

Free-Living Protozoa with Endosymbiotic Methanogens

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Abstract Methanogenic bacteria occur in many, but not all free-living obligate anaerobic protozoa. This sort of symbiosis is especially common among anaerobic ciliates, but is also found in a few species of amoebae and flagellates. Protozoa harbouring methanogens have a clostridium-type fermentative metabolism with H₂ as metabolite, the hydrogen generation taking place in special organelles, so called hydrogenosomes. The relation between the host cells and their endosymbiotic methanogens is syntrophic hydrogen transfer. By removing the generated H₂, the methanogens stimulate host H₂-production, thus increasing the energetic yield of the energy metabolism. This sort of symbiosis has evolved independently in many cases and involves representatives of several major groups of methanogenic bacteria. Symbiotic methanogenesis of free-living anaerobic protozoa plays only a modest quantitative role in terms of CH₄-production in most habitats.

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1 Discovery

It was discovered earlier that sulphidic aquatic habitats rich in decaying organic matter – so called sapropel – harbour special and characteristic protozoan biota (Lauterborn 1901), and throughout the twentieth century a number of such sapropelic protozoa were described including flagellates, amoeboid organisms and, not least, ciliates from such habitats including stratified water columns with an anaerobic hypolimnion, aquatic sediments beneath a certain depth, accumulations of sulphur bacteria and sewage tanks. It later became clear that what characterises these habitats is primarily absence of oxygen. Later, it was demonstrated that many of these protozoa are true anaerobes in that they lack cytochrome oxidase (Fenchel et al. 1977) and in general they are sensitive to the presence of oxygen. Through motile chemosensory behaviour, they even avoid trace concentrations of O_2 . The ciliates in particular are capable of O_2 -uptake that is not coupled to energy conservation, but it allows the ciliates to maintain intracellular anaerobic conditions (Fenchel and Finlay 1990b). This is shown by the fact that low O_2 -tension in the environment (up to 3–4% atmospheric saturation) does not entirely block methane production of the symbiotic methanogens (Fenchel and Finlay 1992). It was also found that anaerobic ciliates were characterised by the presence of ectosymbiotic or endosymbiotic bacteria and sometimes both (Fenchel et al. 1977). It was later demonstrated that the endosymbiotic bacteria are methanogens – which is evident by their blue fluorescence in violet light due to the presence of the coenzyme F_{420} (van Bruggen et al. 1983, Fig. 1a), and later CH_4 -production by these symbiotic consortia could be demonstrated directly (e.g., van Bruggen et al. 1986). Altogether some 40 species of anaerobic free-living ciliates are known to harbour methanogenic bacteria (Fenchel and Finlay 1991c).

The ectosymbiotic bacteria are never methanogens. They occur only in marine species, except for some anaerobic ciliates collected in a sulphate-rich solution lake (Fenchel and Finlay 1995). In two cases (the anaerobic ciliates *Metopus contortus* and *Caenomorpha levanderi*), it has been shown that the ectosymbionts are sulphate reducers and this is also likely to be the case for other ectosymbionts of marine anaerobic ciliates (Fenchel and Ramsing 1992). They probably serve the same purpose for the host cells as do the methanogens, that is, to consume hydrogen which is produced as a metabolite of the host's fermentative metabolism.

An organelle originally found in the anaerobic parasitic flagellate *Trichomonas* (Müller 1980) was named the 'hydrogenosome'. The function of this organelle is in principle to ferment pyruvate into acetate and H_2 , thus enhancing energy yield from fermentation. Hydrogenosomes have since been shown to be widely distributed and they occur in most groups of anaerobic protozoa and also in some chytrids (see Hackstein and Tielens 2010). With respect to the fermentative pathways, there is some variation among different groups. There is now evidence to show that hydrogenosomes derive from mitochondria although how certain enzymatic components such as hydrogenase were incorporated in them remains an open question (Akhmanova et al. 1998; Biagini et al. 1997; Embley and Martin

