Molecular Community Analysis of Acetogenic Bacteria in the Guts of Higher Termites: Community Structure in Termites with Diverse Feeding Strategies

Abstract

CO_2-reductive acetogenesis is a key bacterial activity in the termite hindgut, capable of fueling up to 30% of the metabolism of wood- and grass-feeding termites. In wood-feeding lower termites, acetogenesis is known to be carried out by acetogenic spirochetes. However, the acetogens of higher termites have not been extensively characterized. In this study, we examine the acetogenic bacteria hosted by 6 higher termites species through preparation and phylogenetic analysis of functional gene inventories for formyl-tetrahyrofolate synthetase (FTHFS), a key enzyme in the acetyl-CoA pathway. In wood-, palm-, and litter-feeding higher termites, the dominant acetogens appear to be termite gut *Treponemes* similar to those found in wood-feeding lower termites. However, in subterranean termites, whose diet likely includes some degree of soil-feeding, the dominant acetogens were represented by a novel clade of *Firmicute*-like FTHFS sequences. *Firmicute* acetogens are widespread in the environment, whereas acetogenic *Treponemes*, to date, have only been identified in the guts of termites and wood-feeding roaches. The relative dominance of acetogenic *Firmicutes* in the guts of termites utilizing alternate substrates suggests that the fermentation of wood polysaccharides (and similar substrates) in the termite hindgut establishes a uniquely favorable environment of acetogenesis by spirochetes.

Introduction

The symbiosis between termites and their gut microbes is a highly complex, obligate mutualism. The hindgut community acts as a highly efficient bio-reactor, converting complex substrates to acetate, the principle source of energy for the termite (27). In wood-feeding termites, H_2 is the central free intermediate in the degradation of lignocellulose, representing 22%–26% of the respiratory activity of the termites (31). Microtracer experiments suggest that rates of CO_2-reductive acetogenesis represent 83-100% of hydrogen turnover in these experiments, corresponding to 18%–26% of the termite's respiratory activity (31).

Isoptera is divided into 7 major families. 6 of these families are comprised of "lower termites," which are exclusively wood and/or grass feeders. The "higher termites" are a single family (*Termitidae*), which nonetheless contains about 85% of known genera (13). Higher termites are able to utilize a much broader range of substrates than lower termites; in addition to wood- and grass-feeding, higher termite species have evolved fungus-cultivating, litter- and soil-feeding lifestyles. In lower termites, microbial fermentation of cellulose takes place in a single hindgut paunch. Many higher termites have a more complex gut structure; the five gut compartments of soil-feeding *Cubitermes* sp. have been shown to have distinct physical conditions (pH, metabolite concentrations) (6) and bacterial communities (39). Higher termites with different feeding habits have been found to have vastly different complements of symbiotic bacteria (24, 39, 41, 46).

This diversification of feeding habits and prokaryotic community structure corresponds with altered patterns of acetogenesis and methanogenesis. While wood- and grass-feeding higher termites were found to have high rates of acetogenesis and low rates of methanogenesis, this relationship was reversed in soil-feeding and fungus-cultivating termites (4). Additionally, domain-level phylogenetic profiling found that soil-feeding higher termites have a lower ratio of *Bacteria* to *Archaea* than wood-feeding termites, suggesting a larger methanogenic population (3). However, it has been demonstrated that soil-feeding termites with low rates of *in situ* CO_2 reduction to acetate nonetheless have substantial populations of acetogenic bacteria ($>10^6$ cells/mL) (42). As a result, it has been hypothesized that acetogens in this environment subsist on alternative substrates or within microniches.

While most termite gut acetogens remain uncultured, the diversity of organisms capable of carrying out this activity can be assessed using molecular ecology-based techniques. Leaphart and Lovell (14, 15) have designed primers that target the gene for formyl-tetrahydrofolate synthetase (FTHFS), a key enzyme in the Wood-Ljungdahl pathway of reductive acetogenesis (16). In lower termites, the dominant FTHFS types group phylogenetically with FTHFS genes from acetogenic spirochetes of the genus *Treponema* (30, 37).

The recent metagenome of microbes inhabiting the gut of the wood-feeding higher termite *Nasutitermes* revealed the presence of termite *Trepoene*-like FTHFS genes (46). However, the fragmentary nature of that data precludes detailed phylogenetic analysis of

these sequences. An exhaustive survey of acetogenesis genes in other species of higher termites has not yet been presented. Here, we will explore the diversity of acetogenic organisms present in 6 species of higher termites with diverse feeding regimes.

Materials and Methods

Insect Collection

Nasutitermes sp. Cost003 and *Rhynchotermes* sp. Cost004 were collected in the INBio forest preserve in Guápiles, Costa Rica. Cost003 was collected at a height of 1.2 m in a *Psidium guajaba* tree and appeared to be feeding on deadwood. Cost004 was collected in the same area, from a nest located under an unidentified *Bromeliad*. Extensive feeding trails led from this nest to a large pile of decaying wood and plant material, suggesting a litter-feeding lifestyle. *Microcerotermes* sp. Cost008 was collected from the base of a palm tree about 100 m from the beach at Cahuita National Park in Costa Rica, and appeared to be feeding on dead portions of the same plant. *Amitermes* sp. Cost010 was collected from the roots of dead sugar cane plants at a Costa Rican plantation. Costa Rican termite derived materials were collected, processed, exported, and imported under existing permits between INBio (Costa Rica) and Diversa Corporation (Verenium). Work with these samples at Caltech was subject to guidelines established within a material transfer agreement between the three parties. *Amitermes* sp. JT2 and *Gnathamitermes* sp. JT5 were collected from subterranean nests at Joshua Tree National Park.

DNA Extraction

Guts were extracted from termites within 48 hours of collection. Whole guts were collected from 20 workers of each species. Extracted whole guts were suspended in 500 µL 1X TE (10mM Tris, 1mM EDTA, pH 7.4) and stored at –20 ºC until DNA purification. DNA was purified from gut samples as described by Matson, Ottesen and Leadbetter (20). The purified DNA was quantified using the Hoefer DyNAQuant 200 fluorometer and DNA quantification system (Amersham Pharmacia Biotech) using reagents and procedures directed in the manual (DQ200-IM, Rev C1, 5-98).

