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(Endo)symbiotic Methanogenic Archaea

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Preface

Methanogens are prokaryotic microorganisms that produce methane as an end-product of their metabolism. They are strictly anaerobic archaea belonging to the taxon Euryarchaeota. Methanogens occupy a wide variety of anaerobic environments, even extreme habitats characterized by high temperature, salinity, and extreme pH (Liu and Whitman 2008). More temperate habitats include marine and freshwater sediments, flooded soils, landfills, anaerobic digesters, geothermal systems, and the heartwood of trees. Notably, methanogens also thrive in the cytoplasm of anaerobic unicellular eukaryotes and in the gastrointestinal tracts of animals and humans. Frequently, they attach to the internal surfaces of the gastrointestinal tracts and of the protists living in intestinal environments with the aid of special adhesion-like proteins (Liu and Whitman 2008). In insect guts, methanogens have to cope with a very special environment, since they are exposed to a continuous influx of oxygen through the gut wall that challenges the strictly anaerobic symbionts. Notwithstanding that methanogens are very diverse, they can use only a very restricted number of substrates. They are unable to use organic substances (with the exception of acetate and formate), and consequently, methanogens must rely on CO_2 and methyl-group containing compounds and acetate, which are provided by the fermentations performed by complex anaerobic bacterial communities. The methanogenic substrates are predominantly metabolized with the aid of H_2 that is provided by syntrophic bacterial communities or, in the case of certain anaerobic protists, by the action of specialized mitochondrion-derived organelles, the hydrogenosomes. Since both hydrogenosomes and syntrophic bacterial communities rely on a low concentration of H_2, the interspecies hydrogen transfer that is mediated by methanogens is crucial for the proper functioning of hydrogenosomes and syntrophic anaerobic bacterial communities.

The synthesis of methane follows a complex biochemical pathway that is characterized by a number of unique coenzymes and membrane-bound enzyme complexes. It has been reviewed recently by Hedderich and Whitman (2006). CO_2 is reduced to methane by H_2 in hydrogenotrophic methanogens, which represent the majority of the methanogens living in symbiosis with protists and multicellular animals. The second type of substrate, methylgroup containing compounds

including methanol, methylated amines, and methylated sulfides, is used by methylotrophic methanogens, which are predominantly found in the gastrointestinal tracts of mammals and insects. The third type of substrate is acetate, which is metabolized by acetoclastic methanogens. Although only two genera (Methanosarcina and Methanosaeta) use acetate as substrate, as much as two-thirds of the biologically generated methane is derived from acetate. Notably, acetoclastic methanogens are rare among the symbiotic methanogens. This is not surprising since symbiotic acetoclastic methanogens compete with their hosts for acetate. Notwithstanding, an acetoclastic methanogen has recently been identified as endosymbiont in the free-living protist *Metopus es* (Narayanan et al. 2009).

Methanogens are abundant in habitats where electron acceptors such as O_2, NO_3^-, Fe^{3+}, and SO_4^{2-} are limiting (Liu and Whitman 2008). In such methanogenic habitats, complex organic matter is degraded to methane by the syntrophic action of different groups of anaerobic bacteria. Organic polymers are degraded initially by specialized bacteria to sugars, lactate, short-chain fatty acids, and alcohols. These products are further fermented by other bacteria to acetate, formate, H_2, and CO_2, which are the substrates used by methanogens. These methanogens catalyze the terminal step in the anaerobic food chain by converting the various methanogenic substrates to methane, which is released into the atmosphere. More than 70% of the annual global methane emission (ranging from 500 to 600 Tg CH_4/year) stem from biological methanogenesis and contribute significantly to global warming. (Whitman et al. 2006). The symbiotic methanogens in the gastrointestinal tract of ruminants and other “methanogenic” mammals contribute significantly to the global methane budget. Especially the rumen hosts an impressive diversity of methanogens, which have been studied using culture-independent 16S rRNA methods (Janssen and Kirs 2008; Wright et al. 2004). Insects, particularly termites, also host very complex methanogenic communities, but they release much less methane due to the concomitant oxidation of methane in the soil and the termite mounds. On the other hand, the contributions by the methanogenic endosymbionts of protists living in freshwater sediments can be neglected (van Hoek et al. 2006).

This monograph deals with methanogenic endosymbionts of free-living and symbiotic protists, episymbionts of rumen ciliates, methanogenic endosymbionts of ciliates, and termite flagellates, which are accompanied by bacterial endosymbionts, and methanogens in the gastrointestinal tract of vertebrates and arthropods. One review summarizes our knowledge about the genomic consequences of living together in symbiotic associations; another review discusses the role of methanogens in syntrophic degradation. Finally, the current state of information about hydrogenosomes has been reviewed.

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Free-Living Protozoa with Endosymbiotic Methanogens

Tom Fenchel and Bland J. Finlay

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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 H_2 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 H_2, the methanogens stimulate host H_2-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 CH_4-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

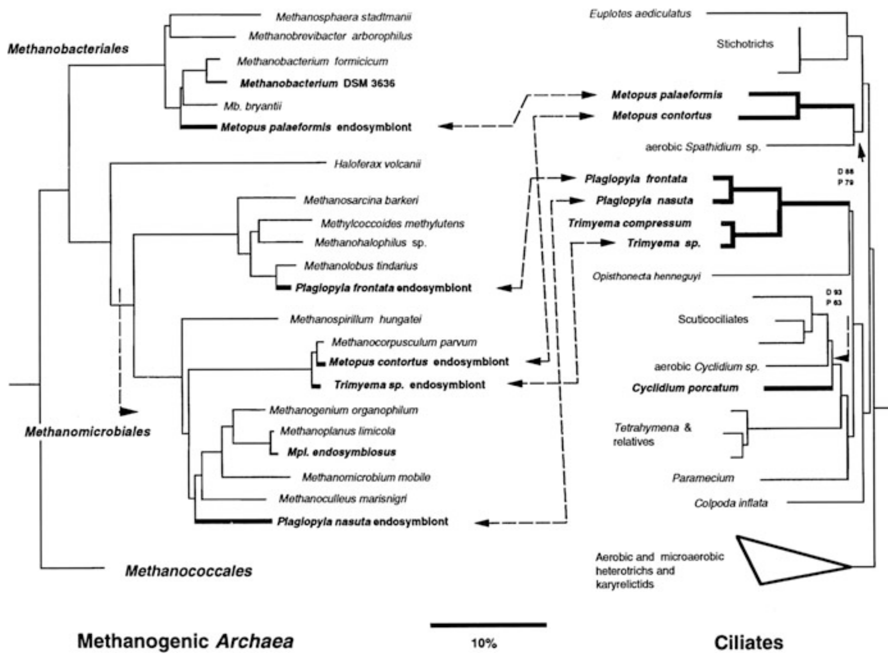

Fig. 1 Phylogenetic trees based on SSU rRNA for ciliates and methanogens. Anaerobic ciliates with hydrogenosomes and methanogens that occur as endosymbionts are printed with bold characters. From Embley and Finlay (1994)

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*, *Caenomorpha*), 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

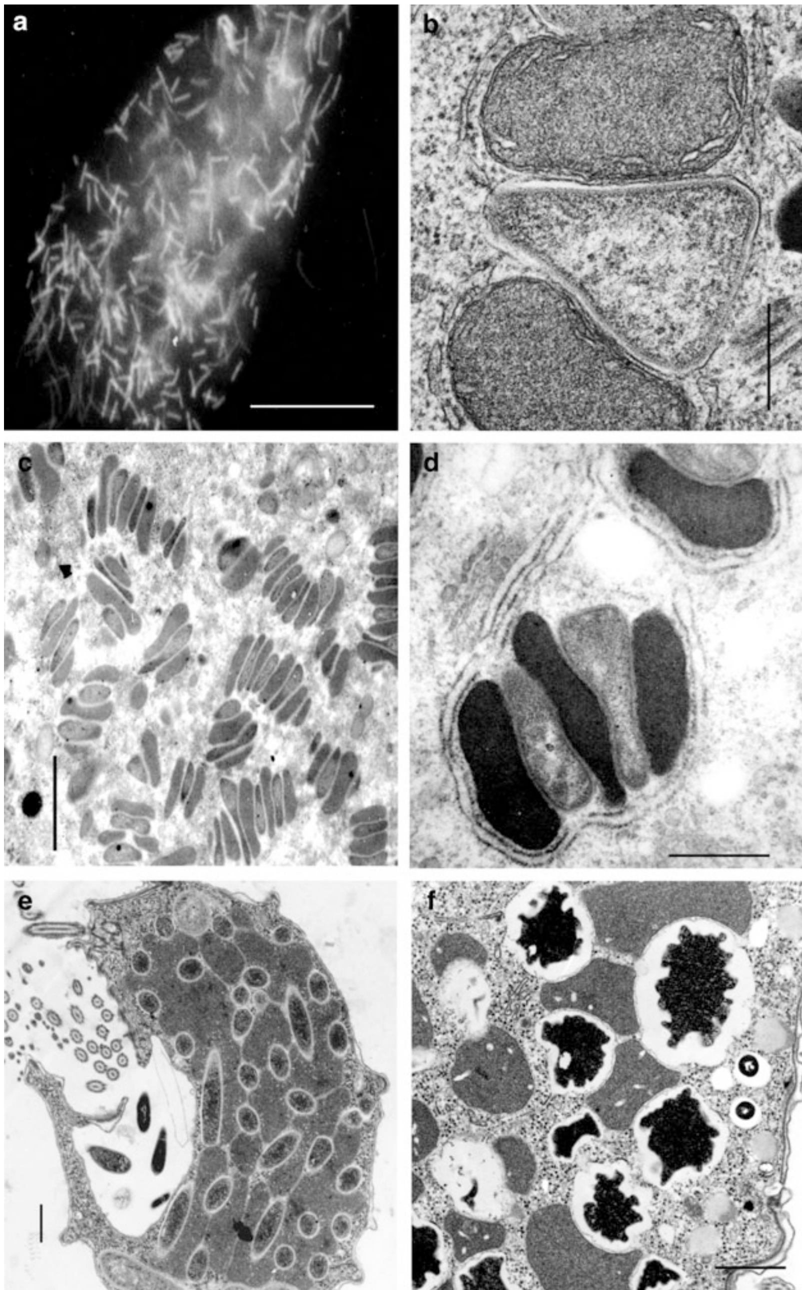

Fig. 2 (**a**) Fluorescence of methanogens in *Metopus palaeformis*; scale bar: 10 μm. (**b**) A methanogen sandwiched between two hydrogenosomes in *Metopus contortus*; scale bar: 0.5 μm. (**c**, **d**) Stacks of alternating hydrogenosomes (*darker*) and methanogens in *Plagiopyla frontata*; scale bars 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 H_2-Tension and Methanogens

The production of methane by the ciliates is closely coupled to their growth rate. In *Plagiopyla frontata*, CH_4-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 CH_4-production rate must be a measure of the H_2-production of the hydrogenosomes: it takes four H_2 to produce one CH_4. Measuring CH_4- and H_2-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 H_2-production of aposymbiotic (previously BES-treated) cells could not, however, account for the CH_4-production of cells with active methanogens: in *Metopus* the measured H_2-production could account for about 70% of the CH_4-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, H_2-tension will build up in the ciliates and thus inhibit H_2-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 H_2-transfer (Worm et al. 2010).

This is supported by simple calculations of the concentration of H_2 in a spherical cell in the absence of methanogens so that H_2 is lost only through diffusion. Using parameter values for a *Plagiopyla* cell, that is, its volume, its H_2-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 CH_4-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 CH_4-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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Anaerobic Ciliates and Their Methanogenic Endosymbionts

Johannes H. P. Hackstein

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Abstract Many anaerobic ciliates possess hydrogenosomes, and consequently, they have the potential to host endosymbiotic methanogens. The endosymbiotic methanogens are vertically transmitted and even the cyst stages carry methanogens. Accordingly, the analysis of the SSU rRNA genes of ciliates and their methanogenic endosymbionts revealed that the endosymbionts are specific for their hosts and not identical with free-living methanogens. Notably, the endosymbionts of a monophyletic group of ciliates that thrive in either freshwater environments or intestinal tracts are substantially different. Ciliates from freshwater sediments host methanogens belonging to the Methanomicrobiales, while ciliates thriving in the intestinal tracts of cockroaches, millipedes and frogs host methanogens that belong to the Methanobacteriales. Comparative analysis of free-living and gut-dwelling ciliates and their corresponding endosymbionts reveals only a limited co-evolution suggesting infrequent endosymbiont replacements. Such an endosymbiont replacement is clearly the reason for the very distant endosymbionts of free-living and gut-dwelling ciliates: the endosymbionts are related to the methanogens in the particular environments, in which the hosts live.

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1 Introduction

Anaerobic protists with hydrogenosomes have the potential to host endo- or episymbiotic methanogens (Hackstein et al. 2002; Hackstein and Tielens 2010; Fenchel and Finlay 2010; Ushida 2010). Anaerobic ciliates, in particular, are well known to have evolved hydrogenosomes repeatedly (7 out of 22 ciliate taxa, see Fenchel and Finlay 1995), and all of them seem to host endosymbiotic methanogens (Hackstein et al. 2002; Fig. 1). A few methanogenic endosymbionts have been isolated and cultured in vitro (van Bruggen et al. 1984, 1986; Goosen et al. 1988): these endosymbionts were found to be similar to free-living methanogens such as for example *Methanocorpusculum parvum* or *Methanobacterium formicicum*. However, the culturing techniques did not allow to decide whether the endosymbionts were specific for their hosts or identical with their free-living relatives. Analysis of the small subunit of the ribosomal genes (SSU rDNA) eventually revealed that the methanogenic endosymbionts were similar, but not identical to their free-living relatives (Embley and Finlay 1994; Embley et al. 1995; Fenchel and Finlay 1995, 2010). The endosymbionts belonged to different taxa of methanogens, and even the endosymbionts of closely related host species appeared to be very different. It was concluded that the observed symbioses were established several times independently, most likely along with the evolution of hydrogenosomes (Embley and Finlay 1994; Embley et al. 1995; Fenchel and Finlay 1995).

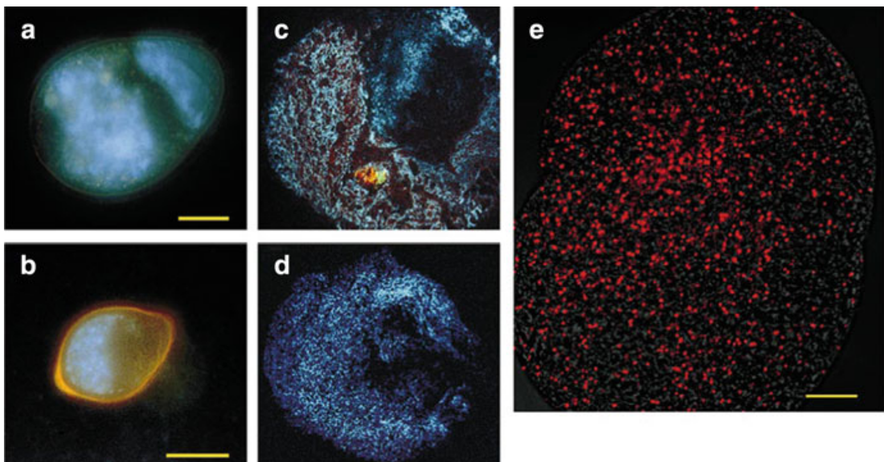

Fig. 1 Endosymbiotic methanogens of *Nyctotherus ovalis*. (**a–d**) F_{420} autofluorescence. (**e**) In situ hybridization. (**a**) *Nyctotherus ovalis* from *Blaberus* sp. var. Amsterdam. (**b**) Cyst of *N. ovalis* from the same isolate. (**c**) Squash preparation of *N. ovalis* from *Blaberus* sp. var. Amsterdam. (**d**) Squash preparation of *N. ovalis* from *Periplaneta americana* var. Amsterdam; note the rod shape of the methanogens. (**e**) part of *N. ovalis* from *Blaberus* sp. var. Nijmegen; in situ hybridization with a probe specific for methanogenic archaea, labelled with Cy5. Confocal LSM. Bars indicate 25 μm in (**a**), (**c**), and (**d**), 20 μm in (**b**), and 10 μm in (**e**). Reproduced with permission by Oxford University Press from van Hoek et al. (2000)

The major conclusion was that the endosymbionts were specific for the particular host species and not representatives of opportunistic methanogens that could thrive in both aquatic and intracellular environments.

2 Methanogenic Endosymbionts Are Transmitted Vertically

This fits well with the observation that the methanogenic endosymbionts are "vertically" transmitted: at mitosis, the endosymbionts are distributed to the daughter cells and even at encystation the endosymbionts are retained (van Hoek et al. 2000; Fig. 1b). The behaviour of the endosymbionts at conjugation has not been studied to my knowledge, but it is likely that both exconjugants possess endosymbionts. If a species is known to host methanogenic endosymbionts, all members of an uncultivated population possess these endosymbionts. However, there are several reports that ciliates kept in culture tend to lose their endosymbionts (Shinzato and Kamagata 2010). Certain strains of cultured ciliates lost their endosymbionts completely after some time, while other strains belonging to the same species retained the symbionts. Interestingly, Wagener et al. (1990) succeeded to re-infect such a symbiont-free strain of *Trimyema compressum* with *M. formicicum.* This experiment revealed that *M. formicicum* can be regarded as an opportunistic methanogen that can be taken up by a symbiont-free ciliate. This consortium was functional, albeit with a lower efficiency than the original methanogenic endosymbionts (Wagener et al. 1990; Shinzato and Kamagata 2010). The analysis of the SSU rRNA genes has shown that the uptake of methanogens from the environment is not a general phenomenon, but the experiments of Wagener et al. (1990) have shown that it is possible.

3 Studies of the SSU rRNA Genes of Host and Symbiont

In order to analyse the "vertical" inheritance of the methanogenic endosymbionts in more detail, van Hoek et al. (2000) studied the methanogenic endosymbionts of closely related anaerobic ciliates that thrive either in freshwater sediments or in the intestinal tracts of cockroaches, millipedes and frogs. Van Hoek et al. (2000) amplified the SSU rRNA genes from both the endosymbionts and their hosts, using single cell PCR. The phylogenetic analysis of the SSU rRNA genes of the hosts revealed the anticipated monophyly of the various cockroach-dwelling *Nyctotherus* species and strains that thrive in the guts of millipedes and frogs. Notably, the free-living species *Metopus* sp., *Brachonella* sp., and "*Caenomorpha*-like" belonged to the same monophyletic cluster. The *Caenomorpha* species formed a closely related but paraphyletic cluster (Fig. 2). The monophyly of the *Nyctotherus*/*Metopus*/*Brachonella* cluster was confirmed by the phylogenetic analysis of the 12S (SSU) rRNA genes located on the genomes of the hydrogenosomes (Boxma

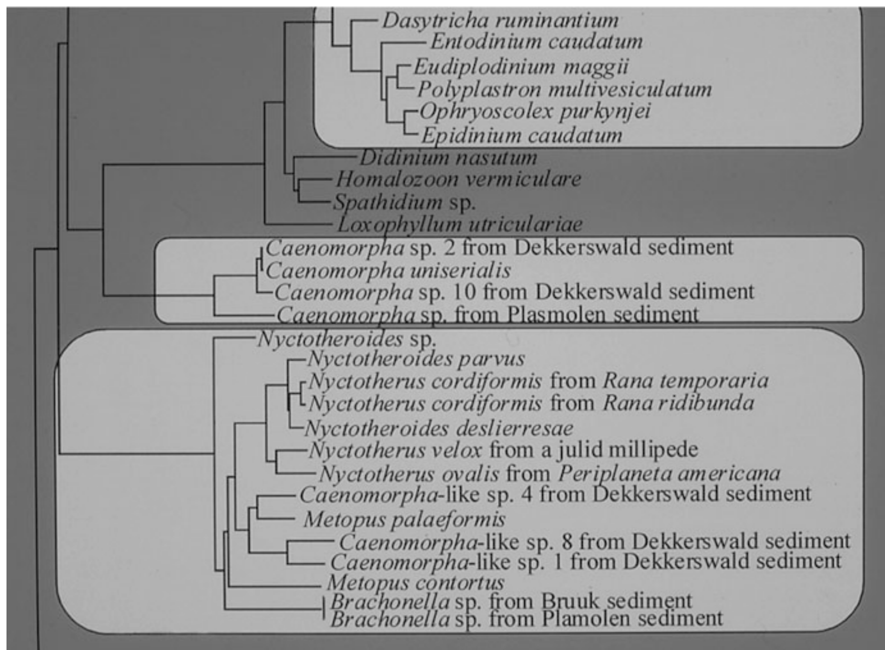

Fig. 2 Detail of a phylogenetic tree (Molphy Star Decomposition, Adachi and Hasegawa 1996) demonstrating the evolution of the relevant ciliate taxa on the basis of their 18S rDNA. *Boxed* species are anaerobes and possess hydrogenosomes. The *unboxed* species are aerobes with mitochondria. The *Nyctotherus*/*Nyctotheroides* and *Metopus*/*Brachonella*/*Caenomorpha-like* cluster is monophyletic (Armophoridae and Clevelandellids). The *Caenomorpha* species form a paraphyletic cluster

et al. 2005) and the corresponding hydrogenases (not shown). This means that the hydrogenosomes of these ciliates are monophyletic and consequently, that these organelles had been acquired by the last common ancestor of this clade – before the various ciliate species adapted to their particular freshwater- or gut-environments.

However, the analysis of the SSU rRNA genes of the methanogenic endosymbionts revealed an unexpected result (Fig. 3). The endosymbionts formed two clusters that belong to two different orders of methanogens (Methanobacteriales vs. Methanomicrobiales). One cluster contained the endosymbionts of the free-living ciliate species; the other contained the endosymbionts of the gut-dwelling ciliate species. Notably, the endosymbionts of the freshwater ciliates clustered among methanogens (Methanomicrobiales) living predominantly in freshwater sediments, whereas the endosymbionts of the intestinal ciliates clustered among predominantly intestinal or faecal methanogens (Methanobacteriales). Each group of endosymbionts was monophyletic, and each endosymbiotic methanogen was distinct from any known environmental methanogen. The endosymbionts were different from each other, given the fact that they were from a different ciliate ribotype. Also, endosymbionts from different ciliate ribotypes living in the same

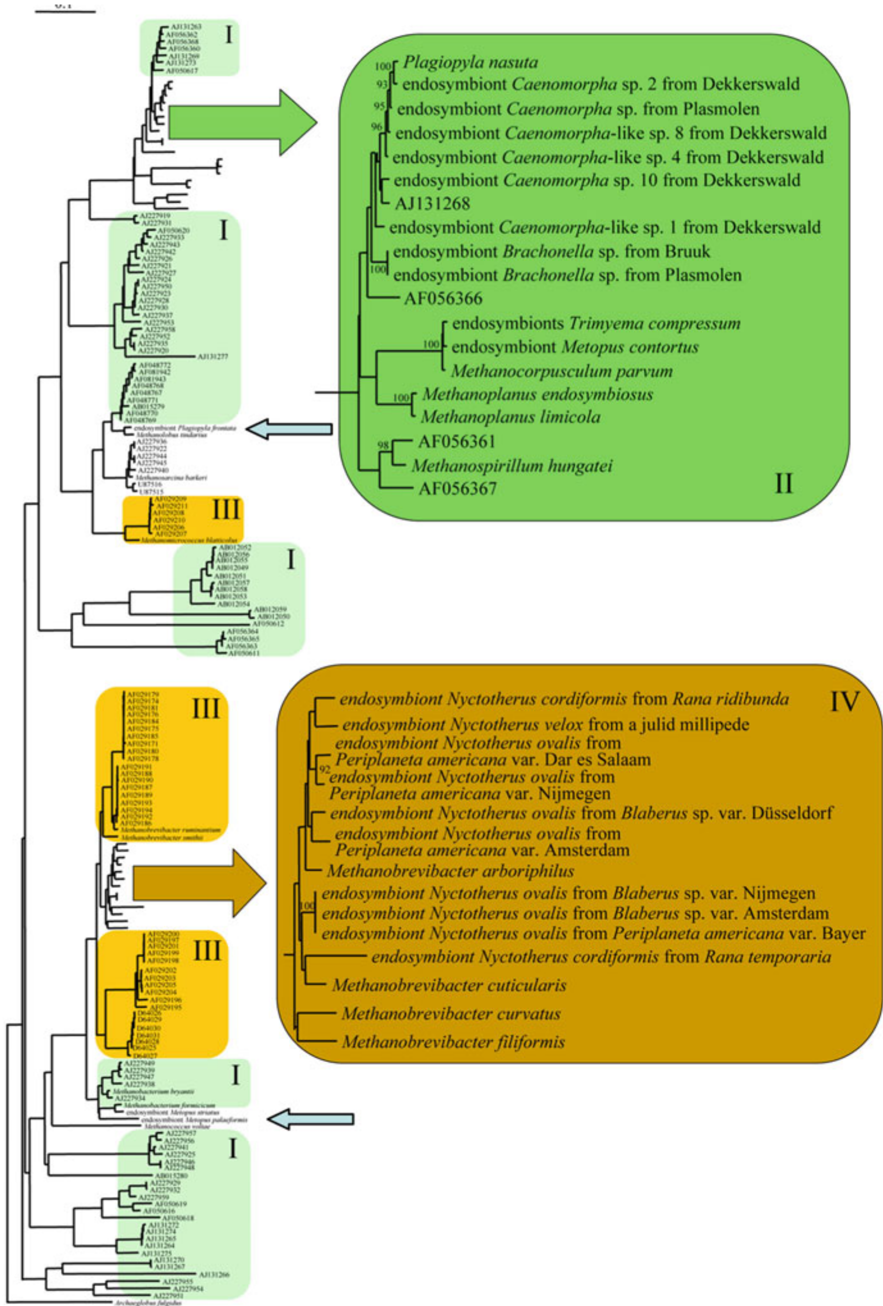

Fig. 3 Neighbour-joining tree (Saitou and Nei 1987) inferred from approximately 770 positions of the 16S rDNA of methanogenic archaea. The clades with the endosymbionts from freshwater (*box* II) and intestinal ciliates (*box* IV) are *highlighted* and enlarged. The *boxes* (I) indicate predominantly free-living methanogens from environmental sources such as sediments and rice fields. The *boxes* (III) mark predominantly uncultured intestinal methanogens. The *small arrows*

pond were different, but endosymbionts from the same ciliate ribotype were identical – regardless of the sampling place. Methanogenic endosymbionts from earlier studies (all from free-living ciliates) clustered at different positions in the phylogenetic tree (Fig. 3), but always among methanogens from freshwater environments. There is one potential exception: the endosymbiont of *T. compressum* strain S10 appeared to be similar to *Methanobrevibacter arboriphilus* that clusters among the endosymbionts of gut ciliates (Fig. 3; Shinzato et al. 2007; Shinzato and Kamagata 2010), whereas endosymbionts from other *Trimyema* strains, cluster among the freshwater methanogens (Fig. 3). However, the ciliate strain S10 had been isolated from a sewage installation, which is likely to harbour *M. arboriphilus*-like methanogens. Also, Narayanan et al. (2009) provided evidence for the presence of an acetoclastic *Methanosaeta* species as endosymbiont of *Metopus es*. This endosymbiont might be derived from an environmental free-living member of the Methanosetaceae, which thrive in anaerobic digesters just as *Metopus es*.

Thus, there is a clear correlation between the methanogenic endosymbionts and the free-living methanogens from the corresponding environments in which the ciliate host lives. This suggests that the endosymbionts stem from the environment, but the fact that the SSU rDNA sequences from the endosymbionts and the free-living methanogens are different argues against the existence of opportunistic symbionts. The substantial times of evolutionary divergence that result in a significant sequence divergence from environmental methanogens also argue for specific, long-lasting symbiotic associations. Other arguments against opportunistic symbionts are provided by the already mentioned vertical transmission of the symbionts and the failure to demonstrate an endosymbiont exchange in transfaunation experiments with *Nyctotherus* ciliates from different cockroach strains (van Hoek et al. 1999, 2000).

4 Endosymbiont Replacements

To study this dilemma further, van Hoek et al. (2000) analysed the potential co-evolution between ciliates and their methanogenic endosymbionts at the level of their SSU rRNA genes. It had been shown earlier with the analysis of symbiotic associations between bacteria and insects that these symbioses exhibited a complete congruency between host and symbiont phylogenies (Baumann et al. 1995, 1997; Bandi et al. 1994, 1997). With respect to the ciliates there was clearly no congruency between the phylogenies of the free-living and gut-dwelling ciliates and their

Fig. 3 (continued) indicate the endosymbionts of the free-living ciliates *Plagiopyla frontata* (*upper*) and of *Metopus striatus* and *Metopus palaeformis* (*lower*).The endosymbiont of *Trimyema compressum* strain S 10 is similar to *Methanobrevibacter arboriphilus* that is located in *box* IV. The distance data were bootstrap resampled 100 times (Felsenstein 1985). Only bootstrap values above 90% are displayed in the *highlighted*, *enlarged boxes* II and IV. Reproduced with permission by Oxford University Press from van Hoek et al. (2000)

endosymbionts. As already mentioned, the host environment determined the phylogenetic position of the endosymbiont (Fig. 3). To circumvent this problem, van Hoek et al. (2000) constructed separate phylogenetic trees for the free-living and intestinal ciliates and their endosymbionts (Fig. 4). Also these trees did not provide

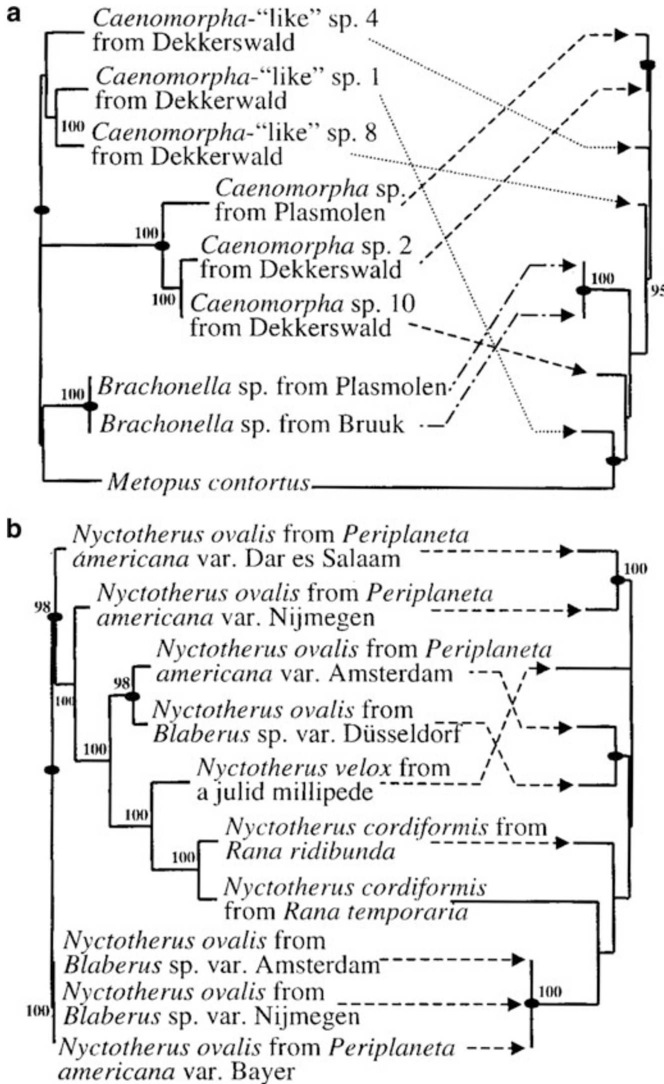

Fig. 4 TreeMap trees of hosts and symbionts (Page 1995) based on 460 positions of the 18S rDNA sequences of the ciliate hosts and 770 positions of the 16S rDNA sequences of the methanogenic endosymbionts. (**a**) Freshwater ciliates (*left tree*) and their methanogenic endosymbionts (*right tree*). (**b**) Intestinal ciliates (*left tree*) and their methanogenic endosymbionts (*right tree*). Corresponding pairs of ciliates and their endosymbionts are indicated by *arrows*. Only bootstrap values above 90% are displayed. Presumed co-speciation events are indicated by *bullets*. Reproduced with permission by Oxford University Press from van Hoek et al. (2000)

evidence for a strict congruency between host and symbiont trees. Only a few potential co-speciation events could be identified. The use of different tree-building algorithms and user-defined trees did not lead to a better match between host and symbiont phylogenies. Thus, the evolution of the anaerobic ciliates and their endosymbionts studied here cannot be completely vertical. Several times in the history of evolution, a horizontal transfer of symbionts must have taken place, i.e. the evolution of the intestinal ciliates must have included a minimum of one endosymbiont replacement, and potentially some more. As has been already mentioned, the last common ancestor of both the free-living and the intestinal ciliates hosted hydrogenosomes and consequently, methanogenic endosymbionts. The nature of these ancestral endosymbionts is unknown, but one might assume that

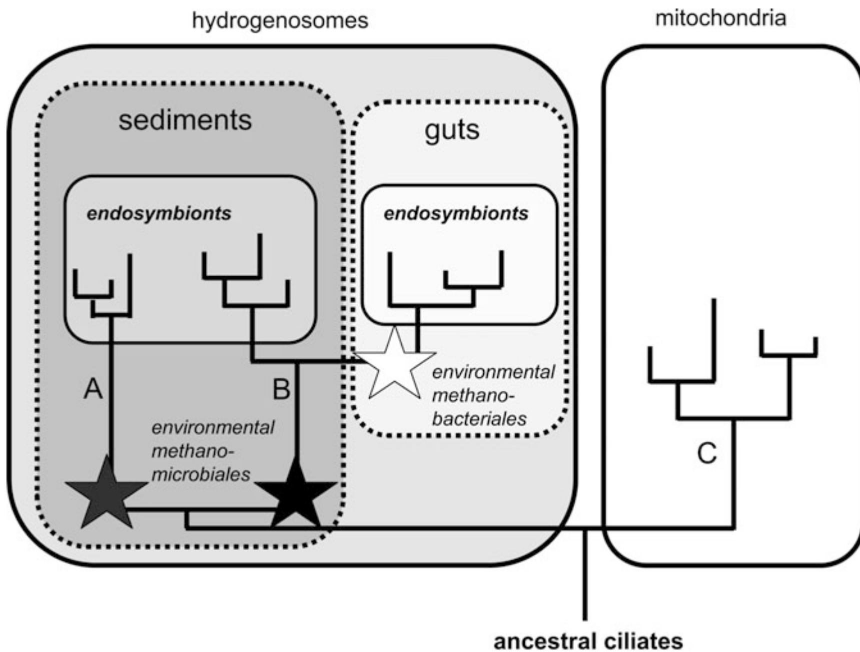

Fig. 5 Cartoon summarizing the evolution of anaerobic heterotrichous ciliates [(**a**) Caenomorphidae, (**b**) Armophoridae and Clevelandellids] and their endosymbiotic methanogens. Ancestral ciliates diverged into aerobic, mitochondria-bearing ciliates [most likely the Stichotrichs (**c**)] and anaerobic, hydrogenosome-bearing heterotrichs (**a**, **b**). The *black asterisks* identify the first acquisition of methanogenic endosymbionts that precedes the adaptation of the ciliates to the various ecological niches. Because it is not known whether the evolution of hydrogenosomes preceded the divergence of Caenomorphidae and Armophoridae and Clevelandellids, two different, independent acquisitions are possible (*black asterisks*). Subsequently, the ciliates diverge (*black lines*), and both Caenomorphids and part of the Armophoridae and Clevelandellids radiate in freshwater sediments. Their endosymbionts are closely related to environmental, free-living Methanomicrobiales. Those Armophoridae and Clevelandellids (**b**) that adapt to life in the gastrointestinal tract acquire endosymbionts that are related to intestinal Methanobacteriales thereby replacing the ancestral endosymbionts (*white asterisk*). Redrawn after Hackstein et al. (2002)

these endosymbionts were related to environmental methanogens. Adaptation of the ciliates to a different environment must have involved an endosymbiont replacement, since it has been shown that all ciliates studied so far possess endosymbionts that are related to free-living methanogens thriving in the corresponding environment (Fig. 5). Notably, the endosymbionts of ciliates living in the guts of frogs and their larvae are of the "intestinal" type, although the hosts of the ciliates, the frogs and their larvae, thrive in an environment that is crowded with free-living methanogens of the "freshwater sediment" type.

Ciliates radiating in the same ecological niche host methanogens that are distinct and different in DNA sequence from all known environmental methanogens. As already mentioned, the endosymbionts do not strictly co-speciate with their hosts, a trait that might be caused by accidental endosymbiont replacements within one and the same environment. However, the genetic distance to environmental methanogens suggest that such endosymbiont replacements are infrequent and followed by regular periods of strictly vertical transmission. A similar phenomenon has been observed in the symbiosis between proteobacteria and certain bivalves belonging to the genus *Solemya* (Krueger and Cavanaugh 1997; Distel 1998). Also here, endosymbiont replacements have been postulated. Since grazing ciliates regularly take up bacteria and free-living methanogens, it is reasonable to assume that one or the other methanogen will escape digestion and survive in the cytoplasm of the ciliate. Eventually such a methanogen might replace an aged population of endosymbionts suffering from its genetic load due to the action of "Muller's ratchet" (c.f. Doolittle 1998). The successful introduction of *M. formicicum* into symbiont-free cells of *T. compressum* shows that such a mechanism must be possible (Wagener et al. 1990). Thus, this scenario can explain both the limited co-evolution between ciliates and their methanogenic endosymbionts and the obvious relationship between endosymbionts and environmental methanogens.

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Symbiotic Methanogens and Rumen Ciliates

Kazunari Ushida

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Abstract Ciliated protozoa are the principal component of the rumen microbiota. They contribute significantly to the digestion of ruminants. As anaerobic fermentative micro organisms, rumen ciliated protozoa produce a significant amount of hydrogen and formate. Methanogenic archaea therefore associate closely with rumen ciliated protozoa. The presence of episymbiotic methanogens in rumen ciliated protozoa has been demonstrated as early as 1980s by microscopy. The number of ciliate-associated methanogens increases from the 10^0-level to 10^4-level per cell of ciliate after feeding. Enhancement of hydrogen and/or formate production from the ciliates by feeding attracts free-living methanogens. There are a couple of studies about the phylogeny of the ciliate-associated methanogens based on a molecular ecological approach. A range of methanogenic archaeal 16S rDNA, representing Methanobacteriales, Methanomicrobiales and Methanosarcinales, has been detected as ciliate-associated methanogens. However, it is still difficult to draw a conclusion about a potentially specific interaction between a particular ciliate species and a species of methanogenic archaea from these limited studies.

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1 The Rumen and Ciliated Protozoa

The rumen has a great capacity to digest plant polymers with the aid of anaerobic microbiota (Hungate 1966). This microbial ecosystem allowed ruminant animals to evolve into the predominant mammals in particular environments such as the semi-arid savannas (Hoffman 1973).

It is well established that the rumen ciliate protozoa have a significant impact on feed digestion in the ruminant animals, although the elimination of the ciliated protozoa does not impair the survival of the ruminant (Ushida et al. 1991).

As an anaerobic environment, the rumen microbial ecosystem requires an electron sink other than oxygen (Wolin 1975). Methane is the prevalent electron sink in this particular ecosystem (Hungate 1966). Fermentative micro organisms, therefore, create a specific relationship with hydrogenotrophic organisms to perform the different fermentation steps.

In the rumen, ciliated protozoa are known as potent hydrogen and formate producers. One cell of an axenic culture of rumen protozoa can produce 5 nmol of hydrogen per day (Ushida and Jouany 1996; Tokura et al. 1997). Potentially, this corresponds to a daily hydrogen production of approximately 50 L in the rumen of a sheep (Ushida et al. 1996). Besides hydrogen, one axenic ciliate cell produces 100 nmol of formate, which corresponds to a daily production of about 50 mol of formate in the rumen of a sheep. Such a concentration of hydrogen and formate attracts methanogens and makes the ciliate/methanogen consortium a predominant contributor for the ruminal methanogenesis. Consequently, elimination of the ciliated protozoa from the rumen, the defaunation, is associated with a 30–45% reduction of ruminal methanogenesis (Ushida et al. 1996).

In the case of rumen ciliates, the elimination of methanogens causes a decrease in the degradative capacities to some extent (Table 1) (Ushida and Jouany 1996). In particular, the elimination of methanogenesis increases the hydrogen and formate production from the ciliates at least by a factor of two, but sometimes to a level several times higher. This slows the fermentation process down (Wolin 1975).

Table 1 Elimination of methanogenesis from ciliated protozoa affects the apparent dry matter degradation (%) in vitro (Ushida and Jouany 1996)

Fauna type	+*M*	−*M*
Mixed type A[a]	67.7	61.1
Epidinium spp.	50.4	42.4
Polyplastron multivesiculatum	49.2	47.0
Isotricha prostoma	27.8	22.7

[a]Mixed type A was defined by Eadie (1967) in which the rumen harbours *Polyplastron multivesiculatum* as a particular organism with common Entodinia and Isotrichids

2 Methanogens Associated with Rumen Ciliates

Methanogenic archaea associate closely with the rumen ciliates to facilitate the inter-species hydrogen transfer in the form of an episymbiosis or an endosymbiosis.

No free-living methanogens were detected in the protozoal fraction prepared by sedimentation (Sharp et al. 1998). Therefore, it was believed that all methanogens that are metabolically associated with the ciliates are present inside the cell or intimately attached to the cell surface of the ciliates.

Episymbiotic methanogens of rumen ciliates were microscopically observed as early as 1980 by their characteristic F_{420} autofluorescence (Vogels et al. 1980). Endosymbiotic methanogens were observed by an Archaea-specific oligonucleotide probe approach (Finlay et al. 1994). It has been shown that these endosymbiotic methanogens are localized in the cytoplasm, not in digestive vacuoles, and adjacent to the hydrogenosomes. Interestingly, the number of the endosymbiotic methanogens exceeds the number of those attached on the cell surfaces of ciliates.

The number of ciliate-associated methanogens increased from the level of 10^0 to 10^4 the most probable number (MPN) per cell of ciliate after feeding (Tokura et al. 1997). When the ciliated protozoa engulfed and fermented feed particles, the number of ciliate-associated methanogens increased. Since the maximal level was recorded shortly (1–2 h) after feeding, it is unlikely that endosymbionts grow to this level in this short period of time. Accordingly, such a rapid increase in the numbers of ciliate-associated methanogens may reflect the active attachment or vigorous engulfment of free-living methanogens. Indeed, the hydrogen supply from the ciliates strongly attracted free-living methanogens (Stumm et al. 1982).

Ciliated protozoa predate and digest engulfed bacteria as a major prey. If engulfed methanogens would be the source of the endosymbiotic methanogens, these methanogens need to be resistant against protozoal lytic activity or they may escape from the digestion within food vacuoles. This point may be supported by the fact that anaerobic ciliates, *Metopus* spp. and *Nyctotherus* spp., which harbour methanogenic symbionts closely related to the free-living organisms (Embley and Finlay 1993; van Hoek et al. 2000). One study evaluated the resistance of methanogenic archaea against the lytic activity of rumen protozoa. It was found that some of the methanogens are relatively resistant against the protozoal lytic activities; i.e. *Methanosarcina barkeri* DSM 800 was more resistant than *Methanobrevibacter* sp. MF1 (Newbold et al. 1996). DSM 800 could established the interspecies hydrogen transfer with *Polyplastron multivesiculatum* (Ushida et al. 1997).

3 Detection of Methanogens Associated with Ciliates

There are a couple of studies about the phylogeny of the ciliate-associated methanogens based on molecular phylogenetic approaches (Sharp et al. 1998; Tokura et al. 1999b; Chagan et al. 1999; Ohene-Adjei et al. 2007;

Regensbogenova et al. 2004; Irbis et al. 2004). However, little information is available about the methanogens isolated from ciliates. An isolation of methanogens from washed ciliated protozoa was tried, and the strain *Methanobrevibacter* sp. MB9 was isolated. This isolate was phylogenetically close to *Methanobrevibacter ruminantium*, on the basis of morphology and 16S rRNA phylogeny (Tokura et al. 1999a). Other attempts for the isolation of symbiotic methanogens have not been found in the literature probably due to the difficulty and tediousness of the isolation procedure. Even for the free-living methanogens, relatively few had been isolated from the rumen (Janssen and Kirs 2008). The isolate *Methanobrevibacter* sp. MB9 uses hydrogen, formate and small amounts of 2-propanol. This substrate use, 2-propanol, is not common for ruminal *Methanobrevibacter* species.

Partial sequences of 16S rDNA of ciliate-associated methanogens are available from studies in Japan and the UK (Chagan et al. 1999; Tokura et al. 1999b; Irbis and Ushida 2004; Regensbogenova et al. 2004). DNA was extracted from washed cells of ciliate protozoa. In some studies, DNA was extracted from a single cell of the ciliated protozoa. Table 2 shows the distribution of archaeal 16S sequences retrieved from the cells of ciliated rumen protozoa. The phylogenetic analyses are also shown in Fig. 1a, b. In this table, there are some unidentified strains of which strain 1Y is phylogenetically close to *Methanobrevibacter gottschalkii*, strain SM9 is close to *Methanobrevibacter millerae*, strain OCP is close to *Methanobrevibacter olleyae*, and strain Z8 is close to *M. ruminantium* (Rea et al. 2007; Evans et al. 2009).

Methanobrevibacter-like sequences and those similar to *Methanomicrobium* were the predominant sequences detected in different studies. *Methanobrevibacter* sp. 1Y-like sequences were found in a range of protozoal species. Lastly, *Methanomicrobium mobile* (AY196679)-like sequences were detected in a range of protozoa both in Japanese and British studies. Methanogens belonging to the Methanobacteriales were detected predominantly in Japanese studies (Accession numbers start with AB; see Tokura et al. 1997, 1999; Chagan et al. 1999; Irbis and Ushida 2004), while those belonging to Methanomicrobiales were predominantly detected in a British study (Accession numbers start with AJ; see Regensbogenova et al. 2004). Interestingly enough, *Ophryoscolex caudatum* was studied in both the Japanese and British studies. This particular rumen protozoon harboured a variety of methanogens such as Methanobacteriales, Methanomicrobiales and Methanosarcinales. In addition to these methanogens, *O. caudatum* harboured also Thermoplasmatales. Other Entodiniomorphs like *P. multivesiculatum*, *Eudiplodinium maggii*, *Diplodinium dentatum*, *Metadinium medium* and *Entodinium furca* harboured relatively limited numbers of species of methanogens. In the case of holotrichs, *Isotricha intestinalis* harboured a phylogenetically broad range of methanogens similar to that shown in *O. caudatum*. An aquatic ciliate, *Metopus contortus* can host a broad range of methanogens. Accordingly, this aquatic ciliate is defined as the generalist host for methanogens (Embley and Finlay 1993). Rumen ciliated protozoa like *I. intestinalis* and *O. caudatum* can also be a generalist host for methanogens. However, it is still difficult to draw a conclusion

Table 2 Registered archaeon 16S rDNA retrieved from rumen ciliate protozoa

Suggested nearest known isolate	Registered archaeon 16S rRNA sequence retrieved from rumen ciliate protozoa								
	P. multivesiculatum	*Eu. maggii*	*O. caudatum*	*D. dentaum*	*E. furca*	*M. medium*	*I. prostoma*	*I. intestinalis*	Mixed population
Methanobacteriales									
Methanobrevibacter ruminantium MB9 (isolate)									AB017514
Methanobrevibacter smithii ALI	AB189858								
Methanobrevibacter millerae ZA-10							AB026169		AB022182, AB022185, AB022181
Methanobrevibacter wolinii SH	AB189856		AB189866					AB189861	
Methanobrevibacter sp. 1Y	AB026173–74	AB026171	AB189865					AB189859	AB022183–84
Methanobrevibacter sp. SM9	AB189857		AB189864					AB189861	
Methanobrevibacter sp. OCP							AB026168		
Methanobrevibacter sp. Z8		AB026170							
Methanobacterium sp. GH	AB026175								
Methanosphaera stadtmaniae DSM 3091		AB026172							AB022186

(*continued*)

Table 2 (continued)

Suggested nearest known isolate	Registered archaeon 16S rRNA sequence retrieved from rumen ciliate protozoa								
	P. multivesiculatum	*Eu. maggii*	*O. caudatum*	*D. dentaum*	*E. furca*	*M. medium*	*I. prostoma*	*I. intestinalis*	Mixed population
Methanomicrobiales									
Methanomicrobium mobile DSM 1539			AJ606400 AJ606411			AJ606405–10			
Methanomicrobium mobile (AY196679)			AJ606402 AJ189867	AJ606418	AJ606412–17	AJ606401		AB189862	
Methanosarcinales									
Methanimicrococcus blattieola PAT			AJ606403						
Methanimicrococcus blatticola DSM 13328			AJ606404						
Methanosarcina sp. 2214B				AJ606419					
Thermoplasmatales									
Thermoplasma sp. XT107			AB189868					AB189863	

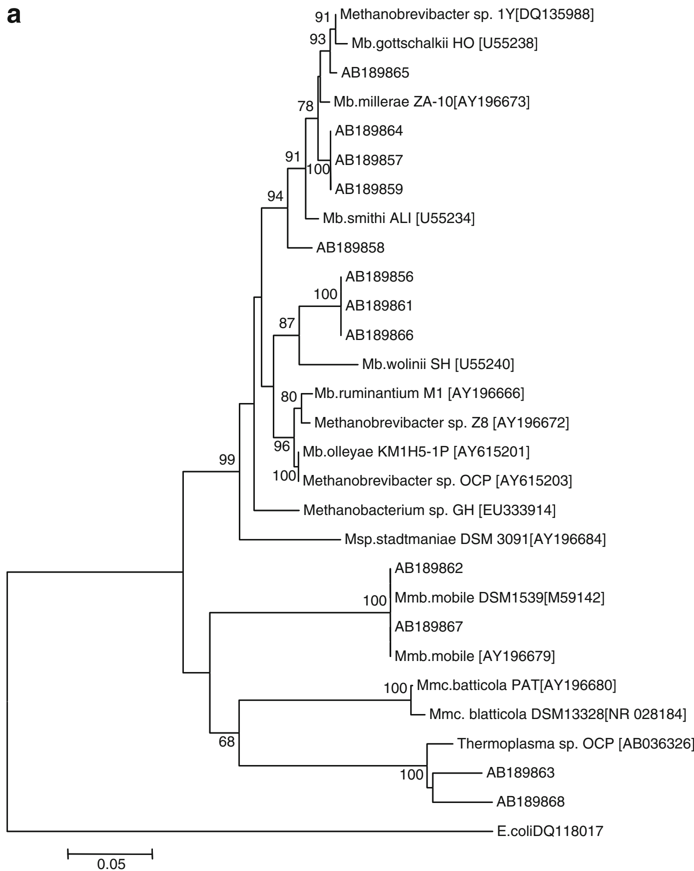

Fig. 1 Neighbour-joining tree computed from partial 16S DNA of methanogens associated with rumen ciliated protozoa by MEGA 4.0 (Tamura et al. 2007) with 500 replicates for bootstrap. (**a**) Partial sequences (*E. coli* [DQ118017] 16S rDNA position 781–1233) are used to analyse

about a potentially specific interaction between a particular ciliate species and a species of methanogenic archaea from these limited studies.

