INTRODUCTORY BACKGROUND

1.1. Preface to topics

Microbes represent most of life's genetic and physiological diversity and are the major drivers of global biogeochemical cycles (57, 124). Yet the vast majority of microbes on Earth have not been successfully cultured (57). In the absence of pure cultures, many scientists rely on genetic and isotopic signatures in the environment to identify and investigate the ecological roles of uncultured microbes (1, 39). While such studies are commonly termed "culture-independent," interpretations ultimately rely on detailed pure culture investigations of phylogenetically or physiologically related microbes. My graduate work at Caltech has focused on two subjects in environmental science that highlight the synergism between environmental and pure culture studies.

Topic I. Formate dehydrogenase gene diversity in lignocellulose-feeding insect gut microbial communities

The majority of this thesis focuses on formate dehydrogenase gene diversity in the symbiotic gut microbial communities of lignocellulose-feeding termites and roaches. Formate dehydrogenase enzymes are crucial for autotrophic CO_2-reductive acetogenesis, a bacterial process that significantly impacts insect nutrition, and, by way of host abundance, impacts the global carbon cycle. Chapters 2 – 4 describe studies of these functional genes in uncultured acetogenic bacteria, which relied on traditional and emerging molecular techniques in microbial ecology and were leveraged by pure culture

studies of a termite gut acetogen. The results shed light on the diversity, evolutionary biology, and activity of an important group of insect gut bacteria.

Topic II. Metabolic impacts on the hydrogen isotope content of bacterial lipids

The second topic of this thesis focuses on the biological determinants of bacterial lipid hydrogen stable isotope composition (H^2/H^1, D/H). Environmental measurements show lipid D/H values vary dramatically in ways that can not be explained by changes in the D/H of water, a source of lipid hydrogen. These data implicate biological processes as the sources of variation. However, such processes have remained almost completely unstudied. In Chapter 5 of this thesis, I describe studies on the relationship between energy metabolism and D/H of fatty acids in pure cultures of physiologically diverse bacteria. The results suggest lipid D/H may be a useful isotopic marker for energy metabolism.

The remainder of this chapter provides background information and a brief chapter outline for each topic.

1.2. Introduction to formate dehydrogenases in insect guts

CO_2-reductive acetogenesis is an anaerobic process used by certain microbes to gain energy, synthesize the key metabolic intermediate, acetyl-CoA, as well as cell carbon (34, 36). Of five known autotrophic mechanisms in nature, the reductive synthesis of one molecule of acetate from two molecules of CO_2 via acetogenesis is the only linear process for CO_2 fixation (34, 117). Its simplicity relative to other CO_2 fixation pathways has prompted some to consider it as the earliest evolved form of autotrophy (83). For these and other reasons, it has caught the interest of microbiologists, biochemists, environmental scientists, and geobiologists over the past ~80 years.

My work seeks to clarify the form, function, and evolution of microbes impacting CO_2 reductive acetogenesis in the guts of lignocellulose-feeding insects, where rates of acetate synthesis from H_2 + CO_2 are characteristically high and impact the global carbon cycle. Specifically, I use the gene for hydrogenase-linked formate dehydrogenase, a key enzyme in fermentative and acetogenic metabolism, as a "hook" to study uncultured acetogens inhabiting the guts of termites and roaches. In this introduction, I first present key microbiological, biochemical, and ecological aspects of acetogenesis. I then outline the biology of termites (and related insects) and termite gut microbial community composition before focusing on termite gut acetogenesis and acetogens. Finally, I introduce a genetic and transcriptional study of a specific termite gut acetogen, which formed the basis for the majority of my thesis work.

1.2.1. The discovery of acetogenesis: early microbiology and biochemistry

Studies of acetogenesis began in 1932 when Fischer *et al.* reported the H_2-dependent synthesis of acetate from CO_2 in sewage sludge [(36) and references therein]. Four years later, the microbiologist Wieringa isolated the first acetogen (125), a Gram-negative spore forming bacterium of the phylum *Firmicutes*, which could grow via the complete synthesis of acetate (CH_3COOH) from CO_2 and H_2 following the reaction:

$$4\ H_2 + 2\ CO_2 \longrightarrow CH_3COOH + 2\ H_2O \qquad \text{(reaction 1)}$$

This organism, *Clostridium aceticum*, was lost, but a second *Firmicute* acetogen, *C. thermoaceticum* (renamed *Moorella thermoacetica*), was isolated a few years later from horse manure by Fontaine *et al.* (44). As no other acetogenic bacteria were isolated for several years, *M. thermoacetica* became the model bacterium for almost all biochemical and enzymological studies of acetogenesis.

In contrast to *C. aceticum*, acetogenesis in *M. thermoacetica* was ironically identified in the context of its heterotrophic metabolism of glucose ($C_6H_{12}O_6$). In 1942, Fontaine and colleagues (44) noted that this bacterium's metabolism of glucose to acetate yielded a novel stoichiometry (reaction 2), which precluded the typical 3-3 split of glucose typical in glycolysis:

$$C_6H_{12}O_6 \longrightarrow 3\ CH_3COOH \qquad \text{(reaction 2)}$$

Later, in 1944, Barker (3) employed fermentation balances to propose that reaction 2 was actually a sum of two reactions, whereby the reducing equivalents from glucose oxidation (reaction 3, where "2H" represents a reducing equivalent such as H_2) are used to generate an additional molecule of acetate via CO_2 reductive acetogenesis (reaction 4):

$$C_6H_{12}O_6 \longrightarrow 2\ CH_3COOH + 2\ CO_2 + 8\ H \qquad \text{(reaction 3)}$$

$$2\ CO_2 + 8\ H \longrightarrow CH_3COOH + 2\ H_2O \qquad \text{(reaction 4)}$$

Barker and Kamen (4) confirmed this reaction scheme by demonstrating that *M. thermoacetica* produces acetate labeled in both carbon positions when grown with $^{14}CO_2$. This landmark study not only refined the concepts of autotrophy and heterotrophy, it was also the first biological study performed which utilized ^{14}C as a metabolic tracer. H. G. Wood, in a mass-balance study using ^{13}C , provided definitive isotopic evidence for the complete synthesis of acetate from CO_2 via reaction 4 (126).

