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NATURE AND IMPORTANCE OF OXYGEN-CONSUMING MICROBES IN TERMITE GUTS

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NATURE AND IMPORTANCE OF OXYGEN-CONSUMING MICROBES IN TERMITE HINDGUTS

By

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ABSTRACT

NATURE AND IMPORTANCE OF OXYGEN-CONSUMING MICROBES IN TERMITE HINDGUTS

By

John Timothy Wertz

In termite hindguts, the fermentative production of acetate – a major carbon and energy source for the termite – depends upon the efficient removal of inwardly diffusing O_2 by microbes residing on and near the hindgut wall. However, little is known about the identity of these microorganisms or the substrates used to support their respiratory activity. A cultivation-based approach was used to isolate potentially important O_2 consuming organisms within the hindgut of *Reticulitermes flavipes*. Highest recoveries of colonies were obtained on plates incubated under hypoxia (2% O_2); and the increased recoveries were attributed to novel, rod-shaped, obligately microaerophilic *Neisseriaceae* that shared 99.7% 16S rDNA identity, but were <95% similar to any other known bacteria. Nearly identical organisms were isolated or their 16S rRNA genes amplified from geographically separated and genetically distinct populations of *Reticulitermes*. PCR-based procedures implied that the isolates were autochthonous to the hindgut, were associated with the hindgut wall, and comprised ca. 3% of the hindgut bacterial community.

Further characterization of *Neisseriaceae* representative strain TAM-DN1 revealed that the isolate used a limited range of energy sources that included acetate. On solid medium, the optimal O_2 concentration for growth was 1%, with no growth above 4% or in the absence of O_2, though TAM-DN1 could adapt to higher (16%) oxygen concentrations in liquid medium and expressed both catalase and superoxide dismutase enzymes. These data suggest TAM-DN1 and related strains

warrant recognition as a new genus and species, for which the name "*Stenoxybacter acetivorans*" gen. nov., sp. nov. is proposed.

Enzymes expressed by TAM-DN1 indicative of growth on acetate included acetate kinase (ACK; EC 2.7.2.1) and phosphotransacetylase (PTA; EC 2.3.1.8), but not acetyl-CoA synthetase (EC 6.2.1.1). TAM-DN1 did not appear to possess typical glyoxylate cycle enzymes, suggesting that it has an alternative pathway to replenish TCA cycle intermediates or can obtain these compounds *in situ*. All "*Stenoxybacter*" isolates possessed *ccoN*, which encodes the oxygen-reducing subunit of the high-affinity cbb_3-type cytochrome oxidase. "*Stenoxybacter*"-specific transcripts of *ccoN*, *ack* and *pta* were detected in hindguts of *R. flavipes* by RT-PCR, suggesting the population is oxidizing acetate and consuming oxygen *in situ*. The maximum contribution of the "*Stenoxybacter*" strains to total hindgut O_2 consumption was approximately 0.1 – 2.0%, similar to other hindgut isolates. Experiments designed to estimate the relative contribution of major microbial groups to total hindgut O_2 consumption suggested that protozoa may be more important to O_2 reduction than previously thought.

Concurrent with the above work, a novel method for the detection and isolation of specific microorganisms, termed "Plate Wash PCR," was developed. A result of this effort was the isolation of novel acidobacteria and verrucomicrobia from termite guts and from soil. Such microbes have often been detected in these environments but few have been previously isolated. Further investigation revealed that only the verrucomicrobia were autochthonous to the termite gut, and that both the verrucomicrobia and acidobacteria isolates are minor members of the gut community and probably have little to no impact on overall hindgut O_2 consumption.

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Chapter 1

Introduction

"*The termite hindgut is filled wall-to-wall with these fascinating morphologies of organisms. I was really gripped with it in the same way you might be interested in going through a rainforest - you don't have to be a scientist to appreciate that this is a beautiful place.*"

- J. Leadbetter (as quoted in 47)

Global Impact of Termites

Termites are one of the most abundant and ecologically relevant soil-dwelling insects on earth. In some areas, such as tropical forests, termite biomass (39-110 g/m^2) surpasses that of grazing herbivores (0.013 – 17.5 g/m^2)(60). Together with their gut-associated microbial symbionts, termites have evolved to thrive on the most abundant form of biomass on the planet, "lignocellulose" (lignin, cellulose, and hemicellulose), the primary components of wood and other terrestrial plant material, and residues derived from it (e.g. humus). Mineralization of a significant portion of the ingested plant material, chiefly cellulose and hemicellulose, has significant ecological consequences. It is estimated that globally, termites consume about 2.2 – 5.1% of the 136 x 10^{15} g of dry plant material produced from photosynthesis (11), contributing 2% and 4% to the biogeochemical cycling of the greenhouse gasses CO_2 and CH_4, respectively (60). Less obvious, but locally dramatic, are termite-mediated changes in the

physical, chemical and biological properties of soils that may contribute significantly to biogeochemical cycling of carbon, nitrogen, and nutrients (25).

Termites can be categorized into two groups, the so-called "lower" termites, which are phylogenetically basal, feed on sound or degraded wood, and harbor protozoan symbionts within their hindguts, or the "higher" termites, which are phylogenetically derived, include wood, soil, litter, and fungus-feeders, have highly compartmentalized guts, and typically lack gut protozoans. Seventy-five percent of the estimated 2,600 species of termites belong to a single family of higher termite, the *Termitidae* (31). The remaining 25% of termite species are distributed among six recognized families of lower termites (*Termopsidae*, *Hodo-*, *Kalo-*, *Masto-*, *Rhino-* and *Serritermitidae*). The gut microbiota of the lower termite *Reticulitermes flavipes* (Kollar, *Rhinotermitidae*) is perhaps the most well studied (7, 8, 17), and so it was used as a model in this dissertation (Figure 1.1).

Nature of Termite Gut Prokaryotes

The ability of termites to thrive on a relatively refractory and nitrogen-poor (approx 0.05% N w/w) food resource is due to the dense populations of symbionts harbored in their guts. Only recently, through cultivation-independent 16S rDNA-based techniques, has the full scope of prokaryotic diversity in the termite gut begun to be revealed. In two of the most comprehensive studies of termite gut microbial diversity thus far, Hongoh *et al.* (26, 27) analyzed a total of 2304 bacterial 16S rRNA gene clones from 32 termite colonies representing four species of *Reticulitermes* and four species of the higher termite,

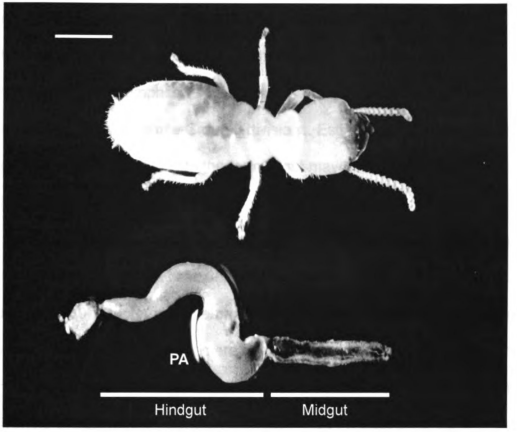

Figure 1.1. A worker larva of the eastern subterranean termite, *Reticulitermes flavipes*, is pictured above an extracted midgut and hindgut from a separate worker. The hindgut paunch is labeled PA. Scale bar = 1 mm. Adapted from Breznak, 1984.

Microcerotermes. In total, these studies have identified 367 bacterial phylotypes (using the criterion of 97% sequence identity) from *Reticulitermes* sp. and 228 phylotypes from *Microcerotermes* sp. The 16S rRNA gene sequences from both termites together encompass 17 bacterial divisions, including the candidate Termite Group 2 and Termite Group 3 divisions. Estimates of overall diversity based on these studies indicate the termite gut may harbor over 700 different phylotypes (27).

At the division level, the dominant phylotypes in guts of lower termites appear to be similar. In the case of *Reticulitermes speratus* (26, 27), *R. santonensis* (69), *Coptotermes formosanus* (56), and *Cryptotermes domesticus* (44), the 16S rRNA gene libraries are dominated by members of the divisions *Spirochaetes*, *Bacteroidetes*, and *Firmicutes*. These divisions appear to be the most phylotype-rich; in *R. speratus*, the *Clostridiales* (*Firmicutes*) were represented by 100 unique phylotypes, the *Spirochaetes* by 61 phylotypes, and the *Bacteroidetes* by 31 phylotypes. Also consistently detected were members of the bacterial divisions TM7, OP11, *Acidobacteria*, *Planctomycetes*, *Verrucomicrobia* and *Endomicrobia* that are represented by few, or no, cultivars (28, 59).

The guts of four species of the wood-feeding higher termite, *Microcerotermes*, were also dominated by members of the divisions *Spirochaetes*, *Bacteroidetes*, and *Firmicutes* (26). However, statistical comparison by ∫-LIBSHUFF revealed these clones were quite different at the nucleotide sequence level from the clones from *Reticulitermes* gut homogenate

(P = 0.0001). Analysis of 16S rDNA clones from guts of the soil-feeding higher termite, *Cubitermes orthognathus*, revealed that *Clostridiales* (approximately 70% of the total number of clones), instead of *Spirochaetes* (10%) or *Bacteroidetes* (10%) appeared to dominate (53). Clustering analysis between conspecific and congeneric termites has revealed that the gut bacterial composition appeared to be determined by (in order of importance): (i) the feeding habit; (ii) the genus of the termites; and (iii) the location of the termite colony (26).

Microscopic examination of the microbiota near the hindgut epithelia and within the hindgut fluid of lower termites like *Reticulitermes flavipes* and *Coptotermes formosanus* clearly reveal radial differences in community structure (12). In *R. flavipes*, a distinctive, epithelium-associated community consists of mostly rod- or filament-shaped prokaryotes, whereas the fluid chiefly consists of spirochetes and protozoa (Figure 1.2) (8, 12). Using fluorescent *in situ* hybridization (FISH) with group-specific probes, Berchtold *et al.* also demonstrated differences in the axial distribution of microbiota in the gut of *Mastotermes darwiniensis* (4). Whereas the posterior region was colonized preferentially by Gram-positive cocci and rod-shaped and filamentous bacteria of the Cytophaga–Flexibacter–Bacteroides (CFB) division, morphologically different microorganisms were located in the anterior paunch and were mostly associated with the flagellates.

Yang *et al.* (69) and Nakajima *et al* (38) dissected and separated hindguts of *R. santonensis* and *R. speratus*, respectively, into two fractions: a "wall

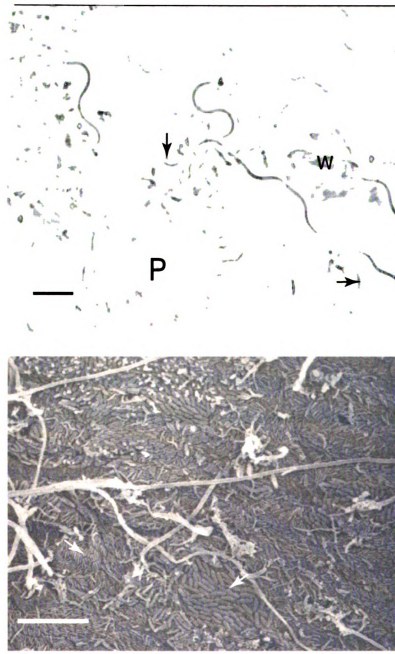

Figure 1.2. Phase-contrast light (top) and scanning electron (bottom) micrographs of microorganisms within the hindgut paunch fluid (A) and associated with the epithelial wall (B). The hindgut fluid harbors protozoa (P), a remarkable morphological diversity of spirochetes (white arrows) including those attached to some protozoans, as well as other rod- or vibrioid- shaped prokaryotes (black arrows). Undigested wood particles can also be seen (W). Associated with the epithelia are a diversity of mostly non-protozoal and non-spirochetal microbes, some of which form microcolonies (arrows). Scale bars represent 0.01 mm (A) and 10 μm (B). Adapted from Breznak, 2000 (A) and Breznak and Pankratz, 1977 (B).

fraction" that represented the hindgut epithelium and associated organisms; and a "fluid fraction" primarily consisting of lumen-associated microbes. From each fraction, 16S rRNA gene clone libraries were constructed. For *R. speratus*, the wall fraction was dominated by clones grouping with the *Actinobacteria*, *Firmicutes* and *Bacteroidetes*. *Spirochaetes* and members of the candidate phylum *Endomicrobia* were more numerous in the hindgut luminal fraction (38). Statistical analysis of the libraries by LIBSHUFF revealed that the two libraries were quite distinct ($P = 0.0001$). Similar libraries from *R. santonensis* had less distinction between wall and fluid communities, however (69). Clones related to *Spirochaetes* and *Mycoplasmatales* were clearly localized to the fluid fraction, but there appeared to be no uniquely wall-associated phylotypes. A LIBSHUFF comparison of the libraries (not reported) revealed a statistically significant difference between the fluid library and the wall ($P = 0.02$), but not vice-versa ($P = 0.3$), indicating the wall-associated community is a subset of the luminal microbiota. This may be due to insufficient separation of the gut fractions, a continual sloughing-off of wall-associated microbes into the lumen, or as ability of facultative anaerobes or aerotolerant anaerobes in the lumen to also colonize the gut epithelium.

Unfortunately, the number of pure culture isolates from termite guts is a poor reflection of the diversity revealed by microscopic and 16S rRNA gene analyses (8, 14). The more frequently encountered isolates, obtained from a number of different termites, include *Enterobacteriaceae* (mostly *Citrobacter* or *Enterobacter* sp.), members of *Firmicutes* (including species of *Lactococcus,*

Enterococcus, *Staphylococcus* and *Lactobacillus*), and *Bacteroides* (1, 2, 21, 55, 62). More recently, sulfate-reducing bacteria and methanogenic *Archaea* have been isolated (33-35). However, it is painfully apparent that about 90% of all prokaryotes seen by direct microscopic counts, or 99% of all bacterial phylotypes found by 16S rRNA methods (26), still elude cultivation. This underscores the fact that new strategies for isolation of phylotypes not-yet-represented in culture (e.g. provision of nutrients and incubation in atmospheres that better mimic the *in situ* environment) and new ways to rapidly differentiate between successful and unsuccessful enrichment or isolation attempts continue to be necessary. Only after painstaking, long-term development of habitat-simulating enrichment media were the first termite gut spirochetes, *Treponema primitia* and *T. azotonutricium* coaxed into pure culture (23, 36). The effort was worthwhile: subsequent studies revealed in the isolates metabolic pathways previously unknown in spirochetes – CO_2-reductive acetogenesis and nitrogen fixation – that would have continued to go unnoticed without the availability of pure cultures for study (36, 37).

Function of Termite Hindgut Microbial Symbionts

Though pure culture isolates of termite gut microbes are limited in number, more than a century of elegant and detailed research has provided significant insights into the basis for the symbiotic interaction between the termite and its hindgut microbial symbionts: to facilitate decomposition of plant material into utilizable sources of carbon and energy; and to provide fixed or recycled nitrogen necessary for termite growth and survival.

Immediately upon ingestion of woody material, endogenous cellulases secreted primarily by the midgut epithelium begin to hydrolyze a portion of the cellulose into soluble oligosaccharides (30, 39, 64, 65) (Figure 1.3). Some of the released glycosides cross the midgut epithelium and can proceed through glycolysis within the termite tissue, but limited activity of pyruvate dehydrogenase prevents further oxidation of pyruvate (41, 58). Instead, pyruvate and glycosides may be stored, used as biosynthetic precursors, or transported into the hindgut and metabolized by the gut microbiota (58). The main energy requirements of the termite are met by oxidation of other metabolites produced fermentatively by hindgut symbionts (see below).

A majority of ingested cellulose passes through the midgut into the hindgut, and in lower termites it is then phagocytosed by anaerobic protozoa (11). Using their own cellulase enzymes, the protozoa hydrolyze cellulose to soluble oligosaccharides, which are largely fermented by the protozoa to acetate according to equation 1. Some oligosaccharides, possibly from hemicellulose degradation as well, may also be secreted by the protozoa to support a population of microbes upon which they feed (42, 66-68).

1) $[C_6H_{12}O_6] + 2H_2O \longrightarrow 2CH_3COOH + 2CO_2 + 4H_2$

Thus the hindgut protozoa fulfill two essential roles in lower termites: (i) their phagocytic activity increases cellulose retention in the hindgut, allowing a

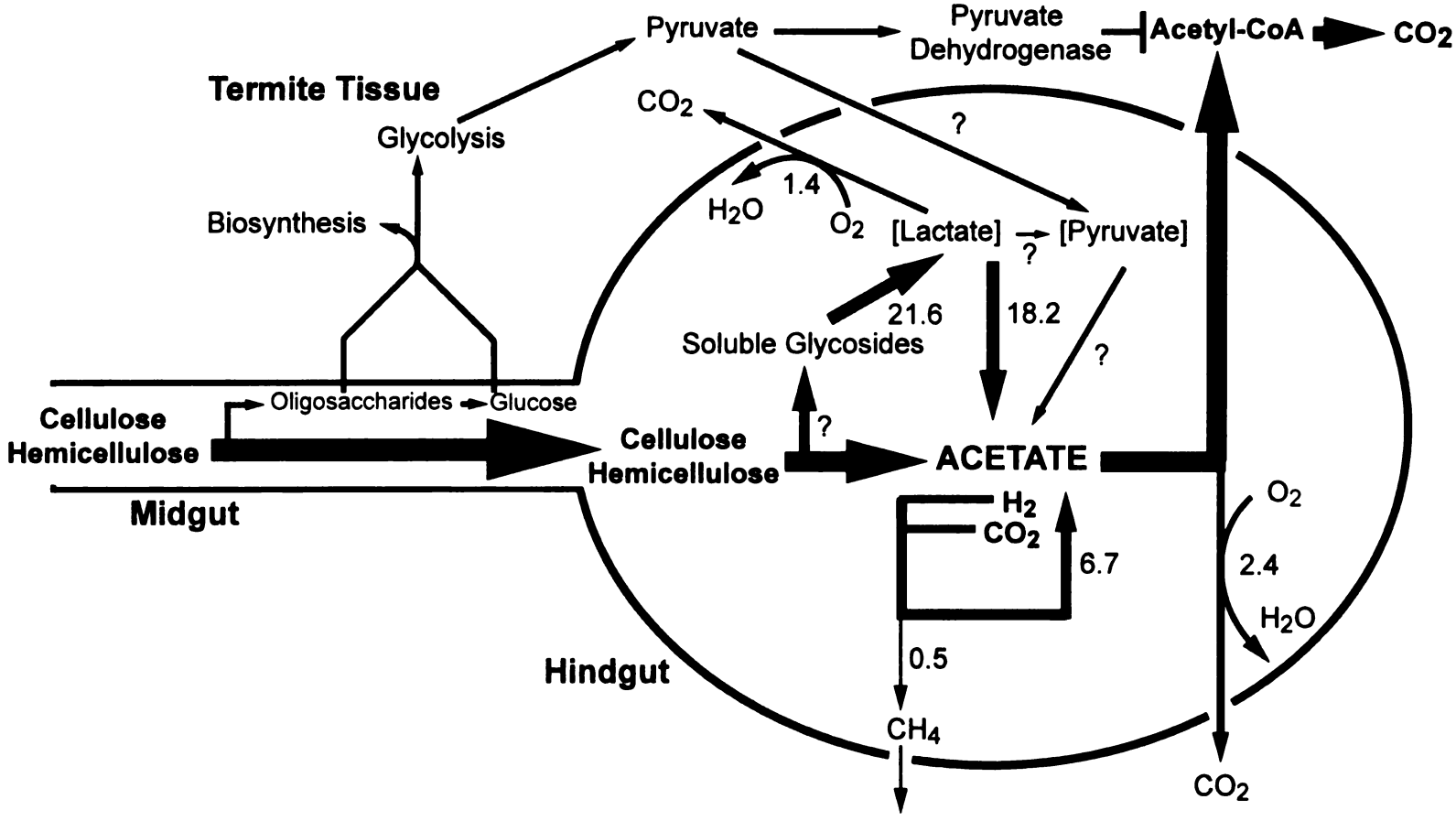

Figure 1.3. Diagrammatic representation of carbon flow in the termite *Reticulitermes flavipes*. Numbers indicate the flux rates (in nmol C h^{-1} $termite^{-1}$) estimated from microinjection of radiolabeled substrates (Tholen *et al*, 2000) or direct measurements of CO_2 and CH_4 emission (Odelson and Breznak, 1983). Lactate and pyruvate have not been directly measured in hindgut fluid and are therefore represented in brackets. Question marks indicate hypothetical pathways for which little or no experimental support exists. The thickness of arrows indicates the relative significance of the pathway.

majority of cellulose to be metabolized; and (ii) produce acetate, a utilizable source of carbon and energy for the termite.

Fermentation of released glycosides by protozoa and anaerobic bacteria is a major source of acetate in the hindgut (48, 51, 54, 55). Additional acetate is produced by CO_2-reductive acetogenesis by bacteria such as spirochetes (6, 13, 36) using H_2 and CO_2 released from glucose fermentation according to equation 2:

2) $4H_2 + 2CO_2 \longrightarrow CH_3COOH + 2H_2O$

Recently, Tholen *et al.* provided evidence for acetate production from lactate. By microinjecting radiolabeled substrates into intact hindguts they were able to demonstrate that up to one-third of the carbon flux in living termites proceeds via a previously unrecognized pool of lactate (61). An absence of measurable lactate within the hindgut implies a very rapid turnover of this small pool to acetate, possibly by lactate-fermenting homoacetogens (54, 55) according to equation 3:

3) $2C_3H_6O_3 \longrightarrow 3CH_3COOH$

As the above pathways indicate, the hindgut system is almost completely homoacetogenic. Acetate dominates the hindgut fatty acid pool, comprising up to 98 mol% of all volatile fatty acids with concentrations as high as 80 mM (43). Acetate is absorbed through the hindgut epithelium, oxidized in termite tissues,

and serves as the major energy source for the insect (24, 43). By measuring the rate of acetate production in the hindgut, Odelson and Breznak clearly demonstrated that microbially produced acetate could account for 71-100% of the CO_2 produced by termites, thereby supporting up to 100% of their daily respiratory requirements (43). Thus the overall sum of reactions in the hindgut and termite tissue can be summarized in equation 4:

4) $$[C_6H_{12}O_6] + 6O_2 \longrightarrow 6CO_2 + 6H_2O$$

Utilization of lignocellulose as the primary source of carbon and energy also requires supplementation of this nitrogen-poor diet with fixed or recycled nitrogen. Breznak *et al.* (10) and Benemann *et al.* (3) were the first to demonstrate nitrogen fixation in termites and attribute it to hindgut prokaryotes. Subsequently, N_2 fixation has been confirmed in more than 20 species of termites (8). Several strains of N_2-fixing bacteria, including enterobacteria as well as spirochetes, have been isolated from a number of different termites (22, 33, 37, 50), and molecular biological studies suggest that additional, not-yet-cultured nitrogen-fixing organisms remain to be identified. For example, cultivation-independent experiments have demonstrated that a diversity of *nifH* genes (encoding dinitrogenase reductase) present in termite hindguts resemble homologous genes in clostridia, γ- and δ-*Proteobacteria* and *Archaea* (40, 45, 46). However, the relative importance to termite nitrogen requirements of microbes bearing these genes remains to be established. An assessment of *in*

situ expression of *nifH* genes in the hindgut of *Neotermes koshunensis* by Noda *et al.* demonstrated that while several phylogenetic classes of *nifH* genes were represented among the hindgut microbiota, only a few were actually expressed *in situ* (40). It now appears that termite gut treponemes may be the origin of such expressed genes (37).

Other members of the hindgut community can also contribute to termite nitrogen economy by recycling the nitrogen in the excretory product uric acid which is secreted into the midgut-hindgut junction through Malpighian tubules (48, 49). Potrikus and Breznak isolated *Streptococcus*, *Bacteroides,* and *Citrobacter* strains from hindguts of *R. flavipes* that could anaerobically degrade uric acid to acetate, CO_2 and NH_3 (48, 49, 51, 52). Additionally, they showed that microbial degradation of ^{15}N-uric acid *in situ* resulted in liberation of ^{15}N that was subsequently assimilated into termite macromolecules (49).

The Importance of O_2 Consumption in Termite Guts

The demonstration that approximately 60% of the termite hindgut is persistently hypoxic (<4% O_2 v/v) has been described as "the most significant recent advance in our understanding of metabolism in the hindgut" (57). Historically, the termite gut was considered to be a completely anoxic system (9, 11), an assumption dating to early observations of the O_2-sensitivity of the protozoa (19). Subsequent observations of termite dependency upon the O_2-sensitive microbial processes of carbohydrate dissimilation (29), reductive acetogenesis (13), and in some cases nitrogen fixation (10), solidified this notion.

Other data, however, seemed to suggest at least some portion of the hindgut was oxic. For example: (i) aerotolerant (21, 55), facultative, or O_2-requiring (62) microorganisms were frequently represented among hindgut isolates; (ii) termites exposed to hyperbaric levels of O_2 resulted in the death of hindgut spirochetes and protozoa (both groups known to be anaerobes), but increased the viable cell counts of bacteria 6- to 10-fold on culture plates incubated in air (63); and (iii) the large surface area-to-volume ratio of the hindgut indicates equilibrium with atmospheric oxygen should occur throughout the gut unless mechanisms existed to block or remove it (5, 15).

The anoxic gut paradigm was not challenged, however, until Veivers *et al.*, employing redox indicator dyes, noted that termites fed antibiotic drugs were no longer able to maintain the low redox potential (Eh = -150 to -250 mV) typical of normal guts (63). These results suggested that the gut bacteria may be oxygen consumers that retain anoxic conditions in the hindgut lumen by removing inwardly diffusing O_2. However, experiments meant to directly detect oxygen in the gut were not done until the observation that *in situ* degradation of lignin model compounds was O_2-dependent (18, 32) prompted Brune and coworkers (16, 20) to use microelectrodes to provide a micrometer scale resolution of O_2 gradients within agarose-embedded, intact hindguts of *Reticulitermes flavipes* and *Nasutitermes lujae* (Figure 1.4). The results clearly revealed the presence of a peripheral, hypoxic zone in the hindgut with O_2 partial pressures of 30 mbar (30% air saturation) at the epithelial surface that rapidly decrease to anoxia 150-200 µm inward. The measurements indicated that a majority of the hindgut

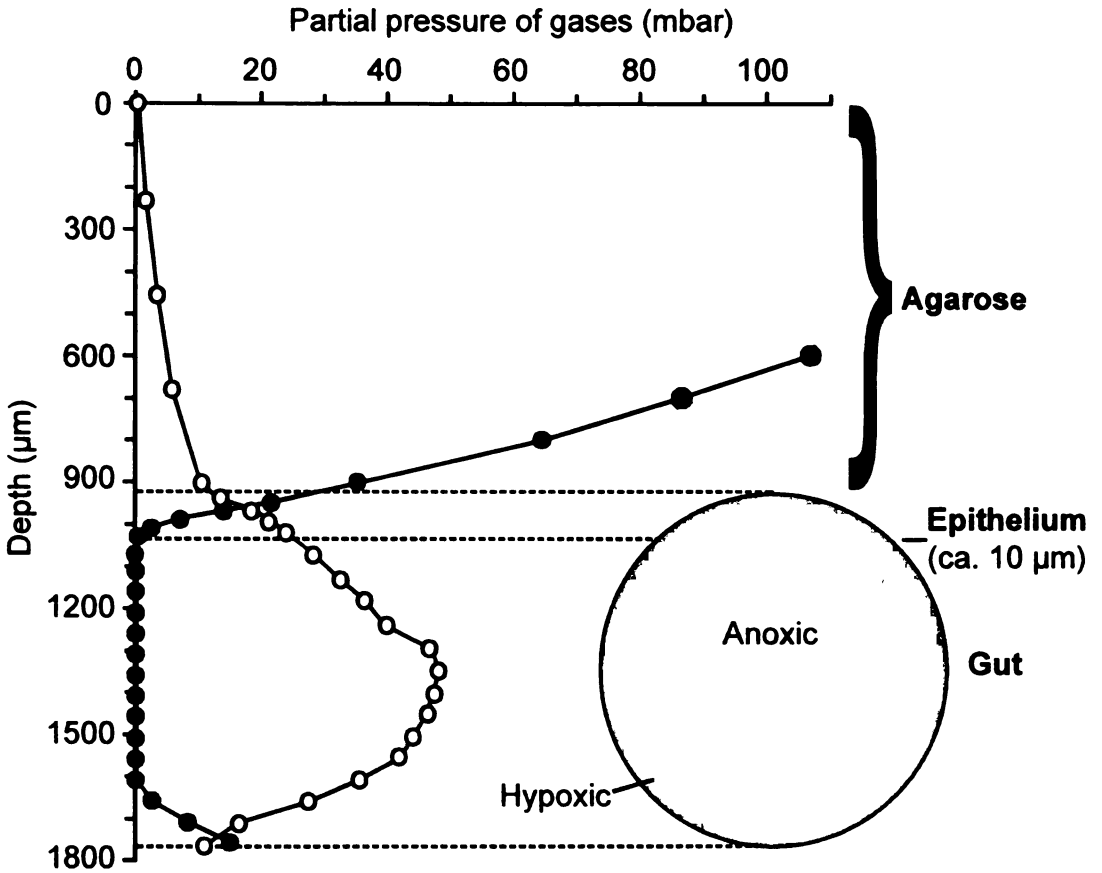

Figure 1.4. Radial gradients of oxygen (•) and hydrogen (○) in an agarose-embedded hindgut of *Reticulitermes flavipes* as measured by microelectrodes. The insert represents the hindgut paunch and indicates the zones of hypoxia and anoxia. Adapted from Brune, 1998.

