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Genome of '*Ca.* Desulfovibrio trichonymphae', an H_2-oxidizing bacterium in a tripartite symbiotic system within a protist cell in the termite gut

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The cellulolytic protist *Trichonympha agilis* in the termite gut permanently hosts two symbiotic bacteria, '*Candidatus* Endomicrobium trichonymphae' and '*Candidatus* Desulfovibrio trichonymphae'. The former is an intracellular symbiont, and the latter is almost intracellular but still connected to the outside via a small pore. The complete genome of '*Ca.* Endomicrobium trichonymphae' has previously been reported, and we here present the complete genome of '*Ca.* Desulfovibrio trichonymphae'. The genome is small (1 410 056 bp), has many pseudogenes, and retains biosynthetic pathways for various amino acids and cofactors, which are partially complementary to those of '*Ca.* Endomicrobium trichonymphae'. An amino acid permease gene has apparently been transferred between the ancestors of these two symbionts; a lateral gene transfer has affected their metabolic capacity. Notably, '*Ca.* Desulfovibrio trichonymphae' retains the complex system to oxidize hydrogen by sulfate and/or fumarate, while genes for utilizing other substrates common in desulfovibrios are pseudogenized or missing. Thus, '*Ca.* Desulfovibrio trichonymphae' is specialized to consume hydrogen that may otherwise inhibit fermentation processes in both *T. agilis* and '*Ca.* Endomicrobium trichonymphae'. The small pore may be necessary to take up sulfate. This study depicts a genome-based model of a multipartite symbiotic system within a cellulolytic protist cell in the termite gut.

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Introduction

Termites require symbioses with gut microbes, in order to digest dead plant matter and obtain nitrogenous compounds (Brune, 2014; Hongoh, 2011). Members of phylogenetically basal ('lower') termite taxa harbour in their guts a dense community of protists, bacteria and archaea. The protists generally establish a symbiotic relationship with multiple species of prokaryotes, which reside in their cytoplasm, nucleoplasm or attach onto the cell surface (Brune, 2014; Sato *et al.*, 2014). Although metagenome, metatranscriptome and metabolome analyses of the microbiota in the gut of lower termites have been performed (Tartar *et al.*, 2009; Do *et al.*, 2014; Tokuda *et al.*, 2014), the functions of individual microbial species and their interrelationships mostly remain unclear. In particular, the multipartite symbiotic system comprising cellulolytic protists and their multiple prokaryotic endo- and/or ectosymbionts has not been characterized in detail.

Trichonympha agilis, a cellulolytic parabasalid protist that is present in the gut of the termite *Reticulitermes speratus*, hosts two bacterial symbionts, '*Candidatus* Endomicrobium trichonymphae' phylotype Rs-D17 (class *Endomicrobia*; Stingl *et al.*, 2005; Ohkuma *et al.*, 2007) and '*Candidatus* Desulfovibrio trichonymphae' phylotype Rs-N31 (class *Deltaproteobacteria*; Sato *et al.*, 2009). The cellular association between *T. agilis* and these two bacteria species is permanent: *ca.* 4,000 and 1,800 cells of '*Ca.* Endomicrobium trichonymphae' and '*Ca.* Desulfovibrio trichonymphae', respectively, always inhabit the *T. agilis* cell in specific subcellular locations, as shown in Figures 1a and b (Sato *et al.*, 2009). These bacteria account for *ca.* 4% and 2% of the total prokaryotic cells in the *R. speratus* gut, respectively (Sato *et al.*, 2009).

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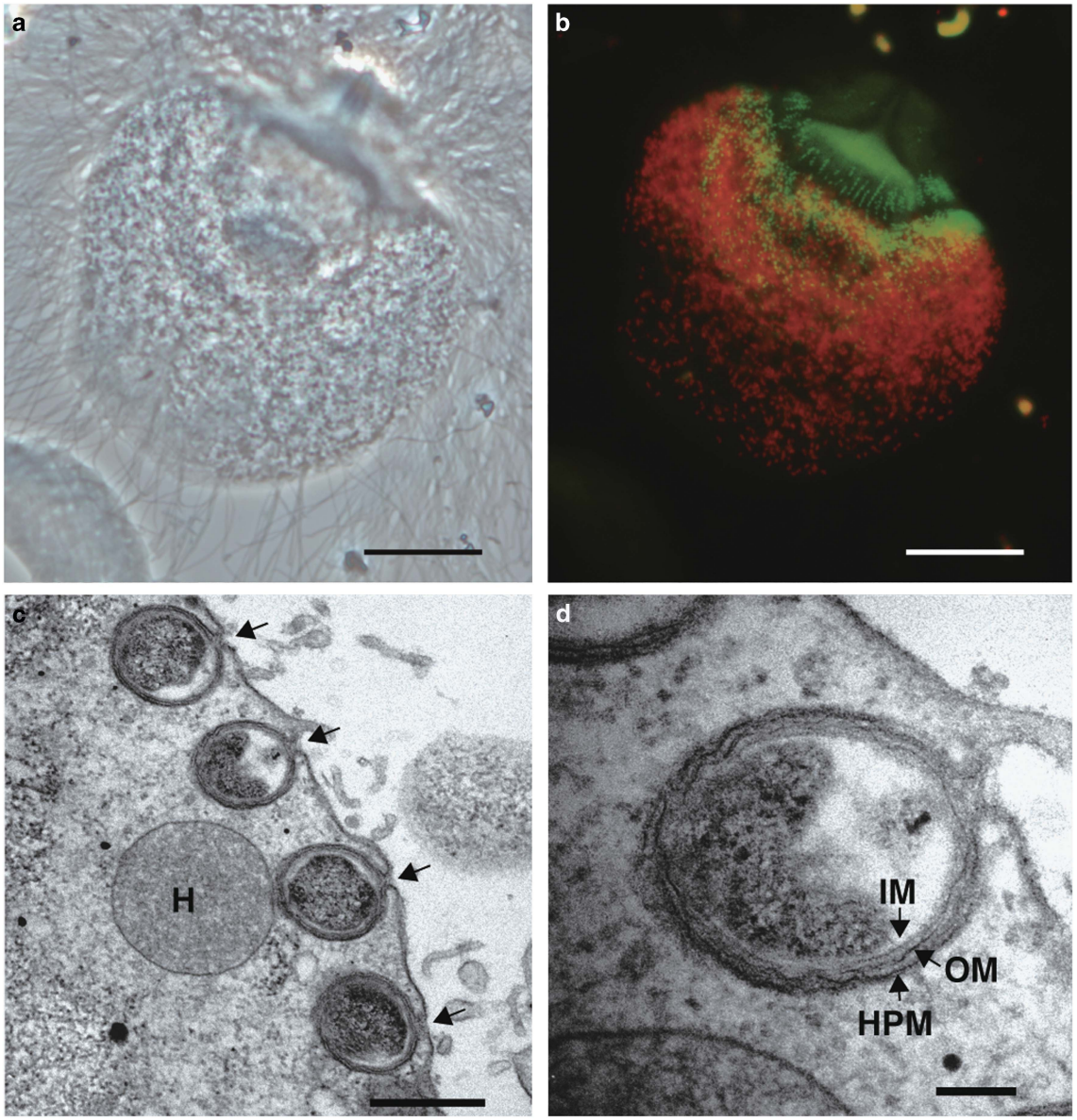

