The Methanogenic and Eubacterial Endosymbionts of *Trimyema*

Naoya Shinzato and Yoichi Kamagata

Contents

1 Monoxenic and Axenic Cultures of *Trimyema* 36
2 Metabolic Features of *Trimyema* 39
3 Methanogenic Symbionts 42
4 Bacterial Symbionts 46
5 Perspectives 50
References 50

Abstract *Trimyema* ciliates thrive in various anoxic environments in which they prey on bacteria and grow with fermentative metabolisms. Like many anaerobic protozoa, instead of mitochondria, *Trimyema* possess hydrogenosomes, which are hydrogen-producing, energy-generating organelles characteristic of anaerobic protozoa and fungi. The cytoplasm of *Trimyema* harbours hydrogenotrophic methanogens that consume the hydrogen produced by these organelles, which confers an energetic advantage to the host ciliate. Symbiotic associations between methanogenic archaea and *Trimyema* ciliates are thought to be established independently and/or repeatedly in their evolutional history. In addition to methanogenic symbionts, it has been shown that *Trimyema compressum* houses bacterial symbionts. Although almost nothing is known about the symbionts except for their phylogeny, this intriguing multi-symbiosis would be a good model for investigating symbiotic interactions among bacteria, archaea, and eukaryotes. In this chapter, we summarise

N. Shinzato
Tropical Biosphere Research Center, University of the Ryukyus, Nishihara, Okinawa 903-0213, Japan

Y. Kamagata (✉)
Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), 2-17-2-1, Tsukisamu-Higashi, Toyohira-ku, Sapporo, Hokkaido 062-8517, Japan
e-mail: y.kamagata@aist.go.jp

J.H.P. Hackstein (ed.), *(Endo)symbiotic Methanogenic Archaea*,
Microbiology Monographs 19, DOI 10.1007/978-3-642-13615-3_4,

the early works and recent progress of studies on *Trimyema* ciliates, in particular *T. compressum*, and discuss the nature of this symbiosis.

1 Monoxenic and Axenic Cultures of *Trimyema*

Trimyema species are anaerobic ciliates that are frequently encountered in various anoxic environments in which they prey on bacteria and grow with fermentative metabolisms. *Trimyema* was first found in sewage tanks and thereafter reported to be present in various aquatic environments, including marine, saltern and hydrothermal vents (Lackey 1925; Augustin et al. 1987; Nerad et al. 1995; Baumgartner et al. 2002; Cho et al. 2008). The genus *Trimyema* is characterized by the following morphological features: (1) the presence of a prominent caudal cilium, (2) a cytostome near the apical end of the cell, (3) somatic kineties in longitudinal rows forming several oblique ciliary girdles and (4) a semicircular structuring of the oral ciliature (Augustin et al. 1987; Nerad et al. 1995; Baumgartner et al. 2002). Thus far, eight species of *Trimyema* have been identified on the basis of morphological features.

Trimyema compressum is frequently found in anoxic freshwater sediments and is the best-studied species in *Trimyema* (Fig. 1). *T. compressum* has been cultured monoxenically or axenically using synthetic media supplemented with living or dead bacteria as food (Wagener and Pfennig 1987; Goosen et al. 1990a; Yamada et al. 1994; Shinzato et al. 2007). The first monoxenic culture of *T. compressum*, strain K (Konstanz), was established from a polluted ditch in West Germany through single-cell isolation by using a micropipette and antibiotic treatment (Wagener and Pfennig 1987). The ciliate could grow in the temperature range of 15–35°C, the optimum being 28°C at which the doubling time was 13 h with *Bacteroides* sp. as food. The highest cell yield obtained under the optimum growth conditions was 2,100 cells ml^{-1}. Freshly cultured strain K cells possessed both

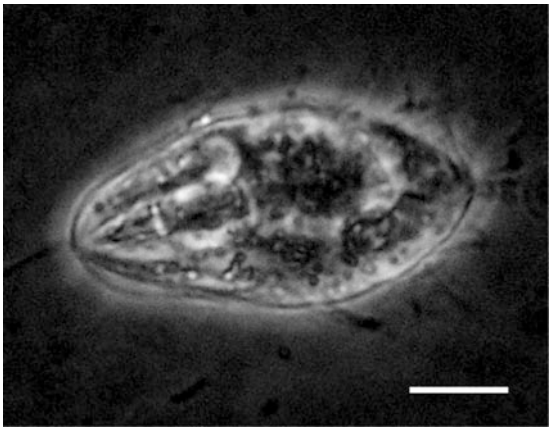

Fig. 1 Phase contrast image of living *T. compressum*. *T. compressum* swims actively in the medium and preys food bacteria. Bar represents 10 μm

methanogenic and non-methanogenic (bacterial) symbionts in their cytoplasm; however, they were lost during continued cultivation (Goosen et al. 1990a). In a similar manner, the second monoxenic culture of *T. compressum* strain N (Nijmegen), was established from a sludge backing pond of a wastewater treatment plant in the Netherlands with *Bacteroides* sp. as food (Goosen et al. 1990a). Although methanogenic and bacterial symbionts were also found in strain N at the beginning, methanogenic symbionts disappeared from the ciliate cells during continued cultivation (Fig. 2). The growth of strain N was observed within a temperature range from 10°C to 30°C with the optimum being between 25°C and 30°C. Strain N reached cell densities of approximately 2–3 $\times$ 10^3 cells ml^{-1}. Following the previous studies, strain NIES and strain S10 were also cultured axenically from an experimental anaerobic filter sludge and a sewage treatment reactor in Japan, respectively (Yamada et al. 1994; Shinzato et al. 2007). *T. compressum* cultures and their features reported to date are summarised in Table 1.

Some researchers have examined the bacterial species suitable as food for *T. compressum* and showed that this species has some food selectivity as reported in many other protozoa (Small 1973; Curds 1977; Fenchel 1980; Liu et al. 2006; Murase and Frenzel 2008). Schulz et al. (1990) tested the preferential use of various chemolithotrophic and phototrophic bacteria by *T. compressum* strain K and concluded that only gram-negative bacteria supported the ciliate's growth. On the other hand, Yamada et al. (1994) examined food selectivity of strain NIES by using various bacterial species and showed that *T. compressum* could prey on various types of bacteria and archaea belonging to the genera *Lactobacillus*, *Clostridium*, *Desulfovibrio*, *Enterobacter*, *Escherichia*, *Pelobacter*, and *Methanoculleus,* but

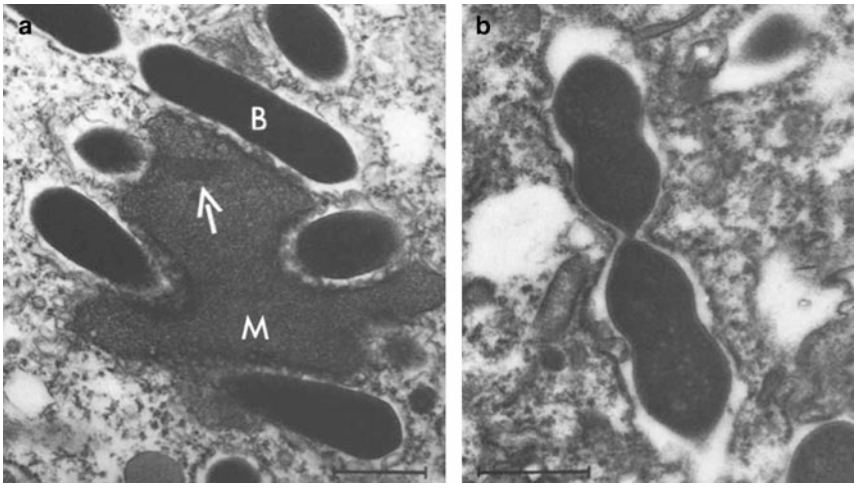

Fig. 2 Transmission electron micrograph of an ultra-thin section of *T. compressum* strain N. (**a**) Methanogenic symbionts (B) associate with microbodies (M: hydrogenosomes). (**b**) Dividing cell of non-methanogenic (bacterial) symbiont, not associated with microbodies. Bars represent 0.5 μm [reprinted from Figs. 1 and 2 of Goosen et al. (1990a) with permission of the publisher]

