APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 2004, p. 1307–1314 Vol. 70, No. 3
0099-2240/04/$08.00+0 DOI: 10.1128/AEM.70.3.1307–1314.2004

Physiology and Nutrition of *Treponema primitia*, an H_2/CO_2-Acetogenic Spirochete from Termite Hindguts

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Received 12 August 2003/Accepted 27 November 2003

Treponema primitia **strains ZAS-1 and ZAS-2, the first spirochetes to be isolated from termite hindguts (J. R. Leadbetter, T. M. Schmidt, J. R. Graber, and J. A. Breznak, Science 283:686-689, 1999), were examined for nutritional, physiological, and biochemical properties relevant to growth and survival in their natural habitat. In addition to using H_2 plus CO_2 as substrates, these strains were capable of homoacetogenic growth on mono- and disaccharides and (in the case of ZAS-2) methoxylated benzenoids. Cells were also capable of mixotrophic growth (i.e., simultaneous utilization of H_2 and organic substrates). Cell extracts of *T. primitia* possessed enzyme activities of the Wood/Ljungdahl (acetyl coenzyme A) pathway of acetogenesis, including tetrahydrofolate-dependent enzymes of the methyl group-forming branch. However, a folate compound was required in the medium for growth. ZAS-1 and ZAS-2 growing on H_2 plus CO_2 displayed H_2 thresholds of 650 and 490 ppmv, respectively. Anoxic cultures of ZAS-1 and ZAS-2 maintained growth after the addition of as much as 0.5% (vol/vol) O_2 to the headspace atmosphere. Cell extracts exhibited NADH and NADPH peroxidase and NADH oxidase activities but neither catalase nor superoxide dismutase activity. Results indicate that (i) *T. primitia* is able to exploit a variety of substrates derived from the food of its termite hosts and in so doing contributes to termite nutrition via acetogenesis, (ii) in situ growth of *T. primitia* is likely dependent on secretion of a folate compound(s) by other members of the gut microbiota, and (iii) cells possess enzymatic adaptations to oxidative stress, which is likely to be encountered in peripheral regions of the termite hindgut.**

Spirochetes are among the most abundant microbial groups in termite hindguts, accounting for as much as one-half of the prokaryotic community (42). 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 survival (20), the specific roles of spirochetes and the factors contributing to their abundance in the hindgut have remained obscure.

Over the past 10 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 rRNA gene clones form a phylogenetically discrete cluster (the "termite cluster") within this genus (34). These studies also revealed a striking degree of phylogenetic diversity among termite gut treponemes, with as many as 21 distinct species occurring within a single termite host species (34). 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 plus CO_2 (31) and N_2 fixation (33). 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, 46). Acetogenesis plays a particularly prominent role in termite nutrition: 71 to 100% of the insect's energy requirements can be met by oxidation of acetate produced by hindgut microbes, and 10.5 to 33% of this acetate production is attributable to H_2-CO_2 acetogenesis (6, 41, 51).

The availability of pure cultures of termite gut spirochetes has enabled us to explore properties relevant to their growth and survival in situ. In this paper, we report on the nutritional, physiological, and biochemical properties of *Treponema primitia* strains ZAS-1 and ZAS-2, H_2/CO_2-acetogenic spirochetes isolated from the hindgut of the California dampwood termite, *Zootermopsis angusticollis* (Hagen) (31). Additional information regarding the taxonomy, nomenclature, and genomic properties of these strains is reported in a companion paper (23).

