

Review article

The flagellates of the Australian termite *Mastotermes darwiniensis*: Identification of their symbiotic bacteria and cellulases

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Abstract

Termites are among the most important wood- and litter-feeding insects. The gut microbiota plays an indispensable role in the digestion of food. They can include Bacteria, Archaea, flagellates, yeasts, and fungi. The unique flagellates of the termite gut belong to the Preaxostyla (Oxymonadida) and Parabasalia (Cristamonadida, Spirotrichonymphida, Trichomonadida, Trichonymphida), which have branched off very early in the evolution of the eukaryotes. The termite *Mastotermes darwiniensis* is the only species of the most primitive termite family Mastotermitidae. It harbors four large flagellate species in the hindgut: the cristamonads *Koruga bonita*, *Deltotrichonympha nana*, *Deltotrichonympha operculata* and *Mixotricha paradoxa*. Two small flagellate species also thrive in the intestine: the cristamonad *Metadevescovina extranea* and the trichomonad *Pentatrichomonoides scroa*. The flagellates themselves harbor extracellular and intracellular prokaryotes. From cell extracts of the not yet culturable symbiotic flagellates two endoglucanases with a similar apparent molecular mass of approximately 36 kD have been isolated. They exhibited significant homology to cellulases of termite origin. The corresponding genes were detected not in the mRNA pool of the flagellates, but in the salivary glands of *Mastotermes darwiniensis*. These results indicated that the intestinal flagellates of *Mastotermes darwiniensis* also use the termite's cellulases for cellulose hydrolysis. In total extracts of the intestine of wild termites also three cellulases were detected, which should originate from flagellates.

Keywords: Termites, *Mastotermes*, intestinal microbiota, flagellates, cellulases, movement symbiosis

1. The Digestive System of *Mastotermes darwiniensis*

Termites are assigned to 13 families and 282 genera (Myles, 1999). The lower wood-feeding termite *Mastotermes darwiniensis* Froggatt (Fig. 1) is the only living member of the family Mastotermitidae. It is believed to be the most primitive existing termite species (Gay and Calaby, 1970). Today, this species is restricted to Northern Australia, but mastotermitid fossil specimens from the Eocene and Miocene have been found in Central America,

the Caribbean region, Europe and Australia (Thorne et al., 2000). The lower termite *Mastotermes darwiniensis* developed a complex symbiotic hindgut flora, which consists of protozoa (formerly named Archaezoa; Cleveland and Grimstone, 1964; Brugerolle et al., 1994; Berchtold and König, 1995; Fröhlich and König, 1999a,b), Bacteria (Berchtold and König, 1996; Berchtold et al., 1999), Archaea (Fröhlich and König, 1999a,b) and yeasts (Prillinger et al., 1996; Schäfer et al., 1996). Defaunation experiments showed that the protozoa appeared to be essential for the termites' survival (Veivers et al., 1983). In amber, containing the Miocene termite *Mastotermes electrodominicus* a 20-million-year old fossil microbial

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Figure 1. Colony of *Mastotermes darwiniensis*. Soldiers and workers are present. Bar = 1.2 cm.

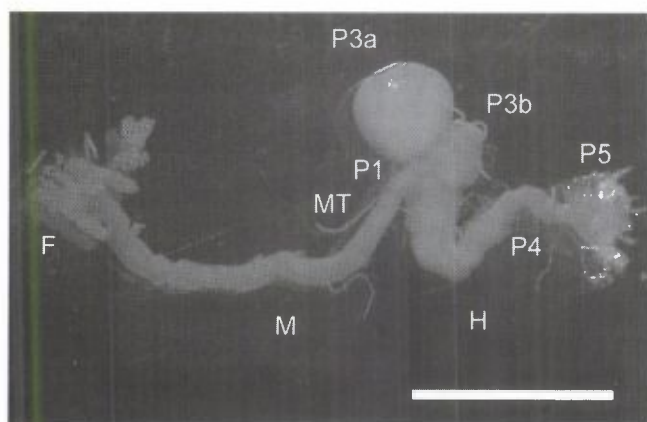


Figure 2. Light micrograph of the gut of *Mastotermes darwiniensis*. F = foregut, M = midgut, H = hindgut, MT = malpighian tubules, P1 = proctodeal segment, followed by the enteric valve (P2, not marked), P3a = thin-walled part of the paunch, P3b = thick-walled part of the paunch, P4 = colon, P5 = rectum. Bar = 4 mm.

community consisting already of protists, spirochetes, and other bacteria has been observed (Wier et al., 2002).

The digestive system of *Mastotermes darwiniensis* consists of the foregut with the crop and the gizzard, the midgut, and the hindgut (Noirot and Noirot-Timotheé, 1969; Noirot, 1995). The hindgut consists of five segments (P1–P5): the proctodeal segment, the enteric valve, the paunch, the colon and the rectum. The paunch is the main microbial fermentation chamber, but the colon also contains microorganisms. The first two hindgut segments P1 and P2 are very small. P3, the paunch, is subdivided into a dilated, thin-walled region (P3a) and a thick-walled, more tubular region (P3b), which is followed by a thick-walled, tubular colon (P4) and the rectum (P5) (Fig. 2). The paunch could be described as an anaerobic gradient system, which is constantly supplied with oxygen. In the case of

Mastotermes darwiniensis oxygen diffusion gradients could be detected up to 100 μm below the epithelium (Berchtold et al., 1999).

2. Handling of Single Microorganisms

A prerequisite for the biochemical and physiological investigation of microorganisms is the isolation and management of pure cultures. The principal procedures for obtaining pure cultures of bacterial strains have not been largely improved since Robert Koch. The isolation according to conventional methods, like the separation of microorganisms by agar plates (Koch, 1881) and agar shake tubes (Pfennig and Trüper, 1981) cannot avoid that individual colonies are formed by cell aggregates. More sophisticated electronic enumeration and sampling systems such as Coulter Counter or flow cytometry can not prevent the formation of cell aggregates. In addition to the isolation of single cells with optical tweezers (Huber, 1999) the "Bactotip" and "Membrane"-method are alternative approaches for the micromanipulation of individual microorganisms. With the aid of these methods single cells can be picked out of a mixed population under direct visual control. The isolated aerobic or anaerobic species can be grown in pure culture or can be subjected to single cell PCR (Fröhlich and König, 1999a, 2000; Prescott et al., 2002; Fröhlich et al., 2002).

Since the sixties the technical equipment of micromanipulators has been greatly improved. A long distance objective (Zeiss, Oberkochen, Germany) with a magnification of hundredfold is now available. This allows manipulation at a magnification of a thousandfold and more with an inverse microscope. The capillary tools can be positioned quickly and precisely. The available pneumatic or hydraulic systems are very accurate pressure devices.

For the isolation of single microbial cells a commercial micromanipulator (Eppendorf model 5171; Fig. 3) equipped with a pressure device (Eppendorf model 5246 plus or CellTram Oil) and mounted onto an inverse phase contrast microscope (Axiovert 25; objective CP "Achromat" 100x/1.25 Oil Ph2; Zeiss) is used (Bactotip method, Figs. 3A,C) (Fröhlich and König, 1999a,b). The magnification is adjusted from 400-fold to 1000-fold. The micromanipulator is used according to manufacturer's instructions (micromanipulator 5171: Operating Manual; Cell Tram Oil: Operating Manual, Transjector 5246: Operating Manual; Eppendorf, Hamburg, Germany). The diameter of the opening of the capillary tip can be adjusted to the size of the bacterial cell of interest. For the isolation of bacteria a sterile capillary tube ("Bactotip"; Figs. 3A,B,D,E) is used, which preferably possesses a bevelled tip (angle 45°) usually with an opening of about 5–10 μm at the anterior end. The sterile Bactotips can be manufactured with a capillary-puller (Saur, Reutlingen, Germany) and a micro-