4 The Effect of Ciliated Protozoa on the Composition of Methanogenic Archaea in the Rumen

As indicated above, a cell of ciliated protozoa can harbour up to 10^4 methanogens. Since the number of ciliates ranges from 10^5 to 10^6 cells/ml rumen fluid (Williams and Coleman 1991), they may encompass a methanogenic population as large as 10^{10} methanogens/ml. If there is a specific relationship between the ciliate species and their methanogenic symbionts, an increase in the number of ciliated protozoa

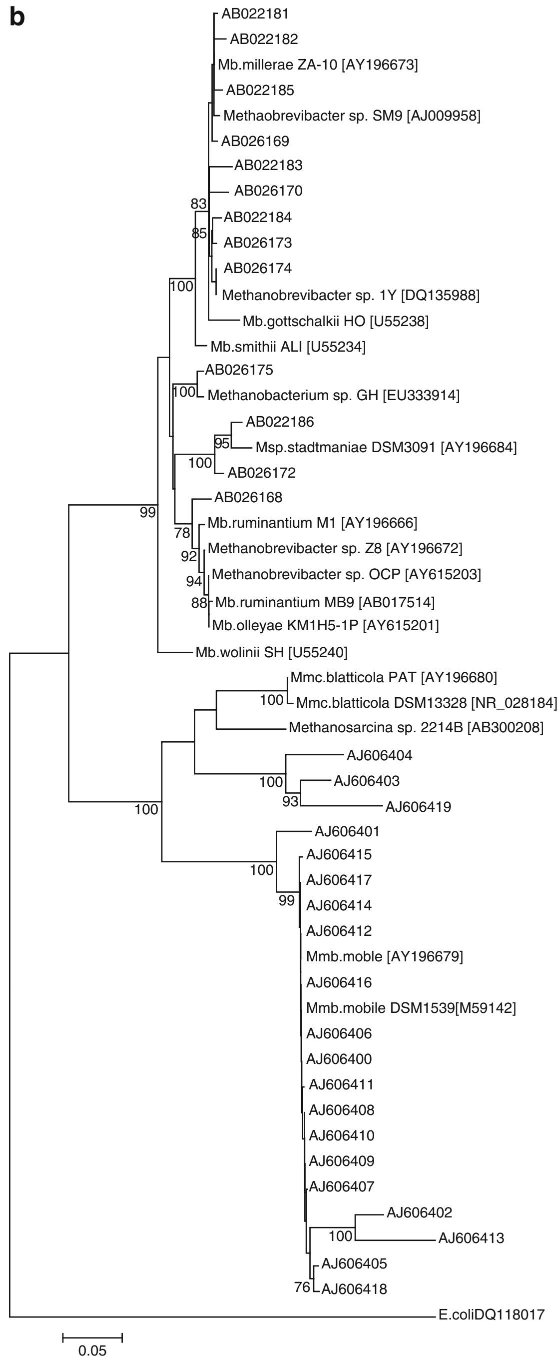

Fig. 1 (Continued) ciliate-associated archaea (AB189856–AB189868); (**b**) (*E. coli* [DQ118017] position 218–798) are used to analyse ciliate-associated archaea (AB022181–AB022186, AB026168–026175, AJ606400–AJ606419)

should affect the methanogenic archaeal population as a selective pressure upon the methanogenic population. In an in vivo study, Ohene-Adjei et al. (2007) indicated that an inoculation of *P. multivesiculatum* into the rumen predominantly associated with the detection of methanogens closely related to *Methanobrevibacter bryantii*, *M. ruminantium* and *Methanosphaera stadtmaniae*. These authors also showed that inoculation of holotrich protozoa (Isotrichidae) into the rumen was primarily associated with the detection of methanogens closely related to *Methanobrevibacter smithii*. Although this Canadian in vivo study appears not to agree with the results shown in Table 2, it is likely that the presence of a particular ciliate protozoa may promote the predominance of particular species of methanogens.

Again, the specificity of the host-methanogenic symbiont relationship is still difficult to be proven, because a long term pure culture system for rumen ciliates has not been established so far. Without a pure culture of rumen ciliated protozoa, consisting of aposymbiotic ciliates, an inoculation study as reported for *Trimyema compressum* cannot be realized (Wagener et al. 1990; Holler and Pfennig 1991).

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The Methanogenic and Eubacterial Endosymbionts of *Trimyema*

Naoya Shinzato and Yoichi Kamagata

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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

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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

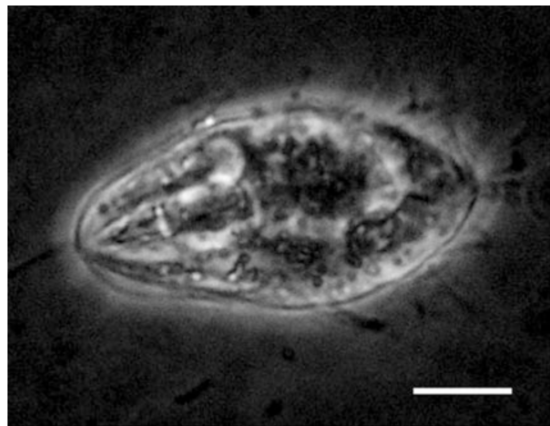

Fig. 1 Phase contrast image of living *T. compressum*. *T. compressum* swims actively in the medium and preys food bacteria. Bar represents 10 μm

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 × 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

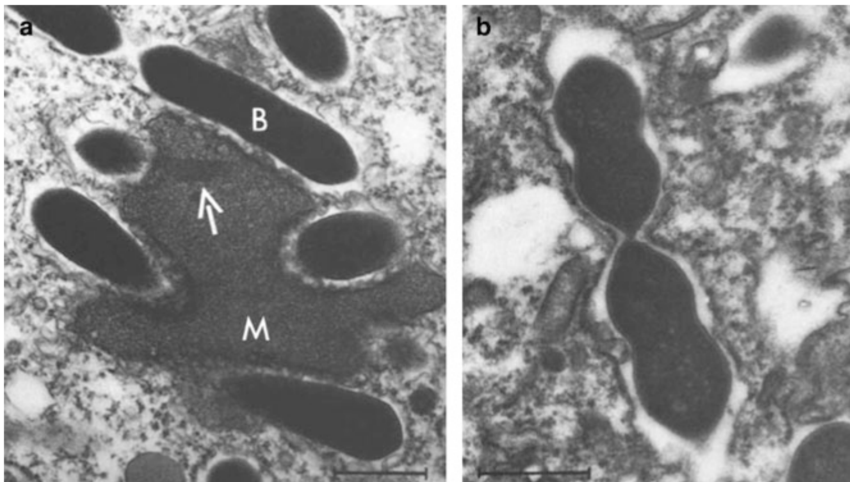

Fig. 2 Transmission electron micrograph of an ultra-thin section of *T. compressum* strain N. (**a**) Methanogenic symbionts (B) associate with microbodies (M: hydrogenosomes). (**b**) Dividing cell of non-methanogenic (bacterial) symbiont, not associated with microbodies. Bars represent 0.5 μm [reprinted 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

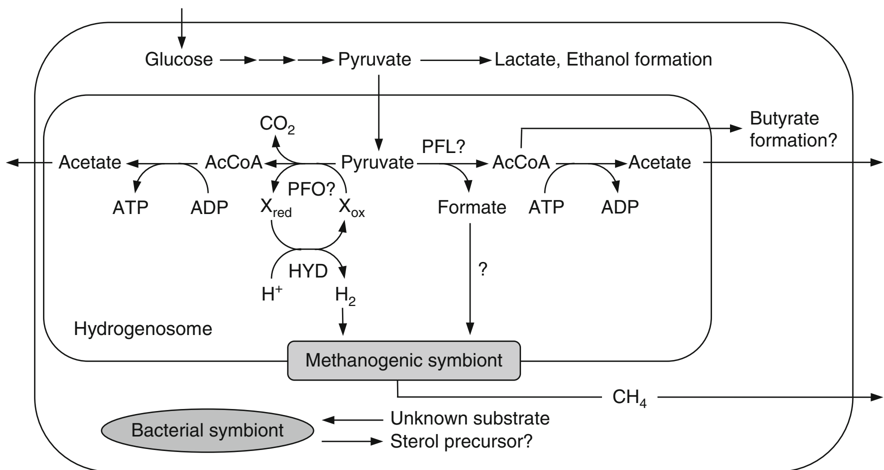

Fig. 3 Speculative metabolic schemes of carbohydrate metabolism in the symbiotic consortium of *T. compressum*. Abbreviations: *AcCoA* acetyl-CoA, *HYD* hydrogenase, *PFL* pyruvate:formate lyase, *PFO* pyruvate:ferredoxin oxidoreductase. Xox, red, unknown electron carrier. Methanogenic symbionts are 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

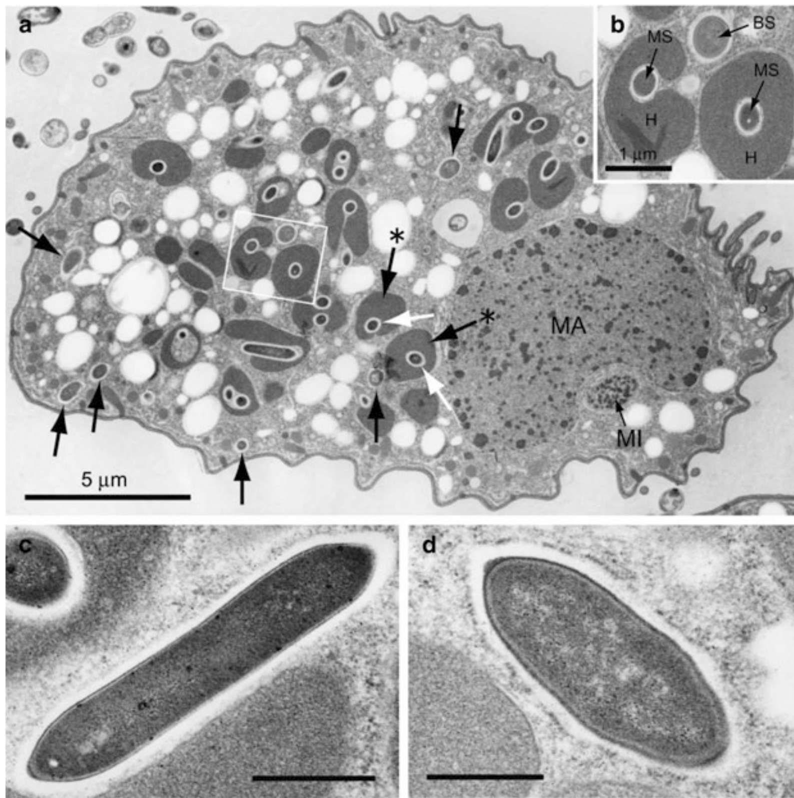

Fig. 4 Transmission electron micrographs of ultra-thin sections of *T. compressum*. (**a**) Whole view of a *T. compressum* cell. The methanogenic symbionts (*white arrows*) were closely associated with hydrogenosomes (*black arrows* with *asterisks*), while the bacterial symbionts (*black arrows*) were 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.

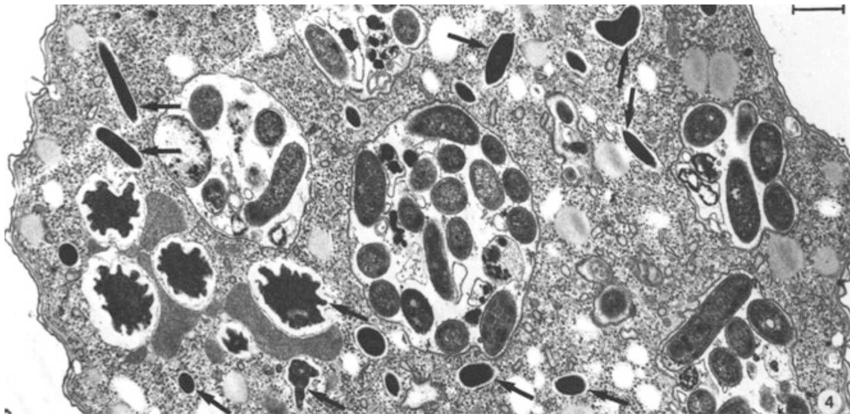

Fig. 5 Transmission electron micrographs of ultra-thin sections of *Trimyema* sp. Electron-dense methanogens with various shapes are enclosed individually in vacuoles within the ciliate cytoplasm (*arrows*). Polymorphic transformation of methanogens are observed from the disc-shaped to the stellate form, 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

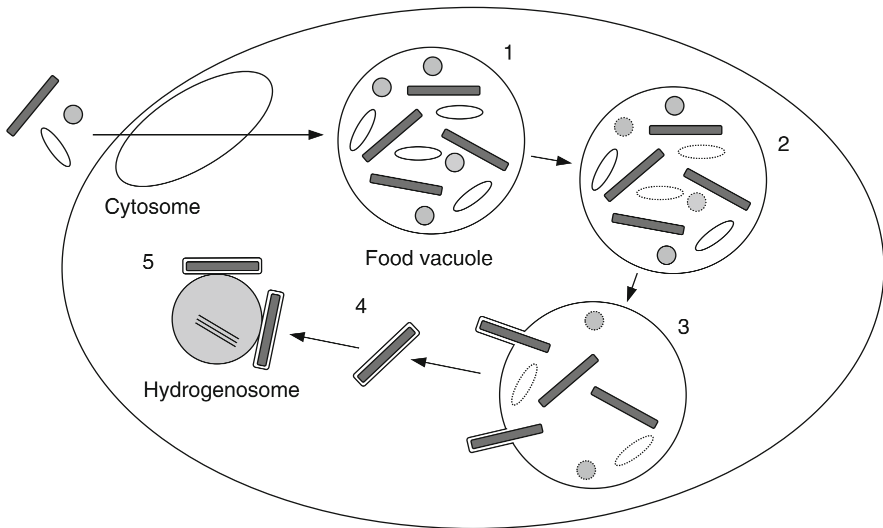

Fig. 6 Proposed process of establishment of a symbiotic association between *T. compressum* and methanogens. (1) uptake of bacteria into food vacuoles; (2) digestion of food bacteria; (3) separation of methanogens into the cytoplasm; (4) transport of the methanogens surrounded by a membrane; (5) localization 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.

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Termite Gut Flagellates and Their Methanogenic and Eubacterial Symbionts

Yuichi Hongoh and Moriya Ohkuma

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Abstract Termites harbor an abundance and diversity of symbiotic microbes in their gut that comprise all the three domains of life: Eucarya, Bacteria, and Archaea. One of the most prominent features of this microbiota is the cellular association of the gut flagellates with eubacteria and/or methanogenic archaea. The eubacterial and methanogenic symbionts are observed both inside and on the surface of the host flagellate cells. Although molecular approaches have gradually revealed the phylogenetic and spatial structures of these as-yet-uncultivable symbiotic complexes, their functions remain largely unknown. Recently, a method to acquire the complete genome sequence of uncultured bacterial species from a small number of cells

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has been developed; two complete genome sequences of endosymbiotic eubacteria of termite gut flagellates have been decoded. This novel genomic approach is expected to provide a great progress in the studies of this multilayered symbiotic system in termite gut.

1 Introduction

Termites are one of the most important decomposers in temperate to tropical regions (Sugimoto et al. 2000). Their ability to thrive on recalcitrant, nitrogen-poor lignocellulose is mostly attributable to the activities of the microbial community in the gut (Fig. 1) (Cleveland 1923; Eutick et al. 1978; Yoshimura 1995). In phylogenetically "lower" termites, the gut microbiota comprises both eukaryotes and prokaryotes, whereas most "higher" termites (family Termitidae) harbor only prokaryotic gut microbes. The majority of these gut microbes are as yet uncultivable; their phylogenetic and spatial distributions have been studied mainly by small subunit rRNA-based molecular analyses (Berchtold et al. 1994; Ohkuma and Kudo 1996; Ohkuma et al. 1998; Lilburn et al. 1999; Iida et al. 2000; Hongoh et al. 2003; Nakajima et al. 2005; Yang et al. 2005; Hongoh et al. 2006b). However, the detailed symbiotic mechanism remains unclear due to lack of effective methodologies for functional analysis of uncultivable microbes.

The eukaryotic gut symbionts comprise two distinct lineages of flagellated protists, belonging to either the phylum Parabasalia or the order Oxymonadida in the phylum Preaxostyla. They are unique to termites and the wood-feeding cockroach *Cryptocercus*, and each termite species possesses a specific set of flagellate species (Yamin 1979; Kitade 2004). Although the cultivation of these flagellates is very difficult, several studies on axenic or mixed cultures demonstrated that the

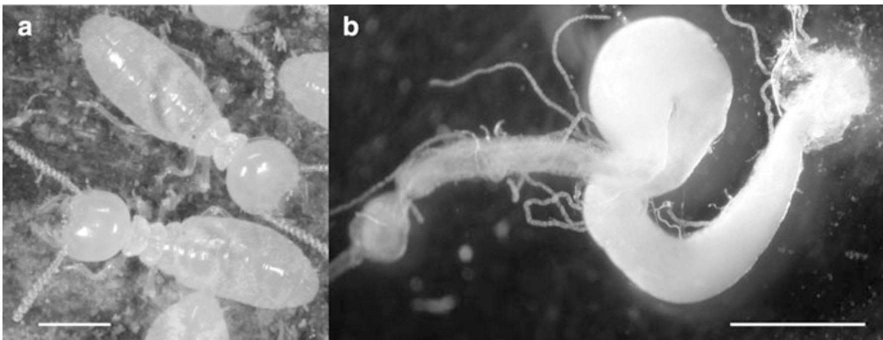

Fig. 1 Termites and a removed gut. (**a**) The lower termite *Reticulitermes speratus*. (**b**) A removed gut from *R. speratus*. Bars = 1 mm. Panel (**b**) was originally published in Hongoh et al. (2008a) as supporting information

flagellates are strictly anaerobic and ferment cellulose: $n(C_6H_{12}O_6) + n(2H_2O) \rightarrow n(2CH_3COOH + 2CO_2 + 4H_2)$ (Yamin 1980, 1981; Odelson and Breznak 1985b).

In addition to the cellulolytic flagellates, lower termites harbor a diversity of eubacteria in their gut. Several hundred or more species of eubacteria inhabit the gut of a single termite species, and the community structure is basically consistent within a host species (Hongoh et al. 2005, 2006a). The eubacterial gut symbionts, distributed among 25 phyla, constitute one or more monophyletic clusters in each phylum, suggesting that they are not allochthonous, but autochthonous symbionts, inherited from parents to offspring through the proctodeal trophallaxis (i.e., transmission of gut contents from the anus of a donor to the mouth of a recipient) (Andrew 1930; Kitade et al. 1997; Hongoh et al. 2005).

Another domain of life, Archaea, is also found in termite gut. The majority are methanogens, which are less abundant and less diverse compared to the eubacterial gut symbionts (Ohkuma et al. 1999; Shinzato et al. 1999; Brauman et al. 2001; Friedrich et al. 2001; Donovan et al. 2004; Brune 2010). To date, three methanogenic strains have been isolated from a lower termite, *Reticulitermes flavipes*, and described as *Methanobrevibacter cuticularis*, *Methanobrevibacter curvatus* (Leadbetter and Breznak 1996), and *Methanobrevibacter filiformis* (Leadbetter et al. 1998). All these methanobrevibacters attach to the gut epithelium. The occurrence of methanobrevibacters and their attachment to the gut wall are observed in both lower and higher termites (Fig. 2a, b) (Tokura et al. 2000; Pester and Brune 2007). From a wood-feeding higher termite, a methanobrevibacter strain closely related to *Methanobrevibacter arboriphilus* has been isolated, and from higher termites of various feeding habits, three strains of the genus *Methanobacterium*, closely related to *Methanobacterium bryantii*, have also been isolated (Deevong et al. 2004).

In general, soil- and litter-feeding higher termites emit much more methane than wood-feeding higher and lower termites (Brauman et al. 1992; Sugimoto et al. 1998). In the gut of wood-feeding termites, unlike many other anoxic environments, H_2-dependent acetogenesis outcompetes methanogenesis as "H_2-sink" (Odelson and Breznak 1983; Breznak and Switzer 1986; Pester and Brune 2007). Methanogens account for 0–10% of the gut prokaryotic population (Leadbetter and Breznak 1996; Brauman et al. 2001) and the rate of CH_4 emission is only 10% of that of CO_2-reductive acetogenesis in the gut of lower termites (Pester and Brune 2007). Although acetogenesis from H_2 plus CO_2 appears nutritionally more beneficial to termites than methanogenesis because acetate is their chief energy and carbon source, the physiological and physicochemical basis for the outcompetition of methanogenesis by acetogenesis is unclear. The localization of methanogens in the gut might account in part for this outcompetition (Breznak 2000; Tholen and Brune 2000), but the question why the localization of methanogens is restricted to the gut wall and the cells of the relatively few species of flagellates in the gut of lower termites remains unanswered. Methane oxidation has never been observed in termite gut (Pester et al. 2007).

Whereas numerous eubacteria and methanogens reside as free or wall-attached forms in the termite gut, it is known that the majority of the prokaryotic members in

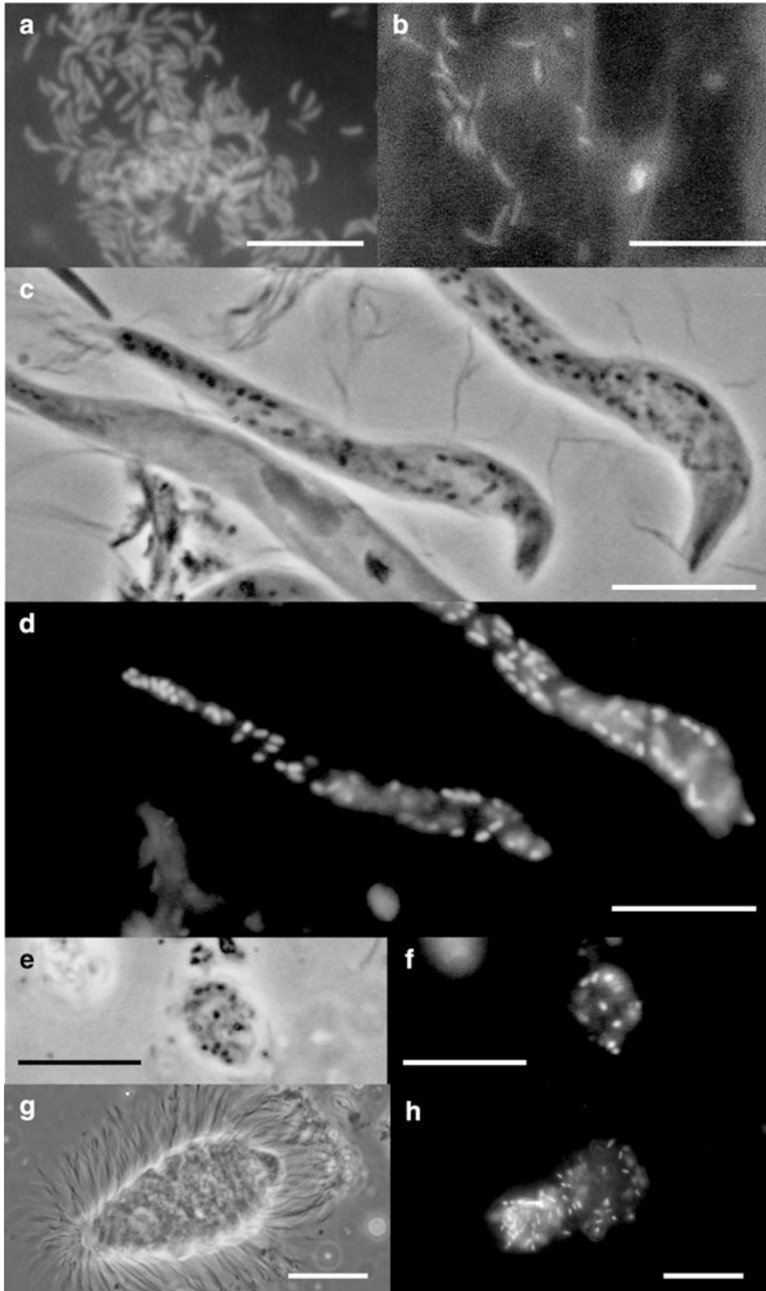

Fig. 2 Localization and morphology of methanogens detected by epifluorescence microscopy. (**a**) Epifluorescence image of methanogens on the gut epithelium of the lower termite *Reticulitermes speratus*. (**b**) Epifluorescence image of methanogens on the gut epithelium of the higher termite *Microcerotermes* sp. (**c**) Phase contrast image of the oxymonad protist *Dinenympha parva*

the gut of lower termites exist as endo- or ectosymbionts of the gut flagellates (Berchtold et al. 1999). Indeed, the physical association of cells between the flagellates and prokaryotes is one of the most prominent features of the termite gut microbiota (Brune and Stingl 2006; Ohkuma 2008). In this chapter, the studies on the cellular association between the flagellates and methanogenic archaea, as well as between the flagellates and eubacteria, in termite gut are reviewed and future perspectives for the functional analysis of the endosymbiotic prokaryotes are presented.

2 Methanogenic Endosymbionts of Termite Gut Flagellates

This section describes the phylogeny and predicted roles of the methanogenic endosymbionts of flagellates in the gut of lower termites. Although there have been fewer reports on the endosymbiotic methanogens of termite gut flagellates compared to those on the eubacterial endosymbionts, the reports contain valuable data, which help us to capture a general tendency on the phylogeny and host specificity and to elucidate their functional roles in the symbiosis with the flagellate host.

2.1 *Phylogeny of Endosymbiotic Methanogens*

The presence of methanogens inside the cells of certain flagellate species in the termite gut was evidenced for the first time by Lee et al. (1987). *Methanobrevibacter*-like rod-shaped cells were detected on the basis of the autofluorescence from the cofactors F_{420} and F_{350}, inside the cells of the parabasalid flagellates *Trichomitopsis termopsidis*, *Tricercomitus termopsidis*, and *Hexamastix termopsidis* from the gut of the termite *Zootermopsis angusticollis*. The corresponding rod-shaped cells associated with these small parabasalids had been described by Kirby (1930). Endosymbiotic methanogens in these three flagellate species have also been reported in the congeneric termite *Zootermopsis nevadensis* (Pester and Brune 2007). No molecular data exist for these methanogens to date.

Tokura et al. (2000) discovered endosymbiotic methanogens inside the cells of the parabasalid flagellate *Microjoenia* sp. and the oxymonad flagellate *Dinenympha parva* in the gut of the termite *Reticulitermes speratus*, on the basis of the F_{420} and

Fig. 2 (continued) from the gut of *R. speratus*. (**d**) Epifluorescence image of endosymbiotic methanogens in the *D. parva* cells. (**e**) Phase contrast image of the parabasalid flagellate *Microjoenia* sp. from the gut of *R. speratus*. (**f**) Epifluorescence image of endosymbiotic methanogens in the *Microjoenia* cell. (**g**) Phase contrast image of the parabasalid flagellate *Spirotrichonympha leidyi* from the gut of the lower termite *Coptotermes formosanus*. (**h**) Epifluorescence image of endosymbiotic methanogens in the *S. leidyi* cell. Bars = 10 µm. Panels (**a**) and (**c–f**) were originally published in Tokura et al. (2000) and slightly modified. Panels (**g**) and (**h**) were kindly provided by Jun-Ichi Inoue and a related study was published in Inoue et al. (2008)

F_{350} autofluorescence (Fig. 2c–f). About 10–50 cells of methanogens were found constantly inside the cells of *Microjoenia* sp. and *D. parva*, respectively (Hara et al. 2004). The total numbers of methanogens associated with *Microjoenia* sp. and *D. parva* were 7.9×10^3 and 1.3×10^5 per gut, respectively. Methanogens were also observed in other species of *Dinenympha* and the oxymonad *Pyrsonympha* sp., but the association was occasional. In total, approximately 4% of the flagellate cells in *R. speratus* guts were found to be associated with methanogens (Tokura et al. 2000).

In the termite *Hodotermopsis sjoestedti*, all the cells of *Dinenympha* and *Microjoenia* were found to be associated with methanogens. The population of the methanogen-associated flagellates was much larger than that in *R. speratus*; they accounted for 42% of the total flagellate cells. In both termite species, *R. speratus* and *H. sjoestedti*, methanogens free in the gut luminal fluid were rarely found (Tokura et al. 2000), while many were observed on the gut epithelium as in *R. flavipes*. *R. flavipes* and *Reticulitermes santonensis* [synonym of *R. flavipes*, found in European countries (Jenkins et al. 2001)] harbor no gut flagellates that are associated with methanogens (Leadbetter and Breznak 1996; Pester and Brune 2007).

To identify the phylogenetic position of these endosymbiotic methanogens in *R. speratus* and *H. sjoestedti*, clone analyses of archaeal 16S rRNA genes were performed. About 50 cells of *Dinenympha* and *Microjoenia*, respectively, were physically isolated by micromanipulation and directly used for PCR amplification. All of the sequenced clones were affiliated with the genus *Methanobrevibacter* (Fig. 3). In *R. speratus*, two phylotypes were obtained from *D. parva*, whereas a single phylotype was found from *Microjoenia* sp. Similarly, two phylotypes were from *Dinenympha* spp. and a single phylotype from *Microjoenia* sp. in *H. sjoestedti*. These phylotypes showed only 94.6–97.3% sequence identity to the closest cultured species, suggesting that they are novel *Methanobrevibacter* species (Tokura et al. 2000). It is unclear whether the two methanogen phylotypes from *D. parva* inhabit the cells of an identical host strain or of distinct strains of *D. parva*.

Interestingly, the methanogen phylotypes LRsD3 from *D. parva*, LRsM1 from *Microjoenia* sp., and RsW10 from the gut wall fraction of *R. speratus* showed >99.7% sequence similarity to one another (cluster A in Fig. 3) (Tokura et al. 2000). The localizations of cluster A methanogens inside the cells of *D. parva* and *Microjoenia* sp. were confirmed by fluorescent in situ hybridization (FISH) analysis (Hara et al. 2004). It remains unknown whether these phylotypes represent an identical species that can change its habitat or represent similar but distinct lineages that have adapted specifically to the respective habitats. No evidence of cospeciation between the endosymbiotic methanogens and the flagellate hosts has been found.

Another endosymbiotic methanogen is observed inside the cells of the parabasalid flagellate *Spirotrichonympha leidyi* in the gut of the termite *Coptotermes formosanus* (Fig. 2g, h) (Tsunoda et al. 1993; Inoue et al. 2008). A single *S. leidyi* cell harbors about 80 cells of methanogens, accounting, in total, for 0.3% of the prokaryotic community in *C. formosanus* guts. The archaeal 16S rRNA sequences obtained from *S. leidyi* cells consisted of a single phylotype, SlMeN10, sharing >99% similarity with one another and 98% similarity with those of cluster A in Fig. 3 (Inoue et al. 2008).

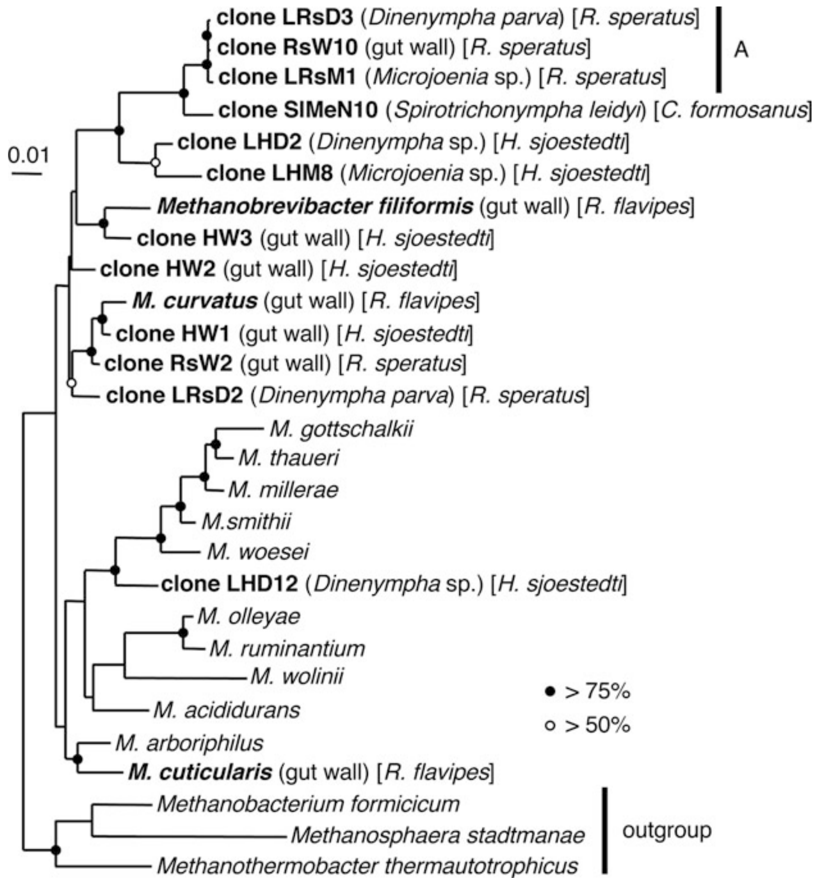

Fig. 3 Phylogenetic position of methanogens found from the gut of lower termites. Uncultured clones obtained by PCR from the gut of lower termites and type strains of described species belonging to the genus *Methanobrevibacter* were used to construct a phylogenetic tree. The localization or host protist species are shown with the host termite species in *parentheses*. Clones and isolates deriving from termite guts are shown in *bold*. The tree was constructed using PhyML v2.4.4 (Guindon and Gascuel 2003) with an HKY base substitution model. Bootstrap confidence values were calculated by 100 resamplings

2.2 Predicted Functions of Endosymbiotic Methanogens

Endosymbiotic methanogens inside flagellate cells in termite gut have never been cultured. In addition, no functional gene has been obtained from them. Therefore, there is no direct evidence for their functions. However, it is still possible to predict some of their functions from their taxonomic positions, localizations, and the data reported by Odelson and Breznak (1985a), who investigated the physiology of a

cultured flagellate, *Trichomitopsis termopsidis*, with and without "endogenous" methanogens.

The cultured strains closest to the endosymbiotic methanogens are *M. curvatus* and *M. filiformis*, which have been isolated from the gut of *R. flavipes*. The energy source of these isolates is restricted to H_2 plus CO_2, yielding CH_4 as the sole product: $4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$. They require one or more complex nutrients such as yeast extract and rumen fluid (Leadbetter and Breznak 1996; Leadbetter et al. 1998). Assuming that the endosymbiotic methanogens share basic physiologies with these isolates, a simple interpretation of the endosymbiosis is mutualism: the flagellate host provides H_2, CO_2, and other nutrients to the endosymbiotic methanogens, and in turn, the methanogens enhance the growth of the host flagellate by promoting the lignocellulose fermentation through the elimination of excess H_2.

The symbiosis mediated by interspecies transfer of H_2 between H_2-evolving fermentative anaerobes and H_2-consuming methanogens is not rare. In general, a high concentration of H_2 suppresses fermentation; the concentration of H_2 must be kept low (Worm et al. 2010). Actually, the growth rate of *Trichomitopsis termopsidis* 6057C, the sole axenic flagellate culture from the termite gut to date, was much enhanced when cocultured with an H_2-consuming methanogen, *Methanospirillum hungatii* (Odelson and Breznak 1985a). In the cocultivation, the produced gas was shifted from H_2 to CH_4, clearly suggesting the interspecies transfer of H_2. Heat-killed *M. hungatii* could not enhance the growth rate. Thus, it seems likely that the endosymbiotic methanogens benefit the flagellate host by lowering the H_2 concentration together with other H_2-consuming prokaryotes in the gut.

H_2 partial pressures, measured using agarose-embedded guts with microelectrodes, were very high in the dilated portion (paunch) of termite hindguts. In the paunch region, the values were 15–30 kPa in *R. santonensis*, 30–72 kPa in *Z. nevadensis* (Pester and Brune 2007), and 2–5 kPa in *R. flavipes* (Ebert and Brune 1997). However, since the hydrogen emission of the embedded guts was 30- to 50-fold higher than that in living termites (Pester and Brune 2007), the actual values in vivo might be much lower. This discrepancy was possibly caused by the damages of the H_2-consuming bacteria and a limited concentration of O_2 utilized to oxidize H_2 compared to living termites (Pester and Brune 2007). It has been demonstrated that the Fe-hydrogenases of a termite gut flagellate retain more than a half of their maximum H_2-evolving potential if the H_2 partial pressure can be kept lower than 20 kPa (Inoue et al. 2007).

Considering that the gut flagellates are the major source of H_2 and CO_2, produced during lignocellulose fermentation, it is reasonable that the methanogens have exploited the cytoplasm of the flagellates as their habitat. While the H_2 partial pressure is very high in the central region of a termite hindgut, it decreases toward the gut peripheral regions (Ebert and Brune 1997; Pester and Brune 2007). Messer and Lee (1989) demonstrated that exogenously supplied H_2 greatly enhanced the methanogenic activity of the termite *Z. angusticollis*, which harbors methanogen-associated flagellates as the main sites for CH_4 emission in the gut. In *Z. nevadensis*, a significant increase of CH_4 emission was also observed, even though the extent was smaller than that in *Z. angusticollis* (Pester and Brune

2007). These findings suggest that the H_2 concentration is a limiting factor for methanogenesis by the endosymbionts. In addition, the gut peripheral regions contain oxygen that suppresses methanogenesis, while the central region is almost completely anoxic (Ebert and Brune 1997). Therefore, the cytoplasm of the H_2-evolving flagellates, a habitat in close proximity to the H_2 source and probably protected from penetrating oxygen, seems an ideal habitat for stable, highly active methanogenesis. Indeed, anaerobic flagellates in various environments occasionally harbor endosymbiotic methanogens as described elsewhere in this book (Fenchel and Finlay 2010). The endosymbiosis seems also beneficial to the methanogens in the termite gut for avoiding washout because methanobrevibacters are generally nonmotile.

Although these factors reasonably explain the merit of the endosymbiosis to the methanogens, the interspecies H_2 transfer cannot fully explain the benefit to the flagellate hosts. Since H_2 diffuses rapidly through the hindgut of termites, it is questionable that the endosymbiotic methanogens can create a boundary layer with a significantly lower H_2 partial pressure around the host flagellate cells within such a high concentration of H_2 (Breznak 2000). While the elimination of H_2 by H_2-consuming prokaryotes including methanogens seems crucially important in the gut symbiotic system, the flagellates do not need to harbor them as intracellular symbionts that occupy large spaces in the cytoplasm. Actually, in *R. flavipes* and *R. santonensis*, the majority of H_2-consuming methanogens were found on the gut epithelium and not associated with the flagellate cells (Leadbetter and Breznak 1996; Pester and Brune 2007; Brune 2010).

A clue to a factor that may benefit the flagellate hosts by the endosymbiosis with methanogens has been implied in the study of the cultured flagellate *Trichomitopsis termopsidis* derived from the gut of *Z. angusticollis*. The cultivation of *Trichomitopsis termopsidis* had been achieved by Yamin (1978). He treated a mixed culture comprising *Trichomitopsis termopsidis* and diverse gut bacteria with penicillin and streptomycin to acquire an axenic culture of *Trichomitopsis termopsidis*. Odelson and Breznak (1985a) used this *Trichomitopsis termopsidis* 6057 culture provided by Yamin, and they unexpectedly found that the "axenic" culture produced both CH_4 and H_2. This clearly indicated that the *Trichomitopsis termopsidis* 6057 culture contained methanogenic archaea, which are insensitive to penicillin and streptomycin.

In their study, the coexistence of the methanogens persisted for a long time, and the emission of CH_4 resumed even after 1 year suppression of methanogenesis by changing nutrients in the culture medium. Their attempts to isolate methanogens from the *Trichomitopsis termopsidis* 6057 culture failed, although methanogens were readily isolated from another "axenic" culture of a parabasalid flagellate, *Trichonympha sphaerica*, which had also been established from a *Z. angusticollis* gut by Yamin (1981) using the same culture condition and medium. Epifluorescence microscopy of the *Trichomitopsis termopsidis* culture failed to detect F_{420}-fluorescent cells. From these results, Odelson and Breznak (1985a) speculated that the *Trichomitopsis termopsidis* culture 6057 contained a

small amount of "endogenous" methanogens inhabiting the cytoplasm of *Trichomitopsis termopsidis* as "energy parasites similar to chlamydiae".

To eliminate the "endogenous" methanogens, Odelson and Breznak (1985a) treated *Trichomitopsis termopsidis* 6057 with bromoethanesulfonate (BES), an analog of a cofactor 2-mercaptoethanesulfonate (CoM), which inhibits methanogenesis, and they succeeded in establishing the putatively axenic culture *Trichomitopsis termopsidis* 6057C exhibiting no methanogenic activity. As described above, Lee et al. (1987) discovered that *Trichomitopsis termopsidis* in *Z. angusticollis* guts permanently harbors endosymbiotic methanogens. Hence, assuming that the "endogenous" methanogens were the intracellular symbionts that Lee and coworkers found later, the comparative experiments between *Trichomitopsis termopsidis* 6057 and 6057C conducted by Odelson and Breznak (1985a) should provide crucial information on the roles of the intracellular methanogens in the symbiosis with the flagellate host.

The elimination of methanogens with the BES treatment exhibited a drastic change in the growth of *Trichomitopsis termopsidis*. The growth rate of *Trichomitopsis termopsidis* 6057C decreased to one-eighth of that of *Trichomitopsis termopsidis* 6057. Interestingly, replacement of heat-killed rumen bacteria as a food source in the culture medium by a heat-killed eubacterial strain, *Bacteroides* sp. JW20, recovered the growth rate of *Trichomitopsis termopsidis* 6057C to a level comparable to that of *Trichomitopsis termopsidis* 6057. Various other strains of prokaryotes, including *M. hungatii*, could not replace *Bacteroides* sp. JW20. This implies that the endosymbiotic methanogens provide certain essential growth factors to the flagellate host, which could be replaced to some extent by a specific strain of heat-killed bacteria as a nutritional source. Thus, it is likely that endosymbiotic methanogens are nutritionally essential to the efficient growth of the flagellate host. A hypothesized symbiotic system between intracellular methanogens and their flagellate host is outlined in Fig. 4.

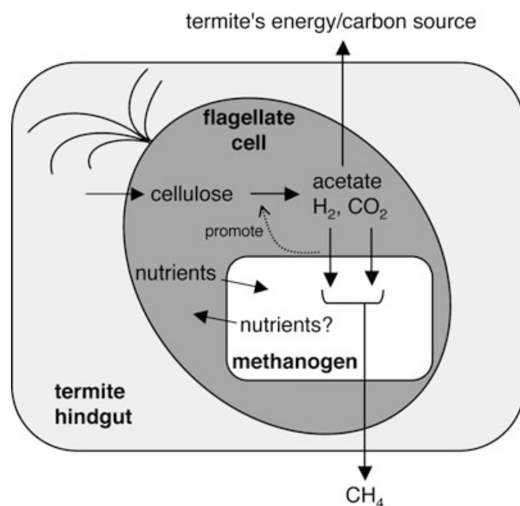

Fig. 4 Hypothesized roles of endosymbiotic methanogens. Endosymbiotic methanogens consume H_2 and CO_2, which are abundantly produced during the lignocellulose fermentation by the flagellate host, and they emit CH_4. The elimination of H_2 stimulates the fermentation process of the flagellate host. The host supplies certain nutrients to the methanogens and, in turn, the latter supply essential growth factors to the former

3 Eubacterial Symbionts of Termite Gut Flagellates

Numerous reports have been published on the eubacterial symbionts that are physically associated with the cells of the flagellates in the gut of lower termites. This section reviews the recent achievements in the study of those ecto- and endosymbionts of the flagellates on their phylogeny, localization, coevolutionary history, and functions. The functional analysis using genomics is described in Sect. 4.

3.1 Phylogeny of Ectosymbiotic Eubacteria

Most of the flagellates in the termite gut harbor ectosymbiotic eubacteria, which attach to the surface of the host cells laterally or with a tip. Numerous morphological studies have described the presence of ectosymbionts on the gut flagellates (Radek 1999), and a specific apparatus to hold the ectosymbionts has occasionally been found (Tamm 1980; Radek et al. 1996; Radek and Tischendorf 1999). The application of culture-independent molecular techniques has enabled researchers to identify these ectosymbionts phylogenetically, which is otherwise almost impossible due to the unculturability.

Among the ectosymbionts, the most frequently found groups are the genus *Treponema* in the phylum Spirochaetes and the order Bacteroidales in the phylum Bacteroidetes. In various flagellate species in the termite gut, both groups of ectosymbionts are observed on a single host cell simultaneously (Fig. 5a, b) (Hongoh et al. 2007b). Further, multiple phylotypes of treponemes have occasionally been detected on a single host flagellate cell (Noda et al. 2003), whereas only a single phylotype of Bacteroidales ectosymbionts is found on a single host cell in most cases (Stingl et al. 2004; Noda et al. 2006b, 2009; Desai et al. 2010). In the parabasalid flagellate *Caduceia versatilis* from the termite *Cryptotermes cavifrons*, an eubacterial phylotype belonging to the phylum Synergistetes has been discovered as a motility ectosymbiont (Hongoh et al. 2007a). All the cells of the host *C. versatilis* are covered with this ectosymbiont, named "*Candidatus* Tammella caduceiae", and also simultaneously covered with a Bacteroidales phylotype (Fig. 5c–f).

Two studies attempted to elucidate the evolutionary history between the Bacteroidales ectosymbionts and their flagellate hosts. Noda et al. (2009) surveyed the gut flagellate community in several termite species for the presence of Bacteroidales ectosymbionts by FISH analysis using a Bacteroidales-specific probe. The Bacteroidales-associated flagellate species were collected using a micromanipulator and subjected to 16S rRNA gene clone analysis. In total, combined with previously published data, 31 taxa of Bacteroidales ectosymbionts from 17 flagellate genera in 10 families were used for phylogenetic analysis. The results clearly indicated multiple, independent acquisitions of the Bacteroidales ectosymbionts by different

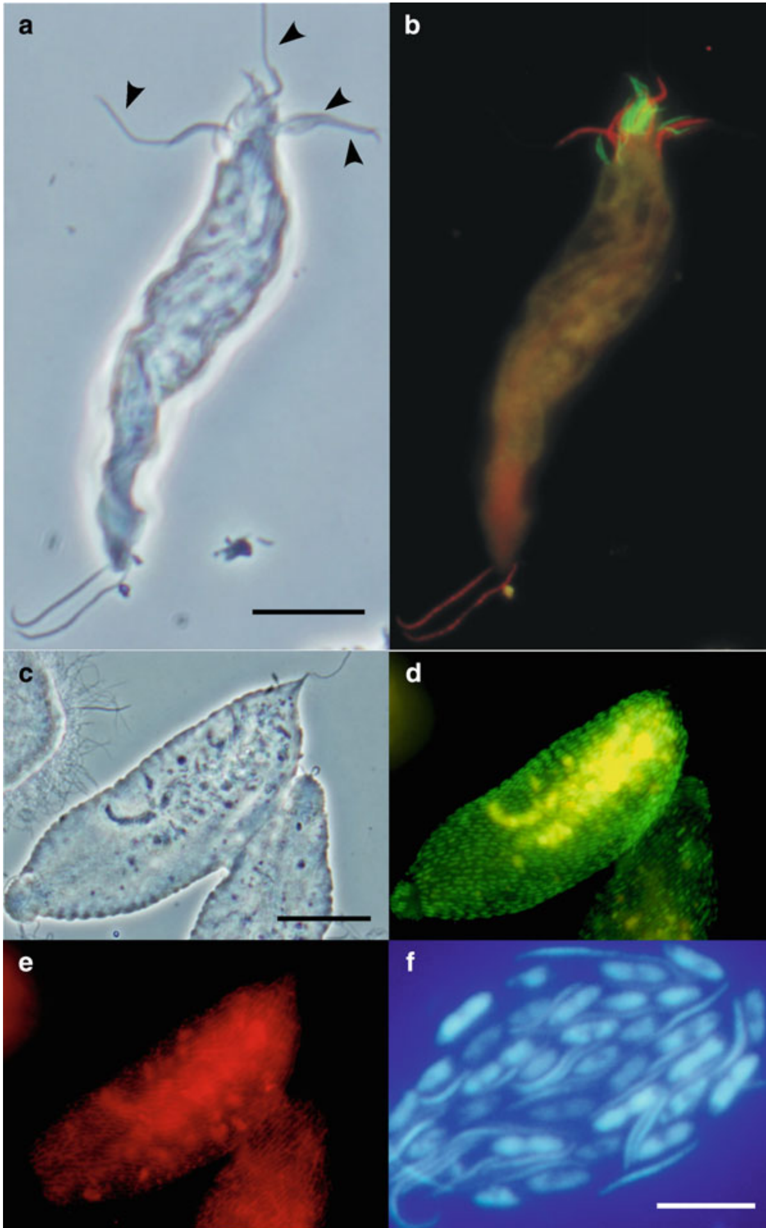

Fig. 5 Ectosymbiotic eubacteria discovered on the surface of flagellate cells from the gut of lower termites. (**a**) Phase contrast image of the oxymonad flagellate *Dinenympha porteri* from the gut of the termite *Reticulitermes speratus*. (**b**) FISH analysis of the ectosymbiotic spirochetes (*red*) and Bacteroidales bacteria (*green*) using taxon-specific probes. *Arrowheads* in panel (**a**) indicate the true flagella of the flagellate host. Bar = 10 μm in panel (**a**). (**c**) Phase contrast image of the parabasalid flagellate *Caduceia versatilis* from the gut of the termite *Cryptotermes cavifrons*.

flagellate genera. Within a host genus, however, the ectosymbionts appear to have cospeciated with their host flagellates.

The cospeciation within a host genus was examined in detail by Desai et al. (2010). They focused on the relationship between the parabasalid flagellates belonging to the genus *Devescovina* and their Bacteroidales ectosymbionts and demonstrated that the devescovinids and the ectosymbionts have strictly cospeciated. On the other hand, they also found that the Bacteroidales ectosymbionts of the oxymonad flagellates *Oxymonas* spp. do not constitute a monophyletic cluster but are derived from distantly related lineages. The latter result was consistent with a previous report that *Oxymonas* sp. from the termite *Neotermes koshunensis* harbors two distinct lineages of Bacteroidales ectosymbionts (Noda et al. 2006a). Hongoh et al. (2007b) found that a single phylotype, designated as "*Candidatus* Symbiothrix dinenymphae", resides on the cells of several distinct flagellate species belonging to the genus *Dinenympha*. These data indicate a complex evolutionary history of the symbiosis between the gut flagellates and their ectosymbiotic eubacteria.

3.2 Phylogeny of Endosymbiotic Eubacteria

In addition to the ectosymbionts, the majority of the termite gut flagellates harbor endosymbiotic eubacteria. A novel-uncultured phylum-level cluster, named Termite Group 1 (TG1), was reported for the first time in 1996 by Ohkuma and Kudo, based on 16S rRNA sequences that were obtained by PCR amplification from the gut homogenate of *R. speratus*. The specific localization of a TG1 phylotype, later named as Rs-D17 (Hongoh et al. 2003), was identified and reported in the 97th Annual Meeting of the American Society for Microbiology by Eldridge et al. (1997) and also in the 9th International Symposium on Microbial Ecology by Ohkuma et al. (2001): the TG1 phylotype is an endosymbiont of the parabasalid flagellate *Trichonympha agilis* (Fig. 6a–c).

