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Physiological Ecology of Termite Gut Spirochetes

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PHYSIOLOGICAL ECOLOGY OF TERMITE GUT SPIROCHETES

By

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ABSTRACT

PHYSIOLOGICAL ECOLOGY OF TERMITE GUT SPIROCHETES

By

Joseph Rex Graber

Treponema sp. strains ZAS-1 and ZAS-2 were the first spirochetes to be isolated from termite hindguts and are also the first known spirochetal H_2/CO_2-acetogens. The ZAS strains were examined for nutritional and physiological properties relevant to *in situ* growth and survival. In addition to using H_2+CO_2 as growth substrates, both strains grew on a variety of organic compounds and were capable of mixotrophic growth (i.e. the simultaneous use of both H_2 and organic substrates). Enzyme activities of the Wood/Ljungdahl pathway of acetogenesis were detected in both strains, whose H_2 thresholds were within the range typical of acetogens (650 and 490 ppmv, respectively). Both strains were able to maintain growth in the presence of small amounts of O_2 (0.5%, vol/vol) and possessed enzyme activities which could mediate protection from O_2. These results demonstrate that *Treponema* strains ZAS-1 and ZAS-2 are nutritionally versatile, perform acetogenesis by the Wood/Ljungdahl pathway, and are likely able to tolerate oxidative stress in the partially hypoxic hindgut environment.

Treponema strain ZAS-9, an additional spirochete from *Z. angusticollis* hindguts, was not a homoacetogen, and in fact produced H_2 as a major product during sugar fermentation. This strain also differed from ZAS-1 and ZAS-2 in a variety of other properties, suggesting that strains ZAS-1 and ZAS-2 be assigned to a single new species of *Treponema*, whereas ZAS-9 should be considered a separate new *Treponema* species.

Strains ZAS-1 and ZAS-2 had requirements for folate, and important cofactor in acetogenesis. On the notion that other termite gut microbes must supply folate *in situ,* heterotrophic organisms were isolated from the guts of the termite *Zootermopsis angusticollis* and screened for folate secretion. Two folate-secreting isolates (*Serratia* strain ZFX-1 and *Lactococcus* strain ZFX-2) were identified; both strains produced a compound that could replace the folate requirements of ZAS-1 and ZAS-2. The folate produced by both ZFX strains was identified as folinate. These results suggest that strains ZFX-1 and ZFX-2 are capable of supplying folinate to acetogenic spirochetes in the termite gut, and may also be important in providing folates to other members of the gut microbiota, as well as to the termite host.

Despite the fact that methanogenesis is the more energetically favorable process, H_2/CO_2-homoacetogens are the primary H_2-consumers in the guts of wood-feeding termites. To explain this observation, I hypothesized that gut methanogens are inhibited by pteridine compounds. The pteridine compound lumazine is an inhibitor of methanogenesis, and pteridines are known to be unusually prevalent in insect physiology. A variety of pteridines, as well as termite gut extracts, were tested for inhibition of cultures of the termite methanogen *Methanobrevibacter filiformis*; only lumazine showed significant inhibitory activity. No lumazine was detected in termite hindguts by TLC and HPLC analysis. These results indicate that gut methanogens are not inhibited by pteridine compounds. The marginalization of methanogenesis in wood-feeding termites must be explained by other factors.

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Introduction

Termite Gut Spirochetes: A Historical Perspective

In 1877, Joseph Leidy published his first observations on the strikingly complex community of microbes inhabiting the intestines of the wood-feeding termite *Reticulitermes flavipes* (56) (Figure 1.1). Leidy initially sought to discover the food source of the insects by examining their gut contents. However, upon microscopic examination of fluid collected from hindguts of worker termites, he reports:

> *"The brownish matter proved to be the semi-liquid food; but my astonishment was great to find it swarming with myriads of parasites, which indeed actually predominated over the real food in quantity. Repeated examination showed that all individuals harbored the same world of parasites wonderful in number, variety, and form."*

Leidy reported detailed observations on a variety of protozoa inhabiting the gut fluid and also observed an abundant and diverse population of somewhat smaller organisms possessing a distinctive undulate morphology and rapid motility (Figure 1.2). Initially referred to by Leidy as *Vibrio termitis*, these organisms would subsequently be recognized as spirochetes (35).

Spirochetes belong to an independent division (Spirochaetes) within the domain Bacteria. Nearly all representatives share a set of distinctive morphological features, including helical or undulate protoplasmic cylinder and periplasmic flagella located within the outer cell membrane (23, 25, 43). Spirochetes are generally

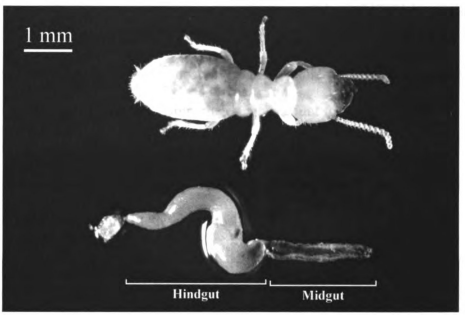

Figure 1.1. *Reticulitermes flavipes*, a wood-feeding lower termite, pictured with a gut extracted from a separate individual. The volume of the hindgut is approximately 1 μl. Adapted from reference 18.

chemoheterotrophs, consuming carbohydrates, amino acids, long chain fatty acids, or long chain fatty alcohols as energy substrates (24). Despite these points of similarity, the various spirochetal species are physiologically diverse and capable of colonizing a wide range of habitats. This is illustrated by the differences between the free-living members of the genus *Spirochaeta* (57), most frequently found in aquatic or sedimentary environments, and the generally host-associated genus *Treponema*, which form relationships (ranging from commensal to pathogenic) with a wide range of vertebrate and invertebrate animals (67).

Termite hindguts harbor the most diverse and abundant population of spirochetes found in any known environment. Spirochetes can account for up to 50% of the prokaryotic cells observed in the small but densely packed termite hindgut (10^9-10^{11} total prokaryotic cells/ml) (74) (Figure 1.2). Termite gut spirochetes are morphologically diverse with cells ranging in length from 3 to 100 μm and displaying a variety of wavelengths or body pitches (10); individual termite species typically harbor from 12 to 15 visibly distinguishable morphotypes (94). These cells were readily identified as true spirochetes by the *in situ* observation of characteristic periplasmic flagella in tranmission electron micrographs (3, 9, 10). Spirochetes appear to colonize the central lumen of the hindgut almost exclusively and are only rarely observed among the dense biofilm of organisms on the hindgut epithelial surface (9). In the lumen, spirochetes are observed both as free-swimming individuals and as epibionts attached to the surfaces of various gut protozoa (26, 83-85). In the case of the protozoa *Mixotricha paradoxa*, regularly arranged rows of spirochetes cover most of the cell surface. The coordinated motion of these epibionts propels the host cell in a unique motility symbiosis (26).

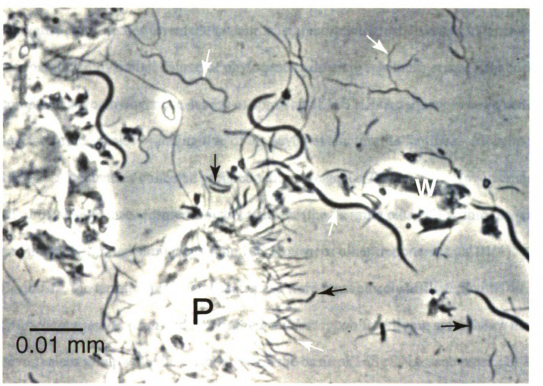

Figure 1.2. Phase contrast micrograph of diluted hindgut contents collected from the termite *Reticulitermes flavipes*. Spirochetes, including some attached to protozoa (P), are indicated by white arrows. Non-spirochetal prokaryotes are indicated by black arrows. Also labeled is an undigested wood particle (W). Adapted from reference 18.

Despite the conspicuous abundance of spirochetes in termite guts, attempts to isolate these organisms in pure culture met with repeated failure. To circumvent this problem, many researchers turned to cultivation independent molecular methods to survey the composition of the spirochetal community (1, 2, 70, 72, 74). By cloning and analyzing spirochetal 16S rDNA genes in community DNA collected from termite hindguts, these studies revealed that spirochetal morphological diversity in termite guts was paralleled by a high degree of phylogenetic diversity. Lilburn *et al.* (59) undertook a comprehensive phylogenetic survey of spirochetal 16S rDNA sequences collected from seven termite species spanning five of the seven extant termite families. All spirochetal 16s rDNA sequences collected grouped within the genus *Treponema*. However, nearly all of these sequences formed a distinct cluster (the "termite cluster") within the genus and had only limited similarity to the 16S sequences other treponemes ($\leq 91\%$). Curiously, the termite spirochetal sequences were most closely affiliated with *Spirochaeta stenostrepta* and *Spirochaeta caldaria*, two free-living spirochetes that nevertheless group with the treponemes on the basis of 16S rDNA sequences. In a single termite species (*R. flavipes*), it was estimated that 26 distinct spirochetal phylotypes were present, corresponding to at least 21 new species. FISH analysis of spirochetes in situ with a series of specific rRNA targeted probes resulted in labeling of both free-swimming spirochetes and protozoan epibionts, but individual phylotypes appeared to be restricted to one of the two life-styles (66).

While these studies provided new information on spirochetal phylogeny, definitive information on the physiological properties of the termite gut spirochetes remained elusive. However, the observation that spirochetes were consistently present in

seemingly healthy termites suggested a non-pathogenic or even beneficial relationship. This hypothesis was tested by Eutick *et al.* (40), using antibiotic treatments to selectively eliminate spirochetes from the guts of termites. While these treatments led to a reduction in termite vitality, it was impossible to determine if this was the direct result of spirochete elimination (as opposed to the loss of some other non-spirochetal member of the community) and if so, what specific benefits the host had derived from the spirochetal population. Until a representative could be isolated in pure culture, the role of spirochetes in the termite hindgut would remain a matter of speculation.

Nature & Function of the Termite Hindgut Community

Studies of the termite hindgut symbiotic system have most frequently focused on two problems posed by the utilization of wood as a primary food source by termites: First, how is this relatively refractory biopolymer converted to a metabolically useful source of carbon and energy? Second, how do termites thrive on a nutrient source containing low (0.05%) nitrogen? Accordingly, the roles of the hindgut microbial community in the digestion of lignocellulose and provision of carbon, energy, and nitrogen to their termite hosts evolved as central areas of research interest.

Carbon flow in the hindgut of lower (i.e. evolutionarily basal termites comprising six of the seven termite families) wood-feeding termites is illustrated in Figure 1.3. Cellulose hydrolysis occurs by the combined actions of termite cellulases secreted from the salivary glands (45, 96, 97) and cellulases of anaerobic protozoa (69, 98-100), the latter of which ferment the liberated glycosyl units within phagosomes by anaerobic protozoa residing within the hindgut:

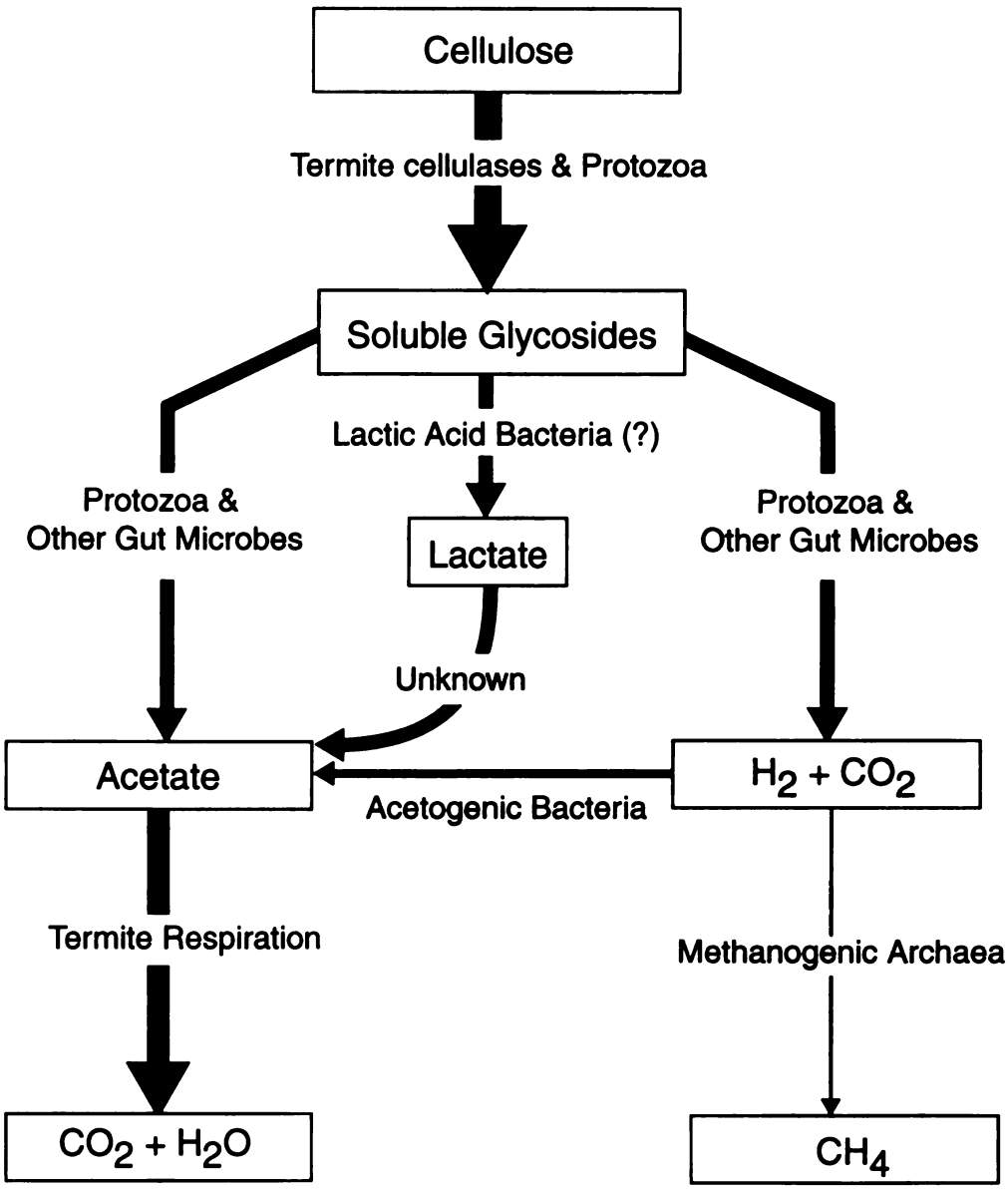

Figure 1.3. Carbon flow during cellulose digestion in wood-feeding termites. The thickness of arrows indicates the relative significance of the indicated microorganims in the hindgut.

1.) $[C_6H_{10}O_5]_n + 3n\ H_2O \rightarrow 2n\ CH_3COOH + 2n\ CO_2 + 4n\ H_2$

The H_2 and CO_2 produced can serve as substrates for homoacetogenic bacteria or methanogenic archaea, which produce acetate or methane, respectively:

2.) $4\ H_2 + 2\ CO_2 \rightarrow CH_3COOH + 2\ H_2O$

3.) $4\ H_2 + CO_2 \rightarrow CH_4 + 2\ H_2O$

Acetate is absorbed through the gut epithelium and oxidized to fuel the insect's energy metabolism (42), while methane and unused H_2 passively diffuses out of the gut. In the context of the symbiosis, methanogens could be viewed as parasites that consume reducing equivalents that could otherwise be directed towards acetate production.

Acetate dominates the hindgut pool of volatile fatty acids, accounting for 94-98 mol% of all VFAs at concentrations ranging 58 to 81 mM (68). Oxidation of microbially produced acetate was estimated to satisfy between 71 and 100% of the host termite's energy requirements (68). Given this observation, and the fact that methanogenesis is severely limited in the termite hindgut (see below), carbon flow in the hindgut was thought to be dominated by reactions 1 and 2, with bacterial acetogenesis accounting for up to 33% of total acetate production (15). However, recent studies using microinjection of radiolabelled substrates into intact guts have revealed that up to one-third of the total carbon flux passes through a previously unrecognized pool of lactate. The low standing concentration of lactate measured in the gut reflects the rapid turnover of this substrate to acetate (93). This observation has revised the estimated carbon flux accounted for by H_2/CO_2-acetogenesis to 10.5% of the total.

Although the polysaccharide components of lignocellulose are very efficiently digested during passage though the hindgut (74-99% of cellulose, 65-87% hemicellulose)

(38), high-molecular-weight core lignin does not appear to be significantly degraded in wood-feeding termites (16, 32, 38, 44). There is, however, evidence for some modification of lignin during gut passage via demethylation of the aromatic moieties of lignin sidechains (38), and a number of organisms capable of degrading aromatic lignin monomers have been isolated from termite hindguts (20, 51, 92). The contribution of these lignin-consuming activities to termite nutrition is currently unknown, but is likely to be relatively minor in comparison to cellulolytic activities.

Hindgut microbes also play important roles in the nitrogen economy of termites (90). Reduction of N_2 to ammonia (NH_3) via the enzyme nitrogenase is a property unique to prokaryotes, and any N_2 fixation measured in termites (either by the acetylene reduction assay or incorporation of $^{15}N_2$ isotope) is thus attributable to bacteria or archaea. N_2 fixation has been detected and quantified in a large number of different termite species (91), and a number of N_2-fixing bacteria have been isolated from termites (41, 52, 77). A highly diverse set of *nifH* genes (encoding dinitrogenase reductase) have been detected in the hindguts a number of termite species (71, 73), and direct amplification of *nif* genes from community mRNA allowed an elegant assessment of which phylogenetic classes of nifH homologues were being expressed in the gut of the termite *Neotermes koshunensis* (65). In addition to providing newly fixed nitrogen to termites, members of the hindgut community act to prevent the loss of fixed nitrogen by recycling the nitrogen present in the excretory product uric acid. Potrikus and Breznak (78-81) demonstrated that uric acid is transported to the hindgut via the Malpighian tubules and isolated a variety of bacteria capable of anaerobically degrading uric acid with the liberation of ammonia (which is presumably reabsorbed by the termite).

Physiochemical Gradients in the Termite Hindgut

Early studies of redox conditions existing within termite hindguts indicated an effectively anoxic environment (5, 6). While this result was in keeping with the presence of strict anaerobes in the hindgut, such as cellulolytic protozoa and methanoarchaea, a number of other observations suggested a more complex oxygen status. In cultivation studies of gut bacteria, a large number of the isolates were observed to be aerotolerant, facultatively aerobic (39, 87), or even strictly aerobic (92). Moreover, the ready mineralization of lignin monomers fed to termites was shown to be dependent on hindgut bacteria and the presence of O_2, and a variety of bacteria isolated from hindguts were shown to be capable of O_2-dependent *in vitro* degradation of aromatic monomers of lignin (20, 51, 92).

An elegant series of studies by Brune and coworkers (19, 21, 37) addressed the issue of O_2 in termite guts by making fine scale microelectrode measurements of the *in situ* physiochemical characteristics of termite guts. These studies revealed a highly structured environment with distinct radial gradients of H_2 and O_2 concentration (Figure 1.4). O_2 partial pressures were highest near the gut epithelium and rapidly decreased to non-detectable levels within 20-200 μm of the periphery. Conversely, hydrogen partial pressure was very high (approx. 50 mbar, or 50,000 ppmv) in the central luminal portion of the gut, and decreased steeply towards the gut periphery. Two main zones of H_2 consumption were observed; one in the center of the lumen, and a second directly at the gut epithelium. The hindguts of both lower and higher termites had pH values of around 7. These results provided convincing new evidence that as much as 60% of the

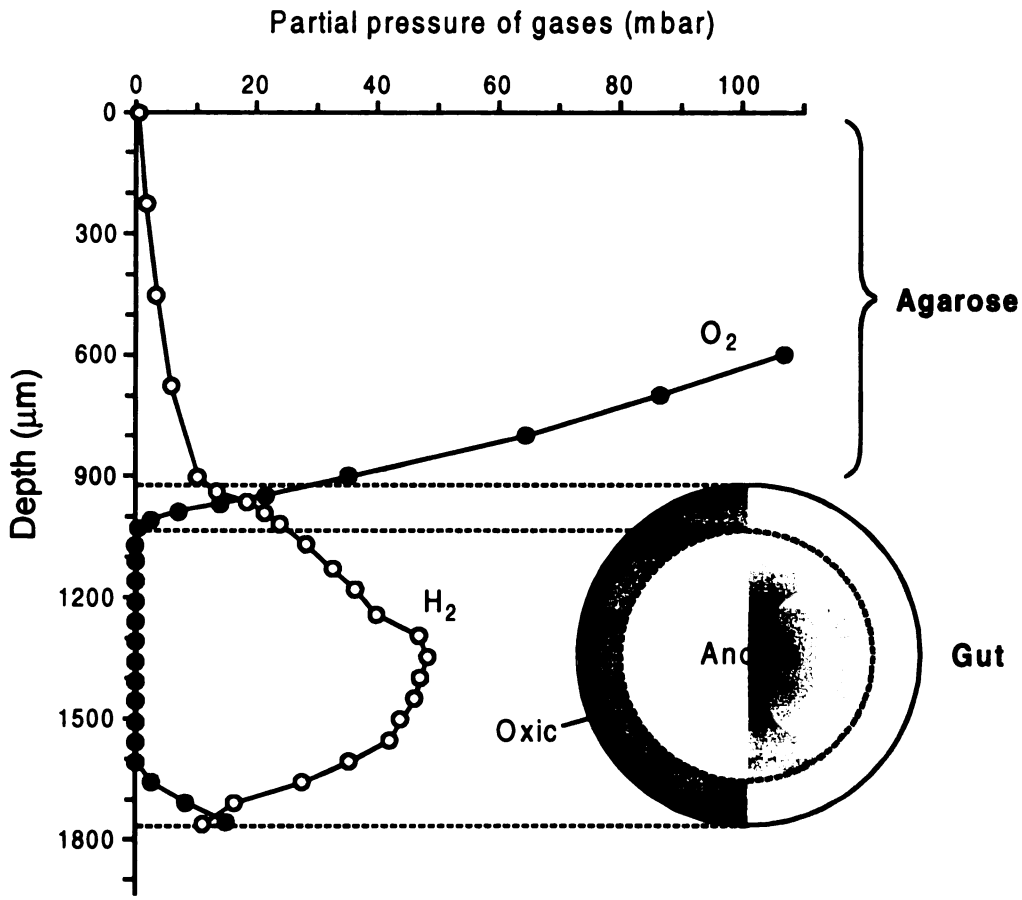

Figure 1.4. Radial profiles of oxygen (●) and hydrogen (O) in an agarose-embedded hindgut from *Reticulitermes flavipes*. The circular inset indicates the relative sizes of the oxic and anoxic zones in the gut (left half) and the hydrogen concentration gradient (right half). Adapted from reference 21.

gut volume is in fact hypoxic in character and also demonstrated that the luminal region of termite hindguts is among the most H_2-rich environments observed in nature (62).

