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Cysts and symbionts of *Staurojoenina assimilis* Kirby from *Neotermes*

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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.

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 (Hollande 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.

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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

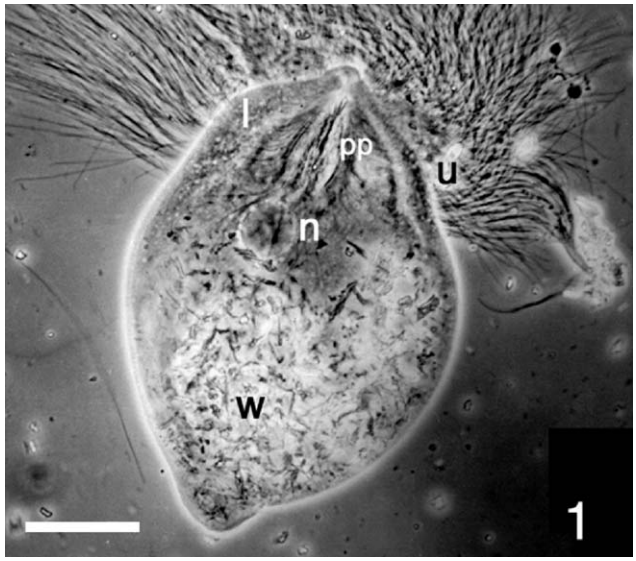

Fig. 1. *S. assimilis* with undulipodiated rostrum, large, spherical nucleus (n) and posterior wood particles (w) seen by phase contrast light microscopy in a live cell. The conspicuous parabasal plates (pp) characterize the genus. The ectosymbiotic bacteria cover the four lobes (l) that protrude between the four bundles of undulipodia (bar = 50 μm).

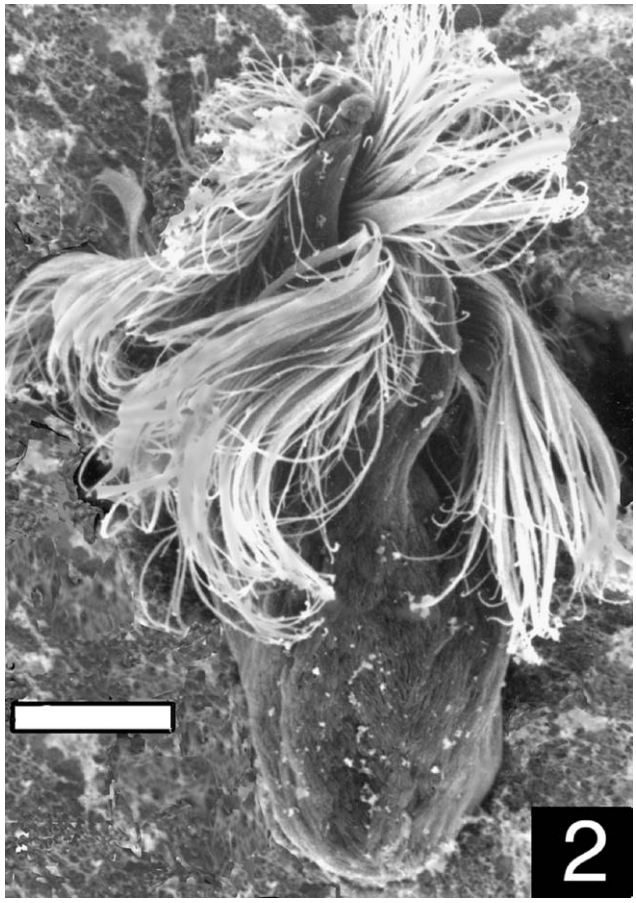

Fig. 2. *S. assimilis* with three of the four undulipodia bundles seen with SEM (bar = 50 μm).