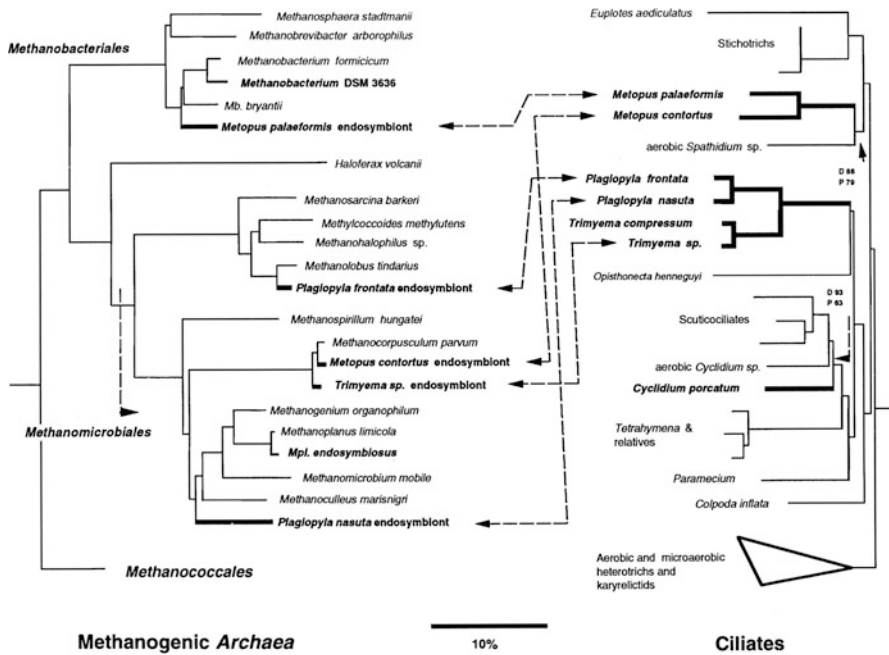


Fig. 1 Phylogenetic trees based on SSU rRNA for ciliates and methanogens. Anaerobic ciliates with hydrogenosomes and methanogens that occur as endosymbionts are printed with bold characters. From Embley and Finlay (1994)

1998; Finlay and Fenchel 1989; Hackstein et al. 1999). The fact that in many cases related sister groups of protozoa may include both aerobic forms with normal mitochondria and anaerobes with hydrogenosomes indicate that they have evolved independently within many groups of protozoa (e.g., Embley and Finlay 1994). The presence of hydrogenosomes and methanogens has now also been established among different anaerobic protozoan symbionts in animals (see Hongoh and Ohkuma 2010 and Ushida 2010).

Fermentation involving hydrogen production is thermodynamically feasible only if the external hydrogen tension does not exceed a certain level and if the presence of methanogens in anaerobic protozoa is understood as syntrophic hydrogen transfer (see Worm et al. 2010).

2 Distribution

Among protozoan groups that have anaerobic representatives, the ciliates have been studied in most detail. Following the most recent systematics of ciliates (Lynn 2008), free-living anaerobic representatives are found in at least eight orders and

among them six have representatives with methanogenic symbionts. Three of these orders apparently include only anaerobes: Armophorida (e.g., *Metopus*, *Caenomorphia*), Plagiopylida (*Plagiopyla*, *Sonderia*, *Trimyema*) and Odonstomatida (e.g., *Myelostoma*, *Saprodinium*). Within five other orders, anaerobes are sister groups with aerobes or in some cases (*Cyclidium* and other genera within Pleurostomatida and *Lacrymaria* within Haptorida) the genera include both aerobic forms and anaerobes with hydrogenosomes and symbiotic methanogens (Esteban and Finlay 1994; Esteban et al. 1993; Fenchel and Finlay 1995). This taxonomic diversity of ciliates with methanogenic symbionts is further increased if some symbiotic ciliates are included such as the intestinal commensal ciliate *Nyctotherus* and ciliates of the rumen (see Ushida 2010). Sequencing of rRNA genes provides an evolutionary tree that is rather consistent with ciliate taxonomy based on morphological criteria and also shows that adaptations to anaerobic life including hydrogenosomes have evolved independently within different groups (Embley and Finlay 1994; Hackstein and Tielens 2010).