Table 1 *T. compressum* cultures and their origin, intracellular symbionts, and sterol requirement

Strain	Culture	Origin	Endosymbiont		Sterol[a]	Reference
			Methanogen	Bacteria		
K	Monoxenic	Polluted ditch	Absent[b]	Absent[b]	Positive	Wagener and Pfennig (1987)
N	Monoxenic	Sludge backing pond	Absent[b]	Present	Negative	Goosen et al. (1990a)
N	Axenic	Strain N	Absent[b]	Absent[b]	Positive	Broers et al. (1991)
NIES	Axenic	Anaerobic filter sludge	Absent[b]	n.e.	Positive	Yamada et al. (1994)
S10	Axenic	Sewage treatment reactor	Present	Present	Negative	Shinzato et al. (2007)
S10C	Axenic	Strain S10	Present	Absent	Negative[c]	Shinzato et al. (2007)

[a]Sterol requirement for maintaining cultures
[b]Symbionts were present in the freshly cultured ciliates, but lost after continued cultivation
[c]Only stigmasterol was tested
n.e. not examined

that some other bacteria and archaea were not ingested. The maximum number of *T. compressum* cells varied depending on the species of food bacteria supplemented. The highest number of ciliates reached 9,300 cells ml^{-1} after feeding on cells of *Desulfovibrio vulgaris* (Yamada et al. 1994).

The observed food selectivity of *T. compressum* might be related to the nutritional effect of food bacteria on the ciliate. Broers et al. (1991) treated strain N cultures with antibiotics (penicillin and streptomycin) and established an axenic culture of strain N by using heat-killed (65°C, 1 h) *Klebsiella pneumoniae* as food. *Bacteroides* and *Klebsiella* cells inactivated by γ-irradiation could support the ciliate growth as well; however, γ-irradiated bacteria could not be replaced with autoclaved bacteria, suggesting the involvement of unidentified heat-labile growth factors in these bacteria (Broers et al. 1991). Indeed, several sterols have been reported to enhance the growth of monoxenic and axenic cultures of *T. compressum*. The first monoxenic culture, strain K, also required at least one sterol such as stigmasterol, stigmastanol, or ergosterol for reproducible growth (Wagener and Pfennig 1987). Addition of stigmasterol to monoxenic cultures of strain N could enhance the growth of the ciliate, although it was not necessary for maintaining cultures (Goosen et al. 1990a; Broers et al. 1991). Such growth enhancing effects of sterols have been seen in the strain NIES in which stigmasterol addition stimulated weakened cell growth and markedly increased the maximum cell number of the ciliate (Yamada et al. 1994). As mentioned, some sterols undoubtedly affect growth stimulation of *T. compressum* cultures. However, it is unclear if such sterols are indispensable for the survival of *T. compressum* in natural environments since some *T. compressum* cultures such as strain N and S10 could grow reproducibly without sterol administration (Goosen et al. 1990a; Shinzato et al. 2007). The sterol requirement of *T. compressum* cultures will be discussed again in a later section of this chapter, as this phenomenon might relate to the presence or absence of their intracellular symbionts.

2 Metabolic Features of *Trimyema*

All known *Trimyema* species live in anoxic habitats and their energy metabolisms are highly adapted to oxygen-free environments. The metabolic features of *Trimyema* have been studied using monoxenic and axenic cultures of *T. compressum*. *T. compressum* has long been known to possess microbodies resembling hydrogenosomes in their cytoplasm. Hydrogenosomes, organelles of mitochondrial origin, ferment pyruvate with substrate-level phosphorylation and hydrogen generation. The hydrogen is formed by hydrogenase activity to dispose off the excess reducing equivalents derived from fermentative metabolism (Müller 1993; Boxma et al. 2005). Some anaerobic protozoa and fungi are known to possess hydrogenosomes instead of mitochondria (Hackstein et al. 2008a, b). To clarify the nature of the *T. compressum* microbodies, several investigations were performed, and cytochemical and immunological staining proved the localization of hydrogenase, a hallmark enzyme of hydrogenosomes, in the microbodies (Zwart et al. 1988; Goosen et al. 1990a; Broers et al. 1991).

On the other hand, *T. compressum* was shown to have some oxygen tolerance up to 0.5 mg l^{-1}. Furthermore, under microaerobic conditions, formate and CO_2 were produced as major end products accompanying oxygen consumption, while no hydrogen, ethanol or succinate was formed (Goosen et al. 1990b). These observations suggested that, to some extent oxygen could act as a terminal electron acceptor instead of protons. Goosen et al. (1990a) examined enzyme activities characteristic of mitochondria and the responses to inhibitors of mitochondrial functions to investigate whether the microbodies retained mitochondrial functions. Representative mitochondrial enzymes, cytochrome oxidase and catalase, were not detected but superoxide dismutase was found. In addition, antimycin A and chloramphenicol did not influence the growth of the bacterial symbiont-free strain K. On the other hand, KCN and NaN_3 also reduced the growth of the ciliate both under aerobic and anaerobic conditions. However, since these inhibitors not only inhibit respiration but also enzyme activities involved in fermentative metabolism, these results suggested that the microbodies in *T. compressum* are defined as hydrogenosome that lacks mitochondrial features (Goosen et al. 1990b).

The studies that analysed the metabolites of *T. compressum* indicated that they gain energy by digestion of food bacteria via fermentative metabolisms. Goosen et al. (1990b) examined fermentation products of *T. compressum* strain N fed *Bacteroides* sp. and detected ethanol, acetate, lactate, formate, CO_2 and hydrogen under anaerobic conditions in which ethanol was formed in large amounts representing 44% of the total carbon excreted. On the other hand, Holler and Pfennig (1991) reported that lactate, acetate and formate were major end products of strain N, grown with *Bacteroides* sp., while no ethanol production was found. However, profiles of the metabolites appeared to vary depending on the growth conditions such as anaerobicity or the species and the amount of food bacteria. For instance, under micro-oxic conditions, strain N did not produce ethanol, while formate was formed as a major end product together with CO_2 evolution (Goosen et al. 1990b).

Holler and Pfennig (1991) also reported a significant decrease in organic acid formation under available oxygen conditions. The shift of the fermentation profile under micro-oxic conditions suggested that oxygen is likely to be used as a terminal electron acceptor, although the nature of the terminal oxidase has not been identified. The species of food bacteria also can influence the fermentation profile of *T. compressum*. For example, when strain N was cultured using *Rubrivivax gelatinosus* as food, the total production of organic acids lowered and acetate became the most dominant metabolite, in contrast with lactate being the most abundant when the ciliate was grown with *Bacteroides* sp. (Holler and Pfennig 1991). The observed shift in metabolites indicated that the fermentation profiles could be influenced by the efficiency of food utilization of the ciliate because the number of *Rubrivivax* cells consumed was suspected to be lower than that of *Bacteroides* cells due to the difference in digestibility (Holler and Pfennig 1991).