FTHFS Amplification, Cloning, and RFLP Analysis

FTHFS genes were amplified from insect guts as described in Leaphart and Lovell (15). Primers with 5´ phosphate groups were purchased from Integrated DNA Technologies. Amplification reactions for cloning contained 1 µM each primer, 1X Failsafe Premix D (Epicentre), and 0.0525 U/µL Expand High Fidelity Taq polymerase (Roche). FTHFS was amplified from Cost003 in reactions containing 1 ng/µL template and following the recommended step-down protocol (15) followed by 25 cycles at 55 ºC. All other termite samples contained low levels of PCR-inhibiting compounds and required further dilution; these reactions contained 0.1 ng/µL template and were amplified for an additional 5 cycles at 55 ºC to generate a similar final concentration of product. PCR reactions were purified using QIAquick PCR purification kits (Qiagen), and cloned using a GC Cloning and amplification kit with LC-Kan vector (Lucigen).

Cloned PCR products were screened by RFLP analysis. Isolated colonies were picked into 10 µL 1X TE, then incubated at 95 ºC for 5 min. This lysate was used to provide template for amplification reactions generating both RFLP analyses and sequencing template. Inserts were amplified using vector primers SL1 and SR2 (from GC Cloning Kit manual), FailSafe Premix A (Epicentre), and 0.05 U/µL Taq polymerase (New England Biolabs). The thermocycling protocol was as follows: 3 min as 95 ºC, 30 cycles of (95 ºC 30 s, 55 ºC 30 s, 72 ºC 1 min 30 s), then 10 min at 72 ºC. RFLP typing used the enzyme HinP1I (New England Biolabs): 6 µL of the PCR product was added to 0.4 µL 10X NEB buffer 2, 0.3 µL HinP1I (New England Biolabs), and 3.3 µL H_2O, then digested at 37 ºC for 4 hr. Digested product was analyzed by gel electrophoresis using a 2.5% agarose gel. A single representative clone of each RFLP type was amplified for sequencing using the protocol above and substituting Expand high fidelity polymerase (Roche) for Taq DNA polymerase.

COII Identification of Termites

Termites identifications were confirmed using insect mitochondrial cytochrome oxidase subunit II (COII) gene sequences. COII genes were amplified directly from the DNA samples used for FTHFS analysis for the Costa Rican termites. JT2 and JT5 COII sequences were amplified from single termites. Single termites were placed in 2 mL microcentrifuge tubes with 50 µL, then crushed using a sterile glass rod. The supernatant from this disruption was transferred to a 200 µL PCR tube, then incubated at 95 ºC for 10 min to lyse suspended cells and inactivate cellular protein. The resultant solution was clarified by centrifugation 1 min at 13,000 x g, and the resultant supernatant used directly

for COII amplification. Termite COII was amplified using the primers CI-J-1773 and B-tLys and cycling conditions described in Miura et al. (22). Reactions included FailSafe Premix D (Epicentre) and Expand high fidelity Taq (Roche).

Sequence Analysis

Cycle sequencing was carried out by Laragen (Los Angeles, CA). Sequence reads were assembled and edited using the Lasergene software package (version 7.2.1, DNASTAR). FTHFS protein sequences were aligned using MUSCLE (8), and phylogenetic analyses were carried out using the ARB software package (18). Libraries were screened for chimeric sequences using the Bellerophon program (11); single RFLP types from Cost003 and Cost004 were eliminated from further analysis.

Results

FTHFS libraries were constructed from 4 species of higher termite from Costa Rica and 2 desert-adapted species from California (Table 3.1). *Nasutitermes* sp. Cost003, collected in the mountains of central Costa Rica, was clearly wood-feeding. *Rhynchotermes* sp. Cost004 was collected in the same area, and appears to be a litter feeder. *Microcerotermes* sp. Cost008, collected on the eastern coast of Costa Rica, was found feeding on dead portions of a palm tree. *Amitermes* sp. Cost010 was collected from the roots of a decaying sugarcane plant in Costa Rica. *Amitermes* sp. JT2 and *Gnathamitermes* sp. JT5 were collected from subterranean nests in the Mohave Desert. It has not been determined whether these three termites were feeding on soil or on nearby plant material.

Table 3.1. FTHFS libraries constructed in this study

Species	Full-Length Clones[a]	RFLP Types[a]	OTU (98% AA)
Nasutitermes sp. Cost003	52	19	14
Rhynchotermes sp. Cost004	63	42	30
Microcerotermes sp. Cost008	27	16	12
Amitermes sp. Cost010	27	18	17
Amitermes sp. JT2	90	24	20
Gnathamitermes sp. JT5	60	24	22

[a] Excludes RFLP types and clones determined to be chimeric

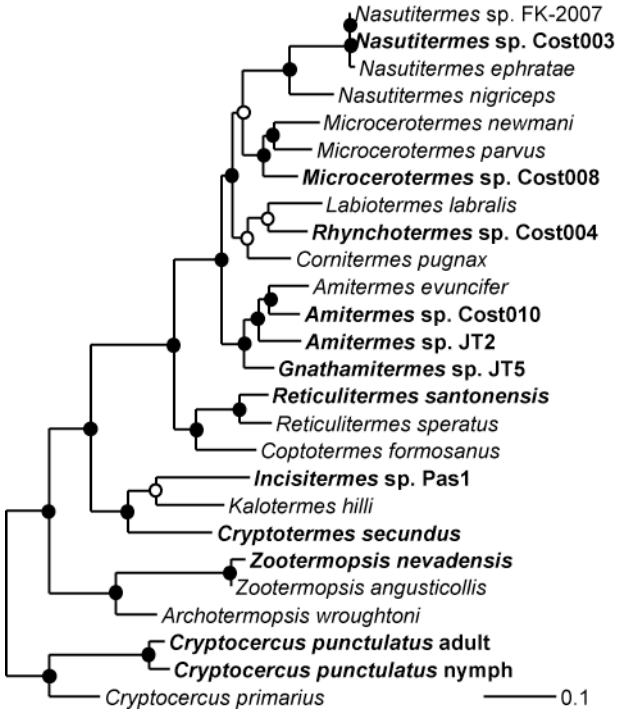

Figure 3.1. Mitochondrial cytochrome oxidase II phylogeny of termites and roaches. Species from which gut FTHFS diversity has been examined are marked in bold. Tree calculated using AxML and 396 unambiguously aligned DNA bases. Open circles mark groupings also supported by either Phylip DNAPARS parsimony or Fitch distance algorithms. Closed circles identify clusters grouped by all three treeing methods. Scale bar represents 0.1 base pair changes per alignment position.

Mitochondrial cytochrome oxidase II (COII) phylogeny was used to help identify collected termites (Figure 3.1). Cost003 was collected within 30 ft of *Nasutitermes* sp.

