Identification and localization of the multiple bacterial symbionts of the termite gut flagellate *Joenia annectens*

Jürgen F. H. Strassert,¹ Mahesh S. Desai,² Renate Radek¹ and Andreas Brune²

Correspondence
Andreas Brune
brune@mpi-marburg.mpg.de

¹Institute of Biology/Zoology, Free University of Berlin, Königin-Luise-Str. 1–3, 14195 Berlin, Germany

²Max Planck Institute for Terrestrial Microbiology, Karl-von-Frisch-Str. 10, 35043 Marburg, Germany

The hindgut of wood-feeding lower termites is densely colonized by a multitude of symbiotic micro-organisms. While it is well established that the eukaryotic flagellates play a major role in the degradation of lignocellulose, much less is known about the identity and function of the prokaryotic symbionts associated with the flagellates. Our ultrastructural investigations of the gut flagellate *Joenia annectens* (from the termite *Kalotermes flavicollis*) revealed a dense colonization of this flagellate by diverse ecto- and endosymbiotic bacteria. Phylogenetic analysis of the small-subunit rRNA gene sequences combined with fluorescence *in situ* hybridization allowed us to identify and localize the different morphotypes. Furthermore, we could show that *K. flavicollis* harbours two phylotypes of *J. annectens* that could be distinguished not only by their small-subunit rRNA gene sequences, but also by differences in their assemblages of bacterial symbionts. Each of the flagellate populations hosted phylogenetically distinct ectosymbionts from the phylum Bacteroidetes, one of them closely related to the ectosymbionts of other termite gut flagellates. A single phylotype of 'Endomicrobia' was consistently associated with only one of the host phylotypes, although not all individuals were colonized, corroborating that 'Endomicrobia' symbionts do not always cospeciate with their host lineages. Flagellates from both populations were loosely associated with a single phylotype of Spirochaetales attached to their cell surface in varying abundance. Current evidence for the involvement of Bacteroidales and 'Endomicrobia' symbionts in the nitrogen metabolism of the host flagellate is discussed.

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INTRODUCTION

Bacterial symbionts have been described for diverse groups of protists living in completely different habitats. The symbionts are most common in anaerobic ciliates from marine and limnic sediments (Fenchel & Ramsing, 1992), in aerobic dinoflagellates from freshwater and saltwater environments (Gold & Pollinger, 1971; Schweikert & Meyer, 2001), and in various lineages of anaerobic flagellates inhabiting the hindguts of lower termites (Kirby, 1964; Brune & Stingl, 2005; Brune, 2006).

The symbiosis between termite gut flagellates and their numerous bacterial symbionts is particularly intriguing. While it is well established that the flagellates contribute to the digestion of lignocellulose in the termite gut

(Cleveland, 1926; Hungate, 1955; Radek, 1999; Inoue *et al.*, 2000), little is known about the functions of their bacterial symbionts. Possible functions range from an involvement in catabolic processes, such as hydrogen metabolism, to anabolic processes, such as nitrogen fixation (Brune & Stingl, 2005; Ohkuma, 2008). Another function, shown in some cases, is the involvement of ectosymbiotic bacteria in motility, with the flagella of the symbionts propelling their eukaryotic host (Cleveland & Grimstone, 1964; Tamm, 1982). To understand the nature of the numerous unknown interactions, the characterization of the uncultivable partners on a phylogenetic and ultrastructural level is indispensable.

The presence of diverse bacterial lineages closely associated with a single flagellate host species suggests complex functional interactions between the partners (e.g. Wenzel *et al.*, 2003; Hongoh *et al.*, 2007a), but only one such multiple symbiosis has been studied from a functional perspective, that of *Trichonympha agilis* and its symbionts.

Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; FISH, fluorescence *in situ* hybridization; SSU, small subunit.

The GenBank/EMBL/DBJ accession numbers for the sequences obtained in this study are GQ469992–GQ469996.

The complete genome sequence of the 'Endomicrobia' endosymbionts suggests that they have the capacity to provide amino acids and cofactors to the host flagellate (Hongoh *et al.*, 2008a), and the removal of hydrogen by the *Desulfovibrio* symbionts coinhabiting the same host flagellate may benefit the fermentative processes of the host flagellate or the 'Endomicrobia' symbionts (Sato *et al.*, 2009). Apparently, the symbiotic interactions are not restricted to the bacterial symbionts and their host flagellate, but occur also between the bacterial symbionts themselves.

