Folate Cross-Feeding Supports Symbiotic Homoacetogenic Spirochetes

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***Treponema primitia*, an H₂-consuming CO₂-reducing homoacetogenic spirochete in termite hindguts, requires an exogenous source of folate for growth. Tetrahydrofolate (THF) acts as a C₁ carrier in CO₂-reductive acetogenesis, a microbially mediated process important to the carbon and energy requirements of termites. To examine the hypothesis that other termite gut microbes probably supply some form of folate to *T. primitia* in situ, we used a bioassay to screen for and isolate folate-secreting bacteria from hindguts of *Zootermopsis angusticollis*, which is the host of *T. primitia*. Based on morphology, physiology, and 16S rRNA gene sequences, the major folate secretors were identified as strains of *Lactococcus lactis* and *Serratia grimesii*. During growth, these isolates secreted 5-formyl-THF at levels up to 146 ng/ml, and their cell-free culture fluids satisfied the folate requirement of *T. primitia* strains in vitro. Analysis of *Z. angusticollis* hindgut fluid revealed that 5-formyl-THF was the only detectable folate compound and occurred at an in situ concentration (1.3 µg/ml) which was more than sufficient to support the growth of *T. primitia*. These results imply that cross-feeding of 5-formyl-THF by other community members is important for growth of symbiotic hindgut spirochetes and thus termite nutrition and survival.**

Spirochetes are a major component of the hindgut microbiota of termites, accounting for as many as 50% of all prokaryotic cells (37), but for many years their role in the gut remained obscure as none had ever been isolated and studied in vitro. Recently, the first pure cultures of the following termite gut spirochetes were obtained: *Treponema primitia* strains ZAS-1 and ZAS-2 and *Treponema azotonutricium* strain ZAS-9 (15, 29, 30). These organisms are believed to be important for termite nutrition because of their ability to fix dinitrogen and produce acetate, a major carbon and energy source for termites. In fact, *T. primitia* is a homoacetogen and possesses the Wood-Ljungdahl (acetyl coenzyme A) pathway for CO₂-reductive acetogenesis (14). This enables *T. primitia* to conserve energy by homoacetogenesis from H₂ plus CO₂, as well as from carbohydrates and methoxylated benzenoids, and to use CO₂ as a carbon source for growth. The Wood-Ljungdahl pathway is undoubtedly important in situ, because a significant portion of acetate formed in the hindgut arises from CO₂ reduction (5, 56). Given the abundance and species diversity of treponemes in termite hindguts (31), it is likely that many of the other not-yet-cultivated spirochetes are also CO₂-reducing acetogens. This notion is supported by the large number of formyltetrahydrofolate synthetase (a key enzyme of the Wood-Ljungdahl pathway) genes similar to those of *T. primitia* detected in hindguts of *Zootermopsis angusticollis*, the termite that is the host of *T. primitia* (45).

During recent studies of the physiology and nutrition of *T. primitia*, cells were found to require folate for growth (14). Requirements for exogenous folate compounds are not uncommon among host-associated bacteria and have been observed in nonhomoacetogenic host-associated spirochetes,

such as *Treponema bryantii* (49) and *Treponema phagedenis* (51). However, tetrahydrofolate (THF) is an important C₁ carrier in the methyl group-forming branch of the Wood-Ljungdahl pathway (32), and THF-dependent enzyme activities of this pathway have been demonstrated in *T. primitia* cell extracts (14). This prompted us to examine the folate requirement of *T. primitia* in more detail and to search for a source of folate in situ. In this paper, we present evidence that strains of *Lactococcus lactis* and *Serratia grimesii* isolated from hindguts of *Z. angusticollis* are important in the secretion of 5-formyl-THF (i.e., folinate). 5-Formyl-THF is the major folate compound detected in gut fluid of *Z. angusticollis* and occurs at a concentration well above that needed to satisfy the folate requirement of *T. primitia* strains in vitro. These results suggest that synthesis and secretion of folate compounds by gut bacteria are important to the functional integrity of the overall hindgut microbiota and, hence, to termite nutrition and survival.