Stingl et al. (2005) confirmed the result and further identified another TG1 phylotype as an endosymbiont of the oxymonad flagellate *Pyrsonympha veterans* in the gut of *R. flavipes*. Moreover, they found that diverse lower termites and a *Cryptocercus* cockroach harbor TG1 phylotypes specific to each host species. On the basis of the phylogenetic distance from other bacterial groups, they described this group as the candidate phylum "Endomicrobia" (later corrected from phylum to

Fig. 5 (continued) (**d**) FISH analysis of ectosymbiotic eubacteria belonging to a phylotype in the phylum Synergistetes (*green*). (**e**) FISH analysis of ectosymbiotic eubacteria belonging to a phylotype in the phylum Bacteroidetes. Bar = 50 μm in panel (**c**). (**f**) DAPI-stained ectosymbiotic bacteria on the surface of a host flagellate cell. Bar = 5 μm in panel (**f**). Panels (**a**) and (**b**) were originally published in Hongoh et al. (2007a). Panels (**c–f**) were originally published in Hongoh et al. (2007b)

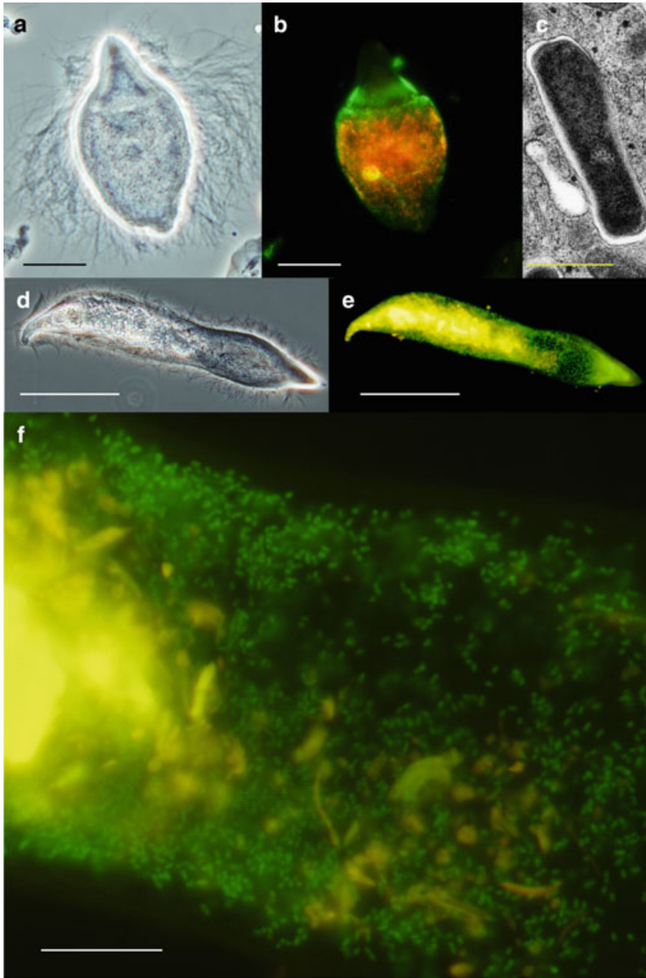

Fig. 6 Endosymbiotic eubacteria discovered inside the cells of flagellate in the gut of lower termites. (**a**) Phase contrast image of the parabasalid flagellate *Trichonympha agilis* from the gut of the termite *Reticulitermes speratus*. (**b**) FISH analysis of the endosymbiotic Termite Group 1 ("Endomicrobia") bacteria (*orange*) and other eubacteria (*green*) using taxon-specific probes. The majority of the eubacteria detected with the green signals comprise endosymbiotic *Desulfovibrio* bacteria. Bars = 20 μm. (**c**) Transmission electron microscopy of the TG1 bacteria. Bar = 0.5 μm. (**d**) Phase contrast image of the parabasalid flagellate *Pseudotrichonympha grassii* from the gut of the termite *Coptotermes formosanus*. (**e**) FISH analysis of the endosymbiotic Bacteroidales bacteria (*green*). The *yellow* color indicates the autofluorescence from phagocytosed wood particles. Bars = 50 μm. (**f**) Magnified image of (**e**). Bar = 10 μm. Panels (**a–c**) were originally published in Hongoh et al. (2008a) and panels (**d–f**) were originally published in Hongoh et al. (2008b) as supporting online materials

class; see below), and on the basis of 16S rRNA sequences and transmission electron micrographs, the phylotypes found inside *T. agilis* and *P. veterans* in *R. flavipes* guts as "*Candidatus* Endomicrobium trichonymphae" and "*Candidatus* Endomicrobium pyrsonymphae", respectively.

The localizations of TG1 phylotypes were further examined by FISH and clone analysis, and it is now recognized that TG1 bacteria are species-specific endosymbionts of various flagellates in diverse lower termites (Ikeda-Ohtsubo et al. 2007; Ohkuma et al. 2007). In general, a single host flagellate cell contains 10–1,000 of the cells of a single TG1 phylotype (Stingl et al. 2005; Ohkuma et al. 2007). Similar to the case of the Bacteroidales ectosymbionts, the evolutionary history of the symbiosis between TG1 bacteria and their flagellate hosts appears to be complicated. While it has been demonstrated that TG1 bacteria have strictly cospeciated with their host *Trichonympha* flagellates (Ikeda-Ohtsubo and Brune 2009), multiple acquisitions or horizontal transfers of TG1 bacteria are suggested in other host lineages and at the level of host families (Ikeda-Ohtsubo et al. 2007; Ohkuma et al. 2007; Desai et al. 2010).

In contrast to the TG1 bacteria, which are endosymbionts of various species of gut flagellates, the endosymbionts belonging to the Bacteroidales inhabit only the cells of the parabasalid flagellates belonging to the genus *Pseudotrichonympha* (Fig. 6d–f) (Noda et al. 2007). Further, *Pseudotrichonympha* flagellates are unique to termites in the family Rhinotermitidae (Yamin 1979; Kitade and Matsumoto 1998). Hence, the distribution of the endosymbiotic Bacteroidales bacteria is restricted. Their population, however, is huge and they are predominant in the prokaryotic gut microbiota of rhinotermitid termites. In the Formosan subterranean termite *C. formosanus*, a single cell of *Pseudotrichonympha grassii* contains up to 10^5 cells of the Bacteroidales endosymbiont, designated as phylotype CfPt1-2, and in total, it accounts for two-thirds of the prokaryotic cells in *C. formosanus* guts (Noda et al. 2005).

Noda et al. (2007) examined the evolutionary history of the symbiosis among rhinotermitid termites, the *Pseudotrichonympha* flagellates, and their Bacteroidales endosymbionts. Phylogenetic analyses showed that these three have almost completely cospeciated, implying the importance of the Bacteroidales endosymbionts to the host *Pseudotrichonympha*, as well as the importance of the *Pseudotrichonympha* to the host termites. Indeed, it has been demonstrated by selective elimination of *P. grassii* from the gut microbiota that *P. grassii* is essential for the host *C. formosanus* to feed on wood materials (Yoshimura 1995).

As in the ectosymbiosis, multiple species of endosymbionts occasionally coinhabit a single flagellate cell. For example, a *Desulfovibrio* phylotype, Rs-N31, always coexists with the TG1 phylotype, Rs-D17, inside the cells of the flagellate *T. agilis* in *R. speratus* guts (Fig. 6b) (Sato et al. 2009). A similar association among *Trichonympha* flagellates, TG1 endosymbionts, and *Desulfovibrio* endosymbionts has also been discovered in *H. sjoestedti* and *Z. nevadensis* guts. The Rs-N31 bacterium has been described as "*Candidatus* Desulfovibrio trichonymphae" on the basis of the 16S rRNA sequence and transmission electron micrographs (Sato et al. 2009).

3.3 Predicted Functions of Eubacterial Symbionts

Although the eubacterial symbionts associated with gut flagellates have never been cultured thus far, certain functions and symbiotic roles have been proposed on the basis of their taxonomic positions, localizations, functional gene marker analyses, and fragmental physiological information.

The most intriguing and clearly demonstrated function of ectosymbionts is to provide the host flagellates with the motility. The parabasalid flagellate *Mixotricha paradoxa* in the gut of the termite *Mastotermes darwiniensis* harbors three species of treponemes on the surface of its cell (Wenzel et al. 2003). Surprisingly, *M. paradoxa* cannot swim by its own flagella, but is propelled solely by a synchronized movement of the ectosymbiotic treponemes (Cleveland and Grimstone 1964). Similarly, the parabasalid flagellate *C. versatilis* in the gut of *C. cavifrons* swims only by the movement of the bundled flagella of the Synergistetes ectosymbionts "*Candidatus* Tammella caduceiae" (Fig. 5c–f) (Tamm 1982; Hongoh et al. 2007a). The detailed symbiotic mechanism, however, is totally unknown due to the unculturability of these flagellates and bacteria.

The motility symbiosis has never been observed in other treponemal ectosymbionts, and instead, other functions have been hypothesized on the basis of the physiological data obtained from cultured strains of the genus *Treponema* isolated from termite guts. Leadbetter et al. (1999) have succeeded in axenically culturing *Treponema* strains from termite gut for the first time in the world. The strains, ZAS-1 and ZAS-2 isolated from the gut of *Z. angusticollis*, later described as *Treponema primitia* (Graber and Breznak 2004; Graber et al. 2004), are homoacetogens that grow by mixotrophy using sugars or H_2 plus CO_2, and exhibit a low level of nitrogen-fixing activity. Another strain, ZAS-9 isolated also from a *Z. angusticollis* gut, later described as *Treponema azotonutricum* (Graber et al. 2004), grows by heterotrophy fermenting sugars to acetate, ethanol, CO_2, and H_2, and it exhibits a strong activity of nitrogen fixation (Lilburn et al. 2001). Recently, the third species, *Treponema isoptericolens*, has been isolated from the gut of the termite *Incistermes tabogae*, which grows by heterotrophy, fermenting sugars to ethanol and CO_2 as main products (Dröge et al. 2008).

Functional gene marker analyses suggested that the H_2-dependent reductive acetogenesis, as exhibited by *T. primitia*, is mainly conducted by treponemes in termite guts. Genes encoding formyl tetrahydrofolate synthetase (FTHFS), a key enzyme in the reductive acetogenesis, have been detected by PCR from the gut of diverse lower termites, and the majority of the FTHFS genes were identified to be originating from treponemes (Salmassi and Leadbetter 2003; Ottesen et al. 2006; Pester and Brune 2006). From these data, one can speculate that the ectosymbiotic treponemes might be involved in mutualism mediated by interspecies H_2 transfer as suggested in the endosymbiosis with methanogens and/or they might contribute to the nitrogen metabolism of the host flagellates and termites by nitrogen fixation, though no direct evidence exists.

The functions of the Bacteroidales ectosymbionts are more difficult to predict because they are only distantly related to cultured Bacteroidales strains (Noda et al. 2009). Since phagocytosis of Bacteroidales ectosymbionts by the host *Devescovina* flagellates have been observed (Noda et al. 2006b), it is conceivable that they serve as a nutrient source for the flagellate hosts. Other hypothesized functions are maintenance of the cell structure of the flagellate host by acting as exoskeletons (Radek et al. 1996; Leander and Keeling 2004) and protection of the anaerobic hosts from penetrating oxygen (Noda et al. 2006b). Inoue et al. (2007) tested hydrogenase activity of the hydrogenosome fraction and endosymbiont fraction, respectively, for the flagellate *P. grassii* from *C. formosanus* guts. As a result, the fraction of the Bacteroidales endosymbiont CfPt1-2 (Fig. 6d–f) exhibited a strong uptake-type hydrogenase activity. Thus, phylotype CfPt1-2 may contribute to the elimination of H_2 together with other H_2-consuming prokaryotes.

Sato et al. (2009) described the uncultured endosymbiotic species "*Candidatus* Desulfovibrio trichonymphae" and characterized its basic properties by functional gene marker analysis. They demonstrated that *D. trichonymphae* has potential to conduct sulfate respiration that uses H_2 as an electron donor. In addition, they speculated that *D. trichonymphae* might contribute to the detoxification of penetrating oxygen as suggested in desulfovibrios isolated from termite guts (Kuhnigk et al. 1996), which share 95% sequence similarity with *D. trichonymphae*. The physiological functions of the coexisting TG1 endosymbionts within the cells of *T. agilis* (Fig. 6b), however, had been impossible to predict until the genome analysis was performed (see below), because there has been no cultured strain closely related to the phylotypes, whereas a distantly related strain has recently been cultured and described as *Elusimicrobium minutum*, isolated from the gut of a larva of the cetoniid beetle *Pachnoda ephippiata* (Geissinger et al. 2009). Upon the description of *E. minutum*, the TG1 phylum has been described as the phylum Elusimicrobia and the endosymbiotic cluster as the candidate class "Endomicrobia" ("TG1" is used throughout this text to avoid confusion) (Geissinger et al. 2009; Herlemann et al. 2009).

4 Genomics of Endosymbionts

To analyze the functions of uncultivable microbiota, metagenomics has been applied to diverse environmental samples, including the hindgut luminal fluid of a higher termite, *Nasutitermes ephratae*. In the metagenomic study, Warnecke et al. (2007) discovered numerous genes of eubacterial origins involved in degradation of cellulose and hemicellulose, H_2 production, reductive acetogenesis, and nitrogen fixation. However, although this type of conventional metagenomics can provide a comprehensive view of the whole microbiota, the functions of individual species in the community remain almost unknown due to the enormous diversity of bacterial species and strains.

Hongoh et al. (2008a,b) proposed an alternative method, aiming to acquire the complete genome sequence of an uncultivable bacterial species from a complex community. They applied isothermal whole genome amplification (WGA) technique using Phi29 DNA polymerase (Dean et al. 2002) to obtain enough DNA from only 10^2 to 10^3 bacterial cells, while $\geq 10^{10}$ cells are required for a standard genome analysis. Two species of endosymbiotic eubacteria, Rs-D17 in the TG1 phylum (Fig. 6a–c) and CfPt1-2 in the order Bacteroidales (Fig. 6d–f), were targeted in the genome analysis.

Single cells of the host flagellates, *T. agilis* from a *R. speratus* gut and *P. grassii* from a *C. formosanus* gut, respectively, were physically isolated by micromanipulation, and the membrane was ruptured using detergent. The bacterial cells that leaked out from the single host cell were collected and subjected to WGA. From the amplified samples, a single circular chromosome (1.1 Mb) and three circular plasmids for Rs-D17 and a single circular chromosome (1.1 Mb) and four circular plasmids for CfPt1-2 were successfully reconstructed, respectively, without ambiguity (Hongoh et al. 2008a,b).

The functional annotation of both genomes revealed their basic metabolic pathways and suggested the roles in each symbiotic system. In Rs-D17, the chromosome encodes 761 protein-coding genes and additionally 121 pseudogenes. The pseudogenes comprise genes involved in functions such as DNA replication/repair, lipopolysaccharide (LPS) biosynthesis, transports, and defense mechanisms. In contrast, genes required for biosynthesis of amino acids and cofactors are abundantly retained (Hongoh et al. 2008a). These characteristics are consistent in the genome of CfPt1-2, but in addition, CfPt1-2 possesses genes for nitrogen fixation from the atmosphere and those for recycling putative nitrogen waste products of the flagellate host. Thus, it is strongly suggested that these endosymbionts of the termite gut flagellates play essential roles in the nitrogen metabolism of the flagellate hosts. Based on the genome analysis and other information, phylotype CfPt1-2 has been described as "*Candidatus* Azobacteroides pseudotrichonymphae" (Hongoh et al. 2008b).

The process of nitrogen fixation and biosynthesis of amino acids and cofactors conducted by the endosymbionts are considered to be much more stable and efficient than those conducted by free-swimming gut bacteria. The endosymbionts can utilize the ample carbon and energy sources without competition and their genomes have been reduced, streamlined, and specialized for nitrogen metabolism. Particularly in CfPt1-2, its ability to directly couple nitrogen fixation to cellulolysis enables a highly efficient growth of the host cellulolytic protist, the termite, and the termite colony, without the limitation of a nitrogen deficiency. The schematic view of the predicted roles of the endosymbiotic eubacteria is shown in Fig. 7.

5 Concluding Remarks and Future Perspectives

Most of the flagellates in termite guts harbor ecto- and endosymbiotic eubacteria and/or methanogenic archaea. During this decade, their phylogenetic and spatial structures have been revealed to some extent by using molecular analyses based on

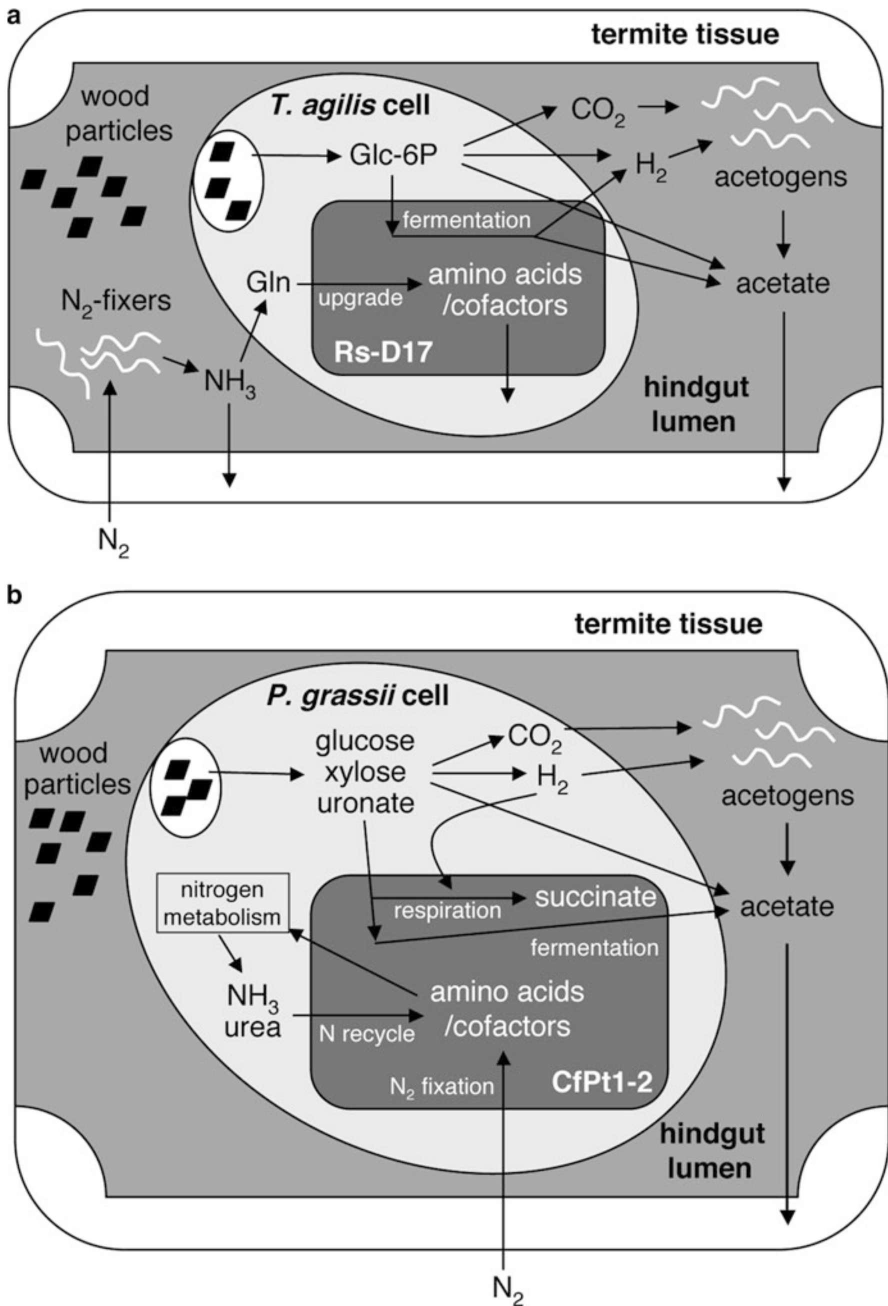

Fig. 7 Predicted functions of endosymbiotic eubacteria based on the complete genome sequences. (**a**) Predicted functions of phylotype Rs-D17 belonging to the phylum Termite Group 1 (Hongoh et al. 2008a). (**b**) Predicted functions of phylotype CfPt1-2 belonging to the order Bacteroidales (Hongoh et al. 2008b)

16S rRNA sequences. However, for these as-yet-uncultivable microbes, effective methodologies to analyze their functions had not been well developed. To overcome the difficulty, metagenomics has recently emerged and been a powerful tool to unveil the functions of bacterial communities comprising both uncultivable and cultivable strains, and it is greatly useful to capture a comprehensive view of the microbiota for their functions and diversity.

More recently, another innovative methodology for the functional analysis of uncultivable microbiota has been developed: whole genome amplification to acquire the complete genome sequence of an individual strain or a cluster of very similar strains from a small number of cells. Applying this strategy, hitherto unknown functions of eubacterial endosymbionts of termite gut flagellates have successfully been revealed: the endosymbionts contribute to the nitrogen metabolism of the flagellate host by provision of amino acids and cofactors and by nitrogen fixation from the atmosphere.

Although "single-cell genomics", which aims to obtain the complete genome sequence from a single bacterial cell, is the best way to analyze the functions of uncultured bacterial species, it takes more time (hopefully a short time) to be established for practical use. However, there are diverse endo- and ectosymbionts of flagellates in termite guts from which 10–100 cells comprising a single strain or very similar strains can be collected. Using a similar method to that described by Hongoh et al. (2008a,b), the complete genome sequences of these symbiotic prokaryotes including the endosymbiotic methanogens might be acquired. It is expected that this novel genomic approach will provide a great progress in understanding this complex, multilayered symbiotic system in termite gut.

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Methanogens in the Digestive Tract of Termites

Andreas Brune

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Abstract Methanogenesis in the enlarged hindgut compartments of termites is a product of symbiotic digestion, fueled by hydrogen and reduced one-carbon compounds formed during the fermentative breakdown of plant fiber and humus. Methanogens are not always the predominant hydrogenotrophic microorganisms, especially in wood-feeding termites, but are restricted to particular microhabitats within the gut. The methanogens in lower termites belong to different lineages of Methanobacteriales that either are endosymbionts of flagellate protists or colonize the periphery of the hindgut, a habitat that is not fully anoxic. The oxygen-reducing capacities of the few isolates so far available indicate that they are well adapted to the continuous influx of oxygen across the gut wall. Higher termites, which lack gut flagellates, often have highly compartmented guts with highly dynamic physico-chemical conditions, including redox and pH. The differences between the micro-environments are most pronounced in the soil-feeding species, where each

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compartment houses a characteristic archaeal community, comprising Methanobacteriales, Methanosarcinales, Methanomicrobiales, and a novel, deep-branching lineage of putative methanogens distantly related to the Thermoplasmatales. All clades form distinct phylogenetic clusters unique to the intestinal tract of insects, but with the exception of several *Methanobrevibacter* species, none of these archaea have been isolated in pure culture. The high methane emissions of termites, together with their enormous biomass in the tropics, make them a significant natural source of this important greenhouse gas.

1 Introduction

Most insects thriving on a fiber-rich diet harbor microbial symbionts that participate in digestion, but only termites, cockroaches, and the larvae of scarab beetles have been found to emit methane (Hackstein et al. 2006). Methane is the product of methanogenic archaea, which are the last link in an anaerobic feeding chain of microorganism located in the enlarged hindgut of these insects – a microbial bioreactor that transforms lignocellulosic matter to short-chain fatty acids, the major energy source for the host (Brune 2009b; Fig. 1).

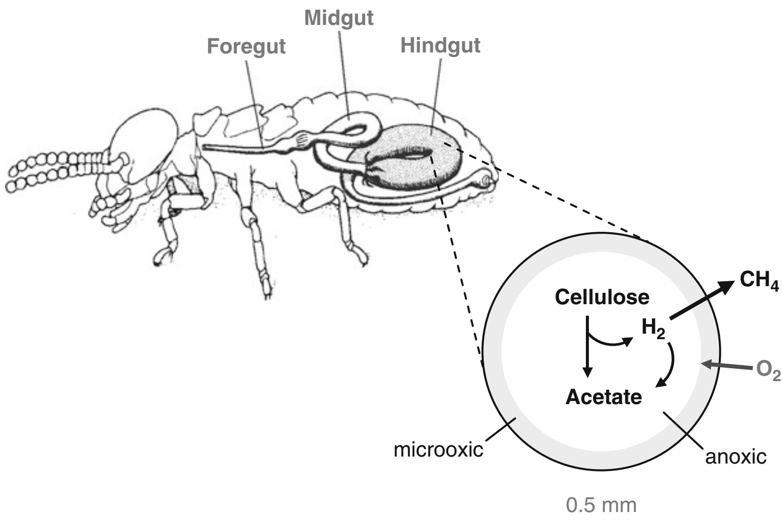

Fig. 1 The hindgut of termites is a microbial bioreactor that transforms lignocellulose to acetate and other short-chain fatty acids, which are the major energy source of the host. Hydrogen is an important intermediate of the microbial fermentations. It is converted either to acetate by homoacetogenic bacteria or to methane by methanogenic archaea. The anoxic status of the hindgut lumen is maintained by the microorganisms colonizing the microoxic hindgut periphery, which consume the oxygen that constantly diffuses into the gut [adapted from Brune and Ohkuma (2010)]

This chapter will provide an overview of the diverse aspects of methanogenesis in termites, including the role of methanogens in symbiotic digestion, the diversity and structure of the methanogenic community in different termite taxa, and recent conceptual advances concerning the interactions of methanogens with other gut microbiota and the physicochemical gut microenvironment. A more detailed treatment of certain aspects can be found in previous reviews of the literature (e.g., Breznak 2000; Brune 2006, 2009a; Purdy 2007; Brune and Ohkuma 2010).

2 Methane as a Product of Symbiotic Digestion

Methane formation in the guts of termites had been suspected already almost 80 years ago. When Cook (1932) studied the respiratory gas exchange of the lower termite *Zootermopsis nevadensis*, he found that the termites continued to form substantial amounts of an unidentified gas when the oxygen in the vessel was depleted. He was not able to analyze the gas, but inspired by the situation in ruminants, proposed that the gas was most likely hydrogen or methane, or a mixture of both. However, it took more than 40 years after Cook's initial observation until methane production in termite guts was finally recognized by Breznak and coworkers. While demonstrating nitrogenase activity in living termites and wood-feeding cockroaches with the acetylene reduction assay, the authors identified methane as an additional peak present in the gas chromatograms (Breznak et al. 1973, 1974) – a classical case of serendipity in science (see Brune 2009a for historical details).

Breznak (1975) had pointed out that the amount of methane produced by termites, if based on body weight, is in the same order of magnitude as that of ruminants. This observation immediately aroused the interest of atmospheric chemists studying the role of methane in radiative forcing of the atmosphere, and termites were identified as a potential source of considerable strength of this greenhouse gas (see also below). In the following years, methane production was found among almost all termite species investigated (e.g., Brauman et al. 1992; Shinzato et al. 1992; Wheeler et al. 1996; Bignell et al. 1997; Sugimoto et al. 1998b), although there were marked differences between taxa that seem to be related to the composition of the diet (wood vs. humus; Fig. 2).

Methane is formed by methanogenic archaea by two fundamentally different processes: (1) the reduction of CO_2 or other C_1 compounds to CH_4 via the C_1 pathway (hydrogenotrophic methanogenesis), and (2) the cleavage of acetate to CH_4 and CO_2 via the acetyl-CoA pathway (aceticlastic methanogenesis) (Hedderich and Whitman 2006; Liu and Whitman 2008). Interestingly, there is no evidence for aceticlastic methanogenesis in termite guts. As in the human gut and in the rumen, it is assumed that the relatively slow-growing aceticlastic species cannot cope with the short retention times of intestinal habitats (Lange et al. 2005; Liu and Whitman 2008). However, this does not explain why they could not avoid washout by attaching to intestinal surfaces (see below).

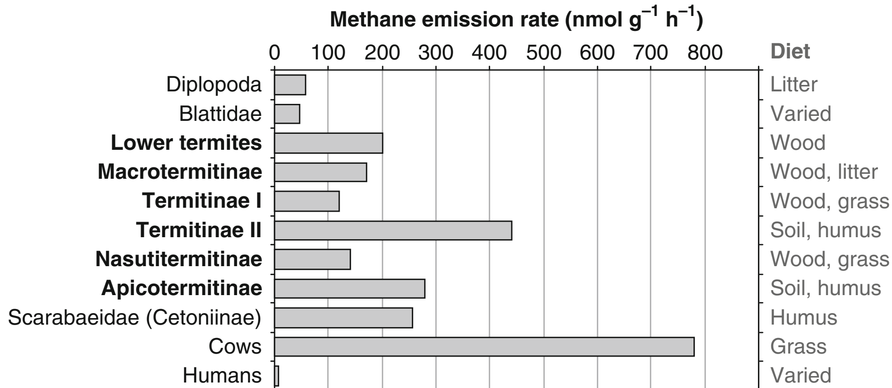

Fig. 2 Methane emission rates of termites (taxa shown in *bold*) in comparison to that of other invertebrates, cows, and humans, and the typical diet of the respective taxon. Termitinae were grouped into wood-feeding (I) and humus-feeding (II) species. Values are averages, based on fresh weight, and were compiled from various sources [for details, see Brune (2009a)]

The most important electron donors of hydrogenotrophic methanogenesis are hydrogen and reduced C_1 compounds, such as methanol and formate, which are formed during the fermentative breakdown of organic matter. In the hindguts of lower termites, hydrogen is a major fermentation product of cellulolytic flagellates and can accumulate to substantial concentrations (Ebert and Brune 1997; Pester and Brune 2007). Although methane production strictly depends on the presence of (hydrogen-producing) gut flagellates (Odelson and Breznak 1983; Rasmussen and Khalil 1983; Messer and Lee 1989), the rates are much lower than one would expect based on the large amount of hydrogen presumably formed by these protists. If the termites are fed with antibacterial drugs, both hydrogen and methane emission rates increase strongly, which suggests that the methanogenic archaea compete with bacteria for the hydrogen formed by the flagellates (Odelson and Breznak 1983). In the phylogenetically higher termites (family Termitidae), which lack gut flagellates, the substrates of methanogens are most likely formed by fermenting bacteria. Also in these taxa, methanogenesis in intact guts or gut homogenates is strongly stimulated by the supply of external hydrogen, as well as by formate (Brauman et al. 1992; Schmitt-Wagner and Brune 1999).

The process responsible for bacterial hydrogen oxidation in termite guts is reductive acetogenesis (Breznak and Switzer 1986). It is a unique feature of termite guts that the bacteria responsible for this activity are members of the Spirochaetes (Leadbetter et al. 1999). While CO_2-reductive acetogenesis seems to predominate over methanogenesis as the hydrogenotrophic process in most wood-feeding termites, the opposite is true for most fungus-cultivating and soil-feeding termite species, both in gut homogenates and in situ (Breznak and Switzer 1986; Brauman et al. 1992; Tholen and Brune 1999, 2000; Pester and Brune 2007). Despite the apparent substrate limitation of methanogenesis in termite guts, termites emit hydrogen in considerable amounts (Zimmerman et al. 1982; Odelson and Breznak 1983; Ebert and Brune 1997;

Sugimoto et al. 1998b; Schmitt-Wagner and Brune 1999; Pester and Brune 2007), which indicates that production and consumption of hydrogen in the hindgut are not tightly coupled (see below).

3 Diversity of Methanogens in Termite Guts

The methanogens in termite guts comprise representatives from almost all major lineages of methanogenic archaea; only Methanococcales seem to be absent (Fig. 4). In view of this considerable diversity, the number of methanogens from termite guts ever isolated in pure culture is quite disenchanting. There are only three described species, all of which belong to the genus *Methanobrevibacter* (Methanobacteriales), and all have been obtained from the same host species, the lower termite *Reticulitermes flavipes* (Leadbetter and Breznak 1996; Leadbetter et al. 1998; Fig. 3). Like other *Methanobrevibacter* species isolated from the human gut or the rumen, the isolates show a very restricted substrate spectrum, growing exclusively on $H_2 + CO_2$ (*Methanobrevibacter cuticularis* also grows, albeit poorly, on formate). The only other methanogen isolated from insect guts is *Methanomicrococcus blatticola* from the cockroach *Periplaneta americana*, the first cultivated representative of a novel lineage of Methanosarcinales that is present also in higher termites (see below). It differs from the *Methanobrevibacter* species in its inability to grow on $H_2 + CO_2$. Instead, it is specialized in the obligately hydrogen-dependent reduction of methanol or methylamines to methane (Sprenger et al. 2000). The strict requirement for hydrogen in methanogenesis is explained

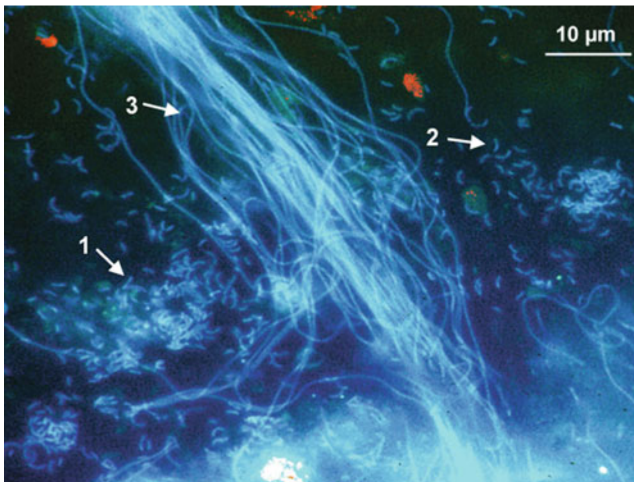

Fig. 3 Methanogens associated with the hindgut wall of *Reticulitermes flavipes*, photographed by the autofluorescence of their cofactor F_{420}. The *arrows* point to the characteristic morphotypes of *Methanobrevibacter cuticularis* (1), *Methanobrevibacter curvatus* (2), and *Methanobrevibacter filiformis* (3). Microphotograph courtesy of J.R. Leadbetter and J.A. Breznak

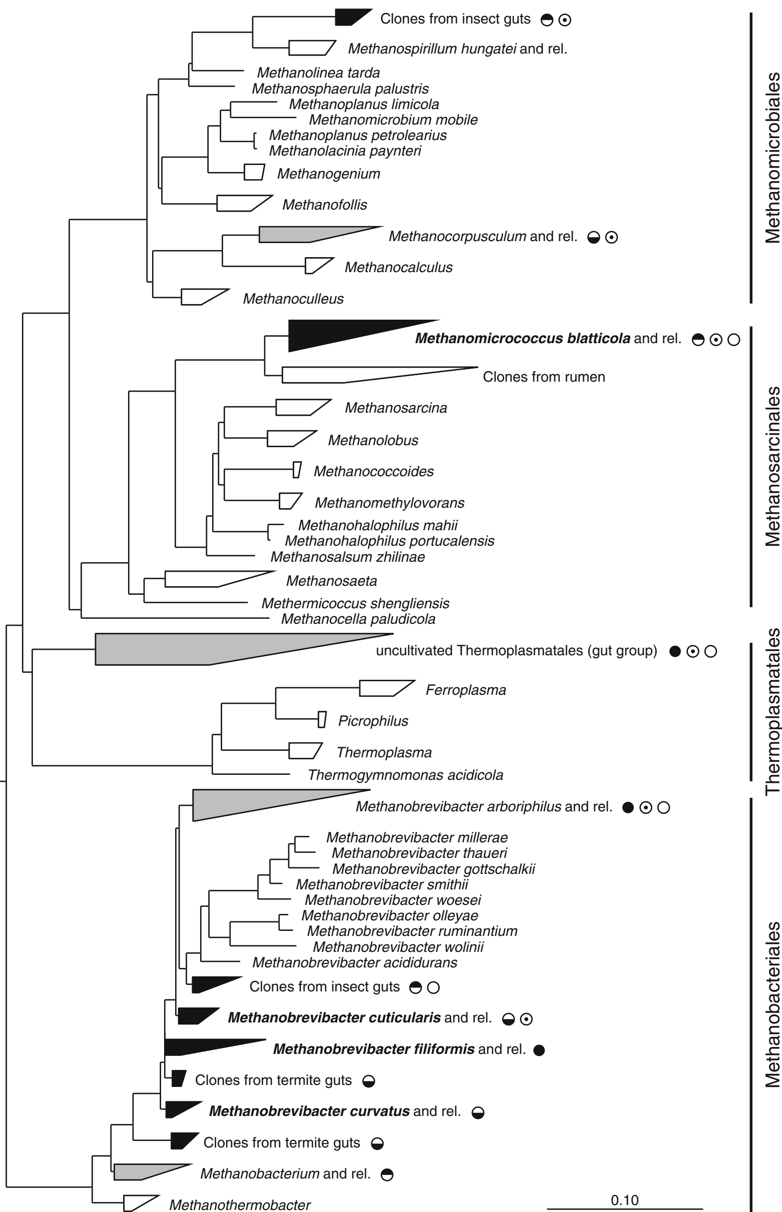

Fig. 4 Major lineages of Euryachaeota harboring representatives of putative methanogens from termite guts. Lineages marked in *black* consist exclusively of clones from insect guts and those marked in *gray* also contain clones or isolates from other environments. *Symbols* indicate the origin of the clones (*Circle with lower half black*, lower termites; *circle with upper half black*, higher termites; *filled circle*, both lower and higher termites; *circled dot*, cockroaches; *open circle*, other insects). The species isolated from insect guts are listed in *bold*

by its inability to oxidize methyl groups to carbon dioxide (Sprenger et al. 2005). The substrate affinities of *M. blatticola* for hydrogen and methanol are higher than those of other methylotrophic methanogens (*Methanosphaera stadtmanae*, *Methanosarcina barkeri*), and since the use of methanol as the terminal electron acceptor is thermodynamically more favorable than the use of carbon dioxide, *M. blatticola* may have a competitive advantage over other methanogens at low hydrogen concentrations (Sprenger et al. 2007).

The methanogens colonizing the hindgut of lower termites belong almost exclusively to the genus *Methanobrevibacter* (Methanobacteriales; Fig. 4). Many lineages of *Methanobrevibacter*-related sequences have been detected by cultivation-independent, 16S rRNA-based surveys (Ohkuma et al. 1995, 1999; Ohkuma and Kudo 1998; Shinzato et al. 1999, 2001), documenting the presence of unique *Methanobrevibacter*-related phylotypes in each lower termite investigated (reviewed by Dighe et al. 2004). Many termites harbor more than one lineage of *Methanobrevibacter*, and selective cloning of archaeal 16S rRNA genes from capillary-picked suspensions of gut flagellates (Tokura et al. 2000) revealed that the phylotypes associated with the flagellates are phylogenetically distinct from those that are attached to the hindgut cuticle or to filamentous bacteria at the gut wall. The *Methanobrevibacter* phylotypes associated with distantly related flagellates form a monophyletic cluster, which indicates that each of the *Methanobrevibacter* lineages within the same termite may have a preference for a particular microhabitat (Tokura et al. 2000; Hara et al. 2004; Inoue et al. 2008).

The methanogenic communities in the hindgut of higher termites are much more diverse. 16S rRNA-based analyses revealed the presence of Methanomicrobiales, Methanosarcinales, and Methanobacteriales in the wood-feeding species *Nasutitermes takasagoensis* (Nasutitermitinae) (Ohkuma et al. 1999; Miyata et al. 2007) and the soil-feeding species *Pericapritermes nitobei* (Ohkuma et al. 1999), *Cubitermes orthognathus* (Friedrich et al. 2001), and *Cubitermes fungifaber* (Donovan et al. 2004) (all Termitinae). The fungus-cultivating *Odontotermes formosanus* (Macrotermitinae) is an exception in that only Methanosarcinales have been recovered (Ohkuma et al. 1999). A general absence of methanogens of other orders from the Macrotermitinae is corroborated by the finding that the amount of rRNA of Methanosarcinales recovered from the gut of the fungus-cultivating *Macrotermes subhyalinus* was in the same range as the total amount of archaeal rRNA obtained from this termite (Brauman et al. 2001). The microbial diversity in representatives of the fourth subfamily of higher termites (Apicotermitinae), which are mostly soil-feeding, has not been investigated. The Methanobacteriales clones obtained from higher termites fall into the radiation of the genus *Methanobrevibacter*, but are phylogenetically distinct from their relatives in the lower termites and other insects. The clones of Methanomicrobiales and Methanosarcinales recovered from higher termites are not represented among the lower termites, but cluster with clones obtained from cockroaches and scarab beetle larvae (e.g., Hara et al. 2002; Egert et al. 2003), including also *M. blatticola* isolated from the cockroach *P. americana* (Fig. 4).

In a comprehensive survey of numerous species of lower and higher termites using dot-blot hybridization with group-specific probes, rRNA of Methanobacteriales was detected in the guts of most termite species studied, regardless of diet or taxonomic classification (Brauman et al. 2001). However, Methanosarcinales were detected only in about half of the species, and a signal for Methanomicrobiales was not obtained. The reasons for these discrepancies are not clear, but the large gap between the combined hybridization signals of the group-specific probes and the archaeal domain probe observed with almost all termites investigated may reflect the presence of other (nonmethanogenic) archaea, which were presumably not covered by the group-specific probes. In several of the clone-based studies of archaeal diversity in termites and other insects (see above), an additional, deep-branching clade of uncultivated archaea only distantly related to cultivated members of the Thermoplasmatales was discovered (Fig. 4); this clade formed a substantial fraction of the clones in the respective libraries and also comprises clones obtained from the guts of mammals. It is not clear whether the clade represents methanogenic or nonmethanogenic archaea.

4 Differences in Methanogenic Activities and Populations

Information on the population sizes of methanogens in insect guts is scarce. Cultivation-based studies indicate that *R. flavipes* harbors about 10^6 methanogens per gut, which would represent about 5% of the total prokaryote cell count (Leadbetter and Breznak 1996; Tholen et al. 1997). Such numbers are not very accurate because of the difficulties in enumeration created by the attachment of methanogens to intestinal surfaces and the uncertainties surrounding the determination of the total cell number of prokaryotes, most of which are intimately associated with the flagellate cells that occupy the bulk of the hindgut volume. Hybridization of RNA extracted from the guts of a wide range of termite species with domain-specific oligonucleotide probes indicated that archaeal rRNA was on average only 1.5% of all prokaryotic rRNA (Brauman et al. 2001). Although not all the archaea in termite guts are necessarily methanogenic (see above), the higher fraction of archaeal rRNA in soil-feeding species ($2.3 \pm 0.5\%$) than in wood-feeding and fungus-cultivating species ($0.9 \pm 0.5\%$) is in agreement with a general trend toward higher methane emission rate among termites with a humivorous lifestyle (Fig. 2).

Since soil-feeding termites, in contrast to their wood- and litter-feeding relatives, digest peptide-rich soil organic matter (Ji and Brune 2006), it is tempting to suggest that these differences are diet related. However, information on the fermentative processes in the hindguts of humivorous insects is sparse, and also the biology of the mostly uncultivated methanogens in higher termites has to be better understood before a reasonable hypothesis can be proposed. Such knowledge may also help to clarify whether the presence of methanogens provides a competitive advantage to their respective hosts. Elimination of methanogens from *Zootermopsis angusticollis* by feeding with bromoethanesulfonic acid (BES) did not affect the survival of the

termites (Messer and Lee 1989). In some species of lower termites, not all colonies were colonized by methanogens, and trends in methane emission among members of the same genus or even species were not always consistent (e.g., Shinzato et al. 1992; Wheeler et al. 1996).

5 Coexistence with Homoacetogens

The predominance of reductive acetogenesis over methanogenesis in most wood-feeding termites has puzzled microbiologists for the longest time. For thermodynamic reasons, methanogens should always outcompete homoacetogens for hydrogen, their common substrate — at least in a well-mixed system. However, the introduction of microsensor techniques into termite gut research led to the recognition that termite guts are spatially structured microenvironments characterized by steep diffusion gradients of metabolites (see Brune 1998; Brune and Friedrich 2000), which brought conceptual advances that allowed the coexistence of methanogens and homoacetogens in this habitat to be explained.

First, it turned out that hydrogen concentrations in termite guts are much higher than originally considered — far above the threshold concentrations at which methanogens can outcompete homoacetogens for hydrogen. At the hydrogen partial pressures observed in the hindgut proper of several lower termites (1–100 kPa; Ebert and Brune 1997; Pester and Brune 2007), both processes would operate at substrate saturation, and a direct competition for hydrogen cannot occur. Therefore, explanations of the predominance of reductive acetogenesis as the hydrogenotrophic process that are based on the ability of homoacetogens to grow mixotrophically on H_2 and other substrates (Breznak 1994) are no longer applicable.

Second, high-resolution profiles of hydrogen concentration in the intestinal tracts of lower and higher termites (Ebert and Brune 1997; Schmitt-Wagner and Brune 1999; Pester and Brune 2007) and rate measurements of reductive acetogenesis by microinjection of radiotracers (Tholen and Brune 1999, 2000; Pester and Brune 2007) documented that sources and sinks are not evenly distributed within the termite gut. The high hydrogen concentrations in the hindgut paunch and the steep hydrogen gradients toward the gut periphery of *Reticulitermes* spp. are in agreement with the location of hydrogen-producing flagellates and (in part) homoacetogenic spirochetes in the gut lumen, and with the absence of any stimulatory effect of externally supplied hydrogen on the in situ rates of reductive acetogenesis. In contrast, the strong hydrogen sink at the hindgut wall of *R. flavipes*, which is clearly caused by an anaerobic process (Ebert and Brune 1997), together with the dense colonization of the cuticle with *Methanobrevibacter* species (Leadbetter and Breznak 1996; Leadbetter et al. 1998), is in agreement with the strong stimulation of methanogenesis by externally supplied hydrogen in this and other termites.

The spatial separation of the two hydrogenotrophic processes – reductive acetogenesis in the hydrogen-rich gut lumen and methanogenesis in the hydrogen-poor gut periphery – precludes any direct competition for hydrogen between

homoacetogens and methanogens (Fig. 5). Although this scenario provides the answer to the original question concerning the basis for the apparent outcompetition of methanogens by homoacetogens for their common substrate, it remains to be explained why the homoacetogens are able to colonize the hydrogen-rich gut lumen, whereas the methanogens (unless associated with gut flagellates) are not. In this context, it is important to recall that the termite gut is unusual not only with respect to the predominance of reductive acetogenesis over methanogenesis but also in the abundance of spirochetal life forms (Lilburn et al. 1999; Breznak 2000). So far, the termite gut is also the only habitat that harbors spirochetes capable of reductive acetogenesis (Leadbetter et al. 1999; Breznak and Leadbetter 2006). Diversity studies and expression analysis of FTHFS genes, the functional markers of reductive acetogenesis, have revealed that termite gut treponemes predominate over homoacetogenic firmicutes in all termites studied (Salmassi and Leadbetter 2003; Ottesen et al. 2006; Pester and Brune 2006; Warnecke et al. 2007). Apparently, these highly motile spirochetes are well adapted to actively maintain their position in the hindgut lumen, whereas methanogens must attach to surfaces to prevent washout – they can colonize the gut lumen only by associating with the gut flagellates or (in higher termites) with cuticular spines protruding from the gut wall into the lumen (Bignell et al. 1980).

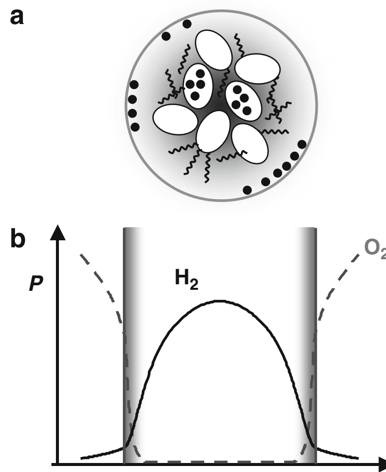

Fig. 5 Schematic cross section (**a**) of an agarose-embedded hindgut (paunch region) of a wood-feeding lower termite (*Reticulitermes* spp.), illustrating the location of methanogens (*filled circles*) attached to the hindgut wall and homoacetogenic spirochetes (*spirals*) within the gut proper. In some species, methanogens are also associated with the gut flagellates (*white ovals*). Radial profiles (**b**) of oxygen and hydrogen partial pressure (P) document that the respiratory activity of the gut microbiota maintains steep oxygen gradients (*dashed line*) within the gut periphery, rendering the center anoxic. Hydrogen (*solid line*) formed by the flagellates accumulates at the gut center but is consumed throughout the entire gut. The strong hydrogen sink below the gut wall is probably caused by methanogens, which prevent larger amounts of H_2 from escaping into the atmosphere [Scheme from Brune and Ohkuma (2010)]

6 Association with Gut Flagellates

In many anoxic environments, methanogens are associated with anaerobic protists (van Hoek et al. 2000; Hackstein et al. 2001; Fenchel and Finlay 2010). The association of methanogens with the gut flagellates of lower termites is a common phenomenon (Odelson and Breznak 1985; Messer and Lee 1989; Shinzato et al. 1992; Hackstein and Stumm 1994; Radek 1994, 1997; Tokura et al. 2000; Hara et al. 2004; Hongoh and Ohkuma 2010), although the hindgut cuticle or the surface of filamentous bacteria colonizing the gut wall remain their typical habitats (Hackstein and Stumm 1994; Leadbetter and Breznak 1996; Leadbetter et al. 1998). Since methanogens located in the gut periphery are clearly hydrogen limited (see above), Sugimoto et al. (1998b) suggested that the rates of hydrogen and methane emission of different termite species may depend on the particular location of methanogens relative to the hydrogen source.

Generally, only the smaller species of termite gut flagellates are associated with methanogens. Lee et al. (1987) investigated the colonization of gut flagellates by methanogens in the hindgut of *Z. angusticollis* by epifluorescence microscopy and reported that only the small trichomonadid flagellates *Trichomitopsis termopsidis*, *Tricercomitus termopsidis*, and *Hexamastix termopsidis* were associated with cells showing the characteristic F_{420} autofluorescence of methanogens. The larger hypermastigotes, which appeared to be the major hydrogen source (Messer and Lee 1989), usually lacked methanogenic symbionts. Similar observations were made by Tokura et al. (2000) with *Reticulitermes speratus*, where the methanogens were regularly associated with the oxymonadid *Dinenympha parva* and a small hypermastigote *Microjoenia* sp., and with *Hodotermopsis sjoestedti*, where the methanogens were associated with *Dinenympha* sp. and *Microjoenia* sp. in large abundance. In all cases, the methanogens seemed to be located within the host cells, which is in agreement also with ultrastructural data reported by Lee et al. (1987).

Odelson and Breznak (1985) were the first to note that a putatively axenic culture of *Trichomitopsis termopsidis*, a gut flagellate isolated from a *Zootermopsis* species, contained a methanogenic symbiont. The symbiosis was not obligate because cultures continued to grow after they were cured of the methanogenic symbiont. Nevertheless, growth yields of *Trichomitopsis termopsidis* increased when the flagellate was cultivated in the presence of the methanogen *Methanospirillum hungatei*, which suggested that the flagellates may benefit in a similar manner from their methanogenic symbiont. There are reports from other environments that indicate that methanogens associated with eukaryotic partner organisms may benefit from an interspecies hydrogen transfer, and the stimulation of fermentative processes by end product removal (hydrogen, formate) may even result in a mutual advantage (see Schink 1997; Worm et al. 2010). However, considering the high hydrogen concentrations throughout the gut lumen of lower termites, it is not clear whether termite gut flagellates indeed benefit from the hydrogen-consuming activity of their methanogenic symbionts under in situ conditions. At the same time, this

would mean that the methanogens associated with gut flagellates are never hydrogen-limited as long as they can maintain their position in the hydrogen-rich gut lumen, no matter whether their particular host is producing hydrogen or not. From that perspective, the association of *Methanobrevibacter* species with gut flagellates may simply serve to maintain a stable position in the anoxic and hydrogen-rich hindgut lumen, an argument that may apply also to other, nonmethanogenic prokaryotes commonly associated with such protists (Brune and Stingl 2005; Hongoh and Ohkuma 2010).

7 Intercompartmental Transfer of Hydrogen

The gut of higher termites is characterized by the absence of cellulolytic flagellates and shows (with the exception of the fungus-cultivating species) also a pronounced compartmentation, which goes hand in hand with a remarkable dynamics of intestinal pH and redox potential (Fig. 6). In soil-feeding *Cubitermes* species, hydrogen production and consumption are spatially separated in different gut compartments (Schmitt-Wagner and Brune 1999; Tholen and Brune 1999). The strong stimulation of both methanogenesis and reductive acetogenesis by external hydrogen added to intact gut compartments led to the hypothesis that hydrogen diffuses across the gut epithelia between hydrogen-producing and hydrogen-consuming gut regions, which are in close contact in situ. Such cross-epithelial transfer of reducing equivalents has been documented in detail in cockroaches and scarab beetle larvae (Lemke et al. 2001, 2003) and would explain the low hydrogen and high methane emissions of such soil-feeding termites. Since methanogenesis in the posterior hindgut is stimulated not only by hydrogen but also by formate, which accumulates to considerable concentrations in other gut compartments, there may also be an intercompartmental transfer of reducing equivalents via the hemolymph (Schmitt-Wagner and Brune 1999).

A detailed analysis of the archaeal community structure in the different gut compartments of *C. orthognathus* showed that the different phylogenetic groups are not evenly distributed among the different compartments (Friedrich et al. 2001). Each of the individual gut compartments harbors a distinct assemblage of euryarchaeota (Fig. 6d). Methanosarcinales colonize the anterior, extremely alkaline hindgut compartment (P1), whereas Methanobacteriaceae and Methanomicrobiales predominate in the posterior compartments (P3 and P4). Members of a deep-rooting clade of putative methanogens distantly related to the Thermoplasmatales (see above) increase toward the rectum (P5). Many of the microbial cells attached to the gut wall or cuticular spines projecting from the hindgut wall into the lumen are putative methanogens based on their characteristic UV-fluorescence (Schmitt-Wagner and Brune 1999), but are yet to be assigned to the different phylogenetic groups.