1.2.2. The Wood-Ljungdahl pathway for acetogenesis

In the next 40 years, Wood and his student L. G. Ljungdahl led efforts to elucidate the biochemistry and enzymology of CO_2 reductive acetogenesis. As a result, the enzymatic steps underlying CO_2 reductive acetogenesis have been collectively termed the "Wood-Ljungdahl" pathway (Figure 1.1). The pathway, also known as the acetyl-CoA pathway, was first proposed in 1969 by Wood and Ljungdahl and later summarized by the same

authors in 1991, after its resolution in *M. thermoacetica* — and the demonstration that this organism could, in fact, grow as an autotroph on $H_2 + CO_2$ (31, 79, 127). From here on, I will use the term "acetogen" to describe microbes that utilize the Wood-Ljungdahl pathway for energy conservation, acetyl-CoA production, and assimilation of CO_2 into cell carbon, either during heterotrophic metabolism (e.g., *M. thermoacetica*) or autotrophic metabolism (e.g., *C. aceticum*).

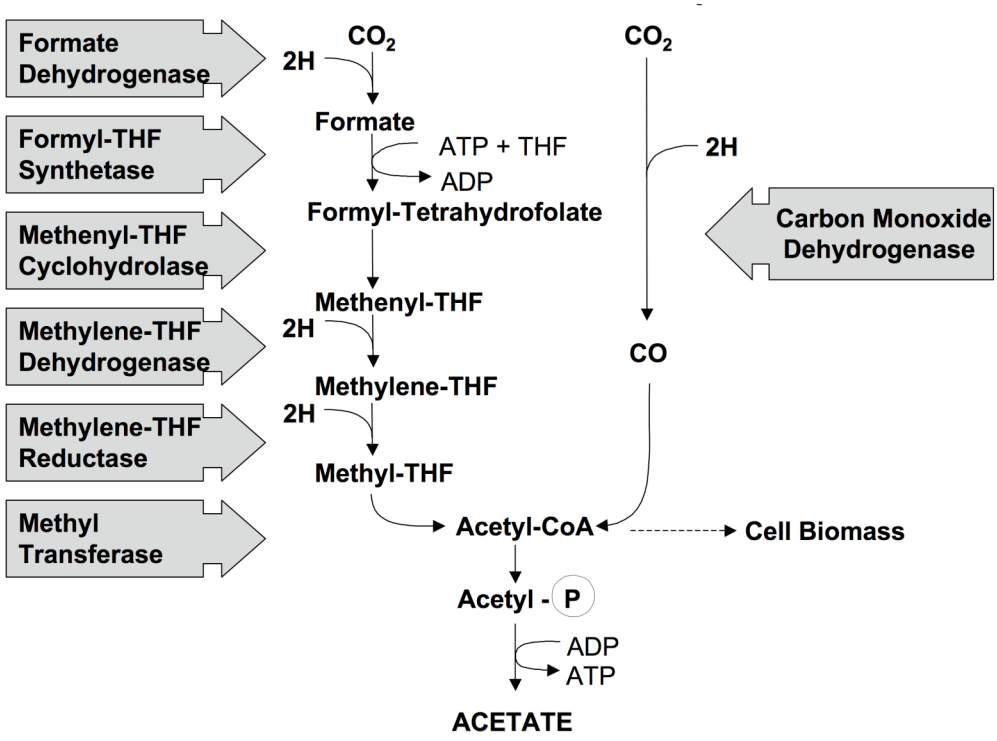

Figure 1.1. Wood-Ljungdahl pathway for CO_2-reductive acetogenesis (adapted from a personal communication by J. R. Leadbetter and (96)). CO_2 is the terminal electron acceptor for reducing equivalents, 2H, which are typically H_2 or derived from it (32). Carbohydrates, CO, methanol, and other incompletely oxidized one-carbon and two-carbon compounds can also serve as sources of 2H (32, 33). The major site of chemiosmotic energy conservation is thought to be the methyl-transferase reaction (32). THF, tetrahydrofolate; Acetyl-P, acetyl-phosphate. Dashed line indicates intermediate drawn off for biosynthetic processes.

The heterotrophic capacity of acetogens is quite diverse. Sources of reductant include carbohydrates, one-carbon (C1), and even two-carbon compounds (36). These substrates interact with the Wood-Ljungdahl pathway in two conceptually different ways. As observed in *M. thermoacetica*, carbohydrate fermentation provides reducing equivalents but no reduced carbon skeletons for acetogenesis. In contrast, acetogens using C1 compounds obtain reducing equivalents, as well as incompletely oxidized carbon substrates, by fully oxidizing a portion of their C1 substrates to CO_2 for reductant, and then channeling the remaining C1 molecules into the acetogenesis pathway at intermediates of similar redox state.

1.2.3. Acetogenesis from H_2 and CO_2

The autotrophic growth of acetogens depends on chemiosmotic energy generation, since there is no net substrate level phosphorylation of ATP (Figure 1.1) when H_2 and CO_2 are substrates for acetogenesis. Two types of chemiosmotic mechanisms have been identified in acetogens. Organisms like *M. thermoacetica,* which possess membranous electron transport proteins, perform proton-based chemiosmosis [(34) and references therein] when they catalyze the methyl transferase reaction in acetogenesis. The autotrophic acetogen, *Acidobacterium woodii*, lacks such membrane features and instead uses the methyl transferase reaction (Figure 1.1) to create a sodium ion-based membrane gradient that drives chemiosmotic ATP production [(34) and references therein].

