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Diversity of Formyltetrahydrofolate Synthetases in the Guts of the Wood-Feeding Cockroach *Cryptocercus punctulatus* and the Omnivorous Cockroach *Periplaneta americana*▽†

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We examined the diversity of a marker gene for homoacetogens in two cockroach gut microbial communities. Formyltetrahydrofolate synthetase (FTHFS or fhs) libraries prepared from a wood-feeding cockroach, *Cryptocercus punctulatus*, were dominated by sequences that affiliated with termite gut treponemes. No spirochete-like sequences were recovered from the omnivorous roach *Periplaneta americana*, which was dominated by *Firmicutes*-like sequences.

The guts of wood-feeding termites and *Cryptocercus punctulatus* cockroaches share an unusual pattern of electron flow, as high rates of CO_2-reductive acetogenesis typically supplant methanogenesis as the terminal electron sink (2, 3). Past studies have shown that from 10 to 30% of gut acetate produced in environments of termites and wood-feeding cockroaches is microbially generated from CO_2 (3, 28), ultimately powering 18 to 26% of the host insect's own respiratory energy metabolism (25). Nevertheless, most termites emit methane (2), and termite emissions constitute approximately 4% of the global methane budget (27). Cockroaches have been proposed to represent an additional source of note (9). Interestingly, methanogenic termites and cockroaches exhibit increased acetogenesis following addition of exogenous H_2 (3, 29). This suggests that these insects are host to a robust population of bacteria that are capable of homoacetogenesis but may be primarily using alternative electron donors (and other substrates and pathways) *in vivo*.

Acetogenic bacteria belonging to two bacterial phyla, *Firmicutes* and *Spirochaetes*, have been isolated from the guts of termites (1, 4, 11, 12, 14). Several surveys have targeted and used the gene for formyltetrahydrofolate synthetase (FTHFS), a key gene in the Wood-Ljungdahl pathway of acetogenesis (16), as a potential marker for the pathway (15, 18). For the wood-feeding termites that have been examined, the studies have revealed an abundance of FTHFS sequences that form a coherent phylogenetic cluster, together with genes from homoacetogenic termite gut spirochetes belonging to the genus *Treponema* (24, 26, 30). This suggests that treponemes may be among the more abundant of the homoacetogens active in these environments.

Little is known about the population structure and biology of CO_2-reducing, acetogenic bacteria in the guts of either omnivorous or wood-feeding cockroaches. The wood-feeding cockroach *Cryptocercus* hosts an abundance of flagellate protozoa closely related to those believed to dominate polysaccharide fermentation in the guts of termites (5, 6, 22), suggesting that at least one key environmental niche is filled by similar microbes in both termites and *Cryptocercidae*. Additionally, *Cryptocercidae* cockroaches, like termites, house diverse spirochetes and are the site of intense CO_2 reduction into acetate (3, 7). Possibly, spirochetes capable of CO_2 reduction into acetate are present in the hindguts of cockroaches. However, no evidence has yet been presented for the existence of homoacetogenic treponemes in environments other than the guts of termites, and FTHFS surveys of human (21) or horse (15) fecal matter and bovine rumen samples (20) revealed only *Firmicutes*-like and other FTHFS alleles that are distinct from those comprising the termite treponeme cluster.

Here, by examining FTHFS gene diversity in *Cryptocercus punctulatus* and *Periplaneta americana* guts, we endeavored to learn more about the distribution and origins of homoacetogenic treponemes (and their genes) that are found in wood-feeding termites. In particular, we wished to ascertain whether FTHFS genes present in either of the two cockroaches are termite treponeme-like and, if so, whether analysis reveals any obvious signal indicating recent or ancient lateral community transfer events between insect lineages.

Analysis of FTHFS diversity in the guts of cockroaches. We catalogued FTHFS gene sequence diversity present in the guts of a wood-feeding cockroach of the family *Cryptocercidae* (*Cryptocercus punctulatus*) and an omnivorous cockroach of the family *Blattidae* (*Periplaneta americana*) in an effort to shed light on the evolutionary origins of the homoacetogenic spirochetes thought to dominate acetogenesis in the guts of wood-feeding termites.