(approx. 60% of the total hindgut volume) was hypoxic, whereas the luminal portion of the hindgut paunch remained anoxic (15). The presence of a steep O_2 gradient at the epithelial wall supported the previous conclusion by Veivers *et al.* that some members of the microbiota on or near the hindgut wall were an important "oxygen sink" (15, 16).

The realization that a majority of the hindgut may be exposed to some level of oxygen caused a paradigm shift in which the essential role of O_2 consuming microorganisms to termite vitality became clear (16, 57). The termite is dependent upon the O_2 consuming members of the peripheral gut microbiota for the creation and maintenance of anoxic conditions within the hindgut lumen so that the incomplete oxidation of dietary polysaccharides to acetate can occur. If oxygen were allowed to diffuse in too far, as with incubation of termites under a hyperbaric (30% O_2) atmosphere or removal of the O_2 consuming bacteria with antibiotic drugs (63), acetate production would cease and the termite would eventually die. Though the importance of the O_2 consuming microbiota to the continued survival of the termite was clear, upon initiation of this Dissertation, little was known about the identity of the microbes responsible for O_2 consumption in the hindgut or the substrate(s) used to support their *in situ* activity.

Linking Community Structure and Function

Elegant physiological studies have appraised the symbiotic function and importance of the termite gut microbiota *in toto*. Molecular techniques such as

16S rRNA-based identification have considerably advanced our knowledge of the nature of the hindgut symbiotic community. However, our ability to relate a particular function to a specific microbial population has been hampered by a lack of a sufficient number of pure culture isolates. Though molecular methods exist that allow metabolic, phylogenetic, and localization information to be combined (e.g. catalyzed reporter deposition FISH (CARD-FISH), large-insert libraries, functional gene arrays), these methods are encumbered by technical difficulty and high cost. The most cost-effective and direct route to linking structure and function is to use a combination of *in vivo* molecular techniques with *in vitro* physiological studies; such methods first require, however, the isolation of target microorganisms in culture.

Dissertation Research

The research presented in this dissertation focuses on the nature and importance of O_2 consumption within termite hindguts. Chapter 2 discusses the isolation, enumeration, and identification of aerobic organisms from *R. flavipes* guts, including a group of abundant, but hitherto unrecognized, obligately microaerophilic *β-Proteobacteria*. The characterization and classification of these novel microorganisms as a new genus and species within the *Neisseriaceae* is discussed. Chapter 3 presents evidence for *in situ* acetate oxidation and O_2 consumption by these microaerophilic *β-Proteobacteria* within the guts of *R. flavipes*, and analyzes, through feeding experiments, the importance of the bacterial and protozoal hindgut populations to overall hindgut O_2 consumption.

Chapter 4 summarizes the main conclusions of these studies. Finally, an appendix chapter, based upon a recent publication, reports the development of a novel method ("Plate Wash PCR") for the detection and isolation of previously uncultivated microorganisms, including potentially important O_2 consumers from termite guts.

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Chapter 2

"*Stenoxybacter acetivorans*" gen. nov., sp. nov., an Acetate Oxidizing Obligate Microaerophile Among Diverse O_2 Consuming Bacteria from Termite Guts

Introduction

Wood-feeding termites depend upon a dense and phylogenetically diverse community of hindgut-associated microbes to contribute to the insect's nitrogen economy, as well as to assist in the degradation of wood polysaccharides (cellulose and hemicelluloses) into short-chain fatty acids used by the host for energy (10, 12). In most termites, microbially-produced acetate dominates the hindgut fatty acid pool, comprising up to 98 mol% of all volatile fatty acids and existing at concentrations as high as 80 mM (34). Acetate is absorbed through the hindgut epithelium and serves as the major oxidizable energy source for the insect, capable of supporting up to 100% of the termites' daily respiratory requirements (34). However, production of acetate depends on the maintenance of anoxia in the hindgut lumen (9).

Early measurements of the redox potential of hindguts, taken together with the presence of strictly anaerobic microbes, led to the hypothesis that termite guts were devoid of oxygen (7, 50). By contrast, other data suggested that some portion of the hindgut contained O_2. For example, aerotolerant (41, 51), facultative, or O_2-requiring (14, 49) hindgut microorganisms were frequently

represented among isolates, and degradation of lignin model compounds *in vivo* was found to be O_2-dependent (15, 28). However, it was not until recently that direct measurements of O_2 concentrations in hindguts were made with microelectrodes, thereby providing a fine-scale resolution of O_2 gradients within intact hindguts embedded in agarose (14, 18). Results clearly demonstrated the presence of a peripheral hypoxic zone, with O_2 at partial pressures of about 30 mbar (30% air saturation) at the epithelial surface decreasing steeply to anoxia a distance of 150-200 μm inward. This indicated that a majority (approx. 60%) of the hindgut contains significant levels of O_2, undoubtedly a result of its small size and large surface area to volume ratio (13), and it implied that some members of the microbiota on and/or near the hindgut wall constituted an important "oxygen sink" (13, 14). Consistent with this interpretation were earlier experiments showing that termite hindguts rapidly become completely oxic (implied by the color of redox dyes) when termites were fed antibacterial drugs (51), and termites exposed to hyperbaric levels of O_2 results in the death of hindgut spirochetes and protozoa (both groups known to be anaerobes), but leads to a 6- to 10-fold increase in overall hindgut bacterial colony counts on culture plates incubated in air (51). However, both treatments decrease the ability of termites to survive on a diet of wood or cellulose.

It seems clear that the O_2-consuming members of the peripheral hindgut microbiota are critical in creating anoxic conditions for the incomplete oxidation of wood polysaccharides to acetate (the main energy source for the insect) by carbohydrate fermenters, and for the anaerobic metabolism of CO_2-reducing

acetogens. However, our understanding of this O_2-consuming microbiota is meager, as is the nature of the substrate(s) that fuels their respiratory activity, although acetate itself seems a likely candidate given its high rate of oxidation to CO_2 in termite hindguts (31) and the inferred abundance of (not-yet-characterized) acetate oxidizers from most-probable-number enumerations (32). Accordingly, we sought to explore the nature and activity of such microbes by using a cultivation approach that included acetate as an oxidizable substrate in isolation media, as well as incubation atmospheres that included hypoxia (2% O_2) based on the hypothesis that specialized members of the O_2-consuming microbiota are so highly adapted to life in hypoxia that they cannot grow in air (21% O_2). Results of that effort have led to the isolation of several previously uncultivated bacteria from termite guts, including members of the *Verrucomicrobia* and *Acidobacteria* (47), and a dominant, novel group of microaerophilic, acetate-oxidizing *β-Proteobacteria*, "*Stenoxybacter acetivorans*" gen. nov. sp. nov., whose description constitutes the subject of the present chapter.

Materials and Methods

Termites

Reticulitermes flavipes (Kollar) (*Rhinotermitidae*) were collected near Dansville, MI., Spring Arbor, MI., Raleigh, NC., Woods Hole, MA., and Janesville, WI. *Reticulitermes santonensis* (Feytaud) was collected near Forêt de la Coubre,

France. *Zootermopsis angusticollis* were from laboratory cultures (31) and *Coptotermes formosanus* were collected near Ft. Lauderdale, FL.

R. flavipes and *C. formosanus* termites were either degutted within hours of collection or maintained in laboratory nests as described previously (11, 34). *R. santonensis* were maintained in the laboratory as described in (53) for one year previous to use.

Isolation and cultivation of bacteria.

Guts from approx. 50 worker termites were extracted with sterile forceps under a hypoxic, CO_2 enriched atmosphere (2% O_2, 5% CO_2, 93% N_2) within a flexible vinyl chamber equipped with an oxygen sensor and controller (Coy Laboratory Products, Grass Lake, MI). Extracted guts were transferred to and homogenized in a sterile glass tissue homogenizer containing 2 ml of a buffered salts solution (BSS) composed of (per liter): KH_2PO_4, 0.2 g; NH_4Cl, 0.25 g; KCl, 0.5 g; $CaCl_2 \cdot 2H_2O$, 0.15 g; NaCl, 1.0 g; $MgCl_2 \cdot 6\ H_2O$, 0.62 g; Na_2SO_4, 2.84 g; and 3-(N-morpholino)propanesulfonic acid (MOPS), 10 mM. The BSS was adjusted to pH 7.0 and was always pre-incubated under the hypoxic atmosphere for at least 12 hours before use. Serial 10-fold dilutions of gut homogenate were prepared in BSS, and 0.1 ml aliquots of each dilution were spread onto six plates of ACY medium, which consisted of BSS amended with 10 mM sodium acetate, 0.05% w/v Bacto casamino acids, 0.05% w/v Bacto yeast extract, 0.01% v/v each of a trace element solution and a vitamin solution (47), and 1.5% v/v Bacto agar. The pH of ACY medium was adjusted to 7.0. After plating, three of the plates

from each dilution were retained within the hypoxic chamber; the remaining three plates of each dilution were removed from the chamber and placed in a glass dessicator jar that was subject to six cycles of evacuation and filling with CO_2-enriched air (5% v/v CO_2, balance air). Every two days the CO_2-enriched air atmosphere in the dessicator jars was replaced. All incubations were held at room temperature (22-23°C)

After approximately 20 days, when new colony formation was subsiding, plates that contained well-separated colonies were selected for colony enumeration, isolation, and identification. Isolates were obtained by using two methods. First, plates were examined under a dissecting microscope and individual colonies were picked with a sterile inoculating loop and streaked for isolation on homologous medium. The number of colonies of similar morphology on each plate was noted, and an effort was made to have at least one representative of each colony type streaked for isolation. For each colony, two duplicate plates were streaked: one was incubated under hypoxia, whereas the companion plate was incubated in CO_2-enriched air. Subcultures were continually checked for purity and the ability to form colonies under hypoxia and/or CO_2-enriched air. Second, specific groups of organisms were targeted for cultivation by using the Plate Wash PCR method described previously (47), with the following forward primers: Acd31f (5' – GAT CCT GGC TCA GAA TC – 3', for members of the *Acidobacteria* domain (4)); End197f (5' – GCA GCA ATG CGT TTT GAG – 3', for members of the *Endomicrobia* domain (this study)); Pla40f (5' - CGG RTG GAT TAG GCA TG – 3', for members of the *Planctomycete* domain

(Kristin Huizinga, personal communication)); and Ver53f (5' – TGG CGG CGT GGW TAA GA– 3', for members of the *Verrucomicrobia* domain (47)). The reverse primer in all cases was the general bacterial reverse primer 1492r (below). Colonies were re-streaked for isolation until they were determined to be pure based on uniform colony and cell morphology, the latter determined by examination of wet mounts by phase contrast microscopy. Preliminary screening of pure cultures for acetate utilization was done by streaking colonies onto plates of ACY medium with and without acetate. Robust growth on acetate-containing medium, but little or no growth on medium lacking acetate, was a presumptive indicator of acetate utilization. Isolated strains belonging to the *Verrucomicrobia* division begin with the letters TAV, and strains of the novel acetate-oxidizing, microaerophilic *β-Proteobacteria* described herein (i.e. "*Stenoxybacter acetivorans*" gen. nov. sp. nov.) begin with the letters TAM.

Bacterial DNA extraction and PCR-related procedures

For rapid phylogenetic identification of isolated strains, a loopfull of colony material was removed with a sterile inoculating loop and placed in 500 µl of sterile BSS in a 1.5 ml polypropylene centrifuge tube and centrifuged for 10 min at 4000 x *g*. The supernatant liquid was removed, and total DNA in the cell pellet was extracted by using the Bactozol DNA extraction kit (Molecular Research Center, Cincinnati, Ohio). Briefly, the cell pellet was suspended in 100 µl of 1x Bactozyme lysis solution and allowed to incubate at room temperature or 50°C until the suspension cleared. Four hundred microliters of DNAzol was added, and

the lysate was incubated at room temperature for 10 minutes. The DNA was precipitated by adding 300 µl absolute ethanol and incubating at room temperature for an additional 10 minutes. The precipitated DNA was harvested by centrifugation at 14,000 x g for 15 minutes, washed in one milliliter of 75% ethanol, re-centrifuged, and allowed to air-dry. The DNA pellet was then redissolved in 250-500 µl of sterile water, pH 7.5. Occasionally, DNA redissolution was aided by incubation at 60°C for 1-12 hours.

For each isolate, the 16S rRNA gene was amplified via the polymerase chain reaction (PCR) using the general primers 8f (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492r (5'-GGT TAC CTT GTT ACG ACT T-3'), which target regions of the gene common to most *Bacteria* (52). The 25 µl reaction mixture contained: 25 – 100 ng DNA template, 1x reaction buffer (Invitrogen, Carlsbad, Calif.), 1.5 mM $MgCl_2$, 0.25 mM of each deoxynucleoside triphosphate, 0.2 µM of each primer, and 0.625 U *Taq* polymerase (Invitrogen). PCR mixtures were incubated in a model PT-100 thermal cycler (MJ Research, Inc., Watertown, Mass.) as follows: (i) 3 min at 95°C; (ii) 30 cycles of 45 sec at 95°C, 45 s at 56°C, 45 s at 72°C; and (iii) 5 min at 72°C. Five-microliter samples of each PCR reaction mixture was analyzed by electrophoresis on 1.0% agarose gels prepared in 0.5x Tris-borate-EDTA gel with 10 µg/ml ethidium bromide (39). Fluorescent bands of the PCR products were visualized by UV transillumination, and images were captured by using a Kodak electrophoresis documentation and analysis system 290 (Eastman Kodak).

To estimate the total number of "*S. acetivorans*", *Acidobacteria* or *Verrucomicrobia* cells in *R. flavipes* termites, as well as their primary anatomical location, a dilution-to-extinction PCR approach was used. DNA was extracted from 50-100 freshly collected intact termites, termite guts, and degutted termite bodies by using a MoBio Ultraclean Soil DNA extraction kit (MoBio Laboratories, Carlsbad, CA.) after homogenization in a Mini-BeadBeater-8 (BioSpec Products, Inc., Bartlesville, OK.) operating at full speed for 45 s according to the DNA isolation protocol. Purified DNA was normalized on a per-termite equivalent basis and serially diluted in dilution buffer (10 mMTris-HCl (pH 8.0) containing 50 ng/µl calf thymus DNA as a carrier). As a control, 100 ng purified DNA from TAM-DN1 or *Verrucomicrobia* strain TAV-1 (47) was also serially diluted in dilution buffer. Each dilution was used as template in a PCR reaction using either the forward primer TAM203f (5' - GCT TCG CAA GGA CCT CAC – 3'; specific for 16S rRNA gene of "*S. acetivorans*" strains based on phylogenetic analysis, below), Ver53f or Acd31f combined with the general reverse primer 1492r (above). The PCR mixture was identical to that described above, and a total of 30 PCR cycles was done. Five-microliter samples of each PCR reaction mixture was analyzed by electrophoresis on 1.0% agarose-0.5x Tris-borate-EDTA gel stained with 1x Gelstar nucleic acid stain (Cambrex, East Rutherford, N.J.). Fluorescent bands of the PCR products were visualized, captured and analyzed as above.

An estimate of the *in situ* abundance of the organisms was based on comparison of the extinction point (lowest amount of DNA that resulted visually obvious amplification) of the known quantity of purified TAM-DN1 or TAV-1 DNA

to the extinction point of corresponding target DNA obtained from termite samples, taken together with the determined genome size and 16S rRNA gene copy number for "*S. acetivorans*" (3.2 Mb and 4 copies, respectively (below)) and *Verrucomicrobia* strain TAV-1 (4.0 Mb and 1 copy, respectively (below)).

To evaluate the autochthony of "*S. acetivorans*" for *R. flavipes*, termites were freshly collected from a forested natural preserve in Dansville, MI, along with termite "nest soil" (i.e. soil at the interface of a fallen log on which termites were feeding and through which they were actively tunneling), and separate, "non-nest soil" that showed no evidence of termites or termite activity. Soil samples were collected with sterile spatulas and placed in Whirl-Pak bags (Nasco, Fort Atkinson, WI). DNA was extracted from termite guts (100 guts) and soil samples (1g) within hours of collection by using the MoBio Ultraclean Soil DNA extraction kit as described above. The purified DNA was used in PCR reactions as above, with the "*S. acetivorans*"-specific 16S rRNA gene forward primer TAM203f and general reverse primer 1492r. PCR amplified DNA was cloned into TOP10 *E. coli* using the plasmid vector pCR2.1 (TA Clone Kit, Invitrogen). The partial sequence of randomly selected clones was determined using the TAM203f primer. Only sequences longer than 500 bp were used for subsequent analyses. The sequences were imported into the ARB software (32), aligned, and phylogenetic trees constructed (as described below) using 503 shared nucleotide positions. The statistical program LIBSHUFF was used to determine statistical relatedness of the clones (40).

Sequencing and Phylogenetic analysis.

Prior to sequencing, unreacted dNTP's and primers in the PCR reactions were digested and dephosphorylated using ExoSap-IT (USB, Cleveland, Ohio). The reaction mixture contained 1.3 µl PCR amplified DNA, 0.5 µl ExoSAP-IT enzyme mix, and 3.2 µl sterile water. Incubation was according to the ExoSAP-IT protocol.

Partial 16S rRNA gene sequences for each isolate was determined with Applied Biosystems cycle sequencing technology (Applied Biosystems, Foster City, Calif.), with the general bacterial primer 8f. Sequence chromatograms were checked for quality, and the initial identification of each isolate was determined by using the BLAST search tool in the Genbank nucleotide database (2) or the Ribosomal Database Project (http://cme.rdp.msu.edu) (17). From the 29 total isolates representing "*S. acetivorans*", a subset of 15 that represented the apparent phylogenetic breadth of the group were chosen for nearly-full length sequencing of the 16S rRNA gene. Fourfold coverage of each nucleotide position was obtained by using the same method described previously (47), but omitting primers F2, R4*, Acd31f, and Ver53f, and adding primer 8f. Individual 16S rRNA gene sequence reads for each isolate were manually edited and assembled by using the Contig Assembly Program tool contained within the program BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

For phylogenetic analyses, the partial or nearly full 16S rRNA gene sequence of each isolate or clone was aligned against a 16S rRNA gene sequence database in the ARB software package (http://www.arb-home.de/)

(32). Ambiguities in the sequence alignments were corrected manually, where possible. For each phylogenetic tree, only unambiguous alignment positions present in every sequence were used. Maximum likelihood phylogenetic trees were constructed in ARB using the FastDNAML routine (32).

Termite microsatellite DNA analysis.

Eight worker termites were selected from each of the six collection sites (Dansville, MI; Spring Arbor, MI; Janesville, WI; Raleigh, NC; Woods Hole MA; and Forêt de la Coubre, Fra.) and individual degutted bodies were placed in 550 µl Bead solution (MoBio Ultraclean Soil DNA kit, MoBio Labs) in separate Bead tubes and homogenized in a Mini-BeadBeater-8 operating at full speed for 1 min. Purified termite body DNA was obtained according to the MoBio Ultraclean Soil DNA kit protocol. The resulting DNA was used in individual, non-multiplexed polymerase chain reactions using 5'-Hex-labeled PCR primers 5-10, 6-1, 11-1, 11-2, and 21-1 as described by Vargo (50). Each 50 µl reaction contained: 10 ng of termite DNA per reaction, 1x reaction buffer (Invitrogen, Carlsbad, Calif.), 1.5 mM $MgCl_2$, 0.25 mM each deoxynucleoside triphosphate, 0.2 µM each primer, and 0.625 U Taq polymerase (Invitrogen). PCR mixtures were incubated in a model PT-100 thermal cycler (MJ Research, Inc., Watertown, Mass.) as follows: (i) 3 min at 94°C; (ii) 35 cycles of 30 sec at 94°C, 50 s at 60°C, 1.0 min at 72°C, and (iii) 5 min at 72.0°C. The PCR products were precipitated by using 1/10 volume 3 M sodium acetate (pH 5.0) with 2 volumes 100% ethanol. The DNA pellet was washed in 75% EtOH, air-dried, and suspended in 50 µl sterile water,

pH 7.0. Two to three hundred nanograms of the purified PCR product were separated by using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, Calif.). Peak sizes were quantified with the Genotyper software (Applied Biosystems), and alleles at each locus were binned according to fragment size ± 1.5 bp to accommodate variability during electrophoretic separation of DNA fragments.

The total number of alleles at each locus for all termites was categorized using the Microsoft Excel spreadsheet. For all possible alleles, a "1" was denoted where an allele was present in a given termite, and a "0" was denoted where an allele was absent. Based on this table, the Jaccard coefficient (Jc) was calculated for all pairwise comparisons using EstimateS (16). A distance matrix was created using Microsoft Excel where the Jaccard distance (Jd) was calculated as 1-(Jc). This matrix was uploaded into MEGA (29) for cluster analysis.

Cultivation, Nutrition and Physiological studies.

Routine cultivation of TAM isolates was carried out in liquid medium that contained: BSS, 0.05% Bacto yeast extract, 10 mM sodium acetate (BYA medium). Isolates were typically inoculated into 15 ml medium in a 50 ml sterile Erlenmeyer flask capped with a sterile, plastic 30 ml beaker (Nalgene) and incubated at 22-23°C with shaking (250 rpm). Though CO_2 was not required for growth, the TAM strains were incubated in a CO_2-enriched hypoxic atmosphere

(2% O_2, 5% CO_2, balance N_2) within a flexible vinyl chamber. Final pH of media in the CO_2-enriched atmosphere was 6.5.

Substrate utilization of TAM-DN1 was performed by using butyl rubber-stoppered, 18 mm anaerobe tubes (Bellco, Vineland, NJ; no. 2048-00150) containing 5 ml of BSS supplemented with 0.05% Bacto yeast extract and 10 mM of the test substrate. The headspace of tubes (ca. 22 ml) consisted of 2% O_2, 5% CO_2 and 93% N_2. Test cultures were inoculated with 1% v/v exponential phase culture growing in BYA medium and incubated at 22-23°C with the tubes held horizontally and shaken at 150 rpm. The cell yield was determined by measuring the optical density of the cultures at 600 nm with a Milton Roy Spectronic 20 colorimeter. A 50% increase of cell yield above the "No Substrate" after passage through two successive transfers was considered evidence of the ability to utilize the substrate.

The ability of TAM-DN1 to utilize acetate and succinate simultaneously was tested in 500 ml screw-cap glass media storage bottles (Bellco no.5636-00533) to which an 18 mm anaerobe tube (described above) was permanently affixed. Bottles containing 100 ml of BYA medium supplemented with 10 mM succinate were inoculated with a 1% v/v exponential phase culture growing in BYA medium and were incubated within a flexible vinyl chamber containing a 2% O_2, 5% CO_2, 93% N_2 atmosphere (described above) with shaking at 250 rpm. Growth was monitored by measuring the optical density of cultures at 600 nm as described above. Simultaneous with growth measurements, 1 ml of culture fluid was removed, centrifuged at 12,000 x *g* for 10 minutes, filtered through a 0.2 µm

pore size filter, and stored at -20 until used for organic acid analysis. Organic acids were quantified by high performance liquid chromatography (HPLC) (Waters, Milford, MA) on a 300- by 7.8-mm Aminex HPX-87H column (Bio-Rad, Hercules, CA) at 23°C with 4 mM H_2SO_4 as the eluent at a flow rate of 0.6 ml/min. Organic acids were detected with a Waters 2487 UV detector at 210 nm and calibrated with homologous standards.

The oxygen tolerance of TAM-DN1 was determined by two approaches. First, the ability of cells to grow in liquid medium under defined headspace concentrations of O_2 was evaluated. To do this, butyl rubber-stoppered 18 mm anaerobe tubes containing 5 ml of anoxic BYA medium were prepared in air (21% O_2) or under a headspace of 100% N_2. To the tubes that contained a 100% N_2 headspace, air was injected to attain a final headspace percentage of 0.5, 1, 2, 4 or 8% O_2 (after the overpressure was released). To attain a headspace percentage of 12 or 16% O_2, pure oxygen was injected. The tubes were then sterilized by autoclaving. Sterilized tubes were inoculated with a 5% inoculum of exponential phase TAM-DN1 cells growing in BYA medium, and incubated horizontally at 22-23°C with shaking at 150 rpm. Growth was monitored spectrophotometrically as described above. Time in lag phase was estimated as described in Lenski *et al.* (30) and total protein was quantified by the BCA method (45) with bovine serum albumin as a standard. Final cell yield was based on the assumption that protein constitutes 55% of cell dry weight (33). In the second approach, the ability of cells to grow on the surface of agar medium under various concentrations of O_2 was evaluated. To do this, 1.5% agar was

incorporated into liquid BYA medium, which was then heated to dissolve the agar, and 4 ml was dispensed into each of a number of Wolfe Anaerobic Agar Bottles fitted with a screw cap (Bellco no. 2535-50020). The bottle plates were then autoclaved, placed on their sides, and the medium allowed to solidify. Bottles were then brought into an anoxic chamber (10% H_2, 5% CO_2, 85% N_2)(Plas Labs, Lansing, MI), uncapped, and streaked with an exponential-phase culture of TAM-DN1. The bottles were then stoppered with a butyl-rubber stopper held in place by a screw cap possessing a small hole as an injection port. Pure, sterile O_2 was then injected into the bottles (60 ml average headspace volume) to attain final concentrations of 0.5, 1, 2, 4, 6, 8, 12, 16 and 21% O_2 in the headspace (after release of overpressure), after which they were incubated in an upright position at 22-23°C.

The ability of strain TAM-DN1 to grow under anoxia was tested by using 5 ml BYA medium that had been deoxygenated under vacuum and added to butyl-rubber stoppered 18 mm anaerobe tubes under 100% N_2. After autoclaving, the BYA medium was supplemented with 10 mM (final conc.) of one of the following from a sterile stock solution: potassium nitrate, potassium nitrite, sodium sulfate, sodium fumarate or D-glucose. For tubes containing sodium fumarate, 10 ml of 100% hydrogen was also added to the headspace of some tubes. The tubes were inoculated with a 2% v/v exponential phase culture growing on BYA medium under hypoxia. Tubes were then incubated at 22-23°C horizontally, shaking at 150 rpm.

Genomic properties.

The genome size of TAM-DN1 and TAV-1 was estimated by pulsed-field gel electrophoresis of restriction endonuclease digestions of total DNA according to previously published protocols (8, 22). Quantification of 16S rRNA gene copy number for TAM-DN1 and TAV-1 was determined according to the methods described in (26, 27).

Enzyme Assays

"*S. acetivorans*" strain TAM-DN1 cells were grown in 100 mL BYA medium in 500 ml sidearm bottles (above) under a hypoxic atmosphere shaking at 250 rpm. At mid-log phase (at which growth was known to be acetate-dependent, representing approximately 5 x 10^8 cells/ml) the entire culture volume was centrifuged at 10,000 x g for 10 min at 4°C, washed with 20 ml sonication buffer (10 mM EDTA, 50 mM Tris-HCl, pH 7.0), re-centrifuged and resuspended in 10 ml of the same buffer. Cells were disrupted in an ice water bath by sonication (3 x 30 s each) with a Branson Model 450 sonifier (power setting of 5, 50% duty cycle) equipped with a ½" threaded-body step horn with flat tip. To remove undisrupted cells and debris, the sonicate was centrifuged at 12,000 x *g* for 60 min at 4°C. The resulting supernatant liquid was considered to be the crude cell extract and was distributed into a Slide-A-Lyzer dialysis cassette (3 ml to 12 ml capacity, 3500 molecular weight cutoff; Pierce, Rockford, IL) and dialyzed for 12 hours in two liters dialysis buffer (50 mM Tris-HCl, pH 7.0). The dialyzed crude

extract was removed from the cassette and used immediately for assays of enzyme activities.

Catalase was assayed by measuring the rate of decrease in A_{240} of H_2O_2 according to Beers *et al.* (6). Superoxide dismutase activity was measured by the xanthine/xanthine oxidase-cytochrome c reduction method (20). NAD(P)H oxidase and peroxidase activities were assayed as described previously (46).

A qualitative, colorimetric test for cytochrome C oxidase was done by spreading TAM-DN1 colony material onto a piece of generic filter paper wetted with a 1% solution of tetramethyl-*p*-phenylenediamine dihydrochloride (Sigma) as described previously (44).

Microscopy

Phase-contrast micrographs were prepared from wet mounts on agar-coated slides (36). Images were captured on a Zeiss Axioskop microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with a SPOT charge-coupled-device digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI). Cells were prepared for electron microscopy by the staff at the Center for Advanced Microscopy at Michigan State University. Electron micrographs were obtained with a JEOL 6400V scanning electron microscope with a LaB6 emitter or JEOL 2200FS 200 kV field emission transmission electron microscope (JEOL-USA, Inc., Peabody, MA).

Results and Discussion

Isolation and enumeration of putative O_2-consuming organisms.

For termites collected from Dansville, MI, the total CFU appearing on plates of ACY isolation medium was 9.2 (± 1.0) x 10^5 per gut equivalent for plates incubated in 5% CO_2-enriched air and 12 (± 2.4) x 10^5 per gut equivalent for plates incubated in hypoxia (5% CO_2, 2% O_2, balance N_2). The consistent and noticeable, though not statistically significant, increase in total CFU seen with plates incubated under hypoxia was also observed with termites collected from Spring Arbor, MI, (3% increase); Janesville, WI, (29% increase); Raleigh, NC, (8% increase); and Forêt de la Coubre, Fra., (12% increase) (Table 2.1). In every case, this increase was due to a single colony type whose morphology was distinct enough to be easily differentiated from the rest (Figure 2.1). Such colonies accounted for as many as 10% of all CFU appearing on plates incubated under hypoxia and were especially abundant in guts of *R. flavipes* collected from the Michigan sites. Phase contrast, scanning and transmission electron microscopy of cells comprising such colonies revealed that they were thin, nonmotile rods (0.5 x 5 μm) with a Gram-negative type cell wall and outer membrane (Figure 2.2a-c). They also accumulate intracellular granuals that appear morphologically similar to poly-β-hydroxybutrate (PHB). Similar granules accumulate in organisms like *Azospirillum* under conditions of low O_2 and a high C/N ratio (25, 48). The isolates were cytochrome C oxidase positive and could only be subcultured on plates incubated in hypoxia. Moreover, the isolates also displayed robust colony growth only if acetate was included in ACY medium.

