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Cortical Symbionts and Hydrogenosomes of the Amitochondriate Protist *Staurojoenina assimilis*

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Abstract

Adhering epibionts with distinctive attachment structures form surface striations on *Staurojoenina*, a termite hindgut hypermastigote. These striations, first called "cuticle thickenings" by Kirby, are always present on *Staurojoenina assimilis* and are intrinsic to the description of the species in this genus of amitochondriate protists. Studies of the live protist by videomicroscopy, fluorescence light microscopy, scanning and transmission electron microscopy as well as 16S rRNA analysis confirm their identity. Each of the conspicuous, uniformly distributed, surface striations (0.2–0.3 μm × 2.5–7.0 μm) is attached longitudinally by a "hook" to the protist membrane. They contain nucleoids that fluoresce with SYTOX, a DNA stain. The striations are rod-shaped, gram-negative enterobacteriaceae. Phylogenetic analysis using the 16S rRNA genes show that the epibionts can be placed firmly within the *Citrobacter* clade. Although they have not been isolated and grown in culture we argue that enough morphological, ecological and molecular biological information about these surface symbionts of *Staurojoenina assimilis* exist for them to merit identification by name. Accordingly we formally describe the epibionts as "*Candidatus* Cuticobacterium kirbyi". These specimens were taken from a newly discovered source of *Staurojoenina assimilis* from two Caribbean islands.

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The bacterial-protist association has been found consistently in the intestines of *Neotermes mona*, dry wood-eating termites of the family Kalotermitidae both from Puerto Rico and St. John, U.S. Virgin Islands. Our work therefore extends early descriptions by Kirby and Hollande. "Rostral granules" identified by Kirby fit the description of hydrogenosomes. Each has a dense tip that resembles the calcium-rich portion of the hydrogenosome known from the literature. The putative calcium-accumulating part of the hydrogenosome faces the parabasal plate in the rostrum of the protist. "*Candidatus* Cuticobacterium kirbyi" is inherited on the surface of dividing cells and through walled but translucent cysts.

Keywords: Archaeprotists, Caribbean termites, *Candidatus* Cuticobacterium kirbyi, *Citrobacter* epibionts, hypermastigotes, *Neotermes*, parabasal plates, protist surface striations

1. Introduction

The hypermastigote genus *Staurojoenina* Grassi (1917) was discovered in the dry wood-ingesting termite *Epicalotermes aethiopicus* (family Kalotermitidae) from the northwest coast of Africa. All the literature on the genus can be found in two reviews (Kirby, 1926; Hollande, 1986) and a recent report of walled cysts and *Caryoletira*-like symbionts in the nucleus of *S. assimilis* Kirby (Dolan et al., 2004). Four species of *Staurojoenina* have been described in addition to *S. assimilis* Kirby 1926, the subject of this paper. The five species all inhabit the intestines of kalotermitids where they are among the largest gut protists. The symbiotic protist genus has a cosmopolitan distribution; the termites in which they are found are listed with their geographical locations (Table 1). As members of the Archaeprotista (Margulis and Schwartz, 1998) by definition they lack mitochondria at all stages, but contain hydrogenosomes.

Regularly adhering epibionts associated with insect intestinal protists have been seen since the early reports of these organisms (Kirby, 1934, 1941, 1944, 1950; Grassé, 1938; Grassé and Hollande, 1942; Hollande and Carruette-Valentin, 1971; Hollande, 1986). In *Staurojoenina* (Class Parabasalidida) the surface bacteria have specific attachment structures and are intrinsic to the description of the five species. The epibiotic bacteria have not been isolated, grown in culture and described according to the regulations of the bacterial code of nomenclature. Other epibiotic spirochetes have been studied (Ohkuma et al., 1999; Iida et al., 2000) including in a recent report of several spirochetes and a rod bacterium on the surface of *Mixotricha paradoxa* (Wenzel et al., 2003). Although the later study demonstrates the usefulness of applications of *in situ* molecular biological probe techniques for analyses of microbial symbioses, it is limited in usefulness because the morphological correlations of the probes with the epibionts cannot be made with certainty.

Table 1. Species of *Staurojoenina*.