MATERIALS AND METHODS

Media and cultivation methods. Routine cultivation of *T. primitia* strains ZAS-1 (DSMZ 12426) and ZAS-2 (DSMZ 12427; ATCC BAA-887) was carried out in butyl rubber-stoppered anaerobe tubes or bottles (30) containing 2YACo medium (31) at no more than one-fifth their volume under an atmosphere of 80% H_2–20% CO_2 (vol/vol). 2YACo medium consisted of inorganic salts including trace elements, a vitamin mixture (10 ng of biotin/ml, 40 ng of 4-aminobenzoic acid/ml, 50 ng [each] of calcium–D-(+)-pantothenate and vitamin B_{12}/ml, 150 ng of pyridoxamine · HCl/ml, and 100 ng [each] of nicotinic acid and thiamine · HCl/ml [final concentrations]) (55), a mixture of 11 cofactors (50 μg [each] of folic acid and thiamine pyrophosphate/ml, 10 μg of riboflavin/ml, 5 μg [each] of pyridoxal · HCl and pyridoxal phosphate/ml, 1.3 μg of hemin/ml, 1 μg [each] of calcium folinate, β-NAD, flavin adenine dinucleotide, and coenzyme A (CoA)/ml, and 0.5 μg of nicotinamide/ml [final concentrations]) (31), and 2% (vol/vol) laboratory-prepared yeast autolysate (equivalent to ca. 2.2 mg of dry solids/ml) (31). The medium was buffered by inclusion of 70 mM $NaHCO_3$ and 10 mM 3-*N*-[morpholino] propanesulfonic acid (MOPS) and was reduced with

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dithiothreitol (final concentration,1 mM). 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 strokes per min) with vessels held in a horizontal position.

Nutritional and growth studies. The ability of commercial yeast extracts to replace laboratory-prepared yeast autolysate in 2YACo medium was tested by using the following products at a final concentration of 6 mg/ml: Tastone 900, 154, and 310; Amberex 1003 and 695; and Amberferm 5925, 5902, and 5021 (all from Red Star Bioproducts, Juneau, Wis.). Cofactor requirements were evaluated by testing for attenuation of growth in 2YACo medium lacking one of the 11 cofactors (listed above). Candidate individual cofactors were then tested in reciprocal experiments in which they were incorporated alone into 2YACo medium instead of the 11-cofactor mixture.

Substrate utilization studies were performed with cells growing under an N_2–CO_2 (80:20 [vol/vol]) atmosphere. For ZAS-1, 2YACo medium was modified to contain Amberferm 5902 (4 mg/ml) in place of yeast autolysate to minimize background growth in medium lacking a test substrate. Unmodified 2YACo medium was used for ZAS-2, with a small amount of H_2 added to the N_2–CO_2 headspace (final concentration, 16 mM H_2; ca. 95,000 ppmv) 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, compared to its absence, was taken to indicate utilization of the substrate as an energy source. Cell growth was determined by measuring the optical densities (OD) of cultures at 600 nm with a Milton Roy Spectronic 20 colorimeter. OD readings were converted to cell numbers 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 on the basis of the acetate production expected from demethylation of the aromatic (*R*) substrate according to the following equation: $R(-OCH_3)_n + 0.5n\ CO_2 \rightarrow R(-OH)_n + 0.75n\ CH_3COOH + 0.5n\ H_2O$. Organic acid production was determined by using a high-performance liquid chromatograph (HPLC) with refractive index detection (4). Aromatic compounds were analyzed by using a Beckman model 127 HPLC equipped with a model 168 photodiode array detector and an Alltech Lichrosorb RP-18 column (250 by 4.6 mm; particle size, 10 μm). The mobile phase was 0.1% phosphoric acid with a methanol gradient, increasing linearly from 48 to 55% in 30 min. The flow rate was 1.5 ml/min.

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

Determination of hydrogen thresholds. Hydrogen thresholds were determined as described by Lovley (36). In brief, cultures were grown under H_2–CO_2 (80:20 [vol/vol]) in 2YACo medium that was either left unmodified (ZAS-2) or modified to contain 4 mg of Amberferm 5902/ml 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 [vol/vol]), followed by the introduction of ca. 6,000 ppmv of H_2 into the gas phase. 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 measured by using a Trace Analytical RGA2 gas chromatograph equipped with an RGD2 trace gas detection unit.

Enzyme assays. Cells from mid-log-phase cultures growing on H_2 plus CO_2 (OD, 0.15 to 0.3) were harvested by centrifugation (at 16,000 × *g* for 10 min) and resuspended at 10 times their original concentration in an appropriate assay buffer (as cited below) containing 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and leupeptin (10 μg/ml) to inhibit proteases. While held at 4°C, cells were disrupted by sonication three times for 30 s each time with a Branson model 450 Sonifier (power setting, 5; 50% duty cycle) equipped with a stepped microtip. The resulting crude cell extracts were assayed for enzyme activities.

