Cysts and symbionts of Staurojoenina assimilis Kirby from Neotermes

Michael F. Dolan\(^a\), Andrew M. Wier\(^b\), Hannah Melnitsky\(^a\), Jessica H. Whiteside\(^c\), Lynn Margulis\(^a\,\ast\)

\(^a\) Department of Geosciences, University of Massachusetts, Amherst, Massachusetts, MA 01003, USA
\(^b\) Department of Biological Sciences, Box 413, University of Wisconsin, Milwaukee, Wisconsin, WI 53211, USA
\(^c\) Lamont-Doherty Earth Observatory of Columbia University, Palisades, New York, NY 10964, USA

Received 13 August 2003; accepted 21 January 2004

Abstract

**Staurojoenina assimilis** Kirby, a hindgut hypermastigote parabasalid symbiont in two kalotermitids (**Neotermes** mona St. John, US Virgin Islands and **N. jouteli** Puerto Rico) was studied live and by electron microscopy. In this first description of hypermastigote protist cysts in termite intestines we report numerous of these translucent walled spheres in a population of **S. assimilis** in the hindgut of one pseudergate from a Puerto Rican mangrove community. Tightly adhering, regularly spaced rod bacteria were observed on the surfaces of all live **S. assimilis** cells. The bacterial nature of these ectosymbiotic rods was verified by TEM and SEM. They are present on the four anterior lobes and most of the rest of the surface of this hypermastigote. The processes by which these ectosymbionts may be retained after ingestion, propagated and transported to the protist's outer membrane are suggested. The ultrastructure of other unknown symbionts, endonuclear microbes that resemble *Caryococcus*, perhaps pleiomorphic Gram negative bacteria, is also described.

© 2004 Published by Elsevier GmbH.

**Keywords:** Bacterial cortical symbionts; *Caryococcus*; Cysts; Nuclear symbionts; *Staurojoenina*; Termite flagellate

Introduction

The hypermastigote genus *Staurojoenina* Grassi, 1917 was discovered in the kalotermitid (dry wood-ingesting termite) **Epicalotermes aethiopicus** from Africa. It was named for its distinctive cross-like (‘stauro’) arrangement of its four anterior lobes alternating with bundles of undulipodia (eukaryotic flagella, Margulis et al. 1993). In addition to *Staurojoenina assimilis* Kirby, 1926 (North America; this paper) four species of *Staurojoenina* have been described: S. mirabilis Grassi, 1917 (Africa), S. caulleryi Grassé and Hollande, 1942, 1945 (Island of Madeira), S. corbeli Hollande, 1986 (Canary Islands), and S. gracilis Hollande, 1986 (Madagascar). All inhabit the intestines of kalotermitids, and are among the largest gut protists. Like all parabasalids they lack mitochondria at all stages, but contain hydrogenosomes (Holland and Carruette-Valentin 1971).

Although genetic and even ultrastructural data are woefully sparse, the protistological community shares a growing awareness of the genetic, morphological, ecological and evolutionary importance to protists of ecto- and endosymbiotic bacteria (Sapp 2004). Indeed several termite gut protist species are diagnosed by characters conferred upon them by stable and specific bacterial symbionts (Dolan 2001). *S. assimilis* is an example of such a composite amitochondriate large protist.
This study of live and fixed populations of the *S. assimilis* intestinal symbiotic microbial community includes the first report of any type of cyst of any hypermastigote from a termite. We also extend the previous description of two other symbionts of *S. assimilis* on the basis of their ultrastructure. One, a surface rod, is compared to similar ectosymbiotic bacteria. We suggest the means by which this cortical rod bacterium-protist association is consistently maintained. The second *S. assimilis* symbiont, a polymorphic, endonuclear microbe is described here for the first time at the electron microscopic level.

**Materials and methods**

**Collection**

These *Staurojoenina* were found in the intestines of dry wood-ingesting termites *Neotermes mona* and *N. jouteli* collected near the seashore on two Caribbean islands. *N. mona* were collected from red mangroves (*Rhizophora mangle*) at Lameshur Bay, St. John (United States Virgin Islands). *N. jouteli* were collected 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. Rudolf Scheffrahn (University of Florida Fort Lauderdale Research and Education Center). Unpublished transmission electron micrographs of *S. assimilis* from *Incisitermes minor* from Newbury Park, CA, the work of our late colleague Dr. David Chase, University of California, Davis, were also used in this analysis.