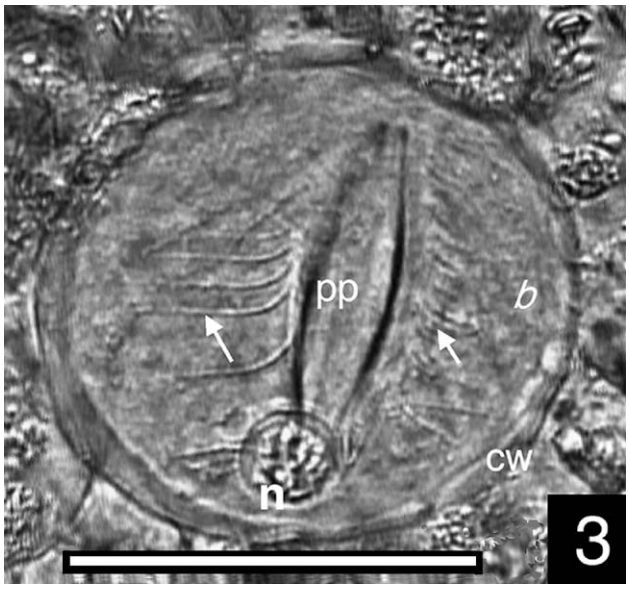

Fig. 3. A single *S. assimilis* translucent cyst in which the nucleus (n), parabasal plates (pp), and ctenofilaments (arrows) are seen in the live protist. The cyst wall and putative epibiotic bacteria (b) can be seen (Nomarski differential interference phase contrast micrograph; bar = 70 μm).

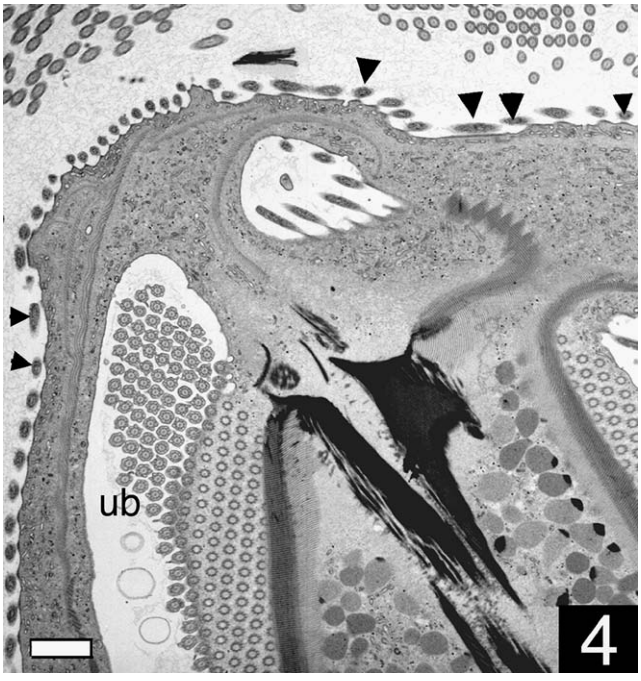

Fig. 4. Two of the four lobes studded with evenly spaced transverse sections of the epibiotic bacterium (arrowheads) interspersed with undulipodial bundles (ub) (TEM, bar = 3 μm).

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

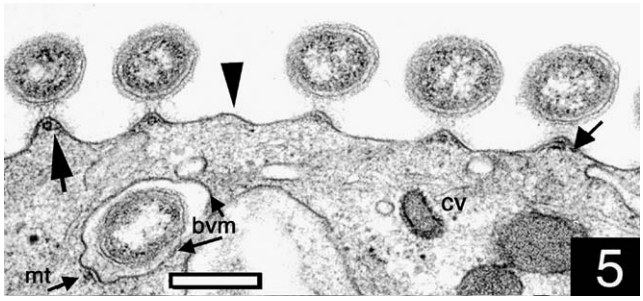

Fig. 5. Five epibionts, each with its single tubule and hook (arrow) attached to membrane to the right of the tubule. At the left the bacterial vacuole membrane (bvm) surrounds the presumed incipient ectosymbiont. Its unhooked microtubule (mt) is seen (small arrow). The bacterium may be transported to the vacant surface ridge (arrowhead) where it will attach like the others (arrows). A coated vesicle (cv) of unknown function is also seen. *S. assimilis* from *Incisitermes minor* preparation made by David Chase. (TEM, bar = 250 nm).