Formyltetrahydrofolate (formyl-THF) synthetase, methenyl-THF cyclohydrolase, and methylene-THF dehydrogenase (40) and methylene-THF reductase (38) were assayed as described previously. 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). Rates of O_2-dependent and H_2O_2-dependent oxidation of reduced pyridine nucleotides (oxidase and peroxidase activities, respectively) were determined as described by Stanton (49). Superoxide dismutase (SOD) was assayed by the xanthine-xanthine oxidase-cytochrome *c* reduction method (21). The protein content of cell extracts was measured by the Lowry assay (37). 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 were grown under H_2–CO_2 with reciprocal shaking (above) in 18-mm-diameter anaerobe tubes containing 5 ml of 2YACo medium modified by the inclusion of 10 mM maltose but containing no dithiothreitol reducing agent. When cells reached mid-log phase, the headspace (ca. 21 ml) 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% (vol/vol). Cultures were then immediately reincubated in a horizontal position with shaking to facilitate equilibration of gaseous O_2 with the liquid phase, and further growth was monitored as described above.

RESULTS

Nutrition and growth of *T. primitia* strains ZAS-1 and ZAS-2. Cells of both strains grew in 2YACo medium under H_2 plus 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 of ≤6.0 or ≥8.0. Both strains grew well within a temperature range of 23 to 32°C, with an optimum at 30°C. No growth occurred at 4 or 34°C. Under optimum conditions, the shortest doubling time of cells was 22 h for ZAS-1 (with glucose [Table 1]) and 29 h for ZAS-2 (with H_2 plus CO_2 plus maltose [see below]).

A search for commercial yeast extracts to replace laboratory-prepared yeast autolysate in 2YACo medium was expanded from an unsuccessful earlier attempt (31). Tastone 900, Amberferm 5925, and Amberferm 5902 could replace yeast autolysate for the growth of strain ZAS-1, whereas Tastone 154, Tastone 310, Amberex 1003, and Amberex 695 were suitable replacements for the 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 (6 mg/ml) were similar to those measured in 2YACo medium.

Besides H_2 plus CO_2, a variety of hexoses, pentoses, and disaccharides were also used as energy sources 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 (≥72 h) prior to exponential growth. The provision of small amounts of H_2 to the headspace (ca. 95,000 ppmv) or the use of larger inocula (>5% [vol/vol]) eliminated such lags (Fig. 1). Strain ZAS-2 was additionally able to utilize methoxylated aromatic compounds (syringate, ferulate, vanillate, and trimethoxybenzoate) as energy sources when they were supplied at ≤2.5 mM (higher concentrations inhibited growth). Cell doubling times were typically longer than 100 h 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 grew on other C_1 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 (vol/vol) to the headspace of actively growing cultures resulted in the immediate cessation of growth.

It was noticed previously that the growth of ZAS-1 and ZAS-2 on H_2 plus CO_2 in the medium used for enrichment (JM4 medium [30]) was markedly improved if the nutrient broth and bovine rumen fluid present in JM4 were replaced by laboratory-prepared yeast autolysate (2 or 4% [vol/vol]) and a

TABLE 1. Substrates utilized by *T. primitia*

Substrate[a]	Net yield (10^8 cells/ml)[b]		Acetate recovery (%)[b,c]		$Y_{substrate}$[d]		Doubling time (h)	
	ZAS-1	ZAS-2	ZAS-1	ZAS-2	ZAS-1	ZAS-2	ZAS-1	ZAS-2
H_2 (+ CO_2)	1.2	1.3	92	96	0.1	0.2	26	48
Glucose	3.5	1.5	92	93	7.9	6.3	22	71
Mannitol	2.9	1.1	94	91	6.5	4.6	23	70
Arabinose	2.5	NU	98		5.6		26	
Xylose	2.7	1.8	91	89	6.1	7.6	26	101
Maltose	6.7	4.7	95	95	15.1	19.8	27	69
Cellobiose	4.5	NU	96		4.5		27	
Trimethoxybenzoate	NU	1.1		96[e]		4.6		127
Syringate	NU	0.9		90[e]		3.8		154
Ferulate	NU	0.5		93[e]		2.1		315
Vanillate	NU	0.7		96[e]		2.6		135

[a] All substrates were provided at a final concentration of 5 mM except for the methoxylated aromatic compounds (2 mM), CO (8 mM), and H_2 (136 mM). NU, substrates tested but not utilized. 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 plus CO_2 have been published previously (31) and are included here for comparison.

