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ARTICLE

Food selectivity of anaerobic protists and direct evidence for methane production using carbon from prey bacteria by endosymbiotic methanogen

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Abstract

Anaerobic protists are major predators of prokaryotes in anaerobic ecosystems. However, little is known about the predation behavior of anaerobic protists because almost none have been cultured. In particular, these characteristics of anaerobic protists in the phyla Metamonada and Cercozoa have not been reported previously. In this study, we isolated three anaerobic protists, *Cyclidium* sp., *Trichomitus* sp., and *Paracercomonas* sp., from anaerobic granular sludge in an up-flow anaerobic sludge blanket reactor used to treat domestic sewage. Ingestion and digestion of food bacteria by anaerobic protists with or without endosymbiotic methanogens were demonstrated using tracer experiments with green fluorescent protein and a stable carbon isotope. These tracer experiments also demonstrated that *Cyclidium* sp. supplied CO_2 and hydrogen to endosymbiotic methanogens. While *Cyclidium* sp. and *Trichomitus* sp. ingested both Gram-negative and -positive bacteria, *Paracercomonas* sp. could only take up Gram-negative bacteria. Archaeal cells such as *Methanobacterium beijingense* and *Methanospirillum hungatei* did not support the growth of these protists. Metabolite patterns of all three protists differed and were influenced by food bacterial species. These reported growth rates, ingestion rates, food selectivity, and metabolite patterns provide important insights into the ecological roles of these protists in anaerobic ecosystems.

Introduction

Anaerobic protists are major predators of prokaryotes that affect the abundance, structure, and diversity of prokaryotic communities in various anaerobic environments such as lakes, groundwater, rumina, and bioreactors [1–6]. Bacterivorous protists have different hunting characteristics and species-specific prey preferences, and each of these protists has its own ecological niche [7–9]. Protist prey preferences results in distinct responses in the composition of prokaryotic community such as disappearance of specific prokaryotes and dominance of bacteria that have resistant to predation (e.g., filamentous bacteria, microcolony-forming bacteria, and Gram-positive bacteria) [10]. On the other hand, some protist species can ingest grazing-resistant bacterial cells mentioned above [11, 12]. The prey preferences of protist against various prokaryotes cell type (shape, size, and cell-wall structure) are key factor to control prokaryotic community structures.

Many types of anaerobic protists such as amoeba, ciliates, and flagellates have been observed in anaerobic environments under a microscope [13–16] and predation by protists strongly affect bacterial and archaeal community structures in anaerobic ecosystems [17, 18], but few studies that focused on the predation behavior of and metabolites from these organisms though the aerobic protists are well studied. Among anaerobic protists, only *Trimyema compressum* has been investigated for its food selectivity [19, 20]. These studies showed that *T. compressum* could ingest both bacteria and archaea, and that Gram-negative bacteria, specifically, supported its growth. However, food selectivity of other anaerobic protists remains largely

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unknown. In addition, molecular methods based on 18S rRNA gene sequencing revealed that protist communities in anaerobic environments contain a substantial number of unknown species that are distantly related based on sequences available in genetic databases [21, 22]. Thus, because they have been overlooked and remain uncultured, the ecological roles of most anaerobic protists are poorly understood.

In wastewater treatment processes, anaerobic protists contribute to sludge reduction by predation, degrading particulate organic matter, including bacterial cells [23, 24]. Anaerobic protists produce various metabolites through fermentative metabolism of food bacteria, with major products that include ethanol, fatty acids, hydrogen, and carbon dioxide [25, 26]. Our previous study suggested that the supply of metabolites produced by anaerobic protists increased when they fed on anaerobic syntrophic bacteria that preferred fatty acids, resulting in high microbial diversity [1]. Thus, metabolites produced by anaerobic protists are important factors that shape microbial communities in anaerobic ecosystems.

In addition, some anaerobic protists harbor endosymbiotic methanogens in their cytoplasm [14, 27, 28]. Several reports have indicated that these endosymbiotic methanogens are closely associated with protist metabolism because they scavenge the hydrogen produced by host protists [29–31]. However, the metabolism of anaerobic protists, including endosymbiotic methanogens, have not yet been investigated. Stable isotope tracer techniques have been used to examine the decomposition pathways of certain substrates [32, 33]. However, although ^{13}C tracer techniques have been used to investigate the metabolism of parasitic protists [34, 35], no study has examined anaerobic protists with endosymbiotic methanogens in their cells.

In this study, we isolated anaerobic protists from anaerobic granular sludge in a domestic wastewater treatment plant to investigate the food selectivity of anaerobic protists with or without endosymbiotic methanogens. Further, we established monoxenic cultures of ciliate *Cyclidium* sp. strain YH (phylum Ciliophora) that harbor endosymbiotic methanogens, flagellate *Trichomitus* sp. strain YH (phylum Metamonada), and *Paracercomonas* sp. strain YH (phylum Cercozoa). Although, protists belonging to these three phyla were detected throughout the year in a UASB reactor used to treat domestic sewage [36], the food selectivity of and metabolites from these protists have not been investigated. In particular, these characteristics of anaerobic protists in the phyla Metamonada and Cercozoa have not been reported previously. Therefore, ingestion and digestion of bacteria by three protists and methane production by endosymbiotic methanogens were examined through a tracer experiment using green fluorescent protein (GFP) and stable isotope ^{13}C. In addition, the effects of food bacteria on the growth, food selectivity, and metabolites of these protists were investigated through feeding experiments with various food bacteria.

Materials and methods

Isolation and cultivation of protists

Anaerobic granular sludge containing protist cells was obtained from the sampling port at the bottom of a UASB reactor at a domestic sewage treatment center in Nagaoka City, Japan [36]. These cells were cultured anoxically at room temperature (25 °C) in a ciliate mineral medium as described previously [27]. *Escherichia coli* strain K-12 was used as the food bacteria for cultivation. *E. coli* cells were grown overnight in M9 minimal medium [37] containing 10 mM of glucose and 0.03% yeast extract at 37 °C with shaking at 200 rpm. The cells were harvested by centrifugation at 8000 rpm, washed three times with 0.1 M phosphate-buffered saline (PBS), and resuspended in the ciliate mineral medium. Stigmasterol and ergosterol dissolved in ethanol were also added to the culture media at a concentration of 1 μg mL^{-1} each as previously described [38]. Monocultures of anaerobic protists were obtained by transferring individual cells to culture bottles of fresh ciliate mineral medium containing food bacteria (i.e., *E. coli* strain K-12) from an enriched culture using MM-89 and IM-9B micromanipulators (Narishige, Tokyo, Japan). Each protist was subcultured every 2 or 3 weeks.

