

Symbiosis in Subterranean Termites: A Review of Insights From Molecular Studies

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ABSTRACT The symbiotic relationship of termites and their eukaryotic and prokaryotic gut microbiota is a focal point of research because of the important roles symbionts play in termite nutrition. The use of molecular methods has recently provided valuable insights into the species diversity and the roles of microorganisms in the guts of termites. This paper provides a review of the current knowledge of symbiont species inventories, genome analysis, and gene expression in the guts of subterranean termites. Particular emphasis is given to the termite genera *Reticulitermes* and *Coptotermes* (Isoptera: Rhinotermitidae), because they contain pest species of global impact in their native and invasive range.

KEY WORDS protozoa, bacteria, gut, 16S rRNA gene, cellulase

Subterranean termites have a significant ecological and economical impact in urban and agricultural settings because of their role in biodegradation of plant material and nutrient cycling. These termites feed predominately on woody tissue, which is rich in difficult-to-digest lignocelluloses, but deficient in vitamins and essential components for protein and fat synthesis. Subterranean termites produce their own cellulases (Yamaoka and Nagatani 1975, Inoue et al. 1997), but not in sufficient amounts to sustain their nutritional needs. Therefore, subterranean termites are dependent on a beneficial symbiosis with a dense and diverse flora of microorganisms in the hindguts of the workers to digest lignocellulosic compounds and to acquire supplemental nutrition (Breznak 2000, Brune 2006, Fig. 1). The symbionts in termite guts combine all three domains of life, namely the Eukarya (protozoa, yeasts, fungi), the Archaea, and Eubacteria (true bacteria) (Breznak 2000, König et al. 2002, 2006).

The association with symbionts predates the origin of the termites. The symbionts were acquired during the evolution of the cockroach-like termite ancestors most likely by feeding on dead plant material colonized by microbes (Grimaldi 2001, Nalepa et al. 2001) and transferred by coprophagy and trophallaxis among gregarious-living conspecifics (Nalepa et al. 2001). When symbionts colonized guts of insects, they entered a mutualistic beneficial relationship, supplementing nutrients and energy of their host and in return, gaining a steady food supply and protection in the constant environment of the gut. With increasing

interdependency of hosts and symbionts, vertical transmission became the predominant way of acquiring the symbiotic inoculation, and co-evolution began leading to “a continuity in the identity of the microbes passed between generations” (Nalepa et al. 2001). The mutualistic beneficial relationship of termites with intestinal symbionts has been suggested as one of the fundamental factors predisposing termites to a social life-style (Thorne 1997). Each worker termite must acquire an initial inoculum of symbionts from parents or nest mates after hatching, and again after each molt. Therefore, dependence on the symbionts requires extended parental care, group living, and overlapping generations; these requirements set the stage for termites to become eusocial (Wilson 1971).

Termite gut symbionts were found in 20-million year old termite fossils preserved in amber (Wier et al. 2002) and have been studied for over a century (Leidy 1877, Koidzumi 1921, Cleveland 1924), yet understanding of symbiont community structure and function is still incomplete. The ecology and metabolism of the termite gut and the symbionts' roles in cellulose degradation, nitrogen fixation and recycling, vitamin production, and acetogenesis for energy production, among others (Fig. 1), have been studied in numerous termite species and are extensively reviewed elsewhere (Breznak 1982, Breznak and Switzer 1986, Breznak and Brune 1994, Bignell 2000, Breznak 2000, Brune and Friedrich 2000, Inoue et al. 2000, König et al. 2006, Ohkuma 2003, Potrikus et al. 1981).

Only a small fraction of the symbiont diversity—at most 10% of the microscopic count (Ohkuma et al. 2006)—is typically obtained in pure culture (Breznak 2000). Therefore, during the past two decades culture-