[b] Cell yields and carbon recoveries were determined under conditions of substrate-limited growth and are corrected for growth and acetate formation in medium lacking the test substrate.

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

[d] Grams of cell yield (dry mass) per mole of substrate consumed. The assumption is that protein makes up 55% of dry cell mass (39) in spirochetes; 10^8 cells correspond to 6.2 (ZAS-1) or 11.6 (ZAS-2) μg of protein.

[e] Based on acetate production expected from demethylation of the aromatic substrate (see Materials and Methods).

mixture of 11 cofactors, resulting in 2YACo or 4YACo medium, respectively (31). However, little or no growth was obtained if either yeast autolysate or the cofactor mixture was omitted from the medium. In order to determine which cofactors were required, ZAS-1 was grown through three successive transfers in 2YACo medium lacking one of the 11 cofactors. Cultures deficient in folinate (formyl-THF) displayed successively decreasing growth yields with each transfer. Further testing demonstrated that calcium folinate alone (final concentration, 500 ng/ml; equivalent to 0.98 μM) could replace the 11-cofactor mixture for both strains. Folate (the unsubstituted, unreduced form of the cofactor, at the same final concentration) could replace folinate for strain ZAS-2 but not for ZAS-1. If yeast autolysate contains folate compounds, they are obviously present at a concentration insufficient to support detectable growth of ZAS-1 and ZAS-2 in 2YACo medium. However, yeast autolysate contains at least one other required factor not present in most commercial yeast extracts or autolysates or in the 11-cofactor or 7-vitamin mixture included in 2YACo medium (described above).

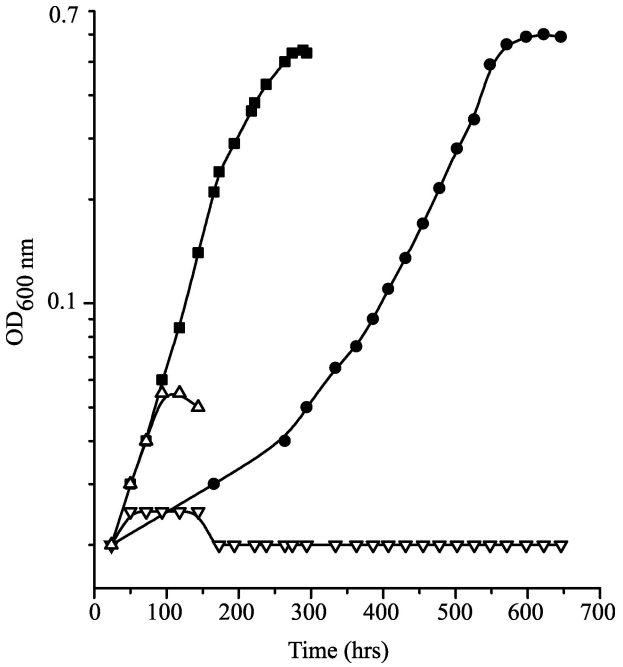

FIG. 1. H_2 stimulation of chemoorganotrophic growth of *T. primitia* strain ZAS-2. Either 10 mM maltose (●), 16 mM H_2 (△), 10 mM maltose plus 16 mM H_2 (■), or no substrate (▽) was added to 2YACo medium under N_2–CO_2 (80:20 [vol/vol]).

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

Hydrogen thresholds. H_2 thresholds of mid-log-phase cultures growing on H_2 plus CO_2 were 490 ± 40 ppmv for ZAS-1 and 650 ± 50 ppmv for ZAS-2 (means ± standard deviations [SD]; $n = 3$). The H_2 threshold of ZAS-2 decreased moderately, to 350 ± 30 ppmv, when ZAS-2 was grown mixotrophically on H_2 plus CO_2 plus trimethoxybenzoate (Fig. 3).