Confirmation of protist feeding on GFP-expressing *E. coli* cells

Chemically competent *E. coli* TOP10 (Invitrogen, Waltham, MA, USA) was transformed with vector pUC18-GFP (Nippon Gene, Tokyo, Japan) following the procedure recommended by Invitrogen. *E. coli* transformants were selected for growth in Luria broth supplemented with ampicillin 100 mg ml^{-1}. GFP-expressing *E. coli* cells were harvested, washed two times, and resuspended in fresh ciliate mineral medium in a culture bottle. After incubation for 30 min, protists in suspension were removed and checked for ingested bacteria under a fluorescence microscope (BX51, Olympus, Tokyo, Japan).

Tracer experiment using ^{13}C-labeled *E. coli*

The *E. coli* cells were cultured overnight in M9 minimal medium containing 10 mM of fully ^{13}C-labeled glucose (Wako Chemicals, Tokyo, Japan) as the sole carbon source at 37 °C with shaking at 200 rpm [39]. Then, the ^{13}C-labeled *E. coli* cells were harvested, washed two times, and

resuspended in fresh ciliate mineral medium in a culture bottle (ca. 10^8 cells mL^{-1}). The initial bacterial density was 5.0×10^8 cells mL^{-1}. Culture bottles were incubated in triplicate at 25 °C in the dark. Serum vials containing medium with ^{13}C-labeled *E. coli* without protist cells were incubated in parallel as a control.

Feeding experiments of anaerobic protists on various food bacteria

Propionibacterium acnes (strain UasXy-5) and *Trichococcus flocculiformis* (strain UasXy-4) were isolated in our laboratory, and *Bacteroides luti* strain UasXn-3 (JCM 19020) was isolated from anaerobic granular sludge as described previously [40]. *Bacteroides graminisolvens* (JCM 15093T), *Methanospirillum hungatei* (JCM10133), *Clostridium acetobutylicum* (JCM 1419), and *Moorella thermoacetica* (JCM 9319) were purchased from the Japan Collection of Microorganisms (JCM, RIKEN, Saitama, Japan). *Methanobacterium beijingense* strain 8-2 (DSM15999) was obtained from the German Resource Centre for Biological Material (DSMZ). In addition, *T. flocculiformis*, which has a filamentous form with chains of coccoid cells, was separated into single coccus cells by sonication and used as a food bacterium (designated *T. flocculiformis*-b). Characteristics of each food bacteria used in the protist cultures are shown in Table S1. Microorganisms other than *E. coli* were cultivated anoxically at 37 °C in anaerobic basal medium as described previously [40] with the following substrate: 10 mM glucose and 0.03% yeast extract for *B. luti, B. graminisolvens, C. acetobutylicum, M. thermoacetica, C. acnes*, and *T. flocculiformis*; and H_2/CO_2 (80/20, v/v) for *M. beijingense* and *M. hungatei*.

Food bacterial cells were harvested by centrifugation at 8000 rpm, washed two times with 0.1 M PBS, and resuspended in fresh ciliate mineral medium in culture bottles. The initial cells concentration was adjusted to 10^8 cells mL^{-1}. Protist cells from monocultures were inoculated into these culture bottles. The experiments were performed more than three times, and each food bacterial culture was subcultured at least three times.

Analytical procedures

The number of protists was determined by manual counts of the numbers of ciliate cells in a Neubauer chamber (ERMA, Tokyo, Japan) under an IX71 light microscope (Olympus, Tokyo, Japan). To count total bacteria, the samples were filtered on a black polycarbonate membrane after sonication. Then, the membrane was air-dried and mounted with 4,6-diamidino-2-phenylindole (DAPI). DAPI-stained bacterial cells from at least three randomly selected visual fields were enumerated.

The growth rate and ingestion rates of each protist were calculated based on time courses of the protists and bacterial numbers during exponential phase growth with the following formula: growth rate = (log final protist number − log initial protist number)/time interval, ingestion rate = (final bacterial number − initial bacterial number)/((final protist number − initial protist number)/(log final protist number − log initial protist number))/time interval [41, 42].

Methane was detected using a gas chromatograph equipped with a flame ionization detector (GC-2014, Shimadzu, Kyoto, Japan), and concentrations of $^{13}CO_2$ and $^{13}CH_4$ were determined by GCMS-QP2010SE gas chromatography (Shimadzu, Kyoto, Japan).

After filtration of the samples through 0.2-μm pore-size membranes (Advantec, Tokyo, Japan), fermentation products were analyzed by capillary electrophoresis (Agilent 7100 Photal, Otsuka Electronics, Osaka, Japan).

DNA extraction, PCR amplification, and 18S rRNA sequencing

Genomic DNA was extracted from cultured cells using a FastDNA SPIN Kit for soil (MP Biomedicals, Carlsbad, CA, USA), and DNA concentrations were determined using a NanoDrop Spectrophotometer ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA). Amplification of eukaryotic 18S rRNA was performed using universal eukaryote-specific primer pairs EukA and EukB [43]. Premix Ex Taq Hot Start Version (TaKaRa Bio, Shiga, Japan) was used for PCR under the following conditions: 5 min at 94 °C; 40 cycles of 1 min at 94 °C, 1 min at 58 °C, and 2 min at 72 °C; and a final 10-min extension step at 72 °C. PCR products were purified using a QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany), and concentrations were measured using a Qubit dsDNA HS assay kit with a Qubit fluorometer (Thermo Fisher Scientific, MA, USA). After purification, PCR products were sequenced using EukA, Euk-555F [43], and EukB primers and a 3730xl DNA Analyzer (Applied Biosystems, CA, USA).

The nucleic acid sequences obtained were aligned using ClustalW software, and a phylogenetic tree was constructed in MEGA 7 [44] using maximum likelihood (Jones–Taylor–Thornton model), neighbor joining (Poisson model), maximum parsimony (close neighbor interchange in the random-tree search algorithm), and unweighted pair group methods with an arithmetic mean (a maximal composite likelihood model) approaches with *B. graminisolvens* (GenBank accession number NR_041642) 16S rRNA gene as an outgroup.