Figure 1 Fluorescence *in situ* hybridization (FISH) and transmission electron microscopy (TEM) of '*Ca.* Desulfovibrio trichonymphae' phylotype Rs-N31 cells associated with a *Trichonympha agilis* cell. (**a**) Phase-contrast image of *T. agilis*. (**b**) FISH analysis using oligonucleotide probes specific to '*Ca.* Desulfovibrio trichonymphae' (6-carboxyfluorescein-labelled, green) and to '*Ca.* Endomicrobium trichonymphae' (Texas red-labelled, red), respectively (Sato *et al.*, 2009). (**c**) TEM image of '*Ca.* Desulfovibrio trichonymphae' cells in a *T. agilis* cell. Pores opening to the outside of the *T. agilis* cell were observed (arrows). H, hydrogenosome. (**d**) Magnified image of a '*Ca.* Desulfovibrio trichonymphae' cell in panel **c**. IM, inner membrane; OM, outer membrane; HPM, host plasma membrane. Bars: 20 μm (**a**, **b**); 500 nm (**c**); 100 nm (**d**).

The complete genome sequence of the uncultured, intracellular symbiont '*Ca.* Endomicrobium trichonymphae' (1.15 Mb, including plasmids) was previously obtained using a whole genome amplification (WGA) technique (Hongoh *et al.*, 2008a). Its small genome showed the potential to synthesize various amino acids and cofactors and to ferment monosaccharides to acetate, lactate, ethanol, CO_2 and H_2 (Hongoh *et al.*, 2008a).

'*Ca.* Desulfovibrio trichonymphae' is uncultured and was previously considered to be an intracellular symbiont (Sato *et al.*, 2009). However, transmission electron microscopy (TEM) of *Trichonympha globulosa* in the gut of the termite *Incisitermes marginipennis* revealed that its *Desulfovibrio* symbionts are localized in deep invaginations of the host (*Trichonympha*) plasma membrane that are open to the exterior of the host cell (Strassert *et al.*, 2012). Our re-examination showed that '*Ca.* Desulfovibrio trichonymphae' phylotype Rs-N31 cells were almost completely buried in the host cytoplasm but still connected to the outside through a small pore (Figures 1c and d). Analyses using the reverse transcription polymerase chain reaction (RT-PCR)

showed that '*Ca.* Desulfovibrio trichonymphae' phylotype Rs-N31 transcribed the *dsrAB* and *apsA* genes that are responsible for sulfate reduction and *hynA* for hydrogen oxidation (Sato *et al.*, 2009). No other information on the functions of '*Ca.* Desulfovibrio trichonymphae' has been available hitherto.

In this study, we attempted to acquire the complete genome sequence of '*Ca.* Desulfovibrio trichonymphae' phylotype Rs-N31, in order to predict its functions and roles in the symbioses with *T. agilis* and '*Ca.* Endomicrobium trichonymphae' phylotype Rs-D17. Our results provide a genome-based model of a tripartite symbiotic system within a cellulolytic protist cell in the termite gut.

Materials and methods

Termites, Fluorescence in situ *hybridization (FISH) and TEM analysis*

The wood-feeding termite *R. speratus* (family Rhinotermitidae) was collected in Saitama Prefecture, Japan. After rearing with cellulose powder for three days, worker termites were subjected to experiments. FISH and TEM were performed as described previously (Sato *et al.*, 2009, 2014). The wood-feeding termite *Hodotermopsis sjostedti* (family Termopsidae) was collected in Kagoshima Prefecture, Japan.

Collection of 'Ca*. Desulfovibrio trichonymphae' cells and WGA*

The gut of *R. speratus* was removed from a worker termite, and the gut contents were suspended in buffer solution U (Trager, 1934). A single cell of *T. agilis* was physically isolated using a TransferMan NK2 micromanipulator (Eppendorf, Hamburg, Germany) with a glass capillary. The collected *T. agilis* cell was washed several times in buffer and dissected into the anterior and posterior parts using the micromanipulator equipped with a Feather blade handle MF-130S and a K-730 micro-blade. The anterior part containing '*Ca.* Desulfovibrio trichonymphae' cells was collected in a 0.2 ml PCR tube and subjected to WGA, using the illustra GenomiPhi HY DNA Amplification Kit (GE Healthcare, Little Charlfont, UK), as described previously (Hongoh *et al.*, 2008a, b).

Genome analysis

Sequencing libraries for the '*Ca.* Desulfovibrio trichonymphae' genome were prepared using the TruSeq DNA PCR-free Sample Prep Kit and the Nextera Mate Pair Sample Prep Kit (Illumina, San Diego, CA, USA). Sequencing was performed using the MiSeq Reagent Kit v3 (600 cycles) on an Illumina MiSeq platform. The generated reads were processed for adapter and quality trimming using programs cutadapt and prinseq, respectively (Martin, 2011; Schmieder and Edwards, 2011). The reads were assembled into contigs, using SPAdes 3.0 (Bankevich *et al.*, 2012), and the contigs and the mate-pair reads were used to generate scaffolds with SCARPA 0.241 (Donmez and Brudno, 2013). Finding and functional annotation of genes were performed using MiGAP (http://www.migap.org), and the result was curated manually. Pseudogenes were manually identified as described previously (Hongoh *et al.*, 2008a). Metabolic pathways were reconstructed using the KEGG automatic annotation server (KAAS; Moriya *et al.*, 2007). Genes were assigned to functional categories based on non-supervised orthologous groups (NOG) (Powell *et al.*, 2014). Clustered regularly interspaced short palindromic repeat (CRISPR) loci were identified using CRISPRFinder (Grissa *et al.*, 2007).