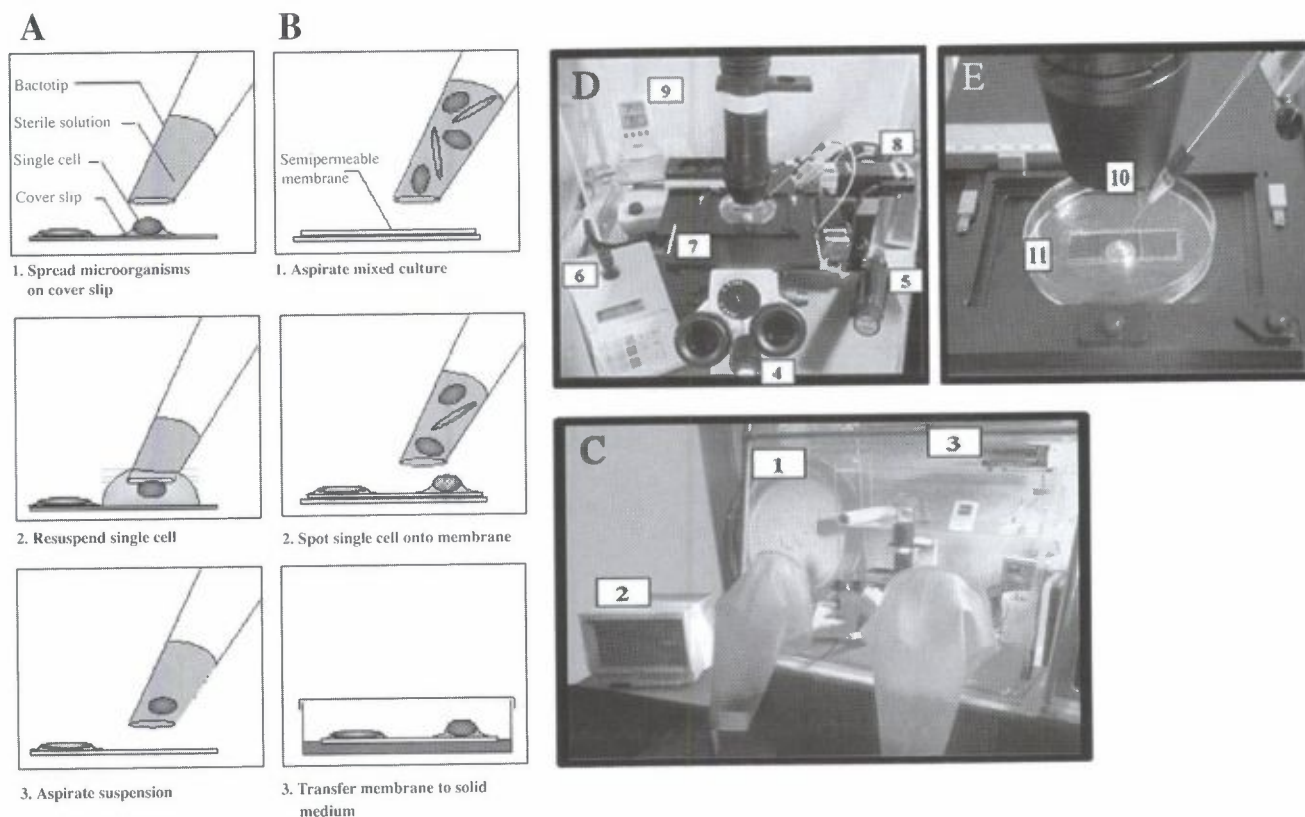


Figure 3. Isolation of single prokaryotic cells. Schematic drawings of the (A) Bactotip- and (B) Membrane-methods. Working station for the manipulation of single cells with a COY-chamber (C), the inverse microscope and a micromanipulator device (D), and the capillary tube (E). (1) COY-chamber, (2) monitor, (3) O₂/H₂-electrode, (4) camera, (5) Cell-Tram Oil, (6) joystick, (7) inverse microscope, (8) micromanipulator, (9) thermometer/hygrometer, (10) Bactotip, (11) cover slip with spread bacteria.

grinder (Saur, Reutlingen) using capillary tubes type GB 100 TF-8P (Science Products GmbH, Hofheim, Germany). The posterior end of the Bactotip is sealed with a droplet of sterile oil. If desired, the inner surface of the tip can be siliconized with dichlorodimethylsilane (Fluka Chemie AG, Buchs, Switzerland). This is advisable, if the bacteria tend to adhere to glass surfaces. Desiccation and oxygen stress (Krämer, 1997) for the isolation of anaerobic and aerobic microorganisms can be avoided by using a glove box with a N₂/H₂ (95:5 v/v) atmosphere (COY chamber, Toepfer Lab Systems, Göppingen, Germany; Fig. 3C). The relative humidity in the chamber is adjusted from 95% to 100%. The microscope bulb is replaced by an optical fibre device (Schott, Mainz, Germany) which reduces the IR-radiation. The microscope is equipped with a CCD camera (Type AVTBC12CE (Zeiss) and a monitor (Type PM 95 B, Zeiss; Fig. 3C).

In contrast to the Bactotip-method, an appropriate dilution (ca. 0.1 µl) of a mixed culture is sucked into the capillary tube (Fig. 3B). The tip is brought close to the surface of a semi permeable membrane (dialysis hose; Roth) and single cells are spotted under visual control on

the membrane in a distance of 5 to 10 mm to each other. Subsequently, the membrane will be removed with sterile tweezers and transferred onto a solid medium. Nutrients diffuse through the membrane and enable individual cells to grow up to colonies. The use of a dialysis membrane has the advantage of a very smooth surface compared to the application of agar-layers, so that very small microorganisms can be separated without limitation of the visual control.

Ashkin et al. (1987) described the use of infrared laser beams (1064 nm) for trapping and manipulation of biological specimens such as the single cells of *Escherichia coli* and *Saccharomyces cerevisiae*. This method was improved and successfully applied for the isolation of hyperthermophilic Bacteria and Archaea (Huber, 1999; Huber et al., 1995). A neodymium-laser is focused by a microscope objective. The movement of the microscope stage is computer-controlled. A rectangular glass capillary with a predetermined breaking point is used as separation chamber (inside dimensions: 0.1 mm × 1 mm, length: 10 cm), which is filled with fresh sterile medium (90%) and the mixed microbial population (10%). A single selected

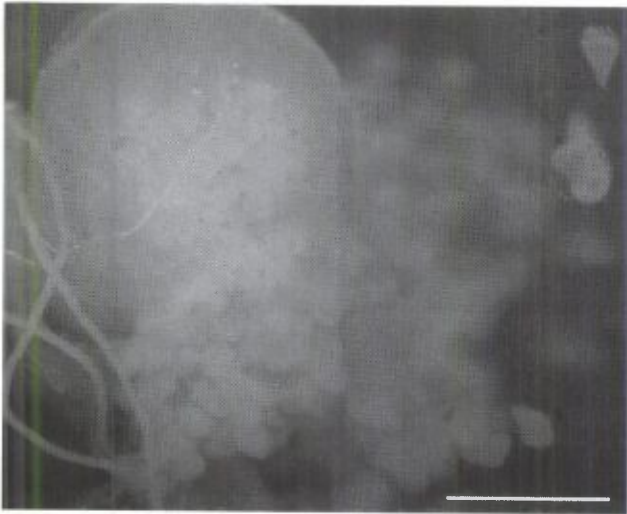


Figure 4. Light micrograph of the paunch of *Mastotermes darwiniensis*. Slashed paunch (P3 region) showing the dense flagellate population. Bar = 1 mm.

Table 1. Intestinal flagellates of *Mastotermes darwiniensis*.