In addition to growth conditions, the presence or absence of methanogenic symbionts could influence the metabolic profiles of the host ciliate. The methanogenic symbiont-bearing strains NIES and S10 of *T. compressum* produced acetate as the dominant end product with a small amount of propionate and butyrate, but formate, lactate, and ethanol were not detected under anaerobic conditions (Yamada et al. 1994, 1997; Shinzato et al. 2007). Yamada et al. (1994) examined the relationship between the species of food bacteria and the fermentation products of strain NIES and showed that the influence of the bacterial species was not significant, but a slight fluctuation in the amount of propionate and butyrate was found. These results suggest that in the presence of methanogenic symbionts the influence of the species of food bacteria on the host metabolism might be insignificant compared with methanogen-free strains such as strains K and N.

The hydrogen evolved from the hydrogenosomes attracts hydrogen-consuming microbes, and an interspecies hydrogen transfer is believed to be the basis of methanogen symbiosis in anaerobic eukaryotes (Embley and Finlay 1994). Maintenance of hydrogen concentrations at very low levels is indispensable for continuous digestion and fermentation of food bacteria since proton reduction is energetically favourable at low hydrogen concentrations only (Stams 1994). Thus, fermentation profiles of methanogen-bearing ciliates can be greatly influenced by the presence or absence of the methanogenic symbionts. Yamada et al. (1997) examined the metabolic profiles of methanogen-free cultures of strain NIES, the derivative of the methanogen-bearing original strain, to evaluate the contribution of methanogenic symbionts to the fermentative metabolism of host ciliates. The maximum cell yield of methanogen-free ciliates decreased by 80% and the major end products changed from acetate and methane to butyrate. These observations supported the idea that methanogenic symbionts confer an energetic advantage on the host ciliate by enhancing the acetogenic reaction (Yamada et al. 1997).

The detailed schemes of carbohydrate metabolism in *Trimyema*, that of hydrogenosome in particular, remain to be elucidated since no biochemical or molecular studies have been performed. Therefore, metabolite profiles are the only available information to allow speculation about the metabolic features of this ciliate. As mentioned previously, ethanol, lactate, acetate, formate, CO_2 and hydrogen

are considered to be major fermentative products of *T. compressum*. On the basis of the metabolic profiles, Hackstein et al. (2008a) presented a speculative metabolic scheme of carbohydrate degradation pathway in *Trimyema* (Fig. 3). In this scheme, pyruvate:formate lyase (PFL) is hypothesized to be involved in pyruvate metabolism since formate production has been found in *T. compressum* cultures. This type of carbohydrate metabolism resembles that of some anaerobic chytridiomycete fungi (Boxma et al. 2004; Hackstein et al. 2008b).

Within anaerobic ciliates, the hydrogenosomes of *Dasytricha ruminantium* and *Nyctotherus ovalis* living in the rumen and hindgut of the cockroach, respectively, have been extensively studied. The key enzyme for pyruvate degradation to acetyl-CoA in *D. ruminantium* is suggested to be pyruvate:ferredoxin oxidoreductase (PFO) (Yarlett et al. 1981, 1982, 1985). Some fractions of acetyl-CoA appear to be exported to cytosol for butyrate formation and accompanied by ATP production (Yarlett et al. 1985; Ellis et al. 1991a,b,c). In addition to *Dasytricha*, the energy metabolism of the *N. ovalis* hydrogenosome has been thoroughly investigated by enzymatic and radioactive tracer experiments (Boxma et al. 2005). *N. ovalis* is the only organism whose hydrogenosome has a genome (Akhmanova et al. 1998; van Hoek et al. 2000a; Boxma et al. 2005). This "missing-link" organelle has conserved many mitochondrial traits in its metabolic features and genome. The pyruvate oxidation in the hydrogenosome of *N. ovalis* is likely to occur with pyruvate dehydrogenase (PDH), as the genes for PFO and PFL have not been detected. The reducing equivalents derived from substrate oxidation are speculated to be used not only for proton reduction but also for fumarate reduction via the electron

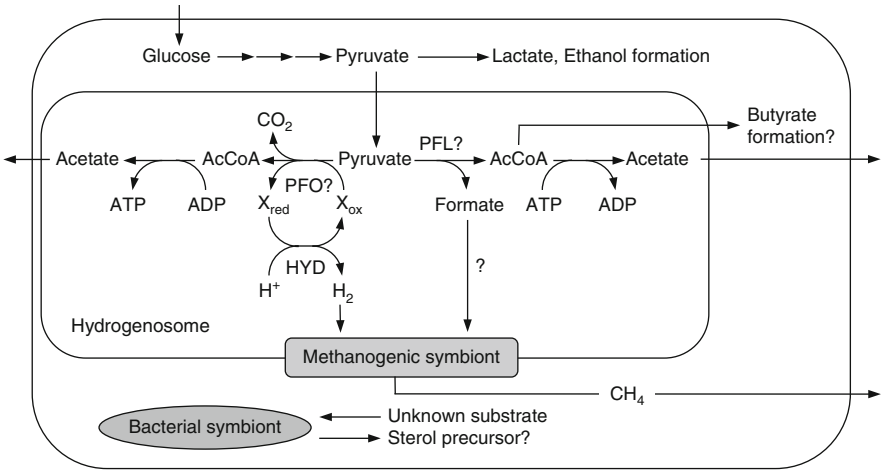

Fig. 3 Speculative metabolic schemes of carbohydrate metabolism in the symbiotic consortium of *T. compressum*. Abbreviations: *AcCoA* acetyl-CoA, *HYD* hydrogenase, *PFL* pyruvate:formate lyase, *PFO* pyruvate:ferredoxin oxidoreductase. Xox, red, unknown electron carrier. Methanogenic symbionts are capable to use both hydrogen and formate as the substrate for methanogenesis. Substrate and contribution of bacterial symbionts are unknown [modified from Fig. 5 of Hackstein et al. (2008a)]

transport chain. In the course of electron transport, proton motive force could be generated at the mitochondrial complex I, although F_0F_1-ATP synthase has not been discovered (Hackstein et al. 2008a).

3 Methanogenic Symbionts

Symbiotic associations between protozoa and methanogenic archaea are found in various anoxic environments (Hackstein and Vogels 1997). Methanogenic symbionts in protozoa can be easily detected by a bluish-green fluorescence of coenzyme F_{420}, which is characteristic of methanogens (Doddema and Vogels 1978). The association of methanogenic symbionts is normally found in hydrogenosome-bearing protozoa and the hydrogen evolved from the organelles is believed to be consumed by the endosymbiotic methanogens. As the oxidation of NADH and $FADH_2$ coupled to proton or bicarbonate reduction is thermodynamically feasible only at low hydrogen concentrations, methanogenic symbionts could facilitate the anaerobic metabolism of the host protozoa by the scavenging hydrogen (Stams 1994). Many free-living and intestinal species of anaerobic protists have been reported to harbour methanogenic symbionts (Embley and Finlay 1993, 1994; van Hoek et al. 2000b). Thus far, *Methanobacterium formicicum* and *Methanoplanus endosymbiosus* have been isolated from anaerobic ciliates and an amoeba (van Bruggen et al. 1984, 1986, 1988; Goosen et al. 1988).

