995). Our goal here is to interpret structural variations in both the spirochetes and the cells to which they attach in a specific evolutionary context.

In study of hindgut microbes of the unique wood-feeding termite *Mastotermes darwiniensis* (Froggatt), the only extant member of its genus, we sought ecto-and endosymbionts in and on one of its protists: the giant trichomonad *Mixotricha paradoxa* (Sutherland). *M. paradoxa*, which ingests pieces of wood through its posterior although it swims with its anterior forward, comprises a motility symbiosis with at least three different types of spirochetes and their attachment structures (Cleveland and Grimstone, 1964; Wier *et al.,* 2001; Wenzel *et al.,* 2003). These spirochetes form a fringe over the entire surface of the cell. The coordinated beating of this fringe of motile cells propels *M. paradoxa* through the liquid that fills the termite's gut. The spirochetes include a thin treponeme morphologically indistinguishable from *Treponema pallidum*, a *Borrelia*-like me-

dium-sized 5:4:5 or 4:8:4 spirochete attached at the posterior periphery of the cell's wood ingestive zone, and *Canaleparolina* sp., a larger and longer spirochete (26–28 μm length $\times$ 0.4–0.7 μm width) known only from two remotely separated localities (Darwin, Australia, and St. John, US Virgin Islands, in the Caribbean; Wier *et al.,* 2001). These species designations are based on transmission electron microscopic cross-sections of the spirochetes, not on gene-sequence-based identification, although our results are consistent with other studies of this microbial symbiosis (Wenzel *et al.,* 2003; Brugerolle, 2004). Our plan was to study, by morphological correlation at the transmission electron microscopic level, endo- or ectonuclear bacterial symbionts and/or contractile karyomastigont that we saw in living *M. paradoxa* cells. The karyomastigont, an organellar system that includes the nucleus, the nuclear connector, and kinetosome-centrioles in many protoctist and animal cells, is a conspicuous component of the cytoskeleton. While neither bacterial nuclear symbionts (Dolan *et al.,* 2004) nor the morphological basis of the contractile portion of the nuclear connector/"rhizoplast" (of the karyomastigont) was imaged, we discovered a plethora of different spirochetes in contact with the outer membranes of protists. We interpreted these images as putative stages of increasing intimacy of the helical, motile eubacteria attached to amitochondriate parabasalids. We describe and interpret here fewer than a dozen from a larger set of micrographs in an attempt to reconstruct a plausible evolutionary sequence.

Materials and Methods

Mastotermes darwiniensis is the remarkable sole survivor of Paleocene termites of the family Mastotermidae. Mastotermitids, like their wood-feeding cockroach blatterid ancestors, lay eggs in packaged masses rather than singly; therefore zoologists consider this family of "lower termites" to be the earliest evolved in the Isoptera lineage. Individual insects, members of the mastotermitid genus, are well preserved as abundant fossils, especially in amber. They are known worldwide—for example, *Mastotermes mexicanus* and *M. dominicus* from the American tropics. The relevance of the availability of limited live mastotermitids with an immense fossil record was brought to our attention by D. Grimaldi of the American Museum of Natural History (Wier *et al.,* 2002).

M. darwiniensis worker termites were obtained in November 2005 from a laboratory culture established by Theodore A. Evans from termites collected in Darwin (CSIRO Entomology, Canberra, Australia). The termites were kindly identified, maintained in the laboratory, and some of their hindguts fixed and embedded by Dr. Nathan Lo, Behavior and Genetics of Social Insects Laboratory, School of Biological Sciences, The University of Sydney, Australia. By February 2006 these studies were underway when the termite guts were dissected and their contents harvested from live specimens of *M. darwiniensis* workers (pseudergates). The head of the termite was held in blunt forceps as fine forceps were used to extract whole intestines that were immediately prefixed in Karnovsky's fixative in cacodylate buffer (pH 7.2). After postfixation in 2% OsO_4 for 1.5 h, samples were washed in cacodylate buffer, dehydrated through an ethanol series, transferred to propylene oxide, and embedded in Epon 812. The embedded samples sent to the first author (A.M.W.), University of Wisconsin, Madison,Wisconsin, had been fixed in 2.5% glutaraldehyde in phosphate buffered saline. Within 4 weeks after fixation they were postfixed in osmium and embedded in Epon resin polymerized for 48 h at 60 °C. Three blocks were retained by L.S. (Pavia) and two mailed to A.M.W.

Thin sections, cut with a diamond knife on a Reichert-Jung ultracut microtome, were stained with saturated uranyl acetate followed by Reynolds lead citrate and examined with a Zeiss 900 (at 80 kV) or a Siemens AMW2 (at 30 kV) electron microscope.

Results

The preservation and fine structure resolution of the tissue are excellent. However, in most cases the genus and species of protists to which the spirochetes are attached are not known. Nor have the bacterial species been identified below the level of phylum. The eukaryotic microbes, amitochondriate heterotrophs, are wood-ingesting motile protists (phylum Archaeprotista, class Parabasalia, kingdom Protoctista), whereas the attached prokaryotes are recognized by morphology and motility (live, videography, light and electron microscopy) as spirochetes: phylum Spirochaetae of the kingdom Prokaryotae (Margulis, 2000; Margulis and Chapman, 2010). The cellulolytic protists and their attached motile eubacteria here share the same habitat— the intestine of the Australian dry-wood-ingesting termite *Mastotermes darwiniensis*. The observations of live organisms are confirmed by ultrastructure. The eubacterial-parabasalid physical associations must be permanent, or at least of long duration, because they have been consistently observed since 16-mm black-and-white ciné films were made of *M. darwiniensis* microbes by Harvard University Professor of Biology L.R. Cleveland in 1956, and observations were published by Grimstone and Cleveland in 1964.