Species	Length (μm)	Titer (mL^{-1})
Cristamonads ¹⁾ (<i>Deltotrichonympha nana</i> , <i>Deltotrichonympha operculata</i> , <i>Koruga bonita</i>)	100–550	10^4 – 10^5
<i>Mixotricha paradoxa</i>	300–500	10^3 – 10^4
<i>Metadevescovina extranea</i>	15–20	ca. 10^7
Trichomonads <i>Pentatrichomonoides scroa</i>	25	ca. 5×10^6

¹⁾Combined titer of the three larger cristamonads.

cell is fixed with the laser beam and is separated from the mixed culture by moving the microscope stage. The capillary is broken at the predetermined breaking point and the single cell is transferred to the culture medium. The culture efficiency after an incubation time of up to 5 days was 20–100%. The isolation of dead cells could be prevented by application of fluorescent dyes staining viable cells for example with bis-(1,3-dibutylbarbituric acid) trimethine oxonol (Beck and Huber, 1997). Photo damage can be reduced to background level under anaerobic conditions (Neumann et al., 1999). This method is a promising tool for the isolation of microorganisms, which cannot be obtained in pure culture by conventional methods.

Meanwhile, a great variety of different microorganisms have been isolated by these methods. The application of both techniques is limited, when larger highly motile spirochetes should be isolated.

Table 2. Distinguishing features of the intestinal flagellates of *Mastotermes darwiniensis*.

A. Length above 50 μm

I. Anterior part is covered by a coat of spirochetes propelling the cells (movement symbiosis), four anterior flagella, size of the nucleus: ca. $33 \mu\text{m} \times 16 \mu\text{m}$, the nucleus can be located in the papilla or the cell body¹⁾. Length: 500 μm , width: 250 μm ²⁾.

Mixotricha paradoxa.

II. Peritrichous flagellation of the anterior cell part.

1. Dense coat of bacteria¹⁾ at the posterior end, size of the nucleus¹⁾: ca. $58 \mu\text{m} \times 25 \mu\text{m}$; length: 300 μm , width: 65–100 μm ³⁾; length: >400 μm ¹⁾. Appearance of *Koruga bonita* is more slender than that of *Deltotrichonympha*.

Koruga bonita.

2. No dense coat of bacteria at the posterior end¹⁾.

Deltotrichonympha nana (length: 300–360 μm , width: 188 μm ; size of the nucleus: ca. $41 \mu\text{m} \times 66 \mu\text{m}$)¹⁾ (length: 289 μm , size of the nucleus: ca. $26 \mu\text{m} \times 42 \mu\text{m}$)⁴⁾.

Deltotrichonympha operculata (length: 450–550 μm , width: 350–450 μm , size of the nucleus: $36 \mu\text{m} \times 57 \mu\text{m}$)⁴⁾.

D. nana and *D. operculata* can not be distinguished easily. Except for being less than half the size *D. nana* differs too little from *D. operculata* to warrant a detailed description⁴⁾. *Deltotrichonympha* sp. and *Koruga bonita* can also occur in a amoeboid stage.

B. Length below 50 μm

I. Five free anterior flagella, one recurrent flagellum, undulating membrane, striated costa present, axostyl does not project at the posterior cell end, cresta absent.

Pentatrichomonoides scroa.

II. Three anterior flagella, one trailing flagellum, cresta present, axostyl projects at the posterior cell end, costa absent.

Metadevescovina extranea.

¹⁾Fröhlich and König, 1999a, cf. Fig. 7; ²⁾Cleveland, 1966c;

³⁾Cleveland, 1966b; ⁴⁾Cleveland, 1966a.

3. The Intestinal Flagellates of *Mastotermes darwiniensis*

Six species of parabasalid flagellates (cristamonads and trichomonads) inhabit the gut of *Mastotermes darwiniensis* (Tables 1 and 2). The flagellates preferentially colonize the paunch (P3 region; Figs. 2 and 4), while low numbers are found in the colon (P4 region) (Berchtold et al., 1999). About 95% of the anterior part of the paunch (P3a region) of *Mastotermes darwiniensis* is tightly packed with large flagellates (1285 ± 244 cells of *Deltotrichonympha operculata*, *Deltotrichonympha nana*, *Koruga bonita*, and *Mixotricha paradoxa* per termite; Fig. 4; Table 1) (Berchtold et al., 1999; cf. König et al., 2002, 2005). Diameter and length of the hindgut compartments (P3a, P3b, P4, colon) and cells of the flagellates as well as their number were determined microscopically using an ocular micrometer. For the determination of the surface area and volume P3a was approximated by a sphere, P3b/P4 by a

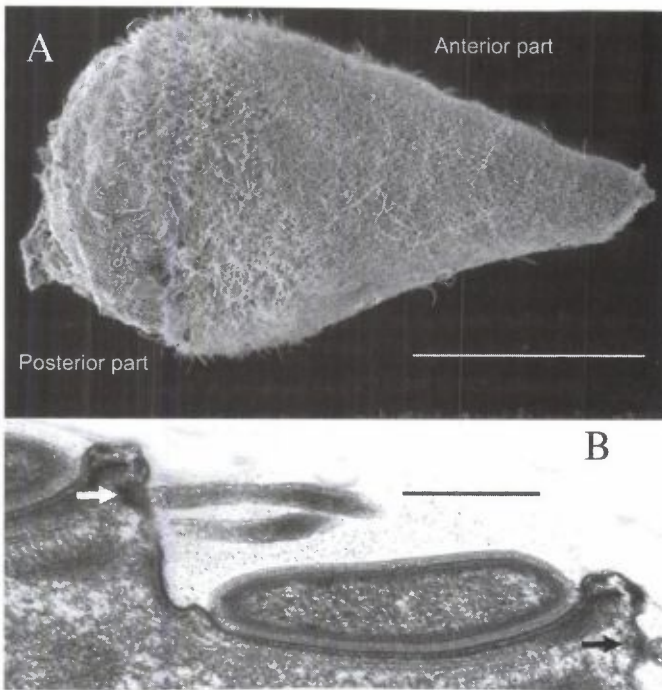


Figure 5. Electron micrograph of the larger cristamonad *Mixotricha paradoxa*. A. Scanning electron micrograph of a whole cell. Bar = 100 µm. B. Thin section showing the surface area of a flagellate: the brackets, a rod-shaped cell between two brackets and the attachment sites of the spirochetes (arrows) at the posterior part of the brackets. Bar = 500 nm.

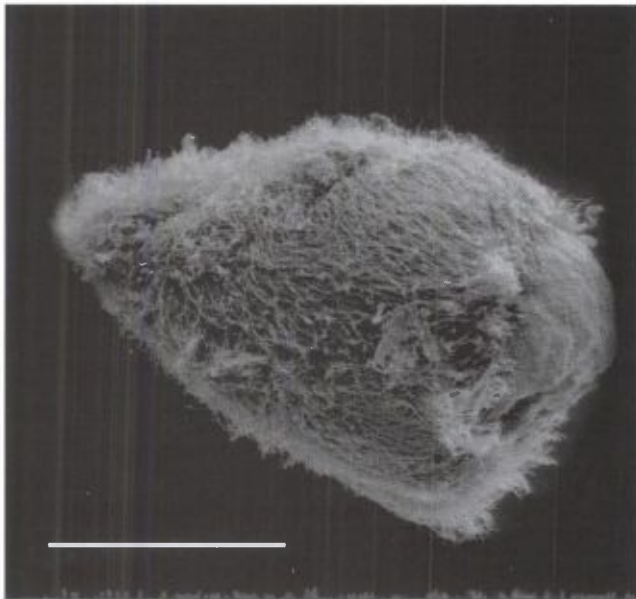


Figure 6. Scanning electron micrograph of the cristamonad *Deltotrichonympha nana*. Bar = 150 µm.

cylinder and the larger flagellates as a cone attached to a hemisphere (Berchtold et al., 1999). Approximately 90% of the DAPI-stained bacterial cells were associated with the protozoa in the P3a region, only 2% were attached to the

gut wall and the rest were found in the residual liquid volume of the lumen fraction. The potentially colonizable surface area provided by the flagellates in the P3a region exceeds that of the wall by a factor of 18. In contrast to the P3a region, about 85% of the prokaryotes of the P3b region are attached to the wall. Besides the larger flagellates the two smaller species *Metadevescovina extranea* and *Pentatrichomonoides scroa* thrive also in the gut of *Mastotermes darwiniensis*.

