General Introduction

The study of acetogenesis in the termite hindgut began with two key papers on methane production. The first was the 1982 proposal by Zimmerman et al. that the world termite population (2.4×10^{17}) consumed 28% of the total biomass produced each year, and could be responsible for 15%–56% of global yearly methane production (97). The second was Odelson and Breznak's 1983 study of fatty acid production in termite guts, and their observation that the ratio of CH_4 to CO_2 emitted by termites was far lower than that expected based on the current understanding of acetate fermentation in that system (66).

Later studies challenged Zimmerman's estimate; it is currently accepted that termites are responsible for up to 2% of global CO_2 and 2%–4% of global CH_4 production (81). However, Odelson and Breznak's observation has withstood the test of time; as they hypothesized, the dominant H_2 sink in wood-feeding termites is not methanogenesis but CO_2-reductive acetogenesis. As a result of acetogenesis, wood-feeding termites emit only trace quantities of methane (68), in stark contrast to the superficially similar cellulose-fermenting ecosystem of the cow rumen, the source of 8% of global methane production (48) (ruminants in general are responsible for 15%–19% (30)).

My work focuses on the bacteria responsible for acetogenesis in the termite gut: their evolutionary history, the effect of termite lifestyle on acetogen population structure, and

the development of molecular techniques for improved enumeration and identification of uncultured bacteria affiliated with this group.

In the introductory section of this thesis, I will briefly summarize key elements of termite phylogeny and nutritional ecology, the microbes present in the termite gut, and the roles played by gut microbes in termite nutrition. The focus will then shift to acetogenesis, its relationship to termite nutrition, the biology of acetogenic isolates from termites, and the results of previous molecular characterizations of the termite gut acetogens.

Termite Phylogeny and Biology

Termites are insects of the order *Isoptera*. *Isoptera* encompasses over 281 genera and 2,600 species (50). There are 7 generally accepted termite families, 6 of lower termites and the "higher termite" family *Termitidae*. Termites associate phylogenetically with the roach and mantid insect orders (46).

Given the focus of this work on the gut microbiota, it is important to discuss briefly the gut morphology of termites and its relationship to termite diet and microbial composition. The termite gut is divided into the foregut, which contains crop and gizzard, the midgut and the hindgut, which is the major site of microbial activity. The hindgut is divided into P1–P5 sections: the P1, a chamber of greater or lesser size, the P2, a valve between P1 and P3, the P3 paunch, the largest chamber of the hindgut, the P4 colon, and the P5 rectum.

Lower termites feed exclusively on dead plant material, primarily wood (some species eat grass) (50). They have a relatively simple gut structure, with a minimal P1 and the bulk of the symbiotic microbial community housed in a single, large chamber that encompasses both the expanded paunch (P3) and a tapering colon (P4) (63). All lower termites have symbiotic protists in their guts, which are thought to aid cellulose digestion (discussed in detail in later sections).

Higher termites are divided into 4–6 subfamilies. The *Macrotermitinae*, thought to be the most basal group of higher termites (47), are the fungus-cultivating termites. These termites harvest plant material and build it into combs for fungal growth; the fungus-degraded material is then digested by the termite (70). *Macrotermitinae*, likely due to the externalization of many symbiotic functions, have expanded midguts and reduced, relatively simple hindguts (64).

The remaining 3–5 subfamilies of higher termite make use of a diversity of feeding strategies, including wood-, grass-, litter-, and soil-feeding. These higher termites have complex hindguts, with well-defined P1 and P3 segments and frequently at least one additional segmentation in the P4/P5 region (64). Each of these chambers is relatively independent, with distinctive pH (4, 17, 18) and microbial communities (34, 73, 74, 85). In wood-feeding termites, the P1 segment has a pH 10-11 and a circumneutral P3 segment (17). Soil-feeders have P1 segments with pH 11-12.5, P3 with pH > 10, and neutral P4b (18).

Termite Gut Microorganisms

Termite guts contain complex microbial communities that span all three domains of life (13, 16). This assemblage represents a stable association; termite gut microorganisms are distinct from those present in the food supply and immediate environment of the host, and organisms found in one termite species are generally most closely related to microbes associated with other termites. In this work, I refer to this association as a *symbiosis* according to the original definition of that term, a close association of two or more organisms; this does not necessarily imply a beneficial or mutualistic relationship.

Termite Gut Protists

Termite gut protists are among the most visually striking and longest-studied termite gut symbionts. All lower termites harbor from 1–11 species of protists, which are key to the ability of these termites to digest wood (44). Protist species composition is generally host specific (53). These protists fall into three orders: *Hypermastigida, Trichomonadida*, and *Oxymonadida*. Hypermastigotes and Trichomonads have been shown to digest cellulose in axenic culture (93, 94). The only evidence for cellulose digestion by Oxymonads is the differential survival of some species in xylan fed vs. cellulose fed *Reticulitermes speratus* (79).

