ORIGINAL ARTICLE

Bacteroidales ectosymbionts of gut flagellates shape the nitrogen-fixing community in dry-wood termites

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Although it is well documented that the lack of nitrogen in the diet of wood-feeding termites is compensated by the nitrogen-fixing capacity of their gut microbiota, the bacteria responsible for this activity are largely unknown. Here, we analyzed the diversity and expression of nitrogenase genes (homologs of *nifH*) in four species of dry-wood termites (*Kalotermitidae*), which thrive on a particularly nitrogen-poor resource. Although each species harbored a highly diverse suite of termite-specific homologs in their microliter-sized hindgut, only a core set related to *nifH* genes of *Treponema* and *Azoarcus* spp., '*Azobacteroides pseudotrichonymphae*', the first member of the *Bacteroidales* identified as a diazotroph, and termite-gut-specific *anfH* genes of hitherto unknown origin were preferentially expressed. Transcription patterns corroborated that the populations of active diazotrophs differ fundamentally between termite genera. Capillary-picked suspensions of the flagellates *Devescovina arta* and *Snyderella tabogae* revealed that their bacterial ectosymbionts each possess two paralogs of *nifH*, which apparently have been acquired consecutively during evolution of *Bacteroidales*, but only one of them (*anfH*) is actively expressed. Transcription patterns correlated neither with the molybdenum content of the diet nor with intestinal hydrogen concentrations, measured with microsensors. We propose that the nitrogen-fixing community in different dry-wood termites is shaped by the symbionts of their specific flagellate populations. Our findings suggest that the diazotrophic nature of '*Armantifilum devescovinae*' has an important role in the nitrogen metabolism of dry-wood termites and is the driving force of co-evolution with its flagellate host.

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Introduction

The hindguts of lower termites are microliter-sized bioreactors that convert lignocellulose to short-chain fatty acids with the help of symbiotic bacteria and flagellate protists (Breznak, 2000; Brune and Ohkuma, 2011). In view of the extremely low nitrogen content of wood (<0.05% dry weight), Cleveland had suggested already almost a century ago that the severe nitrogen limitation in the diet of termites may be compensated by a nitrogen-fixing capacity of their gut microbiota (Cleveland, 1925). Nitrogen fixation in termite guts was eventually demonstrated in the 1970s (Benemann, 1973;

Breznak *et al.*, 1973), and subsequent studies revealed that termites thriving on sound wood obtain 30–50% of the nitrogen in their colonies through this pathway (Tayasu *et al.*, 1998).

Numerous nitrogen-fixing bacteria (diazotrophs) have been isolated from termite guts, but with the exception of spirochetes (Lilburn *et al.*, 2001), none of the isolates is abundant enough to account for the nitrogen-fixing activities in the hindgut (for reviews, see Breznak, 2000; Brune, 2006). This view is supported by a number of cultivation-independent studies that characterized the homologs of the *nifH* gene present in termite guts (Ohkuma *et al.*, 1996, 1999; Noda *et al.*, 1999, 2002; Lilburn *et al.*, 2001; Yamada *et al.*, 2007), a well-conserved marker of diazotrophy (Zehr *et al.*, 2003). A lack of congruence between the *nifH* genes of the isolates and the *nifH* homologs retrieved from the same environments indicates that the predominant diazotrophs in termite guts remain uncultivated (Brune and Ohkuma, 2011).

The *nifH*-like sequences in termite guts represent numerous novel and highly diverse lineages in all

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clusters (I–IV; nomenclature of Zehr *et al.*, 2003) of *nifH* homologs. Many of the sequences in clusters II and III are closely related to *nifH* homologs of cultured *Treponema* species from the gut of *Zootermopsis* spp. (Lilburn *et al.*, 2001; Graber *et al.*, 2004), which suggests that the abundant termite gut spirochetes comprise many potential diazotrophs. This is supported by a metagenomic analysis of the hindgut community of a *Nasutitermes* species, where phylogenetic binning of nitrogenase genes implicated members of *Spirochaetes* and *Fibrobacteres* as the predominant microorganisms capable of N₂ fixation (Warnecke *et al.*, 2007). Other sequences in Cluster III are closely affiliated with *nifH* homologs of cellulolytic clostridia, which indicates that also uncultivated *Firmicutes* may be involved in this process (for details, see Ohkuma *et al.*, 1999; Yamada *et al.*, 2007).

However, not all of the diverse nitrogenase genes present among the gut microbiota seem to be expressed. Using the presence of *nifH* mRNA as an indicator of *nifH* gene expression and diazotrophic activity, Noda *et al.* (1999) demonstrated that the most highly expressed nitrogenases in the dry-wood termite *Neotermes kosshunensis* (*Kalotermitidae*) were so-called alternative nitrogenases (*anf*) in Cluster II, paralogs of classical nitrogenases (*nif*) that contain neither molybdenum nor vanadium cofactors (Eady, 1996; Sicking *et al.*, 2005). Closely related *anfH* genes are present also in *Cryptotermes domesticus* and *Glyptotermes fuscus* (Ohkuma *et al.*, 1999; Yamada *et al.*, 2007), but the general pattern of *anfH* expression in *Kalotermitidae* and the identity of the bacterium carrying these genes remain unknown.