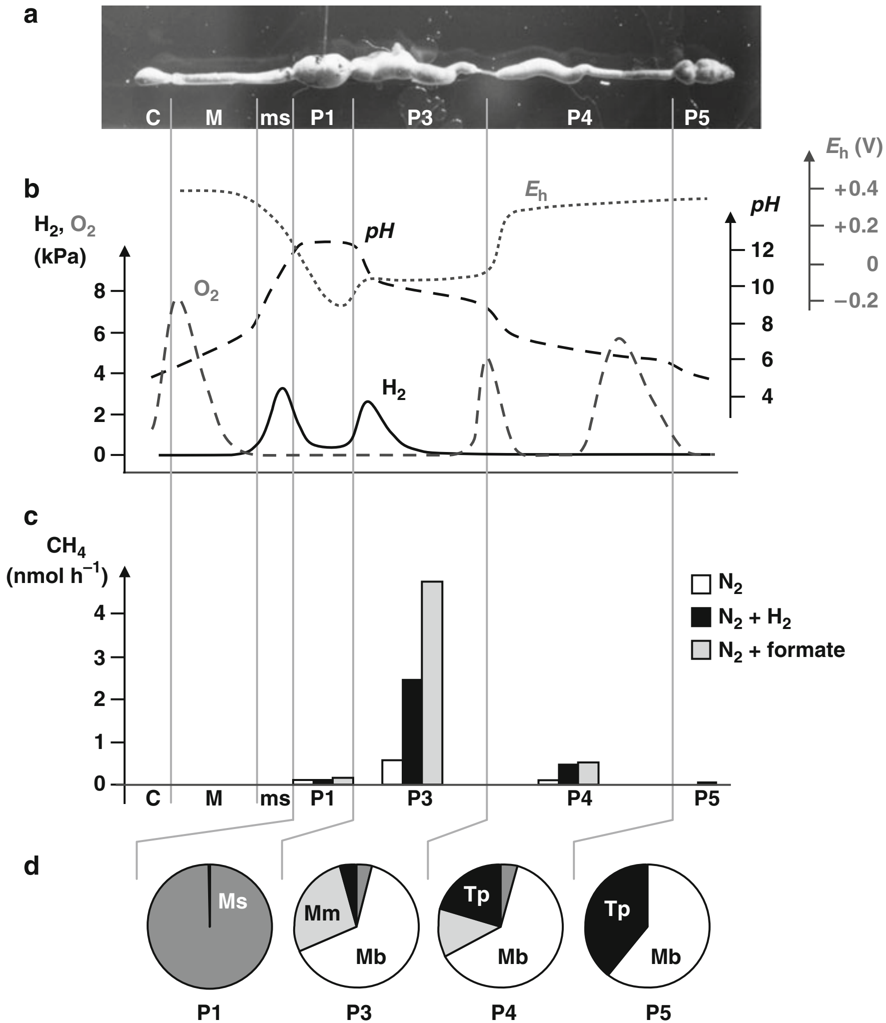

Fig. 6 Gut morphology (**a**) and axial profiles (**b**) of different physicochemical parameters along the gut axis of a soil-feeding termite (*Cubitermes* spp.). Oxygen and hydrogen partial pressures, intestinal pH, and apparent redox potential (against a standard hydrogen electrode) were measured with microsensors. The gut was stretched out and embedded in agarose-solidified Ringer's solution. Methane emission rates (**c**) were determined with isolated gut sections incubated under a N_2 headspace with or without addition of H_2 or formate. Relative abundance of euryarchaeotal clones in 16S rRNA gene libraries of the respective sections (*Ms* Methanosarcinales, *Mb* Methanobacteriales, *Mm* Methanomicrobiales, *Tp* Thermoplasmatales-related clade). The borders between the different gut regions are indicated by the *vertical lines* [scheme based on data from Brune and Kühl (1996), Schmitt-Wagner and Brune (1999), Friedrich et al. (2001) and Kappler and Brune (2002)]

8 Relationship to Oxygen

As obligate anaerobes, the methanogens in termites are restricted to the hindgut, the only gut region characterized by a negative redox potential (Ebert and Brune 1997; Kappler and Brune 2002). It is not clear why they are regularly (in some cases exclusively) located at the hindgut wall, a microhabitat that is characterized by the constant influx of oxygen across the epithelium (Brune 1998). Like all other methanogens, the three *Methanobrevibacter* species colonizing the gut epithelium of *R. flavipes* (Leadbetter and Breznak 1996; Leadbetter et al. 1998) (and also *M. blatticola* colonizing the hindgut epithelium of cockroaches; Sprenger et al. 2000) do not grow in media containing even traces of oxygen and are much more sensitive to oxygen accumulation than the homoacetogenic *Sporomusa* species isolated from termite guts (Boga and Brune 2003). However, *Methanobrevibacter* species remain metabolically active in dense cell suspensions that are exposed to controlled oxygen fluxes as long as the influx of oxygen does not exceed their capacity for oxygen removal (Tholen et al. 2007), whereas reductive acetogenesis of *Sporomusa* species is inhibited even at the lowest oxygen fluxes. It has been proposed that the redirection of electron flow from methanogenesis toward oxygen reduction enables *Methanobrevibacter* species to colonize the hindgut periphery of termites. The mechanisms of tolerance to reactive oxygen species and the biochemistry of oxygen reduction in *Methanobrevibacter* species have been discussed elsewhere in detail (see Brune 2009a).

Nevertheless, the location of methanogens at the gut wall of lower termites, at the unfavorable end of the outwardly directed hydrogen gradient, remains enigmatic. It has been suggested that an attachment to the hindgut cuticle may protect against predation or prevent washout from the gut, which may compensate methanogens for the negative effects of hydrogen limitation and exposure to inflowing oxygen (Breznak 2000). In higher termites, the explanation for the colonization of the hindgut cuticle may lie also in the putative transfer of hydrogen between different compartments. The microorganisms located at the gut wall may be at the bottom end of the radial hydrogen flux from the gut proper, but may benefit from external hydrogen entering the hindgut by cross-epithelial transfer from other compartments (see above).

9 Termites as a Source of Atmospheric Methane

Although the countergradients of methane and oxygen in the hindgut periphery provide seemingly ideal conditions for aerobic methane oxidation (Brune et al. 2000), there is no evidence for the presence of methanotrophic bacteria or their activities in termite guts (Pester et al. 2007). This means that the different methane emission rates of termites from different feeding guilds directly reflect differences in methane production within their intestinal tract. In the past, many attempts were made to extrapolate from the results of laboratory measurements of methane emissions to the contribution of termites to the global methane budget, but even

the most recent estimates are still far from accurate and suffer from numerous biases (see Sanderson 1996; Bignell et al. 1997). Sugimoto and colleagues demonstrated that it is very important to consider methane oxidation in the mound material and the surrounding soil as an important factor mitigating methane production by termites at the environmental level (Sugimoto et al. 1998a, 2000). As a consequence, the net emissions of methane from intact colonies of soil-feeding termites are much lower than those of wood-feeding termites, although the opposite would be predicted from the gross methane emission rates determined with individual termites in the laboratory.

In view of the grossly overestimated contribution of termites to global methane emissions into the atmosphere propagated in the older literature (see Collins and Wood 1984), it is important to note that the most recent estimates place these rates at probably less than 10 Tg per year (1.5–7.4 Tg; Sugimoto et al. 1998b) and almost certainly below 20 Tg per year (a number that is still used in the last global budget published by the IPCC; Denman et al. 2007). Nevertheless, termites remain the second largest natural source of methane on the planet, although their contribution to the total source strength (ca. 600 Tg per year) is certainly dwarfed by the sources under anthropogenic influence (such as the ruminants). A more detailed review of the literature on this subject can be found elsewhere (Brune 2009a).

10 Conclusions

Termites are a significant source of methane in tropical ecosystems. Methane and short-chain fatty acids are formed from lignocellulosic matter by an anaerobic feeding chain of microorganisms located in the highly enlarged hindguts. However, termite hindguts are not purely anoxic fermentors. The gut habitat is characterized by the continuous influx of O_2 across the gut wall and steep hydrogen gradients between gut lumen and periphery. Despite the high hydrogen concentrations in the gut lumen, methanogens are not the predominant hydrogenotrophic microorganisms in lower termites. The ability to attach to biotic or abiotic surfaces or to colonize the cytoplasm of flagellate protists may be an important factor in the successful colonization of the intestinal tract. In higher termites, which lack gut flagellates, the increased methane production is correlated with a dietary shift from wood to humus. The assemblage of methanogenic archaea is more diverse and distributed among several consecutive gut compartments characterized by pronounced axial dynamics of physicochemical parameters, including redox and pH, and (in the case of soil-feeding termites) a cuticle sometimes adorned with cuticular spines. The drivers determining archaeal community structure in the different microhabitats are not clear, but may involve the availability of and competition for methanogenic substrates and differences in adaptation to oxidative stress and other factors imposed by the respective environments. Since many methanogens in termite guts belong to taxa without any cultured representatives, more isolates are sorely needed to address these questions.

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Methanogenic Archaea in Humans and Other Vertebrates

Everly Conway de Macario and Alberto J.L. Macario

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Abstract The presence of methane in biological samples had been detected many years ago and it was believed that the gas could be either of chemical or microbial origin. Detection of methane-producing microbes (methanogens) in samples from animals intensified since the last part of the previous century, going from cultural-physiological characterization and isolation of microbes to further characterization of the isolates at the biochemical, immunological, molecular biologic-genetic, and phylogenetic levels. In this Chapter, we report about methanogens identified at least at the genus level in samples from humans and other vertebrates, focusing on findings at the species levels. The data show that although relatively few vertebrate species have been examined, methanogens are most likely widespread among them and quite diverse if examined at the subspecies level.

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1 Objective and Scope

The work presented in this Chapter was aimed at examining from published reports and our own experience the range of vertebrates that have been found to carry methanogens and the diversity of methanogens uncovered. This review extends and updates previous ones published by us and others (Conway de Macario et al. 1987; Eckburg et al. 2003; Conway de Macario and Macario 2009, 2010) and focuses on the work in which the microbes have been identified at least at the genus level. Methanogens inside protozoa that might inhabit the intestinal tract of man and animals (van Hoeck et al. 2000), are not included

The findings were grouped into two categories, one pertaining to humans and the other pertaining to all other vertebrates studied.

2 Methanogens in Humans

Examples of methanogens found in humans are displayed in Table 1 and Fig. 1. The data reported in the papers cited show that methanogens are widespread in humans, occurring in individuals of all categories, young and old, female and male, healthy and ill. Anatomically, the presence of methanogens is also varied, including habitats such as the large intestine, periodontal space, and vagina. This general view concerning distribution of methanogens indicated by the data in Table 1 is also supported by our previous work (Conway de Macario and Macario 2009).

Another feature that emerges from the data in Table 1 and the other previous searches mentioned above is that the diversity of methanogens in humans is very restricted with only one species, *M. smithii* (*Methanobrevibacter smithii*), being largely predominant. This methanogen was the only one found in all individuals tested or in the few instances in which more than one species were detected in the same individual *M. smithii* was largely predominant. (See references in Table 1.)

The possible roles of methanogens in humans, in health and disease, have been extensively discussed recently (Conway de Macario and Macario 2009). The main conclusions are that methanogens seem to be important, albeit frequently forgotten, components of the human microbiota and that they participate in the mechanism of certain diseases as key members of foodwebs that favor the growth of other microbes, which are the ones that directly cause the disease. For instance, methanogens consume hydrogen and thereby activate hydrogen-producing pathogens, leading to disease indirectly. An example is the promotion of obesity by methanogens via facilitation of utilization of high energy molecules by other microbes that metabolize, for instance, fiber-rich food with great efficiency and thus produce an excess of calories (DiBaise et al. 2008; Samuel et al. 2008; Zhang et al. 2009). Another example is the enhancement by methanogens in the mouth of infection and tissue invasion by pathogenic microbes residing in the periodontal space, thus aggravating periodontitis (Belay et al. 1988; Lepp et al. 2004; Vianna et al. 2008).

Table 1 Examples of methanogens found in humans

Anatomic location	Organism	Reference	Effect/finding	Method
Intestine	Methano-bacteriales	Zhang et al. (2009)	Obesity	PCR-Pyrosequencing 16S rRNA
	M. smithii; *M. stadmanae*	Mihajlovski et al. (2008)	n.a.[a]	*mcrA* and 16S rRNA
	M. smithii	Miller et al. (1982)	n.a.	Antigenic fingerprinting
	M. smithii	Weaver et al. (1986)	Diverticulosis	Culture
	Methanogens	Ansorg et al. (2003)	Methanogens eliminated by metronidazole	Culture (Hungate technique)
	M. smithii	Armougom et al. (2009)	Obesity	RT-PCR
	M. smithii; *M. stadmanae*	Dridi et al. (2009)	n.a.	RT-PCR 16S rRNA and *rpoB*
	M. oralis	Scanlan et al. (2008)	n.a.	PCR *mrcA*
	M. smithii	Conway de Macario et al. (1985)	Several immunotypes	Antigenic fingerprinting; Monoclonal antibodies
Mouth	*M. oralis*	Vianna et al. (2009)	Periodontitis	Restriction Fragment Length Polymorphism (RFLP)
	M. smithii; *M. stadtmanae*	Belay et al. (1988)	Dental plaque and periodontal disease	Antigenic fingerprinting
	M. oralis	Lepp et al. (2004)	Periodontal disease	PCR (SSU rDNA)
	Methanogens	Vianna et al. (2008)	Periodontal disease	Culture
Vagina	*M. smithii*	Belay et al. (1990)	Vaginosis	Antigenic fingerprinting

[a]Abbreviations: *n.a.* not available; Organism names: *M. smithii*, *Methanobrevibacter smithii*; *M. stadtmanae*, *Methanosphera stadtmanae*; *M. oralis*, *Methanobrevibacter oralis*

Other examples are the development of vaginosis (Belay et al. 1990) and diverticulitis (Weaver et al. 1986) facilitated by methanogens in the vagina and the human colon, respectively.

3 Methanogens in Nonhuman Vertebrates

Table 2 displays examples of vertebrate animals in which methanogens have been found, of which some examples are shown in Fig. 2. Ruminants are abundant by comparison with the rest of vertebrates studied, which include a variety of species from animals such as mouse and rat that are frequently used in laboratory

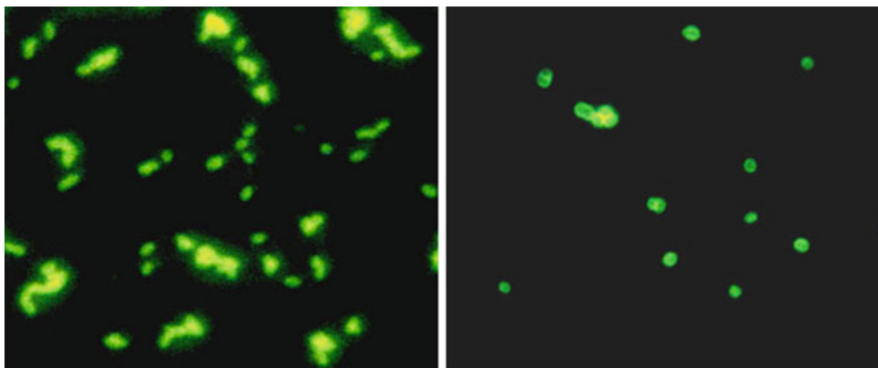

Fig. 1 *Methanobrevibacter smithii* (*left*) and *Methanosphaera stadtmanae* (*right*) are shown stained by indirect immunofluorescence with calibrated antibody probes for each organism. Both organisms appear as cocci (0.7–0.8 μm) and elongated cocci (0.7 × 0.8–1.8 μm) alone, or in chains or clusters. Cocci (sometimes 1.0 μm in diameter) are more common in *M. stadtmanae* while elongated cocci are more common in *M. smithii*

experiments to exotic species such as rhinoceros and hippopotamus, passing through pets like the rabbit (also typically used in laboratory experiments) and some common birds like chicken, turkey, and goose (see references in Table 2).

Interestingly, the diversity of methanogens identified in nonhuman vertebrates is considerably larger than that found in humans.

A variety of methods have been used to detect the presence of methanogens in vertebrates (Table 3). A considerable amount of work, typically early work, was done with methods that detected methane gas emission in breath and intestinal excreta, e.g., feces (Hackstein et al. 1995; Hackstein and van Alen 1996; Florin et al. 2000). Subsequently, other methods were applied aiming at characterizing the microbes emitting methane. The data show that a progression occurred from the original procedures based on cultivation and determination of crucial physiological properties (e.g., preferred growth substrate and methane production) along with morphological characterization using classical techniques (determination of Gram staining properties) and assessment of F420 fluorescence to more precise identification methods. The latter include isolation of methanogens with elucidation of physiological features pertinent to methanogenesis, antigenic fingerprinting with calibrated antibody probes (Macario and Conway de Macario 1983), and phylogenetic classification with nucleic acid probes (Lin and Miller 1998).

This article focuses on work done with methods that actually revealed the microbes in one or more of these characteristics: morphology, physiology, antigenic fingerprint, and phylogenetic classification with nucleic acid probes.

With the advent of monoclonal antibodies and the development of procedures to calibrate both mono- and poly-clonal antibodies (determination of antigenic specificity spectrum with regard to a set of standard antigens) and elucidation of immunochemical specificity with regard to a series of compounds of known structure (Conway de Macario and Macario 1986, 2010), a new era began during which it

Table 2 Examples of methanogens found in various animal species

Animal species	Methanogen/location	Reference	Method
Baboon	*M. smithii*/Feces[a]	Conway de Macario (unpublished)	Antigenic fingerprinting
Buffalo	*M. mobile*/Rumen	Chaudhary and Sirohi (2009)	16S rRNA
	Methanogens/Rumen	Morvan et al. (1996)	Counts
Chicken	Methanobacteriales/Feces	Saengkerdsub et al. (2007)	RT-PCR 16S rDNA
Cow	*M. smithii*/Feces	Lin and Miller (1998)	16S rRNA; genomic DNA reassociation
	Methanogens/Rumen	Morvan et al. (1996)	Counts
	M. arboriphilus/Rumen	Conway de Macario et al. (1987)	Antigenic fingerprinting
	M. ruminantium/Rumen	Wright et al. (2007)	16S rRNA
	M. marisnigri/Feces	Conway de Macario et al. (1987)	Antigenic fingerprinting
Deer	Methanogen/Rumen	Morvan et al. (1996)	Counts
	Methanobacteriaceae, Methanosarcinaceae, *Methanobrevibacter*/ Rumen	Sundset et al. (2009)	16S rRNA Denaturing Grading Gel Electrophoresis (DGGE)
Goat	*Methanosarcina*/Feces	Mukhopadhyay et al. (1991)	Antigenic fingerprinting
Fish	*M. aquaemaris*	Lai and Chen (2001)	16S rDNA
Goose	*Methanosarcina* sp./Feces	Conway de Macario et al. (1987)	Antigenic fingerprinting
	M. smithii/Feces	Lin and Miller (1998)	16S rRNA; genomic DNA reassociation
Hippopotamus	*M. smithii*/Feces	Conway de Macario (unpublished)	Antigenic fingerprinting
Horse	*M. smithii*/Feces	Lin and Miller (1998)	16S rRNA; genomic DNA reassociation
Llama	Methanogens/Rumen	Morvan et al. (1996)	Counts
Panda	*M. smithii*/Feces	Conway de Macario (unpublished)	Antigenic fingerprinting
Pig	*M. smithii*/Feces	Lin and Miller (1998)	16S rRNA; genomic DNA reassociation
Rabbit	Methanogens/Fecal	Marounek et al. (1999)	Methane and Hydrogen production
Rat	*M. smithii*/Feces	Lin and Miller (1998)	16S rRNA; genomic DNA reassociation
Rhinoceros	*M. smithi*/Feces	Conway de Macario (unpublished)	Antigenic fingerprinting
Sheep	*M. gottschalkii*/Rumen	Wright et al. (2008)	Denaturing Grading Gel Electrophoresis (DGGE)
	Metanomicrobiales, Methanobacteriales/ Rumen	Wright et al. (2006)	16S rRNA
	Methanogens/Rumen	Morvan et al. (1996)	Counts

(*continued*)

Table 2 (continued)

Animal species	Methanogen/location	Reference	Method
	Methanogens/Feces	Williams et al. (2009)	16S rRNA
	M. ruminantium, *M. thaueri*, *M. millerae*, *M. olleyae*/Feces	Rea et al. (2007)	16S rRNA; DNA–DNA hybridization
Turkey	*M. marisnigri*/Feces	Conway de Macario et al. (1987)	Antigenic fingerprinting
Wallaby	Methanobacteriales/ Rumen	Evans et al. (2009)	16S rRNA

[a]*M. mobile*, *Methanomicrobium mobile*; *M. smithii*, *Methanobrevibacter smithii*; *M. arboriphilus*, *Methanobrevibacter arboriphilus*; *M. ruminantium*, *Methanobrevibacter ruminantium*; *M. gottschalkii*, *Methanobrevibacter gottschalkii*; *M. thaueri*, *Methanobrevibacter thaueri*; *M. millerae*, *Methanobrevibacter millerae*; *M. olleyae*, *Methanobrevibacter olleyae*; *M. marisnigri*, *Methanoculleus* (ex *Methanogenium*) *marisnigri*; *M. aquaemaris*, *Methanofollis aquaemaris*

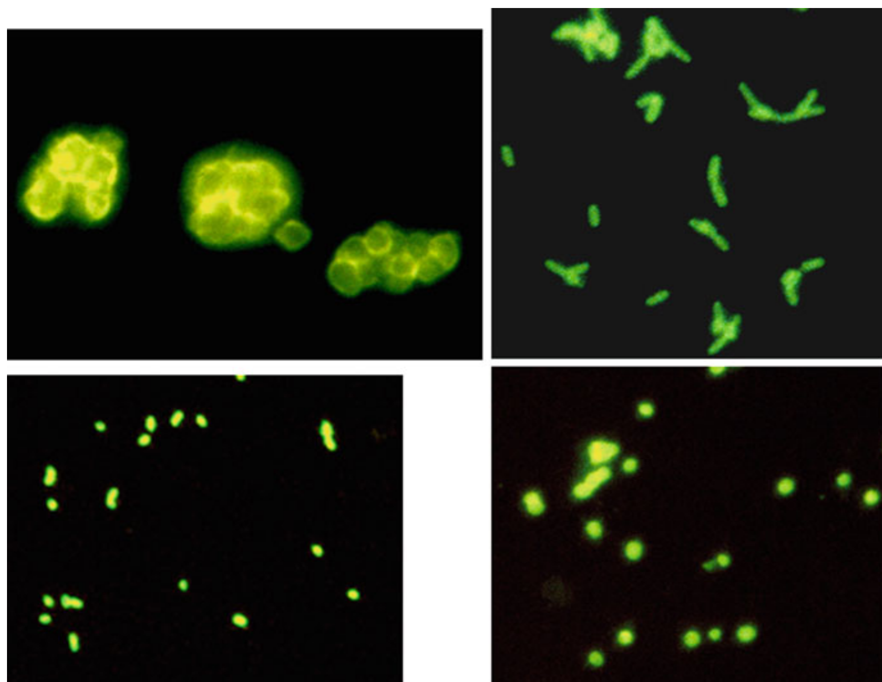

Fig. 2 *Methanosarcina barkeri* (*top left*), *Methanobrevibacter arboriphilus* (*top right*), *Methanobrevibacter ruminantium* (*bottom left*), and *Methanoculleus* (previously *Methanogenium*) *marisnigri* (*bottom right*). Indirect immunofluorescence with calibrated antibody probes for each organism. *M. barkeri* appears as large, irregular cocci (2–4 μm) alone, in packets of various sizes and in laminae of various thicknesses (from one to four cells thick). *M. arboriphilus* appears as elongated cocci and coccobacilli (0.8–1.2 μm), sometimes quite long to appear as short bacilli (2 μm), alone or in very short chains. *M. ruminantium* appears as elongated coccii or coccobacilli (0.7 × 0.8–1.8 μm), alone or in very short chains. *M. marisnigri* appears as irregular cocci of various sizes (1.3–2.6 μm)

Table 3 Methods used over the years to detect presence of methanogens in samples from vertebrates

Determination of:	Method
Methane gas emission	Breath analysis: Gras chromatography
Physiological and morphological properties	Cultivation of organisms: Methods based on the Hungate's technique for anaerobes with physiological characterization combined with microscopy, including F_{420}
Antigens	Antigenic fingerprinting: Indirect immunofluorescence and slide-immnuoenzymatic assay (SIA) with calibrated poly- and mono-clonal antibody probes
Nucleic acids	16S rRNA, 16S rDNA and SSU-rRNA probes and/or sequencing, pyrosequencing technology; sequence comparison and construction of phylogenetic trees
	DNA–DNA hybridization
	Genomic DNA reassociation
	RT-PCR (quantitative)
	Restriction Fragment Length Polymorphism (RFLP)
	Denaturing Gradient Gel Electrophoresis (DGGE)
	Specific gene detection (e.g., *mcrA* gene)

was possible to ascertain the species, strain, and immunotype of the methanogens present in biological samples. Calibrated antibody probes, both mono- and polyclonal, were utilized in conjunction with semiquantitative indirect immunofluorescence (Macario and Conway de Macario 1985) and with quantitative slide immunoenzymatic assay (Conway de Macario et al. 1983), all of which provided for the first time insight into the true identity at the species and subspecies levels, and at the immunotype level of the methanogens occurring in animals. This information also revealed for the first time the extent of the diversity of the methanogens occurring in animals (Conway de Macario et al. 1987), and in nature as well as in manufactured ecosystems such as anaerobic bioreactors of waste-treatment plants receiving municipal sewage with human and animal excreta (Macario and Conway de Macario 1988).

4 Diversity of Methanogens in Vertebrates

Table 4 summarizes the overall results to show the variety of vertebrate species in which methanogens have been identified, and the variety of methanogenic species found. It can be seen that in addition to ruminants, which as mentioned above have the highest representation, there are animals that belong to various groups quite different in physiology and eating habits and also quite separate in terms of phylogeny. It has been shown many years ago, measuring methane gas emission, that over 250 vertebrate species carry methanogens in their intestinal tracts, and that this property of being able to carry methanogens is most likely linked to phylogeny rather than to eating habits: it would be a property shared from the earliest evolutionary times of reptiles, birds, and mammals (Hackstein and van Alen 1996, 2010).

Table 4 Examples of vertebrates found to carry methanogens and the methanogenic species identified

Vertebrate group	Species, family, order[a]
Mammals	
Carnivores (Bears)	Panda (*Ailuropoda melanoleuca*; Carnivora, Ursidae, Ailuropoda)
Equine	Horse (*Equus ferus-caballus*; Equidae, Perissodactyla)
Hippos	Hippopotamus (*Hippopotamus amphibius*; Hippotamidae, Artiodactyla)
Marsupials	Wallaby (Tammar Wallaby, *Macropus eugenii*; Macropodidae; Diprotodontia)
Porcine	Pig (*Sus*; Suidae, Artiodactyla)
Primates	Human (Hominidae, Primates)
	Babboon (Cercopithecidae, Primates)
Rabbits	Rabbit (Leporidae, Lagomorpha)
Rhinos	Rhinoceros (*Rhinoceros unicornis*; Rhinocerotidae, Perissodactyla)
Rodents	Mouse (*Mus musculus*; Muridae, Rodentia)
	Rat (*Rattus norvegicus*; Muridae, Rodentia)
Ruminants	Buffalo (*Bubalus bubalis*; Bovidae,Artiodactyla)
	Cow (*Bos taurus*, Bovidae, Artiodactyla)
	Deer (Cervidae, Artiodactyla)
	Goat (*Capra hircus*, Bovidae, Artiodactyla)
	Llama (*Lama glama*, Camelidae, Artiodactyla)
	Sheep (*Ovis aries*, Bovidae, Artiodactyla)
Birds	Chicken (*Gallus gallus domesticus*; Phasianidae, Galliformes)
	Goose (Anatidae, Anseriformes)
	Turkey (*Meleagris gallopavo*, *M. ocellata*; Phasianidae, Galliformes)
Fish	n.a.
Methanogen	
Genus	Species
Methanobrevibacter	*arboriphilus*; *gottschalkii*; *millerae*; *olleyae*; *oralis*; *ruminantium*; *smithii*; *thaueri*
Methanoculleus (ex *Methanogenium*)	*marisnigri*
Metanomicrobium	*mobile*
Methanosarcina	*barkeri* (?)
Methanosphera	*stadtmanae*

[a]The information on species, order, family, is included here when it was possible to infer them from the published reports; *n.a.* not available

Although the sample of animals (including humans) studied and reported in the literature is still very small and acknowledging that the list in Table 4 may be incomplete, one may predict that methanogens do occur in a great variety of vertebrates, but their diversity is limited. For example, only three species have been identified in humans of which one, *M. smithii*, is highly predominant and of the other two one being *Methanobrevibacter* and the other *Methanosphera* (Conway de Macario and Macario 2009). In nonhuman vertebrates a greater diversity than in humans has been unveiled encompassing 11 species, seven of

which belong to the genus *Methanobrevibacter* and the other four belong one each to the genera *Methanomicrobium*, *Methanoculleus*, *Methanofolis*, and *Methanosarcina*. As with humans, the predominant methanogen in the gastrointestinal tract of other vertebrates identified so far is either *M. smithii* or another *Methanobrevibacter* species (Figs. 1 and 2), while the other genera are considerably less common.

If we consider methanogens at the family level, only three families are represented in vertebrates: Methanobacteriaceae, Methanomicrobiaceae, and Methanosarcinaceae. Members of the other families, Methanospirillaceae, Methanocorpusculaceae, Methanosaetaceae, Methanothermaceae, Methanocaldococcaceae, and Methanococcaceae have not yet been found to inhabit vertebrates.

5 Diversity at the Subspecies Level

It is likely that the few species found in human and animals would display considerable diversity at the subspecies level, especially in relation to the characteristics of the host's type of intestinal system, diet, health vs. disease status, ingestion of chemicals polluting the environment, and other factors, including genetic make- up.

The diversity of *M. smithii* immunotypes was investigated and found to be quite wide (Conway de Macario et al. 1985). So, considering the earlier work measuring methane emission together with more recent research aimed at identifying methanogens at the subspecies level, it can be concluded that methanogens are very widespread in vertebrates, and are more diverse than it can be assumed by just considering genus, or family, or even species as the end point of identification.

6 Methanogens in Vertebrates and Atmospheric Methane

It is very likely that occurrence of methanogens in animals of all kinds is widespread, therefore, methane emission by animals is likely to contribute significantly to atmospheric methane and, thus, add to the greenhouse effect and climate change, a conclusion also advanced by others (Hackstein et al. 1995, 1996; Hackstein and van Alen 1996; Pinares-Patino et al. 2009; Williams et al. 2009). Hence, means are being developed to control methane emission from animals, called vaccines (Williams et al. 2009). It is clear from these concerns and from the strategies thought out to reduce methanogens in animals that a detailed knowledge of the methanogenic flora in animals at the subspecies level is necessary. This knowledge will provide the clues necessary for developing antimethanogen vaccines or compounds with the required specificity and efficacy. Vaccines, or compounds targeted specifically to a methanogenic strain or immunotype that must be eradicated will avoid damage to other microbes that are necessary for the host's health.

7 Conclusions

1. Studies on the presence of methanogens in animals were performed mainly in Australia, India, The Netherlands, New Zealand, and the UK, while studies of methanogens in humans were done chiefly in the USA. Since this a very brief list of countries that represent only certain geographical areas, what we know now on the distribution of methanogens in vertebrates may not be a representative sample of the entire Earth.
2. Typically, studies that identified one or more methanogens in animals were done with a single sample from a single individual. Therefore, the results may not provide an accurate picture of the methanogenic flora of animals valid for many individuals of the same species and for various environmental and corporal conditions that any given species might encounter during its life.
3. In relation to point 2, above, in general no time course studies were carried out on a single individual or on a representative sample of individuals of any given species. This precludes derivation of general conclusions about the methanogenic flora of any given species, in any given geographical location (see also point 1, above). One exception to consider is one study carried out in rabbits of 4, 6, 8, and 11 weeks old (Marounek et al. 1999). It was found that methanogenesis in the intestinal tract of the rabbits examined started at the age of 6 weeks.
4. More than one species/strain can occur in the bovine rumen and large bowel of rat. This finding demonstrates that a plurality of species, albeit limited, can occur in a single individual (Conway de Macario et al. 1987). However, the general trend observed was that only a single species or very few inhabit any given individual.
5. In goat, abundant methanogenic species of the genus *Methanosarcina* were found (Mukhopadhyay et al. 1991). It could be estimated that the *Methanosarcina* organisms made up a considerable portion of the rumen microbial biomass in the goat examined. This would indicate that these organisms could, due to their aceticlastic ability, play a determinant role in nutrient utilization in ruminants and, thereby, could affect body weight. However, this observation was limited to a single individual and generalizations are not fully warranted (see points 1–3, above).
6. Although methanogens are not pathogens for humans by themselves (as far as we can tell at the present time), their presence in humans has been associated with periodontal disease, vaginosis, diverticulosis, and other pathological conditions (Conway de Macario and Macario 2009). In these conditions it was seen as a direct positive correlation between presence and amount of methanogens in the lesion and gravity of the disease. Furthermore, in periodontitis, when treatment was administered and the lesion subsided, methanogens decreased. Samples from healthy vaginas did not contain detectable methanogens but these became abundant in samples from patients with vaginosis. Patient with diverticulosis and diverticulitis contained more methanogens than patients free of these pathological features.

7. The overall data thus far indicate that methanogens inhabit most if not all vertebrate species, although not necessarily all individuals of each species, and are of restricted diversity at the family and genus levels, and even at the species level. However, it is likely that the diversity at the subspecies level of methanogens in vertebrates is relatively great in comparison with their diversity at higher taxonomic levels.

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Methanogens in the Gastro-Intestinal Tract of Animals

Johannes H.P. Hackstein and Theo A. van Alen

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Abstract Nearly all vertebrates host methanogens in their gastro-intestinal tracts. However, a great fraction of vertebrates emits only traces of methane from their faeces (~1 nmol/g faeces/h) and has no significant amounts of methane in their breath. In contrast, many animals host some 100 times more methanogens in their gastro-intestinal tract and emit methane in their breath. These substantial differences are not caused by different feeding habits; rather a genetic factor controls the presence of large amounts of methanogens. The attribute "methane production" is evolutionarily stable, and the loss of this character obeys Dollo's law: once lost in the course of evolution, this character cannot be acquired another time.

Also invertebrates can host methanogens in their gastro-intestinal tract. In contrast to the vertebrates, only a few taxa of arthropods emit methane: millipedes, termites, cockroaches and scarab beetles. All other arthropods in our study did not emit methane and did not host even traces of methanogens. As in vertebrates, the diet of the animals is not crucial for the presence of methanogens. Again, a genetic factor seems to control the presence or absence of methanogens. Methanogenesis is also a prerequisite for the presence of intestinal anaerobic protozoa with endosymbiotic methanogens, but not for the presence of impressive structural

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differentiations of the hindgut epithelium, which – in methanogenic taxa – host enormous amounts of methanogens.

1 Introduction

Methanogens are the dominating archaeal organisms and they possess a great phylogenetic and ecological diversity (Woese et al. 1990; Liu and Whitman 2008). They occupy a broad spectrum of ecological niches, including the cytoplasm of unicellular anaerobic eukaryotes and the gastro-intestinal tract of various animals (Lange et al. 2005). Most methanogens have not been cultured yet, and their diversity can only be deduced from the analysis of their 16S rRNA genes. Methanogens in complex anaerobic environments are frequently involved in interspecies hydrogen transfer thereby improving fermentations and electron transfer in syntrophic communities of bacteria and archaea (Schink 1997; Stams and Plugge 2009; Worm et al. 2010). Earlier studies have suggested that the endosymbiotic methanogens of protozoa occur only in protists with hydrogenosomes, i.e. hydrogen-producing organelles of mitochondrial descent (Fenchel and Finlay 1995; Hackstein et al. 2006a; Hackstein and Tielens 2010). On the other hand, hydrogenosomes are not the same, and methanogens are not always present in protists with hydrogenosomes; sometimes, endosymbiotic methanogens are present in protists without hydrogenosomes (Fenchel and Finlay 2010; Hackstein and Tielens 2010). It has been shown that methanogens in protists exhibit a certain host specificity (Fenchel and Finlay 1995), but on the other hand, endosymbiont replacements seem to be possible, especially in evolutionary timescales (van Hoek et al. 2000). Notably, methanogens in the gastro-intestinal tracts of animals are not found everywhere; in some animals methanogens are abundant, in others they are only of very low abundance or even completely absent. In this chapter, we will discuss the elusive distribution of methanogens in the gastro-intestinal tract of vertebrates and arthropods.

2 Vertebrates

Vertebrates are born (or hatch from the egg) with a sterile gastro-intestinal (GI) tract. Soon after birth or hatching the GI tract becomes colonized by bacteria and archaea. Eventually, after reaching adulthood, vertebrates, and especially mammals, host a very complex and numerous microbiota in their guts (Zoetendal et al. 2006; Liu and Whitman 2008). These microbiota are host-specific and clearly different from free-living bacterial communities (Ley et al. 2008a, b). Virtually all these microbiota include methanogens (Miller and Wolin 1986; Hackstein and van Alen 1996; Hackstein et al. 1996). However, the number of methanogens varies at least by two orders of magnitude between species, with the consequence that the faeces of certain species emit less than 1 nmol/g/h of methane while the faeces of

other species produce much more than 100 nmol/g/h. A systematic analysis of more than 250 species of vertebrates reveals a bimodal distribution of the methane emissions from faeces with about 85 species producing less than 5 nmol/g/h and about 123 producing more than 50 nmol/g/h (Fig. 1; Hackstein and van Alen 1996). Only a few species produce intermediate amounts of methane. If one assumes that one methanogenic archaeon produces approximately 1 fmol methane per hour (10^{-15} mol/h) then one gram of faeces of the low producers could host not more than 10^6 methanogens. Accordingly, high methane producers could host more than 10^8 methanogens in one gram of faeces. This range has been confirmed by enumberation (Miller and Wolin 1982; Doré et al. 1995; El Oufir et al. 1996; Liu and Whitman 2008). These differences have physiological consequences: while the high methane producers emit significant amounts of methane with their breath, the concentrations of methane in the breath of low producers do not exceed the atmospheric background concentration (Hackstein and van Alen 1996). Therefore, we will name the species with faecal emissions of less than 3 nmol/g/h "non-producers" and those with emissions above 20 nmol/g/h "producers".

Methane producers and non-producers are not randomly distributed (Table 1). With a few exceptions, individuals of the same species share their methane status, as well as representatives of closely related species. This raises the question as to whether it is possible to identify the reasons for the presence or absence of high amounts of methanogens in the GI tract. It had been assumed that a plant-based diet rich in fibres provides the basis for the presence of high numbers of methanogens (Miller and Wolin 1986). This is clearly the case in ruminants such as cattle, sheep and goats. These animals, but also hindgut-fermenters such as horse and elephant produce high amounts of methane (Table 1). On the other hand, bamboo-eating pandas do emit only traces of methane, but vegetarian chiropters do not produce methane. In contrast, carnivorous crocodiles, giant snakes and ant-eating species such as the great ant-eater, the tamandua and the aardvark release large amounts of

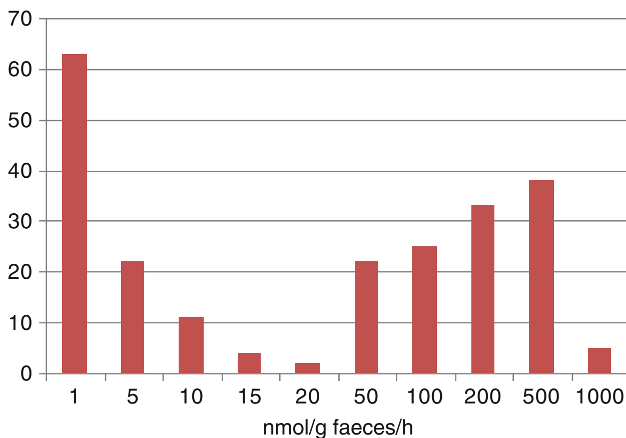

Fig. 1 Histogram of the mean methane emission rates (nmol/g faeces/h, abscissa) by the faeces of 225 amniotes. Ordinate: number of species

Table 1 Methane production in vertebrates

	No. of species	Methane producer	Non-producer	Mean methane production producer/non-producer[a]
Xenopus laevis	1	1	–	Methane in breath
Emydidae	1	1		Methane in breath
Testudinae	4	4	–	30–109/–
Caiman crocodilus	1	1	–	181/–
Iguanidae	3	2	1	275–322/5
Pythonidae	3	3	–	59/–
Ratites	8	4	4	93–414/0.2–0.7
Anser anser	1	1	–	79/–
Anas platyrhynch.	1	1	–	70/–
Galli	2	2	–	102–142/–
Columba livia	1	–	1	–/0.3
Passer domesticus	1	–	1	–/0.7
Strigiformes	3	–	3	–/0.2–0.5
Tachyglossus	1	1	–	44/–
Marsupialia	9	8	1	6–499/0.03
Choleopinae	2	2	–	7–290/–
Myrmecophagidae	2	2	–	134–208
Tenrecidae	2	–	2	–/0.1–0.4
Erinaceinae	2	–	2	–/0.2–0.8
Sorex spec.	1	–	1	No methane in breath
Talpa europaea	1	–	1	No methane in breath
Tupaia bergeri	1	–	1	–/1
Chiroptera	4	–	4	–/1–2
Macroscrelides	1	–	1	–/0.2
Cheirogaleus med.	1	1	–	5[b]/–
Lemuridae	10	10	–	7–505/–
Loridae	3	3	–	11–79/–
Galagonidae	2	1	1	4/3
Alouatta caraya	1	1	–	73/–
Aotinae	3	3	–	113–373/–
Atelinae	3	3	–	155–347/–
Cebinae	2	–	2	–/0.2–0.4
Callimico goeldii	1	–	1	–/1
Pitheciinae	2	2	–	129–433/–
Callithricidae	10	4	6	7–77/0.5–3
Cercopithecinae	11	11	–	31–530/–
Colobinae	6	6	–	219–459/–
Hylobatidae	4	4	–	234–433/–
Hominidae	4	4	–	135–417/–
Canidae	4	–	4	–/0.1–4
Felix silvestris	1	–	1	–/0.1
Procyon lotor	1	–	1	–/0.3
Ailurinae	4	–	4	–/0.2–2
Ursinae	6	–	6	–/0.3–3
Viveridae	4	–	4	–/0.1–1
Delphinapterus l.	1	–	1	No methane in breath
Tursiops truncatus	1	–	1	No methane in breath
Trichecus manatus	1	1	–	51/–

(*continued*)

Table 1 (continued)

	No. of species	Methane producer	Non-producer	Mean methane production producer/non-producer[a]
Proboscidea	2	2	–	9–41/–
Equidae	2	2	–	30–118/–
Tapiridae	2	2	–	66–311/–
Rhinoceros unicor.	1	1	–	8/–
Procavia capensis	1	1	–	257/–
Orycteropus afer	1	1	–	15/–
Suidae	3	3	–	30–68/–
Tayassu tajacu	1	1	–	329/–
Choeropsis liber.	1	1	–	76/–
Camelidae	3	3	–	73–121/–
Giraffidae	2	2	–	21–41
Ovibos moschatus	1	1	–	72/–
Cervinae	5	5	–	69–423/–
Odocoileinae	3	3	–	53–138
Alces alces	1	1	–	110/–
Cephalophus mon.	1	1	–	29/–
Tragelaphinae	3	3	–	66–435/–
Bovinae	2	2	–	116–226/–
Caprinae	6	6	–	21–4,230/–
Reduncinae	2	2	–	14–59/–
Manis tricuspis	1	–	1	–/0.2
Sciuridae	17	7	10	8–142/0.01–4
Castor fiber	1	–	1	–/1
Jaculus jaculus	1	–	1	–/0.3
Cricetinae	8	2	6	9/0.3–2
Cricetomys gamb.	1	1	–	100/–
Gerbellinae	2	–	2	–/0.1–0.4
Murinae	7	1	6	26/0.1–2
Myoxidae	2	–	2	–/0.1–2
Graphiurus murin.	1	-	1	–/0.6
Hystricidae	3	3	–	29–108/–
Thryonomys swin.	1	1	–	3[b]/–
Erethizontidae	2	2	–	90–583/–
Chinchilla laniger	1	1	–	128/–
Caviinae	4	4	–	7–237/–
Dolichotis patago.	1	1	–	25/–
Hydrochorus hyd.	1	1	–	311/–
Dasyproctidae	3	3	–	87–176/–
Octodontidae	2	2	–	2[b]–8/–
Capromys pilorid.	1	1	–	28/–
Myocastor coypus	1	1	–	440/–
Lagomorpha	3	3	–	4[b]–42/–

For a more extended version of this Table see Hackstein and van Alen (1996)
[a]Range of the mean emissions of producers/mean emissions of non-producers as nmol methane/g faeces/h
[b]Classified as methane producer on the basis of their maximal emissions

methane from their faeces; armadillos emit low, but still significant concentrations of methane. Only one species of ant-eating animals, the pangolin, is a non-producer (Table 1). Therefore, a plant-based, fibre-rich diet cannot be the primary reason for the presence of large numbers of methanogens. Also the presence of a highly differentiated GI tract does not necessarily predispose for methane production: while African and South American ostriches do produce methane, their Australian/New Zealandian relatives emu and cassowary, which possess a GI tract of similar complexity and use a comparable diet, are non-producers (Table 1; Fig. 2). Also dolphins and whales, which possess complex foregut differentiations, do not produce methane (Table 1). Thus, neither the presence of a highly differentiated GI tract nor a fibre-rich diet predispose automatically for the presence of high numbers of methanogens.

The just-mentioned example of the methane-producing and non-producing ostriches provides evidence for the intrinsic reasons for the presence/absence of methanogens. A phylogenetic tree of the ostriches based on the mitochondrial 12S rRNA genes (Fig. 2) reveals that these birds are monophyletic. The methane-emitting African/South American ostriches occupy a basal position in the phylogenetic tree, while the non-producing Australian/New Zealandian ostriches and ratites are found in the top of the tree. The latter ostriches and ratites share a recent common ancestor, and this ancestor obviously lost the property to host methanogens, a property that is shared by all its descendents. This argues that a heritable, genetic property provides the basis for the presence of large numbers of methanogens in the GI tract. Also the study of South American apes supports this interpretation. While all old-world apes and monkeys are methane producers, the methane status of the New World apes can differ by species, even by subspecies. For example, the marmoset *Leontopithecus rosalia rosalia* produces only 1 nmol/g/h methane, whereas the closely related *Leontopithecus rosalia chrysomelas* is a producer of some 70–500 nmol/g/h faecal methane. Among the Callitricidae (marmosets and tamarins), which include the two *Leontopithecus* species, four species

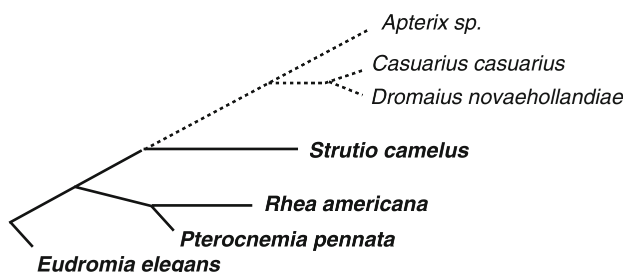

Fig. 2 Evolution of ratites: Faeces of emu (*Dromaius novaehollandiae*), cassowary (*Casuarius casuarius*), and kiwi (*Apteryx* sp.) do not emit significant amounts of methane (max. 2 nmol/g/h). Faeces of ostrich (*Strutio camelus*), nandu (*Rhea americana*), Darwin nandu (*Pterocnemia pennata*), and tinamou (*Eudromia elegans*) produce between 137 and 414 nmol/g/h methane. The tree is based on mitochondrial 12S rDNA sequence data of Cooper et al. (1992). Redrawn after Hackstein and van Alen 1996

are producers and six species are non-producers in our screen (Table 1; Hackstein and van Alen 1996). Moreover, seven species of monkeys belonging to the Cebidae produce large amounts of methane through their faeces, while two *Cebus* species emit only traces. This means that the property "methane production" can be lost at the species level in the absence of any significant differences in diet or other physiological parameters.

Also, among the Rodentia the character "methane production" can be lost at the species, subspecies, or even at the population level. For example, 7 of the 17 species of sciurids studied produced methane while ten did not (Table 1). The species *Sciurus vulgaris* was identified as methane producer, but a highly inbred population of ten individuals did not produce methane. Since differences in the diet can be excluded, a dietary basis for the character "methane production" can be excluded.

This holds also true for the Muridae, where non-producers predominate. From the nine species of Critecinae and Cricetomyinae studied, only three species (golden hamster, muskrat and giant pouched rat) emitted methane. From the seven species belonging to the Murinae, only one, *Leopoldamys sabanus*, produced methane (Table 1). Recently, it has been shown that also certain strains of laboratory rat are methanogenic while other strains are non-methanogenic (Florin et al. 2000). In clear contrast, all species of carnivores, chiropters and insectivores did not produce methane irrespective of their diet, whereas all artiodactyls and perissodactyls studied produced methane. Also all caviomorphs, hystricomorphs and lagomorphs tested produced methane (Table 1).

Thus, a direct correlation between diet and methane production can be excluded, and an alternative explanation for the significant differences found among the species examined seems difficult. Notably, a lack of infection by methanogens in "non-producers" can also be excluded since virtually all species tested produce at least traces of methane indicating the presence of methanogens (Table 1). However, if we incorporate the property "methane producer"/"non-producer" in a phylogenetic tree that is based on the analysis of mammalian protein sequences (Miyamoto and Goodman 1986), a rationale for the phenomenon "methane production" becomes evident (Fig. 3). Non-producers cluster as whole branches, or they are found in terminal positions of the tree. In other words, methane production is a primitive-shared (plesiomorphic) character, while the loss of methane production is a shared-derived (synapomorphic) character. The loss of methane production clearly obeys Dollo's rule: once lost in the course of evolution, the competence for methanogenesis cannot be restored. This holds also true for those losses at lower taxonomic levels (e.g. species level) that could not be included into the phylogenetic tree. The integration of the methane data into other phylogenetic trees, for example the "classical" tree of Novacek (1992) or the molecular trees of Li et al. (1990) or of Janke et al. (1994) does not lead to a different interpretation. Consequently, the loss of the competence to host large numbers of methanogens is an evolutionary stable character that must have a heritable, genetic basis. This can also explain the loss of methanogenesis in populations where the Hardy-Weinberg equation describes the distribution of genetic characters. Evidence for the presence of producers and non-producers in a species have been described here for the species *S. vulgaris* (Table 1),

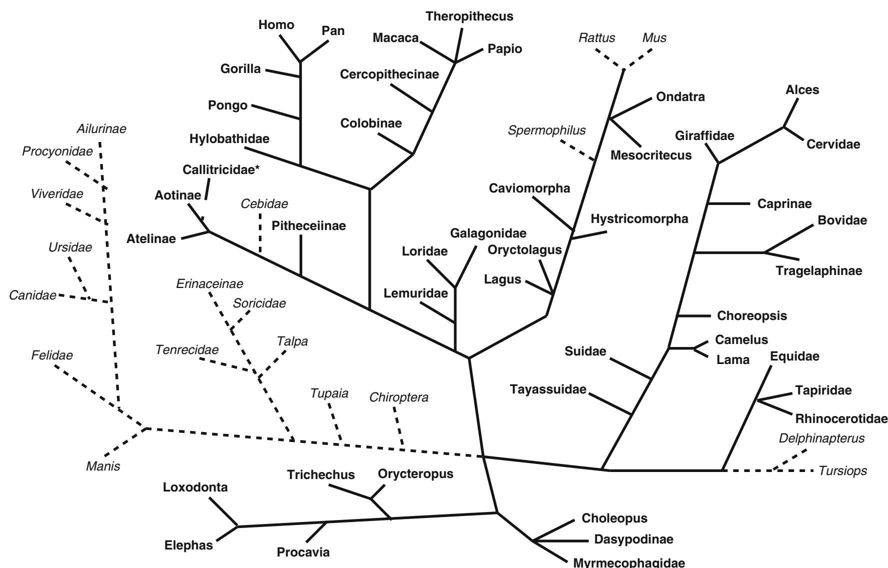

Fig. 3 Integration of information about methane production into the protein sequence based phylogenetic tree of Miyamoto and Goodman (1986). Producers: *roman*, non-producers: *italics*; *asterisk* only four out of the ten species are methane producers. Redrawn after Hackstein and van Alen (1996)

and are also well known for strains of laboratory rats and notably, human populations (Miller and Wolin 1982; Segal et al. 1988; Brusa et al. 1993; Hudson et al. 1993; Doré et al. 1995; Florin et al. 2000; Levitt et al. 2006).

