

HYDROGENASES AND HYDROGEN
SENSORS IN THE SYMBIOTIC
MICROBIAL COMMUNITIES OF WOOD-
FEEDING TERMITES

Thesis by

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In Partial Fulfillment of the Requirements for the

degree of

Doctor of Philosophy

CALIFORNIA INSTITUTE OF TECHNOLOGY

Pasadena, California

2011

(Defended August 16, 2010)

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ACKNOWLEDGEMENTS

I am grateful to everyone who has helped me, in any way, to reach this climactic point in my education. I do not know where to begin. The list is endless. It seems any attempt at thanks is destined for oversight and inadequacy, but here goes.

I can't say I thought I'd be working with termites when I left Michigan Tech. But, here I am, a fan of a biological system among the most fascinating and relevant microbial symbioses and bioreactors known in nature. I am thankful to Jared Leadbetter for allowing me to join his lab and discover this little world. It has been a wonderful journey. I am also thankful for his holistic philosophy that one's legacy encompasses more than his publication record.

A big "thank you" to my co-workers at Kimberly-Clark, especially Dave Fell and Amy Weinheimer, for inspiring me to go a bit deeper into the rabbit hole. And to the folks at the University of South Carolina and Montana State University's Center for Biofilm Engineering, especially Robin Gerlach, for allowing me to try my hand at academic research.

I am indebted to Donald Lueking for taking me under his wing at Michigan Tech. His confidence in me, and the amazing opportunities he entrusted to me, was instrumental to mobilizing me as a scientist. I am so blessed to have known this professor at the peak of his outstanding teaching career in molecular biochemistry. I am also thankful to Susan Bagley and John Adler from Michigan Tech. They were a tremendous help as I ventured to transition from the world of chemical engineering to the biological sciences. I also want to give a nod to the chemical engineering department at Michigan Tech. for helping me

weed my way through the process of forming a solid intellectual foundation and selflessly making education a first priority.

My labmates throughout the years have helped me thorough the daily grind. My professional development has been a community endeavor. I am grateful to all who had the patience to help me along the way.

Of course, I would be remiss not to thank my parents and family. They have been tremendously supportive through the years. They have always encouraged me to go beyond my comfort zone and to place high value on reason and education.

I am also grateful to the many friends who have helped me along the way.

This research was partially supported by an NSF Graduate Student Research Fellowship.

ABSTRACT

The termite gut is an ideal ecosystem for studying hydrogen ecophysiology. Hydrogen is central to the obligate mutualism between termites and their gut microbes and is turned over at rates as high as 33 m³ H₂ per m³ hindgut volume daily and maintained near saturation in some species. Acetogenic bacteria use hydrogen to produce up to 1/3 of the total flux of the termite's primary carbon and energy source, acetate. We have taken a three-fold approach to investigate the hydrogen ecophysiology of the termite gut. In our first approach (Chapter 2) we completed a bioinformatic analysis of [FeFe] hydrogenase-like (H domain) proteins encoded in the genomes of three termite gut treponemes. Treponemes are among the most highly represented groups of gut bacteria. The remarkable diversity of H domain proteins encoded accentuates the importance of hydrogen to their physiology. Moreover, they encoded a poorly understood class hydrogen sensing H domain proteins and thereby present a unique opportunity for their further study. In our second approach (Chapters 3 and 4) we analyzed molecular inventories prepared from termite gut microbiomes of a class of [FeFe] hydrogenases found highly represented in a termite hindgut metagenome. The libraries of peptide sequences clustered with one another in a manner congruent with termite host phylogeny suggesting co-evolution. Interestingly, we observed that higher termite guts may harbor higher sequence diversity than lower termites. In our third approach (Chapter 5) we used microfluidic digital PCR to identify bacteria in the gut of *Reticulitermes tibialis* encoding [FeFe] hydrogenases. The majority of the 16S rRNA gene phlotypes observed to co-amplify with hydrogenase sequences were treponemal, and the only observed instances of the same 16S rRNA-hydrogenase gene pair co-amplifying in multiple microfluidic chambers corresponded to treponemal phlotypes. Therefore, treponemes may be an important or predominant bacterial group encoding an important family of [FeFe] hydrogenases in the termite gut. The above results provide support for an important role for treponemes in mediating hydrogen metabolism in the termite gut and accentuate the intimacy and stability of the association termites have maintained over the course of their evolution with their gut microbial communities.

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Chapter 1

BACKGROUND

Hydrogen in the Termite Gut

Hydrogen is of central importance to the symbiotic community residing in the termite gut (1, 5, 15, 35, 36, 41). This obligate mutualism, in many instances comprising microbes from all three domains of life, enables termites to derive carbon and energy from wood (2-4, 6, 11, 12, 17, 19, 20, 35, 36, 56, 61, 62).