Species	Location	Termite	Reference
S. assimilis	North America: California, San Salvador, Puerto Rico and St. John U.S. Virgin Islands	*Incisitermes* (=*Kalotermes*) *minor*, *Neotermes mona*	Kirby, 1926 Dolan and Margulis, 1997
S. caulleryi	Island of Madeira	*Calotermes*	Grassé and Hollande, 1945
S. corbeli	Canary Islands	*Calotermes*	Hollande, 1986
S. gracilis	Nosy Bé (northeast Madagascar)	*Bifiditermes madagascarensis*	Hollande, 1986
S. mirabilis	Northwest coastal Africa	*Epicalotermes aethiopicus*	Grassi, 1917

Epibionts on uncultivated protists in principle, especially those that require anoxia, are excellent candidates for symbiosis analysis but for the molecular biological probes to be maximally meaningful the morphologically distinctive microbes themselves must be better known (Sapp, 2004). Besides the spirochetes only a few reports of epibiotic eubacteria are available; these include abstracts that claim to identify the rods on the surface of a devescovinid (Goss and Gunderson, 2000) and on a hypermastigote, *Barbulanympha*, from the wood-feeding cockroach *Cryptocercus punctulatus* (Merritt et al., 1996).

The symbiotic association between the cortical epibiotic bacteria and the parabasalid is so specific, regular and predictable that the bacteria deserve to be named as part of the taxonomic description of the protist. Therefore we describe, name and identify the epibionts of *Staurojoenina assimilis* Kirby here. This *Staurojoenina assimilis* was taken from dry wood-eating termites (Kalotermitidae) *Neotermes mona* on two Caribbean islands (Puerto Rico and St. John, U.S. Virgin Islands). Studies of the live protist by videomicroscopy, scanning and transmission electron microscopy and 16S rRNA analysis extended early descriptions by Kirby and Hollande.

2. Materials and Methods

Collection

These *Staurojoenina assimilis* were found in the dry wood-ingesting termite

Neotermes mona near the seashore. *N. mona* were collected from red mangroves (*Rhizophora mangle*) at Lameshur Bay, St. John (U.S. Virgin Islands) and from a red mangrove stand about 20 m from the water's edge at Bahía Fosforescente in southwest Puerto Rico. Termite identifications were confirmed by Dr. Rudolph Scheffrahn (University of Florida, Fort Lauderdale Research and Education Center).

Light microscopy

Hindguts were extracted from live termites and punctured in a few drops of insect Ringer's solution. Some protist samples were fixed in 0.5% glutaraldehyde and stained for 1 h with 1 μM DAPI or SYTOX (Molecular Probes, Inc.) for observation with phase-contrast/epifluorescence confocal microscopy. Some unstained protists fixed in 1% glutaraldehyde and spread on poly L-lysine coated coverslips were measured by use of a stage micrometer.

Electron microscopy

For transmission electron microscopy extirpated hindguts were ruptured in 2% glutaraldehyde in phosphate buffered saline (PBS) for 1 h and post fixed in 1.0% osmium tetroxide (1 h, 22°C), suspended in PBS and washed by centrifugation and resuspension at least three times. The sample, dehydrated in an alcohol series (50%, 70%, 80%, 90%, and 100%) was subject to three changes, 15 min, at each concentration. After immersion in propylene oxide (three changes, 15 min each) it was left overnight in a mixture of equal parts propylene oxide and Spurr's embedding medium to harden at 60°C. The blocks were mounted and sectioned with a glass knife on an MT 2B Porter Blum ultramicrotome. Sections collected on 200-mesh copper grids were stained for five minutes in uranyl acetate followed by five minutes in lead citrate. The preparations were viewed in a Phillips electron microscope 410 at 60 kv.

The resuspended hindgut protist sample was fixed in 1.0% glutaraldehyde in PBS for scanning electron microscopy. The protists were washed again by centrifugation and resuspended three times. They were post-fixed in 2.0% osmium tetroxide for 1 h at room temperature, suspended in PBS, and washed by centrifugation and resuspended three times. After they were washed on 0.45-μm Millipore filter paper and dehydrated through an alcohol series (30%, 50%, 70%, 90%, 100%) they were exposed to critical point drying. The cells were then mounted, using an eyelash, onto metal stubs and sputter-coated with carbon. Samples were viewed with a JEOL JSM-5400 scanning electron microscope at 15 kv.