Spirochetes are motile, helical gram-negative eubacteria whose flagella rotate between the outer and inner plasma membrane (in the periplasm). The liquid anoxic-to-microoxic habitat is the hypertrophied hindgut (= intestine or paunch) of the insect. The micrographs (Figs. 1–9, center panel in 10), selected from a much larger set, show how the evolution of cilia may have occurred.

The motile surface structures were ignored or assumed, with good reason, to be cilia or undulipodia (eukaryotic

"flagella") until careful study by superb observers (Kirby, 1941; Copeland, 1956) or electron microscopic studies (*e.g.,* Grimstone and Cleveland, 1964) demonstrated otherwise (Fig. 1). In many fringes both undulipodial and ectosymbiotic spirochetes abound on the same protist (*i.e., Deltatrichonympha* sp.) or on different protists in the same thin section. Whereas the fringes in Figure 1 are composed of both undulipodia and spirochetes in specific patterns, those on the anterior portion of the *Deltatrichonympha* (Fig. 1, left; Fig. 2), for example, are nearly exclusively standard [9(2)+2] undulipodia. Undulipodia, mistakenly still called flagella among eukaryotic cell biologists, comprise many familiar microtubular cell protrusions: sperm tails, "mastigote flagella," epithelial cilia, algal swimming organelles, *etc.* These are evolutionary homologs with animal tissue cell processes: they have canonical [9(2)+2] microtubular substructure, are intracellular, covered by cell membrane, and underlain by the basal [9(3)+0] kinetosome-centrioles that are constant in width. Invariably the kinetosome-centriole ("basal body") from which the axoneme (shaft) grows distally is of constant diameter (0.25 μm), whereas the length varies from barely visible to extremely long (<1 to over 200 μm; Fig. 1, center). The kinetosome-centriole is embedded in the kinetid, an organellar system that ranges from complex elaboration in myriad ciliates and mastigote algae to minimal fibers and ciliary necklaces in animal tissue cells. Kinetids, intracellular systems that are diverse but conserved in various protoctist taxa, provide clues to identification of cells and whole organisms.

Whereas undulipodia in their kinetids are beneath the eukaryotic plasma membrane, by contrast both ecto-and endosymbiotic spirochetes are covered by their own cell walls (Figs. 2, 4, 5). The walls in spirochetes consist of the inner (plasma membrane) and the outer membrane specific to gram-negative bacteria. The peptidoglycan layer and the rotary-motor flagella, in the characteristic spirochete-specific "n:2n:n" patterned array lie in the periplasm (pe at arrowhead in Fig. 5) between the protein-embedded cell membranes (Margulis, 2000).

The undulipodial striated root fiber (Fig. 3), the kinetosome at the protist cell surface, and the portion of the axoneme illustrate the typical position of standard eukaryotic mature undulipodia at the membrane (pcm). The axoneme, beneath the plasma membrane as usual in protoctist (including algae) and animal cells (including sperm), is emergent. Identification of the granules as ribosomes or organelles involved in motility (as detected in large spirochetes such as *Spirosymplokos*; Guerrero *et al.,* 1993) needs investigation. Cell biologists might make the unwarranted assumption that the protrusion from the surface (Fig. 4) is a cilium. The bifurcated structure that so resembles the typical "striated root fiber of the kinetid" (Margulis *et al.,* 1993a) in Figure 4, and at higher magnification in Figure 5, is the basal (proximal) portion of an undulipodium-like structure. Although the ultrastructure in Figure 5 appears typical of the striated-root (sr) 20-kD calcium-sensitive contractile protein (Salisbury and Floyd, 1978; later named "centrin"), we must question this identification in the absence of cytological and chemical compositional verification. The prokaryotic nucleoid, site of the bacterial DNA, as well as the gram-negative cell wall of the adhering spirochete, and the lack of kinetosome make the structure in Figures 4 and 5 easily distinguished from *bona fide* undulipodia. Other loosely attached or unassociated walled spirochetes of the same morphology also lead us to infer that the bifurcated structure with its two spheres (at arrows) are not components of kinetids. The second distinctive unidentified spirochete with its crenulated wall (sp1) in the same thin section (Fig. 4) shows that the micrograph depicts a fringe on the surface of a protist in which spirochetes are attached or cruise between undulipodia. The crenulated spirochetes are also seen in Figures 2, 4, 7, and 10 (top, center panel).

The most striking new results are in Figures 4–8. Not only do spirochetes form internalized attachment structures that greatly resemble cilia, but spirochete round bodies (RBs) attach regularly to protist cell membranes. RBs are less motile, more resistant stages of the polymorphic life history than standard swimming helices. Here they develop and stabilize attachments to eukaryotic cell membranes. The development of the attachment structure probably begins with fuzzy bulbous protrusions (arrow, as in Figs. 4, 5). The attachment structure fuzz is apparent in at least three spirochetes in a row at a single protist surface: Fig. 9. Our decade-old suggestion of a putative evolutionary sequence that closely resembles these micrographs did not predict spirochete RBs (Margulis, 1991; Fig. 10). Spirochete RBs had not yet been identified as viable propagules. Furthermore, although they were well known in free-living marine spirochetes from microbial mats (Guerrero *et al.,* 1993; Margulis *et al.,* 1993b), they were not known in the termite intestinal microbiota before they were first found as *Spirochaeta coccoides* in *Neotermes castaneus* (Dröge *et al.,* 2006) and proved to be propagules (Brorson *et al.,* 2009).