Anaerobiosis in the gut lumen appears to be maintained largely by respiratory activity of gut bacteria, and elimination of these organisms with antibiotic treatments renders the gut fully oxic (95). This observation fits well with the epithelial zone of O_2 consumption observed by Brune *et al.* (19) (Figure 1.4). High rates of O_2-dependent acetate oxidation have been observed in the gut (93), suggesting that acetate produced in the central lumen may be one of the major substrates of O_2-consuming epithelial biofilm. While this acetate consumption would represent a loss of oxidizable substrate for the host, acetate oxidizers (and other O_2-consuming bacteria) could be viewed as playing an essential symbiotic role in maintaining conditions of anoxia in the lumen, which favors the continued homoacetic fermentation of cellulose.

Acetogenesis vs. Methanogenesis in Wood-feeding Termites

A significant role for H_2-consuming, CO_2-reducing homoacetogens in termite hindguts was first suggested by the dominance of acetate in the hindgut VFA pool and the observation of relatively low rates of H_2 and CH_4 emission from termites (68). It was also observed that H_2 and CH_4 emission rates were significantly increased in termites fed antibacterial drugs (68). These observations suggested that H_2 consumption in the hindgut was dominated by bacterial homoacetogens. This hypothesis was confirmed by the demonstration of H_2-dependent fixation of $^{14}CO_2$ to acetate by termite gut homogenates and the inhibition of this activity by antibacterial drugs (11). Brauman *et al.* (7) found that acetogens out-process methanogens as H_2-consumers in the majority of

wood-feeding termite species examined (although the opposite was true in termites from other feeding guilds, such as the soil-feeders). This situation, while unusual, is highly beneficial to the termite due to the significant contribution of acetogens to termite nutrition (15, 93).

The dominance of acetogenesis over methanogenesis in the termite hindgut is a puzzling situation. In anoxic habitats, various metabolic classes of organisms (sulfate-reducing bacteria, methanogens, H_2/CO_2-acetogens, etc) compete for reducing potential, usually in the form of H_2 produced by fermentative organisms. For a given electron donor (in this case, H_2) thermodynamics suggests that the group of organisms using the most exergonic terminal electron-accepting metabolic process (i.e. the process with the most positive redox potential, $E^{o'}$) should dominate other competitors, as implied by the equation: $\Delta G^{o'} = -n\ F\ \Delta E^{o'}$ [n = number of e^- transferred, F = Faraday constant, $\Delta E^{o'} = E^{o'}$ redox compound - $E^{o'}$ (H^+/H_2)]. This correlates to progressively lower minimum thresholds values of H_2 (33). The minimum H_2 threshold is the H_2 concentration at which an organism's metabolic reaction is still sufficiently exergonic to produce a useful amount of energy. This amount, referred to as the critical Gibbs free energy (ΔG_c), is the amount of energy required to translocate 1 mol of protons through a full energized cell membrane, which in turn correlates to the minimum energy quanta sufficient to drive the synthesis of ATP (approximately -23 kJ/mol substrate, or 1/3 of an ATP) (30, 31, 33). Thus, sulfate-reducing bacteria (average $E^{o'}$= -217 mV) are able to lower the H_2 concentration to a point at which methanogens (average $E^{o'}$= -238) can no longer derive useful energy from their metabolism. Table 1.1 provides a list of Gibbs free energy

Table 1.1. Redox potentials, Gibbs free energy changes, and H_2 thresholds for various anaerobic hydrogen consuming processes[a].

Metabolic Process:	$E^{o\prime}$ [b] (mV)	$\Delta G^{o\prime}$/reaction (kJ)	H_2 Threshold[c] (ppmv)
Acetogenesis:			
$4\ H_2 + 2\ HCO_3^- + H^+ \rightarrow\ CH_3COO^- + 4\ H_2O$	-279	-104.4	260 - 950
Methanogenesis:			
$4\ H_2 + HCO_3^- + H^+ \rightarrow\ CH_4 + 3\ H_2O$	-238	-135.6	25 - 100
Sulfate Reduction:			
$4\ H_2 + SO_4^{2-} + H^+ \rightarrow\ HS^- + 4\ H_2O$	-217	-152.0	8 - 16

[a]Adapted from values presented in reference (33).

[b]$E^{o\prime}$ of the couples accepting electrons from H_2 calculated relative to the redox potential of H_2 (-414 mV)

[c]Range of H_2 threshold values observed for representatives of each of the metabolic types grown in pure culture.

values, redox potentials, and H_2 thresholds of some typical groups of hydrogen consuming anaerobes. This model of competition is referred to as the threshold model (29, 31, 86), and is generally in good agreement with actual H_2 concentrations measured in natural habitats in which the dominant H_2-consuming process was known (27, 49, 62, 63). Methanogens, as would be predicted, dominate as the terminal electron sink organisms in habitats in which CO_2/HCO_3^- is the main terminal electron acceptor, including gastrointestinal systems such as bovine rumen (4, 13, 34, 64, 82).

In the termite hindgut, CO_2 appears to be the primary electron acceptor. Under these conditions, methanogens should out-process acetogens due to the more positive E°' value of the CO_2/CH_4 redox couple, which equates to a more exergonic reaction and lower H_2 threshold (see Table 1). This is certainly the case for most other gastrointestinal systems, including the bovine rumen (4, 13, 34, 64, 82). Figure 1.5 shows free energy values of acetogenesis and methanogenesis calculated for pH levels and concentrations of acetate, bicarbonate, and methane typical of the termite gut environment and are calculated across the range of radial hydrogen concentrations measured in the termite hindgut. While methanogenesis is clearly the more favorable reaction at all H_2 concentrations, acetogens appear to effectively dominate H_2 consumption in the termite hindgut.

A variety of homoacetogenic bacteria have been isolated from several species of termites, including members of the genera *Sporomusa*, *Acetonema*, and *Clostridium* (12, 47, 48). These organisms, however, did not seem to colonize the hindgut at population densities sufficient to support measured rates of H_2/CO_2 acetogenesis (15) and molecular phylogenetic studies of hindgut microbial communities of other termite species have never detected phylotypes closely related to the isolated termite acetogens (70, 72).

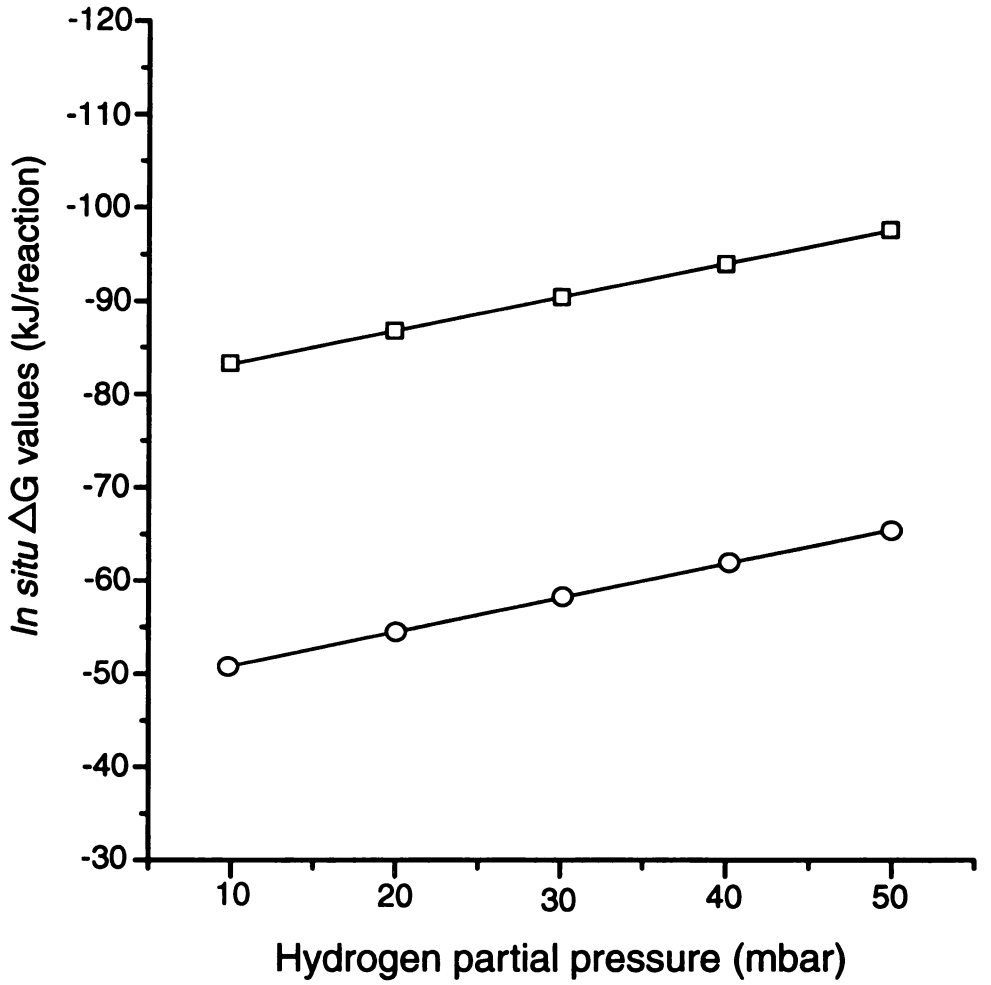

Figure 1.5. *In situ* Gibb's free energies for methanogenesis (□) and acetogenesis (○) under termite hindgut conditions. Values were calculated at 25°C for pH 7.2 and the following substrate and product concentrations: acetate, 80 mM; methane, 1 mM; bicarbonate, 60 mM.

Furthermore, the H_2 thresholds of the isolated acetogens (251-380 ppmv) were significantly higher than those of the cultured termite methanogens (36-45 ppmv) (17). In coculture competitions for limited H_2, the termite acetogen *Sporomusa termitida* was consistently dominated by the termite methanogen *Methanobrevibacter cuticularis* (17). Given these observations, it was considered doubtful that the cultured termite acetogens represented the dominant acetogens in the termite hindgut.

It is unknown what factors in the termite gut favor acetogenesis over the more energetically favorable process of methanogenesis. There are other habitats in which acetogens dominate methanogens, including mildly acidic (76) or carbon limited (46) freshwater sediments. Acetogenesis is also favored over methanogenesis at low temperatures (28, 88, 89). None of these conditions, however, would appear to be relevant to the termite hindgut habitat. Mixotrophic growth by the simultaneous utilization of organic substrates and H_2 has also been proposed to increase energy yields and competitive ability of acetogens (50, 60, 61), and mixotrophic growth has been demonstrated in the termite acetogen *Sporomusa termitida* (14). Hydrogen thresholds of acetogens growing mixotrophically are indeed lower than those measured under unitrophic growth, but are still significantly higher than those characteristic of methanogenesis (75), making the significance of mixotrophy in acetogen/methanogen competitions somewhat debatable.

The Spatial Resource Partitioning Hypothesis

The puzzling dominance of acetogenesis over methanogenesis in the termite hindgut has led to speculation that direct competition for H_2 does not actually occur between the

two groups. This was first suggested by the localization of methanogens in the hindgut of the termite *R. flavipes*: F420 autofluorescent cells (a diagnostic characteristic of methanogens) were rarely observed in the luminal gut fluid and instead appeared to colonize the gut epithelium almost exclusively (53, 54). Considered in light of the physiochemical gradient data of Ebert and Brune (37) and Brune *et al.* (19), this observation suggests an alternative model of H_2 consumption in termite guts referred to as the spatial resource partitioning hypothesis (22, 37).

In this model, methanogens and acetogens occupy distinct physiochemical niches within the hindgut, precluding any direct competition for H_2 (see Figure 1.6). Methanogens appear to be restricted to the region of lowest hydrogen concentration on the gut epithelium, where they may be additionally inhibited by inwardly diffusing O_2. If acetogens were to inhabit the central lumen, they would have access to hydrogen produced by gut protozoa at levels 100 to 1000 fold higher than their minimum thresholds (37). This would be consistent with the two regions of H_2 consumption (one in central lumen, and one near the gut epithelium) observed in the lumen by Ebert and Brune (37) (Figure 1.4).

As promising as this hypothesis may seem, it is not without shortcomings. First, it offers no explanation for the counterintuitive restriction of methanogens to the gut wall, where substrate H_2 is scarce and inhibitory O_2 is abundant. Also, in freshly prepared gut homogenates (in which all microbial spatial associations have been effectively disrupted), H_2-dependent acetogenesis from CO_2 still consistently dominates methanogenesis (11). This would imply that other, as yet unknown, factors also contribute to the dominance of

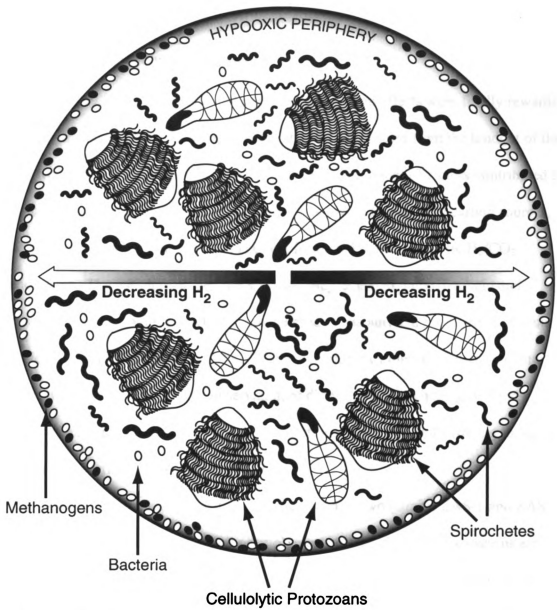

Figure 1.6. Diagram of the termite hindgut in radial cross-section, showing the spatial distributions of various microbial groups and gradients of O_2 and H_2.

homoacetogens over methanogens as H_2-consumers in the hindguts of wood-feeding termites.

Isolation of Termite Gut Spirochetes

In 1999, years of periodic but persistent cultivation efforts were finally rewarded with the first successful enrichment and isolation of spirochetes from the hindgut of the dampwood termite *Zootermopsis angusticollis* (55). A number of factors contributed to the success of these enrichments: the medium was low in fermentable carbon sources, was supplemented with rumen fluid, and was incubated under an anoxic H_2/CO_2 atmosphere. The enrichment medium was also supplemented with rifamycin and phosphomycin (antibiotics to which spirochetes are resistant) and bromoethanosulfonate (a selective inhibitor of methanogens). The development of this enrichment medium was the result of patient microscopic observation of differential spirochetal growth in a variety of exploratory media of varying constitutions over 2-3 months (owing to the slow growth of the termite gut spirochetes) (17, 18).

Of the dozen initially isolated spirochete strains, two (strains ZAS-1 and ZAS-2) grew to sufficient densities to permit physiological studies (55). The two strains are similar in size (0.2 μm by 3 to 7 μms) and display the characteristic morphological features of spirochetes (i.e. undulate cell shape and periplasmic flagella) (Figure 1.7). The 16S rDNA sequences of ZAS-1 and ZAS-2 are 98% similar to each other and, like other termite gut spirochetes, group within the so-called "termite cluster" of the genus *Treponema* (Figure 1.8). The most similar 16S rDNA sequences among cultivated spirochetes were those of *S. stenostrepta* and *S. caldaria* (92-93% similarity). Both

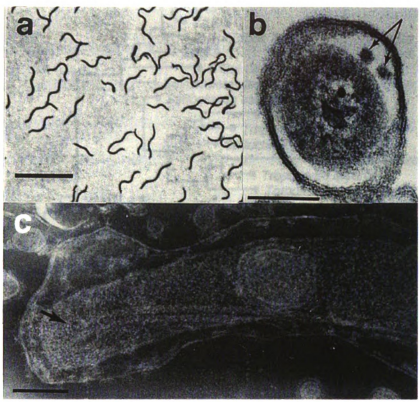

Figure 1.7. Morphology of termite gut *Treponema* strain ZAS-1 by phase contrast (a) and transmission electron microscopy of intact (c) and transverse sectioned (b) cells. The morphology of strain ZAS-2 is virtually identical to that of ZAS-1. Arrows indicate periplasmic flagella, whose insertion points are subterminal (c). Scale bars = 10 μm (a) and 0.1 μm (b, c).

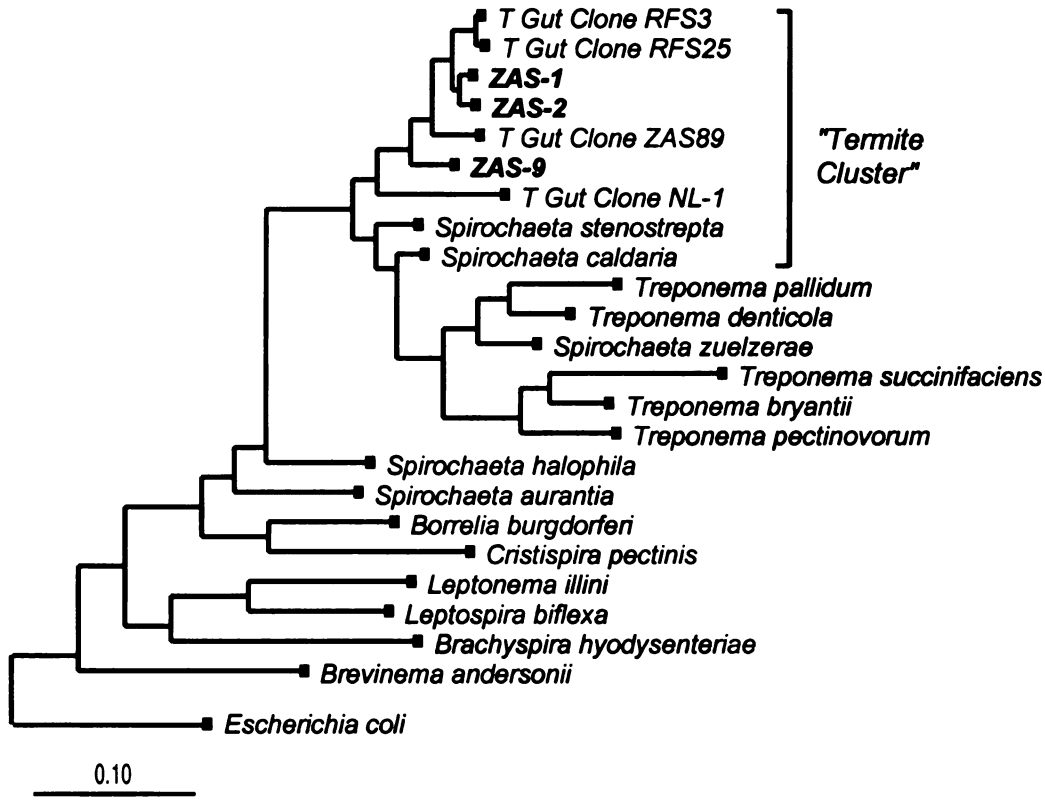

Figure 1.8. Phylogenetic tree inferred from 16S rDNA sequences of termite gut treponemes (strains ZAS-1, ZAS-2, and ZAS-9), representative known spirochetes, and spirochetal 16S rDNA clones generated directly from termite gut contents (T gut clones). A maximum likelihood technique (fastDNAml) was used to generate the tree. The vertical line on the right delimits a distinct subgroup (the "termite cluster") within the genus *Treponema*. Scale bar represents units of evolutionary distance and is based on sequence divergence.

strains required anoxic conditions and provision of yeast autolysate and an 11-cofactor mixture in the growth media (55).

The most interesting property of ZAS-1 and ZAS-2, however, was their ability to carry out H_2/CO_2 acetogenesis. This capability was first suggested by the consumption of headspace H_2 and CO_2 in culture tubes with the coincident production of stoichiometric amounts of acetate as the sole end product. Subsequently, it was shown that pure cultures of strains ZAS-1 and ZAS-2 incorporated $^{14}CO_2$ into both C atoms of acetate. To further test for the presence of the characteristic Wood/Ljungdahl pathway of acetogenesis (Figure 1.9) (36), both strains were successfully assayed for the presence of three characteristic enzyme activities (carbon monoxide dehydrogenase, formate dehydrogenase, and hydrogenase) of the pathway. While the possibility that termite hindgut spirochetes were acetogens had been hypothesized many years ago (8), the confirmation of this metabolic activity in ZAS-1 and ZAS-2 was still something of a surprise, as no other spirochete had ever been shown to be capable of H_2/CO_2 acetogenesis.