Table 2.1. Total cultivable bacteria from the guts of geographically separated *Reticulitermes* workers incubated under CO_2-enriched oxic and hypoxic atmospheres.

Termites (Collection location)	Total[a] CO_2-Enriched Air	Total[a] Hypoxia
R. flavipes		
Dansville, MI	9.2 ± 1.0	12 ± 2.4
Spring Arbor, MI	6.2 ± 2.7	6.4 ± 1.0
Janesville, WI	10 ± 0.8	14 ± 1.7
Raleigh, NC	12 ± 1.5	13 ± 2.6
R. santonensis		
Forêt de la Coubre, Fra.	15 ± 5.4	17 ± 3.2

[a] Total based on direct colony counts of isolation media and are given as colony forming units (CFU) per gut equivalent $x10^5$. Mean ± s.d. n=3.

[b] 5% CO_2, 95% Air

[c] 2% O_2, 5% CO_2, 93% N_2

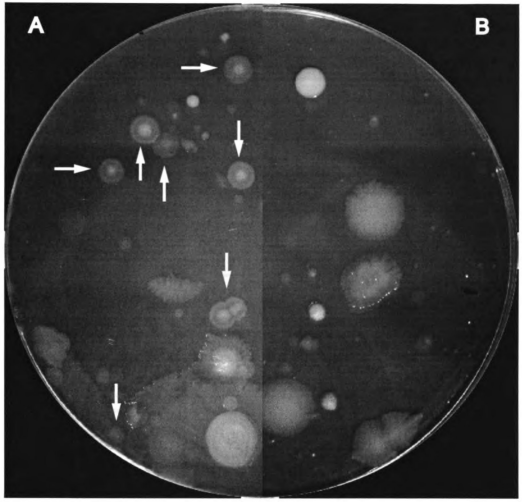

Figure 2.1. Composite photograph showing colony morphotypes on two separate isolation plates. Arrows point to the unique colony morphotype present on the plate incubated in a hypoxic atmosphere (A) that were not on the plate incubated in CO_2-enriched air (B).

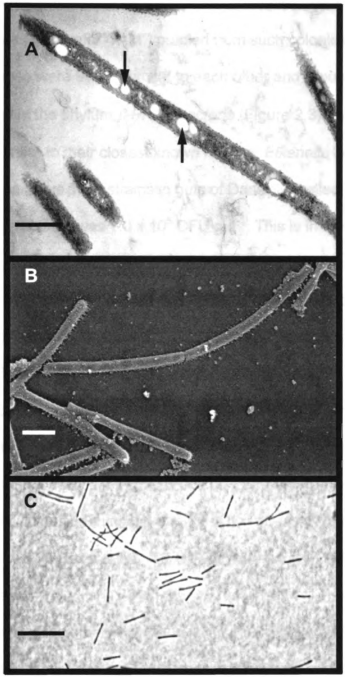

Figure 2.2. Transmission electron (A), scanning electron (B) and phase-contrast light (C) micrographs of termite gut *Neisseriaceae* strain TAM-DN1. Arrows point to intracellular granules that resemble poly-β-hydroxybutyrate. Scale bars are 1 μm (A, B) and 10 μm (C).

Preliminary phylogenetic identification of 18 randomly selected strains (designated with the prefix "TAM") purified from such colonies revealed that their 16S rRNA genes were 99.7% similar to each other and grouped within the family *Neisseriaceae* in the phylum *β-Proteobacteria* (Figure 2.3). However, they were only 94.1% similar to their closest known relative, *Eikenella corrodens*. The *in situ* abundance of the TAM strains in guts of Dansville-collected *R. flavipes,* based on plate counts, was 1.0×10^5 CFU·gut^{-1}. This is in close agreement with the 2.2×10^5 cells·gut^{-1} estimated by quantitative dilution-to-extinction PCR (based upon the estimated 3.2 Mb genome size and 4 rrs copies present in TAM-DN1 (data not shown)). Therefore, the TAM strains are estimated to comprise between 1.6% and 3.5% of the total prokaryotic population in *R. flavipes* guts (49). Owing to their abundance, their phylogenetic novelty, and their apparent ability to oxidize acetate only under hypoxic conditions, suggesting that they might be true microaerophiles specialized to live within the hypoxic peripheral region of *R. flavipes* hindguts, strains of these bacteria were chosen for further examination, with representative strain TAM-DN1 subject to the most detailed characterization.

The bulk of the other colonies present on isolation plates proved to be members of the families *Streptococcaceae*, *Enterococcaceae*, and *Enterobacteriaceae* (Figure 2.3), however these occurred with equal frequency on plates incubated under CO_2-enriched air or the hypoxic gas mixture. Not surprisingly, only colonies of the latter bacteria displayed more robust growth on acetate-containing medium. Members of the *Streptococcaceae* and

Enterococcaceae are capable of growth in the presence of oxygen and can use O_2 as an electron acceptor (49), but are not known to oxidize acetate as an energy source; their appearance on isolation plates was almost certainly supported by energy-yielding nutrients (e.g. sugars, amino acids) present in the yeast extract and casamino acids components of ACY isolation medium. Based on 528 aligned 16S rDNA nucleotides, the *Streptococcaceae* isolates were most closely related to the genus *Lactococcus*, and were 97.1% identical to each other. The *Enterococcaceae* had a 98.0% 16S rRNA gene sequence identity to each other and grouped with the genus *Enterococcus* (564 positions). The *Enterobacteriaceae* isolates grouped most closely to the genera *Citrobacter* and *Enterobacter*, all known to be capable of acetate oxidation, and were 96.8% similar based on 457 nucleotide positions.

With the exception of the *Neisseriaceae*, the isolates above were typical of organisms isolated from termite guts previously or whose 16S rRNA genes were represented in clone libraries prepared from gut homogenates of various termite species (1, 5, 19, 23, 24, 37, 38, 41, 42, 49, 53). A single 16S rRNA gene clone closely related to the *Neisseriaceae* isolates obtained here was obtained from a gut wall fraction prepared from *R. santonensis* (53). Otherwise, these organisms have not previously been encountered or isolated from termite guts, perhaps because most previous cultivation efforts have employed incubations in air or under anoxia.

Recent molecular phylogenetic methods have revealed the presence of not-yet-cultivated organisms related to members of the *Verrucomicrobia*,

Acidobacteria, *Planctomyces* and candidate phylum *Endomicrobia* in the hindgut of termites (24, 35). As these organisms might also be important to O_2 consumption *in situ*, we used "Plate Wash PCR" (47) in early experiments to screen for the presence of such bacteria on plates inoculated with diluted gut homogenates from Dansville, MI specimens of *R. flavipes*. By using this technique, members of the phyla *Verrucomicrobia* and *Acidobacteria* were isolated and were described previously (47) (Figure 2.3). However, dilution-to-extinction PCR implied that the *in situ* abundance of the *Verrucomicrobia* was relatively low (ca. $2x10^3$ cells·gut^{-1}), albeit localized to the gut (Figure 2.4). With *Acidobacteria*-specific primers, PCR products were obtained from both termite gut DNA as well as DNA from degutted bodies, suggesting that the *Acidobacteria* isolates cannot be localized strictly to the gut region. Furthermore, PCR amplification of *Acidobacteria* from gut DNA was only successful with DNA concentrations representing ≥ ¼ of a gut equivalent (Figure 2.4). These results suggest the gut-associated *Verrucomicrobia* and *Acidobacteria* in *R. flavipes*, while intrinsically interesting, are but minor members of the microbial community.

Autochthony of the TAM-strain community

In order to determine whether the TAM strains were autochthonous members of the *R. flavipes* gut community as opposed to allochthonous, transient inhabitants, 16S rRNA gene clone libraries were prepared from termite guts, termite nest soil, and non-termite associated forest soil by using PCR with TAM specific primer sets, and the libraries were analyzed by the LIBSHUFF

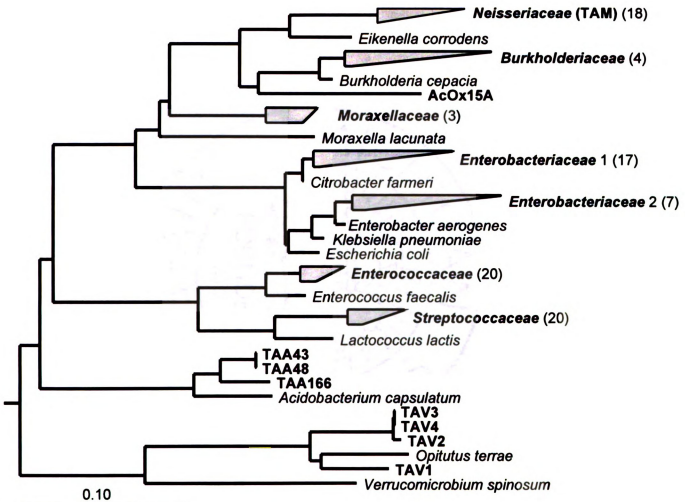

Figure 2.3. Maximum likelihood-based 16S rRNA gene phylogeny of isolates obtained from *Reticulitermes flavipes* collected from Dansville, MI. Isolates obtained in this study are shown in boldface. Many closely related isolates are condensed as gray trapezia with the number of sequences in parentheses. *Aquifex pyrophilus* was used as an outgroup (not shown).

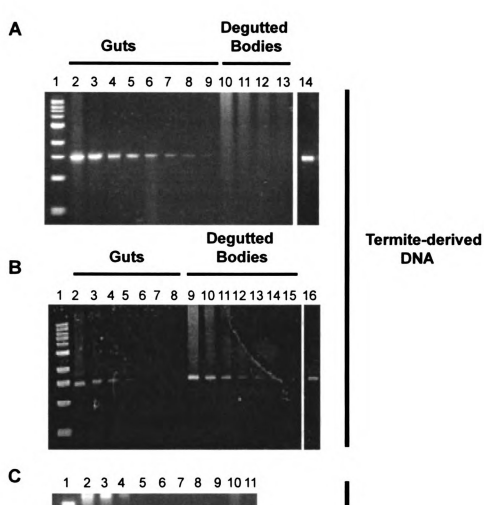

Figure 2.4. Dilution-to-extinction PCR of DNA from termite hindguts and degutted termite bodies. A. *R. flavipes* DNA amplified with *Verrucomicrobia*-specific 16S rDNA primers. (1) 1 kb DNA ladder; (2-9) one gut-equivalent DNA serially diluted 1:2 8x; (10-13) one body-equivalent DNA serially diluted 1:2 4x; (14) positive control *Verrucomicrobia* st. TAV-1 DNA. B. *R. flavipes* DNA amplified with *Acidobacteria*-specific 16S rDNA primers. (1) 1 kb DNA ladder; (2-8) two gut-equivalents' DNA serially diluted 1:2 7x; (9-15), two body-equivalents' DNA serially diluted 1:2 7x; (16) positive control *Acidobacterium capsulatum* DNA. C. Purified DNA from *Verrucomicrobia* strain TAV-1 amplified with *Verrucomicrobia*-specific 16S rDNA primers as a control. (1) 1 kb DNA ladder; (2-9) 100 ng DNA serially diluted 1:16 8x; (10) 100 ng *E. coli* DNA; (11) negative control without DNA.

Figure 2.5. Maximum likelihood-based phylogeny of PCR-generated 16S rRNA gene clones obtained with a termite gut *Neisseriaceae*-specific primer set from *R. flavipes* termite guts (51 clones; black rectangle), termite nest soil (29 clones; gray rectangles), and adjacent but non-termite inhabited forest soil (25 clones; white rectangles). Closely related clones were condensed into a single rectangle, with the number of clones given in parentheses. Rectangles are aligned for ease of comparison only; such alignment is not intended to imply equal evolutionary time. Scale bar represents 0.1 change per nucleotide.

program (40). Results clearly indicate the presence of a closely related (average 98.4% 16S rDNA identity) population in the termite gut that is phylogenetically distinct from 16S rRNA genes amplified from the termite nest soil or surrounding non-termite associated forest soil (P = 0.001) (Figure 2.5). Interestingly, however, there appears to be a cluster of clones from termite nest soil that are genetically related to the termite gut clones. No clones from the forest soil clustered with this group, suggesting they are termite-nest specific.

Geographical Distribution

In order to determine whether TAM-like strains were present in guts of geographically and genetically distinct populations of *R. flavipes*, TAM isolates or TAM-like 16S rRNA gene clones were obtained from hindguts of *Reticulitermes* specimens collected from different sites, which were themselves examined by microsatellite DNA analysis. Five separate microsatellite loci for eight individual worker termites collected from each of the six different sites were amplified by PCR as described previously (50). Combining all termites, forty-four distinct alleles were amplified from the 5 different loci. Clustering analysis of the alleles revealed that most of the termites clustered according to their geographical origin (Figure 2.6). Not surprisingly, termites collected from Raleigh, NC and Woods Hole, MA clustered more closely together, as did those collected from Dansville and Spring Arbor, MI. Interestingly, *R. flavipes* collected in Janesville, WI, clustered with *R. santonensis* collected in France. However, recent studies have suggested the synteny of *R. flavipes* and *R. santonensis* (3), which our data

would seem to support. Between collection sites the results clearly indicate the genetic distinctiveness of the termites, presumably the result of divergent evolution over long periods of time. Within a collection site, there is also a high degree of variation. This may be because worker termites were collected while foraging, and termites from several genetically different colonies may have been collected together from the same food source. No isolates related to the TAM organisms were obtained from the two non-*Reticulitermes* genera, *Zootermopsis angusticollis* (Hagen) (*Termopsidae*) and *Coptotermes formosanus* (Shiraki) (*Rhinotermitidae*) tested in this study.

The TAM isolates and 16S rRNA gene sequences obtained from the genetically distinct and geographically separated *Reticulitermes* are closely related (99.8% 16S rRNA gene identity) (Figure 2.7). This further supports the autochthony of the TAM isolates and suggests these symbionts occupy an important ecological niche within hindguts of *Reticulitermes* termites.

Relationship to oxygen

One of the most striking and readily apparent properties of the TAM-strains was their robust growth under hypoxia, but inability to grow anaerobically (by fermentation or with alternate electron acceptors, below) or in air (or CO_2-enriched air). Accordingly, experiments were done with strain TAM-DN1 to examine the O_2-sensitivity of cells by monitoring their growth on or in acetate-containing solid or liquid medium under various concentrations of oxygen. On solid medium, where cells are in direct contact with the headspace gas phase,

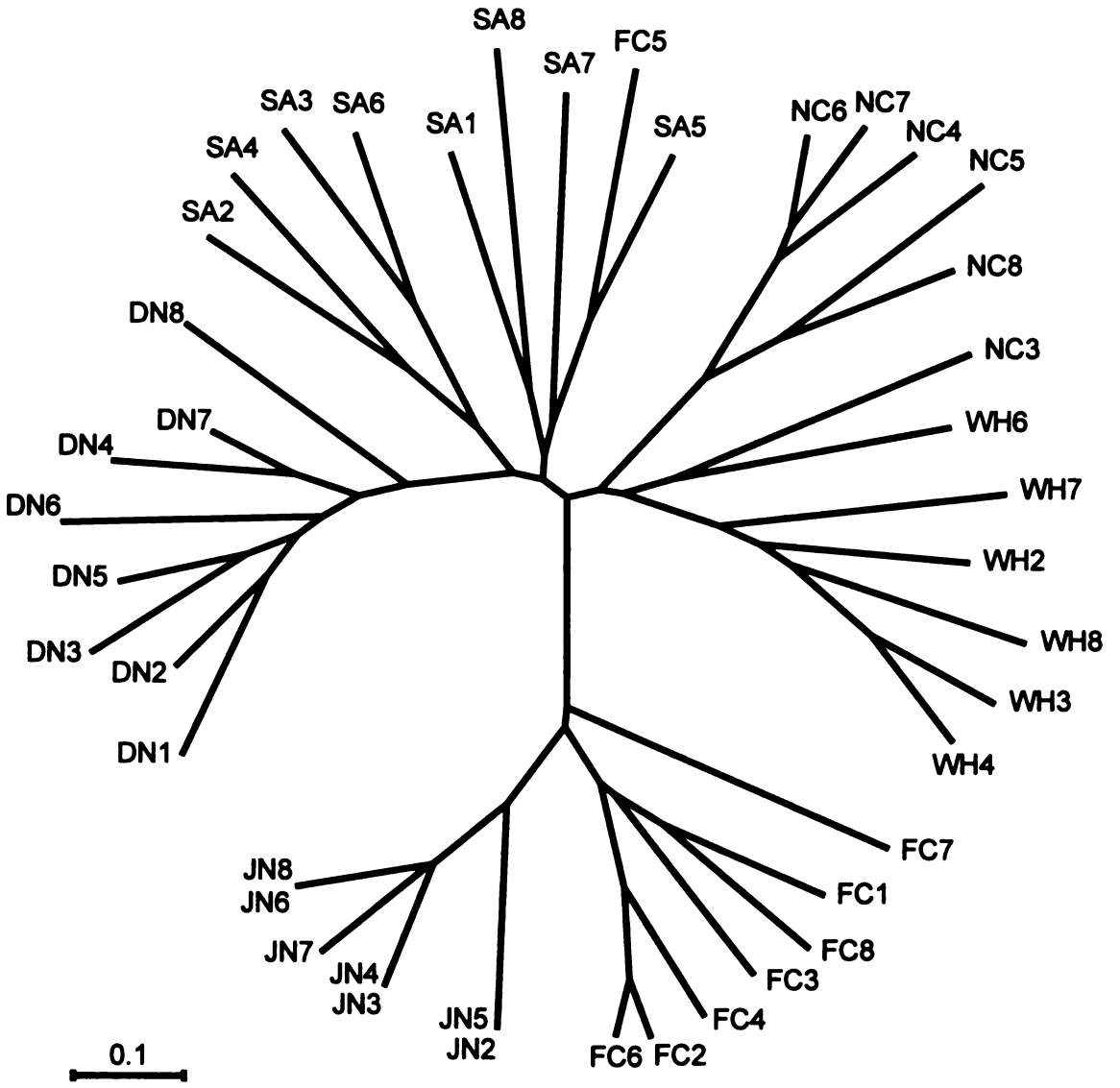

Figure 2.6. Neighbor-joining analysis of 5 microsatellite loci (44 alleles) of 43 individual worker termites collected at different geographical locations. *R. flavipes* workers were collected from Dansville, MI (DN); Raleigh, NC (NC); Spring Arbor, MI (SA); Janesville, WI (JN); and Woods Hole, MA (WH). *R. santonensis* workers were collected from Forêt de la Coubre, France (FC).

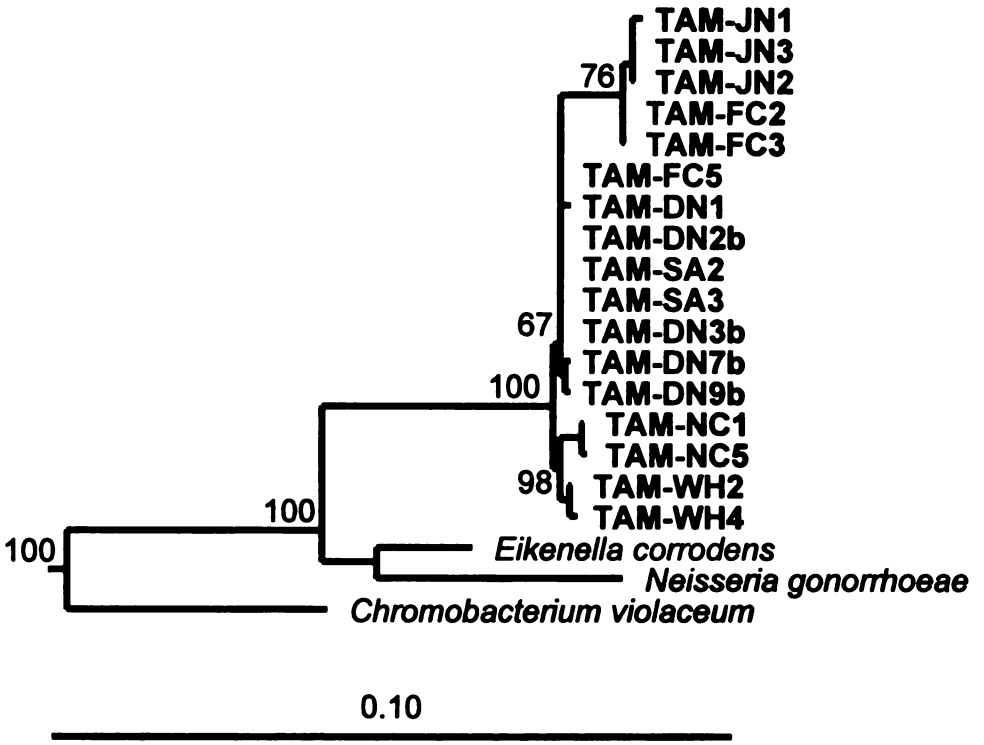

Figure 2.7. Maximum likelihood-based 16S rRNA gene phylogeny of termite gut *Neisseriaceae* isolates and clones. Isolates were obtained from *R. flavipes* collected at: Dansville, MI (DN); Raleigh, NC (NC); Spring Arbor, MI (SA); and Janesville, WI (JN). 16S rRNA gene clones were obtained from *R. flavipes* collected at Woods Hole, MA (WH). Isolates were also obtained from *R. santonensis* from Forêt de la Coubre, Fra (FC). *Lactococcus lactis* was used as an outgroup (not shown). Numbers at nodes represent percentage of topology conservation after 100 bootstrap samplings. Bar represents 0.1 change per nucleotide.

TAM-DN1 was unable to form colonies at O_2 concentrations higher than 4% (Figure 2.8a). Based on the number of colonies on these plates, the optimum O_2 concentration for growth on solid media is between 1 and 2%. By contrast, in broth cultures, where oxygen diffusion to cells is more limited by a relatively large volume of liquid, a 1% v/v inoculum of 2% O_2-grown TAM-DN1 was able to initiate growth at O_2 concentrations at least as high as 16% (Figure 2.8b). Under these conditions, however, the duration of the lag phase increased linearly with the initial O_2 concentration (Figure 2.9). Nevertheless, the final cell yield also increased proportionally with increasing O_2 concentration (Figure 2.9). This observation suggests that, within limits, cells require a period of adaptation to deal with elevated concentrations of O_2 (or reactive oxygen species generated by metabolism in the presence of elevated concentrations of O_2), and the duration of that adaptation is proportional to the O_2 concentration to which they are initially exposed, but once adapted they can consume all of the O_2 for energy generation, which is translated into a corresponding increase in cell biomass.

This microaerophilic nature of TAM-DN1 prompted assays for oxyprotective enzymes. Under *in vitro* cultivation conditions TAM-DN1 does express catalase (99 U·mg. prot.$^{-1}$) and superoxide dismutase (32 U·mg. prot.$^{-1}$) enzymes. No NAD(P)H oxidase or peroxidase activity was detected.

These observations indicate that TAM-DN1 and related strains are obligate microaerophiles and probably reside in the hypoxic region of hindguts, on or near the gut wall. Expression of oxyprotective enzymes like catalase and superoxide dismutase, as well as their ability to adapt in liquid medium to

headspace O_2 concentrations up to 16% may be important for transfer to, and colonization of, guts of newly hatched larvae and recently-molted colony mates, whose microbiota is essentially absent or drastically reduced, respectively, and whose O_2 content is almost certainly substantially higher than when fully colonized.

Substrate Utilization by TAM-DN1

Substrates utilized by strain TAM-DN1 for growth included acetate, acetyl-acetate, succinate, butyrate, glutamate, glutamine, fumarate and casamino acids. Of these, acetate (60-80 mM), butyrate (2 mM) (34), and a number of free amino acids including glutamate (1.7 mM) and glutamine (1.0 mM) (21, 43) have been detected in termite hindgut fluid. HPLC analysis of substrate utilization by TAM-DN1 revealed that the isolate was able to co-utilize acetate and succinate when both were available in the culture medium (Figure 2.10). TAM-DN1 was able to achieve approximately twice the final cell yield (2.3×10^9 cells/ml) when grown on both substrates than on acetate alone (1.2×10^9 cells/ml)(data not shown). The rate of acetate utilization by TAM-DN1 was 0.09 $\mu mol \cdot min^{-1} \cdot mg\ protein^{-1}$ when succinate was present in the medium, and 0.12 $\mu mol \cdot min^{-1} \cdot mg\ protein^{-1}$ without added succinate. Substrates not utilized for growth included cellobiose, maltose, glucose, xylose, arabinose, lactate, pyruvate, citrate, formate, propionate, 2-oxoglutarate, malate, maleic acid, benzoate, threonine, glycine. TAM-DN1 was not able to grow anaerobically on acetate using fumarate (with or without H_2 in the headspace), nitrate, nitrite, or sulfate as electron acceptors.

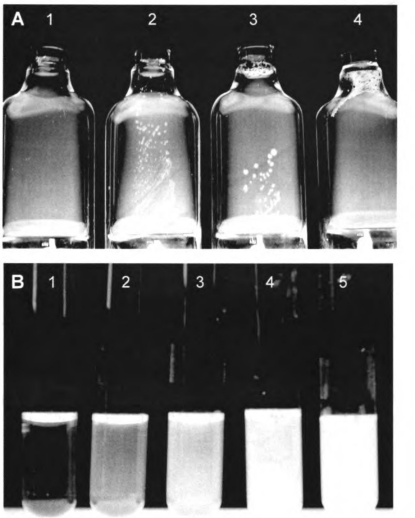

Figure 2.8. O_2 tolerance of *Neisseriaceae* st. TAM-DN1. (A) Growth on solid medium in Wolfe bottles containing a headspace of: 0% (1), 2% (2), 4% (3), 6% (4) v/v oxygen. (B) Growth in liquid medium under an atmosphere of: 0% (1), 2% (2), 4% (3), 8% (4) and 16% (5) v/v oxygen.

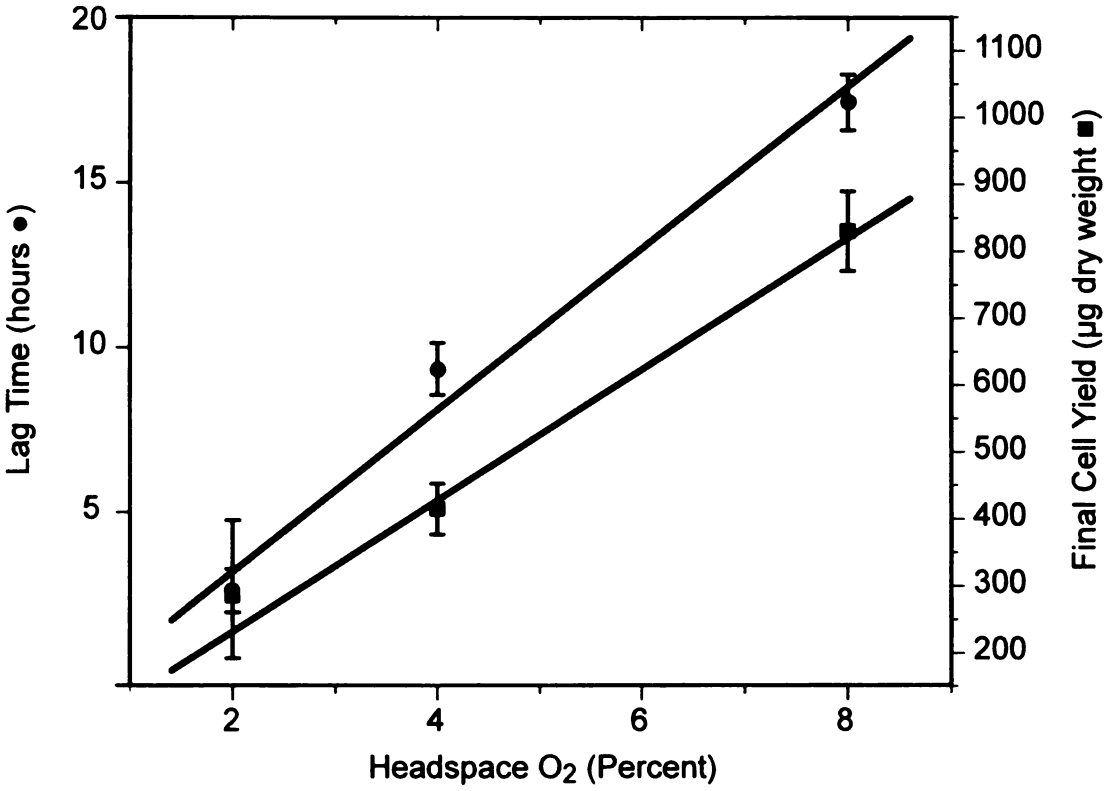

Figure 2.9. Final cell yield (■) and lag time (●) of TAM-DN1 cells grown in liquid medium under a headspace of 0% to 8% oxygen.