4. Morphology of Intestinal Flagellates

One of the larger flagellates in the hindgut of *Mastotermes darwiniensis* is *Mixotricha paradoxa*, a member of the Devescovinidae (cristamonads) (Adl et al., 2005) (Tables 1 and 2). The pear-shaped cells are about 500 µm long and 250 µm in diameter (Fig. 5). They ingest wood particles. The surface of *Mixotricha paradoxa* shows a highly ordered pattern of rod shaped bacteria and in addition it is covered by a dense carpet of spirochetes with exception of the posterior ingestive zone (Cleveland and Grimstone, 1964; Cleveland and Cleveland, 1966; Wenzel et al., 2003; Brugerolle, 2004). The rod shaped bacteria and the spirochetes are attached to regularly arranged protrusions (brackets) of the cell surface. Interestingly, Cleveland and Grimstone (1964) found that the spirochetes and not the relatively small four flagella propel the cells. It is still unknown how the flagellates and the spirochetes communicate and coordinate the direction of movement. For hydro-mechanical reasons it seems that cilia, flagella, sperm tails and spirochetes should automatically synchronize their movement when undulating in close proximity (Machin, 1963). Brugerolle (2004) also described the fine-structure of the cytoskeleton and the organisation of the prokaryote-eukaryote cell junctions. So far, it is not possible to cultivate neither *Mixotricha paradoxa* nor its ectosymbiotic spirochetes and rod-shaped bacteria.

An other examples of symbiotic cristamonad flagellates are *Deltotrichonympha operculata*, *Deltotrichonympha nana* (Figs. 6 and 7; Table 2) and *Koruga bonita* (Fig. 7). The cells of *Deltotrichonympha operculata* and *Deltotrichonympha nana* are characterized by numerous flagella, which cover the anterior part of a cell. At the posterior part the ingestive zone is located. In contrast to *Deltotrichonympha* sp., *Koruga bonita* possesses no dense population of ectosymbiotic spirochetes at the posterior cell part (Fröhlich and König, 1999a).

One of the smaller symbiotic trichomonad flagellates of *Mastotermes darwiniensis* is *Pentatrichomonoides scroa* (Fig. 8). This species is characterized by the presence of 5 anterior flagella originating from a groove and an un-

Figure 7. See page 59.

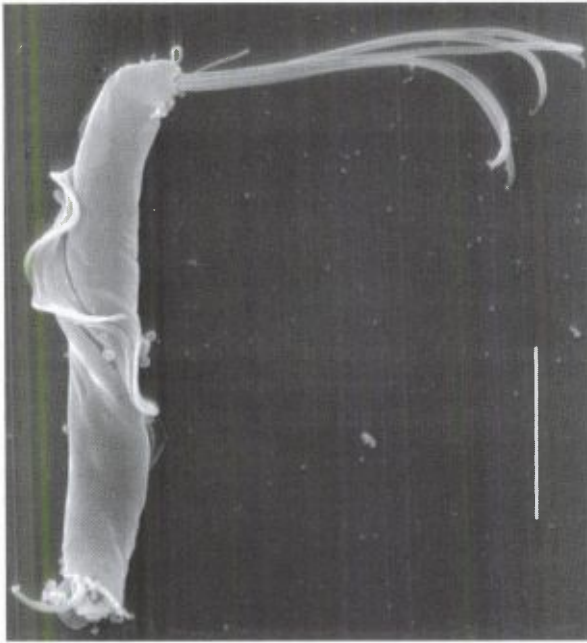


Figure 8. Scanning electron micrograph of the smaller trichomonad *Pentatrichomonoides scroa*. Bar = 5 μm .



Figure 9. Light micrograph of the small cristamonad *Metadevescovina extranea*. The three anterior flagella and the protruding axostyle are visible but not the trailing flagellum. Differential interference contrast. Bar = 10 μm .

dulating membrane with a recurrent flagellum. The cell body is generally slender with a truncated posterior end and a spiralling undulating membrane around the cell body, but

also more massive forms exist. The slender forms have a length of about 30 μm and a width of 5 μm . The five flagella beat synchronously (Brugerolle et al., 1994). About 50 methanogens of the genus *Methanobrevibacter* were found in *Pentatrichomonoides scroa* (Fröhlich and König, 1999a). The other smaller flagellate *Metadevescovina extranea* possesses three anterior flagella and a trailing flagellum (Fig. 9).

5. The Phylogenetic Position of the Intestinal Flagellates of *Mastotermes darwiniensis*

Fig. 10 shows a phylogenetic tree of parabasalids (König et al., 2005). The phylogenetic tree shows that four hindgut protozoa of *Mastotermes darwiniensis* form one subdivision: the small flagellate *Metadevescovina extranea* and the three large flagellates *Koruga bonita*, *Deltotrichonympha nana* and *Deltotrichonympha operculata*. They form a monophyletic group with a quite high bootstrap support. Parallel to this subdivision, *Mixotricha paradoxa* exhibits an earlier emergence, indicating its primitive position among the intestinal flagellates of *Mastotermes darwiniensis* and among the family Devescovinidae. *Foaina* has been proposed to be a primitive genus among the Devescovinidae and Calonymphidae (Gerbod et al., 2002). *Koruga bonita*, *Deltotrichonympha nana* and *Deltotrichonympha operculata* classified in the Deltotrichonymphidae. This indicates that the hypermastigid flagellates are not a monophyletic branch (Fröhlich and König, 1999b) and that the hypermastigont system was invented several times (e.g. Deltotrichonymphidae, Trichonymphidae) in the evolution of the flagellates (Fig. 10). *Pentatrichomonoides scroa*, the second small intestinal flagellate of *Mastotermes darwiniensis* is not closely related to the other five symbiotic flagellates, because it clusters within the Trichomonadinae.

6. Endosymbiotic Archaea of *Pentatrichomonoides scroa*

While the small wood feeding flagellate *Metadevescovina extranea* seems to harbor a few intracellular bacteria *Pentatrichomonoides scroa* (Fig. 7) always possesses prokaryotes in the cytoplasm (Fröhlich and König, 1999a,b). The trichomonad *Pentatrichomonoides scroa* (Brugerolle et al., 1994; Berchtold and König, 1995), one of the smaller gut flagellates of *Mastotermes darwiniensis*, harbours about 50 endosymbiotic methanogens, which are recognized by their greenish fluorescence under ultraviolet irradiation (Fröhlich and König, 1999a). Ten single cells of the endosymbiotic methanogens were isolated by micromanipulation and subjected to SSU rDNA sequence analysis (Fröhlich and König, 1999b). The endosymbiont of *Pentatrichomonoides*

