Acetogenesis from H_2 plus CO_2 and nitrogen fixation by an endosymbiotic spirochete of a termite-gut cellulolytic protist

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Symbiotic associations of cellulolytic eukaryotic protists and diverse bacteria are common in the gut microbial communities of termites. Besides cellulose degradation by the gut protists, reductive acetogenesis from H_2 plus CO_2 and nitrogen fixation by gut bacteria play crucial roles in the host termites' nutrition by contributing to the energy demand of termites and supplying nitrogen poor in their diet, respectively. Fractionation of these activities and the identification of key genes from the gut community of the wood-feeding termite *Hodotermopsis sjoestedti* revealed that substantial activities in the gut—nearly 60% of reductive acetogenesis and almost exclusively for nitrogen fixation—were uniquely attributed to the endosymbiotic bacteria of the cellulolytic protist in the genus *Eucomonympha*. The rod-shaped endosymbionts were surprisingly identified as a spirochete species in the genus *Treponema*, which usually exhibits a characteristic spiral morphology. The endosymbionts likely use H_2 produced by the protist for these dual functions. Although H_2 is known to inhibit nitrogen fixation in some bacteria, it seemed to rather stimulate this important mutualistic process. In addition, the single-cell genome analyses revealed the endosymbiont's potentials of the utilization of sugars for its energy requirement, and of the biosynthesis of valuable nutrients such as amino acids from the fixed nitrogen. These metabolic interactions are suitable for the dual functions of the endosymbiont and reconcile its substantial contributions in the gut.

endosymbiosis | spirochetes | single-cell genomics | adaptive evolution | metabolic interaction

Endosymbiotic associations between eukaryotic cells and bacteria, in which these partners share their unique abilities, have had a profound impact on ecological adaptation and niche expansion. In the gut of termites, there are various examples of species-specific symbiotic associations between protists (single-cell eukaryotes) and bacteria, and most, if not all, have cospeciated (1–6). The social behavior of termites has promoted the stable and sustained vertical inheritance of gut microbes between termite generations, which may be advantageous for the emergence and development of these symbiotic associations in the gut community (7). The gut microbial community is responsible for the utilization of nutritionally unbalanced and recalcitrant woody cellulose (6, 8). Owing to this ability, termites are a keystone of global carbon cycles, are economically detrimental as pests to timber constructions, and as such are expected to be useful in the application of producing biofuel from cellulosic biomass (5, 6, 8). However, the gut community comprises unique and diverse species, which are mostly yet-uncharacterized due to our historical inability to culture them in the laboratory.

In wood-feeding "lower" termites that harbor unique flagellated protists in their guts, these protists play a central role in cellulose digestion. The protists phagocytose ingested wood particles and almost completely decompose and ferment the cellulose to produce acetate, H_2, and CO_2 (6, 8). The host termite uses the produced acetate as a major carbon and energy source. The produced H_2 is a key metabolic intermediate that fuels many bacteria in the gut (9). The gut bacteria are also important for the nutrition of the host termite, carrying out both CO_2-reducing acetogenesis (hereafter, reductive acetogenesis) and nitrogen fixation (6, 10). The H_2 and CO_2 produced from the cellulose fermentation are converted by the gut bacteria via reductive acetogenesis to acetate, which accounts for up to one-third of the carbon and energy demand of the host termite (11). Termites thrive on dead wood extremely poor in nitrogen so the fixation of atmospheric N_2 is crucial for acquisition of nitrogen for the termite (10). The bacteria responsible for these two functions are inferred based on the characterizations of cultured isolates of spirochetes in the genus *Treponema*, the most abundant constituent of the gut bacterial microbiota in many wood-feeding termites (10). One species, *Treponema primitia*, is known to carry out reductive acetogenesis and another, *Treponema azotonutricium*, is a nitrogen fixer (12–15). Indeed, gene-based analyses suggest that treponemes are often major contributors for reductive acetogenesis and, to a lesser extent, nitrogen fixation in the gut microbial community (16–19).

The genome sequences of the two endosymbiont bacteria of two different gut protists have been reported, and their roles are inferred (20, 21). These endosymbionts have the genetic potential to use sugars produced during cellulose degradation in the protist cells and to upgrade nitrogenous nutrients for the host protists. In one of these endosymbionts, an ability of nitrogen

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fixation is also predicted (20). Furthermore, gene inventory studies suggest the importance of protist-associated bacteria for H_2 metabolism or nitrogen fixation in termite guts (19, 22). Therefore, the protist-associated bacteria are very likely to play important roles in the gut metabolism, but their actual activities and contributions are rarely evaluated and our knowledge of the symbiotic relationships is still fragmentary.

In this study, we localized activities of both reductive acetogenesis and nitrogen fixation in the gut of a wood-feeding termite, *Hodotermopsis sjoestedti* (Termopsidae), and identified key genes in the responsible bacterial species. We demonstrate that both functions were attributed to a single *Treponema* species endosymbiotic for a cellulolytic protist in the genus *Eucomonympha* (phylum Parabasalia). To further understand this endosymbiotic relationship, we examined the single-cell genome sequences of the endosymbiont and reconstructed its metabolic ability.