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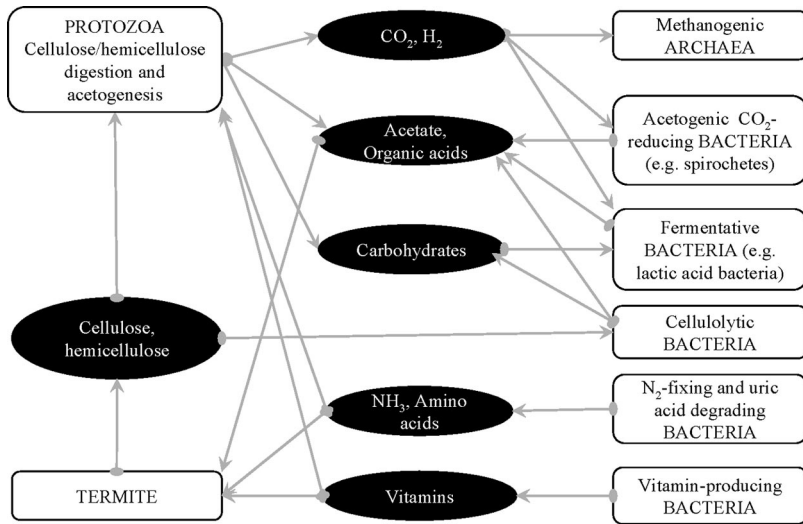


Fig. 1. Simplified diagram of the functional groups of symbionts and their roles and interactions in the hindguts of subterranean termites.

independent molecular methods have been increasingly used to describe microbial assemblages and their roles in insect guts in general (reviewed by Dillon and Dillon 2004) and termite guts in particular (Treusch and Schleper 2006).

The following sections describe the identification of symbionts in the guts of subterranean termites, particularly species of *Reticulitermes* and *Coptotermes* (Isoptera: Rhinotermitidae), with emphasis on the nonculturable majority. Generally, species inventories alone do not provide detailed information about physiological capabilities of symbionts. Recent advances in molecular techniques make it possible to identify genes encoding metabolically important enzymes to determine the role of microorganisms. Characterization of symbionts and their roles in the termite gut may result in beneficial uses, such as biofuel production (Warnecke et al. 2007, Scharf and Tartar 2008), and also lead to novel approaches to effective subterranean termite control (Zhou et al. 2008, Husseiner and Collier 2009) as discussed at the end of this review.

Symbiont Inventories in the Gut of Subterranean Termites

Culture-Independent Methods for Describing Symbiont Communities in the Termite Gut. The most widely used approach to describe symbiotic communities, for example in termite guts, is to sequence the small-subunit rRNA genes (16S rRNA for prokaryotes and 18S rRNA for eukaryotes) in clones from total extracted DNA. Certain regions in rRNA genes accumulate variation as the organisms diverge into different species, while other regions remain conserved. Conserved regions are used to develop universal primers for amplification and sequencing of the rRNA gene (Clarridge 2004).

The use of phylogenetic marker genes, such as rRNA genes, refined the morphological classification of symbiotic Eukarya species (protozoa, yeasts, and fungi) and established the taxonomic relationships among species. Species identification of prokaryotic bacteria, however, turned out to be challenging. Because of the genetic plasticity of bacteria, traditional species definitions do not apply (Clarridge 2004). When using 16S rRNA gene sequence data, the term ribotype signifies a unique sequence; ribotypes with sequence identity of 97% and higher are commonly grouped into phylotypes. Both of these terms are often used to designate taxonomic placement rather than the term species. Organisms and their phylogenetic affiliation can be identified by comparing their rRNA gene sequences to those compiled in public databases, such as NCBI GenBank, the Greengenes 16S rRNA gene database, and the Ribosomal Database Project.

Even undescribed organisms can be grouped into taxa according to their sequence similarities. If only species diversity without species identity is of interest, DNA fingerprinting methods, such as denaturant or temperature gradient gel electrophoresis (DGGE, TGGE), amplified ribosomal DNA restriction analysis (ARDRA) and/or terminal restriction fragment length polymorphism (t-RFLP) can be used to visualize a bacteria community profile (Table 1).

Although molecular methods have provided a wealth of new information on symbiont species, they have their own limitations because of biases that are introduced through differences in lysis efficiency and DNA degradation in different bacteria cell types (Kirk et al. 2004, Ho 2008) and through differential polymerase chain reaction (PCR) amplification caused, for example, by different primer specificity and affinity (Suzuki and Giovannoni 1996), or different copy numbers of the RNA gene (Wintzingerode et al. 1997).