Fluorescence in situ hybridization (FISH)

Protists used for FISH analyses were cultured with CMV medium without resazurin to minimize the amount of autofluorescence [45]. Protist cells were collected by

centrifugation (1000 × *g*) and fixed with 4% paraformaldehyde for 1 h at 4 °C, followed by two washes with 10 mM PBS. Fixed protist cells were embedded in low-melting agarose (Sigma-Aldrich, Steinheim, Germany) in each well of a 10-well glass slide. After drying, the cells on slides were dehydrated in 50%, 80%, and 100% ethanol for 4, 2, 1 min, respectively and dried again. Then, the fixed cells were hybridized with the oligonucleotide probe Arc915 for methanogenic archaea [46]. The samples were counterstained with DAPI before observation under a fluorescence microscope (BX51, Olympus, Tokyo, Japan).

Nucleotide sequence accession numbers

Sequence data were deposited in the DDBJ nucleotide sequence database under accession numbers LC497866 to LC497868.

Results

Isolation of protists

Three monoxenic cultures of protists were obtained and identified as belonging to the genera *Cyclidium*, *Paracercomonas*, and *Trichomitus* based on morphological features reported previously [13, 47, 48]. *Cyclidium* sp. YH was a ciliate, and *Paracercomonas* sp. YH and *Trichomitus* sp. YH were flagellates. *Cyclidium* sp. YH and *Paracercomonas* sp. YH grew with food bacteria alone. In contrast, *Trichomitus* sp. YH required food bacteria with ergosterol and stigmasterol as co-substrates for growth. The 18S rRNA sequences of *Cyclidium* sp. YH, *Paracercomonas* sp. YH, and *Trichomitus* sp. YH showing 97% nucleotide identity with *Cyclidium porcatum* (accession number Z29517), 97% identity with *Paracercomonas anaerobica* (AF411272), and 94% identity with *Trichomitus batrachorum* (AF124610), respectively (Fig. 1). Although *C. porcatum* was described by morphology-based taxonomy at first [48], molecular phylogeny revealed that the species is not member of the genus *Cyclidium* and the family Cyclidiidae [45]. Therefore, detailed taxonomic assignment of *C porcatum* and our isolate *Cyclidium* sp. YH could be discussed and reclassified in the future. In addition, endosymbiotic methanogens inside of *Cyclidium* sp. YH cells were confirmed by FISH (Fig. S1) and were not detected in *Paracercomonas* sp. YH and *Trichomitus* sp. YH cells.

Confirmation of protist feeding on GFP-expressing *E. coli*

Internalization of GFP-expressing *E. coli* by all protist cells was confirmed under a fluorescence microscope (Fig. 2). *Cyclidium* sp. YH, the largest protist of the three tested, ingested larger numbers of bacterial cells than *Trichomitus* sp. YH and *Paracercomonas* sp. YH. In addition, GFP-expressing *E. coli* cells localized inside of *Cyclidium* sp. YH cells, suggesting the formation of food vacuoles. However, *E. coli* cells were not found localized inside of *Trichomitus* sp. YH and *Paracercomonas* sp. YH cells.

Growth characterization and metabolite profiling using ^{13}C-labeled *E. coli*

After cultivation for 10 days, *Trichomitus* sp. YH and *Paracercomonas* sp. YH reached maximum numbers of 1.2×10^4 cells mL^{-1} and 6.2×10^4 cells mL^{-1}, respectively (Fig. 3). *Cyclidium* sp. YH grew to a maximum number of 2.2×10^3 cells mL^{-1} after 18 days of cultivation (Fig. 3). The generation time of flagellates was faster than that of the ciliate. Generation times of the ciliate *Cyclidium* sp. YH ranged from 38.2 to 96.8 h, which was similar to that of other anaerobic ciliates such as *Metopus contortus* (45–60 h), *Metopus palaeformis* (35 h), and *Plagiopyla frontata* (34 h), but faster than Scuticociliatia strain GW7 (112.8 h) and slower than *T. compressum* (10–33 h) [38, 45, 49, 50]. The generation times of the two isolated flagellates *Paracercomonas* sp. YH (27.1–35.5 h) and *Trichomitus* sp. YH (29.2–46.7 h) were also close to those previously reported for anaerobic flagellates such as *Psalteriomonas lanterna* (38 h) [51]. However, the generation times of *Trichomitus* sp. YH were longer than the minimum generation times of 4–6 h for parasite species such as *Trichomonas vaginalis* in axenic culture [52]. This could be because ingestion of bacterial cells by phagotrophy requires more energy than parasites' absorption of soluble nutrients by osmotrophy under nutrient-rich environments. This phenomenon is observed in mixotrophic algae [53, 54].

After exponential growth, the number of cells of each protist rapidly decreased, probably because accumulation of metabolites such as VFA to toxic levels in each culture [42]. During the exponential growth phase, *Cyclidium* sp. YH, *Trichomitus* sp. YH, and *Paracercomonas* sp. YH ingested 1.8×10^3, 1.2×10^2, and 0.6×10^2 cells of food bacteria per protist per hour, respectively.

The metabolite profiles of each protist culture were clearly different (Fig. 4). The major metabolite of *Cyclidium* sp. YH was acetate, with propionate, butyrate, and valerate detected in smaller amounts. In contrast, acetate and butyrate were the major metabolites of *Trichomitus* sp. YH, with smaller amounts of propionate found in this culture, and acetate and propionate were the major products of *Paracercomonas* sp. YH. Lactate, formate, and ethanol were not detected in any of the cultures. Hydrogen was increased in cultures of *Trichomitus* sp. YH and *Paracercomonas* sp. YH. However, methane, rather than hydrogen, was detected in cultures of