At the moment, one can only speculate about the physiological or biochemical basis for the presence of large amounts of methanogens in the guts and faeces of many animals and their significantly lower number in certain other species. Different levels of bile acids have been assumed as modulators of the methane production (Florin and Jabbar 1994), but a general function of bile acids as a physiological control of methanogenesis seems unlikely. The presence of a receptor for methanogenic archaea or some other adherence mechanism could potentially explain the prolonged persistence of high numbers of methanogens in the gut. In the absence of the receptor-mediated adhesion to the gut wall, methanogens are easily removed from the G-I tract in the course of digestion, compensated only by high division rates of the methanogens. This might allow maintaining only titres of methanogens that are 30–100 times lowerthan in species with an adhesion mechanism for methanogens.

It is surprising that so many animals host high numbers of methanogens that cause a significant emission of methane. Most of these methanogenic animals possess “alloenzymatic” intestines that depend in their function on the presence of complex symbiotic microbial associations (Langer 1988, 1991, 1994; Langer and Snipes 1991). Alloenzymatic intestines are characterized by well developed hindguts and caeca and also by the evolution of rumina and other forestomach fermenting organs without rumination. Recently, the analysis of the microbiomes of the

various animals has provided evidence for the existence of characteristic "foregut" and "hindgut" microbiota (Ley et al. 2008b) supporting the anatomical studies. Notably, there is evidence that the postnatal development of these differentiations depends on the presence of effective microbial fermentations, in particular the presence of certain fermentation products such as propionate and butyrate (Jesse et al. 1994). In contrast, "autoenzymatic" intestines do not have the need for microbial symbionts for their digestions, and consequently, they do not possess the differentiations that are characteristic for the alloenzymatic guts. However, they host specific microbial communities that are characteristic for "simple" GI tracts (Ley et al. 2008b). Most of these intestines are found among the animals that belong to the category of "non-producers" of methane. This does not mean that monogastric, autoenzymatic intestines are devoid of any significant microbiota. Our measurements have shown that virtually all of the non-producers of methane emit significant amounts of hydrogen (Hackstein and van Alen 1996), which is indicative of intensive microbial fermentations. But as mentioned above, these fermentations do not contribute to the digestion of resistant biopolymers, and they do not induce any of the intestinal differentiations that are found in the methanogenic animals with alloenzymatic digestion. Even a fibre-rich diet of a non-methanogenic animal does not correlate with any alloenzymatic differentiation of the G-I tract. The best example is the giant panda, which relies completely on a bamboo diet but does not possess any fermentative intestinal differentiation. His gut microbiome classifies his GI tract as belonging to the "simple" type (Ley et al. 2008b). Notably, evolution allowed the development of panda's famous additional thumb, but not of a well-developed caecum or colon. On the other hand, the leaf-eating, methanogenic colobid monkeys evolved a foregut fermenting structure similar to the rumen of the ruminants with a microbiome that resembles that of ruminants (Ley et al. 2008a, b).

A secondary loss of methanogenesis is possible in alloenzymatic animals with foregut differentiations, e.g. dolphins and whales (Fig. 3), but also in animals with hindgut differentiations, e.g. many murids and certain New World monkeys and apes. Monogastric, autoenzymatic animals are primarily non-methanogenic (Fig. 3). The correlation between the presence of high numbers of methanogens and the presence of intestinal differentiations is striking and for sure not accidental. Obviously, methanogens fulfil a crucial role in intestinal fermentations that allow the digestion of plant polymers (Schink 1997; Stams and Plugge 2009). It is likely that this role cannot be taken over by other hydrogen-consuming bacteria. Notably, the presence of high numbers of methanogens must be controlled by one or several genetic factors, since the evolutionary loss of the capacity to host high numbers of methanogens cannot be restored. Also the population-specific distributions of methane producers and non-producers in human populations are indicative of a genetic basis (Segal et al. 1988; Hudson et al. 1993; Brusa et al. 1993; Levitt et al. 2006). Furthermore, the analysis of the trait "methane production" in pedigrees reveals inheritance patterns that are compatible with the interpretation of an autosomal dominant inheritance (Hackstein et al. 1995). Twin studies that rejected a genetic influence on the methane status of humans might be erroneous due to a

statistical analysis that seems not suitable for the detection of different classes of methane producers/non-producers (Florin et al. 2000). It has also been discussed above that a lack of proper infection with methanogens can be excluded for the explanation of the bimodal distribution of producers and non-producers, since also non-producers exhibit a low level of methanogenesis in their faeces. Thus, the presence of high numbers of methanogens in the G-I tract of vertebrates is still elusive, but obviously under the control of one or several genetic factors. It is for sure not the consequence of particular dietary habits or the presence of intestinal differentiations.

3 Arthropods

Arthropods represent by far the largest global biodiversity of all multicellular animals. Despite their small size and the tiny volumes of their intestinal tracts many arthropods host a complex microbiota in their guts (Bayon 1980; Hackstein and Stumm 1994; Cazemier et al. 1997; Hackstein 1997; Egert et al. 2003; Brune 2006; Hackstein et al. 2006b; Warnecke et al. 2007). Already in 1953, Paul Buchner in his seminal monograph (Buchner 1953) described the fascinating world of symbiotic associations between arthropods and bacteria. He emphasized not only the enormous diversity of differentiations of the intestinal tract, but also the more direct associations between bacteria and their hosts involving specialized tissues and organs (e.g. "bacteriomes"). There were a lot of speculations about the contribution of the symbionts to the host's nutrition, but only recently the progress in molecular biological techniques and bioinformatics allowed unravelling of the molecular basis of some of these symbiotic associations (Moran 2003, 2007; Moran and Baumann 2000; Hoffmeister and Martin 2003; Canback et al. 2004; Dillon and Dillon 2004; Dale and Moran 2006; Moya et al. 2008; Ruby 2008). In remarkable contrast to the situation in vertebrates, the role of methanogenic archaea is very limited in the arthropod world. While nearly all vertebrates host at least traces of methanogens, the vast majority of the arthropod taxa is completely devoid of methanogens. In principle, the detection of methanogens in the GI tract of arthropods is easy, since arthropods exhale intestine-born methane with their breath (Bijnen et al. 1996). Due to their small size, the methane production of arthropods can be measured non-invasively by incubating the intact specimen in stoppered glass vials. With a standard gas chromatograph, it is possible to detect sub-nanomolar concentrations of methane after the prolonged incubation of individual or several specimens. In this way, the presence of less than 10^6 methanogens in the GI tract of a single arthropod can be detected.

In a first experiment, we screened more than 110 representatives of 35 higher taxa of terrestrial arthropods for methane and hydrogen emissions (Hackstein and Stumm 1994; Table 2). In a second experiment, we analysed some 70 strains of cockroaches representing 44 different species (Hackstein 1997; Hackstein et al. 2006b; Table 3). To confirm the presence or absence of methanogens in the GI

Table 2 Methane and hydrogen production in invertebrates

	Common name	Methane	Hydrogen	Protists
Araneae	Spiders			
Araneus diadematus[a] (A)		–	+	–
Acari	Mites and ticks			
Boophilus microplus		–	–	–
Isopoda	Sawbugs			
Oniscus asellus[a] (A)		–	–	–
Porcellio scaber (A)		–	–	–
Chilopoda	Centipedes			
Lithobius forficatus (A)		–	–	–
Diplopoda	Millipedes			
Chicobolus sp. (J)		+	+	–
Mestosoma hylaeicum (A)		–	–	–
Orthoporus sp. (J)		+	+	C
Pycnotropis acuticollis (A)		+	+	nd
Rhapidostreptus virgator (A)		+	+	C
Unidentified A (J)		+	+	–
Unidentified B (J)		+	+	–
Unidentified D (J)		+	+	C
Unidentified K (J)		+	+	C
Glomeris sp.[a] (A)		–	+	–
Julus sp.[a] (A)		–	–	–
Polydesmus sp.[a] (A)		–	–	–
Tachypodojulus niger[a] (A)		–	–	–
Thysanura	Bristletails			
Lepisma saccharina (A)		–	–	–
Collembola	Springtails			
Folsmia candida (J, A)		–	–	–
Acrididae	Short-horned grashopper			
Locusta migratoria (A)		–	–	–
Schistocerca gregaria (A)		–	–	–
Unidentified[a] (A)		–	–	–
Gryllidae	Crickets			
Achaeta domesticus (A)		–	–	–
Decticus sp.[a] (A)		–	–	–
Gryllus bimaculatus (A)		–	–	–
Ventralla quadrata (A)		–	–	–
Phasmidae	Stick and leaf insects			
Eurycantha calcerata (A)		–	–	–
Pharnacia acanthopus (A)		–	–	–
Sipyloidea sipylus (A)		–	–	–
Mantidae	Mantids			
Hierodula membranacea (A)		–	–	–
Blattidae	Cockroaches			
Blaberus craniifer (A)		+	+	C
Blaberus fuscus (L, A)		+	–	C
Blaberus giganteus (L)		+	+	C
Blatta orientalis (A)		+	+	–
Blatella germanica (A)		+	+	–
Ectobius sp.[a] (A)		–	–	–
Gromphodorhina port. (L; A)		+	+	C
Leucophaea sp. (A)		+	+	–

(*continued*)

Table 2 (continued)

	Common name	Methane	Hydrogen	Protists
Panchlora nivea (A)		–	–	–
Periplaneta americana (L, A)		+	–	C
Periplaneta australasia (L, A)		+	+	C
Pycnoscelus suriname. (L, A)		+	+	C
Supella supellectilium (L, A)		+	–	F
Isoptera	Termites			
Cryptotermes brevis (A)		+	–	nd
Heterotermes indicola (A)		+	–	F
Mastotermes darwiniensis (A)		+	–	F
Reticulotermes santonen. (A)		+	–	F
Dermaptera	Earwigs			
Forficula auricularia[a] (A)		–	–	–
Heteroptera	Bugs			
Dysdercus intermedius (L, A)		–	+	–
Oncopeltus fasciatus (L, A)		–	–	–
Platymerus biguttata (A)		–	–	–
Pyrrhocoris apterus (L, A)		–	–	–
Cicadoidea	Cicadas			
Nephotettis cincticeps (A)		–	–	–
Aphididae				
Aphis fabae (L, A)		–	–	–
Apidae				
Apis mellifera (A)		–	–	–
Carabidae	Ground beetles			
Carabus sp.[a] (A)		–	–	nd
Pterostichus niger[a] (A)		–	–	nd
Silphidae	Carrion beetles			
Necrophorus vespillo[a] (A)		–	–	nd
Dermestidae	Dermestid beetles			
Dermestes frischi (A)		–	–	nd
Tenebrionidae	Darkling beetles			
Oryzaephilus sp. (L, A)		–	–	nd
Scarus tristis (L)		–	–	nd
Tenebrio sp. (L)		–	–	nd
Tribolium confusum (L, A)		–	–	nd
Zophobas morio (L, A)		–	–	nd
Cryptophagidae	Silken fungus beetles			
Alphitobius diapecur. (L, A)		–	–	nd
Bostrychidae	Branch and twig borers			
Acanthocelides panac. (L, A)		–	–	nd
Rhizopertha dominica (L, A)		–	–	nd
Sitophilus graminarius (L, A)		–	–	nd
Anobiidea	Death-watch beetles			
Anobium punctatum (L)		–	–	nd
Oligomerus ptilinoides (L)		–	–	nd
Ptilinus pectinicorni (L)		–	–	nd
Stegobium panaceum (A)		–	–	nd
Xestobium rufovillosum (L)		–	–	nd
Lyctidae	Powder-post beetles			
Luctus africanus (L)		–	–	nd
Luctus brunneus (L)		–	–	nd

(*continued*)

Table 2 (continued)

	Common name	Methane	Hydrogen	Protists
Minthea rugicollis (L)		–	–	nd
Dynastinae	Rhinocer beetles			
Dynastes hercules (L)		+	+	F
Cetoniidae	Rose chafers			
Cetonia aurata (L)		+	+	F
Dicronorrhina micans (L)		+	+	F
Eudicella gralli (L, A)		+	+	F
Eudicella smittii (L)		+	+	F
Pachnoda bhutana (L, A)		+	+	F
Pachnoda ephippuata (L)		+	+	F
Pachnoda marginata (L)		+	–	F
Pachnoda nachtigalli (L, A)		+	–	F
Pachnoda savignyi (L, A)		+	–	F
Potassia cuprea (L, A)		+	+	F
Phyllopertha horticola[a] (A)		–	–	–
Geotrupinae	Dung beetles			
Geotrupes sp.[a] (A)		–	+	nd
Geotrupes sp[a] (A)		–	–	nd
Cerambycidae	Longhorn beetles			
Hylotrupes bajulus (L)		–	–	–
Chrysomelidae	Leaf beetles			
Crioceris asparag[a] (A)		–	–	nd
Diabrotica baltea (A)		–	–	nd
Leptinotarsa decemlinea. (A)		–	+	nd
Phaedon cochleariae (L, A)		–	+	nd
Cucurlionidae	Weevils			
Otiorrhynchus sulcatus (A)		–	+	nd
Lepidoptera	Butterflies and moths			
Aphomia sociella[a] (L)		–	+	–
Bombyx mori (L)		–	–	nd
Caligo memnon (L)		–	–	nd
Danaus plexippus (L)		–	+	nd
Ephestia kühniella (L)		–	–	nd
Galleria mellonella[a] (L, A)		–	–	–
Heliotis virescens		–	–	nd
Pieris brassicae[a] (L)		–	–	nd
Plutella xylostella (L)		–	–	nd
Spodoptera frugiperda (L)		–	–	nd
Trabala vishnou (L)		–	–	nd
Diptera	Flies			
Hylemyia antiqua (L)		–	–	–
Musca domestica (P, A)		–	–	–
Tipula sp.[a] (L)		–	+	–
Siphonaptera	Fleas			
Ctenocephalides felis (L).		–	–	nd

nd not determined, *C* ciliates, *F* flagellates, *L* larva, *A* adult
[a]Endemic European species from the field

Table 3 Methane emission in cockroaches

Species	Methane emissions	Hindgut differentiation	Protists in hindgut
Blattoidea			
Blattinae			
Blatta orientalis	+	+	C
Deropeltis sp.	+	+	C
Periplaneta americana	+	+	C
Periplaneta australasiae	+	+	C
Periplaneta brunnea	+	+	C
Periplaneta fulginosa	+	+	C
Polyzosteriinae			
Eurycotis floridana	+	+	C
Blaberoidea			
Polyphagidae			
Polyphaginae			
Polyphaga aegyptiaca	+	+	C
Blattellidae			
Plectopterinae			
Eudromiella sp. (Costa Rica)	−	−	−
Lupparia sp. (Luzon, Philippines)	−	−	−
Supella longipalpa	−	−	−
Supella supellectilium	+	+	F
Blattellinae			
Blattella germanica	+ and −	+/−	−
Ischnoptera sp.	nd	−	−
Loboptera decipiens	nd	−	−
Parcoblatta lata	+	nd	nd
Shawella couloniana	−	−	−
Symplece pallens	−	nd	nd
Ectobiinae			
Ectobius sylvestris	−	−	−
Ectobius sp.	−	−	−
Nyctiborinae			
Nyctibora sp. *(Costa Rica)*	+	+	−
Blaberidae			
Blaberoid complex			
Zetoborinae			
Schultesia lampyridiformis	+	+	−
Blaberinae			
Archimandrita sp.	+	+	−
Blaberus craniifer	+	nd	nd
Blaberus fuscus	+	+	C
Blaberus discoidalis	+	+	C
Blaberus giganteus	+	+	C
Blaberus sp. *CR*	+	nd	nd
Byrsotria fumigata	+	+	C
Eublaberus distanti	+	+	−

(*continued*)

Table 3 (continued)

Species	Methane emissions	Hindgut differentiation	Protists in hindgut
Eublaberus posticus	+	+	–
Blaptica sp.	+	+	C + F
Panchloroid complex			
Pycnoscelinae			
Pycnoscelus surinamensis	+	+	C + F
Diplopterinae			
Diploptera punctata	+	+	C
Panchlorinae			
Panchlora nivea	–	–	–
Oxyhaloinae			
Gromphodorhina chopardi	+	+	C
Gromphodorhina portentosa	+	+	C
Leucophaea maderae	+	+	–
Nauphoeta cinerea	+	+	F
Gen. near Griffiniella	+	+	C
Epilamproid complex			
Epilamprinae			
Rhabdoblatta sp.	+	+	–

nd not determined, *C* ciliates, *F* flagellates, *hindgut differentiations* presence of an enlarged, well differentiated hindgut

tracts, individual arthropods were dissected and subjected to an analysis with the aid of epifluorescence microscopy (Hackstein and Stumm 1994). Epifluorescence microscopy allows the detection of individual methanogenic archaea due to their blue F_{420} autofluorescence that is characteristic for methanogens (Doddema and Vogels 1978; Figs. 4 and 5).

Our analysis revealed that only representatives of four out of the 35 taxa studied emitted methane: millipedes, cockroaches, termites, and scarab beetles. All other species did not produce methane, but sometimes hydrogen instead (Table 2). Also the microscopical inspection did not provide evidence for the presence of any methanogen in the arthropods belonging to a non-methanogenic taxon (Hackstein and Stumm 1994). A correlation with the diets of various arthropods was not evident since even species with a strong plant- and fibre-rich diet were completely amethanogenic (e.g. crickets, locusts, stick insects). A size factor could also be excluded since even tiny larvae of methanogenic species with a gut volume of about 1 μL emitted methane. Thus, as in vertebrates, methane production is characteristic for certain taxa, and therefore, controlled by an intrinsic, hereditary, genetic property of the host. This assumption is supported by the observation that certain species of cockroaches (which belong to a methanogenic taxon) have lost the capacity to host methanogens. These species belong predominantly to the Blattellinae and Plectopterinae (Table 3). Even amethanogenic strains of *Blattella germanica* and *Periplaneta americana* were found. These amethanogenic strains

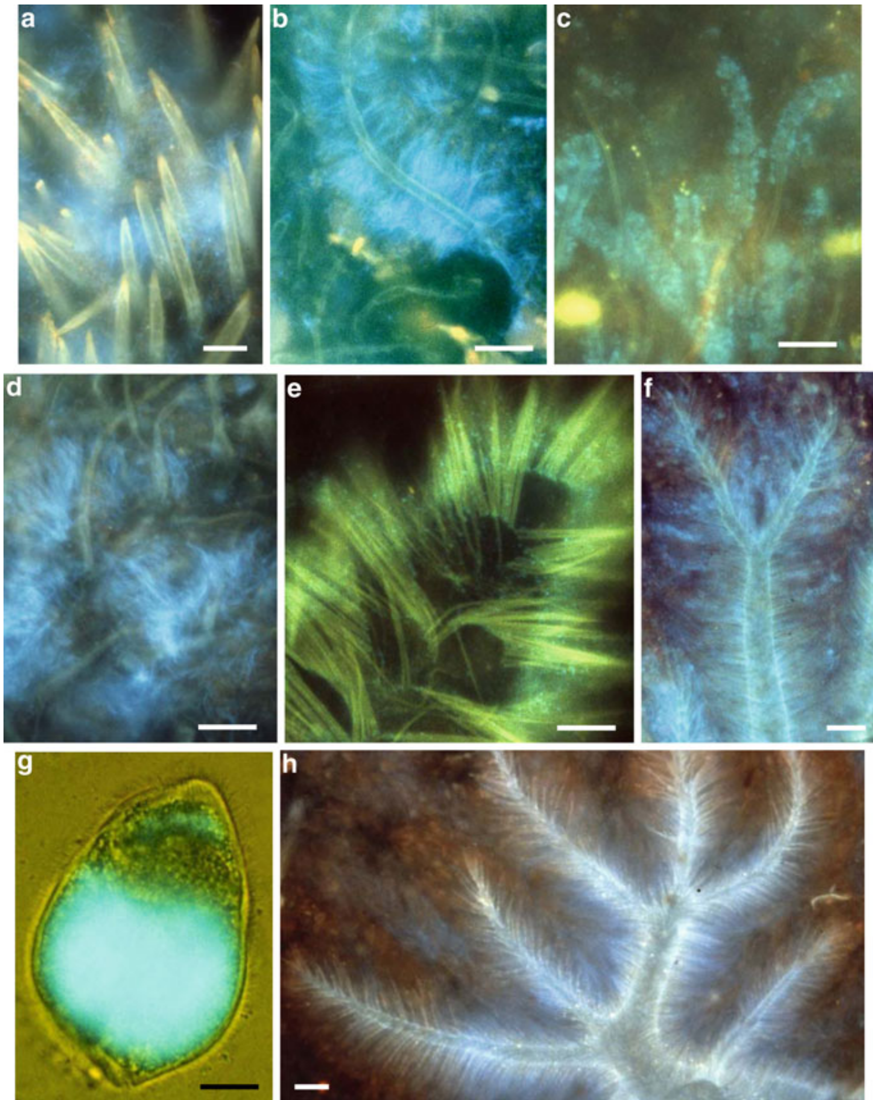

Fig. 4 Methanogenic archaea in the hindguts of the various arthropods, detected by fluorescence microscopy. The *blue* autofluorescence caused by the cofactor F_{420} indicates the presence of methanogens. The *yellowish/greenish* fluorescence originates from the chitinous cuticular structures of the arthropod hosts. (**a**) Filamentous methanogens loosely associated with cuticular hairs in the anterior part of the hindgut of the cockroach *Diploptera punctata*. Bar: 10 µm. (**b**) Filamentous methanogens adhering with their tips to cuticular hairs of the hindgut of the cockroach *Nauphoeta cinerea*. Bar: 10 µm. (**c**) Coccoid methanogens closely associated with cuticular hairs of the hindgut of the cockroach *Leucophaea maderae*. Bar: 10 µm. (**d**) Filamentous methanogens in the posterior hindgut of *Diploptera punctata*. There is no evidence for a close association with cuticular hindgut structures. Bar: 10 µm. (**e**) Small, coccoid methanogens between cuticular hairs (*yellowish* autofluorescence) covering the hindgut of *Nyctibora* sp. Note that many

could be transiently infected with methanogens by co-culture with methanogenic species. However, soon after the removal of the donor insects, the methanogens in the recipients were lost. This means that these amethanogenic strains had definitively lost the capacity to maintain permanently methanogens in their GI tract. Trials to infect amethanogenic species that belong to an amethanogenic taxon were unsuccessful: saw bugs and crickets could not be infected with methanogens – not even transiently (Hackstein and Stumm 1994; Hackstein et al. 1996).

Therefore, we analysed the GI tract and the intestinal surfaces in more detail. The complete GI tracts of the arthropods were dissected and studied by epifluorescence, phase contrast, and differential interference contrast (DIC) microscopy. Arthropod guts are clearly compartmentalized in the anterior–posterior direction. In general, it is possible to identify an oesophagus, crop, midgut and hindgut (Dettner and Peters 2003). The intestinal tract of millipedes and cockroaches is structured relatively simple (Fig. 6a), while the GI tract of cetonid and scarabeid larvae is dominated by a voluminous midgut and hindgut (Fig. 6c). The GI tract of termites, especially of humivorous species is highly structured and consists of compartments with a very variable pH value (Fig. 6b). Notably, in all methanogenic arthropods studied so far, the methanogens are restricted to the hindgut (Hackstein and Stumm 1994), also the methanogens, which are associated with anaerobic, gut-dwelling protozoa. Only parasites, such as gregarines (lacking methanogenic endosymbionts) can be found in the midgut, which, however, in the methanogenic arthropods hosts a complex and numerous microbiota of (facultatively) anaerobic bacteria. The strongly alkaline pH in the midgut of humivorous insects could explain the absence of methanogens and symbiotic protozoa, which depend on habitats with a moderate, near neutral pH as found in the hindguts. However, the midguts of cockroaches possess a more or less neutral pH. Nevertheless, methanogens are completely absent from this compartment. Whether the peritrophic membrane (Dettner and Peters 2003), which wraps the gut contents during their passage through the anterior parts of the GI tract, prohibits the colonization by methanogens, remains unclear. However, it is noteworthy to mention that the peritrophic membrane becomes disintegrated in the hindgut.

The methanogens occur free-floating in the hindgut lumen, attached to food particles, adhering to the gut wall, or as endosymbionts of protists. In certain insects, cuticular differentiations such as trichomes or complex epithelial differentiation of the gut wall (e.g. "pseudosetae", Figs. 4f, h, 7 and 8) enlarge the inner

Fig. 4 (continued) methanogens are found at the basis of the hairs, adhering to the cuticle of the hindgut, at a distance of only a few micrometers to the tracheoles, which support aerobic mitochondrial metabolism in the hindgut epithelium. Bar: 10 µm. (**f**) Coccoid methanogens closely associated with a "pseudoseta" from the hindgut of a larva of the scarab beetle *Pachnoda marginata*. Bar: 10 µm. (**g**) An anaerobic nyctotheroid ciliate from the hindgut of the cockroach *Byrsotria fumigata*. Note the intensive autofluorescence of F_{420} originating from endosymbiotic methanogens. Bar: 10 µm. (**h**) Filamentous methanogens closely associated with a pseudoseta from the hindgut of a larva from the scarab beetle *Pachnoda bhutana*. Bar: 10 µm. Reproduced with permission from Hackstein et al. (2006b)

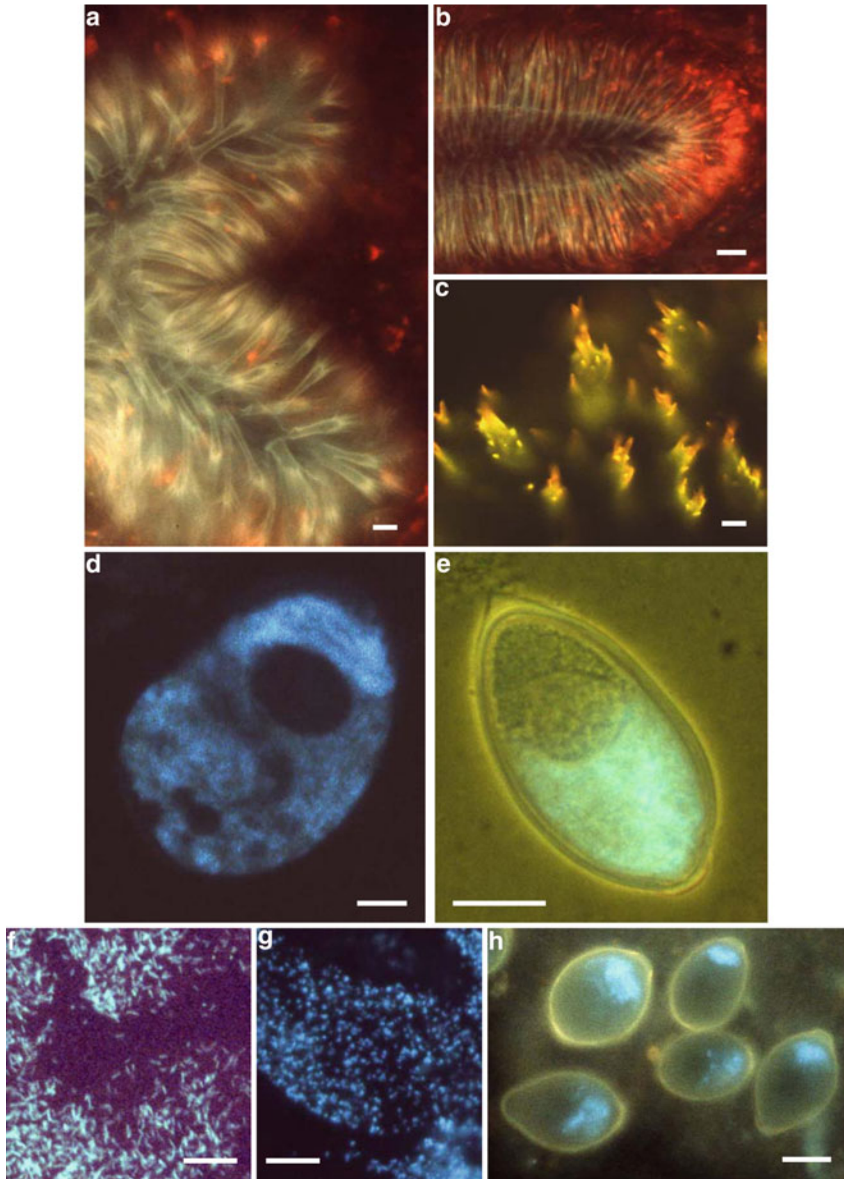

Fig. 5 (**a**, **b**) Differentiations of the hindgut epithelium of short-horned grasshoppers and crickets. Note the complete absence of blue-fluorescing methanogens. Both taxa do not produce methane. (**a**) *Phaeophylacris bedoides*, a cave-dwelling cricket (Bar 10 μm). (**b**) Unidentified, European short-horned grasshopper (Bar: 10 μm). (**c**) Cuticular structures at the junction between midgut and hindgut of the cockroach *Rhabdoblatta* sp. These chitinous bracts are likely to have a function in disrupting the peritrophic membrane before the gut contents enter the hindgut (Bar: 10 μm). (**d**) An anaerobic nyctotheroid ciliate from the hindgut of the cockroach sp. The blue autofluorescence stems from numerous endosymbiotic methanogens. The *dark spot* identifies the location of the

surface of the hindgut by several orders of magnitude. These structures provide attachment sites for a complex microbiota, which includes methanogens as a dominant component (Figs. 4f, h and 7). However, the presence of such elaborated differentiations of the gut wall does not per se enable the colonization by methanogens. Also non-methanogenic insects possess such structures – without any trace of a methanogenic archaeon (Fig. 5a, b). Notably anaerobic protozoa with endosymbiotic methanogens were also found exclusively in the hindgut of many (but not all) methanogenic arthropods (Figs. 4g and 5d–h). Such protozoa were never found in the GI tract of non-methanogenic animals.

The morphology of both the intestinal and the endosymbiotic methanogens is rather variable suggesting the presence of various species of methanogens. Only three species from termite guts and one from a cockroach gut have been isolated and cultured in vitro (Leadbetter and Breznak 1996; Leadbetter et al. 1998; Sprenger et al. 2000). All four species of methanogens adhere to the internal surface of the hindgut. PCR – and T-RFLP guided profiling studies in termites and cetonids confirmed the anticipated diversity of intestinal archaea, which are clearly different from non-gut communities (Ohkuma et al. 1995, 1999; Shinzato et al. 1999, Tokura et al. 2000; Brauman et al. 2001; Friedrich et al. 2001; Egert et al. 2003; Donovan et al. 2004; Miyata et al. 2007). Also the *Nyctotherus*-like ciliates from the hindgut of methanogenic cockroaches and millipedes and their methanogenic endosymbionts are different at the 16S rDNA level from each other and from free-living gut methanogens (van Hoek et al. 1998, 2000). The endosymbiotic methanogens are similar to, but distinct from gut-dwelling *Methanobrevibacter* species. The free-living relatives of *Nyctotherus* host different methanogens belonging to the Methanomicrobiales (van Hoek et al. 2000). The ciliates and their endosymbionts predominantly co-speciate, suggesting a vertical inheritance of the endosymbionts. The exceptions from the co-speciation argue for infrequent endosymbiont replacements (van Hoek et al. 2000; Hackstein et al. 2002).

The adherence of the methanogens to the internal surfaces and the supporting structures of the hindgut might explain the persistence of methanogens in the arthropod guts. It is conceivable that their adherence is under genetic control explaining the occurrence of methanogens in certain taxa, their absence in other taxa and the amethanogenic strains of otherwise methanogenic arthropods.

Fig. 5 (continued) macronucleus, which does not contain methanogens (Bar: 10 μm). (**e**) Cyst (resting stage) of the ciliate shown in Fig. 4g (i.e. from the hindgut of the cockroach *Byrsotria fumigata*). The blue autofluorescence discloses the presence of methanogens also in cysts (Bar: 10 μm). (**f**, **g**) Endosymbiotic methanogens from ciliates thriving in the hindgut of the cockroach *Periplaneta americana* (strain Amsterdam) (**f**), and the cockroach *Blaberus* sp. (strain Amsterdam) (**g**). The methanogens were released from the ciliates by gentle squashing. Note the different shapes of the methanogens (Bar: 5 μm) (**h**) Cysts of ciliates from the hindgut of a cockroach belonging to the Oxyhaloinae (Genus near Griffiniella) containing endosymbiotic methanogens (blue autofluorescence) (Bar: 10 μm). Reproduced with permission from Hackstein et al. (2006b)

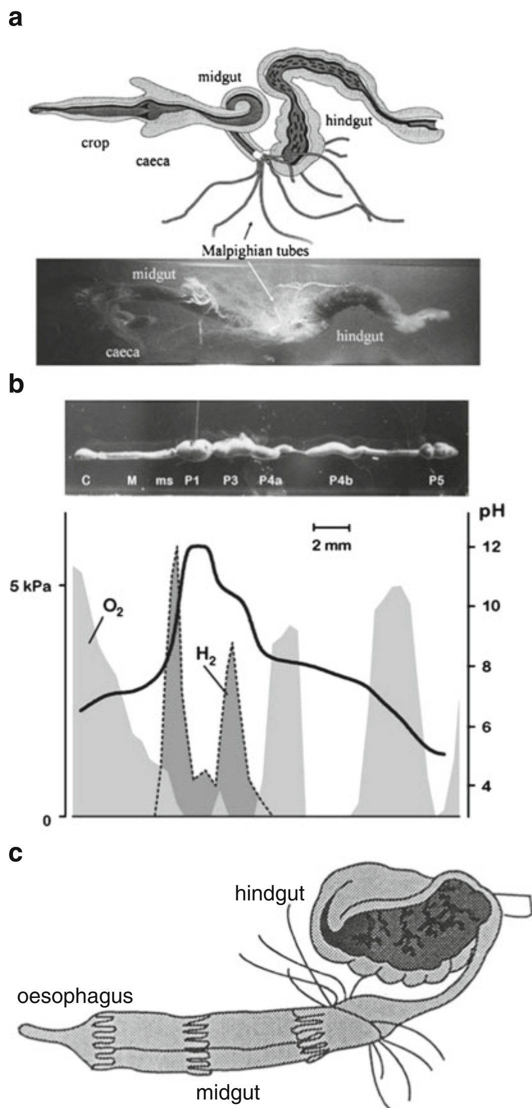

Fig. 6 Macroscopical views of the intestinal tract of cockroaches (**a**), termites (**b**) and larvae of scarab beetles (**c**). (**a**) *Above*: cartoon of the intestinal tract of a cockroach (*Periplaneta americana*). *Below*: a picture of a gut of *Periplaneta americana*, which has been embedded into agarose for microsensor measurements (after removal of the crop). (**b**) The unravelled intestinal tract of a termite (*Cubitermes* sp.) to demonstrate the complex longitudinal compartmentalisation of the termite gut. A microsensor is inserted into compartment P1. The plot below displays the longitudinal variations in pH (*solid line*) and the partial pressures of O_2 and H_2. *C* Crop, *M* midgut, *ms* mixed segment, P1-5: proctodeal regions. (**b**) Reproduced with permission from Brune and Friedrich (2000). (**c**) A cartoon demonstrating the gross organisation of the intestinal tract of the larva of the scarab beetle *Pachnoda* spec. The midgut is highly alkaline. The interior of the hindgut is shown to indicate the location of the pseudosetae (*black* structures; c.f. Figs. 4f, h and 7). Reproduced with permission from Hackstein et al. (2006b)

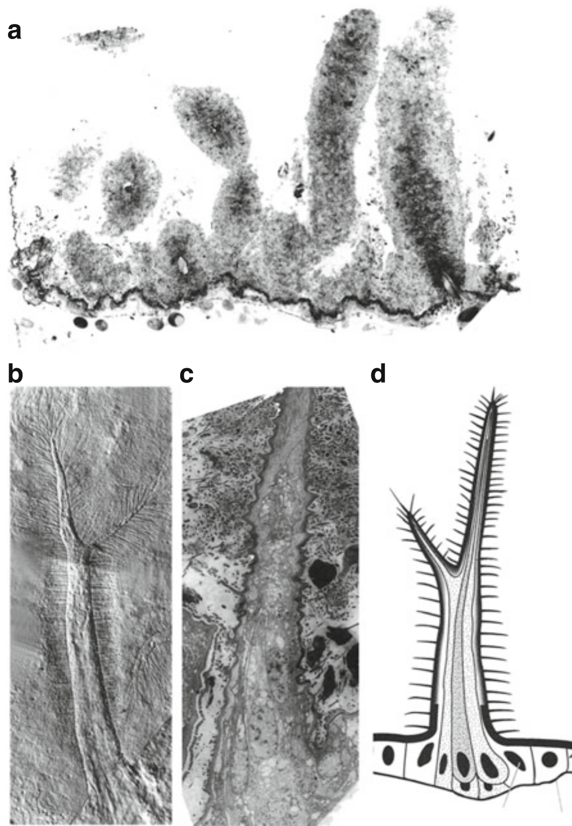

Fig. 7 (**a**) Light micrograph (semi-thin section) of the hindgut epithelium of a larva of *Dynastes hercules* (Scarabaeidae). Villus-like structures, measuring between 200 and 500 μm protrude into the lumen of the hindgut. These structures, which we have named "pseudosetae", are composed of several, elongated cells of the hindgut epithelium and covered by a complex prokaryotic microbiota, including methanogens (c.f. Fig. 4f, h). (**b**) Light micrograph (differential interference contrast) of a single pseudoseta from the hindgut of a *Pachnoda marginata* (Scarabaeidae) larva after the removal of the bacteria adhering to this structure. The surface of the pseudoseta is covered with a cuticle, which carries numerous hairs (trichomes) enhancing the surface by about two orders of magnitude. Sizes 100–300 μm. (**c**, **d**): Electron micrograph (**c**) and cartoon (**d**) of a single pseudoseta. Note that tracheae and tracheoles as well as mitochondria are lacking in the distal parts of the pseudosetae. The vacuoles are most likely involved in the transport of fermentation products (mainly short chain fatty acids) generated in the lumen of the hindgut to the hindgut epithelium, and eventually to the hemolymph. *Black* ovals in (**d**) indicate the nuclei of the hindgut epithelium and the pseudoseta. Reproduced with permission from Hackstein et al. (2006b)

The adherence of the methanogens to the gut wall and the small size of the guts create a problem for the survival of the methanogens. Methanogens are strictly anaerobic (Liu and Whitman 2008), but at the gut wall they experience a continuous influx of oxygen. Due to their small size, arthropod guts possess a large surface to volume ratio (Brune and Friedrich 2000, Hackstein et al. 2006b) that makes it

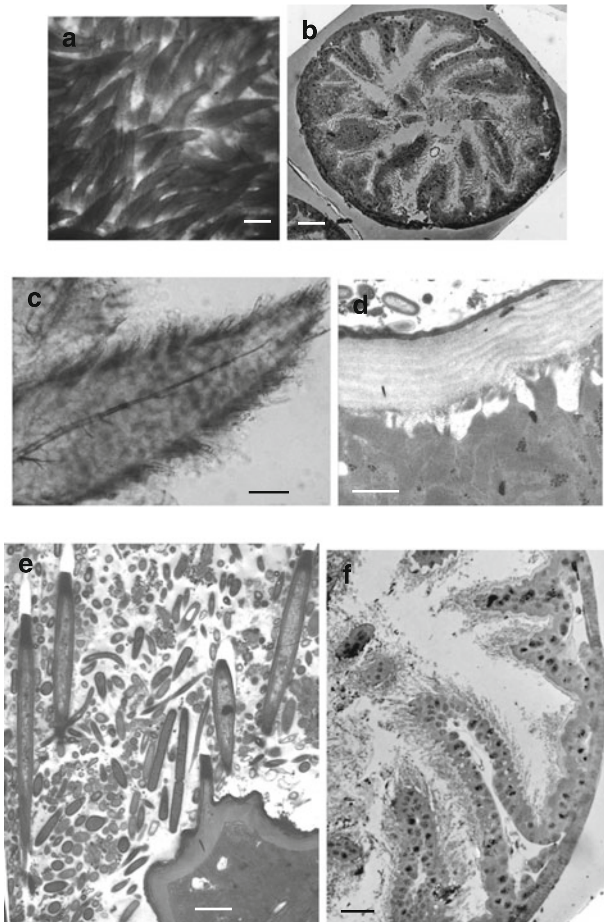

Fig. 8 (**a–f**) Various aspects of the hindgut epithelium of the cockroach *Nyctibora* sp. (**a**) Low magnification light microscopy reveals that the inner surface of the hindgut is covered by villus-like protrusions of the hindgut epithelium (Bar 200 μm). (**b**) A cross-section of the hindgut shows that these villi fill nearly the whole volume of the gut (Bar 200 μm). (**f**) The same aspect at higher magnification (Bar: 100 μm) reveals the presence of tracheae inside of these villi. (**c**) A light micrograph at higher magnification (Bar: 100 μm), which shows that tracheae and tracheoles are present in each of the villi. (**d**) Mitochondria with many cristae are found just below the cuticle, which covers the epithelial cells at the luminal side (Bar: 1 μm). (**e**) Electron micrograph of a villus, which is associated with numerous bacteria forming a complex microbiota strongly adhering to the villus with its trichomes (several of which are cut). Note the trachea inside the epithelial cell (Bar: 2 μm). Reproduced with permission from Hackstein et al. (2006b)

difficult to maintain anaerobic conditions inside the gut. However, aerobic and facultatively anaerobic bacteria located close to the gut wall sequester the oxygen and generate a steep oxygen gradient across the gut wall with the consequence that the lumen, but not the wall of the gut becomes completely anaerobic. In lower termites, the centre of the gut is populated by flagellates that generate hydrogen,

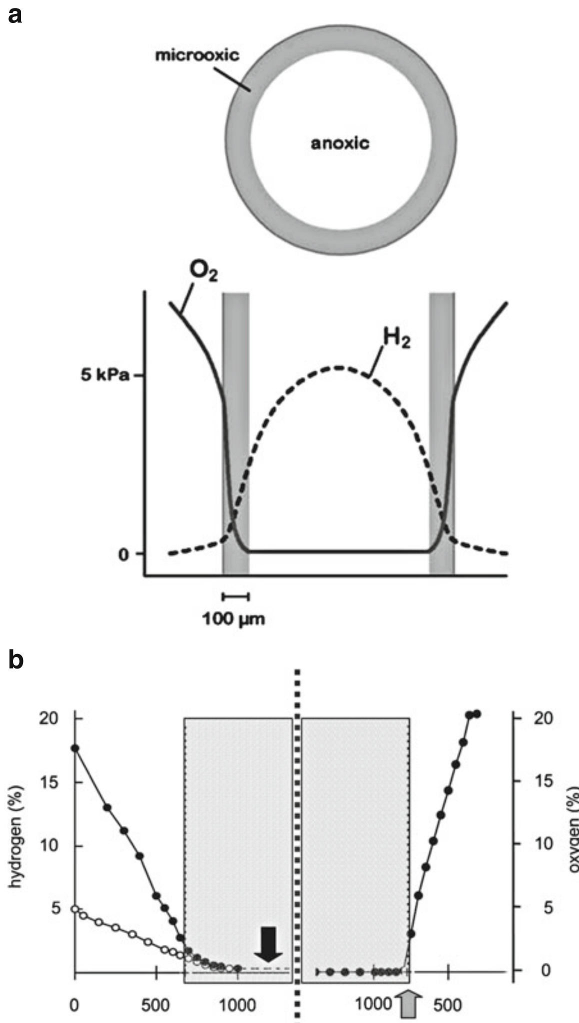

Fig. 9 Cartoons illustrating the radial profiles of H_2 and O_2 partial pressures in termite (**a**) and cockroach guts (**b**), respectively, as measured with the aid of microsensors at explanted guts, which had been embedded into agarose (Brune et al. 1995; for such a set-up, see Fig. 6a, b). Very steep O_2 gradients (**a**, and right part of **b**) are caused by the respiration of facultatively aerobic microbiota with the consequence of small microoxic zones at the periphery of the hindgut lumen (cross-section in **a**, and *grey arrow* in **b**, *right panel*). The H_2 peak in the termite hindgut is caused by hydrogen-producing protozoa. The hydrogen diffuses out of the gut, being partially consumed by methanogens colonizing the hindgut wall. In (**b**), hydrogen is generated throughout the hindgut lumen, but the presence of methanogens throughout the lumen keeps the partial pressure of hydrogen low (*black arrow* at the *left panel*). The hydrogen-consuming communities are not saturated, since even the application of external hydrogen at a partial pressure of 18% does not cause higher than background levels of hydrogen in the gut lumen [*left panel*, *open circles* (5% H_2) and *black circles* (18% H_2)]. The *shaded areas* indicate the location of the left and right halves of the hindgut, respectively. Abscissa: distance to the surface of the agarose in micrometers. (**a**) Reproduced with permission from Brune and Friedrich (2000). Reproduced with permission from Hackstein et al. (2006b)

which accumulates in the centre of the gut and diffuses outwards through the gut wall, where methanogens and other bacteria create a hydrogen sink (Fig. 9a). Thus, in lower termites the methanogens at the gut wall occupy a position between an inside-directed oxygen gradient and an outside-directed hydrogen gradient. In cockroaches, there is no outside directed hydrogen gradient, since the gut ciliates possess endosymbiotic methanogens and since the free-living methanogens are more evenly distributed throughout the hindgut (Fig. 9b). Nevertheless, as also in cockroaches the hindgut microbiota generate a steep oxygen gradient and produce hydrogen in the lumen of the gut that is consumed in situ by interspecies hydrogen transfer. The lack of accumulation of hydrogen even after incubation of hindguts in a hydrogen atmosphere indicates that hydrogen is limiting methanogenesis in the hindgut (Fig. 9b).

Interestingly, we detected hydrogen emissions in many of the methanogenic species in our screen (Table 2; Hackstein and Stumm 1994). The explanation for this paradox lies in the fact that dense populations of (facultatively) anaerobic bacteria in the midgut generate substantial amounts of hydrogen that – in the absence of methanogens – diffuses out of the midgut. Part of this hydrogen is exhaled with the breath, while another substantial part is transferred to the methanogenic hindgut by intercompartment hydrogen transfer (Lemke et al. 2001). The anatomy of the intestinal tract of termites, cockroaches, and scarab beetle larvae favours such an intercompartment hydrogen transfer (Lemke et al. 2001). We estimated that the hydrogen, which is transferred to the hindgut, contributes to some 25–30% of the methane production in the hindgut (Lemke et al. 2001; Hackstein et al. 2006b).

4 Conclusions

Methanogenic archaea in the intestinal tract of vertebrates and arthropods fulfil an important role in interspecies hydrogen transfer (Schink 1997; Stams and Plugge 2009). Remarkably, significant amounts of methanogens are only present in part of the vertebrate taxa and in four of the many orders of arthropods. Obviously, neither diet nor structure of the GI tract can explain the presence of significant amounts of methanogens in certain taxa and their absence in others. Notably, the taxonomic position of the host or population constraints is crucial for the methane status. This means that hereditary, genetic factors of the host control the presence of symbiotic methanogens in the GI tract of animals.

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Syntrophy in Methanogenic Degradation

Petra Worm, Nicolai Müller, Caroline M. Plugge, Alfons J.M. Stams, and Bernhard Schink

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Abstract This chapter deals with microbial communities of bacteria and archaea that closely cooperate in methanogenic degradation and perform metabolic functions in this community that neither one of them could carry out alone. The methanogenic degradation of fatty acids, alcohols, most aromatic compounds, amino acids, and others is performed in partnership between fermenting bacteria and methanogenic archaea. The energy available in these processes is very small,

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attributing only fractions of an ATP unit per reaction run to every partner. The biochemical strategies taken include in most cases reactions of substrate-level phosphorylation combined with various kinds of reversed electron transport systems in which part of the gained ATP is reinvested into thermodynamically unfavourable electron transport processes. Altogether, these systems represent fascinating examples of energy efficiency at the lowermost energy level that allows microbial life.

1 Introduction

In oxygen-limited environments, such as lake sediments or the lower layers of eutrophic lakes in summertime, biomass oxidation has to be coupled to alternative electron acceptors such as nitrate, Mn(IV), Fe(III), sulfate, or CO_2 (which is reduced to methane) (Zehnder 1978; Schink 1989). The relative importance of these alternative electron acceptors depends on their availability in the respective habitat; most freshwater sediments are rich in iron oxides, and marine sediments are well supplied with sulfate due to the high sulfate content of seawater (28 mM). Only methanogenesis is independent of external electron acceptors because the methanogenic degradation of biomass is actually a dismutation of organic carbon:

$$C_6H_{12}O_6 \rightarrow 3CH_4 + 3CO_2 \quad \Delta G^{0'} = -390\ \text{kJ/mol}$$

Whereas aerobic, nitrate-reducing or manganese-reducing bacteria typically are able to degrade polymeric organic compounds via the respective monomers to CO_2 and other inorganic products in one single cell, the conversion of complex organic matter by iron reducers or sulfate reducers requires a cooperation with fermenting bacteria, which feed the respective terminal oxidizers with classical fermentation products such as fatty acids, alcohols, and others. Methanogenic degradation of organic matter is even more complex and requires cooperation of three different metabolic groups (guilds) of bacteria, including primary fermenters, secondary fermenters, and methanogens (Bryant 1979; Fig. 1a). Primary fermenting bacteria are known for long times and have been isolated by classical procedures with all kinds of polymeric or monomeric substrates. Also anaerobic protozoa, including flagellates and ciliates, can operate in this manner (see Fenchel and Finlay 2010). Different from iron reducers or sulfate reducers, methanogenic archaea use only very few substrates, including hydrogen, CO_2, other C_1-compounds, and acetate. In one exceptional case, a methanogen can also oxidize isopropanol and ethanol (Widdel et al. 1988). Thus, the majority of classical fermentation products such as alcohols, fatty acids, branched-chain fatty acids and aromatic fatty acid residues from partial degradation of amino acids, long-chain fatty acids from lipid hydrolysis, and heterocyclic aromatic compounds deriving from nucleic acids all need to be fermented further to those substrates that methanogens can use (Bryant 1979; Schink 1997; Schink and Stams 2002; McInerney et al. 2008; Stams and Plugge 2009).

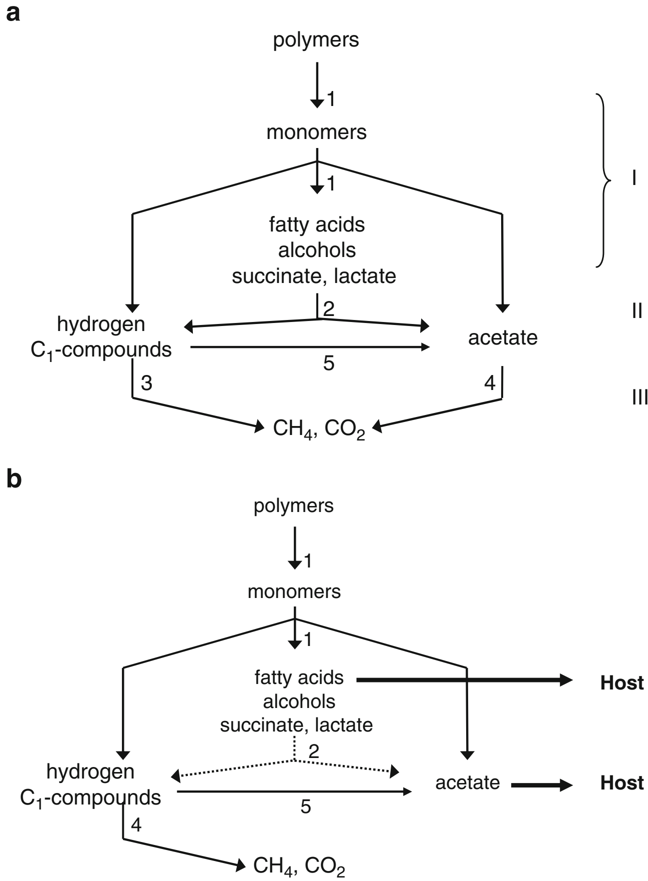


Fig. 1 Methanogenic degradation of complex organic matter by cooperation of different metabolic groups. (**a**) Electron flow in a freshwater sediment or biogas reactor. (**b**) Electron flow in plant digestion in the rumen. Metabolic groups of organisms involved: primary fermenters (1), secondary fermenters (2), hydrogen and C1-compounds-using methanogens (3), acetoclastic methanogens (4), and homoacetogenic bacteria (5) (modified after Schink 1997)

This is the function of the secondary fermenting bacteria that depend on close cooperation with methanogenic partners and are the subject of this chapter.