Formate dehydrogenase and carbon monoxide dehydrogenase are critical enzymes for the

pathway's CO_2 assimilating capacity (Figure 1.1). Formate dehydrogenase from *M. thermoacetica* was identified as an NADPH-linked cytoplasmic protein containing catalytic tungsten and selenium moieties (128). These latter aspects are notable as they represent discoveries of the first tungsten protein and one of the first selenoproteins in nature [(127) and references therein].

Lastly, autotrophic growth of acetogens depends on H_2-oxidizing hydrogenases. These enzymes either "activate" H_2 into physiological forms (e.g., ferredoxin, NADPH) that are the direct reductants for acetogenesis, or transduce H_2 to a reducing enzyme, like formate dehydrogenase, when they are physically linked to other enzymes in a multi-enzyme complex (36). The latter has not been biochemically established in any acetogen to date, but may be relevant in H_2 rich environments. Hydrogenases are not explicitly shown in Figure 1.1 but are nonetheless just as important as CO_2 "activating" enzymes like formate dehydrogenase for acetogenesis from $H_2 + CO_2$.

1.2.4. Phylogeny of acetogenic bacteria

Acetogens, as a group, represent over 20 bacterial genera [(34) and references therein]. Almost all acetogens belong to the phylum *Firmicutes*, but many of these genera are not monophyletic (i.e., sister taxa of acetogens may not be acetogenic) (101). The majority of acetogen isolates belong to the *Firmicute* genera *Acetobacterium* and *Clostridium*. Acetogen isolates outside the *Firmicutes* include two species of spirochete that belong to the genus *Treponema*, within the phylum *Spirochaetes* (34); δ-*Proteobacteria* like the sulfate reducer, *Desulfotignum phosphitoxidans*, which grows as an acetogen in the

absence of sulfate (107); and *Holophaga foetida*, in the phylum *Acidobacteria* (76).

No known acetogenic *Archaea* have been identified, although the use of a major portion of the Wood-Ljungdahl pathway (i.e., all enzymes except formate dehydrogenase and formyl-tetrahydrofolate synthetase) for synthesis of cell carbon in autotrophic methanogenic *Archaea* is well-established (36). Theoretically, a bonified *Archaeal* acetogen should possess better energetics (i.e., conserve more ATP per acetate formed) than bacterial acetogens, which burn additional ATP to active THF with formyl-tetrahydrofolate synthetase (Figure 1.1). The Wood-Ljungdahl pathway is also employed by anaerobes like the sulfate reducing bacterium *Desulfobacterium autotrophicum* for anabolism. In addition, acetate oxidizing methanogens (e.g., *Methanosarcina barkeri*) and sulfate reducers (e.g., *Desulfotomaculum actetoxidans*) can use the Wood-Ljungdahl pathway in reverse to fuel catabolism. However, it is worth emphasizing that the aforementioned microbes are not true "acetogens" even though they may employ all or parts of the Wood-Ljungdahl pathway in their metabolism.

1.2.5. Ecology of acetogenic bacteria

Many acetogens are able to grow in a non-acetogenic capacity; for example, *M. thermoacetica* can use nitrate as a terminal electron acceptor instead of CO_2 (37). This physiological diversity translates into a wide distribution in nature (35, 38). Bacteria with acetogenic capabilities have been isolated from diverse anaerobic environments, including sewage, sediments, animal waste, hot springs, rumen fluid, and termite hindguts (36), but our knowledge of the ecological role such bacteria play as acetogens

within their respective environments remains fairly limited. As a consequence, there are few estimates for the impacts of acetogenesis outside environments such as certain sediments (49, 81) and termite hindguts (20). Nevertheless, these estimates, which credit acetogens for generating ~ 10% of sediment acetate production (127) and ~ 10^{12} kg of acetate annually in termite guts (20, 36), indicate acetogenesis plays an important role in the global carbon cycle.

1.2.6. Ecological impacts of termite gut acetogenesis

The global significance of acetogenesis is clearest in termites. These insects are best known for their ability to consume cellulose and lignocellulose, the most abundant biopolymers on land. These, and other activities, confer termites with the status of ecologically important arthropods that mediate carbon turnover and maintain soil fertility in terrestrial ecosystems (7, 9). Globally, termites consume ~ 4% of terrestrial plant biomass and account for 2 – 5% of terrestrial CO_2 and 3 – 5% of methane emitted to the atmosphere (10, 15, 67, 84, 102, 104, 116). Regionally, termites may be responsible for as much as 20% of carbon mineralization (9). These ecological roles are associated with one of nature's most striking nutritional mutualisms, wherein a complex community of obligate symbiotic microbes inhabiting termite hindguts degrades lignocellulose and other recalcitrant food substrates into carbon forms like acetate which fuel the host termite's metabolism. Among the most important termite gut symbionts are CO_2 reductive acetogens, which generate nearly 1/3 of the host's fuel (92). Globally termite gut acetogens generate ~ 1.22 x 10^{12} kg acetate annually (20); this is ~ 10% of all acetate (~ 10^{13} kg) metabolized in anaerobic environments (36, 127).

Acetogenesis in termite guts also has implications for global climate. Acetogenesis is typically outcompeted by methanogenesis for reductant generated from anaerobic degradation (e.g., H_2) in environments that are poor in electron acceptors other than CO_2. The best example can be found in animal rumens where methanogenic *Archaea* dominate H_2 consumption and generate enough methane, a potent greenhouse gas, to account for 13 – 15% of global emissions (114). In contrast, the dominant H_2 consuming process in termite guts is bacterial CO_2-reductive acetogenesis (21). As a result, termite emissions account for 3 – 5% of total methane emissions (114), rather than upwards of ~ 10%, as predicted based on ruminant-like carbon flows in termite guts (14).

1.2.7. Termite Biology

Termites are eusocial animals that belong to the insect order *Isoptera*, which means "equal winged" and describes the fact that fore- and hind-wings are of approximately equal size in reproductive castes (48). They constitute one of our planet's most diverse and abundant animal groups, with ~ 3000 extant species (41) representing at least 10^{18} individuals (7). The majority of termites inhabit tropical environments (9), where they account for as much as 95% of insect biomass in soils (9). Termite abundance, biomass, and species diversity tend to decline with distance from the equator (40) .

