Molecular Community Analysis of Acetogenic Bacteria in Roaches and Lower Termites: Evolution of the Symbiosis between Termites and Acetogenic *Spirochetes*

Abstract

The termite gut is host to a highly active population of acetogenic bacteria, which can fuel up to 1/3 of the energy metabolism of the host insect. In order to shed light on the roots of this symbiosis, we carried out molecular community analysis of acetogens present in the guts of the wood-feeding roach *Cryptocercus punctulatus* and lower termites of the genus *Incisitermes*. Acetogenesis in the termite gut is carried out primarily by spirochetes from the genus *Treponema.* Termite *Treponemes* appear to have acquired the ability to carry out acetogenesis by lateral gene-transfer from acetogenic *Firmicutes*, and this capacity has not yet been identified in free-living spirochetes. Phylogenetic analysis of the gene for formyl-tetrahydrofolate synthetase (FTHFS) suggests that spirochetes acquired this component of the acetogenic pathway prior to the roach-termite divergence, and that at least three species of FTHFS-bearing spirochetes were present in the last common ancestor of *Cryptocercus* and *Isoptera*.

Introduction

Molecular phylogeny of *Isoptera* and related insect species confirms that eusocial termites diverged from wood-feeding roaches whose modern representatives exist as the roach family *Cryptocercidae* (9, 16). Wood-feeding *Cryptocercus* are known to possess

complex microbial communities that share many characteristics with the microbiota of termites, including the presence of cellulolytic flagellates (5) and high rates of reductive acetogenesis (3). However, a long-standing question remains as to whether this similarity is due to vertical transmission from a wood-feeding common ancestor or whether the roach hindgut community was acquired from true termites via a lateral community-transfer event (23, 36, 37). Phylogenetic examination of ribosomal RNA genes from cellulolytic flagellates and gut bacteria have yet to yield clear evidence of either vertical or horizontal transmission of symbionts (i.e., branch patterns that robustly demonstrate congruent (or incongruent) host-symbiont evolutionary histories) (4, 8, 25, 33).

CO_2-reductive acetogenesis is a major electron sink in the guts of wood-feeding termites, accounting for 18%–26% of respiratory electron flow (2, 3, 29) and generating 10%–30% of the acetate produced in this environment (3, 34). Microbially generated acetate is the principal source of carbon for oxidation and biosynthesis for the termite host (24). The diversity of acetogens present in an environmental sample can be investigated using the gene for formyl-tetrahydrofolate synthetase (FTHFS), a key enzyme in the Wood-Ljungdahl pathway of reductive acetogenesis (14), as a marker of acetogenic capability (12, 17).

FTHFS diversity has been examined in three lower termites: *Zootermopsis nevadensis, Cryptotermes secundus*, and *Reticulitermes santonensis* (28, 31). The hindgut communities of all three termites were dominated by FTHFS sequences that cluster phylogenetically with the FTHFS genes of acetogenic spirochetes isolated from the

termite *Zootermopsis angusticolis* (11, 31). Fragmentary FTHFS gene sequences present in the metagenome of *Nasutitermes* termites also fell within this "termite *Treponeme* cluster" (38). Termite *Treponeme* FTHFS sequences cluster more broadly with FTHFS sequences from acetogenic *Firmicutes*, rather than an FTHFS from *Treponema denticola*, suggesting that this gene may have been acquired via lateral gene-transfer (31).

To date, the guts of termites are the only environment in which acetogenic spirochetes have been identified. This suggests that the acquisition of acetogenic capability by members of the genus *Treponema* occurred within the context of this symbiosis. Additionally, the presence of acetogenic spirochetes in members of four major lineages of termites on four continents suggests that this lateral gene-transfer event took place early in the evolutionary history of this insect. FTHFS and 16S rRNA phylogenies both suggest some degree of coevolution between lower termites and their symbiotic spirochetes (1, 28). However, within the termite gut *Treponeme* clade, each lower termite carries multiple, polyphyletic FTHFS sequence groups. This diversity might have been generated by either repeated horizontal symbiont-transfer or by vertical transmission from a common ancestor with multiple FTHFS-bearing spirochetes.