The transformation of *Borrelia burgdorferii* helically motile spirochetes to RB propagules was induced by penicillin and other unfavorable conditions in pure cultures *in vitro.* Resuspension of these spirochetes in growth media that includes serum predictably induces reversion to the helical swimming form in over 90% of RB propagules (Brorson *et al.,* 2009). However, here we demonstrate for the first time the transformation of RBs to helices (and, by inference from many thin-section electron micrographs, from helices to RBs) while attached to the surfaces of nucleated cells in nature.

Discussion

This favorite habitat of spirochetes between the undulipodia of the dense populations of archaeprotists in termite

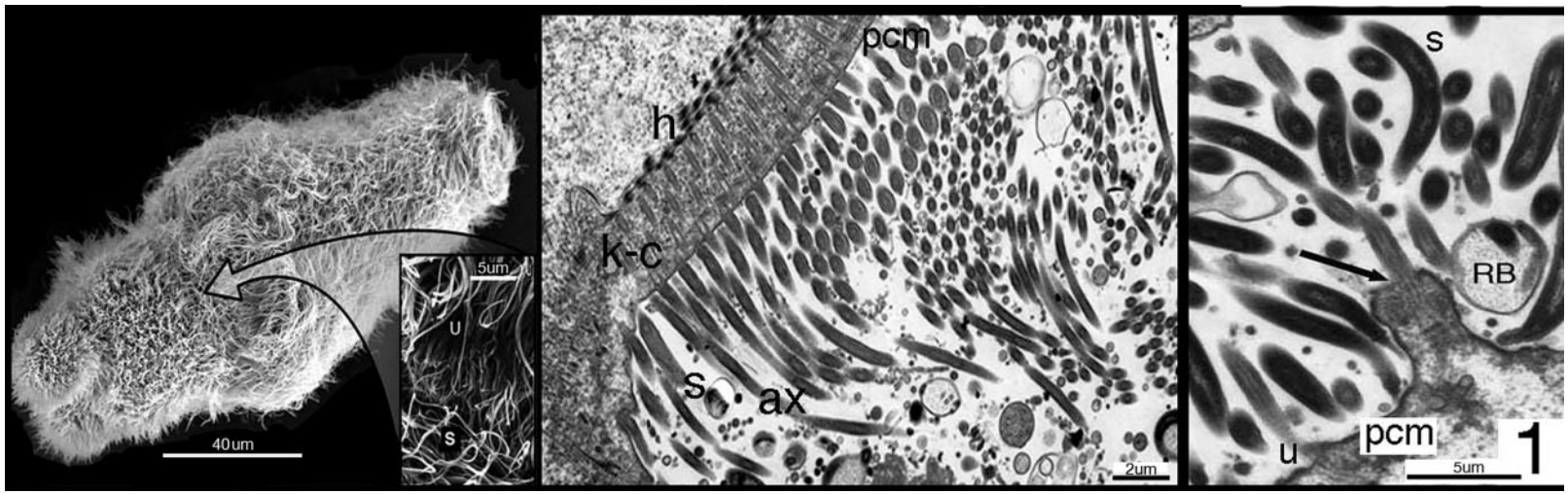

Figure 1. Fringes on *Mastotermes*' protists: difficulty of distinguishing undulipodia (intracellular) from ectosymbiotic spirochetes. Left panel: *Deltatrichonympha* sp., whole cell. The anterior portion of the cell to the upper right is covered by undulipodia. The arrow indicates the transition zone from longer undulating undulipodia to the posterior vestment of shorter, more helical spirochetes (magnified in the inset). Scanning electron micrograph by Dean Soulia (taken while a Master's student at UMASS Amherst). Center panel: unidentified large archaeprotist, in which the fringe comprises both an undulipodial layer at the protist membrane surface (pcm)—with typical kinetosome-centrioles (k–c), [9(2)+(2)]-axonemal microtubule arrays (ax)—and nearly no ectosymbiotic spirochetes (s). Structures that may be hydrogenosomes (h) underlie the kinetosome-centrioles. Right panel: The protist fringe includes standard undulipodia-like structures (u), spirochetes (s), and protrusions with properties of both the cell organelles and the ectosymbiotic bacteria (arrow). Both typical motile forms, with nipple-like attachment structures, and round body propagule forms of spirochetes are seen. A more inclusive portion of this same transmission electron micrograph provides context and is interpreted in Fig. 2.