Videomicroscopy

An Optronix camera mounted on a Nikon Optiphot microscope fitted with fluorescence, Nomarski differential interference, and phase contrast microscopy was used for videomicroscopy of live material. The video images and commentary were recorded on three-quarter inch Sony U-matic 60 min tapes and were confirmed by still photographs taken with 160 ASA 35 mm Ektachrome film through the same microscope.

DNA isolation, extraction and16S ribosomal DNA analysis

Approximately 100 *Staurojoenina* cells were hand picked and frozen at –20°C. When ready for extraction the sample was washed three times in 1 ml of 40 mM EDTA, 0.75 M sucrose, 50 mM Tris-HCL buffered to pH 8.3, and then subjected to three freeze/thaw cycles. The sample was treated with proteinase K, CTAB and extracted in chloroform. Nucleic acids were precipitated by the addition of isopropanol followed by pelleting by centrifugation. After an ethanol wash the DNA was resuspended in TE buffer (10 mM Tris-EDTA pH 8.0).

Near complete 16S rDNA genes were chosen for the construction of the 16S rDNA gene library. The forward bacterial primers BAC-11F

(5′ AGRGTTTGATCCTGGCTCAG-3′) and BAC-1492R

(5′-CGGCTACCTTGTTACGCTT-3′) were used to produce an approximately 1450 base pair stretch of DNA.

The amplified 16S DNA was gel purified and cloned into pCRII-TOPO plasmid vectors (Invitrogen, Carlsbad, CA) using the T-overhang method. Recombinant colonies were identified by the blue/white color selection (Sambrook et al., 1989). Insert size was determined by colony PCR using SP6 and T7 primers directed against the plasmid, followed by electrophoresis to confirm the presence of the appropriate insert size. Plasmid inserts amplified from 50 selected clones were screened via Amplified rDNA Restriction Analysis (ARDRA) (Moyer et al., 1994).

The ARDRA analysis was conducted on 50 clones using tetrameric cutting restriction endonucleases and gel electrophoresis (Maidek et al., 2000). The restriction endonucleases used were AluI and MspI. Colony PCR products (10 µl) were digested for 1 h at 37°C. The digests were run on a 4% agarose gel for one hour.

The insert was sequenced on a Beckman-Coulter DNA Sequencer. The alignment of DNA sequences, made with Sequencer and Clustal X software, was subjected to phylogenetic analysis using PAUP 4.08b (Hall, 2001).

3. Results and Discussion

Morphology

Whether the termites were collected in Newbury Park, California or San Salvador Island in the Bahamas (Dolan and Margulis, 1997), from Puerto Rico or the U.S. Virgin Islands (this study), at all times under all circumstances the surfaces of *S. assimilis* that we observed were replete with the striations reported by Kirby (1926). The striations were confirmed in all subsequent studies as gram negative epibiotic bacteria (Hollande, 1986; Dolan et al., 2004). The bacteria are seen as tiny striations with the DNA stain SYTOX especially by use of confocal epifluorescence light microscopy (Fig. 1). Their nucleoids extend most of the length of the rods. The casually associated surface spirochetes are easily distinguishable from the regular pattern of the epibionts.

Both scanning (Fig. 2) and transmission electron microscopic images (Fig. 3) revealed the epibiotic bacteria to be long thin rods. They generally measured 0.2–0.3 μm in width by 2.5–5.0 μm in length although some as long as 7 μm, presumably bacteria prior to division, were occasionally seen. The bacteria tightly and systematically adhere to the cortex in all preparations. The centrifugation and resuspension steps of the ultrastructural preparations did not dislodge them. Sporadic but abundant surface spirochetes (Fig. 2, black arrows) were never neatly and regularly aligned on the cortex as were the rod epibionts.