RT-PCR

Gut contents of ten *R. speratus* workers were suspended in solution U in a 1.5 ml tube, and centrifuged at a low speed to collect cells of large protists including *T. agilis*. The sedimented cells were subjected to RNA extraction, using the RNA PowerViral Environmental RNA/DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA), and contaminating DNA was digested using the TURBO DNA-free Kit (Ambion, Waltham, MA, USA). Extracted RNA was reverse-transcribed using the SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Generated cDNA was used as template for PCR amplification, using the Phusion High-Fidelity DNA Polymerase (NEB) with primers listed in Supplementary Table S1. No specific amplification was detected from the RNA samples without reverse transcription in RT-PCR. Amplification products were cloned using the Zero Blunt TOPO PCR Cloning Kit for Sequencing (Invitrogen) and Competent Quick DH5α (TOYOBO, Tokyo, Japan), and were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosciences, Foster City, CA, USA) on an ABI 3730xl DNA Analyzer, as described previously (Sato *et al.*, 2014).

Phylogenetic analysis and codon adaptation index (CAI) calculation

Maximum-likelihood trees were constructed using MEGA6 (Tamura *et al.*, 2013). Sequences were aligned using MUSCLE (Edgar, 2004) with manual corrections, and ambiguously aligned sites were removed using Gblocks (Talavera and Castresana, 2007). CAI was calculated using CAIcal (Puigbo *et al.*, 2008). The gene coding for AroP was identified by a BLAST search using the *aroP* gene of '*Ca.* Endomicrobium trichonymphae' phylotype Rs-D17 as the query, in our ongoing genome analysis of '*Candidatus* Endomicrobium sp.' HsTcC-Em16 from a single cell of *Trichonympha* sp. HsjTcC in the gut of the termite *H. sjostedti*.

Accession numbers
The sequences reported in this study have been deposited in DDBJ under accession numbers AP017368 for the '*Ca.* Desulfovibrio trichonymphae' phylotype Rs-N31 genome and LC122271 for *aroP* of '*Ca.* Endomicrobium sp.' HsTcC-Em16.

Results

Morphology of 'Ca. Desulfovibrio trichonymphae' phylotype Rs-N31
We re-investigated the morphology of '*Ca.* Desulfovibrio trichonymphae' phylotype Rs-N31 associated with *T. agilis* cells in the gut of *R. speratus*. TEM analysis showed that the majority of the '*Ca.* Desulfovibrio trichonymphae' cells were coccoid, embedded in the peripheral region of the host cytoplasm, and connected to the outside through a small pore with 41.4 ± 5.1 nm diameter (mean ± s.d., $n = 14$; Figures 1c and d). When '*Ca.* Desulfovibrio trichonymphae' cells were present deeper inside the host cytoplasm, the surrounding host membrane extended like tubes, which connected adjacent '*Ca.* Desulfovibrio trichonymphae' cells to each other (Supplementary Figure S1). The tube-like structures possibly lead to the exterior, although not confirmed. The bacterium possessed inner and outer membranes, and its lipopolysaccharide layer was not prominent (Figure 1d).

General features of the 'Ca. Desulfovibrio trichonymphae' genome
We reconstructed the complete genome sequence of '*Ca.* Desulfovibrio trichonymphae' phylotype Rs-N31 with no gaps or ambiguous nucleotide sites, from a WGA product of the anterior part of a single *T. agilis* cell. The genome consisted of a circular chromosome of 1 410 056 bp (Supplementary Figure S2); no plasmids were found. The chromosome contains 1082 putative protein-coding sequences (CDSs), two rRNA operons, and 49 tRNA genes corresponding to codons for all 20 amino acids (Table 1). In addition, 188 pseudogenes were identified (Supplementary Table S2) and classified into NOG (Supplementary Figure S3). Of the 188 pseudogenes, 55 (29.3%) were assigned to category [L] (replication, recombination and repair) including transposase, integrase, DNA methyltranferase and CRISPR-associated proteins, and 14 (7.4%) were to category [V] (defense mechanisms) including DNA restriction-modification systems (Supplementary Figure S3). A remnant of the CRISPR region was identified. The biosynthetic pathway for lipidA is missing as seen in a free-living relative, *Desulfovibrio desulfuricans* ATCC 27774, and several other genes involved in lipopolysaccharide biosynthesis are pseudogenized (Supplementary Table S2).

A maximum-likelihood tree based on concatenated sequences of 30 ribosomal proteins showed that *D. desulfuricans* ATCC 27774 was the closest relative to '*Ca.* Desulfovibrio trichonymphae' among the genome-sequenced bacteria, and these two bacteria clustered with *Lawsonia intracellularis* PHE/MN1-00, which is an intracellular pathogen of swine intestine that causes proliferative enteropathy (Mcorist *et al.*, 1995; Supplementary Figure S4). The genome size of '*Ca.* Desulfovibrio trichonymphae' is approximately half that of *D. desulfuricans* ATCC 27774, and similar to that of *L. intracellularis* PHE/MN1-00 (Table 1). The G+C content (55%) was comparable with that of *D. desulfuricans* ATCC 27774 and much higher than that of *L. intracellularis* PHE/MN1-00 (Table 1).

Carbon and energy metabolism
Predicted metabolic pathways of '*Ca.* Desulfovibrio trichonymphae' are outlined in Figure 2. The genome retains pathways for gluconeogenesis, non-oxidative pentose phosphate biosynthesis, and a partial tricarboxilic acid (TCA) cycle, allowing the biosynthesis of various compounds including amino acids, cofactors, nucleotides, and peptidoglycans. In addition, '*Ca.* Desulfovibrio trichonymphae' encodes genes for tricarboxylate transporter (TctABC), which possibly imports citrate (Winnen *et al.*, 2003). Citrate can be converted to 2-oxoglutarate, which is a precursor of amino acids.