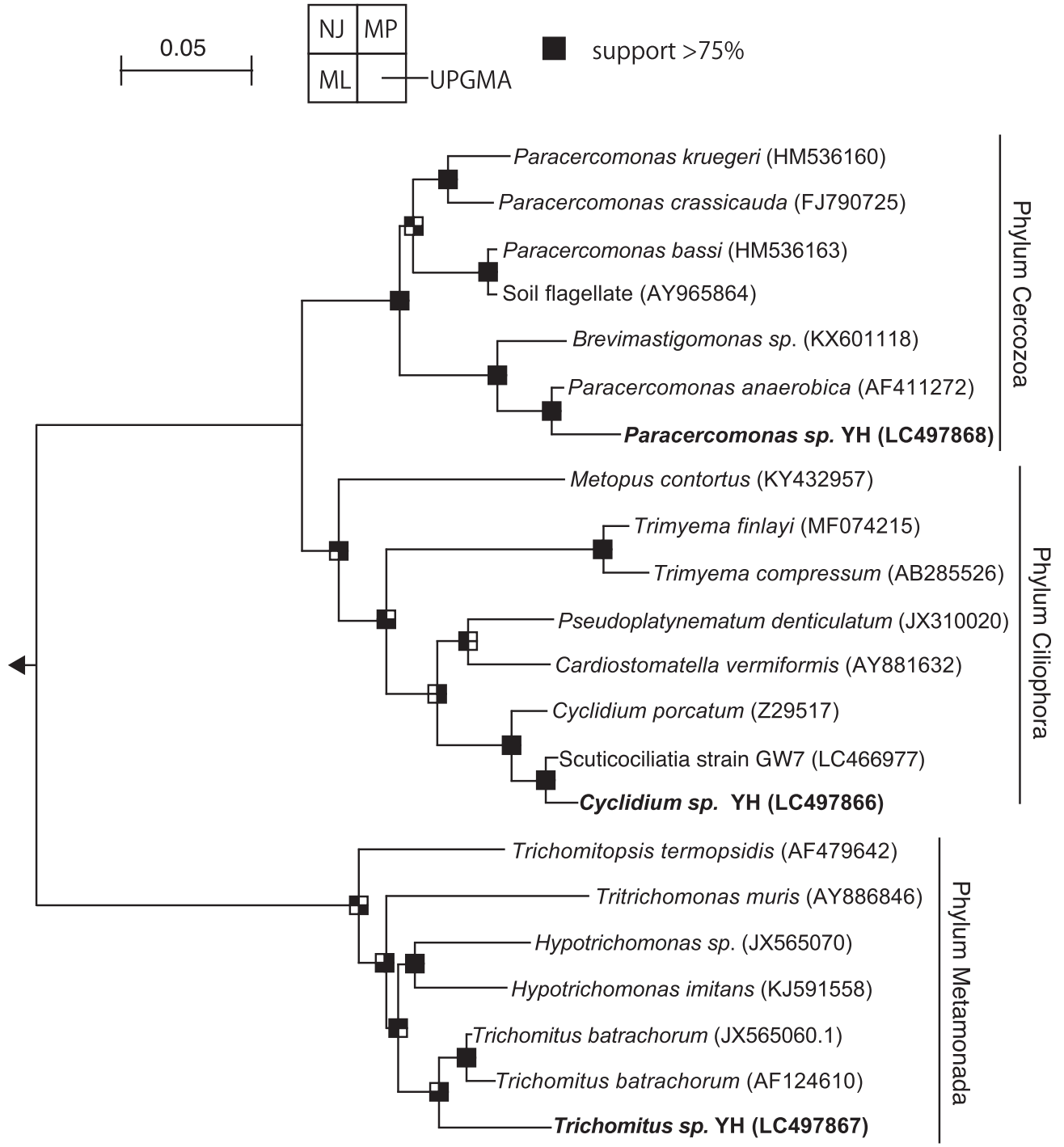

Fig. 1 Neighbor-joining tree showing the phylogenetic affiliation of *Cyclidium* sp. YH, *Paracercomonas* sp. YH, and *Trichomitus* sp. YH isolated in this study. Branching points that support a probability of >75% in the bootstrap analyses (based on 1000 replications, estimated using the NJ method for the upper-left sector, the MP method for the upper-right sector, the ML method for bottom-left sector, and the UPGMA method for the bottom-right sector) are shown as black squares. The scale bars represent sequence divergence.

Cyclidium sp. YH that harbored an endosymbiotic methanogen. *Cyclidium* sp. YH produced 0.34 pM of CH_4 per protist per hour, on average. *Trichomitus* sp. YH and *Paracercomonas* sp. YH produced hydrogen 0.63 and 0.06 pM-H_2 per protist per hour, respectively. Acetate increased in the cultures of food bacteria without protists, perhaps due to autolysis of *E. coli* cells.

To examine digestion of ^{13}C-labeled *E. coli* by protists, $^{13}CO_2$ and $^{13}CH_4$ were measured in the protist cultures (Table 1). After cultivation for 21 days, the percentage of

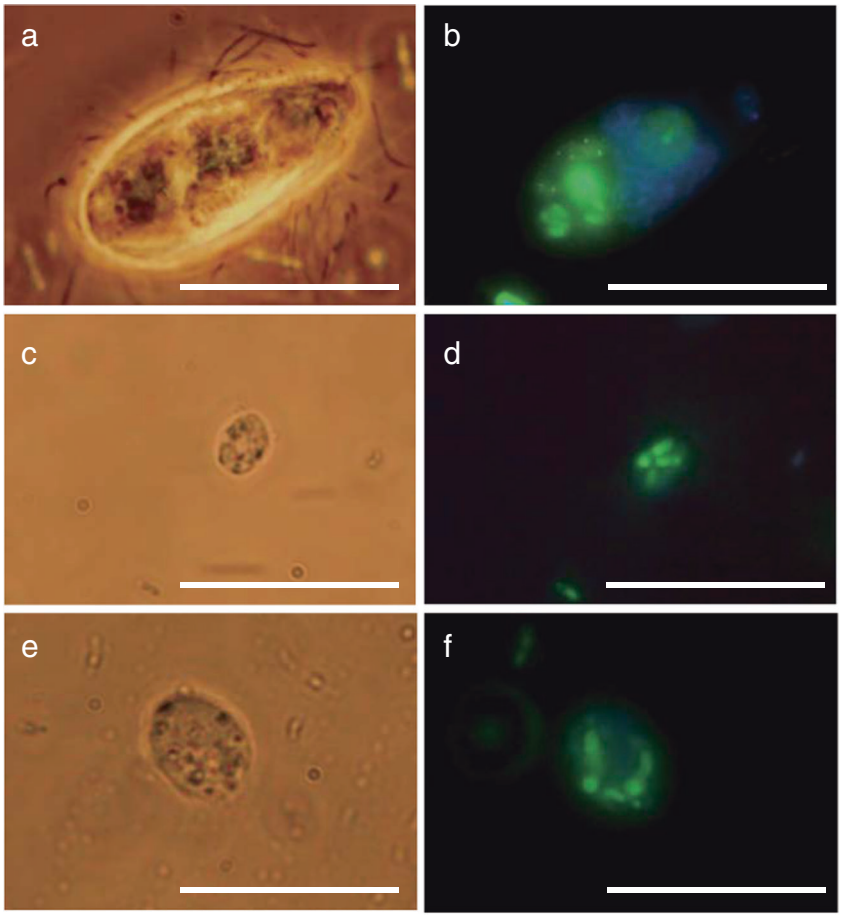

Fig. 2 Internalization of GFP-expressing *E.coli* by each protist. a, **b** *Cyclidium* sp. YH; **c**, **d** *Paracercomonas* sp. YH; **e**, **f** *Trichomitus* sp. YH. The images were obtained 30 min after inoculation of GFP-expressing *E.coli* to protists culture. Panels **a**, **c**, and **e** are bright field. Panels **b**, **d**, and **f** are fluorescence field. Panels **a** and **b**, **c** and **d**, **e** and **f** were taken at same location. The scale bar represents 20 µm.