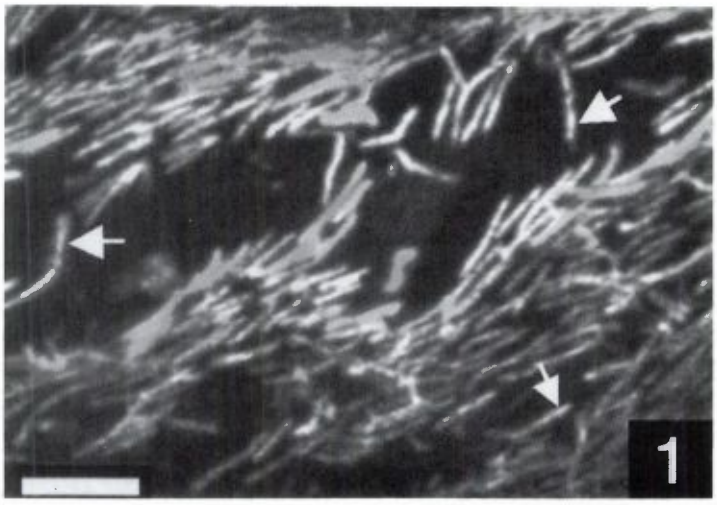

Figure 1. SYTOX stained long thin epibionts cover the surface of *S. assimilis*. The nucleoid fluorescence, for example at the arrows, is conspicuous. (Confocal light micrographic image, bar = 5 μm).

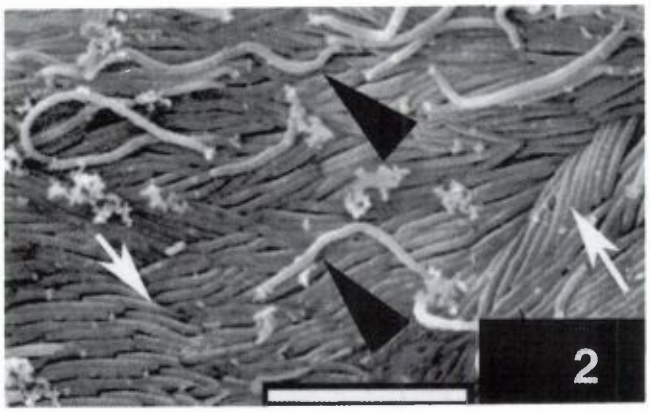

Figure 2. Cortical striations at this resolution are the rod bacteria (white arrows). Casually associated surface spirochetes are indicated by black arrowheads. (SEM, bar = 3 μm).

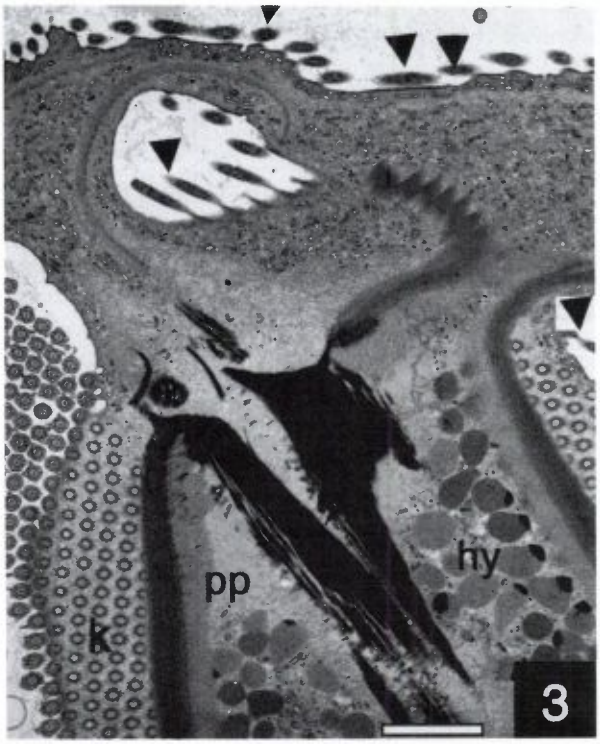

Figure 3. Rostrum distribution of putative hydrogenosomes (hy) oriented with their opaque tips facing the striated parabasal plate (pp) on its proximal side. Distal to the plate are rows of kinetosomes (k). Epibiotic bacteria indicated by black arrowheads. (TEM, bar = 2.5 μm).