The general scheme underlying the symbiosis between wood-feeding termites and their gut microbes is shown in Figure 1-1. Termites ingest wood and microbial symbionts then ferment its component polysaccharides to produce primarily acetate, carbon dioxide, and hydrogen (1, 5, 12, 19, 35-37, 41, 54, 56, 62). Acetate is absorbed by the termite and used for energy and biosynthesis (37). The carbon dioxide and hydrogen produced in this initial fermentation are used by bacteria in reductive acetogenesis to produce more acetate— up to 1/3 of the total pool in the gut (1, 5, 27, 37, 41). With these high rates of reductive acetogenesis, the termite gut is “the smallest and most efficient natural bioreactor currently known” (41). Only a small portion of the hydrogen is used by methanogens to produce methane (1, 25, 41). The total daily production of hydrogen in this environment can be up to 9-33 m³ H₂ per m³ hindgut volume (41). Hydrogen partial pressures have been measured in the termite gut that exceed those measured for any other biological system (15, 18, 41, 45, 47, 49, 53). Figure 1-2 presents a comparison of partial pressures of hydrogen measured in representative microbial communities.

A complex matrix of microenvironments characterized by different hydrogen concentrations are maintained in the termite gut (6-8, 15, 25, 26, 41). Hydrogen partial

Figure 1-1.

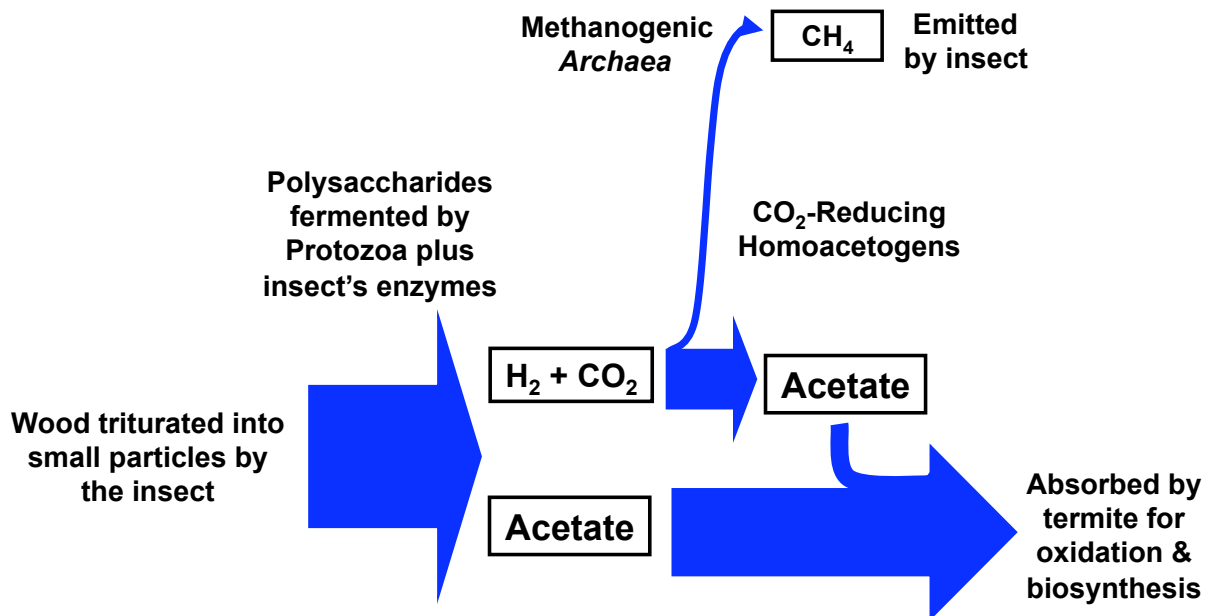


Figure 1-1. General scheme underlying symbiosis between anaerobes and wood-feeding lower termites. Termites consume wood and break it down into small particles. The particles are degraded and their component polysaccharides fermented by the symbiotic microbial community in the termite gut (12, 19, 56, 62). The acetate produced in this fermentation is absorbed by the termite and used for respiration and biosynthesis (37). The H_2 and CO_2 formed in the initial fermentation is used primarily by homoacetogenic bacteria in reductive acetogenesis to produce more acetate (1, 5, 41). A small fraction of the H_2 and CO_2 is used by methanogenic archaea to produce methane that is emitted by the insect (41, 52, 54). Figure was kindly provided through a private communication by Jared R. Leadbetter.