Table 1. Molecular methods used in the description of species inventories and gene function of symbionts in the gut of subterranean termites

Method	Main components of method	Target	Purpose	Termite species	References
Fluorescent in situ hybridization (FISH)	Designing taxa specific probes, hybridization, fluorescent imaging, flow cytometry	Small subunit rRNA and other genes	Enumeration, localization of taxa on particular genes/ gene families	Cf, Rs, Rs = f	Nakashima et al. 2005, Noda et al. 2005, Shimzato et al. 1999, Stingl et al. 2005
Amplified ribosomal DNA restriction analysis (ARDRA)	PCR with universal primers, cloning, restriction of PCR product	Small subunit rRNA genes	Species no., abundance and changes in symbiont communities	Cf, Rf	Fisher et al. 2007, Ho 2008, Shimzato et al. 2005
Terminal restriction fragment length polymorphism (t-RFLP)	PCR of total symbiont community with marked primer, restriction of PCR product, electrophoresis for detection of labeled terminal restriction fragment	Small subunit rRNA genes	Comparison of community profiles Species numbers relative abundance	Ra, Ro, Rs = f, Rs	Hongoh et al. 2005, Nakajima et al. 2005, Yang et al. 2005
Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR)	PCR of repetitive sequence elements	Repetitive sequences in bacteria genome	Profiling bacteria communities	Rf	Bauer et al. 2000
Denaturing gradient gel electrophoresis (DGGE)	PCR with universal primers, separation of PCR products on gels containing denaturants	Small subunit rRNA genes	Profiling bacteria communities	Rf	Bauer et al. 2000, Hayashi et al. 2007
Gene sequencing	PCR with taxa specific, gene specific or universal primers, sequencing of PCR product for gene detection, sequencing cDNA as template for gene expression	Small subunit rRNA genes	Species numbers and phylogeny	Cf, Ra, Ro, Rf, Rs = f, Rs, (Md)	Fisher et al. 2007, Ho 2008, Hongoh et al. 2003, 2005, Husseneder et al. 2005, Kudo et al. 1998, Noda et al. 2005, Ohkuma et al. 1995, 1999a, 2006, Ohkuma and Kudo 1996, Shimzato et al. 1999, 2001, 2005, Yang et al. 2005
Arrays	Bacteria clones (macroarray) or oligonucleotide probes (microarray) hybridization	Nitrogen fixation genes cellulase genes	Gene identification	Cf, Rs, (Cd, Cf, Hs, Nk) Rs	Matsui et al. 2009, Noda et al. 1999, Ohkuma et al. 1999b, Ohtoko et al. 2000, Todaka et al. 2007
Genome sequencing	Pyrosequencing (454, Life Sciences)	Array with target genes	Screening for taxa/species families	Not yet applied in termite symbiosis	
Metagenomics/Environmental genomics	Shotgun sequencing of metagenomic libraries, assembly of genome fragments of the symbiont community	Symbiont genome	Identification of genes, pathways	Cf, Rs	Hongoh et al. 2008a,b
Quantitative real-time PCR (qRT-PCR)	PCR amplification for gene enumeration reverse transcription of mRNA for gene expression	Community genome ('metagenome')	Species composition, functional groups of microbial communities	(N asutitermes sp. higher termite)	Warnecke et al. 2007
CDNA cloning and Heterologous gene expression	PCR amplification of genes cloning gene into expression vector	Genes, transcripts	Enumeration of genes quantitative gene expression	Cf, Rf. (Nk)	Nakashima et al. 2002a,b, Noda et al. 1999, Zhou et al. 2007
		Genes	Gene function and expression confirmation of gene function	Cf, Cl	Inoue et al. 2005, Nakashima et al. 2002a, Watanabe et al. 2002

Species of the Rhinotermitidae and other families of the lower termites (in brackets): Cf, *Cryptotermes formosanus*; Cl, *C. laetaus*; Cd, *Cryptotermes domesticus*; (GF), *Glyptotermes fuscus*; (Hs), *Hodotermopsis sjoestedti*; (Md), *Mastotermes darwiniensis*; (Nk), *Neotermes koshunensis*; Ra, *Reticulitermes amannianus*; Rf, *R. flavipes*; Ro, *R. okinawanus*; Rs = f, *R. santonensis*, the European synonym for *R. flavipes*; Rs, *R. speratus*.

