Conclusions

This work focused on the biology and community structure of CO_2-reducing acetogens in the guts of termites. Using the gene for formyl-tetrahydrofolate synthetase (FTHFS) as a genetic marker of acetogenic capability, I explored the diversity of uncultured acetogens present in wood-feeding roaches and diverse termite species. I also used this symbiosis as a platform for the development of microfluidic techniques that allowed molecular characterization of single bacterial cells.

The best-known and longest-studied acetogens are bacteria associated with the phylum *Firmicutes*. These bacteria are widespread in the environment, and can be found even in ecosystems where acetogenesis is not a major H_2 sink. Acetogenic spirochetes, though dominant in the guts of wood-feeding termites, have been found nowhere else on Earth.
In chapter two of this thesis, I present the discovery that the guts of wood-feeding roaches, like those of wood-feeding lower termites, are dominated by acetogenic *Treponemes*. Phylogenetic analysis of roach-derived FTHFS types reveal a cluster of *Treponeme*-like FTHFS genes that represent a basal radiation to the termite *Treponeme* cluster. This suggests that they represent the modern descendents of an ancient divergence, and can be taken as evidence that acetogenic *Treponemes* were present in the last common ancestor of termites and roaches.

In chapter three, I present FTHFS community profiles of higher termite guts. Previous examinations of termite gut acetogens focused on wood-feeding lower termites. To complement these studies, I examined FTHFS genes present in the gut of a wood-feeding

higher termite species (*Nasutitermes* sp. Cost003). This termite was found to be dominated by termite gut *Treponemes*, as were the guts of a palm-feeding *Microcerotermes* sp. and litter-feeding *Rhynchotermes* sp. The story changed, however, when three subterranean termite species were examined. The guts of these species are dominated by a novel group of *Firmicute*-like FTHFS types. Rates of acetogenesis and methanogenesis were not measured for these species, and the exact composition of their diets remains unknown. However, the subterranean lifestyle of these termites suggests a higher degree of exposure to soil. Soil-feeding termites host robust populations of acetogenesis-capable bacteria but generally have low rates of CO_2-reductive acetogenesis. The dramatic alteration of acetogen population structure in subterranean termites (as opposed to wood-feeding termites) suggest environmental conditions that favor acetogenic *Firmicutes* over *Treponemes*. This shift in population structure seems likely to be related to the low rates of CO_2-reductive acetogenesis observed in soil-feeding termites.

Chapters four and five present the development of techniques for microfluidic PCR-based techniques for multiplex PCR from single cells. We developed these techniques in order to facilitate the species-level identification of uncultured acetogens. Using microfluidic devices, we carried out multiplex PCR on hundreds of individual environmental bacteria in parallel. PCR product retrieval and characterization allowed the establishment of FTHFS and 16S rRNA gene pairs derived from uncultured bacteria.

The ability to establish 16S rRNA sequence identities of uncultured, FTHFS-bearing bacteria opens a whole new window into the biology of termite gut acetogens. Much of the information derived from molecular community assays is based on hypotheses derived from phylogenetic inference. However, phylogenetic inference should be taken as circumstantial evidence at best, particularly as regards metabolic genes such as FTHFS. A phylogenetic inference is only as good as your closest cultured representative, which in the case of termite gut *Treponemes* consists of a grand total of two gene sequences (from *T. primitia* ZAS-1 and ZAS-2). Using the microfluidic digital PCR techniques presented herein, it is now possible to establish the species identities of uncultured FTHFS-bearing bacteria.

The first targets for microfluidic digital PCR characterization of environmental acetogens should be the novel sequence clusters we have identified in wood-feeding roaches and higher termites. The evolutionary hypotheses presented in chapter two would be greatly strengthened by definitive evidence that the FTHFS sequences discovered indeed belong to acetogenic *Treponemes*. Of particular interest is the basal "roach group III" cluster, for which the phylogenetic evidence of *Treponemal* derivation is weakest. If this sequence cluster does indeed represent acetogenic spirochetes, it will be interesting to discover where they fall within the termite *Treponeme* 16S rRNA cluster. If these sequences belong to a non-spirochetal organism, it will most likely represent the bacterial lineage from which acetogenic *Treponemes* acquired their FTHFS gene.

The most interesting target for microfluidic digital PCR in higher termites is the "Amitermes clade" of FTHFS sequences that dominates the guts of subterranean higher termites. This group was hypothesized to represent acetogenic *Firmicutes,* as the most closely related cultured organism was *Ruminococcus productus*. However, the distances involved are at least as great as those between *R. productus* and the termite *Treponeme* cluster. An exciting alternate hypothesis would be that this represents a novel lineage of termite *Treponemes* that arose following a lateral gene acquisition of FTHFS from a different acetogenic *Firmicute*.

In summary, this work presents new insights into the evolutionary history of the symbiosis between termites and CO_2-reducing acetogens and the relationship between host diet and acetogen community structure. Furthermore, it presents new microfluidics-based techniques for molecular characterization of uncultured, environmental bacteria. However, the work is not done. The microfluidic approach we developed has great power to expand our understanding of the novel acetogens discovered in studies of acetogen community structure. Furthermore, the stage is now set for expansion into many avenues of scientific research. Ongoing research in this laboratory involves the use of microfluidics for single cell whole genome amplification; any of our newly discovered acetogenic bacteria would make interesting targets for this approach. The dominance of non-spriochetal acetogens in the guts of subterranean targets suggests that these environments might be good targets for cultivation-based characterizations, as attempts to cultivate acetogenic *Firmicutes* have in the past proven more fruitful than those targeting acetogenic *Spirochetes*. Finally, the microfluidic digital PCR approach we have

developed can and should be utilized for other molecular community assays. Likely targets within termite guts include bacterial cellulases discovered in wood-feeding higher termites and genes involved in bacterial nitrogen fixation. Likely targets in other environments include genes involved in sulfate reduction and methanotrophy, both of which feature large clusters of sequences identified in molecular community analyses that cannot be classified based on comparison to cultured strains.