In *Trimyema* species, *T. compressum* and *Trimyema* sp. were found to possess methanogenic symbionts in their cytoplasm (Wagener and Pfennig 1987; Finlay et al. 1993). *T. compressum* appeared to harbour methanogenic symbionts by nature because the ciliates freshly cultured from environmental samples were always accompanied by them (Wagener and Pfennig 1987; Goosen et al. 1990a; Yamada et al. 1994; Shinzato et al. 2007). The cell size of the methanogenic symbionts in *T. compressum* were reported to be 0.65 μm wide and 1.6–3.3 μm long (Wagener and Pfennig 1987), and another study reported that they were 0.3–0.4 μm wide and 1.3–2.0 μm long (Shinzato et al. 2007). The number of methanogenic symbionts reported from strain K was up to 340 cells per single ciliate and varied between zero and several hundred in different cells (Wagener and Pfennig 1987). In contrast, single cells of strain S10 contained an average of 436 cells of methanogenic symbionts ($N = 20$) ranging from 272 to 769 (Shinzato et al. 2007). Transmission electron microscopic observations of freshly cultured strains N and S10 demonstrated that the methanogenic symbionts were located nearby or embedded in hydrogenosomes (Figs. 2 and 4). Such a characteristic proximity between methanogens and hydrogenosomes has been found in other anaerobic protozoa harbouring methanogenic symbionts (Embley and Finlay 1994). Hydrogen has a large diffusion flux; this is as a result of maximising the efficiency of interspecies hydrogen transfer. The molecular phylogenetic identification based on 16S rRNA genes of the symbionts in strain S10 showed that they were closely related to *Methanobrevibacter arboriphilus* with 97.2% sequence similarity (in ca. 1,300 bp) (Shinzato

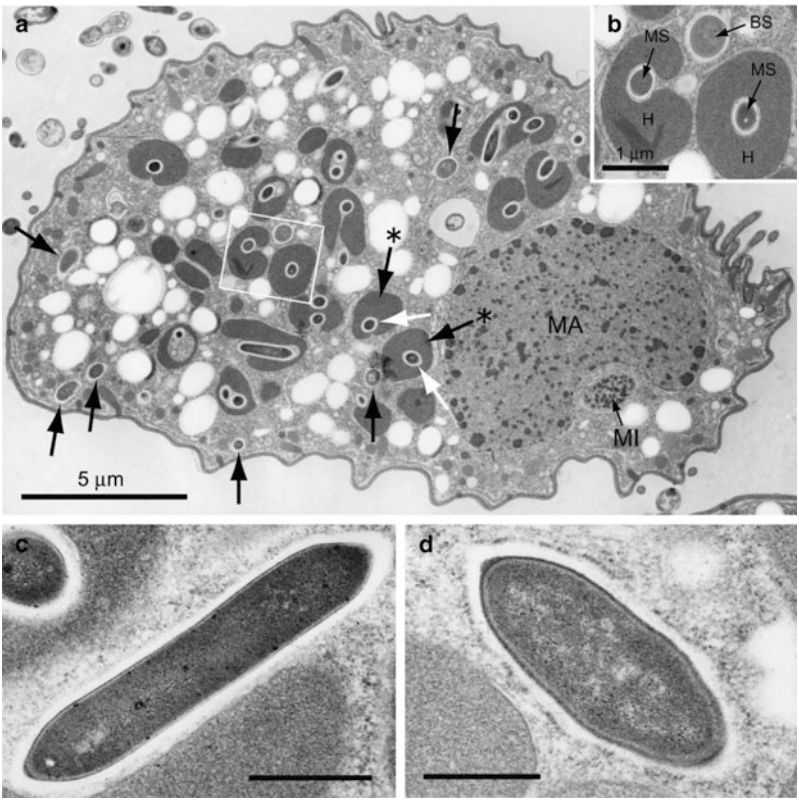

Fig. 4 Transmission electron micrographs of ultra-thin sections of *T. compressum*. (**a**) Whole view of a *T. compressum* cell. The methanogenic symbionts (*white arrows*) were closely associated with hydrogenosomes (*black arrows* with *asterisks*), while the bacterial symbionts (*black arrows*) were distributed over the cytoplasm independent of hydrogenosomes. The macronucleus and micronucleus are shown with MA and MI, respectively. (**b**) Enlarged view of the boxed region in (**a**). Methanogenic symbionts (MS) are surrounded by hydrogenosomes (H). Bacterial symbionts are also shown (BS). (**c**) Longitudinal section of the methanogenic symbiont. (**d**) Longitudinal section of the bacterial symbiont. Bars represent 0.5 µm unless stated otherwise [reprinted from Fig. 1 of Shinzato et al. (2007) with permission of the publisher]

et al. 2007). An attempt to isolate the methanogenic symbionts from squashed *T. compressum* strain K cells using a deep agar method was unsuccessful (Wagener and Pfennig 1987).

Trimyema sp. was cultured from the sediments of a productive pond in England together with the living mixed microbial flora, and, the association with methanogenic symbionts was investigated by electron microscopy and molecular approaches (Finlay et al. 1993). Although this strain was identified as a member of the genus *Trimyema* according to its general morphological characteristics, it was obviously different from *T. compressum* in terms of the number of longitudinal somatic kineties, kinetosomes in some kineties, structure of brosse and so on.

Trimyema sp. contained up to 300 cells per ciliate of methanogenic symbionts in their cytoplasm. The methanogenic symbionts were relatively small and irregularly disc-shaped and distributed over the cytoplasm (Fig. 5). However, they appeared to show polymorphic traits, transforming according to the degree of association with the hydrogenosome, and those that were attached to the hydrogenosome were significantly larger and profusely dentate. This morphological change is thought to facilitate efficient capture of hydrogen evolved from the hydrogenosome. Molecular phylogenetic inspections of the methanogenic symbionts in *Trimyema* sp. based on 16S rRNA gene sequences revealed that they were closely related to the free-living methanogen *Methanocorpusculum parvum* with a 10-base difference in 840 compared bases. A relative of *M. parvum*, which was isolated and described as *M. endosymbiosus* (van Bruggen et al. 1986), has also been reported from the marine ciliate *Metopus contortus*.

The association between *T. compressum* and methanogenic symbionts seems to be somewhat unstable and capricious, even though *T. compressum* freshly isolated from the environment was always accompanied by methanogenic symbionts. Indeed, most monoxenic and axenic cultures of *T. compressum* lost their symbionts during continued cultivation as mentioned earlier (Wagener and Pfennig 1987; Goosen et al. 1990a; Yamada et al. 1997). The strain K lost its symbionts during continued cultivation, especially under conditions of an abundant supply of food bacteria. In contrast, the continued presence of methanogenic symbionts in the ciliate could be enhanced under food-limited conditions (Wagener and Pfennig 1987). The loss of symbionts seems to be a result of the outgrowth of the host cells, which disturbs the synchronisation of the growth of the host and the symbiont.