The bulk of the hindgut volume in all lower termites is occupied by cellulolytic flagellates—strictly anaerobic protists that are densely colonized by diverse bacterial symbionts (for reviews, see Hongoh and Ohkuma, 2010; Ohkuma and Brune, 2011). Although the flagellate symbionts can represent the vast majority of the bacteria in the hindgut, their contribution to the metabolic processes had long been completely unknown (Brune and Stingl, 2005; Brune and Ohkuma, 2011). Recently, Hongoh *et al.* (2008b) demonstrated that the genome of ‘Candidatus *Azobacteroides pseudotrichonymphae*’, the endosymbiont of *Pseudotrichonympha grassi* of the subterranean termite *Coptotermes formosanus* (Rhinotermitidae), encodes a complete *nifHDK* operon and all other genes essential for N₂ fixation. This was not only the first report of nitrogen fixation in the *Bacteroidales*, but also the first time that the bulk of nitrogen-fixing activity in a lower termite could be attributed to the symbionts of their gut flagellates.

As specific associations between gut flagellates and symbiotic *Bacteroidales* are common also in dry-wood termites (Noda *et al.*, 2006; Desai *et al.*, 2010; Strassert *et al.*, 2010), it seemed possible that

Figure 1 Differential interference contrast photomicrographs of the termite gut flagellates *Devescovina arta* (a) from *Neotermes castaneus* and *Snyderella tabogae* (b) from *C. longicollis*. Scale bars, 30 µm.

the high activities of nitrogen fixation present in this termite family are due to the symbiont of their flagellates. Here, we identified the active diazotrophs in representatives of four major genera of *Kalotermitidae* (*Neotermes castaneus*, *Cryptotermes longicollis*, *Kalotermes flavicollis* and *Incisitermes marginipennis*). We characterized the diversity of *nifH* genes and the actively transcribed homologs in the hindgut community by phylogenetic analysis of *nifH* clone libraries and fingerprinting analysis of the hindgut community. In addition, we investigated the identity and expression of *nifH* homologs associated with the bacterial symbionts of *Devescovina arta* and *Snyderella tabogae* (Figure 1), numerically predominant gut flagellates of the termite species *N. castaneus* and *C. longicollis*, in suspensions of capillary-picked flagellates. Finally, we used hydrogen microsensors to determine the hydrogen partial pressure in the gut of these dry-wood termites and investigated possible correlations of intestinal hydrogen concentration, molybdenum availability and nitrogen limitation with the transcription of specific *nifH* homologs.

Materials and methods

Termites and feeding trials

The four species of *Kalotermitidae* used in this study were obtained from the Federal Institute of Materials Research and Testing (BAM) (Berlin, Germany). Termites were maintained on a diet of pinewood in temperature- and humidity-controlled chambers (25 °C, 72% relative humidity). *C. longicollis* and *I. marginipennis* were kept exclusively on dry wood without any additional water, whereas *N. castaneus* and *K. flavicollis* were also provided with a layer of moist vermiculite. Prior to the acetylene reduction assay (see below), batches of termites (40–80 animals) were fed filter paper for 7–10 days. To test the effect of environmental factors on *nifH* transcription, smaller batches (15–20 animals) were placed on filter paper soaked with (NH₄)₂SO₄ (120 mM), Na₂MoO₄ (1 mM) or water for

the same duration (Noda *et al.*, 1999), or were incubated under an air headspace amended with 40% H₂. Pseudergates (worker larvae) were used for all experiments.

Nucleic acid extraction

Ten to 15 termites were dissected, and whole guts were immediately placed into an ice-cold solution of sodium phosphate (0.1 M), Tris-HCl (125 mM), NaCl (25 mM) and sodium dodecyl sulfate (2.5%, w/v) (pH 8.0), and homogenized by bead beating (Henckel *et al.*, 1999). Total nucleic acids (DNA and RNA) were co-extracted with phenol–chloroform–isoamyl alcohol and purified as described previously (Lueders *et al.*, 2004). The nucleic acids of the flagellates *D. arta* from *N. castaneus* and *S. tabogae* from *C. longicollis* were extracted from capillary-picked flagellate suspensions (Desai *et al.*, 2010). To test the effect of environmental conditions on *nifH* transcription, guts of all termites were prepared in the same manner, and total RNA was extracted using the Trizol method (Invitrogen, Karlsruhe, Germany). DNA extraction, PCR amplification, cloning and sequencing of small-subunit (SSU) rRNA genes of flagellates and their bacterial symbionts were performed as described previously (Desai *et al.*, 2010). DNA and cDNA clone libraries (see below) were obtained from the same nucleic acid preparations, and flagellate suspensions were prepared from the same batch of termites.

PCR, RT-PCR and cloning of *nifH* homologs

The *nifH* homologs were amplified from total nucleic acids using universal *nifH* primers (IGK and YAA), recombinant *Taq*DNA polymerase (Invitrogen) and the PCR conditions described by Ohkuma *et al.* (1999). When DNA from gut homogenates was used as a template, 0.4 mg ml⁻¹ bovine serum albumin was included in the reaction mixture.

For reverse transcription (RT)-PCR, nucleic acid extracts were digested with RNase-free DNase (RQ1; Promega, Mannheim, Germany) and subjected to a single-tube, one-step, two-enzyme RT-PCR (Access RT-PCR System kit; Promega) using the primers IGK and YAA, including 0.4 mg ml⁻¹ bovine serum albumin, and following the manufacturer's instructions. The RT-PCR program started with a reverse transcription step for 45 min at 48 °C, immediately followed by an initial denaturation step at 94 °C for 2 min, and a total of 30 cycles (denaturation at 94 °C for 30 s, extension at 68 °C for 2 min). The first nine cycles were operated with a touchdown program (annealing at 57 °C for 45 s, decreasing 1 °C per cycle); the remaining cycles had an annealing temperature of 48 °C. Final extension was at 68 °C for 10 min. Specificity of the RT-PCR was validated by standard agarose gel electrophoresis. Parallel assays without reverse transcriptase did not yield any product, confirming the absence of DNA template in the RNA samples.