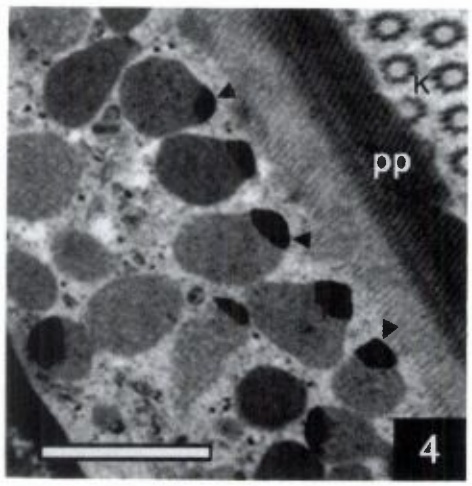

Figure 4. At least six putative hydrogenosomes with electron dense tips (black arrowheads) oriented toward the parabasal plate (pp) are seen here. Some seven kinetosomes (k) at the base of one of the four undulipodial bundles lie on the outside of the plate. (TEM, bar = 2 μm).

The attachments for each cortical bacterium were distal to a raised portion of the *S. assimilis* surface (Fig. 3) with its subtending cytoplasmic microtubule and hook (Dolan et al., 2004). *Staurojoenina*'s ridge, since it is attached beneath the rod-shaped bacterium, resembles a keel in SEM images; it extends longitudinally beyond both ends of the rod (Dolan et al, 2004). The attachment structures are indicated by black arrowheads in the electron micrograph of an actively swimming fixed cell (Fig. 3). The attachment between the cortical bacterium and the cell membrane appears stretched at the right edge of the figure consistent with our observations of living cells. The bacteria tightly adhere to *S. assimilis* even though movement never seems to cease.

In videos of live cells, correlated with fine structure, the densely packed cortical rods are visible on the four protoplasmic lobes between which lie the four bundles of undulipodia (eukaryotic flagella). The large ratio between the number of cortical bacteria per single protist cell implies that the epibionts divide somehow in association with *Staurojoenina*. The symbiosis is stable in that the ratio of surface bacteria to the protist cell persists through all stages of development. The epibiont is probably present on the protist in its walled cyst and on cells with short, newly developing rows of undulipodia (Dolan et al., 2004). The even spacing and abundant covering of epibiotic bacteria was predictably similar in all cells studied.

Whereas both kinetosomes and epibionts were distal to the striated parabasal plates (pp, Fig. 3) other regularly distributed bodies were proximal. We interpret the cell inclusions within the rostrum as hydrogenosomes. These bodies, 0.5–2.0 μm in diameter, were included in Kirby's description of the species. He called them "deeply staining granules" and noted that they lie within the rostral "square" of the anterior protoplasmic lobes (Fig. 5, Plate 1, p. 90 in Kirby, 1926). We found them to align the periphery of the "square" and from the rostral region proceed posteriorly (hy, Fig. 3). The inclusions, that seem to line the interior of the parabasal plates, extend from the anterior most rostral region toward the posterior. Aligned in single or double rows, many display dark tips oriented toward the inside walls of the parabasal plates. Their electron lucent ends face the rostral lumen (Fig. 4). The faint border around these inclusions is interpreted to be poorly fixed hydrogenosome membrane. As micron-sized membrane-bounded organelles with electron dense inclusions at one end they resemble the hydrogenosomes of other anaerobic protists, the parabasalid *Tritrichomonas foetus* (Benchimol et al., 1982; Benchimol et al., 1996; Benchimol, 2000) and the chytrid *Neocallimastix frontalis* (Biagini et al., 1997).

Whether or not the electron dense material in the putative hydrogenosomes is located in a peripheral vesicle, as in these other anaerobic protists, is difficult to assess. Nevertheless, the observations here are consistent with the concept that the dark tips oriented toward the parabasal plates are related to calcium ion metabolism as has been shown for the others. Calcium ions may be sequestered and released in parabasal plate-related motility systems analogous to the sarcoplasmic reticulum of vertebrate cells; in any case significance of the orientation of the these inclusions and their exact identification is unknown at this time.

Molecular phylogenetic affiliation

Molecular biological analysis indicates the epibionts are gamma proteobacteria related to other citrobacters including the arsenate reducing gut isolate from the subterranean termite *Reticulitermes flavipes* (Herbel et al., 2002; Fig. 5). Like the free-swimming bacterium in *R. flavipes*, the *Staurojoenina* epibiont is closest to *Citrobacter braakii.* Members of the genus, which includes *Citrobacter freundii* and *Citrobacter murliniae* all belong to the enterobacteriaceae, a huge group of symbiotic and free-living gram-negative bacteria that have been isolated from the intestines of insects, mammals and many other animals. The morphology of the epibiont corresponds well to their identification by 16S rRNA techniques. The Gen Bank accession number of the 16S rRNA sequence of our *Staurojoenina* epibiont is AY567708.