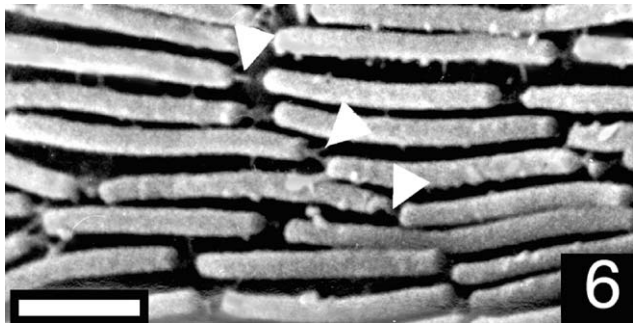

Fig. 6. Epibiotic rods longitudinally aligned on an *S. assimilis* anterior lobe. This SEM corresponds to the bacteria seen in transverse thin section in Fig. 5. Attachment structures extend beyond the bacteria (arrowheads; bar = 3 μm).

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.

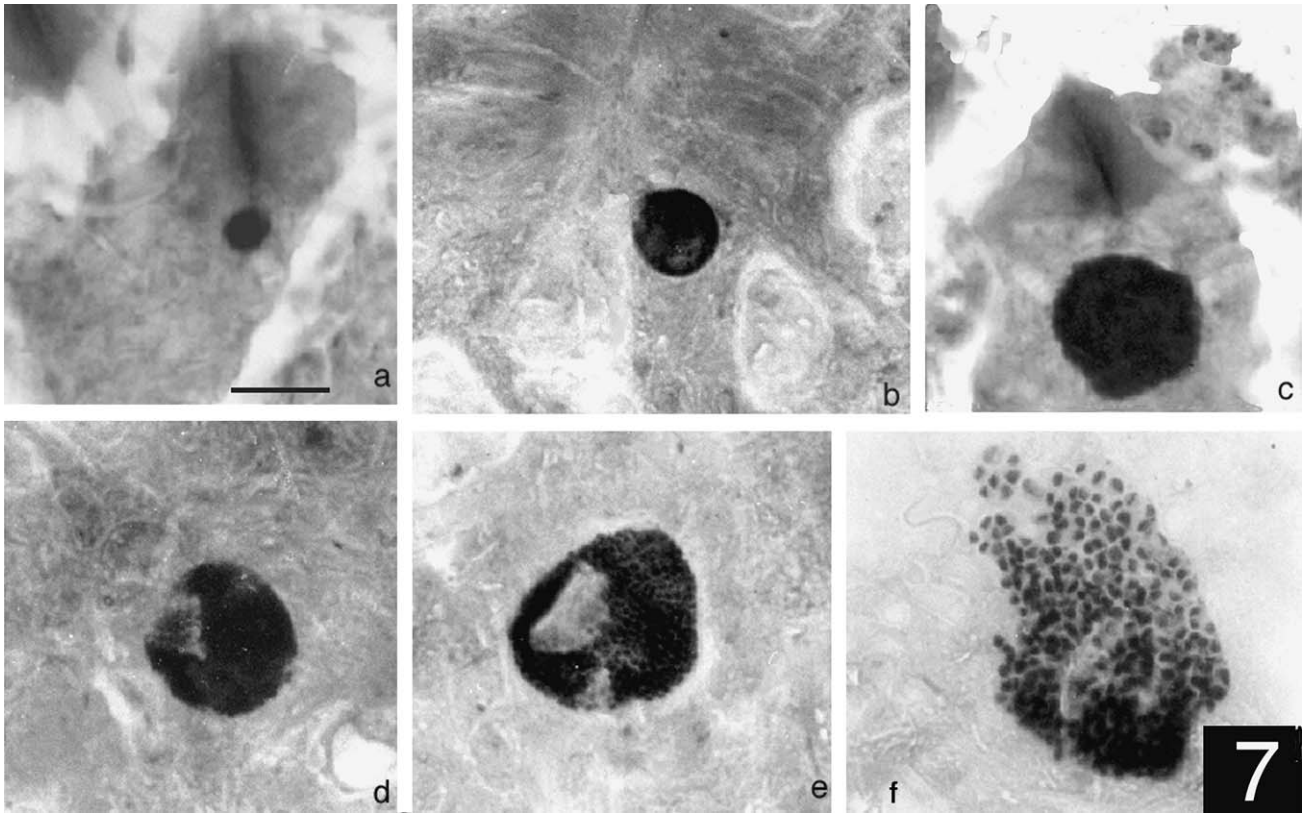

Fig. 7. Developmental sequence of nuclear symbiont. (a) uninfected nucleus, (b) early stage with 0.2–0.5 μm particles, (c) hypertrophied stage with large opaque particles, (d)–(e) differentiation into translucent and stainable portions, (f) rupturing of nucleus and release of over a hundred mature symbionts up to 4 μm in diameter (Hematoxylin stain, bar = 15 μm).

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 35 mm 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