Amplicons were cloned with the pGEM-T easy cloning kit (Promega) according to the manufacturer's instructions. Inserts were commercially sequenced using M13 primers (GATC, Konstanz, Germany). Nucleic acid sequences have been submitted to the EMBL/GenBank database under accession numbers FN649270–FN649328, FN649412 and FN649413.

Phylogenetic analysis

The SSU rRNA genes of flagellates and bacteria were phylogenetically analyzed as described previously (Desai *et al.*, 2010). For phylogenetic analysis of *nifH* homologs, we built a comprehensive database including representatives of all established lineages (Zehr *et al.*, 2003) and all sequences previously obtained from termite guts (Yamada *et al.*, 2007), using the ARB software suite (<http://www.arb-home.de>). Novel sequences were aligned both at the nucleotide and the amino-acid level. Phylogenetic trees (amino-acid alignments) were constructed using the maximum-likelihood algorithm implemented in ARB (PhyML with Dayhoff PAM, 100 bootstraps); tree topology was confirmed with maximum-parsimony analysis (PHYLP Protein parsimony; 1000 bootstraps).

T-RFLP analysis

The PCR and RT-PCR conditions were the same as described above, except that the forward primer (IGK) was fluorescence-labeled with pentamethine carbocyanine (IRD 700; MWG Biotech, Ebersberg, Germany). Amplicons were digested with the restriction enzyme *Hha*I (Promega), and terminal restriction fragment (T-RF) length polymorphism (T-RFLP) was analyzed as described previously (Pester and Brune, 2006). T-RF length was calculated by comparison with molecular size markers (50–700 bp, LI-COR) using the Gel-Pro Analyzer software (version 4.5; Media Cybernetics, Bethesda, MD, USA).

Hydrogen microsensor measurements

Polarographic hydrogen microsensors had a tip diameter of 10 µm and were purchased from Unisense (Aarhus, Denmark). Microsensor calibration and the experimental setup for hydrogen measurement in termite guts have been described in detail elsewhere (Ebert and Brune, 1997; Pester and Brune, 2007).

Acetylene reduction assay

Nitrogenase activity in living termites was determined using rubber-stoppered glass vials (10 ml) containing 40–90 termites under air amended with 16% acetylene at 30 °C (Breznak *et al.*, 1973; Noda *et al.*, 1999). Samples (0.1 ml) of headspace gas were analyzed for ethylene by gas chromatography over several hours. The gas chromatograph (GC 14b; Shimadzu, Griesheim, Germany) was equipped with a stainless-steel column filled with Porapak R

(Macherey-Nagel, Darmstadt, Germany; 80/100 mesh, $6' \times 1/8''$) and a flame ionization detector, using nitrogen as carrier gas and isothermal conditions (40 °C).

Results

PCR of total nucleic acids from the guts of the four termite species using *nifH*-specific primers yielded products of the expected length (~470 bp). Amplicons of the same length were obtained also by RT-PCR of total RNA in DNase-treated extracts, and by PCR of DNA extracted from capillary-picked flagellate suspensions of *D. arta* and *S. tabogae* from the termite species *N. castaneus* and *C. longicollis*. Clone libraries were constructed for each sample, and the sequences of the *nifH* homologs in the libraries were compared (Table 1).

Diversity of *nifH* homologs in gut homogenates

The clone libraries revealed a high diversity of *nifH* homologs in the hindguts of the four termite species. Alignment of the sequences showed that each termite species harbored numerous phylotypes that clustered with the sequences of other *nifH* homologs previously obtained from termite guts. We extended the nomenclature of Yamada *et al.* (2007) to indicate their phylogenetic affiliation (Figure 2). Clones sharing the same amino-acid signature were considered as one phylotype. Many phylotypes obtained from several or all of the termite species were highly similar, but members of each cluster had a characteristic amino-acid signature and differed considerably in their DNA sequence. The amino-acid sequences of all *nifH* homologs (groups I–III) showed the four cysteine and one arginine residues typically conserved in proteins of the NifH family (Dean and Jacobson, 1992). A few

Table 1 Phylotypes of *nifH* homologs obtained from hindgut homogenates of the four termite species and from flagellate suspensions of *Devescovina arta* (from *N. castaneus*) and *Snyderella tabogae* (from *C. longicollis*)