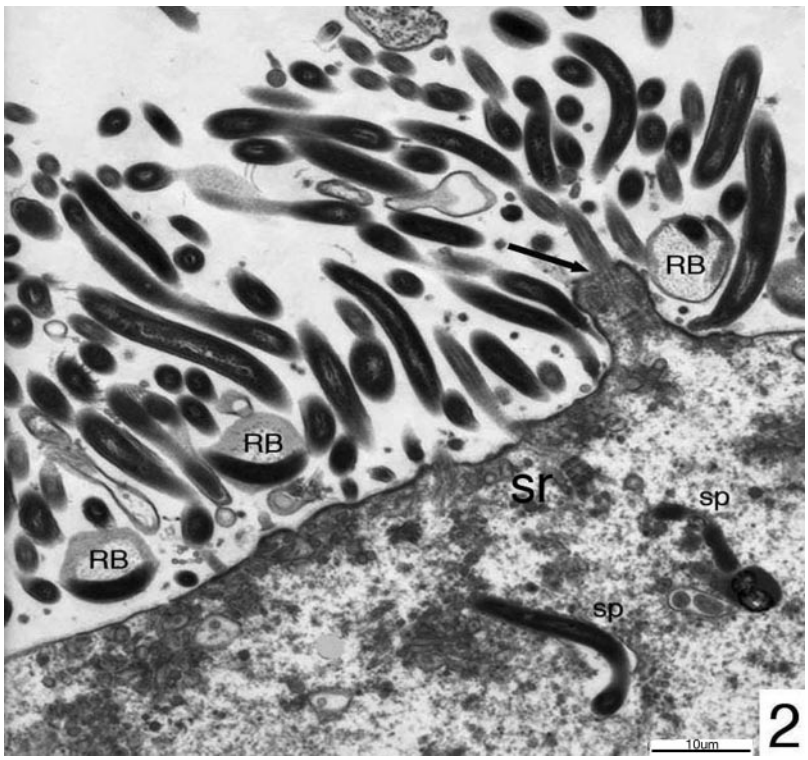

Figure 2. Fringe comprising intracellular protrusions that resemble an undulipodium with an aberrant base (no kinetosome) with a bifurcated striated root fiber (arrow, sr). The fringe contains unattached spirochetes, attached spirochetes with attachment structures, round bodies (RB), an unattached crenulated spirochete (another in Fig. 7 at higher magnification), other bacteria, and at least two spirochetes (sp) inside the protist cell.

organic-rich viscous intestinal fluid has been described for years (Bloodgood and Fitzharris, 1976). The formation of a "bacterial rootlet" beneath an attached spirochete was first reported by Smith and Arnott in 1974. The spirochete symbiotic associations shown here are likely to have evolved convergently under anoxic conditions from unassociated free-swimmers normally seen in hindgut preparations and independently of Proterozoic eukaryosis (Margulis *et al.,* 2006). These microbial associates (spirochetes, parabasalids) apparently have repeated the ancient process as they became progressively integrated.

The micrographs in this *Mastotermes darwiniensis* series may be interpreted as legacies of steps in the evolutionary integration of motile (free-swimming) gram-negative eubacteria into the surface of the organism to which they are *en masse* attached. The bacteria are attached to at least one distinctive unknown amitochondriate cellulolytic protist covered with undulipodia and likely to be uninucleate, suggesting that it is a hypermastigote class Parabasalia, order Hypermastigida. The attachment structures here most likely evolved inside isopteran insects or their blatterid ancestors in the Phanerozoic Eon since termites are unknown in the fossil record prior to the Mesozoic's Cretaceous Period. Many of the epibionts are spirochetes or spirochete round bodies (RBs), integrated into the surface of protists. The concept that the nucleus, recombinant from archae- and eubacterial genomes, evolved by liberation from the karyomastigont cytoskeletal organellar system (Margulis *et al.,* 2005, 2008) is strongly supported by the ultrastructural observations here. Our karyomastigont model of the origin of the nucleocytoskeletal system is consistent with observations both on confusions between undulipodia and spirochetes (Fig. 9) and on parabasalid, algal, and other mitoses (Fig. 10) made by Edouard Chatton (France, 1882–1947)*, Harold Kirby (California, 1900–1950; Kirby, 1944), André Lwoff (France, 1902–1994)†, and Karl Belar (Germany, 1895–1931; Belar, 1926).

From independently motile spirochetes, including their RB propagules and their symbiogenetic associations, the micrographs help reconstruct an evolutionary sequence. Spirochetes, once free-living and free-swimming, became

* Edouard Chatton (1883–1947). Exhibition and slide show presentation by M.-O. Soyer-Gobillard and J. Schrével at the Muséum d'histoire naturelle, Perpignan, Laboratoire Arago, Banyuls-sur-mer, France, 2005–2009. [Illustrated book in preparation.]

† André Michel Lwoff (1902–1994). Exhibition and slide show presentation by M.-O. Soyer-Gobillard and J. Schrével at the Muséum d'histoire naturelle, Perpignan, Laboratoire Arago, Banyuls-sur-mer, France, 2005–2009. [Illustrated book in preparation.]

