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Symbionts of the gut flagellate *Staurojoenina* sp. from *Neotermes cubanus* represent a novel, termite-associated lineage of *Bacteroidales*: description of '*Candidatus* Vestibaculum illigatum'

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The symbioses between cellulose-degrading flagellates and bacteria are one of the most fascinating phenomena in the complex micro-ecosystem found in the hindgut of lower termites. However, little is known about the identity of the symbionts. One example is the epibiotic bacteria colonizing the surface of hypermastigote protists of the genus *Staurojoenina*. By using scanning electron microscopy, it was shown that the whole surface of *Staurojoenina* sp. from the termite *Neotermes cubanus* is densely covered with long rod-shaped bacteria of uniform size and morphology. PCR amplification of 16S rRNA genes from isolated protozoa and subsequent cloning yielded a uniform collection of clones with virtually identical sequences. Phylogenetic analysis placed them as a new lineage among the *Bacteroidales*, only distantly related to other uncultivated bacteria in the hindgut of other termites, including an epibiont of the flagellate *Mixotricha paradoxa*. The closest cultivated relative was *Tannerella forsythensis* (<85 % sequence identity). Fluorescence *in situ* hybridization with a newly designed clone-specific oligonucleotide probe confirmed that these sequences belong to the rod-shaped epibionts of *Staurojoenina* sp. Transmission electron microscopy confirmed the presence of a Gram-negative cell wall and revealed special attachment sites for the symbionts on the cell envelope of the flagellate host. Based on the isolated phylogenetic position and the specific association with the surface of *Staurojoenina* sp., we propose to classify this new taxon of *Bacteroidales* under the provisional name '*Candidatus* Vestibaculum illigatum'.

INTRODUCTION

Wood-feeding termites depend on their symbiotic gut microbiota for lignocellulose digestion (Brune, 2003). In their guts, an impressively dense assemblage of unusual flagellate protists and prokaryotic symbionts contribute to the digestion of their chemically recalcitrant diet (Breznak & Brune, 1994; Radek, 1999; Inoue *et al.*, 2000). Although the role of the flagellates in cellulose degradation was discovered almost 80 years ago (Cleveland, 1926), and the fermentative nature of these symbionts had already been outlined in the 1940s (for a review, see Hungate, 1955), many details still remain to be resolved. In particular, the nature and function of the prokaryotic epibionts associated with most of the flagellates (e.g. Ball, 1969; Lavette, 1969; Starr, 1975; Dolan, 2001) is far from clear. The presence of special attachment sites on the cell envelope of the flagellates (Smith & Arnott, 1974; Tamm, 1980; Radek *et al.*, 1992, 1996) indicate tight interactions, but only in the case of *Mixotricha paradoxa* (Cleveland & Grimstone, 1964) and a devescovinid flagellate (Tamm, 1982), has a function of the epibionts in the motility of the host cell been demonstrated. Apart from some epibiotic spirochaetes (Iida *et al.*, 2000; Noda *et al.*, 2003) of several other gut flagellates, the epibionts of *M. paradoxa* (Wenzel *et al.*, 2003) are also among the few instances where the phylogenetic affiliation of the prokaryotic partner has been substantiated (Dolan, 2001).

Flagellates of the genus *Staurojoenina* are found only among the 'dry-wood termites' (family Kalotermitidae) (Yamin, 1979; Dolan & Margulis, 1997). In their paper, Dolan & Margulis (1997) published excellent electron micrographs

The GenBank/EMBL/DDBJ accession number for the sequence reported in this paper is AY540335.

prepared by the late David Chase, which show that the surface of *Staurojoenina assimilis* from *Incisitermes minor* is covered with unusual epibiotic bacteria. The ultrastructure of the junctional complexes is completely different from that of the motility symbionts of *Caduceia versatilis* (Tamm, 1980; d'Ambrosio *et al.*, 1999), and only remotely resembles the situation of the second epibiont of *M. paradoxa* (Cleveland & Grimstone, 1964; Wenzel *et al.*, 2003). It is likely that also the nature of the association between *Staurojoenina* species and their epibiotic bacteria differs from those examples. In this study, we present a detailed analysis of the identity, phylogenetic position and ultrastructure of the epibionts associated with a hitherto undescribed *Staurojoenina* species colonizing the gut of *Neotermes cubanus*, and classify them in a provisional *Candidatus* taxon.