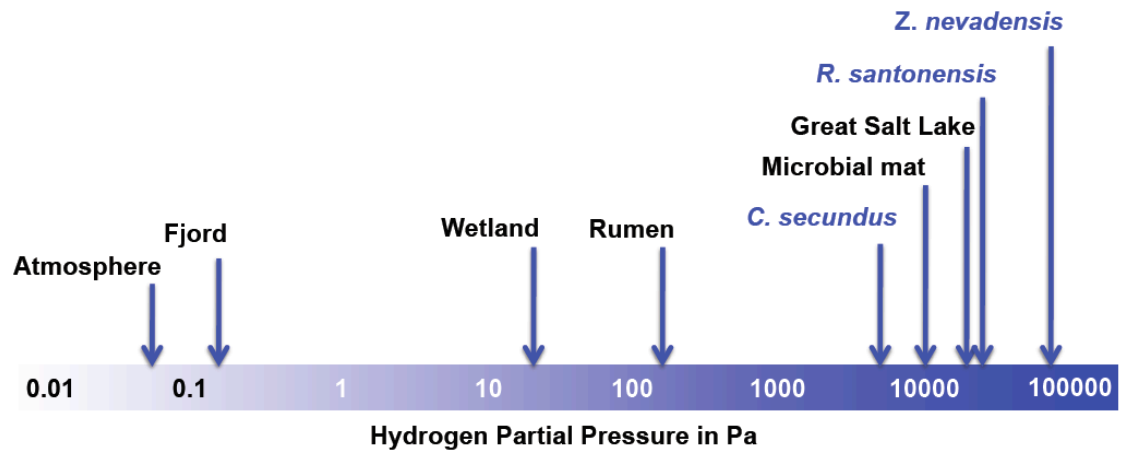
Figure 1-2.

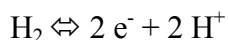
Figure 1-2. Representative biologically produced hydrogen partial pressures. The partial pressure of hydrogen in the atmosphere is given as a reference. Hydrogen pressures were measured in the bubble gas of a natural wetland (53); in a surface layer of intertidal mats dominated by *Lyngbya* spp. (18); in the hindguts of termites, shown in blue (41); in the open waters of Saanich Inlet, an anoxic fjord in British Colombia (47); in the rumen of a steer (49); and in the pycnocline of the Great Salt Lake in Utah (45).

pressures in the guts of termites have been found to vary dramatically with position, see Figure 1-3 (15, 41). Hydrogen partial pressure reaches a maximum in the hindgut paunch, see Figures 1-3A, and decreases as you approach the axial extremities of the gut, see Figure 1-3B (7). Hydrogen partial pressures also vary radially being highest at the center of the gut and decreasing symmetrically to near zero at the epithelium (7).

The work presented in the following chapters addresses questions remaining unanswered about genes encoding proteins that eubacteria use for producing, consuming, and monitoring hydrogen in the termite. The objective has been to advance our understanding of the nature of the symbiosis in the termite gut. Study of the rich diversity of hydrogenase-like proteins (H domain proteins) endemic to the termite gut is a unique opportunity to provide substantial contributions to our understanding of these proteins.

Hydrogenases

H domain proteins are used to make, break, or sense hydrogen. Hydrogenases catalyze the following reaction:



There are four major classes of hydrogenases – all named according to the metal composition of their catalytic sites: the evolutionarily related nickel iron (NiFe) hydrogenases and nickel iron selenium (NiFeSe) hydrogenases, and two evolutionarily distinct classes called [FeFe] hydrogenases and metal-free hydrogenases, for a review see Schwartz *et al.* (46).

The structures of two [FeFe] hydrogenases, CpI from *Clostridium pasteurianum* (42) and the heterodimeric [FeFe] hydrogenase from *Desulfovibrio vulgaris* (32), have been solved, and the catalytic site, or H cluster, of CpI from *C. pasteurianum* is shown in Figure 1-4.

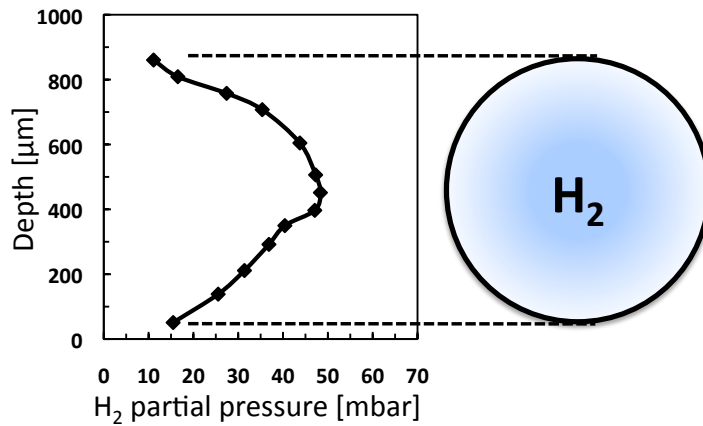
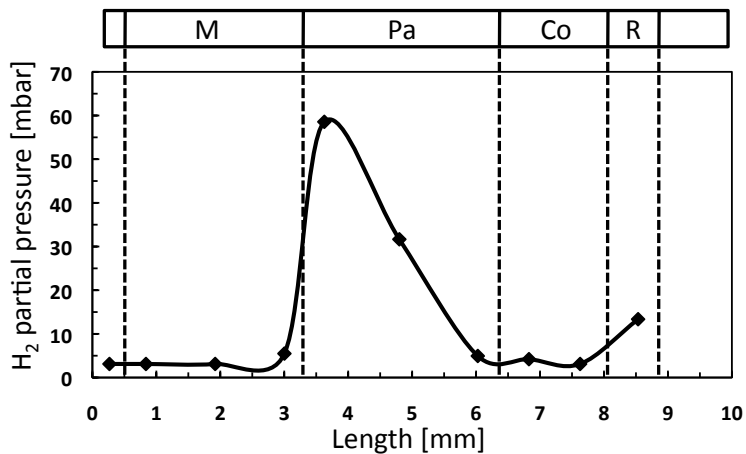
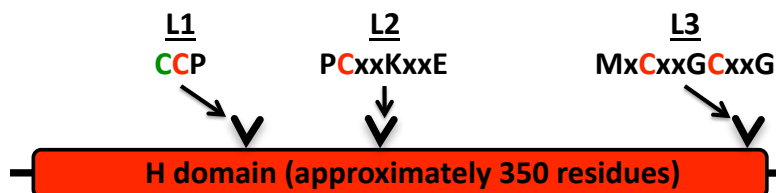
Figure 1-3.**A.****B.**