Given their abundance in the hindgut microbial community, the observation of acetogenesis in the ZAS strains suggests that spirochetes may be the dominant acetogens in the termite hindgut. This hypothesis is supported by the primarily luminal localization of spirochetes, which places them among (or attached to) H_2-producing protozoa and within the luminal zone of H_2-consumption observed by Ebert *et al.* (37). This interpretation, however, should be viewed with a note of caution. It is presently unknown what percentage of spirochetes in the termite hindgut are actually acetogens and whether acetogenic spirochetes exist in termites other than *Z. angusticollis*. An

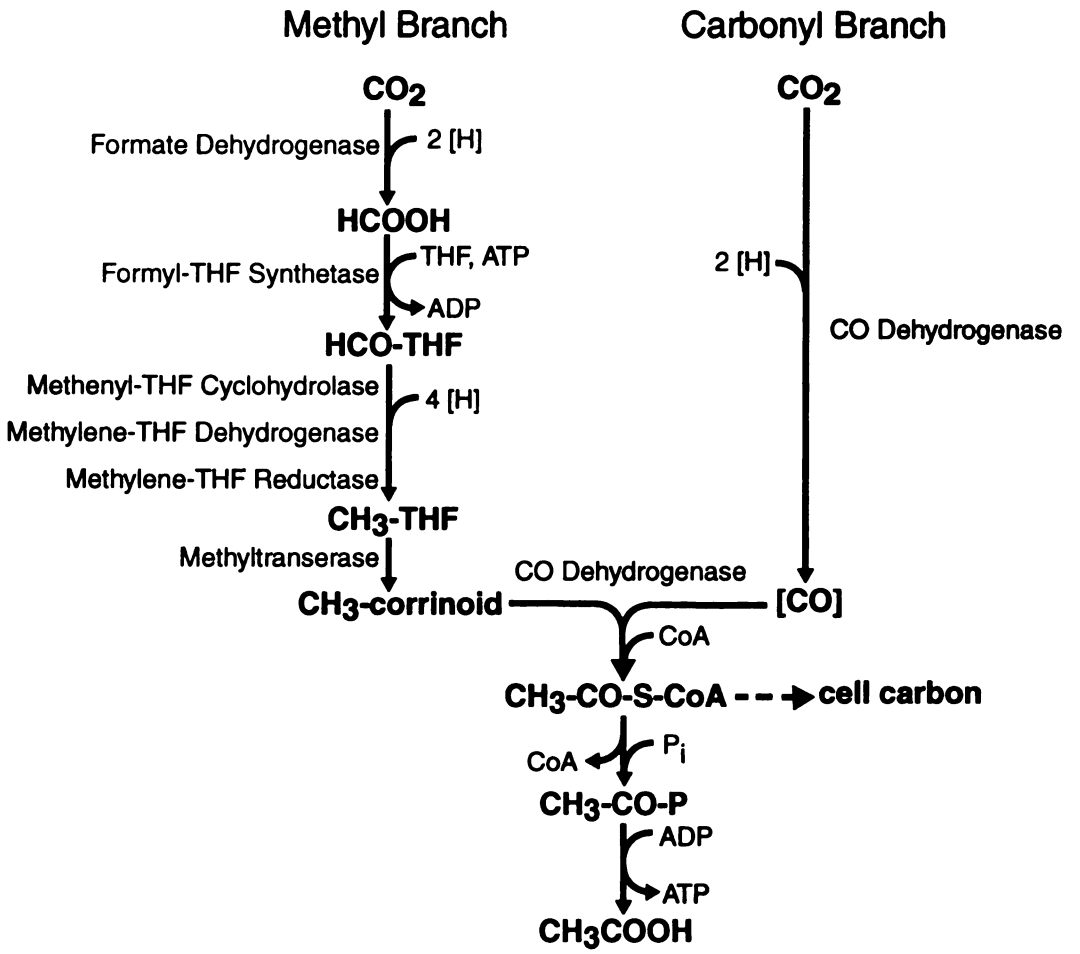

Figure 1.9. The Wood/Ljungdahl pathway of acetogenesis, highlighting enzymes of the methyl and carbonyl branches. THF=Tetrahydrofolate. Adapted from reference 36.

additional spirochete subsequently isolated from *Z. angusticollis*, *Treponema* strain ZAS-9 (more thoroughly described in Chapter 3), is not capable of H_2/CO_2-acetogenesis. This observation suggests that the morphological and phylogenetic diversity observed in termite-associated spirochetes is likely paralleled by a similarly high degree of physiological diversity. In addition to their role in the provision of acetate to their hosts, termite spirochetes are likely to perform a variety of other functions in the termite hindgut.

In keeping with this hypothesis, strain ZAS-9 was recently determined to be capable of N_2 fixation (58), another property not previously observed in spirochetes. Strains ZAS-1, ZAS-2, and ZAS-9 each possess two distinct *nifH* homologs, several of which were nearly identical to *nifH* sequences known to be expressed in termite hindguts (58, 65). Nitrogen fixation was unambiguously demonstrated in ZAS-9 by the acetylene reduction assay, by the fixation of $^{15}N_2$ into cellular ^{15}N, and by growth with N_2 as the primary nitrogen source. ZAS-1 and ZAS-2, however, showed only low levels of nitrogenase activity and no enhancement of nitrogen limited growth by the provision of N_2. These results strongly suggest that distinct populations of spirochetes make significant contributions to host nutrition both in terms of carbon (via acetogenesis) and nitrogen provision.

Dissertation Research

The research presented in this dissertation focuses on the physiological ecology of termite gut spirochetes. Chapter 2 presents a more thorough physiological characterization of

strains ZAS-1 and ZAS-2, with special attention focused on those properties relevant to survival within the termite hindgut and their role as symbiotic homoacetogens. Chapter 3 provides further description of strain ZAS-9, explores the taxonomic and genomic properties of all three ZAS strains, and proposes Latinate species epithets. Chapter 4 examines the requirement of strains ZAS-1 and ZAS-2 for the vitamin folate (a critical cofactor in acetogenesis) and the role of other members of the hindgut microbiota in providing this factor *in situ*. Chapter 5 revisits the issue of acetogenesis vs. methanogenesis in termite hindguts, and tests an alternative to the spatial resource partitioning hypothesis. Finally, chapter 6 summarizes the main conclusions of these studies.

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Chapter 2

Physiology and Nutrition of *Treponema* strains ZAS-1 and ZAS-2, H_2/CO_2-Acetogenic Spirochetes from Termite Hindguts

Introduction

Spirochetes are among the most abundant microbial groups in termite hindguts, accounting for up to one-half of the prokaryotic community (43). For more than a century, however, our knowledge of these organisms was largely limited to sporadic reports of their presence in various termite species, their morphological diversity, and their physical association with termite gut protozoa (8). Although elimination of spirochetes from the termite gut led to a decrease in termite survivorship (21), specific roles of spirochetes and factors contributing to their abundance in the hindgut have remained obscure.

Over the past ten years, our understanding of termite hindgut spirochetes has advanced dramatically. Cultivation-independent molecular approaches revealed that they group within the genus *Treponema* and that the large majority of 16S rDNA clones form a phylogenetically discrete cluster [the "termite cluster"] within this genus (35). These studies also revealed a striking degree of phylogenetic diversity amongst the termite gut treponemes, with as many as 21 distinct species occurring within a single termite host species (35). A few years ago, the first pure cultures of these organisms were isolated in our laboratory and were found to possess metabolic capabilities hitherto unknown in the *Spirochaetes* division of the *Bacteria*, including acetogenesis from $H_2 + CO_2$ (32) and N_2

fixation (34). Both of these processes are unique to prokaryotes, and have been demonstrated to be important in the provision of carbon, nitrogen, and energy to termites (6, 47). Acetogenesis plays a particularly prominent role in termite metabolism: 71-100% of the host's energy requirements can be met by oxidation of acetate produced by hindgut microbes, and it has been estimated that from 10.5% to 33% of this acetate production is attributable to H_2/CO_2 acetogenesis (6, 42, 52).

The availability of pure cultures of termite gut spirochetes has allowed the exploration of properties relevant to their growth and survival *in situ*. In this paper, we report on the nutritional, physiological and biochemical properties of *Treponema* strains ZAS-1 and ZAS-2, H_2/CO_2-acetogenic spirochetes isolated from hindguts of the California dampwood termite, *Zootermopsis angusticollis* (Hagen) (32). Additional information regarding the taxonomy, nomenclature, and genomic properties of these strains is included in chapter 3.

Material & Methods

Media and cultivation methods

Routine cultivation of strains ZAS-1 and ZAS-2 was in butyl rubber stoppered tubes or bottles containing ca. one-fifth their volume of medium 2YACo (32) under an atmosphere of 80% H_2 / 20% CO_2 (v/v). Medium 2YACo consisted of a mixture of inorganic salts, vitamins, cofactors, and 2% (v/v) yeast autolysate (equiv. to ca. 2.2 mg dry solids/ml). The medium was buffered by inclusion of 70 mM $NaHCO_3$ and 10mM 3-N-[morpholino] propanesulfonic acid (MOPS) and reduced with dithiothreitol (DTT) (1 mM final conc.). The pH of the medium prior to inoculation was 7.2. Unless otherwise

noted, cultures were grown at 30°C on a reciprocal shaker (50 oscillations per min.) with vessels held in a horizontal position.

Nutritional and growth studies

The ability of commercial yeast extracts to replace yeast autolysate in 2YACo medium was tested by using the following products at 6 mg/ml final concentration: Tastone nos. 900, 154, and 310; Amberex nos.1003 and 695; and Amberferm nos. 5925, 5902, and 5021 (Red Star Bioproducts, Juneau, Wisconsin). Cofactor requirements were evaluated by testing for an attenuation of growth in 2YACo medium lacking one of the eleven cofactors present in the cofactor stock solution (32). Candidate compounds were then tested in reciprocal experiments in which they alone were incorporated into medium 2YACo instead of the cofactor mixture.

Substrate utilization studies were performed with cells growing under an atmosphere of N_2/CO_2 (80/20, v/v). For ZAS-1, medium 2YACo was modified to contain Amberferm 5902 (4 mg/ml) in place of yeast autolysate. Unmodified medium 2YACo was used for ZAS-2, with a small amount H_2 (16 mM) added to the N_2/CO_2 headspace to aid in the initiation of growth (see below). H_2 was not added to cultures grown on methoxylated aromatics. An increase in cell yield (>20%) in the presence of a substrate, as compared to its absence, was taken to indicate utilization of the substrate as an energy source. Cell growth was determined by measuring the optical density (OD) of cultures at 600 nm with a Milton Roy Spectronic 20 colorimeter. OD readings were converted to cell number by reference to a standard curve relating these quantities. Substrate carbon balances were determined under conditions of substrate-limited growth.

Carbon recoveries for methoxylated aromatic compounds were calculated based on acetate production expected from demethylation of the aromatic (R) substrate according to the following equation:

$$R(-OCH_3)n + 0.5n\ CO_2 \rightarrow R\ (-OH)n + 0.75n\ CH_3COOH + 0.5n\ H_20$$

Organic acid production was determined by using a high performance liquid chromatograph (HPLC) with refractive index detection (4). Aromatic compounds were analyzed using a Beckman model 127 HPLC equipped with a model 168 photodiode array detector and an Alltech Lichrosorb RP-18 column (250 x 4.6 mm, 10 μm particle size). The mobile phase was 0.1% phosphoric acid with a methanol gradient, linearly increasing from 48 to 55% in 30 minutes. The flow rate was 1.5 ml/min.

To test for mixotrophic growth, strain ZAS-2 was grown in 750 ml bottles containing 100 ml 2YACo media with 2 mM maltose and a 650 ml headspace composed of 20% H_2, 20% CO_2, 60% N_2 (vol/vol). Consumption of H_2 was followed by gas chromatography (5). Maltose consumption and organic acid production were followed by using the anthrone assay (1) and HPLC analysis (above), respectively.

Determination of hydrogen thresholds

Hydrogen thresholds were determined as described by Lovley (37). In brief, cultures were grown under H_2/CO_2 (80/20, v/v) in medium 2YACo unmodified (ZAS-2) or modified to contain 4 mg/ml Amberferm 5902 in place of yeast autolysate (ZAS-1). When cultures reached mid-log phase (at which point further growth of both strains was strictly dependent on the presence of H_2), the headspace was replaced with N_2/CO_2 (80:20, v/v), followed by the introduction of a small amount of H_2 (ca. 6000 ppmv). The

basal level to which this H_2 was consumed (i.e. the H_2 threshold) was determined through three cycles of H_2 addition and consumption for each culture. H_2 was monitored by using a Trace Analytical RGA2 gas chromatograph equipped with a RGD2 trace gas detection unit.

Enzyme Assays

Cells from mid-log phase cultures (OD_{600} values of 0.15 to 0.3) were harvested by centrifugation (16,000 x g for 10 min) and resuspended at 10x their original concentration in appropriate assay buffer (as cited below) containing 10 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethanesulfonyl fluoride (PMSF), and leupeptin (10 μg/ml) to inhibit proteases. While held at 4°C, cells were disrupted by sonication 3 times for 30 s each with a Branson Model 450 sonifier (power setting of 5; 50% duty cycle) equipped with a stepped micro-tip. The resulting crude cell extracts were assayed for enzyme activities.

Formyltetrahydrofolate (formyl-THF) synthetase, methenyl-THF cyclohydrolase and methylene-THF dehydrogenase (41) and methylene-THF reductase (39) were assayed as described. THF and THF derivatives used in the assays were obtained from Schircks Laboratories (Jona, Switzerland). Catalase was assayed by measuring the rate of decrease in the A_{240} of H_2O_2 (2). Oxidase and peroxidase activities coupled to the oxidation of NADH or NADPH were assayed as described by Stanton (50). Superoxide dismutase was assayed by the xanthine/xanthine oxidase-cytochrome c reduction method (22). Protein content of cell extracts was measured by the Lowry assay (38).

Absorbance measurements were made using a Perkin-Elmer Lambda 14 UV/VIS spectrophotometer.

Oxygen Tolerance

Cells growing under anoxic conditions were tested for the ability to maintain growth after the addition of various concentrations of O_2 to the headspace. Replicate cultures (n = 3) were grown under H_2/CO_2 with oscillation (above) in 18 mm anaerobe tubes containing 5ml of medium 2YACo modified by the inclusion of 10 mM maltose, but containing no DTT reducing agent. When cells reached mid-log phase, the headspace was balanced to atmospheric pressure with H_2/CO_2, and sterile O_2 was injected to a final headspace concentration of 0.5%, 1%, 2.5%, or 5% (v/v). Cultures were then reincubated, and further growth was monitored as described above.

Results

Nutrition and growth of *Treponema* strains ZAS-1 and ZAS-2

Cells of both strains grew in medium 2YACo under H_2+CO_2 within an initial pH range of 6.5 to 7.8, with an optimum at pH 7.2. No growth was observed in media with an initial pH $\leq$6.0 or $\geq$8.0. Both strains grew within a temperature range of 23°C to 32°C, with an optimum at 30°C. No growth occurred at 4°C, or 34°C. Under optimum conditions (H_2/CO_2 + organic substrates), the shortest doubling time of cells was 24 hrs (ZAS-1) and 29 hrs (ZAS-2).

A variety of commercial yeast extracts (6 mg/ml final concentration) were tested for their ability to replace yeast autolysate in medium 2YACo. Tastone 900, Amberferm 5925, and Amberferm 5902 could replace yeast autolysate for growth of strain ZAS-1, whereas Tastone 154, Tastone 310, Amberex 1003, and Amberex 695 were suitable replacements for growth of strain ZAS-2. No single product was effective for both organisms. Growth rates and cell yields of ZAS-1 and ZAS-2 in media containing commercial yeast extracts were similar to those measured in media 2YACo.

Besides $H_2 + CO_2$, a variety of hexoses, pentoses, and disaccharides were also used as energy sources by both strains and were fermented homoacetogenically (Table 1). Curiously, however, when grown on organic substrates under N_2/CO_2, strain ZAS-2 displayed prolonged lag phases ($\geq$72 h) prior to the initiation of growth. The provision of small amounts of H_2 (16 mM) or the use of larger inocula (> 5% v/v) eliminated such lags (Figure 2.1). Strain ZAS-2 was additionally able to utilize methoxylated aromatic compounds (syringate, ferulate, vanillate, and trimethoxybenzoate) as energy sources when supplied at $\leq$2.5 mM (higher concentrations inhibited growth). Cell doubling times were typically greater than 100 hours on these substrates, and acetate production was consistent with demethylation of the compounds (i.e. the aromatic ring did not appear to be cleaved) (Table 1). This was confirmed for trimethoxybenzoate, which was quantitatively converted to gallic acid and acetate. Neither strain was capable of growth on other C1 compounds or methyl group donors tested (methanol, formate, CO, betaine, or choline). Relatively low concentrations of CO were inhibitory to both strains; addition of 1% CO (v/v) to the headspace of actively growing cultures resulted in the immediate cessation of growth.

Table 2.1. Substrate Utilization by *Treponema* strains ZAS-1 and ZAS-2

	Net Yield (cells/ml)[b]		Acetate Recovery[b,c]		Doubling Time (hrs)	
Substrate[a]	**ZAS-1**	**ZAS-2**	**ZAS-1**	**ZAS-2**	**ZAS-1**	**ZAS-2**
H_2/CO_2	$1.2x10^8$	$1.3x10^8$	92%	96%	26	48
Glucose	$3.5x10^8$	$1.5x10^8$	92%	93%	22	71
Mannitol	$2.9x10^8$	$1.1x10^8$	94%	91%	23	70
Arabinose	$2.5x10^8$	n.u.	98%	-	26	-
Xylose	$2.7x10^8$	$1.8x10^8$	91%	89%	26	101
Maltose	$6.7x10^8$	$4.7x10^8$	95%	95%	27	69
Cellobiose	$4.5x10^8$	n.u.	96%	-	27	-
Trimethoxybenzoate	n.u.	$1.1x10^8$	-	96%[d]	-	127
Syringate	n.u.	$9.3x10^7$	-	90%[d]	-	154
Ferulate	n.u.	$4.7x10^7$	-	93%[d]	-	315
Vanillate	n.u.	$7.2x10^7$	-	96%[d]	-	135

[a]All substrates were provided at a final concentration of 5mM except for the methoxylated aromatic compounds (2mM), CO (8 mM) and H_2 (136 mM). Substrates tested but not utilized are indicated by n.u. Ribose, methanol, formate, CO, lactate, pyruvate, glycine, betaine, and choline were not utilized by either strain. Data for growth of both strains on H_2/CO_2 was originally published in reference (32) and is presented here for comparison.

[b]Yields and carbon recoveries were determined under conditions of substrate-limited growth and are corrected for growth and acetate formation in modified 2YACo medium (see methods) lacking the test substrate.

[c]Percentage of substrate carbon recovered as acetate, or H_2-derived electron recovery as acetate for H_2/CO_2.

[d]Based on acetate production expected from demethylation of the aromatic substrate (see methods).

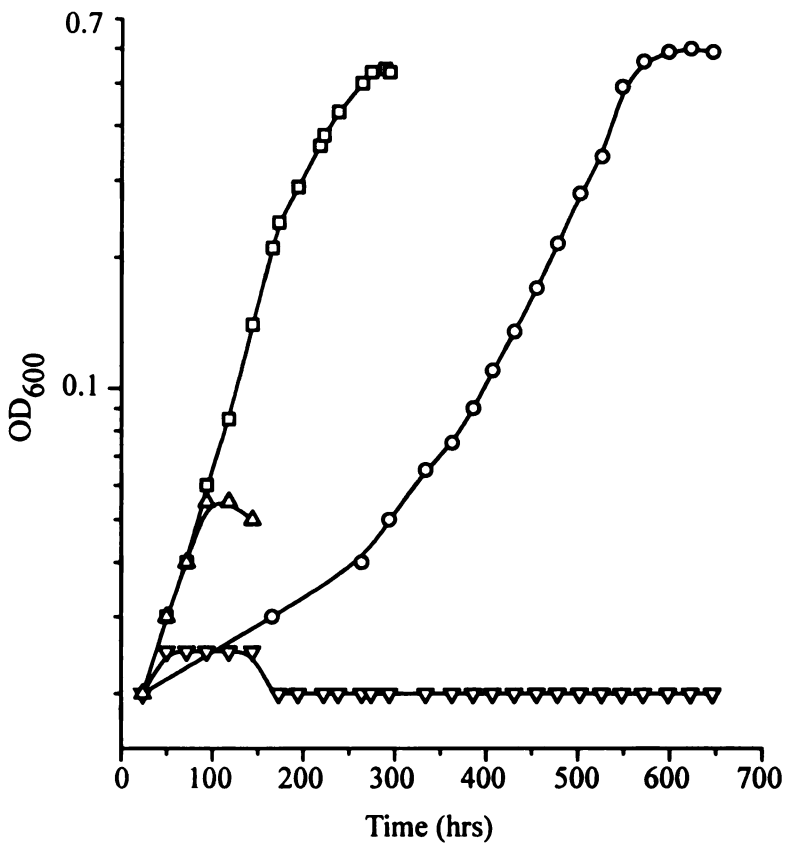

Figure 2.1. H_2 stimulation of chemoorganotrophic growth of *Treponema* strain ZAS-2. Substrates added to 2YACo medium under N_2/CO_2 head spaces (80/20, v/v) were: 10 mM maltose (○), 16 mM H_2 (△) 10 mM maltose plus 16 mM H_2 (□), and no added substrates (▽).

In order to determine which cofactors in 2YACo medium were required for growth, ZAS-1 was grown through 3 successive transfers in 2YACo media lacking one of the eleven cofactors. Cultures deficient in folinate (formyltetrahydrofolate) displayed successively decreasing growth yields with each transfer. Further testing demonstrated that folinate alone (500 ng/ml, final concentration) could replace the eleven cofactor mixture for both strains.

Mixotrophic growth

Strain ZAS-2 was capable of mixotrophic growth (i.e. the simultaneous utilization organic substrates and H_2 for energy). Growing in the presence of both maltose and H_2 + CO_2, strain ZAS-2 displayed a significantly shorter doubling time (29.2 ± 1.2 hrs.) than when growing on maltose (65.9 ± 2.4 hrs.) or H_2 + CO_2 (50.3 ± 2.1 hrs.) alone. Moreover, maltose and H_2 were consumed simultaneously during growth (Figure 2.2), and cell yields and acetate production from maltose plus H_2 + CO_2 were close to the sum of cell yields and acetate production when grown on either substrate alone (Table 2.2). Strain ZAS-2 also appeared to be capable of mixotrophy with H_2 + CO_2 plus other organic substrates: doubling times during growth on H_2 + CO_2 plus trimethoxybenzoate (38.1 ± 2.5 hrs) or xylose (35.2 ± 1.5 hrs) were significantly shorter than during unitrophic growth on either organic substrate alone (Table 1).