METHODS

Termites. A colony of *Neotermes cubanus* (Freytaud), originally collected in Topes de Collantes, Cuba, was provided by Dr I. Hrdy, Praha. The species identification was confirmed by Drs J. Krecek, Fort Lauderdale, and K. Krishna, New York. Termites were maintained in polyethylene containers on a diet of pine wood and water.

Scanning and transmission electron microscopy. For scanning electron microscopy, worker larvae of *N. cubanus* were dissected, and the contents of the hindgut paunch were released into 0·2 M sodium phosphate buffer (pH 7·0) containing 2·5 % glutaraldehyde and 4 % OsO_4. The samples were fixed for 1 h on ice, washed three times in the same buffer, dehydrated in a series of ethanol, and critical-point dried with a Bal-Tec CPD 030. Prior to the investigation with a Philips SEM 515 or a Fei Quanta scanning electron microscope, the samples were sputtered with gold in a Balzers Union SCD 040. For transmission electron microscopy, the flagellates were pre-fixed for 1 h in 0·05 M sodium cacodylate buffer (pH 7·0) containing 2·5 % glutaraldehyde, washed three times in the same buffer, and post-fixed in reduced OsO_4 (a fresh 1 : 1 mixture of 2 % OsO_4 and 3 % $K_4[Fe(CN)_6]$) for 1 h on ice. After several further rinses in buffer, the cells were embedded in 3 % agar, dehydrated in a series of ethanol, and embedded in Spurr's resin. Ultrathin sections were stained with saturated aqueous uranyl acetate for 30 min, followed by lead-citrate staining according to Reynolds (1963), and observed using a Philips EM 208 electron microscope.

Preparation of flagellates for DNA extraction. Termite hindguts were carefully ruptured, and a suspension of gut flagellates was prepared as described previously (Stingl & Brune, 2003), except that the cells were not fixed with formaldehyde. An aliquot (15 µl) of the suspension was spotted into the first well of a 10-well Teflon slide (Roth). Using an inverted microscope, 50–60 unambiguously identified flagellates of the genus *Staurojoenina*, which were identified by their characteristic morphology (Fig. 1A), were aspirated from the suspension with a micropipette and transferred to a well containing 15 µl sterile PBS (Stingl & Brune, 2003). This procedure was repeated twice to minimize the amount of loosely attached bacteria in the sample. Finally, 30–40 flagellates were transferred to a sterile Eppendorf tube with 200 µl PBS. DNA was extracted with the NucleoSpin kit (Macherey-Nagel), according to the manufacturer's instructions, and eluted in 50 µl sterile water.

Cloning of 16S rRNA genes. PCR with primers 27F (Edwards *et al.*, 1989) and 1492R (Weisburg *et al.*, 1991) was performed with a Mastergradient thermocycler and the MasterTaq DNA polymerase kit (both Eppendorf) in a total reaction volume of 50 µl, following the protocol of Henckel *et al.* (1999) and using 1 µl of the extracted DNA as template. After purification (EZNA Cycle-Pure kit, peqlab, Erlangen, Germany), the PCR products (3 µl) were cloned in *Escherichia coli*, using the TA cloning kit (Invitrogen). Clones with correct-size inserts were sorted by RFLP analysis (Stingl & Brune, 2003).

Sequencing and phylogenetic analysis. The inserts of three randomly chosen clones were sequenced on both strands using primers 27F (Edwards *et al.*, 1989), 533F, 907R (Lane *et al.*, 1985; used also reverse complementary), and 1492R (Weisburg *et al.*, 1991) by GATC (Konstanz, Germany). Sequences were checked and assembled using DNAStar software (http://www.dnastar.com). Phylogenetic analysis was done as described elsewhere (Stingl & Brune, 2003), using the ARB program package (http://www.arb-home.de; Ludwig & Strunk, 1996).