Figure 1-3. Distribution of hydrogen partial pressures in the gut of *Reticulitermes flavipes*. (A) Radial distribution of hydrogen in the hindgut paunch of *Reticulitermes flavipes* with an image illustrating how hydrogen concentrations are maximal at the center of the hindgut paunch (dark blue) and diminish symmetrically (fading blue) toward the epithelium. (B) Distribution of hydrogen partial pressures along the axis of the gut of *Reticulitermes flavipes*. M – midgut; Pa – hindgut paunch; Co – colon; R – rectum. Measurements were taken using a microsensor. Figure is based upon a figure created by Ebert and Brune (15), which has been borrowed with permission.

Figure 1-4.

A.



B.

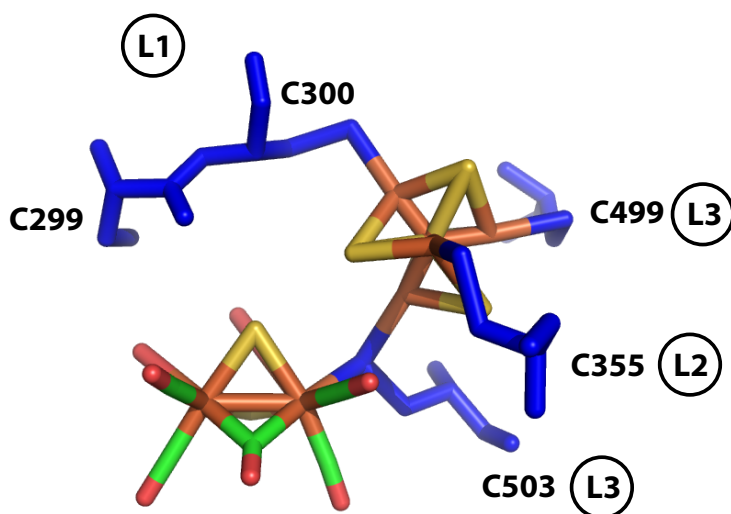


Figure 1-4. H domain and its conserved sequence signatures. (A) [FeFe] hydrogenases can be identified by the three conserved sequence signatures in their H domain (31). Each signature contains cysteine residues essential for catalysis (31). The cysteines in red are involved in coordinating the [4Fe-4S] cluster or bridging the cluster to the 2Fe cluster (31). The cysteine in green is believed to act as an acid/base in catalysis (31). (B) The H domain of *C. pasteurianum*. The cysteines of the H domain sequence signatures are in blue and the name of the sequence signature to which each belongs is indicated. The image was prepared using MacPyMOL and structure 1feh from the PDB database. C299 may participate in catalysis as an acid/base. C300, C499, C355 and C503 coordinate the [4Fe-4S] cluster domain (31). C503 bridges the [4Fe-4S] cluster to the 2Fe cluster domain (31). The 2Fe cluster is coordinated by CO and CN ligands and the two atoms are bridged by a carbon monoxide atom (31).

The cluster is made of a diatomic cluster of two iron atoms bridged by a cysteine to a [4Fe-4S] iron sulfur cluster (32, 42). These two iron atoms interact directly with hydrogen and are the namesake of this class of hydrogenases (42). Three proteins, or maturases, HydE, HydG and HydF, are necessary for assembling the H cluster of [FeFe] hydrogenases (24).

An analysis of hydrogenase-like proteins encoded in a termite gut metagenome sequence revealed that the vast majority are H domain proteins (60). It is unclear why only one of the over 100 hydrogenases identified was a [NiFe] hydrogenase. This may be a consequence of the high specific molar activities of [FeFe] hydrogenases (16). The metagenome paper provided initial experimental evidence that the termite gut microbial community is a rich reservoir of H domain proteins.

The multitude and diversity of [FeFe] hydrogenases observed in the termite gut metagenome enabled the definition of families of H domain proteins based upon phylogenetic and primary sequence character analyses. This was the first effort to classify these proteins based upon evolutionary relationships. Because of their relevance to the termite hindgut and evolutionary significance, these family designations have been used in the following chapters. Classifications based upon sequence characteristics have also been proposed by others (31, 57).