Another example of multiple symbionts associated with a single host species is the flagellate *Joenia annectens* and its numerous prokaryotic ectosymbionts and endosymbionts. *J. annectens* is a large parabasalid flagellate from the dry-wood termite *Kaloterms flavicollis*. This flagellate is easily identified among the other small flagellates living in the same host termite by its single tuft of flagella at the anterior cell pole. The different morphotypes and ultrastructural characteristics of the prokaryotes intimately associated with *J. annectens* have been investigated in detail by Radek *et al.* (1992). Although the multitude of bacterial morphotypes present in and on the eukaryotic cell makes *J. annectens* a favourable subject for studying the diversity of prokaryotic associations, the few reports of bacteria associated with *J. annectens* have only addressed the presence of individual symbionts (Ikeda-Ohtsubo *et al.*, 2007; Ohkuma *et al.*, 2007; Noda *et al.*, 2009).

Here, we present the results of a detailed analysis of the multiple symbioses of *J. annectens* and its associated bacteria based on the small-subunit (SSU) rRNA genes of symbionts and host flagellate. We identify the symbionts, document their phylogeny and, using fluorescence *in situ* hybridization (FISH) with specifically designed oligonucleotide probes, their subcellular location, and report on the specificity of the associations.

METHODS

Termites. Specimens of *Kaloterms flavicollis* were obtained from the Federal Institute for Materials Research and Testing in Berlin, where they are in culture. The termites were maintained in plastic boxes at 25 °C on a diet of pine wood. To remove autofluorescent wood particles from the gut, the termites were shifted to a diet of cellulose powder (microcrystalline cellulose, Sigma-Aldrich) 3–4 weeks prior to preparation for *in situ* hybridization. Only false workers (pseudergates) were used for all analyses.

DNA extraction and PCR amplification. Two termite hindguts were removed with fine-tipped forceps, and the contents were suspended in solution U (Trager, 1934). Approximately 70 flagellates identified as *Joenia annectens* based on morphological characteristics were collected by micropipetting and disrupted by three cycles of freeze–thawing. DNA was extracted with the NucleoSpin kit (Macherey-Nagel) following the manufacturer's instructions. The SSU rRNA genes of the flagellates were amplified by PCR with flagellate-specific primers (Ohkuma *et al.*, 1998). The PCR started with a denaturing step at 94 °C for 3 min, followed by 30 cycles at 94 °C for 20 s, 50 °C for 20 s and 72 °C for 50 s, and a final extension

step at 72 °C for 7 min. The bacterial SSU rRNA genes were amplified using the primers 27F (Edwards *et al.*, 1989) and 1492R (Weisburg *et al.*, 1991). The PCR conditions were as described above, except that annealing was at 48 °C.

Cloning and sequencing. PCR products were purified using the MinElute PCR Purification kit (Qiagen). The products were cloned with the pGEM-T vector kit (Promega). Positive clones (transformants) were amplified using M13 primers and checked for inserts by gel electrophoresis. Clones with correctly sized inserts were sorted by RFLP analysis after double-digesting with the restriction enzymes *MspI* and *HhaI*. Clones that showed identical patterns with both enzymes were considered as one ribotype. Several representatives of each ribotype were sequenced using M13 primer sets and the internal primers 343F and 1100R (Ikeda-Ohtsubo, 2007). Clones showing a sequence similarity of $\geq 99.5\%$ were defined as one phylotype. Sequences obtained in this study have been submitted to GenBank under accession numbers GQ469992–GQ469996.

Phylogenetic analysis. The near-full-length SSU rRNA gene sequences were imported into the current Silva database (version 96; <http://www.arb-silva.de>) using the ARB software package (Ludwig *et al.*, 2004). After automatic alignment with the most similar sequences in the database using the ARB Fast_Aligner tool, the alignment was manually refined. The datasets were filtered by base frequency ($>50\%$), and phylogenetic trees were calculated using maximum-likelihood analysis (AxiML). Tree topology was tested with RAxML and maximum-parsimony analysis implemented in ARB (100 or 1000 bootstraps, respectively).

Whole-cell *in situ* hybridization. Oligonucleotide probes were designed with the Probe_Design tool in ARB; probe specificity was tested using the Probe_Match function. Probes had at least two mismatches against any other bacterial phylotype in the Silva database that had been previously detected in termite guts. The probes were 5'-labelled with carbocyanine (Cy3) or fluorescein. For each labelled probe, up to two unlabelled helper probes were designed to increase the accessibility of the labelled probe to the SSU rRNA (Fuchs *et al.*, 2000; Table 1).

Hindguts were opened in PBS (137 mM NaCl, 4.3 mM Na₂HPO₄, 2.7 mM KCl, 1.47 mM KH₂PO₄; pH 7.4). The sample was fixed with 4% (v/v) paraformaldehyde in PBS (3 vols paraformaldehyde solution to 1 vol. sample) for 5 h at 4 °C. After fixation, the sample was centrifuged (50 g, 3 min), and the supernatant was replaced with PBS. This step was repeated three times. The sample was resuspended in PBS with an equal volume of 96% (v/v) ethanol, and stored at –20 °C.