Genes coding for glucokinase, diphosphate-fructose-6-phosphate 1-phosphotransferase, and pyruvate kinase are pseudogenized; the bacterium has lost the glycolytic pathway (Figure 2). Consistently, it lacks genes for mannose permease, which are commonly found in the genomes of desulfovibrios

Table 1 General features of the '*Ca.* Desulfovibrio trichonymphae' phylotype Rs-N31 and other genomes

	'Ca. *Desulfovibrio trichonymphae' Rs-N31*	*Desulfovibrio desulfuricans* ATCC 27774	*Lawsonia intracellularis* PHE/MN1-00	'Ca. *Endomicrobium trichonymphae' Rs-D17*
Life style	surface-embedded symbiont	free-living	intracellular pathogen	Intracellular symbiont
Chromosome size (bp)	1 410 056	2 873 437	1 457 619	1 125 857
Plasmid size (bp)	none	none	27 048; 39 794; 194 553	11 650; 5 701; 5 362
CDS	1082	2356	1340	776
GC (%)	54.8	58.1	33.1	35.2
rRNA genes	6	9	6	3
tRNA genes	49	52	43	45
Pseudogenes	188	–	–	121
Coding density (%)	79.8	83.0	83.7	66.9

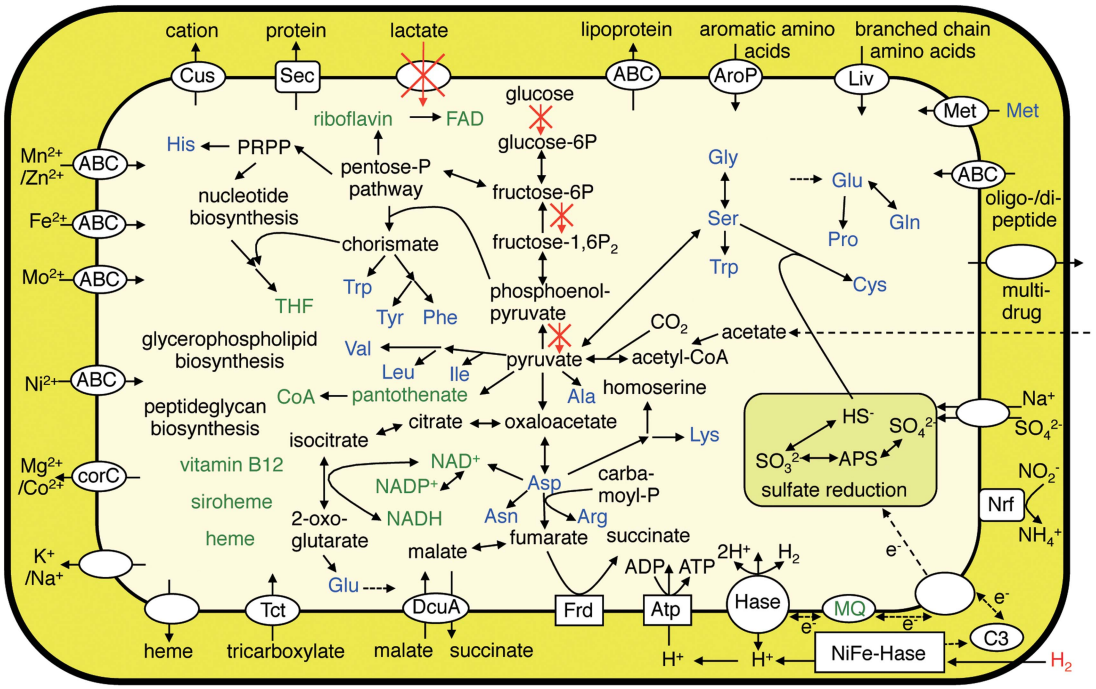

Figure 2 Predicted metabolic pathways of '*Ca.* Desulfovibrio trichonymphae' phylotype Rs-N31. Genes responsible for the pathways marked with red X's are pseudogenized.

(Santana and Crasnier-Mednansky, 2006). In addition, the genome lacks the genes for lactate dehydrogenases, and genes for lactate utilization proteins (LutABC) and lactate permease are pseudogenized; thus, it cannot use lactate as an electron donor nor a carbon source although this ability is common in desulfovibrios (Heidelberg *et al.,* 2004; Keller and Wall, 2011). A gene coding for a candidate pyruvate transporter, LctP-2 (Meyer *et al.*, 2014), is absent. Pathways for using malate, fumarate, and/or succinate as carbon sources were not found. Since the genome encodes genes involved in conversion of acetate to acetyl-CoA, and acetyl-CoA plus CO_2 to pyruvate, the main carbon sources appear to be acetate and CO_2 (Badziong *et al.*, 1979). Genes coding for the complete components of F_oF_1-ATPase, dissimilatory sulfite reductase, fumarate reductase and several hydrogenases were found, while genes for formate dehydrogenase and alcohol dehydrogenase are absent. These indicate that the bacterium generates energy via anaerobic respiration using H_2 as the electron donor and sulfate and/or fumarate as the electron acceptors (Figures 2 and 3).

Hydrogen metabolism

Predicted mechanisms to oxidize H_2 in '*Ca.* Desulfovibrio trichonymphae' are shown in Figure 3. '*Ca.* Desulfovibrio trichonymphae' has genes for three hydrogenases: periplasmic (NiFe) hydrogenase (HynAB) and two membrane-bound H^+-translocating (NiFe) hydrogenase complexes (CooFHKLMUX and EchABCDEF) that have active sites facing the cytoplasm. Considering the consistent supply of H_2 from the host hydrogenosomes and formation of H^+-membrane potential by the action of HynAB, it is likely that one or both of the membrane-bound hydrogenases oxidize H_2 with inward H^+-translocation (Pereira *et al.*, 2011). This should be coupled with the formation of a reduced form of ferredoxin and/or nicotinamide adenine dinucleotide (NADH), which are required to fix CO_2 and also possibly to reduce sulfate (Figure 3; Ramos *et al.*, 2015). Genes for (FeFe) hydrogenase and (NiFeSe) hydrogenase are absent (Supplementary Tables S2 and S3).

The genome encodes genes for periplasmic cytochrome *c* class III (c_3), membrane-bound high-molecular-weight cytochrome *c* (HmcABCDEF) and membrane-bound Hdr-like menaquinol oxidoreductase (DsrMKJOP). These transmit electrons produced by the action of HynAB to the dissimilatory sulfite reductase subunit DsrC (Figure 3; Keller and Wall, 2011). The genome possesses genes for the biosynthesis of menaquinone, which is an electron carrier in the plasma membrane.