Several otherwise aerobic ciliates belonging to different taxonomic groups are capable of slow balanced growth under strict anaerobic conditions (Bernard and Fenchel 1996). It is therefore not particularly strange that strict anaerobes have evolved independently within different groups.

A phylogenetic tree for the symbiotic methanogens also indicates that the association between the methanogenic symbionts and their anaerobic hosts has evolved independently on several occasions (Embley and Finlay 1994; Fig. 1). The symbionts belong to different cardinal groups of methanogens, but they are not identical to any sequenced species of free-living methanogens. Even within ciliate genera of anaerobic ciliates (*Metopus* and *Plagiopyla*), different species may harbour methanogens belonging to different major groups. The results of rRNA-gene sequencing of symbiotic methanogens of *Metopus contortus* and *Plagiopyla nasuta* are at variance with earlier claims that their methanogenic symbionts are 'opportunistic symbionts', that is, otherwise free-living methanogens, and that they have been isolated into pure cultures (van Bruggen et al. 1986; Goosen et al. 1988).

Among other groups of protozoa, the amoebae *Pelomyxa* and *Mastigella* harbour methanogenic symbionts (van Bruggen et al. 1985, 1988). This is strange in that these organisms do not have hydrogenosomes. *Pelomyxa* seems to have more types of endosymbiotic bacteria, of which one is not a methanogen. It has been speculated that this organism is responsible for producing the necessary H_2 on the basis of fermentative metabolites of the amoeba so that the protozoan–bacteria consortium should represent a three-step food chain, but this warrants closer investigation.

While there are several free-living anaerobic flagellates, there is only one example, the genus *Psalteriomonas*, of a flagellate that has hydrogenosomes and harbours symbiotic methanogens. The genus includes two species and belongs to the family Vahlkampfiidae; the species occur in eutrophic ponds (Broers et al. 1993; van Bruggen et al. 1988). The symbiotic flagellates in the termite gut also possess symbiotic methanogens.

3 Morphology and Life Cycles

There is variation with respect to morphology and behaviour among the symbiotic methanogens, but it is a common theme that they tend to remain in close contact or even attached to hydrogenosomes (Fig. 2b–f) and that they are not enclosed in a membrane-covered vacuole. In the limnic *Metopus palaeformis*, the symbiotic methanogens, 300–400 per host cell, appear as long rods that are mainly found in the vicinity, but not attached to the hydrogenosomes (Finlay and Fenchel 1991). In the marine *Metopus contortus*, there are 6,000–10,000 methanogens per host cell. They appear to undergo a polymorphic life cycle and they seem to start off as ordinary short rods with a typical bacterial cell wall. When they make contact with a hydrogenosome, the cell wall is at least partially lost. The cells become larger and attain an irregular shape (Fig. 2b). Sequencing of rRNA genes shows that there is only a single species of methanogens related to the genus *Methanocorpusculum* (Finlay and Fenchel 1991; Embley et al. 1992). A related species occurs as the symbiont of a *Trimyema* sp. It has a similar life cycle, and the irregularly shaped bacteria eventually become embedded in aggregations of hydrogenosomes (Fig. 2f; Finlay et al. 1993). A similar arrangement of hydrogenosomes and methanogens is found in the anaerobic ciliate *Cyclidium porcatum* (Fig. 2e; Esteban et al. 1993).

The most intimate relation between hydrogenosomes and methanogens is found in the marine ciliate *Plagiopyla frontata* (Fenchel and Finlay 1991b; Fig. 2c, d).

The hydrogenosomes and the methanogens are both disk shaped and they are arranged like a stack of coins with alternating methanogens and hydrogenosomes; the stacks are capped with hydrogenosomes at either end. There are altogether about 3,500 methanogens and a similar number of hydrogenosomes per *Plagiopyla* cell during the entire growth phase of the ciliate (generation time 35–36 h). Prior to the division of the host cells, the hydrogenosomes divide, followed by a division of the methanogens, so that the aggregates double, but they retain the characteristic arrangement of hydrogenosomes and symbionts.