The Eukaryotes: Protozoa, Yeasts, and Fungi. The xylophagous flagellate protozoa located in the hindgut paunch of the workers are the major source of cellulose and hemicellulose hydrolysis of subterranean termites (reviewed by Brugerolle and Radek 2006). Protozoa also ferment the hydrolysis products of cellulose and hemicelluloses to acetate (Odelson and Breznak 1985, Bignell 2000) which is used by the termite as an energy source. The strictly anaerobic flagellates can occupy over 90% of the paunch volume (König et al. 2002). Yamin (1979) listed 205 species of lower termites containing flagellates of the two protozoan lineages Oximonada and Parabasalia.

The inventory of protozoa species is generally specific to the termite host species, but the proportions of protozoa species can vary within a species according to geographic location, diet, season, and temperature as has been shown for several *Reticulitermes* species (Mannesmann 1972, 1974, Mauldin et al. 1981, Kitade and Matsumoto 1993). The taxonomy of protozoa in *Reticulitermes* has been revised many times since the first description by Leidy (1877). Flagellates have been described in the hindgut of workers of *R. flavipes* (Kollar) (at least 12 species), *R. speratus* (Kolbe) (11), *R. virginicus* (Banks) (8), and *R. hageni* (Banks) (9) (Yamin 1979, Belitz and Waller 1998, Cook and Gold 1998, 1999, Lewis and Forschler 2004). Workers of *Coptotermes formosanus* (Shiraki) have only three species of protozoa in their guts, *Pseudotrichonympha grassii* (Koidzumi), *Holomastigotoides hartmanni* (Koidzumi), and *Spirotrichonympha leidy* (Koidzumi) (Lai et al. 1983, Koidzumi 1921, Yamin 1979, Yoshimura 1995). The three species form a "disassembly line" for lignocellulose digestion in the gut; their specific location in the gut correlates with their capability of decomposing mainly highly polymerized cellulose in the anterior hindgut or low molecular weight cellulose in the posterior region (Yoshimura 1995, Inoue et al. 2000). When termites were fed on diets containing only carbohydrates with low molecular weight, both *P. grassii* and *H. hartmanni* disappeared, which suggested that both of these species play important roles in the digestion of high molecular weight carbohydrate polymers (Tanaka et al. 2006).

While the protozoan gut symbionts of subterranean termites are comparatively well described (Yamin 1979) other Eukarya are still being newly discovered. Yeasts and fungi are believed to play a role in termite nutrition as direct source of food and by modifying wood for consumption (Beard 1974). Fungi have been isolated from colonies of subterranean termites and their environment, their integument, and their guts (e.g., *R. hesperus* (Banks), Hendee 1933; *R. flavipes*, Zoberi and Grace 1990; *C. formosanus*, Rojas et al. 2001, Jayasimha and Henderson 2007). Yeasts that were isolated from *R. flavipes* are possibly involved in xylan and cellulose hydrolysis (Prillinger et al. 1996, Schäfer et al. 1996). In addition to classic culturing methods, molecular screening using phylogenetic markers and sequencing of the termite gut community

may reveal the presence of additional yet uncultured species of Eukarya in the termite gut.

The Prokaryotes: Archaea and Eubacteria. The number of prokaryotes in termite guts exceeds the number of protozoa by orders of magnitude. Microscopic cell counts estimated 10^6 to 10^7 bacteria compared with 4×10^4 protozoa per gut of *R. flavipes* (Schultz and Breznak 1978, Breznak 1982, O'Brien and Slaytor 1982). The prokaryote groups consist mainly of methanogens (Archaea) and the phyla Proteobacteria, Actinobacteria, Firmicutes, Bacteroidetes, and Spirochaeta (Eubacteria).

Three species of methanogenic Archaea of the genus *Methanobrevibacter* have been cultured from *R. flavipes* (Leadbetter and Breznak 1996, Leadbetter et al. 1998). Methanogens have also been detected in the gut of *R. speratus* using culture-independent sequencing of archaeal 16S rRNA genes (Ohkuma et al. 1995, 1999a, Shinzato et al. 1999, 2001). Some methanogens are ecto- or endosymbionts of the protozoa (Tokura et al. 2000). Methane emission, however, is secondary to acetate production in wood-feeding termites, because acetogenic bacteria outcompete methanogens for the access to hydrogen (Brauman et al. 1992).