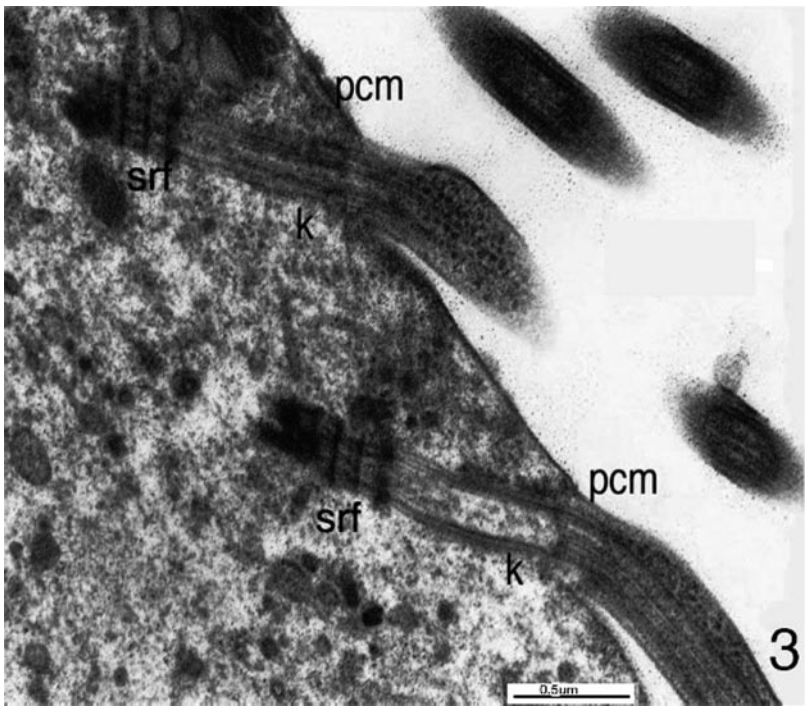

Figure 3. Undulipodia with deep striated kinetids at surface membrane (pcm) of an unidentified archaeprotist cell. Conspicuous granules attached to the kinetosome (k), striated root fibers (srf), resemble structures in some surface spirochetes attached to termite intestinal protists. However, the absence of the periplasm and its flagella, nucleoids, and outer membranes with peptidoglyan wall material definitely distinguishes these cell protrusions as eukaryotic cell motility organelles and not ectosymbiotic spirochetes.

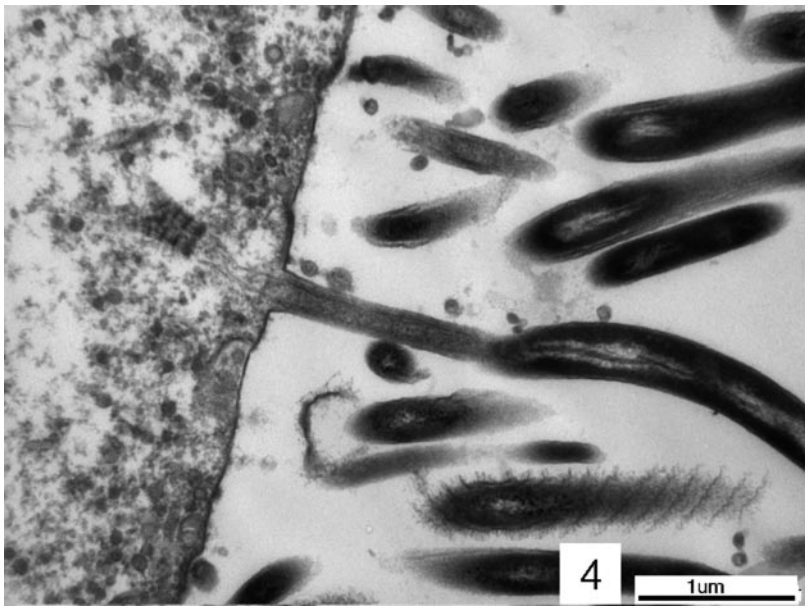

Figure 4. Abberant undulipodium-like structure lacking central tubules and kinetosome, surrounded by an electron-dense area (arrow) like that of the spirochete attachment sites (as). Thin fibers appear to attach the "axoneme" to a bifurcating striated rootlet (sr) 0.5 μm inside the protist cell membrane (pcm). Also seen are two different spirochetes (sp, sp1), one with its nucleoid (n).

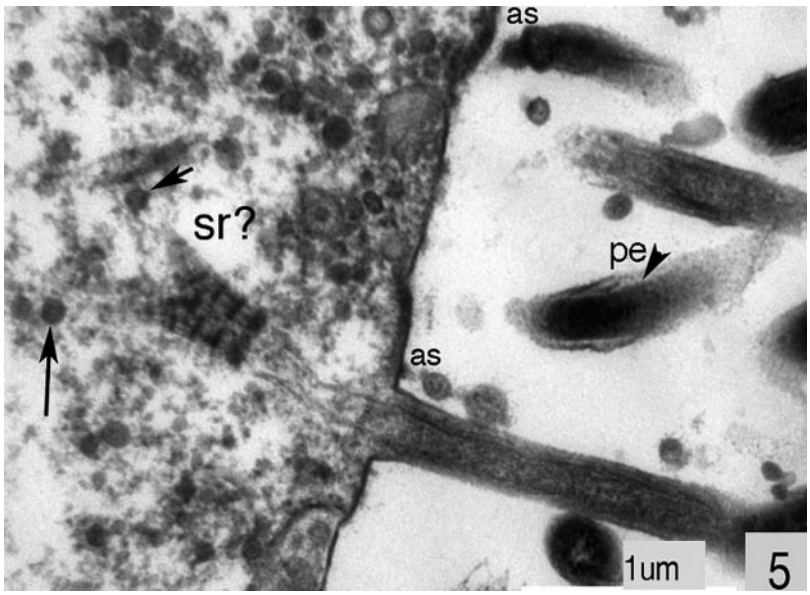

Figure 5. Spheres (arrows) at each of the two proximal tips of the bifurcated striated root (sr) root fiber–kinetid-like basal apparatus (in Fig. 4). as, attachment site; pe, periplasm.