In these and other examples, it is obvious that there is always a close physical contact between hydrogenosomes and the symbiont cells.

4 Significance of the Association

The compound 2-bromoethanesulfonic acid (BES) is a specific inhibitor of methanogenesis (Oremland and Capone 1988). When applied to ciliates with methanogens, methane evolution stops immediately. The fluorescence of the methanogens is not affected, but the bacteria no longer divide, and so their number is halved for every subsequent division of the host cells. After eight cell divisions, the host cells are aposymbiotic and the methanogens do not recover when the ciliates are transferred to a medium without BES. It has also proven impossible to re-infect the ciliates with water from the sampling locality or culture fluid from non-treated

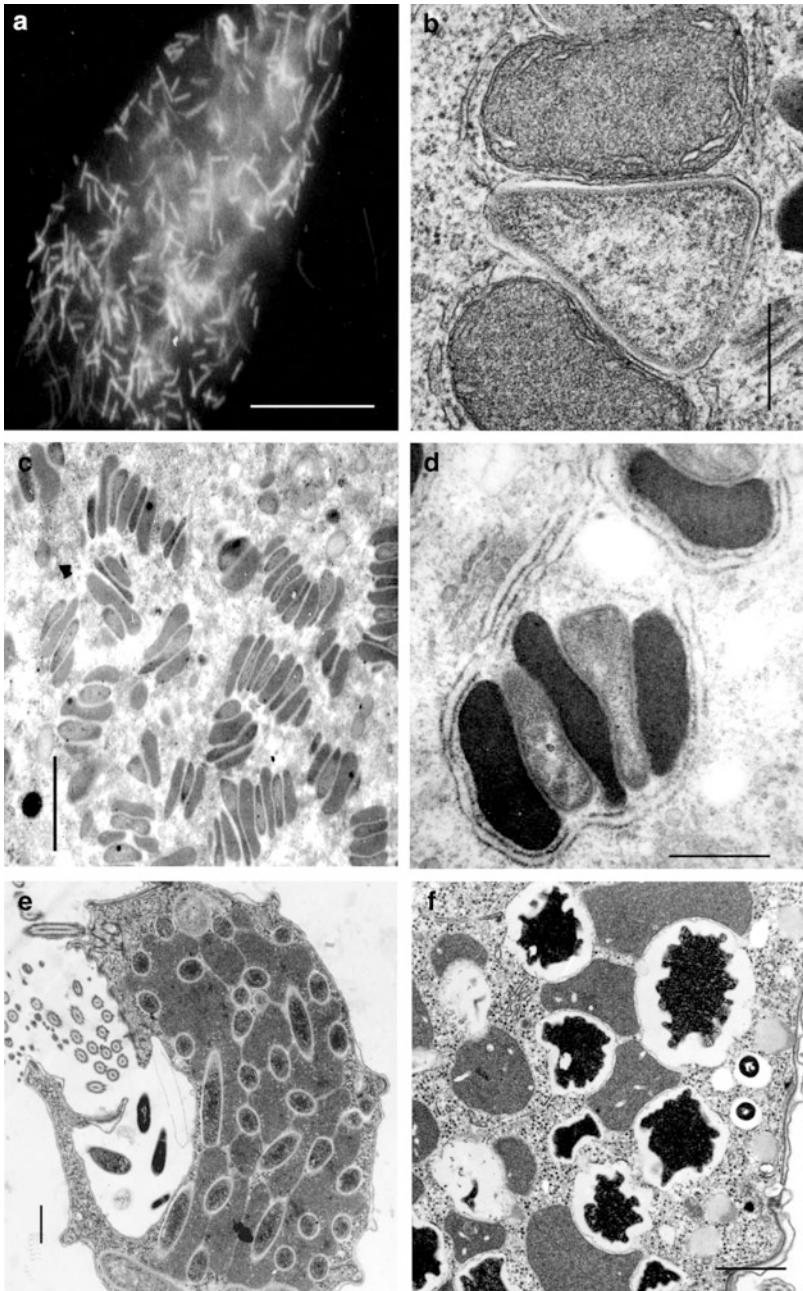


Fig. 2 (a) Fluorescence of methanogens in *Metopus palaeformis*; scale bar: 10 μm . (b) A methanogen sandwiched between two hydrogenosomes in *Metopus contortus*; scale bar: 0.5 μm . (c, d) Stacks of alternating hydrogenosomes (darker) and methanogens in *Plagiopyla frontata*; scale bars 5 and 0.5 μm , respectively. (e) Complex of methanogens and hydrogenosomes in *Cyclidium porcatum*; scale bar: 0.5 μm . (f) Irregularly shaped methanogens in vacuoles surrounded by hydrogenosomes in *Trimyema* sp.; scale bar: 0.5 μm

cultures filtered through a 5 μm filter – nor from extract of homogenised ciliates with intact methanogens. The aposymbiotic cells survive and grow indefinitely, but they seem to have lost the capability to attain methanogenic symbionts again (Fenchel and Finlay 1991a). Taking into consideration (1) that methanogenic symbionts do not apparently occur as free living, (2) that they have many special adaptations to life as endosymbionts, and (3) that aposymbiotic ciliates apparently cannot be re-infected with methanogens, it seems to indicate that the endosymbiotic methanogens have approached the status of organelles.

When BES is added to cultures of growing *Metopus contortus* or *Plagiopyla frontata*, the exponential growth rate constant immediately decreases to about 70% of the previous value. Aposymbiotic cells that have been kept without BES also grow with a growth rate constant that is 70% of that of cells with active methanogens and the growth yield is also about 70% of that of normal cells. However, in similar experiments with *Metopus palaeformis*, BES did not seem to affect the growth rate constant significantly.