Hydrogen thresholds

The lowest level to which H_2 could be consumed (i.e. the H_2 threshold) was determined for mid-log phase cultures of H_2/CO_2 grown cells, after replacing the

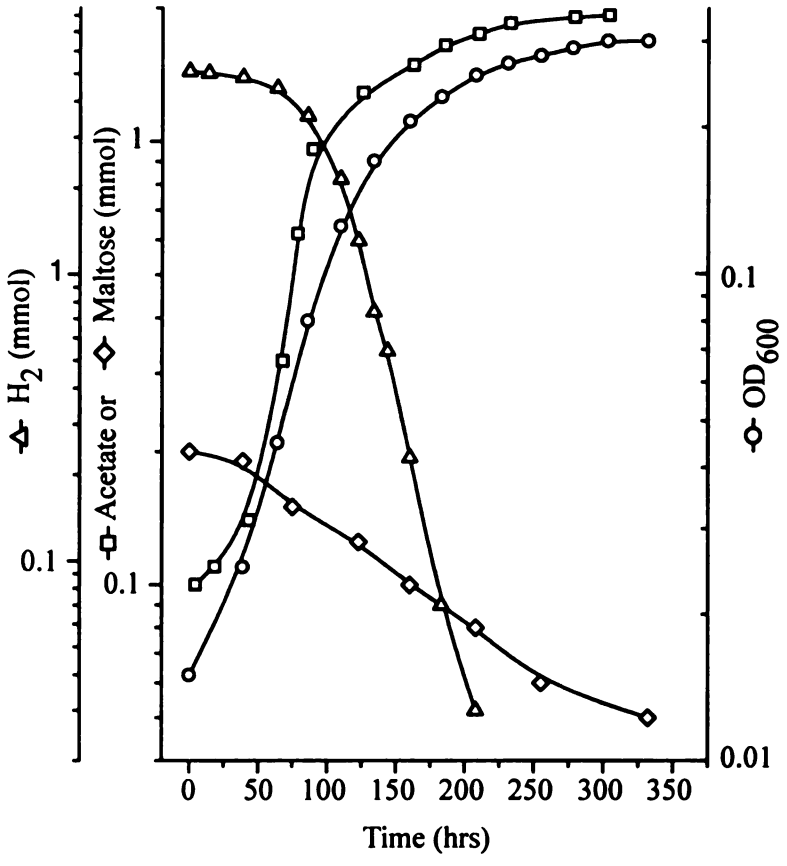

Figure 2.2. Mixotrophic growth of *Treponema* strain ZAS-2 on H_2+CO_2 and maltose.

Table 2.2. Cell yields and acetate production by *Treponema* strain ZAS-2 growing under unitrophic and mixotrophic conditions.

Substrate	Substrate Consumed[a] (mmol)	Acetate Produced[a] (mmol)	Recovery[b]	Cell Yield[a] (cells/ml)
Maltose	0.15 ± 0.04	0.84 ± 0.19	93%	2.4×10^8
H_2+CO_2	5.23 ± 0.35 (H_2)	1.23 ± 0.13	94%	1.7×10^8
Maltose plus H_2+CO_2	0.15 ± 0.01 (maltose) plus 5.20 ± 0.19 (H_2)	1.95 ± 0.03	89%	4.3×10^8

[a]Averages derived from replicate cultures (n = 3).

[b]Acetate recovery as a percentage of substrate carbon (maltose) or electron (H_2) consumption.

headspace with N_2/CO_2 (80/20, v/v) and measuring the extent of consumption of three successive additions of small amounts (ca. 6000 ppmv) of H_2. ZAS-1 and ZAS-2 displayed H_2 thresholds of 490 ppmv (s.d. 40 ppmv) and 650 ppmv (s.d. 50 ppmv) respectively. The H_2 threshold of ZAS-2 decreased moderately to 350 ppmv (s.d. 30 ppmv) when growing mixotrophically on H_2 + CO_2 plus trimethoxybenzoate (Figure 2.3).

H_2/CO_2-acetogenic enzyme activities

Previous work in our laboratory demonstrated that strains ZAS-1 and ZAS-2 possessed hydrogenase, formate dehydrogenase, and CO dehydrogenase, the latter a characteristic enzyme of CO_2-reductive acetogenesis via the Wood/Ljungdahl pathway (32). To further confirm the presence of this pathway in ZAS-1 & 2, enzymes associated with the conversion of formate to the methyl-group of acetate were assayed. Cell extracts of ZAS-1 and ZAS-2 displayed activities for formyl-THF synthetase, methenyl-THF cyclohydrolase, methylene-THF dehydrogenase, and methylene-THF reductase, the four characteristic enzymes of the methyl group-forming branch of the Wood/Ljungdahl pathway (Table 2.3). The formyl-THF-synthetase activity of both strains was dependent on the presence of ATP, formate, and THF. The activity of methylene-THF-dehydrogenase was NADP dependent in both strains; no activity was observed with NAD. In all cases, enzyme activity was abolished in cell extracts that had been boiled for 10 minutes.

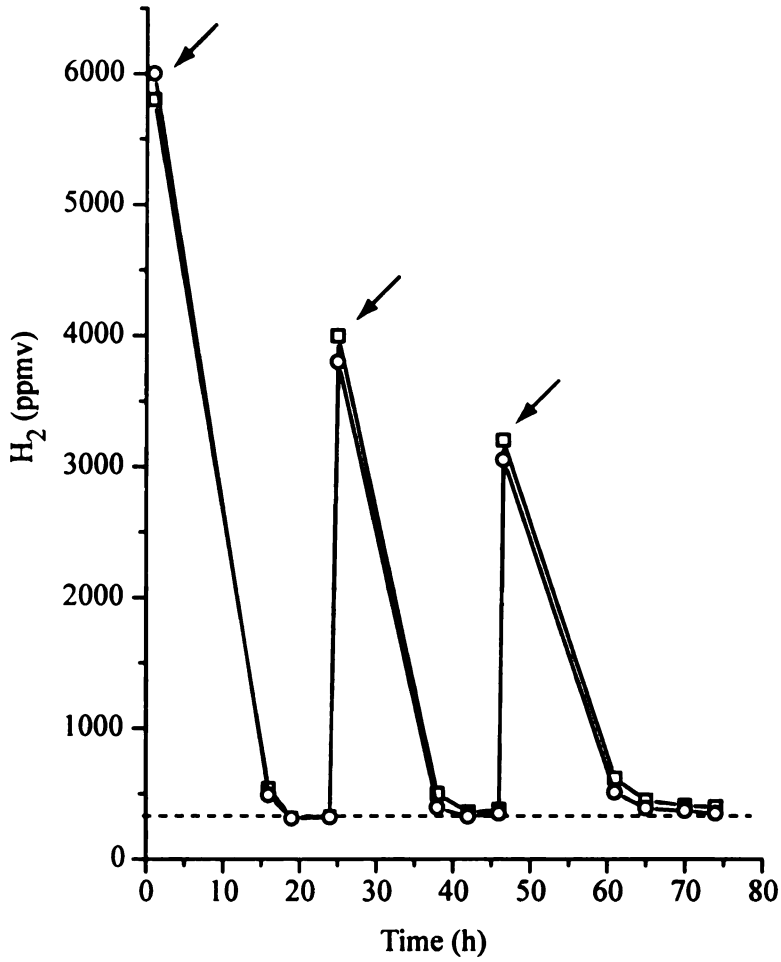

Figure 2.3. Determination of H_2 threshold for replicate cultures (□ and ○) of *Treponema* strain ZAS-2, growing under mixotrophic conditions (H_2 + CO_2 plus trimethoxybenzoate). H_2 additions are indicated by arrows. The threshold value is indicated by a dashed line.

Table 2.3. Enzyme activities relevant to H_2/CO_2-acetogenesis in *Treponema* str. ZAS-1 and ZAS-2

	Specific Activity[a]	
Enzyme	ZAS-1	ZAS-2
Hydrogenase[b]	0.47	0.45
CO dehydrogenase[b]	1.28	0.64
Formate dehydrogenase[b]	0.13	0.20
Formyl-THF-synthetase	0.76	0.71
Methenyl-THF-cyclohydrolase	0.12	0.11
Methylene-THF-dehydrogenase	0.39	0.33
Methylene-THF-reductase	0.18	0.22

[a]Specific activities are expressed as micromoles of substrate used (or product formed) per minute per milligram of protein.

[b]Activities for these enzymes were previously published in reference (32), and are included here for comparison.

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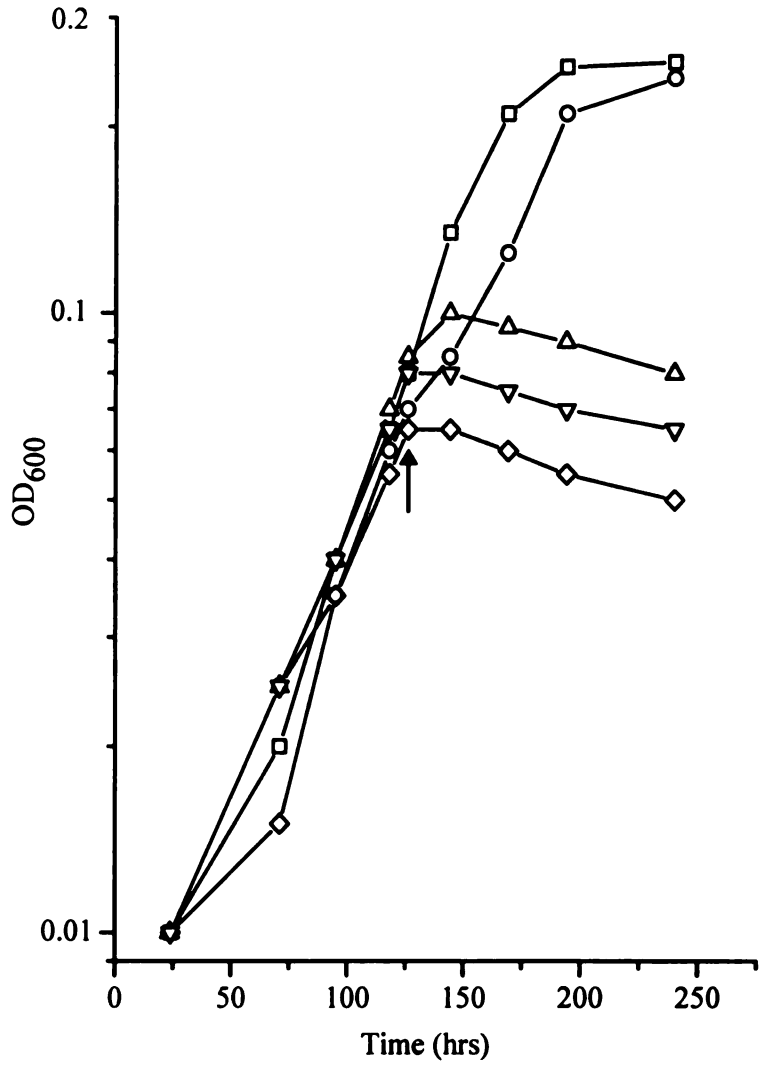

Figure 2.4. Effect of O_2 on the growth of *Treponema* strain ZAS-2. Cultures were initially grown under 80% H_2:20% CO_2, with O_2 additions made at 125 hours (indicated by arrow). The resulting concentration of O_2 in the culture tube headspace (percentage by volume) was: 0 (□), 0.5 (○), 1 (△), 2.5 (▽), and 5 (◇).

Oxygen tolerance and oxidative stress enzymes

Mid-log phase cultures of strains ZAS-1 and ZAS-2 were able to maintain growth after the addition of 0.5% O_2 to the headspace atmosphere, albeit with a slight and transient decrease in growth rate (Figure 2.4). Addition of ≥1% O_2, however, led to the rapid cessation of growth. The tolerance of cells to limited O_2 exposure prompted assays for enzymes associated with oxidative stress protection. Cell extracts of both strains showed low levels of NADH- and NADPH peroxidase, whereas neither exhibited activities of catalase or superoxide dismutase (SOD). Both strains had low levels of NADPH oxidase, but relatively high levels of NADH oxidase activity was seen only in ZAS-2 (Table 2.4). Exposure of cultures to 0.5% O_2 12 hours prior to enzyme assays did not significantly alter the levels of any of the tested enzyme activities in either of the strains, and activity of all enzymes tested was abolished in boiled cell extracts.

Discussion

Treponema strains ZAS-1 and ZAS-2 possess a variety of properties that are typical of traditional "acetogens", including the use of the acetyl-CoA (Wood/Ljungdahl) pathway for reductive synthesis of acetyl-CoA/acetate from H_2 + CO_2 for energy generation and carbon fixation (16). Operation of this pathway, first suggested by the presence of CO dehydrogenase in cells (32), was further supported in this study by the demonstration of the corresponding enzymes catalyzing conversion of CO_2 to the methyl group of acetate (Table 3). Strains ZAS-1 and ZAS-2 are also similar to other homoacetogens in being nutritionally versatile (17) and capable of homoacetogenic

Table 2.4. Oxygen and peroxide detoxifying enzyme activities in *Treponema* str. ZAS-1 and ZAS-2.

	Specific Activity[a]	
Enzyme	ZAS-1	ZAS-2
NADH Oxidase	n.d.	560
NADPH Oxidase	7	10
NADH Peroxidase	10	40
NADPH Peroxidase	3	5
Catalase	n.d	n.d
Superoxide Dismutase	n.d.	n.d.

[a]Specific activities are expressed as nanomoles pyridine nucleotide oxidized per minute per milligram protein. n.d., activity not detected.

growth on a variety of organic substrates likely to be present in the termite gut, including hexoses, pentoses, disaccharides, and (in the case of ZAS-2) the methyl group of methoxylated aromatic compounds. Although the high-molecular-weight core lignin of lignocellulose does not appear to be degraded significantly during passage through the guts of wood-feeding termites (7, 12, 20, 25), there is evidence for the demethylation of aromatic lignin moieties (20). As such, it seems likely that acetogenic spirochetes contribute to termite nutrition via acetogenesis from fermentation and demethylation of organic substrates in situ, as well as from H_2 and CO_2 produced by other members of the gut microbiota.

Strain ZAS-2 [and apparently also ZAS-1 (32)] was capable of mixotrophy, i.e. simultaneous use of $H_2 + CO_2$ and organic substrates as energy sources (Figure 2; Table 2). This capability has been demonstrated in other acetogens, including those isolated from termite guts (5). Given the seemingly constant and relatively high standing pool of H_2 in hindguts (18), mixotrophy may well be a frequent, if not constant, mode of growth for the ZAS strains in situ. Indeed, some strains may have become so adapted to the relatively high concentration of H_2 in termite hindguts that they now require it for optimal growth. This was suggested by the stimulatory effect of H_2 on utilization of organic substrates by strain ZAS-2 (Figure 1), which may be indicative of a requirement for H_2 as a low potential electron donor for some step(s) in biosynthesis. Although ZAS-2 possesses a hydrogenase (Table 3) and presumably forms some H_2 during fermentation of organic substrates, at low cell densities such free H_2 may diffuse away from cells rapidly and thereby limit the rate of growth. Only as cell density (and hence H_2-forming capacity per ml) increases and H_2 accumulates would the specific growth rate increase

incrementally. This interpretation is supported by the broadly concave shape of the growth curve of ZAS-2 on maltose in the absence of added H_2 (Figure 1).

The H_2 thresholds of ZAS-1 and ZAS-2 (490 ± 40 ppmv and 650 ± 50 ppmv, respectively) were also well within the range reported for other H_2-utlilizing acetogens (13). For ZAS-2, the H_2 threshold was somewhat lower during mixotrophic growth on H_2 plus trimethoxybenzoate (350 ± 30 ppmv), but was not lowered to a level which would make ZAS-2 competitive with H_2-utilizing methanogens under H_2-limited growth conditions. In any event, direct competition with methanogens for H_2 may be largely irrelevant; given the high H_2 concentrations measured in the central region of termite hindguts where spirochetes are most abundant [ca. 50,000 ppmv (18)], it is difficult to imagine that spirochetes are ever seriously limited for H_2 in vivo.

In contrast to other homoacetogens, the amount of energy conserved by strains ZAS-1 and ZAS-2 during H_2/CO_2 acetogenesis is rather low. Assuming that the protein content of cells is 55% of the dry mass (40), it can be calculated from the information in Table 1 that the cell yields of ZAS-1 and ZAS-2 (mg dry mass/mmol substrate) are respectively 0.1 and 0.2 during growth on $H_2 + CO_2$, and 7.9 and 6.3 for glucose. This is substantially lower than that of other acetogens, whose cell yields range from 0.5 to 4.2 with H_2 and 32.7 to 53.0 with hexoses (14, 19, 46, 53). However, this seemingly inefficient coupling of acetogenesis to growth may contribute to the effectiveness of the spirochetes as symbionts. Acetate produced by gut microbes is the primary energy source for termites (6), and ZAS-1 and ZAS-2 will generate acetate as their sole end product without a concomitantly large increase in biomass and nutrient demand that could be detrimental to the host.

Previous studies have shown that the peripheral region of termite hindguts is microoxic (9, 18). O_2 concentrations near the gut epithelium are 50-100 μM, and decrease steeply to anoxia within about 200 μm of the gut wall. It seems reasonable to assume that spirochetes inhabiting the hindgut lumen are likely to encounter this microoxic zone periodically. Although ZAS-1 and ZAS-2 are "strict anaerobes" in the traditional sense (i.e. they are incapable of growth in air), their ability to tolerate low levels of oxygen exposure could be of adaptive value in their natural habitat. Both ZAS-1 and ZAS-2 were capable of maintaining growth in the presence of 0.5% (vol/vol) oxygen (equivalent to a dissolved O_2 concentration of 6 μM). This ability suggests that the spirochetes could survive transient exposure to substantially higher concentrations of O_2 (a situation that is perhaps more analogous to that which they would encounter in vivo). Both strains possessed activities for NADH and NADPH peroxidase activities that could afford protection against H_2O_2. ZAS-2 also exhibited activity of the O_2-consuming enzyme NADH oxidase. The activities of peroxidase and oxidase were similar to those of *Brachyspira hyodysenteriae*, an aerotolerant anaerobic spirochete that colonizes the gastrointestinal tract of pigs (50). Studies have shown that free-living acetogens display varying degrees of aerotolerance and can grow under atmospheres ranging from 0.3% (*Acetobacterium woodii*) to 6% (*Clostridium glycolicum* RD-1) oxygen (28, 30). A recent study revealed a similar degree of aerotolerance, as well as active reduction of O_2 using H_2 or organic compounds as reductants, in a variety of non-spirochetal acetogens isolated from termite hindguts (3). Like the acetogens examined in these studies, both strains lack superoxide dismutase (SOD) activity. The ability to tolerate low levels of O_2 has also been observed in the termite hindgut-associated methanogens

Methanobrevibacter cuticularis and *M. curvatus* (31), suggesting that aerotolerance may be a common trait among other anaerobes in the termite hindgut.

Studies of the nutritional requirements of ZAS-1 and ZAS-2 revealed a strict requirement for folate compounds. This observation was surprising, given the fact that tetrahydrofolate plays a critical role as a one-carbon carrier in the methyl-group-forming branch of the Wood/Ljungdahl pathway (36). Most bacteria are capable of de novo folate biosynthesis, and numerous homoacetogens grown in defined media have no apparent folate requirements (23, 33, 45). By contrast, many host-associated bacteria require an exogenous source of folate, including ruminal [*Treponema bryantii* (49)] and genital-associated [*T. phagedenis* (51)] spirochetes and numerous nonspirochetal members of the bovine rumen microbial community (24, 48). The source of folate compounds for the ZAS strains in vivo is currently unknown. Although provision of folates by the termite host is a possibility (folate synthesis has been demonstrated in the mosquito, *Aedes aegypti* (27, 54, 55)), most insects require a dietary source of pre-formed folate. A more likely source of folate compounds are other members of the complex hindgut community. A variety of bacteria have been shown to secrete folate compounds in vitro (15, 26), as well as in gastrointestinal tracts (10, 11, 29, 44). This hypothesis is further explored in chapter 4.

In summary, *Treponema* strains ZAS-1 and ZAS-2 possess an assortment of nutritional and physiological properties that would appear to make them well-adapted to life as termite hindgut symbionts. In light of the phylogenetic diversity of termite gut spirochetes revealed by cultivation-independent molecular analyses (35), it seems certain that the few strains currently in culture (32, 34) offer only an introductory glimpse into

the physiological diversity of termite gut spirochetes and their importance to termite nutrition.

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Chapter 3

Taxonomy of *Treponema* stains ZAS-1, ZAS-2, and ZAS-9

Introduction

Treponema strains ZAS-1, ZAS-2, and ZAS-9 are currently the only termite gut spirochetes isolated and studied in pure culture (4, 5). The results reported by Leadbetter *et al.* (4) and in Chapter 2 of this dissertation established that strains ZAS-1 and ZAS-2 are highly similar to each other in terms of morphology, 16S rRNA similarity (98%), and in exhibiting a homoacetogenic metabolism. By contrast, strain ZAS-9 is readily distinguishable from ZAS-1 and ZAS-2 morphologically (ZAS-9 cells are longer and have shorter wavelengths) and ZAS-9 is not a homoacetogen. Moreover, the 16S rRNA sequence of ZAS-9 is only 93% and 92% similar to that ZAS-1 and ZAS-2, respectively (5). ZAS-9 also shows 10-fold higher nitrogenase activities than either of the other strains in vitro and is capable of unambiguous N_2-dependent growth, suggesting that ZAS-9 may be a more significant provider of fixed nitrogen to termites than ZAS-1 or ZAS-2 (5).

This chapter presents a more thorough characterization of the nutritional and physiological properties of strain ZAS-9 and compares a number of taxonomically relevant properties between the three ZAS strains. Overall, the results suggest that ZAS-1 and ZAS-2 should be regarded as two strains of single new species in the genus *Treponema*, whereas strain ZAS-9 should be considered a separate new species of *Treponema* distinct from that accommodating ZAS-1 and ZAS-2.

Material and Methods

Media and Cultivation Conditions

Routine cultivation of *Treponema* strains ZAS-1 and ZAS-2 was as described in Chapter 2 with one exception: in medium 2YACo, the cofactor solution was replaced with folinate (500 ng/ml). Cultivation of strain ZAS-9 was identical to the other strains, except that medium 2YACo supplemented with fermentable sugars was used.

Substrate Utilization Studies

Substrate utilization by strain ZAS-9 was determined with cells growing under an atmosphere of 80% N_2/20% CO_2 (vol/vol) except during testing of H_2 as a growth substrate (80% H_2/20% CO_2). Cultures were grown at 30°C. An increase in cell yield (>20%) in the presence of a substrate, as compared to its absence, was taken to indicate utilization of the substrate as an energy source. Cell growth was determined by measuring the optical density (OD) of cultures at 600 nm on a Milton Roy Spectronic 20 colorimeter. OD readings were converted to cell number by reference to a standard curve relating these quantities. Substrate-product carbon balances were determined under conditions of substrate-limited growth. Organic acid production was determined by using high performance liquid chromatograph (HPLC) with refractive index detection (2). Ethanol production was enzymatically measured (ethanol assay kit 332-A; Sigma-Aldrich, St. Louis, Missouri). Production of H_2 was determined by gas chromatography (3).