MATERIALS AND METHODS

Termites. Specimens of *Z. angusticollis* were collected in the San Gabriel Mountains of southern California and were maintained in the laboratory on pieces of *Pinus ponderosa* in polypropylene containers. Specimens were used within 1 month of collection.

Folate compounds. Folic acid, dihydrofolate (DHF), THF, 5-methyl-THF, 5,10-methylene-THF, 5-formyl-THF (i.e., folinate), 10-formylfolate, and di- and triglutamate forms of folic acid were obtained from Schirck's Laboratories (Jona, Switzerland). The purity of all folate standards was 95% or greater and was confirmed by high-performance liquid chromatography (HPLC) analysis (see below). Standard solutions of folate compounds were prepared as described by Könings (25) and contained 10 mM (final concentration) 2-mercaptoethanol and 2% (wt/vol) sodium ascorbate to prevent oxidation. 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 *T. primitia* strains ZAS-1 and ZAS-2 was performed as described previously (29), with the following exceptions: medium 2YACo was modified to obtain medium 2YAFo by replacing the 11-cofactor mixture with 5-formyl-THF alone (final concentration, 500 ng/ml). Medium 2YA was identical to medium 2YAFo but lacked added

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5-formyl-THF. Medium 2YA was used to examine the folate requirement of *T. primitia*, as well as the ability of culture fluids of putative folate secretors (below) to satisfy this requirement when the fluid was incorporated at a concentration of 10% (vol/vol). Cultures of *T. primitia* ZAS-1 and ZAS-2 used for such experiments were grown in medium 2YA through two transfers, at which point the residual 5-formyl-THF carried over with the inoculum permitted detectable, but limited, growth. The growth substrates provided to *T. primitia* strains in all experiments were 10 mM maltose and H₂-CO₂ (80:20 in the culture headspace).

Folate secretion bioassay plates were prepared as described by Hewitt and Vincent (20) and were seeded with the folate-requiring organism *Enterococcus hirae* ATCC 8043. The culture media for folate-secreting isolates (ZFX strains) varied according to the experimental conditions. Trypticase-Phytone-yeast extract (TPY) medium (2) was used as a relatively nonselective medium for isolation of termite gut heterotrophs. Medium GM2 contained the following components (per liter): NaCl, 1.0 g; KCl, 0.5 g; MgCl₂ · 6H₂O, 0.4 g; CaCl₂ · 2H₂O, 0.1 g; NH₄Cl, 0.3 g; KH₂PO₄, 0.2 g; Na₂SO₄, 0.15 g; NaHCO₃, 5.8 g; 3-*N*-(morpholino)propanesulfonic acid (MOPS), 10 mmol; yeast autolysate (29), 5 ml; and trace element solution SL11, a selenite-tungstate solution, a seven-vitamin solution, and vitamin B₁₂ (59), 1 ml each. Medium GM3 contained 0.2% yeast extract, 0.2% peptone, and 20 mM glucose. Medium GM4 contained the following components (per liter): NaCl, 1.0 g; KCl, 0.5 g; MgCl₂ · 6H₂O, 0.4 g; CaCl₂ · 2H₂O, 0.1 g; NH₄Cl, 0.3 g; KH₂PO₄, 0.2 g; Na₂SO₄, 0.15 g; NaHCO₃, 5.8 g; Casamino Acids (Difco), 5 g; and MOPS, 10 mmol. After autoclaving, medium GM4 was supplemented with 0.01 g of glutathione per liter, 0.04 g of asparagine per liter, 0.04 g of glutamine per liter, 0.02 g of uracil per liter, 0.01 g of adenine per liter, 0.01 g of guanine per liter, 3.6 g of glucose 3.6 per liter, and trace element and vitamin solutions described by van Neil and Hahn-Hagerdal (57). Routine cultivation of ZFX strains was performed on plates containing reinforced clostridial medium (Difco) with 2% agar at 30°C. The initial pH values for all media used were pH 7.2 to 7.4.