Results

Fractionation of Reductive Acetogenesis. To localize reductive acetogenesis activity in the gut community, the reduction of $^{14}CO_2$ to acetate was measured. Using a differential low-speed centrifugation technique, the gut contents were fractionated into the large protist-enriched fraction and the fraction containing small protists and freely swimming bacteria (small protist/bacteria fraction). The former fraction was extensively washed to remove free-swimming bacteria. Because reductive acetogenesis is sensitive to oxygen, all of the sample preparations and assays were performed under anoxic conditions. The large-protist fraction constituted up to 63% of the whole-gut activity whereas only less than 15% was detected in the small protist/bacteria fraction (Table 1). The supply of H_2 stimulated reductive acetogenesis activity in all of the fractions. Enzymes in the acetyl-CoA (Wood–Ljungdahl) pathway for reductive acetogenesis (23) showed a higher level of activity in the large protist fraction (Table S1).

The large-protist fraction almost exclusively consisted of the cellulolytic protists of the genera *Eucomonympha* and *Trichonympha*. The ^{14}C radio isotope assay under the presence of exogenously supplied H_2 was sensitive enough to allow the analysis of pools of manually isolated cells of each protist species independently. Strong activity was observed for *Eucomonympha* (9.5 ± 2.6 pmol/h per cell, $n = 3$) whereas only weak activity was detected in *Trichonympha* (0.31 ± 0.27 pmol/h per cell, $n = 3$). The activity associated with *Eucomonympha* was estimated to be ∼58% of the whole gut. Thus, the substantial activity of reductive acetogenesis in the gut was associated with the *Eucomonympha* protists.

Genes involved in the acetyl-CoA pathway, *acsA* (also known as *cooS*) and *acsB*, encoding two subunits of the acetyl-CoA synthase/carbon monoxide dehydrogenase complex (ACS/CODH), respectively, a gene encoding formyltetrahydrofolate synthetase, and a gene, *acsF*, encoding an Ni-insertion protein to ACS/CODH were successfully PCR-amplified and identified from associated bacteria of manually isolated *Eucomonympha* cells (*SI Text*). Transcribed sequences corresponding to the identified *acsA* and *acsB* were detected in RNA extracted from the gut community (*SI Text*). An in situ hybridization experiment against mRNA of the *acsB* confirmed expression and specific localization to the *Eucomonympha* cells (Fig. S1).

Table 1. Fractionation of $^{14}CO_2$-reducing acetogenic activity of the *H. sjoestedti* gut contents

	Exogenous H_2		Endogenous H_2	
Fraction	Activity	Ratio, %	Activity	Ratio, %
Whole gut	0.52 ± 0.23	100	0.23 ± 0.12	100
Large protists	0.33 ± 0.15	63	0.14 ± 0.12	61
Small protists/bacteria	0.08 ± 0.04	15	0.03 ± 0.03	13

Activity (μmol/h/g termite) is the mean ± SD for $n = 7$ experiments. The atmosphere in the reaction vials was replaced to 100% H_2 (exogenous H_2) or to 100% N_2 (endogenous H_2). In the latter condition, gut microorganisms endogenously supplied the reductant (e.g., H_2, formate, or others produced during their fermentation). The ratio of activity after fractionation was calculated with the activity in the whole gut as 100%. Total bacterial cells in the small protists/bacteria fraction were 3.7 times as many as protist-associated bacterial cells in the large protist fraction whereas the amount of total protein in the former fraction was approximately one-half of that in the latter fraction that contains large protists.

Table 2. Fractionation of $^{15}N_2$ incorporation and effect of H_2

		$^{15}N_2$ incorporation	
Fraction	Gas phase	δ ^{15}N, ‰	Atom % excess
Large protists	$^{15}N_2$	38.3	0.014
Large protists	$^{15}N_2 + H_2$	43.7	0.016
Large protists	$^{15}N_2$ + Ar	28.7	0.011
Large protists	Ar	−5.5	−0.002
Large protists	Ar + H_2	−5.5	−0.002
Large protists	–	−6.8	−0.003
Small protists/bacteria	$^{15}N_2$	−5.5	−0.002
Small protists/bacteria	Ar	−2.7	−0.001
Small protists/bacteria	–	−2.7	−0.001

^{15}N incorporation was measured in duplicate mass spectrometry measurements, and the mean values were used for the calculations. The differences in the duplicate measures of the ^{15}N atom % was <0.3%. The reactions were prepared under N_2, and then the gas phase of the reaction vials was replaced with $^{15}N_2$ gas [$^{15}N_2$ (99.7 atom %): Ar = 4:6] or Ar gas, or none (indicated by –). In the experiments of $^{15}N_2 + H_2$, $^{15}N_2$ + Ar, and Ar + H_2 gas phases, 40% (vol/vol) of the gas phase was replaced with H_2, Ar, and H_2, respectively.