Genomic G+C Content Determination

The guanosine plus cytosine (G+C) content of genomic DNA was calculated from the apparent ratios of deoxyguanosine and deoxythymidine (dGuo/dThd), which were determined by using the HPLC method of Mesbah *et al.* (6), with the following modifications: genomic DNA was harvested from mid-log phase cultures by using a Qiagen genomic DNA isolation kit with a 500/G purification column (Qiagen Inc., Chatsworth, California). A Shimadzu HPLC equipped with a model SPD-10A UV/VIS detector (Shimadzu Corp., Kyoto, Japan) and an Alltech Alltima C18 column (250 x 4.6 mm, 5 μm particle size) (Alltech Assoc., Deerfield, Illinois) were used. Data was analyzed by using the Shimadzu EZ Start (v. 7.1) software package.

Results

Morphology of *Treponema* strains ZAS-1, ZAS-2, and ZAS-9

The morphology of strains ZAS-9 and ZAS-2 is depicted in Figure 3.1. Cells of ZAS-2 were 3 to 7 μm in length and had a wavelength of 2.3 ± 0.2 μm (n = 15). Strain ZAS-1 was virtually indistinguishable from ZAS-2 on a morphological basis.
In contrast, cells of ZAS-9 were longer (10 to 12 μm in length by 0.2 to 0.3 μm in width) than cells of ZAS-1 and ZAS-2 and displayed a shorter wavelength [1.1 ± 0.1 μm (n = 15)], giving the cells a more tightly coiled appearance.

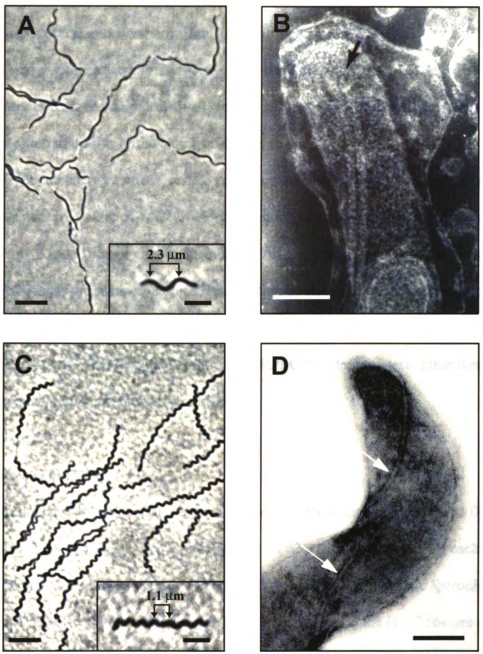

Figure 3.1. Morphology of *Treponema* strains ZAS-2 (A & B) and ZAS-9 (C and D) by phase contrast (A & C) and transmission electron microscopy (B & D). Insets in panels A and C show cells of each strain, with wavelengths indicated by arrowed bars. Periplasmic flagella are indicated by arrows in panels B & D. Strain ZAS-1 is morphologically indistinguishable from strain ZAS-2. Bars, 5 μm (A and C), 2.5 μm (A and C insets), and 0.1 μm (B & D). Panel B is adapted from reference 4, and is presented here for comparison.

Nutrition, Growth, and Fermentation Products of strain ZAS-9

Energy sources used by ZAS-9 for growth are presented in Table 3.1. Carbohydrates were the only growth-supportive substrates; no growth occurred in the presence of organic acids or the amino acid glycine. ZAS-9 was also incapable of using H_2 (+ CO_2) as an energy sources, and no enhancement of growth (in terms of rate or cell yield) was observed when H_2 was provided along with a fermentable carbohydrate (data not shown). ZAS-9 did not grow in medium 2YACo in the absence of added growth substrates. Major products of maltose fermentation by ZAS-9 were acetate, ethanol, H_2, and CO_2 (Table 3.2). The carbon and electron recoveries (90% and 88%, respectively), and the lack of any other detectable products in HPLC profiles, suggested that these were the only fermentation products of strain ZAS-9.

G+C Content of DNA

Genomic G+C content of the three ZAS strains were similar: ZAS-1, 51.04 ± 0.09 mol%; ZAS-2, 50.87 ± 0.17 mol%; ZAS-9, 50.02 ± 0.11 mol% (n=6 for each strain). G+C content was also measured for the DNA of the free-living spirochete *Spirochaeta aurantia* J1 (65.60 ± 0.05 mol%) for comparison. As a control, the G+ C content was determined for *Myxococcus xanthus* DK1622, a strain previously assayed by the same method (6); the value determined here (67.41 ± 0.07 mol%) was in good agreement with that reported in the previous study (67.55 ± 0.02 mol %). For all strains examined, peaks corresponding to the four unmodified deoxynucleosides (dGua, dThy, dAde, and dCyt) were the only peaks observed in HPLC chromatograms.

Table 3.1. Substrates Used as Energy Sources by *Treponema* strain ZAS-9

Substrate[a]	Doubling Time (h)	Yield (cells/ml)
Glucose	35	$3.1x10^8$
Fructose	37	$2.6x10^8$
Ribose	42	$1.4x10^8$
Xylose	35	$2.8x10^8$
Maltose	47	$5.6x10^8$
Cellobiose	45	$4.8x10^8$

[a]All substrates were provided at a final concentration of 5 mM except for H_2 (see methods). H_2, mannitol, arabinose, sucrose, trehalose, glycine, lactate, pyruvate, and uric acid were not utilized. No growth was observed in medium 2YACo in the absence of added substrates.

Table 3.2. Fermentation Products of *Treponema* strain ZAS-9

Product	mmol per 100 mmol maltose[a]
Acetate	280
Ethanol	80
H_2	520
CO_2	360[b]

[a]Carbon recovery = 90%; electron recovery = 88%.

[b]Assumed to be equal to the sum of acetate plus ethanol

Discussion

As reported by Leadbetter *et al.* (4) and in Chapter 2, ZAS-1 and ZAS-2 are both homoacetogens capable of growth on H_2 + CO_2 as well as a variety of organic compounds. The only significant differences observed between the two strains have been the ability of ZAS-2 to grow on methoxylated aromatic compounds (albeit very slowly) and its apparent reliance on H_2 as a growth stimulant. ZAS-1 and ZAS-2 are otherwise highly similar to each other in terms of morphology, physiology, 16S rRNA sequence, and genomic G+C content. Given these results, it seems reasonable to conclude that ZAS-1 and ZAS-2 represent two strains of a single species.

By contrast, strain ZAS-9 differs substantially from ZAS-1 and ZAS-2 in a number of taxonomically relevant properties, including cell morphology and its capacity for nitrogen fixation and N_2-dependant growth. Unlike ZAS-1 and ZAS-2, ZAS-9 is not a homoacetogen, and does not consume H_2 during growth. In fact, H_2 is a major product of fermentation by ZAS-9. ZAS-9's non-homoacetogenic nature of ZAS-9 is also supported by the fact that cells possess a hydrogenase activity (1.15 U/mg protein), but no formate dehydrogenase or CO dehydrogenase (J. A. Breznak, personal communication). The demonstration of H_2 evolution by ZAS-9 and H_2/CO_2-acetogenesis by ZAS-1 and ZAS-2 suggests that interspecies H_2 transfer between spirochetes may be an important component of H_2 turnover in the termite hindgut.

In addition to the genomic G+C content data reported here, other genomic properties of the ZAS strains have been the subject of studies by other members of our laboratory. As with G+C content, genome sizes determined by pulsed field gel electrophoresis of macrorestricted genomic DNA were fairly similar between the three

strains: ZAS-1, 3.46 Mb; ZAS-2, 3.84 Mb; ZAS-9, 3.9 Mb (Brendan Keough & Kwi Kim, personal communication). Each strain also possessed two copies of the small subunit rRNA-encoding gene (Kwi Kim, personal communication).

The *S. aurantia* J1 genomic G + C content determined here (65.6 mol%, as measured by direct HPLC analysis of deoxynucleosides) was in relatively good agreement with value previously reported by Breznak and Canale-Parola (1) for this organism as measured by the equilibrium density centrifugation (ρCsCl) method (64.5 mol%). Both values, however, were significantly higher than the G+C content inferred by the thermal denaturation (T_m) method (60.4 mol%) in the same study, in which a discrepancy of approximately 4 to 6 mol% G+C content between the ρCsCl and T_m methods was also reported for the spirochetes *S. stenostrepta* and *S. litoralis* (1). While the basis for this discrepancy is unknown, the results of this study suggest that genomic G+C contents for free-living spirochetes are more accurately measured by direct HPLC quantification of deoxynucleosides or density centrifugation than by thermal denaturation.

In summary, *Treponema* strains ZAS-1 and ZAS-2 are sufficiently similar to be considered two strains of a single new species, whereas the morphological and physiological differences displayed by strain ZAS-9 support the assignment of this strain to a separate new species. Latinate species epithets for these organisms are currently under consideration. These results also further our knowledge of diversity in termite-associated spirochetes; the previously reported morphological and phylogenetic diversity of these organisms appears to be paralleled by physiological diversity (of an as yet unknown extent). Our current understanding of the ZAS strains suggests that the primary

contribution of ZAS-1 and ZAS-2 to termite nutrition is through H_2/CO_2-acetogenesis, while ZAS-9 is likely to be of greater importance in the provision of fixed nitrogen to their hosts.

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Chapter 4

Interspecies Cofactor Transfer Supports the Folate Requirement of Homoacetogenic Termite Gut Spirochetes

Introduction

Folate is found in all living cells, playing a central metabolic role as a carrier of one-carbon (C1) groups. In particular, the synthesis of purines, pyrimidines, and several amino acids require folate cofactors (9). "Folate" is actually a generic term referring to folic acid (i.e. pteroylpolyglutamate) compounds, all of which are composed of a pteridine ring, a *p*-aminobenzoate moiety, and one or more glutamate residues (Figure 4.1). Individual folate derivatives vary in reduction state, nature and position of C1 substitution, and degree of polyglutamation. Intracellular folates generally occur at the biologically active tetrahydrofolate (THF) level of reduction, but are otherwise diverse in form and highly variable between organisms (8). While the majority of animals require a dietary source of folates, plants and bacteria are generally capable of *de novo* folate synthesis.

In Chapter 2, *Treponema* strains ZAS-1 and ZAS-2 were shown to have a strict requirement for folate. This was particularly surprising for homoacetogenic organisms, since three enzymes on the methyl-forming branch of the Wood/Ljungdahl pathway of acetogenesis require THF as a cofactor (27). Other homoacetogens appear to be capable of synthesizing folate, and a number of acetogens grown in defined media have no

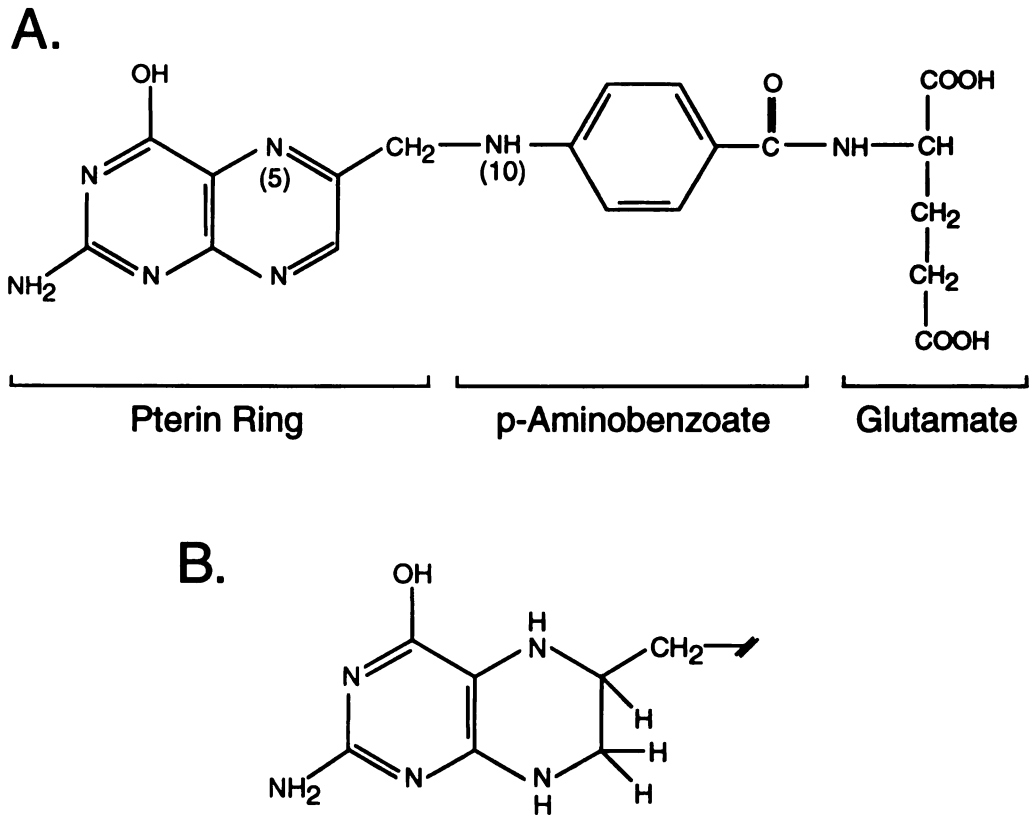

Figure 4.1. Generalized structure of folate compounds (A) and the pteridine portion of the reduced form of folate compounds, tetrahydrofolate (B). Individual derivatives vary in reduction state, C1 substitution (at N^5 or N^{10}), and number of glutamyl residues (generally from 1 to 9).

apparent folate requirements (12, 26, 39). The absence of the ability to synthesize folate, however, is not uncommon in host-associated organisms, including the spirochetes *Treponema bryantii* (42) and *T. phagedenis* (43).

In considering potential sources of folate compounds for the ZAS strains in the termite hindgut, it was hypothesized that the most likely candidates would be other members of the gut microbial community. Folate secretion has been observed in members of diverse bacterial genera, including *Bacillus*, *Pseudomonas*, *Aeromonas*, *Serratia* (18), *Propionibacterium* (16), and *Bifidobacterium* (10). Moreover, production of folate by bacteria has long been thought to play an important role animal nutrition (37) and has been clearly demonstrated in a number of gastrointestinal systems (6, 7, 22, 29, 37, 38).

Research presented in this chapter examines the folate requirements of *Treponema* strains ZAS-1 and ZAS-2 in greater detail and identifies folate-secreting bacteria from the hindgut of the termite *Zootermopsis angusticollis* (the original source of the ZAS strains) that are likely to provide folate to the ZAS strains *in vivo*.

Material and Methods

Termites

Specimens of *Zootermopsis angusticollis* were collected in the San Gabriel Mountains of southern California and maintained in the laboratory on *Pinus ponderosa* stump material in polypropylene containers. Specimens were used within one month of their collection.

Folate Compounds

Folic acid, dihydrofolate (DHF), tetrahydrofolate (THF), 5-methyltetrahydrofolate (5-CH_3-THF), 5-10-methylenetetrahydrofolate (5-10-CH_2-THF), 5-formyltetrahydrofolate (5-HCO-THF; folinate), 10-formylfolate (10-HCO-folate), and di- and triglutamate forms of folic acid were obtained from Schirck's Laboratories (Jona, Switzerland). Standard solutions of folate compounds were prepared as described in Könings *et al.* (21) and contained 10 mM 2-mercaptoethanol (final concentration) and 2% Na · ascorbate (w/v) to prevent the oxidation of reduced folate compounds. All preparative and analytical procedures involving folate compounds or culture filtrates were carried out under reduced lighting conditions to minimize photochemical degradation.

Media and Cultivation Methods

Routine growth of *Treponema* strains ZAS-1 and ZAS-2 was as described in Chapter 2, with the following exception: medium 2YACo was modified to medium 2YAFo by replacing the 11-cofactor mixture (25) with 500 ng/ml folinate (final concentration). Medium 2YA was identical to 2YAFo, but lacked folinate. Culture media for folate-excreting strains (ZFX strains) varied according to experimental conditions. Medium GM1 was adapted from a bifidobacterial enrichment medium TPY (2) and consisted of (in grams per liter): glucose, 15; tryptone (Difco), 10; phytone (BBL), 5; yeast extract (Difco), 2.5; cysteine · HCl, 0.5; K_2HPO_4, 2; $MgCl_2 \cdot 6\ H_2O$, 0.5; $ZnSO_4 \cdot 7\ H_2O$, 0.25; $CaCl_2$, 0.15; $FeCl_3$, 0.001; Tween 80 (1 ml/L). Plates for anoxic incubations were supplemented with sterile $PdCl_2$ (30 mg/L final concentration) after autoclaving. Medium GM2 contained (g/l): NaCl, 1.0; KCl, 0.5; $MgCl_2 \cdot 6H_2O$, 0.4;

$CaCl_2 \cdot 2H_2O$, 0.1; NH_4Cl, 0.3; KH_2PO_4, 0.2; Na_2SO_4, 0.15; $NaHCO_3$, 5.8; 3-N-[morpholino] propanesulfonic acid (MOPS) (10 mM final concentration) and 0.5% (v/v) yeast autolysate. Trace element solution SL11, selenite-tungstate solution, 7-vitamin solution, and vitamin-B_{12} solution were added as described by Widdel and Bak (50). Complex medium GM3 contained 0.2% yeast extract, 0.2% peptone, and 20 mM glucose. Semi-defined medium GM4 contained (g/l): NaCl, 1.0; KCl, 0.5; $MgCl_2 \cdot 6H_2O$, 0.4; $CaCl_2 \cdot 2H_2O$, 0.1; NH_4Cl, 0.3; KH_2PO_4, 0.2; Na_2SO_4, 0.15; $NaHCO_3$, 5.8; casamino acids (Difco), 5; MOPS (10 mM). After autoclaving, medium GM4 was supplemented with (g/l): glutathione, 0.01; asparagine, 0.04; glutamine, 0.04; uracil, 0.02; adenine, 0.01; guanine, 0.01; glucose (20 mM final concentration) and trace element and vitamin solutions described in Van Neil *et al.* (47). Routine cultivation of ZFX strains was on plates of Reinforced Clostridial Medium (Difco) containing 2% agar at 30°C. All described were at an initial pH of 7.2-7.4.

Enrichment, Enumeration, and Isolation of Termite Hindgut Heterotrophs

Worker larvae of *Z. angusticollis* were degutted with forceps, removing any attached midgut segments from hindguts. Two extracted hindguts were placed in 5 ml of dithiothreitol (DTT)-reduced buffered salt solution (24) and homogenized while held in an anoxic glove box (3). Enumerations were performed by preparing a serial 10-fold dilution series of gut homogenate in tubes of anoxic ZFX medium. Aliquots (0.1 ml) of the resulting dilutions were spread in triplicate on plates of GM1 medium containing 2% agar and incubated under anoxic (95% N_2: 5% H_2), hypoxic (98.5% N_2: 1.5% O_2), or oxic (air) conditions at 25°C. Colonies on the high dilution plates were enumerated, then

grouped by colony and cell morphology, and representatives were selected for folate-secretion bioassays.

Folate Secretion Bioassays

The folate secretion bioassay was a modification of the folate diffusion bioassay described by Hewitt and Vincent (15). The folate-requiring bioassay organism, *Enterococcus hirae* was obtained from the American Type Culture Collection (ATCC 8043). Liquid cultures of *E. hirae* were grown overnight in brain heart infusion (Difco) at 37°C, and 0.25 ml of the culture was transferred to 25 ml of 50% strength AOAC folate assay medium (AOAC-FA) (Difco) and was again incubated at 37°C overnight. Twenty ml of the resultant culture was added to 250 ml AOAC-FA medium containing 2% agar, which had been autoclaved and cooled to 50°C. This suspension was dispensed into Petri plates and used for bioassays. To test for folate secretion, colonies of interest were picked and patched onto the assay plates and incubated overnight under oxic, hypoxic, or anoxic conditions. Formation of satellite *E. hirae* colonies around colonies of the test organism was taken to indicate secretion of a putative folate compound(s). *Bifidobacterium infantis* strain S12 (ATCC 15697), a known folate secretor (10), was used as a positive control. Strains yielding a positive test result were designated ZFX strains, streaked for isolation, and subjected to further characterization.

Nucleotide Sequence Analysis of 16s rDNA

The 16s rDNA gene was PCR amplified from putative folate-secreting strains using primers 8f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-

GGTTACCTTGTTACGACTT-3'). Each 100-μl PCR reaction mixture contained the primers (30 pM each), deoxynucleoside triphosphates (Boehringer-Mannheim; 50 μM each), $MgCl_2$ (2 mM), 2.5 U of *Taq* polymerase (Gibco), 10 μl of PCR buffer (supplied with enzyme) and a small amount of colony material. The reactions were performed with a Gene Amp model 9600 thermocycler (Perkin-Elmer). PCR amplifications consisted of a 3-min hold at 95°C, followed by 30 cycles consisting of 30 s at 95°C, 30 s at 55°C, and 45 s at 72°C. After thermocycling, an additional extension was performed for 10 min at 72°C. Positive controls contained *E. coli* genomic DNA and negative controls contained no template. After amplification, PCR products were purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA). and subjected to restriction fragment length polymorphism (RFLP) analysis to eliminate redundancy (23). Each amplimer was digested with *Rsa*I and *Hpa*II (New England Biolabs) and the resulting fragments were resolved on 2.5% NuSieve gel (FMC BioProducts) to yield a restriction fragment length polymorphism (RFLP) pattern. One representative of each RFLP banding pattern was selected for sequencing. Nearly full length (~1500 nt) 16S rDNA sequences were determined using an ABI PRISM 3100 Genetic Analyzer and overlapping eubacterial sequencing primers: 8f, 339f, 515f, 700f, 776f, 934f, 1100f, 337r, 531r, 685r, 1100r, and 1492r. Phylogenetic analysis was performed using the Ribosomal Database Project sequence database (28) and the ARB software package (www.biol.chemie.tu-muenchen.de). Sequences were aligned using the ARB automatic aligner, followed by manual correction of ambiguous regions. A maximum likelihood method (fastDNAml) was used to generate phylogenetic trees (34).

Nutritional and growth studies

Substrate utilization was assessed by using medium GM2 as a basal medium to which test substrates were added. Media were held in rubber stoppered 18 mm anaerobe tubes (Bellco Glass; Vineland, NJ) under atmospheres of 100% N_2 or in foam-stoppered nephlometry flasks under air. Incubations were at 30°C. An increase in cell yield (>20%) in the presence of a substrate, as compared to its absence, was taken to indicate utilization of the substrate as an energy source. Cell yield was determined by measuring the optical density (OD) of cultures at 600 nm with a Milton Roy Spectronic 20 colorimeter. Soluble metabolic products were detected by high performance liquid chromatography (HPLC) with refractive index detection (3), and H_2 production was measured by gas chromatography (4).