Isolation and identification of folate secretors. The hindguts of two worker larvae of *Z. angusticollis* were extracted with sterile forceps under anoxic conditions, any attached midgut segments were removed from the hindguts, and the hindguts were placed in a small glass tissue homogenizer containing 5 ml of dithiothreitol-reduced buffered salts solution (3, 28). The hindguts (total volume, ca. 5 µl) were then homogenized, and a serial 10-fold dilution series of hindgut homogenate was made in tubes containing anoxic TPY medium (headspace, 100% N₂). Aliquots (0.1 ml) of the resulting dilutions were spread in triplicate on plates containing TPY medium with 2% agar and incubated under anoxic (95% N₂-5% H₂), hypoxic (98.5% N₂-1.5% O₂), or oxic (air) conditions at 25°C. Colonies that developed were enumerated and grouped by colony and cell morphology. Representatives of each group were then picked and patched onto folate secretion bioassay plates, which were incubated under the same atmospheres used for the original isolations. Formation of satellite *E. hirae* colonies around patches 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. Putative folate-secreting strains were streaked for isolation prior to further characterization.

To test folate-secreting isolates for production of folate compounds useful to *T. primitia*, subsamples of cultures grown in medium GM2 were collected at the onset of the stationary phase, and the pH was adjusted to 7 prior to centrifugation at 10,000 × *g* for 10 min. Supernatants were degassed with a vacuum and flushed with N₂ several times, passed through a 0.2-µm-pore-size filter, and added to *T. primitia* cultures in medium 2YA at a concentration of 10% (vol/vol). Cultures of *T. primitia* that served as inocula in these experiments were grown in 5-formyl-THF-free medium 2YA through two transfers to reduce the residual amount of 5-formyl-THF carried over with the inoculum.

Identification of isolates was based on the 16S rRNA gene sequence (see below), as well as physiological and nutritional properties. Substrate utilization was examined by using basal medium GM2 to which test substrates were added. The media were kept in 18-mm anaerobe tubes (Bellco Glass, Vineland, N.J.) with rubber stoppers under 100% N₂ or in nephelometry flasks with foam stoppers under air. Most substrates were provided at a final concentration of 10 mM; the only exceptions were methoxylated aromatic compounds, which were provided at a final concentration of 2 mM. Incubation was at 30°C. A 20% or greater increase in the cell yield in the presence of a test substrate compared to the cell yield in its absence was taken to indicate that the substrate was utilized as an energy source. The cell yield was determined by measuring the optical densities of cultures at 600 nm with a Milton Roy Spectronic 20 colorimeter. Soluble metabolic products were analyzed by HPLC with refractive index detection (3), and H₂ production was measured by gas chromatography (4).

Analysis of 16S rRNA genes. Genomic DNA was prepared by suspending 10 µl of a stationary-phase culture in 1.0 ml of sterile distilled water and centrifuging

the preparation at 15,000 × *g* for 1 min. The supernatant was removed, and the resultant pellet was resuspended in 200 µl of 5% Chelex DNA extraction reagent (Perkin-Elmer) and incubated for 30 min at 56°C. Samples were then incubated at 100°C for 8 min and centrifuged at 15,000 × *g* for 2 min. The DNA in supernatants was quantified by measuring the absorbance at 260 nm. The 16S rRNA gene was amplified by PCR from putative folate-secreting strains by using primers 8f (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492r (5'-GGT TAC CTT GTT ACG ACT T-3'), a Gene Amp model 9600 thermocycler (Perkin-Elmer), and previously described reaction conditions (58). PCR products were purified with a QIAquick PCR purification kit (QIAGEN, Valencia, Calif.) and were subjected to restriction fragment length polymorphism (RFLP) analysis (27) by using the restriction enzymes RsaI and HpaII (New England Biolabs). One representative of each RFLP banding pattern was selected for sequencing. Nearly full-length (~1,500-nucleotide) 16S rRNA gene sequences were determined by 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 by using the ARB software package (www.biol.chemie.tu-muenchen.de) after incorporation of comparative rRNA gene sequences from the Ribosomal Database Project (33). Sequences were aligned by using the ARB automatic aligner, followed by manual correction of ambiguous regions. A maximum-likelihood method (fastDNAmI) was used to generate phylogenetic trees (36).