'*Ca.* Desulfovibrio trichonymphae' retains genes for sodium:sulfate symporter, dissimilatory sulfite reductase (DsrAB), adenylylsulfate reductase (AprAB), quinone-modifying oxidoreductase (QmoABC), heterodisulfide reductase (HdrABC) and Hdr-coupled NADH dehydrogenase (FlxABCD) (Ramos *et al.*, 2015) (Figure 3 and Supplementary Table S3). Thus, '*Ca.* Desulfovibrio trichonymphae' most likely has the ability to reduce sulfate to sulfide, which can be assimilated into cysteine and/or diffused toward the outside of the cell (Grein *et al.*, 2013). In addition, the bacterium has genes for membrane-bound cytochrome *c* nitrite reductase (NrfAH),

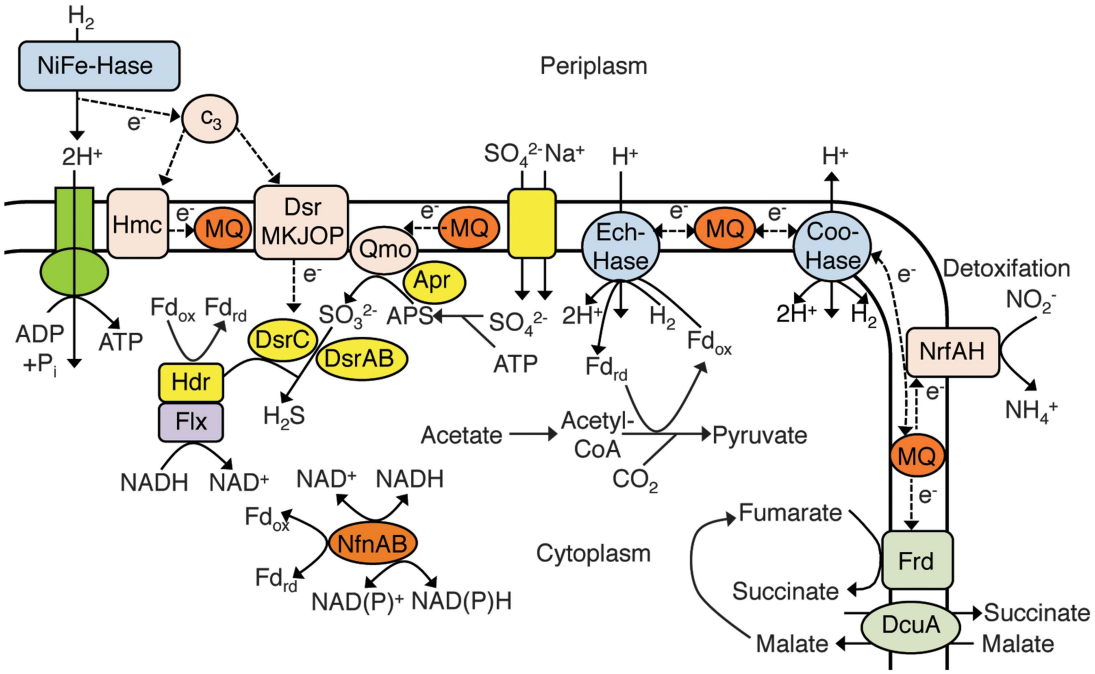

Figure 3 Predicted electron flows of '*Ca.* Desulfovibrio trichonymphae' phylotype Rs-N31. Hmc, high-molecular-weight cytochrome *c*; c_3, cytochrome *c* class III; MQ, menaquinone; Dsr, dissimilatory sulfite reductase; Qmo, quinone-modifying oxidoreductase; Apr, adenylylsulfate reductase; APS, adenylyl sulfate; Hdr, heterodisulfide reductase; Flx, NAD(P)H dehydrogenase; Nfn, ferredoxin-NADP(+) reductase; Coo-Hase, CO-induced membrane-bound hydrogenase; Ech-Hase, energy-conserving membrane-bound hydrogenase; Nrf, cytochrome *c* nitrite reductase; Frd, fumarate reductase; DcuA, anaerobic C_4-dicarboxylate antiporter.

which protects sulfate-reducing activity from strong inhibition caused by nitrite (Greene *et al.*, 2003). The genome possesses genes for fumarate reductase (FrdABCD), fumarase, aspartase, and anaerobic C_4-dicarboxylate (fumarate, succinate, malate and aspartate) antiporter (DcuA). Since in other parabasalids, such as *Trichomonas vaginalis*, malate is produced in the cytoplasm as a metabolic intermediate in its fermentation process (Müller *et al.*, 2012), '*Ca.* Desulfovibrio trichonymphae' possibly takes up malate from the host *T. agilis* cell, converts it to fumarate, conducts fumarate respiration, and exports succinate (Figure 3; Ullmann *et al.*, 2000).

We performed RT-PCR for *cooH*, *echE*, *hdrA*, *frdA* and *nrfA*, to verify expressions of each of these genes. All of their transcripts were detected. Transcripts of the *dsrAB*, *aprA* (*apsA*) and *hynA* genes were detected previously (Sato *et al.*, 2009). Thus, the sulfate-reducing system using H_2 as an electron donor is probably functional. The gene for the redox-sensing transcriptional repressor Rex, which downregulates the expression of genes and operons for sulfate respiration (Ravcheev *et al.*, 2012; Christensen *et al.*, 2015), is pseudogenized. The predicted regulon governed by Rex in desulfovibrios includes genes for sulfate adenyltransferase and adenylate kinase, and operons for *dsrAB*, *aprAB*, *dsrMKJOP*, *qmoABC*, *cooFHKLMUX* and F_oF_1-ATP synthase subunits, according to the RegPrecise database (Novichkov *et al.*, 2013). These genes might be constitutively expressed.