Methanogenic environments are widely distributed in nature. Wetlands, freshwater sediments, swamps, and digestive tracts of ruminants and insects are environments that produce high amounts of methane. Man-made systems, such as rice paddies and anaerobic bioreactors and landfills, are other important sources of methane production.

Methanogenic archaea catalyze the final step in the overall anaerobic degradation of organic material to methane and CO_2. One metabolic group of methanogenic archaea converts CO_2 plus hydrogen or formate to methane, whereas others use acetate or methanol. Acetate, the most important intermediate in anaerobic

digestion, accounts for approximately two-thirds of all methane produced, while the last third is produced from the reduction of CO_2 with electrons derived from the oxidation of hydrogen or formate (Ferry 1992; Liu and Whitman 2008). Currently, only two types of acetoclastic methanogens have been identified: *Methanosaeta* sp. and *Methanosarcina* sp. *Methanosarcina* sp. is a genus of versatile methanogens, including species capable of growing with different substrates including acetate, methanol, methylamines, and H_2/CO_2, whereas *Methanosaeta* sp. uses only acetate. *Methanosaeta* sp. is widely distributed in nature and, because of its high affinity for acetate, it outcompetes *Methanosarcina* sp. in low-acetate environments (Conklin et al. 2006). In rumen and other animal gastrointestinal tracts, however, *Methanosarcina* sp. are typically present, due to the high acetate concentrations occurring in these environments (see Conway de Macario and Macario 2010). Both acetoclastic archaea grow very slowly, with doubling times of 1–12 (*Methanosaeta*) and 0.5–2 (*Methanosarcina*) days (Jetten et al. 1992). Despite their restricted substrate range (H_2/CO_2, formate, and methylated C_1-compounds), methanogenic archaea are phylogenetically very diverse. They are classified into five orders (Whitman et al. 2006). Representatives of the orders Methanobacteriales and Methanomicrobiales are commonly present in animal gastrointestinal tracts.

The importance of the secondary fermenting bacteria varies with the kind of substrate utilized and the efficiency of the methanogenic partners at the end of the anaerobic feeding chain. If the methanogens maintain a low concentration of hydrogen and acetate, numerous classical primary fermentations are shifted to the formation of hydrogen, CO_2, and acetate and produce much less reduced side products such as fatty acids than they do in pure culture (Iannotti et al. 1973; Tewes and Thauer 1980; Schink and Zeikus 1982). Thus, the majority of electrons from substrate degradation will flow through the outer lines of the scheme depicted in Fig. 1a, and the electron flow through the central part may be only of minor importance to ensure complete degradation of biopolymers as this is typical for freshwater lake sediments, swamps, or sewage sludge digesters.

The situation is basically different in the fermentations proceeding inside the gastrointestinal tracts of animals. There, the host organism is not favoured by complete degradation of biopolymers inside the gut to methane and CO_2 but uses a substantial part of the overall electron input for its own support, e.g., in the form of fatty acids. Since the retention time of the feed inside the guts is limited to a few hours or 2 days at maximum, neither the slow-growing acetate-utilizing methanogens nor the fatty acid-degrading syntrophic associations will establish. Therefore, these fatty acids accumulate in the gut and are taken up by the host. In ruminants, this acid transfer proceeds at concentrations of 60 mM acetate, 20 mM propionate, and 10 mM butyrate (Bryant 1977); in termite guts, only acetate is produced to major amounts (Breznak and Kane 1990; Brune 2007). Methanogenesis in the intestinal tracts is restricted to hydrogen utilization, in order to shift the overall electron flow mainly towards fatty acids production and to minimize unwanted side fermentations such as alcohol formation (Fig. 1b).

The ability to transfer electrons to a partner organism is an important metabolic feature associated with many physiologically diverse microorganisms. This trait is

usually referred to as syntrophism. Syntrophism is a special type of symbiosis between two microorganisms in which growth of one organism depends on supply of growth factors or nutrients or removal of products by a partner organism. Especially among anaerobic microorganisms, cooperation of several metabolic types of bacteria in the feeding chain is a common feature. The mutual dependence can be explained calculating the changes in Gibbs' free energy ($\Delta G^{0'}$) for the oxidation of, e.g., ethanol to hydrogen, CO_2, and acetate (Bryant et al. 1967). Under defined standard conditions with gases at 10^5 Pa pressure, 1 M concentration of products/substrates at pH 7.0 and 298 K, the Gibbs' free energy value for ethanol oxidation is positive with +9.6 kJ/reaction (Table 1). This indicates that the reaction

Table 1 Equations and standard free energy changes for relevant reactions described in the chapter (Gibbs' free energy changes are taken from Thauer et al. 1977)

Reaction	$\Delta G^{0\prime}$ (kJ/reaction)
For secondary fermentation reactions	
Glucose → 2 Acetate$^-$ + 2H$^+$ + 2CO_2 + 4H_2	−216
Ethanol + H_2O → Acetate$^-$ + H$^+$ + 2H_2	+9.6
Propionate$^-$ + 2H_2O → Acetate$^-$ + CO_2 + 3H_2	+76
Butyrate$^-$ + 2H_2O → 2 Acetate$^-$ + H$^+$ + 2H_2	+48
Crotonate$^-$ + 2H_2O → 2 Acetate$^-$ + H$^+$ + H_2	−6.2
Acetate$^-$ + 2H_2O → 2CO_2 + 4H_2	+96
Benzoate$^-$ + 6H_2O → 3 Acetate$^-$ + CO_2 + 2H$^+$ + 3H_2	+49.5
Phenol + 5H_2O → 3 Acetate$^-$ + 3H$^+$ + 2H_2	+5.7
Acetone + CO_2 → 2 Acetate$^-$ + 2H$^+$	−34
Alanine + 3H_2O → Acetate$^-$ + H$^+$ + HCO_3^- + NH_4^+ + 2H_2	+7.5
Isoleucine + 3H_2O → 2-Methylbutyrate$^-$ + H$^+$ + HCO_3^- + NH_4^+ + 2H_2	+7.5
Valine + 3H_2O → Isobutyrate$^-$ + H$^+$ + HCO_3^- + NH_4^+ + 2H_2	+9.7
Leucine + 3H_2O → Isovalerate$^-$ + H$^+$ + HCO_3^- + NH_4^+ + 2H_2	+4.2
Leucine + 3H_2O → α-Ketoisocaproate$^-$ + NH_4^+ + H_2	+51
α-Ketoisocaproate$^-$ → Isovalerate$^-$ + H_2	−56
Glutamate$^-$ + 2H_2O → Acetate$^-$ + 0.5H$^+$ + HCO_3^- + NH_4^+ + 0.5 Butyrate$^-$	−58
Glutamate$^-$ + 4H_2O → Propionate$^-$ + 2HCO_3^- + H$^+$ + NH_4^+ + 2H_2	−5.8
Glutamate$^-$ + 3H_2O → 2 Acetate$^-$ + H$^+$ + HCO_3^- + NH_4^+ + H_2	−34
Aspartate$^-$ + 3H_2O → Acetate$^-$ + H$^+$ + 2HCO_3^- + NH_4^+ + 2H_2	−14
Serine + 2H_2O → Acetate$^-$ + H$^+$ + HCO_3^- + NH_4^+ + H_2	−90
For reactions of methanogenic archaea	
4H_2 + CO_2 → CH_4 + 2H_2O	−131
4 Formate$^-$ + 4H$^+$ → CH_4 + 3CO_2 + 2H_2O	−145
4CO + 2H_2O → CH_4 + 3CO_2	−211
Acetate$^-$ + H$^+$ → CH_4 + CO_2	−35
4 Methanol → 3CH_4 + CO_2 + 2H_2O	−106
H_2 + Methanol → CH_4 + H_2O	−113
CO_2 + H_2O → H$^+$ + HCO_3^-	+4.8
CO_2 + H_2 → Formate$^-$ + H$^+$	−4.5
For hydrogen-consuming reactions	
Crotonate + H_2 → Butyrate$^-$	−75
Pentenoate + H_2 → Valerate$^-$	−75
Glycine + H_2 → Acetate$^-$ + NH_4^+	−78
CO_2 + H_2 → Formate$^-$ + H$^+$	−4.5

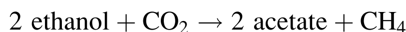
cannot take place nor can any microbe gain energy from this oxidation. However, the Gibbs' free energy becomes negative when the hydrogen partial pressure (pH_2) decreases. This example of interspecies hydrogen transfer is characteristic for the way how organic matter is degraded in methanogenic habitats.

2 Syntrophic Culture Systems, Microbiology, Biochemistry, and Molecular Biology

Life depends on the availability of energy, which is stored inside the cell in the form of ATP. Under physiological conditions, including heat losses, the synthesis of ATP requires 60–70 kJ/mol (Thauer et al. 1977). Membrane-bound ATPases couple the hydrolysis or synthesis of ATP to the transport of protons (in some cases also Na^+ ions) across the cytoplasmic membrane. Depending on the stoichiometry of the ATPase system in question, the ratio of ions translocated versus ATP synthesized or hydrolyzed may vary between 3 and 5; in most cases, a ratio of 3–4 appears to be justified (Engelbrecht and Junge 1997; Cherepanov et al. 1999). As a consequence, the smallest amount of energy that can still be converted to ATP – and with this into metabolic activity and growth – is equivalent to one-third or one-fourth of an ATP unit, i.e., in the range of −15 to −20 kJ/mol reaction (Schink 1997; Schink and Stams 2002). It is this minimum increment of energy with which syntrophically fermenting methanogenic communities have to operate.

2.1 Ethanol

The biochemistry of syntrophic oxidation of ethanol, although the oldest syntrophic system is known, has still not been elucidated in detail. Early work on the so-called S-organism indicated that ethanol is oxidized via acetaldehyde to acetyl-CoA and further to acetate, including ATP synthesis by acetate kinase (Reddy et al. 1972a, b, c). This concept was confirmed by similar studies on the ethanol-oxidizing bacteria *Pelobacter acetylenicus* and *Pelobacter carbinolicus* (Schink 1985, Eichler and Schink 1986). Nonetheless, the energetics of this reaction chain is still unclear. The overall reaction



$$2 \text{ ethanol} + CO_2 \rightarrow 2 \text{ acetate} + CH_4$$

yields $\Delta G^{0\prime} = -112$ kJ/mol, which leaves a total of about −40 kJ per ethanol oxidation reaction for the syntrophic ethanol oxidizer, indicating that part of the ATP formed by substrate-level phosphorylation has to be invested into reversed electron transport. Oxidation of acetaldehyde to acetyl-CoA ($E^{0\prime} = -370$ mV) can be coupled to hydrogen formation via ferredoxin at a sufficiently low hydrogen

pressure. A ferredoxin-like electron carrier has been purified from *P. acetylenicus* (Kowalski and Schink, unpublished). The energetically difficult reaction is the transfer of electrons from the acetaldehyde/ethanol coupled (−196 mV) to hydrogen formation. Such a reaction requires energy input in the form of, e.g., a reversed electron transport, a feature that is common to all syntrophically fermenting bacteria studied so far (Fig. 2). Hydrogen formation from ethanol in crude extracts of *P. acetylenicus* is stimulated by ATP (Hauschild 1997). Since *P. acetylenicus* also contains a menaquinone-like electron carrier (Strohm and Schink, unpublished), a basically similar reversed electron transport system as suggested for *Syntrophomonas wolfei* (discussed in Sect. 2.2) can be anticipated, but experimental evidence has not been provided yet. A comproportionating [FeFe] hydrogenase as described for *Thermotoga maritima* (Schut and Adams 2009) could finally release the electrons from NADH and ferredoxin towards proton reduction. *T. maritima* ferments glucose to acetate, CO_2, and H_2 via the Embden–Meyerhof pathway, generating two NADH and four reduced ferredoxins per molecule of glucose. In order to re-oxidize these carriers, a proposed bifurcating [FeFe] hydrogenase uses electrons from NADH and reduced ferredoxin in a 1:1 ratio to produce H_2. Schut and Adams (2009) found genes with sequence similarity to this [FeFe] hydrogenase in several other microorganisms, including the ethanol-degrading *P. carbinolicus*, the butyrate-degrading *S. wolfei* (discussed in Sect. 2.2), and the propionate-degrading *Syntrophobacter fumaroxidans* (discussed in Sect. 2.3).

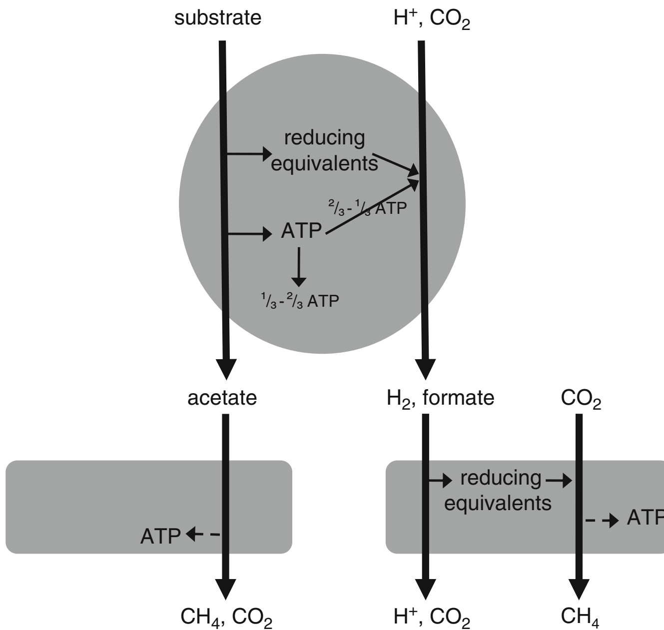


Fig. 2 Conversions performed by a secondary fermenting bacterium (*top*), a hydrogen- and formate-using methanogen (*bottom right*), and an acetoclastic methanogen (*bottom left*)

Also sulfate reducers like *Desulfovibrio vulgaris* have been shown to couple ethanol oxidation to acetate with electron transfer to methanogenic partners (Bryant et al. 1977). However, this activity appears not to be coupled to ATP synthesis by the sulfate reducer because *D. vulgaris* does not grow in such co-cultures (Kremer et al. 1988). The classical sulfate reducers oxidize ethanol via acetaldehyde directly to acetate without intermediate formation of an activated acetyl residue; thus, no ATP is formed by substrate-level phosphorylation and the cultures do not grow (Kremer et al. 1988). Nonetheless, syntrophic growth of an ethanol-degrading sulfate reducer in the absence of sulfate with a methanogen as electron scavenger has been documented (Walker et al. 2009). Ethanol-degrading sulfate reducers that grow in the absence of sulfate in co-culture with methanogens have to form ATP through substrate-level phosphorylation via acetyl-CoA or acetyl phosphate as intermediates or the electron transfer from the intermediate carriers to hydrogen or formate as extracellular electron carriers has to be coupled to some kind of net ion translocation, which is coupled to ATP synthesis.

2.2 Butyrate

Anaerobic butyrate degraders known to date belong to only two groups of bacteria: the genus *Syntrophomonas* within the phylum Firmicutes and the genus *Syntrophus* within the order Syntrophobacterales of the phylum Proteobacteria (Table 2). Fermentation of butyrate to acetate and hydrogen is endergonic (Table 1) and occurs only at very low hydrogen partial pressures, e.g., in the presence of methanogenic archaea (Schink 1997). Syntrophic butyrate oxidizers use only very few substrates. Beyond oxidation of saturated fatty acids in co-culture with methanogens, axenic growth is possible only with unsaturated fatty acids such as crotonate (Schink 1997; McInerney et al. 2008). They cannot use external electron acceptors for growth, thus reflecting the high degree of specialization of these bacteria for syntrophic cooperation (Schink 1997).

Butyrate is oxidized via β-oxidation to acetate yielding one mole ATP per mole of butyrate. The reducing equivalents are transferred to flavoenzymes and NAD^+ (Wofford et al. 1986). Re-oxidation of these electron carriers of a relatively positive redox potential with protons to form hydrogen is energetically difficult. Of course, a low hydrogen partial pressure helps to facilitate those reactions, but no known methanogen is able to maintain a hydrogen partial pressure low enough (10^{-10} atm) to allow direct proton reduction with these electrons (Thauer and Morris 1984; Schink 1997). Therefore, it was postulated that syntrophic butyrate degraders have to invest energy into a reversed electron transport, thus leaving only a fraction of an ATP for growth of the bacterium (Thauer and Morris 1984).

Recently, Müller et al. (2009) showed that an enzyme system similar to the comproportionating [FeFe] hydrogenase of *T. maritima* is essential in butyrate oxidation by *S. wolfei*. The comproportionating [FeFe] hydrogenase of *T. maritima* drives the endergonic reduction of protons to hydrogen with NADH by exergonic

Table 2 Propionate-degrading syntrophic bacteria (modified after McInerney et al. 2008)

Organism	Growth possible with			Phylogenetic position	Reference
	Propionate + sulfate	Propionate + fumarate	Propionate + syntrophic partner		
Syntrophobacter fumaroxidans	+	+	+	δ-Proteobacteria	Harmsen et al. (1998)
Syntrophobacter pfennigii	+	−	+	δ-Proteobacteria	Wallrabenstein et al. (1995)
Syntrophobacter sulfatireducens	+	−	+	δ-Proteobacteria	Chen et al. (2005)
Syntrophobacter wolinii	+	ND	+	δ-Proteobacteria	Boone and Bryant (1980)
Pelotomaculum schinkii	−	−	+	Low G + C Gram positives	De Bok et al. (2005)
Pelotomaculum thermopropionicum	−	+	+	Low G + C Gram positives	Imachi et al. (2002)
Pelotomaculum propionicicum	−	−	+	Low G + C Gram positives	Imachi et al. (2007)
Smithella propionica	ND	ND	+		Liu et al. (1999)
Desulfotomaculum thermobenzoicum thermosyntrophicum	+	−	+	Low G + C Gram positives	Plugge et al. (2002b)

reduction of another couple of protons with reduced ferredoxin, which is produced in pyruvate oxidation during growth on glucose (Schut and Adams 2009). In butyrate oxidation by *S. wolfei*, no such ferredoxin-reducing reaction is involved. Nonetheless, hydrogen formation from NADH is likely catalyzed by a [FeFe] hydrogenase homologue in *S. wolfei*. This reaction is possible already at a hydrogen partial pressure of 10^{-3} atm (Schink 1997; Müller et al. 2009). Since the enzyme found in *S. wolfei* is associated with a formate dehydrogenase-like protein analogous to its homologue in *Eubacterium acidaminophilum,* interspecies electron transfer may occur via hydrogen and/or formate, depending on the environmental conditions (Graentzdoerffer et al. 2003; Müller et al. 2009).

The thermodynamically most difficult step in butyrate oxidation is the transfer of electrons derived from butyryl-CoA oxidation to NAD^+, for which a redox potential difference of at least +200 mV has to be overcome (Schink 1997). It was hypothesized that electrons from butyrate oxidation are transferred to quinones in the membrane, and that the reduced quinones are re-oxidized with NAD^+ (Wallrabenstein and Schink 1994). Such a reaction would require energetization by, e.g., a proton gradient, which was found to be essential for hydrogen formation from butyrate by *S. wolfei* (Wallrabenstein and Schink 1994).

The *S. wolfei* [FeFe] hydrogenase catalyzes the reduction of quinones with NADH, indicating that, besides forming hydrogen from NADH oxidation, this enzyme also catalyzes the proton gradient-driven endergonic oxidation of quinones with NAD^+ (Müller et al. 2009). However, a direct linkage between quinol oxidation and proton translocation has not been demonstrated so far.

Another possible mechanism for reversed electron transport during butyrate oxidation was postulated for *Syntrophus aciditrophicus* based on genome data. Here, an Rnf complex could oxidize NADH and transfer electrons to ferredoxin, driven by influx of protons or sodium ions into the cell (McInerney et al. 2008). Electrons that arise during butyryl-CoA oxidation could be transferred to components of the membrane, where NAD^+ is reduced in a similar manner as postulated for *S. wolfei* (McInerney et al. 2008). With the Rnf complex, *S. aciditrophicus* has the potential prerequisites for producing reduced ferredoxin during butyrate degradation, which may drive comproportionating reactions such as NADH oxidation by [FeFe] hydrogenases or bifurcating reactions such as butyryl-CoA oxidation by the Bcd/EtfAB complex (Li et al. 2008; Herrmann et al. 2008). In contrast, genes that encode for the Rnf complex are not present in the genome of *S. wolfei*, indicating that the pathway of butyrate degradation is different in both organisms and does not include reduced ferredoxin in *S. wolfei* (Müller et al. 2009).

2.3 Propionate

All currently identified syntrophic propionate-oxidizing bacteria are affiliated with the class of Deltaproteobacteria within the phylum of Proteobacteria (McInerney et al. 2005), or the low G + C Gram-positive bacteria in the class Clostridia within

the phylum Firmicutes (Imachi et al. 2002; Plugge et al. 2002b; de Bok et al. 2005) (Table 3). Some of the *Syntrophobacter* sp. are able to use sulfate as the electron acceptor for propionate oxidation (McInerney et al. 2005) and can grow in pure culture by propionate oxidation with sulfate. In addition, they can grow by fermentation of pyruvate or fumarate. *Smithella propionica* is phylogenetically related to the genus *Syntrophus* (Liu et al. 1999) and lacks the ability to reduce sulfate. *S. propionica* does not oxidize propionate but ferments it to acetate plus butyrate and grows in pure culture on crotonate (de Bok et al. 2001; Liu et al. 1999). These substrates or substrate combinations have been used to obtain axenic cultures of the syntrophs since they bypass the energetically unfavourable steps in propionate oxidation. *Pelotomaculum schinkii*, however, could not be obtained in pure culture until today nor could it grow on any other compound but propionate. As such, this organism remains a true obligately syntrophic bacterium (de Bok et al. 2005).

All species described to date were isolated from anoxic reactors, indicating the importance of these organisms in those types of reactors. The significance of these

Table 3 Fatty acid-degrading syntrophic bacteria (modified after McInerney et al. 2008)

Organism	Substrate range	Phylogenetic position	Reference
Syntrophomonas wolfei subsp. *wolfei*	$C_{4:1}$–$C_{6:1}$, C_4–C_8	Low G + C Gram positives	McInerney et al. (1979, 1981)
Syntrophomonas sapovorans	$C_{16:1}$, $C_{18:1}$, $C_{18:2}$, C_4–C_{18}	Low G + C Gram positives	Roy et al. (1986)
Syntrophomonas bryantii	$C_{4:1}$, C_4–C_{11}, 2-methylvalerate	Low G + C Gram positives	Stieb and Schink (1985)
Syntrophomonas curvata	$C_{4:1}$, $C_{18:1}$, C_4–C_{18}	Low G + C Gram positives	Zhang et al. (2004)
Syntrophomonas erecta subsp. *erecta*	$C_{4:1}$, C_4 + $C_{5:1}$, C_4–C_8	Low G + C Gram positives	Zhang et al. (2005)
Syntrophomonas zehnderi	$C_{16:1}$, $C_{18:1}$, $C_{18:2}$, C_4–C_{18}	Low G + C Gram positives	Sousa et al. (2007)
Syntrophomonas cellicola	$C_{4:1}$, C_4–C_8, C_{10}	Low G + C Gram positives	Wu et al. (2006)
Thermosyntropha lipolytica	$C_{4:1}$, C_4–C_{18}, $C_{18:1}$, $C_{18:2}$, triglycerides, yeast extract, tryptone, casamino acids, betaine, pyruvate, ribose, xylose	Low G + C Gram positives	Svetlitshnyi et al. (1996)
Syntrophothermus lipocalidus	$C_{4:1}$, C_4–C_{10}, isobutyrate	Low G + C Gram positives	Sekiguchi et al. (2000)
Syntrophus aciditrophicus	$C_{4:1}$, fatty acids	δ-Proteobacteria	Jackson et al. (1999)
Syntrophus gentianae	$C_{4:1}$	δ-Proteobacteria	Szewzyk and Schink (1989)
Syntrophus buswellii	$C_{4:1}$	δ-Proteobacteria	Mountfort and Bryant (1982)

bacteria in rumen or animal gastrointestinal tracts maybe very limited because they compete with the feeding interests of the host (see Sect. 1).

The question whether hydrogen or formate is transferred in syntrophic co-cultures has been studied in detail in propionate-degrading *S. fumaroxidans* co-cultures (Fig. 3). Thermodynamic calculations, flux measurements in defined co-cultures, and enzyme measurements all confirmed that interspecies formate transfer is an essential mechanism in syntrophic propionate degradation in suspended cultures (Dong et al. 1994a; Dong and Stams 1995). The terminal reductases were studied in detail and biochemical evidence for formate transfer was found (De Bok et al. 2002). Two formate dehydrogenases were isolated and characterized. In contrast to most formate dehydrogenases that contain molybdenum, one formate dehydrogenase (CO_2-reductase) of *S. fumaroxidans* contains tungsten and has an unusually high specific activity both in the formate oxidation and in the CO_2 reduction assay (Reda et al. 2008). When syntrophic co-cultures of *S. fumaroxidans* and *Methanospirillum hungatei* were grown with limiting amounts of tungsten, the propionate degradation activity decreased. This decrease coincided adequately with decreased formate dehydrogenase activity while the hydrogenase activities remained almost unchanged (Plugge et al. 2009). In their natural habitats, syntrophically propionate-degrading bacteria form mixed microcolonies with methanogens in which interspecies distances are much shorter. Under these conditions, interspecies hydrogen transfer may become more important than interspecies formate transfer. In syntrophic propionate-degrading microcolonies, *Syntrophobacter*-like bacteria are often surrounded by *Methanobrevibacter* sp., methanogens that can use only hydrogen but not formate (Grotenhuis et al. 1991). Also in thermophilic sludge, interspecies hydrogen transfer appears to be the preferred path of electron transfer (Schmidt and Ahring 1993).

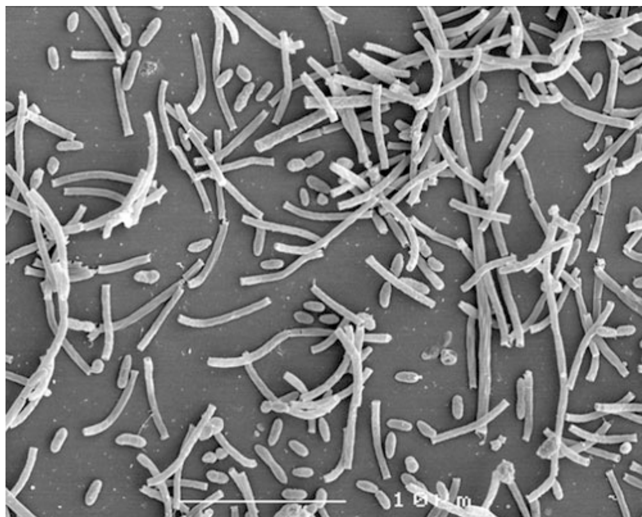


Fig. 3 Scanning electron micrograph of a syntrophic propionate-degrading coculture of *S. fumaroxidans* (*lemon- or oval shaped*) and *M. hungatei* (*rod shaped*)

In addition, slow propionate degradation was observed, also in very concentrated cell suspensions of *S. fumaroxidans* and the hydrogen-oxidizing *Methanobrevibacter arboriphilus* (Dong et al. 1994a). See also Sect. 3.

The organisms involved in propionate degradation are genuine specialists in obtaining metabolic energy for growth, since they have to grow under thermodynamically very unfavourable conditions. The standard Gibbs' free energy change of the complete degradation of propionate to methane and CO_2 is about −60 kJ, which is approximately equivalent to the amount of energy needed to produce 1 mol of ATP. A community of three microorganisms brings about this conversion: one bacterium that degrades propionate to acetate, CO_2, and hydrogen, and two methanogenic archaea: one that cleaves the acetate and another one that uses hydrogen to reduce CO_2 to methane. The actual energy that is available for each member of the community depends on the in situ concentrations of substrate, intermediates, and products and will vary during growth. Moreover, it depends also on the enzyme repertoire the microbes have.

Our model organism *S. fumaroxidans* degrades propionate via the methylmalonyl-CoA pathway. One ATP is harvested in the conversion of pyruvate to acetate via substrate-level phosphorylation. Reducing equivalents are released at three different redox potentials. Reduced ferredoxin is formed in the conversion of pyruvate to acetate, whereas NADH and $FADH_2$ are formed in the oxidation of malate and succinate, respectively. These intracellular redox mediators need to be re-oxidized by reduction of protons or CO_2. The oxidation of reduced ferredoxin ($E^{0\prime}$ Fd(ox)/Fd(red) = −398 mV) and NADH ($E^{0\prime}$ NAD^+/NADH = −320 mV) can be coupled to reduction of protons ($E^{0\prime} = -414$ mV) or CO_2 (−432 mV) only if the hydrogen or formate concentration is kept low by methanogens.

The disposal of reducing equivalents generated in pyruvate oxidation to acetyl-CoA is rather straightforward because most strictly anaerobic bacteria contain pyruvate: ferredoxin oxidoreductases (Chabrière et al. 1999). Here, electrons travel via ferredoxin to hydrogenases or formate dehydrogenases to produce hydrogen or formate, respectively. Hydrogen and formate are scavenged by the methanogens, thus enabling an efficient re-oxidation of the ferredoxin.

The oxidations of succinate and malate with protons are endergonic even at a hydrogen partial pressure as low as 1 Pa (the minimum level that can be achieved by methanogens). To drive these reactions, input of metabolic energy via reverse electron transport is required. The mechanism that drives succinate oxidation to fumarate ($E^{0\prime} = +33$ mV) during syntrophic growth may be similar to the mechanism of energy conservation in fumarate respiration by *Wolinella succinogenes* (Kröger et al. 2002), but operating in reverse. Experimental evidence was obtained that 2/3 ATP is needed to drive this conversion (van Kuijk et al. 1998). As such, the net ATP gain for the bacterium is 1/3 mol ATP per mole of propionate converted. However, until present, the molecular mechanisms involved in *S. fumaroxidans* and other syntrophic propionate oxidizers using the methylmalonyl-CoA pathway are not fully understood.

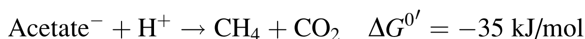
Also the oxidation of malate to oxaloacetate with NAD^+ is an endergonic reaction. Nonetheless, the purified malate dehydrogenase of *S. fumaroxidans*

exhibits a very high K_m value towards oxaloacetate and NADH and as such the organism may be able to efficiently perform this conversion (van Kuijk and Stams 1996). Still, the exact mechanism of NADH re-oxidation remains unclear.

S. fumaroxidans and *Pelotomaculum thermopropionicum* contain [FeFe] hydrogenases that are homologues to the comproportionating [FeFe] hydrogenase of *T. maritima*. This suggests that NADH and ferredoxin that are generated in the methylmalonyl-CoA pathway are simultaneously re-oxidized with the reduction of protons. These novel bifurcating enzyme complexes may be essential in these syntrophic fermentations. Additionally, the Rnf complex in *S. fumaroxidans* might use the membrane potential to reduce NAD^+ with ferredoxin re-oxidation in order to stimulate NADH re-oxidation of the comproportionating [FeFe] hydrogenase.

2.4 Acetate

Although acetate can be used directly by certain methanogens such as *Methanosarcina* spp. and *Methanosaeta* spp. and is converted by these organisms to methane and CO_2, this situation is typical only for systems at moderate temperature and low salt content. At enhanced temperature, acetate can be oxidized to 2 CO_2 + 4 pairs of reducing equivalents (H_2 or formate) in a reaction analogous to a reversal of homoacetate fermentation (see Table 1), and the electrons thus released are used by a methanogenic partner. This phenomenon has been observed first in a thermophilic reactor system (Zinder and Koch 1984), later also at lower temperature in sludge of enhanced ammonia content (Schnürer et al. 1996). The overall reaction



$$\text{Acetate}^- + H^+ \rightarrow CH_4 + CO_2 \quad \Delta G^{0\prime} = -35 \text{ kJ/mol}$$

can hardly feed two organisms. The energy yield increases with rising temperature (Schink 1997); at 60°C, $\Delta G'$ is −42 kJ/mol, which is just sufficient to allow two organisms to grow with this process. At lower temperatures, the energy supply becomes a serious problem, and doubling times increase to the range of several weeks (Schnürer et al. 1996). Indications were reported recently that also at slightly acidic conditions, e.g., in bogs, acetate is degraded in a syntrophic cooperation (Metje and Frenzel 2007); at pH 5.0, the $\Delta G = -46$ kJ/mol.

The biochemistry of syntrophic acetate oxidation appears to be basically a reversal of the homoacetogenic acetate formation pathway (so-called Wood–Ljungdahl pathway or CO-dehydrogenase pathway). Acetate is activated to acetyl-CoA and cleaved by a CO-dehydrogenase/acetate synthase to a methyl and a carbonyl residue, which are oxidized separately through well-described pathways (Schnürer et al. 1997; Hattori et al. 2000, 2005). The question remains how the bacterium couples this pathway to ATP synthesis, especially since it can run the reaction chain also backwards to form acetate and to grow this way, at least to a minor extent.

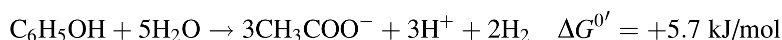
2.5 Branched Fatty Acids

Branched-chain fatty acids are formed during degradation of amino acids. Oxidative decarboxylation of valine leads to 2-methylpropionate (isobutyrate), leucine forms 3-methylvalerate (isovalerate), and isoleucine 2-methylbutyrate (neovalerate). Whereas neovalerate can be degraded easily by beta-oxidation to an acetyl and a propionyl residue, the other two acids pose some mechanistic difficulties. While isobutyrate is isomerized to butyrate in a B_{12}-dependent reaction and subsequently cleaved to two acetyl residues (Stieb and Schink 1989), isovalerate degradation includes a carboxylation and subsequent formation of three acetyl residues (Stieb and Schink 1986). In all cases, the degradation of the branched carbon skeletons is slow and these branched fatty acids, similar to the corresponding residues of aromatic amino acids, accumulate in anoxic environments to a certain extent and may be taken up again by other anaerobic bacteria for reductive synthesis of amino acids (Allison and Bryant 1963), thus saving a lot of biosynthetic effort into amino acid synthesis.

2.6 Benzoate

Aromatic compounds were considered for a long time not to be degradable in the absence of oxygen, and reliable reports on their degradation in methanogenic ecosystems date back only into the late 1970s (Healy and Young 1978). The best-studied system is the syntrophic oxidation of benzoate by species of the genus *Syntrophus*, i.e., *S. buswellii*, *S. aciditrophicus*, and *S. gentianae*. Benzoate degradation in these bacteria proceeds via an initial activation to benzoyl-CoA by a ligase reaction, partial reduction to a cyclohexene derivative, addition of water to form a 2-hydroxylated cyclohexane carboxyl-CoA, and subsequent beta-oxidative ring cleavage and degradation to three acetyl moieties plus CO_2 (Schöcke and Schink 1997). Preliminary evidence indicates that the primary product of benzoyl-CoA reduction in syntrophically fermenting bacteria is cyclohexene carboxyl-CoA, different from the corresponding reaction observed in nitrate-reducing bacteria which forms a cyclohexadiene derivative (Fuchs 2008), and also the biochemistry of the reduction reaction appears to be different (Boll 2005). The overall ATP yield of the entire reaction chain has been calculated for *S. gentianae* to be 1/3 to 2/3 ATP equivalents, in accordance with the calculated energy yields (Schöcke and Schink 1999).

Phenol is another important aromatic compound that is degraded anaerobically through carboxylation to a 4-hydroxybenzoyl derivative and subsequent dehydroxylation to benzoyl-CoA. In nitrate-reducing bacteria, the initial carboxylation consumes the equivalent of two ATP units. The energetic situation of fermentative phenol degradation is very tight:



$$C_6H_5OH + 5H_2O \rightarrow 3CH_3COO^- + 3H^+ + 2H_2 \quad \Delta G^{0\prime} = +5.7\ \text{kJ/mol}$$

Even in syntrophic cooperation with a hydrogen-oxidizing partner, the phenol degrader obtains only little energy (approximately −40 kJ/mol phenol; Schink 1997), thus keeping the overall energy budget small and hardly allowing to spend two ATP into the initial carboxylation reaction. So far, the details of the biochemistry of syntrophic phenol degradation have not been studied in detail; only recently, a defined co-culture of a syntrophically phenol-degrading bacterium has been isolated (Qiu et al. 2008).

2.7 Sugars

Sugars can be fermented by numerous groups of bacteria and archaea. The biochemical pathways of sugar oxidation are diverse but in most cases end up with pyruvate as a key metabolite. Most bacteria degrade sugars by converting mono- or disaccharides from polysaccharide cleavage into fructose or glucose, which are oxidized to pyruvate through the Embden–Meyerhof–Parnas pathway. Pyruvate can be further oxidized to acetate or CO_2 by anaerobic respiration or be used as internal electron acceptor for fermentative production of a variety of acids or solvents.

Fermentation of sugars via the Embden–Meyerhof–Parnas pathway with subsequent acetyl-CoA phosphorylation usually yields acetate, CO_2, and hydrogen. Formation of only acetate, CO_2, and hydrogen would require formation of 4 mol ATP per mole of glucose: two in glycolysis and two in the acetate kinase reaction. However, the reaction provides a negative-free reaction enthalpy of only −216 kJ/mol (Table 1), which is not sufficient for the formation of 4 ATP. This fermentation would need to re-oxidize the glycolysis-derived NADH with protons, which is endergonic under standard conditions. Most mesophilic sugar fermenters cope with this problem by releasing various reduced side products such as organic acids or alcohols. Thus, NADH can be re-oxidized without hydrogen formation, but on the other hand, only 2–3 mol of ATP per mole of glucose can be gained. In the presence of hydrogen-scavenging methanogenic partners, the formation of only acetate, CO_2, and hydrogen is favoured (Schink 1997). For example, the glucose-fermenting *Ruminococcus albus* shifts its fermentation pattern from acetate plus ethanol under axenic growth conditions to acetate, CO_2, and hydrogen in syntrophic co-culture (Iannotti et al. 1973). Obviously, the bacterium optimizes its ATP gain that is maximal if the hydrogen partial pressure is low enough to shift the thermodynamic equilibrium of glucose oxidation towards a more negative-free reaction enthalpy, thus allowing the formation of 4 ATP.

The facultatively anaerobic *Bacillus* sp. BoGlc83 grows anaerobically only in the presence of a methanogenic partner (Müller et al. 2008). At first, this organism did not release reduced side products and appeared to be forced to cooperate with methanogens, therefore. Later, it was shown that in co-cultures with *M. hungatei*, traces of lactate and succinate are formed besides acetate and methane at temperatures higher than 20°C and at glucose concentrations higher than 2 mM.

Nonetheless, no growth occurs in the absence of methanogens (Müller et al. 2008). Regarding that the natural habitat of *Bacillus* sp. BoGlc83 are cold profundal sediments, e.g., of Lake Constance, it seems likely that production of lactate and succinate are stress responses to unusual heat and high substrate concentrations much different from the cold and nutrient-poor natural environment of this organism.

2.8 Amino Acids

Much of our knowledge on anaerobic protein and amino acid degradation has been obtained through studies on ruminants, since protein is an important dietary product for ruminants (Allison 1970; Bryant 1977; Hobson and Wallace 1982). Proteins in the rumen are hydrolyzed by extracellular proteases and intracellular peptidases (Hazlewood and Nugent 1978) to single amino acids, peptides, and ammonia. Also in anaerobic digesters, the input of proteins coming from different sources of waste (e.g., slaughterhouses, beer breweries, and dairy industries) can be large. Proteins are composed of about 20 structurally different amino acids which require distinct biochemical pathways for degradation. As such, anaerobic degradation of amino acids by mixed methanogenic consortia is very complex and is performed by many fermentative microorganisms. Degradation involves oxidation and reduction reactions of one or more amino acids. Some amino acids are degraded preferentially via oxidation and others can also serve as electron acceptors. The combined oxidation and reduction of pairs of amino acids (Stickland reaction) is a well-known mechanism by which proteolytic clostridia degrade amino acids (Stickland 1934; Barker 1981; Stams 1994). In the Stickland reaction, the oxidation of one amino acid is coupled to the reduction of another one. In the oxidative branch, alanine and many other amino acids can be partly degraded; glycine is a classical electron acceptor in the reductive branch via a selenium-dependent glycine reductase (Andreesen 1994, 2004) (Table 1). Other couples have been described in the past (Barker 1981). Amino acid degradation is significantly affected by the presence of methanogens. Methanogens can act as scavengers of reducing equivalents in the oxidation of amino acids, taking over the role of the reductive branch of the Stickland reaction. Nagase and Matsuo (1982) observed that in mixed methanogenic communities, the degradation of alanine, valine, and leucine was inhibited by inhibition of methanogens, and Nanninga and Gottschal (1985) could stimulate the degradation of these amino acids by the addition of hydrogen-utilizing anaerobes. Several anaerobic bacteria have been described that grow syntrophically on amino acids in co-culture with methanogens (McInerney 1988; Stams 1994; Plugge and Stams 2005).

Usually, the first step in the degradation of amino acids is a deamination (Barker 1981; McInerney 1988; Andreesen et al. 1989). Deamination can be performed by anaerobic bacteria in three ways. Oxidative NAD(P)-dependent deamination of alanine, valine, leucine, or isoleucine leads to the corresponding keto acid. The $\Delta G^{0\prime}$ of the deamination of alanine, valine, leucine, and isoleucine to the corresponding keto

acids when coupled to hydrogen formation is around +60 kJ/mol (Table 1). As a consequence, methanogens are needed to pull the reaction in a similar fashion as described for other syntrophic oxidations above. The keto acid is then converted via oxidative decarboxylation to a fatty acid releasing electrons at $E^{0\prime} = -470$ mV, which can easily be transferred via ferredoxin to protons. Overall, the oxidative deamination of the four mentioned amino acids to fatty acids, ammonia, and hydrogen is slightly endergonic. The second mechanism is a reductive deamination and is found only in anaerobes (McInerney 1988; Andreesen et al. 1989; Andreesen 1994). Reducing equivalents are used to convert the amino acid to its corresponding fatty acid, with concomitant production of ammonia. An example is the reduction of glycine to acetate via the selenenium-dependent glycine reductase (Stickland 1934; Andreesen 2004). The third mechanism, a redox-neutral reaction, results in the production of the corresponding keto acid. An example is the conversion of serine to pyruvate plus ammonia by the action of serine ammonia lyase or the C–C rearrangement of glutamate to 3-methylaspartate (Buckel and Barker 1974).

Glutamate is an abundant amino acid in proteins (McInerney 1988). In methanogenic habitats, glutamate can be metabolized in several different ways, leading to different growth yields. The effect of hydrogen removal by methanogenic partners on the metabolism of amino acid-fermenting anaerobes has been studied best with glutamate. Glutamate fermentation is carried out by a variety of anaerobes, including a number of *Clostridium* species, *Peptostreptococcus asaccharolyticus*, and *Acidaminococcus fermentans* (Gottschalk 1986; Boiangiu et al., 2005). These microorganisms ferment glutamate to acetate and butyrate by either the β-methylaspartate or the hydroxyglutarate pathway (Buckel and Barker 1974). In this fermentation, reducing equivalents formed in the oxidation of glutamate to acetate are disposed of, either partly or completely, by reductive formation of butyrate from acetyl residues. *Anaeromusa acidaminophila* ferments glutamate to acetate plus propionate (Nanninga et al. 1987). In this bacterium, reducing equivalents are disposed of by reduction of pyruvate to propionate. Besides acetate, butyrate, and propionate also traces of hydrogen (up to 20 kPa) are formed during glutamate fermentation via the β-methylaspartase and the hydroxyglutarate pathway. Work in the laboratory of W. Buckel has recently unravelled the mechanisms underlying this hydrogen production (Buckel 2001a, b; Boiangiu et al. 2005). Pyruvate is oxidatively decarboxylated to acetyl-CoA by pyruvate:ferredoxin oxidoreductase. Re-oxidation of reduced ferredoxin proceeds in two ways: the majority (up to 80%) is re-oxidized during the synthesis of butyrate from two acetyl-CoA and the remaining 20% is used to reduce protons to hydrogen. This reaction is catalysed by an iron-only hydrogenase. For butyrate synthesis from two acetyl-CoA, however, reduced NADH is necessary. For *Clostridium tetanomorphum*, it was postulated that NAD^+ is reduced by a membrane-bound NADH-ferredoxin oxidoreductase (Fig. 4) (Buckel 2001b; Boiangiu et al. 2005).

Several Bacteria have been isolated that during growth on glutamate release reducing equivalents exclusively as hydrogen, in the formation of acetate, propionate, or both. Microorganisms that ferment glutamate to acetate only include *Caloramator coolhaasii* (Plugge et al. 2000) and *Caloramator proteoclasticus*

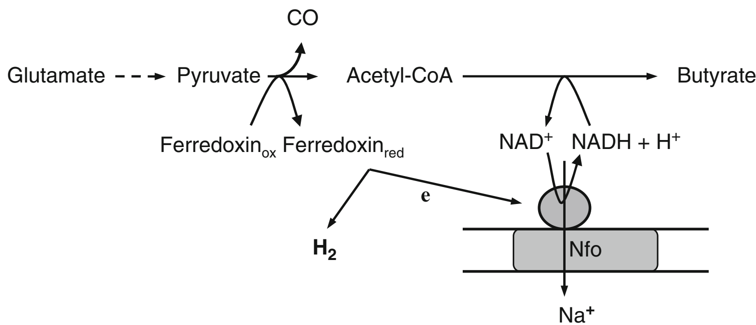


Fig. 4 Model of NADH-ferredoxin oxidoreductase (after Boiangiu et al. 2005)

(Tarlera et al. 1997). Propionate as the only product is formed from glutamate by *Aminobacterium colombiense* (Baena et al. 1998) and *Gelria glutamica* (Plugge et al. 2002a). *Acidaminobacter hydrogenoformans* (Stams and Hansen 1984; Meijer et al. 1999), *Thermanaerovibrio acidaminovorans* (Cheng et al. 1992; Baena et al. 1999a), and *Aminomonas paucivorans* (Baena et al. 1999b) form both acetate and propionate from glutamate. In syntrophy with methanogens, the hydrogen pressure can be lowered to 1 Pa and glutamate degradation to CO_2, acetate, or propionate, and hydrogen becomes feasible (Plugge et al. 2002a). Hence, both under these conditions in the acetate and in the propionate-forming pathway, energy conservation to the extent of 1 ATP per mole glutamate is thermodynamically possible.

According to ^{13}C-labelling studies with 1-^{13}C- and 3-^{13}C-glutamate, the pathway of glutamate fermentation to acetate in *C. coolhaasii* proceeds via 3-methylaspartate and pyruvate. *T. acidaminovorans* forms propionate by oxidation of glutamate followed by decarboxylation of succinyl-CoA via methylmalonyl-CoA to propionyl-CoA (Plugge et al. 2001). Operation of the citric acid cycle can be excluded since no 2,3-double labelled propionate could be detected; obviously, neither fumarate nor free succinate was formed as intermediates. The formation of [2,3-^{13}C] succinate indicated that glutamate is directly oxidized to succinyl-CoA, in which part of the succinyl-CoA is converted to succinate and excreted, and the majority is further converted to propionate.

2.9 Carrier Systems

Depending on the type of syntrophic conversion, the carrier system that transfers electrons from the producer to the consumer may vary. The best-studied and best-accepted electron carrier is hydrogen. However, formate is considered to be an important agent in interspecies electron transfer during propionate conversion as already discussed in Sect. 2.3. Formate can also act as electron carrier in syntrophic butyrate conversion by *S. wolfei* since this bacterium contains a formate

dehydrogenase with high homology to a formate dehydrogenase of *E. acidaminophilum* (FdhA-II) that was suggested to play a role also in interspecies formate transfer (Müller et al. 2009).

In syntrophic acetone-degrading methanogenic cultures, acetate was identified as the only interspecies carrier compound (Platen and Schink 1987; Platen et al. 1994). In this syntrophic culture, growth and conversion of acetone to acetate proceeded until acetate had accumulated to ~10 mM. Addition of an active acetoclastic methanogen (*Methanosaeta* sp.) greatly enhanced the acetone degradation rate. In addition, experiments with ^{14}C-labelled CO_2 showed that CO_2 is stoichiometrically incorporated into the formed acetate (Platen and Schink 1987).

Interspecies electron cycling through sulfur and sulfide has been described for *Desulfuromonas acetoxidans* in syntrophic cultures with *Chlorobium limicola*, a phototrophic green sulfur bacterium (Pfennig and Biebl 1976; Biebl and Pfennig 1978). Acetate oxidation by *D. acetoxidans* and electron transfer to the phototrophic green sulfur bacterium *C. limicola* (Biebl and Pfennig 1978) occurred in the presence of small amounts of sulfide (53–92 μM) in the light (Biebl and Pfennig 1978). A similar sulfur cycle mediated electron transfer was described in an artificial co-culture, which syntrophically oxidized acetate to CO_2 with concomitant reduction of nitrate (Kaden et al. 2002).

The discovery of bacterial nanowires and identification of presumed electron transfer components required for electrical conductivity in these pili-like structures provided a novel view on mechanisms involved in interspecies electron transfer (Gorby et al. 2006; Reguera et al. 2005). Pili-like structures have been identified in a number of pure and mixed cultures, and also syntrophic co-cultures of propionate-oxidizing *P. thermopropionicum* and *Methanothermobacter thermoautotrophicus* produced these pili-like structures. Analysis of the conductive properties of pili indicated that they could transfer electrons between cells of *Geobacter sulfurreducens* and the surface of Fe(III) oxides (Reguera et al. 2005). These pili were not required for attachment to the insoluble electron acceptor; rather they are interpreted to function as channels for electron transfer to the Fe(III) oxides, extending the electron transfer capabilities of the cells well beyond their outer surface (Reguera et al. 2005). Pili "nanowires" also served as electric conduits to mediate long-range electron transfer across biofilms formed on anode electrodes in microbial fuel cells, which could maximize current production per unit of anode surface area (Reguera et al. 2005).

2.10 Alternative Substrates for Pure Cultures and Technical Systems to Replace Methanogens

Outside the laboratory, bacterial communities are nearly always communities composed of a wide variety of species. It is appropriate to consider the relevance of these interspecies interactions to the outcome of activity assays and the cultivability in the laboratory. Defined cultures of syntrophically fermenting bacteria are

required for detailed physiological and molecular studies and to understand their significant role in nature. To obtain such cultures, technical systems can be used to replace the methanogenic partner or alternative substrates can be supplied to bypass the energetically unfavourable steps occurring in syntrophic conversions.

The first axenic culture of an obligatory syntrophic bacterium was *S. wolfei* (Beaty et al. 1987). Studies on the butyrate metabolism of syntrophic co-cultures of *S. wolfei* and *M. hungatei* revealed a high activity of β-oxidation enzymes (Wofford et al. 1986). With this knowledge, Beaty and co-workers grew *S. wolfei* on agar plates containing crotonate as the sole source of carbon and energy. The pure culture obtained dismutated crotonate to butyrate and acetate, but exhibited butyrate oxidation only after re-association with a syntrophic partner. Later, it was shown that *S. wolfei* and *Syntrophospora bryantii* could grow in pure culture on butyrate plus 3-pentenoate (Amos and McInerney 1990; Dong et al. 1994b). Butyrate plus 3-pentenoate were converted to valerate, acetate, and propionate.

The first successful axenic culture of a syntrophically propionate-degrading bacterium was obtained from an enrichment culture by inhibiting the methanogens with bromoethanesulfonic acid (an analogue of coenzyme M) and subsequently adding fumarate as external electron acceptor. This allowed to isolate *S. fumaroxidans* (Stams et al. 1993; Harmsen et al. 1998) and to study the pathway of propionate oxidation (Plugge et al. 1993). Phylogenetically, *S. fumaroxidans* is very closely related to sulfate-reducing bacteria.