Analysis of folate compounds. To prepare culture filtrates for folate analysis, samples of cultures pregrown in medium GM4 were collected at the end of logarithmic growth and amended with 2-mercaptoethanol (final concentration, 10 mM) and 2% (wt/vol) sodium ascorbate, and the pH was adjusted to 7 prior to centrifugation at 10,000 × *g* for 10 min. Supernatants were degassed and flushed with N₂ several times and then were passed through a 0.2-µm-pore-size filter and stored under N₂ at -80°C until they were processed for analysis (see below). To collect *Z. angusticollis* gut fluid, worker termites were placed in an anoxic glove box (85% N₂, 10% H₂, 5% CO₂), in which their guts were extracted (see above). After removal, the guts were pooled in groups of 10 on a small square of dry Parafilm, after which each hindgut was punctured at least once with the tip of the forceps. The punctured guts with adherent gut fluid were then transferred into a 1.5-ml Eppendorf centrifuge tube. When about 50 punctured guts had been collected in a single tube, they were centrifuged at 11,000 × *g* for 10 min to pellet the gut tissue and resident microbiota and, in the process, to express the remaining gut fluid, which constituted the supernatant liquid. A measured volume of the supernatant was removed and supplemented with 2-mercaptoethanol and sodium ascorbate as described above and then processed for analysis immediately.

Rat plasma conjugase (RPC) was used to remove excess glutamate residues (i.e., more than a single glutamate) from folate compounds prior to HPLC analysis. RPC was prepared as described by Pfeiffer et al. (38) by using rat plasma obtained from Pel-Freez Biologicals (Rogers, Ark.). The activity of the crude RPC preparation was confirmed as described previously (38). RPC was added to samples (either culture filtrates or termite gut preparations) at a concentration of 5% (vol/vol), and the mixture was incubated at 37°C for 1 h; this was followed by heating to 100°C for 5 min and then cooling on ice for 5 min. RPC-treated samples were centrifuged at 10,000 × *g* for 10 min at 5°C, and the supernatants were collected and stored at -80°C until HPLC analysis.

Folate compounds were identified by using a Shimadzu HPLC system (Shimadzu Corp., Kyoto, Japan) equipped with an Alltima C₁₈ column (250 by 4.6 mm; particle size, 5 µm; Alltech Associates, Deerfield, Ill.) and a protocol similar to that described by Pfeiffer et al. (38). Gradient elution was performed at 25°C with acetonitrile and 33 mM phosphoric acid (pH 2.3) as follows: the acetonitrile concentration was kept at 5% (by volume) for the first 9 min, then raised linearly to 7% over the next 13 min, then raised linearly to 16% over the next 9 min, then kept at 16% for 14 min, then raised to 25% over 2 min, then kept at 25% for 5 min, and finally decreased to 5% within 3 min. The flow rate was 0.8 ml/min, and the sample injection volume was 20 µl. Separated folate compounds were detected by using a Shimadzu model SPD-10A UV/VIS detector set at 280 nm and a model RF-10A fluorescence detector set at an excitation wavelength of 280 nm and an emission wavelength of 356 nm for reduced folate compounds and at an excitation wavelength of 360 nm and an emission wavelength of 460 nm for 10-formylfolate (24). Compounds were identified by comparison of the retention times of unknown compounds with the retention times of authentic folate standards. Concentrations were estimated from the areas under HPLC peaks and by reference to standard curves relating the fluorescence detector response (in millivolts) to known amounts of folate standards. The folate concentrations reported below for culture filtrates were corrected for folate carried over in the inocula.