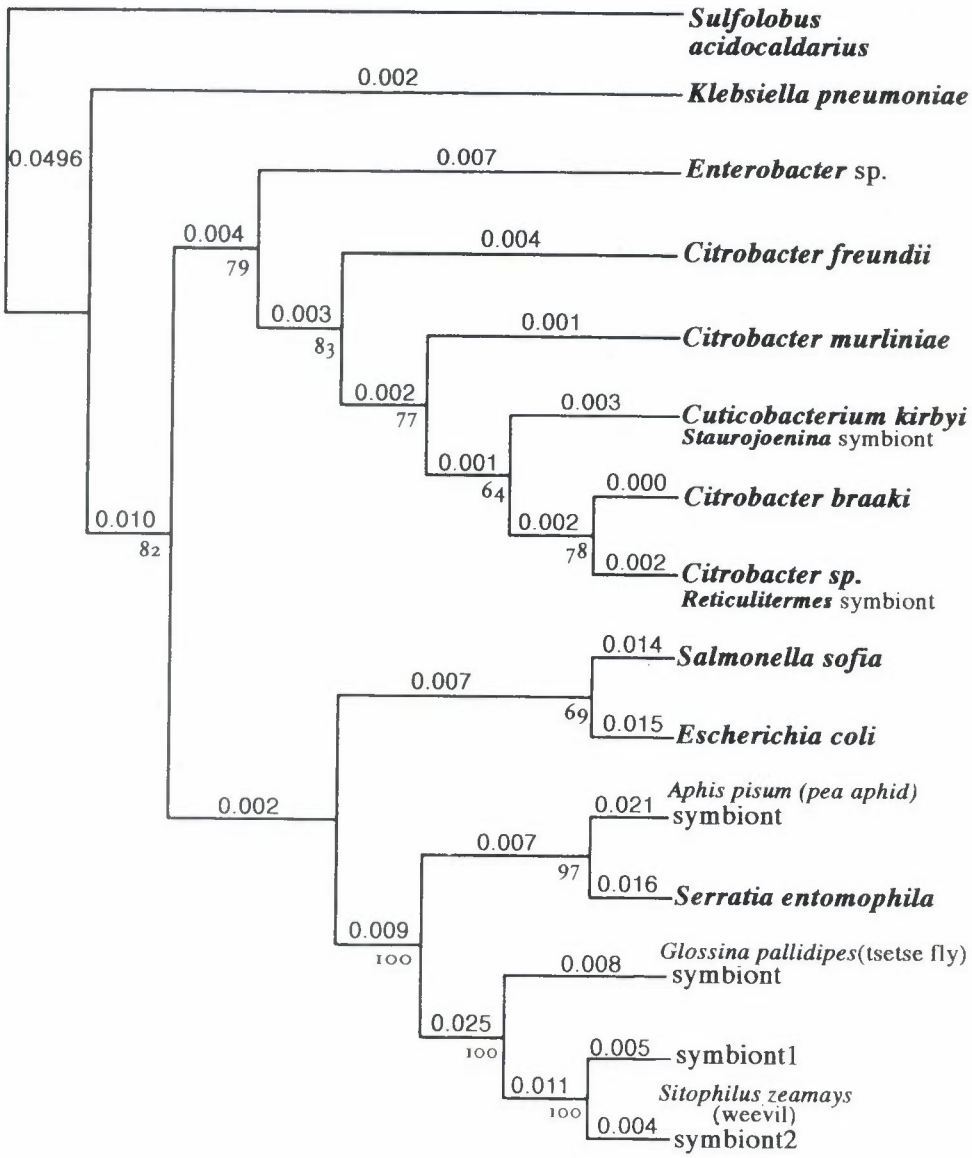

Figure 5. Neighbor joining tree based on 16S rDNA sequence analysis (PAUP) that shows taxonomic affiliation of "*Candidatus* Cuticobacterium kirbyi" and closely related citrobacters. Branch lengths are shown above branches and bootstrap values beneath nodes.