Nitrogen Fixation Associated with *Eucomonympha* Protist. Nitrogen fixation activity was investigated after fractionation of the gut contents as in the reductive acetogenesis assay described above. The acetylene reduction assay for living termites showed an activity of 171.3 ± 92.5 nmol/h/g fresh-weight termite ($n = 4$). Substantial activity was observed in the large protist fraction (105.5 ± 27.4 nmol/h/g fresh-weight termite; $n = 6$) whereas no activity was detected at all in the small protist/bacteria fraction ($n = 4$). The nitrogen fixation ability was then examined with $^{15}N_2$ stable isotope incorporation in the cell mass (Table 2), and, again, the activity was detected with the large protist fraction but not with the small protist/bacteria fraction. When the termites were fed on starch, the condition known for cellulolytic protists to disappear, no acetylene reduction was detected. The results indicate that bacteria associated with large cellulolytic protists are responsible for nitrogen fixation in the gut.

Nitrogenase, the enzyme catalyzing nitrogen fixation, is generally inhibited by the presence of H_2 (24). Considering this inhibitory effect of H_2, nitrogen fixation in the termite gut is enigmatic because H_2 is known to accumulate at a high partial pressure in the gut lumen (6, 9). Therefore, we examined the effect of H_2 on ^{15}N-incorporating nitrogen fixation activity (Table 2; compare the activities of the large protist fraction under $^{15}N_2$ and $^{15}N_2$+H_2). The activity detected in the large protist fraction was not inhibited, but rather seemingly stimulated, by the presence of H_2. Acetylene-reducing activity in the large protist fraction under the presence of 20% H_2 also significantly increased (133.6 ± 40.2 nmol/h/g fresh-weight termite; $n = 4$) compared with that under the presence of argon gas instead (62.5 ± 13.3 nmol/h/g fresh-weight termite; $n = 3$).

Nitrogen fixation associated with large cellulolytic protist species was further investigated by the presence of a nitrogen fixation gene, *nifH*, which encodes nitrogenase reductase and is used as a molecular marker for nitrogen-fixing bacteria in

termite guts (18, 22, 25). The *nifH* sequence was successfully amplified by PCR from manually isolated cells of *Eucomonympha* whereas no amplification was detected with isolated *Trichonympha* cells. The control amplifications of bacterial 16S rRNA gene sequence were detected in both protist species. The results strongly suggest that the *Eucomonympha*-associated bacteria are responsible for the nitrogen fixation activity in the gut.

The *nifH* sequences of the associated bacteria of *Eucomonympha* belonged to a so-called alternative nitrogenase group of *nifH* (hereafter designated as *anfH*) represented by the previously reported HSN10 sequence (AB011910) from the gut microbial community of *H. sjoestedti* (25). The repertoire of transcribed *nifH* were investigated by RT-PCR using RNA extracted from the gut community. Although PCR often introduces some biases depending on the primer applied, the majority of the detectable transcribed sequences corresponded to the *anfH* sequence of the *Eucomonympha*-associated bacteria (23 of 25 clones) under the condition applied here. The specific localization of the *anfH* mRNA with *Eucomonympha* cells was confirmed by in situ hybridization (Fig. S1). The other transcribed sequences belonged to an ordinary nitrogenase group of *nifH* and encoded identical protein to the previously reported HSN20 sequence (AB011918) (25). No *nifH* or *anfH* sequence has been detected so far from the gut community of this termite except for those of the HSN10 and HSN20 groups, and their corresponding gene sequences were found in the same genome (see *Genome of the Endosymbiont*). The results support the major contribution of the *Eucomonympha*-associated bacteria to nitrogen fixation in the gut.

Identification of the Bacterial Endosymbiont of *Eucomonympha*. *Eucomonympha* cells were numerous in the gut, observed at 1,935 ± 206 cells per termite, with each *Eucomonympha* cell harboring a dense population of rod-shaped endosymbiotic bacteria amounting to $>10^4$ bacterial cells per single protist cell (Fig. 1). Therefore, the protist and its bacterial endosymbionts represent abundant species in the gut community. The bacterial endosymbionts of *Eucomonympha* were observed to be separated from the host protist cytoplasm by an electron sparse intermembrane space and were often found in the proximity of hydrogenosome-like organelles surrounded by a single membrane.

PCR-amplified bacterial 16S rRNA gene sequences from carefully isolated *Eucomonympha* cells were clonally analyzed, and a single clone group with minimal sequence variation (<1.3% nucleotide difference) was obtained (*SI Text*). The sequence information was used to design specific probes for fluorescence in situ hybridization (FISH). The FISH gave a specific signal to the majority of rod-shaped endosymbiotic bacteria of *Eucomonympha* (Fig. 1 *B–E* and *SI Text*). An analysis of bacterial 16S rRNA gene sequences in the gut community showed that the endosymbionts were the most abundant bacteria (21 of 218 clones) (Fig. S2), supporting its dominance in the gut. Phylogenetic analyses (Fig. 2) revealed that the sequences of the endosymbionts were derived from spirochetes of the genus *Treponema* and belonged to the previously defined termite *Treponema* cluster II (26), which consists of the ectosymbionts attached to the cell surface of gut protists as far as their localizations were examined (27, 28). This cluster formed a clade with saccharolytic treponemes that contained *Treponema brennaborense*, an isolate from a digital dermatitis ulcer of a cow (29), as a close cultured relative, but distantly related to the termite *Treponema* cluster I that comprises isolates and clone sequences from termite guts. We propose a species, "*Candidatus* Treponema intracellularis" for this previously unidentified endosymbiont (*SI Text*).