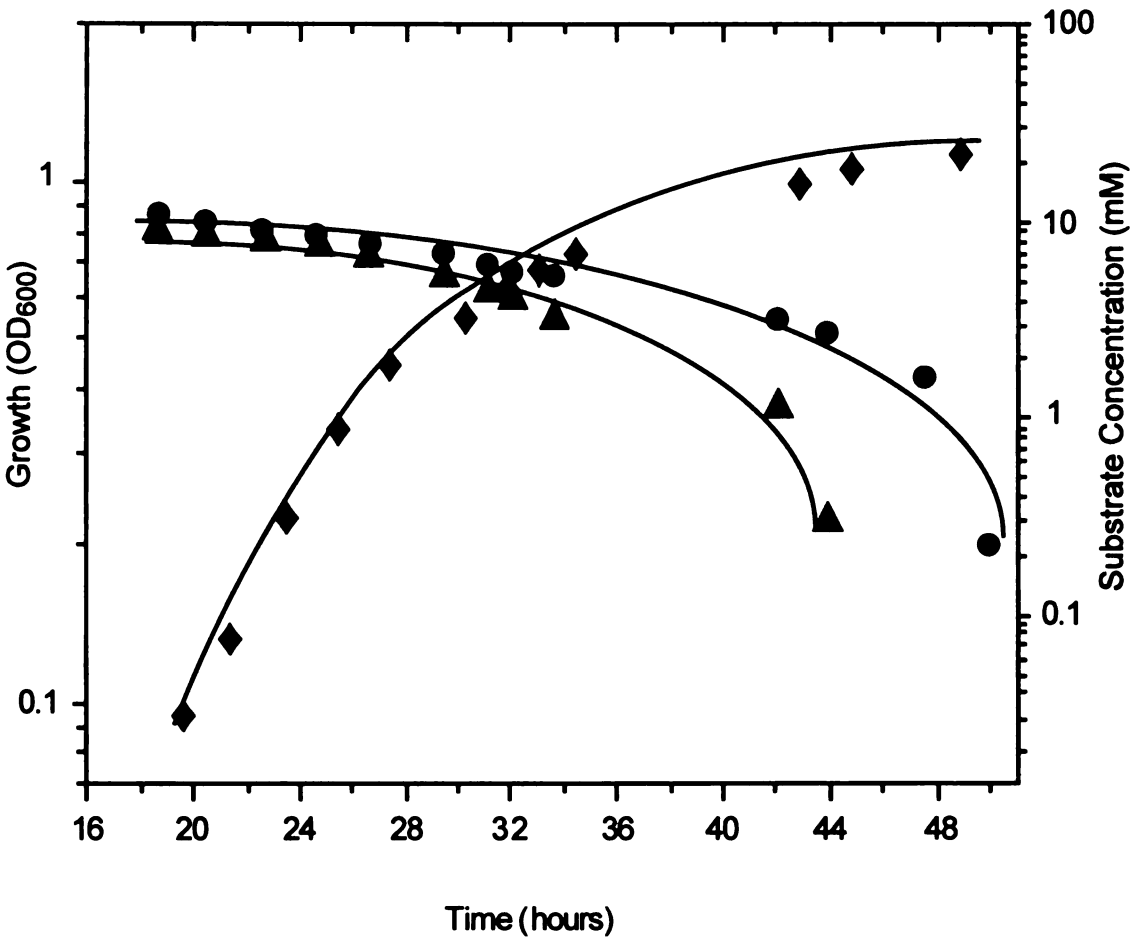

Figure 2.10. Co-utilization of acetate (●) and succinate (▲) by TAM-DN1 during growth (♦).

TAM-DN1 was not able to grow fermentatively on glucose. In BYA medium, TAM-DN1 grown at 22°C, 30°C, 37°C and 42°C had the shortest generation time (3.4 hours) at 30°C. No growth occurred above 37°C.

Rationale for the proposal of TAM-DN1 and related strains as a new genus and species

The molecular and physiological characterization of isolate TAM-DN1 and related strains suggests the cultivars are sufficiently distinct from any other known bacteria to warrant their classification as a novel genus and species within the domain *Bacteria*. The distant (94.1%) 16S rRNA gene identity to their closest cultivated relative, coupled with their microaerophilic phenotype, substrate utilization spectra, autochthony to the termite gut and apparent specialization to the hypoxic periphery has distinguished these isolates from any other known genus and species of *Bacteria*. The proposed name "*Stenoxybacter acetivorans*" appears in quotations throughout this dissertation to signify that the name has not been validly published, and to reflect a desire that the first description of "*Stenoxybacter acetivorans*", for comparative work or bibliographies not be cited as this chapter but rather the paper derived from it that will be published within the primary literature.

Description of "*Stenoxybacter*" gen. nov.

Stenoxybacter (Sten.o.xy.bac' ter. Gr. adj. stenos narrow, Gr. adj. oxys acid/sour, in combined words indicating oxygen (N.L. *oxygenium*), NL. masc. n. bacter rod/bacterium, N.L. masc. n. *Stenoxybacter,* rod with a narrow oxygen range). The genus description is, at present, the same as the type species "*Stenoxybacter acetivorans*".

Description of "*Steoxybacter acetivorans*" sp. nov.

Stenoxybacter acetivorans (a.ce.ti.vo' rans. L. neut. n. *acetum* vinegar/acetic acid. L. pres. part. *vorans* devouring, N.L. pres. part. *acetivorans*, acetate consuming). The type strain is TAM-DN1.

Cells are thin (0.5 µm x 5 µm) rods with an undulating, Gram-negative type outer membrane and cell wall. Cells contain electron translucent inclusions that resemble poly-β-hydroxybutyrate. Cytochrome C oxidase, catalase, and superoxide dismutase positive. On solid BYA medium, colonies have a crenate, low convex morphology. Optimum oxygen concentration for growth on solid medium is between 1 and 2% O_2. Optimum temperature for growth in liquid medium is 30°C. The cells have a 3.2 Mb genome with 4 16S rRNA gene copies per cell.

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I would like to thank Ed Vargo for sending *R. flavipes* termites from North Carolina, Andreas Brune for providing *R. santonensis* from France, and Nan-Yao Su for providing a collection of *C. formosanus*. I would also like to thank Bradley Stevenson for assistance with Plate Wash PCR, hypoxic cultivation, and molecular techniques, James McKinlay for help with HPLC analyses, Kwi Kim for determining the genome size and rrs copy number of TAM-DN1, Joseph Graber for assistance with anoxic cultivation techniques, and Thomas Schmidt for help with primer design and microsatellite analyses. My gratitude as well to Hans Trüper for providing help with nomenclature and etiology.

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Chapter 3

Physiological Ecology of "*Stenoxybacter acetivorans*" in Termite Guts

Introduction

In termite hindguts, fermentative production of acetate – a major carbon and energy source for the insect – depends upon the efficient removal of inwardly diffusing O_2 by microbes residing on and near the hindgut wall (11). Acetate, present in abundance (60-80 mM) within the hindgut fluid, is the most likely electron donor supporting the respiratory activity of such microorganisms (40). Chapter 2 discussed the isolation and identification of microorganisms from *Reticulitermes flavipes* guts that may be important O_2 consumers *in situ*. Abundant among these isolates were strains of a novel, obligately microaerophilic *β-Proteobacterium*, "*Stenoxybacter acetivorans*" gen. nov. sp. nov., that appeared to be autochthonous in hindguts of *R. flavipes* and present in genetically distinct populations of *Reticulitermes* collected from different geographical locations. Owing to their abundance, obligately microaerophilic nature, and ability to utilize a relatively narrow range of substrates, including acetate, as energy sources, it seemed reasonable to hypothesize that "*S. acetivorans*" resides in the hypoxic, peripheral region of *R. flavipes* hindguts and uses a high-affinity terminal oxidase for acetate-driven respiratory activity *in situ*. Among the information needed to test this hypothesis, however, were the nature

of "*S. acetivorans*" enzymes (and their encoding genes) specific to acetate utilization and oxygen consumption.

Key enzymes specific to acetate utilization by microbes include those that activate acetate to acetyl-CoA prior to its entry into dissimilatory and assimilatory pathways. Among these are acetate kinase (ACK; EC 2.7.2.1) and phosphotransacetylase (PTA; EC 2.3.1.8), which catalyze the conversion of acetate first to acetyl-phosphate, then to acetyl-CoA, respectively (Figure 3.1). ACK and PTA are the primary enzymes of acetate activation in organisms such as *Corynebacterium glutamicum* and *Methanosarcina thermophila* (1, 20). By contrast, in many other organisms such as the *Enterobacteriaceae*, the ACK/PTA enzyme pair functions in the reverse direction, primarily during anaerobic growth on carbohydrates wherein acetate is a major fermentation product and is excreted. This allows additional ATP production by substrate-level phosphorylation (14, 27, 28, 41). When grown on acetate as a carbon and energy source, the *Enterobacteriaceae* and *Bacillus subtilis* express an acetate-inducible acetyl-CoA synthetase (ACS; EC 6.2.1.1) which catalyzes the conversion of acetate, ATP and CoA, to acetyl-CoA, AMP, and inorganic pyrophosphate (PPi) (27, 28) (Figure 3.1). Kinetic data have demonstrated that acetyl-CoA synthetase has a high affinity for acetate (Km = 0.2 mM), whereas acetate kinase is a low affinity enzyme (Km = 7.0 - 22 mM) (1, 28). However, inasmuch as PPi is often hydrolyzed by endogenous pyrophosphatase, which serves to pull the ACS-mediated reaction, acetate activation to acetyl-CoA by ACS consumes two high energy phosphate bonds compared to one consumed

by the ACK/PTA enzyme pair. Other, less widely distributed acetate-activating enzymes include a pyrophosphate-dependent acetylkinase (EC 2.7.2.12) found in *Entamoeba histolytica* (61) and an ADP-forming acetyl-CoA synthetase (EC 6.2.1.13) found in *Archaea* and some *Eukarya*, and which is structurally and evolutionarily unrelated to ACS (25, 37).

When acetate is the only carbon and energy source for a bacterium such as *Escherichia coli*, further catabolism of acetyl-CoA occurs via the TCA cycle. However, the TCA cycle alone does not allow any net assimilation of acetate carbon. Under these conditions, the anapleurotic glyoxylate cycle is derepressed and serves to replenish TCA cycle intermediates derived from acetate catabolism, but drawn off for biosynthesis of cell material (14, 17) (Figure 3.1). The two characteristic and key enzymes of the glyoxylate cycle are isocitrate lyase (ICL; EC 4.1.3.1) and malate synthase A (MSA; EC 2.3.3.9). Each turn of the cycle results in the net formation of one molecule of malate from two molecules of acetyl-CoA. Malate can then be oxidized to oxaloacetate, an important precursor for amino acid synthesis and gluconeogenesis.

Among high-affinity oxidases used to mediate terminal electron transfer to oxygen, especially during aerobic respiration at low ambient oxygen concentrations, are the cbb_3-type cytochrome oxidases. These have been referred to as "specialized enzymes at the heart of microbial metabolism" (43). Many so-called microaerophilic microorganisms or organisms that spend at least part of their life in low O_2 environments, such as the N_2-fixing root nodule symbiont *Bradyrhizobium japonicum*, or the gastric mucosa-colonizing pathogen

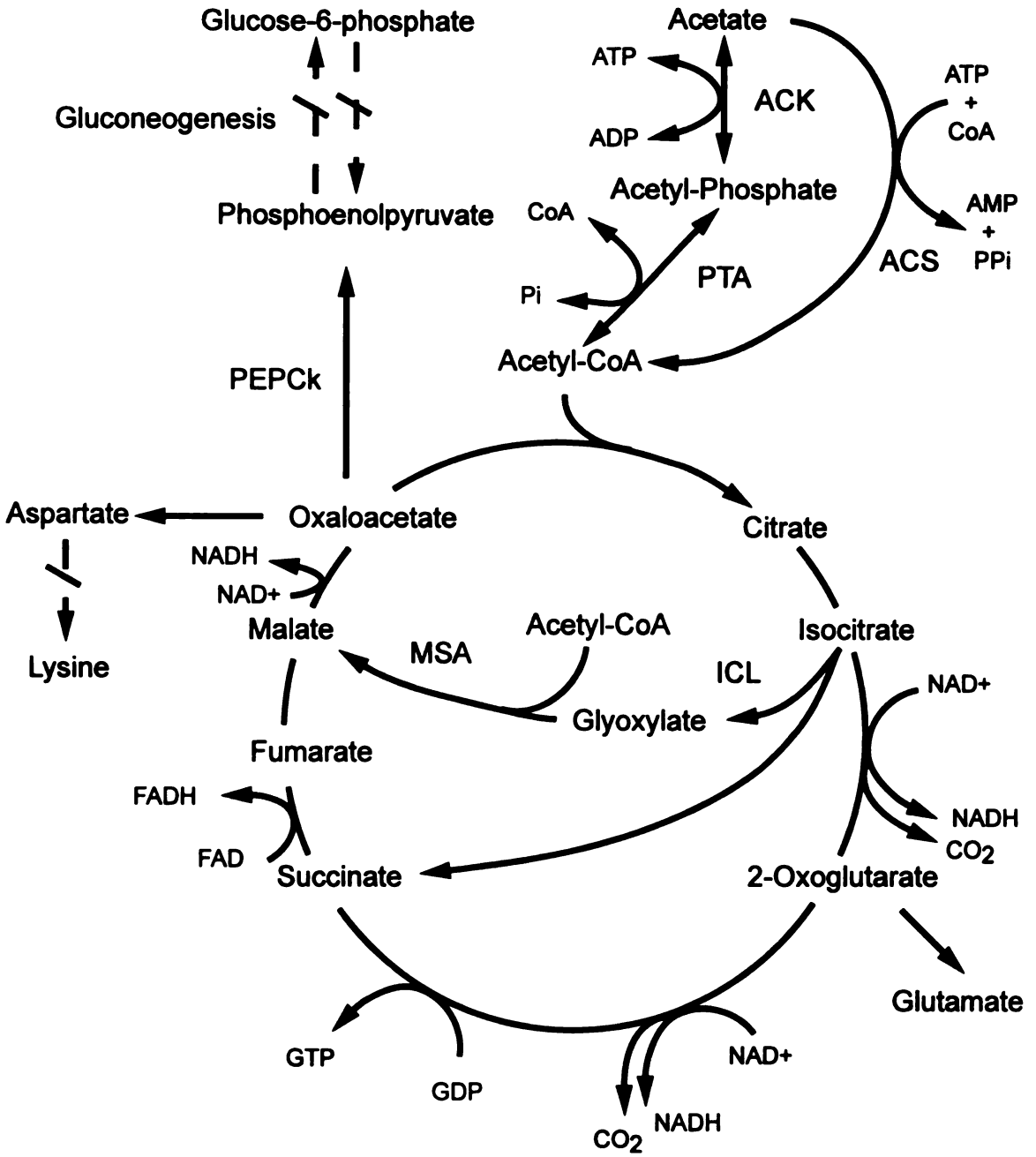

Figure 3.1. Common pathways for acetate activation and metabolization. Enzymes involved in acetate activation are: acetate kinase (ACK), phosphotransacetylase (PTA), or acetyl-coA synthetase (ACS). Once activated, acetyl-coA proceeds through the TCA cycle. If acetate is the sole carbon source, replenishment of TCA cycle intermediates drawn off for biosynthesis such as 2-oxoglutarate or oxaloacetate is accomplished through the action of the glyoxylate cycle enzymes isocitrate lyase (ICL) and malate synthase A (MSA). For biosynthesis of glucose from acetate, the anapleurotic enzyme phosphoenolpyruvate carboxykinase (PEPCk) is used.

Helicobacter pylori, utilize a cbb_3-type terminal cytochrome oxidase (34, 46). In contrast to the well-studied cytochrome aa_3 oxidase, which has an estimated Km for oxygen of 0.1 to 1 μM, the cbb_3 oxidases have estimated Km's in the nanomolar range (e.g. 7 nM for *B. japonicum*) (46). The ability of organisms utilizing these high-affinity enzymes to "pull" oxygen down to very low concentrations presumably minimizes the production of reactive oxygen species (to which microaerophiles may be particularly sensitive) and also allows O_2-labile processes such as N_2-fixation to occur, as with *B. japonicum* bacteroids in root nodules (46). The typically greater energy yield of aerobic vs. anaerobic metabolism may give a competitive edge to organisms with a cbb_3 -type terminal oxidase in low-O_2 environments. However, physiological studies with cells, as well as experiments with purified cbb_3 oxidase reconstituted into phospholipid vesicles, suggest that the cbb_3-type oxidase is significantly less efficient at transducing energy than cytochrome aa_3 (51).

In this chapter, a combination of molecular and physiological approaches were used to: (i) determine the primary location of "*S. acetivorans*" *in situ*; (ii) identify key genes and gene products involved in acetate utilization and oxygen consumption by "*S. acetivorans*" *in vitro*; and (iii) determine whether these same genes (and by inference, the gene products) are being expressed by cells of "*S. acetivorans*" *in situ*. Experiments were also done to estimate the potential contribution of "*S. acetivorans*" to overall O_2 consumption in guts of *R. flavipes*, as well as the relative contribution of major microbial groups (i.e. bacteria and protozoa) to O_2 consumption.

Materials and Methods

Termites.

Reticulitermes flavipes (Kollar) (*Rhinotermitidae*) were collected near Dansville, and Spring Arbor, MI. The termites were either degutted within hours of collection or maintained in laboratory nests as described previously (8, 40).

Microorganisms and Growth Conditions

"*Stenoxybacter acetivorans*" strain TAM-DN1 and *Citrobacter* strain RFC-10 were isolated from the guts of *Reticulitermes flavipes* collected in Dansville, MI, as described in Chapter 2. Routine cultivation was carried out in liquid BYA medium under a hypoxic (2% O_2, 5% CO_2, 93% N_2) atmosphere (Chapter 2).

Optical density readings of cells were made in an 18 mm anaerobe tube using a Spec-20 spectrophotometer at a wavelength of 600 nm. A standard curve relating optical density to cell number was done by using a Petroff-Hauser counting chamber. Cells were counted at 400x magnification. Optical density (and hence cell number) was related to protein concentration by centrifuging 1 ml of cells at a given optical density in a 1.5 ml centrifuge tube for 5 min at 4000 x g. The supernatant was removed, and the cells were suspended in 100 µl lysis buffer (1% v/v sodium dodecyl sulfate (SDS) in BSS, (above)). Complete lysis of the cells was observed microscopically. Total protein was measured by the BCA method (52) using bovine serum albumin (dissolved in lysis buffer) as a standard.

In Situ Location of the "*Stenoxybacter*" population

To evaluate the approximate location of the TAM community within the termite hindgut, four *R. flavipes* termites were degutted and the guts pooled in 100 µl of BSS on one-half of a 100 x 15 mm sterile Petri dish under a dissecting microscope. With a razor blade, the midgut was separated from the hindgut as close to the midgut-hindgut junction as possible. The guts were then transferred into a fresh 100 µl drop of BSS, sliced longitudinally, and washed by gentle agitation using sterile forceps. The 100 µl of BSS (now visibly dense with gut microbes) was transferred into a sterile, 10 ml conical centrifuge tube. The remaining sliced gut fragments were transferred to a separate 1.5 ml centrifuge tube containing 0.5 ml of BSS and vigorously agitated for 60 s with the aid of a Vortex mixing device. Gut fragments were allowed to settle, the supernatant was removed and added to the previous 10 ml conical tube. This portion of the microbiota was referred to as the "fluid fraction". The gut fragments were transferred, using sterile forceps, to a 1 ml Bead Tube (Mo-Bio Ultraclean Fecal DNA isolation kit, Mo Bio Laboratories, Carlsbad, CA) containing 500 µl of Bead Solution for DNA extraction. These gut fragments constituted the "wall fraction." This process was repeated for a total of 50 guts. The fluid fraction was centrifuged at 4000 x g for 10 minutes to pellet cells, the supernatant was removed, the pellet suspended in 500 µl of Bead Solution, and transferred to a dry 1 ml Bead Tube for DNA extraction.

DNA was obtained from the fluid and wall fractions using a Mo-Bio Ultraclean Fecal DNA Isolation Kit after bead homogenization as described above. "*Stenoxybacter*" or spirochete-specific 16S rRNA genes were PCR amplified from each fraction using primers TAM203f or 63f (5'-CAT GTC GAC GTY TTA AGC ATG CAA GT-3'; domain *Spirochaetes* (32)) as described above. Each reaction contained one gut-equivalent termite DNA made up to 50 ng with calf thymus DNA. After PCR incubation, five-microliter samples of each reaction mixture was analyzed by electrophoresis on 1.0% agarose-0.5x Tris-borate-EDTA gel stained with 1x Gelstar nucleic acid stain. The fluorescent bands of the PCR products were digitally captured as described above. The amount of PCR product in each band was quantified using the Kodak 1D electrophoresis analysis software by dividing the total, background-subtracted intensity of each band by the total band area (in pixels).

Enzyme assays.

"*S. acetivorans*" strain TAM-DN1 cells were grown in 100 ml BYA medium under a hypoxic atmosphere (2% O_2, 5% CO_2, 93% N_2) shaking at 250 rpm in a 500 ml media bottle with an 18 mm anaerobe tube attached as described in Chapter 2. When the culture had reached mid-log phase (during which growth was known to be acetate-dependent and which represented approximately 5 x 10^8 cells/ml) the cells were centrifuged at 10,000 x g for 10 min at 4°C, washed with 20 ml sonication buffer (10 mM EDTA, 50 mM Tris-HCl, pH 7.0), re-centrifuged and suspended in 10 ml of the same buffer. Cells were disrupted in

an ice water bath by sonication 3 x 30 s each with a Branson Model 450 sonifier (power setting of 5, 50% duty cycle) equipped with a ½" threaded-body step horn with flat tip. To remove undisrupted cells and debris, the sonicate was centrifuged at 12,000 x g for 60 min at 4°C. The resulting supernatant liquid was considered to be the crude cell extract and was distributed into a Slide-A-Lyzer dialysis cassette (3 ml to 12 ml capacity, 3500 molecular weight cutoff ; Pierce, Rockford, IL) and dialyzed for 12 hours in two liters dialysis buffer (50 mM Tris-HCl, pH 7.0). The dialyzed crude extract was removed from the cassette and used immediately for assays of enzyme activities.

Acetyl-CoA synthetase (AMP-forming, EC) was assayed by two different methods: (i) measurement of acetate- and CoA-dependent formation of AMP from ATP according to Oberlies *et al.* (38) and (ii) measurement of acetate- and CoA-dependent formation of inorganic pyrophosphate from ATP, according to the protocol supplied with the kit for enzymatic detection of pyrophosphate (Sigma-Aldrich, St. Louis, MO). Pyrophosphate-acetate phosphotransferase was assayed according to the same method, except that the rate of acetate-dependent disappearance of PPi was followed. Acetyl-CoA synthetase (ADP-forming) was measured by determining the rate of acetate and CoA-dependent ADP formation from ATP to the oxidation of NADH in the presence of phosphoenolpyruvate, pyruvate kinase and lactate dehydrogenase according to (48). Acetate kinase activity was determined by using the hydroxylamine method of Jones and Lipmann (24) as modified by Brown (10). Phosphotransacetylase was determined according to the method of Whiteley (59). Malate synthase

activity was assayed according to the method of Carpenter and Merkler (13). Isocitrate lyase was assayed according to the same protocol as malate synthase, substituting isocitrate for glyoxylate. Protein content of cell extracts was measured by the BCA assay (52) with bovine serum albumin as a standard. All absorbance measurements were made using a Perkin-Elmer Lambda 14 UV/VIS spectrophotometer and Perkin-Elmer UVWinLab software.

PCR primer design

(i) Broad specificity primers.

Deduced amino acid sequences for genes encoding known acetate kinase (*ack*), phosphotransacetylase (*pta*), acetyl-CoA synthetase (*acs*), isocitrate lyase (*aceA*), malate synthase A (*aceB*), malate synthase G (*glcB*), and the O_2-reducing subunit of the cbb_3-type cytochrome oxidase (*ccoN*) were obtained from completed, annotated microbial genomes through the Comprehensive Microbial Resource at The Institute for Genomic Research (TIGR, http://cmr.tigr.org/tigr-scripts/CMR/CmrHomePage.cgi) based on a gene name search. Additional sequences were obtained though Genbank (3). The amino acid sequences for each enzyme of interest were aligned using ClustalW, and the alignments were uploaded into the Blocks Multiple Alignment Processor of the CODEHOP online primer design tool (47). The blocks were then imported into the CODEHOP program. For all primers, the default search criteria were used, except that *Ralstonia eutropha* replaced *Homo sapiens* as a model for codon bias. Suggested primers from the CODEHOP output were selected with an effort to

obtain a maximum distance between the forward and reverse primers in order to allow amplification of as much genetic information as possible (Table 3.1).

(ii) "*S. acetivorans*"-specific primers.

The ClustalW protein alignment tool inside the ARB software package (http://www.arb-home.de/) (33) was used to align the deduced amino acid sequences of the functional genes from known organisms with those amplified by PCR from the "*S. acetivorans*" strains using the broad specificity primers. Visual inspection of the "*S. acetivorans*" strain sequences typically revealed areas of conservation specific to the "*S. acetivorans*" clade. Additional primer pairs were then designed to target these specific regions (Table 3.1). The sequence specificity of candidate primers was checked by searching the ARB database and performing a BLAST search.

PCR

The optimal annealing temperature for each primer pair was determined by temperature gradient PCR with DNA from "*S. acetivorans*" TAM-DN1 or *E. coli* DNA. The 25 µl reaction mixture contained: 25 – 100 ng DNA template, 1x reaction buffer (Invitrogen, Carlsbad, CA), 1.5 mM $MgCl_2$, 0.25 mM each deoxynucleoside triphosphate, 0.2 µM each primer, and 0.625 U Taq polymerase (Invitrogen). PCR mixtures were incubated in a model PTC-200 DNA Engine gradient thermal cycler (MJ Research, Watertown, MA) as follows: (i) 3 min at 95°C; (ii) 30 cycles of 45 s at 95°C, 45 s at 50-62°C, 45 s at 72°C, and (iii) 5 min at 72°C.The PCR products were then visualized on a 0.5x TBE, 1% agarose gel

Table 3.1. Gene-targeted broad-range and "*Stenoxybacter acetivorans*"-specific PCR primers used in this study.

Primer	Primer Sequence[a] 5'-3'	Amplicon Size[b] (bp)	Targeted Gene	Annealing Temperature[c] (°C)
Broad Specificity:				
Ack312F	TCC CGC TGG CCC CNY YNC AYA AYC	657	Acetate Kinase EC 2.7.2.1	56
Ack969R	GAG TTC TCG CCG ATG CCN SCN GTR AA			
Pta1138F	CCG ACA AGC GCA TCG TGY TNC CNG ARG G	848	Phosphotrans-acetylase EC 2.3.1.8	56
Pta1986R	GCG CAT GCC CTG CAR CAT NGG NCC			
Acs840F	GAC CCG CTG TTC ATC CTG TAY CAN WSN GG	735	Acetyl-CoA Synthetase EC 6.2.1.1	52
Acs1575R	TGG CCG GAC ACG TTG AND ACR TCR TC			
AceA328F	TGG CCG GCC ACA TGT AYC CNG AYC A	728	Isocitrate Lyase EC 4.1.3.1	56
AceA1056R	TGT GGA AGC CGG CCA GNG TRA TRA AC TG			
AceBGenF	CGG CCT GAA TTG CGG NVG NTG GGA	315	Malate Synthase A EC 2.3.3.9	56
AceBGenR	CGG AAC CAG ACC CGG ATG NGC NRY CCA			
GlcBGenF	CGC GTT GGG GCT CCY TNT AYG AYG C	1155	Malate Synthase G[d]	56
GlcBGenR	CCA TCA GAT CCG GCA TCG SCC ACA TNC C			

Table 3.1. (cont.)

CcoNGenF	CTC CAA GCG CAC GGG NCC NGA YYT	2000	N Subunit, cbb_3-type Cytochrome Oxidase	53
CcoNGenR	CGT GCA CGT GGC CGA YNR TCC ART C			
***"Stenoxybacter acetivorans"*-specific:**				
AckTAMF	CAA CAC SCT TTT CCT GAA C	600	Acetate Kinase	56
AckTAMR	CCA CAY GCC ACA GTG ATG GC			
PtaTAMF	GAC ACC ATT CGC ASC AAT T	450	Phosphotrans-acetylase	58
PtaTAMR	CAA TTC CWC TSC TTT GGC TAC			
GlcBTAMF	GCC CGA CGG CAA AAC CAC ATT CAA ATT GC	500[e]	Malate Synthase G	56
CcoNTAMF	GTG CCT TCT ACC TTC ACC	700	N Subunit cbb_3-type Cytochrome Oxidase	58
CcoNTAMR	GCG ATA GAA AAT CAT ACT TTG			

[a] Symbols for incompletely specified bases follow IUPAC / IUBMB recommendations.

[b] Estimated amplicon size based on enzyme sequence for *Escherichia coli*.

[c] Annealing temperatures optimized for conditions in this study with *E. coli* genomic DNA for general bacterial primers and TAM-DN1 genomic DNA for "*Stenoxybacter*"-specific primers.

[d] An EC number has not yet been established for malate synthase G.

[e] When paired with GlcBGenR.

with ethidium bromide stain. Once the optimal annealing temperature was determined, the amplicon was cloned by using the TA Cloning Kit (Invitrogen) and transformed into TOP10 *E. coli*. Several clones were selected for sequencing.

Sequencing and Phylogenetic analysis.

Prior to sequencing, unreacted dNTP's and primers from the PCR reactions were removed using ExoSap-IT (USB). The reaction mixture contained 1.3 µl PCR-amplified DNA, 0.5 µl ExoSAP-IT enzyme mixture, and 3.2 µl sterile water. The reaction was incubated according to the ExoSAP-IT protocol.