Termites belong to the detritovorous insect superorder *Dictyoptera*, which also includes cockroaches and mantids (48, 64). The general consensus is that termites descend from a wood-feeding cockroach, whose extant representatives belong to the sub-social, wood-

feeding cockroach genus *Cryptocercidae* (64, 80). The split between cockroach and termite lineages is estimated to have occurred ~ 140 million years ago (mya) in the early Cretaceous (48, 119). Within *Isoptera*, termites are traditionally classified into seven families (Figure 1.2) based on morphology and molecular data (64, 69).

Termites are also classified into two broad taxonomic groups, termed "lower" and "higher" termites. Members of six families are considered "lower" termites: *Mastotermididae, Hodotermitidae, Termopsidae, Kalotermitidae, Rhinotermitidae*, and *Serritermitidae*. Members of the seventh family, *Termitidae*, are "higher" termites. This single family comprises the most termite individuals and > 85% of all termite genera. It is further classified into four sub-families: *Macrotermitinae, Apicotermitinae, Termitinae,* and *Nasutitermitinae*. The relationships between the subfamilies are still being debated, but taxonomists generally agree that fungus-cultivating *Macrotermitinae* are basal to other higher termite lineages and that *Nasutitermitinae* and *Termitinae* are the most derived (41, 64). Fossil remains date higher termite evolution to ~ 50 mya in the Eocene (48) .

Apart from molecular phylogeny, important characteristics related to diet, gut structure, and gut microbial community composition also distinguish lower termites from their higher termite relatives. The diet of lower termites is comprised mainly of wood but can also include grass (64). The gut tracts of lower termites are relatively simple, consisting of foregut (crop and gizzard), midgut (site of digestive enzyme secretion), hindgut (largest compartment where most microbes are located and where digestion occurs),

colon, and rectum [(16, 89), see diagrams in (25)]. Microelectrode measurements have revealed that the hindgut is circumneutral and features steep radial gradients of O_2 (microoxic at the epithelial surface, anoxic in the luminal center), H_2 (~ 70 kPa in the center, decreasing outwards), and redox potential (-130 to -290 mV in the center, increasing outwards) (8, 26, 100). Axial variations in pH and redox have also been documented (6, 8). Physicochemical variations correlate with host activity (i.e., host-controlled tracheal gas exchange) and microbial distribution within the hindgut (77). Overall, the hindgut harbors a dense microbial community composed of morphologically diverse prokaryotes and cellulose fermenting flagellate protozoa that together enable lower termites to thrive on wood (16, 24).

Higher termites, on the other hand, are able to eat a wider range of substrates. These include wood in various stages of decay, grass, dung, leaf-litter, soil rich in humus, and fungi (9). They also possess multi-chambered hindguts with varying levels of segmentation (i.e., segments P1-P5) (24, 90). Degree of hindgut segmentation varies among higher termite species; soil-feeding termites possess the most highly differentiated hindguts, whereas fungus-cultivating termites have the least complex gut tracts. Physicochemical parameters such as pH and redox potential vary between and within hindgut chambers (26), and have been related to host factors and prokaryotic community composition along axial and radial axes of the gut tract (109, 111). Higher termites harbor *Bacteria* and *Archaea* in their hindguts (like lower termites) but do not possess any gut flagellates. The complete lack of flagellate protozoa is perhaps the most striking feature of the higher termite gut microbial community.

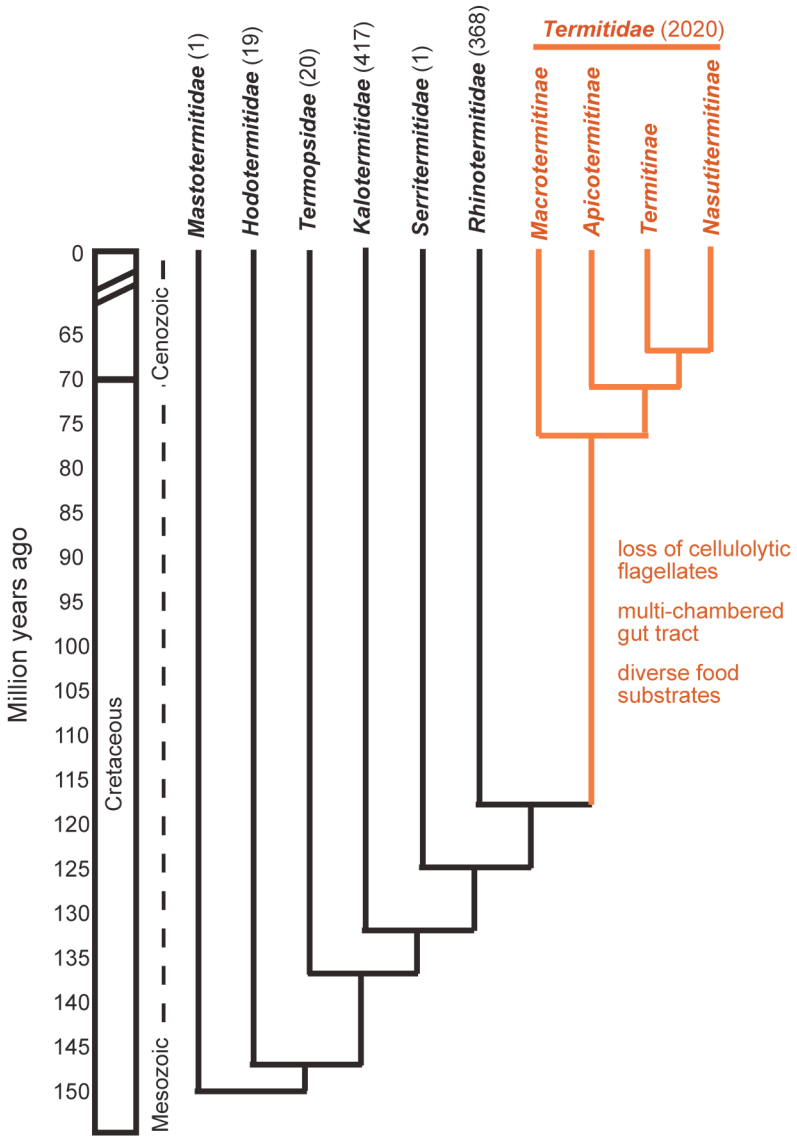

Figure 1.2. Phylogenetic reconstruction of termite family and subfamily evolution using extant and fossil insect data [adapted from Figure 7.88 in (48)]. Species number and key characteristics distinguishing higher (in red) from lower termites (in black) are listed.