Genome of the Endosymbiont. Single cells of the endosymbiotic treponeme species of *Eucomonympha* were sorted, and, after whole genome amplification, the genome sequences of five single cells were examined individually. These five single cells showed

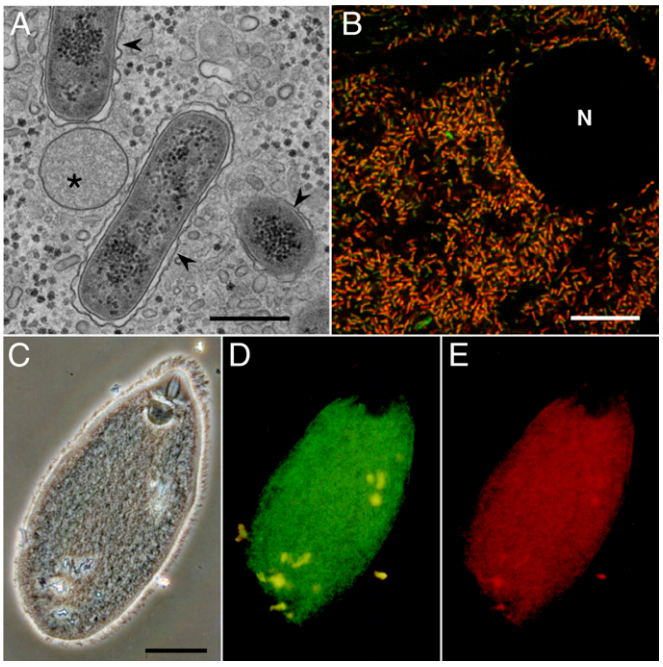

Fig. 1. Rod-shaped treponeme endosymbionts filling the *Eucomonympha* cell. (*A*) Transmission electron micrograph of the endosymbiont cells (arrowheads) within a *Eucomonympha* cell and its hydrogenosome-like organelle (asterisk). (Scale bar: 0.5 μm.) (*B–E*) FISH of the *Eucomonympha* endosymbionts. (*C*) Phase contrast image of a *Eucomonympha* cell. (Scale bar: 50 μm.) (*B*, *D*, and *E*) Simultaneous hybridizations with two probes specific for the endosymbiont (*D*; in green) and with a general bacterial probe (*E*; in red). Amorphous yellow signals in *D* and corresponding red signals in *E* were derived from autofluorescence of ingested wood particles. (*B*) Merge image of laser-scanning confocal microscope of the endosymbiont cells. Almost all of the intracellular bacteria were hybridized with both the specific and the general bacterial probes (in orange-yellow). N indicates the host nucleus where no bacterium is detected. (Scale bar: 10 μm.)

less than 0.8% nucleotide difference in their 16S rRNA gene sequences, well within a species-level difference. The completeness of the single-cell genomes, judged on the presence of conserved single copy genes (30), ranged from 40% to 85% (Table S2). The estimated genome sizes were 2.67–3.21 Mb, which was slightly smaller than the genome size of the isolated *Treponema* strains from the termite gut (3.79–4.06 Mb) and nearly equivalent to *T. brennaborense* (3.06 Mb). The GC content was 55.2 ± 0.28.

Among the five single-cell genomes, all of the acetyl-CoA pathway genes were compositely detected although no single genome harbored all these genes (Table S3). All acetyl-CoA pathway genes encoded proteins homologous to those of *T. primitia* and those detected in the termite-gut microbial communities (Table S3 and Figs. S3–S6). Two gene sets encoding alternative and ordinary nitrogenases and genes involved in metal cofactor assembly for nitrogenase were also found in the single-cell genomes (Fig. S7). The encoded alternative nitrogenase subunits were closely related to those of *T. primitia* strain ZAS-1. In contrast, the ordinary nitrogenase subunits and the metal cofactor assembly proteins were distantly related to those found in the genomes of *Treponema* species, and rather they were similar to those of some firmicutes (Figs. S8 and S9), implying the acquisition of the genes by a horizontal gene transfer.