In this study, we examine the acetogenic bacteria present in roaches of the family *Cryptocercidae*. By examining the diversity and phylogeny of FTHFS genes present in wood-feeding roaches and lower termites, we hope to trace the evolutionary history of this symbiosis, shedding light on the mechanisms that have generated and maintained this remarkable association.

Materials and Methods

Insect Collection

Incisitermes minor termites were collected from a wood pile in Pasadena. *Cryptocercus punctulatus* were collected by Christina Nalepa (NC State University). The adult sample was from a roach collected at Mt. Collins, the nymphs from the South Mountains. The insects were shipped priority mail; upon receipt, they were maintained in glass jars in the dark.

DNA Extraction

DNA was extracted from whole dissected guts. The *C. punctulatus* adult sample contained a single gut, the *C. punctulatus* nymph sample contained three pooled guts, and the *Incisitermes* sample contained the guts of 7 workers. *C. punctulatus* guts were prepared within a week of receipt, *Incisitermes* within 24 hours of collection. DNA was purified 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 (12). 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), 0.0525 U/μL Expand High Fidelity Taq polymerase (Roche) and 1 ng/μL template, using the recommended step-down protocol (12) and 25 cycles at 55 ºC. 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. PCR product was analyzed by gel electrophoresis to verify the presence of full-length insert. 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 of each RFLP type was selected for sequencing. Samples were amplified using vector primers SL1 and SR2 as described above, but with the substitution of EXPAND high fidelity polymerase. Cycle sequencing reactions were carried out by Laragen (Los Angeles, CA).

COII Identification of Termites and Cockroaches

Roach and termite identifications were confirmed using insect mitochondrial cytochrome oxidase subunit II (COII) gene sequences (Figure 2.1). COII genes were amplified directly from the DNA samples used for FTHFS analysis. *Cryptocercus punctulatus* COII was amplified using primers and cycling conditions described in Park et al. (27). *Incisitermes* COII was amplified using the primers CI-J-1773 and B-tLys and cycling conditions described in Miura et al. (21). FailSafe Premix D (Epicentre) and Expand high fidelity Taq (Roche) were substituted for the polymerase and buffers described.