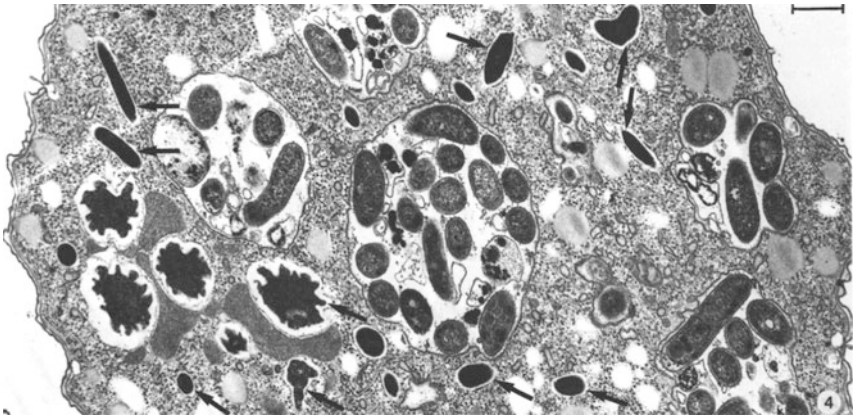

Fig. 5 Transmission electron micrographs of ultra-thin sections of *Trimyema* sp. Electron-dense methanogens with various shapes are enclosed individually in vacuoles within the ciliate cytoplasm (*arrows*). Polymorphic transformation of methanogens are observed from the disc-shaped to the stellate form, at which the methanogens are completely surrounded by hydrogenosomes. Several large vacuoles, each containing many (non-methanogen) food bacteria, are also shown. Bar represents 1 μm [reprinted from Fig. 4 of Finlay et al. (1993) with permission of the publisher]

The loss of symbionts is also reported in other anaerobic ciliates. Finlay et al. (1993) mentioned the experience that some species of anaerobic ciliates lost methanogenic symbionts when the cultures were held at a high temperature (27°C), whereas methanogenic symbionts tended to be maintained in the ciliate when the cultures were kept at a low temperature (10°C). This observation indicates that vigorous growth of the ciliates at high temperature might surpass the growth of methanogenic symbionts.

In the case of strain N, the observed loss of methanogenic symbionts occurred without apparent effect upon the ciliate growth, although methanogenic symbionts are believed to provide metabolic advantages to the host ciliate (Holler and Pfennig 1991). However, Yamada et al. (1997) examined the effect of methanogenic symbionts on the host and demonstrated that in the absence of symbionts, the major fermentation products shifted from acetate to butyrate and, the maximum cell yield decreased from 3,300 to 2,700 cells ml^{-1}. In anaerobic metabolism such as fermentation of carbohydrate, the formation of more oxidized product yields more energy. Therefore, methanogenic symbionts in *T. compressum* appeared to contribute to further substrate oxidation of the host by scavenging hydrogen thus maintaining the hydrogen concentration at a very low level. The significance of the methanogenic symbionts for the host growth has also been examined in two hydrogenosomal ciliates, *Plagiopyla frontata* and *M. contortus,* by methanogen-curing experiments using bromoethane sulfonic acid (BES), a specific inhibitor for methanogen. The results showed that the absence of methanogenic symbionts reduced the growth yield by approximately 30% in both ciliates (Fenchel and Finlay 1991).

To the best of our knowledge, only the cultures of *T. compressum* strain S10 have been stably maintaining methanogenic symbionts for a long period (more than 10 years, unpublished data). The reason for such a stable co-existence is unknown. However, the culture of strain S10 has been routinely transferred to a new medium at the declining stage (10 days after reaching maximum cell density), hence, the ciliates surviving at the stage are exposed to food-limited conditions. The majority of the ciliate population possesses methanogenic symbionts, probably because it would be advantageous in such starvation conditions. If this is the case, a short-interval culture transfer may result in the loss of methanogenic symbionts. Considering the numbers of facts as mentioned above, methanogenic symbionts are undoubtedly beneficial to the survival of the host ciliates in food-limited natural environments.

As described earlier, it appears that two types of methanogens have established symbiotic associations with *Trimyema* species. The first one is a rod-shaped methanogen found in *T. compressum*, which was a relative of *Methanobacterium* or *Methanobrevibacter* in the order *Methanobacteriales* (Wagener and Pfennig 1987; Shinzato et al. 2007). The other one is not a rod-shaped, polymorphic methanogen that was found in *Trimyema* sp. It was identified as a close relative of *M. parvum* in the order *Methanomicrobiales* (Finlay et al. 1993). This indicates that *Trimyema* ciliates have established symbiotic associations with different species of methanogens independently in their particular niches as hypothesized in other anaerobic ciliates (Embley and Finlay 1993). In the case of strain S10, the *Methanobrevibacter* species is known as a major phylogenetic group of

methanogens present in the intestinal tracts of animals including humans (Lin and Miller 1998). Probably, in sewage treatment reactors where strain S10 was isolated, it could have been one of the candidates as a symbiotic partner of the anaerobic protozoa living in such environments. Indeed, the methanogenic symbionts found in the anaerobic ciliate *N. ovalis* that resides in a cockroach hindgut has been identified as *Methanobrevibacter* (van Hoek et al. 2000b).

Trimyema is not the only ciliate known to harbour phylogenetically distantly related methanogens as endosymbionts. *M. contortus*, a marine ciliate, harboured polymorphic methanogens closely related to the symbionts found in *Trimyema* sp., while *Metopus palaeformis*, another species isolated from a municipal landfill, was found to be associated with rod-shaped, non-transforming *Methanobacterium* species (Embley et al. 1992). Likewise, relatives of the genera *Methanolobus* and *Methanoculleus* have been reported as endosymbionts in *P. frontata* and *Plagiopyla nasuta*, respectively (Embley and Finlay 1994). These disorderly combinations of methanogenic symbionts and host ciliates suggest that these symbioses may have been established independently in their particular niches after the diversification of the ciliate species (Embley and Finlay 1994; van Hoek et al. 2000b). In addition, these events may have been accompanied by the replacement of methanogenic symbionts.

The possibility of symbiont replacement has been examined using an aposymbiotic strain of *T. compressum*. Wagener et al. (1990) attempted to re-infect aposymbiotic ciliates with two strains of *M. formicicum*, DSM3636 and 3637, which had been originally isolated from *Metopus striatus* and *Pelomyxa palustris*, respectively, and successfully constructed a new symbiosis with these exogenous methanogens. In the course of symbiosis formation, methanogens ingested by food vacuoles were surrounded by a cytoplasmic membrane and eventually separated from the vacuoles (Fig. 6). The newly established consortium produced methane, and the growth of the host ciliate was significantly stimulated under food-limited conditions (Wagener et al. 1990). However, this consortium was readily dissolved by abundant food supply, which indicated a low interdependency between the methanogens and the host ciliates. The success of symbiosis reconstruction suggests that methanogenic symbiont and host ciliate might recognize each other by some means but not by highly specific ways, which could allow a relatively easy symbiont replacement of anaerobic ciliates. However, it is still unclear which factors are involved in the establishment and perpetuation of symbiosis in anaerobic protozoa. Furthermore, methanogens engulfed in food vacuoles must be taken out and brought close to the hydrogenosome, for which a specialized recognition and transport mechanism would be needed.

4 Bacterial Symbionts

Of the known species of *Trimyema*, only *T. compressum* has been reported to harbour bacterial and methanogenic symbionts. The bacterial symbionts were first reported from two strains of *T. compressum*, strains K and N, which were isolated from