Analyses of the single-cell genomes suggested that the endosymbiotic treponeme of *Eucomonympha* is able to use glucose, maltose, mannose, fructose, and glucuronate and ferment them through glycolysis to produce acetate although no gene for

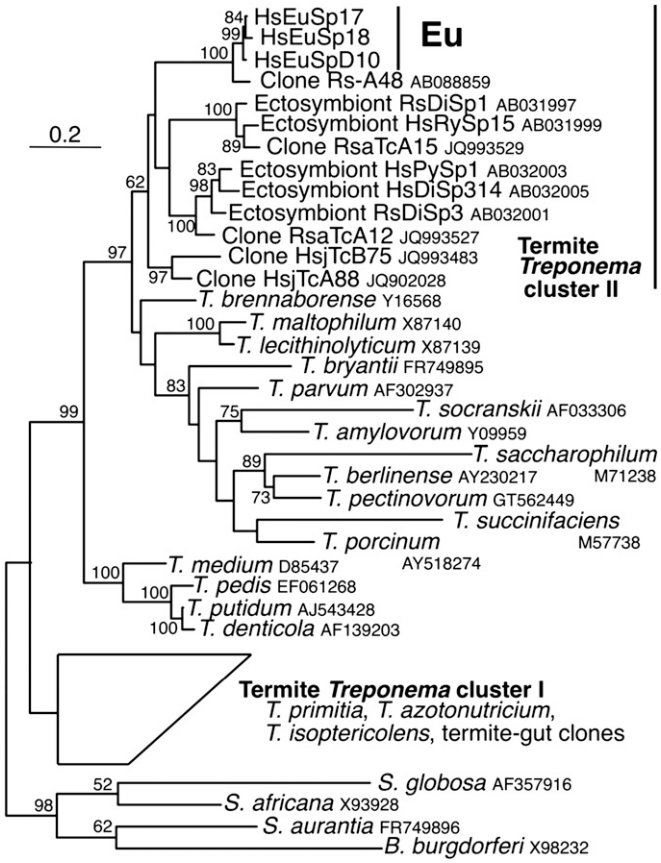

Fig. 2. Phylogenetic relationship of the endosymbiont of *Eucomonympha* and representatives of termite-gut and described treponemes, inferred based on the 16S rRNA gene sequences. The sequences identified from the endosymbionts of *Eucomonympha* are indicated by "Eu." The previously described termite *Treponema* clusters I and II (26) are indicated. Ectosymbiont sequences are identified from termite-gut oxymonad protists (27). Clones RsaTcA12, RsaTcA15, HsjTcA88, and HsjTcB75 were obtained from cell suspensions of *Trichonympha* protists (39). *B.*, *Borrelia*; *S.*, *Spirochaeta* for *S. africana* and *S. aurantia*, and *Sphaerochaeta for S. globosa*; *T.*, *Treponema*. Bootstrap values over 50% are shown at nodes. (Scale bar corresponds to 0.2 substitutions per nucleotide position.)

enolase in the glycolytic pathway was found (Table S4). The genomes have genes for synthesis and utilization of glycogen for storing energy-rich carbohydrates. The presence of urease and ammonium transporter genes in the genomes suggested that the endosymbionts are enabled to use urea and ammonia. The fixed or used nitrogen is likely assimilated first by glutamine synthetases and then used for biosynthesis of nitrogenous nutrients. The genomes were found to compositely harbor most of the genes involving biosynthesis of amino acids and many genes for biosynthesis of cofactors (Fig. S10).

Discussion

The dual functions of reductive acetogenesis and nitrogen fixation discovered in the endosymbiotic treponeme of the *Eucomonympha* protist, together with the cellulolytic ability of the protist, clearly benefit the host termite in terms of the efficient carbon, nitrogen, and energy metabolism. The cells of the cellulolytic protist occupy a large volume of the gut, and their cytoplasm provides a considerable space and a safe niche for the bacteria to proliferate. Indeed the endosymbiont is predominant in the gut community, which reconciles its contribution to the substantial biochemical activities in the gut. Probably, the functional potentials for both reductive acetogenesis and nitrogen fixation optimally match the intracellular niche provided by the cellulolytic host. It is noted that the previously isolated termite-gut treponemes exhibit either only one or the other of the two phenotypes (12–14) although their genomes predict these potentials. Therefore, there should be important endosymbiotic interactions suitable for these dual functions (Fig. 3).

The host *Eucomonympha* protist degrades cellulose, and, as in other parabasalian protists, very likely ferments it to acetate, H_2, and CO_2. H_2 is particularly important as the sink of reducing equivalents produced during the fermentation and effective removal of H_2 theoretically enhances the fermentation of cellulose. Therefore, endosymbionts that are capable of using the produced H_2 and CO_2 for reductive acetogenesis would help enhance cellulose decomposition by consuming H_2 and gain its own energy source. However, availability of H_2 may not be a limiting factor for gut bacteria because H_2 partial pressures are high throughout the gut lumen in most termites investigated so far (6, 9) and indeed exogenous supply of H_2 does not stimulate reductive acetogenesis in intact guts (31). Nevertheless, H_2 consumption within the H_2-producing protist cells may have some impacts on the protist metabolism. As intracellular populations of gut protists, methanogens that produce methane presumably from H_2 plus CO_2 are well-known and distributed widely, but generally limited only to smaller protist species (32). Indeed, in the termite *H. sjoestedti*, associated methanogens are observed with the gut small-protist species (33). Possibly, the niche segregation of reductive acetogens and methanogens determines their relative activities in the gut.