Biosynthesis of amino acids, cofactors and nucleotides

'*Ca.* Desulfovibrio trichonymphae' retains biosynthetic pathways for 18 amino acids and possesses genes for transporters of methionine (MetNIQ), aromatic amino acids (AroP), and branched-chain amino acids and possibly threonine (LivKHMGF) (Figure 2). Among the 18 amino acids, five (asparagine, cystein, glutamine, proline and serine) cannot be synthesized by '*Ca.* Endomicrobium trichonymphae' (Hongoh *et al.*, 2008a). Conversely, '*Ca.* Desulfovibrio trichonymphae' does not possess biosynthetic pathways for methionine and threonine, both of which can be provided by '*Ca.* Endomicrobium trichonymphae' (Hongoh *et al.*, 2008a); thus, the capacity of amino acid biosynthesis is partially complementary (Supplementary Table S4). Furthermore, '*Ca.* Desulfovibrio trichonymphae' has the potential to synthesize various cofactors, including heme and cobalamin (vitamin B_{12}) (Figure 2). The cofactor-biosynthesis capacity is also partially complementary with that of '*Ca.* Endomicrobium trichonymphae' (Supplementary Table S5). '*Ca.* Desulfovibrio trichonymphae' retains nucleotide biosynthetic pathways. Genes for nitrogen fixation and transporters for ammonium or urea are absent.

Comparative genome analysis

We classified genes into NOG and compared the genome of '*Ca.* Desulfovibrio trichonymphae' with *D. desulfuricans* ATCC 27774, *L. intracellularis*

PHE/MN1-00, and '*Ca*. Endomicrobium trichonymphae' (Figure 4 and Supplementary Figure S5). As in '*Ca*. Endomicrobium trichonymphae', the number of genes involved in signal transduction is reduced, and genes for motility and chemotaxis are missing or pseudogenized; '*Ca*. Desulfovibrio trichonymphae' has lost its motility, which is normally a characteristic of the genus *Desulfovibrio* including *L. intracellularis*. '*Ca*. Desulfovibrio trichonymphae' should thus be vertically transmitted and have no free-living phase. The proportion of genes categorized in energy production and conversion (C), amino acid transport and metabolism (E), and coenzyme transport and metabolism (H) were similar to or higher than that found in *D. desulfuricans* ATCC 27774, and much higher than in *L. intracellularis* (Figure 4).

'*Ca*. Desulfovibrio trichonymphae' shared 906 CDSs with *D. desulfuricans* ATCC 27774, while 176 and 1,450 CDSs were unique in the former and the latter, respectively (Supplementary Figure S6). Among them, genes for superoxide dismutase and cytochrome *bd* are missing in the '*Ca*. Desulfovibrio trichonymphae' genome, and genes coding for dye-decolorizing peroxidase, catalase, and rubredoxin-oxygen oxidoreductase are pseudogenized (Supplementary Table S6). Thus, the ability to detoxify oxygen is compromised in '*Ca*. Desulfovibrio trichonymphae', and the bacterium cannot use oxygen as an electron acceptor unlike certain desulfovibrios (Kuhnigk *et al*., 1996; Cypionka, 2000). This is in contrast to *D. desulfuricans* ATCC 27774, which can grow even at 18% O_2 (Lobo *et al*., 2007).

Lateral gene transfer (LGT)

Genes of '*Ca*. Desulfovibrio trichonymphae' showing the highest sequence similarity to those of non-desulfovibrio bacteria are listed in Supplementary

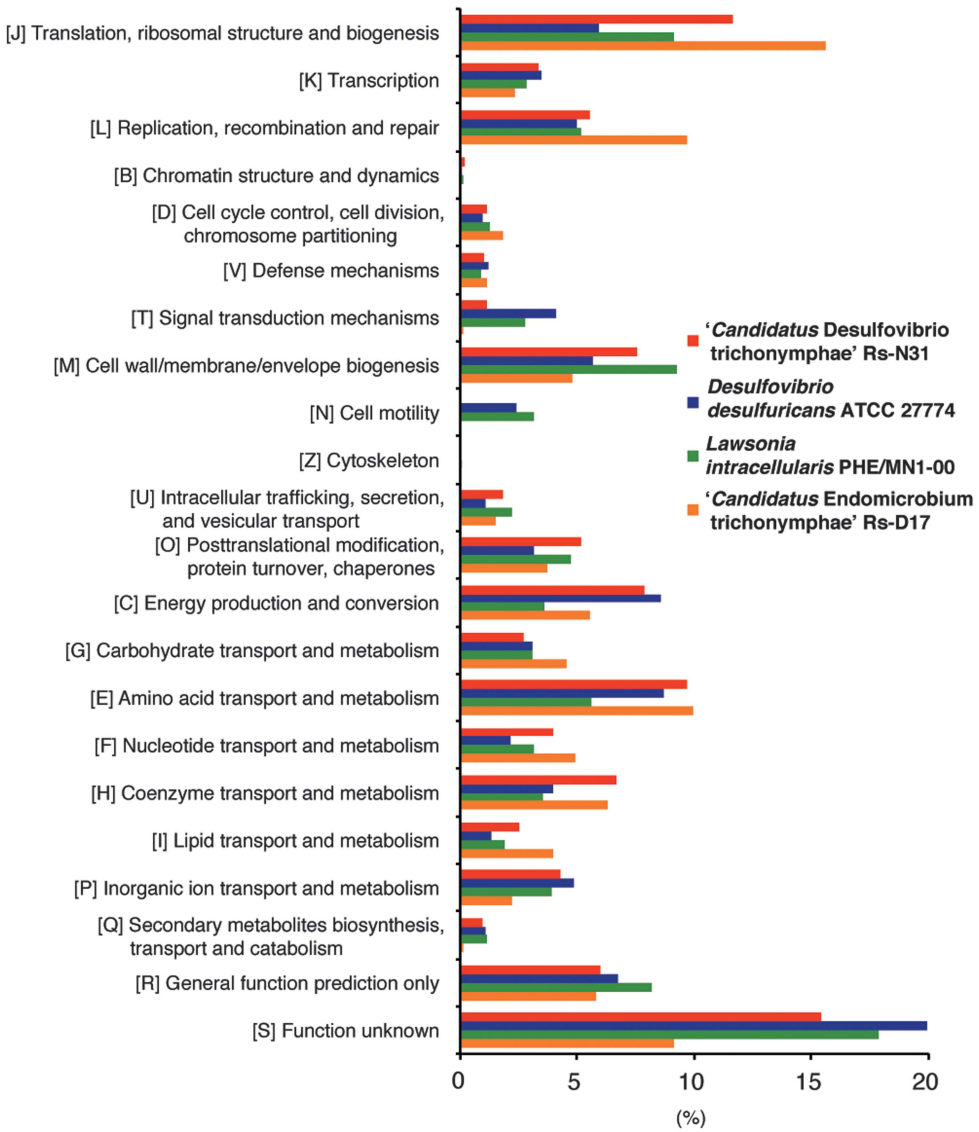

Figure 4 Non-supervised orthologous groups (NOG) classification of genes in the genomes of '*Ca*. Desulfovibrio trichonymphae' phylotype Rs-N31 and reference organisms.