Many protist species within the termite have further symbioses with bacteria. Several termite gut flagellates have endosymbionts that may provide amino acid and cofactor synthetic capabilities (see comments on *Endomicrobia* in the gut bacteria section). Other prokaryote-protist symbioses include endosymbiotic methanogens (presumably involved

in H_2 transfer) (58), the use of ectosymbiotic bacteria to provide motility (22, 82), and the use of ectosymbiotic bacteria for osmotic regulation and sensory functions by *Streblomastix strix* (29).

Archaea in the Termite Gut

Archaea appear to represent a minor but constant population within the termite gut. In a dot-blot analysis, Archaea represented 0.83%–1.78% of the prokaryotic SSU rRNA in lower termites, 0.13%–1.68% in wood-feeding higher termites, and 1.42%–3.22% in soil-feeding higher termites (8). The best-studied archaeal group in the guts of termites are the methanogens. Wood-feeding termites produce little methane, but a few (presumably specialized) methanogens are present. They have been observed as symbionts of certain protist species (58) and colonizing the gut wall of *R. flavipes* (55). Soil-feeding termites, on the other hand, produce on average more methane (9); this is most likely reflected in the increased abundance of Archaea listed above. In these termites, methanogens are specifically associated with P4 and P5 gut compartments (85). Nonmethanogenic archaea are also abundant in the guts of some termites (8, 25, 34, 76), where their function remains ambiguous.

Bacteria of the Termite Hindgut

The guts of termites, like most animals, host a large diversity of bacteria. A summary of 16S rRNA analyses of gut bacterial diversity in representatives of each of the major feeding classes (wood-feeding lower termite, wood-feeding higher termite, fungus cultivating, soil-feeding) is presented in Table 1.1.

Table 1.1. Abundance of key bacterial phyla in termites of different feeding groups[a]

Species	Food	*Spirochetes*	*TG1*	*Fibrobacter*	*Firmicutes*	*Proteobacteria*	*CFB*	Other
Reticulitermes speratus[b]	Wood	42–63	4–11	–	3–19	–	6–16	–
Nasutitermes takasagoensis[c]	Wood	62	–	10	10	3	8	–
Odontotermes formosanus[d]	Fungus Cultivating Soil	–	–	–	54	14	31	–
Cubitermes sp.[e]								
P1		–	–	–	96	–	4	–
P3		–	8	–	72	4	12	4
P4		–	10	–	50	20	10	10
P5		–	–	–	48	21	28	3

[a] Abundance given as percent of total bacterial 16S rRNA sequences, N.D. not detected
[b] From Hongoh, Ohkuma, and Kudo (40)
[c] From Miyata et al. (61)
[d] From Shinzato et al. (77)
[e] From Schmitt-Wagner et al. (74)

The most abundant bacterial group in wood-feeding termites are the *Spirochetes*. Termite gut spirochetes largely affiliate with the genus *Treponema*; termite gut *Treponemes* have been implicated in acetogenesis (56), nitrogen fixation (59), and lignocellulose degradation (91). Bacteria from TG1 (Termite Group 1, sometimes referred to as *Endomicrobia*) are largely present as endosymbionts of gut protists (43, 80), and may be involved in amino acid and cofactor synthesis to supplement host nutrition (41). *Fibrobacter*-like bacteria (including the TG3 group) make up approximately 10% of the bacterial complement of higher termites (39), and may be involved in cellulose degradation (91). *Firmicutes* are abundant in the guts of many animals; termite-relevant physiological capabilities include acetogenesis (6, 15, 51, 52) and cellulose degradation (38). CFB group bacteria (mainly *Bacteroides*) and *Proteobacteria* are present in many termite species, but little is known about their physiologies in this environment.

Roles for Microbes in Termite Nutrition

Gut microbes play several important roles in termite nutrition. Given the emphasis of this work on CO_2-reductive acetogenesis, I will focus on cellulose fermentation, the major source of available reducing power (H_2). The nature of the soil components utilized by soil-feeding termites is still poorly understood as is the role of gut bacteria in substrate transformations (reviewed in (7)). Gut microbes have also been implicated in termite nitrogen balance, specifically nitrogen acquisition through nitrogen fixation and reclamation of nitrogenous waste through uric acid degradation (reviewed in (11)).