Nitrogen fixation by the endosymbiont is clearly advantageous for the host protist not only because the protist can use the fixed nitrogen but also because the protist can monopolize the supply

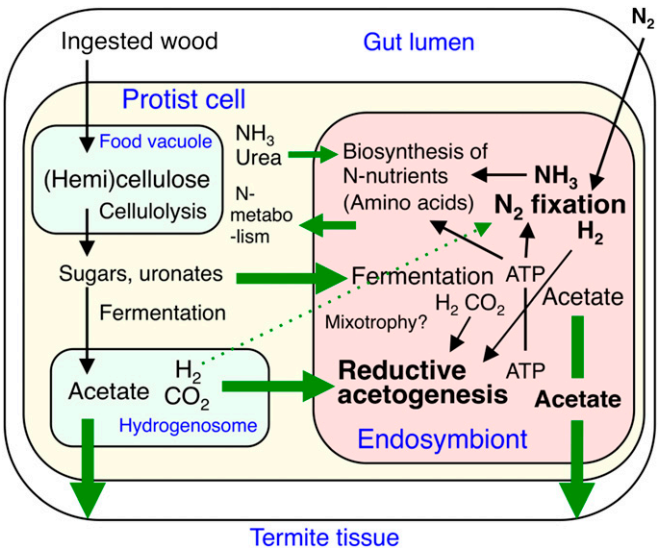

Fig. 3. A schematic view of the endosymbiotic relationships. The *Eucomonympha* protist endocytoses, degrades, and ferments (hemi) cellulose to acetate, H_2, and CO_2 through metabolisms in food vacuole, cytoplasm, and possibly hydrogenosome. The produced H_2 and CO_2 are used by the endosymbiont for reductive acetogenesis. Probably, H_2 is also used by nitrogen fixation as the reductant (dotted arrow). The degradation intermediates, sugars (such as glucose), and uronates are also used by the endosymbiont for fermentation. Termite absorbs acetate produced by both protist and endosymbiont as the major carbon and energy source. The endosymbiont fixes N_2 and uses NH_3 and urea, if available, and biosynthesizes nitrogenous nutrients that are possibly supplied to the host protist. H_2 produced by nitrogen fixation and fermentation of the endosymbiont is probably reused by reductive acetogenesis. The products of reductive acetogenesis and nitrogen fixation are shown in bold. The arrows in green indicate interspecies transfers of metabolic compounds.

of more valuable nitrogenous nutrients converted by the endosymbiont, such as amino acids directly usable for host protein synthesis. This ability is expected to give the protist a selective advantage and allow the protist to grow efficiently and stably, independently of other gut bacteria, which, in turn, benefits the host termite for stable maintenance of the essential cellulolytic protists. As shown in this study, the nitrogen fixation activity of the endosymbiont is resistant to H_2. This nature, probably as the consequence of adaptive evolution in this niche where H_2 accumulates abundantly, is required for the endosymbiosis within the protist cell. The nitrogen fixation reaction itself is also known to produce H_2. Possibly, H_2 serves as a reductant of the nitrogenase of the endosymbiont through its hydrogenase function, as in the case of CO_2 reduction in acetogenesis, because the activity is seemingly stimulated by the presence of H_2, although the electron transfer mechanism needs further study.

The endosymbiont of *Eucomonympha* could use sugars and uronates, both abundant substrates available from the (hemi) cellulose degradation in the protist cell. This ability suggests the mixotrophic nature of simultaneous utilizations of H_2 plus CO_2 for reductive acetogenesis and sugars (or uronates) for fermentation because these substrates coexist and are available abundantly for the endosymbiont unless the host termite starves. Such mixotrophy is shown in *T. primitia* ZAS-2, which can use maltose and H_2 plus CO_2 simultaneously to increase cell yield and acetate production when compared with the growth on either substrate alone (34). The mixotrophic nature of the endosymbiont is especially advantageous for satisfying the high-energy demand of the nitrogen fixation reaction. Acetogenesis solely from H_2 plus CO_2 is energy-conservative (23), but the net energy yield may not be enough for the high level of nitrogen fixation activity that supports the nitrogen demands of the entire gut community and the host termite. The almost exclusive contribution of the endosymbiont to the gut nitrogen fixation activity indicates that the intracellular location is an ideal niche for this function, and, once endosymbiotic nitrogen fixation is established, other nitrogen fixers may have become obsolete with gradual loss of this ability.

Spirochetes are generally characterized by their conspicuous spiral morphology and vigorous motility. Flagella located in the periplasmic space are wrapped around the cytoplasm to produce the unique helical locomotion. However, the rod-shaped morphology of the endosymbiont of *Eucomonympha* is unique among spirochetes although nonhelical spirochete species of the genus *Sphaerochaeta* have been isolated from the termite gut and other environments (35, 36). By localizing within the protist cell, motility has become unnecessary, which likely has caused the dispensing of its spiral morphology, because both the motility and the spiral morphology are tightly linked (37). Indeed in the transmission electron microscopy (TEM) observations of the endosymbiont cells, no periplasmic or other flagellum was detected (Fig. 1*A*). Furthermore, there are no genes for flagellum and its biosynthesis found in the genome sequences of the endosymbiotic treponeme (*SI Text*).