Some sulfate-reducing bacteria can alter their metabolism and act as syntrophically fermenting partners if sulfate becomes depleted (see above; Bryant et al. 1977; Scholten et al. 2007; Walker et al. 2009). Although this metabolic flexibility may be helpful for the enrichment and isolation of syntrophic bacteria, it can be applied only to already highly enriched syntrophic cultures. A strategy for isolation of syntrophs could be stepwise: from enrichment culture via molecular characterization (16S rRNA based) to a strategic choice of substrate, electron acceptor, or unsaturated compound for the isolation of the microorganism. Examples of unsaturated compounds used are fumarate, crotonate, pentenoate, and benzoate.

A cultivation apparatus capable of maintaining very low H_2 (<0.01 Pa) pressures by mechanical means was developed by Valentine et al. (2000). This apparatus provided a method to study interspecies hydrogen transfer by externally providing the thermodynamic requirement for very low hydrogen concentrations, thus preventing the need for use of co-cultures to study the metabolic pathways. The culture vessel is constructed of glass and operates by sparging a liquid culture with purified gases, which remove hydrogen directly as it is produced. The culture device was constructed to decouple the syntrophic relationship in an ethanol-oxidizing methanogenic enrichment culture, allowing ethanol oxidation to dominate the methane production. Moreover, the culture apparatus was successfully used to grow pure cultures of the ethanol-oxidizing, proton-reducing *P. acetylenicus* (Valentine et al. 2000). This culture apparatus may have a potential to study also other forms of syntrophic metabolism; however, we have to realize that fatty acid oxidation requires hydrogen pressures substantially lower than ethanol oxidation.

2.11 Anaerobic Methane Oxidation

Although not a part of the methanogenic feeding chains discussed here, it is worth mentioning that also anaerobic oxidation of methane with sulfate as electron acceptor is, according to our present understanding, catalyzed by a syntrophic association of two organisms. One of the partners appears to be similar to methanogens but operates in reverse, i.e., it oxidizes methane by a reversal of the methane formation reaction (methyl-CoM reductase). The partner is a sulfate reducer that uses the intermediates released by its partner to reduce sulfate to sulfide. The overall reaction releases only little energy:

$$CH_4 + SO_4^{2-} + H^+ \rightarrow CO_2 + HS^- + 2H_2O, \Delta G^{0\prime} = -18 \text{ kJ/mol}$$

The reaction has been observed mainly in coastal shelf areas of the oceans at water depths between 800 and 1,000 m, at methane pressures of 80–100 atm over gas hydrate deposits (Boetius et al. 2000). Under these conditions, the reaction energetics are slightly more favourable (up to −40 kJ/mol), thus barely feeding the two organisms involved with a minimum energy supply. Although this process is probably the most important reaction mitigating methane emissions to the atmosphere worldwide, it is still only barely understood. Especially, the identity of the electron carrier between the two partner organisms is still entirely enigmatic; from feeding experiments, we know that it is none of the "usual" carriers to be considered such as hydrogen, formate, methanol, or acetate (Nauhaus et al. 2002).

3 Spatial Organization of Syntrophic Communities

The close cooperation of two metabolically different organisms during syntrophic degradation requires short transport paths between the partners to optimize metabolite transfer, especially at low overall energy yields. The metabolite flux from one organism to the other is an inverse linear function of the diffusion distance (Schink and Thauer 1988). One should assume, therefore, that optimal transfer is ensured in mixed communities in which the partners are homogeneously mixed. Syntrophic co-cultures show a defined tendency to form mixed aggregates also in defined laboratory cultures (Fig. 5). However, since the respective partners are different organisms, they multiply separately and will form sooner or later nests of genetically identical organisms that compete with each other within the nests and have only limited exchange to the partner nests outside. One has to assume that such communities should mix through each other to maintain optimal metabolite transfer at short distances. Microscopic pictures of methanogenic communities in biogas reactors have shown that nests, as described, really do exist within such structures, but that in other areas, the partners appear to be fairly well mixed (Grotenhuis et al. 1991; Fang et al. 1995; Harmsen et al. 1996). It is still an open question how such mixing can be accomplished by organisms that appear to be basically immotile and do not show any means of gliding motility.

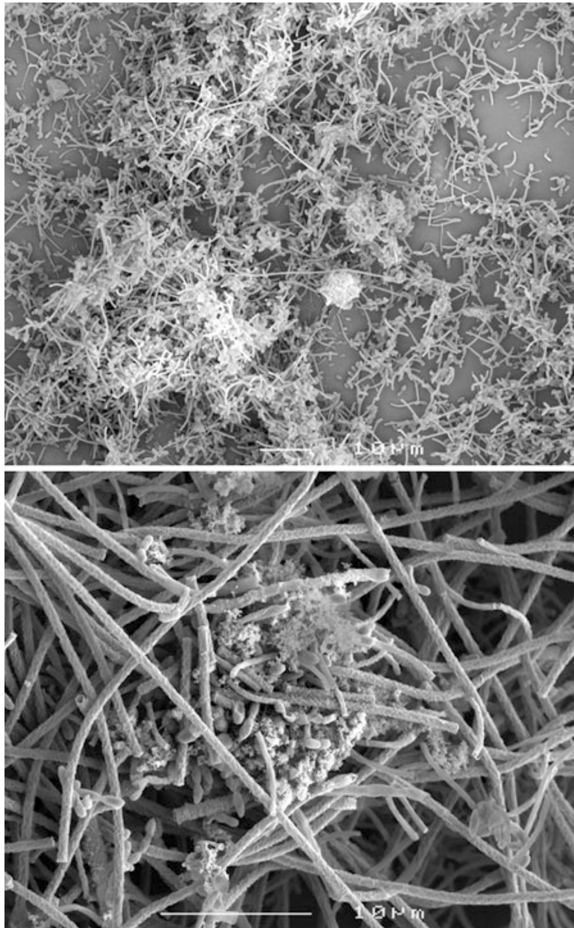


Fig. 5 Scanning electron micrographs of a propionate-converting (**a**) coculture of *S. fumaroxidans* (*oval shaped*) and *M. hungatei* (*rod shaped*) and (**b**) triculture of *S. fumaroxidans* (*oval shaped*), *M. hungatei* (*rod shaped*), and *M. concilii* (*long filaments*) showing the close proximity of the syntrophic partners

4 Concluding Remarks

The interrelationship of different trophic groups (guilds) within methanogenic microbial communities is a fascinating object to study, with perspectives to ecology, physiology, biochemistry, and energetics. These organisms cooperate in a very complex process, and they do so with minimum increments of energy for sustainment of life. These energy increments are at the lowermost range of energy that can be converted into ATP at all, and with this, these organisms are interesting model subjects to study energy starvation on a broader basis.

The question arises why nature designed methanogenic degradation in such a modular structure instead of having few types of organisms, which could convert polymeric substrates all the way down to methane plus CO_2. Theoretical considerations suggested that metabolic pathways can be efficient only up to a limited length of reaction chains (Costa et al. 2006) and this may apply as well to, e.g., cellulose degradation to methane. One can argue as well that the strategy taken in these anaerobic communities is simply to establish a complex network of functions by independent modular units. This makes regulation easy for every single unit that acts only in a single function rather than combining many different metabolic tasks into one.

Methanogenesis in bioreactors is a sustainable technology to produce biogas from organic waste. More than 80% of the chemical energy in organic waste components is conserved as methane, which in aerobic conversion would have been lost. Presently, much research is done to replace fossil fuels to alternative sustainable (CO_2-neutral) energy sources. Microbial methane formation from waste and wastewater will contribute to this development. From the technological viewpoint, it will be important to produce methane at a high rate and to convert all organic compounds to biomass. The proper functioning and structuring of syntrophic communities of anaerobic bacteria and archaea will be important in this respect. Further research is needed to get insight into the factors that regulate methane formation by syntrophic communities.

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Hydrogenosomes

Johannes H.P. Hackstein and Aloysius G. M. Tielens

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Abstract "Hydrogenosomes" are mitochondrion-derived, double membrane-bounded organelles that produce hydrogen and ATP. These properties discriminate them from the likewise mitochondrion-derived "mitosomes" that produce neither hydrogen nor ATP. The only character that is most likely shared by mitochondria, hydrogenosomes, and mitosomes is their involvement in the Fe–S metabolism.

Hydrogenosomes and mitosomes are found in a broad spectrum of rather unrelated species of unicellular, anaerobic eukaryotes, suggesting that hydrogenosomes and mitosomes evolved repeatedly and independently in the various taxonomic groups. With the exception of two hydrogenosomes, all these organelles lack a genome and an electron transport chain, which makes it sometimes difficult to trace their origins back to their mitochondrial origins. However, genomic evidence, EST studies, and the analysis of the organellar metabolism clearly reveal both a mitochondrial descent and individual differences in the properties of the various organelles. In this paper, we describe the diversity of hydrogenosomes based predominantly on information that became available recently. We also pay attention to the fact that certain hydrogenosomes are found in close association with endosymbiotic methanogens.

1 Introduction

It is now generally accepted that at least three different types of organelles evolved from one ancestral endosymbiont: mitochondria, hydrogenosomes, and mitosomes (Embley and Martin 2006; Hackstein et al. 2006; Howe 2008; van der Giezen 2009; Hjort et al. 2010). Mitochondria typically possess a genome that encodes components of an electron transport chain used in oxidative phosphorylation for the production of ATP. Usually, mitochondria are depicted as organelles that use oxygen as terminal electron acceptor in their process of ATP production. However, many mitochondria exist that can produce ATP without using any oxygen. The mitochondria of anaerobic eukaryotes produce ATP with the help of proton-pumping electron transport (i.e., oxidative phosphorylation), but they use terminal electron acceptors other than oxygen, such as fumarate (Tielens et al. 2002). In addition to anaerobic mitochondria, another type of anaerobic ATP-producing organelle exists, the hydrogenosome. Hydrogenosomes are double membrane-bounded organelles of a size of 0.3–2 μm that are characterized by the production of hydrogen with the aid of a hydrogenase that donates electrons originating from the oxidation of substrates to protons (Müller 1993). In contrast to mitochondria and hydrogenosomes, mitosomes are not involved in the production of ATP. They are double membrane-bounded and present only in anaerobic eukaryotic organisms that lack mitochondria and hydrogenosomes. Mitosomes do not produce hydrogen. Their function is elusive and most of them share with mitochondria and hydrogenosomes only the presence of components of an iron–sulphur cluster synthesizing machinery (Tachezy and Dolezal 2007). Mitosomes and hydrogenosomes are found exclusively in anaerobic, unicellular organisms. Accumulating evidence suggests

that all these organelles evolved from mitochondrial precursors, accompanied by a loss of the organellar genome and the electron transport chain (Embley and Martin 2006; Hackstein et al. 2006; Tielens and van Hellemond 2007; van der Giezen 2009; Hjort et al. 2010). The discovery of a hydrogenosome with a mitochondrial genome and parts of an electron transport chain in the ciliate *Nyctotherus ovalis* discloses the existence of a hydrogen-producing mitochondrion, a "missing link" between hydrogenosomes and mitochondria (Boxma et al. 2005; Martin 2005). Notably, another missing link with a genome and an electron transport chain has been recently discovered in the unrelated Stramenopyle *Blastocystis* sp. (Perez-Brocal and Clark 2008; Stechmann et al. 2008; Wawrzyniak et al. 2008).

Hydrogenosomes and mitosomes are found in a broad spectrum of species, suggesting that hydrogenosomes and mitosomes evolved repeatedly in rather unrelated taxonomic groups (Fig. 1). Even within the Excavata, at least three different rather unrelated species with hydrogenosomes were identified, i.e., the heterolobosean amoeboflagellate *Psalteriomonas lanterna*, the preaxostylid flagellate *Trimastix pyriformis*, and the parabasalid flagellate *Trichomonas vaginalis* with its relatives *Tritrichomonas foetus, Monocercomonas* sp., and *Histomonas meleagridis*. In addition, *Giardia lamblia* (Excavata) is a species belonging to the diplomonads that hosts mitosomes. Among the Chromalveolata, various anaerobic ciliate species with hydrogenosomes evolved repeatedly from different aerobic ciliate progenitors (see below). One of them, *N. ovalis*, possesses a hydrogenosome with a genome (Akhmanova et al. 1998a; Boxma et al. 2005). Also, the Stramenopile *Blastocystis* sp. possesses a hydrogenosome-like, genome-bearing organelle (Perez-Brocal and Clark 2008; Stechmann et al. 2008; Wawrzyniak et al. 2008) and *Cryptosporidium* sp., which belongs to the apicomplexa and hosts a mitochondrion remnant (mitosome) that lacks a genome and a hydrogenase (Keithly 2008). Among the Unikonta, the anaerobic chytridiomycete fungi *Piromyces* sp. and *Neocallimastix* sp. possess hydrogenosomes (Müller 1993), while fungi-related Microsporidia such as *Encephalitozoon cuniculi* (Katinka et al. 2001), *Antonospora locustae* (Williams and Keeling 2005), and *Trachipleistophora hominis* (Williams et al. 2002) possess mitosomes. Mitosomes are also found in the rather unrelated species *Entamoeba histolytica* (Tovar et al. 1999) and *Mastigamoeba balamuthi* (Gill et al. 2007). These obviously diverse origins of the organelles (and hydrogenosomes in particular) strongly suggest that neither the various mitosomes nor the hydrogenosomes are the same. It is anticipated that the various organelles, even if they belong to the same type, are structurally and metabolically different. Lastly, on the basis of electron microscope studies, quite a number of potential hydrogenosome/mitosome-like organelles have been identified in anaerobes such as *Spironucleus elegans*, *Chilomastix cuspidata*, *Andalucia incarcerata*, *Lyromonas vulgaris*, *Monopylocystis visvesvarai*, *Sawyeria marylandensis*, *Carpediemonas membranifera*, *Dysnectes brevis*, *Vahlkampfia anaerobica*, *Percolomonas descissus*, *Postgaardi mariagerensis*, and *Breviata anathema* (Hampl and Simpson 2008).

In this review, we focus on the hydrogenosomes and describe the different hydrogenosomes in the following in some detail.

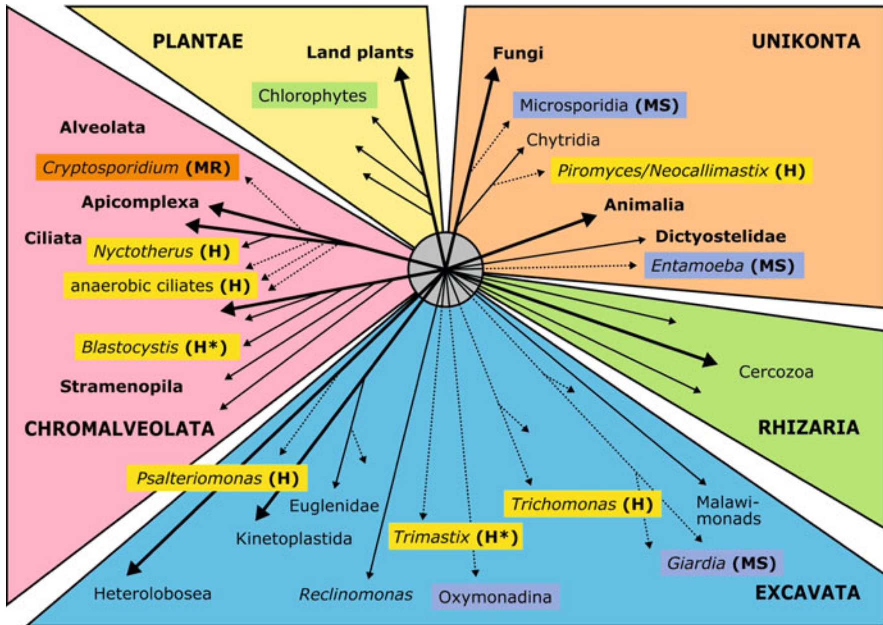

Fig. 1 Cartoon interpreting the evolution of mitochondria and related organelles (*H* hydrogenosomes, *MS* mitosomes, *MR* mitochondrial remnants). *H** indicates that hydrogenase activity has not yet been demonstrated in *Blastocystis* and that the hydrogenase has not yet been localized to the organelle of *Trimastix*. The *solid lines* represent the evolutionary descent based on the presence of an organellar genome and the *broken lines* indicate the absence (loss) of a genome. The monophyly of the mitochondria has been derived from the phylogenetic analysis of more than 444 alpha-proteobacterial and 2061 mitochondrial genomes (February 2010). Since the branching order is not resolved so far, the cartoon indicates solely the common origin by the "origin" in the centre. A later loss of the organellar genome is only indicated if additional data argue for a loss of the organelle genome after the diversification of the hosts. The phylogenetic relationships between the hosts are still discussed controversially. Therefore, the smallest common denominator is used to display an unrooted "tree" of the most basic taxonomic arrangement agreed by most biologists: plantae; uniconta (animals and fungi); rhizaria; chromalveolata; excavata. No attempts were made to root this tree, nor to give any indication of a potential branching order. Certain green algae possess "normal" mitochondria, but express a plastidic hydrogenase under anaerobic conditions (chlorophytes). Substantial differences in metabolism have been established for the hydrogenosomes of *Trichomonas*, *Piromyces*/*Neocallimastix*, and the various ciliates. From Hackstein et al. (2006), modified

2 The Hydrogenosomes of the Trichomonadina

Trichomonads are microaerophilic, parasitic flagellates that belong to the Parabasalia. The hydrogenosomes of Trichomonads are the best-studied organelles of this kind. They were discovered in 1973 in *T. foetus*, 1974 in *Monocercomonas* sp., 1975 in *T. vaginalis*, and 2008 in *H. meleagridis* (Lindmark and Müller 1973, 1974; Lindmark et al. 1975; Mazet et al. 2008). These organelles are double

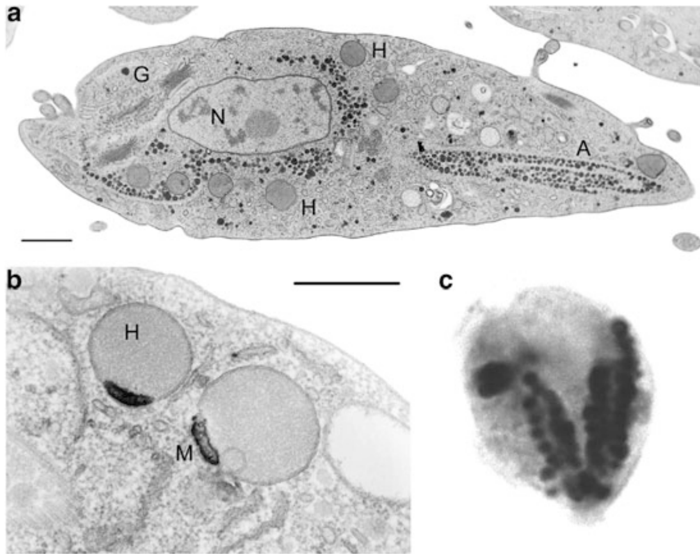

Fig. 2 (**a**) Electron micrograph of *Tritrichomonas foetus:* seven hydrogenosomes (*H*) can be identified in the cytoplasm (*N* nucleus, *G* Golgi apparatus, *A* axostyl). (**b**) A higher magnification reveals that a double membrane surrounds the hydrogenosomes (*M* marginal plate). **a** and **b** were kindly provided by M. Benchimol, Rio de Janeiro. Bar: in **a** and **b** 1 µm. (**c**) *Trichomonas vaginalis*, light microscopical picture of cell stained with BSTP following Zwart et al. (1988) to demonstrate hydrogenase activity; natural size approximately 10 × 45 µm. Courtesy C.K. Stumm, Nijmegen

membrane- bounded and about 0.3 µm in diameter in *T. foetus* and *T. vaginalis* (Fig. 2), 0.3–0.6 µm in diameter in *H. meleagridis* (Mazet et al. 2008), and up to 2 µm in length in *Monocercomonas* (Benchimol 2008). They do not contain an organellar genome (Clemens and Johnson 2000). Most of the recent studies focussed on the human parasite *T. vaginalis.* In 2007, the complete (nuclear) genome has been published allowing a reconstruction of the hydrogenosomal metabolism that corroborates the earlier enzymatic studies (Carlton et al. 2007). In addition, the proteome of the hydrogenosomes has been analysed. Together with the genome data, the analysis of the proteome suggests that the hydrogenosome of *T. vaginalis* consists of at least 200 different proteins (Henze 2008). This is considerably less than the 700–800 proteins predicted for yeast mitochondria (Sickmann et al. 2003) and suggests a significantly lower complexity of the hydrogenosomes in comparison to mitochondria.

The metabolism of the hydrogenosomes of *T. vaginalis* has been described in detail by Carlton et al. (2007) and Hrdý et al. (2008); it will be summarized below. The trichomonad hydrogenosomes import pyruvate and malate (Hrdý et al. 2008). The latter is decarboxylated to pyruvate by a NAD-dependent malic enzyme inside the hydrogenosome. The initial step in the catabolism of pyruvate is the oxidative decarboxylation by the pyruvate:ferredoxin oxidoreductase (PFO) (Fig. 3) to

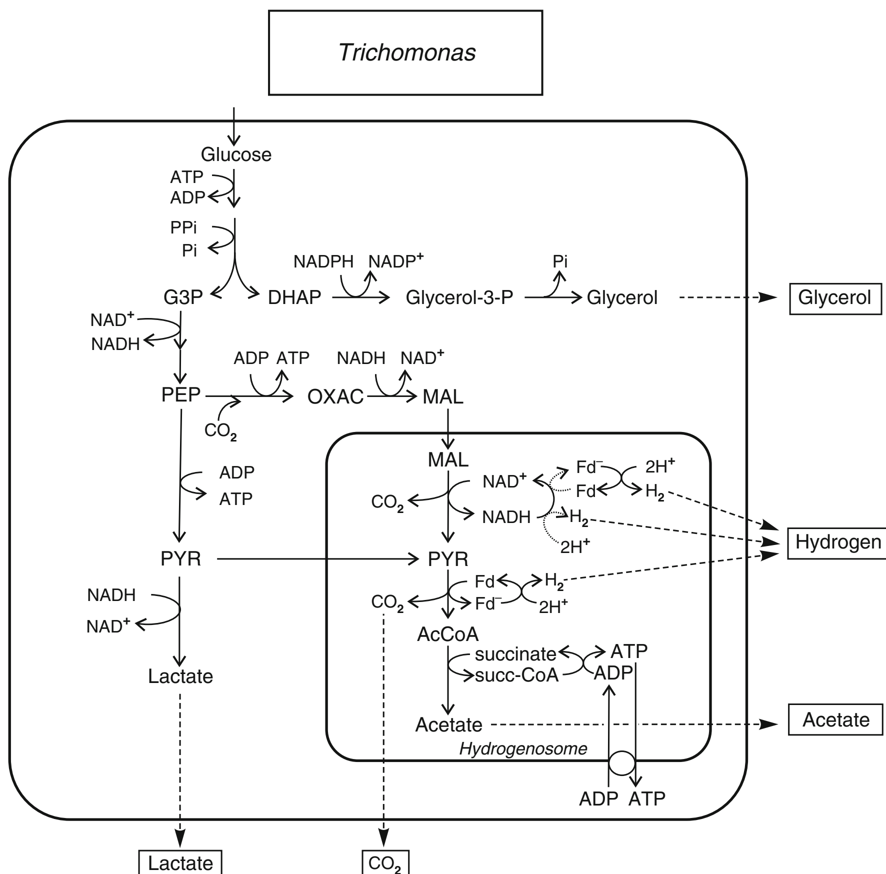

Fig. 3 Energy metabolism of *Trichomonas vaginalis*. Shown is a scheme of the metabolic pathways involved in the production of the major end products. End products are in *boxes*. Abbreviations: *AcCoA* acetyl-CoA; *DHAP* dihydroxyacetonephosphate; *Fd* ferredoxin; *G3P* glyceraldehyde-3-phosphate; *MAL* malate; *OXAC* oxaloacetate; *PEP* phosphoenolpyruvate; *PYR* pyruvate; *Succ-CoA* succinyl-CoA [adapted from Carlton et al. (2007) and Hrdý et al. (2008)]

acetyl-CoA and CO_2. The reduced ferredoxin is reoxidized by a [FeFe] hydrogenase; the genome of *T. vaginalis* encodes five hydrogenases with hydrogenosomal targeting signals. It is assumed that one or the other hydrogenase reacts directly with NADH, alternatively under the involvement of ferredoxin and the 24 kDa and 51 kDa proteins that are homologous to the corresponding subunits of a mitochondrial Complex I.

The next step in the catabolism of pyruvate is the formation of acetate from acetyl-CoA with the simultaneous transfer of the CoA moiety to succinate (Fig. 3). This reaction is catalysed by the enzyme ASCT (acetate:succinate CoA-transferase). The corresponding gene was not identified in the draft genome version.

However, recently, the gene and the enzyme have been identified and characterized in detail (van Grinsven et al. 2008).

The succinyl CoA synthetase (SCS, also known as succinate thiokinase) uses the energy-rich CoA bond for the generation of ATP/GTP from ADP/GDP. It is the only enzyme of *Trichomonas* also known from the TCA cycle of aerobic mitochondria. It regenerates succinate for the reaction with ASCT. It is unknown to date whether the acetate, which is formed by the action of ASCT, is excreted via diffusion or by a so far unidentified transporter.

ATP and ADP are exchanged by a member of the mitochondrial carrier family, HMP 31 (Tjaden et al. 2004). Besides the energy-generating pathway, several other metabolic pathways have been identified. Most importantly, several components of the Fe–S cluster synthesizing machinery were identified – a function that is shared with mitochondria, hydrogenosomes of *Blastocystis*, and several mitosomes (Burri et al. 2006; Tachezy and Dolezal 2007). In addition, a glycine–decarboxylase complex (GDC, also known as glycine cleavage system, GCS) has been found. It allows the formation of THF-CH_2 (Methylene tetrahydrofolate), which is a key compound in the C_1 metabolism. Also this enzyme complex is found in mitochondria and the hydrogenosomes of *Nyctotherus* and *Blastocystis*. Furthermore, several enzymes with a function in the protection against reactive oxygen species (ROS) have been identified. Lastly, quite a number of proteins (Hmp35, HPP, Sam50, Pam18, Mdj1, Mge, Hsp10, Hsp60, and Hsp70) involved in the import of proteins into the organelle were detected (Hrdý et al. 2008).

3 The Hydrogenosomes of *T. pyriformis*

The anaerobic flagellate *T. pyriformis* belongs to the Preaxostyla. It is rather unrelated to the trichomonads although both genera belong to the supergroup Excavata. Electron microscopy has revealed the presence of double membrane-bounded organelles of a size of 0.3–1.2 μm, which, on the basis of their morphology, have been interpreted as hydrogenosomes (Brugerolle and Patterson 1997; O'Kelly et al. 1999; Simpson et al. 2000). Recently, a highly expressed [FeFe] hydrogenase has been identified in an EST study (Hampl et al. 2008). Also two enzymes involved in the maturation of [FeFe] hydrogenases were detected in that screen as well as a PFO. The localization of both enzymes has not yet been studied. Thus, it remains unclear as to whether the hydrogenase and the PFO are localized in the putative hydrogenosomes or in the cytoplasm. Notwithstanding, the presence of highly expressed hydrogenase and PFO genes suggests that the double membrane-bounded organelles are bona fide hydrogenosomes that have a metabolism similar to trichomonads. Notably, four proteins belonging to the GCS have been identified; a GCS is characteristic for mitochondria and also present in the hydrogenosomes of *Trichomonas*, *Blastocystis*, and *Nyctotherus*. Further support for a mitochondrial/hydrogenosomal nature of the organelles comes from the presence of three genes encoding mitochondrial carriers, three genes encoding components of an organellar

(mitochondrial) import machinery (TOM40, MPP, Hsp60), a lipoyl transferase, and a pyridine nucleotide transhydrogenase alpha. In addition, a gene encoding the TCA cycle enzyme aconitase was found that contrasts with the hydrogenosomes of *Trichomonas*, which do not possess TCA cycle enzymes except SCS (Carlton et al. 2007; Hampl et al. 2008; Hrdý et al. 2008).

Thus, there is no doubt that *T. pyriformis* hosts mitochondrion-derived organelles. The definitive classification of these organelles as hydrogenosome or mitosome depends on the localization of the hydrogenase and the PFO, i.e., if these enzymes are localized inside the organelles, they are hydrogenosomes, but if the enzymes are located in the cytoplasm, the organelles are mitosomes. Regardless of whether the organelles will be classified as hydrogenosomes or mitosomes, the available data clearly show that these organelles contain a unique set of proteins. Therefore, *T. pyriformis* harbours a unique version of anaerobic mitochondrion-related organelles.

4 The Hydrogenosomes of *P. lanterna*

The third representative of the Excavata with hydrogenosomes is the microaerophilic amoeboflagellate *P. lanterna* (Broers et al. 1990; Broers 1992; Fig. 4). It belongs to the Heterolobosea, which are rather unrelated to both *T. pyriformis* and *T. vaginalis*. *P. lanterna* possesses full-fledged hydrogenosomes: hydrogen

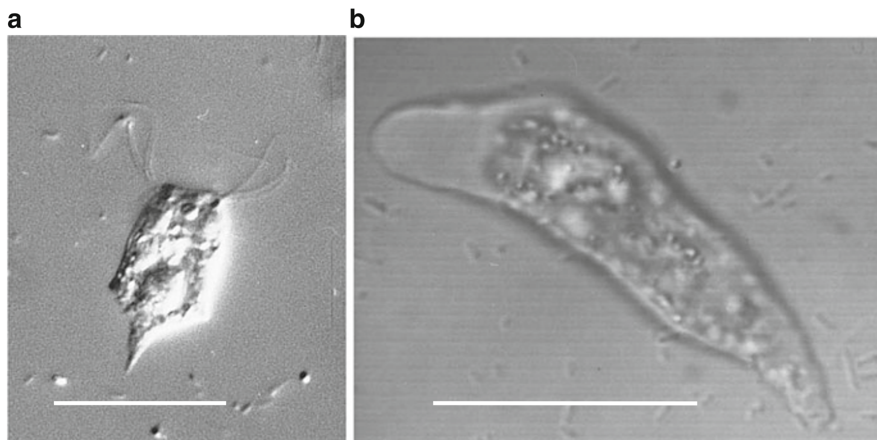

Fig. 4 Light microscopy of *Psalteriomonas lanterna*. (**a**) Flagellate stage of *Psalteriomonas lanterna* DIC-microscopy. At the apical side of the cell, two of the four flagella clusters can be seen. The globule in the centre of the cell is the hydrogenosomal complex. Courtesy C.K. Stumm, Nijmegen. (**b**) Amoeba stage of *Psalteriomonas lanterna*. CLS-microscope. Bars: 30 µm. Reproduced from de Graaf et al. (2009)

formation has been demonstrated, and hydrogenase activity was shown to be localized to the hydrogenosomes (Broers 1992).

Electron microscopy revealed the presence of two types of double membrane-bounded organelles (1) single, predominantly dumbbell-shaped organelles that are scattered in the cytoplasm and surrounded by 1–2 cisterns of rough ER and (2) stacks of up to 20, more or less sausage-shaped organelles, which are located in the centre of the cell (de Graaf et al. 2009; Fig. 5). At the isolation of *P. lanterna* from anaerobic sediments, these stacks of hydrogenosomes were "spiked" with methanogenic archaea (Broers et al. 1990), which were indicative of an intracellular source of hydrogen (and potentially other end products of the hydrogenosomal metabolism). The endosymbiotic methanogens were lost in the course of the prolonged in vitro culture of *P. lanterna*.

The cytoplasmatic organelles are very similar to the mitochondria of certain related aerobic Heterolobosea because of their dumbbell shape and their close association with the rough ER (Fig. 5). Both types of hydrogenosomes are about 0.3–0.6 μm in diameter and up to 3 μm in length (de Graaf et al. 2009). There is no evidence for the presence of a genome.

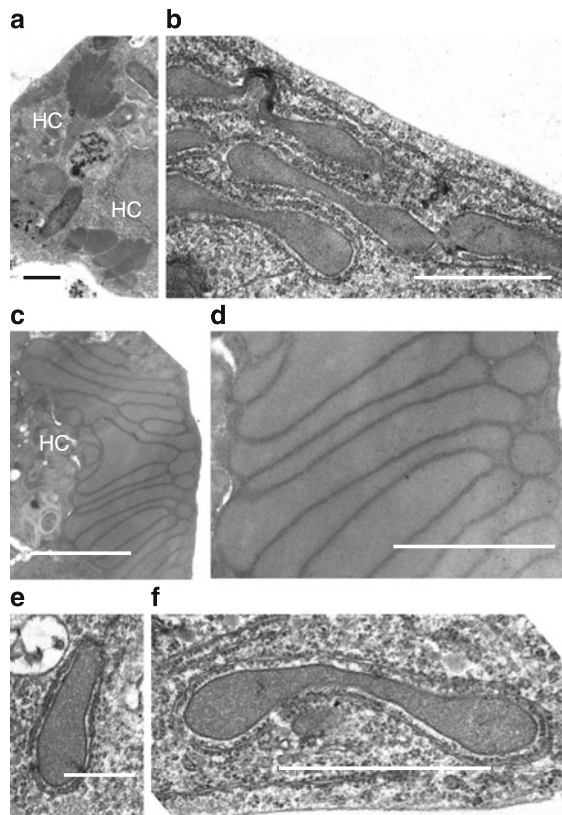

Fig. 5 Electron microscopy of the hydrogenosomes of *Psalteriomonas lanterna* flagellates. (**a**) Cell with two small stacks of hydrogenosomes. *HC* hydrogenosomal complex. (**b**) Group of dumbbell-shaped hydrogenosomes in the periphery of the cell. The hydrogenosomes are surrounded by cisterns of rough endoplasmatic reticulum (rough ER). These organelles have been named "modified mitochondria" by Broers (1992). (**c**) Large stack of hydrogenosomes (HC). (**d**) Detail of the hydrogenosomal complex shown in **c**. (**e**) "Single" hydrogenosome surrounded by rough ER. (**f**) Dumbbell-shaped hydrogenosome ("modified mitochondrion") Bars **a**–**d**, **f**: 1 μm; **e**: 0.5 μm. Reproduced from de Graaf et al. (2009)

The analysis of about 480 ESTs allowed the identification of a number of hydrogenosomal/mitochondrial genes. Besides a [FeFe] hydrogenase of the long type that is phylogenetically related to the hydrogenases of anaerobic chytrids and certain green algae (de Graaf et al. 2009), a PFO with significant sequence similarity to the PFOs of *T. vaginalis* and *Blastocystis* sp. was found. In addition, a gene with sequence similarity to the 51 kDa subunit of a mitochondrial Complex I has been identified, which is phylogenetically related to the 51 kDa subunit of *T. vaginalis* (de Graaf et al. 2009). Furthermore, a member of the mitochondrial carrier family has been identified. Phylogenetic analysis revealed that this carrier does not cluster with the true mitochondrial AACs and not with the alternative ATP/ADP carriers of *Trichomonas*. Its phylogenetic position suggests that it is another type of an alternative AAC. Lastly, a Hsp60 and a PCCB (propionyl-CoA carboxylase beta) gene were found among the ESTs. Both genes are characteristic for mitochondria. In the phylogenetic analysis, both genes cluster with their homologs from the related aerobic heterolobosean amoeboflagellate *Naegleria gruberi*. Also the phylogenetic analysis of the 18S rRNA genes of *P. lanterna* and its relatives shows that the few anaerobic Heterolobosea cluster among their many aerobic relatives (Fig. 6; Weekers et al. 1997; O'Kelly et al. 2003; Moon-van der Staay et al. 2006).

The morphology of the hydrogenosomes and the genes that were identified in the EST screen prove the mitochondrial ancestry of the hydrogenosomes of *P. lanterna*. Although the number of genes studied is very small, it was possible to reconstruct a rudimentary scheme of the hydrogenosomal metabolism (de Graaf et al. 2009). The PFO decarboxylates pyruvate to acetyl-CoA and CO_2. The electrons are transferred to the hydrogenase either with the aid of a so far unidentified ferredoxin or by the 51 kDa subunit. It is likely that ATP is formed in a similar way as in *T. vaginalis* and that the ATP is exported by the potential mitochondrial ATP/ADP carrier. Thus, the key enzymes of this metabolism are similar to those known from the hydrogenosomes of *Trichomonas* and *Trimastix*. However, the phylogenetic analysis has shown that the peculiar hydrogenosomes of *P. lanterna* derived from the mitochondria of its aerobic relatives among the Heterolobosea (Fig. 6) – clearly distinct from the descent of the hydrogenosomes of *Trichomonas* and *Trimastix* that do not have close aerobic relatives.

5 The Hydrogenosomes/Mitochondrion-like Organelles (MLOs) of *Blastocystis* sp.

The Stramenopile *Blastocystis* sp. is an anaerobic gut parasite that belongs to the supergroup Chromalveolata. It possesses double membrane-bounded organelles with both mitochondrial and hydrogenosomal properties. The organelles have been studied by enzymatic and molecular (EST) methods (Lantsman et al. 2008; Stechmann et al. 2008). These analyses had results that are sometimes controversial. For example, a [FeFe] hydrogenase has been identified among the ESTs and localized to the organelles with the aid of histocytochemistry using an antiserum

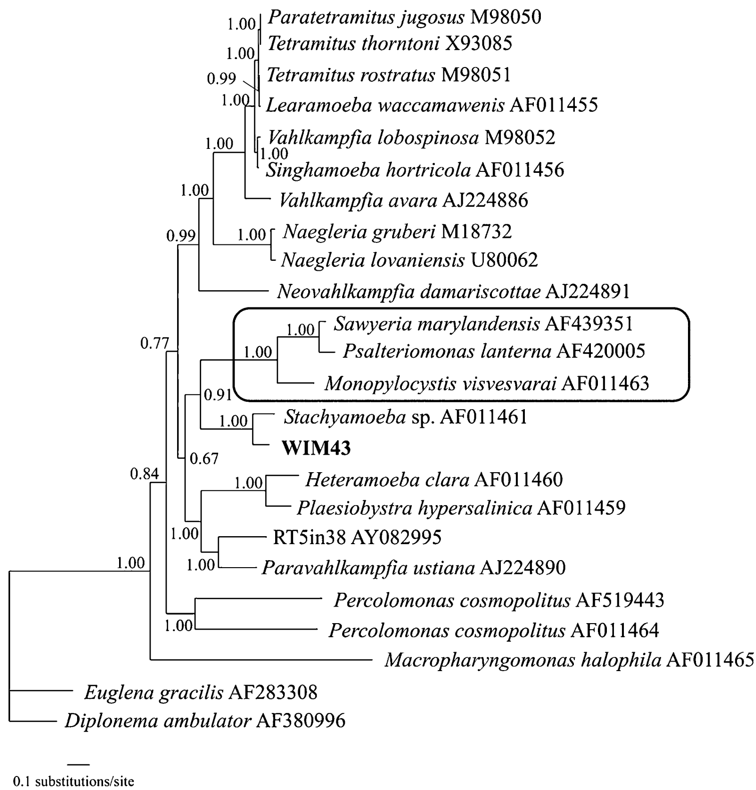

Fig. 6 Bayesian tree of Heterolobosea based on nuclear SSU rRNA gene sequences using 1698 positions. Numbers at nodes represent the posterior probability. Anaerobic Heterolobosea are *boxed*. After Moon-van der Staay et al. (2006), modified

against the *Blastocystis* hydrogenase. Therefore, the organelles can be regarded as hydrogenosomes (Stechmann et al. 2008). However, enzymatic studies failed so far to provide evidence for hydrogenase activity (Lantsman et al. 2008). Also, the EST analysis provided evidence for the presence of a PFO. Enzymatic studies, however, did reveal pyruvate:NADP oxidoreductase (PNO) activity instead of PFO activity. PNO is as PFO, a strictly anaerobic enzyme that decarboxylates pyruvate. It has been found also in the mitochondrial remnants (mitosomes) of *Cryptosporidium* sp. and the mitochondria of *Euglena gracilis*. In *Blastocystis*, PNO decarboxylates pyruvate to acetyl-CoA and CO_2; acetyl-CoA is metabolised to acetate by ASCT, an enzyme also present in hydrogenosomes. The CoA moiety is transferred to succinate that is recycled via a SCS (Lantsman et al. 2008; Stechmann et al. 2008). This is a pathway present in all hydrogenosomes studied up to now, and the genes ASCT and PFO in *Blastocystis* have significant sequence similarity to the homologous genes of *Trichomonas*.

The organelles host an incomplete TCA cycle. The EST studies provided evidence for SCS, succinate dehydrogenase (SDH), fumarase (Fum), and malate

dehydrogenase (MDH) genes, but the enzymatic studies revealed enzymatic activity of aconitase, isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase in addition to the SCS activity. A SDH activity was not observed, although the EST studies revealed the presence of all four subunits of a mitochondrial Complex II (Lantsman et al. 2008; Stechmann et al. 2008).

EST analysis and sequencing of the organellar genome (Perez-Brocal and Clark 2008; Stechmann et al. 2008; Wawrzyniak et al. 2008) identified 16 genes encoding subunits of a mitochondrial Complex I. The sequencing of the organellar genome disclosed a typical mitochondrial genome with 41 genes – with the notable exception of genes encoding subunits of mitochondrial Complex III, IV, and V, which were lacking. Since an alternative oxidase (AO) has been found among the ESTs, it is likely that *Blastocystis* possesses an electron transport chain consisting of Complex I, Complex II, and an AO. This is in remarkable agreement with the electron transport chain in the hydrogenosomes of *N. ovalis*, which, however, lack typical AO activity (Boxma et al. 2005). It is likely that Complex I is functional and – in the absence of Complex III/IV – accounts for the transmembrane potential that has been observed in the organelles of *Blastocystis* (Stechmann et al. 2008).

The EST analysis provided also evidence for the presence of various components of an amino acid metabolism, including a GCS that is found in mitochondria and many hydrogenosomes. Also six components of a Fe–S assembly pathway have been found, and parts of an urea cycle.

Thus, the metabolism of the *Blastocystis* organelles has mitochondrial, but also many hydrogenosomal traits. The metabolism is a remarkable example of convergent evolution if compared with the hydrogenosomal metabolism of *N. ovalis*. It represents a blueprint for the adaptation to anaerobic environments.

6 The Hydrogenosomes of Chytridiomycete Fungi

Fungi form a very diverse group of eukaryotes belonging to the Unikonta. The majority of investigated fungi contain mitochondria and are capable of oxidative phosphorylation. On the other hand, there are anaerobically functioning chytridiomycete fungi that contain hydrogenosomes (Hackstein et al. 2008a).

Anaerobic chytridiomycete fungi are important symbionts in the gastrointestinal tract of herbivorous mammals. A flagellated rumen-dwelling organism, *Neocallimastix frontalis*, was described in 1975 by Colin Orpin (1975). Two years later, Orpin published a report showing that *N. frontalis* and two other anaerobes had cell walls that contained chitin, indicating that these rumen-dwelling organisms are fungi (Orpin 1977).

The diversity of the anaerobic chytridiomycete fungi is large and they are found in the gastrointestinal tract of nearly all large herbivores, ranging from ruminants such as cattle, sheep, goat, deer, and antelopes to the foregut-fermenting marsupials and camelids on the one hand, and hindgut-fermenting species such as horse, elephant, rhinoceros, mara (Patagonian hare), and capybara ("water pig," the world's

largest rodent) on the other. Anaerobic chytridiomycetes can be isolated from rumen fluid or faeces, and they are maintained in anaerobic culture, most of them as pure axenic cultures. In the rumen of cattle or sheep, these anaerobic fungi can be as frequent as 7.6×10^8 thallus-forming units; in the faeces, there are still 4.2×10^4 units per g dry weight (Trinci et al. 1994). Anaerobic chytrids are not truly host specific since it is possible to transfaunate various host animals with isolates from different hosts. On the other hand, the various isolates are not the same, even if collected from the same host species and assigned to the same chytrid species. The patterns of utilization of substrates and the metabolic properties are different from isolate to isolate (Trinci et al. 1994).

6.1 Mitochondria versus Hydrogenosomes

The majority of the cultured fungi belonging to the taxa Ascomycota, Basidiomycota, and Zygomycota contain mitochondria. These mitochondria host a genome of varying size, which characteristically encodes only a handful of proteins (Bullerwell and Lang 2005). This implies that the vast majority of the 700–800 mitochondrial proteins (Sickmann et al. 2003) is nuclear encoded, synthesized in the cytoplasm, and imported into the organelles. Interestingly, certain cultivars of mitochondriate species are able to maintain mitochondria in the absence of a mitochondrial genome. Such yeasts are known as “petites”; they are viable but respiration deficient and, consequently, incapable of growing on non-fermentable substrates (Contamine and Picard 2000). In this respect, these mitochondria are similar to the genome-less chytrid hydrogenosomes.

On the other hand, two natural isolates of fission yeasts, *Schizosaccharomyces japonicus* var *japonicus* and *S. japonicus* var *versatilis*, lack detectable cytochromes and are respiration deficient, but nevertheless retained fully functional mtDNA (Bullerwell and Lang 2005). These fission yeasts are considered to be an intermediate evolutionary stage in between respiratory-competent fungi and those that completely lack mitochondrial DNA. The mitochondria of these yeast species might be similar to the genome-containing hydrogenosomes of the anaerobic ciliate *N. ovalis* that are described in detail below. Therefore, these respiration-deficient mitochondria represent an evolutionary intermediate between classical mitochondria and the hydrogenosomes of the chytridiomycete fungi.

6.2 Chytrids Perform a “Mixed Acid Fermentation”

Notably, the members of the phylum chytridiomycota such as *Piromyces* (Fig. 7) and *Neocallimastix*, which possess hydrogenosomes, lack both mitochondria and an organellar genome (van der Giezen et al. 1997). These hydrogenosomes of chytrid fungi are double membrane-bounded compartments up to 1 μm in size (Fig. 8) that

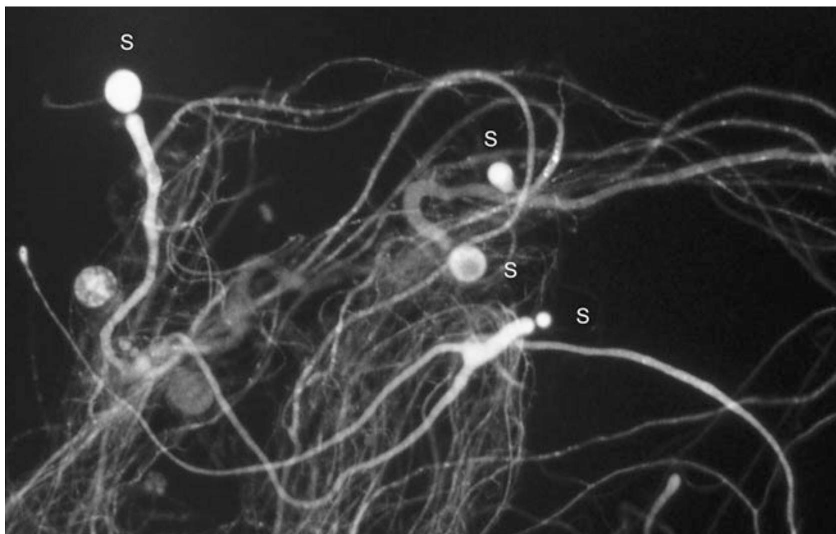

Fig. 7 Epifluorescence micrograph of *Piromyces* sp. E2 originally isolated from the faeces of an Indian elephant. Magnification about ×400. The organism was vitally stained with Rhodamine 123. S: young sporangia. Reproduced with permission from Hackstein et al. (2008a)

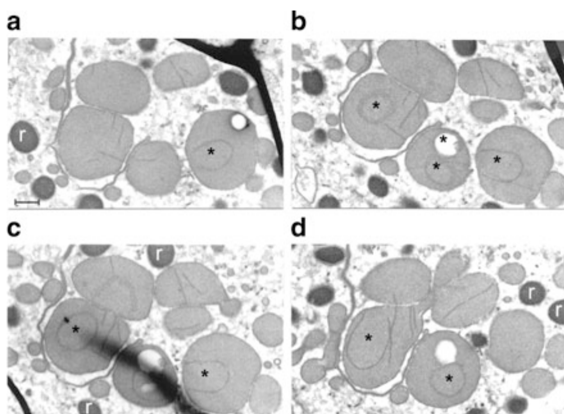

Fig. 8 Electron micrographs of the hydrogenosomes of *Neocallimastix* sp. L2. Serial sectioning, **a**–**d**. Bar = 0.5 μm; *r* ribosome globules (Munn et al. 1988). *Asterisk*, internal vesicular structures of the hydrogenosomes. Reproduced with permission from Hackstein et al. (2008a)

produce ATP by substrate-level phosphorylation together with hydrogen, CO_2, formate, and acetate as end products of the organellar metabolism (Marvin-Sikkema et al. 1993, 1994; Akhmanova et al. 1999; Hackstein et al. 2001; Voncken 2001). The intact organism produces succinate, lactate, and ethanol in addition when growing on cellulose, glucose, or fructose as a carbon source (Julliand et al. 1998). Such a "mixed acid fermentation" is very similar to bacterial mixed acid fermentations that are, for example, well known for facultative anaerobic enteric bacteria, such as *Escherichia coli*.

6.3 The Hydrogenosomal Metabolism

The hydrogenosomal metabolism has been studied in more detail in the chytridiomycetes *Piromyces* and *Neocallimastix*. Notably, the hydrogenosomes of these organisms are clearly different from those known of Trichomonads and anaerobic ciliates, structurally (Fig. 8) and metabolically. Most importantly, the hydrogenosomes of *Neocallimastix* sp. L2 and *Piromyces* sp. E2 contain PFL as key enzyme (Akhmanova et al. 1999), and not PDH (as in *N. ovalis*) or PFO (as in *T. vaginalis* and many other anaerobic organisms). Accordingly, PFO is lacking as the analysis of a large collection of ESTs reveals.

As discussed above, these chytridiomycetes produce formate, acetate, succinate, lactate, ethanol, hydrogen, and carbon dioxide (Fig. 9). However, the ratio of these

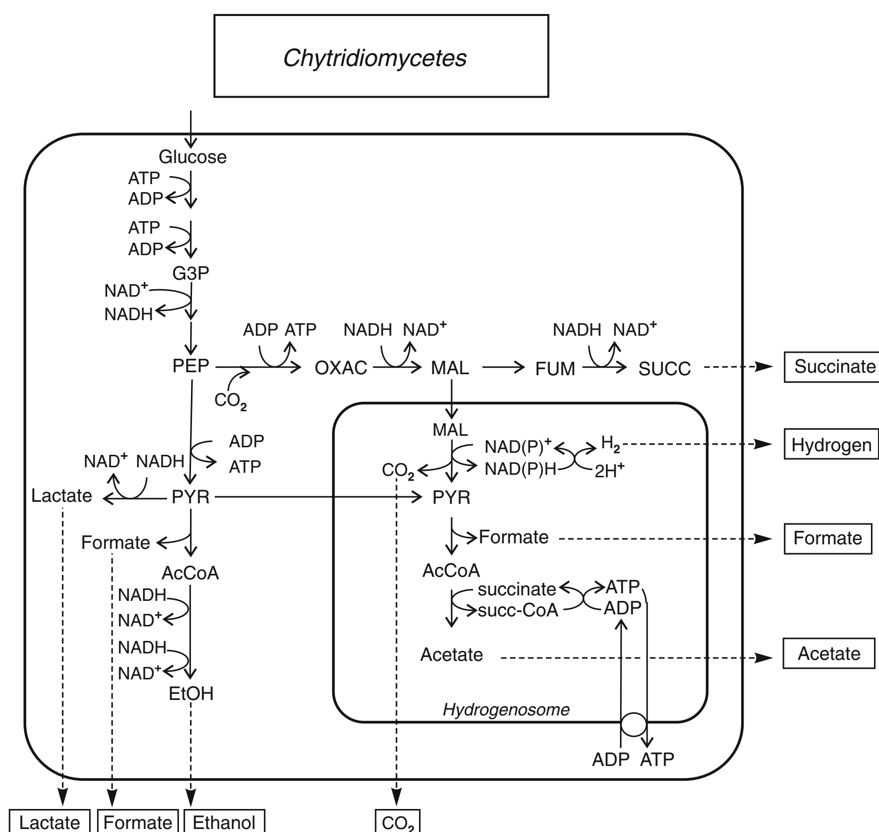

Fig. 9 Energy metabolism of chytridiomycetes. Shown is a scheme of the metabolic pathways involved in the production of the major end products. End products are in *boxes*. Abbreviations: *AcCoA* acetyl-CoA; *EtOH* ethanol; *FUM* fumarate; *G3P* glyceraldehyde-3-phosphate; *MAL* malate; *OXAC* oxaloacetate; *PEP* phosphoenolpyruvate; *PYR* pyruvate; *SUCC* succinate [adapted from Boxma et al. (2004)]

excreted end products is not constant, as it was shown that the growth of *Piromyces* sp. E2 in the presence of increasing concentrations of fructose is accompanied by changes in the fermentation pattern (Boxma et al. 2004). Increasing the fructose concentration from 0.1 to 0.5% resulted in a threefold increase in degradation of this substrate to end products. It is remarkable that the relative fluxes of fructose degradation through the various pathways were not constant during changing fructose concentrations. Although the absolute amounts of hydrogen formed in the incubations during growth at these increasing concentrations of fructose remained constant, the relative flux of malate into the hydrogenosomes and hence the relative flux to hydrogen decreased from 47 to 15% (Boxma et al. 2004). In contrast, the relative fluxes in the formation of the cytosolic end products lactate, ethanol, and succinate increased several fold. These observations show that increasing amounts of a fermentable carbon source result in an increased metabolism without an increased production of hydrogen. This implicates a relative shift from a hydrogenosomal carbon metabolism to a cytosolic one.