5 Intracellular H₂-Tension and Methanogens

The production of methane by the ciliates is closely coupled to their growth rate. In *Plagiopyla frontata*, CH₄-production is about 4.5 pmol per cell h⁻¹ during exponential growth. This figure decreases to about half that value during the last two cell divisions in batch cultures and during the stationary phase it drops to the detection limit after about 100 h (Fenchel and Finlay 1991b).

The CH₄-production rate must be a measure of the H₂-production of the hydrogenosomes: it takes four H₂ to produce one CH₄. Measuring CH₄- and H₂-production of *Plagiopyla frontata* and *Metopus contortus* simultaneously showed that some hydrogen (about 5%) is not consumed by the methanogens, but diffuses out of the ciliates. Measuring H₂-production of aposymbiotic (previously BES-treated) cells could not, however, account for the CH₄-production of cells with active methanogens: in *Metopus* the measured H₂-production could account for about 70% of the CH₄-production of normal cells and in *Plagiopyla* the corresponding figure was only about 45%. It is possible that some of the reduction equivalents produced by the hydrogenosomes is in the form of formate as has been shown for the anaerobic ciliate *Trimyema* (Goosen et al. 1990; Holler and Pfennig 1991). This was not tested in Fenchel and Finlay (1991b), but it is likely that in the absence of methanogens, H₂-tension will build up in the ciliates and thus inhibit H₂-production in the hydrogenosomes which will instead excrete more reduced end products than acetate, such as lactate or propionate, and that the significance of the association between the host cells and their methanogenic symbionts is one of syntrophic H₂-transfer (Worm et al. 2010).

This is supported by simple calculations of the concentration of H₂ in a spherical cell in the absence of methanogens so that H₂ is lost only through diffusion. Using parameter values for a *Plagiopyla* cell, that is, its volume, its H₂-production rate

under exponential growth and the diffusion coefficient and solubility of H_2 in water, it could be shown that in the absence of methanogens, the H_2 -tension would increase to about 1.3 kPa – a value around a thousand fold higher than the ambient H_2 -tension and this is a H_2 -tension that would be inhibitory to fermentative processes involving H_2 -release (Fenchel and Finlay 1992, 1995).