entirely integrated motile/sensory extensions of eukaryotic cells. This integration led to Darwin's "imperfections and oddities" (Margulis *et al.,* 2005) as precursor protoctists of ciliated animal cells and plant sperm. The integrated spirochete that evolved to become the undulipodium is, by hypothesis, the evolutionary antecedent of mitotic cell division and intracellular motility systems generally. Intracellular motility at the light microscopic level in live organisms distinguishes even tiny eukaryotes from any prokaryotes (Margulis, 1993a; Margulis *et al.,* 2005, 2006.) Hints of this concept were already apparent in the "course boards" that Chatton produced for his students (Fig. 10, right). Our

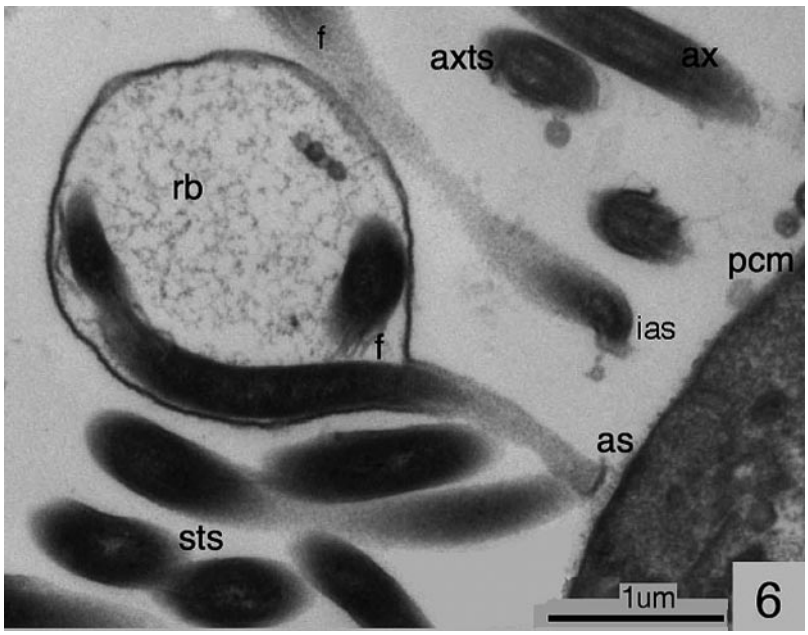

Figure 6. Attachment structure of a spirochete round body (rb). The spirochete is transforming from the round body propagule to a motile form with flagella (f). The attachment site (as) is an electron-dense portion of the protist cell membrane (pcm) at which a truncated end of the bacterium is attached by fibrous material. Transverse sections of spirochetes (sts) and axonemes (axts) are seen. ax, axonemal microtubule array.

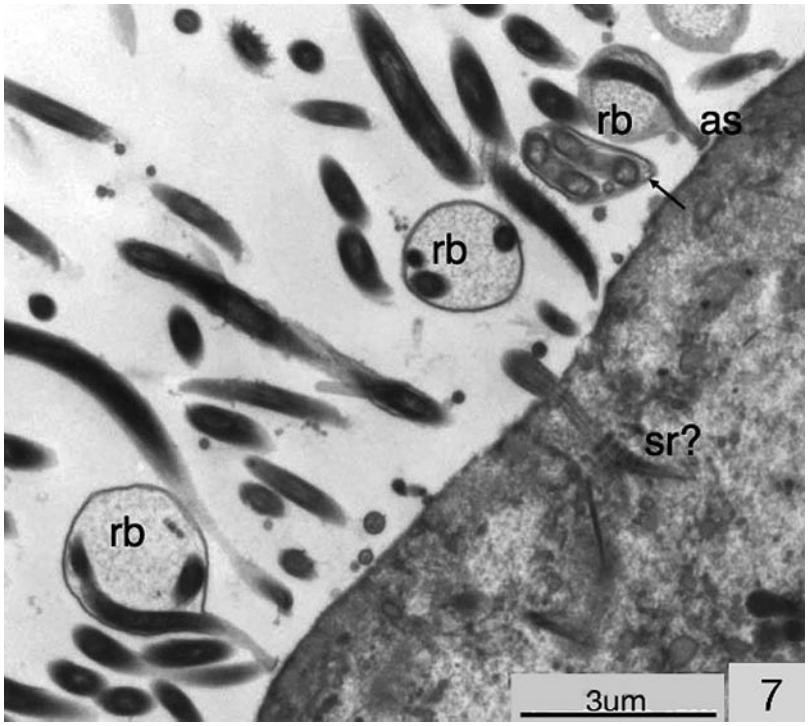

Figure 7. Round body (rb) distribution on protist surface. Spirochete sections are visible inside unattached rb at arrow. An undulipodium-like structure is seen in the center of the figure. It lacks a kinetosome, but has a central fiber that continues from the "axoneme" through the cytoplasm to the striated root (sr). All attachment sites (as) are electron-dense regions of the protist cell membrane.