Table S7. Among them, the aromatic amino acid permease AroP was phylogenetically closest to those of '*Ca.* Endomicrobium trichonymphae' and '*Ca.* Endomicrobium sp.' HsTcC-Em16 (Supplementary Figure S7A). The codon adaptation indices (CAI) of *aroP* were 0.69 and 0.73 in '*Ca.* Desulfovibrio trichonymphae' and '*Ca.* Endomicrobium trichonymphae', respectively. These values were within the range of 95% confidence intervals (0.65–0.79 and 0.69–0.82, respectively; Supplementary Table S7). These indicate that an LGT of *aroP* has occurred between the ancestor of '*Ca.* Desulfovibrio trichonymphae' and the common ancestor of the endosymbiotic *Endomicrobium* species, and that the LGT was not a very recent event. The putative malate/succinate antiporter DcuA, which plays a crucial role in fumarate respiration (Ullmann *et al.*, 2000), and the fumarate reductase subunits were probably acquired through LGT (Supplementary Table S7 and Supplementary Figures S7B and C).

Discussion

The present study revealed that '*Ca.* Desulfovibrio trichonymphae' retains the complex system required to oxidize H_2 by sulfate and/or fumarate in spite of its reduced genome size. In contrast, the bacterium has lost the ability to utilize other electron donors common in desulfovibrios, such as lactate, formate, and ethanol. The corruption of the repressor gene *rex* suggested that '*Ca.* Desulfovibrio trichonymphae' constitutively expresses genes for sulfate respiration. Since the protist host and the co-inhabiting '*Ca.* Endomicrobium trichonymphae' most probably generate H_2 during the fermentation of sugars (Yamin, 1980; Odelson and Breznak, 1985; Hongoh *et al.*, 2008a; Zheng *et al.*, 2016), we suggest that interspecies H_2 transfer is one of the driving forces for the evolution of this tripartite symbiosis. Consistently, '*Ca.* Desulfovibrio trichonymphae' cells are localized immediately adjacent to hydrogenosome-like compartments (Figure 1; Sato *et al.*, 2009), organelles that produce H_2 (Müller *et al.* 2012).

Although the H_2 partial pressure in the protist-inhabiting portion (paunch) of agarose-embedded termite guts is extremely high (for example, 15–30 kPa in *Reticulitermes santonensis*), the H_2-emission rate of living termites is 30 to 50 fold lower (Ebert and Brune, 1997; Pester and Brune, 2007). In addition, the H_2 partial pressure steeply decreases toward the peripheral gut region (Ebert and Brune, 1997; Pester and Brune, 2007). These indicate that H_2 is rapidly removed by H_2-oxidizers, especially in the gut of living termites, and imply that competition for H_2 can occur among those microbes. Indeed, exogenously-supplied H_2 greatly enhances the methanogenic activity of the termites *Zootermopsis angusticolis* and *Reticulitermes flavipes*, both of which harbour methanogens that produce CH_4 from H_2 and CO_2 in their gut (Messer and Lee, 1989; Ebert and Brune, 1997). Therefore, it is tempting to infer that an ancestor of '*Ca.* Desulfovibrio trichonymphae' might have colonized the surface of a *Trichonympha* cell for an abundant H_2 supply, and subsequently evolved into a vertically transmitted, almost intracellular symbiont. The small pore may be necessary for sulfate uptake from the outside of the host protist cell. The colonization on the *Trichonympha* cells should also have eliminated the cost and need for its own motility that is otherwise required to keep the bacterium at nutritionally optimal sites in the gut and to prevent washout from the gut.

The removal of H_2 by '*Ca.* Desulfovibrio trichonymphae', in turn, likely benefits the protist host and the co-inhabiting '*Ca.* Endomicrobium trichonymphae' by decreasing the inhibitory effect of H_2 against their fermentation processes. The fact that all the *T. agilis* cells in the *R. speratus* gut harbour '*Ca.* Desulfovibrio trichonymphae' strongly suggests that they have a mutualistic relationship. On the other hand, certain *Trichonympha* species do not possess *Desulfovibrio* symbionts (Strassert *et al.*, 2012). Other endo- and/or ectosymbionts might substitute the role of '*Ca.* Desulfovibrio trichonymphae'. Otherwise, since H_2 diffuses rapidly in the gut environment, a portion of the gut protist community might not need to house H_2-oxidizers if there are enough H_2-oxidizing activities in total in the gut. Thus, the need for cellular association between a protist and H_2-oxidizers might also depend on the total abundance of H_2-oxidizers in the gut.

Trichonympha-associated *Desulfovibrio* phylotypes are not monophyletic (Supplementary Figure S8), implying that independent acquisitions of *Desulfovibrio* symbionts by *Trichonympha* protists have occurred (Sato *et al.*, 2009; Strassert *et al.*, 2012; Ikeda-Ohtsubo *et al.*, 2016). *Trichonympha collaris* in the gut of *Zootermopsis nevadensis* harbours rod-shaped *Desulfovibrio* ectosymbionts, which are laterally attached to the host cell surface (Ikeda-Ohtsubo *et al.*, 2016). The *Desulfovibrio* ectosymbionts of *Trichonympha globulosa* are held by invaginations much deeper than those of *T. collaris* (Ikeda-Ohtsubo *et al.*, 2016; Strassert *et al.*, 2012). These might be in intermediate stages in the evolution to the nearly intracellular symbiont like '*Ca.* Desulfovibrio trichonymphae' phylotype Rs-N31. Since the concentration of sulfate in the termite gut is not high (for example 0.00–0.01 mM in *R. speratus* and 0.09–0.33 mM in *H. sjostedti*) (Sato *et al.*, 2009), the supply of malate from the host cytoplasm for fumarate respiration may be another driving force for the symbioses between these *Trichonympha* and *Desulfovibrio* species.