Hybridization was performed according to Stingl & Brune (2003) with minor modifications. Prior to hybridization, the cells were only air-dried; the dehydration in an ethanol series was omitted. After the hybridization procedure, one or two drops of a DAPI solution (1 µg ml^{–1}) were added to each well of the Teflon-coated slides, then the samples were rinsed for 5–10 s with 80% ethanol, air-dried, and embedded in Citifluor AF1 (Citifluor, London, UK). To identify bacterial cells and to distinguish non-specific binding, *in situ* hybridization was also performed with the Bacteria probe EUB338 (Amann *et al.*, 1990) and the nonsense probe NON338 (Wallner *et al.*, 1993). For the newly designed probes (Table 1), optimal hybridization conditions were tested by increasing formamide concentrations from 0 to 60% in increments of 10%, and then fine-tuned in 5% increments.

Scanning electron microscopy. The cells were fixed for 1 h in 2.5% glutaraldehyde in 100 mM sodium cacodylate buffer. After three rinses using the same buffer, the cells were post-fixed on ice for 1 h with 1% OsO₄ in 100 mM sodium cacodylate. The samples were washed again three times and transferred into small cups covered with planktonic gauze. After dehydration in a series of ethanol, the cells

Table 1. Newly designed oligonucleotide probes used for whole-cell *in situ* hybridization of the bacterial symbionts of *J. annectens*

Probe	Target/comment	Sequence (5' to 3')	Formamide concn (%)
Bact1029	Bacteroidales phylotype JoeBact-1	CTCGCAATAGGCCATTGC	20–40
Bact1029-h	Unlabelled helper probe of Bact1029	CGTCCTAATGCGTTCAAACC	
Endo728	Endomicrobium phylotype JoeEndo-1	TTGGGCCAGAAAATTGCCTT	20–40
Endo1023	Endomicrobium phylotype JoeEndo-2	GCTAACTCTCYTGCGAGTCA	≠
Endo1023-h	Unlabelled helper probe of Endo1023	TCCGACTTTTACCATTAGCAGTTCA	
Endo126	Endomicrobium phylotype JoeEndo-2	CACATTCTGAGGAAGGTTTCCC	≠
Endo126-h1	Unlabelled helper probe of Endo126	ATGTGTTACTCACCCGTTTGCC	
Endo126-h2	Unlabelled helper probe of Endo126	GGACCATCTCAAAACGCATTGC	

≠No signal using formamide concentrations from 0 to 60 %.

were dried with a Balzer CPD 030 and coated with gold using a Balzer SCD 040. Samples were examined with an FEI Quanta 200 ESEM.

Transmission electron microscopy. Cells were fixed as for scanning electron microscopy. Prior to dehydration in a series of ethanol, the cells were rinsed three times in 100 mM sodium cacodylate buffer and embedded in 1 % agar. The samples were then embedded in Spurr's resin (Spurr, 1969). Ultrathin sections were stained with saturated uranyl acetate and lead citrate according to Reynolds (1963) and examined using a Philips EM 208.

RESULTS

Joenia annectens: clone library and phylogeny

A clone library (29 clones) of eukaryotic SSU rRNA genes obtained from a suspension of about 70 capillary-picked individuals of *J. annectens* yielded two closely related phylotypes (99.2 % sequence similarity). The sequences of the two phylotypes were identical to the sequence variants previously reported by Ikeda-Ohtsubo *et al.* (2007), corroborating that the termite *Kaloterms flavicollis* harbours two distinct populations of this flagellate species. Whole-cell *in situ* hybridization of hindgut contents with probe Flg-Joe-Kfl (Ikeda-Ohtsubo *et al.*, 2007), which specifically hybridizes with only one of the two phylotypes (AB326381), revealed that both are present in similar abundance. Moreover, careful inspection revealed that the two populations showed slight differences in size and cell shape. The flagellates that showed a bright FISH signal were smaller and had a conical, slender body (phylotype Joe-1; AB326381; mean values $68 \times 30 \mu\text{m}$; $n=60$). The flagellates that did not hybridize with the probe were typically longer and broader, and more oval (phylotype Joe-2; AB326382; mean values $79 \times 44 \mu\text{m}$; $n=60$). However, since the size range of the two populations overlaps (Fig. 1), it is not possible to differentiate them based solely on morphometric analysis.