H_2/CO_2-acetogenic enzyme activities. Previous work in our laboratory demonstrated that ZAS-1 and ZAS-2 possessed hydrogenase, formate dehydrogenase, and acetyl-CoA synthase (measured as CO dehydrogenase); the latter is a key enzyme of CO_2-reductive acetogenesis via the Wood/Ljungdahl pathway (31). To further confirm the presence of this pathway in *T.*

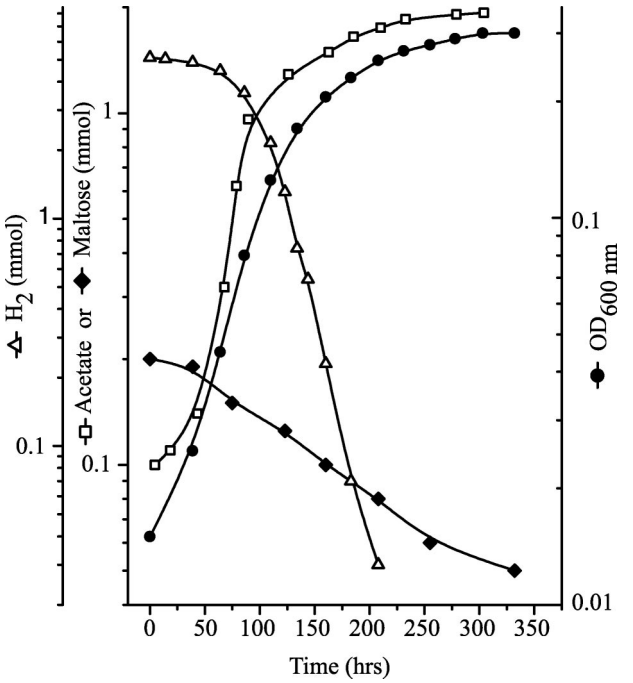

FIG. 2. Mixotrophic growth of *T. primitia* strain ZAS-2 on H_2 (plus CO_2) and maltose.

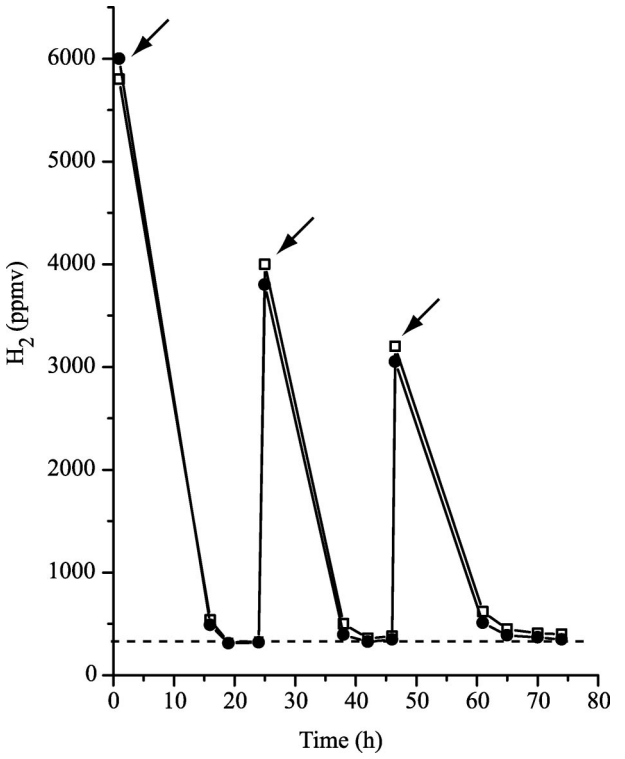

FIG. 3. Determination of H_2 thresholds for two (of three) replicate cultures of *T. primitia* strain ZAS-2 growing mixotrophically on H_2 plus CO_2 plus trimethoxybenzoate (● and □). H_2 additions are indicated by arrows. The H_2 threshold is indicated by a dashed line. Symbols stand for two of three replicate cultures.

primitia, 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 3). The formyl-THF synthetase activities of both strains were 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 min.

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 the growth rate (Fig. 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 peroxidases, whereas neither exhibited catalase or SOD activity. Both strains had low levels of NADPH oxidase, but relatively high levels of NADH oxidase activity were seen only in ZAS-2 (Table 4). Exposure of cultures to 0.5% O_2 12 h prior to enzyme assays did not significantly alter the levels of any of the enzyme activities tested in either of the strains, and for all enzymes tested, activity was abolished in boiled cell extracts.