FIG. 1. Requirement of folate compounds for growth of *T. primitia* strains ZAS-1 and ZAS-2. All folate compounds were provided at a final concentration of 500 ng/ml. Symbols: ■, folic acid; ○, dihydrofolate; ▲, tetrahydrofolate; ▽, 5-formyl-THF; ◇, no addition. OD₆₀₀, optical density at 600 nm.

Nucleotide sequence accession numbers. The 16S rRNA gene sequences of *S. grimesii* ZFX-1 and *Lactococcus lactis* subsp. *lactis* ZFX-2 have been deposited in the GenBank database (www.ncbi.nlm.nih.gov/GenBank/index.html) under accession no. AY789460 and AY789461, respectively.

RESULTS

Folate requirement of *T. primitia*. *T. primitia* strain ZAS-2 was able to use folic acid, DHF, THF, or 5-formyl-THF (i.e., folinate) for growth, whereas strain ZAS-1 used only THF or 5-formyl-THF (Fig. 1). Growth of ZAS-1 was actually inhibited in the presence of folic acid or DHF compared to the folate-free control cultures. Addition of 500 ng of 5-formyl-THF per ml to these seemingly inhibited cultures permitted normal growth (data not shown), suggesting that the relatively large pool of added folic acid or DHF interfered with uptake and/or utilization of the otherwise growth-limiting amount of 5-formyl-THF carried over with the inoculum. For both strains, approximately half-maximal growth yields (1.1×10^9 cells/ml

for ZAS-1 and 2.9×10^8 cells/ml for ZAS-2) were attained in medium 2YA supplemented with 100 ng of 5-formyl-THF per ml. Provision of the folate precursors *p*-aminobenzoate, glutamate, GTP, and various pteridines (pterin, 6-hydroxymethylpterin, pteric acid), individually or together, did not alleviate the requirement of either strain for a preformed folate.

Folate-secreting bacteria from termite hindguts. The numbers of CFU recovered from the hindguts of *Z. angusticollis* were 5.0×10^5 CFU per hindgut for oxic conditions, 1.1×10^6 CFU per hindgut for hypoxic conditions, and 6.5×10^5 CFU per hindgut for anoxic conditions. Of 32 colonies picked from the highest-dilution plates and sorted on the basis of colony and cell morphology and incubation atmosphere (oxic, anoxic, or hypoxic), 18 (designated ZFX strains) were determined to be putative folate secretors, i.e., they were surrounded by a corona of satellite colonies when they were patched onto folate-free bioassay medium seeded with *E. hirae*, the folate-requiring bioassay organism. A similar result was obtained with *B. infantis* S12, a known folate secretor (10) used as a positive control. The diameter of the coronas surrounding ZFX strains (ca. 2 cm) was similar to the diameter of the coronas surrounding *B. infantis* S12. Of the 14 isolates that yielded negative results, 13 failed to grow on the assay plates (possibly indicating that the isolates themselves required folate), and 1 grew but did not support the development of *E. hirae* satellite colonies.