Cellulose Fermentation in Lower Termites

Early studies of cellulose fermentation focused on lower termites. In 1924, Cleveland demonstrated that termites could not survive on wood or cellulose when their symbiotic protozoa were removed (20, 21). Trager (89) and Hungate (42) extended Cleveland's work with demonstrations of cellulose decomposition by termite gut protozoa in mixed cultures. In 1978 and 1981, Yamin reported the first axenic cultures of *Trichomitopsis termopsidis* (93) and *Trichonympha sphaerica* (94) from *Z. angusticollis*, which allowed the unambiguous demonstration of cellulose degradation by these protists.

Cellulose Fermentation in Higher Termites

Wood-feeding higher termites harbor few gut protists. As a result, it has been proposed that these termites have gained the capability of digesting cellulose without the aid of digestive symbionts (78). Several endoglucanases have indeed been isolated from termite tissues (35, 45, 86, 88). However, transcriptional studies show that in wood-feeding

higher termites these enzymes are largely expressed in the salivary glands and midgut, with only low levels of expression in the hindgut (35).

Alternatively, symbiotic bacteria may have replaced protists as the primary cellulose fermenters in higher termites. This hypothesis was initially dismissed due to a lack of cultivated cellulose-degrading bacteria from termite guts and low rates of cellulase activity in the hindgut (78, 88). In 2007, Tokuda and Watanabe demonstrated bacteria-associated cellulase activity in the hindguts of two *Nasutitermes* species; the methodological change associated with this discovery was the performance of cellulase assays utilizing bacterial cell pellets rather than crude lysates (87). The recent metagenomic analysis by Warnecke et al. (91) demonstrated that in another wood-feeding *Nasutitermes* species: 1, the genomes of gut bacteria encode numerous putative endoglucanases and xylanases, 2, several of these genes have demonstrable activity when expressed in *E. coli*) and 3, proteins corresponding to these genes can be detected in P3 fluid (host-derived enzymes were not detected). This suggests that termite gut bacteria indeed play a significant role in degradation of wood polysaccharides by higher termites.

Hydrogen Production and Cellulose Degradation

The major products of cellulose degradation by termite gut protists are H_2, CO_2, and acetate according to the equation below (42, 65, 95).

$$C_6H_{12}O_6 + 2\ H_2O \longrightarrow 2\ CH_3COOH + 2\ CO_2 + 4H_2$$

The stoichiometry of cellulose fermentation within the guts of wood-feeding higher termites is unknown, but presumed to follow a similar pattern.

Acetate has been shown to accumulate in the guts of both higher and lower termites at concentrations of up to 80 mM, and oxidation of this acetate can account for up to 100% of the respiratory activity of the termite host (66). However, in 1983 Odelson and Breznak (66) observed that H_2 emissions from live termites were not sufficient to balance the observed rates of acetate production according to the equation above. While low rates of methane emission (a key H_2 sink in anaerobic ecosystems) were present, they too were insufficient to account for the missing electrons. As a result, they proposed that the H_2 generated during cellulose fermentation was being utilized for the reduction of CO_2 to acetate by acetogenic bacteria.

Introduction to Acetogenesis

H_2-mediated reduction of CO_2 is an important electron sink in many anaerobic ecosystems. In most environments, this niche is dominated by methanogenic archaea. CO_2-reductive acetogenesis is less energetically favorable ($\Delta G^{\circ\prime}$ = –94.9 kJ/mol for acetogenesis vs. –131.0 for methanogenesis) (72). However, for unknown reasons, acetogens can coexist with and even outcompete methanogenic archaea in some environments, including the termite gut (9, 49, 69).

Acetogenesis by bacteria from H_2 and CO_2 was first reported in 1932 by Fischer et al. (reported in German (31), reviewed in (26)). The model acetogen, *Moorella thermoacetica*, was at first characterized as a glucose-fermenting organism that produced acetate as the sole end product with stoichiometry 3 mol/mol glucose (32).

$$C_6H_{12}O_6 \longrightarrow 3\ CH_3COOH$$

The discrepancy was proposed, and later demonstrated (2, 3), to be due to the pairing of glucose fermentation to acetate and CO_2 with reduction of that CO_2 to acetate, wherein:

$$C_6H_{12}O_6 + 2\ H_2O \longrightarrow 2\ CH_3COOH + 2\ CO_2 + 8\ [2H]$$

$$8\ [2H] + 2\ CO_2 \longrightarrow CH_3COOH$$

The fermentation in the termite gut was projected to follow a similar pattern, but with protists carrying out the glucose fermentation and transferring the 8 reducing equivalents to acetogenic bacteria for the reduction of CO_2 to acetate (66).