Library preparation and sequence analysis. FTHFS gene diversity was examined in DNA extracted from pooled whole guts of two *P. americana* adults, from a single whole-gut sample

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TABLE 1. Abundance of FTHFS sequence clades in libraries constructed from *P. americana* and *C. punctulatus* gut extracts[a]

Host insect	Abundance of FTHFS sequence clades				
	Treponeme affiliated	*Clostridium* sp. M62/1	Clone F group	Clone E group	Other
P. americana	0	41	31	0	28
C. punctulatus adult	88	0	0	2	10
C. punctulatus nymph	50	2	0	41	7

[a] Clades defined as marked in Fig. 1 and 2. Abundance given as percentage of full-length clones.

from a *C. punctulatus* adult, and from three pooled whole-gut samples from *C. punctulatus* nymphs. *P. americana* specimens were collected on the grounds of the California Institute of Technology (Pasadena, CA), and gut DNA was extracted within 24 h of collection. *Cryptocercus punctulatus* specimens were collected and supplied by Christina Nalepa (North Carolina Department of Agriculture and the North Carolina State University). The *C. punctulatus* adult used in this study was collected from a site on Mt. Collins (TN) and the nymphs from the South Mountains of North Carolina. Upon receipt, they were maintained in the laboratory in glass jars in the dark, and gut DNA was extracted within a week of receipt (*C. punctulatus*) or within 24 h of collection (*P. americana*). DNA was purified as described elsewhere (30). Cockroach and termite identifications were confirmed using insect mitochondrial cytochrome oxidase subunit II (COII) gene sequences (see Fig. S1 in the supplemental material). COII genes were amplified from whole-gut DNA extracts, using primers and cycling conditions described by Park et al. (23).

FTHFS genes were amplified from insect guts by using primers developed by Leaphart and Lovell (15) with the recommended step-down protocol (15) and 25 cycles at 55°C. PCR products were purified, cloned, and screened via restriction fragment length polymorphism (RFLP) analysis using the restriction enzyme HinP1I. A single representative of each RFLP type was selected for sequencing. Deduced FTHFS protein sequences were aligned using MUSCLE (8), and phylogenetic analyses were carried out using the ARB software package (19). The Bellerophon program (10) was used to examine the library for possible chimeric sequences.

Sequences generated in this study have been deposited into GenBank under accession numbers GU552320 through GU552435. This collection also includes previously unpublished FTHFS sequences from a library constructed and analyzed in the same manner from hindguts of dry-wood (*Kalotermitidae*) termites collected in Pasadena, CA, belonging to an *Incisitermes* species, most likely to be *I. minor*.

RFLP analyses revealed 34, 26, and 29 nonchimeric phylotypes from *P. americana*, the *C. punctulatus* adult, and the *C. punctulatus* nymphs, representing 86, 60, and 88 full-length clones, respectively. These phylotypes were further binned into operational taxonomic units (OTUs) by using a cutoff of 98% amino acid sequence similarity and characterized by phylogenetic analysis (Table 1; see Table S1 in the supplemental material). Three OTUs from *P. americana*, represented by clone 1A, clone 2B, and clone 7A could not be reliably placed by phylogenetic analysis and are not included in presented trees.

Termite treponemas and the "*Treponeme*-affiliated" FTHFS cluster. Gut bacteria carrying termite treponeme-like FTHFS genes are commonly encountered in termite gut communities. In the four termite species (representing four of the six termite families) examined previously, sequences that affiliated with the termite treponeme group were more frequently encountered than were those from putative homoacetogenic *Firmicutes* (24, 26, 30). Here, this pattern is shown to extend to the flagellate- and spirochete-rich hindgut communities of the wood-feeding cockroach, *C. punctulatus* (Fig. 1). In contrast, treponeme-like FTHFS sequences were not identified in libraries prepared from *P. americana*.

The termite treponeme cluster of FTHFS gene sequences is defined as encompassing FTHFS alleles from termite *Treponema* isolates and termite gut environmental sequences that phylogenetically affiliate with them and share a conserved hexapeptide insert (26). Two major groups of FTHFS alleles (cockroach groups I and II) from *C. punctulatus* fell within the termite treponeme cluster (Fig. 1). These cockroach-derived sequences were distinct from the FTHFS variants that originated from termite hindgut communities, with less than 93% amino acid similarity to the most closely related termite-derived sequences. Cockroach group II comprised three alleles affiliated with a set of FTHFS gene sequences (from *Zootermopsis nevadensis* and dry-wood termites) lying at the base of the termite treponeme cluster. The FTHFS alleles that comprise "cockroach group I" are more numerous than those representing cockroach group II and fell well within the termite treponeme FTHFS radiation. The robustness with which the cockroach group I sequences cluster to the exclusion of termite community-derived sequences, and the long branch length separating this group from the others, is inconsistent with it having arisen after a recent lateral acquisition from termites. However, the short branch lengths between the alleles comprising this group, compared to most others on the tree, including those of cockroach group III, suggest that a breakout radiation of diversity has occurred in cockroach group I after either a lateral acquisition event occurred or an evolutionary bottleneck was resolved. As a result, lateral transfer of cockroach group I genes, or the organisms that encode them, from a termite hindgut community cannot be ruled out.