6 Symbiotic Consortia as Natural Chemostats

It was noted that the volume fraction of methanogens in host cells is remarkably constant when comparing different species and in individual species under different growth conditions, that is, around 2%. This can be explained by describing the symbiotic consortium as a kind of chemostat (Finlay and Fenchel 1992, Fenchel and Finlay 1995).

It is assumed that the growth rate of the symbiont is dependent only on the H_2 -production of the host and also that the cells are 'diluted' due to the growth, that is, increase in cell volume of the host, which is also coupled to H_2 -production. The system deviates from a real chemostat, in that some H_2 is not diluted at the same rate as the bacteria, but is lost through diffusion across the cell surface of the host cells. Cell yield of methanogens (in terms of dry weight production per unit CH_4 produced) and maximum growth rate constants for methanogens were taken from the literature. Applying the model to data on *Plagiopyla frontata* and its methanogen symbionts predicted realistic values for the volume fraction of methanogens and also showed that over a rather wide range of host growth rates (up to 80% of the maximum growth rates for the methanogens), the volume fraction constituted by the symbionts is relatively stable (Fenchel and Finlay 1995).

An interesting aspect of the model is that it shows an association between host cells and an intracellular bacterium that is solely dependent on some host metabolite that can instantaneously become stable, and the generation time of the symbionts becomes identical to that of the host cell. As in a real chemostat, the bacteria will increase in number until competition for the substrate lowers their growth rate constant to become identical of that of the host. It is, therefore, not so difficult to imagine the origin of such associations. Once a bacterial cell has somehow evaded a food vacuole, it can grow and multiply in the cytoplasm of its future host on the basis of a host metabolite, then the association will be stable.

7 The Role of Symbiotic Methanogenesis in Natural Habitats

It can be asked what is the quantitative role of symbiotic methanogenesis in natural systems. Some theoretical consideration would suggest that in the case of anaerobic freshwater systems, this role is small. In such systems, in the absence of sulphate, the terminal mineralisation process is methanogenesis. Anaerobic protozoa are

phagotrophs and they have low growth efficiencies in comparison to aerobic phagotrophs – and consequently the biomass relative to the biomass of their food bacteria is low (Fenchel and Finlay 1990a). It was calculated that in such methanogenic systems, symbiotic methanogenesis could at most contribute about 3% of the produced methane (Fenchel 1993). This was demonstrated directly for lake sediments where it was found that the methanogenesis of anaerobic ciliates was negligible compared to methanogenesis caused by free-living bacteria (van Hoek et al. 2006).

The situation may be different in marine habitats. Seawater has a high content of sulphate, so the dominating terminal mineralisation process under anaerobic conditions is sulphate reduction. In a sense, the host cells can be considered as a refuge for methanogens in anaerobic, but sulphate-rich habitats. Otherwise, methanogenesis plays a significant role only when sulphate has been depleted and this happens only when there is a very high input of degradable organic matter or at considerable depths in sediments. In seawater, symbiotic methanogenesis could therefore potentially play a larger relative role. Fenchel (1993) tested this by measuring total methanogenesis and that of methanogenic ciliates for different marine shallow water habitats and at different depths in sediments. In sandy sediments, methanogenic ciliates contributed at the most 2–3% of the total CH₄-production. In masses of photosynthetic sulphur bacteria and especially in an accumulation of decaying sea grass leaves, higher values were found. In the latter case, where there were about 200 ciliates with methanogenic symbionts ml⁻¹ down to about 20-cm depth, symbiotic methanogenesis contributed to >80% of the total CH₄-production at one occasion, but in most cases it was around 20%. But this, first of all, reflects that the dominating terminal mineralisation process was sulphate reduction at this site.

In general, it can be concluded that symbiotic methanogenesis plays a modest role in a biogeochemical context – primarily because phagotrophy plays a modest quantitative role in anaerobic habitats.

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