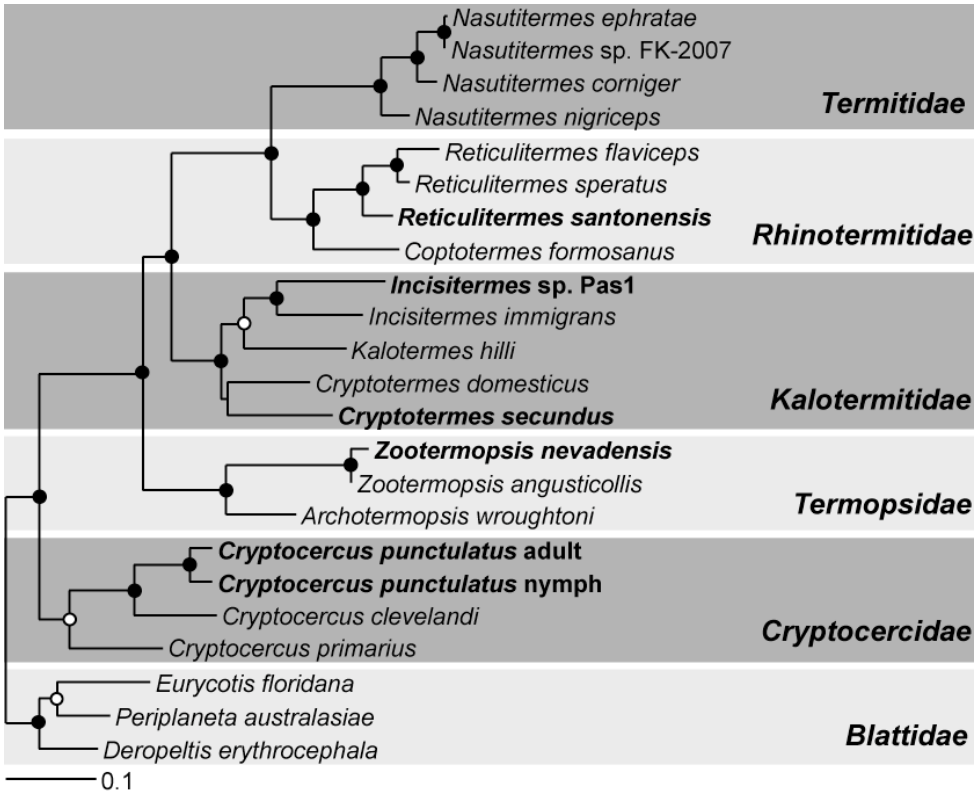

Figure 2.1. Mitochondrial cytochrome oxidase II phylogeny of termites and roaches. Species from which gut FTHFS diversity has been examined marked in bold. Tree calculated using AxML and 396 unambiguously aligned DNA bases. Open circles mark groupings also supported by either Phylip DNAPARS parsimony 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. Alternate *R. santonensis* and *C. secundus* COII sequences used to represent those from Pester and Brune (28), which were truncated.

Sequence Analysis

Sequence reads were assembled and edited using the Lasergene software package (version 7.2.1, DNASTAR). FTHFS protein sequences were aligned using MUSCLE (6), and phylogenetic analyses were carried out using the ARB software package (18). A single chimeric sequence was identified in the *Incisitermes* library using the Bellerophon program (7), and eliminated from further analysis. Sequence similarities given in the text of this paper represent amino acid similarities calculated using an ARB neighbor joining matrix and 352 alignment positions.

Results

DNA was extracted from the pooled guts of 7 *Incisitermes* workers and from 2 *C. punctulatus* whole gut samples, one containing a single adult gut and the other 3 pooled nymph guts. FTHFS sequences from each sample were amplified and cloned to generate libraries that represent a snapshot of the acetogenic diversity in these environments. The libraries were sorted by restriction fragment length polymorphism (RFLP), and a single representative of each RFLP type sequenced, generating 26, 29, and 16 nonchimeric phylotypes corresponding to 60, 88, and 90 total clones, respectively. These phylotypes were characterized by phylogenetic analysis (Table 2.1), and were further binned into operational taxonomic units with a cutoff of 98% amino acid sequence similarity (Table 2.2).

Table 2.1. Composition of FTHFS libraries constructed from *C. punctulatus* and *Incisitermes* sp. Pas1[a]

Species	*Treponemes*			Acetogenic Firmicutes	Clone E / *Streptococcus*	Non-acetogenic
	I	II	III			
C. punctulatus adult	49	3	27	6	2	6
C. punctulatus nymph	22	6	8	6	36	2
Incisitermes sp. Pas1	37	63	-	-	-	-

[a] Abundance given as percentage of full-length clones

All of the FTHFS sequences from *Incisitermes* termites, 79% of those from the adult roach, and 36% of those amplified from the roach nymph fell within the termite *Treponeme* cluster (Figure 2.2). Many of the FTHFS sequences recovered from *Incisitermes* grouped closely with sequences identified in *C. secundus*, which also falls within the *Kalotermitidae* family of lower termites. The *C. punctulatus* individuals contained three major groups of termite *Treponeme*-like FTHFS sequences. Roach group III formed a cluster that was identified as basal to the termite *Treponem*e radiation by two of the three treeing methods used (the Fitch distance algorithm clustered Roach group II and several associated lower termite sequences with Roach group III). Sequences of Roach group III lacked the hexapeptide insert characteristic of the termite *Treponeme* clade. Roach group II was the least abundant of the three groups, and affiliated with sequences basal to most of the termite *Treponeme* cluster. Roach group I lies near the middle of the termite *Treponeme* radiation, but is basal to a radiation that includes the cultured acetogen *Treponema primitia* strain ZAS-1 and sequences from representatives of all three families of lower termites examined.