1.2.8. Termite gut microbiota

All termites harbor a complex community of microbes in their gut tracts. Wet mount preparations of hindgut contents reveal an environment densely packed with microbes that have diverse morphologies and exhibit varying levels of motility. Microscopy counts indicate at least 10^9 cells are present in every milliliter of hindgut fluid [(16) and references therein]. This is 3-orders of magnitude greater than the microbial density of seawater (124).

Lower termite gut microbiota include members of all three domains of life. Higher termites, as previously described, have robust prokaryotic gut populations but do not possess eukaryotic gut protists. In the following section, I provide a summary of current knowledge for each microbial group.

Eukaryotic Flagellates

Gut flagellates are perhaps the most visible members of the gut community in lower termites and wood-feeding roaches. They dominate ~ 90% of hindgut volume, have striking morphologies, and interesting patterns of movement [(16) and references therein]. In addition, flagellates which are morphologically and phylogenetically similar to those in termites and roaches do not appear to exist anywhere else in nature (23, 55). As such, they have been the focus of over a century of study (74).

Taxonomic studies indicate gut flagellates represent over 400 different species of *Oxymonadida* and *Parabasalia* (23, 55, 62, 132). All but two species, *Trichonympha*

termosidis and *Trichonympha sphaerica* isolated by Yamin in the 1980s (129, 130), remain uncultured. Difficulties in isolation result from incomplete knowledge of the nutritional requirements for different protists — it is likely many obtain unidentified nutrients from other microbes. Indeed, prokaryotes are known to colonize protist cell surfaces as ectosymbionts and even exist inside protozoa as endosymbionts (27, 61, 62, 105). The recently published genome sequences of two endosymbionts, a putative nitrogen fixing *Bacteroidete* (53) and a member of the *Endomicrobia* (previously known as phylum TG1) thought to produce amino acids for their host protist (52), support the concept that termite gut protists form nutritional symbioses with other gut microbes. The associations between endosymbionts and their hosts appear to be fairly stable based on recent phylogenetic evidence indicating protist-endosymbiont cospeciation (88, 122).

Archaea

Termite guts harbor significantly fewer Archaea than Bacteria (i.e., ~ 5% versus 95%) (13). In addition, Archaeal populations in wood- and litter-feeding termites are consistently lower than in soil-feeding termites (13). Phylogenetic analyses have indicated that Archaea are methanogens that belong to the genus *Methanobrevibacter* in the order *Methanobacteriales* (24). Only two termite gut methanogens (both *Methanobrevibacter sp.*) have been isolated to date (71, 72). These appear to be attached or in close proximity, to the gut wall of the lower termite *Reticulitermes* and are tolerant of microoxic levels of O_2 (71, 72). The latter finding is significant as all methanogens were once considered strict anaerobes. Non-methanogenic Archaea exist in termites (e.g., *Thermoplasmales* and *Crenarchaeota*) but have been much less studied (45, 113).

A recent review highlights developments in our understanding of these gut community members (24).

Bacteria

Bacteria are, by far, the most abundant prokaryotes in termite guts (13). 16S rRNA gene studies have greatly aided efforts to define this population. Extensive surveys from the wood-feeding lower termite *Reticulitermes speratus* (50, 54) have shown that members of the phylum *Spirochaetes* are the most dominant (~ 50% of clones), followed by *Cytophaga-Flexibacter-Bacteroides* (CFB group, ~ 20%), low G+C *Firmicutes* (~ 15%), and *Endomicrobia* (~ 10%). The remaining ~ 5% of clones in these studies affiliated with *Proteobacteria*, *Actinobacteria*, *Mycoplasma* and other phyla. Practically all phylotypes recovered in 16S rRNA surveys represent uncultured species; many appear to be unique to termite gut environments.

Wood-feeding higher termite *Nasutitermes* (subfamily *Nasutitermitinae*) and *Microcerotermes* (subfamily *Termitinae*) bacterial communities have also been investigated with 16S rRNA gene inventory methods (51, 86). The prevalent gut bacteria are *Spirochaetes* (~ 60%), *Firmicutes* (~ 10%), and CFB group (~ 10%) bacteria, similar to lower termites. But two differences are worth noting: Higher termite guts lack *Endomicrobia* and harbor a new group of bacteria, the *Fibrobacters* (phylum TG3, ~10%). The absence of *Endomicrobia* is expected given the loss of cellulolytic gut protists by higher termites. The presence of fibrobacters in higher termites is more intriguing as the ruminant isolate *Fibrobacter succinogenes* is a well-known cellulolytic

bacterium (115). A recent metagenomic analysis of the gut community in a wood-feeding higher termite not only confirmed previous gene inventory studies, but also implicated fibrobacters and, surprisingly, spirochetes as functional replacements for cellulolytic flagellate protozoa (123).