**Light microscopy**

Hindguts were extracted from live termites and punctured in a few drops of insect Ringer’s solution. Unstained protists, fixed in 0.5% glutaraldehyde, were measured with a stage micrometer. The gut contents of a single termite were fixed in 1.0% glutaraldehyde, washed in PBS, stained for 1 h with 0.1 μg/ml DAPI and observed with phase-contrast/epifluorescence microscopy. Other cells were spread on poly L-lysine coated coverslips fixed with 1.0% glutaraldehyde and stained with iron hematoxylin following Kirby’s protocol (1950).

**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 each, 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 5 min in uranyl acetate followed by 5 min in lead citrate, and viewed in a Phillips electron microscope 410 at 60 kV.

For scanning electron microscopy protists were fixed in 1.0% glutaraldehyde in PBS, suspended in PBS and washed by centrifugation and resuspension 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 resuspension 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 protist 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 stored on three-quarter inch Sony U-matic 60-min tapes and confirmed by still photographs taken with 160ASA 35mm Ektachrome film through the same microscope. Live images were captured and stored in digital format with the use of the Final Cut Pro program on a G5 Macintosh computer.

Results

The hindguts of all termites sampled were replete with the large, conspicuous, wood-digesting parabasalid *S. assimilis* (Figs. 1 and 2). In all of the more than three dozen insects sampled populations of *S. assimilis* were present along with smaller protists and bacteria. No other large hypermastigotes or comparably sized intestinal symbionts were seen. The rostrum, or anterior portion of *Staurojoenina* with its parabasal plates and four-fold symmetry easily distinguishes this hypermastigote from trichonymphids and other large parabasalids.

Cysts

Two percent of the *S. assimilis* cells from one termite from the Puerto Rico locale had formed rounded cysts prior to examination (Fig. 3). These were mixed in amongst hundreds of normal, swimming cells of the same species. When subjected to osmotic stress *S. assimilis* cells may bloat and burst, and produce finger-like processes of cytoplasm. The fact that cysts were present among many normal swimming *S. assimilis* cells precluded osmotic stress as the cause of the formation of these spherical structures. The cysts were 60–80 μm in diameter and contained the rostral parabasal plates, ctenofilaments (Kirby 1926; Hollande 1986), and the nucleus (Fig. 3). Inside the cysts neither undulipodia nor wood particles were seen, nor was there any internal motility. The faintly dotted pattern inside the cyst is interpreted to indicate the presence of epibiotic rod
The cysts developed a 4–6 μm thick hyaline wall, which became stiffer and thicker over time as judged by the effect of swimming cells bouncing off it. No cell division was observed in the cysts nor was excystation observed.

**Bacterial epibionts**

Long (2.5–6.0 μm), thin (0.2–0.5 μm) rods cover the surface of *S. assimilis* cells (Figs. 4–6). The indisputably bacterial nature of the rods on *Staurojoenina* was first confirmed by Hollande (1986) in European termites. Each rod is attached to a ridge on the surface membrane of the protist. Under each bacterium on its cortical ridge lies a single submembranous tubule. We interpret these tubules to be standard microtubules because they have the same diameter (c. 24 nm) and transverse cross-section appearance as do tubules in the hundreds of axonemes seen in many fixed and stained thin sections. The microtubule is attached to the plasma membrane by a very fine 30 nm-long “hook” that invariably comes off to one side (Fig. 5 arrows). The distinctive ridges are evenly spaced. The protist component of the attachment structure is the ridge that longitudinally subends the bacterium; outside the protist at each bacterial site fuzz invariably extends 30–40 nm from the protist plasma membrane to the outer bacterial wall. The fuzz, which connects along at least a 20° angle of the proximal wall of each bacterium is probably a glycocalyx like that reported for similar prokaryotes.

The ridge to which the rod-shaped bacterium is attached, extends longitudinally beyond both ends of the rod (Fig. 6, arrowheads). The ends of each rod bacterium are blunt not fusiform. The presence of dumbbell-shaped bacteria on the protist’s surface indicates that these rod-shaped bacteria may divide there.

The epibionts were also found enclosed in the cytoplasm (bacterial vacuole membranes, in Fig. 5). Their Gram-negative staining pattern was confirmed by their wall morphology i.e. persistence of both an inner and outer membrane. Dumbbell-shaped bacteria indistinguishable from those found on the protist surface were also seen in cytoplasmic vacuoles.