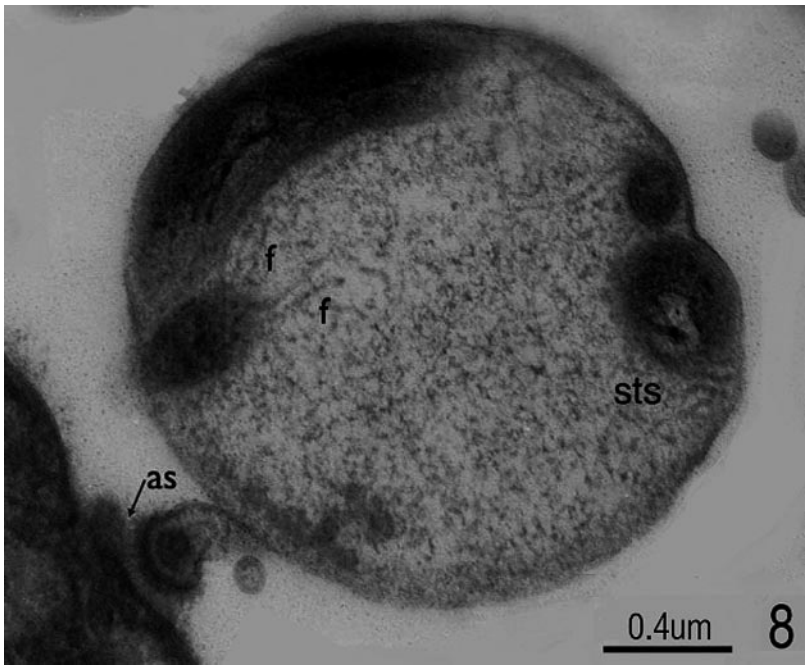

Figure 8. Spirochete round body with its attachment structure (as). In this case the attachment structure is only 0.2 μm wide. The protist cell membrane and the tip of the round body are electron dense and connected by a fibrous material. f, flagella; sts, transverse sections of spirochetes.

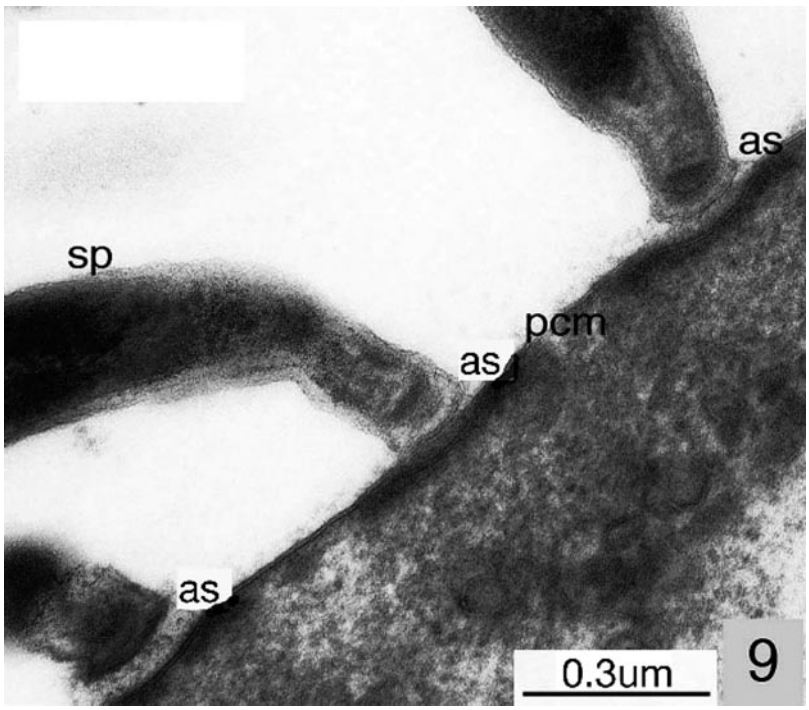

Figure 9. Attachment structures (as) of three linearly aligned surface ectosymbiotic spirochetes (sp) on the protist cell membrane (pcm) of an unidentified archaeprotist in *Mastotermes darwiniensis* intestine. pcm, protist cell membrane.

hypothesis has continued to develop on two fronts: (1) Microtubular structures, generically undulipodia—that is, [9(2)+2] homologs that include cilia, eukaryotic "flagella," macrocilia of ctenophores, haptonemes of prymnesiophytes, axopods, axostyles, and many kinds of sperm tails—originated symbiogenetically from spirochetes and diversified. In the eukaryosis process, motile spirochetes integrated with pleiomorphic sulfidogenic archaebacteria in Proterozoic Eon seas. (2) The karyomastigont organellar system (a chromosome-containing membrane-bounded nucleus with a proteinaceous connector that attaches to the centriole-kinetosomes at the base of the undulipodia) evolved in response to selection pressures that tended to separate attached spirochetes (eubacteria that became undulipodia) from their sulfidogenic archaebacterial partners (the rest of the cytoplasm). The tethered nucleus and its kinetosome-centriole connector, the stabilized, permanent karyomastigont, is—in our view—the organellar system from which intracellular motility of protoctist, animal, plant, and fungal mitosis evolved. Protoctist mitotic (and other cell) motility patterns gave rise to the less variant mitotic patterns of liberated nuclei typical of the earliest animals, plants, fungi, and their later descendants.