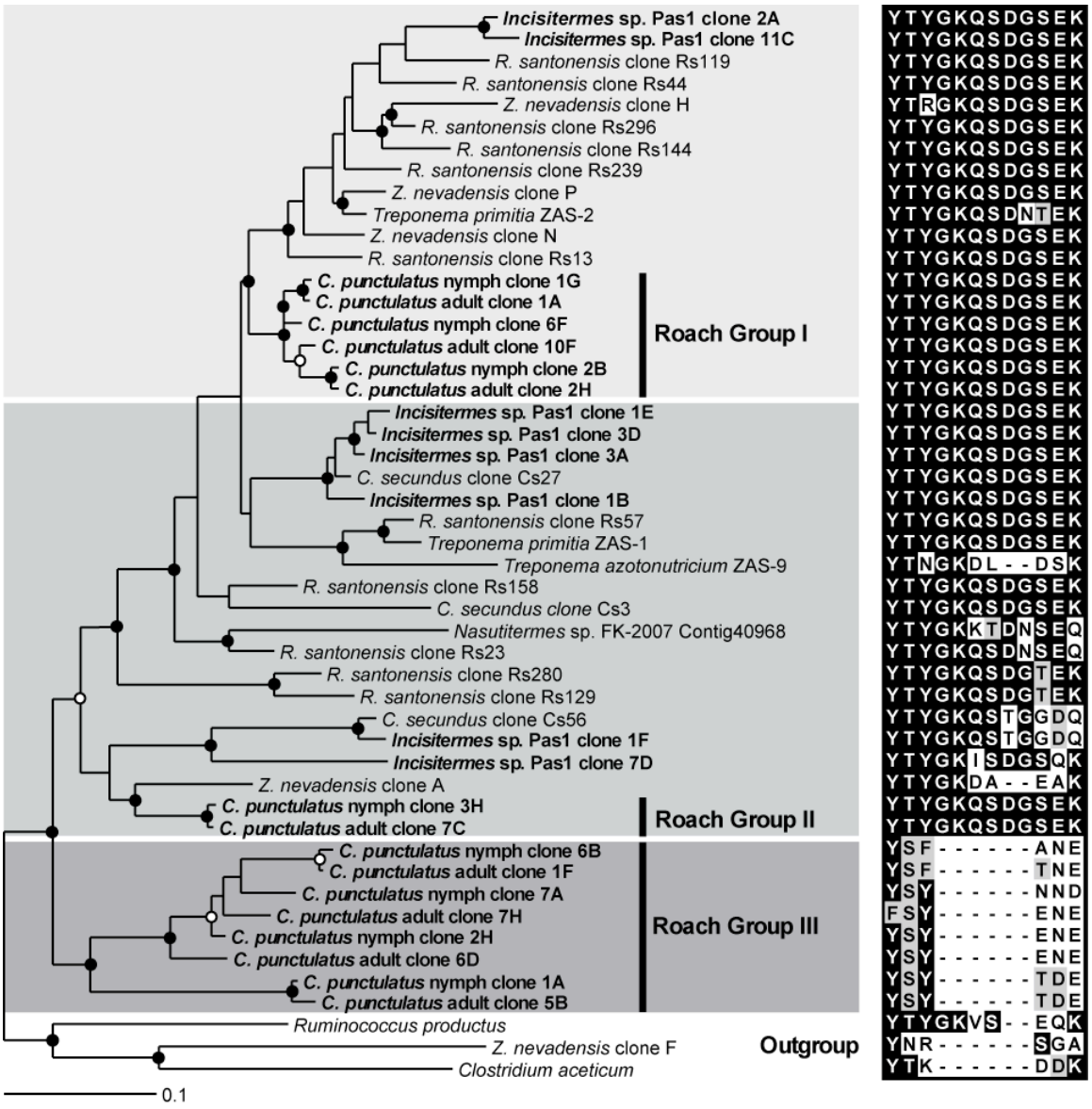

Figure 2.2. Phylogenetic analysis of termite *Treponeme* FTHFS sequences. ***Left***. Tree constructed using Phylip PROML algorithm and 351 unambiguously aligned amino acid positions. Open circles mark groupings also supported by either Phylip PROTPARS parsimony or Fitch distance algorithms. Closed circles identify clusters grouped by all three treeing methods. Scale bar represents 0.1 amino acid changes per alignment position. ***Right***. A highly variable region of the protein sequence, corresponding to residues 229–234 in *M. thermoacetica.* Each line of the alignment corresponds to a sequence in the tree at left.

Six percent of the FTHFS sequences in each *C. punctulatus* individual group phylogenetically with FTHFS sequences from acetogenic *Firmicutes* (Figure 2.3). Two

of these sequence types contained clones from both adult and nymph, and may represent small but stable populations within the roach gut. While the phylogenetic analysis presented in Figure 2.3 suggests that *Z. nevadensis* clone F and affiliated sequences may be basal to FTHFS sequences present in *C. punctulatus*, this relationship is not supported when the analysis is carried out with a larger sequence library.