A list of Eubacteria cultured from termite guts, including several *Reticulitermes* and *Coptotermes* species can be found in König et al. (2006). Many of the pure isolates are unique to termite guts and have been shown to catalyze key metabolic functions, including acetogenesis, nitrogen fixation, uric acid fermentation, and sulfate reduction (Brune and Stingl 2005). The main culturable flora in the guts of wood-feeding termites consists of lactic acid bacteria (Schultz and Breznak 1978, Bauer et al. 2000). Culture dependent methods, however, provide only a limited and biased bacterial inventory because culture is inherently selective (Breznak 2000, Ohkuma et al. 2006). To obtain a comparatively unbiased bacterial inventory, culture independent molecular methods are increasingly used to describe species richness, relative abundance, phylogenetic affiliation, and putative functions (Tables 1 and 2).

The most extensive inventory of bacteria in subterranean termite guts has been established for the Japanese species *R. speratus* using 16S rRNA gene sequencing (Ohkuma and Kudo 1996, Kudo et al. 1998, Hongoh et al. 2003). To date, over 312 phylotypes have been identified from worker guts. The phylum Spirochaeta was the most dominant and a fairly diverse group (approximately half of the gut flora and over 60 ribotypes), the majority of which belonged to the genus *Treponema* (Hongoh et al. 2003, Ohkuma et al. 2006). Similar diversity of spirochaetes has been confirmed in *R. flavipes* by screening for spirochaete-specific 16S rRNA genes (Lilburn et al. 1999). Spirochaetes significantly contribute to the carbon, nitrogen, and energy requirements of termites via acetogenesis and nitrogen fixation (Leadbetter et al. 1999, Breznak 2002). The most diverse phylum was the Firmicutes (the low G+C gram-positive bacteria), which contain the clostridia, the lactic acid bacteria and *Mycoplasma*

Table 2. Major bacterial phyla in subterranean termites (Rhinotermitidae) identified with culture-independent methods and their putative functions

Bacteria phylum	Subterranean termite species			Suggested function
	<i>R. speratus</i>	<i>R. flavipes</i>	<i>C. formosanus</i>	
Spirochaeta	Dominant phylum			Acetogenesis, N ₂ -fixation
Firmicutes	Most species diversity			Acetogenesis, sugar degradation and fermentation
Bacteroidetes	Dominant phylum			Fermentation of sugars and nitrogenous compounds, uric acid degradation
Endomicrobia	Endosymbionts of flagellates			Biosynthesis of amino acids and cofactors
Proteobacteria	20% or less of the bacterial inventory			N ₂ -fixation, sulfate reduction
Actinobacteria				N ₂ -fixation, plant polymer degradation

Functional groups have been inferred based on experiments with species cultured from termite guts and/or animal intestines (Ohkuma and Kudo 1996, Breznak 2002) or hypothesized based on identification of genes involved in metabolic pathways (Hongoh et al. 2008a). Thus, the list of functions will likely grow and become more detailed and accurate with future research.

(134 ribotypes, Hongoh et al. 2003). Other predominant phyla were the Bacteroidetes and the candidate phylum Endomicrobia (Stingl et al. 2004) formerly named Termite group 1 (Hongoh et al. 2003, 2008a), which are endosymbionts of the flagellates. Additional phyla, namely the Proteobacteria and Actinobacteria, were only represented by a few clones. Nakajima et al. 2005 studied the bacteria community associated with the gut wall of *R. speratus* and found that the bacteria from the gut wall fraction represented 19% of the whole hindgut community with the major phyla present, but with the Firmicutes and the order Bacteroidales as the most dominant groups as opposed to the spirochaetes in the whole gut. The unequal distribution of the bacteria community among the gut habitats, such as gut wall, fluid and protozoa, was confirmed in *R. santonensis*, the European synonym of *R. flavipes*, by Yang et al. (2005), which led to the conclusion that niche heterogeneity determines the spatial organization of the symbionts in the termite gut.

The bacterial gut flora of *R. flavipes* from North America was described by Fisher et al. (2007) using ARDRA and 16S rRNA gene sequencing. The authors found 33 phylotypes from six phyla. Similar to *R. speratus* clone libraries, spirochaetes (mostly *Treponema*) were the most dominant phylum. In decreasing order of abundance followed the phyla Firmicutes, Endomicrobia, Proteobacteria, Bacteroidetes, and Actinobacteria. Sulfate-reducing bacteria, such as *Desulfovibrio* spp., had only a very low population density in *Reticulitermes* spp. (Hongoh et al. 2003, Yang et al. 2005).