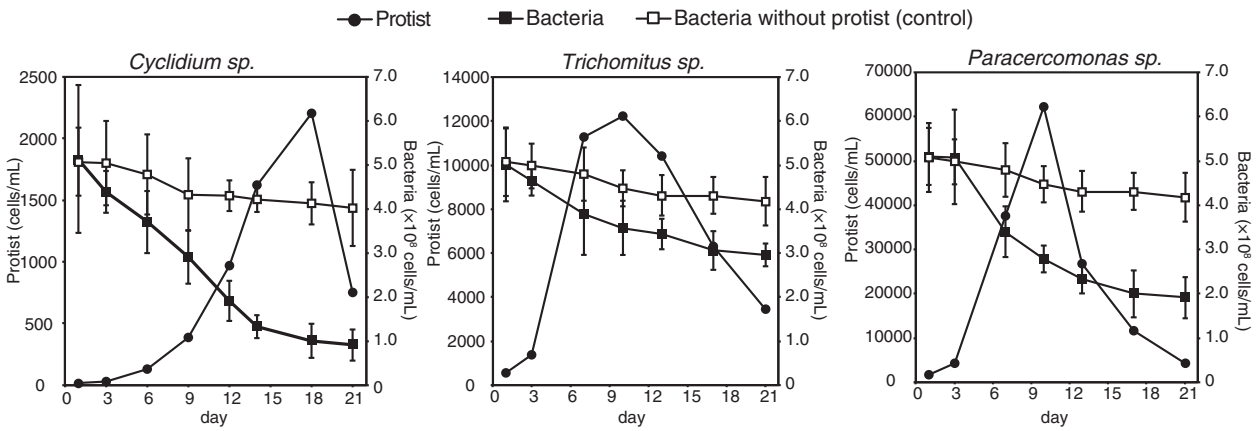

Fig. 3 Growth of each protists with ^{13}C-labeled *E. coli* as a food bacteria. Error bars represent the standard deviation.

$^{13}CO_2$ increased in all cultures relative to that of the control. The abundance of $^{13}CO_2$ reflected the amount of ^{13}C-labeled *E. coli* ingested by each protist. In addition, $^{13}CH_4$ was only detected in the *Cyclidium* sp. YH cultures, indicating that endosymbiotic methanogens convert CO_2 produced by protists into methane gas.

Food selectivity of each anaerobic protists

Table 2 shows the growth rate and generation times of each protist during the exponential growth phase during culture with various food bacteria. The protists did not grow when

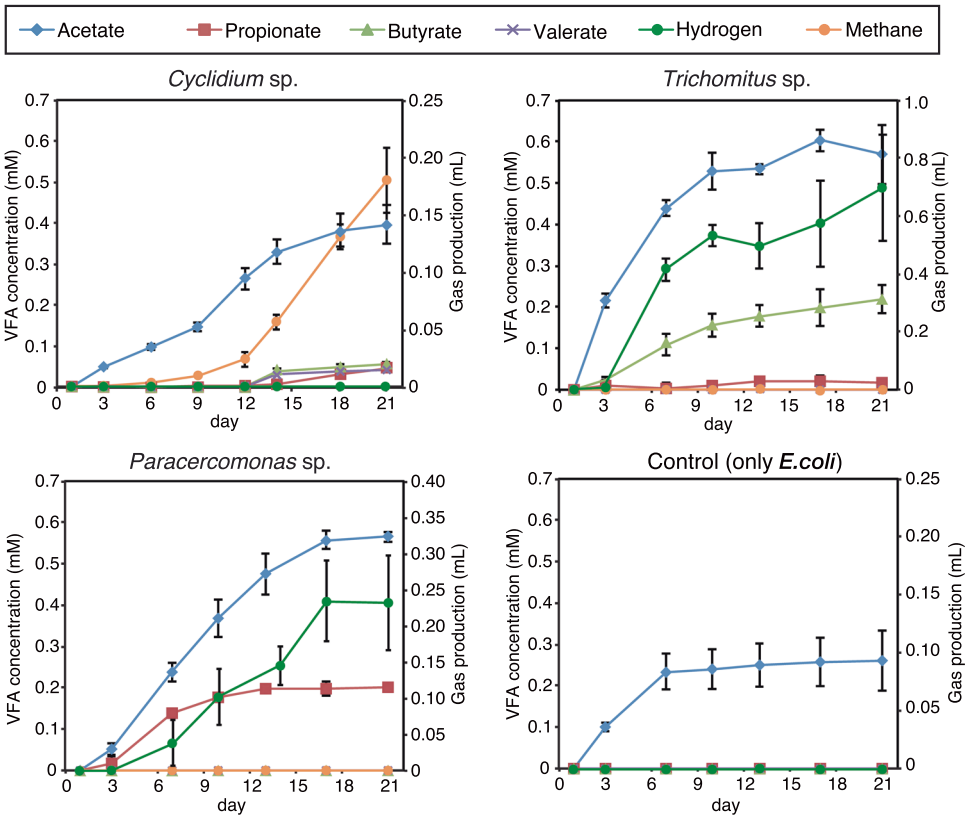

Fig. 4 Production of fermentation products by each protist with ^{13}C-labeled *E. coli* as a food bacteria. Error bars represent the standard deviation.

Table 1 Percentage of $^{13}CO_2$ and $^{13}CH_4$ after cultivation.