The partial gene sequence was determined with Applied Biosystems cycle sequencing technology (Applied Biosystems), using the broad specificity or "*S. acetivorans*"-specific forward primer corresponding to the gene of interest (Table 3.1). Sequences were quality checked by hand, and the initial identification of each sequence was determined by using the BLASTx search tool in the Genbank protein database. Each nucleotide sequence was converted to the deduced amino acid sequence by using Transeq (www.ebi.ac.uk/emboss/transeq).

For phylogenetic analyses, the sequences were aligned against a database populated with protein sequences from TIGR as well as Genbank by using the ClustalW protein alignment algorithm within the ARB software package (33). Ambiguities in the sequence alignments were corrected manually. Only alignment positions present in every sequence were used in subsequent

phylogenetic analyses. Phylogenetic trees were constructed in ARB using the maximum likelihood routine for protein sequences.

RNA extraction and purification

All RNA work was done with reagents, pipette tips, and tools that were RNase-free. Approx. 100 *R. flavipes* termites were degutted and the guts immediately placed in a 1.5 ml centrifuge tube on dry ice. One ml of RNA protect reagent was then added, and the contents were immediately transferred into a sterile glass tissue homogenizer followed by thorough homogenization for 3 min. RNA was purified from the homogenate according to the protocol for bacteria in the RNeasy RNA purification kit (Qiagen, Valencia, CA). The RNA was quantified by optical density at 260 nm measured using a Perkin-Elmer Lambda 14 UV/VIS spectrophotometer. After quantification, the RNA was treated with DNase I as follows: to a 0.5 ml RNase-free microcentrifuge tube, 1 µg RNA, 1 U DNase I (amplification grade; Invitrogen), and RNase-free water was added for a final volume of 10 µl. The reaction was incubated at room temperature for 15 minutes, after which the DNase was inactivated by the addition of 1 µl of 25 mM EDTA and incubation for 10 min at 65°C. The DNA-free RNA was stored at -80°C until used.

Reverse Transcriptase PCR

First-strand cDNA synthesis was done following the protocol described for Superscript III reverse transcriptase (Invitrogen). To a nuclease-free 0.5 ml

centrifuge tube, 2 pmol of gene-specific reverse primer, 0.5-1 µg *R. flavipes* gut RNA, 1 µl 10 mM dNTP mix (Roche, Indianapolis, IN), and sterile, RNAse-free water was added for a total volume of 13 µl. The mixture was heated to 65°C for 5 minutes, then placed on ice for >1 min. After brief centrifugation, 4 µl buffer, 1 µl 0.1 M DTT, and 200 U Superscript III reverse transcriptase (Invitrogen) were added. The reaction was then incubated at 55°C for 1-2 hours, heated to 70°C for 15 minutes, then stored at -20°C until PCR amplification.

PCR amplification of the first-strand cDNA was done according to the PCR methods described above, using 2-4 µl of the first-strand cDNA synthesis reaction mixture, the annealing temperature of the specific primer set (Table 3.1), and extending the total number of PCR cycles to 35.

Oxygen Uptake Measurements

"Stenoxybacter acetivorans" strain TAM-DN1 and *Citrobacter* sp. RFC-10 were grown on BYA media, or BYA media modified to contain 0.75 mM NH_4 (a concentration known to limit growth to approximately half the cell yield otherwise attained with excess NH_4) and 20 mM sodium acetate. These modifications were made so that *in vitro* growth of termite gut isolates would more closely mimic the low-nitrogen, acetate-rich environment of the termite gut. The cells were harvested upon entry into stationary phase by centrifugation (10,000 x g, 10 min, 4°C), washed 2x in insect Ringer's solution (per liter; 7.5 g NaCl, 0.35 g KCl, 0.21 g CaCl, pH 7.0) and resuspended at 10x initial concentration in the same buffer. Oxygen uptake rates were measured under rapid stirring in a 2-ml glass HPLC

vial fitted with a screw-cap having a central hole through which a narrow Clark-type oxygen electrode (Diamond General, Ann Arbor, MI) was placed and sealed with dental wax. Prior to use, the oxygen electrode was calibrated by immersion in insect Ringer's solution that had been vigorously bubbled with air for >15 min (100% air saturation), or that had been degassed under vacuum and bubbled with 100% N_2 (0% air saturation). The cell suspensions were vigorously aerated by shaking, added to the O_2 uptake chamber and oxygen consumption was measured before and after the addition of substrate. Sodium acetate, sodium succinate, sodium lactate or D-glucose were the test substrates and were added individually to a final concentration of 10 mM.

For measurements of oxygen uptake by termite guts, guts were removed from *R. flavipes* worker termites that had been maintained in the laboratory for approximately 8 months. Such termites were either untreated (controls) or were fed (for eleven days prior to gut removal) on diets intended to eliminate major components of the gut microbiota (bacteria or cellulolytic protozoa; below). Extracted guts were immediately placed in 2 ml insect Ringer's solution within the O_2 uptake chamber, as described above. For each experiment, 10-20 guts were used. The guts were first allowed to settle, then the overlying buffer was aspirated by using an 18-guage needle attached to a 5 ml syringe. The aspirated buffer was then immediately replaced with fresh, fully aerated buffer, and O_2 uptake was measured as described above, with and without the addition of 10 mM (final concentration) sodium acetate.

Elimination of Gut Microbes

To determine the relative contribution of major components of the gut microbiota (cellulolytic protozoa and bacteria) to oxygen consumption by termite guts, either or both groups of microbes were largely eliminated from guts by pre-feeding the termites on agarose food cubes containing starch (known to eliminate cellulolytic protozoa (58)), a mixture of antibacterial drugs, or a combination of both. Control termites for these experiments were fed for the same period of time on agarose food cubes containing microgranular CC41 cellulose powder (Whatman, Brentford, UK). To prepare such food cubes, a solution of 1% w/v agarose in insect Ringer's solution (above) was heated to boiling, and 10% w/v cellulose or 5% w/v cornstarch was added under rapid stirring. When desired, the antibiotics ampicillin, cefoperazone and vancomycin (800 µg/ml each, final conc.) were incorporated after the agarose had cooled but before it solidified. The agarose suspensions were allowed to solidify in trays, and the final thickness of the suspension was approximately one-half inch. Once solidified, the agarose was cut into two-inch squares, and each square was placed within a 100 x 15 mm sterile Petri dish to which 20-40 termites were added. The Petri dishes were placed within a humid chamber for 11 days, with the agarose food squares replaced every three days.

To determine the efficacy of such treatments in removing gut microbes, viable cell counts were of bacteria were made every second day of treatment, as were direct microscopic counts of protozoa. For viable cell counts, twelve termites from each treatment were degutted and the guts were placed into 2 ml

of 1x basal salts solution (53) buffered with 10 mM MOPS (pH 7.0). The guts were thoroughly homogenized with a sterile glass tissue homogenizer, and the homogenate was serially diluted in 10-fold increments in the same buffer. Samples from each dilution were plated onto a complex medium containing (per liter): KH_2PO_4, 0.2 g; NH_4Cl, 0.25 g; KCl, 0.5 g; $CaCl_2 \cdot 2H_2O$, 0.15 g; NaCl, 1.0 g; $MgCl_2 \cdot 6\ H_2O$, 0.62 g; Na_2SO_4, 2.84 g; brain heart infusion medium (BD Franklin Lakes, NJ) 3.7 g; casamino acids 1.0 g; and cellobiose, D-glucose, D-xylose, maltose, sodium pyruvate, sodium lactate, and sodium acetate, 1 mM each. The medium was buffered by inclusion of morpholinopropanesulfonic acid (MOPS; 10 mM final conc.) and adjusted to pH 7.0 prior to being autoclaved. Plates were incubated at 23°C under hypoxia (93% N_2, 5% CO_2, 2% O_2) for 15 days before colonies were counted.

For quantification of protozoa, four termites were degutted, and the guts placed within 100 µl insect Ringer's solution on a sterile Petri dish under a dissecting microscope. Each gut was sliced longitudinally with a razor blade, and the gut contents were washed out of the gut and into the Ringer's solution briefly by agitation while being held with sterile forceps. The 100 µl suspension containing gut fluid and microorganisms, but not sliced guts, was added to a sterile, 1.5 ml centrifuge tube and briefly centrifuged to pellet the cells. The pellet was resuspended in 10% neutral buffered formalin (per liter: 37% formalin, 100 ml; Na_2HPO_4, 6.5 g; NaH_2PO_4, 4.0 g; pH 7.0) and incubated at 4°C overnight. The fixed cells were again collected by centrifugation and then resuspended in a mixture of equal volumes of insect Ringer's and absolute ethanol and placed at -

20°C until counted. For counting, 10 µl of the cell suspension was transferred onto a well of an 8-well Teflon coated slide (each well having an area of 28.26 mm), allowed to dry, and washed briefly in water. The cells were stained with freshly-prepared (5-[4,6-dichlorotriazin-2-yl] aminofluorescein (DTAF, 0.2 mg/ml; prepared in a buffer containing 0.05 M Na_2HPO_4 and 0.15 M NaCl; pH 9.0) for 30 minutes followed by 3 washes in the same buffer for 30 minutes each. Slides were air-dried, coverslips were mounted with Entellan (Merck) preservative, and cells were visualized at 100X magnification by UV epifluorescence with a Zeiss Axioskop equipped with a DTAF-specific filter set. Protozoa in at least 20 fields of view were counted.

Results

In situ location of "*S. acetivorans*"

Results of the PCR-based method for assessing the *in situ* location of the TAM organisms suggested that they are more closely associated with the epithelial wall as opposed to the gut fluid (Figure 3.2a). DNA extracted from the epithelium and adherent microorganisms (wall fraction) amplified with TAM-specific 16S rDNA primers required 24 amplification cycles before a quantifiable product could be detected. Twenty-five cycles were required before a product could be detected using DNA from non- or loosely-adherent wall-associated microbes ("fluid fraction") with the same primer set, suggesting less TAM-specific 16S rRNA genes were present in the "fluid fraction" of the gut. This is supported

by the fact that a significantly greater amount of PCR product was obtained from the wall fraction at least through 27 PCR cycles. Clone libraries prepared from PCR products resulting from amplification with wall fraction DNA confirmed the reaction was TAM-specific (data not shown).

To test the validity of this method, the TAM-specific primers were replaced by primers specific for the 16S rRNA gene of spirochetes, bacteria known to be present primarily in the gut fluid rather than attached to the epithelium (8). As expected, a quantifiable PCR product was obtained after 13 cycles from fluid fraction DNA, but not wall fraction DNA (Figure 3.2b). A greater amount of PCR product was obtained from the fluid fraction than the wall fraction at least through 17 PCR cycles.

Enzymes and Genes Relevant to Acetate Oxidation *In Vitro*

In an effort to identify enzymes specific to acetate oxidation by "*S. acetivorans*", crude extracts of acetate-grown cells were examined for enzyme activities associated with the activation of acetate to acetyl-CoA. The results revealed the presence of acetate kinase and phosphotransacetylase, but not acetyl-CoA synthetase (AMP-forming) or the less widely distributed ADP-forming synthetase, nor PPi-acetate phosphotransferase (Table 3.2). However, AMP-forming acetyl-CoA synthetase activity could be readily detected in reaction mixtures to which authentic acetyl-CoA synthetase (purified from *Saccharomyces cerevisiae*, Sigma) was added. Acetate kinase activity was dependent on the presence of both acetate and ATP in the reaction mixture; and

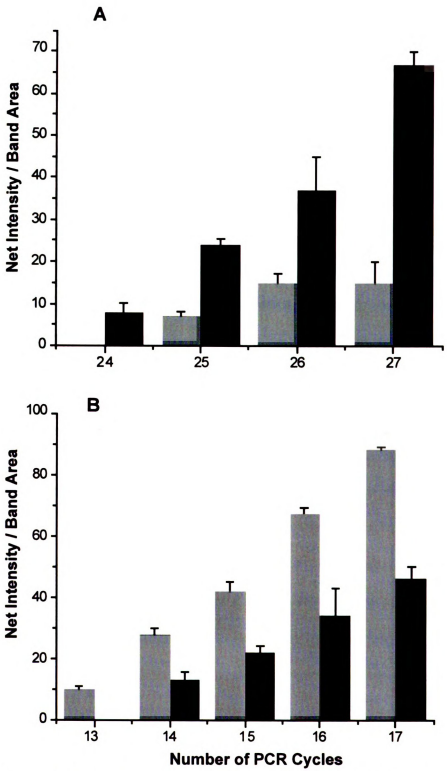

Figure 3.2. *In situ* association of "*S. acetivorans*" with the gut epithelial wall. Quantification of product intensity after PCR of termite gut fluid (gray bars) or sliced and washed gut epithelium (black bars) with "*Stenoxybacter*"- specific (A) or spirochete-specific (B) 16S rDNA primers. Error bars represent standard deviation (n=3).

Table 3.2. Enzymes and genes relevant to acetate oxidation and O_2-consumption in acetate-grown cells of "*S. acetivorans*".

Enzyme	Specific Activity[a]	Gene Amplified
Acetate Activation		
Acetate Kinase	3.7 ± 0.7	*ack*
Phosphotransacetylase	1.1 ± 0.5	*pta*
Acetyl-coA Synthetase (AMP-forming)	0.0	none
Acetyl-coA Synthetase (ADP-forming)	0.0	n.d.
PPi-Acetate Phosphotransferase	0.0	n.d.
Glyoxylate Cycle		
Isocitrate Lyase	0.0	none
Malate Synthase	0.5 ± 0.3	*glcB*
Oxygen Reduction		
cbb_3-type Cytochrome Oxidase	n.d.	*ccoN*

[a] Specific activities are expressed as micromoles product formed per minute per milligram protein (mean ± sd)(n=3). n.d., not determined.

phosphotransacetylase activity was dependent on the presence of both acetyl-phosphate and coenzyme A. Interestingly, robust acetate kinase activity was also observed in crude extracts of succinate-grown cells (9.4 ± 1.9 U·mg prot.$^{-1}$), suggesting that this enzyme may be constitutively synthesized in "*S. acetivorans*".

Not surprisingly, PCR amplification of "*S. acetivorans*" genomic DNA with broad specificity primers readily revealed the presence of genes encoding acetate kinase (*ack*) and phosphotransacetylase (*pta*), but not acetyl-CoA synthetase (Table 3.2). However, the *acs* gene was readily amplified by using the same primer set and *Escherichia coli* genomic DNA as a control. Upon translation, the deduced amino acid sequence of the *ack* PCR product was 69% identical to its closest relative, an acetate kinase from *Neisseria meningitidis* Z2491 (Genbank accession number CAB84946) (Figure 3.3). Moreover, the ACK sequences from 11 different strains of "*S. acetivorans*" were 99.8% identical to each other, (based on 178 amino acids).

The same broadly specific acetate kinase primers were also used to PCR amplify *R. flavipes* gut homogenate DNA, and a clone library was constructed. Phylogenetic analysis of the deduced amino acid sequence from 44 clones revealed that a majority formed a distinct cluster (*R. flavipes* ACK cluster 1, 26 clones) whose closest relative was an acetate kinase from *Clostridium thermocellum* (Figure 3.3). Other clones grouped together with an acetate kinase from *Treponema denticola* (*R. flavipes* ACK clusters 2.1 and 2.2; 7 and 3 clones,

respectively). Less abundant were clones related to *Treponema pallidum*, *Bacteroides thetaiotaomicron*, and *Mycobacterium vanbaalenii*.

The ~800 nucleotide amplicon from "*S. acetivorans*" TAM-DN1 DNA obtained by using the broad specificity phosphotransacetylase primers bore 75% deduced amino acid identity to its closest known relative, a phosphotransacetylase from *Neisseria meningitidis* MC58 (AAF41056) (Figure 3.4). The deduced amino acid sequences of phosphotransacetylases from different TAM strains were 93% identical (based on 201 amino acids).

Bacteria that oxidize acetate aerobically usually employ the TCA cycle to do so, and such bacteria typically possess a glyoxylate bypass for replenishment of TCA cycle intermediates drawn off for biosynthesis. Accordingly, and as potential targets for inferring *in situ* activity, two key enzyme activities of the glyoxylate bypass were sought in crude extracts of TAM-DN1, i.e. isocitrate lyase and malate synthase A (Fig. 3.1). Although glyoxylate- and acetyl-CoA-dependent putative malate synthase A activity was detected, isocitrate lyase activity was not (Table 3.2). Moreover, despite multiple attempts to PCR amplify the genes encoding these two enzymes, including a re-design of primers and application of less stringent amplification conditions, no amplification products resulted from PCR with primers targeted to isocitrate lyase or malate synthase A, although amplification of these genes always occurred with *E. coli* control DNA. However, TAM-DN1 DNA amplified with malate synthase G primers resulted in a ~1100 nucleotide product bearing 64% deduced amino acid sequence identity to malate synthase G from *Pseudomonas syringeae* pv. tomato st. DC3000

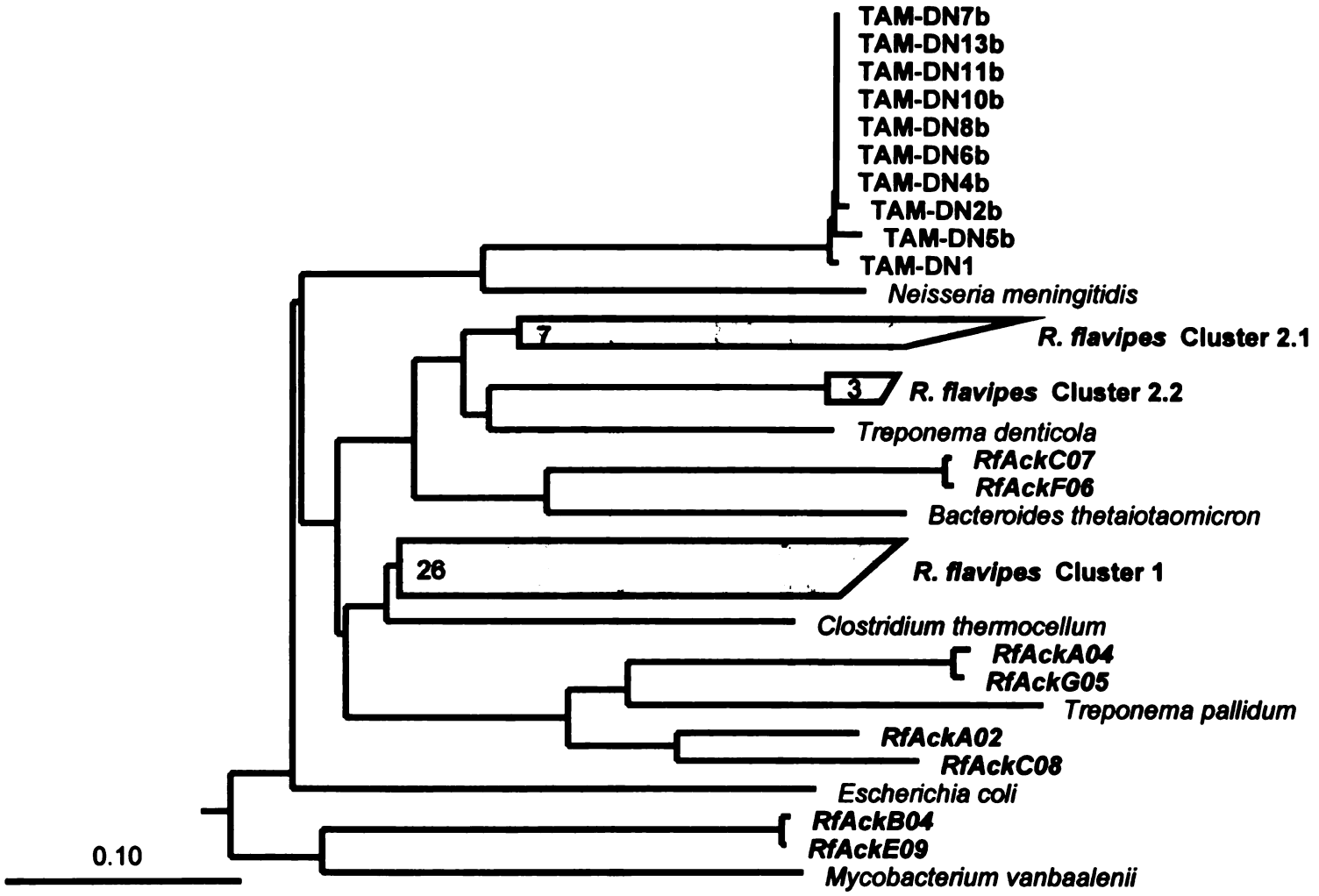

Figure 3.3. Maxiumum likelihood-based phylogenetic analysis of the deduced amino acid sequence(178 positions) of acetate kinase from selected "*S. acetivorans*" isolates (boldface) and *R. flavipes* guts (boldface+italics). Numbers within *R. flavipes* clusters represent the number of sequences within that cluster. The acetate kinase from *Neurospora crassa* was used as an outgroup. Scale bar represents 0.1 change per amino acid.

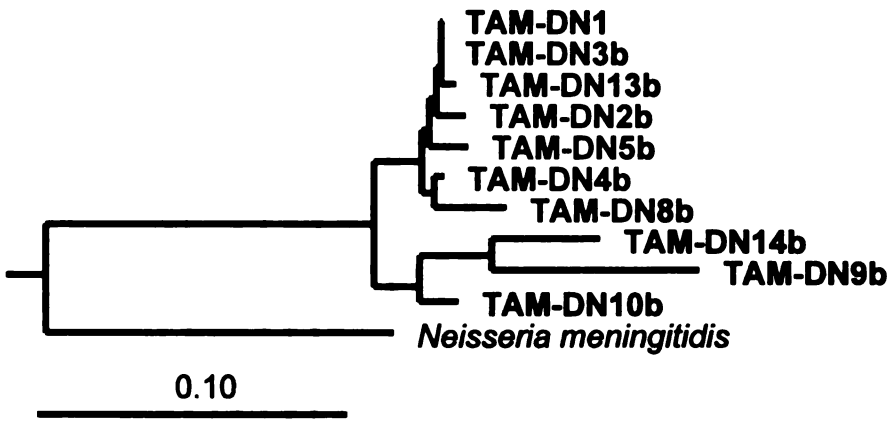

Figure 3.4. Maximum likelihood-based phylogenetic analysis of the deduced amino acid sequence (201 positions) of phosphotransacetylase from selected "*S. acetivorans*" isolates (boldface). *Escherichia coli* PTA is used as an outgroup (not shown). Scale bar represents 0.1 change per amino acid.

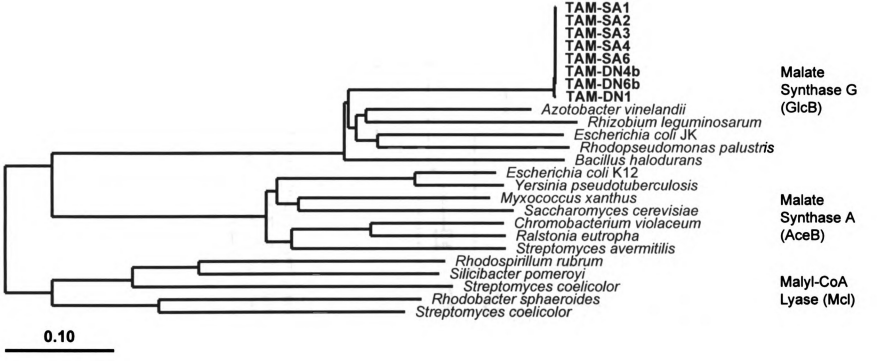

Figure 3.5. Maxiumum likelihood-based phylogenetic analysis of the evolutionarily-related malate synthase A, malate synthase G and malyl-CoA lyase proteins. The deduced amino acid sequences (170 positions) from selected "*S. acetivorans*" isolates (boldface) group within the malate synthase G cluster. The malyl-CoA lyase cluster is used as an outgroup. Scale bar represents 0.1 change per amino acid.

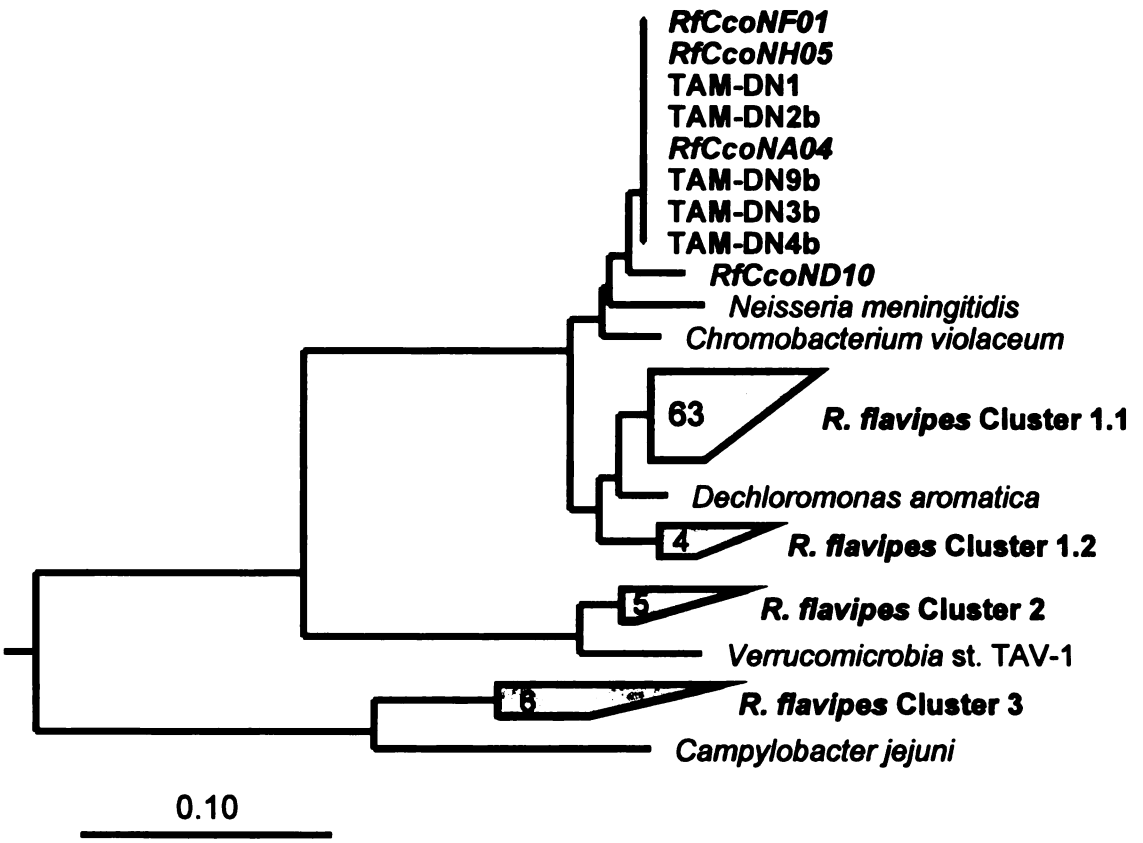

Figure 3.6. Maximum likelihood-based phylogenetic analysis of the deduced amino acid sequence (136 positions) of the O_2-reducing (*ccoN*) subunit of the cbb_3-type cytochrome oxidase from selected "*S. acetivorans*" isolates (boldface) and *R. flavipes* guts (bold+italics). The *ccoN* subunit from *Magnetospirillum magnetotacticum* is used as an outgroup (not shown). Scale bar represents 0.1 change per amino acid.

(AAO54024) (Figure 3.5). Phylogenetic analysis revealed that the deduced amino acid sequences of malate synthase G PCR products from 8 different "*S. acetivorans*" strains were 99.5% identical and grouped specifically with malate synthase G enzymes, not the phylogenetically related malate synthase A or malyl-CoA lyase enzymes (Figure 3.5) (35). In light of the apparent absence of isocitrate lyase, the role of malate synthase G in "*S. acetivorans*" remains to be determined.

Inasmuch as "*S. acetivorans*" strains were shown to be microaerophilic (Chapter 2), it was hypothesized that the cells possessed a high-affinity cbb_3-type oxidase as their terminal respiratory enzyme. To explore this possibility, broad specificity primers were designed that targeted *ccoN*, the gene encoding the O_2-reducing subunit of the cbb_3 cytochrome oxidase (Table 3.1). PCR amplification of "*S. acetivorans*" TAM-DN1 genomic DNA yielded a 2 kb product whose deduced amino acid sequence was 96% identical (based on 125 amino acids) to a cbb_3-type cytochrome oxidase from *Chromobacterium violaceum* ATCC 12472 (NP_900844). Sequencing and phylogenetic analysis revealed the *ccoN* subunit to be highly conserved among the TAM strains (99.8% sequence identity).

The same broad specificity *ccoN* primers were also used to construct a clone library from *R. flavipes* gut homogenate DNA. Phylogenetic analysis of 82 clones from this library revealed that a majority of the clones fell within distinct groups (*R. flavipes* clusters 1.1 and 1.2; 63 and 4 clones, respectively), whose closest relative was the *ccoN* from *Dechloromonas aromatica* (Figure 3.6). Other

clones grouped with *ccoN* from *Verrucomicrobia* isolate TAV-1, also cultivated from termite guts (*R. flavipes* cluster 2, 5 clones) (See Appendix and (53)). Six clones (*R. flavipes cluster* 3) formed a cluster related to *Campylobacter jejuni*, and four grouped together with the *ccoN* genes from the TAM isolates.