All 18 ZFX strains grew well under oxic, hypoxic, and anoxic conditions and could be easily placed into one of two groups based on two distinct RFLP patterns of the PCR-amplified 16S rRNA genes. Analysis of a nearly full-length sequence of this gene from a representative of each RFLP group (strains ZFX-1 and ZFX-2) revealed that strain ZFX-1 was closely related to the *Serratia liquefaciens* complex (16), with 99.7% 16S rRNA sequence similarity to *S. grimesii*. Hence, strain ZFX-1 was provisionally identified as a strain of this species. Strain ZFX-2 was a *Lactococcus* strain whose 16S rRNA sequence exhibited 99.9% similarity to the 16S rRNA sequence of *L. lactis* subsp. *lactis* (Fig. 2). All isolates in the ZFX-1 RFLP group were motile gram-negative rods that were approximately 0.6 to 0.8 by 1.5 to 2 μ m; all isolates in the ZFX-2 group produced nonmotile coccoid cells that were approximately 0.5 to 0.8 by 0.9 to 1.1 μ m and occurred in short chains. Together, these results suggest that all folate-secreting isolates were similar, if not identical, to either *S. grimesii* ZFX-1 or *L. lactis* ZFX-2. Both of these strains have been deposited in the American Type Culture Collection, Manassas, Va. (accession no. BAA-1054 and BAA-1055, respectively). The mean numbers of ZFX-1-like and ZFX-2-like strains in *Z. angusticollis* were 5.1×10^5 and 5.4×10^5 CFU per hindgut, respectively. Given a mean hindgut fluid volume of about 3.2 μ l (i.e., 63.2% of the total hindgut volume [35]), this translates to approximately 3.3×10^8 total folate secretor CFU/ml of hindgut fluid.

Under aerobic and anaerobic conditions, *S. grimesii* ZFX-1 and *L. lactis* ZFX-2 were capable of growth on glucose, mannitol, ribose, xylose, maltose, trehalose, and, in the case of the *L. lactis* strain, cellobiose. During aerobic growth, *S. grimesii* also utilized acetate, pyruvate, lactate, fumarate, malate, and succinate as energy sources. Arabinose, glycine, formate, methanol, ferulate, syringate, and trimethoxybenzoate were not utilized by either strain. During anaerobic growth on glucose, *S.*

FIG. 2. Phylogenetic trees inferred from 16S rRNA gene sequences (~1,500 nucleotides) of strain ZFX-1 (A) and strain ZFX-2 (B) and related organisms. A maximum-likelihood technique (fastDNAm1) was used to generate the trees. The homologous sequence of *Desulfovibrio senexii* was used as an outgroup in the tree in panel A (data not shown). The scale bars indicate the evolutionary distance per nucleotide and are based on sequence divergence.

grimesii ZFX-1 produced a mixture of succinate, acetate, ethanol, 2,3 butanediol, and H_2 . No organic products were detected during aerobic growth of strain ZFX-1 on glucose, implying that glucose was completely oxidized under these conditions. Under both aerobic and anaerobic conditions in medium GM2, strain ZFX-2 carried out a homolactic fermentation of glucose, which is consistent with its identification as *L. lactis* (54). The fermentation pattern was not altered during growth in a complex medium (medium GM3).

Folate secretion by *S. grimesii* ZFX-1 and *L. lactis* ZFX-2. Culture filtrates of ZFX-1 and ZFX-2, when incorporated into folate-free medium 2YA at a concentration of 10% (vol/vol), increased the growth of *T. primitia* to levels well above the level permitted by the 5-formyl-THF carried over with the inoculum (Fig. 3). The cell yields of *T. primitia* ZAS-1 in media supplemented with culture filtrates from *S. grimesii* ZFX-1 and *L. lactis* ZFX-2 (8.3×10^8 and 11.1×10^8 cells/ml, respectively) were similar to the yields in media supplemented with 100 ng of 5-formyl-THF/ml (12.8×10^8 cells/ml). However, addition of *T. primitia* culture filtrates to media used to grow the ZFX strains did not elicit any discernible enhancement of folate production by either ZFX strain (data not shown).