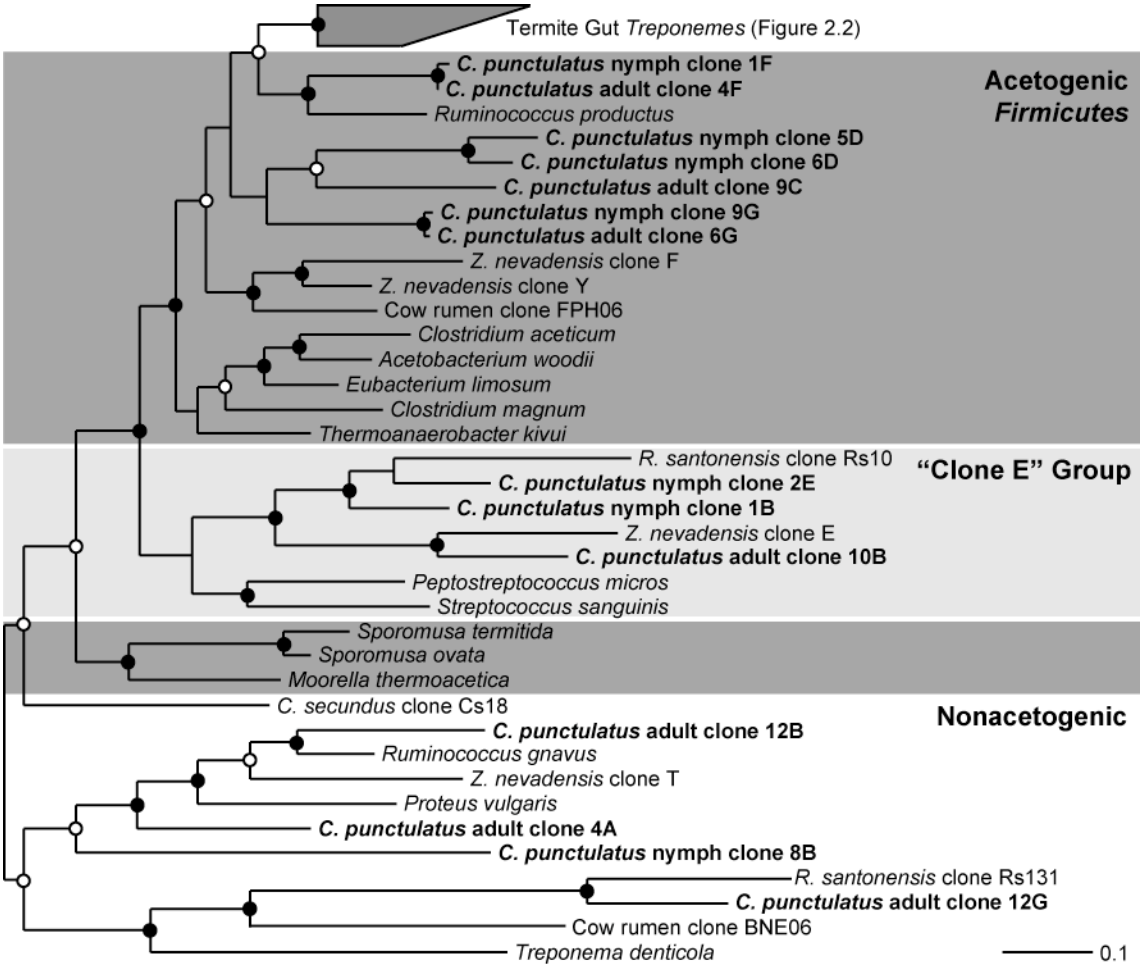

Figure 2.3. Phylogenetic analysis of FTHFS sequences from roaches and lower termites. Tree constructed using Phylip PROML algorithm and 317 unambiguously aligned amino acid positions. Open circles mark groupings also supported by either Phylip PROTPARS parsimony or Fitch distance algorithms. Closed circles identify clusters grouped by all three treeing methods. Scale bar represents 0.1 amino acid changes per alignment postion. Fifteen sequences from *C. punctulatus* and *Z. nevadensis* were chosen to represent the termite *Treponeme* group in this analysis.

Thirty-six percent of sequences from the roach nymph, and two percent of those from the adult, clustered with *Z. nevadensis* clone E and *R. santonensis* clone Rs10. This group was clustered with sequences from *Peptostreptococcus micros* and *Streptococcus pyogenes*, and is most likely nonacetogenic. Remaining FTHFS types were most likely nonacetogenic but were not closely related to cultured organisms.

Discussion

The presence of acetogenic spirochetes in the hindgut appears to be a common characteristic of termites and wood-feeding roaches. In the three termite species previously examined, *Treponeme*-like FTHFS sequences were more abundant than those from acetogenic *Firmicutes* (28, 31). In this study, we confirmed that this pattern is repeated in the lower termite *Incisitermes* sp. Pas1, and extends to the gut of the wood-feeding roach *C. punctulatus*.