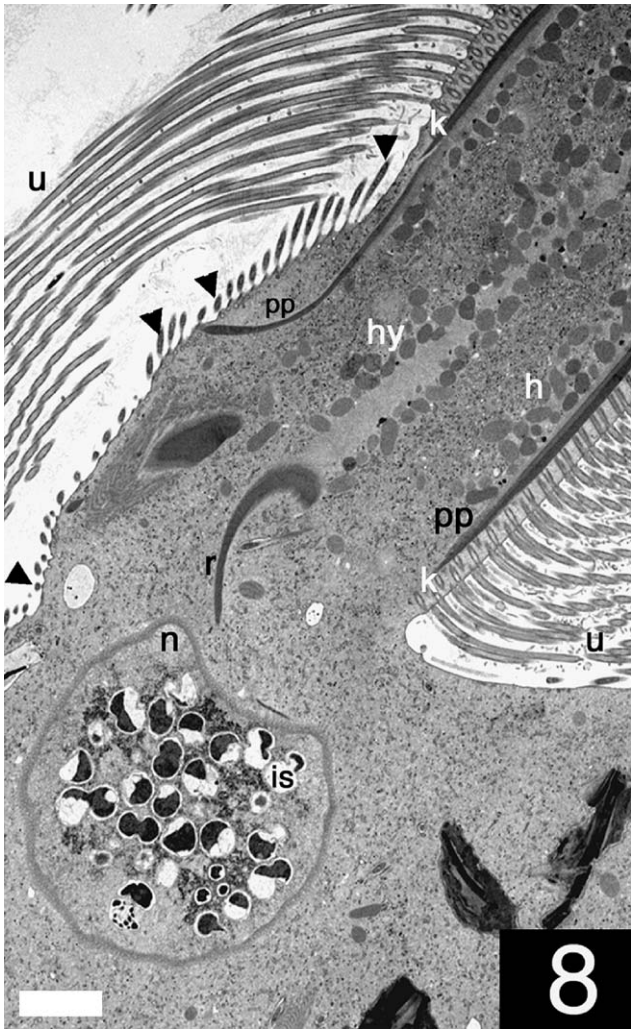

Fig. 8. Longitudinal section of a swimming *S. assimilis* cell. On the membrane posterior to the undulipodial bundles (u) ectosymbionts in oblique to tranverse section are indicated by arrowheads. The nucleus (n) is infected with intranuclear symbionts (is) of different sizes and electron opacities. At least four appear to be dividing by binary fission. Aligned hydrogenosomes (h) underlie the parabasal plates (pp) whereas others (hy) line the lumen proximal to the plate. One of the four rhizoplast bands (r), approaches a concavity in the nucleus (TEM, bar = 10 μm).

bacteria (b in Fig. 3). 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 subtends 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 necrotrophs (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).