Termite	Phylotypes ^a	Phylogenetic group ^b	Number of clones/library			Genotypes ^c	T-RF length (bp) ^d
			DNA	cDNA	Flagellates (DNA)		
<i>Neotermes castaneus</i>							
	Aarc-1	<i>Azoarcus</i> -related (I)	0/23	1/12	0/7	FN649278	58
	Arma-1	<i>Armantifilum</i> (II)	0/23	8/12	4/7	FN649273–74	171
	Bact-1a, b	<i>Bacteroidales</i> (III-3a)	2/23	2/12	3/7	FN649270–72	120 , 142, 269
	Trepo-1a, b	<i>Treponema</i> (III-3b)	6/23	0/12	0/7	FN649275–77	142
	Firmi-1	Subgroup III-1b	2/23	1/12	0/7	FN649283–84	190 , 269
	Others	Group II	13/23	0/12	0/7	FN649279, 88, 89	323, 171, 182
		Subgroup III-1a				FN649280, 82	470, 142
		Subgroup III-2				FN649281, 85	168, 142
		Group IV ^e				FN649286, 87	53
<i>Cryptotermes longicollis</i>							
	Arma-2a, b	<i>Armantifilum</i> (II)	14/20	10/10	7/12	FN649293–97	171
	Bact-2, 1c	<i>Bacteroidales</i> (III-3a)	3/20	0/10	5/12	FN649290–92	120, 168
	Trepo-2	<i>Treponema</i> (III-3b)	2/20	0/10	0/12	FN649298	317
	Firmi-2	Subgroup III-1b	1/20	0/10	0/12	FN649299	269
<i>Kaloterme flavicollis</i>							
	Arma-3	<i>Armantifilum</i> (II)	1/21	0/18		FN649312	182
	Bact-3, 4a, b	<i>Bacteroidales</i> (III-3a)	9/21	17/18		FN649300–11	120 , 142, 168 , 415
	Trepo-3	<i>Treponema</i> (III-3b)	6/21	1/18		FN649313–14	228
	Firmi-3	Subgroup III-1b	1/21	0/18		FN649318	317
	Others	Group II	4/21	0/18		FN649315	275
		Subgroup III-2				FN649316–17	142
		Group IV ^e				FN649319	53
<i>Incisitermes marginipennis</i>							
	Aarc-2	<i>Azoarcus</i> -related (I)	2/22	3/10		FN649326	142
	Trepo-4	<i>Treponema</i> (III-3b)	10/22	7/10		FN649320–25	269
	Firmi-4	Subgroup III-1b	9/22	0/10		FN649328	269
	Others	Group IV ^e	1/22	0/10		FN649327	269

Abbreviation: T-RF, terminal restriction fragment.

The abundance of clones in the respective clone libraries (DNA or cDNA) is given for each phylotype, together with the representative genotypes and corresponding T-RFs.

^aThe phylotypes (clones sharing the same amino acid signature) within each group were typically quite similar (96–99% amino-acid identity; details not shown).

^bThe phylogenetic position of the groups is shown in Figure 2.

^cRepresentative sequences were selected for each phylotype. They typically had more than 99% DNA sequence similarity.

^dT-RFs of clones from the cDNA libraries are shown in bold (see Figure 4).

^eSequences in Group IV (pseudo-*nifH*) were not included in Figure 2.

Figure 2 A maximum-likelihood tree showing the phylogenetic relationship of *nifH* homologs obtained from dry-wood termites to those of other termites and representative isolates. Group nomenclature is based on Yamada *et al.* (2007). Clusters containing sequences obtained in this study are shown in bold; the shaded clusters contain sequences of genes that are preferentially expressed (see Table 1 for details). The marked clusters (*) contain *nifH* homologs from flagellate suspensions (for details, see Figure 3). The tree was rooted using Group-IV (pseudo *nifH*) genes as outgroup. Tree topology is based on maximum-likelihood analysis of 144 unambiguously aligned amino-acid positions and was confirmed by maximum-parsimony analysis; nodes with high bootstrap support are marked (○ > 70%; ● > 90%).

sequences fell into the so-called pseudo-*nif* group (Group IV; Ohkuma *et al.*, 1999) and are not explicitly shown in the tree (for details, see Table 1).

Sequences in Group-I were relatively rare and were found only in *N. castaneus* and *I. marginipennis*, where they formed a termite-specific cluster (Aarc) comprising a previously published *nifH* homolog from *Reticulitermes speratus* (Rs17; Ohkuma *et al.*, 1996). They were most closely related to the *nifH* gene of *Azoarcus* strain BH72 (92.3–93.0% amino-acid identity).

Group II was mostly represented by a cluster of closely related clones (Arma) encountered in three of the four termites species (not in *I. marginipennis*) (Figure 3). They are most closely related to the *anfH* gene of *Clostridium pasteurianum* (80.9–82.3% amino-acid identity). Sequences in this cluster had been previously recovered only from the dry-wood termites *N. koshunensis* and *C. domesticus*, and the wood-feeding cockroach *Cryptocercus punctulatus* (Yamada *et al.*, 2007). The homolog from *N. koshunensis* is part of an operon encoding an alternative nitrogenase (Anf) lacking an Mo cofactor

(Noda *et al.*, 1999). A few other clones were closely related to the *anfH* genes of *Spirochaetes* (including an isolate from the termite gut; Lilburn *et al.*, 2001) and *Rhodobacter capsulatus*.

Sequences from Group III were quite diverse and fell mostly into subgroup III-3. A large number of clones clustered with the *nifH* gene of '*A. pseudotriconymphae*' (93.7–97.8% amino-acid identity), the first member of the *Bacteroidales* identified as a diazotroph (Figure 3; Hongoh *et al.*, 2008b). Clones from this cluster (Bact) were retrieved from the same three termites as those from Group II (Arma), but in contrast to the latter, also comprised many *nifH* homologs previously obtained from other termite families (Yamada *et al.*, 2007). A second cluster (Trepo) of clones was most closely related to a *nifH* homolog of the termite gut spirochete *Treponema azotonutricium* (97.2–98.6% amino-acid identity) and was represented in all four termite species. Three of the four termite species (not *C. longicollis*) also harbored clones from subgroups III-1 and III-2, which have been previously detected in termites of diverse families (Yamada *et al.*, 2007) and are related to the *nifH* homologs of *Clostridiales*.