Whole-cell *in situ* hybridization. Gut contents of five termites were suspended in 900 µl PBS and fixed for 12 h using 3·7 % formaldehyde. Hybridization procedure and conditions were as described in Stingl & Brune (2003). The oligonucleotide probe Bac303, designed to detect the *Bacteroides*/*Porphyromonas* subgroup of the CFB phylum (Manz *et al.*, 1996), was modified to achieve specificity for the sequences obtained in this study. Probe Sym_Stau_303: 5′-CCG GTG TGG GGG ACC TTC-3′ had at least one mismatch to all other sequences available in GenBank (www.ncbi.nlm.nih.gov). Maximal formamide concentration for successful hybridization was 15 %, which was used routinely. Non-specific binding of the probes was excluded by checking every sample also with a nonsense probe (Wallner *et al.*, 1993). The information on probe Sym_Stau_303 has been submitted to probeBase (http://www.microbial-ecology.de/probebase; Loy *et al.*, 2003).

RESULTS

Electron microscopy

Scanning electron microscopy (SEM) showed the presence of rod-shaped bacteria covering the whole cell surface of *Staurojoenina* sp. (Fig. 1A). Groups of 5–10 bacterial cells, oriented in parallel to each other, were arranged in oblique or rectangular position. The total number of epibionts per flagellate was estimated to be approximately 3500. While the diameter of the cells (0·3 µm) was fairly constant, the length of the symbionts ranged between 2·5 and 6 µm (mean 4·0 µm), indicating asynchronous growth and cell division. Spirochaetal cells were occasionally attached between the rod-shaped epibionts, albeit in much smaller numbers (20–90 per flagellate, Fig. 1B).

Transmission electron microscopy (TEM) of ultrathin sections of the flagellates revealed the Gram-negative cell wall of the epibiotic rods (Fig. 1C). In addition to the inner and outer membranes, the cells were surrounded by a diffuse outer layer (capsule). A plaque of electron-dense extracellular material connected the bacteria to the flagellate surface. The attachment sites of the flagellates had the form of elongated ridges, and electron-dense material below the attachment sites apparently serves to support the plasma membrane. Bacterial rods were found not only attached to the surface of the flagellates, but also in vacuoles (Fig. 1D). The vacuoles often showed remnants of the