Protist	$^{13}CO_2$ (%)	$^{13}CH_4$ (%)
Cyclidium sp.	7.0 ± 0.3	12.9 ± 2.3
Trichomitus sp.	3.9 ± 0.7	-
Paracercomonas sp.	4.6 ± 0.3	-
Control[a]	1.7 ± 0.1	-

- No detected.

[a]*E.coli* only (no protists).

Methanobacterium beijingense or *Methanospirillum hungatei* archaeal cells were used as substrates. *Cyclidium* sp. YH grew when all tested bacteria except for *T. flocculiformis* were used as substrates. *Cyclidium* sp. YH, therefore, ingested both Gram-positive and Gram-negative bacteria except for filamentous forms of bacteria. In contrast, *Trichomitus* sp. YH ingested filamentous *T. flocculiformis*. Although, *Trichomonas* sp. YH grew in cultures with either Gram-positive or -negative bacteria, no growth was observed when *P. acnes* or *M. thermoacetica* was used as the food bacterium. Growth of *Paracercomonas* sp. YH was observed only when they fed on Gram-negative bacteria, namely *E. coli*, *B. luti*, and *B. graminisolvens*. No growth of *Paracercomonas* sp. YH was observed when Gram-positive bacteria were used as food bacteria. This showed that bacterial cell-wall structure affects *Paracercomonas* sp. YH food selectivity.

The growth rate and generation times of *Cyclidium* sp. YH were significantly different depending on the food bacterial species ($p < 0.05$). Among the food bacteria tested, growth rates of *Cyclidium* sp. YH were higher with *E.coli*, *B. graminisolvens, T. flocculiformis*-b, *M. thermoacetica*, and *Clostridium acetobutylicum* than with *B. luti* and *P. acnes*. However, the growth rates of *Trichomitus* sp. YH and *Paracercomonas* sp. YH did not differ significantly by food bacteria species in present study.

Ingestion rates when the protists were cultured to the exponential growth phase with various food bacteria were highest for *Cyclidium* sp. YH, followed by *Paracercomonas* sp. YH and then *Trichomitus* sp. YH (Fig. 5a). The ingestion rates of *Cyclidium* sp. YH, *Paracercomonas* sp. YH, and *Trichomitus* sp. YH were 1.5–2.7 × 10^3, 1.3–1.5 × 10^2, and 0.4–0.6 × 10^2 cells of food bacteria per protist per hour, respectively. Ingestion rates did not differ by food bacterial species when all bacteria compared could be ingested by a protist.

The volatile fatty acid composition in each protist culture varied markedly according to the food bacterial species present (Fig. 5b). Although, acetate was detected in all cultures, propionate, butyrate, and valerate were not detected in all cases. In cultures of *Cyclidium* sp. YH fed on

Table 2 Growth rate (d^{-1}) of each protist fed various food bacteria and archaea.

Food	*Cyclidium sp.*	*Paracercomonas sp.*	*Trichomitus sp.*
Gram-negative bacteria			
E.coli	0.33 ± 0.08^{a}	0.48 ± 0.07^{a}	0.59 ± 0.11^{a}
Bacteroides luti	0.17 ± 0.02^{b}	0.51 ± 0.09^{a}	0.42 ± 0.09^{a}
Bacteroides graminisolvens	0.33 ± 0.08^{a}	0.61 ± 0.21^{a}	0.50 ± 0.21^{a}
Gram-positive bacteria			
Propionibacterium acnes	0.20 ± 0.07^{b}	-	-
Trichococcus flocculiformis	-	-	0.61 ± 0.17^{a}
Trichococcus flocculiformis -b	0.44 ± 0.05^{a}	-	0.55 ± 0.07^{a}
Moorella thermoacetica	0.31 ± 0.03^{a}	-	-
Clostridium acetobutylicum	0.34 ± 0.06^{a}	-	0.38 ± 0.10^{a}
Archaea			
Methanobacterium beijingense	-	-	-
Methanospirillum hungatei	-	-	-

Different superscript letters indicate ANOVA grouping with Tukey's test at 95% confidence.
- not growth.

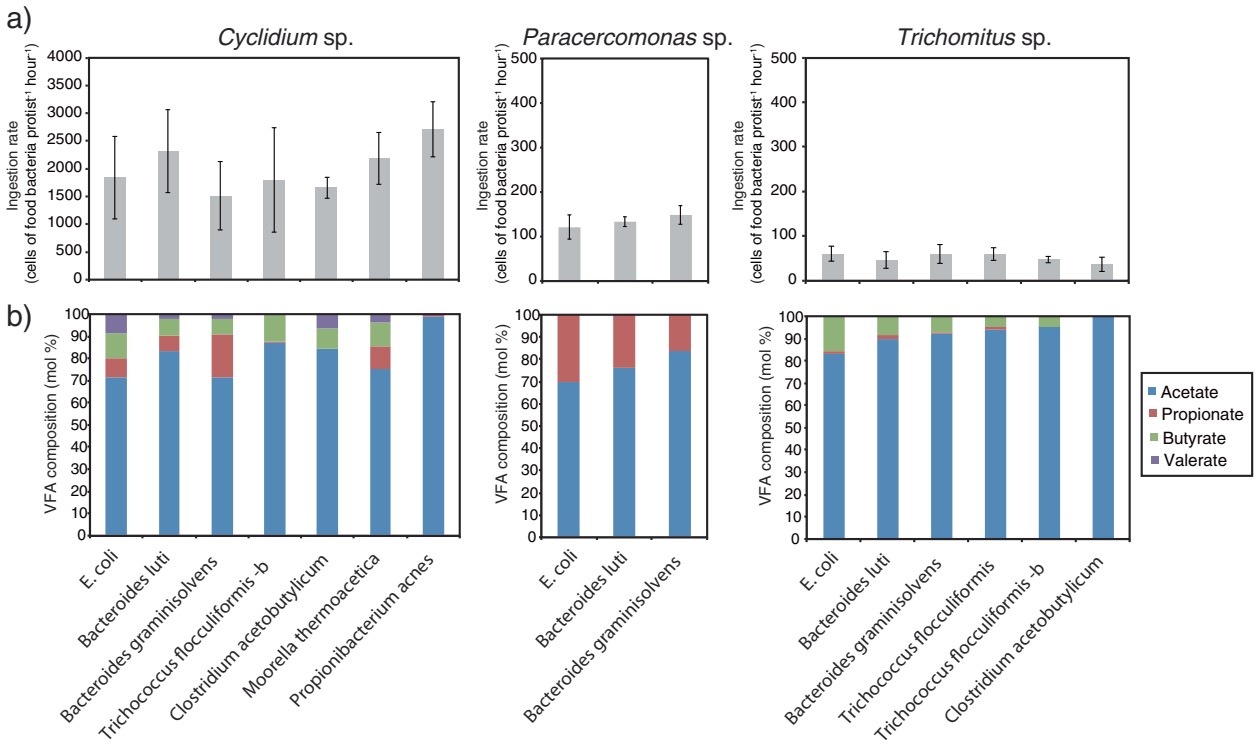

Fig. 5 Ingestion rate (**a**) and fatty acid compositions (**b**) of each protists fed various food bacteria. Error bars represent the standard deviation. **a** Ingestion rates were determined during exponential growth phase of each protists. **b** Fatty acid compositions were determined after the protist number reached maximum number.