Figure 3 Consensus trees showing the phylogenetic relationship of *nifH* homologs in the clusters Arma (II) and Bact (III-3a) containing sequences obtained from flagellate suspensions of *Devescovina* and *Snyderella* spp. Sequences obtained in this study are shown in bold; phylotypes represented also in cDNA libraries are marked (■; see Table 1 for details). The trees were rooted using the next relatives as outgroup (see Figure 2). Tree topology was confirmed by maximum-parsimony analysis; nodes with high bootstrap support are marked (○ > 50%; ● > 90%).

The phylotypes in the mRNA-based clone libraries were typically found also in the DNA-based libraries of the same nucleic acid preparations, but the diversity of the cDNA clones seemed to be lower. Many phylotypes were not represented (Table 1), which indicated that not all *nifH* homologs present in the respective gut communities may be transcribed. Nevertheless, the clusters Arc, Arma, Bact, Firmi and Trepo were present in the cDNA libraries of several or all termite species inspected (see Table 1). The Bact cluster consisted of multiple phylotypes in all termites except *I. marginipennis*, but only Bact-1a (*N. castaneus*) and Bact-3 (*K. flavicollis*) seem to be expressed. The PCR conditions were highly specific for *nifH* homologs; only the cDNA library of *N. castaneus* yielded an additional, non-*nifH* sequence (homologous to a putative ribonucleoside reductase transcript from human feces) that was also represented in the

corresponding T-RFLP profile (labeled non-*nifH* in Figure 4).

nifH homologs in capillary-picked flagellates

Cloning and sequence analysis of the eukaryotic SSU rRNA genes in the capillary-picked suspensions of *D. arta* and *S. tabogae* confirmed the purity of the preparations. The sequence of *D. arta* was identical to that obtained in a previous study of the same termite (FN377761; Desai *et al.*, 2010); the newly obtained SSU rRNA gene sequence of *S. tabogae* has been submitted to GenBank (FN649413).

Analysis of the bacterial 16S rRNA gene sequences obtained from the same suspensions confirmed the association of *D. arta* with 'Candidatus Armantifilum devescovinae' and an 'Endomicrobium' species, the two previously reported symbionts of this flagellate (Desai *et al.*, 2010). The bacterial clones from *S. tabogae* comprised only a single phylotype (FN649412) that was almost identical (99.0% sequence similarity) to the previously reported ectosymbiont (CcSn-04; AB462747) of a *Snyderella* sp. from the termite *Cryptotermes cavirostris* (Noda *et al.*, 2009). Both formed a sister group of 'A. devescovinae' in Cluster V of *Bacteroidales* (see below). Sequences of 'Endomicrobia' were not detected in *S. tabogae*.

The clone libraries of *nifH* genes obtained from the same suspensions each yielded two distinct phylotypes (Arma and Bact) in equal abundance. With both flagellates, the sequences were identical to that of the phylotypes obtained from the DNA-based and/or mRNA-based clone libraries of the respective whole-gut homogenates (Arma-1 and Bact-1a for *D. arta*; Arma-2a and Bact-2 for *S. tabogae*; Table 1).

Community profiles of *nifH* genes and mRNA

To corroborate the relative abundance of *nifH* genes and their transcripts in the hindgut communities, we also obtained DNA-based and mRNA-based T-RFLP profiles of the respective nucleic acid extracts (Figure 4). The results confirmed the high diversity of *nifH* genes among the gut microbiota and the preferential transcription of only a few homologs that was already observed with the clone libraries. Almost all of the peaks in the mRNA-based profiles could be assigned to a corresponding phylotype in the clone libraries, which indicated that the analysis accounted for the majority of the expressed *nifH* homologs. Although most of the phylotypes in the DNA-based clone libraries were represented also in the corresponding T-RFLP profiles, the presence of several additional peaks indicated that *nifH* gene diversity in the clone libraries had not been exhaustively sampled. Replications of the mRNA-based T-RFLP profiles with nucleic acids extracted from different batches

Figure 4 DNA- and mRNA-based T-RFLP profiles of *nifH* homologs from the hindgut of four termite species. PCR products (~470 bp) were digested with *HhaI*. Peak assignment is based on T-RF lengths of the *nifH* homologs obtained from the respective clone libraries (Table 1).

of *N. castaneus*, *K. flavicollis* and *C. longicollis* (*I. marginipennis* was no longer available at this point) yielded almost identical profiles for the respective species (data not shown).

An effect of the slight changes in the protocol used for RT-PCR on the comparison of DNA-based and mRNA-based T-RFLP profiles was not observed. When the nucleic acids extract of *N. castaneus* was subjected to the same protocol as used for RT-PCR except that no reverse transcriptase was added, T-RFLP analysis of the PCR product yielded a T-RFLP profile identical to that of the standard protocol, indicating that inclusion of bovine serum albumin, the different polymerase in the RT-PCR kit and the touchdown protocol did not affect the results. As this control was not conducted for all samples, we cannot exclude with certainty that minor differences between the DNA-based and mRNA-based profiles are caused by experimental bias.