Genes Expressed by "*S. acetivorans*" *In Situ*

Owing to the high degree of within-species similarity of "*S. acetivorans*" genes presumably important to acetate-supported aerobic respiration (i.e. *ack*, *pta* and *ccoN*, above), coupled with their substantial sequence divergence from homologues present in other known organisms or PCR amplified from termite gut homogenates, an effort was made to design "*S. acetivorans*"-specific primers to examine (by reverse transcriptase PCR; RT-PCR) whether these same genes were expressed *in vitro* and *in situ*. To do this, "*S. acetivorans*"-specific primer pairs for these genes were designed (Table 3.1) and used in conventional PCR amplification with purified *R. flavipes* gut homogenate DNA as template. Upon cloning and sequencing of the PCR products, all were identical, or nearly so, to *ack*, *pta*, and *ccoN* genes from the TAM isolates (data not shown).

When these same primers were used in individual RT-PCR reactions with *R. flavipes* gut homogenate RNA as a template, each reaction usually yielded a single PCR product of the same size as the analogous product obtained by using, as a template, DNA from acetate-grown TAM-DN1 (Figure 3.7). Importantly, no products were obtained if reverse transcriptase was omitted from the RT-PCR reactions, indicating that any products formed in the complete

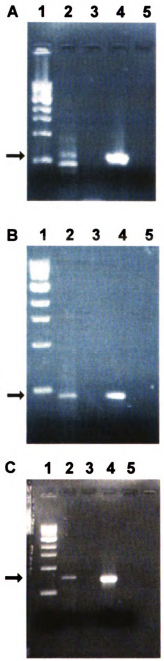

Figure 3.7. Gelstar-stained agarose gel electrophoresis of RT-PCR products for (A) acetate kinase (*ack*); (B) phosphotransacetyalse (*pta*); and (C) the O_2-binding subunit of the cbb_3 terminal oxidase (*ccoN*) from *R. flavipes* gut homogenate RNA with "*S. acetivorans*"-specific, gene-targeted primers. Lane 1, 1 kb DNA ladder. Lane 2, RT-PCR product using *R. flavipes* gut RNA as template. Lane 3, RT-PCR with *R. flavipes* gut RNA without the addition of reverse transcriptase enzyme. Lane 4, RT-PCR with TAM-DN1 DNA as template. Lane 5, RT-PCR without the addition of template.

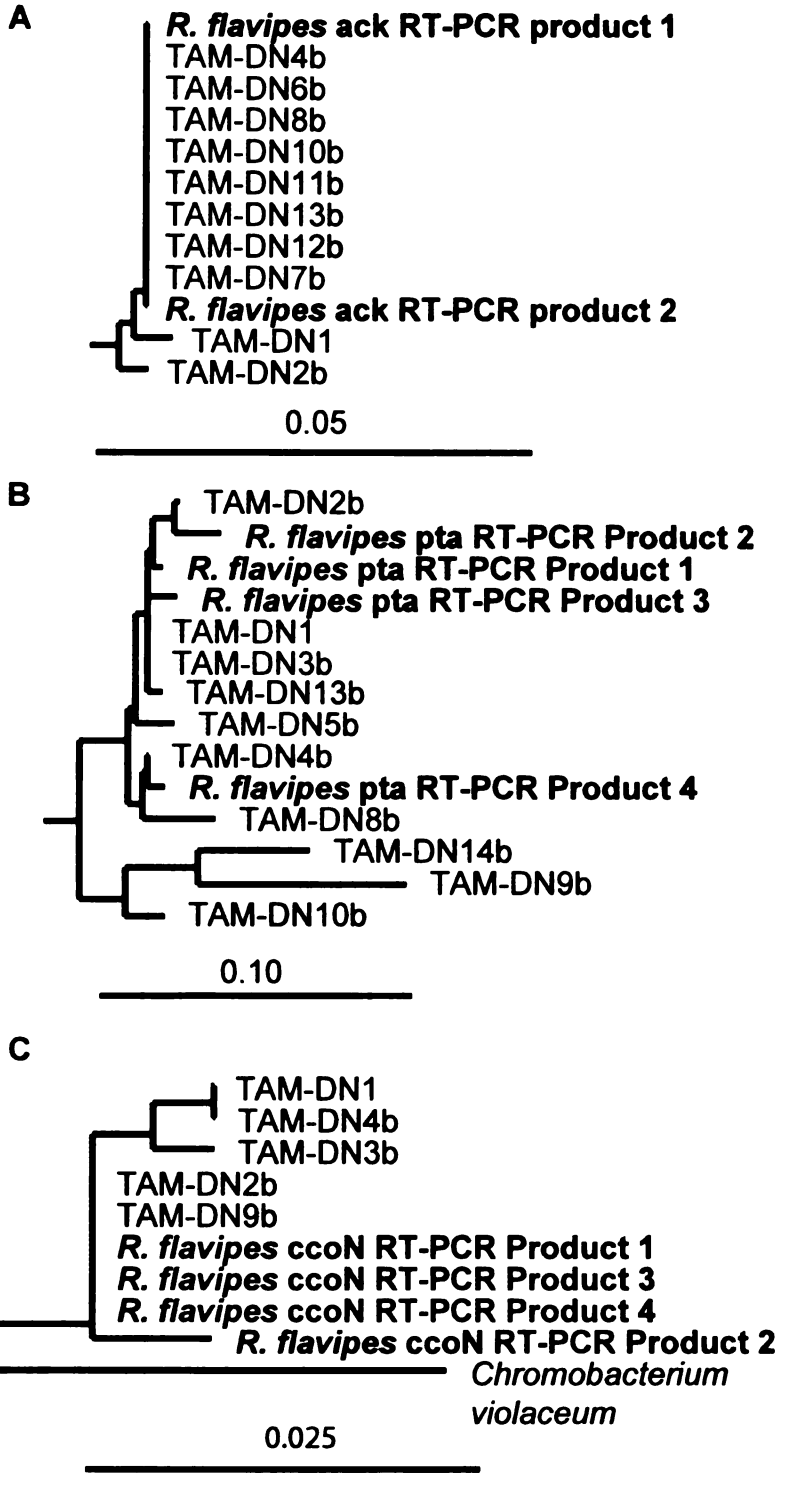

Figure 3.8. Maximum likelihood-based phylogenetic analysis of the deduced amino acid sequences of RT-PCR products depicted in Fig. 3.7. All RT-PCR products clustered specifically with the respective genes from "*S. acetivorans*" isolates. (A) acetate kinase (170 positions); (B) phosphotransacetylase (182 positions); (C) *ccoN* subunit of cbb_3 oxidase (124 positions). *Neisseria meningitidis* was used as an outgroup for all phylogenetic analyses (not shown). Scale bars represent 0.1, 0.05 or 0.025 changes per amino acid.

reaction mixtures were derived from RNA (presumably mRNA) and not from DNA contamination of the template. Upon cloning and sequencing of the correctly-sized RT-PCR products, phylogenetic analysis revealed that they grouped specifically with the *ack*, *pta* and *ccoN* gene products from "*S. acetivorans*" isolates (Figure 3.8). These results indicated that expression of *ack*, *pta* and *ccoN* is not only relevant to acetate-dependent growth of "*S. acetivorans*" *in vitro*, but to growth and/or survival *in situ* as well.

It was noticed that, in RT-PCR reactions with the *ack*-targeting primer pair, an additional product was produced that was slightly smaller (450 bp) than the anticipated *ack* fragment (600 bp)(Figure 3.7). However, cloning and sequencing of material from this band revealed that it was related to a gene encoding a hypothetical protein in *Drosophila melanogaster* and probably arose from non-specific amplification of RNA from termite gut tissue.

Oxygen Consumption by "*S. acetivorans*" and Other Components of the *R. flavipes* Gut

In an effort to estimate the potential contribution of "*S. acetivorans*" to O_2 consumption by guts of *R. flavipes*, per-cell rates of O_2 consumption were determined for "*S. acetivorans*" grown *in vitro* without, and with, NH_4 limitation. The latter condition was imagined to be a possible mimic of what these cells might be experiencing *in situ*, as the food source of *R. flavipes* (wood) is relatively low in N (ca. 0.05% w/v for sound wood). When grown on NH_4 limiting medium, the O_2 consumption rate of TAM-DN1 was 1.5 (± 0.4) x 10^{-5} pmol·min^{-}

^{1}·cell^{-1} (mean ± s.d.) in buffer supplemented with acetate and 1.0 (± 0.2) x 10^{-5} pmol·min^{-1}·cell^{-1} in succinate-supplemented buffer (Table 3.3). The O_2 consumption rates in medium with no NH_4 limitation were similar 9.9 (± 0.6) x 10^{-5} and 9.5 (± 0.4) x 10^{-5} pmol·min^{-1}·cell^{-1} in acetate- or succinate-supplemented buffer, respectively. Little or no oxygen consumption was observed when glucose or lactate was added to the buffer, or in buffer without added substrate. Per-cell rates of O_2 consumption by *Citrobacter* str. RFC-10, another abundant organism isolated with "*S. acetivorans*", were about half that of "*S. acetivorans*" on these same substrates (Table 3.3). However, *Citrobacter* RFC-10 also displayed lactate- and glucose-supported O_2 consumption.

O_2 consumption rates for intact extracted guts of *R. flavipes* in acetate-supplemented buffer were 1013 ± 141 pmol·min^{-1}·gut^{-1}. However, the oxygen consumption rates for guts incubated in buffer without acetate (969 ± 256 pmol·min^{-1}·gut^{-1}) were not significantly different than for guts incubated with acetate ($p=0.80$). These data suggested that respiration by intact guts was probably largely, if not entirely, supported by endogenous acetate, which is present in relatively high concentrations in *R. flavipes* hindgut fluid (ca. 80 mM).

To estimate the relative contribution of bacteria and protozoa to O_2 uptake by whole guts, termites were fed on artificial diets intended to eliminate all or most of these particular microbial communities from the gut, after which measurements were made of whole gut O_2 consumption (Table 3.4). Compared to the normal diet of wood, even a seemingly innocuous shift to a short-term diet

Table 3.3. Substrate-specific oxygen consumption rates of "*Stenoxbacter*" st. TAM-DN1, *Citrobacter* st. RFC-10, and whole *R. flavipes* guts.

Substrate[a]	"*Stenoxybacter*" st. TAM-DN1 (pmol/min/cell x 10^{-5})[b]	*Citrobacter* st. RFC-10 (pmol/min/cell x10^{-5})[b]	*R. flavipes* Whole Guts (pmol/min/gut)
None	0.0	0.0	969 ± 256
Acetate	1.5 ± 0.4	0.6 ± 0.2	1013 ± 141
Succinate	1.0 ± 0.2	0.5 ± 0.2	n.d.
Lactate	0.0	1.0 ± 0.3	n.d.
Glucose	0.0	0.8 ± 0.2	n.d.

[a] Final substrate concentration was 10 mM in fully aerated insect Ringers solution. n.d. not determined. All values represent mean ± standard deviation (n=3).

[b] Cultivated in nitrogen-limited medium that also contained an excess of acetate.

Table 3.4. Effect of diet on gut microbial communities and O_2 consumption rates of whole guts of *R. flavipes*.

Diet[a]	Cultivable bacteria/archaea (CFU/gut x 10^5)[b]	Protozoa (Cells/gut x 10^3)[c]	Whole-gut O_2 Consumption (pmol/min/gut)[d]
Wood (control)	4.5 ± 0.5	9.6 ± 1.5	1013 ± 141
Cellulose	3.2 ± 0.7	6.6 ± 0.3	740 ± 86
Cellulose + Ab	0.004 ± 0.002	3.3 ± 1.5	348 ± 65
Starch	4.0 ± 0.5	2.3 ± 0.3	436 ± 108
Starch + Ab	0.003 ± 0.001	0.7 ± 0.1	286 ± 92

[a] Termites were maintained on diet for 11 days. Ab, antibiotic cocktail [ampicillin, cephoperazone, vancomycin (800 µg/mL each)]. Values are the mean ± standard deviation of the mean.

[b] For each treatment, twelve guts were pooled, homogenized and plated onto carbon-supplemented 1/10 brain-heart infusion media and incubated in hypoxia (2% O_2, 98% N_2). Colonies were counted 14 days after plating (n=4).

[c] Determined by direct counts of DTAF-stained gut fluid (n=3).

[d] Buffer supplemented with 10 mM acetate, pH 7.0 (n=3).

of pure cellulose in agarose resulted in a moderate, but statistically significant drop in both bacterial viable cells ($p = 0.04$) counts and protozoan numbers ($p = 0.008$), as well as a drop in whole gut O_2 consumption ($p = 0.04$). However, incorporation of a triple-antibiotic cocktail (800 µg/ml each ampicillin, cefoperazone, vancomycin) into such a diet drastically reduced bacterial CFU by a factor of 10^3, but it also resulted in a 50% drop in total protozoan numbers. The O_2 consumption rate of whole guts extracted from such termites was a little less than half that of cohorts fed cellulose alone.

Termites fed on a diet of cornstarch retained relatively high numbers of bacterial CFU per gut, but lost about two-thirds of their protozoa compared to cellulose-fed controls. This was expected: starch is known to almost totally eliminate cellulolytic protozoa from termite guts (15, 58), probably because it is so readily hydrolyzed, even by salivary amylases present in the termite itself, that protozoa no longer have a selective advantage for retention in the gut. Moreover, it is a substrate on which wood-feeding termites can survive indefinitely. Nevertheless, the O_2 consumption rate of whole guts from starch-fed termites (436 ± 108 pmol·min^{-1}·gut^{-1}) was only about 60% that of the cellulose-fed controls ($p = 0.003$). Incorporation of antibiotics into the starch diet not surprisingly decreased the bacterial CFU drastically, and it also further reduced protozoan numbers and whole gut O_2 consumption rates to the lowest value seen in the entire experiment (286 ± 92 pmol·min^{-1}·gut^{-1}; Table 3.4).

Although facultative and strictly aerobic bacteria, including obligate microaerophiles like "*S. acetivorans*", would be expected to augment the

respiratory activity of the gut tissue itself and contribute to the O_2 uptake rates seen with extracted whole guts, the data presented in Table 3.4 suggest that protozoa, long thought to be strict anaerobes, also make a substantial contribution to O_2 consumption. This is discussed further below, as are cautionary considerations in interpreting results from feeding experiments in which major components of the gut microbiota are disrupted or eliminated.

Discussion

In situ location of "*S. acetivorans*"

The obligate microaerophilic phenotype of the "*S. acetivorans*" strains suggested that their *in situ* location was within the "hypoxic zone," which extends from the gut epithelial wall to approximately 150 – 200 μm inward (11). However, direct visualization of the cells within the termite hindgut by fluorescent *in situ* hybridization (FISH) (5, 55) with a fluorescently-labeled, "*S. acetivorans*"-specific 16S rRNA probe was not possible owing to the overpowering autofluorescence of the termite tissue. Attempts to (i) decrease the autofluorescence with various fixatives, blocking reagents, HCl and H_2O_2 washes (6, 55); and (ii) to amplify the TAM strain-specific signal by tyramide signal amplification (42) or hybridization with dual fluorescently labeled probes (6) was ultimately unsuccessful. Therefore, an alternative, PCR-based method was used to identify the general (wall- or fluid-associated) location of the "*Stenoxybacter*" cells within the hindgut. By using this method, which involved physical separation of the hindgut epithelium (and its associated microbiota) from the luminal community, the location of the spirochete

population within the gut fluid could be correctly identified (Figure 3.2b). After substitution of the spirochete-specific 16S rDNA primers with "*Stenoxybacter*"-specific primers, the results suggested that the "*Stenoxybacter*" population was primarily wall-associated (Figure 3.2a). Thus, the microaerophilic phenotype of the "*Stenoxybacter*" population is consistent with their *in situ* location on, or associated with, the hindgut wall, an area known to contain oxygen-consuming microorganisms (11).

In vitro and *In situ* Acetate Oxidation by TAM-DN1

Consistent with the acetate-rich environment of the termite gut, "*S. acetivorans*" strain TAM-DN1 expresses low affinity acetate kinase enzyme ($Km_{acetate}$ = 7 - 22 mM) rather than the high-affinity acetyl-CoA synthetase ($Km_{acetate}$ = 200 μM)(60). Given that the phosphotransacetylase reaction (1.1 $\mu mol \cdot min^{-1} \cdot mg\ protein^{-1}$) (most likely the rate-limiting step) can account for the entire experimentally-derived rate of acetate consumption by TAM-DN1 (0.12 $\mu mol \cdot min^{-1} \cdot mg\ protein^{-1}$) (Chapter 2), the ACK/PTA pathway is most likely the sole method for activating acetate in "*S. acetivorans*" cells. The lack of activity for other acetate-activating enzymes such as AMP- or ADP-forming acetyl-CoA synthetase and PPi-acetate phosphotransferase support this conclusion. Furthermore, the expression of acetate kinase by TAM-DN1 cells during succinate-dependent growth suggests the ACK/PTA pathway is constitutive; regulation of this pathway may be unnecessary within the acetate-rich termite hindgut environment.

As mentioned above, the experimentally derived acetate consumption rate for TAM-DN1 was 0.12 μmol·min^{-1}·mg protein^{-1}. By relating cell number to protein content, this rate can be expressed as 1.1 x 10^{-6} nmol·hour^{-1}·cell^{-1}. Assuming the *in situ* "*Stenoxybacter*" population to be approximately 1 x 10^{5} cells·gut^{-1} (Chapter 2), and to the extent that the *in vitro* measurements might reflect the *in situ* conditions, the estimated acetate oxidation rate for the TAM population in the termite gut is 0.1 nmol·gut^{-1}·hour^{-1}. This is approximately 4.6% of the 2.4 nmol·gut^{-1}·hour^{-1} acetate oxidation rate as measured by microinjection of radiolabeled acetate into *R. flavipes* hindguts (56), and 0.5% of the estimated 20.2 nmol·termite^{-1}·hour^{-1} acetate production rate measured in laboratory maintained termites (40). Therefore, though both the "*Stenoxybacter*" population and the termite are sharing the same substrate, the bacteria probably have minimal impact on the acetate resources available to meet the daily energy requirements of the termite host.

PCR amplification of *R. flavipes* gut DNA with the broad-range acetate kinase primers revealed a diversity of *ack* genes (Figure 3.3). Considering that the termite gut is primarily an acetogenic system, and the ACK/PTA pathway is bidirectional (Figure 3.1), these results reflect the fact that many gut organisms may operate this pathway in the direction of acetate and ATP production when fermenting mono- and polysaccharides (60). A majority of *ack* genes amplified from the termite gut clustered with the *ack* genes of known acetate-producing anaerobes, such as *Clostridium* and *Treponema* species (Figure 3.3). However, the distant phylogenetic relationship of the "*Stenoxybacter*" *ack* and *pta* genes to

any other *ack* or *pta* genes in *R. flavipes* hindgut organisms allowed the facile design of "*Stenoxybacter*"-specific gene-targeted primers. By using these "*Stenoxybacter*"-specific primers in reverse-transcriptase PCR reactions with *R. flavipes* hindgut RNA, the *in situ* expression of *ack* and *pta* by the "*Stenoxybacter*" population was confirmed (Figure 3.7 and 3.8). This, coupled with the ability of TAM-DN1 to co-utilize acetate and succinate (Chapter 2), and the inability to grow on other substrates found within the termite gut fluid such as cellobiose, xylose and lactate, supports the conclusion that the "*Stenoxybacter*" strains are almost certainly using acetate *in situ*.

When acetate is the only carbon and energy source for a bacterium the TCA cycle alone does not allow any net assimilation of acetate carbon. Under these conditions, the anapleurotic glyoxylate cycle serves to replenish TCA cycle intermediates derived from acetate catabolism, but drawn off for biosynthesis of cell material (14, 17) (Figure 3.1). As further possible evidence of *in situ* acetate oxidation, the ability of TAM-DN1 to operate a glyoxylate cycle was examined. Activity for the first enzyme in the glyoxylate cycle, isocitrate lyase, was not detected in cell-free extract of TAM-DN1 (Table 3.2), even though cells were harvested during acetate-dependent growth. Attempts to amplify the isocitrate lyase gene from TAM-DN1 using broad-range primers were also unsuccessful. However, apparent malate synthase activity was detected. *E. coli* is known to contain two distinct malate synthase isoforms, malate synthase A (MSA) and malate synthase G (MSG) (36). Malate synthase A is involved in metabolization of glyoxylate formed from the dissimilation of acetate, whereas malate synthase

G metabolizes glyoxylate formed primarily from growth on glycolate (36). Both isoenzymes condense glyoxylate with acetyl-CoA to form malate, and both display similar enzyme kinetics. PCR amplification and sequencing results clearly reveal that TAM-DN1 possesses a malate synthase G, whereas no malate synthase A, or the related malyl-CoA lyase, was identified (Table 3.2, Figure 3.5) (35).

This seeming paradox of growth on C2 compounds without a complete glyoxylate cycle has been reported in other organisms (2, 26, 35), and has led to the proposal of an alternative anapleurotic pathway, the citramalate cycle (22) (Figure 3.9). In this pathway, the requirement for isocitrate lyase is surmounted by the action of β-methylmalyl-CoA lyase. This enzyme cleaves β-methylmalyl-CoA to form propionyl-CoA and glyoxylate. Glyoxylate then condenses with acetyl-CoA to form malate in the typical malate synthase-type reaction, which can involve malate synthase A, G, or malyl-CoA lyase (35).

Enzymatic assays for the key enzymes β-methylmalyl-CoA lyase and malyl-CoA lyase in the citramalate cycle are available in the literature (35). However, the malyl-CoA and β-methylmalyl-CoA substrates are not commercially available and must be synthesized in the laboratory. Therefore, for purposes of timeliness and practicality, these assays with TAM-DN1 extract were not pursued further.

The possibility exists that TAM-DN1 uses a citramalate or similar cycle as an anapleurotic pathway if acetate is the sole carbon and energy source. However, it is likely that the "*Stenoxybacter*" population does not rely solely on

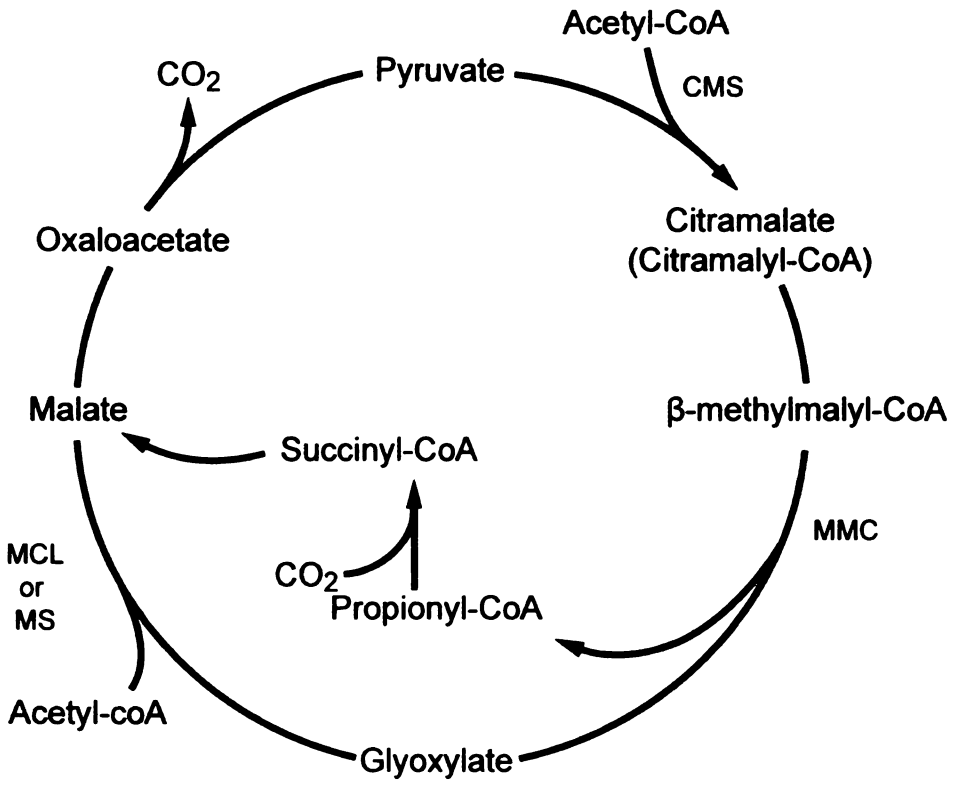

Figure 3.9. The citramalate cycle - an alternative to the glyoxylate cycle for acetate assimilation in organisms lacking isocitrate lyase activity. Key enzymes are citramalate synthase (CMS), β-methylmalyl-CoA lyase (MMC) malyl-coA lyase (MLC) and malate synthase A or G (MS). It is not yet known if the product of the CMS reaction is citramalate or citramalyl-CoA. Figure adapted from Meister *et al.* 2005.

acetate for carbon and energy *in situ*. Compounds such as amino acids and volatile fatty acids that can be used for biosynthesis or regeneration of TCA cycle intermediates have been measured in termite gut fluid (19, 40, 50). Furthermore, lysis of hindgut organisms though normal cell death or predation by protozoa (39) certainly releases some amount of lipids, nucleotides, etc. into the termite fluid upon which the "*Stenoxybacter*" population may rely. This notion is supported by the inefficacy of attempts to cultivate TAM-DN1 on a completely defined medium – the presence of a small amount of yeast extract is thus far required for growth.

In vitro and *In situ* O_2 consumption by TAM-DN1

TAM-DN1 reduces oxygen through the use of at least one terminal cytochrome oxidase, as verified by the PCR amplification of *ccoN*, the O_2-reducing subunit of a cbb_3-type haem-copper oxidase (Figure 3.5). The presence of a high-affinity (Km_{oxygen} = 7 nM) oxidase is not surprising given the microaerophilic nature of the "*Stenoxybacter*" isolates and their phylogenetic relationship to *Eikenella corrodens* and *Neisseria* spp., which also contain cbb_3-type terminal oxidases (23). Given that approximately 60% of the hindgut volume is exposed to a persistently low level of O_2, a diversity of *ccoN* genes within the hindgut is expected. PCR amplified with broad-range primers, a majority of *ccoN* sequences from *R. flavipes* gut DNA clustered with *Dechloromonas aromatica*, an organism known to degrade aromatic compounds (16). Whereas organisms related to *Dechloromonas aromatica* have not been isolated or described from

termite guts, O_2-dependent aromatic ring cleavage of lignin model compounds is known to occur (12).

Though the hindgut contains a diversity of organisms with *ccoN* genes, RT-PCR with *R. flavipes* gut RNA and *ccoN*-targeted "*Stenoxybacter*"-specific primers revealed that the *in situ* population of "*S. acetivorans*" appears to be expressing the O_2 reducing enzyme (Figures 3.7 and 3.8). Though the cbb_3-type cytochrome oxidase has also been implicated in the reduction of nitrate (44), TAM-DN1 is unable to use nitrate for anaerobic respiration *in vitro*. The high-affinity for oxygen (Km_{oxygen} = 7 nM) suggests that locally, the "*Stenoxybacter*" population could be very effective at consuming oxygen to extremely low levels, possibly supporting the O_2-labile processes of N_2-fixation, CO_2-reduction and methanogenesis by other gut organisms.

Given the abundance of "*Stenoxybacter*" cells within the termite gut as measured by colony counts and semi-quantitative PCR (Chapter 2) and insofar as the *in vitro* O_2 consumption rates (Table 3.4) represent maxima not necessarily reflective of the *in situ* situation, a maximum rate of oxygen consumption within the termite gut by the "*Stenoxybacter*" population of 1.4 – 3.6 $pmol \cdot min^{-1} \cdot gut^{-1}$ is estimated. Similarly, quantification of the *in situ* abundance of *Enterobacteriaceae* coupled with the O_2 consumption rate of *Citrobacter* sp. RFC-10 yields a maximum potential O_2 consumption rate of 0.2 $pmol \cdot min^{-1} \cdot gut^{-1}$ for the *Enterobacteriaceae* within the termite hindgut. Estimates of O_2 consumption rates for the lactic acid bacteria, based on *Enterococcus* st. RfL6, were 3.0 $pmol \cdot min^{-1} \cdot gut^{-1}$ (57).

In this study, an O_2 consumption rate for whole termite guts of 1013 pmol·min^{-1}·gut^{-1} was measured. This is approximately 6x higher than the estimated 178 pmol·min^{-1}·gut^{-1} measured by Brune *et al* with microelectrodes (11). Given the fact that two completely different methodologies were used (microelectrode-agarose chamber vs. Clark-type O_2 uptake chamber), this difference should perhaps be expected. Furthermore, O_2 consumption estimates by the microelectrode technique admittedly measured O_2 consumption only within the hindgut paunch region (~1/5 of the total hindgut tissue), and probably underestimates total hindgut respiration. However, the rates measured in this study are, in all likelihood, an over-estimation, as the whole extracted gut (midgut + hindgut) was used. Furthermore, the whole-gut O_2 consumption rates measured in this study were higher than the respiration rate of the entire termite (860 pmol·min^{-1}·termite^{-1}) measured by Odelson *et al.* (40). Because the measurements by Odelson *et al.* did not require dissection of the termite, they are certainly more accurate. Therefore, the true hindgut O_2 consumption rate most likely lies somewhere between 178 and 860 pmol·min^{-1}·termite^{-1}.

Table 3.5 gives the respective contributions of the "*Stenoxybacter*", *Enterobacteriaceae*, and lactic acid bacteria to the total O_2 consumption within the termite gut, based on both microelectrode and O_2 uptake chamber methods. The fact that each population contributes roughly 1% to the total suggests that rather than a single group of microorganisms being responsible for a majority of the oxygen consumption, the task is spread out among many. Therefore, even if a fraction of the estimated 700 phylotypes (21) within the termite gut are

Table 3.5. Estimated contribution to the total hindgut O_2 consumption rate by three hindgut bacterial communities.