A cluster of *C. punctulatus* FTHFS sequences, "cockroach group III," represents a sister branch to the termite *Treponeme* clade. This group fell at the base of the termite treponeme cluster (to the exclusion of all other deduced FTHFS proteins available for analysis), using all three of the treeing algorithms employed. This group lacked the hexapeptide insert (Fig. 1, right panel) previously used to define the termite treponeme cluster (26) but was classified as treponeme-affiliated on the strength of its phylogenetic affiliation with the termite treponeme cluster. This cluster had a high level of within-group diversity, as evidenced by branch lengths. If FTHFS variants belonging to this cluster of sequences were present in the gut communities of the last common ancestor of termites and cockroaches, they appear to have subsequently gone extinct prior to the radiation of the four (of the six) lower termite families that have been examined. The position of this clade basal to the termite treponeme assemblage and its absence from all of the phylogenetic breadth of termites thus far examined suggest that the organisms encoding the cockroach

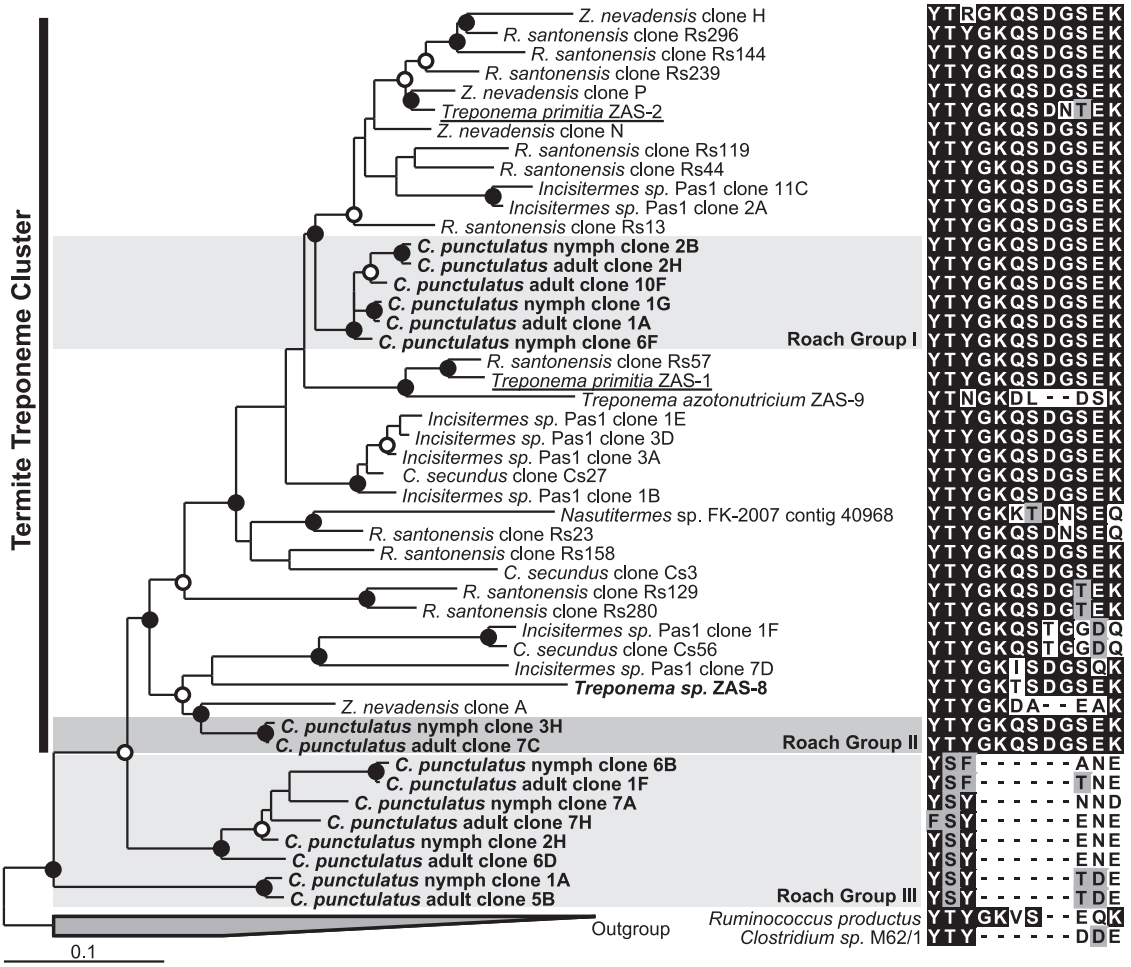

FIG. 1. Phylogenetic analysis of termite treponeme and treponeme-affiliated FTHFS sequences. (Left) Tree constructed using PHYML algorithm and 343 unambiguous, aligned amino acid positions. Open circles mark groupings also supported by either Phylip PROTPARS parsimony or Fitch distance algorithms. Closed circles identify clusters grouped by all three treeing methods. Scale bar represents 0.1 amino acid changes per alignment position. The tree was rooted using FTHFS sequences from *Ruminococcus* and *Clostridium* sp. M62/1 clades in Fig. 2. (Right) A highly variable region of the protein sequence, corresponding to residues 229 to 234 in *Moorella thermoacetica* (ABC18448.1). Each line of the alignment corresponds to a sequence in the tree to the left.

group III alleles should be targets for future study. In particular, it would be of interest to confirm whether these organisms are indeed homoacetogens and/or spirochetes and to examine any similarity that they have to homoacetogenic termite treponemes.