Effect of environmental factors on *nifH* transcription

To understand the effect of environmental factors on the transcription and expression of *nifH* homologs in the four termite species, we conducted several feeding trials with different diets. All termite species produced ethylene from acetylene, which indicated that nitrogenase is synthesized in all termites under normal (wood-feeding) conditions,

Table 2 Acetylene reduction rates of termites kept on a diet of wood or filter paper

Termite	Number of termites	Average weight (mg) ^a	Specific rate (nmol g ⁻¹ h ⁻¹) ^b	
			Wood	Filter paper
<i>Neotermes castaneus</i>	40	16.6	27.1 ± 2.0	39.7 ± 2.1
<i>Cryptotermes longicollis</i>	60	7.2	4.4 ± 1.4	— ^c
<i>Kaloterms flavicollis</i>	80	7.2	4.6 ± 0.2	4.2 ± 0.3
<i>Incisitermes marginipennis</i>	80	27.2	18.3 ± 2.7	40.9 ± 4.7

^aFresh weight, determined with five randomly selected individuals.

^bAverage of two replicates, determined over an interval of 1 h (*I. marginipennis*), 2 h (*N. castaneus*) or 3 h (*C. longicollis*, *K. flavicollis*).

^cNot determined.

albeit at different levels (Table 2). With the exception of *K. flavicollis*, the nitrogenase activity of all species increased almost twofold when termites were fed filter paper for 7–10 days. When *N. castaneus* was fed filter paper soaked with ammonium sulfate solution, acetylene was no longer reduced.

Dietary effects on the preferential transcription of *anfH* genes in the gut of *N. castaneus* and *C. longicollis* were assessed by comparing the mRNA-based T-RFLP profiles under different feeding regimens. In *N. castaneus*, the T-RF corresponding to the *anfH* gene (171 bp) was always the largest peak, but an additional T-RF of 142 bp (corresponding

Table 3 Relative abundance of T-RFs in expression profiles of *nifH* homologs obtained from the guts of *N. castaneus* subjected to different feeding regimen and environmental conditions

Feeding regimen	Total peak height (%) ^a				
T-RF (bp) Phylotype	120 Bact-1a	142 Multiple	171 Arma-1	190 Firmi-1	269 Unknown
Standard conditions ^b	8.3	7.6	77.3	4.5	2.3
Dry wood only ^c		41.0	57.0		2.0
Filter paper ^d		43.3	56.7		
+molybdate	6.0	10.8	74.7		8.4
+molybdate+H ₂	7.1	2.9	90.0		
+H ₂		4.5	95.5		

Abbreviation: T-RF, terminal restriction fragment.

^aExcluding the non-*nifH* peak at 232 bp.

^bKept on dry wood with moist vermiculite.

^cFed exclusively dry wood.

^dFilter paper soaked with sodium molybdate solution and/or termites incubated with H₂.

to several *nifH* homologs from different groups; see Table 1) increased in relative abundance in termites fed a nitrogen-poor diet (filter paper or dry wood without vermiculite). However, the peak did not increase strongly if the filter paper was soaked with sodium molybdate solution, and disappeared almost completely when termites were incubated under H₂ (Table 3). In *C. longicollis*, the T-RF corresponding to the *anfH* gene was the only peak under all treatments. All T-RFs (except the non-*nifH* peak in *N. castaneus*) disappeared from the mRNA-based profiles when *N. castaneus* and *C. longicollis* were fed filter paper soaked with ammonium sulfate solution.

Microsensor measurements of hydrogen partial pressure showed that only *I. marginipennis* and *K. flavicollis* accumulated hydrogen in the hindgut paunch (Figure 5). Highest hydrogen partial pressures were always encountered in the anterior region (*K. flavicollis*, 32.4 ± 12.3 kPa, *n* = 4; *I. marginipennis*, 42.8 ± 4 kPa, *n* = 3). In *N. castaneus* and *C. longicollis*, hydrogen did not accumulate above the detection limit of the sensors (0.1 kPa). However, the preferential transcription of *anfH* genes remained unaffected when filter-paper-fed *N. castaneus* and *C. longicollis* were kept under a headspace supplemented with hydrogen gas for 2 days, both in the presence and in the absence of molybdate (Table 3).

Discussion

Our results document that the nitrogen-fixing populations among the bacterial gut microbiota of dry-wood termites (*Kalotermitidae*) differ between the four termite species investigated in this study. Although all species seem to harbor a similar set of diazotrophs in their hindgut, their *nifH* expression patterns are dominated by different phylogenetic groups. There is strong evidence that the *anfH* genes

Figure 5 Profiles of hydrogen partial pressures in agarose-embedded guts of the four species of dry-wood termites, measured with microsensors. Axial profiles were normalized to account for different gut lengths. Radial profiles were taken from the agarose surface through the middle of the anterior hindgut paunch (indicated by the long arrow) for all termites except *C. longicollis*; the short arrowheads indicate the positions of the proximal and distal hindgut wall. Profiles were selected as typical from three to five replicates; values for the rectum may not be reliable because the content was often lost during dissection.

preferentially expressed in *Neotermes* species, as first reported by Noda *et al.* (1999) for *N. koshunensis*, belong to 'A. devescovinae', the ectosymbiont of the most abundant gut flagellates of many dry-wood termites (*Devescovina* spp.; Desai *et al.*, 2010). Also the *nifH* expression patterns in other dry-wood termites appear to be shaped by flagellate symbionts. After the report of nitrogen fixation in 'A. pseudotrichonymphae' (Hongoh *et al.*, 2008b)—the nitrogen-fixing endosymbionts of *Pseudotrichonympha* spp. from subterranean termites (*Rhinotermitidae*; Noda *et al.*, 2007)—this is the second case of diazotrophy in the phylum *Bacteroidetes*. The apparently consecutive acquisition of two (*nifH* and *anfH*) paralogs during evolution may—as in the case of 'A. pseudotrichonymphae'—also serve

to explain the strict co-speciation of '*A. devescovinae*' with its flagellate host (Desai *et al.*, 2010).