Bacterial symbionts: clone library and phylogeny

A clone library of bacterial SSU rRNA genes was obtained from the same *J. annectens* preparation (see above); 120 clones were sorted according to their RFLP patterns, and

54 representatives were sequenced. The majority of the clones (71 %) fell into the phylum Bacteroidetes. A detailed phylogenetic analysis showed that they consisted of two phylotypes with a sequence divergence of 11.6 %. They both belonged to the so-called Bacteroidales cluster IV (Ohkuma *et al.*, 2002; Noda *et al.*, 2006a, b) but were clearly polyphyletic. One phylotype (JoeBact-1; 41 % of the clones) fell into a subcluster harbouring only uncultivated bacteria from termite guts, most of them identified as symbionts of gut flagellates, with the rod-shaped ectosymbiont of *Mixotricha paradoxa* (Wenzel *et al.*, 2003) as next, albeit distant, relative (91.1 % sequence similarity). The other (JoeBact-2; 30 % of the clones) was virtually identical to a phylotype previously obtained from *J. annectens* (Noda *et al.*, 2009) and loosely affiliated with a separate subcluster of Bacteroidales, which comprises clones from other termites and the isolate *Tannerella forsythia* (Fig. 2), a species originally obtained from the oral microbiota of humans (Tanner *et al.*, 1986).

A smaller fraction of clones (13 %) belonged to the candidate class 'Endomicrobia' in the bacterial 'Termite

Fig. 1. Size measurements of the two phylotypes of *J. annectens*. The size classes of phylotype Joe-1 (●) and phylotype Joe-2 (○) overlap.

Fig. 2. Phylogenetic positions of the Bacteroidales ectosymbionts of *J. annectens* (in bold). Tree topology is based on maximum-likelihood analysis of SSU rRNA gene sequences (>1280 unambiguously aligned base positions). Node support indicates bootstrap values of >50 % (○) and >95 % (●) in both maximum-likelihood and maximum-parsimony analyses. Roman numerals indicate the Bacteroidales clusters defined by Ohkuma *et al.* (2002). All available near-full-length SSU rRNA gene sequences of cluster IV are shown. Names of termite species are given in parentheses. Representatives of other phyla were used as out-group (not shown).

Group 1' (TG-1), which were recently classified as members of the Elusimicrobia phylum (Geissinger *et al.*, 2009). Phylogenetic analysis again revealed the presence of two distinct phlotypes that were only distantly related (9.6 % sequence divergence). One of the phlotypes (JoeEndo-1; 10 % of the clones) was virtually identical (99.7 % sequence similarity) to a phylotype previously obtained from a suspension of *J. annectens* (Kfja16; Ohkuma *et al.*, 2007) and forms a monophyletic group with 'Endomicrobia' phlotypes previously obtained from termite gut flagellates affiliated to the parabasalids, and from gut homogenates of the termites *Mastotermes darwiniensis* and *Neotermes cubanus*. Also the other phylotype (JoeEndo-2; <3 % of the clones) was virtually identical (99.7 % sequence similarity) to another phylotype that had been previously obtained from a suspension of *J. annectens* in our laboratory (Kfje-1; Ikeda-Ohtsubo *et al.*, 2007). It falls into a different cluster comprising symbionts of termite gut flagellates belonging to the oxymonads as well as symbionts previously obtained from the gut homogenate of *Hodotermopsis sjoestedti*. The phylogenies were identical to those previously reported (Ikeda-Ohtsubo *et al.*, 2007; Ohkuma *et al.*, 2007).

Another fraction of clones (13 %) belonged to the phylum Spirochaetes. It consisted of a single phylotype (JoeTrep-1) that fell into the so-called 'termite cluster' of treponemes

(Lilburn *et al.*, 1999), also referred to as *Treponema* cluster I (Ohkuma *et al.*, 1999), which occur exclusively in termite guts. It forms a well-supported monophyletic group (95.9 % sequence similarity) with two other clones previously obtained from the hindgut of *K. flavicollis* (bootstrap values >95 %; Fig. 3).

The rest of the clones (<3 %) represented phlotypes consisting of only one clone each. They were not further investigated because they most probably represent members of the prokaryotic gut microbiota accidentally included during the picking process.