DISCUSSION

T. primitia strains ZAS-1 and ZAS-2 possess a variety of properties typical of traditional "homoacetogens," including enzymes of the acetyl-CoA (Wood/Ljungdahl) pathway for synthesis of acetyl-CoA and acetate from H_2 plus CO_2 for energy generation and CO_2 fixation (15). Operation of this pathway, first suggested by the presence of acetyl-CoA synthase/CO dehydrogenase in cells (31), was further supported

TABLE 2. Cell yields and acetate production by *T. primitia* strain ZAS-2 during unitrophic and mixotrophic growth

Substrate	Amt of substrate consumed (mmol)[a]	Amt of acetate produced (mmol)[a]	Recovery (%)[b]	Cell yield (10^8 cells/ml)
Maltose	0.15 ± 0.04	0.84 ± 0.19	93	2.4
$H_2 + CO_2$	5.23 ± 0.35 (H_2)	1.23 ± 0.13	94	1.7
Maltose + H_2 + CO_2	0.15 ± 0.01 (maltose) 5.20 ± 0.19 (H_2)	1.95 ± 0.03	89	4.3

[a] Means ± SD from three replicate cultures.
[b] Acetate recovery as a percentage of substrate carbon (maltose) or H_2-derived electrons consumed (summed for mixotrophic conditions).

TABLE 3. Enzyme activities relevant to H_2/CO_2 acetogenesis in *T. primitia*

Enzyme[a]	Sp act[b]	
	ZAS-1	ZAS-2
Hydrogenase	0.47	0.45
Acetyl-CoA synthase/CO dehydrogenase	1.28	0.64
Formate dehydrogenase	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 of hydrogenase, formate dehydrogenase, and acetyl-CoA synthase, measured as H_2-, formate-, or CO-dependent reduction of methyl viologen, respectively, have been published previously (31) and are included here for comparison.

[b] Expressed as micromoles of substrate used (or product formed) per minute per milligram of protein.

TABLE 4. Oxygen- and peroxide-detoxifying enzyme activities in *T. primitia*

Enzyme	Sp act[a]	
	ZAS-1	ZAS-2
NADH oxidase	ND	560
NADPH oxidase	7	10
NADH peroxidase	10	40
NADPH peroxidase	3	5

[a] Expressed as nanomoles of pyridine nucleotide oxidized per minute per milligram of protein. No catalase or SOD activity was detected. ND, not detected.

here by demonstration of the corresponding enzymes catalyzing conversion of CO_2 to the methyl group of acetate (Table 3). To the extent that enzyme activities measured with crude extracts and permeabilized cells can be correlated to the rate of acetate formation by growing cells, we compared the activity of the acetate-forming enzyme system, dA_E/xdt, to the rate of acetate production by cells during growth on H_2 plus CO_2, dA_G/xdt, where dA stands for micromoles of acetate produced by x milligrams of cell protein per unit of time (dt, in minutes), as inferred from enzyme activity (E) or growth measurements (G). This comparison was done for strain ZAS-2, which showed no background growth in 2YACo basal medium in the absence of H_2 (31). Inasmuch as optimum conditions for each enzyme activity reported in Table 3 have not yet been established, for dA_E/xdt we used the mean specific activity of all ZAS-2 enzymes, after dividing hydrogenase activity by 4. This gave 0.33 ± 0.25 μmol of acetate · min^{-1} · mg of $protein^{-1}$ (mean ± SD). We calculated dA_G/xdt as being equal to $Y_{A/G} \cdot k$, where $Y_{A/G}$, the acetate yield from growing cells, is calculated as $1/(4 \cdot Y_{\text{hydrogen}})$, expressed as micromoles of acetate · mg of $protein^{-1}$, and k, the specific growth rate constant, is calculated as 0.693/doubling time in minutes (39) (Table 1). For simplicity, we ignored the small fraction of H_2-derived electrons used for biomass synthesis, which probably accounted for an electron recovery, in acetate produced, of only 96% (Table 1, footnote c). This is reflected by using a rounded-off value of 4 in the term $4 \cdot Y_{\text{hydrogen}}$. The calculated value of dA_G/xdt is then 0.55 μmol · min^{-1} · mg of $protein^{-1}$. Thus, even by a rough approximation, the mean activity of measured enzymes could account for as much as 60% of acetate production by growing cells. Hence, it seems reasonable to assume that the acetyl-CoA pathway is probably the major, if not the sole, route of acetate production from CO_2 in these spirochetes.