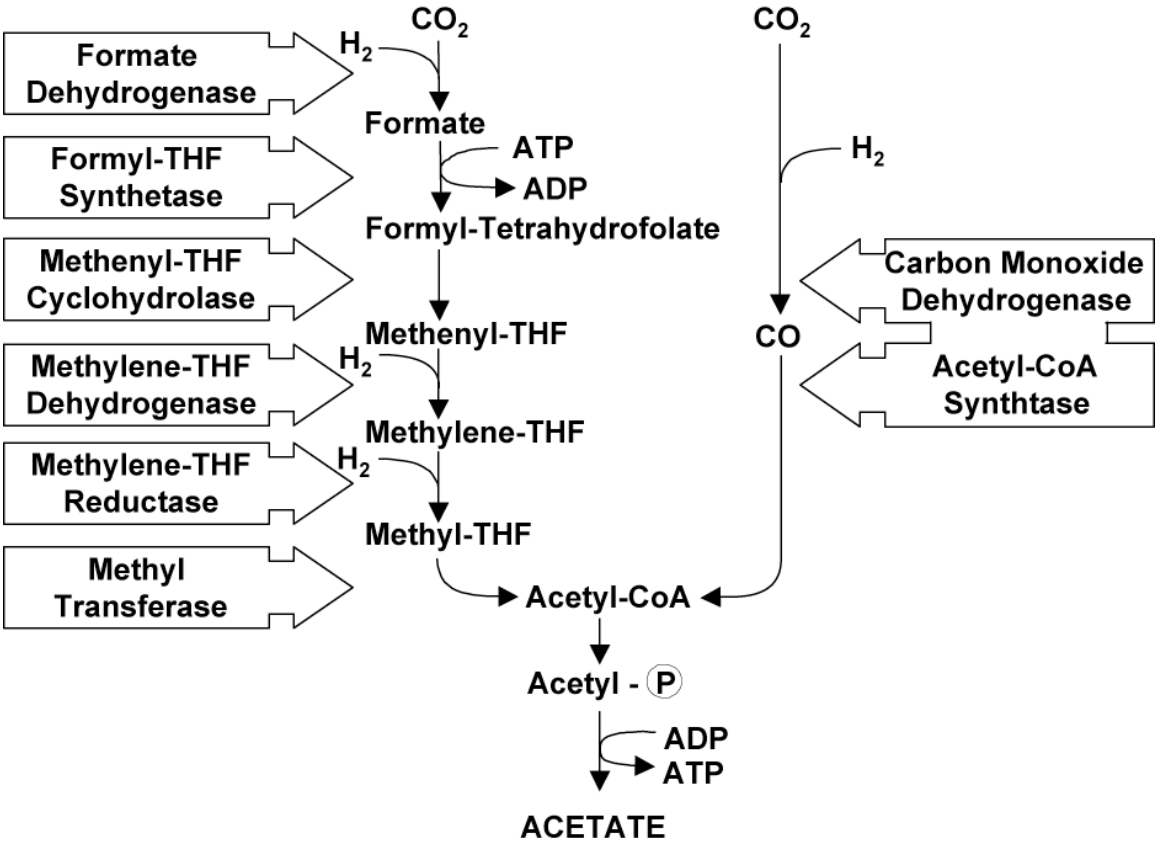

Figure 1.1. Wood-Ljungdahl Pathway for CO_2-reductive acetogenesis. Reducing equivalents depicted as H_2.

CO_2-reductive acetogenesis occurs via the Wood-Ljungdahl or acetyl-CoA cycle (60) (Figure 1.1). *Acetogens* are generally defined as bacteria utilizing this pathway as a major source of energy for growth and for CO_2 fixation into organic carbon (26).

One of the remarkable features of acetogenic bacteria is their metabolic flexibility. Methanogenic archaea are highly specialized, using H_2/CO_2, acetate, and a few other C1 compounds (92). The acetogens, however, make use of a diversity of substrates (26). Most acetogenic bacteria ferment a variety of carbohydrates and funnel the resultant reducing equivalents into the reduction of CO_2 (extrinsic or intrinsically generated during pyruvate conversion to acetate) (26). Additionally, many acetogens can directly feed reduced C1 units such as carbon monoxide, formate, and methanol into the acetyl-CoA pathway according to their redox potentials (24, 26). These reactions generally proceed as a disproportionation, where a subset of substrate molecules are oxidized in order to generate the required reducing equivalents for reduction of CO_2 to the carbonyl group of acetate. An example discussed in later sections is acetogenic o-demethylation of methoxylated aromatics. The methyl groups from these compounds enter the pathway at the level of methyl-THF; one methyl unit is oxidized to CO_2, (generating 3 reducing equivalents), for every 3 methyl units condensed with CO_2 to form acetate (33).

$$CH_3\text{-THF} \longrightarrow CO_2 + THF + 3\ [2H]$$

$$3\ CH_3\text{-THF} + 3\ CO_2 + 3\ [2H] \longrightarrow 3\ CH_3COOH + 3\ THF$$

.