"*Candidatus* Cuticobacterium kirbyi" has been assigned GenBank accession number (AY567708). The following species were used (accession numbers in parentheses) in phylogenetic analysis: *Sulfolobus acidocaldarius* (D14876),

Klebsiella pneumoniae (X87276), *Enterobacter* sp. (U39556), *Citrobacter freundii* (AF025365), *Citrobacter murliniae* (AF025369), *Citrobacter braakii* (AF025368), *Citrobacter* sp. (AF463533), *Salmonella sofia* (X80677), *Escherichia coli* (J01698), *Aphis pisum* symbionts (M27040), *Serratia entomophila* (AJ233427), *Glossina pallidipes* symbionts (M99060), *Sitophilus zeamays* symbiont (M85270), and *Sitophilus zeamays* symbiont (M85269).

The details of the cortical bacteria relation to the protist's metabolism remain unknown of course but possibilities include: oxygen may be scrubbed by the facultative anaerobic *Citrobacter*-like epibiont, the bacterium may aid in digestion of cellulose or other intractable carbohydrate or it may provide specific nitrogen-rich metabolites in the nitrogen-limited environment of the termite hindgut. Carbon from the bacterium may react with hydrogen from hydrogenosomes to produce acetate, a major metabolite in xylophagous intestines that enters the termite epithelium. The new bacterial isolate from the common North American subterranean termite, *Reticulitermes flavipes*, shows 99.5% identity to *Citrobacter braakii* from the intestines of humans and other mammals (Gupta et al., 2003). These data suggest that the *Staurojoenina* epibionts here may also consume acetate (as carbon source) and hydrogen gas with the use of arsenate as terminal electron acceptor. In any case, the idea that the epibionts are capable of arsenate reduction to arsenic (Herbel et al., 2002) deserves investigation.

Our work here on *Staurojoenina* epibionts is evaluated in the context of a previous study of 20 million year old (Miocene) fossil termites (*Mastotermes electrodominicus*) embedded in amber (Wier et al., 2002). There we obtained images of bacteria tightly attached to membrane in well-preserved tissues in a museum specimen. Taken together we conclude that intestinal protist-bacterial symbioses are developmental, permanent and ancient.

Tightly adhering gram-negative rod-shaped bacteria are common in oxymonad (Dyer and Khalso, 1993) and parabasalid archaeprotists as our examples show (Dolan, 2001; Dolan et al., 2004). Literature search, especially from the first half of the twentieth century, will reveal many more protist species that permanently sport adherent rod-shaped surface bacteria. Those that live in high sulfide and/or low oxygen habitats seem especially prone to such regular associations. At least three epibiotic gram-negative rods, on *Barbulanympha* and *Urinympha* from the wood-feeding cockroach *Cryptocercus punctulata* (Bloodgood and Fitzharris, 1976) as well as peritrichous rods in grooves on the surface of *Caduceia versatilis* ("Rubberneckia") from *Cryptotermes cavifrons* (Tamm, 1980, 1982; d'Ambrosio et al., 1999), may also be assignable to *Cuticobacterium*. Some may belong to the gamma-proteobacterial *Bacteroides/Porphyromonas* group as suggested by 16S rRNA sequences in unpublished studies (Merritt et al., 1996; Goss and Gunderson, 2000). The epibiotic rod of *Mixotricha* is also related to *Bacteroides*

(Wenzel et al., 2003). Others may be enterobacteria like that on *S. assimilis*. As *in situ* hybridization techniques are applied to study bacterial DNA in protist associations more and more accurate identification of the components of these microbial communities are expected to be made

Electron microscopic images associated with 16S rRNA *in situ* staining permit the identification of uncultivable bacteria that are integral parts of the surfaces of protists. Distinctive molecular modes of bacterial attachment to protist cell surfaces were reported by Radek and Tischendorf (1999) in other termite intestinal symbionts. Similar analysis is warranted for these attachments, especially since each raised portion of the *S. assimilis* cortex is underlain by a microtubule apparently of protist origin. The detailed morphological and molecular biological studies, we suggest, are adequate and acceptable to name new "candidatus" bacteria until the growth and transfer of these microbes in pure culture is achieved.