FK-2007 (the source of the 2007 metagenome by Warneke et al. (46)), and had an identical COII gene sequence. The identification of Cost008, Cost010, and JT2 termites could be confirmed to genus level with molecular phylogeny. No COII genes were available for *Rhynchotermes* or *Gnathamitermes*, so identification of Cost004 and JT5 relied on morphological characteristics. The COII gene from *Gnathamitermes* genus groups closely with sequences from *Amitermes* termites. The genus *Rhynchotermes* is typically classified as a member of the *Nasutiterminae* subfamily. However, this family is paraphyletic (2, 12), and Cost004 groups phylogenetically with termites from proposed subfamily *Syntermitinae* (9).

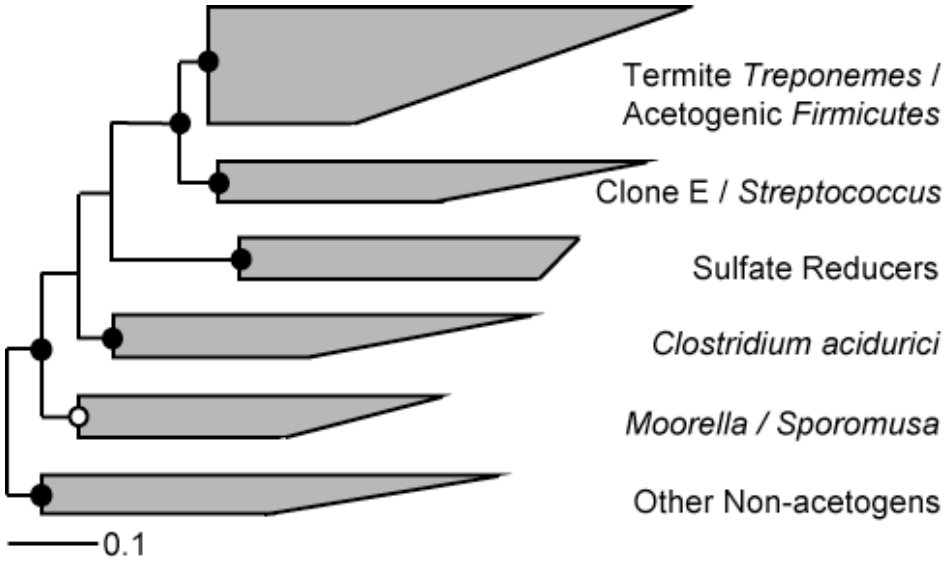

Figure 3.2. Phylogeny of major FTHFS clades found in termites and relatives. Tree calculated using Phylip PROML and 337 unambiguously aligned amino acids. Open circles mark groupings also supported by either Phylip PROTPARS parsimony or Fitch distance algorithms. Closed circles identify clusters grouped by all three treeing methods. Scale bar represents 0.1 amino acid changes per alignment position.

Table 3.2. Composition of FTHFS libraries from the hindgut microbiota of termites and relatives[a]

Species	Food Source	Termite *Treponemes*	*Firmicute* Acetogens	*Moorella / Sporomusa*	Clone E / *Streptococcus*	*Clostridium aciduri*	Other Non-acetogenic
C. punctulatus adult	Wood	78	5		2		5
C. punctulatus nymph	Wood	50	7		41		2
Z. nevadensis[c]	Wood	77	10		4		9
C. secundus[d]	Wood	97					2
Incisitermes sp. Pas1	Wood	100					
R. santonensis[d]	Wood	98			1		1
***Nasutitermes* sp. Cost003**	Wood[b]	98	2				
***Rhynchotermes* sp. Cost004**	Litter[b]	37	6			45	10
***Microcerotermes* sp. Cost008**	Palm[b]	89	11				
***Amitermes* sp. Cost010**	Sugarcane / Soil[b]	12	85	4			
***Amitermes* sp. JT2**	Grass / Soil[b]	1	87	6			3
***Gnathamitermes* sp. JT5**	Grass / Soil[b]	2	28	2	37	10	17

[a] Sequence abundance for each major FTHFS clade is given as percentage of total clones examined
[b] Food source unknown, probable sources based on nest location and/or feeding trails.
[c] From Salmassi and Leadbetter, (37)
[d] From Pester and Brune, (30)

A diversity of FTHFS sequences were identified in higher termites. These FTHFS types were classed into 6 broad categories (Figure 3.2). Sequences from the termite *Treponeme* and acetogenic *Firmicute* groups were considered probable acetogens. The *Spromusa/Moorella* group was considered indeterminate, and all other groups are considered probable nonacetogens. Phylogenetic analysis of FTHFS sequences from higher termites show striking variability in community composition (Table 3.2). Wood-feeding Cost003 and palm-feeding Cost008, similar to lower termites and *C. punctulatus*, are dominated by termite *Treponeme*-like FTHFS sequences. The library generated from Cost004, a litter feeder, was dominated by nonacetogenic FTHFS types, but the majority of acetogenic FTHFS sequences present were *Treponeme*-like. The remaining species of termite were subterranean and appeared to feed on soil and/or plant material. Cost010

and JT2 were both dominated by FTHFS types that grouped with acetogenic *Firmicutes*. Like Cost004, the JT5 library was dominated by nonacetogens. However, the majority of acetogenic FTHFS sequences from JT5 grouped with those from Cost010 and JT2.

FTHFS types from probable acetogens were identified in all higher termite species. Figure 3.3 summarizes the phylogenetic relationships amongst the "Lovell cluster" of probable acetogens (marked as node A). Groups A, B, and C have been termed *Firmicute* acetogens, as those represent the most closely affiliated characterized organisms. However, it should be noted that the distances between termite gut FTHFS types and those from *Firmicute* acetogens are relatively large, and that at least one incidence of horizontal gene-transfer (to generate the termite *Treponeme* clade) has been postulated within this cluster.