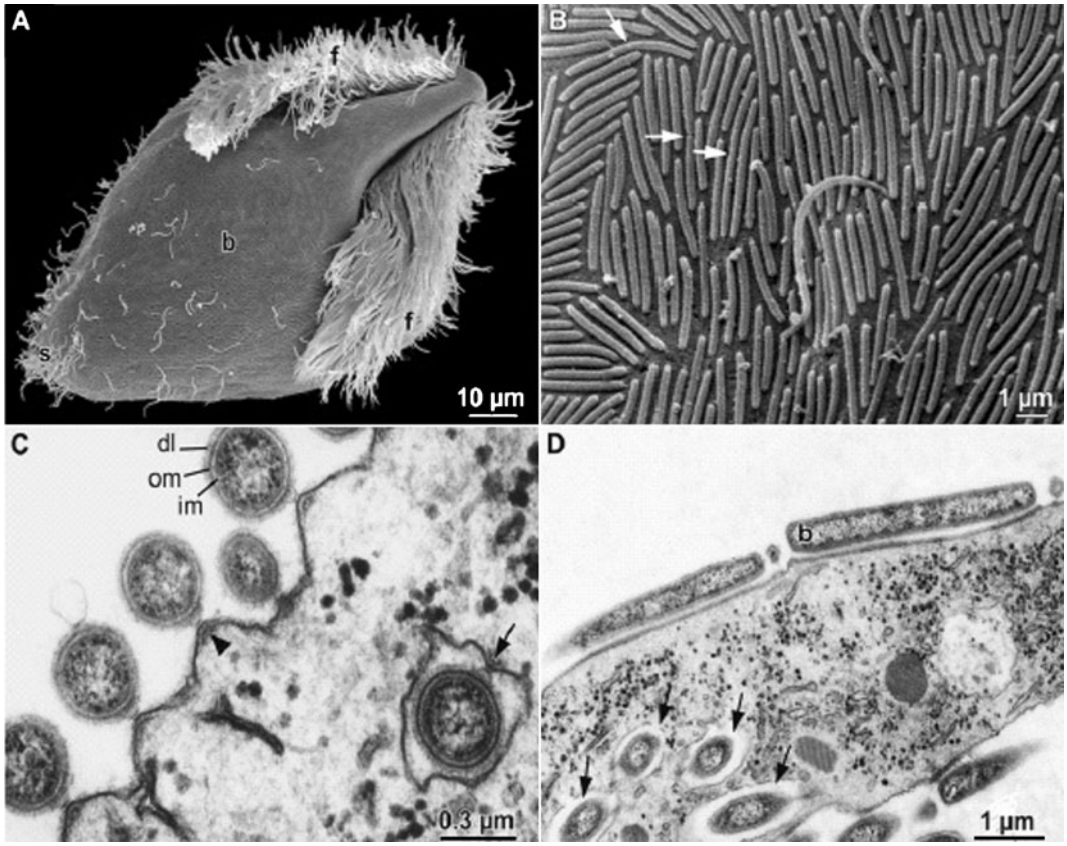

Fig. 1. Scanning (A, B) and transmission (C, D) electron micrographs of *Staurojoenina* sp. from *Neotermes cubanus*. (A) Overview of a *Staurojoenina* cell, showing two of the four flagellar tufts (f), numerous bacterial rods (b), and occasional spirochaetes (s) attached to the surface. (B) Close-up of the cell surface, showing single spirochaetes between the ectobiotic rods. Arrows point to early stages of cell division. (C) Cross-section of the epibiotic rods. In addition to inner membrane (im) and outer membrane (om), the cell is surrounded by a diffuse layer (dl). Electron-dense material supports the plasma membrane of the flagellate below the attachment sites (arrowhead). The arrow points to a phagocytized rod-shaped bacterium with remnants of attachment complex. (D) Longitudinal section showing bacteria attached to the cell surface (b) and phagocytized bacteria (arrows).

attachment structures, which confirmed that the bacteria were taken up by phagocytosis. Although phagocytosis apparently occurs all over the non-flagellated surface, the highest density of vacuoles was observed in the posterior region of the host cell. While the bacteria in the tightly fitting vacuoles located close to the flagellate surface appeared fairly intact, those in loosely fitting vacuoles located deeper within the flagellate cell had a changed morphology and were apparently subject to degradation (not shown). Fusion with lysosomes remains to be demonstrated.

Cloning and phylogenetic analysis

PCR amplification and cloning of 16S rRNA genes from DNA extracted from a suspension of hand-picked *Staurojoenina* cells and subsequent RFLP analysis indicated that the resulting clone library contained only a single ribotype. Almost full-length sequences (1417–1418 bp) were obtained for three randomly chosen clones; since the sequences were virtually identical ($>99{\cdot}5\,\%$ sequence identity), the clone library was considered homogeneous, and only one sequence was submitted to GenBank. The preliminary BLAST searches (Altschul *et al.*, 1997) had already indicated an affiliation of the clones with the *Bacteroidales*. In a detailed phylogenetic analysis (Fig. 2), the clone from *Staurojoenina* sp. always fell within a cluster of clones comprising *Bacteroides*-related sequences from other termites (Cluster 4; Ohkuma *et al.*, 2002), which also included the rod-shaped symbiont of the trichomonad flagellate *Mixotricha paradoxa* (Wenzel *et al.*, 2003). The closest cultivated relative was the human oral bacterium *Tannerella forsythensis*, although the sequence similarity was rather low ($<85\,\%$).