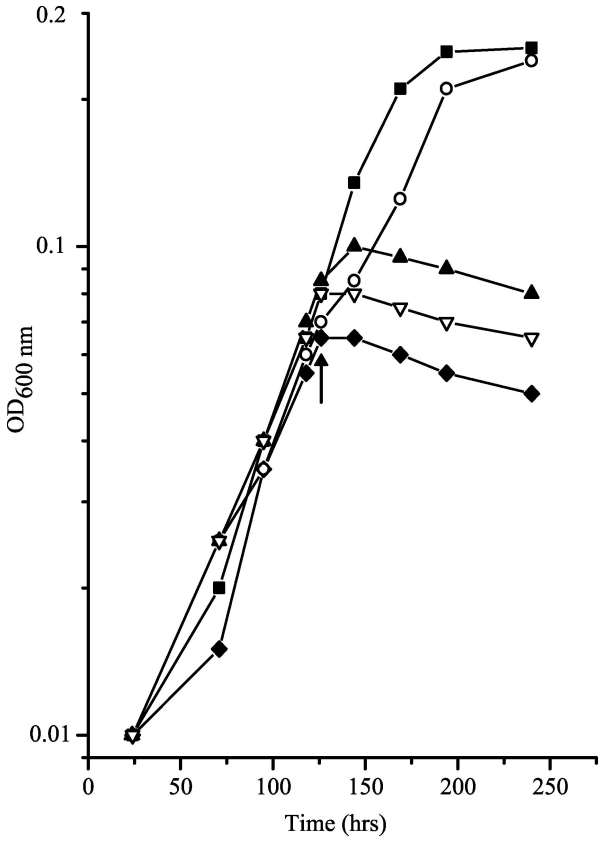

FIG. 4. Effect of O_2 on the growth of *T. primitia* strain ZAS-2. Cultures were grown under H_2–CO_2 (80:20 [vol/vol]), and O_2 was introduced at 125 h (arrow) to yield a concentration (expressed as a percentage of headspace volume) of 0% (control) (■), 0.5% (○), 1% (▲), 2.5% (▽), or 5% (◆).

Strains ZAS-1 and ZAS-2 are also similar to other homoacetogens in being nutritionally versatile (16) and capable of homoacetogenic 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, 11, 19, 25), there is evidence for the demethylation of aromatic lignin moieties (19). As such, it seems likely that *T. primitia* contributes 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 [31]) was capable of mixotrophy, i.e., simultaneous use of H_2 plus CO_2 and organic substrates as energy sources (Fig. 2; Table 2). This capability has been demonstrated in other acetogens, including those isolated from termite guts (5). If H_2 concentrations in the hindgut lumen of *Z. angusticollis* are as high as those of other

xylophagous termites (ca. 5 kPa or 50,000 ppmv in *Reticulitermes flavipes*) (17), mixotrophy may be a common mode of growth for *T. primitia* in situ. Indeed, some spirochetes may have become adapted to the constant presence of H_2 in termite hindguts such that they now require it for optimal growth. This was suggested by the stimulatory effect of H_2 on the utilization of organic substrates by strain ZAS-2 (Fig. 1), which may indicate 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 the 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 milliliter) 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 (Fig. 1).

The mean H_2 thresholds of ZAS-1 and ZAS-2 (490 and 650 ppmv, respectively) were also well within the range reported for other H_2-utilizing acetogens (12). For ZAS-2, the mean H_2 threshold was somewhat lower during mixotrophic growth on H_2 plus trimethoxybenzoate (350 ppmv), but it was not lowered to a level that would make ZAS-2 competitive with H_2-utilizing methanogens under H_2-limited growth conditions. In any case, direct competition with methanogens for H_2 may be largely irrelevant: if high luminal H_2 concentrations exist in *Z. angusticollis*, spirochetes may never encounter seriously H_2 limited conditions in vivo.