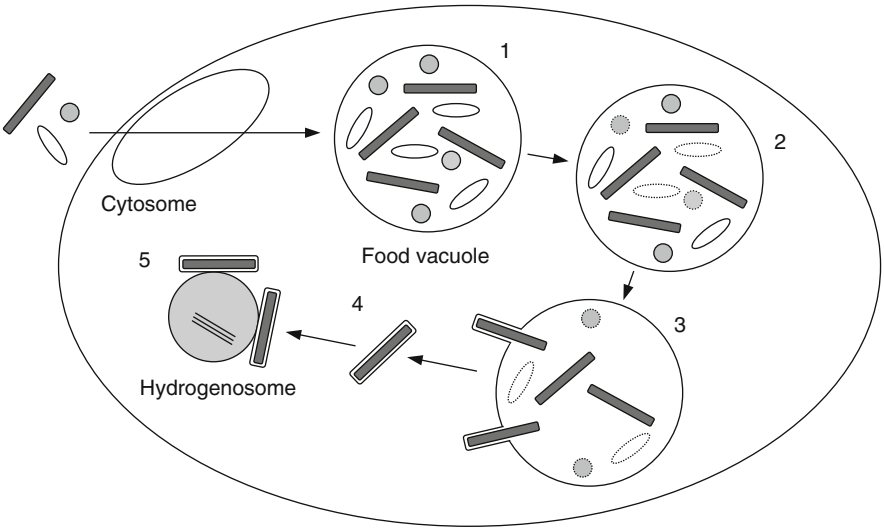

Fig. 6 Proposed process of establishment of a symbiotic association between *T. compressum* and methanogens. (1) uptake of bacteria into food vacuoles; (2) digestion of food bacteria; (3) separation of methanogens into the cytoplasm; (4) transport of the methanogens surrounded by a membrane; (5) localization of methanogens near by hydrogenosome. The last step was not demonstrated in the experiments [redrawn from Fig. 5 of Wagener et al. (1990)]

different habitats in Europe (Goosen et al. 1990a). These rod-shaped bacteria were 0.3–0.4 μm wide and 0.5–0.7 μm long (Fig. 2). Single ciliate cells possessed 20–100 bacterial symbionts in the cytoplasm. In contrast to the methanogenic symbionts, the bacterial symbionts were located in the cytoplasm independently of hydrogenosomes. The bacterial symbionts were persistent during continued cultivation of strain N but were lost in strain K. However, a monoxenic culture of strain K was originally established by Wagener and Pfennig (Wagener and Pfennig 1987) and, no description of these bacterial symbionts was found in the report. One might speculate that they have been eliminated from the ciliate cell during the course of purification with antibiotic treatment (penicillin and streptomycin). On the other hand, scanning electron microscopic observation could not reveal the presence of any episymbiotic bacteria on the surface of *T. compressum* (Wagener and Pfennig 1987).

Bacterial symbionts were also found in *T. compressum* strain S10, which was isolated from a sewage treatment reactor in Japan (Shinzato et al. 2007). Bacterial symbionts designated TC1 were spherical rods 0.3–0.6 μm wide and 0.8–2.0 μm long and persisted stably in the ciliate for over 10 years of cultivation (unpublished result). Transmission electron microscopic observation showed that they were distributed throughout the cytoplasm in contrast to the methanogenic symbionts that were consistently associated with hydrogenosomes (Fig. 4). Molecular phylogenetic identification based on 16S rRNA gene sequence and fluorescence in situ hybridization (FISH) revealed that they are a member of the order *Clostridiales* and

affiliated with the lineage of *Syntrophomonadaceae*. The closest isolate was *Desulfosporosinus* sp. strain A10 with 84.3% of sequence similarity indicating the uniqueness of the bacterial symbiont (unpublished result). The phylogenetic relationships among bacterial symbionts of *T. compressum* strains are unclear because phylogenetic information of the bacterial symbionts found in the two European strains is not available.

Besides *T. compressum*, triplex symbiosis composed of anaerobic protozoa, methanogens and bacteria has been found in some anaerobic ciliates. *Cyclidium porcatum,* an anaerobic scuticociliate, contains both methanogenic and bacterial symbionts in the anterior part of the cell that are associated with the hydrogenosome and form a tightly organized complex (ca. 8 μm) (Esteban et al. 1993). The bacterial symbionts, relatively large and thick rods, were distinguishable from the small methanogenic symbionts. Both prokaryotic symbionts were visualized by simultaneous FISH-staining using archaea- and bacteria-specific probes. However, the molecular phylogeny of these symbionts has not been elucidated. Although the physiological significance of these bacterial symbionts is still unclear, their close association to hydrogenosomes implies that the substrate is supplied by the hydrogenosomes, which is different from the situation of the *T. compressum* bacterial symbionts. As all constituents (hydrogenosome, methanogen, and bacterial symbionts) persisted in mostly the same ratios, these three components are thought to proliferate at the same rate, probably by synchronising with the division rate of the ciliate (Esteban et al. 1993). Such a complex symbiosis has also been reported in the giant amoeba *P. palustris*, in which both methanogenic and bacterial symbionts were held in its cysts (van Bruggen et al. 1983).

The physiological roles of these bacterial symbionts on ciliate survival are of great concern as we attempt to understand the symbiosis. Goosen et al. (1990a) compared strains K and N in terms of the requirement of growth factors and the response to antibacterial drugs. The results showed that only strain K required sterols for growth (stigmasterol, stigmastanol, or ergosterol) and that only strain N was sensitive to chloramphenicol (100 μg ml^{-1}), in which the growth rate of strain N was markedly decreased with a low maximum cell yield (40–50% of untreated control). Likewise, antibiotic treatment (penicillin and streptomycin) for establishing the axenic culture of strain N caused a striking decrease in ciliate cell yield (50% of untreated ciliates) (Broers et al. 1991). Such growth suppression by antibiotic treatment was also reported in the strain S10 cultures. Although the *T. compressum* strain S10C obtained from antibiotic treatment of strain S10 could grow without any growth factors, its maximum cell yield decreased to 30% of that of the original strain (Shinzato et al. 2007). Since antibiotic treatment is expected to eliminate the bacterial symbionts from the ciliates, these observations strongly suggest that the bacterial symbionts support the vigorous growth of *T. compressum*.

The axenic culture of *T. compressum* required C24-alkylated sterols such as stigmasterol, stigmastanol and ergosterol as growth stimulating factors. Strictly anaerobic protozoa cannot synthesize C24-alkylated sterols since this reaction

requires the participation of molecular oxygen (Nes and McKean 1977). On the other hand, a variety of bacteria contain hopanoids (pentacyclic triterpenes), which are structurally similar to sterols, the biosynthesis of which does not require molecular oxygen (Rohmer et al. 1979). Therefore, it has been speculated that hopanoids might be provided from food bacteria as sterol precursors (Wagener and Pfennig 1987). Although the possible contribution of bacterial symbionts as providers of sterol precursors was not considered in previous reports, it must be advantageous for *T. compressum* in particular when they grow in environments scarce in sterols or their precursors. However, it has not been tested whether hopanoids can support the growth of ciliates. Further studies are needed to corroborate this hypothesis.

Another possible contribution of the bacterial symbionts to the host ciliate is the role of a hydrogen-scavenger, which could work as a backup to the methanogenic symbionts. Goosen et al. (1990a) examined hydrogenase activity in both symbiont-bearing strain N and symbiont-free strain K by cytochemical staining and showed that hydrogenase activity was detected only in strain N, although both strains possessed methanogenic symbionts. If the detected hydrogenase activity means hydrogen production in strain N, it may be speculated that the bacterial symbionts may be involved in hydrogen removal from the ciliate cell. On the other hand, the reason for the lack of hydrogenase activity in strain K has not been clarified. If the lack of hydrogenase activity in strain K means no hydrogen production of the ciliate, under these conditions methanogenic symbionts should rely on other substrates such as formate, one of the fermentative products of *T. compressum* and a common substrate for many methanogens. The disposal of reducing equivalents in anaerobic protozoa may occur not only by interspecies hydrogen transfer but also by transfer of formate as proposed in methanogenic syntrophic consortia (Stams and Plugge 2009).