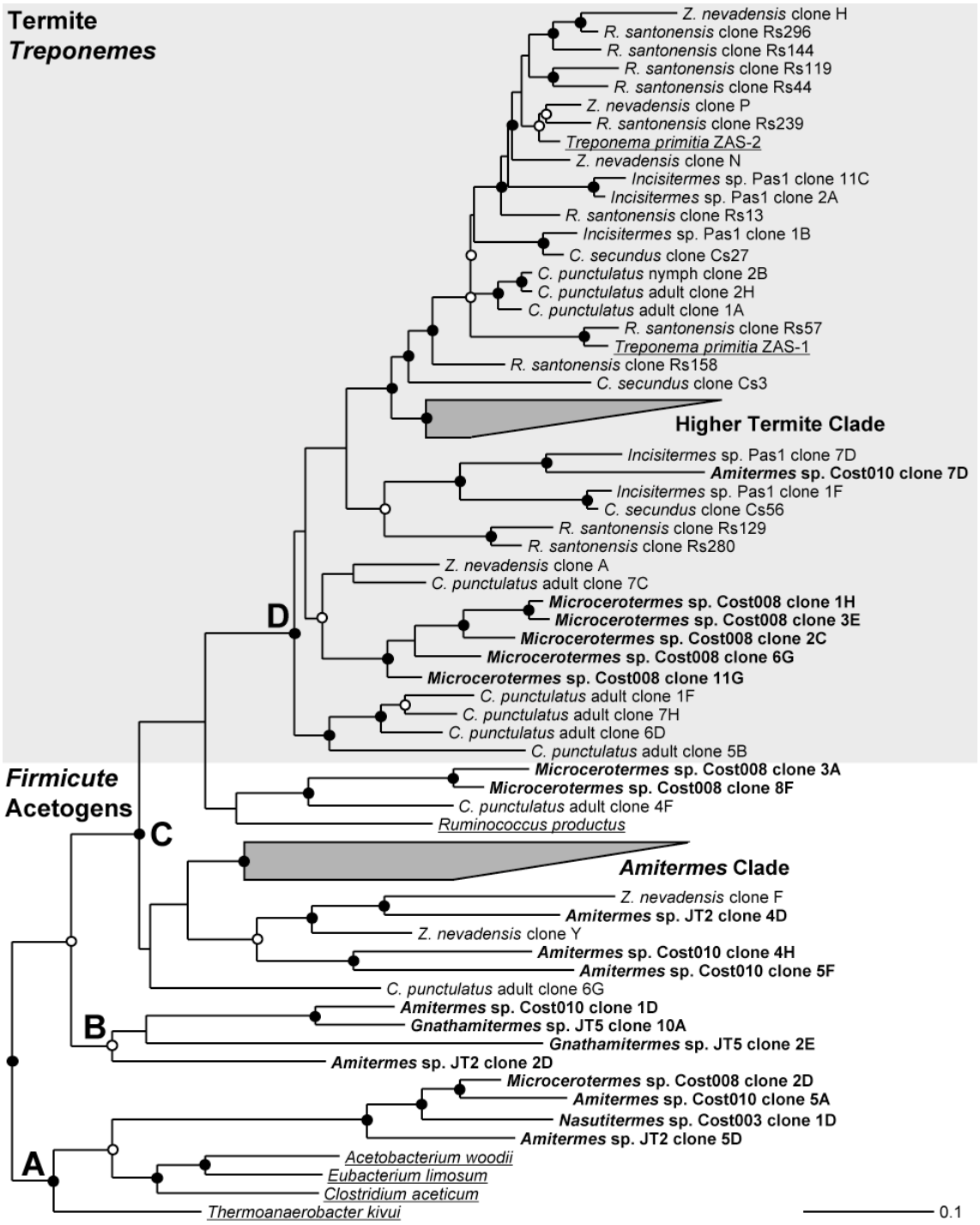

Figure 3.3. FTHFS sequences from potential acetogens. Sequences from this study marked in bold, known acetogens underlined. Tree calculated using Phylip PROML and 339 unambiguously aligned amino acids. Open circles mark groupings also supported by either Phylip PROTPARS parsimony or Fitch distance algorithms. Closed circles identify clusters grouped by all three treeing methods. Scale bar represents 0.1 amino acid changes per alignment position. Tree was rooted using 7 members of the *Moorella / Sporomusa* group of potential acetogens.

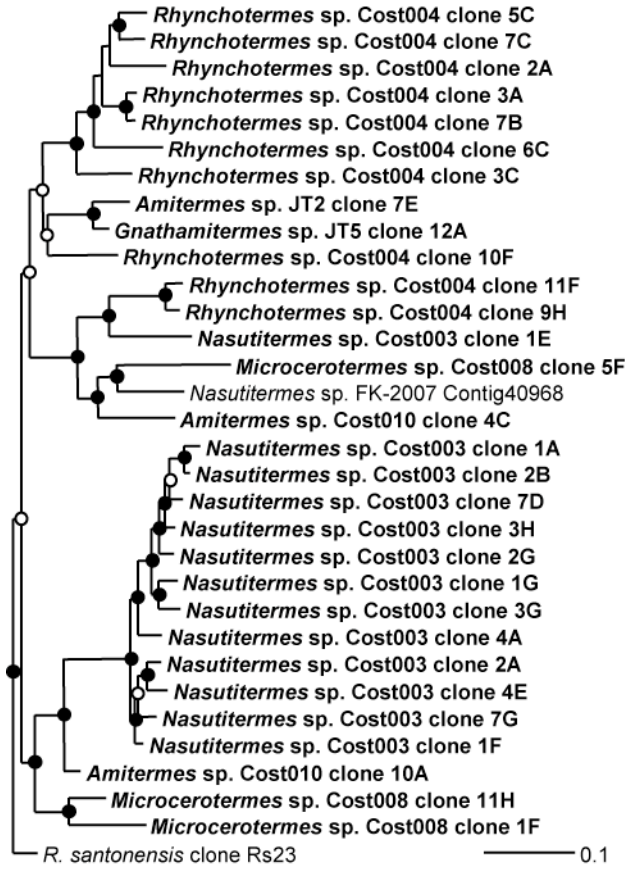

Figure 3.4. Higher termite clade of termite *Treponemes*. Tree calculated using Phylip PROML and 354 unambiguously aligned amino acids. Open circles mark groupings also supported by either Phylip PROTPARS parsimony or Fitch distance algorithms. Closed circles identify clusters grouped by all three treeing methods. Scale bar represents 0.1 amino acid changes per alignment position. An outgroup consisting 3 termite *Treponeme* isolates was used to root the cluster.

Node D was chosen as the boundary for the termite *Treponeme* clade of FTHFS sequences. Most of the sequences included in this group (*C. punctulatus* adult clone 7C and above) share a hexapeptide insert absent from other acetogens; the basal *Microcerotermes* and *C. punctulatus* clusters were included based on the strength of their phylogenetic association with these sequences. While Cost008 was dominated by a distinct group of termite *Treponeme* FTHFS sequences, most of the *Treponeme*-like sequences amplified from higher termites formed a single cluster (Figure 3.4). These sequences grouped to the exclusion of FTHFS types from lower termites. All higher

termites examined hosted sequences that fell within the "higher termite clade"; clones affiliated with this group represented 98% of acetogenic FTHFS sequences retrieved from Cost003, 85% in Cost004, 26% in Cost008, 8% in Cost010, 1% in JT2, and 6% in JT5.