Finally, several acetogens have been reported to utilize alternative electron acceptors, such as nitrate (75) and the C=C double bonds in phenylacrylate derivatives (90).

While patterns of carbon flow during acetogenesis are fairly well understood, the energetics of acetogenesis are a bit harder to pin down. As can be seen in Figure 1.1, no net ATP is generated via substrate-level phosphorylation during acetogenesis from H_2 and CO_2. As a result, ATP must be generated via chemiosmotic phosphorylation. The reactions most likely to yield sufficient energy to translocate ions are the two final methyl transformations, catalyzed by methylene-THF reductase and methyl transferase (23). Acetogens can be grouped into those that depend on a proton or a sodium motive force; methylene-THF reductase has been proposed to drive proton translocation, while methyl transferase is considered a more likely driver of sodium translocation (62). However, the exact patterns of electron flow in these organisms remain unclear.

Acetogenesis in the Termite Gut

In 1986, H_2-dependent $^{14}CO_2$ reduction to acetate was demonstrated in termite gut homogenates, where it was found to occur at rates that were 2- to 33-fold higher than rates of methanogenesis (14). Table 1.2 presents measured rates of acetogenesis and methanogenesis from selected termites examined in this and an expanded study carried out in 1992 (9). A general pattern was observed in which wood-feeding lower and higher termites (represented here by *R. flavipes* and *N. nigriceps*) and the wood-feeding roach *C. punctulatus* had acetogenesis rates that outpaced methanogenesis. However, the reverse was observed in the guts of soil-feeding termites (*C. speciosus*) and the common cockroach (*P. americana*).

Table 1.2. Rates of acetogenesis and methanogenesis in the guts of selected termites

Species	Rate of acetogenesis from CO_2 in gut homogenates (μmol acetate/g/hr)		Rate of CH_4 emission from live animals (μmol CH_4/g/hr)
	Under N_2	Under H_2	
Reticulitermes flavipes[a]	0.09	0.93	0.10
Nausitermes nigriceps[a]	0.89	3.68	0.24
Cubitermes speciosus[a]	0.01	0.02	0.85
Cryptocercus punctulatus[b]	0.04	0.14	<0.01
Periplaneta americana[b]	0.02	0.04	2.02
Beef Cow Rumen	0.00[c]	0.05[c]	0.9-1.1[d]

a. From Brauman et al. (9)
b. From Breznak and Switzer (14) CH_4 production measured as $^{14}CH_4$ production in gut homogenates in presence of $^{14}CO_2$ and N_2 headspace (rather than emission)
c. From Le Van et al. (54)
d. Calculated based on 60-71 kg/cow/yr (48), assumes 450kg animal.

In 2007, Pester and Brune measured acetogenesis rates in three species of wood-feeding lower termites by microinjection of ^{14}C-bicarbonate into guts that had been extracted, intact, from living termites (68). They observed rates of CO_2 fixation to acetate that corresponded to 22%–26% of the respiratory carbon turnover, confirming a major role for acetogenic bacteria in fueling host metabolism.

Acetogenic Bacteria Isolated from the Termite Hindgut

Over 100 species of acetogenic bacteria have been described (26). Of these, the overwhelming majority are *Firmicutes*. However, acetogenic capability is not monophyletic; several different lineages of acetogenic bacteria have been described, and many acetogens are closely related to nonacetogenic strains (26, 83).

Six species of acetogenic bacteria have been isolated from the guts of termites (Table 1.3). Four are acetogenic *Firmicutes*: *A. longum, C. mayombei, S. aerovorans,* and *S. termitida*. *A. longum* was isolated from the gut of a wood-feeding lower termite, and was

isolated from the highest dilution of the six strains. *S. termitida* was isolated from an enrichment using a single whole gut from a wood-feeding higher termite. *C. mayombei* and *S. aerovorans* were both isolated from soil feeders. The remaining two isolates, *T. primitia* strains ZAS-1 and ZAS-2, are acetogenic spirochetes isolated from the guts of the wood-feeding lower termite *Z. angusticollis*. This was the first report of acetogenesis, or chemolithoautotrophy in general, in a spirochete (56).