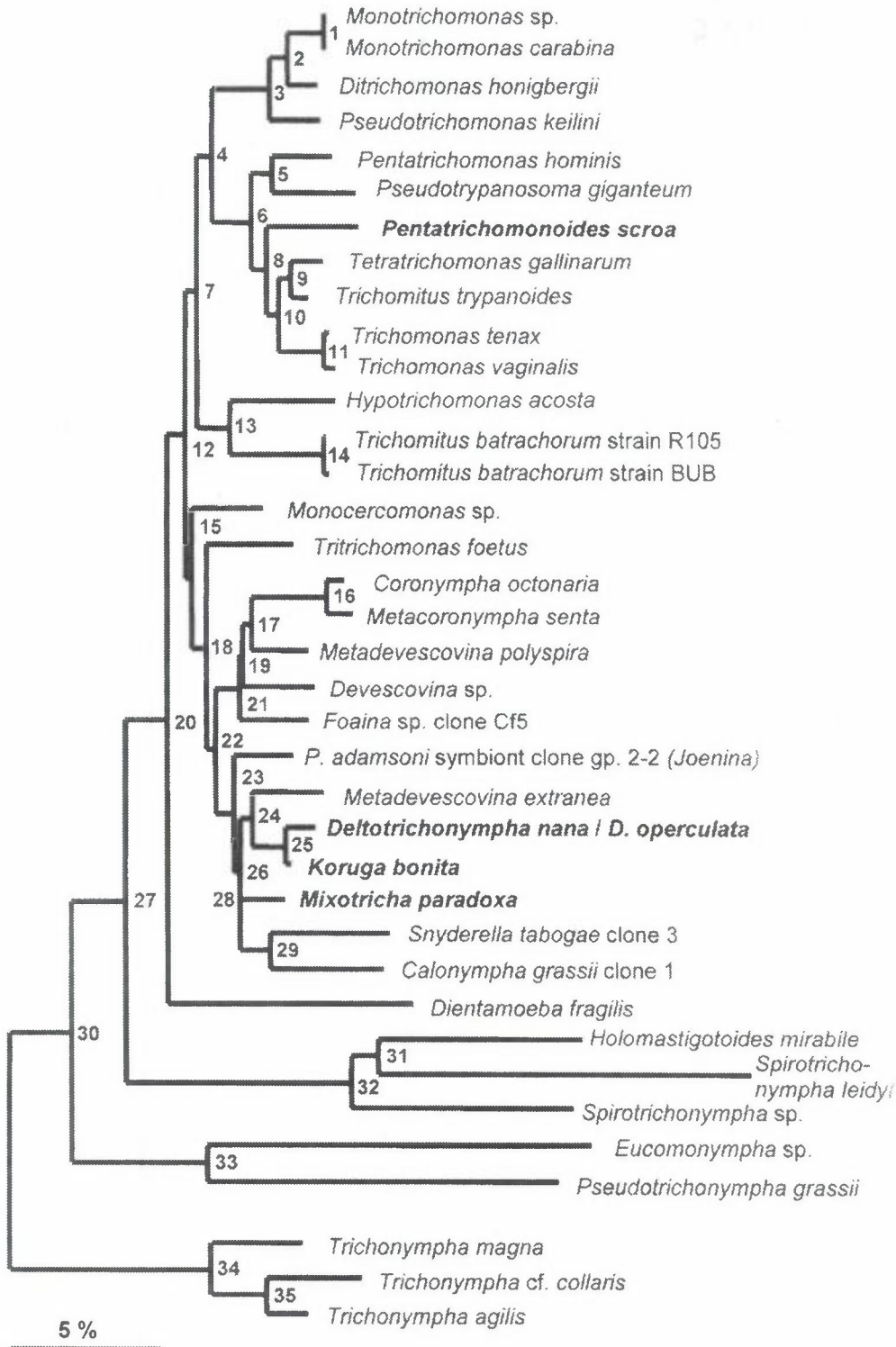


Figure 10. Unrooted phylogenetic tree of the symbiotic parabasalids of *Mastotermes darwiniensis*. Neighbor-joining analysis of SSU rDNA sequences. Bar represents 5 substitutions per 100 nucleotides. The bootstrap values are computed by three different reconstruction methods: distance matrix, maximum parsimony and maximum likelihood. Asterisks designate nodes with bootstrap values below 40% (Li et al., 2003). Bootstrap values: 1 = 100/100/100; 2 = 88/97/92; 3 = 100/100/96; 4 = 64/62/50; 5 = 98/97/98; 6 = 100/99/99; 7 = 88*/43; 8 = 86/80/*; 9 = 79/*/*; 10 = 93/50/*; 11 = 100/100/100; 12 = 54/*/*; 13 = 90/100/92; 14 = 100/100/100; 15 = 49/43/*; 16 = 100/100/100; 17 = 76/43/*; 18 = 83/*/*; 19 = 44/67/*; 20 = 72/45/*; 21 = 89/84/56; 22 = 75/45/*; 23 = 59/58/40; 24 = 65/69/63; 25 = 100/100/99; 26 = 42/71/ 58; 27 = 100/84/*; 28 = 100/48/48; 29 = 100/94/95; 30 = 88/98/100; 31 = 92/83/92; 32 = 100/100/100; 33 = 100/100/100; 34 = 100/100/100; 35 = 100/100/99.

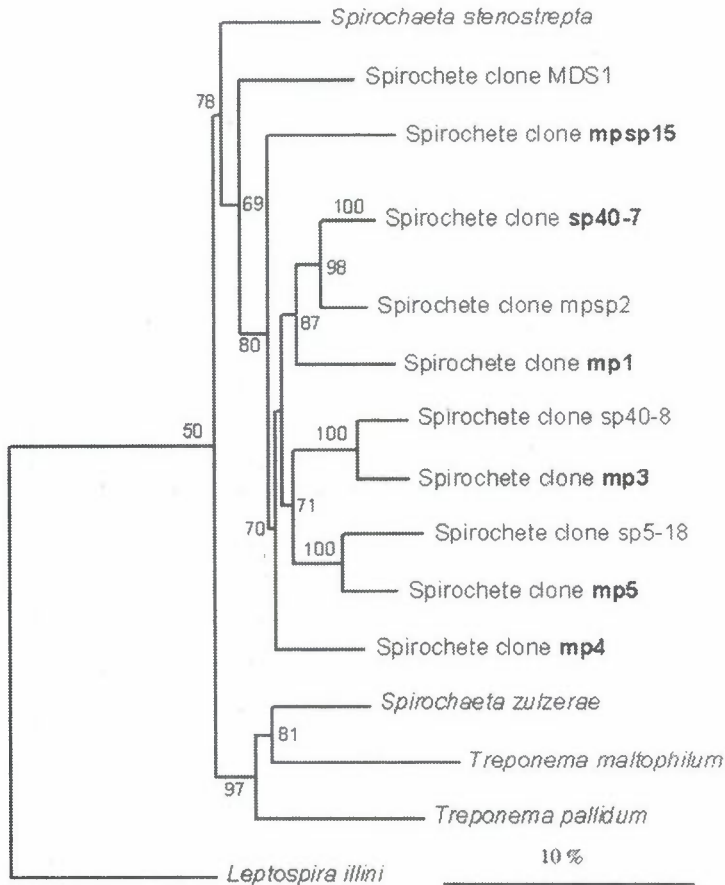


Figure 11. Phylogenetic relationship of *Mixotricha paradoxa*-associated spirochete clones. The relationship was determined by neighbor joining analysis. The data set contained 15 alignment positions and *Leptospira illini* as outgroup. The bootstrap values (100 runs) obtained by using the programme SEQBOOT are inserted at the respective branching points. Bootstrap values under 50% were not shown. Bar represents 10 substitutions per 100 nucleotides.

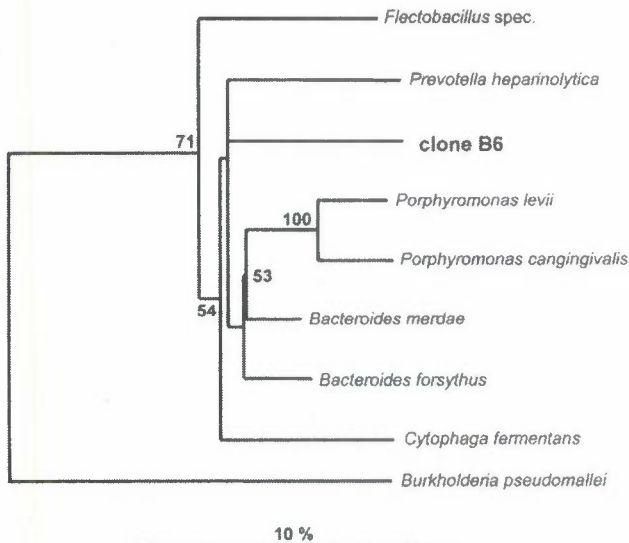


Figure 12. Phylogenetic relationship of rod-shaped clone B6. 16S rDNA sequences were obtained from the EMBL-database (Stoesser et al., 2001). The relationship was determined by neighbor joining analysis. The data set contained 9 alignment positions and *Burkholderia pseudomallei* as outgroup. The bootstrap values (100 runs) obtained by using the programme SEQBOOT inserted at the respective branching points. Bootstrap values under 50% were not shown. Bar represents 10 substitutions per 100 nucleotides.

scroa (*Methanobrevibacter* sp.; AJ132468) was related to clone CD 3 from the termite *Cryptotermes domesticus* (Ohkuma and Kudo, 1998) and *Methanobrevibacter curvatus* (Leadbetter and Breznak, 1996), which was obtained from the gut epithelium of *Reticulitermes flavipes*. Members of *Methanobrevibacter*, especially *Methanobrevibacter smithii*, are widespread in the intestine of humans and animals (Miller and Wolin, 1986).