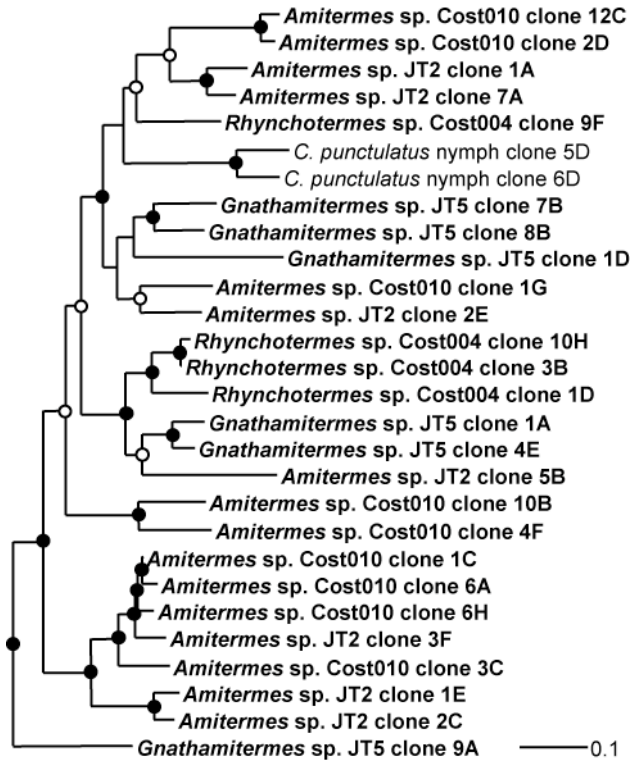

Figure 3.5. Amitermes clade of probable *Firmicute* acetogens. Tree calculated using Phylip PROML and 340 unambiguously aligned amino acids. Open circles mark groupings also supported by either Phylip PROTPARS parsimony or Fitch distance algorithms. Closed circles identify clusters grouped by all three treeing methods. Scale bar represents 0.1 amino acid changes per alignment position. An outgroup consisting of 6 cultured *Firmicute* acetogens was used to root the cluster.

The acetogenic community of the three subterranean termites (Cost010, JT2, and JT5) was dominated by a novel clade of *Firmicute*-like FTHFS sequences (the *Amitermes* clade in Figure 3.3, phylogenetic detail in Figure 3.5). This clade also included sequences from Cost004 and *C. punctulatus*. Sequences affiliated with this cluster

represented 89% of acetogenic FTHFS sequences found in JT2, 83% in JT5, 72% in Cost010, 15% in Cost004, and 4% of those found in the *C. punctulatus* nymph.

Cost010, JT2 and JT5 termites also contained sequences that fell within the *Moorella*/*Sporomusa* FTHFS clade (Figure 3.6). This clade contained FTHFS sequences both from true acetogens such as *Sporomusa termitida* (5) and *Moorella thermoacetica* (32) and from organisms that carry all or some of the machinery for the acetyl-CoA cycle but do not grow as CO_2-reductive acetogens, such as *Desulfitobacterium hafniense* (26), *Carboxydothermus hydrogenoformans* (48), and *Syntrophomonas wolfei* (21). As a result, while these sequences may belong to acetogenic organisms, we chose not to define them as such.

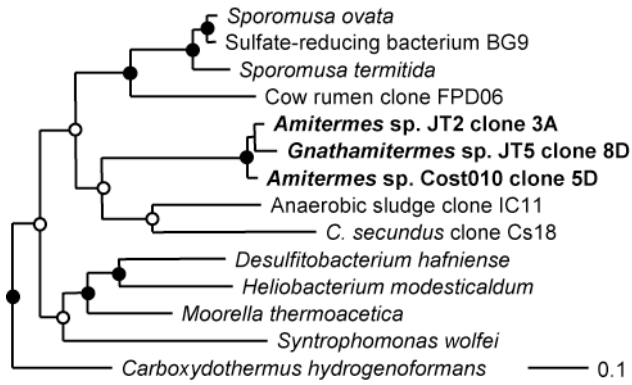

Figure 3.6. Phylogeny of *Moorella / Sporomusa* FTHFS clade. Tree calculated using Phylip PROML and 350 unambiguously aligned amino acids. Open circles mark groupings also supported by either Phylip PROTPARS parsimony or Fitch distance algorithms. Closed circles identify clusters grouped by all three treeing methods. Scale bar represents 0.1 amino acid changes per alignment position. An outgroup consisting of 7 cultured *Firmicute* acetogens was used to root the cluster.

The remaining FTHFS types were identified as probable nonacetogens (Figures 3-7 and 3-8). Two of these sequence groups can be assigned a probable role in amino acid or purine degradation. The first, described as the *Clostridium acidurici* group, included

clones that represent 45% of the FTHFS library from Cost004, and 10% of FTHFS sequences from JT5 (Figure 3.7a). This sequence cluster was closely related to FTHFS sequences from purinolytic *Firmicutes C. acidurici* (1), *Clostridium cylindrosporum* (1), and *Eubacterium acidaminophilum* (50). In these organisms, anaerobic degradation of purines results in the transfer of a formimino group to tetrahydrofolate (THF). Formimino-THF is converted to formyl-THF, and FTHFS is used to couple the release of formate and THF to generation of ATP via substrate-level phosphorylation. Uric acid degradation, which has been hypothesized as a role for gut bacteria in termite nitrogen conservation (33), can proceed via this pathway (45).