Assignment of clonal isolates to morphotype by fluorescence *in situ* hybridization (FISH)

When hindgut suspensions were hybridized with the bacteria probe EUB 338 (Amann *et al.*, 1990), most prokaryotic

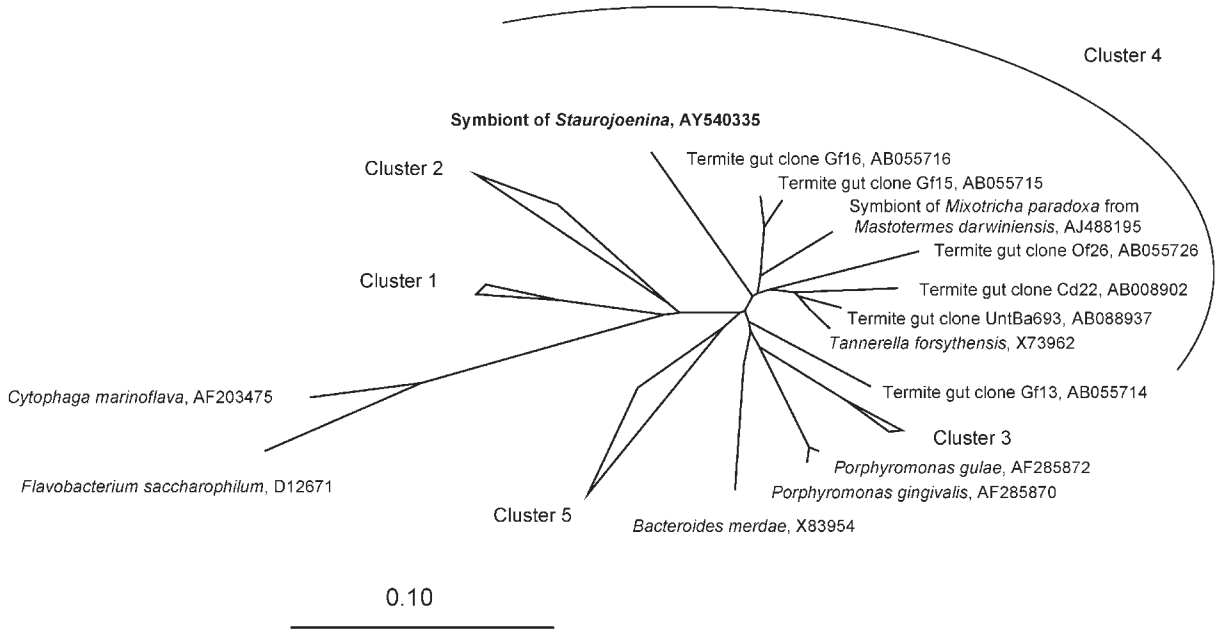

Fig. 2. Phylogenetic tree of SSU rRNA gene sequences of the symbiotic bacteria associated with *Staurojoenina* sp. from *Neotermes cubanus* (marked in bold) and their closest relatives, showing also their relationship to the *Bacteroides*-related sequences from other termites (Clusters 1–5, Ohkuma *et al*., 2002). GenBank accession numbers are included in the tree, which was constructed with the maximum-likelihood algorithm implemented in ARB (fast DNAml; Olsen *et al*., 1994) and is based on 850 unambiguously aligned sequence positions. Bar, 10 substitutions per 100 nt.

cells, including those attached to the surface of *Staurojoenina* sp., were labelled (details not shown). By contrast, the sequence-specific oligonucleotide probe Sym_Stau_303, specifically designed to detect the 16S rRNA gene sequence of the clones obtained in this study, hybridized exclusively with all rod-shaped bacterial cells colonizing the surface of this flagellate (Fig. 3). The probe did not hybridize with any bacteria located on or within other flagellate species or with those freely suspended in the hindgut fluid.

DISCUSSION

The results of this study show that the cell surface of the *Staurojoenina* sp. colonizing the gut of *Neotermes cubanus* is