Table 1.3. CO_2-reducing acetogens isolated from termite guts

Species	Termite	Dilution	Reference
Acetonema longum	*Pterotermes occidentis*	10^{-6} dilution	(52)
Clostridium mayombei	*Cubitermes speciosus*	Not reported	(51)
Sporomusa aerivorans	*Thoracotermes macrothorax*	10^{-3} dilution	(5, 6)
Sporomusa termitida	*Nasutitermes nigriceps*	1 gut/tube	(12, 15)
Treponema primitia ZAS-1	*Zootermopsis angusticollis*	1 gut/tube	(36, 37, 56)
Treponema primtia ZAS-2	*Zootermopsis angusticollis*	1 gut/tube	(36, 37, 56)

Nutritional Characteristics of Termite Gut Acetogens

Acetogenesis from H_2 and CO_2 is the form most discussed in the context of the termite gut. This is due in part to evidence that it does play a major role in carbon cycling in the termite; as discussed above, H_2 is a major product of cellulose fermentation by termite gut protists, and observed rates of $^{14}CO_2$ reduction to acetate are sufficient to account for 22%–26% of the respiratory activity of the termite. However, termite gut acetogens are capable of utilizing a wide range of carbon sources, including mono- and disaccharides such as glucose (*A. longum, C. mayombei,* ZAS-1, ZAS-2), xylose (*C. mayombei*, ZAS-1, ZAS-2), and cellobiose (*C. mayombei*, ZAS-1) (see references in Table 1.3). Lactate and formate were identified by Tholen and Brune (84) as intermediates generated when ^{14}C-glucose was injected into *R. flavipes*; the *Sporomusa* strains used both compounds (*S.*

termitida is noted as growing only weakly on formate), and *C. mayombei* utilized formate but not lactate.

Furthermore, *S. termitida* and *T. primitia* ZAS-2 are both capable of mixotrophic growth, simultaneously utilizing H_2 + CO_2 and organic substrates for carbon and energy (12, 36). This could allow these organisms to increase both the amount of energy per unit time generated by the cell and the amount of energy generated per mol H_2 (12). This ability has been invoked as a possible cause of the ability of acetogenic bacteria to outcompete methanogens in the termite gut (10). While the remaining termite gut acetogens are also capable of utilizing organic compounds, their ability to benefit from mixotrophy has not been investigated.

O-Demethylation of Aromatic Side Chains by Termite Gut Acetogens

In 1981, *Acetobacterium woodii* was shown to be capable of O-demethylation of methoxylated aromatic acids (1), and this activity has since been identified in many acetogens (33). While there is limited evidence for degradation of core lignin compounds in the guts of wood-feeding termites, lignin monomers can be utilized (13, 19). Ring cleavage appears to be minimal in the absence of oxygen, but side chain modifications are carried out under anaerobic conditions (19). Some of this activity might be attributable to acetogens; *S. aerivorans, S. termitida,* and *T. primitia* ZAS-2 are capable of growth by demethylation of methoxylated aromatics (see references in Table 1.3). *A. longum* and *C. mayombei* were each listed as growing consistently but weakly on a single modified aromatic (2,3,5-trimethoxybenzoate and syringate, respectively), while *T.*

primitia ZAS-1 did not utilize any of the four methoxylated aromatics tried (syringate, vanillate, ferulate, 2,3,5-trimethoxybenzoate).

Oxygen Reduction by Termite Gut Acetogens

The traditional view of the termite gut is that of a strictly anaerobic fermentation. This view is based on the oxygen sensitivity of termite gut protists (20) and the importance of anaerobic metabolic activities such as acetogenesis. However, Brune et al. have demonstrated conclusively that the gut epithelium does not serve as a barrier to oxygen diffusion, and that as a result the periphery of the gut may be microoxic (17).

This finding has stimulated research into oxygen tolerance and utilization by gut microbes. Termite gut acetogens *A. longum, S. aerivorans,* and *S. termitida* have been shown capable of growth when inoculated into media with up to 1.5% (*S. aerivorans*) O_2 in the headspace (5). These strains did not grow in the presence of oxygen; rather, the bacteria were able to use H_2 in the headspace to reduce oxygen, and resumed growth once the medium was anoxic. *T. primitia* (both ZAS-1 and ZAS-2) are described as "tolerating" O_2 concentrations of up to 0.5%, but it was not specified whether they grew in the presence of this oxygen or responded by reducing it prior to resuming growth (36).

Molecular Community Analysis of Termite Gut Acetogens

Although four of the six acetogenic isolates are *Firmicutes*, the numerical dominance of spirochetes in the termite gut makes it tempting to suggest *Treponemes* as key acetogens

in this environment. However, the fact that *T. primitia* ZAS-1 and ZAS-2 were not isolated from high dilutions made this difficult to prove.