Coevolution of termites with their gut bacterial communities has been another focus of exploration. Hongoh *et al.* (51) analysed bacterial 16S rRNA genes from 8 different species of Japanese *Reticulitermes* and *Microcerotermes* and showed that communities from termites of the same species are more similar to each other than to communities from termites of different species. This suggests some degree of host-symbiont coevolution (51); the extent of coevolution remains under debate. Lower and higher termite gut 16S rRNA sequences appear interspersed within bacteria phyla (e.g., *Spirochaetes*) suggesting symbiont phylogeny deviates from termite host phylogeny at family scales (5, 78, 93-95, 97). However, Berlanga *et al.* (5) argued against this interpretation with data that indicates 16S rRNA sequences from termites of the same lower termite family (*Kalotermitidae)* are more closely related to each other than to sequences from termites of a different lower termite family (*Rhinotermitidae*). A recent publication concluded the evolutionary history between ectosymbiotic *Bacteroidales* and gut protists has involved multiple instances of symbiont acquisition, suggesting the evolutionary history of termites and their gut symbionts is influenced by additional factors besides coevolution (87). More taxon sampling of bacteria and their hosts is needed for clarification.

Other factors like diet (e.g., soil versus wood) and gut physicochemistry (e.g., pH) also

impact community composition (12, 13, 86). With respect to diet, low G+C *Firmicutes* (~ 70%) are the dominant bacteria in soil-feeding termites but not wood-feeding higher termites, whose bacterial communities are instead dominated by spirochetes (24, 111). With respect to physicochemistry, the highly alkaline anterior gut sections (pH ~ 11, P1) of both wood- and soil- feeding termites are consistently dominated by firmicutes (110, 118); posterior hindgut sections (P3, P4) of soil-feeders are more circumneutral (pH ~ 7 – 10) and harbor CFB group, proteobacteria and spirochetes (111). Circumneutral P3 sections of wood-feeders are dominated by spirochetes. More details on termite gut microbial community structure can be found in several reviews (16, 17, 19, 24, 62).

1.2.9. Termite gut nutritional ecology

All termites engage in obligate nutritional symbioses with a dense and complex community of microbes in their hindguts (16, 19, 24, 62). Symbioses supporting the carbon, energy, and nitrogen metabolism of lignocellulose-feeding termites have been identified (24). These symbioses are not unexpected given the nutritional paucity of the host diet – lignocellulose lacks essential nutrients like amino acids and vitamins and has a C:N ratio 100-fold higher than insect tissue (70). Much less is known about the symbiotic relationships in soil-feeding termites, whose food substrates are very ill-defined (12).

As this thesis focuses on acetogenesis, a process mediating carbon and electron flow, I will only discuss organisms and processes related to lignocellulose degradation in termites. Details on nitrogen symbioses (e.g., N_2 fixation and uric acid recycling) can be found in references (16, 17, 19, 24, 62).

Model of lignocellulose degradation

Lignocellulose is a complex matrix comprised of three biopolymers: cellulose, hemicellulose, and lignin. The relative contribution of each polymer may vary with plant species and tissue, but lignocellulose generally contains 20 – 35% cellulose, 30% lignin, and 5% hemicellulose (82). Several studies have shown that termites and their gut communities only metabolize the cellulose and hemicellulosic fractions (16, 24, 56). Figure 1.3 depicts a schematic of the current model for wood degradation in termites. This degradation is stepwise: (1) termites increase wood particle surface area to volume ratio by maceration; (2) polysaccharides are hydrolyzed and then fermented, yielding the fermentation by-products H_2, CO_2, and acetate; (3) H_2 and CO_2 are converted to additional acetate by acetogenic bacteria. Very little carbon and energy is lost from the system as methane; this has prompted some to consider termite guts as the most efficient bioreactors in nature (100).

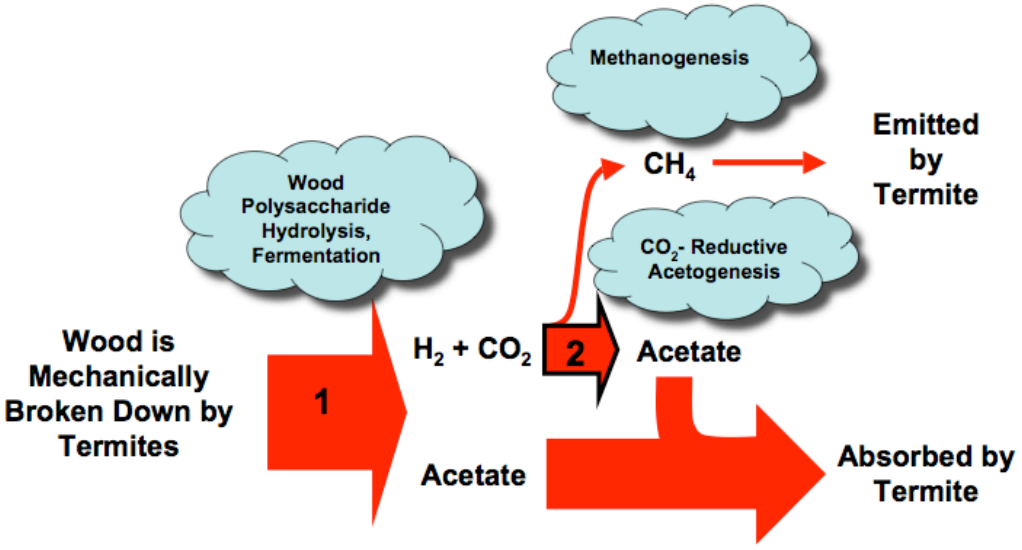

Figure 1.3. General scheme outlining carbon and energy flow in wood-feeding termites. (Adapted from schematic by J. R. Leadbetter, personal communication). Step 1 results from the combined activities of host insect and cellulolytic protozoa in lower termites; spirochete and fibrobacter bacteria are implicated in step 1 in higher termites (123). Spirochetes dominate acetogenesis (step 2) in lower and higher wood-feeding termites (99, 103, 123). Acetogenesis generates up to ~ 1/3 of gut acetate (21), which can accumulate up to 80 mM in the gut and support up to 100% of termite respiratory metabolism (92).