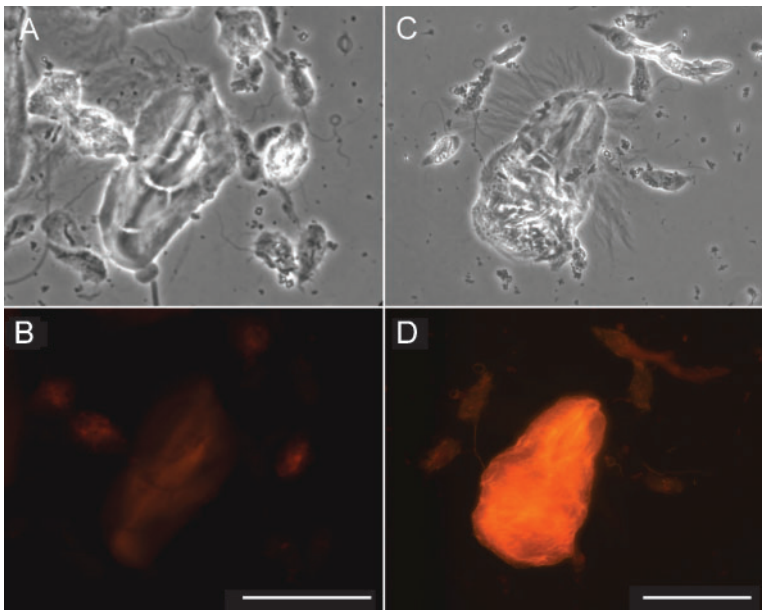

Fig. 3. Phase-contrast photomicrographs (A, C) and corresponding epifluorescence photomicrographs (B, D) of *Neotermes cubanus* hindgut suspensions hybridized with the Cy3-labelled nonsense probe (B) or the sequence-specific oligonucleotide probe Sym_Stau_303 (D). Each image pair shows the same field of view containing several gut flagellates, each including a large *Staurojoenina* cell. Epifluorescence photomicrographs were taken with the same filter sets and exposure time to illustrate the specificity of probe Sym_Stau_303 for the epibionts of *Staurojoenina* sp.; the slight autofluorescence present in all flagellates was not caused by the probes. Bars, 50 μm.

completely covered with rod-shaped micro-organisms. Ultrastructure of the epibionts and their attachment sites closely resembles that of the epibionts of *Staurojoenina assimilis* from *Incisitermes minor* (Dolan & Margulis, 1997). The epibionts represent a homogeneous population related to the *Bacteroidales*, where they form a distinct phylogenetic lineage among the 16S rRNA gene clones of other hitherto uncultivated bacteria from the hindguts of various termites, only distantly related to *Tannerella forsythensis* [formerly *Bacteroides forsythus* (Tanner *et al.*, 1986); see also Maiden *et al.* (2003)].

Although the 16S rRNA genes of bacteria related to the *Bacteroidales* are regularly encountered in termite guts (Schultz & Breznak, 1978; Ohkuma & Kudo, 1996; Kudo *et al.*, 1998; Berchtold *et al.*, 1999), most of them belong to lineages containing no cultured representatives (Ohkuma *et al.*, 2002; Hongoh *et al.*, 2003; Schmitt-Wagner *et al.*, 2003). Considering the results of the present study, one reason for these cultivation problems might lie in the symbiotic association with flagellates and a possibly obligate need for a specific host. Interestingly, the epibionts of *Staurojoenina* sp. fall into the same cluster of the *Bacteroidales* as one of the two epibionts of the trichomonad flagellate *Mixotricha paradoxa* from *Mastotermes darwiniensis* (Wenzel *et al.*, 2003).

There are two preliminary reports on the ectosymbionts of the hypermastigid flagellate *Barbulanympha* spp. from the gut of the wood-eating roach *Cryptocercus punctulatus* (Merritt *et al.*, 1996) and a devescovinid flagellate *Caduceia* species from the gut of *Cryptotermes cavifrons* (Goss *et al.*, 2000), which were reportedly identified as members of the *Bacteroides*/*Porphyromonas* subgroup. Although details were never published, it seems likely that many of the *Bacteroides*-related 16S rRNA genes recovered from the guts of lower termites (Ohkuma *et al.*, 2002; Hongoh *et al.*, 2003) represent epibionts of gut flagellates. A larger dataset might allow an excellent case study of co-evolution between the bacterial symbionts and their flagellate hosts.