Bacterial symbionts: localization by FISH

We designed sequence-specific oligonucleotide probes (Table 1) to localize the major bacterial phlotypes present in the clone libraries and to determine their specificity for the respective *J. annectens* populations by FISH. Probe CF319a (Manz *et al.*, 1996), which matches both Bacteroidales phlotypes obtained in this study, hybridized with the rod-shaped bacteria densely covering the surface of all *J. annectens* flagellates (Fig. 4b). Simultaneous hybridization with probe Bact1029, specific for phylotype JoeBact-1 (Fig. 4a, d), revealed that only about half of the *J. annectens* flagellates carried this phylotype on their cell surface, which means that the Bacteroidales covering the

Fig. 3. Phylogenetic position of the Spirochaetales ectosymbiont of *J. annectens* (in bold) among other members of the genus *Treponema*. The tree was constructed using maximum-likelihood analysis of SSU rRNA gene sequences (>1390 unambiguously aligned base positions). Node support indicates bootstrap values of >50% (○) and >95% (●) in both maximum-likelihood and maximum-parsimony analyses. Roman numerals indicate the *Treponema* clusters following the nomenclature of Ohkuma *et al.* (1999). Not all available sequences of the *Treponema* cluster I are shown. Names of termite species are given in parentheses. Representatives from other genera of Spirochaetes were used as out-group (not shown).

other half of the *J. annectens* cells should represent phylotype JoeBact-2 (Fig. 4a–c).

When we combined probes Flg-Joe-Kfl (specific for flagellate phylotype Joe-1) and Bact1029 (specific for ectosymbiont JoeBact-1) in a double hybridization, we found that the fluorescence signals of the respective probes were associated with the same flagellate cells, indicating that *J. annectens* flagellates of phylotype Joe-1 are consistently associated with ectosymbionts of phylotype JoeBact-1 (Fig. 4d–f). Conversely, one can safely conclude that the epibionts of flagellate phylotype Joe-2 represent the phylotype JoeBact-2. However, in rare cases, we also observed that a few cells on the surface of those flagellates that did not hybridize with probe Flg-Joe-Kfl (presumably representing *J. annectens* phylotype Joe-2) still hybridized with Bact1029 (Fig. 5a, b).

The endomicrobium JoeEndo-1 was detected with probe Endo728, which is specific for this phylotype. The

rod-shaped symbionts were localized in a ring-like cytoplasmic area below the nucleus, surrounding the axostyle (Fig. 4g, j). However, not all *J. annectens* flagellates contained such a ring of endosymbiotic bacteria. Simultaneous hybridization with probe Flg-Joe-Kfl revealed that only host cells that did not hybridize with this probe, i.e. phylotype Joe-2, harboured bacteria hybridizing with probe Endo728 (Fig. 4j–l). They appeared to be completely absent from phylotype Joe-1, but also a considerable fraction of the Joe-2 population did not contain these bacterial symbionts (Fig. 4j–l).

The second 'Endomicrobia' phylotype (JoeEndo-2) could not be located by FISH, either with the probe TG1-Joe-Kf originally designed by Ikeda-Ohtsubo *et al.* (2007) or with two newly designed probes that should be specific for this phylotype. Even when two unlabelled helper probes were added (Table 1), no hybridization signal was detected. The probe TG1-T-287 (Ohkuma *et al.*, 2007), which matches with both 'Endomicrobia' phylotypes obtained in this

Fig. 4. Fluorescence *in situ* hybridization of hindgut preparations of *Kaloterms flavicollis*. Images in each row show the same preparation simultaneously hybridized with Cy3-labelled (orange) and fluorescein-labelled (green) oligonucleotide probes and counter-stained with DAPI (blue) to illustrate presence of flagellates and bacteria not detected with the respective probes. (a–c) Simultaneous hybridization with the probes Bact1029 (a) and CF319a (b) revealed symbionts of phylotype JoeBact-1 attached to the surface of *J. annectens* cells and a second phylotype of Bacteroidales attached to *J. annectens* flagellates not covered by JoeBact-1 (asterisk). (d–f) The combination of the probes Bact1029 (d) and Flg-Joe-Kfl (e) showed that only *J. annectens* flagellates of phylotype Joe-1 are covered by Bacteroidales of phylotype JoeBact-1. An asterisk indicates the position of the second *J. annectens* phylotype (Joe-2); the insets show a less-magnified overview including a larger number of cells. (g–i) Hybridization with the probe Endo728 (g) revealed symbionts of the 'Endomicrobia' phylotype JoeEndo-1 localized in a ring-like cytoplasmic area; simultaneous hybridization with probe TG1-T-287 (h) did not reveal any additional targets, indicating that phylotype JoeEndo-2 is not present in *J. annectens*. (j–l) The combination of probe Endo728 (j) with probe Flg-Joe-Kfl (k) showed that only flagellates of phylotype Joe-2 (marked with a circle) harbour bacteria of phylotype JoeEndo-1; a flagellate of the same phylotype without JoeEndo-1 symbionts is indicated with an asterisk. Scale bars: 50 µm.