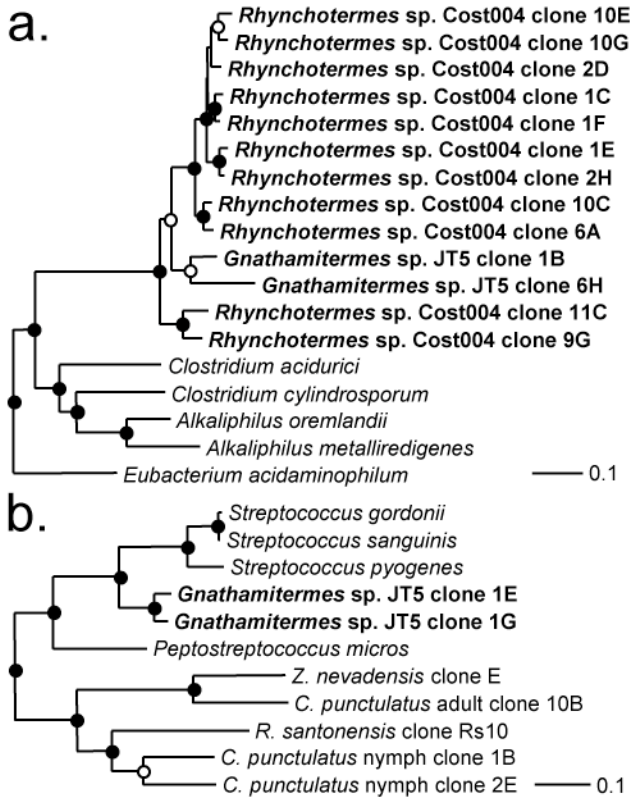

Figure 3.7. Putative amino acid or purine-degrading FTHFS clades. **a.** *C. acidurici* clade. **b.** Clone E/*Streptococcus* clade. Trees calculated using Phylip PROML and 351 unambiguously aligned amino acids. Open circles mark groupings also supported by either Phylip PROTPARS parsimony or Fitch distance algorithms. Closed circles identify clusters grouped by all three treeing methods. Scale bar represents 0.1 amino acid changes per alignment position. Trees rooted using 7 cultured *Firmicute* acetogens.

The second group of FTHFS types linked to purine or amino acid degradation is the Clone E/*Streptococcus* group (Figure 3.7b). Clones that represented 37% of the total FTHFS library from JT5 clustered with FTHFS sequences from *Peptostreptococcus micros* and three species of *Streptococcus* (*S. pyogenes, S. gordonii,* and *S. sanguinis*). While the genomic context of the FTHFS gene from *Peptostreptococcus micros* does not contain obvious functional clues, the Clone E-like FTHFS sequences in the three *Streptococci* are part of a conserved histidine degradation operon. In this context, FTHFS is again being used to generate ATP from the release of formate following the breakdown of a formimino group attached to THF. While the use of FTHFS to generate ATP from the release of formate during histidine degradation has not been formally reported in bacteria, the presence of glutamate formimidoyltransferase in certain bacterial histidine degradation operons has been observed via bioinformatics techniques (29).

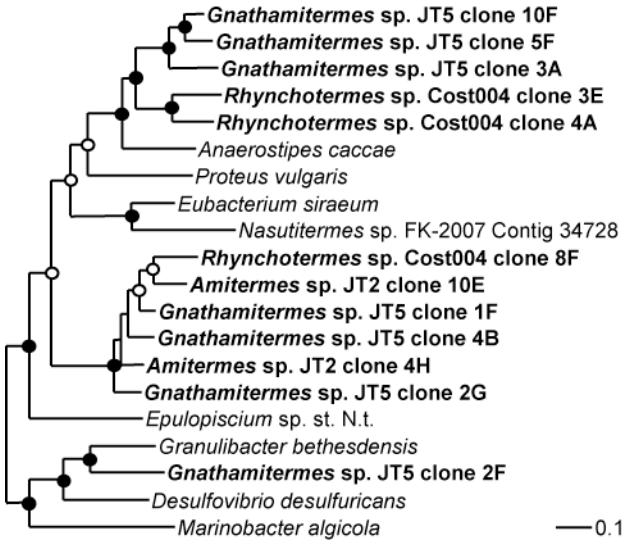

Figure 3.8. Nonacetogenic FTHFS sequences. Tree calculated using Phylip PROML and 350 unambiguously aligned amino acids. Open circles mark groupings also supported by either Phylip PROTPARS parsimony or Fitch distance algorithms. Closed circles identify clusters grouped by all three treeing methods. Scale bar represents 0.1 amino acid changes per alignment position.

Remaining nonacetogenic FTHFS sequences fall into three clusters (Figure 8). JT5 clone 2F appears to belong to a sulfate-reducing *Proteobacterium*. The cluster that includes JT5 clone 10F clone 4A appears to belong to nonacetogenic *Firmicutes*. Finally, a third cluster, including Cost004 clone 8F, does not cluster reliably with currently available FTHFS sequences.

Discussion

The diverse feeding habits of higher termites seem to have dramatic effects on the population of acetogenic bacteria in their guts. FTHFS libraries from lower termites have, without exception, proven to be dominated by sequences from the termite gut *Treponeme* clade. In higher termites, there appear to be two distinct scenarios for acetogenic bacteria. In Cost003, Cost008, and Cost004, the most abundant acetogenic bacteria appear to be termite *Treponemes* (comprising 98%, 89%, and 37% of total FTHFS). These termites appeared to primarily feed on wood, palm, and litter, respectively. In Cost010, JT2, and JT5, the most abundant acetogenic bacteria appear to be *Firmicutes* (85%, 87%, and 28% of FTHFS sequences), most of which fall within the novel "*Amitermes* clade." These termites had subterranean lifestyles that are consistent with increased exposure to humics and a grass- or soil-feeding diet.

Termite *Treponeme* FTHFS sequences from higher termites largely fell within a single "higher termite clade." This finding is a striking contrast to the diversity of FTHFS types found in lower termites, and may indicate an evolutionary bottleneck during which most lines of acetogenic *Treponemes* were lost. The higher termite clade may also represent a

symbiotic innovation that allowed this particular line of acetogens to outcompete other bacteria. The only termite gut *Treponeme* sequences that fell outside this cluster were a group of sequences found in Cost008 and a single sequence identified in Cost010. These may represent either FTHFS types lost from other lines of higher termites but retained in these insects or a reacquisition from lower termites of FTHFS types lost early in the higher termite radiation.

Cost010, JT2, and JT5 represent the first examples of termite gut communities that are not dominated by FTHFS sequences from the termite *Treponeme* clade, but rather by *Firmicute*-associated sequences. Given that *Treponeme*-associated FTHFS types are present in these termites, it seems likely that this shift in community structure is due to the presence of conditions that favor this group over acetogenic *Treponemes*. These termites have subterranean lifestyles and diets that potentially include soil-feeding. Soil-feeding *Cubitermes* spp. have been shown to have low rates of *in situ* CO_2 fixation to acetate. However, a robust population of CO_2-reductive acetogens can be detected in gut homogenates when incubated with inhibitors of methanogenesis (42). While studies in the termite gut have focused on acetogenesis from H_2 and CO_2, acetogens are capable of utilizing a wide variety of substrates (7). The acetogens present in soil-feeding termites may principally subsist on alternative sources of reducing equivalents and/or carbon, such as carbohydrates or methoxylated aromatics. The *Amitermes* clade of FTHFS types may represent organisms better adapted to this lifestyle than termite gut *Treponemes.*

Finally, although the primers utilized in this study were designed for specific detection of acetogenic FTHFS types, several intriguing nonacetogenic FTHFS types were identified in higher termite libraries. A group of FTHFS types was identified in Cost004 and JT5 that cluster with FTHFS sequences from purinolytic *Firmicutes*. Uric acid recycling by gut bacteria has been hypothesized to play a role in termite nitrogen conservation (33), and the presence of this FTHFS clade suggests that *Firmicutes* may be carrying out this activity within the guts of Cost004 and JT5. Litter-feeding *Rhynchotermes* termites have been shown to have lower rates of nitrogen fixation than wood-feeding *Nasutitermes* (35). While this was initially attributed to higher nitrogen content in their food source, uric acid recycling may also play a role. Additionally, these bacteria may aid in release of nitrogen from food material.