Cultivation-based techniques are inherently limited to identification and enumeration of bacteria that will grow in the media chosen. Given the cryptic nutritive requirements of environmental bacteria, it is impossible to say with any certainty that the most abundant organisms cultured are indeed key players in the environment. Indeed, despite the abundance (more than half of the bacterial population in some termites) and importance of spirochetes in the termite gut, only 3 additional species have been successfully cultivated in the over 100 years of termite research; *Treponema azotonutricium* (37), *Spirochaeta coccoides* (27), and *Treponema isoptericolans* (28).

In the section on the bacteria of the termite hindgut, I discussed the results of 16S rRNA molecular community profiling studies carried out on gut bacteria from different termite species (Table 1.1). Acetogenic capability is not restricted to a single bacterial grouping (83), so 16S rRNA is not a suitable tool for characterization of acetogens. In 2001, Leaphart and Lovell discovered that the gene for formyl-tetrahydrofolate synthetase (FTHFS) from acetogenic *Firmicutes* was distinct from that of nonacetogens, and designed primers that would specifically amplify genes that fall within this cluster (57).

Leaphart and Lovell did not design their primer set to target spirochetal acetogens, as the FTHFS sequences from *T. primitia* ZAS-1 and ZAS-2 were not yet known. In 2003, Salmassi and Leadbetter demonstrated that the Lovell primers could amplify FTHFS

genes from ZAS-1 and ZAS-2 (71). Phylogenetic analysis of the recovered sequences found that they were not closely related to the *Treponema denticola* FTHFS sequence, but rather were closely related to FTHFS genes from "Lovell cluster" acetogenic *Firmicutes* (Figure 1.2). This suggests that *T. primitia* acquired its acetogenic capability by lateral gene transfer from a *Firmicute* acetogen.

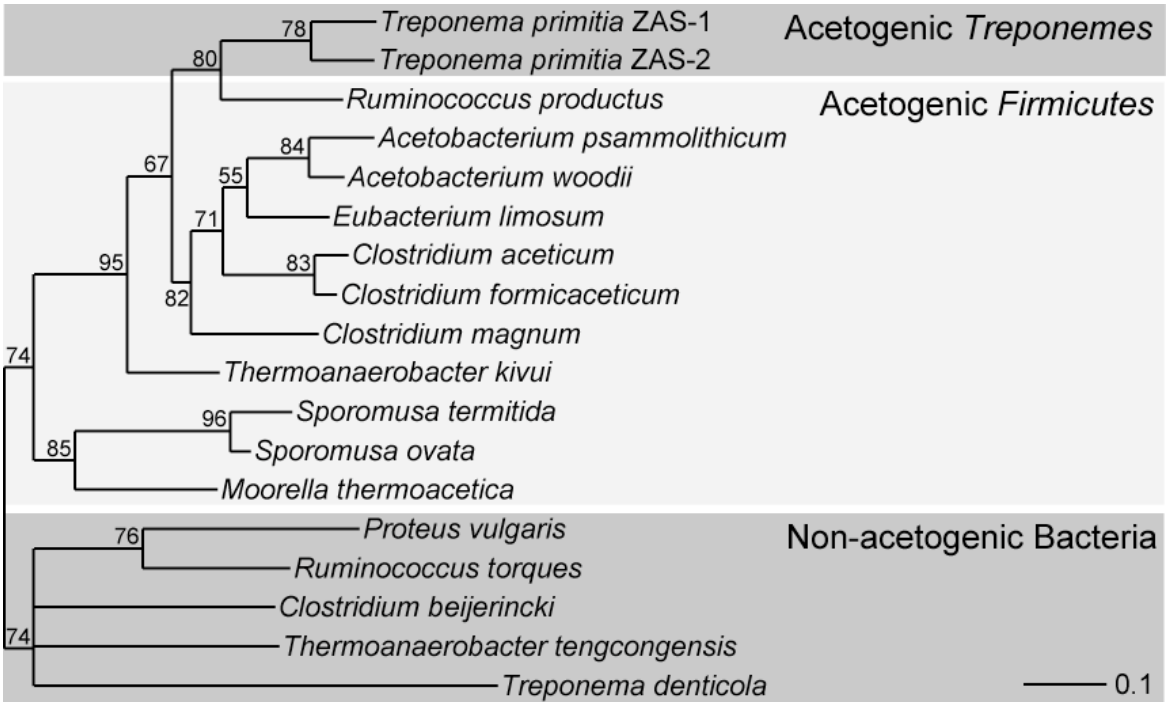

Figure 1.2. Phylogenetic analysis of FTHFS genes from acetogenic bacteria. Tree built using the TreePuzzle algorithm, with 1,000 puzzling steps and 345 unambiguously aligned amino acid positions. Scale bar represents 0.1 amino acid changes per alignment position.