Similarly, using ARDRA and 16S rRNA gene sequencing Shinzato et al. (2005) found 49 phylotypes from ten bacterial phyla in the guts of *C. formosanus* workers in Japan, including 39 novel phylotypes. One of the unknown bacteria species has since been characterized as a novel genus and species of lactic acid bacterium and named *Pilibacter termitis* (Higashiguchi et al. 2006). Thirty-five phylotypes clustered with species known from termite guts, supporting the existence of termite-specific lineages (Hongoh et al. 2003). Most of the clones belonged to the three phyla Bacteroidetes, Firmicutes, and Spirochaetes. A similar

bacteria composition in terms of the three major phyla and the most dominant phylotype was confirmed in *C. formosanus* colonies from the USA and China (Huseneder et al. 2005, Ho 2008). The most dominant member of the Bacteroidetes which comprised 70% of the analyzed clones was characterized as an endosymbiont in the cytoplasm of the gut flagellates by Noda et al. (2005).

Stingl et al. (2005) used 16S rRNA sequencing and fluorescent in situ hybridization to specifically target endosymbionts of gut flagellates. The authors proposed that the diverse endosymbionts in the protozoan cytoplasm, which were only distantly related to other bacteria, are assigned to a novel candidate phylum "Endomicrobia." Members of the Endomicrobia are present and restricted to the guts of lower termites, including *R. flavipes*, *R. speratus* and *C. formosanus*, and the wood-feeding roach *Cryptocercus* (Stingl et al. 2005, Ikeda-Ohtsubo et al. 2007).

In summary, these studies showed that the majority of bacterial phylotypes in the gut of both *Reticulitermes* and *Coptotermes* were novel species; comparison with public gene database sequences showed <97% sequence similarity to 16S rRNA genes of known bacteria. The majority of the termite gut bacteria formed monophyletic clusters with bacteria lineages found only in subterranean termites and not in the environment, suggesting termite specific lineages arising from the interdependence of symbionts and their host (Hongoh et al. 2003, 2005, Shinzato et al. 2005).

Using the 16S rRNA gene to profile bacteria communities has provided a glimpse into the immense diversity of bacteria species in the guts of subterranean termites. However, limited sampling size that usually captures <50% of the total species diversity (Ho 2008), presents a challenge for the investigation of the variability of bacteria diversity among individuals within colonies, among colonies, among populations from different habitats and geographical regions and among termite species. High-throughput sequencing methods, such as pyrosequencing for symbiont communities in the termite gut (Warnecke et al. 2007), and microarrays for species identification and quantification (Zhou

2003) will facilitate the compilation of increasingly complete species inventories.

Genes and Genome Analysis of the Symbiotic Community in Subterranean Termite Guts

Sometimes functions of uncultured species can be inferred from their phylogenetic affiliation alone, for example, in the case of methanogens; however, such inferences of general physiological properties and ecological relationship of phylogenetic groups are ambiguous at best without further confirmation. Identifying genes encoding metabolically important enzymes can be useful in guiding us toward the possible role of microorganisms in the community.

Complete depolymerization of cellulose in lower termites requires multiple functional types of cellulases, that is, endoglucanases, exoglucanases, and beta-glucosidases, from both termites and their symbionts (Breznak and Brune 1994, Li et al. 2006). The endogenous secretion of cellulolytic enzymes was first demonstrated in the salivary glands, foregut, and midgut of *R. speratus* (Yamaoka and Nagatani 1975, Inoue et al. 1997); endoglucanase and beta-glucosidase components were isolated from the same species (Watanabe et al. 1997). Subsequently, several genes encoding endo- and exoglucanases were cloned from termites (Watanabe et al. 1998, Tokuda et al. 1999). In addition to termite-derived cellulases, several genes were isolated from symbiotic protozoa in the hindgut of subterranean termites (*R. speratus*: Ohtoko et al. 2000, *Coptotermes* spp: Nakashima et al. 2002b, Watanabe et al. 2002).