To prepare culture filtrates of ZFX strains, sub-samples of stationary phase cultures grown in medium GM2 were supplemented with 10 mM 2-mercaptoethanol (final concentration) and 2% Na · ascorbate (w/v) and adjusted to pH 7. Samples were centrifuged at 10,000 g for 10 minutes, and supernatants were degassed, flushed with N_2, passed through a 0.2 μm syringe filter, and stored under N_2 at -80°C until analysis. Production of folate compounds supporting the growth of *Treponema* strains ZAS-1 and ZAS-2 was assessed by replacing folinate in medium 2YAFo with 10% (v/v) ZFX culture filtrates. Cultures of ZAS-1 and ZAS-2 that served as inocula in these experiments were grown in folinate-free 2YA medium through two transfers to reduce residual folinate carried over with the inoculum.

Identification of Folate Compounds

Identification of unknown folate compounds was done by comparing HPLC retention times with those of folate standards. The HPLC protocol used was a modification of the procedure described by Pheiffer *et al.* (35). Folates were separated by using a Shimadzu HPLC system (Shimadzu Corp., Kyoto, Japan) equipped with an Alltech Alltima C_{18} column (250 x 4.6 mm, 5 μm particle size) (Alltech Assoc., Deerfield, Illinois). Gradient elution was performed with acetonitrile and 33 mM phosphoric acid (pH 2.3) was performed as follows: acetonitrile was held at 5% for the first 9 minutes, then was raised linearly to 7% over the next 13 min, then raised linearly to 16% for the next 9 min, held at 16% for 14 min, then raised to 25% over two min, held at 25% for 5 min, and finally decreased to 5% acetonitrile within 3 min. The flow rate was 0.8 ml/min, and sample injection volume was 20 μl. Separated folate compounds were monitored by using a Shimadzu model SPD-10A UV/VIS detector set at 280 nm and a model RF-10A fluorescence detector set 280 nm excitation and 356 emission wavelengths for reduced folate compounds and 360/460nm for 10-HCO-folate (20). Data was analyzed with the Shimadzu EZ Start (v. 7.1) software package. Folate concentrations reported for culture filtrates are corrected for folates carried over in culture inocula.

Rat plasma conjugase (RPC) was used to remove excess glutamate residues (i.e. more than a single glutamate) from folate compounds, as these interfere with HPLC analysis. RPC was prepared as described in Pfeiffer *et al.* (35) using rat plasma obtained from Pel-Freez Biologicals (Rogers, AR). Activity of the crude RPC preparation was

confirmed as described previously (35). ZFX strains were grown in medium GM4, and filtrates were collected as described above. RPC preparation was added to filtrates at 5% (v/v), and the mixture was incubated at 37°C for one hour, heated to 100°C for 5 min, and cooled on ice for five minutes. Samples were centrifuged at 10,000 x *g* for 10 minutes at 5°C. Supernatants were collected and stored at -80°C until analysis.

Results

Folate Requirements of *Treponema* strains ZAS-1 and ZAS-2

Treponema strains ZAS-1 and ZAS-2 were tested for their ability to use folates in the three major states of reduction (folic acid, DHF, and THF) and folinate (5-HCO-THF) (Figure 4.2). ZAS-2 was able to use all of the folates tested, whereas ZAS-1 grew only when provided with THF or folinate. Growth of ZAS-1 appeared to be inhibited in the presence of folate and dihydrofolate compared to the limited growth seen in the negative control cultures. Addition of 500 ng/ml folinate to these "inhibited" cultures eliminates this effect (data not shown), suggesting that the relatively large pools of folate and dihydrofolate may bind to (and thus rendered inaccessible) the small amounts of growth supportive folinate carried over with the inoculum (36). Provision of *p*-aminobenzoate, glutamate, various pteridines (pterin, 6-hydroxymethlypterin, pteroic acid), and the pterin precursor guanosine 5'-triphosphate (GTP) did not alleviate the requirement of either strain for preformed folates.

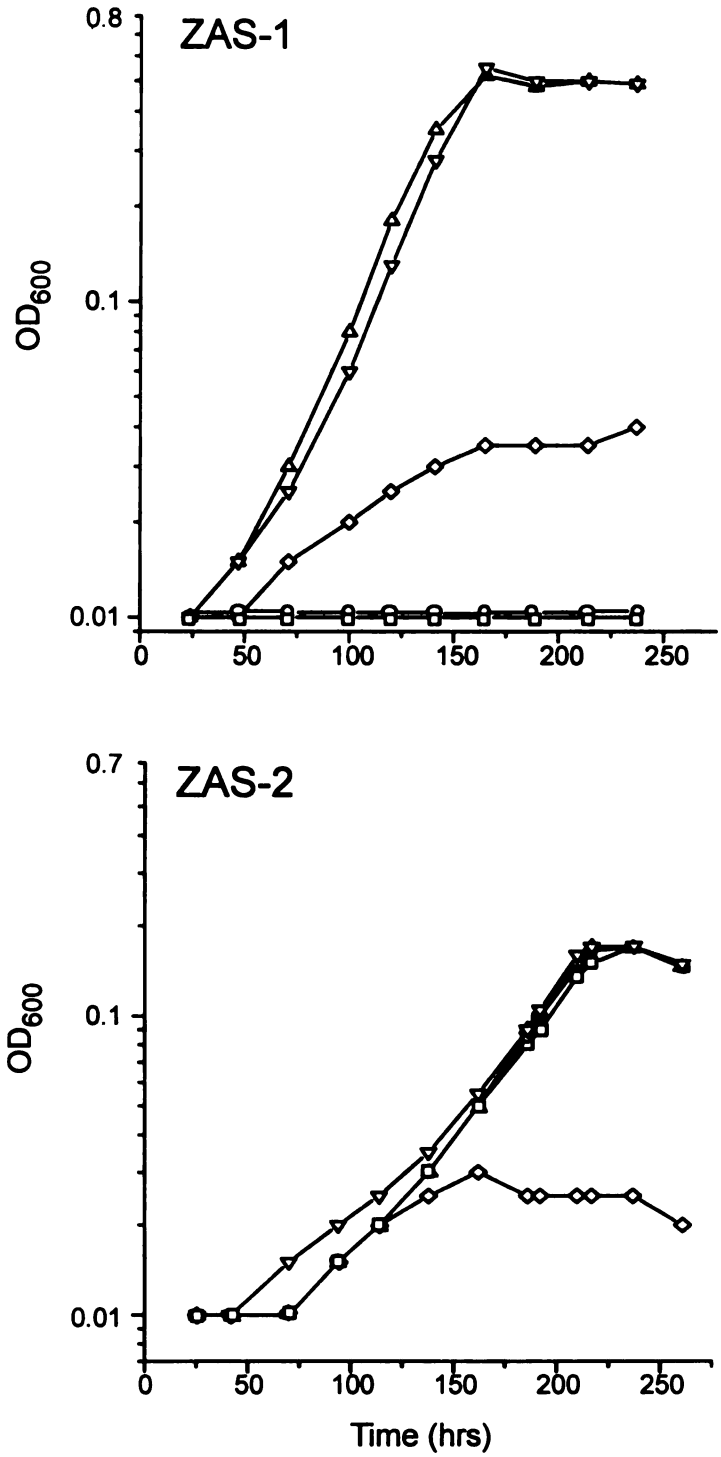

Figure 4.2. Folate utilization by *Treponema* strains ZAS-1 and ZAS-2. All folate compounds were provided at a final concentration of 500 ng/ml. The specific compounds were: folic acid (□), dihydrofolate (○), tetrahydrofolate (△), 5-formyltetrahydrofolate (i.e. folinic acid) (▽), and no addition (◇).

Isolation of Folate-Secreting Bacteria from Termite Hindguts

Colonies derived from the highest dilutions of *Z. angusticollis* gut homogenates were sorted on the basis of morphological characteristics (both colonial and microscopic) and incubation condition (oxic, anoxic, or hypoxic). A total of 32 colonies were then bioassayed for folate secretion by using *Enterococcus hirae*, an organism with a strict folate requirement. Eighteen of the screened colonies supported the growth of satellite colonies of *E. hirae* in the folate-free bioassay medium, implying secretion of a putative folate into the surrounding medium (Figure 4.3). These colonies were designated "ZFX" strains. *Bifidobacterium infantis* str. S12, a known folate secretor (10), was used as a positive control organism in bioassays. The diameter of the zone of *E. hirae* satellite colonies surrounding ZFX colonies was similar to that observed surrounding *B. infantis* S12 colonies. Of the colonies yielding negative results, thirteen failed to grow on the assay plates (perhaps indicating a requirement for folate in these isolates) and one grew but did not support the growth of *E. hirae*. Colonies yielding positive results were streaked for isolation and subjected to further characterization.

Characterization of Folate-Secreting Strains

16S rDNA genes were PCR amplified from genomic DNA of the eighteen putatively folate-secreting isolates using bacteria specific primers. Analysis of the eighteen clones revealed two distinct RFLP banding patterns. Nearly complete 16S rDNA sequences were obtained for one isolate representing each RFLP pattern, designated strains ZFX-1 and ZFX-2. Phylogenetic analysis of these sequences indicated that strain ZFX-1 grouped with the genus *Serratia,* whereas ZFX-2 grouped within the

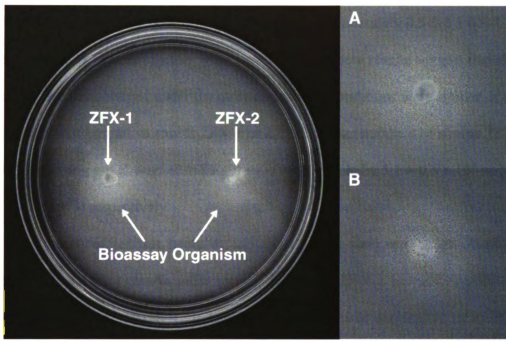

Figure 4.3. Folate secretion assay of strains ZFX-1 and ZFX-2. Putative folate secretion is indicated by the formation of satellite colonies of the folate-requiring bioassay organism, *Enterococcus hirae,* surrounding surface colonies of ZFX-1 (inset A) and ZFX-2 (inset B).

genus *Lactococcus* (Figure 4.4). ZFX-1 had 99.7% 16S rRNA sequence similarity to *Serratia grimesii* and 99.1% similarity of *Serratia liquefaciens*. Strain ZFX-2 was 99.9% similar in 16S rRNA sequence to *Lactococcus lactis* subsp. *lactis*.

All ZFX-1 and ZFX-2 type isolates grew well on plates incubated under oxic, anoxic, and hypoxic conditions. Cells of all strains corresponding to the ZFX-1 RFLP pattern were motile gram-negative rods approximately 0.6-0.8 x 1.5-2 μm in size, and all ZFX-2 type isolates were non-motile coccoid cells approximately 0.5-0.8 x 0.9-1.1 μm in size (Figure 4.5). Taken together with the RFLP data, these results suggest that all folate-secreting isolates obtained under the three enrichment conditions were similar, if not identical, to representative strains ZFX-1 or ZFX-2. Mean numbers of strains ZFX-1 and ZFX-2 occurring per *Z. angusticollis* gut were estimated to be $5.1 \pm 0.6 \times 10^5$ and $5.4 \pm 0.7 \times 10^5$ (n = 3) respectively.

A variety of hexoses, pentoses, and disaccharides were used as growth substrates by both strains during growth under aerobic conditions; ZFX-1 was additionally capable of using organic acids (Table 4.1). During anaerobic growth on glucose, strain ZFX-1 produced a mixture of succinate, acetate, ethanol, 2-3 butanediol, and H_2. No organic products were detected during aerobic growth of ZFX-1 on glucose. Under both oxic and anoxic conditions, ZFX-2 produced lactate as the sole end product from glucose. Lactate production was stoichiometrically consistent with glucose consumption, and the fermentation pattern was not altered during growth in a complex medium (GM3).

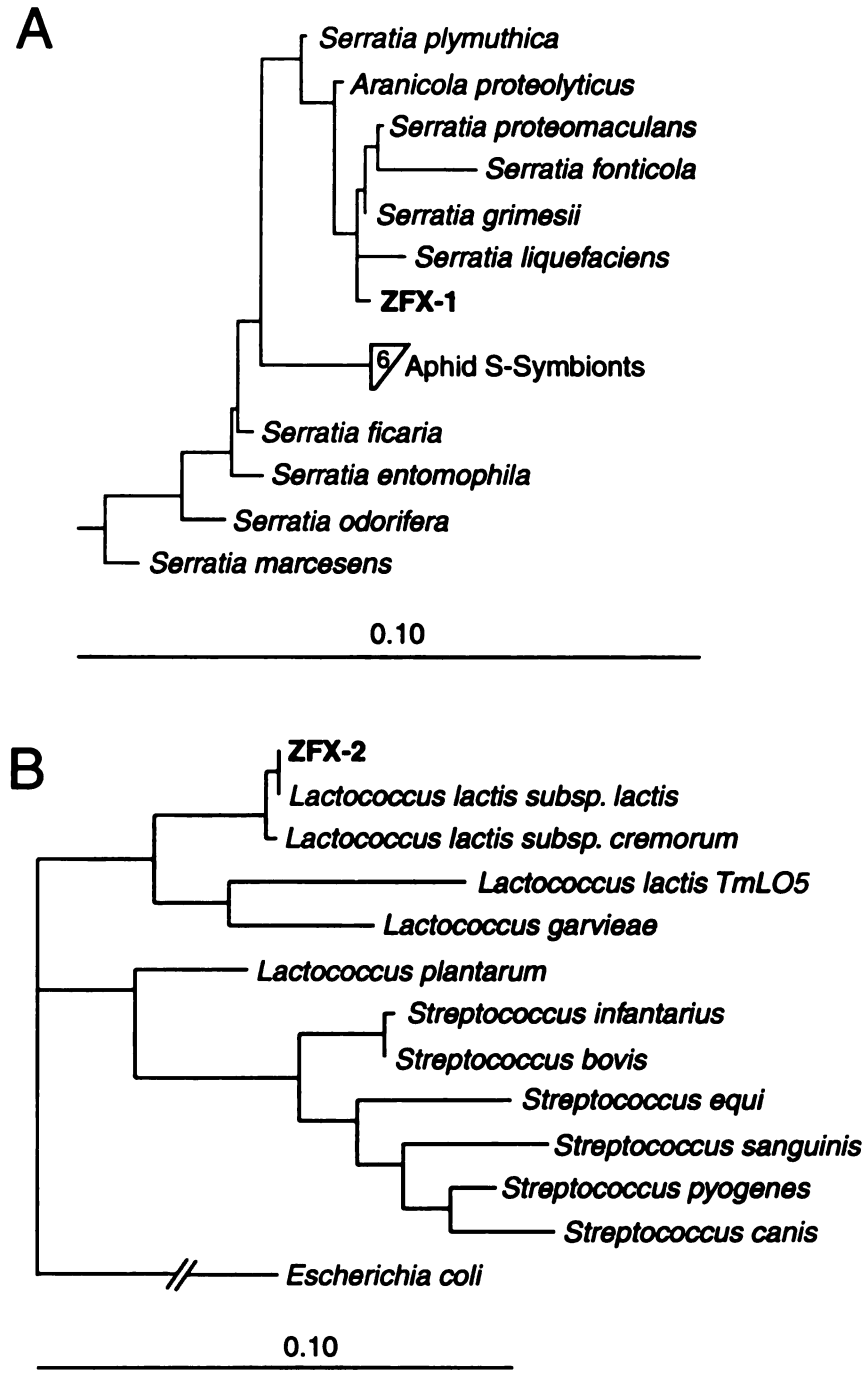

Figure 4.4. Phylogenetic trees inferred from 16S rDNA sequences (~1500 nt) of ZFX-1 (A), ZFX-2 (B), and related organisms. A maximum likelihood technique (fastDNAml) was used to generate the trees. The homologous sequence from *Desulfovibrio senezii* was used as an outgroup in tree A (not shown). The scale bars represent units of evolutionary distance and are based on sequence divergence.

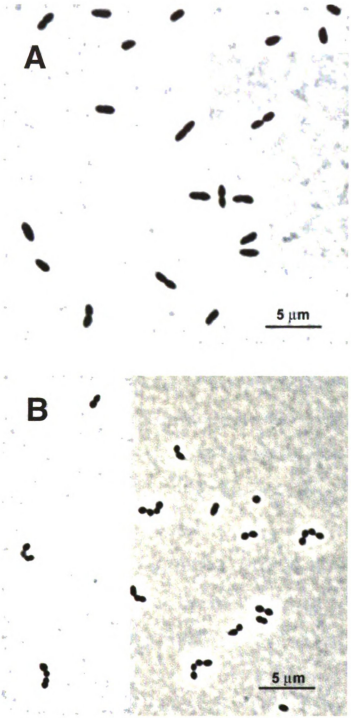

Figure 4.5. Phase contrast micrographs of *Serratia* strain ZFX-1 (A) and *Lactococcus* strain ZFX-2 (B).

Table 4.1. Substrate Utilization by *Serratia* strain ZFX-1 and *Lactococcus* strain ZFX-2

Substrate[a]	Strain ZFX-1 Doubling Time (h)	Strain ZFX-2 Doubling Time (h)
Glucose	1.30	0.73
Mannitol	1.45	0.73
Ribose	2.65	0.81
Xylose	1.92	0.82
Maltose	1.22	0.75
Cellobiose	n.u.	0.85
Trehalose	1.11	0.81
Acetate	1.85	n.u.
Pyruvate	1.58	n.u.
Lactate	1.67	n.u.
Fumarate	1.59	n.u.
Malate	1.50	n.u.
Succinate	1.18	n.u.

[a]All substrates were provided at a final concentration of 10 mM except for methoxylated aromatic compounds (2mM). Substrates tested but not utilized are indicated by n.u. Arabinose, glycine, formate, methanol, ferulate, vanillate, syringate, and trimethoxybenzoate were not utilized by either strain.

Folate Production by *Serratia* strain ZFX-2 and *Lactococcus* strain ZFX-1

To characterize the putative folate compound(s) secreted by the ZFX strains, cultures were grown in anoxic semi-defined medium GM4. Culture filtrates were collected at the time of inoculation (i.e. 0 hrs.) and at the end of logarithmic growth (9 hrs. for ZFX-1, 7 hrs. for ZFX-2). Reducing agents (2-mercaptoethanol and Na·ascorbate) were immediately added to protect folates from oxidative damage, and filtrates were then treated with rat plasma conjugase (RPC) to remove excess glutamate residues prior to analysis.

Good resolution of folate standards was observed in HPLC analysis using fluorescence detection for reduced folates and UV absorbance for unreduced forms (see methods and Figure 4.6). A distinct peak with a retention time identical to that of authentic folinate (i.e. 5-HCO-THF) was observed in culture filtrates of both ZFX strains (Figure 4.6). Concentrations of folinate in culture fluids of ZFX-1 and ZFX-2 at the end of growth were 146 ng/ml and 117 ng/ml, respectively (corrected for folinate carried over in inocula). An additional fluorescent compound with a retention time of 31 minutes was produced during growth of ZFX-1; the identity of this compound is unknown, but it did not correspond to any standard folate compound tested under the three detection methods. No other peaks corresponding to folates were observed using any of the three detection methods, suggesting that folinate is the sole folate compound secreted by strains ZFX-1 and ZFX-2 at significant levels.

Provision of 10% culture filtrates (v/v) of strains ZFX-1 and ZFX-2 supported the growth of *Treponema* strains ZAS-1 and ZAS-2 in the absence of added folate compounds (Figure 4.7). The filtrates used in these experiments were not treated with

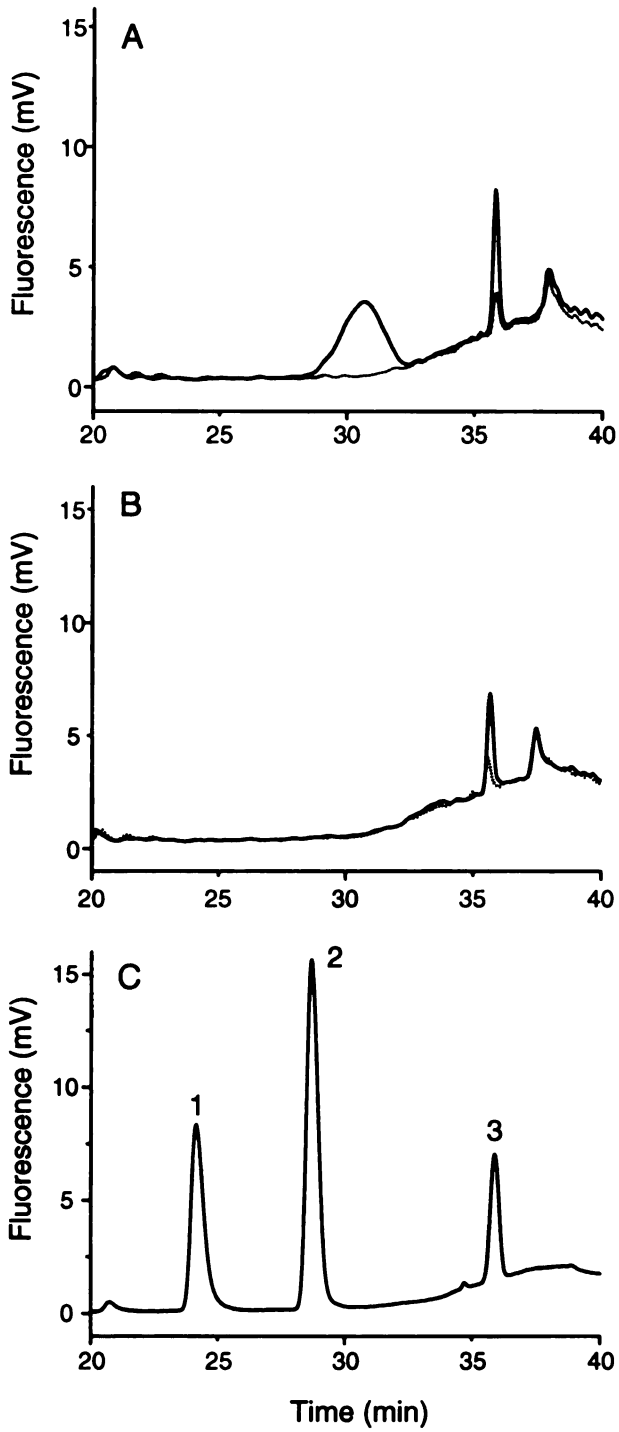

Figure 4.6. HPLC chromatograms showing fluorescent (280/359 nm) compounds present in culture filtrates of strains ZFX-1 (A) and ZFX-2 (B) and for a standard mixture (C) of THF (peak 1, 1 ng), 5-CH_3-THF (peak 2, 1 ng), and 5-HCO-THF (peak 3, 5 ng). In panels A & B, dashed and solid lines indicate samples taken at the time of inoculation and at the end of logarithmic growth, respectively. The peak eluting at 31 minutes in panel A could not be identified, but did not correlate to any folate standard.