Fig. 5. (a, b) Fluorescence *in situ* hybridization showing that also *J. annectens* phylotype Joe-2 (asterisk) is occasionally colonized by Bacteroidales symbionts of the phylotype JoeBact-1 (probes were identical to those used in Fig. 4d, e). (c) Scanning electron micrograph showing the posterior end of a *J. annectens* cell that carries both short (white arrow) and long (black arrow) rod-shaped ectosymbionts. Scale bars: 50 µm (a, b), 10 µm (c).

study, successfully detected phylotype JoeEndo-1 in flagellates of the Joe-2 phylotype (Fig. 4g–i), which indicates the functionality of this probe. However, this probe also did not provide any evidence for the presence of target cells in the Joe-1 flagellates or in the other flagellate species occurring in this termite. It is therefore highly unlikely that phylotype JoeEndo-2 is a consistent symbiont of any of these flagellates, at least in the batch of *K. flavicollis* used in the current study.

Bacterial symbionts: morphological characteristics

Scanning electron microscopy showed mostly rod-shaped bacteria attached to the cell surface of *J. annectens* (Fig. 6a–c). There were two morphotypes that differed considerably in length (3.3×0.2 µm vs 10.0×0.2 µm; Radek *et al.*, 1992). When extremely small or large flagellates were considered as representatives of the two *J. annectens* populations (using also the differences in cell shape to avoid misidentification), it became evident that each of the host cells was associated with a particular symbiont: the small, slender flagellates (phylotype Joe-1) were covered by the short rods, and the longer and broader flagellates (phylotype Joe-2) were covered by the long rods (Fig. 6a–c). Taking into account the results of the FISH analysis, the short rods represent phylotype JoeBact-1 and the long rods represent phylotype JoeBact-2. In agreement with the results obtained by FISH (see above), we occasionally observed flagellates that harboured a small number of short rods among the long rod-shaped ectosymbionts (Fig. 5c).

In transmission electron micrographs of ultrathin sections of the flagellate cells, all ectobiotic rods showed a second, outer membrane in addition to the plasma membrane (Fig. 6d), a feature typical of Gram-negative bacteria. The rods were attached to the flagellates' plasma membrane by their

tips. At the attachment sites, the membrane of the flagellate was supported by an electron-dense layer (Fig. 6d). The gaps between the rods and the flagellate membrane were filled with glycocalyx material (details not shown). The ultrastructure of all attached rods was very similar; they lacked differential cytological features that would allow a clear identification of the two morphotypes by transmission electron microscopy.

Transmission electron microscopy revealed a characteristic morphotype of endobiotic bacteria located in the cytoplasm surrounding the axostyle (Fig. 6e). The spindle-shaped cells (2.0×0.3 µm; Radek *et al.*, 1992) had tapered cell poles and were surrounded by two membranes (Fig. 6e; inset). Longitudinal sections of the flagellates showing the complete axostyle revealed that not all flagellates harboured bacteria of this morphotype. Considering the ring-like arrangement of bacteria below the nucleus, these bacteria can be assigned to 'Endomicrobia' phylotype JoeEndo-1, which showed exactly the same localization pattern in our FISH examinations.

In many sections of *J. annectens*, we also detected spirochaetes attached to the plasma membrane. There were two morphotypes of similar dimensions (20×0.2 µm; Radek *et al.*, 1992). One had a rather straight form and a conspicuously wavy outer membrane (Fig. 6f). The other, which occurred less frequently (approx. 10% of the attached spirochaetes), was slightly undulated. Here, the periplasmic space at the attached cell pole was narrower than elsewhere, where outer membrane and plasma membrane were clearly separated (Fig. 6g, h). In contrast to the observations of Radek *et al.* (1992), the outer membrane was wavy also in this morphotype. While the abundance of the ectobiotic rods and spindle-shaped endosymbionts (if present) was rather constant for individual flagellates, the number of spirochaetes varied considerably. Cells were either covered with a large or a

Fig. 6. Ultrastructure of the bacterial symbionts of *J. annectens*. (a) Scanning electron micrograph showing short rods (phylotype JoeBact-1) attached to the surface of a small flagellate and long rods (phylotype JoeBact-2) attached to the surface of a large flagellate. (b, c) Higher magnifications of the long (b) and short (c) ectosymbiotic rods. (d) Transmission electron micrograph of the ectosymbiotic rods showing inner and outer membrane (black arrows) and the electron-dense layer underlying the plasma membrane of the flagellate at the attachment site (white arrow). (e) Longitudinal section of *J. annectens* showing numerous bacteria of the 'Endomicrobia' phylotype JoeEndo-1 (arrows). The bacteria are arranged in an area below the nucleus (n) surrounding the axostyle (ax). The inset shows the endosymbiont at a higher magnification. The two membranes (white and black arrows) and tapering cell poles are visible. (f) Spirochaetal symbionts attached to the plasma membrane of the flagellate (longitudinal section) showing the wavy outer membrane and the straight-cell form of the first morphotype (arrows). (g) Transmission electron micrograph of the second spirochaetal morphotype showing the wavy outer membrane and the undulated cell form; the narrow periplasmic space of the appendage is indicated by an arrow. (h) The same morphotype attached to the plasma membrane of *J. annectens*. Scale bars: 50 μm (a), 5 μm (b, c), 0.2 μm (d), 4 μm (e), 0.3 μm (e, inset), 0.2 μm (f–h).