All wood-feeding insects examined hosted multiple sequence types within the termite *Treponeme* clade, with the least diverse, *C. secundus*, containing three distinct groups of FTHFS sequence, and the most diverse, *R. santonensis*, containing 11 groups. The most closely related termites, *Incistermes* sp. and *C. secundus*, contained FTHFS types that were 97% similar, despite extensive geographic separation (*Incisitermes* were collected in Pasadena, CA, while *C. secundus* was collected in Darwin, Australia). This tendency of sequences from a single species of termite to group more closely with each other than with those from other termite species, and the tendency of closely related termites to host phylotypes more similar to each other than to distantly related termites, has been

observed in spirochete rRNA analyses (1, 13), and suggests a high degree of host-symbiont coevolution.

The *C. punctulatus* individuals examined in this study contained three novel lineages of termite *Treponeme*-like FTHFS sequences. One, referred to in this study as roach group III, is basal to the termite *Treponeme* clade, and lacked a hexapeptide insert characteristic of other termite gut *Treponeme* FTHFS types. The basal position of this clade and its absence from lower termites suggests that it represents an evolutionarily ancient lineage, present in the last common ancestor of termites and roaches, that was lost prior to the radiation of the lower termites examined. Because roach group III clustered consistently with termite gut *Treponemes* to the exclusion of all other FTHFS types, we propose that it represents a line of *Treponemes* that diverged following the acquisition of acetogenic capability by spirochetes but prior to the acquisition of the hexapeptide insert. Alternatively, this group may represent descendants of the *Firmicute* from which termite *Treponemes* acquired their FTHFS genes.