The Clone E / *Streptococcus* FTHFS clade also likely represents an alternative use of the FTHFS enzyme. The *Streptococci* associated with this clade appear to be utilizing FTHFS in the context of histidine degradation. The entire clade may represent FTHFS types employed in this manner or, alternatively, it may represent FTHFS adapted for formyl-THF metabolism rather than synthesis. One of the uricolytic strains isolated from *Reticulitermes flavipes* by Potrikus and Breznak (34) was a *Streptococcus* species; the termite-derived sequences may represent FTHFS genes from similar organisms.

In conclusion, the diversity of lifestyles and feeding strategies employed by higher termites coincides with a diversity of population structures among symbiotic acetogens. FTHFS sequences amplified from wood-, palm-, and litter-feeding higher termites

affiliate with the acetogenic *Treponemes* that dominate the guts of wood-feeding lower termites. However, subterranean termites, whose diets may include some level of soil-feeding (and who certainly experience greater exposure to soil), yielded a diversity of sequences that affiliate with acetogenic *Firmicutes* but few *Treponeme*-like FTHFS sequences. It has been broadly observed that wood-feeding termites (both higher and lower) have higher rates of acetogenesis than soil feeders; this may correlate with a uniquely favorable environment for acetogenic *Treponemes*.

Chapter Three Appendix

1. Table 3.3. Operational taxonomic unit grouping of FTHFS sequences identified in this study

2. Table 3.4. Sequences used in FTHFS phylogenetic analysis

3. Table 3.5. Sequences used in COII phylogenetic analysis

Table 3.3. Operational taxonomic unit grouping of FTHFS sequences identified in this study

Group	Phylotype	Abundance (%)[a]	Genotypes[b]
***Nasutitermes* sp. Cost003**			
Termite *Treponemes*	1F	23	**1F**
	2B	15	**2B**, 2F
	1A	12	**1A**, 2D, 7B
	2A	10	**2A**, 7A
	1E	6	**1E**
	2G	6	**2G**
	3H	6	**3H**
	1G	4	**1G**
	4A	4	**4A**, 4B
	4E	4	**4E**
	7D	4	**7D**
	3G	2	**3G**
	7G	2	**7G**
Acetogenic *Firmicutes*	1D	2	**1D**
***Microcerotermes* sp. Cost008**			
Termite *Treponemes*	1H	30	**1H**, 5E, 8H
	1F	15	**1F**
	2C	15	**2C**, 11A
	6G	11	**6G**, 4H
	11G	4	**11G**
	11H	4	**11H**
	3E	4	**3E**
	5F	4	**5F**
	9E	4	**9E**
Acetogenic *Firmicutes*	2D	4	**2D**
	3A	4	**3A**
	8F	4	**8F**
***Rhynchotermes* sp. Cost004**			
Termite *Treponemes*	7C	10	**7C**, 2E, 4E, 5A
	3A	8	**3A**, 8C
	6C	5	**6C**
	3C	3	**3C**
	5C	3	**5C**, 11A
	10F	2	**10F**
	11F	2	**11F**
	2A	2	**2A**
	7B	2	**7B**
	9H	2	**9H**
Acetogenic *Firmicutes*	10H	2	**10H**
	1D	2	**1D**
	3B	2	**3B**
	9F	2	**9F**
Clostridium acidiurici	1C	13	**1C**, 2B
	1E	10	**1E**, 7A, 8D, 4C, 6B
	10C	3	**10C**
	10E	3	**10E**, 12E
	1F	3	**1F**
	2H	3	**2H**, 9E
	9G	3	**9G**, 12H

Group	Phylotype	Abundance (%)[a]	Genotypes[b]
	10G	2	**10G**
	11C	2	**11C**
	2D	2	**2D**
	6A	2	**6A**
Nonacetogenic FTHFS	4A	6	**4A**
	3E	2	**3E**
	8F	2	**8F**
***Amitermes* sp. Cost010**			
Termite *Treponemes*	10A	4	**10A**
	4C	4	**4C**
	7D	4	**7D**
Acetogenic *Firmicutes*	2D	23	**2D**, 8H
	4F	15	**4F**
	1G	8	**1G**
	10B	4	**10B**
	12C	4	**12C**
	1C	4	**1C**
	1D	4	**1D**
	3C	4	**3C**
	4H	4	**4H**
	5A	4	**5A**
	5F	4	**5F**
	6A	4	**6A**
	6H	4	**6H**
Moorella / *Sporomusa*	5D	4	**5D**
***Amitermes* sp. JT2**			
Termite *Treponemes*	7E	1	**7E**
Acetogenic *Firmicutes*	1E	48	**1E**, 1B, 8G, 12B
	1A	20	**1A**, 2G, 1F
	2E	6	**2E**
	2C	2	**2C**
	3F	2	**3F**
	5D	3	**5D**, 8A
	7A	2	**7A**
	2D	1	**2D**
	4D	1	**4D**
	5B	1	**5B**
Moorella / *Sporomusa*	2H	6	**2H**, 3A
Nonacetogenic FTHFS	4H	2	**4H**, 3D
	10E	1	**10E**
***Gnathamitermes* sp. JT5**			
Termite *Treponemes*	12A	2	**12A**
Acetogenic *Firmicutes*	4E	7	**4E**
	1D	5	**1D**
	8B	5	**8B**
	1A	3	**1A**
	9A	3	**9A**
	10A	2	**10A**
	2E	2	**2E**
	7B	2	**7B**
Moorella / *Sporomusa*	8D	2	**8D**
Clostridium acidiurici	1B	7	**1B**
	6H	3	**6H**
Clone E / *Streptococcus*	1G	27	**1G**, 8E

Group	Phylotype	Abundance (%)[a]	Genotypes[b]
Nonacetogenic FTHFS	1E	10	**1E**
	3A	7	**3A**, 9F
	10F	2	**10F**
	1F	2	**1F**
	2F	2	**2F**
	2G	2	**2G**
	4B	2	**4B**
	5F	2	**5F**

[a]Defined as percent of full-length, nonchimeric clones
[b]Sequenced RFLP type clones. Group representative marked in bold.