The function of the epibionts of *Staurojoenina* species is not yet clear, but in view of the enormous numbers of bacteria present on each flagellate, it is suggestive that they play an important role for the host. Cleveland & Grimstone (1964) provided an elegant description of the fascinating motility symbiosis between *Mixotricha paradoxa* and its epibiotic spirochaetes, which were recently identified as members of the *Treponema* cluster by 16S rRNA gene sequence analysis (Wenzel *et al.*, 2003). *Mixotricha paradoxa*, which occurs only in the gut of *Mastotermes darwiniensis*, is propelled by the helical undulations of the spirochaetes adhering to the host membrane through specialized cell junctions. The spirochaetes are attached to projecting brackets of the cell surface in a manner that allows the helical movement of the individual cells to travel in metachronal waves along the cell surface of the host, resulting in locomotion (Cleveland & Grimstone, 1964).

Also the devescovinid flagellate 'Rubberneckia', recently described as *Caduceia versatilis* (d'Ambrosio *et al.*, 1999), is densely colonized by two different bacterial epibionts (Tamm, 1982). In this case, the rod-shaped bacteria (2000–3000 per flagellate) are deeply embedded into the cell surface of the host, which is propelled by the self-synchronizing movement of the bacterial flagella (Tamm, 1982). Although the ultrastructure of the junctional complex is completely different, the morphology of these symbionts resembles that of the epibionts of *Staurojoenina*. This agrees with the preliminary report that *Caduceia* spp. from *Cryptotermes cavifrons* are associated with members of the *Bacteroides*/*Porphyromonas* subgroup (Goss *et al.*, 2000). However, our light-microscopy observation of live preparations of *Staurojoenina* flagellates, which are highly motile and possess four large tufts of eukaryotic flagella (Fig. 1), did not yield any evidence for motility of the attached bacteria, and a motility symbiosis seems unlikely.

It is more feasible that the interactions between epibionts and hosts are of a metabolic nature. Many bacteria among the *Bacteroidales* are polysaccharide-fermenting anaerobes, some of them producing cellulases and other fibre-degrading enzymes, which might complement enzyme activities lacking in the host. Although the large phylogenetic distance to the closest cultivated relative ($<85\%$ sequence identity) allows no safe predictions of the physiological properties of the epibionts, the epibionts might benefit from the reduced products of the flagellate's fermentative metabolism. Again, nothing is known about the fermentation products of *Staurojoenina* species, but there is circumstantial evidence that the flagellates in the hindgut of *Reticulitremes flavipes* form lactate as a major product (Tholen & Brune, 2000). The possibility of a cross feeding of lactate between lactic-acid bacteria and a *Bacteroides* isolate from this termite has been previously demonstrated (Schultz & Breznak, 1979).

Another equally plausible function of the epibionts of benefit to the flagellate host might lie in the observation that phagocytized cells of the epibionts were regularly encountered in digestive vacuoles (see Fig. 1D). Since wood is an extremely nitrogen-poor diet, it may be that the protein-rich bacteria on the cell membrane are an excellent nitrogen source for the host. Although it is not known whether the intestinal flagellates require an organic nitrogen source, the epibionts are likely to assimilate ammonia from the gut fluid, and might even fix dinitrogen (Breznak, 2000). Obviously, more work is necessary to understand the nature of this symbiosis and the respective functions of the symbiotic partners.

Description of '*Candidatus* Vestibaculum illigatum'

Vestibaculum illigatum (Ves.ti.ba'cu.lum. L. fem. n. *vestis* the covering for the body, clothing, L. neut. n. *baculum* a (walking) stick, N.L. neut. n. *vestibaculum* a stick-shaped

part of the body cover, L. perf. part. pass. *illigatum* bound, fastened, attached).

Rod-shaped bacterium of constant diameter (0·3 μm) and variable length (2·5–6 μm). Gram-negative cell-wall structure with outer membrane. Colonizes the cell surface of *Staurojoenina* sp., to which it is connected by electron-dense extracellular material. Basis of assignment: 16S rRNA gene sequence (accession number AY540335), hybridization with 16S rRNA-targeted oligonucleotide probe (5′-CCG GTG TGG GGG ACC TTC-3′). Source: epibiont of *Staurojoenina* flagellates from the gut of the termite *Neotermes cubanus* (Freytaud); so far uncultured.

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