Our hypothesis on the origin and evolutionary trajectory of cilia from karyomastigonts, here with enhanced probability that it is correct, provides a framework to interpret the micrographs as extant examples of convergent evolution. Spirochete symbiotic associations, like these Australian ones, evolved in independent, unassociated free-swimmers that progressively integrated until ectobionts eventually became internalized endobionts. Spirochete associations evolved to be cytoskeletal systems. Spirochetes, probably oxygen-tolerant sulfide oxidizers, conferred intracellular motility on the earliest eukaryotes. A Proterozoic evolutionary process by convergence was repeated in relict Paleocene Epoch habitats. The karyomastigont—the organellar system that includes the nucleus, cytoskeleton, paradesmose (thin mitotic spindle of amitochondriate parabasalids and other protists), and, in general, the intracellular microtubular motility system of eukaryotes—is understood as a legacy of the symbiogenetic origin of nucleated cells (Dolan, 2005). Among the modern analogs of spirochete-protist associations under conditions of reduced oxygen concentrations, especially striking is the diversity of spirochete attachment modifications. Some that extend from the surface into the peripheral cytoplasm resemble striated root fibers of ciliate kinetids, reminiscent of their kinetodesmal centrin calcium-sensitive proteins (Salisbury and Floyd, 1978.) We have direct paleontological information from termites embedded in Miocene amber for a Phanerozoic fossil record of spirochetes (Wier *et al.,* 2002, 2007). The "imperfections and oddities" of spirochete-protist attachment surfaces (Margulis *et al.,* 2005) are so ancient (*c.* 20 million years old), distinctive, and interpretable that we feel justified that

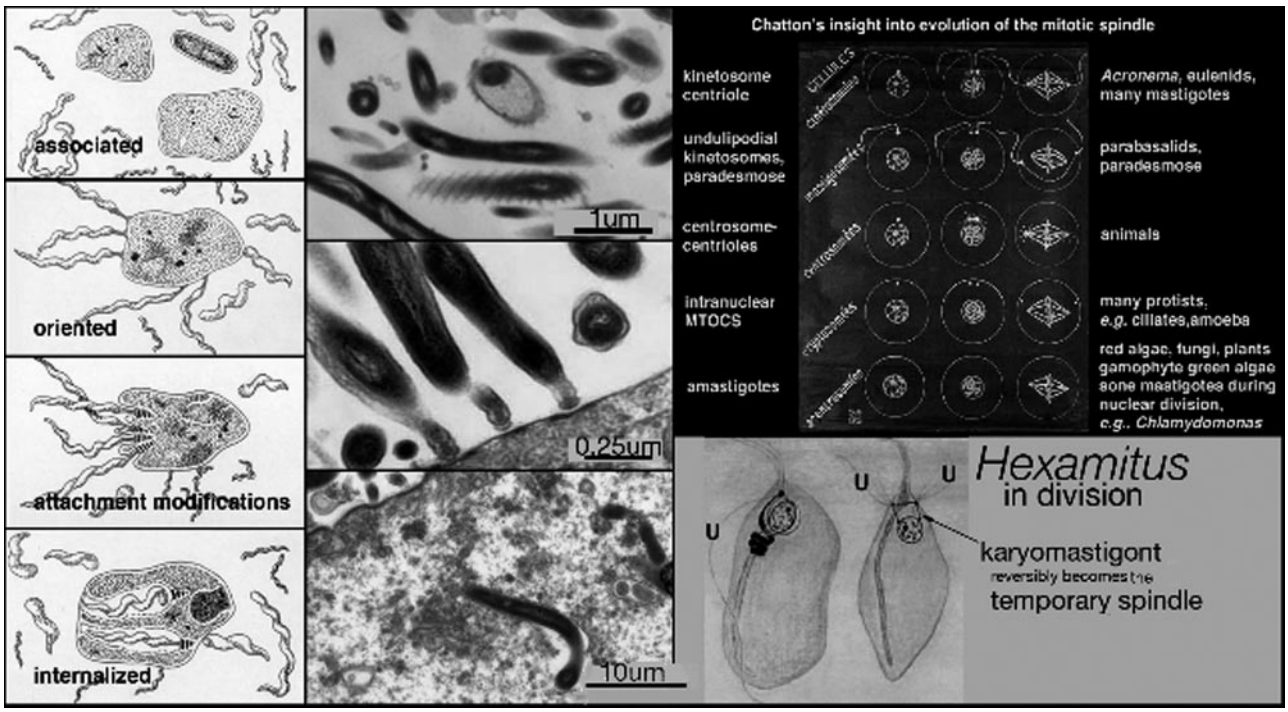

Figure 10. Evolutionary scenario for the origin of the karyomastigont, the mitotic spindle, and surface cilia from spirochetes. Original 20-year-old hypothesis, left panel adapted from drawing by K. Delisle (Margulis, 1991). Sample matching micrographs from this study at center panel (scale bars = 2 μm). Relevant similar protist evolutionary schemes were conceived and used in the teaching and research large "course boards" of Edouard Chatton, director of Laboratoire Arago, Banyuls-sur-Mer, France (right panel, u = undulipodia; courtesy of Dr. Marie-Odile Soyer-Gobilliard). Some of the course boards are on display at the city museum in Perpignan.

modern termite associations help us interpret the evolutionary history of motile anaerobic eukaryotic cells. We propose a testable hypothesis: cilia and other undulipodia evolved from aerotolerant, sulfide-oxidizing, RB-forming free-living mud spirochetes (approximately 0.25 μm wide $\times$10–12 μm long) comparable to extant components of the "*Thiodendron*" consortium of Dubinina and her colleagues (Dubinina *et al.*, 2010; Margulis *et al.,* 2006). The role of attachment structures in establishment of permanent symbiotic associations with eukaryotes and the formation of round body (RB) propagules correlated with disease symptoms in animal tissue (*e.g.,* syphilis, Lyme borreliosis) was recently discussed (Margulis *et al.,* 2009; Brorson *et al.,* 2009).

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