7. Intracellular Mycoplasmas of *Koruga bonita*

Bacterial cells were aspirated from the cytoplasm of the flagellate *Koruga bonita* (Fig. 7) by using a glass capillary with an opening of 0.5 μm in diameter. Partial sequencing of the 16S rRNA gene revealed that the micromanipulated cells belonged to the genus *Mycoplasma* (*Mycoplasma* sp.; AJ132469) (Fröhlich and König, 1999b). The nearest neighbour was *Mycoplasma alvi* (Pettersson et al., 1996).

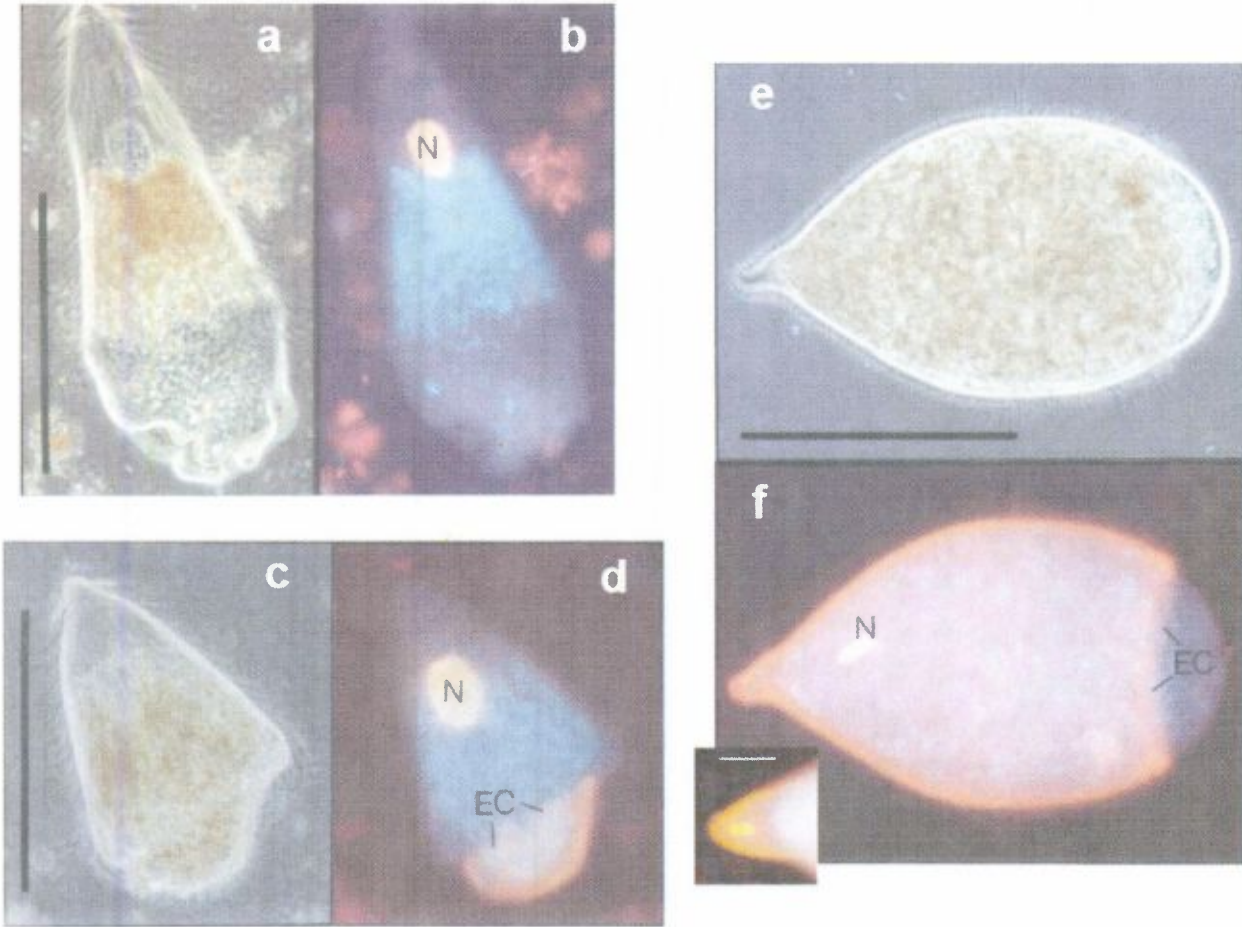


Figure 7. Light micrographs of the larger cristamonads of *Mastotermes darwiniensis*. *Koruga bonita* (a, b), *Deltotrichonympha* sp. (c, d), *Mixotricha paradoxa* (e, f). Phase contrast microscopy (a, c, e), epifluorescence microscopy (ethidium bromide staining; b, d, f). Red layers indicate a dense layer of ectosymbiotic bacteria (d, f). N = nucleus, EC = ectosymbiotic bacteria. Inset (f): the nucleus can also be located in the papilla in some cell stages of *Mixotricha paradoxa*. Bar = 250 μ m (inset: 50 μ m).

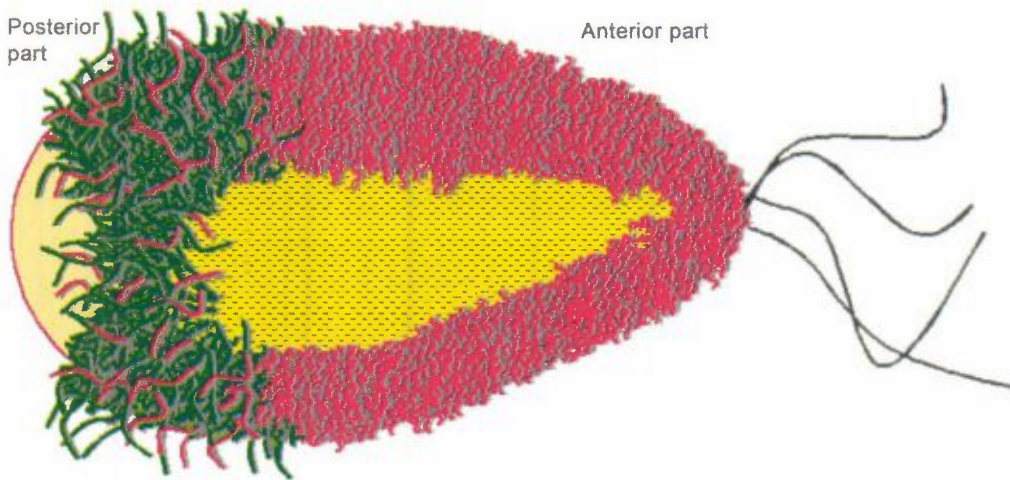


Figure 13. Schematic drawing showing the proposed distribution of the bacterial ectosymbionts on the cell surface of *Mixotricha paradoxa*.
 Spirochete clone mpsp 15
 Spirochete clone mp1
 Spirochete clone mp3
 Rod-shaped clone B6