**Intranuclear bionts**

In at least ten live *S. assimilis* cells, we observed swollen nuclei like those interpreted by Kirby (1944, in species of *Trichonympha*) as “parasitically infected”. Approximately 4% of the cells from the guts of several termites from both locales harboured endonuclear spherical or subspherical particles interpreted to be varying sizes of an intranuclear symbiont (Fig. 7). While an uninfected *Staurojoenina* nucleus is 10 μm in diameter (Fig. 7a), infected nuclei increase in size to over 30 μm before the rupture and release of the full-grown necrocytophs (Fig. 7b–f). The early infection nucleus contains numerous particles that stain darkly with hematoxylin and increase from <0.5 to 3–4 μm as they consume the *Staurojoenina* chromatin (Figs. 7 and 8). With the increase in size they differentiate and appear in the electron microscope to have darkly staining and unstaining portions of varied size and appearance (Fig. 9).
The intranuclear symbionts have typical Gram-negative bacterial cell walls (Fig. 9, upper right). They are situated in direct contact with the chromatin, not bounded by a vacuole membrane. With the uranyl acetate-lead citrate TEM stain the cytoplasm is starkly differentiated between electron-dense and electron-lucent regions. This pattern was also seen in hematoxylin preparations. Several of the intranuclear symbionts were seen in division (Fig. 9). The darkly stained region does not contact the cell wall in any of the cells, rather it is consistently separated by an electron-lucent region of cytoplasm (Fig. 9, right). The infected nucleus displays a conspicuous tubule-studded membranous coat outside the nuclear envelope. The membranous coat is 250–350 nm wide whereas the tubules, 10–20 nm in diameter, are oriented longitudinally, transversely and obliquely (center inset Fig. 9). The membranous coat is unlikely to characterize the normal nuclear membrane since it has not been described in other parabasalids (e.g. Brugerolle and Bordereau 2003). Its relation to the infection requires further investigation.

Discussion

This is the first report of hypermastigote cysts in any kalotermitid. *Trichonympha* cysts have been described from the wood-eating cockroach *Cryptocercus*. They form during gametogenesis (Cleveland 1949; Cleveland et al. 1963). The *Staurojoenina* cysts, in comparison, contain more of the rostral cytoskeleton, and have a thicker cyst wall. We consider Cleveland’s unconfirmed reports of fertilization in the hypermastigote symbionts of termites unconvincing (e.g. Cleveland 1965), and therefore conclude that gametogenesis and fertilization have not been reliably reported in any termite hypermastigote. Cyst formation here may be a relict phenomenon left over from the loss of sex in lineages of termite flagellates. The *Staurojoenina* cysts are much larger and have thicker walls than those of the smaller parabasalids *Trichomitus* and *Monocercomonas* (Brugerolle 1973).

Tightly adhering Gram-negative rod-shaped bacteria are common on the surface of oxymonads and parabasalids (Table 1). Of the ectosymbiotic Gram-negative
rods on the protists of termites and Cryptocercus, those on Barbulanympha from Cryptocercus (Bloodgood and Fitzharris 1976) as well as the peritrichous rods in grooves on the surface of Caduceia versatilis (“Rubberneckia”) from Cryptotermes cavifrons (d’Ambrosio et al. 1999; Tamm 1980) may belong to the α-proteobacterial Bacteroides/Porphyromonas group as suggested by 16S rDNA sequences both presented in abstracts at meetings of the American Society for Microbiology. This phylogenetic affinity is also consistent with that of the ectosymbiotic rods of Mixotricha paradoxa, a trichomonad from the hindgut of the Australian termite Mastotermes darwiniensis (Wenzel et al. 2003). The significance of the presence and form of these rod-shaped bacteria in cytoplasmic vacuoles is controversial. Although it might be generally thought that these bacteria are in the process of digestion, the presence of dumbbell-shaped bacteria indistinguishable from those on the surface suggests that they might reproduce within the vacuoles. We suggest that it is possible that bacteria ingested with wood particles may survive and even divide within vacuoles before being ‘recycled’ to the cell surface (see note added in proof).

Kirby reported three genera of nuclear parasites in Trichonympha: Caryococcus Dangeard, bacteria with a crescentric staining area at the periphery, that may or may not cause nuclear hypertrophy; Caryoletira Kirby, a multinucleate organism that undergoes sporogony and consumes the chromatin causing enlargement of the nucleus, and Nucleophaga-like parasites, poorly understood organisms thought to be chytrids. They consume chromatin and cause enlargement of the protist nucleus (Kirby 1944). Based on the inner and outer membranes of their cell wall, lack of any organelles (e.g., nucleus or kinetosomes) and the peculiar dark- and light-staining pattern, these intranuclear symbionts of Staurojoenina most closely resemble Caryococcus. They are probably pleomorphic Gram-negative bacteria. Necrotrophic intranuclear and cytoplasmic symbionts such as these are widespread in protists of termites’ intestines (Kirby 1941). The significance to metabolism and ecology in xylophagous insects of these associated microbes remains poorly understood.