HPLC analysis of rat plasma conjugase-treated, late-log-phase culture filtrates of the two ZFX strains revealed a dis-

FIG. 3. Growth of *T. primitia* strains ZAS-1 and ZAS-2 in folate-free medium 2YA supplemented with 5-formyl-THF (final concentration, 500 ng/ml) (○) or culture filtrates of *S. grimesii* strain ZFX-1 (◇) or *L. lactis* strain ZFX-2 (■). Culture filtrates were added at a final concentration of 10% (vol/vol). Negative controls (▲) were supplemented with 10% uninoculated medium GM3. OD₆₀₀, optical density at 600 nm.

tinct peak with a retention time (36 min) identical to that of authentic 5-formyl-THF (Fig. 4). The concentrations of 5-formyl-THF in culture fluids of *S. grimesii* ZFX-1 and *L. lactis* ZFX-2 at the end of growth were 146 ± 23 and 117 ± 11 ng/ml, respectively ($n = 3$). No other peaks corresponding to folates were observed when any of the three detection methods was used (see Materials and Methods), suggesting that 5-formyl-THF was the major if not sole folate compound secreted by both strains. The material in the *S. grimesii* ZFX-1 culture fluid responsible for the broad peak at a retention time of 31 min has not been identified yet, but it did not correspond to any of the folate standards (see Materials and Methods). In ZFX-1 and ZFX-2 filtrates not pretreated with conjugase, the

FIG. 4. HPLC chromatograms showing fluorescent (excitation at 280 nm, emission at 359 nm) compounds present in a standard mixture of tetrahydrofolate (1 ng), 5-methyl-THF (5-CH₃-THF) (1 ng), and 5-formyl-THF (5-HCO-THF) (5 ng), culture filtrates of *S. grimesii* ZFX-1 and *L. lactis* ZFX-2, and hindgut fluid of *Z. angusticollis*. In the chromatograms of culture filtrates, the dotted and solid lines indicate samples taken at the time of inoculation and at the end of logarithmic growth, respectively. The material responsible for the broad peak in the ZFX-1 culture filtrate (between 29 and 32 min) was not identified but did not correspond to any folate standard.

retention times of the folates detected were most similar to that of the triglutamate form of folic acid, suggesting that 5-formyl-THF secreted by the ZFX strains is the triglutamate derivative.

Analysis of *Z. angusticollis* hindgut fluid also revealed a single well-defined peak corresponding to 5-formyl-THF (Fig. 4). At a volume of 3.2 μ l of cell-free fluid per hindgut (see above), the concentration of 5-formyl-THF was estimated to be 1.3 μ g/ml, which would be more than sufficient to support the growth of *T. primitia* in situ.

DISCUSSION

Results of this study support our previous hypothesis that in situ the folate requirement of *T. primitia* is met by folate-secreting members of the hindgut microbiota of *Z. angusticollis* (14). Among the observations that make this interpretation compelling are (i) the specific folate secreted by *S. grimesii* ZFX-1 and *L. lactis* ZFX-2 in vitro (i.e., 5-formyl-THF) satisfied the folate requirement of both *T. primitia* strains; (ii)

5-formyl-THF was the only detectable folate compound in *Z. angusticollis* hindgut fluid and was present at a concentration (1.3 μ g/ml) that was 13-fold greater than the concentration required to support half-maximal growth of *T. primitia* strains in vitro; and (iii) the combined population densities of the ZFX strains in situ (2×10^8 CFU per ml of hindgut contents), together with the amount of 5-formyl-THF secreted by cells (ca. 10 to 100 ng/ml/ 10^8 cells), suggested that the ZFX strains made a significant contribution to the 5-formyl-THF pool in situ. Inasmuch as the extent of folate secretion by bacteria can vary with the growth rate, pH, and ionic strength (52), it may well be that the conditions in situ provoke even greater secretion per cell than we observed in vitro. Upon uptake by spirochetes, 5-formyl-THF is presumably converted to 5,10-methylenyl-THF by the physiologically irreversible synthetase (EC 6.3.3.2), which is widely distributed in prokaryotes and eukaryotes (21) and which serves to usher 5-formyl-THF into THF-dependent C₁ interconversion pathways. Although the impetus for this study was to identify an in situ source of folate for symbiotic spirochetes, 5-formyl-THF secretion by ZFX strains may be beneficial to the termites as well. Most insects appear to require a dietary source of folate (13), and the folate content of woody plant tissue on which termites feed is likely to be quite low. Bacterially produced folates might be either taken up from the hindgut directly or acquired by coprophagy.