B. luti, *B. graminisolvens*, and *M. thermoacetica*, acetate, propionate, butyrate, and valerate were detected, similar to that observed when *E. coli* was used as the food bacterium. However, propionate was only slightly detected or not detected when *T. flocculiformis*-b, *Clostridium acetobutylicum*, and *P. acnes* were used as food bacteria in *Cyclidium* sp. YH cultures. Furthermore, valerate was not detected when *T. flocculiformis*-b and *P. acnes* were used as food, and butyrate was not detected with *P. acnes*. Although, major products acetate and butyrate and minor product propionate was found in the *Trichomitus* sp. YH culture, butyrate and propionate were not detected when

C. acetobutylicum was used as the food bacterium. The major products in all cultures of *Paracercomonas* sp. YH were acetate and propionate. Regardless of food bacterial species, hydrogen was detected in cultures of *Trichomitus* sp. YH and *Paracercomonas* sp. YH, and methane was detected in cultures of *Cyclidium* sp. YH (data not shown).

Discussion

Three protist genera isolated in this study belong to the phyla Ciliophora, Cercozoa, and Metamonada, respectively, and these phyla are frequently found in anaerobic environments [36, 55, 56]. Although, most studies have shown that anaerobic protists in these phyla are bacterivorous heterotrophic organisms in various environments [49, 57, 58], very little is known about their specific roles in anaerobic reactors and natural ecosystems [59]. In anaerobic granular sludge of UASB reactors, particularly, the ecological roles and functions of each anaerobic protist have not been characterized. Therefore, the present study provides important information of these protists' physiological characteristics such as growth rate, generation time, ingestion rate, food selectivity, and metabolites.

Hydrogen and CO_2 have been reported to be common metabolites of anaerobic protists [20, 26, 60]. Anaerobic ciliates and trichomonads have unique organelles, hydrogenosomes, instead of mitochondria, in which organic matter is oxidized to volatile fatty acids, hydrogen, and carbon dioxide for ATP synthesis [61]. An anaerobic cercomonad (i.e., *Brevimastigomonas motovehiculus*) was also found to possess anaerobic mitochondrion-related (hydrogenosome-like) organelles [62]. Hydrogen and $^{13}CO_2$ were detected in the *Trichomitus* sp. YH and *Paracercomonas* sp. YH cultures, which suggested that these protists might also possess hydrogenosome-like organelles.

The present study showed that *Cyclidium* sp. YH directly supplied CO_2 and hydrogen to endosymbiotic methanogens because both $^{13}CO_2$ and $^{13}CH_4$ were detected in a tracer experiment using ^{13}C-labeled *E. coli*. The presence of hydrogenosomes and methanogens in cells of the genus *Cyclidium* was observed previously [48 63]. Many researchers have speculated that the relationship between host protist cells and endosymbiotic methanogens is based on syntrophic hydrogen and CO_2 transfer; however, this transfer has not been demonstrated previously [25, 27, 64]. This is the first study to show direct evidence of a symbiotic relationship between an anaerobic protist and endosymbiotic methanogens. However, the percentage $^{13}CH_4$ was low (12.9%), even though ^{13}C-labeled *E. coli* was used as the sole carbon source. This was probably because the endosymbiotic methanogens utilized bicarbonate in the medium and may indicate that the endosymbiotic methanogens used both CO_2 produced from hydrogenosomes and dissolved CO_2 from outside of host protist cells. Thus, endosymbiotic methanogens can contribute to the maintenance of hydrogen concentrations at very low levels by scavenging exterior hydrogen or that produced from hydrolysis.

Feeding experiments in this study showed that the food selectivity of the protists were affected by the cell shape and cell-wall structure of food bacteria. Effects of bacterial cell shape on protist predation are well known; size-selective predation by protists exists, and filamentous bacteria are morphologically resistant to predation [65, 66]. Although, *Trichomitus* sp. YH can take up both Gram-positive and -negative bacteria, *P. acnes* and *M. thermoacetica* did not support growth, suggesting that cell shape and cell-wall structure of food bacteria were not the only factors associated with the growth of *Trichomitus* sp. YH. Yamada et al. [20] also reported that food selectivity of the anaerobic ciliate *T. compressum* depends on the digestibility of food bacteria rather than on differences in general cell-wall structure.

The feeding experiment with *Paracercomonas* sp. YH revealed, however, that the cell-wall structure of food bacteria does influence their food selectivity. *Paracercomonas* sp. YH can grow only on Gram-negative bacteria. This might indicate that this protist does not have enzymes to digest the thick peptidoglycan layer of Gram-positive bacteria. None of the three protists grew when fed archaea. This also could be related to the indigestibility of their cell walls. Archaea possess cell walls like sheaths and pseudomurein as a cell-wall component, which differs from the walls of bacterial cells. Digestive enzymes in protists should be examined in future studies.

Previous studies have suggested that food bacterial species influence fermentation patterns and growth rates of anaerobic protists such as *T. compressum* [20, 38, 64]. We also observed that the metabolites from and growth rates of *Cyclidium* sp. YH changed depending on food bacteria. The metabolite profiles of *Trichomitus* sp. YH also changed depending on food bacterial species, although its growth rates were not affected. Thus, our results support the idea that the metabolisms of anaerobic protists are affected by food bacterial species, and that anaerobic protists can supply various metabolites to environments based on the bacterial species ingested.