Besides hydrogen-scavenging, the proposed physiological roles of bacterial symbionts in anaerobic protozoa include amino acid synthesis (Hongoh et al. 2008), nitrogen fixation (Hongoh et al. 2009), and oxygen removal (Sato et al. 2009). Some of these functions were suggested from the genome sequence information of the symbionts. The recent advance of molecular techniques enables whole genome sequencing of uncultured microbes such as intracellular symbionts conducted by micromanipulation and pyrosequencing coupled with genome amplification using Phi29 DNA polymerase. Comparative genomics between the symbiont and its free-living relatives could highlight the physiological roles of the symbiont since the genes that are not used under symbiotic conditions tend to be eliminated from the symbiont genome due to the necessity of such genes (Moran et al. 2009). Thus, whole genome sequencing would be a powerful tool for elucidating the physiological role of the bacterial symbionts. Since the physiological role of the *T. compressum* bacterial symbionts remains unclear, the whole genome sequence of the bacterial symbionts is highly expected to be analyzed in the nearest future for a better understanding of the physiological basis of this symbiosis.

5 Perspectives

Symbiosis, intracellular symbiosis (endosymbiosis) in particular, drastically accelerates evolutional changes in organisms as the result of the conjugation between distinct living systems. This event has allowed organisms to adapt to environmental changes and expand their niches on the earth. It is evident that mitochondria and chloroplasts, which are believed to originate from bacterial symbiosis, enabled ancient eukaryotes to thrive in oxic environments and considerably increased their energy yield by exploiting the potential of oxygen respiration and photosynthesis. However, the detailed process of these symbiotic events and the following merging process (to becoming an organelle) have not been thoroughly clarified, as it is impossible to witness these events. Nevertheless, we fortunately may obtain important clues to understand the common basis of symbiotic events from the modern symbiotic associations as found in the protozoa. Particularly in anoxic environments, various limitations in yielding energy or certain nutrients are likely to promote the formation of various types of symbiosis to survive in such environments. Thus, the multiplex symbiosis found in *T. compressum* described in this review is an intriguing research model for studying the symbiotic interactions among bacteria, archaea and eukaryotes. However, nothing is known about this symbiosis and a variety of subjects remain to be elucidated, i.e., metabolism of the symbionts, interaction between the symbiont and host, evolutional history of the symbiosis, molecular mechanism of symbiosis formation, etc. Recent advances in sequencing technology and molecular methods enable us to analyse the whole genome sequence of the microorganisms involved in complex symbiotic consortia. The combined approaches using genomics and conventional physiological studies are expected to address the questions concerning the symbiotic associations found in *Trimyema* and to reveal an underlying common philosophy of symbiotic evolution.

References

Akhmanova A, Voncken F, van Alen T, van Hoek A, Boxma B, Vogels G, Veenhuis M, Hackstein JHP (1998) A hydrogenosome with a genome. Nature 396:527–528

Augustin H, Foissner W, Adam H (1987) Revision of the genera *Acineria*, *Trimyema* and *Trochiliopsis* (Protozoa, Ciliophora). Bull Br Mus nat Hist (Zool) 52:197–224

Baumgartner M, Stetter KO, Foissner W (2002) Morphological, small subunit rRNA, and physiological characterization of *Trimyema minutum* (Kahl, 1931), an anaerobic ciliate from submarine hydrothermal vents growing from 28°C to 52°C. J Eukaryot Microbiol 49:227–238

Boxma B, Voncken F, Jannink S, van Alen T, Akhmanova A, van Weelden SW, van Hellemond JJ, Ricard G, Huynen M, Tielens AG, Hackstein JHP (2004) The anaerobic chytridiomycete fungus *Piromyces* sp. E2 produces ethanol via pyruvate:formate lyase and an alcohol dehydrogenase E. Mol Microbiol 51:1389–1399

Boxma B, de Graaf RM, van der Staay GW, van Alen TA, Ricard G, Gabaldon T, van Hoek AH, Moon-van der Staay SY, Koopman WJ, van Hellemond JJ, Tielens AG, Friedrich T, Veenhuis M, Huynen MA, Hackstein JHP (2005) An anaerobic mitochondrion that produces hydrogen. Nature 434:74–79

Broers CA, Stumm CK, Vogels GD (1991) Axenic cultivation of the anaerobic free-living ciliate *Trimyema compressum*. J Protozool 38:507–511

Cho BC, Park JS, Xu K, Choi JK (2008) Morphology and molecular phylogeny of *Trimyema koreanum* n. sp., a ciliate from the hypersaline water of a solar saltern. J Eukaryot Microbiol 55:417–426

Curds CR (1977) Microbial interactions involving protozoa. In: Skinner FA, Shewan JN (eds) Aquatic microbiology. Academic, New York, pp 69–105

Doddema HJ, Vogels GD (1978) Improved identification of methanogenic bacteria by fluorescence microscopy. Appl Environ Microbiol 36:752–754

Ellis JE, McIntyre PS, Saleh M, Williams AG, Lloyd D (1991a) Influence of CO_2 and low concentrations of O_2 on fermentative metabolism of the rumen ciliate *Dasytricha ruminantium*. J Gen Microbiol 137:1409–1417

Ellis JE, McIntyre PS, Saleh M, Williams AG, Lloyd D (1991b) Influence of CO_2 and low concentrations of O_2 on fermentative metabolism of the ruminal ciliate *Polyplastron multivesiculatum*. Appl Environ Microbiol 57:1400–1407

Ellis JE, Mcintyre PS, Saleh M, Williams AG, Lloyd D (1991c) The influence of ruminal concentrations of O_2 and CO_2 on fermentative metabolism of the rumen entodiniomorphid ciliate *Eudiplodinium maggii*. Curr Microbiol 23:245–251

Embley TM, Finlay BJ (1993) Systematic and morphological diversity of endosymbiotic methanogens in anaerobic ciliates. Antonie Leeuwenhoek 64:261–271

Embley TM, Finlay BJ (1994) The use of small subunit rRNA sequences to unravel the relationships between anaerobic ciliates and their methanogen endosymbionts. Microbiology 140:225–235

Embley TM, Finlay BJ, Thomas RH, Dyal PL (1992) The use of rRNA sequences and fluorescent probes to investigate the phylogenetic positions of the anaerobic ciliate *Metopus palaeformis* and its archaeobacterial endosymbiont. J Gen Microbiol 138:1479–1487

Esteban G, Guhl BE, Clarke KJ, Embley TM, Finlay BJ (1993) *Cyclidium porcatum* n. sp.: a free-living anaerobic scuticociliate containing a stable complex of hydrogenosomes, eubacteria and archaeobacteria. Eur J Protistol 29:262–270

Fenchel T (1980) Suspension feeding in ciliated protozoa: functional response and particle size selection. Microb Ecol 6:1–11

Fenchel T, Finlay BJ (1991) Endosymbiotic methanogenic bacteria in anaerobic ciliates: significance for the growth efficiency of the host. J Protozool 38:18–22

Finlay BJ, Embley TM, Fenchel T (1993) A new polymorphic methanogen, closely related to *Methanocorpusculum parvum*, living in stable symbiosis within the anaerobic ciliate *Trimyema* sp. J Gen Microbiol 139:371–378

Goosen NK, Horemans AMC, Hillebrand SJW, Stumm CK, Vogels GD (1988) Cultivation of the sapropelic ciliate *Plagiopyla nasuta* Stein and isolation of the endosymbiont *Methanobacterium formicicum*. Arch Microbiol 150:165–170

Goosen N, Wagener S, Stumm CK (1990a) A comparison of two strains of the anaerobic ciliate *Trimyema compressum*. Arch Microbiol 153:187–192

Goosen NK, van der Drift C, Stumm CK, Vogels CD (1990b) End products of metabolism in the anaerobic ciliate *Trimyema compressum*. FEMS Microbiol Lett 69:171–176