	In situ **abundance** (cells·gut^{-1} x 10^5)	***In situ*** **O_2 consumption** (pmol·min^{-1} ·gut^{-1})	**% contribution to total** [f]	**% contribution to total** [g]
"*Stenoxybacter*" strains	1.0 – 2.2 [a]	1.5 – 3.6 [d]	0.8 – 2.0	0.1 – 0.4
Enterobacteriaceae	0.4 [b]	0.2 [e]	0.1	0.02
Lactic acid bacteria	1.9 [c]	3.0	1.7	0.3

[a] Based upon quantification of colony forming units and semi-quantitative dilution-to-extinction PCR.

[b] Based solely on colony forming units.

[c] Estimated from most-probable number studies by Tholen et al. 1997, using *Enterococcus* sp. RfL6 as a model organism.

[d] Using TAM-DN1 as the model organism for the "*Stenoxybacter*" population.

[e] Using *Citrobacter* sp. RFC-10 as a model organism for the *Enterobacteriaceae* community.

[f] Assuming total gut O_2 consumption is 178 pmol·gut^{-1}·min^{-1} based on microelectrode measurements (Brune et al. 1995).

[g] Assuming total gut O_2 consumption is 1013 pmol·gut^{-1}·min^{-1} based on measurements in this study.

responsible for 1% of the total O_2 consumption, the task of removing O_2 from the hindgut system for the continued fermentative production of acetate is accomplished. It is interesting to note that an ability to consume O_2, through respiratory or other enzymes, has been discovered in many so-called "strict" or "aerotolerant" anaerobes such as members of the *Bacteroidetes*, *Enterococcaceae*, *Desulfovibrio*, and *Methanobrevibacter* (4, 31, 49, 57). Many of these, or closely related species, are termite gut symbionts, some, such as the methanoarcheae are known to inhabit the hindgut epithelial wall (29, 30). Therefore, these atypical O_2 consumers may, together with other wall-associated microbes such as "*S. acetivorans*", contribute significantly to the overall O_2 respiration within the termite gut.

Relative Contribution of Major Microbial Groups to Hindgut O_2 Consumption

Total removal of inwardly diffusing oxygen in the termite gut is most likely accomplished by some combination of O_2 reduction by hindgut bacteria, archaea, protozoa, and epithelial cell mitochondria. To increase our understanding of the microbial contribution to the total O_2 consumption in the gut, termites were maintained on diets that included antibiotic drugs and/or starch that resulted in the removal of a majority of termite gut bacteria and/or protozoa (9, 45). Antibiotics that target the bacterial cell wall were chosen specifically in order to minimize possible damage to the epithelial mitochondria, thereby reducing any confounding variables.

The results reveal that the size of the per-gut protozoan population is highly correlated with the whole-gut O_2 consumption rate ($r^2 = 0.95$, Figure 3.10), whereas the correlation between the whole-gut O_2 consumption rate and the number of cultivable bacteria and archaea is much weaker ($r^2 = 0.56$). These analyses reveal that a disruption to the protozoan population has a greater effect on the whole-gut O_2 consumption rate than does a disruption in the bacterial population.

Given the complexity of the termite gut and the unknown pleiotropic effects of even the smallest disruption, the reason for a high correlation between O_2 consumption rate and number of protozoa is unclear. It may be that protozoa are directly involved in O_2 consumption in the hindgut. This may be analogous to rumen ciliates that are able to use O_2 as a terminal electron acceptor if present in very low concentrations (18). Direct visualization of the attachment of the protozoan *Pyrsonympha vertens* to the epithelium (placing it, at least in part, within the hypoxic zone) was frequently seen in transmission electron micrograph section of *R. flavipes* hindguts (8). Given the microscopic volume of the hindgut, the large number of protozoa, and their constant motion, it would be difficult to imagine that a majority of protozoa would not encounter some level of oxygen, if only periodically. If the protozoa are directly involved in oxygen consumption in the hindgut, a similar analysis using higher termites, which by and large do not contain gut protozoa, would be interesting.

Second, the protozoa may indirectly and selectively affect the O_2 consuming prokaryotes through predation, production of vitamins, cofactors,

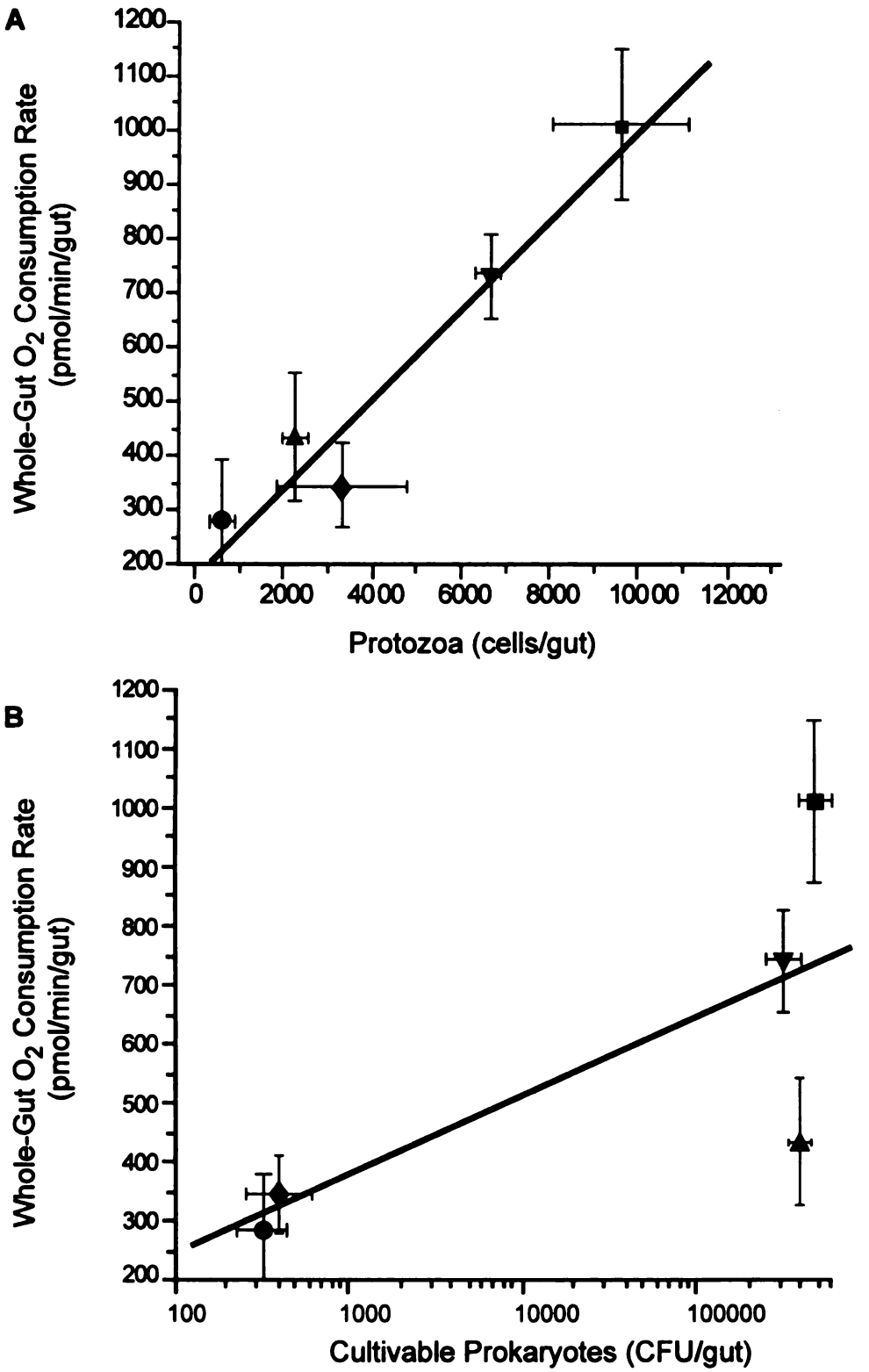

Figure 3.10. Correlation between *R. flavipes* whole-gut oxygen consumption rate and the number of gut protozoa (A) or cultivable prokaryotes (B). r^2 values for the best-fit regression lines are 0.95 (A) and 0.56 (B). Termites were maintained for 11 days of diets of cellulose (▼), cellulose with antibiotics (♦), starch (▲), and starch with antibiotics (●). Wood control (■).

substrates, or some other unknown mechanism. In this case the loss of the protozoa may directly or indirectly affect the O_2 consuming community, resulting in a decrease in whole-gut O_2 consumption.

A final explanation for the seemingly high correlation between O_2 consumption and gut protozoa includes the possibility that some of the protozoan symbionts are O_2 consumers. These symbionts could have a protective role, removing O_2 from the immediate proximity of the protozoans to allow for anaerobic fermentation. Furthermore, aerobic oxidation of protozoan-derived H_2 by ecto- or endosymbionts would improve the relative energy yield to the eukaryote by consumption of a fermentation endproduct (7). Only a low incidence of aerobic oxidation of H_2 by most-probable number studies was found in *R. flavipes* (57), however the homogenization procedure certainly resulted in the lysis of a majority of the protozoa. A lack of pure culture isolates of prokaryotic symbionts of protozoa, including members of the abundant *Candidatus Endomicrobia* (formerly Termite Group 1)(54) division make current testing of this question difficult.

A possible confounding reason for the weak correlation between whole-gut O_2 consumption and the number of bacteria and archaea may be due to a shift in the composition of this community (and hence metabolic rates and capabilities) without a shift in the total number of organisms. 16S rRNA-based identification of the bacteria and archaea within the gut after completion of the feeding experiments would resolve this question.

When introducing "directed" changes into the termite hindgut community, one is always confounded by the complex, immeasurable, and innumerate confounding interrelationships between the microbiota. A disruption to one population, community, or even cell may have a "rippling" effect on the rest of the community that renders any results far from absolute. Until there is a mechanism by which one microbe can be selectively manipulated with the guarantee of having no effect on any other microbe, any conclusions based on disruptions to the gut community will be replete with caveats. However, as that time may never exist, such measurements remain useful as a first approximation of *in situ* behavior that can often offer insights that lead to new, testable hypotheses.

Conclusions

In this chapter the physiological ecology of "*Stenoxybacter acetivorans*" within the *R. flavipes* hindgut is described. The "*Stenoxybacter*" population was primarily associated with a hypoxic area of the hindgut known to contain O_2 consuming microorganisms. Consistent with their existence in a high (60 – 80 mM) acetate-containing environment, these organisms express a low affinity pathway for acetate-activation and a high affinity respiratory oxidase. Detection of "*Stenoxybacter*"-specific expression of these genes *in situ* indicates the population respires O_2 and metabolizes acetate in the termite hindgut. Estimates suggest the maximal *in situ* respiratory capability of the "*Stenoxybacter*" population is 0.1 to 2% of the total O_2 reduction in the hindgut, similar to estimates of other abundant O_2-consuming isolates from the *R. flavipes* hindgut.

Disruption of the gut microbiota by maintaining termites on selective diets revealed that disruption of the protozoal community had a greater effect on the whole-gut O_2 consumption rate than did disruption of the bacterial community. This suggests a need for future studies that further explore the relationship between O_2 consumption and the hindgut protozoa.

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Chapter 4

Summary

The historical view of the termite hindgut as an anaerobic fermentation chamber has led to a greater understanding of O_2-sensitive hindgut processes such as cellulose digestion, acetogenesis, methanogenesis, and H_2 production (2, 4). However, the realization that a majority of the hindgut is exposed to some level of oxygen caused a paradigm shift in which the important role of O_2 consuming microorganisms to termite vitality became clear (3, 11). This dissertation has investigated the nature and importance of the O_2 consuming microbiota in the hindguts of the termite *Reticulitermes flavipes*.

In Chapter 2, a cultivation-based approach, using acetate containing media and incubation conditions that included hypoxia (2% O_2, 5% CO_2, 93% N_2) was used to test the hypotheses that acetate was a likely electron donor for aerobic respiration in the gut, and that some members of the O_2-consuming microbiota were microaerophiles, highly adapted to the "hypoxic zone" on and near the hindgut epithelial wall. Among the numerically dominant isolates were members of the *Enterobacteriaceae*, *Enterococcaceae* and *Streptococcaceae*, closely related to strains previously isolated from termite guts (1, 5, 8-10, 13). However, highest recoveries were obtained on acetate-containing plates incubated under hypoxia, an increase due almost exclusively to a single, easily distinguishable colony type. Phase contrast and electron microscopy of cells comprising such colonies revealed they were thin, nonmotile rods that appeared

to accumulate intracellular poly-β-hydroxybutryate. They were able to be subcultured only on plates incubated under hypoxia and displayed robust colony growth only if acetate was included in the medium, suggesting they were acetate-oxidizing microaerophiles. Phylogenetic identification of these, and similar strains (designated with the prefix "TAM") isolated from *Reticulitermes* workers collected from widely separated locations within the US and one location in France revealed they shared >99% 16S rRNA gene identity, but only were <95% similar to any other known bacteria, including the closest cultivated relative, *Eikenella corrodens*.

Though other intrinsically interesting microorganisms were isolated, including the first *Verrucomicrobia* and *Acidobacteria* isolated from termites (12) (See Appendix), the estimated *in situ* abundance, apparent microaerophilic phenotype, and distant phylogenetic relationship to any other known bacteria suggested the TAM organisms were unique and potentially important O_2 consuming microbes in the guts of *Reticulitermes*. Therefore, the characterization and physiological ecology of the TAM isolates became the focus of this dissertation.

PCR-based procedures implied the TAM isolates were autochthonous to the hindgut and comprised ca. 5% of the hindgut bacterial community. *In vitro* characterization of representative strain TAM-DN1 revealed that besides acetate, acetyl-acetate, succinate, butyrate, fumarate, glutamate, glutamine and casamino acids, few other substrates, including common organic acids and carbohydrates, were growth-supporting. This is perhaps a reflection of the

relatively stable, acetate-rich hindgut environment. When cultivated on solid medium in direct contact with the atmosphere, the oxygen tolerance of TAM-DN1 was equally narrow. Their ability to adapt to higher headspace O_2 concentrations in liquid medium, as well as express of oxyprotective enzymes like catalase and superoxide dismutase, may be important for colonization of the guts of newly hatched larvae or recently-molted colony mates under conditions of substantially higher O_2 content. The proposed classification of the TAM strains as "*Stenoxybacter acetivorans*" gen. nov., sp. nov. is a reflection of their distinct physiology and phylogeny.

The observations described in Chapter 2 suggested that "*Stenoxybacter*" st. TAM-DN1 and related strains were likely to be involved in oxygen consumption and acetate oxidation within the termite gut. Chapter 3 described the combination of *in vitro* and *in situ* molecular and physiological approaches used to test these hypotheses.

As inferred from its obligate microaerophilic phenotype, and confirmed with PCR, the primary location of the "*Stenoxybacter*" cells *in situ* appears to be in close association with the hindgut wall, a hypoxic region characterized by a persistent inward flux of O_2 (3). Through enzyme assays and PCR experiments with gene-specific primers, key genes and gene products involved in acetate utilization and oxygen consumption by "*S. acetivorans*" were detected. The use of an acetate-activating pathway with a low Km for acetate, and a terminal oxidase possessing a high affinity for oxygen, suggested that "*S. acetivorans*" is well-adapted to life within the hypoxic zone of termite hindguts. Detection of key

acetate utilization and O_2 consuming gene transcripts from *R. flavipes* hindguts revealed that the genes (and by inference, the gene products) are being expressed by cells of "*S. acetivorans*" *in situ*. Estimates of the potential contribution of "*S. acetivorans*" to overall O_2 consumption in guts of *R. flavipes* indicate the organisms may be responsible for approximately 0.1 to 2% of the total hindgut O_2 consumption. Finally, feeding treatments that largely eliminated major microbial groups (i.e. bacteria and protozoa) from the *R. flavipes* hindgut revealed the hindgut protozoa may have a more significant role, directly or indirectly, to O_2 consumption within the hindguts of lower termites than was previously recognized. .

This study represents the first dedicated effort to isolate and characterize the O_2 consuming microbiota in termite hindguts. As a result, new, fundamental information about the nature, function, and importance of "*Stenoxybacter acetivorans*" within the hindgut community has been realized. Furthermore, the putative importance of the hindgut *Eukarya*, an abundant but often overlooked potential oxygen sink (owing to their "strict" anaerobic metabolism) was suggested. The discovery of "*Stenoxybacter acetivorans*", an abundant, but heretofore undetected population of O_2 consuming microbes within the termite gut reveals the need for further refinements to cultivation techniques in order to better mimic the *in situ* environment. Further study of these microorganisms will likely reveal new principles of growth and survival under reduced O_2 concentrations, a poorly understood phenomenon often encountered in nature.

As the quotation by Dr. Jared Leadbetter that opened this dissertation suggested, the diversity of termite gut microbes astounds today as much as it did 100 years ago. At once invigorating to behold and challenging to study, fundamental answers to a host of biological, biochemical and ecological questions lie within a microliter of hindgut fluid. After all, Dr. Leadbetter notes "if only one or two of those two hundred species were doing something useful for the termite, evolution would have booted the freeloaders millions of years ago" (7). With such a profusion of complexity, an increase in understanding of only a fraction of the symbionts will serve as a foundation to piece together the whole. Dr. Robert E. Hungate, whom, after 45 years studying the termite gut and bovine rumen advised (6):

> *"The total activity (metabolism) of the ecosystem should be measured, and the precision and validity of the ecological analysis should be tested by the algebraic addition of the individual activities and comparison of the sum with the measured activity of the total system. Adherence to this goal will do much to prevent over-inflation of the ecologist's ego concerning his ecological accomplishments!"*

Study of the termite gut and associated symbionts will, without doubt, lead to new discoveries and surprises, as well as prevent many egos from over-inflation for the next one hundred years.

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Appendix

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Preface

Of particular abundance in soils, members of the *Acidobacteria*, *Planctomycetes* and *Verrucomicrobia* divisions often also comprise a small subset of 16S rRNA gene clones in libraries from termite gut homogenates. Members of the candidate division *Endomicrobia* have not been identified in soil, but are also abundant within the guts of lower termites. To facilitate as thorough an examination of microbial O_2 consumption in termite hindguts as possible, it was desirable to screen for representatives of these divisions during the isolation of O_2-consuming bacteria described in Chapter 2. Thus, in conjunction with ongoing work in the laboratory to isolate *Acidobacteria*, *Planctomycetes* and *Verrucomicrobia* from soil, a team effort was made to develop a facile, high-throughput method to facilitate the detection and isolation of these microbes from soil (B.S. and S.E.) and soil invertebrates (J.W.). The fruit of this effort, the isolation of novel *Verrucomicrobia* and *Acidobacteria* from soil and *R. flavipes* guts, is presented in this Appendix, and is contemporaneous with experiments described in Chapter 2.

New Strategies for the Cultivation and Detection of Previously Uncultured Microbes

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ABSTRACT

An integrative approach was used to obtain pure cultures of previously uncultivated *Acidobacteria* and *Verrucomicrobia* from agricultural soil and from the gut of wood-feeding termites. Some elements of the cultivation procedure included: the use of agar media with little or no added nutrients; relatively long periods of incubation (over 30 days); protection of cells from exogenous peroxides; and inclusion of humics or a humic analogue (anthraquinone disulfonate) and "quorum signaling" compounds (acyl homoserine lactones) in growth media. Air, hypoxic (1–2% O_2, v/v) and anoxic incubation atmospheres were also used, some with elevated concentrations of CO_2 (5%, v/v), the latter of which treatments resulted in a significantly greater occurrence of *Acidobacteria* on isolation plates. A simple, high-throughput, PCR-based surveillance method ("Plate Wash PCR") was developed that greatly facilitated the detection and ultimate isolation of target bacteria from among as many as 1,000 colonies of non-target microbes growing on the same agar plates. Results illustrate the power of integrating culture methods with molecular techniques to isolate bacteria from phylogenetic groups underrepresented in culture.

INTRODUCTION

Cultivation-independent molecular techniques have illuminated the enormous microbial diversity that exists on our planet and have served to define nearly 40 phylum-level divisions existing within the *Bacteria* domain alone (23). Most of these divisions, however, are poorly represented by cultured organisms, and at least 13 remain "candidate divisions" represented only by environmental gene sequences (23). The *Acidobacteria* and *Verrucomicrobia* are among those *Bacteria* divisions represented by a large diversity of 16S rRNA genes, which occur in particular abundance in soils, but contain few cultured members (3, 11, 12, 17, 20, 21, 23, 36, 48). Hence, our appreciation of the physiological diversity of *Acidobacteria* and *Verrucomicrobia* is limited, as is our knowledge of their role in global biogeochemical cycles. Clearly, a better understanding of these divisions would be attained by having a greater diversity of their members available in pure culture for detailed study.

The intrinsic selectivity of any given medium and incubation condition imposes limits on the nature, number, and diversity of microbes recovered from natural samples. It follows, then, that the application of isolation procedures that better mimic conditions existing in the habitat from which the samples are obtained could increase the likelihood of retrieving previously uncultured organisms. Recent efforts to accomplish this have met with some success by using: (i) relatively low concentrations of nutrients (1, 13-15, 19, 43, 45, 50); (ii) non-traditional sources of nutrients, signaling molecules, or inhibitors (of undesired organisms) (9, 10, 13, 31); and (iii) relatively lengthy periods of

incubation (19, 22, 24-26, 33, 39, 40), sometimes directly in the natural environment from which the inoculum was obtained (26).

For soil microbes, some of which may have become adapted to elevated concentrations of CO_2 and lower-than-atmospheric concentrations of O_2 (38), the composition of the incubation atmosphere may be an important consideration. Elevated CO_2 is rarely used in incubation atmospheres for isolation of soil microbes, yet CO_2 could be important for metabolic processes other than pure autotrophy. Likewise, transition of soil microbes to fully aerobic conditions on plating in air may be a stressful event. This would be especially true if cells were not immediately equipped to cope with reactive oxygen species (ROS) like hydrogen peroxide (H_2O_2), superoxide (O_2^-) or hydroxyl radical ($OH\cdot$) produced by their own metabolism or present in media as a result of autoclaving (reviewed in 29). Even with facultative anaerobes like *Escherichia coli*, an abrupt transition of anaerobically-grown cells to aeration can severely retard growth of certain mutants (27). It is also noteworthy that the cultivability (in air) of *E. coli* and *Vibrio vulnificus* following starvation is greatly improved if plating media are supplemented with catalase or pyruvate, two compounds known to eliminate H_2O_2 (5, 34). Such observations suggest that incubation atmospheres enriched with CO_2 and/or limited in O_2, as well as the incorporation of agents to detoxify ROS in the plating media, should be included among treatments seeking to recover previously uncultured microbes.

Whatever cultivation approach is tried, however, one is ultimately confronted with the need to evaluate its success. This is a potentially arduous

task if, as in this study, many different media and incubation conditions are being tested and little or nothing is known about the microbes sought other than their 16S rRNA gene sequences. Accordingly, some high throughput screening method is desirable. To deal with this, we developed a simple, high-throughput, PCR-based procedure, "Plate Wash PCR", that facilitated the surveillance of isolation plates for the presence of target organisms and the ultimate recognition of colonies comprised of them. The results of this endeavor constitute the substance of the present paper.

MATERIALS AND METHODS

Sample collection and manipulation.

Soil samples were collected between August 2001 and October 2002 from the Long Term Ecological Research (LTER) site located at the Michigan State University W.K. Kellogg Biological Station (KBS) in Hickory Corners, Michigan. The KBS-LTER site includes a large-scale replicated field experiment with treatments representing different cropping systems and types of management, several successional sites, and unmanaged forested sites (http://www.lter.kbs.msu.edu). Soil core (2 cm diameter × 10 cm depth) samples were taken from each of five permanent sampling stations distributed across one of four replicate fields (replicate 1) of the Never Cultivated Successional (NCS) treatment, which is representative of "native" soil.

Collected soil cores were stored at 4°C (usually for less than 48 h) until they were homogenized under a hypoxic, CO_2-enriched atmosphere (2% O_2, 5%

CO_2, balance N_2) contained within a flexible vinyl hypoxic chamber fitted with an oxygen sensor/controller (Coy Laboratory Products, Grass Lake, MI). Approximately 30 g of soil was added to 100 ml of phosphate-buffered saline (PBS; pH 7.0) containing 224 mM sodium pyrophosphate as a dispersal agent, and 1 mM dithiothreitol (DTT) as a reducing agent (47). The suspension was stirred vigorously for 30 min and allowed to settle for 30 min. An aliquot of the supernatant was serially diluted in the same buffer and spread onto various media with at least three replicate plates per dilution.

Termites, *Reticulitermes flavipes* (Kollar) (Rhinotermitidae), were collected near Dansville, MI and either used immediately or maintained in the laboratory as described previously (8, 35). Guts from 25-50 worker larvae were extracted under a hypoxic atmosphere (described above) with sterile forceps and pooled in a glass tissue homogenizer containing 2 ml of a sterile basal salts solution based on "freshwater medium" described by Widdel and Bak (49), and contained (per liter): KH_2PO_4, 0.2 g; NH_4Cl, 0.25 g; KCl 0.5 g; $CaCl_2 \cdot 2H_2O$, 0.15 g; NaCl, 1.0 g; $MgCl_2 \cdot 6H_2O$, 0.62 g; Na_2SO_4, 2.84 g; and MOPS (pH 7.0), 10 mM. After homogenization, the homogenate was diluted serially in the basal salts solution and spread onto various media.

The total numbers of microbes per gram (dry wt) soil or per termite gut were determined by direct microscopic count after staining with 5-(4,6-dichlorotriazine-2-yl) aminofluorescein (DTAF) following a protocol described by J. Bloem (4). Soil moisture content was determined by baking three replicate

samples of soil at 80°C to constant mass. Soil moisture content was then used with total direct counts to estimate the number of cells/g (dry wt) of soil.

Cultivation Conditions and Screening.

The basal medium used for cultivation of soil bacteria was a modification of the basal salts solution described above and contained (per liter): KH_2PO_4, 0.2 g; NH_4Cl, 0.25 g; KCl, 0.5 g; $CaCl_2 \cdot 2H_2O$, 0.15 g; NaCl, 1.0 g; $MgCl_2 \cdot 6H_2O$, 0.62 g; Na_2SO_4, 2.84 g; HEPES (pH 6.8), 10 mM; trace element solution (below), 1 ml; vitamin B_{12} solution (50 mg/l), 1 ml; and mixed vitamin solution (below), 1ml; Bacto Agar (Becton, Dickinson, and Company, Franklin Lakes, NJ) , 15 g; final pH adjusted to 6.8 – 7.0. The trace element stock solution contained (per liter): $FeCl_2 \cdot 4H_2O$, 1.5 g; $CoCl_2 \cdot 6H_2O$, 190 mg; $MnCl_2 \cdot 4H_2O$, 100 mg; $ZnCl_2$, 70 mg; H_3BO_3, 6 mg; $Na_2MoO_4 \cdot 2H_2O$, 36 mg; $NiCl_2 \cdot 6H_20$, 24 mg; $CaCl_2 \cdot 2H_2O$, 2 mg; HCl (25% v/v), 10 ml (49). The mixed vitamin stock solution contained (per liter): 4-aminobenzoic acid, 40 mg; D-(+)-biotin, 10 mg; nicotinic acid, 100 mg; Ca-D(+)-pantothenate, 50 mg; pyridoxamine dihydrochloride, 100 mg; and thiamine dihydrochloride, 100 mg (49). All solutions were heat sterilized; except for the trace element and mixed vitamin solutions, which were passed through a 0.22 µm filter. Variations in the medium composition above included the incorporation of some or all of the following (per liter): a mixture of organic carbon substrates (yeast extract, Bacto protease peptone #3, casamino acids, and dextrose (Becton, Dickinson)), 0.05 g each; catalase (bovine liver, Sigma-Aldrich, Inc.), 2,000 U (spread onto individual plates containing 30 ml of solidified medium just

prior to inoculation) or 130,000 U (added to 1 liter of cooled, molten agar just prior to pouring plates); soil extract (44), 100 ml; disodium anthraquinone-2, 6-disulfonate (AQDS), 2 g; and an N-acyl homoserine lactone "cocktail" (acyl-HSLs) prepared in ethyl acetate acidified with 0.1% (v/v) acetic acid and containing N-(butyryl, heptanoyl, hexanoyl, β-ketocaproyl, octanoyl, and tetradecanoyl)-DL-homoserine lactones (Sigma-Aldrich Inc.), used at a final concentration of 1 μM each in the media. The pH range of the prepared media was 5.9 – 6.4, depending upon medium composition. Cultivation of termite gut microbes was done with the same basal medium (above) containing also (per liter): sodium acetate, 2.46 g: and/or a combination of yeast extract and peptone, 0.1 g each.

Incubation atmospheres used were: air (unamended); CO_2-enriched (5% v/v) air; 2% O_2 and 5% CO_2, balance N_2 (termed hypoxic); or 5% CO_2 and 10% H_2, balance N_2 (termed anoxic). Incubations under atmospheres other than air were carried out in glass dessicator jars, the flexible vinyl hypoxic chamber (above), or a Plexiglas anoxic chamber (Plas-Labs Inc., Lansing, MI). All incubations were maintained under low light conditions at room temperature (21-23°C).

Primary screening for growth of target organisms was done after 30 days or more of incubation by sacrificing at least one replicate agar plate from selected treatments containing between 30 and 300 colonies and subjecting it to Plate Wash PCR (PWPCR) with group-specific primers (below). Remaining plates from successful treatments were then used as a source of colonies that were picked

individually, or removed in groups by swabbing sectors of the plate, for patching or streaking on homologous medium. For picking isolated colonies, many of which were invisible to the naked eye, plates were held under a dissecting microscope and illuminated with cool white light from a fiber-optic illuminator positioned at about a 45° angle from the horizontal plate surface. When subcultures were grown, individual colonies or defined pools of them were again subjected to screening by the PCR with specific primers. This process was continued until individual colonies of target organisms were ultimately identified and obtained as pure cultures (Fig. A.1).