The endosymbionts of termite-gut cellulolytic protists in the genus *Pseudotrichonympha* and *Trichonympha* have smaller genomes (1.1 Mb in each case) rich in pseudogenes, and thus their ongoing genome erosion is suggested (20, 21). In contrast, the estimated genome size of the endosymbiont of *Eucomonympha* does not look severely reduced. This genome feature is probably because the dual endosymbiotic roles described in this study require a larger gene repertoire and metabolic networks. Nevertheless, analyses of cluster of orthologous groups (COGs) of protein showed that the endosymbiont of *Eucomonympha* is more similar to the two endosymbionts of the termite-gut protists in the gene content of some COGs than the cultured treponemes (Fig. S11). Commonly, in all of the endosymbionts, relative gene abundance in COGs of carbohydrate metabolism/transport and signal transduction mechanisms, as well as cell motility, decreased, and that in translation and coenzyme metabolism increased, compared with cultured treponemes. Given the genome size and gene content, it is possible that the acquisition of the endosymbiont by *Eucomonympha* was a more recent event and that the endosymbiont is in an initial developmental stage, but in a dynamic process of adaptive evolution as an endosymbiont. To support this assertion, there are transposable elements in high densities in the genomes (e.g., putative transposases amounted to 5.5–9.3% coding sequences) (Table S2). Such sequences are often observed in symbionts and pathogens in initial stages of genome reduction (38).

The protist species *Teranympha mirabilis* in the gut of the termite *Reticulitermes speratus* is the closest known relative of *Eucomonympha* spp. in *H. sjoestedti* (39). From the isolated *T. mirabilis* cells, we successfully identified sequences of the bacterial 16S rRNA gene and genes involved in reductive acetogenesis and nitrogen fixation, all of which were closely related to those of the endosymbiont of *Eucomonympha* (*SI Text* and Fig. S12). The results suggest that a common ancestor of *Eucomonympha* and *Teranympha* acquired an endosymbiotic treponeme species that had the dual functions. The *Eucomonympha–Teranympha* lineage is phylogenetically a sister to *Pseudotrichonympha* (39), but their endosymbionts are completely different. The *Pseudotrichonympha* endosymbiont belongs to the order Bacteroidales (40). It is possible that, after the two lineages separated, they acquired the endosymbionts independently or that the endosymbiont of one lineage was replaced by another. In either case, the endosymbionts of both lineages seem to share many functions, such as nitrogen fixation, utilization of urea and ammonia, glycogen metabolism, and H_2 consumption, although H_2 is not used for reductive acetogenesis in the endosymbiont of *Pseudotrichonympha*, in addition to upgrading nitrogenous nutrients and the utilization of sugars, which are also common to the genome-sequenced endosymbiont of *Trichonympha* that belongs to the phylum Elusimicrobia (21). These common features suggest that different bacterial species have convergently established the similar intracellular niches of the cellulolytic protists and that these functions are important for the establishment of the endosymbiotic relationships. In the case of the endosymbiont of *Trichonympha*, the sequenced genome carries neither acetyl-CoA pathway genes nor nitrogen fixation genes. However, associations of the second bacterial symbionts, which are different species depending on the *Trichonympha* lineages, have been reported, and they likely involve H_2 metabolism (19, 41, 42). One of them belongs to the genus *Desulfovibrio*, and genes for sulfate reduction and hydrogenase are identified (41). Another is an unclassified bacterial species in Deltaproteobacteria, and investigations of its formate dehydrogenase gene imply its importance for gut H_2 economy, possibly as a reductive acetogen (19).

The endosymbiont of *Eucomonympha* is related to lineages of ectosymbiotic treponemes of termite-gut protists. This phylogenetic relationship implies an evolution from ecto- to endosymbioses, as is also suggested in Bacteroidales endo- and ectosymbionts of termite-gut protists (43). The ectosymbiotic attachments of treponemes onto the protist cells have been often observed (27, 28), and they are hypothesized to catalyze reductive acetogenesis (12). Termite-gut microbial communities harbor a variety of endo- and ectosymbiotic relationships and thus provide attractive models for comparative studies to understand how these symbiotic relationships have been established, adapted, and coevolved. Such studies using empirically testable models of endosymbioses can give us fruitful indications for contentious evolutionary trajectories of eukaryotic cells and their organelles from ancient endosymbioses (44).