H domain proteins can be identified by three conserved sequence signatures, see Figure 1-4 (31, 57). Sequences containing these signatures are likely to be H domain proteins (31, 57).

The signatures contain cysteine residues that are essential to the H domain coordinating the H cluster (31, 32, 42).

H domain proteins contain domains that augment the function of the H domain in catalysis. H domain proteins typically have several iron sulfur cluster coordinating sites that mediate electron transfer (31, 32, 42, 57). The most common iron sulfur clusters are [Fe-S], [2Fe-2S], and [4Fe-4S] (31, 57). H domain proteins commonly contain two closely spaced, consecutive [4Fe-4S] clusters near their N-terminus (31, 57). Iron sulfur clusters are usually coordinated by cysteine residues, but it is common to find near the N-terminus of a hydrogenase that the 1st cysteine of a [4Fe-4S] has been replaced by a histidine (31, 57). Some H domain proteins contain domains not involved in electron transfer that, instead, may couple behavioral or transcriptional modifications to hydrogen levels.

Prior to the sequencing of a termite gut metagenome, few (43, 48, 60, 61) H domain proteins were proposed to contain domains normally implicated in cell signalling, for a review see Schwartz *et al.* (46). The only characterized hydrogen sensors are [NiFe] hydrogenase homologues including, most notably, those from *Alcaligenes eutrophus* and *Rhodobacter capsulatus* (9). These two proteins, as shown in Figure 1-5, are components of two-component regulatory systems involved in transcriptional regulation (14, 29). The *Nasutitermes* termite hindgut metagenome paper reported a multitude of H domains fused with domains usually implicated in signal transduction (60). These domains include the PAS domain that, in bacterial systems, is most often found in sensors of two-component regulatory systems, as in the *A. eutrophus* and *R. capsulatus* sensory hydrogenases (55). A response regulator receiver domain typically found in proteins participating in phosphorelays or two component regulatory systems (39, 50, 51) was also observed in some sequences. The final sensory domain observed was the methyl-accepting chemotaxis protein domain that may function in regulating bacterial swimming behavior (59). The

Figure 1-5.