Interestingly, *T. collaris* harbours a third symbiont, '*Candidatus* Adiutrix intracellularis', in addition to an *Endomicrobium* endosymbiont and the *Desulfovibrio* ectosymbiont (Ikeda-Ohtsubo *et al.*, 2016). '*Ca.* Adiutrix intracellularis' is an intracellular symbiont, which belongs to the 'Rs-K70 group', a

deeply branching clade in the class *Deltaproteobacteria*. A draft genome analysis of this bacterium suggested that it produces acetate from H_2 and CO_2 (Ikeda-Ohtsubo *et al.*, 2016). Transcription of the gene coding for a key enzyme, hydrogenase-linked formate dehydrogenase, was detected *in situ* (Rosenthal *et al.*, 2013). Interrelationships among these three bacterial symbionts are unknown. Hydrogen-oxidizing activity has been experimentally demonstrated in bacterial symbionts of other termite-gut protists. *Eucomonympha* sp. in the gut of *H. sjostedti* harbours an intracellular rod-shaped bacterium, '*Candidatus* Treponema intracellularis' (order *Spirochaetales*), which also produces acetate from H_2 and CO_2 (Ohkuma *et al.*, 2015). '*Candidatus* Azobacteroides pseudotrichonymphae' (order *Bacteroidales*), an intracellular symbiont of the protist *Pseudotrichonympha grassii* in the gut of the termite *Coptotermes formosanus*, exhibited a strong H_2-uptake activity (Inoue *et al.*, 2007; Hongoh *et al.*, 2008b).

The ability to synthesize amino acids and cofactors is, in part, complementary between '*Ca.* Desulfovibrio trichonymphae' and '*Ca.* Endomicrobium trichonymphae' (Supplementary Tables S4 and S5). Given that the termite host feeds on nitrogen-poor wood materials, biosynthesis and provision of essential nitrogenous compounds are critically important for the termite, and probably also for the protists (Odelson and Breznak, 1985). In addition, provision of cobalamin from '*Ca.* Desulfovibrio trichonymphae' to '*Ca.* Endomicrobium trichonymphae' should be important because the latter has a cobalamin-dependent methionine synthase and a cobalamin-dependent ribonucleotide reductase (Hongoh *et al.*, 2008a). The synthesized methionine, in turn, can be provided through the host cytoplasm to '*Ca.* Desulfovibrio trichonymphae', which possesses the transporter MetINQ. Whereas '*Ca.* Desulfovibrio trichonymphae' probably takes up amino acids, including methionine, also from the gut lumen through the small pore, provision of nitrogen sources from the host cytoplasm without competition seems to be beneficial to the bacterium. Thus, the complementary supply of nitrogenous compounds may be another driving force for the evolution of this symbiosis. It remains unknown how such compounds are released to the host cytoplasm from these symbiotic bacteria; the protist host may digest the symbionts like exogenously supplied bacterial cells (Odelson and Breznak, 1985).

Because the gene for ammonium transporter is pseudogenized in '*Ca.* Endomicrobium trichonymphae' (Hongoh *et al.*, 2008a) and absent in '*Ca.* Desulfovibrio trichonymphae', amino acids should be their primary nitrogen sources. Interestingly, the aromatic amino acid transporter *aroP* gene has obviously been laterally transferred between the ancestors of '*Ca.* Desulfovibrio trichonymphae' and the endosymbiotic endomicrobia. Genes required for the fumarate respiration of '*Ca.* Desulfovibrio trichonymphae' may have also been laterally acquired from other bacterial lineages (Supplementary Table S7). It has been suggested that '*Ca.* Adiutrix intracellularis' laterally acquired genes required for its reductive acetogenesis (Ikeda-Ohtsubo *et al.*, 2016). Thus, LGT plays important roles in the symbiotic system in the termite gut.

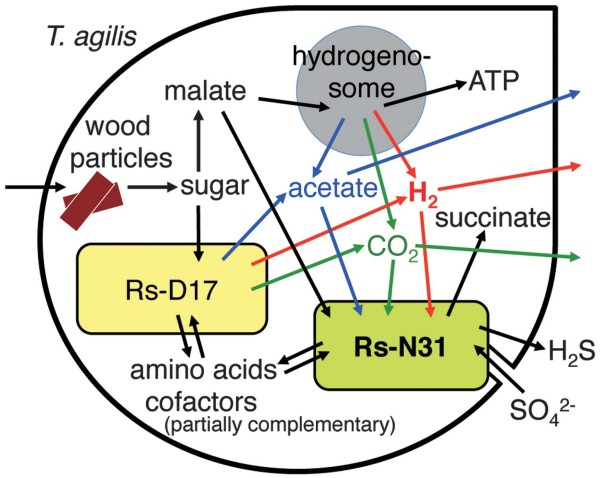

Figure 5 Proposed tripartite symbiotic relationship among '*Ca.* Desulfovibrio trichonymphae' phylotype Rs-N31, '*Ca.* Endomicrobium trichonymphae' phylotype Rs-D17, and *Trichonympha agilis*.

In conclusion, our analyses of the complete genomes of the two symbionts '*Ca.* Desulfovibrio trichonymphae' and '*Ca.* Endomicrobium trichonymphae' unveiled an elaborate mutualism within the protist cell. A schematic tripartite symbiosis is depicted in Figure 5. The gut protists phagocytose wood particles, hydrolyze the cellulose and hemicellulose to monosaccharides, and ferment the monosaccharides to acetate, CO_2 and H_2 (Odelson and Breznak, 1985; Yamin, 1980). A portion of the monosaccharides are imported by '*Ca.* Endomicrobium trichonymphae' and fermented to acetate, CO_2, ethanol, and H_2 (Hongoh *et al.*, 2008a). The produced acetate is the main carbon and energy source of the termite host (Brune, 2014). '*Ca.* Desulfovibrio trichonymphae' takes up the generated H_2 as the energy source and acetate and CO_2 as the carbon sources. Malate, produced during the fermentation process of the protist, enables '*Ca.* Desulfovibrio trichonymphae' to oxidize H_2 even when sulfate is unavailable. The removal of H_2 should promote the fermentation processes of the protist and '*Ca.* Endomicrobium trichonymphae'. The protist provides these bacteria with the metabolites and the habitat, and in turn, obtains various amino acids and cofactors. Future studies of the functions of the uncultivable protist host are needed to fill the picture describing this complex symbiotic system.

Conflict of Interest

The authors declare no conflict of interest.

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