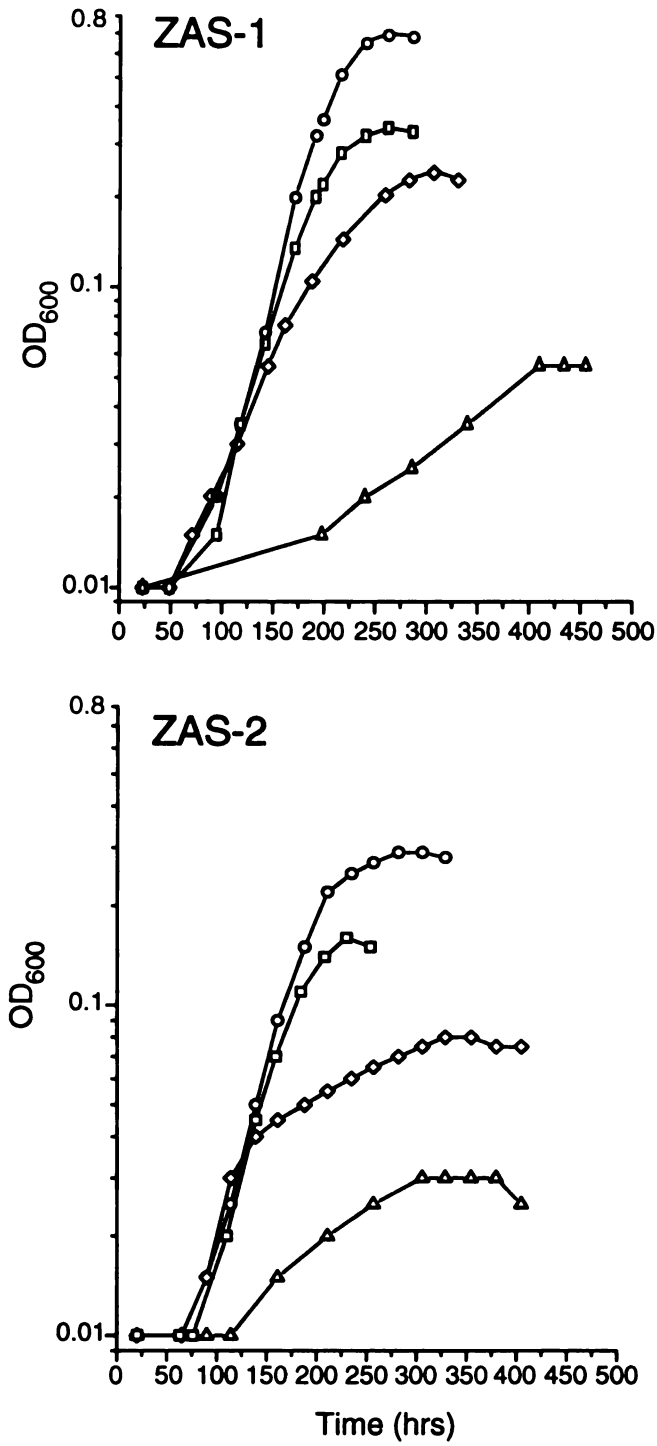

Figure 4.7. Growth of *Treponema* strains ZAS-1 and ZAS-2 in folate free media supplemented with folinate (O), cultures filtrates of *Serratia* strain ZFX-1 (◇) and *Lactococcus* strain ZFX-2 (□). Culture filtrates were added at 10% (v/v) final concentration. Folinate was provided at 500 ng/ml final concentration. Negative controls (△) were supplemented with 10% uninoculated GM3 medium.

RPC, indicating that ZAS-1 and ZAS-2 are capable of using the folinate compounds secreted by the ZFX strains in their native states of polyglutamation. Provision of ZFX culture filtrates did not replace the requirement of either spirochete for yeast autolysate in medium 2YACo, and addition of ZAS culture filtrates did not result in any discernible enhancement of folate production by either ZFX strain (date not shown).

Discussion

The observed folate requirement of *Treponema* strains ZAS-1 and ZAS-2 *in vitro* suggested that a source of folate compounds must exist within the termite hindgut. Given the critical nature of this cofactor in the homoacetogenic metabolism of these organisms [(27); Chapter 2] and the importance of CO_2-reductive acetogenesis in termite nutrition (5, 45), determination of the source of folates *in situ* was deemed important to a better understanding of the physiological ecology of the ZAS strains and the termite hindgut symbiotic system as a whole.

There are several potential sources of folate in termite hindguts. Since plants are capable of folate synthesis, wood ingested by termites is one possibility. However, the primarily structural role of lignocellulose in plants suggests that this material is likely be relatively low in folate, since this cofactor is normally involved in active biosynthetic processes. The termite host itself is also potential source, although the ability of termites to synthesize folate compounds is unknown. *De novo* synthesis of folate has been demonstrated in mosquito muscle tissue (19, 48, 49), but the majority of insects require a dietary source of folates. In any event, it seems unlikely that the gut microbial

community would have significant access to folate pools existing within host cells. As such, the most likely source of folates would be other microbes inhabiting the hindgut.

Folate synthesis is a common capability in bacteria, and secretion of folate compounds has been demonstrated in a variety of bacterial species, including members of the genera *Bacillus*, *Pseudomonas*, *Aeromonas*, *Serratia* (18), *Propionibacterium* (16), and *Bifidobacterium* (10). Bacterial folate production has been observed in a variety of gastrointestinal systems (7, 29, 37), and host uptake of bacterially produced folates has been unambiguously demonstrated in rat and human gastrointestinal systems (6, 38). The presence of folate-requiring bacteria in the bovine rumen (14, 41, 42) and observation of THF production in rumen enrichment cultures (41) suggests that extensive folate cross-feeding also occurs in the bovine rumen.

Folate secretion by *Z. angusticollis* gut isolates *Serratia* strain ZFX-1 and *Lactococcus* strain ZFX-2 was demonstrated by both microbiological assay with *E. hirae* and by direct detection of folate compounds in ZFX culture filtrates. Folate concentrations in ZFX culture fluids were within the range observed for other folate secreting bacteria, which secrete folates to concentrations 20-160 ng/ml in culture fluids (10, 16, 18). Levels of folate production similar to those of ZFX-2 (100 ng/ml) have been reported in *Lactococcus lactis* strain MG1363. In contrast to ZFX-2 though, 90% of the folate produced by strain MG1363 is accumulated intracellularly rather than being secreted into the environment (17). Levels of folate production and secretion also vary widely between individual species and strains of propionibacteria and bifidobacteria (10, 16). It has been hypothesized that strains showing the highest levels of folate secretion

lack feed-back inhibition mechanisms controlling folate release, a trait which may be selected for in densely colonized gastrointestinal environments (10).

Although no *Serratia* strains have been identified in recent culture collections (11, 40, 44) or molecular studies (32, 33) of *Reticulitermes* sp. termite gut bacteria, several strains identified as pathogenic *Serratia marcescens* have been isolated from *Z. angusticollis* (13). However, the termites from which strain ZFX-1 was isolated appeared to be in good health, and no subsequent signs of disease were observed in other members of the same colony. These observations, and the presence of a relatively large population of strain ZFX-1 in the gut (~5 x10^5 cells per gut), suggests that ZFX-1 is more likely to be a beneficial member of the hindgut microbiota. Strain ZFX-1 showed 96% sequence similarity to the 16S rRNAs of a number of secondary symbionts (S-symbionts) of aphids (46) (Figure 4.4). The role of S-symbionts in aphids is currently unknown, but the primary aphid symbiont *Buchnera* has been implicated in the provision of the vitamin riboflavin to their hosts (30). The observation of folate secretion by *Serratia* strain ZFX-1 raises the possibility that the related aphid S-symbionts could also be involved in the provision of vitamins to their hosts.

Lactic acid bacteria (LAB), including *Lactococcus* species similar to strain ZFX-2, are frequently cultured from termite hindguts (1, 11, 40, 44). The population levels determined for strain ZFX-2 in the *Z. angusticollis* hindgut are consistent with numbers of LAB observed in other termite species. LAB are estimated to represent approximately 3% of the total gut bacterial count in the termite *Reticulitermes flavipes* (44). Lactococci isolated from the guts of other termites exhibit a heterolactic fermentation under anoxic conditions (producing lactate plus smaller amounts of ethanol, acetate, and formate) and

shift their fermentation pattern to favor mostly acetate production under oxic conditions (1, 44). In contrast, strain ZFX-2 performs a homolactic fermentation of glucose under both oxic and anoxic conditions. The lack of some medium component required for acetate formation seems unlikely, since medium GM2 is similar in composition to the culture medium used in previous studies of lactococci (1, 44), and growth on a more complex media (GM3) did not result in any change in the fermentation pattern of ZFX-2. The inability of strain ZFX-2 to shift to the more energetically favorable fermentation pattern favoring acetate production could indicate a lesion in a component of the pyruvate dehydrogenase complex. Given the wide distribution of LAB among diverse termite species (1), it is possible that these organisms are significant folate providers in many hindgut systems. Considering to high degree of variability in folate secretion observed among closely related strains of other folate-secreting bacteria though, it would be necessary to evaluate folate secretion in strains isolated from diverse termites to determine if this is a common property of termite-associated *Lactococcus* species.

The relatively high levels of folinate secretion observed in strains ZFX-1 and ZFX-2 suggests that these organisms represent the major source of folate for *Treponema* strains ZAS-1 and ZAS-2 in the *Z. angusticollis* hindgut. *In situ* confirmation of this hypothesis, however, presents a considerable technical challenge due to the very small volume of extracellular fluid in termite hindguts (0.27-3.2 μl) (31) and the likelihood that secreted folates are efficiently absorbed by folate-requiring members of the dense gut microbial community. In mammalian gastrointestinal systems, investigators have tracked the incorporation of radiolabelled *p*-aminobenzoate into folate compounds, demonstrating *in situ* bacterial production and host-uptake of folates (6, 38). This method could prove

useful in confirming bacterial folate synthesis in termite hindguts and determining the *in situ* role of folate-secretors in providing this vitamin to their termite hosts, as well as to other members of termite hindgut microbial communities.

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Chapter 5

Acetogenesis vs. Methanogenesis in the Termite Hindgut: The Lumazine Hypothesis

Introduction

The puzzling dominance of acetogenesis over methanogenesis in guts of wood-feeding termites was discussed in Chapter 1. To summarize, bacterial H_2/CO_2-acetogens and archaeal methanogens are both present in termite hindguts, but the former clearly out-process the latter as H_2-consumers, as indicated by: i) the low rates of H_2 or methane emission measured in most wood-feeding termites (3, 35); ii) the increased rate of methane emission observed after feeding termites antibacterial drugs (35); and iii) the preferential H_2-dependent reduction of $^{14}CO_2$ to acetate, rather than methane, in termite gut homogenates (5). The dominance of H_2/CO_2-acetogenesis in termite hindguts is surprising, since methanogens utilize a more energetically favorable form of metabolism and are the dominant H_2-consumers in most anoxic habitats in which CO_2/HCO_3^- is the primary terminal e^- acceptor (2, 7, 13, 33, 37). While unusual, the dominance of termite CO_2-reductive acetogenesis as an electron sink is highly beneficial to termites: acetate production in the hindgut fuels the majority of the insect's energy metabolism (35), whereas CH_4 emission represents a loss of otherwise useful carbon and energy. Explaining this peculiar situation is important in understanding the functional ecology of the termite hindgut community.

The spatial resource partitioning (SRP) hypothesis (10, 15) discussed in Chapter 1 offers the best current explanation for the dominance of H_2/CO_2-acetogenesis in termite hindguts. In this model, methanogens and acetogens occupy distinct physiochemical niches within the hindgut and hence do not directly compete for H_2. In most termites, methanogens appear to be restricted to the region of lowest hydrogen concentration on the gut epithelium (22, 23), where they may be additionally inhibited by inwardly diffusing O_2 (9). In contrast, homoacetogens such as *Treponema* strains ZAS-1 and ZAS-2 colonize the anoxic lumen of the hindgut, where hydrogen production by gut protozoa and other luminal microbes maintains H_2 at levels 10 to 100 fold higher than the threshold for H_2/CO_2-acetogenesis (15). Although the SRP hypothesis helps to reconcile the prevalence of acetogenesis in termite hindguts, it offers no explanation for the restriction of methanogens to the gut epithelium, nor does it explain why acetogens continue to dominate H_2-consumption in termite gut homogenates, wherein the spatial distribution of microbes is homogenous and all members should have equal access to H_2 (3, 5). While the spatial separation of acetogens and methanogens undoubtedly contributes to the dominance of acetogenesis as the primary H_2-sink, it seems likely that some other factor(s) also affects this outcome. It is possible that methanogens are in some way prevented from colonizing the H_2 rich gut lumen, perhaps due to the presence of some inhibitory compound.

Pteridines are a class of fluorescent compounds possessing a pyrimidine-pyrazine ring structure. Most naturally occurring pteridine compounds are 2-amino-4-hydroxy derivatives and are collectively referred to as pterins (Figure 5.1A). Pterins are involved in a variety of physiological functions, and all living organisms appear to contain at least

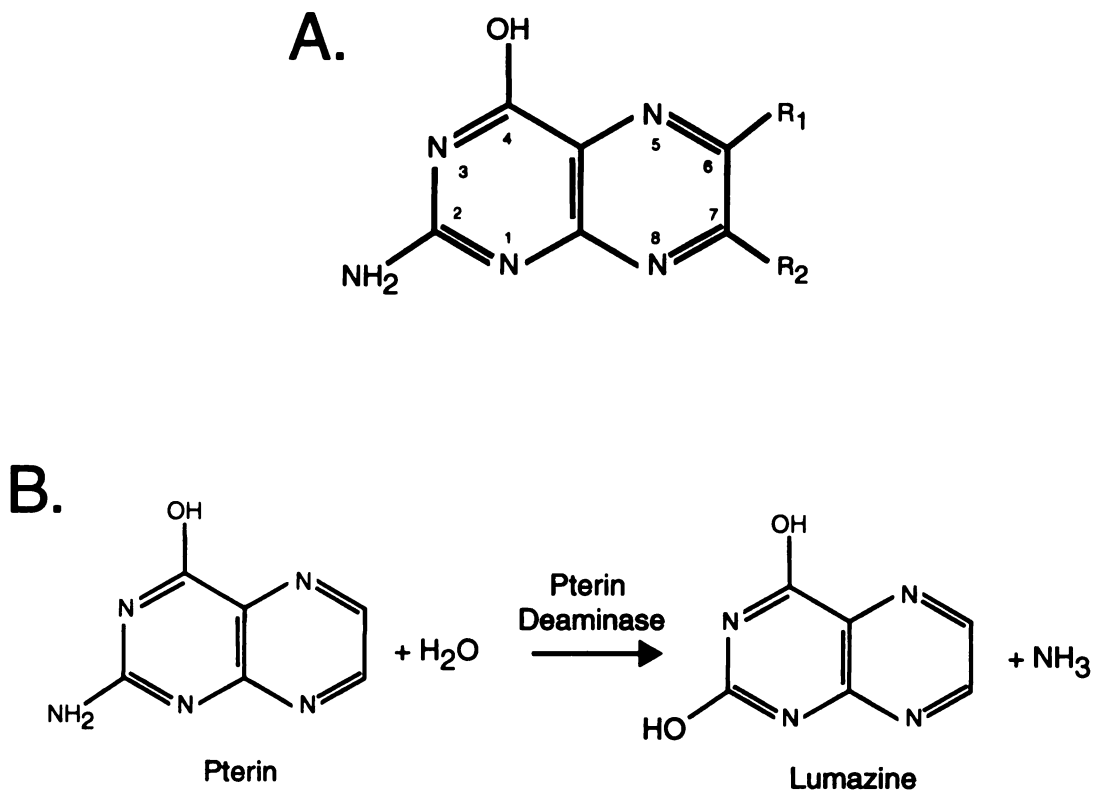

Figure 5.1. Generalized chemical structure of pterin compounds (A) and production of lumazine from pterin via enzymatic deamination (B).

trace amounts of them. Insects, however, contain higher concentrations of pterins than most organisms and seem to have developed more varied physiological functions for these compounds (49). Insects are capable of synthesizing a wide variety of pteridine compounds (19, 46, 47), which play significant roles in pigmentation (1, 29, 31, 40), nitrogen storage and excretion (11, 18), and development (20, 30).

Although there have been no studies of pteridines in termites, these compounds could play a significant role in the competition between hindgut acetogens and methanogens: the pteridine compound lumazine has been demonstrated to be a potent inhibitor of methanogenesis (34). Lumazines are usually formed by the enzymatic deamination of a pterin (Figure 5.1B) (38). Lumazine compounds (1, 16, 17, 36) and pterin deaminase activities (17, 43-45) have been detected in a wide range of insect species, and various bacteria have also been shown to possess pterin deaminase activities (26-28, 41). Nagar-Anthal *et al.* (34) demonstrated that relatively low concentrations of lumazine (0.09-0.6 mM) completely inhibited growth and methanogenesis in phylogenetically diverse methanogens, whereas much higher concentrations had no apparent inhibitory effects on non-methanogenic organisms, including H_2/CO_2-homoacetogens. The compound tested in that study, lumazine (2, 4-pteridinedione), is the deaminated derivative of unsubstituted pterin; other lumazine and pteridine compounds were not examined for anti-methanogenic properties.

The observation that lumazine inhibited methanogenesis, in conjunction with the known importance of pterin metabolism in insects, raises the possibility that lumazine (or other pteridine compounds) function as inhibitors of methanogenesis in hindguts of wood-feeding termites. In this model, the termite host would excrete lumazine (or related

pterins, which could be subsequently deaminated by gut bacteria) into the hindgut. The presence of this compound in the extracellular gut fluid would act to inhibit methanogens in the H_2 rich gut lumen, leaving homoacetogens as the primary H_2-consuming group. This would also potentially explain the restriction of methanogens to the gut epithelium, since organisms living in biofilm communities are typically less susceptible to the action of inhibitory compounds (12, 48).

In this chapter, the lumazine hypothesis is investigated in three ways: i) the susceptibility of the termite gut methanogen *Methanobrevibacter filiformis* to inhibition by lumazine, other pteridine derivatives, and termite gut extracts is evaluated; ii) the acetogenic termite gut spirochetes, *Treponema* strains ZAS-1 and ZAS-2 are screened for excretion of pteridines and the ability to deaminate exogenous pterins; and iii) gut extracts of the termite *Reticulitermes flavipes* are screened for the presence of lumazine and other pteridine derivatives.

Material and Methods

Organisms, Media, and Cultivation Conditions

Treponema strains ZAS-1 and ZAS-2 and *Methanobrevibacter filiformis* (DSM 11501) were grown in medium 2YACo (24) as described in Chapter 2, with the following modification: the cofactor mixture was either omitted (*M. filiformis*) or replaced with 500 ng/ml folinate (strains ZAS-1 and ZAS-2). All strains were cultivated under anoxic conditions with atmospheres containing 80% H_2 and 20% CO_2.

Termite Gut Extract Preparation

Reticulitermes flavipes worker termites were collected in Dansville, Mich. as described in Chapter 4 and used within 24 hours. Termites were chilled to 4°C and degutted by using forceps (4). To estimate the concentration of *R. flavipes* gut extract that would yield measurable amounts of pteridine compounds, termite biomass was compared with that of *Pyrrhocoris apterus*, an insect in which pteridine concentrations have been quantified (36). A total of 300 extracted guts were pooled in a glass tissue homogenizer containing 1.5 ml of 50 mM $Na_2CO_3/NaHCO_3$ buffer (pH 10). The extraction of pteridine compounds from gut homogenates was a modification of the procedure described in Melber *et al.* (30). Homogenates were heated to 100°C for one minute and immediately cooled on ice, followed by the addition of 5 ml of chloroform. Suspensions were mixed by vortexing and centrifuged for 10 min at 3000 x *g*. The upper aqueous phase (containing pterins) was removed and added to 0.2 ml of chloroform, mixed, and centrifuged for 5 min at 10,000 x *g*. The upper aqueous phase was collected and stored at -80°C until analysis. All preparations were carried out under reduced lighting conditions.

***Treponema* strain ZAS-1 and ZAS-2 Pterin Deaminase Assays**

Pterin compounds (0.1 mM, final concentration) were added to cultures of ZAS-1 and ZAS-2 at the time of inoculation. At the onset of stationary phase, culture filtrates were obtained by centrifuging cells for 10 min at 10,000 x *g* and collecting the supernatants, which were then neutralized, degassed, and passed through a 0.2 μm pore

size syringe filter into a pre-sterilized sealed tube filled with N_2. Pterin deaminase activity was assessed by TLC analysis of filtrates (see below) to detect any shifts in R_f value or coloration consistent with conversion of the pterin compound to its lumazine derivative.

Methanogen inhibition assays

The effects of various pteridine compounds and *R. flavipes* gut extracts on *M. filiformis* were tested in replicate (n=2-4) liquid cultures. Methane production was assessed by gas chromatography as described previously (35). Pteridines, gut extracts, or ZAS strain culture filtrates were added to cultures after the onset of growth (as determined by optical density readings) and methane production. Termite gut extracts were added to culture tubes at 5% (vol/vol) final concentration, a dose based on comparison with potentially inhibitory pteridine concentrations measured in *P. apterus* (see above). Pteridines were provided at final concentrations of 0.5 mM (lumazines) and 0.2 mM (pterins) unless otherwise noted. ZAS culture filtrates were provided at a final concentration of 10% (vol/vol). Sterile anoxic water was added to negative control tubes. At the time of additions, culture tubes were wrapped in aluminum foil to protect compounds from photochemical degradation. Subsequent assessments of culture growth were based on methane production alone. All pteridine compounds were obtained from Schirck's Laboratories (Jona, Switzerland).