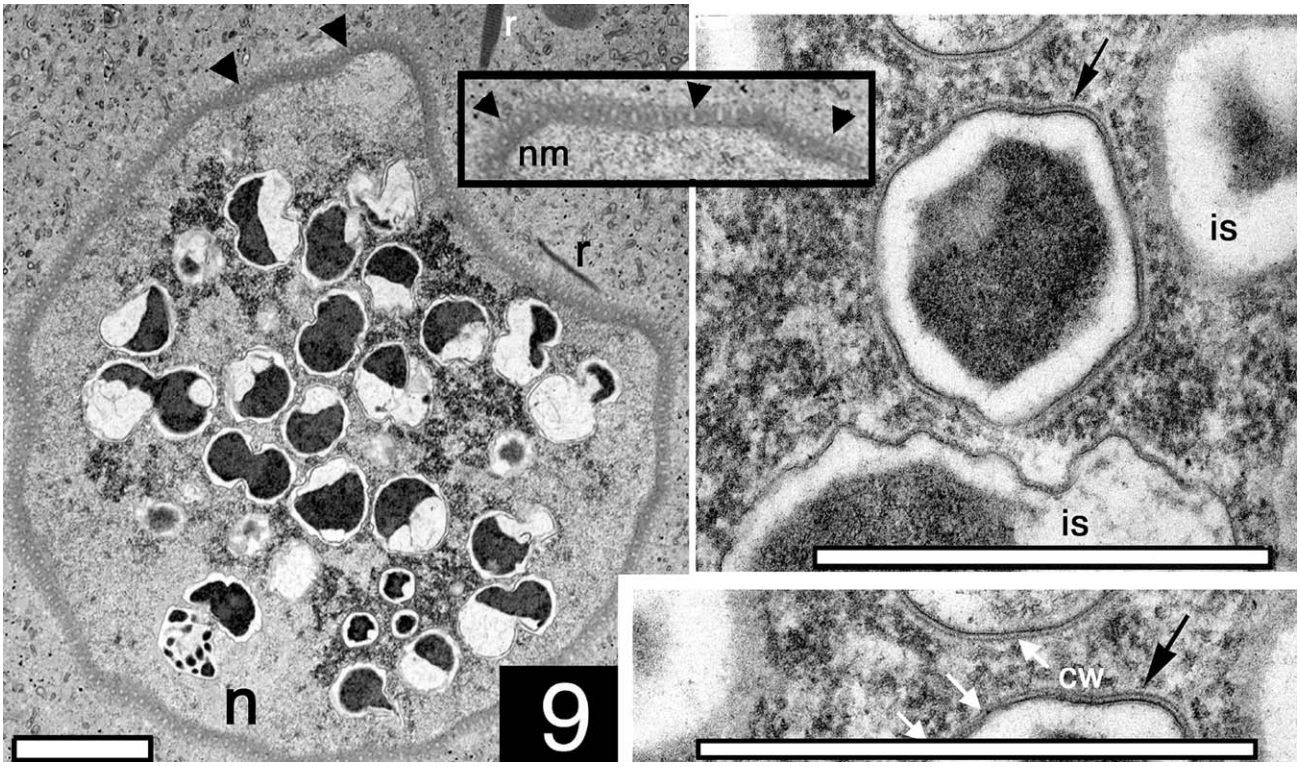

Fig. 9. The nucleus (n) and rhizoplast band (r) of Fig. 8 seen at higher magnification shows the intranuclear symbionts (is) with their Gram-negative cell walls (cw, lower right inset, black arrow). The nucleus contains intact chromatin along with at least 24 symbionts apparently at different stages of development (TEM, bar at left 8 μm). The appearance of the thickened nuclear membrane (250–350 nm) is due to membranous tubules. As seen at arrowheads in the center inset the 10–20 nm tubules may be sectioned longitudinally, obliquely or transversely. The Gram-negative cell wall with its two bounding membranes (inner and outer) that define the periplasm is shown at the arrow in the upper right TEM of the intranuclear symbionts (is). The outer membrane (black arrow) seems to define the cell wall (cw) in the lower right inset that shows the same intranuclear symbiont at higher magnification. This symbiont's outer membrane is in intimate contact with the *Staurojoenina* chromatin (white arrow) (TEM, bars at right = 3 μm).

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 pleiomorphic 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

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

key: *d* = devescovinid, *h* = hypermastigote, *o* = oxymonad, *t* = trichomonad

genus *Citrobacter*, common intestinal enterobacteria (Wier et al. 2004).

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