***Firmicutes* homoacetogens and "Lovell cluster A."** A number of sequences that fell outside the treponeme-affiliated cluster but within the "Lovell cluster A" of potential acetogens (18) were identified in the three cockroach-derived libraries (Fig. 2; see Fig. S2 in the supplemental material). This cluster incorporates all currently sequenced *Firmicutes* acetogens. However, with the addition to the analysis of FTHFS genes from newly sequenced microbial genomes, Lovell cluster A can no longer be considered to represent a single, monophyletic "*Firmicutes* acetogens" cluster. That notwithstanding, within this cluster, three groups with high representation in our clone libraries were identified.

A total of 41% of all clones recovered from the omnivorous cockroach *P. americana* and ca. 2% of those identified in the *C. punctulatus* nymphs clustered phylogenetically within the Lovell cluster A, with an FTHFS sequence from *Clostridium* sp. M62/1, a butyrate-producing *Firmicutes* strain originally isolated from human feces (17). This group represents the most abundant (and most OTU-rich) cluster of FTHFS sequences identified in the gut of the omnivorous cockroach *P. americana.* To our knowledge, *Clostridium* sp. M62/1 has not yet been demonstrated experimentally to be capable of homoacetogenic growth. The FTHFS gene in this organism is located in close proximity to a putative glutamate formiminotransferase and formimidoyltetrahydrofolate cyclodeaminase, which suggests that may be playing a role in other pathways, e.g., in histidine metabolism. However, as this cluster of FTHFS variants was found to be the most highly diverse and abundant sequence cluster in gene libraries of *P. americana*, it may represent the best candidate for the resident population of acetogenic bacteria in that insect's gut environment. CO_2-reductive acetogenesis has previously been demonstrated to occur at low yet significant rates in this insect (3, 13).