Because of the recent development of culture independent approaches for gene identification, including in situ hybridization for gene localization, the development of PCR primers to specifically amplify certain cellulase families (Sheppard et al. 1994), as well as quantitative real-time PCR and cDNA libraries for gene expression analyses (Table 1), a wealth of cellulolytic enzymes have been recently characterized from termite guts. In general, the majority of endogenous termite endoglucanases belong to one glycosyl hydrolase family (GHF 9) and the genes to produce these endoglucanases were likely inherited from a wood feeding roach ancestor (Lo et al. 2000, Zhou et al. 2007). The cellulases of the protozoan symbionts are more diverse. A comprehensive screening for cellulases was undertaken by Todaka et al. 2007, who used an environmental cDNA library to describe lignocellulose digestion genes of protozoa in *R. speratus*. This approach revealed cellulolytic enzymes from ten glycosyl hydrolase families in the protozoa community of *R. speratus*: cellulases (three families), xylanases (3), beta-glucosidases (1), arabinosidase (1), mannosidase (1), and arabinofuranosidase (1). In addition, protozoan cellulases of several glycosyl hydrolase families were detected in *R. flavipes* (Zhou et al. 2007), *Coptotermes lacteus* (Froggatt) (Watanabe et al. 2002), and *C. formosanus* (Nakashima et al. 2002a, Inoue et al. 2005). Whether

termites and symbionts have a “dual cellulose digesting system” (Nakashima et al. 2002b) or a “single unified cellulose digestion system, whereby endogenous and symbiotic cellulases work sequentially and collaboratively across the entire digestive tract” (Zhou et al. 2007) is still controversial (Tokuda et al. 2007). Both hypotheses are not mutually exclusive and agree on the necessity of both the termite-derived and symbiont derived cellulases to digest wood efficiently. While the protozoan symbionts have cellulolytic and xylanolytic capabilities, no lignase-coding genes of symbiont origin were identified (Todaka et al. 2007). To date, only termite-derived putative lignase genes have been identified (Scharf and Tartar 2008), although subterranean termite gut bacteria have been shown to also degrade lignin compounds (Kuhnigk and König 1997).

Similar to elucidating the cellulolytic system, the use of culture-independent amplification and sequencing of nitrogen fixation genes (*nifH*) from the mixed bacterial population in the gut of termites provided evidence for the presence of an unexpected diversity of nitrogen fixing bacteria and nitrogenases in the guts of *Reticulitermes* and *Coptotermes* species (Ohkuma et al. 1996, Kudo et al. 1998, Ohkuma et al. 1999b). Most of the *nifH* genes from lower termites were related to those of clostridia and sulfate reducing bacteria and methanogenic archaea. However, gene expression studies based on quantitative transcript (mRNA) analysis revealed that only a few among the diverse *nifH* sequences found in the gut community are preferentially expressed in the gut (Noda et al. 1999).

The complete genomes for two uncultured bacteria living as endosymbionts of protozoa in the guts of subterranean termites have been sequenced. The genomes of both bacteria are reduced in size compared with those of free-living bacteria species as it would be expected for true endosymbionts (Hongoh et al. 2008a, 2008b). The first bacterium belongs to the Termite group 1 bacteria and was found in *R. speratus* (Hongoh et al. 2008a). Its genome retained pathways for anaerobic energy metabolism (glycolysis, gluconeogenesis, and nonoxidative pentose phosphate biosynthesis, as well as for fermentation of sugars to acetate), and biosynthesis of nucleic acids, cofactors, and nucleotides. The second bacterium is the Bacteroides species that is dominant in the gut of *C. formosanus* (Shinzato et al. 2005, Ho 2008, Hongoh et al. 2008b). Similar to the former bacterium, the Bacteroides genome contains genes for sugar fermentation and carbohydrate storage, but also *nifH* genes, which predict the ability to fix nitrogen. Full genome sequencing of an array of individual symbiont and endosymbiont species in the termite gut will allow comparative genome analyses in the future. In concert with studies that assemble the species inventories and analyze individual genes, genome analyses will provide the genetic basis of function, specialization, and interaction of individual symbionts within the three-way symbiotic network of termite, protozoa, and bacterial endosymbionts.

Beyond analyzing the genomes of single species, environmental or communal genomic studies of symbiont assemblages (often called metagenomics) become increasingly more feasible and affordable thanks to the rapid advances in high-throughput sequencing techniques (Venter et al. 2004). To date, the metagenome of symbionts in the hindgut paunch has been assembled for only one termite species, a wood-feeding higher termite that does not contain flagellate protozoa (*Nasutitermes* sp., Termitidae, Warnecke et al. 2007). While the metagenome of this higher termite obviously lacked the genes of protozoa origin previously described by the gene expression studies in guts of lower termites (Wu-Scharf et al. 2002, Todaka et al. 2007), this first system-wide shotgun sequence analysis of the bacterial community in a termite gut yielded hundreds of bacterial gene fragments involved in cellulose, hemicellulose and xylan hydrolysis, hydrogen production, CO₂-reductive acetogenesis, and N₂-fixation.