Role of termites

Termites contribute to lignocellulose degradation by providing finely macerated wood particles with increased surface area to aid their symbionts in their degradative activities. However, the role of termite-derived cellulase enzymes is more debatable. Termites encode endoglucanase genes which, when expressed, have hydrolyzing activity on crystalline cellulose (120). However, the site of expression (salivary glands and midgut) argues against a driving role for the termite host in cellulose hydrolysis and fermentation, since these processes are thought to occur in the hindgut paunch (121, 133).

Role of flagellate protozoa

The role of flagellate protozoa in termite nutrition has been the subject of study since the 1920s. The first studies, performed by Cleveland (28-30), demonstrated that the selective removal of anaerobic protozoa with hyperbaric O_2 correlates with low survival rates of termites fed wood and cellulose. This was the first hard evidence that flagellate protozoa play a fundamental role in the wood-feeding ability of termites and roaches. Later, Hungate reported the biochemical basis for the termite's dependence on protozoa is protozoal depolymerization of cellulose into glycosyl units and the subsequent fermentation of these units into short chain fatty acids, which could be absorbed by the host for it's metabolism (58-60). Kovoor then identified acetate as the major short chain fatty acid in termite guts [(68), reviewed by (15)]. Measurements of cellulose fermentation stoichiometries in axenic cultures of termite gut protists by Yamin and colleagues (91, 129-132) were consistent with Hungate's calculations.

Odelsen and Breznak's study of volatile fatty acid (VFA) production in wood-feeding termite gut homogenates using gas chromatography/mass spectroscopy confirmed acetate as the dominant gut short chain fatty acid and the major fuel for termite respiration (acetate supported 77-100% of *Reticulitermes flavipes* respiration) (92). Their results supported the idea that protists are the major gut acetate producers. But Odelsen and Breznak's most striking finding was that the concentration of acetate was lower in the guts of antibiotic dosed termites than the control group. This was the first evidence implicating bacteria in the carbon and energy nutrition of their host.

Role of acetogenic bacteria

A landmark study by Breznak and Switzer in 1986 (21) firmly supported Odelsen and Breznak's hypothesis that bacteria contribute to acetate production (and, hence, termite nutrition) via acetogenesis from H_2 + CO_2. More significantly, Breznak and Switzer showed acetogenesis rates in termite guts are high enough to impact the global carbon cycle. Using ^{14}C to trace carbon and electron flows in gut homogenates, they demonstrated that the dominant reductant consuming process in the guts of wood-feeding termites is CO_2-reductive acetogenesis, rather than methanogenesis. A later study by Brauman *et al.* (14), which reports acetogenesis and methanogenesis rates in termites with different feeding habits, confirmed Breznak and Switzer's observations and extended the dominance of acetogenesis to grass-feeding termites. This latter data set indicated that acetogenesis rates could be 15 to 20-fold higher in wood- and grass-feeding termites than soil- and fungus-feeding termites.

Recently, Pester *et al.* (98) showed that H_2 is the central free intermediate in lignocellulose degradation using hydrogen microsensors to infer H_2 flux in three lower termite species. Their measurements indicated termite guts are characterized by high concentrations of H_2 (~ 70 kPa) and rapid turnover, with little H_2 loss from the system. In addition, microinjections of $NaH^{14}CO_2$ into intact hindguts revealed methane emission accounts for only 4% of respiratory electron flow, whereas acteogenesis corresponds to ~ 20%. Their results were consistent with previous estimates (based on gut homogenates) that CO_2-reductive acetogenesis can fuel up to ~ 30% of termite respiration (21).

The finding that acetogens outcompete methanogens for H_2 in termite guts remains curious. Methanogenesis is predicted to dominate acetogenesis as an electron sink in anaerobic environments, based on its energetic favorability (methanogenesis $\Delta G^{o'}$ = -136 kJ/mol, acetogenesis $\Delta G^{o'}$ = -105 kJ/mol), and typically does so in sulfate depleted sediments and the ruminant gut. More study is needed to elucidate reasons underlying robust acetogen and meager methanogen populations in termite guts.

1.2.10. Termite gut acetogens

Firmicutes and Spirochaetes isolates

Only seven termite gut acetogens have been isolated to date. Almost all isolates, at the time, were considered new bacterial species. The first gut acetogen isolate, *Sporomusa termitidae*, was obtained by Breznak and colleagues (22) from the wood-feeding higher termite *Nasutitermes nigriceps*. Later isolates (and their origins) include: *Acetonema longum* (lower termite) (66), *Clostridium mayombei* (soil-feeding higher termite)(65), *Sporomusa termitidis* (wood-feeding higher termite) (47), *Sporomusa aerivorans* (soil-feeding higher termite) (11), *Treponema primitia* str. ZAS-1 (lower termite) (73), and *T. primitia* str. ZAS-2 (lower termite) (73). Most of these isolates can utilize carbohydrate-derived reductants for acetogenesis in addition to H_2; some are capable of mixotrophic growth wherein H_2 + CO_2 and organic substrates are utilized simultaneously for catabolism and anabolism (18, 46). Of the 7 isolates, strains ZAS-1 and ZAS-2 are the only bacteria that are not *Firmicutes*. To this day, they remain the sole examples of chemolithoautotrophy in the phylum *Spirochaetes*.

"Culture-independent" surveys of acetogens implicate spirochetes

The abundance and diversity of spirochetes in termite guts led investigators to speculate that spirochetes might be the dominant acetogens in these environments. Leadbetter's isolation of two acetogenic spirochetes provided the first concrete evidence moving this speculation into the realm of hypothesis (73). However, the in situ relevance of microbial isolates is typically questionable, since major cultivation biases limit isolate studies to those bacteria that manage to thrive in a given enrichment medium. The predictive capacity of pure culture studies, therefore, needs to be corroborated with "culture-independent" molecular profiling techniques, which can survey the entire gut community. Salmassi *et al*. (103) used such an approach to survey the acetogen population inhabiting the guts of the wood-feeding lower termite, *Zootermopsis nevadensis.* They analyzed clone libraries of the Wood-Ljungdahl pathway marker gene, *fhs*, which encodes formyl-tetrahydrofolate synthetase (FTHFS) (Figure 1.2), and discovered that the majority of sequences phylogenetically affiliated with the FTHFS from the spirochete, *T. primitia*. This finding was the first solid molecular evidence supporting the hypothesis that spirochetes are the dominant acetogens in termite guts. Pester and Brune's survey of expressed *fhs* genes in lower termite guts lent additional support to the concept that spirochetes are the major acetogens in wood-feeding termites (99).