8. The Spirochetal Population of *Mastotermes darwiniensis*

Spirochetes possess a cellular ultrastructure which is unique among eubacteria (Canale-Parola, 1991; Holt, 1978). The helical protoplasmic cylinder is encased by an outer envelope, which has some features analogous to the outer membrane of gram-negative bacteria. The spirochetes possess internal organelles of motility called periplasmic flagella, which are located between the protoplasmic cylinder and the outer envelope (Paster et al., 1996). Previously, eleven spirochetal 16S rDNA sequences with similarity values of 81% to 99% to each other originating from the hindgut of *Mixotricha darwiniensis* were published (Berchtold et al., 1994; Berchtold and König, 1996; Wenzel et al., 2003). These clones also belong to a site branch of *Treponema* (Berchtold et al., 1994; Berchtold and König, 1996; Lilburn et al., 1999; Ohkuma et al., 1999). Although spirochetes are always a dominant part of the microflora of all termites (Margulis and Hinkle, 1992), only five species has been obtained in pure culture (Leadbetter et al., 1999; Dröge et al., 2006a,b). The so far identified spirochetal clones cluster with the genera *Treponema* (Berchtold et al., 1994; Berchtold and König, 1996; Lilburn et al., 1999; Ohkuma et al., 1999) as well as with *Spirochaeta* (Dröge et al., 2006a).

9. Ectosymbiotic Spirochetes and Rods of *Mixotricha paradoxa*

One of the first detected symbioses between flagellates (*Pseudodevescovina uniflagellata*) and spirochetes was described by Kirby (1936). *Pseudodevescovina uniflagellata* lives in the gut of the Australian dry wood termite *Neotermes insularis*. Only three years earlier Sutherland (1933) published an article about *Mixotricha paradoxa* where the attached spirochetes were misconceived as cilia. A detailed description of the fine structure of *Mixotricha paradoxa* and the role of the ectosymbiotic bacteria in cell locomotion was provided by Cleveland and Grimstone (1964) as well as by Brugerolle (2004). Over the years more and more examples of surface symbiosis between protists and prokaryotes from the termite gut appeared (Ball, 1969; Bloodgood and Fitzharris, 1976; Dyer and Khalsa, 1993; Smith et al., 1975; To et al., 1980), but examples of motility symbiosis in the termite gut could be rarely detected (Tamm, 1982). Locomotory mechanisms of two larger flagellates from *Mastotermes darwiniensis* have been studied (Cleveland and Cleveland, 1966; Tamm, 1999).

Ectosymbiotic bacteria of flagellates can easily be detected by electron microscopy (Radek et al., 1992; Dyer and Khalsa, 1993; Radek and Tischendorf, 1999; Radek et al., 1996; Brugerolle, 2004) or after staining the cells with

ethidium bromide (Fröhlich and König, 1999a). Ectosymbiotic spirochetes have been identified on the surface of flagellates (Iida et al., 2000; Noda et al., 2003; Wenzel et al., 2003). *Mixotricha paradoxa* is a rare example of a movement symbiosis between eukaryotic and prokaryotic microorganisms (Wenzel et al., 2003; Brugerolle, 2004).

Cleveland and Grimstone (1964) found two spirochete morphotypes on the surface of *Mixotricha paradoxa*, a small one, which covered the surface of the flagellate as a dense carpet and a longer spirochete, which only appeared sporadically. Sometimes, this longer spirochete could also be seen on the anterior part of the cell. It is only loosely bound to the spirochete carpet. Cleveland and Grimstone (1964) described the regular arrangement of the spirochetes and a rod-shaped bacterium attached to the so-called brackets on the cell surface. These brackets seem to be significant for the locomotory function of the spirochetes. They form a regularly posteriorly oriented attachment site for the spirochetes, which allows the spirochetes to propel the flagellate cells forward. The rod shaped-bacteria, which are attached to the anterior site of the brackets, have no part in the locomotion of *Mixotricha paradoxa* (Cleveland and Grimstone, 1964; König and Breunig, 1997; Fig. 5).

Wier et al. (2001) described a pillotinaeous spirochete (*Canaleparolina darwiniensis*) from the surface of *Mixotricha paradoxa*. The spirochete occurs also free-swimming in the paunch lumen of *Mastotermes darwiniensis*. This species possesses 16 periplasmic flagella (16:32:16). Brugerolle (2004) described in addition to the slender spirochetes a larger spirochete with a length of 30 μm and a diameter of 0.5 μm . This spirochete possessed 18–30 flagella arranged in two rows which are located in a ridge on the surface. The morphology is similar to that of the described genus *Canaleparolina*. In addition two as yet undescribed rods (length of about 4 μm , diameter of about 0.5 μm) abundant in the posterior part of the flagellate were observed.

10. Identification of the Ectosymbiotic Spirochetes of *Mixotricha paradoxa*

Berchtold and König (1996) found about 13 different spirochetal 16S rDNA clones in the intestinal tract of *Mastotermes darwiniensis*. Spirochetes constitute a main part of the gut flora of termites (Berchtold et al., 1994; Berchtold and König, 1996). Their function in the gut of termites remained unclear for a long time. Today we know that they ferment mono- and oligosaccharides, form acetate from CO_2 and H_2 and fulfil a locomotory function (Cleveland and Grimstone, 1964; Cleveland and Cleveland, 1966; Leadbetter et al., 1999; Wenzel et al., 2003; Brugerolle, 2004; Dröge et al., 2006a,b).

The bacteria associated with the cell surface of

Mixotricha paradoxa were identified. Six spirochetal 16S rDNA clones (mpsp 15, sp 40-7, mp1, mp3, mp4, mp5) were obtained from the bacteria on the cell envelope of *Mixotricha paradoxa* (Wenzel et al., 2003). Two clones (mpsp15, sp 40-7) were nearly identical (99%) to already described clones (Berchtold et al., 1994; Berchtold and König, 1996), while the 16S rDNA sequences of clones mp1, mp3, mp4 and mp5 have not been found previously.

The "spirochete" tree including the 16S rDNA sequences of the six spirochete clones mpsp15, sp 40-7, mp1, mp3, mp4, and mp5 obtained from the ectosymbiotic bacteria together with some representative spirochetes from the EMBL-database (Berchtold and König, 1995) was constructed. The phylogenetic tree (Fig. 11) shows that the ectosymbiotic spirochetes of *Mixotricha paradoxa* belong all to the *Treponema* branch (Berchtold et al., 1994; Berchtold and König, 1996; Paster et al., 1996; Lilburn et al., 1999; Ohkuma et al., 1999; Iida et al., 2000).

11. Identification of an Ectosymbiotic Rod-shaped Bacterium of *Mixotricha paradoxa*

Wenzel et al. (2003) obtained 16S rDNA amplicates (e.g. clone B6; ca. 400 bp) from *Mixotricha paradoxa*, which were related to *Bacteroides forsythus*. The *Bacteroides forsythus*-related clone B6 and some of its phylogenetic relatives were used to construct the "*Bacteroides*" tree. Fig. 12 shows the phylogenetic relationship of clone B6 to representatives of *Bacteroides*-related species. This indicates that the epibiotic rod-shaped bacterium on the surface of *Mixotricha paradoxa* is a member of the *Bacteroides*-branch with probably *Bacteroides forsythus* as closest described species. The obtained sequence showed similarity to the uncultured clones Gf15 [AB055715; 92% in 857 aligned bp] and Gf16 [AB055716; 92% in 855 aligned bp] from *Glyptotermes fuscus* and *Bacteroides* cf. *forsythus* oral clone BUO63 [AY008308; 94% in 495 bp]. The low phylogenetic relationship indicated that clone B6 originated from a new species.

12. Assignment of 16S rDNA sequences to the Corresponding Ectosymbiotic Bacterial Morphotypes

By using cell envelope preparations for fluorescence *in situ* hybridization an interference with the fluorescence of wood particles was avoided. In addition, the fluorescence signals of the specific Cy3-labelled probes could be enhanced by applying helper oligonucleotides (Fuchs et al., 2000) and the binding of the probes was facilitated by performing a denaturing step.