**Note added in proof**

The tightly adhered Gram-negative rod-shaped bacteria on the four surface lobes of Staurojoenina assimilis have been named “Candidatus Cuticobacterium kirbyi”. These cortical symbionts are related to members of the

**Table 1. Rod-shaped ectosymbiotic gram-negative bacteria on the surface of parabasalids and oxymonads**

<table>
<thead>
<tr>
<th>Protist (group)</th>
<th>Bacterium/host insect</th>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barbulanympha (h)</td>
<td>Undescribed rod</td>
<td>TEM</td>
<td>Bloodgood and Fitzharris (1976)</td>
</tr>
<tr>
<td></td>
<td>Bacteroides-Porphyromonas/</td>
<td>16S rDNA</td>
<td>Merritt et al. ASM abstract, 1996</td>
</tr>
<tr>
<td></td>
<td>Cryptocercus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caduceia versatilis (d)</td>
<td>Flagellated rod</td>
<td>TEM</td>
<td>Tamm (1982)</td>
</tr>
<tr>
<td></td>
<td>Bacteroides-Porphyromonas/</td>
<td>16S rDNA</td>
<td>Goss and Gunderson, ASM Abstract, 2000</td>
</tr>
<tr>
<td></td>
<td>Cryptotermes cavifrons</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Devescovina glabra (d)</td>
<td>Undescribed rod/</td>
<td>TEM</td>
<td>Radek and Tischendorf (1999)</td>
</tr>
<tr>
<td></td>
<td>Cryptotermes dudleyi</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mixotricha paradoxa (t)</td>
<td>Bacteroides group/</td>
<td>16S rDNA</td>
<td>Wenzel et al. (2003)</td>
</tr>
<tr>
<td></td>
<td>Mastotermes darwiniensis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polymastix sp. (o)</td>
<td>Fusiformis/ Parasphaeria boleiriana</td>
<td>TEM</td>
<td>Brugerolle et al. (2003)</td>
</tr>
<tr>
<td>Staurojoenina assimilis (h)</td>
<td>Undescribed rod/</td>
<td>TEM, SEM</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>Neotermes spp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staurojoenina corbelii (h)</td>
<td>Undescribed rods/</td>
<td>SEM</td>
<td>Hollande (1986)</td>
</tr>
<tr>
<td></td>
<td>Bifiditermes rogiera</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Streblomastix strix (o)</td>
<td>Long undescribed rods/</td>
<td>TEM</td>
<td>Dyer and Khalso (1993)</td>
</tr>
<tr>
<td></td>
<td>Zootermopsis sp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urinympha (h)</td>
<td>Undescribed rods/</td>
<td>TEM</td>
<td>Bloodgood and Fitzharris (1976)</td>
</tr>
<tr>
<td></td>
<td>Cryptocercus sp.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

key: d = devescovinid, h = hypermastigote, o = oxymonad, t = trichomonad
genus *Citrobacter*, common intestinal enterobacteria (Wier et al. 2004).

Acknowledgements

We thank Dale Callaham for help with electron microscopy (National Science Foundation grant BBS 8714235), and Sean Werle (University of Massachusetts-Amherst) and Rudolf Scheffrahn (University of Florida) for assistance with termite identification. Thomas H. Teal (University of Washington-Seattle), David Bermudes (Yale Medical School) and Renate Radek (Free University of Berlin) aided with micrograph interpretation. We gratefully acknowledge funding from: the University of Massachusetts-Amherst (Commonwealth College Honors Research Fellowship to H.M.; College of Natural Sciences and Mathematics; The Graduate School), NASA (Office of Space Sciences), and Liberé (Abe Gomel). We thank Professor Wolfgang E. Krumbein who made possible cooperation with our German colleagues. The Alexander von Humboldt Stiftung prize and residence grant from the Hanse Wissenschaftkolleg (Delmenhorst) (L.M.) were essential to the completion of this work. The American Museum of Natural History provided access to Harold Kirby’s specimen collection. This paper is dedicated to the memory of David C. Chase.

References