In contrast to those of other homoacetogens, the substrate-specific biomass yields ($Y_{substrate}$) of *T. primitia* ZAS-1 and ZAS-2 were surprisingly low: 0.1 and 0.2, respectively, during growth on H_2 (plus CO_2) and 7.9 and 6.3, respectively, during growth on glucose (Table 1). These biomass yields are substantially lower than those of other acetogens, which range from 0.5 to 4.2 with H_2 and from 32.7 to 53.0 with hexoses (13, 18, 45, 52). However, this seemingly inefficient coupling of acetogenesis to growth may contribute to the effectiveness of *T. primitia* as a hindgut symbiont. Acetate produced by gut microbes is the primary energy source for termites (6), and low $Y_{substrate}$ values mean that ZAS-1 and ZAS-2 will carry out homoacetogenesis without a concomitantly large increase in biomass and nutrient demand that might be detrimental to the host.

Previous studies have shown that the peripheral region of the termite hindgut is hypoxic (9, 17). O_2 concentrations near the gut epithelium are 50 to 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 zone periodically. Although ZAS-1 and ZAS-2 are "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 could maintain growth in the presence of 0.5% (vol/vol) O_2 in the gas phase (equivalent to a dissolved O_2 concentration of 6 μM). This finding 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 NADH and NADPH peroxidase activities that could afford protection against H_2O_2. ZAS-2 also possessed 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 tracts of pigs (49). Studies have shown that free-living acetogens display various degrees of aerotolerance and can grow under atmospheres ranging from 0.3% oxygen (*Acetobacterium woodii*) to 21% oxygen (*Sporomusa* strain ST-1) (28; K. Küsel, A. Gößner, C. R. Lovell, and H. L. Drake, Abstr. 103rd Gen. Meet. Am. Soc. Microbiol. 2003, abstr. Q-375, p. 582, 2003). A recent study revealed a similar degree of aerotolerance, as well as active reduction of O_2 by using H_2 or organic compounds as reductants, in a variety of nonspirochetal acetogens isolated from termite hindguts (3). Like the acetogens examined in those studies, the *T. primitia* strains lack SOD activity. The ability to tolerate low levels of O_2 has also been observed in the termite hindgut-associated methanogens *Methanobrevibacter cuticularis* and *Methanobrevibacter curvatus* (30), suggesting that aerotolerance may be a common trait among anaerobes in the termite hindgut.

Studies of the nutritional requirements of *T. primitia* revealed a strict requirement for folate compounds. This was surprising given that THF plays a critical role as a one-carbon carrier in the methyl-group-forming branch of the Wood/Ljungdahl pathway (35). Most bacteria are capable of de novo folate biosynthesis, and numerous homoacetogens grown in defined media have no apparent folate requirements (22, 32, 44). By contrast, many host-associated bacteria require an exogenous source of folate or a folate derivative, including ruminal (*Treponema bryantii* [48]) and genital (*Treponema phagedenis* [50]) spirochetes and numerous nonspirochetal members of the bovine rumen microbial community (24, 47). The source of folate compounds for *T. primitia* in vivo is currently unknown. Although provision of folates by the termite host is a possibility (e.g., folate synthesis has been demonstrated in the mosquito, *Aedes aegypti* [27, 53, 54]), most insects require a dietary source of preformed folate. A more likely source of folate compounds is other members of the complex hindgut community. A variety of bacteria have been shown to secrete folate compounds in vitro (14, 26) as well as in gastrointestinal tracts (10, 29, 43), and we have recently isolated several strains of bacteria from the hindguts of *Z. angusticollis* whose extracellular secretions can support the growth of ZAS-1 and ZAS-2 in folate-free medium (J. R. Graber and J. A. Breznak, presented at the 9th International Symposium on Microbial Ecology, Amsterdam, The Netherlands, 26 to 31 August 2001). The nature of these organisms and their putative folate secretions are the subjects of ongoing study in our laboratory.

In summary, *T. primitia* 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 (34), it seems certain that the few strains currently in culture (31, 33) offer only an introductory glimpse

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

ACKNOWLEDGMENTS

This research was funded by grants from the National Science Foundation (IBN97-09000 and IBN01-14505).

HPLC analysis of aromatic compounds was performed with equipment provided by the laboratory of James Tiedje, with the aid of Benjamin Griffin. We are grateful to Tom Schmidt and members of his laboratory for helpful discussions and criticisms.

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