We were quite surprised to recover two nearly identical sets of *nifH* homologs from flagellate suspensions of *D. arta* and *S. tabogae*. The lack of any nitrogenase genes from '*Endomicrobium trichonymphae*' (Hongoh *et al.*, 2008a)—the closest relative of the second symbiont of *D. arta* (Desai *et al.*, 2010)—and the apparent absence of '*Endomicrobia*' from *S. tabogae* strongly suggest that both the Arma-1 (*anfH*) and the Bact-1a (*nifH*) paralogs stem from '*A. devescovinae*' and the closely related symbiont of *S. tabogae*. The origin of both paralogs from the same population of symbionts is further supported by their almost equal abundance in the clone libraries of the respective flagellate suspensions.

The *nifH* homologs (Bact) obtained from '*A. devescovinae*' and the symbiont of *S. tabogae* are closely related to the *nifH* gene of '*A. pseudotriconymphae*', which is an endosymbiont of a different flagellate lineage (Noda *et al.*, 2007) and the first member of the *Bacteroidales* known to possess a *nif* operon, which must have been acquired by horizontal gene transfer from a diazotrophic member of another bacterial phylum (Hongoh *et al.*, 2008b). As all three symbionts belong to the same termite-specific lineage of *Bacteroidales* (Cluster V; Ohkuma *et al.*, 2002), it is possible that a *nifH* gene of subgroup IIIa was present already among the gut microbiota of the dictyopteran ancestor of termites and has been maintained throughout the lineage of Cluster-V *Bacteroidales* (Figure 6). This is supported by the presence of *nifH* subgroup III-3a in many termite species and also in their closest relative, the cockroach *C. punctulatus* (Yamada *et al.*, 2007 and this study).

The *anfH* homolog (Arma), however, was probably acquired at a later time. This termite-specific lineage

of *anfH* genes in Cluster II, originally discovered by Ohkuma *et al.* (1999), so far comprises only sequences from dry-wood termites and *C. punctulatus* (Yamada *et al.*, 2007 and this study). Considering that '*A. devescovinae*' and the symbionts of *Snyderella* spp. are sister groups, the most parsimonious explanation for the presence of two *nifH* paralogs in this apical lineage would be secondary acquisition of an *anfH* gene (Group II) in addition to the *nifH* gene (Cluster III-3a) already present in Cluster-V *Bacteroidales* (Figure 6). However, in the absence of direct evidence (that is, genome analysis or *in situ* hybridization), the possibility that the two *nifH* homologs originated from a minor bacterial population present in both suspensions cannot be eliminated completely.

The origin of the *anfH* genes in *Neotermes* and *Cryptotermes* from the *Bacteroidales* ectosymbionts of these flagellates is in agreement with the virtual absence of *anfH* clones and the lack of detection of transcripts in *K. flavicollis* and *I. marginipennis*, two termites that harbor neither *Devescovina* nor *Snyderella* flagellates (Strassert *et al.*, 2009, 2010). It is possible that the *nifH* homolog (Bact) most highly expressed in *K. flavicollis* originates from one of the two ectosymbionts of *Joenia annectens*, although it has to be considered that these ectosymbionts belong to a different lineage of *Bacteroidales* (Cluster IV; Strassert *et al.*, 2010). Interestingly, the *nifH* homolog (Aarc) that is preferentially expressed in *I. marginipennis* is closely related to the *nifH* genes of *Azoarcus* species, which are known to fix nitrogen only as endosymbionts of grass roots (Hurek and Reinhold-Hurek, 2003). A 16S rRNA-based analysis of the gut microbiota of *I. marginipennis* conducted in our laboratory showed a large abundance of Rhodocyclales (>14% of all pyrotag sequences; T Köhler, unpublished results). It is

Figure 6 Phylogenetic tree of *Bacteroidales* Cluster-V, which consists exclusively of uncultivated bacteria from the gut of many termites. The 16S rRNA gene sequences of flagellate symbionts from dry-wood termites (*Kalotermitidae*) are shown in bold (Desai *et al.*, 2010 and this study). Not all available sequences are shown (for more details, see Noda *et al.*, 2009). Tree topology is based on maximum-likelihood analysis of 1273 unambiguously aligned nucleotide positions and was verified by maximum-parsimony analysis; nodes with high bootstrap support are marked (● >90%). The tree was rooted using other lineages of the *Bacteroidetes* as outgroup. The arrows indicate the hypothetical evolutionary events of *nif* and *anf* gene acquisition (see Discussion).

possible that they represent another lineage of so-far unidentified flagellate symbionts with the capacity to fix nitrogen.

The isolation of the nitrogen-fixing spirochete *T. azotonutricium* and the presence of apparently spirochetal *nifH* homologs in all termites investigated have implicated spirochetes as potential nitrogen-fixing bacteria in termite guts (Lilburn *et al.*, 2001). The results of our study document the preferential transcription of a spirochetal *nifH* gene (Cluster IIIb) in both *K. flavicollis* and *I. marginipennis* (Figure 2), thereby providing the first direct evidence for a key role of termite gut treponemes in N₂ fixation. Termite gut treponemes possess several paralogs of *nifH* (Lilburn *et al.*, 2001), but only the *nifH* genes in subgroup III-3b seem to be expressed in both termites. It remains to be investigated whether the capacity to fix nitrogen correlates with the ability of certain termite gut treponemes to form stable symbioses with flagellates (Noda *et al.*, 2003).