Table 3.4. Sequences used in FTHFS phylogenetic analysis

Source / Sequence Type	Designation	Accession	Reference
Cryptocercus punctulatus adult gut clone	1A		
Cryptocercus punctulatus adult gut clone	1F		
Cryptocercus punctulatus adult gut clone	4F		
Cryptocercus punctulatus adult gut clone	5B		
Cryptocercus punctulatus adult gut clone	6D		
Cryptocercus punctulatus adult gut clone	7C		
Cryptocercus punctulatus adult gut clone	7H		
Cryptocercus punctulatus adult gut clone	10B		
Cryptocercus punctulatus nymph gut clone	1B		
Cryptocercus punctulatus nymph gut clone	2E		
Cryptocercus punctulatus nymph gut clone	5D		
Cryptocercus punctulatus nymph gut clone	6D		
Cryptotermes secundus gut clone	Cs3	DQ278251	(30)
Cryptotermes secundus gut clone	Cs18	DQ278253	(30)
Cryptotermes secundus gut clone	Cs27	DQ278254	(30)
Cryptotermes secundus gut clone	Cs56	DQ278258	(30)
Incisitermes sp. Pas-1 gut clone	1B		
Incisitermes sp. Pas-1 gut clone	1F		
Incisitermes sp. Pas-1 gut clone	2A		
Incisitermes sp. Pas-1 gut clone	7D		
Reticulitermes santonensis gut clone	Rs10	DQ278259	(30)
Reticulitermes santonensis gut clone	Rs13	DQ278232	(30)
Reticulitermes santonensis gut clone	Rs23	DQ278210	(30)
Reticulitermes santonensis gut clone	Rs44	DQ278211	(30)
Reticulitermes santonensis gut clone	Rs57	DQ278215	(30)
Reticulitermes santonensis gut clone	Rs119	DQ278226	(30)
Reticulitermes santonensis gut clone	Rs129	DQ278222	(30)
Reticulitermes santonensis gut clone	Rs144	DQ278223	(30)
Reticulitermes santonensis gut clone	Rs158	DQ278226	(30)
Reticulitermes santonensis gut clone	Rs239	DQ278201	(30)
Reticulitermes santonensis gut clone	Rs280	DQ278207	(30)
Reticulitermes santonensis gut clone	Rs296	DQ278208	(30)
Zootermopsis angusticollis gut clone	A	AY162294	(37)
Zootermopsis angusticollis gut clone	E	AY162296	(37)
Zootermopsis angusticollis gut clone	F	AY162298	(37)
Zootermopsis angusticollis gut clone	H	AY162302	(37)
Zootermopsis angusticollis gut clone	N	AY162306	(37)
Zootermopsis angusticollis gut clone	P	AY162307	(37)
Nasutitermes sp. FK-2007 metagenome	Contig34728	JGI GOI: 2004131907	(46)
Nasutitermes sp. FK-2007 metagenome	Contig40968	JGI GOI: 2004144560	(46)
Cow Rumen clone	FPDO6	AB085528	Database only
Anaerobic sludge clone	IC11	EU009529	Database only
Aceotbacterium woodii		AF295701	(15)
Alkaliphilus metalliredigenes QYMF		CP000724	Database only
Alkaliphilus oremlandii		NC_009922	Database only
Anaerostipes caccae		ABAX03000038	Database only
Carboxydothermus hydrogenoformans Z-2901		ABB16038	(48)
Clostridium aceticum		AF295705	(15)
Clostridium acidurici		M21507	(47)
Clostridium cylindrosporum		L12465	(36)
Clostridium formicaceticum		AF295702	(15)

Source / Sequence Type	Designation	Accession	Reference
Clostridium magnum		AF295703	(15)
Desulfitobacterium hafniense st. Y51		NC_007907	(26)
Desulfovibrio desulfuricans		AJ494753	(14)
Epulopiscium sp. st. N.t. morphotype B		NZ_ABEQ01000077	Database only
Eubacterium acidaminophilum		AY722711	Database only
Eubacterium limosum		AF295706	(15)
Eubacterium siraeum		ABCA03000037	Database only
Granulibacter bethesdensis		NC_008343	(10)
Heliobacterium modesticaldum		NC_010337	(38)
Marinobacter algicola		ZP_01892361	Database only
Moorella thermoacetica		NC_007644	(32)
Peptostreptococcus micros		NZ_ABEE02000017	Database only
Proteus vulgaris		AF295710	(15)
Ruminococcus productus		AF295707	(15)
Sporomusa ovata		AF295708	(15)
Sporomusa termitida		AF295709	(15)
Streptococcus gordonii		NC_009785	(44)
Streptococcus pyogenes st. SSI-1		BAC64868	(25)
Streptococcus sanguinis		NC_009009	(49)
Sulfate-reducing bacterium BG9		AJ494757	(14)
Syntrophomonas wolfei subsp. wolfei str. Goettingen		EAO23711	Database only
Thermoanaerobacter kivui		AF295704	(15)
Treponema azotonutricium st. ZAS-9		AY162316	(37)
Treponema denticola		NC_002967	(40)
Treponema primitia st. ZAS-1	ZAS-1a	AY162313	(37)
Treponema primitia st. ZAS-2		AY162315	(37)

Table 3.5. Sequences used in COII phylogenetic analysis

Source	Accession	Reference
Amitermes evuncifer	DQ442066	(12)
Archotermopsis wroughtoni	DQ442080	(12)
Coptotermes formosanus	AB109529	(28)
Cornitermes pugnax	DQ442106	(12)
Cryptocercus primarius	DQ007644	(17)
Cryptocercus punctulatus adult		
Cryptocercus punctulatus nymph		
Cryptotermes secundus	DQ442111	(12)
Incisitermes sp. Pas1		
Kalotermes hilli	AF189101	(43)
Labiotermes labralis	DQ442149	(12)
Microcerotermes newmani	DQ442166	(12)
Microcerotermes parvus	DQ442167	(12)
Nasutitermes ephratae	AB037328	(23)
Nasutitermes nigriceps	DQ442193	(12)
Nasutitermes sp. FK-2007	EU236539	(46)
Reticulitermes santonensis	AF291742	(19)
Reticulitermes speratus	AB109530	(28)
Zootermopsis angusticollis	DQ442267	(12)
Zootermopsis nevadensis		

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