Hackstein JHP, Vogels GD (1997) Endosymbiotic interactions in anaerobic protozoa. Antonie Leeuwenhoek 71:151–158

Hackstein JHP, de Graaf RM, van Hellemond JJ, Tielens AGM (2008a) Hydrogenosomes of anaerobic ciliates. In: Tachezy J (ed) Hydrogenosomes and mitosomes: mitochondria of anaerobic eukaryotes. Springer, Heidelberg, pp 97–112

Hackstein JHP, Baker SE, van Hellemond JJ, Tielens AGM (2008b) Hydrogenosomes of anaerobic chytrids: an alternative way to adapt to anaerobic environments. In: Tachezy J (ed) Hydrogenosomes and mitosomes: mitochondria of anaerobic eukaryotes. Springer, Heidelberg, pp 148–162

Holler S, Pfennig N (1991) Fermentation products of the anaerobic ciliate *Trimyema compressum* in monoxenic cultures. Arch Microbiol 156:327–334

Hongoh Y, Sharma VK, Prakash T, Noda S, Taylor TD, Kudo T, Sakaki Y, Toyoda A, Hattori M, Ohkuma M (2008) Complete genome of the uncultured Termite Group 1 bacteria in a single host protist cell. Proc Natl Acad Sci USA 105:5555–5560

Hongoh Y, Sharma VK, Prakash T, Noda S, Toh H, Taylor TD, Kudo T, Sakaki Y, Toyoda A, Hattori M, Ohkuma M (2009) Genome of an endosymbiont coupling N_2 fixation to cellulolysis within protist cells in termite gut. Science 322:1108–1109

Lackey JB (1925) Studies on the biology of sewage disposal. N J Agric Exp Sta Bull 417:1–39

Lin C, Miller TL (1998) Phylogenetic analysis of *Methanobrevibacter* isolated from feces of humans and other animals. Arch Microbiol 169:397–403

Liu XY, Shi M, Liao YH, Gao Y, Zhang Z, Wen DH, Wu WZ, An CC (2006) Feeding characteristics of an amoeba (Lobosea: Naegleria) grazing upon cyanobacteria: food selection, ingestion and digestion progress. Microb Ecol 51:315–325

Moran NA, McLaughlin HJ, Sorek R (2009) The dynamics and time scale of ongoing genomic erosion in symbiotic bacteria. Science 323:379–382

Müller M (1993) The hydrogenosome. J Gen Microbiol 139:2879–2889

Murase J, Frenzel P (2008) Selective grazing of methanotrophs by protozoa in a rice field soil. FEMS Microbiol Ecol 65:408–414

Nerad TA, Schaffer SA, Small EB, Mangold LA (1995) *Trimyema shoalsia* sp. n., an anaerobic, microaerotolerant marine ciliate from Appledore Island, Gulf of Maine, USA. Acta Protozool 34:289–298

Nes WR, McKean ML (1977) Biochemistry of steroids and other isopentenoids. University Park Press, Baltimore

Rohmer M, Bouvier P, Ourisson G (1979) Molecular evolution of biomembranes: structural equivalents and phylogenetic precursors of sterols. Proc Natl Acad Sci USA 76:847–851

Sato T, Hongoh Y, Noda S, Hattori S, Ui S, Ohkuma M (2009) Candidatus *Desulfovibrio trichonymphae*, a novel intracellular symbiont of the flagellate *Trichonympha agilis* in termite gut. Environ Microbiol 11:1007–1015

Schulz S, Wagener S, Pfennig N (1990) Utilization of various chemotrophic and phototrophic bacteria as food by the anaerobic ciliate *Trimyema compressum*. Eur J Protistol 26:122–131

Shinzato N, Watanabe I, Meng XY, Sekiguchi Y, Tamaki H, Matsui T, Kamagata Y (2007) Phylogenetic analysis and fluorescence in situ hybridization detection of archaeal and bacterial endosymbionts in the anaerobic ciliate *Trimyema compressum*. Microb Ecol 54:627–636

Small EB (1973) A study of ciliate protozoa from a small polluted stream in east-central Illinois. Am Zool 18:225–230

Stams AJ (1994) Metabolic interactions between anaerobic bacteria in methanogenic environments. Antonie Leeuwenhoek 66:271–294

Stams AJ, Plugge CM (2009) Electron transfer in syntrophic communities of anaerobic bacteria and archaea. Nat Rev Microbiol 7:568–577

van Bruggen JJA, Stumm CK, Vogels GD (1983) Symbiosis of methanogenic bacteria and sapropelic protozoa. Arch Microbiol 136:89–95

van Bruggen JJA, Zwart KB, van Assema RM, Stumm CK, Vogels GD (1984) *Methanobacterium formicicum*, an endosymbiont of the anaerobic ciliate *Metopus striatus* McMurrich. Arch Microbiol 139:1–7

van Bruggen JJA, Zwart KB, Hermans JGE, van Hove EM, Stumm CK, Vogels GD (1986) Isolation and characterisation of *Methanoplanus endosymbiosus* sp. nov. an endosymbiont of the marine sapropelic ciliate *Metopus contortus* Quennerstedt. Arch Microbiol 144:367–374

van Bruggen JJA, van Rens GLM, Geertman EJM, van Hove EM, Stumm CK (1988) Isolation of a methanogenic endosymbiont of the sapropelic ameba *Pelomyxa palustris* Greeff. J Protozool 35:20–23

van Hoek AHAM, Akhmanova AS, Huynen M, Hackstein JHP (2000a) A mitochondrial ancestry of the hydrogenosomes of *Nyctotherus ovalis*. Mol Biol Evol 17(1):202–206

van Hoek AH, van Alen TA, Sprakel VS, Leunissen JA, Brigge T, Vogels GD, Hackstein JH (2000b) Multiple acquisition of methanogenic archaeal symbionts by anaerobic ciliates. Mol Biol Evol 17:251–258

Wagener S, Pfennig N (1987) Monoxenic culture of the anaerobic ciliate *Trimyema compressum* Lackey. Arch Microbiol 149:4–11

Wagener S, Bardele CF, Pfennig N (1990) Functional integration of *Methanobacterium formicicum* into the anaerobic ciliate *Trimyema compressum*. Arch Microbiol 153:496–501

Yamada K, Kamagata Y, Nakamura K, Inamori Y, Nakamura I (1994) Selectivity of food bacteria for the growth of anaerobic ciliate *Trimyema compressum*. Arch Microbiol 161:229–233

Yamada K, Kamagata Y, Nakamura K (1997) The effect of endosymbiotic methanogens on the growth and metabolic profile of the anaerobic free-living ciliate *Trimyema compressum*. FEMS Microbiol Lett 149:129–132

Yarlett N, Hann AC, Lloyd D, Williams A (1981) Hydrogenosomes in the rumen protozoon *Dasytricha ruminantium* Schuberg. Biochem J 200:365–372

Yarlett N, Lloyd D, Williams AG (1982) Respiration of the rumen ciliate *Dasytricha ruminantium* Schuberg. Biochem J 206:259–266

Yarlett N, Lloyd D, Williams AG (1985) Butyrate formation from glucose by the rumen protozoon *Dasytricha ruminantium*. Biochem J 228:187–192

Zwart KB, Goosen NK, van Schijndel MW, Broers CAM, Stumm CK, Vogels GD (1988) Cytochemical localization of hydrogenase activity in the anaerobic protozoa *Trichomonas vaginalis*, *Plagiopyla nasuta*, and *Trimyema compressum*. J Gen Microbiol 134:2165–2170