Fluorescence *in situ* hybridization was performed with specific Cy3-labelled probes derived from 16S rDNA

sequences obtained from the ectosymbiotic spirochetes and rod-shaped bacterium clone B6. Three spirochetal clones could be localized on the cell surface, clone mpsp15 at the anterior and clones mp1 and mp3 at the posterior part. Clone mp1 occurred in a lower number than clone mp3 (Fig. 13). The spirochetal clones which could not be localized on the cell surface form probably no dense population on the cell surface and their weak fluorescence signals might have been overlooked. Cleveland and Grimstone (1964) described only two morphotypes of spirochetes, shorter ones which were attached to the brackets and longer ones which only appeared sporadically and were not linked to the brackets. In the light microscope we also observed the long spirochetes on living flagellate cells. They were not found on the isolated cell envelopes indicating that they are not tightly bound to the cell surface. In contrast, the smaller morphotypes remained at the surface of the envelopes. Spirochete clone mpsp15 possesses one flagellum at each cell pole (Wenzel et al., 2003). After Margulis and Hinkle (1992) the characteristic flagella array is 1:2:1 corresponding to one flagellum at the end of the cell and two overlapping flagella in the middle of the cell.

Rod shaped bacteria (length: 0.8–1.1 μm , width: 0.3 μm) are attached to cell surface brackets in a regular pattern (Cleveland and Grimstone, 1964; König and Breunig, 1997) perpendicular to the cell of *Mixotricha paradoxa*. The distance between the cells in a row is about 0.9 μm and between two adjacent rows is 0.5 μm . The individual rods in the rows are staggered. The fluorescent probes B6.1 and B6.2 were specific for the *Bacteroides forsythus*-related clone B6. Positive hybridization results showed that clone B6 is spread all over the surface of *Mixotricha paradoxa* in a similar regular pattern as found in electron micrographs.

When the cell envelopes were incubated with the probes B6.1 and B6.2 or with the Cy3-labelled universal probe Eub 338 (Amann et al., 1990) the same regular pattern of a rod-shaped bacterium was obtained indicating that clone B6 was the only rod associated with the cell surface.

13. Glycolytic Enzymes of *Mastotermes darwiniensis* and its Flagellates

Several glycolytic activities were found in cell extracts of the flagellates (Table 3). Not the total activity of the cellulases seemed to be formed by the flagellates themselves. Two endoglucanases Cel I and Cel II, with the molecular mass of approx. 48 kD, were isolated from cell extracts of the not yet culturable symbiotic flagellates living in the hindgut of the most primitive Australian termite *Mastotermes darwiniensis* (Li et al., 2003). The N-terminal sequences of these cellulases exhibited significant homology to cellulases of termite origin, which belong to glycosyl hydrolase family 9. The corresponding genes were

Table 3. Glycolytic and laccase activities of the large flagellates.

Enzyme	Activity [μ U/cell] <i>Mixotricha paradoxa</i> (Devescovinidae)	Deltotrichonymphidae
α -L-Arabinosidase	0.3	0.2
β -L-Arabinosidase	–	–
β -D-Cellobiosidase	1.2	0.7
α -D-Galactosidase	0.2	0.2
β -D-Galactosidase	–	–
β -D-Glucosidase	4.0	5.9
β -D-Glucuronidase	–	–
α -D-Mannosidase	–	–
β -D-Mannosidase	0.2	0.4
β -D-Xylosidase	0.5	0.3
Cellulase	169.0	164.0
Xylanase	135.0	98.0
Laccase (ABTS) ¹⁾	0.001	0.001

– = not found; ¹⁾2,2'-azinobis-3-ethylbenzthiazolinesulfonic acid.

not detected in the mRNA pool of the flagellates, but in the salivary glands of *Mastotermes darwiniensis*. A protein with the molecular mass of approx. 48 kD was also detected in crude extract of these flagellates by western blot analysis using a polyclonal antiserum against the cellulase of the termite *Mastotermes darwiniensis*. The results gave evidence that cellulases occurring in the nutritive vacuole of the flagellates partly originated from the termite host. Probably, the cellulases are secreted from the salivary glands of *Mastotermes darwiniensis*. During the mechanical grinding of the wood particles by the termites, the cellulases are attached to wood particles or mixed with them, then the attached cellulases or the mixture move to the hindgut where they are most probably endocytosed by the flagellates.

It has been shown for *Coptotermes formosanus* that the endoglucanases of this termite are restricted to the salivary glands, the foregut, and the midgut (Nakashima et al., 2002). According to our work, a major portion of the endoglucanase activity found in cells of the hindgut flagellates of *Mastotermes darwiniensis* is likely to originate from the termite's cellulases. It has also been found that 40% of the endoglucanase activity of *Mastotermes darwiniensis* is present in the hindgut and most (ca. 84%) of the cellulase activity of the whole hindgut is present in the flagellate extract (Veivers et al., 1982). This implies that a certain amount of termite cellulases, secreted from the salivary glands, moves into the hindgut and enters the flagellate cells. They may be involved in the digestion of cellulose in the flagellate cells.

Using a PCR-based approach DNA encoding cellulases belonging to glycosyl hydrolase family 45 were obtained from micromanipulated nuclei of the flagellates *Koruga*

bonita, *Deltotrichonympha nana*, and *D. operculata* (Li et al., 2003). The cellulase sequences of the termite symbiotic protists were phylogenetically monophyletic, showing more than 84% amino acid identity with each other. The deduced cellulase sequences of termite origin and flagellate origin consist of a single catalytic domain, lacking a cellulose-binding domain (CBD) and a spacer sequence found in most microbial cellulases.

In the gut extracts of wild termites (*Mastotermes darwiniensis*) a cellulase activity (hydrolase family 45) (Watanabe et al., 2006) which was identical to the amino acid sequence of one mRNA sequence isolated by Li et al. (2003) was found in approximately equal magnitude to termite-derived cellulases, indicating that the flagellates produce cellulases.

14. Conclusions and Perspectives

Mixotricha paradoxa, a cristamonad from the hindgut of the Australian termite *Mastotermes darwiniensis* Froggatt, is a rare example of a movement symbiosis between eukaryotic and prokaryotic microorganisms (Cleveland and Grimstone 1964; Wenzel et al., 2003; Brugerolle, 2004). It is known that a lot of symbiotic relationships of protozoa and spirochetes play no role in the locomotion of the protist cell (Bloodgood and Fitzharris, 1976; Breznak, 1984; Iida et al., 2000). This indicates that spirochetes must fulfil also other functions. The spirochetal isolates display physiological pathways which were previously unknown within the spirochetal group, including acetogenesis from H₂ plus CO₂ and nitrogen fixation (Graber and Breznak, 2004; Graber et al., 2004). Both processes are beneficial for termites, because acetate is their major carbon and energy source and N₂ fixation by symbiotic hindgut bacteria can supply up to 60% of the nitrogen in termite biomass. These findings imply an important role of symbiotic spirochetes in the nutrition of the termites. In addition, they form acetate by fermenting monosaccharides and oligosaccharides (Graber et al., 2004; Dröge et al., 2006a,b).

From the movement symbiosis between spirochetes and the flagellate *Mixotricha paradoxa* the hypothesis was derived that eukaryotic locomotory organelles such as flagella and cilia originated from spirochetes (Bermudes et al., 1987; Margulis, 1993), while the cytoplasm is assumed to be of archaeal origin. Our studies showed that several spirochete species synergistically contrive the movement of *Mixotricha paradoxa*. Since *Mixotricha paradoxa* belongs to the early branching flagellates the movement symbiosis should be an early invention during the evolution of the eukaryotic cell. If the above mentioned hypothesis is correct the ancestor of locomotory organelles should have been a relative of *Treponema*. In the same habitat flagellates moving with the aid of spirochetes (*Mixotricha paradoxa*) and flagella (e. g. *Koruga bonita*) are present.

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