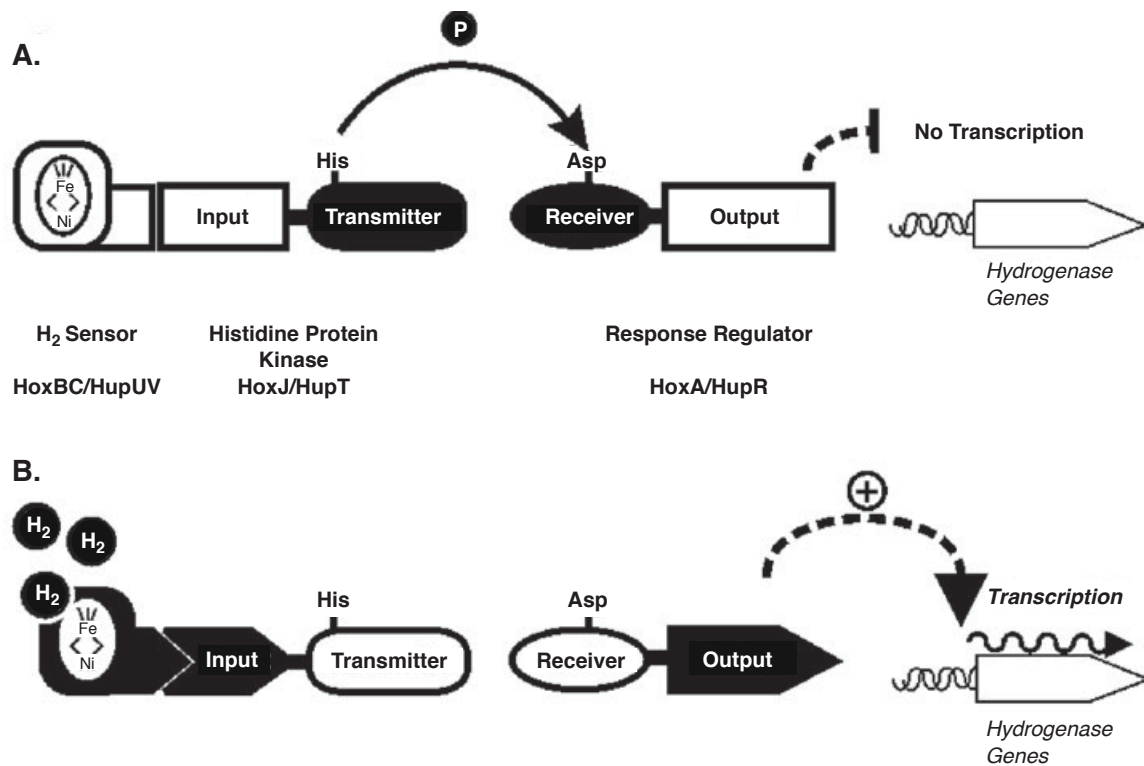


Figure 1-5. Model for the sensory [NiFe] hydrogenases of *Alcaligenes eutrophus* and *Rhodobacter capsulatus*. The sensory [NiFe] hydrogenases are part of a two-component regulatory system. In the absence of hydrogen (A) the sensing system suppresses transcription of target genes, and in the presence of hydrogen (B) transcription of target genes is activated. The input module is a PAS domain. The output module is a transcriptional activator. The transmitter and receiver modules are the canonical histidine-kinase and response regulator receiver modules of two-component regulatory systems. In the presence of hydrogen, the [NiFe] hydrogenase oxidizes hydrogen. The PAS domain then senses a change in electron potential and communicates this signal to the transmitter domain. Figure is taken from Schwartz with permission (46).

discovery of this domain in a putative H domain protein was intriguing because chemotaxis toward hydrogen has not been demonstrated experimentally. Shaw *et al.* have recently reported a PAS domain containing H domain protein from *Thermoanaerobacterium saccharolyticum* and some clostridia encode similar proteins and Posewitz *et al.* have reported proteins in *Halothermothrix orenii* with a region sharing homology simultaneously with PAS and histidine kinase domains (10, 43, 48). The discovery of a multitude of putative sensory H domain proteins in a termite's gut metagenome supports the hypothesis that termites are a rich reservoir of novel [FeFe] hydrogenase homologues and that their study may provide insight into the functional diversity and evolution of this class of proteins.

Some [FeFe] hydrogenases have heteromeric quaternary structures (31, 57, 58). The best studied multimeric [FeFe] hydrogenases are the trimeric complex from *Thermotoga maritima* and the tetrameric complexes from *Thermoanaerobacter tengcongensis*, and *Desulfovibrio fructosovorans*, see Figure 1-6 (13). These complex hydrogenases are believed to couple the oxidation or reduction of NAD(P)(H) to hydrogen production or consumption, respectively.

Levels of Physiological Resolution to Study H Domain Proteins

H domain proteins may be studied at three interdependent levels of molecular resolution: The level of individual genes or proteins, the level of individual cells or cell genomes, and the level of an entire symbiotic microbial community or metagenome. A gene or protein based analysis of H domain proteins provides the highest level of resolution facilitating an understanding of function and evolution on the molecular level. A higher level of complexity and lower level of molecular resolution may be sought through the study of the

Figure 1-6.

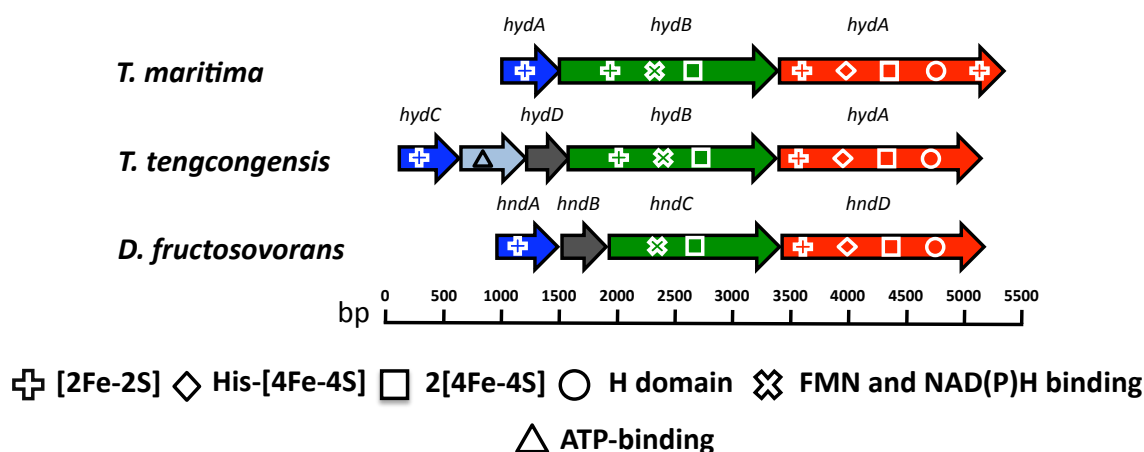


Figure 1-6. Gene organization and domain composition of multimeric [FeFe] hydrogenases. Each arrow represents a gene and homologous genes share the same color. Domain symbols are listed in proper order but are not intended to represent precise locations. Domains were identified using the Pfam server. Domains represented in the figure are: 2[4Fe-4S] – F cluster made up of two adjacent Fer4 domains, PF00037; [2Fe-2S] – PF00111, [2Fe-2S] iron-sulfur cluster binding domain; ATP-binding – PF02518, Histidine kinsae-, DNA gyrase B-, and HSP90-like ATPase; FMN and NAD(P)H binding – PF01512, Respiratory-chain NADH dehydrogenase 51 Kd subunit; H domain – PF02906 and PF02256, iron only hydrogenase large subunit, C-terminal domain, and iron only hydrogenase small subunit; His-[4Fe-4S] – a [4Fe-4S] cluster with the first coordinating cysteine replaced with a histidine.