PCR and Plate Wash PCR.

The polymerase chain reaction (PCR) was carried out with primers targeting regions of 16S rRNA-encoding genes common to nearly all bacteria, or specific to the phyla *Acidobacteria* and *Verrucomicrobia* (Table A.1). Unless otherwise stated, each 25 µl reaction mixture contained approximately 50 ng of template DNA, 1× reaction buffer (Invitrogen, Carlsbad, CA), 1.5 mM $MgCl_2$, 0.25 mM of each dNTP, 0.2 µM of each forward (F) and reverse (R) primer, and 0.625 units of *Taq* DNA polymerase (Invitrogen). Reactions were incubated in a model PT-100 thermal cycler (MJ Research Inc., Watertown, MA) for the following amplification schedule: 95.0°C, 3 min; 30 cycles of [95.0°C, 30 sec; (see Table A.1 for annealing temp), 30 sec; 72.0°C, 45 sec]; and 72.0°C for 10 min.

Preliminary experiments, to determine optimum PCR conditions with the *Acidobacteria*-targeting (Acd31F:1492R) and *Verrucomicrobia*-targeting

(Ver53F:1492R) primer pairs, were done by using template DNA from *Acidobacterium capsulatum* (ATCC 51196) and *Verrucomicrobium spinosum* (ATCC 43997), respectively. Optimum reaction conditions were determined across a gradient of annealing temperatures (50 – 65°C) and $MgCl_2$ concentrations (1 - 2.5 mM) by using a PTC-200 DNA Engine gradient thermocycler (MJ Research, South San Francisco, CA). Sensitivity of target gene detection was determined by performing the PCR reactions with *Acidobacteria*-targeting primers and decreasing amounts of *A. capsulatum* DNA mixed with non-target DNA (*E.coli* K12) to yield 50 ng total DNA per reaction. Sensitivity was also determined by using the *Verrucomicrobia*-targeting primer set with decreasing amounts of genomic DNA from a termite-associated *Verrucomicrobia* division isolate TAV1 (described below) in a 1:2 mass ratio with *E. coli* K12 DNA. Direct, group-specific PCR amplification of 16S rDNA genes in environmental samples was carried out with group-specific primers (see above) and 50 ng of DNA from soil or from 50 termite guts. Genomic DNA was extracted using the Ultraclean Soil or Fecal DNA Kits as per manufacturer's protocols (MoBio Laboratories, Carlsbad, CA).

Plate Wash PCR (PWPCR) was simply the PCR in which template DNA was obtained from the aggregate of colonies present on an isolation plate (Fig. A.1). To do this, the surface of the agar medium was flooded with 2 ml of Bead Solution from the Ultraclean Fecal DNA Kit (MoBio Laboratories), and then a sterile spreader was used to suspend as much colony material as possible. The bead solution with suspended cells was transferred to a dry bead tube from the

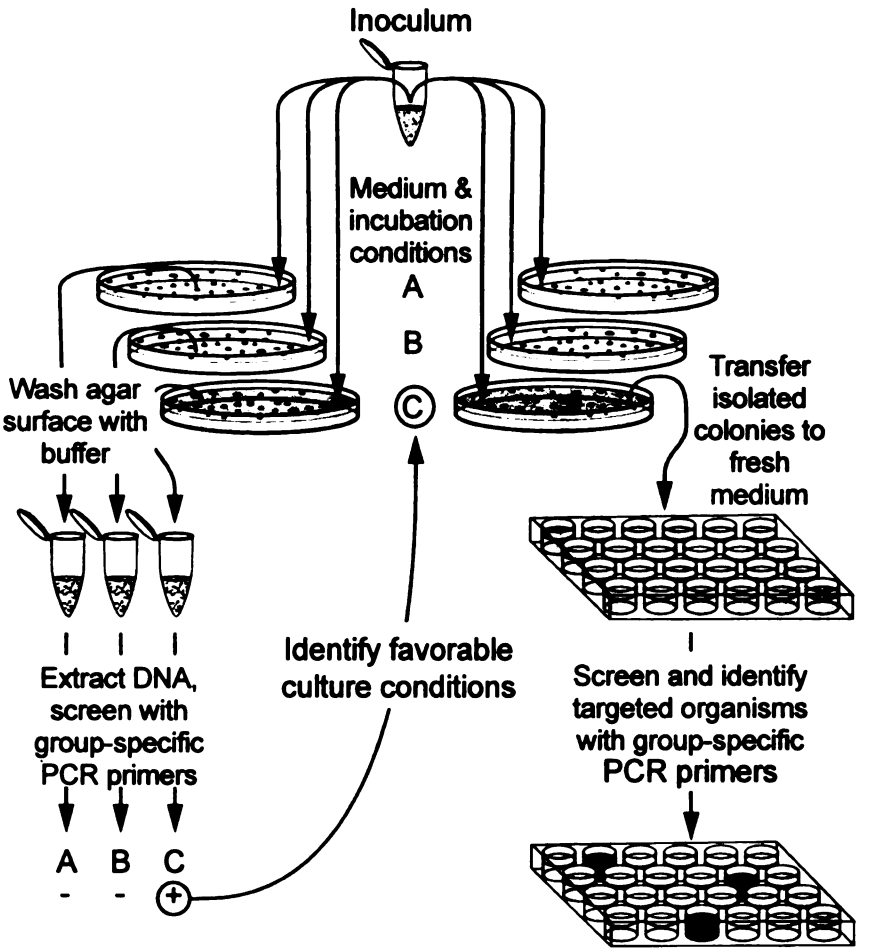

Figure A.1. Plate Wash PCR method to detect growth, and monitor isolation of, targeted bacteria. Of the three media and incubation conditions shown in this diagram (A, B, C), growth of targeted bacteria is represented only in "C".

Table A.1. PCR primers used for Plate Wash PCR

Primer	Position[a]	Sequence (5'-3')	Annealing temp[b]	Source
F2		CAG TCA CGA CGT TGT AAA ACG ACG GC	62.0	(28)
R4*[c]		CAG GAA ACA GCT ATG ACC ATG		(28)
Acd31F	15-31	GAT CCT GGC TCA GAA TC	56.7	(3)
Ver53F	37-53	TGG CGG CGT GGW TAA GA	61.0	D. Buckley; personal communication
1492R	1492-1510	GGT TAC CTT GTT ACG ACT T		(30)

[a] Position of target region is given using *E. coli* numbering system of the 16S rRNA gene. The primers F2 and R4* complement regions of the multiple cloning site of pCR2.1 or pCR4.0.

[b] Annealing temperatures optimized for the conditions used in this study are given for the forward primer of each primer pair.

[c] Modified from (28), by omission of 5 deoxynucleotides from the 5' end.

DNA Kit, 50 µl of lysozyme solution (50 mg/ml, Sigma-Aldrich, St. Louis, MO) was added, and the tube was incubated for 45 minutes in a 56°C water bath. After incubation, DNA extraction was carried out according to the manufacturer's protocol, except that a Mini-BeadBeater-8 (BioSpec Products Inc., Bartlesville, OK) operating at full speed for 45 sec was used for physical disruption of the cells. The concentration and purity of each DNA sample was estimated by absorbance at wavelengths from 220 to 320 nm (41).

The ability of PWPCR to detect a colony of a target organism from among a large excess of non-target organisms was examined by performing PWPCR on simulated isolation plates. A laboratory collection of 26 different bacterial isolates obtained from KBS LTER soil [various α-, β-, and γ-*Proteobacteria*; *Bacillus* spp. (phylum *Firmicutes*); *Arthrobacter* spp. (phylum *Actinobacteria*), and *Cytophaga*- and *Flavobacterium*-like strains (phylum *Bacteroidetes*)], were each inoculated onto 3 or 4 sites (94 total) on plates of R2A agar (Difco, Detroit, MI), a medium commonly used for isolating environmental heterotrophs. Some plates were also inoculated at one site with *V. spinosum* (ATCC 43997), and then all plates were incubated in air, at room temperature, and in the dark for 6 days. At the end of the incubation period, plates with (positive) and without (negative) *V. spinosum* were used for PWPCR individually, and after diluting template DNA extracted from positive plates with DNA extracted from negative plates.

PCR products were analyzed by electrophoresis of 5 µl samples of reaction mixtures on 1% agarose gels at 100 volts in 0.5x TBE. PCR products were visualized by UV illumination after staining with 1x Gelstar nucleic acid stain

(Cambrex, East Rutherford, NJ), and images were captured by using a Kodak Electrophoresis Documentation and Analysis System (EDAS) 290 (Eastman Kodak).

Sequence determination and phylogenetic analyses.

PCR amplified 16S rRNA genes from environmental samples, PWPCR, or bacterial isolates were cloned directly into *E. coli* using the plasmid vector pCR2.1 or pCR4.0 (TOPO TA cloning kit, Invitrogen). Restriction fragment length polymorphism (RFLP) analyses were used to identify common and unique clones. The partial sequence of each clone was determined with the Applied Biosystems cycle sequencing technology (Applied Biosystems, Foster City, CA), the 16S rRNA gene primer 531R (5'-TAC CGC GGC TGC TGG CAC-3'), and/or vector primers. Preliminary phylogenetic affiliation of each clone was determined by sequence comparison to the Genbank nucleotide database using BLAST (2), or to the Ribosomal Database Project II database using the sequence match tool (18). Nearly full-length sequence (at least 4-fold coverage) of the 16S rRNA gene from isolates and selected clones was obtained by using primers complementary to the multiple cloning site of pCR2.1 or pCR4.0 (F2 and R4*, see Table A.1); Acd31F, Ver53F and 1492R (Table A.1); and 338F (5'-CTC CTA CGG GAG GCA GCA GT-3'), 531R (above), 776F (5'-AGC AAA CAG GAT TAG ATA CCC TGG-3'), 810R (5'-GGC GTG GAC TTC CAG GGT ATC T-3'), and 1087F (5'-GGT TAA GTC CCG CAA CGA-3') with the Applied Biosystems cycle

sequencing technology and either an ABI Prism® 3100 Genetic Analyzer or ABI Prism 3700 DNA Analyzer (Applied Biosystems).

Contiguous sequences for each isolate were assembled with the Vector NTI software package (Informax). These were inserted into, and aligned against, a 16S rRNA gene sequence database in the ARB software package (http://www.arb-home.de/) (32), along with any other available phylum-specific sequences (>500 nt) from Genbank (http://www.ncbi.nlm.nih.gov/), the Ribosomal Database Project II (http://rdp.cme.msu.edu/) (18), or our own environmental clones. Aligned *Acidobacteria* and *Verrucomicrobia* sequences greater than 1250 nt in length were used to generate phylogenetic trees using maximum likelihood based on 1097 shared nucleotides for the *Acidobacteria* and 1050 nucleotides for the *Verrucomicrobia*. The minimum evolutionary distance method in PAUP* was used for bootstrap analyses of the same data (46).

Treatment effects on cultivability.

In order to determine which, if any, treatments had a significant impact on overall cultivability or the cultivability of *Acidobacteria* from soil, CFU/g soil (dry wt) and PWPCR results were compared for each treatment and used in a chi square test for goodness of fit with a Bonferroni error rate adjustment (37, 42). Colonies used to determine CFU/g soil (dry wt) had a minimum diameter of 0.2 mm and were visible using a colony counter fitted with a 1.5 × magnifying lens. For overall cultivability, the average CFU/g soil (dry wt) was used as the expected value and that for a particular treatment was used as the observed

value. For *Acidobacteria* cultivability, the expected value was the probability of detection using PWPCR among all treatments multiplied by the number of agar plates used for a given treatment, where as the observed value was the number of times *Acidobacteria* were detected for a particular treatment. A total of 63 treatments were screened for this analysis.

Nucleotide sequence accession numbers.

Partial 16S rRNA gene sequences (ca. 1400 bases) from isolates KBS89, TAA43, TAA48, TAA166, TAV1, TAV2, TAV3, and TAV4 have been deposited in the EMBL, GenBank, and DDBJ nucleotide sequence databases under accession numbers AY587227 through AY587234.

RESULTS & DISCUSSION

Specificity and Sensitivity of PCR and PWPCR

Only targeted 16S rRNA genes were amplified during the PCR with group-specific primers in control reactions run in the presence of *E. coli* DNA, or following PWPCR of a diverse collection of soil bacteria (Fig. A.2). Amplification with the PCR and as little as 16 pg of *A. capsulatum* DNA and 93.75 fg of *V. spinosum* DNA yielded a visible amplimer. The specificity of each primer set was also confirmed by sequence analysis of clones obtained after amplification with the PCR using soil or termite gut community DNA and after PWPCR of simulated or experimental isolation plates. Of more than 100 such clones examined, all corresponded to the 16S rRNA gene targeted by the primer pair.

By using PWPCR, the equivalent of a single *V. spinosum* colony could be detected on plates among a background of at least 940 non-target colonies composed of 26 different soil bacteria from six major phylogenetic groups (Fig. A.2). Considering the small amount of the *V. spinosum* colony material relative to that of the other bacteria, it should be quite possible to detect colonies of targeted microbes among a much larger number of non-target colonies of similar size. RFLP analysis revealed that only *Verrucomicrobia*-specific rDNA was amplified despite the diversity of non-specific DNA in each sample (data not shown).

Treatment Effects and Isolation of *Acidobacteria* and *Verrucomicrobia*.

Based on direct microscopic counts, $1.41 \pm 0.16 \times 10^9$ (n = 3) DTAF-stainable microbes were present in each gram (dry wt) of soil. In cultivation experiments, recoveries ranged from 4.0×10^7 to 9.7×10^7 CFU/gram of soil (dry wt), or roughly 4.0 to 7.0% of the total microbial community based on direct counts. These recoveries were higher than the "1% or less" recoveries commonly cited, but similar to those from other studies that have used low nutrient concentrations and long incubation times (19, 24). No single treatment significantly increased the overall recovery of soil bacteria relative to any other (Fig. A.3a), which suggests that the longer incubation times used for all experiments may be responsible for our higher recoveries of bacteria. When PWPCR results were compiled from the same experiments, however, one treatment, used individually or in combination with other treatments, had a

significant positive effect on the occurrence of soil *Acidobacteria* on plates: this was the presence of 5% CO_2 in incubation atmospheres (Fig. A.3b). Incubation of media in atmospheres with 5% CO_2 resulted in a slight acidification (about half of a pH unit) and, therefore, could also be responsible for the increase in cultivation of *Acidobacteria*. Incubation of plates under hypoxia or supplementation of media with catalase or acyl-HSLs also tended to elicit a greater occurrence of *Acidobacteria*, whereas supplementation of media with an organic nutrient mixture appeared to have the opposite effect. The addition of humics in the form of soil extract or the humic analogue AQDS had no apparent effect on the occurrence of *Acidobacteria* (data not shown). While these latter treatments were not statistically significant in this study, they may ultimately prove to be so if examined individually and systematically in a large-scale experiment.

PWPCR-based identification of primary isolation plates containing *Acidobacteria* enabled us to make an informed selection of companion treatment plates from which to prepare subcultures for additional PCR-based screening (see Methods). Ultimately, soil *Acidobacteria* strain KBS89 was isolated from soil that was plated on basal medium supplemented with catalase, acyl-HSLs, and a mixture of organic carbon substrates (described above), and incubated under an atmosphere of CO_2-enriched air.

A PWPCR-based strategy, similar to that used for the isolation of *Acidobacteria* from soil, was used for the isolation of previously uncultivated microbes from termite guts. The overall recovery of viable prokaryotes from

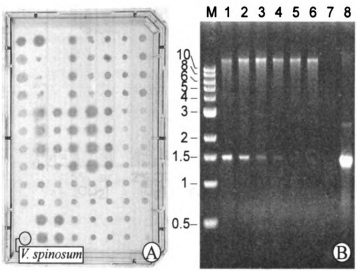

Figure A.2. Detection of *Verrucomicrobium spinosum* within a collection of diverse bacteria isolated from soil. (A) A single *V. spinosum* colony is shown among 94 other colonies growing on an agar plate. (B) Plate Wash PCR with *Verrucomicrobia*-specific primers using template in which *V. spinosum* colony material represented: 1 part in 95 (i.e. plate in panel A; lane 1); 1 part in 189 (lane 2); 1 part in 471 (lane 3); 1 part in 941 (lane 4); and 1 part in 9401 (lane 5). Plate Wash PCR of a control plate lacking *V. spinosum* (lane 6); negative control (no DNA, lane 7); and *V. spinosum* DNA (50 ng; lane 8) are also shown in Panel B. Sizes (kb) of markers in lane M are given to the left.

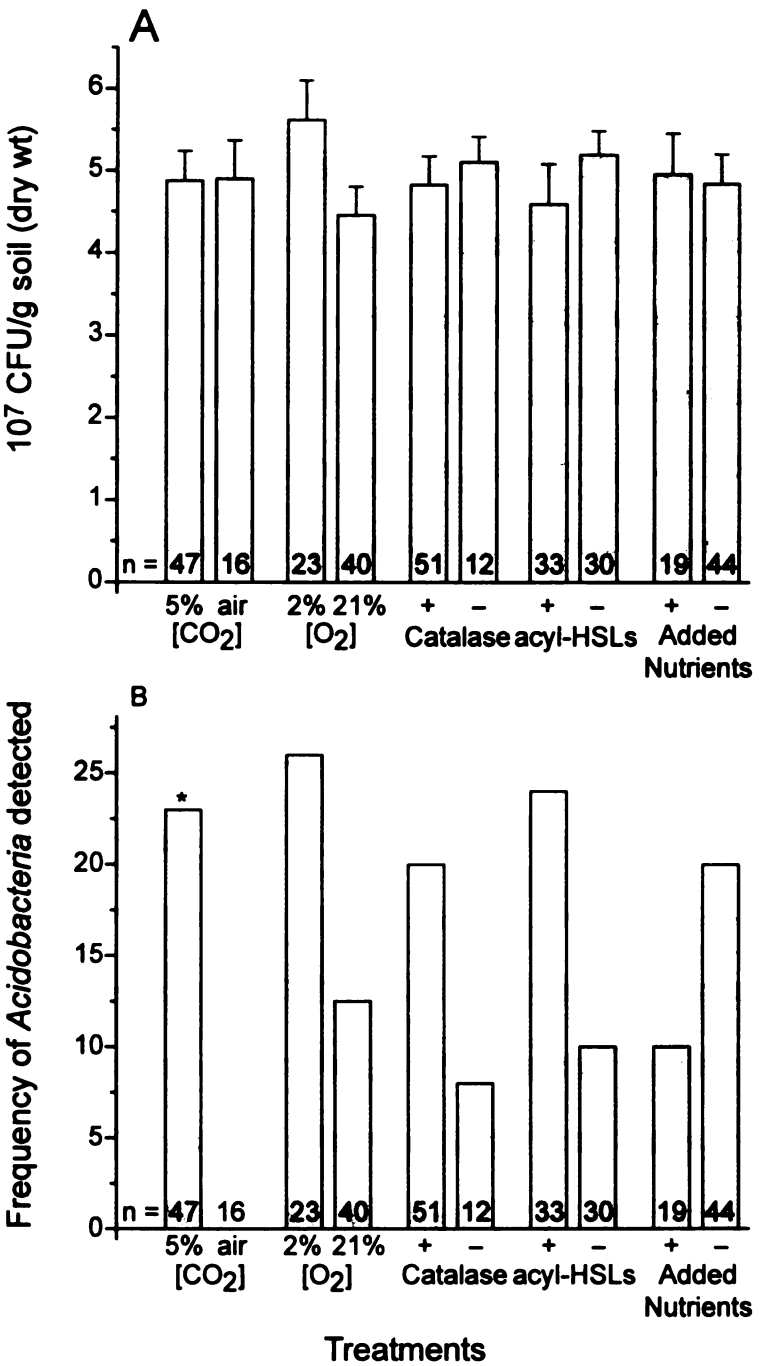

Figure A.3. Influence of medium additives and incubation conditions on CFU recovered from soil (panel A) and on the occurrence of *Acidobacteria* among the isolates (panel B). The label "air" refers to the concentration of CO_2 (0.03% v/v) in normal air. Data in panel (A) represent the mean CFU recovered from soil among n samples. Error bars represent sample standard deviation. Data in panel (B) represent the frequency with which *Acidobacteria* were detected among the plates in panel A using Plate Wash PCR. These data were subjected to chi-square analyses with a Bonferroni error rate adjustment. Statistical significance (a = 0.10, df = 1) is indicated by an *.

termite guts (9.7% of the direct microscopic count) was marginally higher than that from soil, with an estimated 4.5×10^5 CFU per gut equivalent. As with primary isolation plates from soil inocula, PWPCR revealed the presence of *Acidobacteria* on some of the plates, and by using analogous procedures *Acidobacteria* strains TAA43, TAA48, and TAA166 were subsequently isolated on plates containing basal medium supplemented with yeast extract and peptone (0.1% w/v each) and incubated under an atmosphere of CO_2-enriched air. By using *Verrucomicrobia*-specific primers, *Verrucomicobia* were also detected by PWPCR on primary isolation plates of all compositions inoculated with termite gut homogenate and incubated under air and hypoxic atmospheres enriched with 5% CO_2. Media used for cultivation of termite associated microorganisms contained yeast extract and peptone, with or without acetate and/or catalase. From such plates, four termite gut *Verrucomicrobia* (strains TAV1 through TAV4) were isolated, assisted by PWPCR surveillance of subcultures. TAV1 and TAV2 were isolated from plates containing basal medium with yeast extract, peptone, and acetate; TAV3 and TAV4 were isolated from the same medium without acetate. All TAV isolates were obtained from plates incubated under CO_2-enriched air.

Properties of *Acidobacteria* and *Verrucomicrobia* Isolates.

All of the *Acidobacteria* isolates belong to subdivision 1 of the *Acidobacteria* (Fig. A.4) (23). Based on 16S rRNA gene sequence similarity, the nearest cultivated relatives to strains KBS89 and TAA166, are Ellin351 (97%)

and Ellin337 (98%), respectively (40). TAA166 is the nearest cultivated relative to TAA43 and TAA48 (96.1%), the latter of which are identical to each other. All *Acidobacteria* isolates are short rods (0.5 µm × 1 µm) that divide via binary fission and form slightly opaque colonies after 4-5 days, which reach a maximum of 1mm diam in 14-16 days. Soil *Acidobacteria* isolate KBS89 and, to a lesser extent, the termite gut *Acidobacteria* isolates produce copious amounts of an extracellular (apparently capsular) material (Fig. A.4), which made colonies hard to disrupt and was presumably responsible for their flocculent growth in liquid cultures.

The termite-associated *Verrucomicrobia* isolates (TAV1-4) belong to subdivision 4 of the phylum *Verrucomicrobia* (Fig. A.5) (23). Based on 16S rRNA gene sequence similarity, the nearest cultivated relative to TAV1 is *Opitutus terrae* strain VeSm 13 (94.2%). Strains TAV2, TAV3, and TAV4 have 16S rRNA gene sequences virtually identical to each other, and the nearest cultivated relative to these isolates is *Opitutus terrae* strain PB90-1 (93%). TAV1 shares only 92.7% sequence similarity to the other TAV isolates. All of the termite-associated *Verrucomicrobia* isolates are facultative anaerobes, obtaining significantly higher population densities in liquid culture under CO_2-enriched air and hypoxic atmospheres, than under CO_2-enriched anoxic atmospheres. "TAV" cells are 0.25-0.50 µm in diameter and occur almost exclusively in pairs (Fig. A.5b). Additionally, TAV1 produces an abundance of extracellular (apparently capsular) material (Fig. A.5c). The TAV isolates were detected on primary isolation plates after 30 days and subculture plates after 14 days. On the original

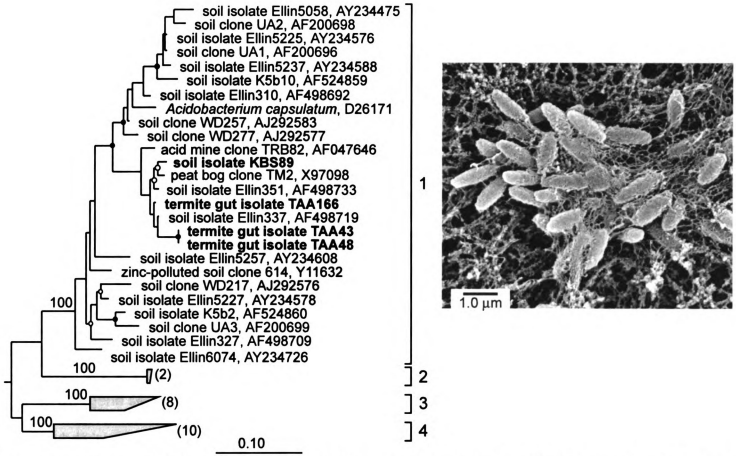

Figure A.4. Maximum likelihood tree (left) of subdivisions 1-4 of the phylum *Acidobacteria* based on 16S rRNA gene sequences from organisms in culture as well as PCR-generated clones from soil. Isolates obtained in this study are shown in boldface. Bootstrap values for branchpoints of the major subdivisions are given. Branchpoints conserved in all analyses with bootstrap values >75% are represented as closed circles; bootstrap values of 50-74% are represented as open circles. Subdivisions 2-4 are labeled, bracketed, and condensed as grey trapezia with the number of sequences represented in parentheses. 16S rRNA gene sequences of members of subdivisions 6-8 were used as outgroups (not shown). The scale bar represents 0.10 changes per nucleotide. The scanning electron micrograph (right) shows KBS89 cells trapped in an extracellular matrix.

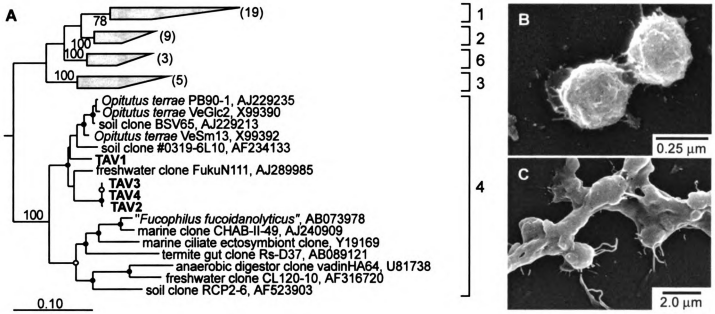

Figure A.5. Maximum likelihood tree of subdivisions 1-4 and 6 of the phylum *Verrucomicrobia* based on 16S rRNA gene sequences from organisms in culture as well as PCR-generated clones from environmental samples (Panel A). Isolates obtained in this study are shown in boldface. Bootstrap values for branchpoints of the major subdivisions are given. Branchpoints conserved in all analyses with bootstrap values >75% are represented as closed circles; bootstrap values of 50 - 74% are represented as open circles. Subdivisions 1-3 and 6 are labeled, bracketed, and condensed as gray trapezia with the number of represented sequences in parentheses to simplify presentation of the tree. 16S rRNA gene sequences of members of the phylum *Planctomycetes* were used as an outgroup (not shown). The scale bar represents 0.10 changes per nucleotide. The scanning electron micrograph of TAV2 (B) shows the doublet cell morphology shared by all TAV isolates; and that of TAV1 (C) shows the encapsulation of the cells in an extracellular matrix, a morphological feature not shared by TAV2, 3, or 4.

isolation media, they formed very small (< 0.5 mm), white, round, mucoid colonies that were only visible with a dissecting microscope. After isolation and several passages in the laboratory, however, all TAV isolates formed larger colonies (2-4 mm) in 2-5 days on R2A in air. Preliminary results from studying the distribution and abundance of these targeted phylogenetic groups suggest that *Verrucomicrobia* are autochthonous to the guts of *R. flavipes* and not allochthonous contaminants derived from soil, whereas the opposite is true for the *Acidobacteria* (J.T. Wertz, B.S. Stevenson, and J.A. Breznak, Abstr. 103rd Gen. Mtg., Am. Soc. Microbiol., abstr. N-223, 2003).

Overview of the PWPCR-Based Isolation Procedure.

Given the variety of individual cultivation treatments and treatment combinations used in this study, as well as the various sources of inocula, the detection and isolation of *Acidobacteria* and *Verrucomicrobia* would have been extremely difficult without the PWPCR method. One of the most time-consuming aspects of any isolation procedure is the screening, picking and subculture of colonies from primary isolation plates, and if low nutrient conditions are used to prevent overgrowth by non-desired organisms, most colonies on such plates will be fairly small. Indeed, colonies of the *Acidobacteria* and *Verrucomicrobia* strains isolated in this study would have been easily overlooked without the aid of a dissecting microscope. However, the PWPCR procedure economizes on time by directing one to treatment plates known to contain the target organism(s). Hence, owing to its simplicity, utility and relatively low cost, we anticipate that PWPCR

will become widely used as an adjunct to creative approaches for isolation of novel, sought-after organisms. The only requirement is at least one specific and reliable primer in the pair used for the PCR.

As with any method, PWPCR also has some limitations. For PWPCR, the sought-after organisms must be capable of growth on plates solidified with agar (or an agar substitute) and also capable of being harvested from such plates. This would eliminate organisms that either cannot grow on solid media or that, like certain spirochetes (7) and spirilla (16), form largely subsurface colonies difficult to harvest by simple plate washing. However, the key element of PWPCR is the PCR with a specific primer pair, so as long as sufficient cell material can be obtained to make a DNA template, either by harvesting cells from liquid cultures or removing subsurface colony material by coring, surveillance of cultures is possible. Thus, our results underscore the power of integrating various cultivation conditions with molecular biology to retrieve some of the "not-yet-cultured majority" of microbes on our planet (6, 39).

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