C. punctulatus hosts two additional groups of *Treponeme*-like FTHFS sequences (roach groups I and II). These were again distinct from termite-derived FTHFS types, with less than 93% amino acid similarity to the most closely related termite-derived sequences. Phylogenetic analysis shows that roach group I is basal to a termite *Treponeme* subclade that encompasses sequences present in all three lower termite families examined. The remaining FTHFS sequences (associated with roach group II) do not appear to be

monophyletic, and may be descendents of a more extensive radiation of acetogenic spirochetes present in the last common ancestor but lost from *C. punctulatus*.

In conclusion, we posit that the acquisition of acetogenic capability by gut spirochetes occurred prior to the divergence of *Cryptocercidae* and *Isoptera*. Furthermore, the three lineages of *Treponeme*-like FTHFS types identified in *C. punctulatus* are proposed to represent an ancestral radiation of acetogenic spirochetes, whose further divergence gave rise to the rich diversity of FTHFS types observed in wood-feeding lower termites.

Chapter Two Appendix

1. Table 2.2. Operational taxonomic unit grouping of FTHFS sequences identified in this study

2. Table 2.3. Sequences used in FTHFS phylogenetic analysis

3. Table 2.4. Sequences used in COII phylogenetic analysis

Table 2.2. Operational Taxonomic Unit Grouping of FTHFS sequences identified in this study

Group	Phylotype	Abundance (%)[a]	Genotypes[b]
***C. punctulatus* adult**			
Treponeme Group I	1A	40	**1A**, 1B, 1C, 3B, 3D, 4B, 5D, 10E
	2H	7	**2H**
	10F	2	**10F**
Treponeme Group II	7C	3	**7C**
Treponeme Group III	1F	20	**1F**, 1G, 6E
	7H	3	**7H**
	5B	2	**5B**
	6D	2	**6D**
Acetogenic *Firmicutes*	4F	2	**4F**
	6G	2	**6G**
	9C	2	**9C**
Clone E Group	10B	2	**10B**
Nonacetogenic	4A	2	**4A**
	12B	2	**12B**
	12G	2	**12G**
***C. punctulatus* nymph**			
Treponeme Group I	1G	16	**1G**, 1E, 11G
	2B	15	**2B**, 1C, 3G, 10H
	6F	2	**6F**, 6E
Treponeme Group II	3H	6	**3H**, 7F
Treponeme Group III	2H	3	**2H**, 1D, 9D
	1A	2	**1A**, 12G
	9C	2	**7A**, 9C
	6B	1	**6B**
Acetogenic *Firmicutes*	1F	3	**1F**, 6A
	5D	1	**5D**
	6D	1	**6D**
	9G	1	**9G**
Clone E Group	1B	35	**1B**, 3A, 3C
	2E	1	**2E**
Nonacetogenic	8B	2	**8B**
***Incisitermes* sp. Pas1**			
Treponeme Group I	2A	34	**2A**, 3C, 3F, 3G, 8B
	11C	2	**11C**, 11G
Treponeme Group II	1B	40	**1B**, 11F
	3D	12	**3D**, 4B, 11B
	1F	4	**1F**
	1E	2	**1E**
	3A	2	**3A**
	7D	2	**7D**