small number of spirochaetes, or seemed to lack spirochaetal epibionts completely. Occasionally, both spirochaetal morphotypes were attached to the same flagellate, and since a reliable differentiation of the morphotypes by light microscopy was not possible, we did not attempt to assign the *Treponema* phylotype present in the clone library by FISH.

DISCUSSION

In this study, we analysed the multiple bacterial associations of the termite gut flagellate *Joenia annectens*, whose phylogenetic position among other parabasalid flagellates has been previously reported by Strassert *et al.* (2009). Our results indicate that *Kalotermes flavicollis* harbours two

closely related populations of *J. annectens*, which are distinguished not only by their SSU rRNA genes but also by the types and numbers of bacterial symbionts colonizing the surface and the cytoplasm of the respective phylotypes.

The rod-shaped ectosymbionts of the two phylotypes of *J. annectens* belong to a lineage of Bacteroidales comprising many hitherto uncultivated bacteria from termite guts (cluster IV), but are only distantly related. Although each host phylotype was preferentially associated with a particular ectosymbiont, a few individuals were colonized by epibionts of both phylotypes. This is in agreement with the observations of Radek *et al.* (1992), who occasionally observed more than one morphotype of ectobiotic rods on the same flagellate cell. Although the canonical ectosymbiont always prevailed over the second type, host specificity of the association is apparently not as strict as in other cases of symbiosis between Bacteroidales and termite gut flagellates (Noda *et al.*, 2007; Desai *et al.*, 2009).

This lack of specificity among the epibionts of *J. annectens* may be related to their mode of attachment. In contrast to the candidate taxa 'Vestibaculum illigatum' and 'Armantifilum devescovinae' and other Bacteroidales ectosymbionts, which are laterally attached to elongated ridges of the surface of the host cell (Stingl *et al.*, 2004; Noda *et al.*, 2006a, b; Desai *et al.*, 2009), the ectosymbionts of *J. annectens* are attached to the flagellates' plasma membrane by one tip of their cells only. This mode of attachment is typical for spirochaetes, but otherwise has been reported only for the bristle-like 'Candidatus Symbiothrix dinenymphae' and other bristle-like Bacteroidales ectosymbionts of various termite gut flagellates (Hongoh *et al.*, 2007b; Noda *et al.*, 2009). Interestingly, also spirochaetal ectosymbionts are not specific for their host flagellates (Noda *et al.*, 2003), whereas the tightly attached ectosymbionts of devescovinids, which show a lateral attachment, even cospeciate with their host (Desai *et al.*, 2009). So far, one can only speculate on the reasons for this particular mode of attachment, which permits a larger number of ectosymbionts per host cell than lateral attachment to the host surface.

The two 'Endomicrobia' phylotypes recovered from the *J. annectens* suspension have already been detected in previous studies (Ikeda-Ohtsubo *et al.*, 2007; Ohkuma *et al.*, 2007), but only one phylotype (JoeEndo-1) could be localized within the flagellate cells by FISH. The characteristic arrangement of the endosymbionts, which form a ring below the nucleus surrounding the axostyle, allowed an unequivocal assignment to the corresponding morphotype in the ultrathin sections of *J. annectens*. Here, the endosymbionts located below the nucleus in the vicinity of the axostyle showed the spindle shape and other morphological features of the 'Endomicrobia' cells previously described by Stingl *et al.* (2005) in ultrathin sections of *Trichonympha* and *Pyrsonympha* species.

Although the exclusive presence of phylotype JoeEndo-1 within flagellates of phylotype Joe-2 agrees with previous reports of host specificity in different 'Endomicrobia'

lineages (Ikeda-Ohtsubo *et al.*, 2007; Ohkuma *et al.*, 2007), the results of the FISH analysis indicate that the situation in *J. annectens* differs from that previously described for *Trichonympha* flagellates. Here, 'Endomicrobia' were invariably encountered in all host cells, and cospeciation of endosymbiont and host indicates a vertical mode of transmission (Ikeda-Ohtsubo & Brune, 2009). However, numerous flagellates in the Joe-2 population lacked 'Endomicrobia', and 'Endomicrobia' were completely absent from the closely related phylotype Joe-1. Moreover, the next relative of the 'Endomicrobia' phylotype associated with the Joe-2 population is most closely related to the endosymbionts of phylogenetically unrelated flagellates (Ohkuma *et al.*, 2007). A similar case has been reported for *Devescovina* flagellates, where the closest relatives of the 'Endomicrobia' are endosymbionts of phylogenetically unrelated flagellates (Desai *et al.*, 2009).