Lactic acid bacteria, including species similar to the folate-secreting *L. lactis* strain ZFX-2, are common members of termite gut microbial communities (1, 12, 46, 55). The population density of ZFX-2-type cells in *Z. angusticollis* is similar to that of lactic acid bacteria in other termite species, in which they represent approximately 3% of the total prokaryotic microbiota (55). Not only are these organisms important in the production of lactate and acetate, the former of which can be converted to acetate by other microbes in situ (47, 55), but they also have been implicated in the recycling of excretory nitrogen (uric acid) back to termites for biosynthesis (39–42). The present results reveal yet another dimension to the importance lactic acid bacteria as termite gut symbionts. Members of the genus *Serratia* are also common associates of insects, including termites such as *Z. angusticollis* (17). Although some species (e.g., *Serratia marcescens*) have been previously implicated as insect pathogens (50), the *S. grimesii* strains isolated here appear to have quite the opposite role. Based on their broad distribution among arthropods, it has recently been suggested that members of the genus *Serratia* play an as-yet-undefined but important role in their hosts (6). As folate secretion has also been reported for other *Serratia* strains (23), provision of this cofactor could represent one potential beneficial role for these organisms in insect gastrointestinal systems.

The amounts of folate secreted by ZFX-1 and ZFX-2 in vitro (117 to 146 ng/ml) were within the range observed for various other folate-secreting bacteria (20 to 160 ng/ml) (10, 22, 23, 52). However, it is interesting that the amount secreted by *L. lactis* ZFX-2 was higher than the amounts observed for 16 strains of *L. lactis* isolated from a variety of sources (5 to 46 ng/ml), which retained the majority of the folate produced intracellularly (52). It has recently been shown that the degree of folate polyglutamation is the primary factor that affects the extent of folate secretion; folate derivatives with longer polyglutamate tails are more likely to be retained intracellularly (53). It

is thus likely that the higher level of folate secretion observed in *L. lactis* ZFX-2 is the result of production of the triglutamate form of 5-formyl-THF rather than the more extensively glutamated forms (four to six residues) common in other *L. lactis* strains (52). Likewise, the nature of the folate secreted by *L. lactis* ZFX-2 (5-formyl-THF) was different from that of the folates produced by other *L. lactis* strains examined (10-formyl-THF and 5,10-methenyl-THF) (52). These observations suggest that the termite-associated *L. lactis* strains may have evolved some degree of specialization as folate-secreting symbionts.

Secretion of folate compounds has been observed in members of diverse bacterial genera (10, 22, 23, 52), and cross-feeding of folate compounds between microbes has been either demonstrated directly or strongly implied in a number of gastrointestinal systems (8, 9, 26, 34, 43, 44), including the bovine rumen (19, 48). Although secretion of folate represents a cost to the organisms synthesizing this cofactor, it benefits the overall symbiosis. It enables homoacetogenic microbes such as *T. primitia* to perform CO₂-reductive acetogenesis, a process that minimizes the loss of carbon and reducing potential (in the form of CO₂, H₂, or CH₄) by production of acetate, the host's primary energy source. Acetate is also likely to be a major substrate supporting the respiratory activity of the gut wall-associated microbiota, which consumes inwardly diffusing oxygen and renders the luminal region of the hindgut anoxic for continued fermentative production of acetate (7, 11).

In recent years increased attention has been focused on the beneficial aspects of microbial communities in gastrointestinal systems (18, 60). The results of this study underscore the importance of considering not only direct benefits to hosts but also the nutritional interactions and interdependencies between individual species that constitute the complex and dynamic, yet highly integrated, consortial communities typical of many animal gastrointestinal tracts.

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