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

Table 2.3. Sequences used in FTHFS phylogenetic analysis

Source / Sequence Type	Designation	Accession	Reference
T. primitia ZAS-1	ZAS-1a	AY162313	(31)
T. primitia ZAS-2	ZAS-2	AY162315	(31)
T. azotonutricium ZAS-9	ZAS-9	AY162316	(31)
Z. angusticollis Gut Clone	A	AY162294	(31)
Z. angusticollis Gut Clone	E	AY162296	(31)
Z. angusticollis Gut Clone	F	AY162298	(31)
Z. angusticollis Gut Clone	H	AY162302	(31)
Z. angusticollis Gut Clone	N	AY162306	(31)
Z. angusticollis Gut Clone	P	AY162307	(31)
Z. angusticollis Gut Clone	T	AY162309	(31)
Z. angusticollis Gut Clone	Y	AY162311	(31)
C. secundus Gut Clone	Cs3	DQ278251	(28)
C. secundus Gut Clone	Cs18	DQ278253	(28)
C. secundus Gut Clone	Cs27	DQ278254	(28)
C. secundus Gut Clone	Cs56	DQ278258	(28)
R. santonensis Gut Clone	Rs10	DQ278259	(28)
R. santonensis Gut Clone	Rs13	DQ278232	(28)
R. santonensis Gut Clone	Rs23	DQ278210	(28)
R. santonensis Gut Clone	Rs44	DQ278211	(28)
R. santonensis Gut Clone	Rs57	DQ278215	(28)
R. santonensis Gut Clone	Rs119	DQ278226	(28)
R. santonensis Gut Clone	Rs129	DQ278222	(28)
R. santonensis Gut Clone	Rs131	DQ278221	(28)
R. santonensis Gut Clone	Rs144	DQ278223	(28)
R. santonensis Gut Clone	Rs158	DQ278226	(28)
R. santonensis Gut Clone	Rs239	DQ278201	(28)
R. santonensis Gut Clone	Rs280	DQ278207	(28)
R. santonensis Gut Clone	Rs296	DQ278208	(28)
Nasutitermes sp. FK-2007	Contig40968	JGI GOI: 2004144560	(38)
Cow Rumen Clone	BNE06	AB085284	Database only
Cow Rumen Clone	FPH06	AB085574	Database only
Acetobacterium woodii		AF295701	(12)
Clostridium aceticum		AF295705	(12)
Clostridium magnum		AF295703	(12)
Eubacterium limosum		AF295706	(12)
Moorella thermoacetica		NC_007644	(30)
Peptostreptococcus micros		NZ_ABEE02000017	Database only
Proteus vulgaris		AF295710	(12)
Ruminococcus gnavus		NZ_AAYG02000005	Database only
Ruminococcus productus		AF295707	(12)
Sporomusa ovata		AF295708	(12)
Sporomusa termitida		AF295709	(12)
Streptococcus sanguinis		NC_009009	(39)
Thermoanaerobacter kivui		AF295704	(12)
Treponema denticola		NC_002967	(32)

Table 2.4. Sequences used in COII phylogenetic analysis

Source	Accession	Reference
Archotermopsis wroughtoni	DQ442080	(10)
Deropeltis erythrocephala	DQ874271	(9)
Coptotermes formosanus	AB109529	(26)
Cryptocercus clevelandi	DQ007626	(15)
Cryptocercus primarius	DQ007644	(15)
Cryptotermes domesticus	AF189086	(35)
Cryptotermes secundus	AF189093	(35)
Incisitermes immigrans	AB109542	(26)
Kalotermes hilli	AF189101	(35)
Nasutitermes corniger	AB037327	(22)
Nasutitermes ephratae	AB037328	(22)
Nasutitermes nigriceps	AB037329	(22)
Nasutitermes sp. FK-2007	EU236539	(38)
Eurycotis floridana	DQ874283	(9)
Periplaneta australasiae	DQ874310	(9)
Reticulitermes flaviceps	AB109532	(26)
Reticulitermes santonensis	AF291743	(19)
Reticulitermes speratus	AB109530	(26)
Zootermopsis angusticollis	DQ442267	(10)

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