The correlation of the surface-adherent bacteria with the archaeprotist morphology in *S. assimilis* is so invariant that a new taxon for the cuticle prokaryotes is necessary. We propose the name *Cuticobacterium kirbyi* for this epibiont and establish a new genus for comparable associations as follows:

Diagnosis. Properties of the genus Cuticobacterium

Rod-shaped gram-negative epibiotic symbiotic bacteria that range from 0.1–0.5 μm in diameter and 1.0–10 μm in length. Ribosomal RNA genetic analysis indicates they are gamma proteobacteria (enterobacteria) closely related to the genus *Citrobacter*. The epibionts are associated with parabasalid protists (trichomonads and hypermastigotes) at well-defined membrane sites by attachment structures. Although the bacteria may be limited to specific identifiable portions of the protist surface they abundantly cover those portions. The attachment establishes a regular and predictable alignment on the protist. The attachment structures, which are strong enough to immobilize the bacteria, persist through pipetting, centrifugation, staining, and other cytological procedures. The association is permanent and present at all times on all members of the protist taxon in question.

Species kirbyi

Cortical gram negative bacterium that measures 0.2–0.3 μm in width and from 2.5–7.0 μm in length and is distributed densely over the entire surface of *S. assimilis*, except between the four lobes where the undulipodial bundles lie. Each bacterium is correlated with a specific attachment site on the surface of the protist.

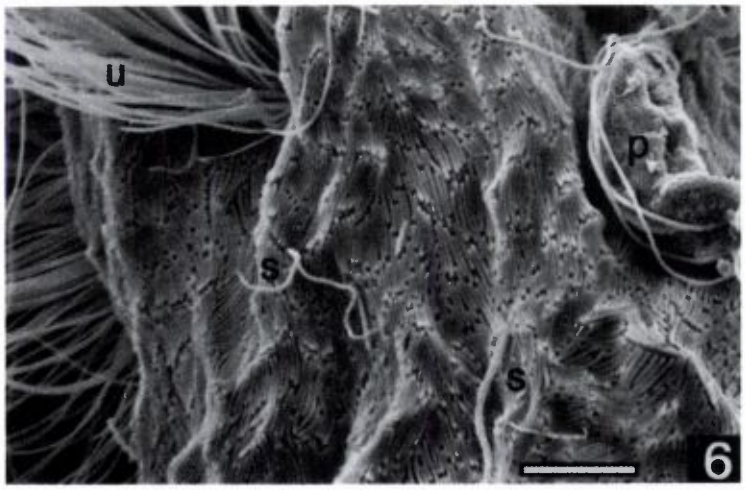

Figure 6. "*Candidatus* Cuticobacterium kirbyi" in its natural habitat as part of the surface of *Staurojoenina assimilis*. (SEM, bar = 10 μm).

Etymology

Cuticobacterium kirbyi [*cutico*, L: little skin, + *bacter*, L: rod]. The first part of the genus name "*Cutico*" is the Latin diminutive of "*cutis*" (skin) and therefore "cutico", little skin"); it refers to the fact that the adherent bacterial covering (Fig. 6) forms a little skin. The second portion of the genus name "*bacterium*" (Latin) refers to its rod shape. The species, "*kirbyi*" is named for Harold Kirby, Jr. (1900–1952), Professor of Zoology, University of California, Berkeley, who described the major features of these organisms and their associations as intrinsic to the host.

Ecology

"*Candidatus* Cuticobacterium kirbyi" is found on the surface of, and in vesicles within, the hypermastigote *Staurojoenina assimilis*. This protist is restricted to the hindgut of kalotermitids such as *Neotermes mona*, *Neotermes luykxii*, and *Incisitermes minor*.

Type information

Neither "*Candidatus* Cuticobacterium kirbyi" nor *Staurojoenina assimilis* have been cultured. The type specimens of the protist have been deposited at the Department of Invertebrates of the American Museum of Natural History.

"*Candidatus* Cuticobacterium kirbyi" are not seen in these protist type specimens, and cannot be resolved by light microscopy staining techniques because of their dense investiture on the protist cell. There are currently no generally accepted procedures for depositing candidatus type specimens or for depositing photographs or video images as type specimen material. We therefore can only report the type locality for the insect that harbors this symbiotic bacterium. We advocate that electron micrographs and video footage submitted with museum type specimens of such protists be acceptable as type material until cultivation of the epibionts is achieved. The 16S rDNA of *C. kirbyi* was obtained from *Neotermes mona* from a red mangrove stand about 20 m from the water's edge at Bahía Fosforescente in southwest Puerto Rico.

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