1.2.11. *Treponema primitia* str. ZAS-2 formate dehydrogenases

Gene surveys of 16S rRNA and FTHFS prompted a recent study by Matson *et al.* (85) of the Wood-Ljungdahl pathway in *T. primitia* str. ZAS-2, in which they report the

discovery of two genes for the CO_2-fixing Wood-Ljungdahl enzyme, formate dehydrogenase (FDH). This finding is significant for two reasons:

First, FDH genes in *T. primitia* strain ZAS-2 are only distantly related to FDH in the model acetogen, *M. thermoacetica*. FDH genes in *T. primitia* phylogenetically group with genes for hydrogenase-linked FDH (FDH_H, *fdhF*) in enteric *Gammaproteobacteria,* which utilize FDH_H for carbohydrate fermentation (43). This is highly unexpected as no Wood-Ljungdahl pathway enzymes in *M. thermoacetica* (or any other acetogen) are known to be hydrogenase-linked (i.e., associated in a multi-enzyme complex like FDH_H in the *Escherichia coli* formate hydrogenase lyase complex) (36). Indeed, the canonical FDH in *Moorella* is a NADPH-linked enzyme that only interacts indirectly with hydrogenase (128). The identification of hydrogenase-linked FDH genes in *T. primitia* therefore suggests that the current model of acetogenesis based *M. thermoacetica* may not have relevance in termite guts, where acetogenesis rates translate into globally relevant carbon fluxes.

Second, the two genes encode selenium-dependent (Sec) and selenium-independent (Cys) FDH_H homologs. This finding is the first indication that the redox active trace element selenium may influence *T. primitia*'s energy metabolism and physiological ecology. The selenium-dependant FDH_H gene contains an in-frame TGA stop codon encoding the non-canonical amino acid, selenocysteine, at the enzyme active site. In contrast the other gene encodes the amino acid cysteine at the corresponding catalytically relevant position. Studies on selenocysteine enzymes show that they are more catalytically active than their

cysteine homologs (2). This led Matson *et al.* (85) to hypothesize that *T. primitia* preferentially utilizes its selenium-dependent FDH_H when selenium is replete and switches to its selenium independent variant as a "back up" when selenium is scarce. They tested this hypothesis with quantitative RT-PCR and confirmed that transcription of the two gene variants varied with selenium concentration in the predicted directions (i.e., Sec variant transcription increased and Cys variant transcription decreased with the addition of selenium). Matson *et al.* (85) therefore posited that selenium may influence the genome content and physiological ecology of uncultured termite gut acetogens. My work on uncultured termite gut acetogens stems from this hypothesis and is presented in Chapters 2 – 4 of this thesis.

1.2.12. Overview of chapters 2 – 4

My work aims at understanding the diversity, evolution, and activity of termite gut acetogens at a functional gene level. I have employed traditional gene inventory, novel sequencing, and single cell techniques to:

(i) assess whether hydrogenase-linked FDH genes (*fdhF*) are relevant for CO_2-reductive acetogenesis in the guts of taxonomically and nutritionally diverse termites,

(ii) determine whether Sec and Cys forms of *fdhF* have relevance in uncultured termite gut microbes,

(iii) discover uncultured acetogenic spirochetes which encode both Sec and Cys *fdhF* like *T. primitia*, and

(iv) identify microbes whose activity dominates *fdhF* transcription in gut communities.

In Chapter 2, I present work related to degenerate *fdhF* primer design and gene inventory analysis from three species of phylogentically lower termite and a wood-feeding roach (objectives i and ii).

In Chapter 3, I discuss surveys of *fdhF* diversity in taxonomically and nutritionally diverse higher termites and compare *fdhF* phylogeny in higher and lower termites (objectives i and ii).

In Chapter 4, I demonstrate that high-throughput sequencing of community mRNA can be leveraged by gene inventory and pure culture data to identify the major transcriptionally active *fdhF* phylotypes in termite gut microbial communities (objective iv). I also show that microfluidic multiplex digital PCR can be used to discover the 16S rRNA identity of transcriptionally active uncultured microbes as well as those that encode both Sec and Cys gene variants (objective iii).

1.3. Introduction to bacterial lipid D/H and metabolism

Compound specific isotope analysis is a powerful tool for identifying sources and inferring processes in the environment (42). Accordingly, compound-specific approaches have gained popularity in a wide variety of fields; these include organic geochemistry, paleoclimate, bioremediation science, and archeology [(42, 108) and references therein]. Many of these studies have relied on stable isotopes of carbon, but recent instrumental developments now allow the stable isotopes of hydrogen to be measured easily and accurately (112).

Compound specific stable hydrogen isotope ratios (D/H) have already proved useful in environmental science. For example, D/H of lacustrine sedimentary lipids have been used to infer D/H of environmental water and reconstruct the geochemistry of past environments (106). Measurements of lipid D/H in marine sediments have also been made but are more difficult to interpret (63, 75). This primarily stems from our limited mechanistic understanding of the factors and processes underlying D/H signals in organic matter. My work seeks to clarify the biological determinants of lipid D/H in microbes, organisms which account for ~ 50% of all living biomass on the planet (124).

Overview of chapter 5

In the last chapter of this thesis, I present my investigation of the relationship between metabolism and lipid D/H in physiologically diverse bacteria. I show evidence that lipid D/H varies systematically with energy metabolism and propose a biological basis for lipid D/H variations.

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