Thin Layer Chromatography

As an initial screen for the presence of pteridine compounds, *R. flavipes* gut homogenates were analyzed by thin layer chromatography (TLC) (30). Gut homogenate (10 µl) was applied to 20 x 20 cm TLC plates of CEL 400-10 0.1 mm microcrystalline cellulose (Machery-Nagle; Düren, Germany). Chromatograms were developed by using an ascending solvent [isopropanol: 2% (w/v) NH_3-acetate (1:1)] in a sealed tank lined with Whatman no. 1 filter paper. For two-dimensional chromatography, plates were developed in the first dimension as described above, allowed to dry, and developed in the second dimension using 3% NH_4Cl. Fluorescent compounds on TLC plates were visualized under a long wavelength (366 nm) U.V. lamp (Black-Ray, Ultra-Violet Products, San Gabriel, CA). The ratio of distance traveled by the solute to that of the mobile phase (R_f value) and visual appearance of fluorescant spots in gut extracts were compared to those of standard pteridine compounds (500 µM concentrations applied as 10 µl spots).

HPLC Analysis

Preparative one-dimensional TLC was performed prior to HPLC analysis of fluorescent compounds in gut extract. A total of 50 µl of *R. flavipes* gut extract was applied to a TLC plate and developed as described above. Each fluorescent spot was scraped from the glass backing with a razor blade and resuspended in 1 ml of 10 mM phosphoric acid buffer (pH 3.2) containing 4% methanol. The resulting suspension was agitated for 5 min, centrifuged for 15 min at 12,000 x *g* to sediment cellulose particles, and the supernatant was collected and used for HPLC analysis.

The HPLC system and column used for analysis was described in Chapter 4. The HPLC protocol was a modification of the method described by Porcar *et al.* (36). The mobile phase consisted of 10 mM potassium phosphate buffer (pH 3.2) with 0.5% methanol (v/v) pumped at a flow rate of 1 ml/min. The injection volume was 20 µl, and each sample was analyzed by fluorescence detection at the following wavelengths (excitation/emission): 335/440 nm, 350/410 nm, and 365/490 nm.

Results

Methanogen Inhibition Assays

Lumazine, other pteridine compounds, and gut extracts of *Reticulitermes flavipes* were tested for the ability to inhibit methanogenesis by *Methanobrevibacter filiformis*, a methanogen previously isolated from the gut of *R. flavipes* (23). The addition of lumazine to actively growing of cultures of *M. filiformis* resulted in a substantial reduction in the rate of methane emission; this effect was consistently observed at final lumazine concentrations ranging from 0.1 to 1 mM (Figures 5.2 and 5.3). In contrast, no inhibition of *M. filiformis* was observed following the addition of 5% (v/v) *R. flavipes* gut extract (Figure 5.2) or extracts of whole *R. flavipes* worker termites (data not shown). Addition of alternatively substituted lumazine derivatives also yielded no significant reduction in CH_4 emission (Figure 5.3). A variety of pterin compounds commonly observed in insects (pterin, biopterin, neopterin, leucopterin, xanthopterin, and isoxanthopterin) added to *M. filiformis* cultures at near saturating concentrations (0.2 mM) also had no inhibitory effects on methane emission. Analogous experiments using culture filtrates of *Treponema* strains ZAS-1 and ZAS-2 provided at 10% (vol/vol) final

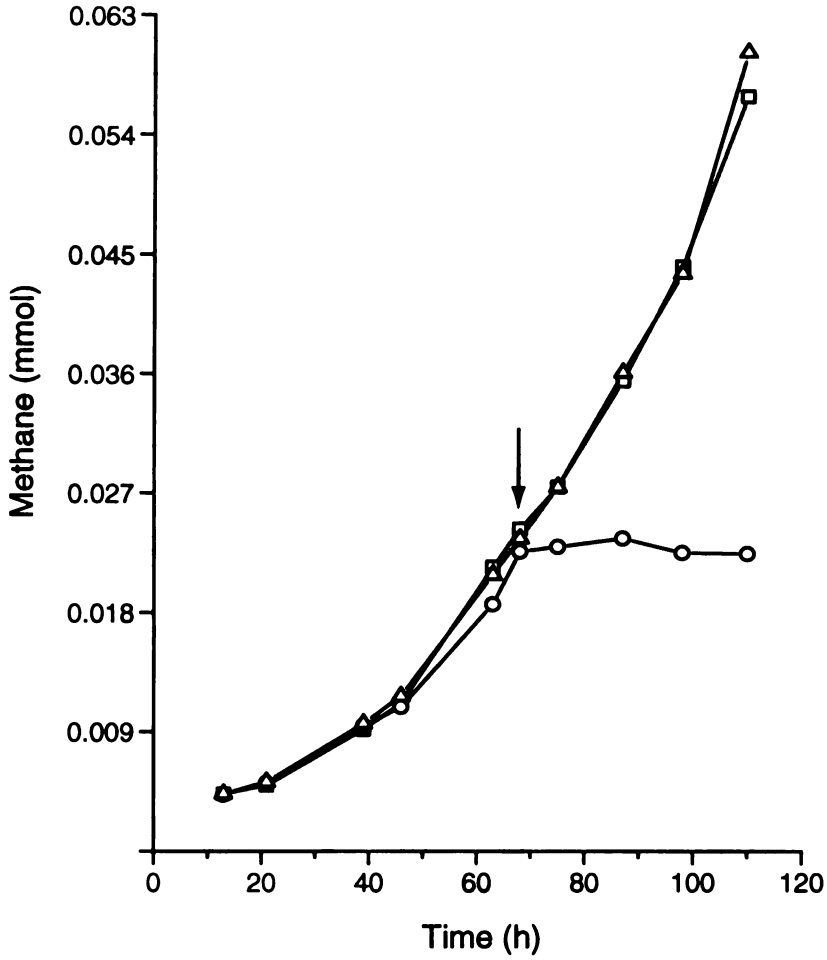

Figure 5.2. Methane production by growing cells of *M. filiformis* following addition of lumazine (O), *R. flavipes* gut extract (△), and anoxic water (□). Lumazine was provided at a final concentration of 1 mM, and gut extract was added at 5% (vol/vol). The time of additions is indicated by the arrow.

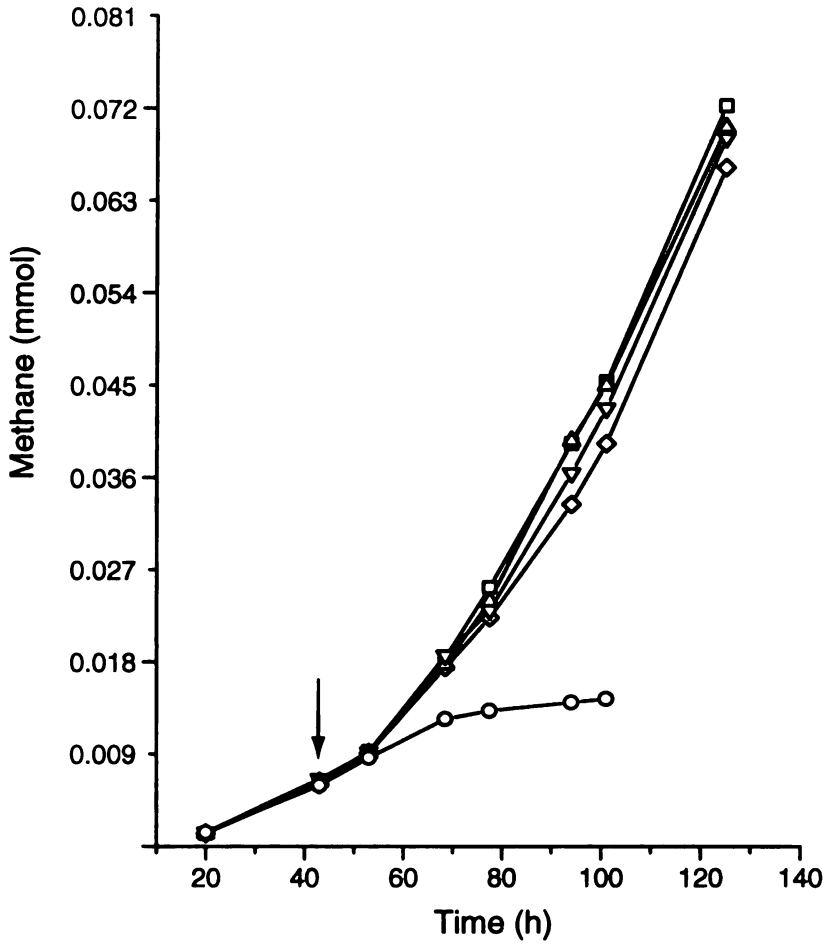

Figure 5.3. Methane production by growing cells of *M. filiformis* following addition of biolumazine (◇), xantholumazine (△), and isoxantholumazine (▽), all provided at 0.5 mM final concentrations. Additions of anoxic water (□) and 0.1 mM lumazine (○) were used as controls. The time of additions is indicated by the arrow.

concentration also had no effect on growth or methanogenesis by *M. filiformis* (data not shown).

Thin Layer Chromatography of *R. flavipes* Gut Extracts

TLC analysis of *R. flavipes* gut extracts revealed six fluorescent spots which were designated A through F (Table 5.1). Two-dimensional TLC separations of gut extract showed no additional spots, suggesting that the six spots represented the only fluorescent compounds present at detectable levels. A variety of pteridine compounds (both pterins and lumazine derivatives) commonly found in insects were also screened. Of the six spots detected in *R. flavipes* gut extracts, five (A-E) were not similar in R_f value or coloration to any screened pteridine. Spot F, however, was similar to leucopterin in both properties (Table 5.1). To assay *Treponema* strains ZAS-1 and ZAS-2 for pterin deaminase activity, the pterins listed in table 5.1 were added to ZAS-1 and ZAS-2 cultures at the onset of growth and culture filtrates were harvested at the end of lag phase. No detectable shifts in R_f value or coloration were observed in any of the compounds, indicating a lack of pterin deaminase activity. TLC analysis of late log phase and stationary phase culture filtrates of *Treponema* strains ZAS-1 and ZAS-2 showed no detectable production of fluorescent compounds during growth.

HPLC Analysis of *R. flavipes* Gut Extracts

To remove potentially interfering compounds present in *R. flavipes* gut extracts, the extracts were pre-purified by TLC prior to HPLC analysis. This purification was first tested by applying a total of 2.5 nmol of lumazine to TLC plates, developing the plate as

Table 5.1. Thin Layer Chromatography of Pteridines and *R. flavipes* Gut Extract

Compound	R_f Value	Color
Gut Extract Fluorescent Spots[a]:		
Spot A	0.99	Whitish-Green
Spot B	0.85	Blue-Green
Spot C	0.66	Blue-Green
Spot D	0.56	Blue-White
Spot E	0.35	Light Green
Spot F	0.13	Green
Pteridine Compound Standards[a]:		
Lumazine	0.52	Light Green
Xantholumazine	0.27	Blue-Green
Isoxantholumazine	0.31	Dark Purple
Pterin	0.50	Blue
Xanthopterin	0.26	Green
Isoxanthopterin	0.24	Purple
Biopterin	0.58	Blue-Purple
Neopterin	0.50	Blue-Purple
Leucopterin	0.14	Green

[a]Gut extract and 0.5 mM pteridine standard solutions were applied to TLC plates as 10 µl spots.

described above, and extracting the resultant spot from material excised from the plate. Recovery of lumazine from this material (as determined by HPLC analysis) was greater than 90%. HPLC analysis of pterin and lumazine standards (listed in table 5.1) resulted in good linearity of response for micro- to nanomolar concentrations of all compounds tested.

The six fluorescent compounds present in *R. flavipes* gut extracts were subjected to HPLC analysis with fluorescence detection. Five of the compounds (A, B, C, D, and E) showed no detectable peaks at any of the three pteridine-specific fluorescence excitation/emission wavelength combinations tested (data not shown). Spots C and E showed U.V. absorbance at 250 nm, suggesting that what had been interpreted as fluorescence on TLC plates may have actually been U.V. absorbance. Compound F yielded a single fluorescent peak with similar retention time to that of standard leucopterin. The amount of leucopterin present in gut extract equates to a concentration of 0.04 nmol per *R. flavipes* gut. If all of the detected leucopterin was present in gut fluid and assuming an approximate volume of 0.27 µl fluid per gut (35), leucopterin concentration in gut fluid would be 0.15 mM.

Discussion

The results of this study demonstrate that neither lumazine nor other pteridine compounds are likely to play a significant role in the inhibition of termite gut methanogens. Of the ten pteridines tested, only unsubstituted lumazine (2, 4-pteridinedione) showed any significant inhibition of the termite methanogen *M. filiformis*. While lumazine has been reported in the fruit fly *Drosophila melanogaster* (21, 39) and

the honeybee *Apis mellifica* (14), this compound was not present at detectable levels in gut extracts of *R. flavipes* by either TLC or HPLC analysis. The only pteridine compound observed in gut extracts was leucopterin, which was shown to be non-inhibitory to *M. filiformis* at higher concentrations (200 μM) than could have been present in *R. flavipes* gut fluid (150 μM at maximum). A more likely explanation for the presence of leucopterin in the gut would be as a nitrogen excretory product, since this is one of roles played by pterin compounds in other insects (49). It remains possible that lumazine (or pteridines) are present in the termite hindgut at concentrations below the detection limits of the assays used in this study, but it is doubtful that such low levels of these compounds would have any significant activity against methanogens in the gut.

In a series of independent experiments conducted by Dr. John Breznak, filter paper containing the methanogen-inhibitor bromoethanosulfonate (BES) or lumazine (5 and 500 μM final concentrations, respectively), were fed to *R. flavipes* worker termites. Methane emission rates measured in live termites fed BES were significantly reduced, whereas lumazine-fed termites actually showed a substantial increase in rates of methane emission (John Breznak, personal communication). The cause of this outcome is as yet unknown, but could be explained by either the degradation of ingested lumazine by the termite or gut microbiota (possibly to methanogenic precursors) or by the presence of a population of gut methanogens more resistant to lumazine inhibition than *M. filiformis*. Regardless of the reason, the failure of exogenous lumazine to inhibit methanogens *in situ* further indicates that this compound is unlikely be significantly involved in methanogen inhibition in the termite hindgut.

At this time, the failure of termite gut methanogens to dominate H_2 consumption in the termite hindgut remains somewhat enigmatic. While it is possible that some non-pteridine inhibitor of methanogens is present in the gut lumen, the abolition of methanogenesis observed in the BES-feeding studies [(32) and those discussed above] suggests that colonization of the gut epithelium affords methanogens no special protection from inhibitory substances. Also, incubation of live termites (32) and agarose embedded termite guts (42) under H_2-enriched atmospheres results in an increase in methane emission, suggesting that substrate limitation is the dominant limiting factor for the population of epithelial methanogens. This inference is further supported by the observation of significantly higher rates of methane emission in *Zootermopsis angusticollis* than in other wood-feeding termites (3). Trichomonad flagellates containing endosymbiotic methanogens colonize the gut of *Z. angusticollis* (25), and by living within these protozoa, intracellular methanogens apparently escape the restriction of others methanogens to the gut wall and gain increased access to H_2 produced by cellulose degradation.

Taken together, these observations suggest that the spatial resource partitioning hypothesis articulated by Brune and coworkers (10, 15) remains the best current explanation for the dominance of H_2/CO_2-acetogens as H_2 consumers in the hindguts of most wood- and grass-feeding termites. Since no termite gut H_2/CO_2-acetogen has yet displayed any property which would make them unusually competitive with methanogens [(6, 8), and Chapter 2], it would be predicted that successful colonization of the gut lumen by methanogenic archaea would result in a methanogenically dominated H_2-flow, equating to a significant loss of energy for the termite host. This makes the

counterintuitive restriction of methanogens to the gut epithelium all the more puzzling. This unusual situation may yet be explained by considering other factors specific to growth and survival in termite hindguts, which could include avoidance of grazing by gut protozoa, avoiding being washed out of the gut, or a reliance on maintaining a close physical association with some other member of the epithelial microflora. Determining which (if any) of these factors plays a role in influencing the localization of methanogens in the hindgut remains a critical unresolved issue in understanding the functional ecology of the termite hindgut symbiotic system.

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Chapter 6

Summary

This dissertation has investigated of the physiological properties of the termite gut spirochetes *Treponema* strains ZAS-1, ZAS-2 (3), and ZAS-9 (5) and their relationships with other members of the termite hindgut microbial community. Although over a hundred years of research had yielded a substantial amount of data on the abundance, diversity, and distribution of termite gut spirochetes, their physiological properties and functional roles in situ were poorly understood, largely due to a lack of any cultured representatives. The availability of pure cultures of *Treponema* strains ZAS-1, ZAS-2, and ZAS-9, the first spirochetes to be isolated from the guts of wood-feeding termites, has finally made in vitro physiological studies possible. Gaining further understanding of the physiology, nutrition, and growth requirements of these organisms in vitro has resulted in the formulation of new hypotheses regarding their in situ roles and relationships with other members of the termite gut microbial community.

The research presented in Chapter 2 focused on the physiological properties relevant to the in situ growth and survival of *Treponema* strains ZAS-1 and ZAS-2, H_2/CO_2-homoacetogenic termite gut spirochetes. Strains ZAS-1 and ZAS-2 proved to be nutritionally versatile and were capable of mixotrophic utilization of H_2 and organic substrates, including various carbohydrates and (in the case of ZAS-2) methoxylated aromatic compounds. These results suggest that the ZAS strains are likely to contribute to termite nutrition via acetogenesis from fermentation and demethylation of organic

substrates in situ, as well as from H_2 and CO_2 produced by other members of the gut microbiota. ZAS-1 and ZAS-2 were shown to be similar to other homoacetogens in possessing enzyme activities of the Wood/Ljungdahl pathway and displaying H_2 thresholds within the range of typical of known acetogens. In comparison to other homoacetogens however, ZAS-1 and ZAS-2 were less efficient in terms of energy conservation; this may represent an adaptation to a symbiotic lifestyle which prevents excessive cell growth (which could be detrimental to the host) while still allowing production of acetate to fuel termite energy metabolism. Both strains had requirements for folate, a curious observation considering the importance of this cofactor in acetogenesis. Although ZAS-1 and ZAS-2 are strict anaerobes, both strains are capable of some degree of O_2 tolerance and detoxification, which is likely an adaptation to the partially hypoxic conditions encountered by spirochetes in termite hindguts. Taken together, these findings demonstrate that *Treponema* strains ZAS-1 and ZAS-1 are well adapted to life in the termite hindgut and suggests that their relationship to their termite hosts is beneficial in nature.

Chapter 3 provided a more thorough description of *Treponema* strain ZAS-9, which differed significantly from ZAS-1 and ZAS-2 in terms of morphology and metabolism. ZAS-9 is not a H_2/CO_2-homoacetogen and in fact produced H_2 as a product of sugar fermentation. This raises the possibility that interspecies H_2 transfer between spirochetes may be an important component of H_2 turnover in the termite hindgut. In terms of taxonomy, the results suggest that ZAS-1 and ZAS-2 should be regarded as two strains of single new species in the genus *Treponema*, whereas strain ZAS-9 should be considered a separate new species of *Treponema* distinct from that accommodating ZAS-

1 and ZAS-2 (Latinate species epithets are currently under consideration). The physiological differences observed among the three strains of termite gut spirochetes currently in pure culture likely offers only an introductory glimpse into the functional diversity of these organisms. Future cultivation efforts should continue to yield new insights into the physiological capabilities of termite gut spirochetes.

The observation of folate requirements in *Treponema* strains ZAS-1 and ZAS-2 in Chapter 2 resulted in an investigation of the in situ source of this cofactor, which was presented in Chapter 4. Since folate-secreting bacteria had been observed in a variety of other gastrointestinal systems(1, 2, 7), it was hypothesized that other members of the termite gut microbial community were providers of folate. Consistent with this hypothesis, two folate-secreting strains were isolated from guts of the termite *Zootermopsis angusticollis*, *Serratia* strain ZFX-1 and *Lactococcus* strain ZFX-2. Provision of culture filtrates of ZFX-1 and ZFX-2 supported the growth of *Treponema* strains ZAS-1 and ZAS-2 in the absence of added folates. The folate compound produced by both ZFX strains was determined to be folinate (5-HCO-tetrahydrofolate), which was demonstrated to be a growth supportive form of the cofactor for ZAS-1 and ZAS-2. These results suggest that folate secreting bacteria such as strains ZFX-1 and ZFX-2 are the most likely providers of folates to the ZAS strains in situ. The ZFX strains may also be important in providing folate to other members of the gut microbial community to the termite host.

Finally, Chapter 5 tested the hypothesis that methanogenic archaea are inhibited by the presence of pteridine compounds in the termite hindgut, allowing H_2/CO_2-homoacetogens to act the primary H_2-consumers in the guts of wood-feeding termites.

The pteridine compound lumazine has been shown to be inhibitory to methanogenesis (6), and pteridine compounds are typically present in insects at relatively high levels (as compared to other animals) (8). The presence of inhibitory pteridines in the lumen of the termite hindgut would help to explain the counterintuitive restriction of methanogens to the region of lowest H_2 concentration in the gut, the epithelium. However, lumazine was the only pteridine which had any inhibitory effect on the termite methanogen *Methanobrevibacter filiformis*, and this compound was not detected in gut extracts of the termite *Reticulitermes flavipes*. It was therefore deemed doubtful that pteridines play a significant role in the inhibition of methanogenesis in the *R. flavipes* hindgut, and the localization of termite methanogens to the gut epithelium must be explained by other factors.

In concluding this dissertation, it seems appropriate to revisit the pioneering work of Dr. Joseph Leidy (4). In 1881, after years of microscopic observations describing the diverse inhabitants of termite hindguts, Leidy makes the following comment:

> *"Termites... are so common, easily obtained and preserved alive, and their parasites are so exceedingly numerous, constant in their occurrence, and curious, that once the fact becomes sufficiently known, the insects will become subjects to illustrate at once the infinity of life and the wonders that are revealed by the microscope."*

Over one hundred years later, the termite hindgut continues to fascinate microbiologists, and it seems safe to conclude that it will continue to do for at least another hundred years to come.

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