physiological context of H domain proteins through the study of the genomes of single cells or metabolic and behavioral responses to hydrogen. At the lowest level of molecular resolution H domain proteins are investigated across an entire bacterial community in its native setting, the termite gut. This last level of resolution introduces the most complexity because it accounts for all interactions, environmental and biological, that occur *in situ*. Each level of resolution complements the understanding of the termite gut symbiosis made accessible by the others. The metagenome sequence of a termite (60) has provided an initial glimpse into hydrogenase-like protein function and phylogeny through complementary analyses at the community-wide and individual gene levels of resolution. The studies reported in the following chapters provide examples of insights obtained using all three levels of resolution to advance our understanding of the function and distribution of H domain proteins in the termite gut.

Termite Species

Over 281 genera comprising at least 2600 species of termites are known (23, 28). Based upon evolutionary relationships, these termites may be divided into six families, see Figure 1-7 (28). Members of Termitidae, the largest family of termite species, are referred to as “higher termites.” All other termites are “lower termites.”

The most commonly referenced distinction between higher and lower termites is that the latter have protozoa in their hindgut and the former do not (11). Higher termites also have a more segmented gut structure, see Figure 1-8 (6, 33, 34). The family Cryptocercidae, or wood roaches, is believed to represent the most recent common ancestor of all termites (20, 27, 30). The six families of termites provide a unique opportunity to investigate the representation of H domain proteins across evolutionarily distinct symbiotic communities.

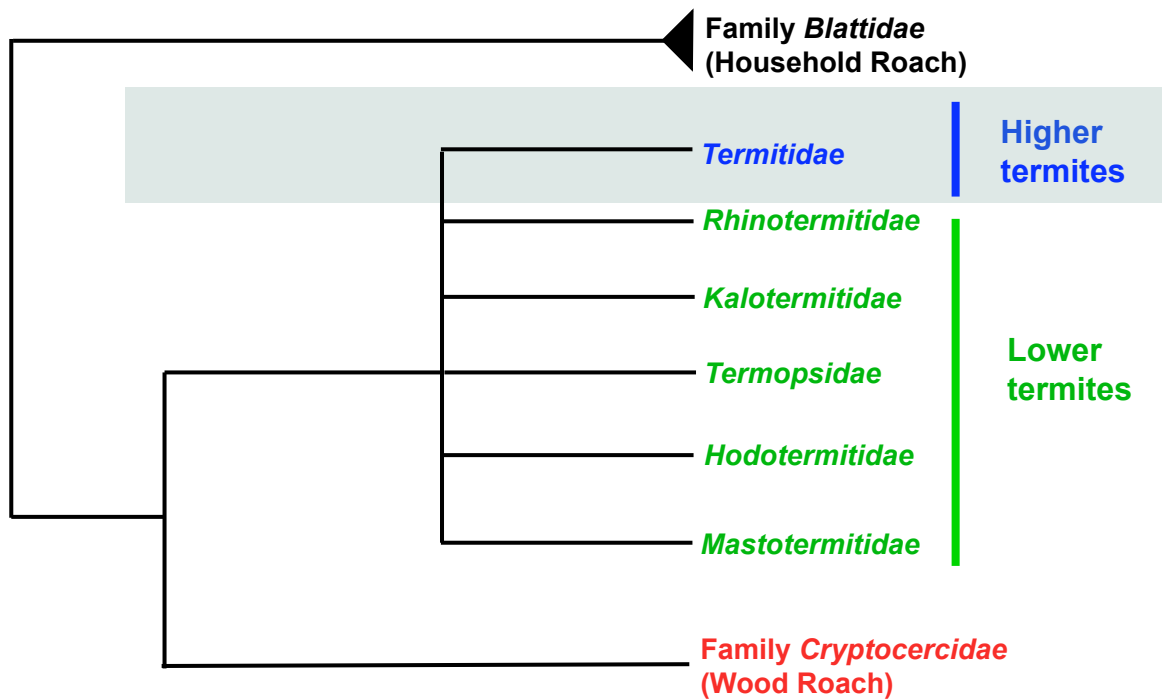
Figure 1-7.

Figure 1-7. Phylogram of termite families and wood roaches. Tree based upon phylogenetic analyses reported by Inward et al. (21, 22).