The strong differences between the nitrogen-fixing populations among the four species of dry-wood termites beg for an explanation. The rate of ethylene production in the acetylene reduction assay, which is directly proportional to the rate of nitrogen fixation (Hardy *et al.*, 1973), allows the investigated termites to be separated into two groups: *N. castaneus* and *I. marginipennis* with high rates, and *C. longicollis* and *K. flavicollis* with low rates of nitrogen-fixing activity. Similar rates reported for *Cryptotermes brevis* (Breznak *et al.*, 1973) and much higher rates in *N. koshunensis* than in *C. domesticus* (Ohkuma *et al.*, 1999) suggest that these are genus-specific patterns. However, as *N. castaneus* and *C. longicollis*—the termite species harboring flagellates with *anfH*-expressing symbionts (*Devescovina* and *Snyderella* spp.)—differ strongly in their acetylene reduction activities, a direct link between the expression of a particular homolog and nitrogenase activity can be excluded. Rather, the expression patterns of the *nifH* homologs in *N. castaneus* and *I. marginipennis* did not change when the termites were fed nitrogen-free filter paper, even though acetylene reduction rates were markedly increased. Apparently, the intestinal community of these termites is well prepared to deal with different levels of nitrogen stress.

It has been shown that bacteria possessing both *nif* and *anf* genes preferentially express their *anf* paralogs in the absence of molybdenum cofactor (Masepohl and Klipp, 1996; Kessler *et al.*, 1997; Braun *et al.*, 1999). Based on their detailed study of nitrogenase activity, *nifH* gene expression and *nif* operon structure in *N. koshunensis*, Noda *et al.* (1999) had suggested that preferential expression of *anfH* in *Kalotermitidae* is related to the lack of molybdenum in sound wood, which may have favored the acquisition of a molybdenum-independent nitrogenase (that is, *anfH*) by their gut symbionts. As the *anfH* expression levels in *N.*

koshunensis were not affected by the addition of molybdenum to the diet, they concluded that either *anf* gene expression is regulated in a different manner than in other bacteria or the symbiont possessing the *anf* genes may have lost the ability to produce the primary, molybdenum-dependent nitrogenase (Noda *et al.*, 1999). The transcript patterns obtained for *N. castaneus* and *C. longicollis*, and the preferential expression of *anfH* over *nifH* genes in the ectosymbionts of their major gut flagellates (this study), show that this phenomenon seems to be common in kalotermitids, and suggest that regulation of *nif* and *anf* gene expression by molybdenum in Cluster-V *Bacteroidales* may indeed differ from that in other prokaryotes.

As hydrogen has been shown to inhibit nitrogenases to various degrees (Guth and Burris, 1983), it seemed possible that differences in hydrogen partial pressure contribute to the particular nitrogenase expression pattern in the hindgut of a given termite species. However, the strong accumulation of hydrogen in *K. flavicollis* and *I. marginipennis*, and its virtual absence in *N. castaneus* and *C. longicollis*, correlate neither with the nitrogenase activity pattern nor the transcription of *anfH* genes, and transcription patterns did not change when *N. castaneus* and *C. longicollis* were incubated under external hydrogen. Rather, the hydrogen concentrations in the gut of the four termite species seem to reflect the types of flagellates present: *Kalotermites* and *Incisitermes* species harbor large hypermastigid flagellates (*Joenia* and *Trichonympha*, respectively), which are absent in *Neotermes* and *Cryptotermes* species (Yamin, 1979), and probably form the bulk of the hydrogen produced in the gut (Messer and Lee, 1989; Inoue *et al.*, 2007). This is in agreement also with previous reports, where *Reticulitermes* and *Zootermopsis* species (harboring *Trichonympha* flagellates) showed high hydrogen partial pressures, and *Cryptotermes secundus* (lacking *Trichonympha* flagellates) showed only little accumulation of hydrogen (Ebert and Brune, 1997; Pester and Brune, 2007).

There is abundant evidence that the bacterial symbionts of most gut flagellates are specific for and even co-speciate with their respective hosts (for example, Stingl *et al.*, 2005; Noda *et al.*, 2006, 2007, 2009; Ikeda-Ohtsubo *et al.*, 2007; Ohkuma *et al.*, 2007; Ikeda-Ohtsubo and Brune, 2009; Desai *et al.*, 2010; Strassert *et al.*, 2010). The nitrogen-fixing nature of the bacterial symbionts has been documented for at least two of these symbiotic pairs (Hongoh *et al.*, 2008b and this study) and may indeed be the evolutionary driver for many of these associations. In view of the lack of correlation between the transcription patterns of *nifH* genes and other parameters that could potentially affect expression or activity of different *nifH* homologs, the species composition of the flagellate community emerges as the most likely determinant of the nitrogen-fixing populations prevailing in the

hindgut of a given termite species. A preferential association of certain flagellates with nitrogen-fixing bacteria would give rise to a pattern of diazotrophs that reflects the species composition of the flagellate assemblage in a particular termite. Consequently, termites of the same genus, which often harbor closely related flagellates, should also share the same lineage of diazotrophs, resulting in phylogenetic clusters of *nifH* homologs specific for these taxa—a phenomenon that had been recognized already by Yamada *et al.* (2007). On the other hand, the assemblages of gut flagellates often differ substantially between different genera of termites within the same family. A prominent case is the family *Kalotermitidae*, where the differences in the distribution of *Devescovina*, *Joenia* and *Trichonympha* species may be responsible for the unique patterns of diazotrophic symbionts.

Conflict of interest

The authors declare no conflict of interest.

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