Materials and Methods

Analytical Methods. The termite specimens were collected and maintained as described previously (25, 27). Termites were introduced into an anaerobic

chamber (Bactron Model II; Sheldon Manufacturing Inc.) with an O_2-free atmosphere of $N_2/H_2/CO_2$ (80/10/10, vol/vol/vol) and all of the following sample preparations were performed under the anoxic condition at ambient temperature. Guts were dissected in 0.5–1.0 mL of buffered saline solution (16.2 mM K_2HPO_4, 10.35 mM KH_2PO_4, 36.75 mM NaCl, 32.25 mM KCl, 0.795 mM $CaCl_2$, 7.95 mM $MgCl_2$, 1.0 mM DTT; pH 7.2). Gut debris was removed by filtration through nylon mesh. The gut content was centrifuged at 23 × *g* for 10–20 s, and the supernatant was transferred to a new tube and centrifuged. After five rounds of the centrifugation, the resulting supernatant was used as the small flagellate/bacteria fraction. The cell pellet of the first centrifugation was washed 7–15 times with fresh buffer and used as the large flagellate fraction. Using a micromanipulation system (Transferman NK2; Eppendorf) operated in the anaerobic chamber, two hundred cells of each *Eucomonympha* and *Trichonympha* protist were collected from the large protist fraction prepared as described above but with the buffer containing 3.25 mM reduced glutathione instead of DTT. After sealing reaction vials with butyl rubber stoppers and removing from the anaerobic chamber, the gas phases were exchanged to H_2 or N_2 for the fractions and to H_2 for the isolated protist cells. The reductive acetogenic activity was measured as described previously (11) with slight modifications. Reaction vials (4.2 mL) had a final liquid volume of 0.25–0.50 mL and contained 1.95 μmol/mL concentration of $NaH^{14}CO_3$ (specific activity, 0.117 MBq/μmol) and were incubated at 30 °C. The incubation time was 2.5–5 h for the fractions and 24–72 h for the isolated protist cells. An aliquot of the reaction sample (10–50 μL) was analyzed by HPLC equipped with an ion-exclusion reverse phase column (RSpak KC-811; Shodex) and an on-line flow scintillation analyzer (Ramona 2000; Raytest). Elusion was isocratic with 0.1% H_3PO_4 (flow rate 1.0 mL/min) at 50 °C. Under this condition, acetate, formate, and other acids were clearly separated. The buffer with glutathione was also used for the fractionation, and the assay of the fractions and the activities showed the similar patterns and equivalent levels to those with the buffer containing DTT instead.

Acetylene reduction assay of a living termite specimen was performed as described previously (25). For the fractionated samples, the gas phase of the cell suspension was replaced first with 100% N_2 and then with N_2 gas containing 16% acetylene. After 30 min to 3 h incubation, 0.1 mL of gas was assayed as described previously (25). The termites were fed on soluble starch (Nacalai Tesque) moistened with distilled water for 7–9 d, and acetylene reduction was also assayed as described above. Microscopic observations of gut contents of the starch-fed termites confirmed that large protists had almost completely disappeared, but some small protists and spirochete-like bacteria were retained. For the ^{15}N incorporation assay, the gas phase of the cell fraction in a 10-mL vial was composed of $^{15}N_2$ (99.7 atom %) or Ar or N_2. After 20 h incubation at ambient temperature, the cells were harvested and dried at 70 °C. ^{15}N concentrations of the dried samples were determined in duplicate measurements by an automatic gas chromatograph-mass spectrometer (EA1110-DELTA plus Advantage ConFloIII system; Thermo Finnigan). Atom % excess was the difference between sample and reference, and $\delta\ ^{15}N$ (‰) was obtained with the notation $[(R_s - R_r)/R_r] \times 1{,}000$, where R_s and R_r are the atomic ratio of the sample and reference, respectively. As the reference, the atmospheric nitrogen was used (^{15}N atom % was 0.366).

Gene Identification and Analyses. The protist cells were manually isolated using a micromanipulator as described previously (27, 28) and were used as templates for PCR of respective genes (*SI Text*). The PCR products were cloned, and their DNA sequences were analyzed. Analyses of *nifH* genes were conducted as described previously (45). Maximum likelihood phylogenetic analyses were conducted with the unambiguously aligned sequences using RAxML MPI version 8.1.2 (46). Confidence of tree topology was estimated with bootstrap analyses of 500 resamplings. FISH and transmission electron microscopy (TEM) were conducted as described previously (27, 40).

Single-Cell Genome Sequencing and Analyses. Manually isolated *Eucomonympha* cells were pooled, ruptured, and treated with DNase I (Promega). After staining with CellTracker Green CMFDA (Life Technologies), single bacterial cells were sorted using a MoFlo XDP (Beckman Coulter) as described previously (47). The genome DNA of the sorted cells was amplified with the REPLI-g UltraFast Mini kit (Qiagen). After checking the identity and purity based on the PCR-amplified 16S rRNA gene sequence, the genome sequencing was conducted on the Illumina MiSeq platform. The generated quality-passed sequence reads were adapter and quality trimmed and assembled using SPAdes 3.1.1 (48). After checking the assembled sequences in the context of GC content and coverage using the blobology bash (49), the reads were filtered and reassembled using the same program. The resulting sequences were annotated with the PROKKA pipeline (50) and uploaded to the RAST server (51), and the annotations were checked manually with BLAST searches against public databases.

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