The apparent absence of cospeciation in several instances of 'Endomicrobia'-flagellate symbioses, pointed out already in earlier studies (Ikeda-Ohtsubo *et al.*, 2007; Ohkuma *et al.*, 2007), might be caused by the horizontal transfer of 'Endomicrobia' between different flagellate species present in the same gut (Ikeda-Ohtsubo and Brune, 2009). However, the inability – despite serious efforts – to localize the second phylotype of 'Endomicrobia' (JoeEndo-2) within any of the *J. annectens* cells offers another explanation. Although the general absence of a symbiont is hard to document, it is safe to conclude that the second 'Endomicrobia' phylotype is not a regular symbiont of *J. annectens*. Rather, it may represent a free-living bacterium that was inadvertently included in the flagellate suspension during micropipetting. There is increasing evidence for the presence of free-living 'Endomicrobia' in flagellate-free higher termites and cockroaches (Ohkuma *et al.*, 2007; Warnecke *et al.*, 2007), and also lower termites harbouring gut flagellates with endosymbiotic 'Endomicrobia' seem to harbour distinct, putatively free-living lineages (Ikeda-Ohtsubo *et al.*, 2010). The fact that we did not detect JoeEndo-2 cells by FISH in any of the preparations may be explained by the apparently low abundance of free-living 'Endomicrobia' in the gut of lower termites (Ikeda-Ohtsubo *et al.*, 2010).

Although the clone library obtained from the *J. annectens* suspension contained only a single phylotype affiliated with the Spirochaetales, we observed two distinct morphotypes of spirochaetes attached to the flagellates' plasma membrane by transmission electron microscopy. Although it cannot be excluded that the same phylotype has different morphotypes, it seems more likely that the second phylotype was not represented in the clone library because of a bias towards the former, probably representing the obviously more abundant morphotype. It is also possible that the second morphotype is not a regular ectosymbiont of *J. annectens*. The hindgut of *K. flavicollis* contains more than a dozen phylotypes of Spirochaetes (Berlanga *et al.*, 2007), and given the promiscuity of many spirochaetes with respect to their target of attachment reported for the

gut flagellates of *Neotermes koshunensis* (Noda *et al.*, 2003), the second morphotype may represent a species that attaches only occasionally to *J. annectens*.

More difficult to explain is the absence of phylotypes representing the three morphotypes of endosymbionts of *J. annectens* described in detail by Radek *et al.* (1992). One possible explanation for this phenomenon is a discrepancy in the assemblages of bacterial symbionts of this flagellate between the two batches of termites used for the ultrastructural and molecular phylogenetic analyses. This would be in agreement with our inability to detect any additional symbionts by FISH with the general Bacteria probe. However, the additional morphotype irregularly scattered in the cytoplasm and one of the two morphotypes in the nucleus possess a thick cell boundary resembling the cell wall structure of Gram-positive bacteria (Radek *et al.*, 1992). Since many Gram-positive bacteria require special protocols for DNA extraction and FISH (e.g. including cell-wall lysing enzymes), both the clone library and the FISH analysis may be biased against Firmicutes.

In this context, it may be worthwhile to speculate about the role of the symbionts in the bacteria–flagellate symbioses in termite guts. Based on the first genome sequence of the ‘Endomicrobia’ from *Trichonympha agilis*, it has been proposed that these endosymbionts are involved in upgrading amino acids provided by the host flagellate (Hongoh *et al.*, 2008a). The same group has shown that the Bacteroidales endosymbionts of *Pseudotrichonympha grassi* are able to fix dinitrogen (Hongoh *et al.* 2008b), suggesting that the fixed nitrogen can be assimilated and used for the synthesis of diverse amino acids that may ultimately benefit the host. If also the Bacteroidales ectosymbionts of *J. annectens* are involved in supplying high-quality nitrogen to the flagellate, the presence or loss of ‘Endomicrobia’ would not be as critical as in flagellates lacking dinitrogen-fixing Bacteroidetes.

Most prokaryotes in the termite gut are uncultivated and their functions in the digestion of lignocellulose remain to be elucidated. Identification of the partners is a first but important step in understanding the complex interactions in this tripartite symbiosis involving bacteria, flagellates and animal host.

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