The growth rates of *Cyclidium* sp. YH were affected by food bacteria, but its ingestion rates did not differ significantly based on food bacterial species. This suggests that the amount of nutrients obtained varies by food bacterium. To compare the relationship between each food bacterium and protist growth, we estimated the cell number and

volume required for protist growth (Fig. S2). Significant differences were observed in the number and volume of cells need for *Cyclidium* sp. YH growth. Significantly more food bacterial cells or cell volume was needed for growth of *Cyclidium* sp. YH with *B. luti* and *P. acens* than *T. flocculiformis*-b. Therefore, it is likely that *T. flocculiformis*-b cells were better substrates for *Cyclidium* sp. YH than *B. luti* and *P. acens* for growth and a source of energy. This is consistent of cell yields (increased number of protist cells/number of ingested bacterial cells) for *Cyclidium* sp. YH. When *T. flocculiformis*-b used as food bacteria the cell yield was $1.1 \times 10^{-5} \pm 3.8 \times 10^{-6}$, this was significantly higher than that of *B. luti* ($2.8 \times 10^{-6} \pm 7.0 \times 10^{-7}$) or *P. acens* ($3.1 \times 10^{-6} \pm 3.1 \times 10^{-7}$) ($p < 0.05$). A previous study showed that use of specific bacteria as a substrate stimulated protist growth and maximum cell number [67, 68]. In addition, protists such as *Trimyema* sp., *Paramecium* sp., and *P. shumwayae* are known to need sterol and fatty acids as a growth factor [38, 69, 70]. Moreover, *T. compressum* cell yields were reported to change based on bacterial cell qualities like carbohydrate and protein contents [50]. Although the metabolite composition of *Cyclidium* sp. YH with each food bacterium had no apparent association with high or low growth rates, our results suggest that differences in the nutrients contained in food bacteria can affect protist growth. This could be occurring in other protists species as well, because the metabolite compositions of *Trichomitus* sp. YH and *Paraceromonas* sp. YH were also altered depending on the food bacteria consumed.

The rate of bacterial ingestion of *Cyclidium* sp. YH was the fastest of the three. *Cyclidium* sp. YH, which is a ciliate, was larger than the two other isolates, which are flagellates, suggesting that the difference in ingestion rates is associated with cell size. Comparing the ingestion rates of 12 aerobic and 6 anaerobic protists that similar to our experimental conditions (Table S2 and Fig. 6), it seems to be logarithmically proportional to the cell volume regardless of either anaerobic or aerobic species. Our data and other anaerobic protist are in the same trends. The slope of regression line is 0.75 (Fig. 6), which is close to slope of respiration rates of aerobic protists against cell volume [71]. Thus, we might be roughly estimate an ingestion rate based on protists cell size. Most ciliates have a large cell size and can consume more prokaryotic cells than flagellates [11, 72, 73]. In particular, members of the genus *Cyclidium* have shown great efficiency in removing organic matter and can contribute to sludge reduction and treatment by predation in anaerobic treatment processes [23, 36].

The food selectivity of *Cyclidium* sp. proved the previous our prediction that the ciliate in UASB reactor was selectively ingesting the rod or coccus-shaped bacteria in outer layer of granular sludge [1]. In UASB reactor, number of ciliates were

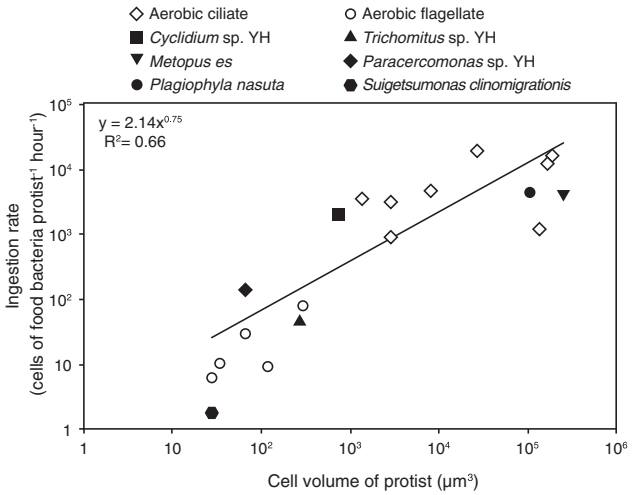

Fig. 6 Ingestion rate of various protists under high prey concentration (10^7 cells mL^{-1}≤) against cell volume. The plotted data are obtained this study and from the references listed in Table S2.

ranged 10^2 to 10^3 cells mL^{-1} [1] and combining the ingestion rate, it is assumed that the maximum contribution of *Cyclidium* sp. for control of bacterial populations is estimated up to 5.8×10^7 cells of bacteria mL^{-1} day^{-1}. Although this ingestion rate is <0.1% day^{-1} of prokaryotes (both bacteria and archaea) of granular sludge (more than 10^{12} cells mL^{-1}), it is increased 1.2% day^{-1} if limited to bacteria located in the outer layer of granular sludge that could be ingested by protist (ca. 5×10^9 cells mL^{-1}) [74]. This bacteria turnover rate is compatible with prokaryotes turnover rate by grazing effect in marine and lake (0.83–49.0% day^{-1}) [75]. This non-negligible bacteria turnover rate by ciliate ingestion could be a reasons for proliferation of syntrophic microorganism in UASB reactor and higher methane concentration though the COD removal is same [1].

In addition, flagellates reach higher cell numbers than ciliates [76], as we found in this study. In natural environments such as lake and marine, flagellates numbers are ranged 10^4–10^6 cells mL^{-1}, which is 10–100 times higher than ciliates [77 78]. Thus, total contributions of *Trichomitus* sp. and *Paracercomonas* sp. to control of bacterial populations could be the same to *Cyclidium* sp. in actual environments. In particular, *Trichomitus* sp. YH can ingest filamentous bacteria, which is important findings because ingestion of filamentous bacteria by anaerobic protist was not reported previously, they might be key factor to prevent bulking caused by filamentous bacteria in UASB reactor [79]. However, limited information about their effects is available although food selectivity of protists expects to change prokaryotes community. These relationships between protists and bacterial community in actual environment need to be examined in future studies.

Conclusion

In the present study, three bacterivorous protists representing different phyla from a UASB reactor were isolated and provide quantitative data for ingestion rate, specific food selectivity, and metabolites. In addition, a tracer experiment using ^{13}C-labeled *E. coli* cells showed that *Cyclidium* sp. YH directly supplied CO_2 and hydrogen to endosymbiotic methanogens. The information provide important insights into the ecological roles of these protists in anaerobic ecosystems and will help to estimate an actual contribution of protozoan community in anaerobic reactor.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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