Salmassi and Leadbetter also used the Lovell primers to build a community gene inventory of FTHFS sequences present in DNA extracted from the hindguts of *Zootermopsis nevadensis* workers (71). The majority of FTHFS types amplified from this environment grouped phylogenetically with ZAS-1 and ZAS-2 sequences, suggesting

that spirochetes are indeed the most abundant acetogens in this termite. Pester and Brune (67) reported similar results for two more wood-feeding lower termites, *Reticulitermes santonensis* and *Cryptotermes secundus*. Furthermore, they demonstrated that termite gut *Treponeme* FTHFS sequences were the most abundant FTHFS types in the community mRNA pool, showing that these organisms were actively utilizing the acetyl-CoA cycle *in situ* (67). Taken together, this evidence suggests a major role for spirochetes in acetate formation within the guts of wood-feeding lower termites.

On the Organization of This Thesis

My work has focused on furthering our understanding of the roles and community structure of acetogenic bacteria in the termite hindgut. In the second chapter of this thesis, I utilize the FTHFS-based community analysis method to examine the diversity of acetogenic bacteria present in the guts of wood-feeding roaches (*C. punctulatus*). These roaches, as shown in Table 1.2, have high rates of acetogenesis, but the nature of the acetogenic bacteria present in their guts was unknown. We demonstrated that wood-feeding roaches, like lower termites, host a diversity of acetogenic spirochetes. This, in addition to phylogenetic evidence placing roach-hosted spirochetes as basal to at least two key radiations of termite-derived sequences, suggests that acetogenic spirochetes arose prior to the roach-termite divergence. Additionally, it suggests that a diversity of sequence types were present in the last common ancestor, and that these bacteria gave rise to the complex species assemblage seen in lower termites today.

The third chapter of this thesis presents work in which I utilize the same techniques to explore the diversity of FTHFS-bearing organisms in higher termites. Higher termites, as mentioned above, have adapted to a variety of lifestyles. Some, like lower termites, feed exclusively on wood, but other termite species have adapted to using food sources at different stages of decomposition, up to and including soil. I explored the acetogenic community of 6 species of higher termite, 4 tropical species collected in Costa Rica and 2 desert-adapted species from California. A striking bifurcation was noted, as wood-, palm-, and litter-feeding species were dominated by *Treponeme*-like FTHFS types, while soil-exposed (and potentially soil-feeding) subterranean termite species were dominated by novel *Firmicute*-like FTHFS types. This suggests that the environmental conditions that allow high rates of acetogenesis in the guts of wood-feeding termites may correspond with those that favor *Treponemes* over other acetogenic bacteria.

In the fourth and fifth chapters of this thesis, I discuss the development of microfluidics-based tools for molecular characterization of uncultured microorganisms. In the previous section, I presented evidence that *Treponemes* are the dominant CO_2-reductive acetogens in the guts of wood-feeding termites. However, this hypothesis is based on the phylogenetic affiliation of a large cluster of FTHFS genes with those from *T. primitia* strains ZAS-1 and ZAS-2. Given that these *Treponemes* are believed to have acquired their FTHFS gene by lateral gene transfer, this affiliation should not be taken as definitive proof of identity. The fourth chapter of this thesis describes the development of technique for highly parallel, multiplex PCR interrogation of single bacterial cells from environmental samples. We used a microfluidic device to separate individual

microbes from the guts of *Z. nevadensis* and perform multiplex PCR reactions for simultaneous amplification and detection of bacterial 16S rRNA genes and a key FTHFS sequence type. Retrieval and analysis of PCR products from successful reactions allowed the rRNA-based species characterization of bacteria that hosted the targeted FTHFS gene, confirming the phylogenetics-based hypothesis that it was derived from a spirochete.

The final chapter of this thesis presents an expansion of the microfluidic technique described in chapter 4. The approach described in chapter 4 utilized sequence-specific Taqman probes to detect on-chip amplification of FTHFS and 16S rRNA genes. However, conventional Taqman probes are limited to detection of simple target populations. The presence of highly conserved sequence regions in bacterial rRNA genes allows the design of *"all-bacterial"* probes, but FTHFS probes were constrained to small clusters of highly similar sequences. In chapter 5, I present a modified "universal template probe" (96) strategy that allows multiplex detection of amplicons generated using degenerate primers. Using this system, we have developed a novel "Lovell cluster" FTHFS assay for detection and characterization of acetogenic bacteria using multiplex microfluidic PCR.

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