The enormous diversity of symbionts in the termite gut will always be a challenge for metagenomic analyses. However, with increasingly powerful sequencing and bioinformatic tools more genomes of symbionts will be assembled and the collection of phylogenetic marker genes will grow. Increasing length of sequence reads will make it easier to link genes of interest to phylogenetic markers. Thus, in time we should be able to characterize functional groups and physiological traits of entire microbial communities within the guts of termites.

Beneficial Uses of Termite Gut Microbiota

One possible outcome of assembling the species inventories, gene sequence-based metagenomes (Warnecke et al. 2007) and gene expression-based metatranscriptomes (Todaka et al. 2007, Scharf and Tartar 2008) of the gut microbiota of many termite species would be the discovery of biochemical catalysts and novel fermentation technology for the generation of sustainable energy sources from lignocellulose, such as glucose, ethanol, methane and hydrogen (Warnecke et al. 2007). The termite gut is a promising source for the discovery of new enzymes that, for example, mineralize organic materials, degrade aromatic compounds for bioprocessing (Harazono et al. 2003) and toxic substances for bioremediation (Hayashi et al. 2007), convert recalcitrant plant fiber into biogas or hydrogen for biofuel (Pester and Brune 2007, Scharf and Tartar 2008) and fixate nitrogen for soil fertilization.

Because subterranean termite colonies are dependent on their symbiotic network to survive, the microbial community itself could also provide much needed tools and targets for termite control. Most studies have failed to identify entomopathogenic microbes in healthy termite guts (Fisher et al. 2007, Shinzato et al. 2005, Ho 2008). Although there is evidence that some entomopathogens kill termites in laboratory studies, most of them have largely failed to meet expectations in field trials (reviewed

in Su and Scheffrahn 1998, Culliney and Grace 2000). Antiseptic behavior for pathogen removal (Logan et al. 1990, Culliney and Grace 2000), an effective immune system (Rosengaus et al. 1999), and the protective effects of a healthy gut fauna (Veivers et al. 1982) limit the effectiveness of bio-control agents in termites. These biological barriers to infection could be circumvented by the use of genetically engineered natural symbionts as "Trojan Horses" that deliver and express toxins in the termite gut and are spread throughout a termite colony by social interactions (Husseneder and Grace 2005, Zhao et al. 2008, Husseneder and Collier 2009). Other novel approaches to termite control include silencing genes of the symbionts that are vital to termite survival. For example, Zhou et al. (2008) provided proof of concept that ingestion of double-stranded RNA by *Reticulitermes* workers reduces the expression of vital gene products, such as endogenous cellulase and the caste-regulatory storage-protein hexamerin. The combination of RNA interference and paratransgenesis as a sustainable delivery system into termite colonies (Husseneder and Collier 2009) could lead to a novel, pesticide-free approach to social insect control.

In conclusion, the gut of subterranean termites provides a model for studying the ecology of a complex symbiotic network and is also a unique bioresource of novel genes and enzymes with potential use for industrial biotechnology. Molecular methods have dramatically increased our knowledge of symbiont species and gene diversity in termite guts during the last two decades. Gene expression and sequence based studies of termite symbionts have identified a myriad of putative genes involved mainly in lignocellulose digestion and nitrogen fixation (Matsui et al. 2009). The actual properties of most of the gene products cloned from termite symbionts, however, have to be further investigated, for example, by using heterologous gene expression in domesticated hosts, such as *Escherichia coli* or laboratory yeast strains (Handelsman 2004).

While recent research accumulated a fair body of knowledge concerning the parts of the multilayered symbiosis in the termite gut (e.g., by compiling gene, genome and species inventories), understanding the ecology of the symbiont community in the biological context of the termite gut requires a more holistic approach. Future research will increasingly focus on interactions in the symbiotic network consisting of competition, cooperation, and communication among symbionts and with their termite hosts to optimize nutrient acquisition and energy production.

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