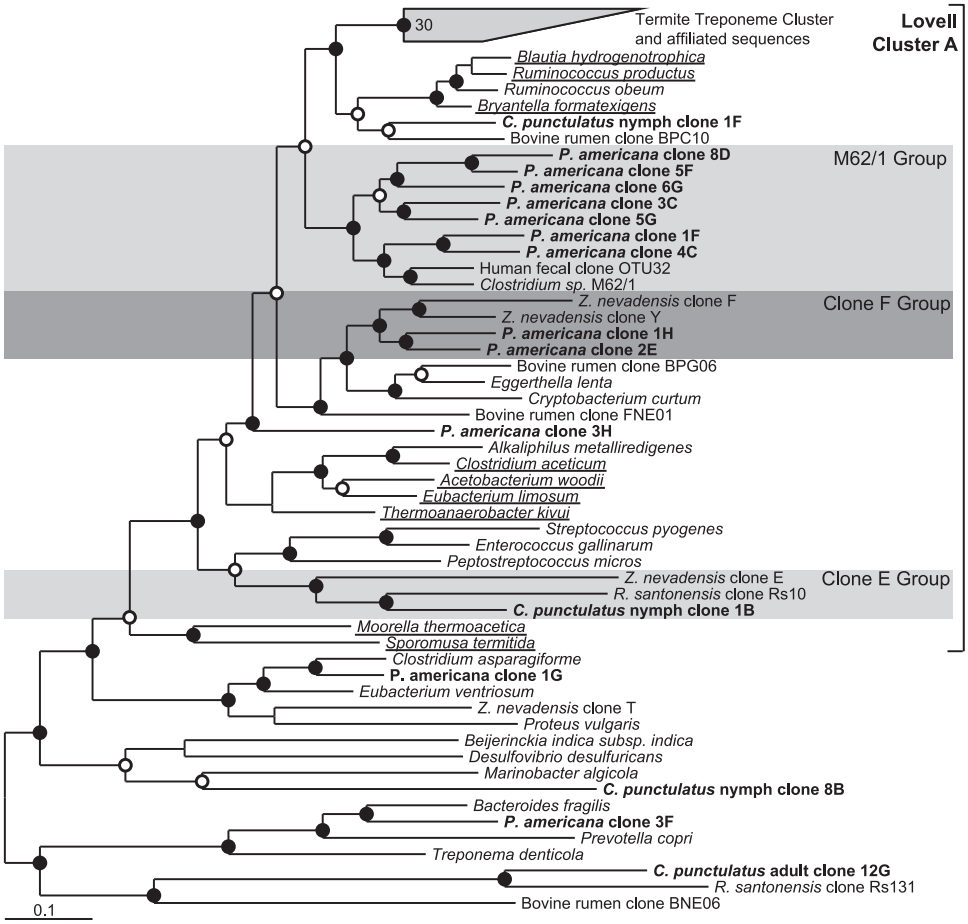

FIG. 2. Phylogenetic analysis of FTHFS sequences representing more than 2% of *C. punctulatus* or *P. americana* clone libraries. The unrooted tree was constructed using PHYML algorithm and 311 unambiguous, aligned amino acid positions. Open circles mark groupings also supported by either Phylip PROTPARS parsimony or Fitch distance algorithms. Closed circles identify clusters grouped by all three treeing methods. Scale bar represents 0.1 amino acid changes per alignment position. The treponeme-affiliated FTHFS cluster is represented by 30 termite and *Cryptocercus* sequences. An expanded tree containing all FTHFS OTUs and additional reference sequences can be found in the supplemental materials.

Two alleles representing 31% of the *P. americana* library clustered with "Clone F" and "Clone Y" FTHFS sequences previously identified in a study on the dampwood termite *Z. nevadensis* and also affiliated more remotely with FTHFS sequences from two *Actinobacteria* species (*Eggerthella lenta* and *Cryptobacterium curtum*). These alleles are borne by organisms of uncertain phylogenetic position and appear to be present at various abundances in the guts of diverse termites and cockroaches.

One final FTHFS clade identified in this study is of potential interest, as it encompasses a diverse group of alleles that group with *Z. nevadensis* clone E and *Reticulitermes santonensis* clone Rs10 (24, 26). Sequences affiliated with this group were identified in both *C. punctulatus* libraries (Table 2; see Fig. S2 in the supplemental material) but were far more abundant in the library prepared from the nymph specimens (41%) than in that prepared from the adult (2%) specimens. The significance of this difference is hard to infer at this juncture, as these were insects from different colonies, geographical locations, and developmental stages. However, the magnitude of this difference suggests that a comprehensive exploration of the variability of FTHFS community composition in *Cryptocercidae* may present a fruitful line of inquiry.

Implications. The hindgut microbial communities of both the omnivorous cockroach *Periplaneta americana* and the wood-feeding cockroach *Cryptocercus* bear a variety of FTHFS genes, However, the phylogenetic profiles of the genes from these two cockroaches are entirely distinct. The FTHFS alleles from the omnivorous cockroach appear to share more commonalities with those from vertebrate gut tracts, whereas those from *C. punctulatus* appear to share more commonalities with those from termite gut communities. In particular, many of the FTHFS alleles recovered from *C. punctulatus* are postulated to be encoded by homoacetogenic spirochetes based on phylogenetic inference. Similar alleles were not recovered from *P. americana*, suggesting that homoacetogenic spirochetes are absent from the gut community of omnivorous cockroaches. This suggests that the emergence of a microbial community rich with homoacetogenic spirochetes may have been coincident with the evolution of the wood-feeding lifestyle and the widespread success of that host and its subsequent lineages. Given that the majority of cockroach species examined have been

methanogenic (9), it seems likely that the common ancestor of *Blattidae* and *Cryptocercidae* had a gut fermentation dominated by methanogenesis as the dominant electron sink. Since methane essentially represents calories lost to the nutrition of the host, the acquisition and/or evolution of the first homoacetogenic treponemes may have helped drive the subsequent success of the progenitor of the *Cryptocercidae* cockroaches and the termites, during the transition from an omnivorous to lignocellulose-based diet.

Nucleotide sequence accession numbers. Sequences generated in this study have been deposited into GenBank under accession numbers GU552320 through GU552435.

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