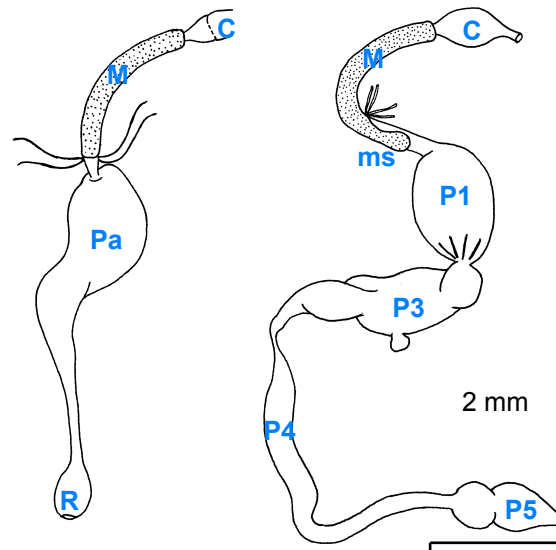
Figure 1-8.

Figure 1-8. Higher and lower termite gut structures. (A) Lower termite, *Reticulitermes*, gut and (B) a higher termite, *Cubitermes*, gut. Pa – Hindgut paunch; R – rectum; C – crop; M – midgut; ms – mixed segment; P1-P5 – proctodeal segments. Images are taken from Brune with permission (6).

The hindgut metagenome has been sequenced for a higher termite, *Nasutitermes*, from Costa Rica (60). Much of the work reported in the following chapters investigates H domain proteins in an untapped and potentially rich reservoir of their sequence diversity, namely lower termites. We have furthered our understanding of factors that may influence the distribution and evolution of hydrogenases in gut communities by cross-comparing representative sets of sequences across termite species. This has advanced our understanding of H domain proteins at the community level of resolution.

Termite Gut Treponeme Isolates

In 1999, Jared Leadbetter was the first to isolate treponemes from the hindgut of a termite, *Zootermopsis angusticolis* (17, 27, 30). Treponemes are helical shaped bacteria belonging to the phylum Spirochaete. Treponemes are among the most abundant groups of bacteria in the guts of termites, constituting up to 50% of the total prokaryotes in some species (6, 40). They may also be a major producer of acetate by reductive acetogenesis (38, 40, 44).

In the following chapter, I will present the results of a bioinformatic analysis of the hydrogenases encoded in the genomes of *Treponema azotonutricium* ZAS-9 and *T. primitia* ZAS-1 and ZAS-2, shown in Figure 1-9. *Treponema primitia* is a hydrogen consuming acetogen (17, 27). *T. azotonutricum* is not an acetogen and, therefore, not believed to be a substantial consumer of hydrogen; rather, it produces hydrogen (17).

These isolates represent a unique opportunity to study hydrogenases in species having distinct and complimentary hydrogen physiologies. Investigating the hydrogenases of these strains has contributed to our understanding of these enzymes at the single cell genome and individual gene or protein levels of resolution.

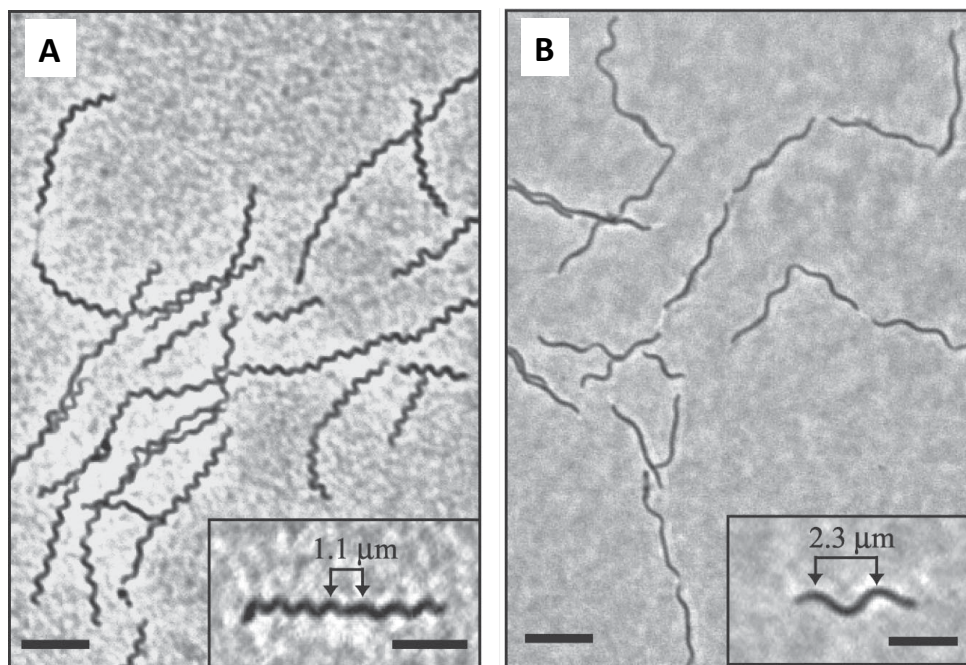
Figure 1-9.

Figure 1-9. Phase-contrast microscopy images of *T. primitia* and *T. azotonutricum*. Insets show single cells of each strain. (A) *T. primitia* ZAS-2, (B) *T. azotonutricum* ZAS-9. Bars, 5 μm for images and 2.5 μm for insets. Images taken from Graber *et al.* with permission (17).

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