Metabolic experiments using labeled glucose indicated that an incomplete TCA cycle operates in the reductive mode allowing the formation of succinate from oxaloacetate via a malate intermediate (Fig. 9). Since the formation of significant amounts of labeled CO_2 could be excluded while formate and acetate plus ethanol were formed in a 1:1 ratio, it must be concluded that PFL and not PFO or pyruvate dehydrogenase (PDH) play the central role in the hydrogenosomal metabolism (Boxma et al. 2004). Moreover, experiments with isolated hydrogenosomes of *Piromyces* have shown that acetate and formate are formed in equimolar amounts confirming the activity of PFL in the hydrogenosomes (Akhmanova et al. 1999).

6.4 *The Role of the Hydrogenosomes in the Energy Metabolism of* Piromyces *sp. E2*

The observation that the hydrogenosomal PFL and the cytoplasmic ADHE are the key enzymes in the degradation of carbohydrates by anaerobic chytrids reveals that the metabolism of these hydrogenosomes is fundamentally different from the hydrogenosomal metabolism in both trichomonads and *N. ovalis*-like ciliates. Obviously, anaerobic chytrids chose their own way to adapt to anaerobic environments by evolutionary tinkering. The metabolic scheme displayed in Fig. 9 shows a generalized metabolism involving substrate-level ATP formation with the aid of ASCT and SCS. A quantitative analysis revealed that (1) PFL must be present and (2) that under certain conditions, hydrogen formation can become marginal (Boxma et al. 2004). The evolutionary strategy of chytrids apparently tends to avoid the formation of reduction equivalents by using PFL instead of PFO or PDH (Akhmanova et al. 1999; Hackstein et al. 1999, 2006; Voncken 2001).

The major role of the chytrid hydrogenosomes seems to be the generation of ATP by substrate-level phosphorylation. The presence of PFL in the absence of

hydrogenosomal ADHE most probably directs all organellar pyruvate into substrate-level ATP formation. A possible presence of ADHE inside the hydrogenosomes would compromise this function of the hydrogenosome as an energy-generating organelle. In the cytoplasm, however, ADHE might allow regulation of PFL activity, thus saving pyruvate (and its metabolites) for anabolic pathways. A partial TCA cycle with links to anabolic pathways operates in the cytoplasm (Akhmanova et al. 1998b) This hypothesis is supported by the observation that several mitochondrial enzymes, which are involved in anabolic reactions, e.g., malate dehydrogenase, aconitase, isocitrate dehydrogenase, and acetohydroacid reductoisomerase, have been retargeted to the cytoplasm in *Piromyces* sp. E2 (Akhmanova et al. 1998b Hackstein et al. 1999). Consequently, compartmentalization of the energy metabolism seems to enhance the possibilities for regulation of the metabolic pathways of this organism.

6.5 The Evolution of Hydrogenosomes from Fungal Mitochondria

Using 18S rDNA phylogenies or the phylogenies of mitochondrial genes from aerobic chytrids, a monophyletic origin of all chytrids becomes evident (Bullerwell and Lang 2005). There is no doubt about a fungal origin of the chytrids – regardless as to whether they are thriving in oxic or anoxic environments. The aerobic representatives possess mitochondria: phylogenetic analysis of their nuclear and mitochondrial genomes reinforces their fungal origin (Bowman et al. 1992; Paquin et al. 1995; Paquin and Lang 1996). Also an analysis of biochemical and morphological traits consistently establishes a close relationship between chytrids and other fungi (Ragan and Chapman 1978). Akhmanova et al. (1998b) demonstrated that several enzymes of mitochondrial origin, which lack putative targeting signals, were retargeted to the cytoplasm (in active form) in the hydrogenosome-bearing chytrid *Piromyces*.

Chytrid hydrogenosomes look rather different from the pictures of mitochondria in textbooks (Fig. 8). However, electron microscopical analysis revealed a structure resembling the ultrastructure of mitochondria from particular diseased human patients (Frey and Mannella 2000; Hackstein et al. 2001; Voncken et al. 2002). Also, the relict mitochondrion (mitosome) of *Cryptosporidium parvum* looks very similar (Keithly et al. 2005). Apparently, in these cases, the inner membrane undergoes a derangement in the mechanism that normally stabilizes the crista junctions (Mannella 2006).

Because of their intrinsic function in the organelle, ADP/ATP carriers (AACs) and chaperonins are the best indicators for the phylogenetic analysis of an organelle of mitochondrial origin. Phylogenetic analysis of the AACs and chaperonins of anaerobic chytrids unequivocally revealed a fungal mitochondrial ancestry (Voncken 2001; Voncken et al. 2002; van der Giezen et al. 2002, 2003). Moreover, the spectrum of responses against the various inhibitors is quite specific and

differentiates these AACs clearly from other adenine transporters – regardless as to whether these transporters are from mitochondrial or hydrogenosomal origin (Hackstein et al. 2006). While the AACs are eukaryotic "inventions" that allowed the exploitation of the ATP formed inside the organelle after the organelle formation, the chaperonins tend to trace the ancestry of the organelle back to the endosymbiont that gave rise to the mitochondrion. Also, the phylogenetic analysis of Hsp 60 and the Hsp 70 clearly reveals a clustering with their fungal mitochondrial relatives and not with the alpha-proteobacterial cluster (Hackstein et al. 1999; Voncken et al. 2002; van der Giezen et al. 2003).

Genomic analyses of the hydrogenosomal enzyme succinyl-CoA synthetase, SCS, (Dacks et al. 2006) and two additional hydrogenosomal enzymes involved in arginine biosynthesis (Gelius-Dietrich et al. 2007) further confirm the fungal mitochondrial origin of the *Neocallimastix* hydrogenosome. Most of the other hydrogenosomal genes have not been identified so far.

We now know that the anaerobic chytrids comprise many species that are integral in the rumen ecosystem and crucial in the digestion of plant material to simple sugars. Moreover, they produce hydrogen needed for the growth of methanogenic bacteria [reviewed in Williams et al. (1994)]. However, there is no evidence for endo- or episymbiotic associations between anaerobic chytrids and methanogenic archaea. Notwithstanding, co-culture of chytrids and methanogens has profound effects on the overall metabolism of the chytrids (Marvin-Sikkema et al. 1990).

7 The Hydrogenosomes of Anaerobic Ciliates

Ciliates represent an extremely species-rich, monophyletic group of highly complex unicellular eukaryotes. They are characterized by a nuclear dimorphism and rather complex patterns of morphologically distinct cortical cilia. Most of the ciliates thrive in aerobic environments and possess mitochondria, but anaerobic species evolved in at least 8 of the 22 orders of ciliates as classified by Corliss (Corliss 1979; Fenchel and Finlay 1995). Certain ciliates in seven of these eight orders possess "hydrogenosomes" (Hackstein et al. 2008b; Fig. 10). However, the identification of many of these hydrogenosomes was based solely on the presence of intracellular methanogenic archaea. Such a symbiotic association is indicative of an inter-species hydrogen transfer and could reveal the presence of intracellular hydrogen sources, i.e., hydrogenosomes (Hackstein et al. 2002).

The development of fluorescence microscopy, electron microscopy, cytobiochemistry, and techniques for cellular fractionation allowed the discovery of hydrogenosomes in free-living anaerobic ciliates such as *Plagiopyla*, *Trimyema*, and *Metopus* (van Bruggen et al. 1983, 1984, 1986; Goosen et al. 1988, 1990; Zwart et al. 1988; Finlay and Fenchel 1989; Fenchel and Finlay 1995, 2010; Biagini et al. 1997; Shinzato and Kamagata 2010). Hydrogenosomes were also identified in ciliates thriving in the gastrointestinal tract of ruminants and marsupials (e.g., *Isotricha*, *Dasytricha*, *Epidinium*, *Eudiplodinium*, *Polyplastron,* and *Amylovorax*)

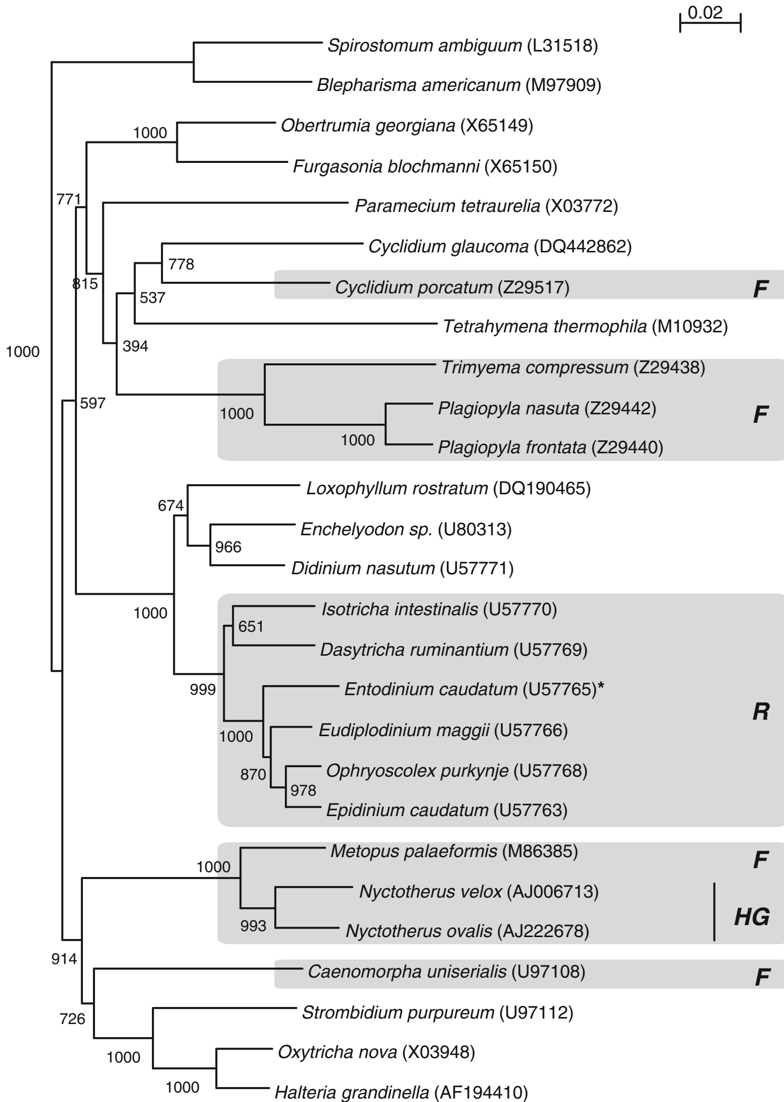

Fig. 10 Neighbour-joining phylogenetic tree of 18S ribosomal RNA of ciliates. Ribosomal RNA sequences were aligned using Clustal X (Jeanmougin et al. 1998) and phylogenetic trees were prepared by neighbour-joining (Saitou and Nei 1987). Shown are the accession numbers of used sequences and the bootstrap values for 1,000 independent analyses. Shaded boxes indicate anaerobic ciliates with hydrogenosomes, whereas all other ciliates contain mitochondria and function aerobically. The natural habitat of the hydrogenosome-containing ciliates is indicated by the following abbreviations: *F* free living; *HG* hindgut; *R* rumen. Ciliate species that might possess mitosomes instead of hydrogenosomes are indicated by an *asterisk*. Reproduced with permission from Hackstein et al. (2008b)

(Vogels et al. 1980; Snyers et al. 1982; Yarlett et al. 1981, 1982, 1983, 1984, 1985; Lloyd et al. 1989; Paul et al. 1990; Ellis et al. 1991a,b,c; Cameron and O'Donoghue 2002a). They were also found in *N. ovalis* that lives in the hindgut of cockroaches (Gijzen et al. 1991; Akhmanova et al. 1998a; Boxma et al. 2005). Figure 10 shows the distribution of hydrogenosomes and mitochondria in the various orders of ciliates. All these hydrogenosomes are surrounded by a double membrane, and under optimal fixation conditions, in a number of ciliate species, cristae-like protrusions can be seen in these organelles, and in that way they clearly resemble mitochondria (Fig. 11).

7.1 N. ovalis

Nyctotherus species (Armophorea) are anaerobic, heterotrichous ciliates with hydrogenosomes that thrive in the intestinal tract of cockroaches, millipedes, frogs, and reptiles. *N. ovalis* from the hindgut of cockroaches is the only species that has been studied in more detail (van Hoek et al. 1998, 1999, 2000b). Notably, the presence of a mitochondrial genome has been demonstrated in the hydrogenosomes of *N. ovalis* (Akhmanova et al. 1998a; van Hoek et al. 2000a; Boxma et al. 2005). This genome was shown to be a typical mitochondrial genome of ciliate origin (Boxma et al. 2005). This ciliate origin is reinforced by the analysis of some 90 genes that encode mitochondrial proteins (Boxma et al. 2005; Ricard 2008, de Graaf et al. unpublished, see below).

Metabolic studies revealed that a small part of the glucose was degraded to typical end products of a glycolytic fermentation: approximately 24% of the degraded glucose was excreted as lactate and 5% as ethanol (Boxma et al. 2005). The major part of the glucose was degraded via the hydrogenosomes to acetate and succinate. Those studies showed that *N. ovalis* does not use a complete TCA cycle for the degradation of glucose and does not use pyruvate formate lyase (PFL) activity in its pyruvate metabolism, as is the case in hydrogenosomes of anaerobic

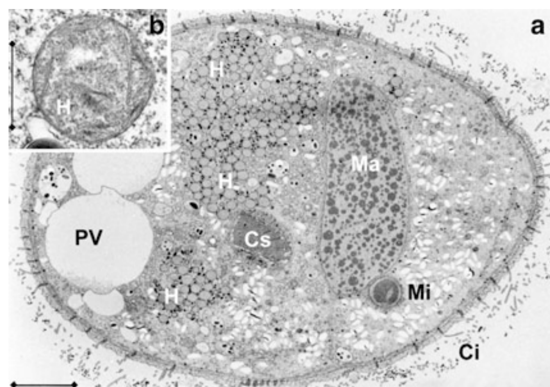

Fig. 11 Electron micrograph of *N. ovalis* (**a**) with a close up view of a hydrogenosome (**b**). Bar in **a**: 10 μm, bar in **b**: 0.5 μm. *H* hydrogenosomes; *Ma* macronucleus; *Mi* micronucleus; *Cs* cytostome; *PV* pulsating vacuole. Reproduced with permission from Hackstein et al. (2008b)

chytrids. The product of glycolysis in the cytosol, pyruvate, is apparently either converted into lactate or ethanol or transported into the hydrogenosome to be converted into acetate or succinate. For the production of acetate, this pyruvate is decarboxylated by a pyruvate dehydrogenase complex (PDH) and not by a PFO (Boxma et al. 2005). The excretion of significant amounts of succinate indicated that endogenously produced fumarate is used as a terminal electron acceptor. Protons act as another hydrogenosomal electron acceptor, which results in the formation of hydrogen. Fumarate reduction is most likely catalysed by a membrane-bound fumarate reductase (an anaerobically functioning variant of Complex II), coupled to Complex I of the electron transport chain via quinones. Consistent with the biochemical/biophysical requirements (Tielens et al. 2002), small amounts of rhodoquinone 9 and menaquinone 8 were detected, whereas ubiquinone 7 and 8 (which are found in large amounts in the aerobic ciliates *Euplotes* and *Tetrahymena*, respectively) were not detected in *N. ovalis* (Boxma et al. 2005).

7.2 In Silico Reconstruction of the Basal Hydrogenosomal Metabolism of N. ovalis

The significance of these experimental data might be circumstantial without molecular support (Boxma et al. 2005). Genes for all four subunits of a PDH are present and are expressed. In addition, a gene was detected for acetyl-CoA synthase, an enzyme for the production of acetate from acetyl-CoA, and also several genes, which are predicted to encode enzymes of the TCA cycle, i.e., malate dehydrogenase, succinate dehydrogenase (2 subunits), succinyl-CoA synthetase, and alpha-ketoglutarate dehydrogenase (1 subunit). Thus, basically the core energy metabolism of a typical ciliate mitochondrion was detected, albeit in an anaerobic version (Fig. 12). In fact, *N. ovalis* contains hydrogen-producing mitochondria (Boxma et al. 2005).

There is no evidence for genes encoding components of mitochondrial Complexes III and IV. Notably, these complexes are also absent in the electron transport chains of anaerobic mitochondria and the hydrogenosomes of *Blastocystis* (Tielens et al. 2002; Perez-Brocal and Clark 2008; Stechmann et al. 2008; Wawrzyniak et al. 2008). Therefore, it is likely that these hydrogenosomes gain their energy by the generation of a PMF through proton pumping by the mitochondrial Complex I. Of the subunits of a mitochondrial Complex I, 12 out of the 14 subunits that form the core of a bacterial Complex I were cloned and sequenced until now (de Graaf unpublished). Accordingly, imaging studies using inhibitors and fluorescent dyes not only demonstrated the presence of a functional Complex I in these hydrogenosomes but also indicated the absence of functional Complexes III and IV and the absence of a plant-like alternative terminal oxidase (Boxma et al. 2005).

Also, no homologs of an F_0F_1-ATP synthase have been discovered so far, as in the organelles of *Blastocystis* and *Trichomonas* (Carlton et al. 2007; Perez-Brocal and Clark 2008; Stechmann et al. 2008; Wawrzyniak et al. 2008).

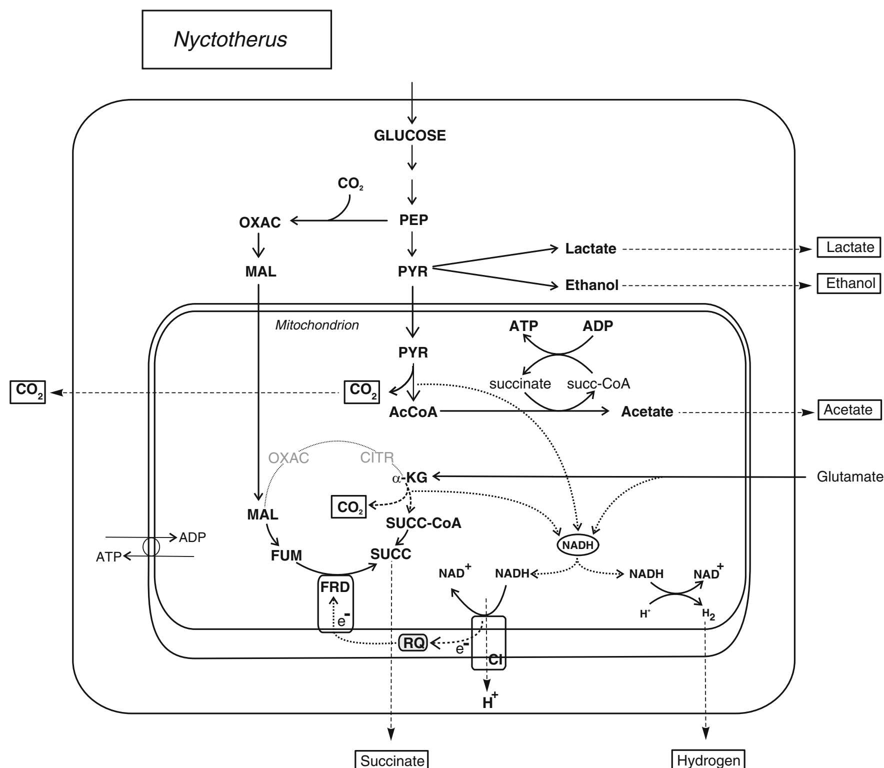

Fig. 12 Speculative metabolic schemes of the main pathways in carbohydrate metabolism in the ciliate *Nyctotherus ovalis*. End products are in *boxes*. Abbreviations: *AcCoA* acetyl-CoA; *CI* Complex I; *FRD* fumarate reductase; *FUM* fumarate; *α-KG* α-ketoglutarate; *MAL* malate; *OXAC* oxaloacetate; *PEP* phosphoenolpyruvate carboxykinase; *PYR* pyruvate; *RQ* rhodoquinone; *SUCC* succinate; *SUCC-CoA* succinyl-CoA

In addition, components of a mitochondrial amino acid metabolism were identified, including a GCS. Moreover, components of fatty acid metabolism, an AAC, several members of the mitochondrial solute carriers family, a malate-oxoglutarate translocator, components of a mitochondrial protein import and processing machinery, components of a protein synthesizing machinery, and proteins belonging to a ROS defence systems were found. Several proteins originated from lateral gene transfer (Ricard 2008). Thus, the hydrogenosome of *N. ovalis* is not simply a rudimentary mitochondrion. It is a highly specialized organelle of considerable complexity.

7.3 The Hydrogenosomes of Other Ciliates

Metabolic studies have also been carried out on the hydrogenosomes of rumen ciliates such as *Dasytricha*, *Isotricha*, *Epidinium*, and *Eudiplodinium*. All rumen ciliates form a monophyletic group (Fig. 10; Strüder-Kypke et al. 2006), but not all

of them possess hydrogenosomes (Yarlett et al. 1984, 1985). Certain rumen ciliates utilize cellulose and starch – others predate on bacteria and smaller protozoa. Glucose is the major monosaccharide liberated by degradation of plant polymers and can be used by these rumen protozoa as fermentation substrate. The main end products of the metabolism of exogenously added glucose as well as of intracellular amylopectine of rumen ciliates with hydrogenosomes are hydrogen, acetate, lactate, butyrate, and CO_2 (Yarlett et al. 1985; Ellis et al. 1991a,b,c). The ratio in which these end products are formed is influenced by O_2 and CO_2 concentrations similar to those present in the rumen. The investigated rumen ciliates are able to use oxygen as terminal electron acceptor. The nature of this terminal oxidase is still unknown, but cytochromes appear not to be involved. *Dasytricha ruminantium* is the best-studied rumen ciliate, but even the knowledge of the metabolism of this rumen ciliate is still far from complete. The enzyme used for the degradation of pyruvate to acetyl-CoA in this protozoon is suggested to be PFO, which has been identified tentatively in the hydrogenosomal fraction (Yarlett et al. 1981, 1982, 1985). This acetyl-CoA is the substrate for the hydrogenosomal formation of acetate, but seems also to be exported from the hydrogenosomes for the formation of butyrate (Yarlett et al. 1985; Ellis et al. 1991b). These aspects make this hydrogenosome of rumen ciliates very different from that of *Nyctotherus* (Fig. 12) and also different from the hydrogenosomes of *Trichomonas* (Fig. 3) and the anaerobic chytrids (Fig. 9).

The only other published metabolic studies on hydrogenosomes of ciliates deal with the free-living Plagiopylid ciliate *Trimyema* (Goosen et al. 1988, 1990; Holler and Pfennig 1991; Shinzato and Kamagata 2010). *Trimyema* consumes oxygen under micro-aerobic conditions and is reported to produce formate as the major end product with minor amounts of acetate and lactate (Goosen et al. 1990; Holler and Pfennig 1991). Under those micro-aerobic conditions, hydrogen and ethanol are not produced. Under strictly anaerobic conditions, however, ethanol is the main end product, while acetate, lactate, formate, and hydrogen are then formed in minor amounts (Goosen et al. 1990; Holler and Pfennig 1991).This pattern of anaerobic fermentation products resembles the one found in anaerobic chytridiomycete fungi (Boxma et al. 2004; see above). These fungi perform a bacterial-type mixed acids fermentation, using PFL for the degradation of pyruvate, instead of PDH or PFO, which is used by *N. ovalis* and Trichomonads, respectively. Albeit that no additional biochemical data are available and that no cell fractionation studies have been performed, it is likely that the Plagiopylids evolved a type of hydrogenosome that is clearly different from those of *Nyctotherus* and *Dasytricha*.

7.4 Can the Methanogenic Symbionts Tell Us More about the Origin and Function of Ciliate Hydrogenosomes?

As mentioned before and described by Fenchel and Finlay (2010) and Ushida (2010), anaerobic ciliates are frequently associated with symbiotic methanogens. The nature of the methanogenic symbionts supports the conclusion that different

ciliates host different types of hydrogenosomes. While *Nyctotherus* and *Metopus*, and also *Plagiopyla* and *Trimyema*, host different endosymbiotic methanogens (Fenchel and Finlay 1995, 2010; van Hoek et al. 2000b; Hackstein et al. 2002), certain rumen ciliates seem to host episymbiotic methanogens. Whether this episymbiotic association is specific and whether there is any rumen ciliate (except *Dasytricha* and *Isotricha*) with *endo*symbiotic methanogens is still a matter of debate (Fenchel and Finlay 1995; Tokura et al. 1999; Regensbogenova et al. 2004; Ushida 2010).

Because the methanogens (regardless of being endo- or episymbiotic) rely on substrates provided by the host, the properties of the endosymbiont might provide some information about the metabolic characteristics of the host. The group of Vogels and Stumm succeeded in cultivating a number of putative methanogenic endosymbionts from the anaerobic ciliates *Metopus striatus*, *Metopus contortus*, and *Plagiopyla nasuta*, from the amoeboflagellate *Psalteriomonas*(*Lyromonas*) *vulgaris* and the giant amoeba *Pelomyxa palustris* (van Bruggen et al. 1984, 1986, 1988; Goosen et al. 1988; see Fenchel and Finlay 1995 for more references and discussion). The conclusion from these studies was that certain endosymbionts were similar if not identical to well-known free-living methanogens, e.g., *Methanobacterium formicicum*. Only the putative endosymbiont from *M. contortus* seemed to represent a new type of methanogen, i.e., *Methanoplanus endosymbiosus*. The latter host, *Metopus*, belongs to the same taxon as *N. ovalis*, which makes it likely that this ciliate (Metopus) possesses a similar mode of pyruvate metabolism. The metabolic properties of the methanogenic endosymbiont *M. formicium*, however, suggested that this methanogen might be capable of using other substrates besides hydrogen and CO_2, e.g., formate (Dong et al. 1994). Notably, Narayanan et al. (2009) provided evidence for the presence of an acetoclastic *Methanosaeta* species as endosymbiont of *Metopus es* suggesting that methanogenic endosymbionts might be able to use acetate excreted by the hydrogenosomes. This argues again for the metabolic diversity among ciliate hydrogenosomes and their methanogenic endosymbionts. This metabolic diversity could provide additional arguments for multiple origins of the hydrogenosomes, but unfortunately, metabolic data of both hosts and symbionts are scarce.

7.5 Evolutionary Aspects

There exists a rather broad agreement that the anaerobic ciliates evolved secondarily from aerobic ancestors since several ciliate taxa comprise both aerobic and anaerobic species. Phylogenetic studies suggest that hydrogenosomes have arisen independently at least three to four times in ciliates (Fig. 10; Clarke et al. 1993; Embley and Finlay 1994; Embley et al. 1995, 2003; Fenchel and Finlay 1995; Hirt et al. 1998; Hackstein et al. 2001, 2002). The existence in *N. ovalis* and *Blastocystis* of a "missing link," an organelle with characteristics of mitochondria as well as hydrogenosomes, demonstrates that hydrogenosome-bearing ciliates can evolve

from mitochondriate ciliates (Martin 2005; Ricard 2008; Perez-Brocal and Clark 2008; Stechmann et al. 2008; Wawrzyniak et al. 2008). Albeit that the patchy distribution of hydrogenosomes alone is not sufficient to prove multiple independent origins of ciliate hydrogenosomes, the existence of a missing link like the hydrogenosome of *N. ovalis* provides a clear scenario for the evolution of hydrogenosomes from mitochondria. Apparently, hydrogenosomes in ciliates can evolve "easily" by evolutionary tinkering from mitochondria in the course of the adaptation of their hosts to anaerobic/micro-aerobic environments. This happened several times independently in the evolution of ciliates – at least three independent origins are supported by the existence of three different types of hydrogenosomes in the few ciliates that have been studied so far.

It remained unclear until now whether or not all anaerobic ciliates possess hydrogenosomes, in particular those anaerobes that do not possess endosymbiotic methanogens. Theoretically, anaerobic ciliates might possess anaerobic mitochondria (Tielens et al. 2002), hydrogenosomes, or they could even have lost ATP-generating organelles completely. In this case, they most likely host mitochondrial remnants, mitosomes, just like *Giardia* and *Entamoeba* spp., which are completely dependent on cytosolic reactions for the production of ATP (see, e.g., Hackstein et al. 2006 and Hjort et al. 2010 for discussion). However, the presence of these elusive organelles has not been studied systematically and in more detail in ciliates so far – with a few remarkable exceptions to be discussed below.

It has already been addressed that at least among the rumen ciliates, species with mitosomes might exist, because there is evidence that certain rumen ciliates, such as *Entodinium simplex*, *Entodinium caudatum*, *Diploplastron affine*, *Ophryoscolex caudatus*, *Eremoplastron bovis*, and *Ostracodinium obtusum bilobum*, did not exhibit detectable hydrogenase activity in the particulate cell fraction (Yarlett et al. 1984). Also, electron microscopy did not reveal the presence of mitochondrial-shaped organelles or typical hydrogenosomes in certain species of rumen and marsupial gut ciliates; a systematic search for mitosomes, however, has not been performed (Williams and Coleman 1992; Cameron and O'Donoghue 2002b). The observation that also PFO and malate dehydrogenase (decarboxylating) activities (Yarlett et al. 1984) are not enhanced in the particulate cell fraction, together with a low cytoplasmic hydrogenase activity, might argue for the absence of hydrogenosomes and potentially for the presence of mitosomes. However, until now, there are no additional data that could support this speculation.

The adaptation to an anaerobic lifestyle with the aid of hydrogenosomes required the acquisition of an (oxygen-sensitive) hydrogenase. The evolution of fumarate respiration in *N. ovalis* shows that an adaptation to life in anaerobic environments can occur in steps – by evolutionary tinkering. Once anaerobiosis could be tolerated by the invention of fumarate respiration, it became possible to acquire a hydrogenase. The [FeFe] hydrogenase of *N. ovalis* most likely has been obtained by lateral gene transfer from anaerobic (sulphate-reducing) bacteria (Boxma et al. 2007; de Graaf et al. 2009). The peculiar 24 and 51 kDa subunits of this complex hydrogenase are paralogous to the corresponding proteins of the mitochondrial Complex I (which is functional in *N. ovalis*), and have a different (most likely beta

proteobacterial) origin. The acquisition of this hydrogenase obviously allows a fine tuning of the NADH pool, which is crucial for the maintenance of homeostasis under anaerobic conditions. Thus, *N. ovalis* not only turns out to be a missing link but also demonstrates that the adaptation to anaerobic environments can involve several steps to allow the evolution of multiple levels for the control of homeostasis.

8 Conclusions

In the recent years, it has become clear that there are many mitochondria that do not function as described in most biochemical textbooks (Tielens et al. 2002). Moreover, there are mitochondrion-related organelles such as hydrogenosomes, mitosomes, mitochondrial remnants, and mitochondrion-like organelles (Fig. 1; Hackstein et al. 2006). All these organelles exhibit a large diversity of metabolic properties (Tielens et al. 2002; Tielens and van Hellemond 2007; van der Giezen, 2009; Hjort et al. 2010). In this review, the diversity of hydrogenosomes, bona fide hydrogenosomes, and hydrogenosome/mitochondrion-like organelles has been described. It has been shown that the metabolism of all these organelles is rather different corroborating their independent evolution from mitochondria and mitochondrion-like organelles. Their diversity is the consequence of their independent evolution in different anaerobic niches from organelles that were already adapted to different aerobic environments. The diversity of the hydrogenosomes described here is remarkable. Since there are many such organelles awaiting a more detailed analysis, a even larger diversity of hydrogenosomes might be expected.

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Evolution of Prokaryote-Animal Symbiosis from a Genomics Perspective

Rosario Gil, Amparo Latorre, and Andrés Moya

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Abstract Symbioses involving prokaryotes living in close relationship with eukaryotic cells have been widely studied from a genomic perspective, especially in the case of insects. In the process toward host accommodation, symbionts experience major genetic and phenotypic changes that can be detected in comparison with free-living relatives. But, as expected, several scenarios allowed the evolution of symbiotic associations, from the first stages of free-living bacteria,

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through secondary and facultative symbiosis, towards the final point of obligate primary endosymbiosis. Particular relevance has the association formed by the coexistence of several symbionts into a given host. A summary of findings in this field, as well as an evolutionary scenario to explain these changes, is presented in this chapter.

1 Introduction

The term symbiosis refers to the close ecological relation between two (or more) species, able to report benefits to all (mutualism) or some of the implied organisms, with or without harm of one of the involved species (parasitism or commensalism, respectively). Symbiosis is an important source of evolutionary innovation, with examples in the entire Biosphere, being even at the origin of the eukaryotic cell (Margulis 1993). Since then, stable symbioses have evolved independently many times in diverse groups of eukaryotes (Moya et al. 2008). Most symbioses have a demonstrated biochemical basis: in some cases one of the partners benefits from organic compounds produced by the other; in others, its waste products (mainly nitrogen compounds) are recycled by the other. In mutualistic symbioses, matter and energy flow in both directions, so that both partners benefit from the association.

Numerous eukaryotic groups maintain a mutualistic relationship with prokaryotic cells, especially because many eukaryotic lineages present limited metabolic capabilities. Animal metabolism, in particular, is relatively narrow, and essential molecules (such as amino acids, vitamins, or fatty acids) must be retrieved from the environment for survival. Animals with specialized feeding behaviors tend to establish symbiotic associations with microorganisms, which provide the nutrients that are deficient in their diets. In fact, most intracellular mutualistic symbioses between bacteria and animals that have been analyzed at the genomic level (involving insects, nematodes and deep-sea animals, Table 1) are related to nutrient provision. Regarding insects, the most studied and diverse invertebrate group on earth, the presence of such associations throughout most of their evolutionary history suggests that symbiosis has been a driving force in the diversification of the group.

A high proportion of mutualistic symbiotic relationships established by insects imply the participation of bacteria. Frequently, the association is so tight that it is called endosymbiosis, when the bacteria (endosymbiont) obligatorily live inside specialized eukaryotic cells (bacterocytes), which can even form a specialized organ (the bacteriome), located inside the abdominal cavity of the insect. It has been estimated that up to a 15% of all insects species carry bacterial endosymbionts (Baumann 2005), attributing to them the great adaptive success of the Insecta class, by making possible the colonization of new ecological niches and allowing them to feed on restricted diets (such as plant sap, cereals or blood), poor in some essential nutrients that are provided by the endosymbionts. The elimination of these bacteria,

Table 1 Remarkable data of the mutualistic animal endosymbiont genomes sequenced as of November 2009

Stage of symbiosis	Organism	Phylum/class	Host	Symbiotic function	Genome size (Kb)	G + C content (%)	Repetitive DNA (%)	Accession number
S-symbionts	*Sodalis glossinidius* str. *morsitans*	Proteobacteria/ γ-Proteobacteria	*Glossina morsitans* (tsetse fly)	Related with trypanosome infections	4,171	54.7	Not indicated[b]	AP008232 AP008233 AP008234 AP008235
	Hamiltonella defense[a] 5AT	Proteobacteria/ γ-Proteobacteria	*Acyrthosiphon pisum* (aphid)	Resistance to parasitoid wasps	2,110	40.0	21	CP001278
	Regiella insecticola	Proteobacteria/ γ-Proteobacteria	*Acyrthosiphon pisum* (aphid)	Resistance to parasitoid wasps and fungal pathogens	>2,035	42.2	>14	ACYF01000000
	Wolbachia pipientis *w*Pip	Proteobacteria/ α-Proteobacteria	*Culex pipiens* (mosquito)	Reproductive parasite	1,482	34.2	Not indicated[b]	AM999887
	Wolbachia pipientis *w*Ri	Proteobacteria/ α-Proteobacteria	*Drosopila simulans* (fruit fly)	Reproductive parasite	1,446	35.0	22.1	CP001391
	Wolbachia pipientis *w*Mel	Proteobacteria/ α-Proteobacteria	*Drosophila melanogaster* (fruit fly)	Reproductive parasite	1,268	35.2	8.9	NC_002978
P-endosymbionts	*Ruthia magnifica*[a]	Proteobacteria/ γ-Proteobacteria	*Calyptogena magnifica* (deep-sea-clam)	Nutrient provision (amino acids, vitamins and cofactors), Nitrogen recycling	1,200	35.0	0	CP000488
	Vesicomyosocius okutanii[a]	Proteobacteria/ γ-Proteobacteria	*Calyptogena okutanii* (deep-sea-clam)	Nutrient provision (amino acids, vitamins and cofactors), Inorganic Carbon fixation, Nitrogen recycling	1,022	31.6	0	AP009247

(*continued*)

Table 1 (continued)

Stage of symbiosis	Organism	Phylum/class	Host	Symbiotic function	Genome size (Kb)	G + C content (%)	Repetitive DNA (%)	Accession number
	*Wolbachia pipientis w*Bm	Proteobacteria/ α-Proteobacteria	*Brugia malayi* (nematode)	Nutrient provision (nucleotides, vitamins and cofactors)	1,080	34	0	AE017321
	Blochmannia pennsylvanicus[a]	Proteobacteria/ γ-Proteobacteria	*Camponotus pennsylvanicus* (carpenter ant)	Nutrient provision (amino acids), Nitrogen storage	792	29.6	0	CP000016
	Blochmania floridanus[a]	Proteobacteria/ γ-Proteobacteria	*Camponotus floridanus* (carpenter ant)	Nutrient provision (amino acids), Nitrogen storage	706	27,4	0	BX248583
	Wigglesworthia glossinidia	Proteobacteria/ γ-Proteobacteria	*Glossina brevipalpis* (tsetse fly)	Nutrient provision (vitamins)	698	22.5	0	BA000021 AB063523
	Buchnera aphidicola BSg	Proteobacteria/ γ-Proteobacteria	*Schizaphis graminum* (aphid)	Nutrient provision (amino acids, vitamins, fatty acids)	653	25.3	0	AE013218 AF041836 Z21938
	Buchnera aphidicola BAp	Proteobacteria/ γ-Proteobacteria	*Acyrthosiphon pisum* (aphid)	Nutrient provision (amino acids, vitamins, fatty acids)	652	26.3	0	BA000003 AP001070 AP001070
	Buchnera aphidicola BBp	Proteobacteria/ γ-Proteobacteria	*Baizongia pistaciae* (aphid)	Nutrient provision (amino acids, vitamins, fatty acids)	618	26.0	0	AE016826 AF492591
	Carsonella ruddi[a]	Proteobacteria/ γ-Proteobacteria	*Pachypsylla venusta* (psyllid)	Nutrient provision?	160	16.0	0	AP009180
	Blattabacterium strain Bge	Bacteroidetes/ Flavobacteria	*Blattella germanica* (cockroach)	Nutrient provision (amino acids) and Nitrogen excretion	637	27.1	0	CP001487

	Blattabacterium strain BPLAN	Bacteroidetes/ Flavobacteria	*Periplaneta americana* (cockroach)	Nutrient provision (amino acids) and Nitrogen excretion[c]	637	28.2	0	CP001429 CP001430
Consortia	*Buchnera aphidicola BCc*	Proteobacteria/γ-Proteobacteria	*Cinara cedri* (aphid)	Nutrient provision (amino acids, vitamins, fatty acids)	422	20.1	0	CP000263 AY438025
	Sulcia muelleri GWSS	Bacteroidetes/ Flavobacteria	*Homalodisca vitripennis* (sharpshooter)	Nutrient provision (amino acids)	246	22.0	0	CP000770
	Baumannia cicadellinicola Hc	Proteobacteria/γ-Proteobacteria	*Homalodisca vitripennis* (sharpshooter)	Nutrient provision (amino acids and vitamins)	686	33.2	0	CP000238
	Sulcia muelleri SMDSEM	Bacteroidetes/ Flavobacteria	*Dieroprocta semicincta* (cicada)	Nutrient provision (amino acids)	276	22.0	0	CP001605
	Hodgkinia cicadicola[a] Dsem	Proteobacteria/α-Proteobacteria	*Dieroprocta semicincta* (cicada)	Nutrient provision (amino acids and vitamins)	144	58.0	0	CP001226

[a]These bacteria are called *Candidatus*

[b]These genomes contain repetitive DNA, but the relative amounts are not indicated on the bibliography

[c]Although the authors postulate a different interpretation of the metabolic capabilities (Sabree et al. 2009), a detailed metabolic analysis reflects that both *Blattabacterium* strains have the same mutualistic role, as described by López-Sánchez and coworkers for the Bge strain (López-Sánchez et al. 2009)

consequently, critically diminishes the biological fitness of the host, affecting to its growth, fertility or longevity.

The first to notice the link between a restricted diet and the presence of endosymbiotic bacteria in insects was Paul Buchner (1965), who coined the terms of primary (P-) endosymbiont and facultative or secondary (S-) symbiont, based on its morphologic characterization and its presence among the individuals of a certain taxonomic group. This classification has later been validated by means of molecular genetics techniques and the complete sequencing of genomes of an increasing number of endosymbiotic bacteria (Baumann 2005). Buchner classified as P-endosymbionts those bacteria of a unique morphological type that are present in all the insects of a defined taxonomic group, confined inside specialized insect cells located in the abdominal cavity. Such P-endosymbionts are essential for its host fitness and survival. On the other side, the S-symbionts were identified as morphologically diverse bacteria, without a defined spatial distribution in the host body, and whose sporadic presence in some individuals of a defined taxon suggested that they were not essential for host survival. In fact, S-symbionts vary in number and distribution among species and among individuals of the same species, and can live outside of the eukaryotic cells. The congruence between the phylogenetic trees based on host and their corresponding P-endosymbionts sequences, respectively, indicate that each endosymbiont derives from a single infection of the ancestor of the host by the ancestors of its P-endosymbiont, and follows a path of vertical evolution, promoted by their exclusively maternal transmission between insect generations (Munson et al. 1992). On the contrary, the topological incongruence between the phylogenetic trees based on sequences of the S-symbionts and their hosts and the polyphyletic character of such bacteria suggest the existence of multiple events of infection and/or the horizontal transfer of these bacteria among insects (Russell et al. 2003).

Buchner and the early researchers of prokaryote–eukaryote symbioses did not differentiate between the two prokaryotic domains, since the existence of archaea was not recognized until the nineties of the past century (Woese et al. 1990). Therefore, certain early symbioses described as involving “bacteria” were, in fact, involving archaea (Hackstein et al. 2006). This was the case for many methanogenic symbionts hosted by protists that were described in termite and cockroach guts in the 1980s. However, symbioses between arthropods and methanogenic archaea do not seem to have a nutritional foundation. The archaea are always restricted to the hindgut, where they can appear free in the gut lumen, attached to digesta or to the hindgut wall, or as endosymbionts of anaerobic ciliated protozoa that occupy the same gut compartment. Little is known about the function of methanogenic archaea in the guts of arthropods, besides their role in lowering H_2 partial pressure by producing methane, while the archaea uses the hydrogen as a source for methane formation, which indicates that the relationship is mutualistic. Phylogenetic studies have been performed on anaerobic heterotrichous ciliates that keep an endosymbiotic association with methanogenic bacteria (van Hoek et al. 2000). This is an interesting study group because they live in the most divergent

niches, such as marine and freshwater sediments and the intestinal tract of animals. The topology of the phylogenetic trees indicates that the coevolution of host and endosymbiont can only be demonstrated in a few analyzed cases, which most likely means that, although probably hydrogenosome-bearing ciliates acquired methanogenic endosymbionts at the very beginning of their evolution towards anaerobiosis, prior to the anaerobic heterotrichous ciliates radiation, endosymbiont replacements must have accompanied the evolution of these protists. In addition to its role in hydrogen transfer, it has also been proposed that artropode intestinal methanogens can contribute to nitrogen–carbon balance in the hindgut by the fixation of atmospheric nitrogen, since these archaea posses a complete gene repertoire needed for nitrogen fixation (Raymond et al. 2004). Whole genome studies will help to identify other possible benefits of these methanogenic archaea to their hosts.

Even though new data are accumulating on archaeal symbionts of animals, most analyses concentrate on nutritional and physiological aspects. At the beginning of the genomics era, research on prokaryote endosymbionts of eukaryotic cells focused on a limited group of arthropods, mostly sap-sucking insects (Hemiptera: Sternorrhyncha), and those have been for quite a while the main models used to define the evolutionary and molecular aspects of prokaryote-animal symbioses. Therefore, we will focus mostly on bacterial endosymbionts of insects to detangle the molecular aspects of these symbioses from a genomics perspective, paying special attention to the genomic changes experienced by the bacterium in their adaptation to an endosymbiotic lifestyle.

The advent of genomics allowed the complete sequencing of genomes and the development of metagenomic methods, making possible the study of environmental samples and non-cultivable microorganisms, thus offering new opportunities for symbiosis research. The availability of many genomes of bacterial endosymbionts, opens the door to comparative analyses among them, unveiling common molecular aspects regarding the establishment and maintenance of symbiotic associations. In order to completely understand the different stages of genomic evolution of bacterial endosymbionts, it became necessary to analyze and compare genome sequences from endosymbionts in different stages of their symbiotic integration. These comparative analyses allowed researchers in the field to define a plausible scenario for the process of symbiotic integration, from a free-living bacterium to an obligate mutualistic lifestyle (Moya et al. 2009) (Fig. 1). The first step towards the establishment of an obligate intracellular mutualistic symbiosis takes place when a free-living bacterium infects an eukaryotic host. From this point, both organisms will co-evolve to adapt to the new situation. The host develops specialized cells to harbor the bacterium, which in turn provides benefits to the host that end up being essential. From an evolutionary point of view, this new stable situation triggers a cascade of changes that model the shape and content of the bacterial genome. In the course of this chapter, we will see how genomics and metagenomics studies helped researchers on the field to detangle the physiological and evolutionary changes that bacteria experience in their way towards an obligatory mutualistic intracellular symbiosis with eukaryotic hosts.

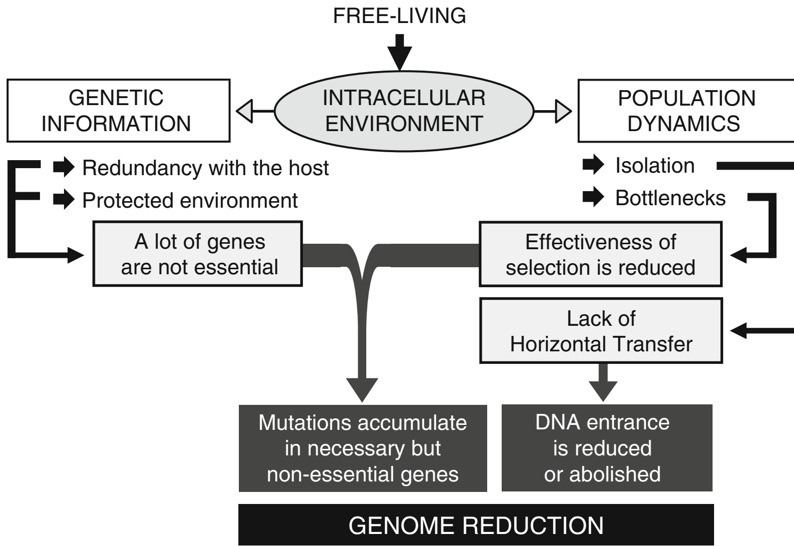

Fig. 1 Genetic and population factors involved in the genome reduction syndrome experienced by mutualistic endosymbionts. At the beginning of endosymbiosis, the new rich, protected and stable intracellular niche provided by the host makes superfluous some gene functions, that become redundant (since they can be contributed by the host) or unnecessary in a stable and protected environment, but forces the preservation of genes required for the maintenance and viability of the partnership. The decreased efficiency of the purifying selection causes a fast accumulation of slightly deleterious mutations on non-essential genes, increasing the rates of genomic evolution. In addition, the drastic reduction of the bacterial effective population size between successive insect generations increases the relative influence of random genetic drift. Furthermore, the obligatory intracellular life-style prevents the entrance of genetic material by horizontal gene transfer, making the losses irreversible

2 Survival, Replication and Transmission, the Three Biological Processes Involved In the Establishment of a Permanent Symbiotic Association

Mutualism and parasitism are two sides of the same coin. At the very beginning, it is not possible to determine if the relationship that would be established will be parasitic or mutualistic, since this distinction is based on the effect of the bacterium in the eukaryotic host but, from the bacterial point of view, the biological processes needed to successfully infect hosts are largely the same for both types of micro-organisms (Gil et al. 2004a). In both cases, it will be necessary to overcome the physical, cellular, and molecular barriers presented by the host, to achieve internalization, survival, and proficient replication of the prokaryote inside the eukaryotic host cell. No matter if the interaction is harmful, neutral, or beneficial to the host,

natural selection will favor the bacteria that achieve this goal (Ochman and Moran 2001). Most evolutionary transitions leading towards symbiotic lifestyles involve gene loss and horizontal gene transfer (HGT) of virulence genes within bacterial lineages. Genomic analyses indicate that, in many cases, the same molecular factors are involved both in pathogenic and mutualistic relationships although, in the case of mutualism, traditionally considered parasitic traits, at some point, became beneficial for both partners. In facultative symbionts, toxins that are known or suspected to target eukaryotic cells are involved in protecting the host against natural enemies (Oliver et al. 2009). Such toxins are encoded by genes present in lysogenic bacteriophages that participate in mutualistic functions but also act as hot spots for non-homologous recombination events that allow gene exchange of virulence cassettes among heritable symbionts (Degnan and Moran 2008). But even endosymbiotic bacteria with a long-time established relationship with their hosts, which have suffered a dramatic genome size reduction (as will be discussed below), maintain genes that encode essential endosymbiotic factors that are proposed to be virulence associated in bacterial pathogens, such as type III secretion systems and urease (Gil et al. 2003; Goebel and Gross 2001; Shigenobu et al. 2000). In many free-living bacteria, genes encoding the type III secretion system are located within pathogenicity islands that have been acquired by HGT. This system is present in many insect endosymbiotic bacteria where it has been proposed to be essential to invade the host cells, thus playing an essential role in the establishment of the symbiosis (Dale et al. 2001, 2002).

The establishment of a permanent intracellular association necessarily implies the development of efficient mechanisms for bacterial survival and replication inside the host cell. The bacteria must adapt their replication, so that their growth rates are coordinated with the development of their hosts in a way that depends on their location inside the host cell. In *Buchnera aphidicola*, which lives confined in vacuole-like organelles inside the aphid bacteriocytes, there is a tight coupling of bacterial cell number and aphid growth, with the bacteria showing a doubling time of approximately 2 days, much longer than the maximum exhibited by many free-living bacteria (Baumann and Baumann 1994). *Blochmannia floridanus* and *Wigglesworthia glossinidia*, which live free in the cytosol of bacteriocytes of their hosts (carpenter ants and tsetse flies, repectively), lack *dnaA*, the gene that encodes the essential DNA replication initiation protein in bacteria. Other alternative mechanisms reported so far for DNA replication initiation are also absent in *B. floridanus*. It has been suggested that this could imply the existence of a more direct control of DNA replication of the symbionts by the host (Gil et al. 2003).

An efficient transmission of the bacteria to the offspring must also be guaranteed. The acquisition of mechanisms ensuring maternal transmission to the host progeny allows the association to be heritable, resulting in the emergence of a new composite organism host-endosymbiont. The fine-tuning of this process detected in long-established obligate mutualistic symbioses suggests a long history of selection favoring host adaptations that help to maintain the association (Moran and Telang 1998).

3 Early Stages In the Symbiotic Relationship

The genomic era has allowed the sequencing of whole genomes of many bacteria living in symbiosis with eukaryotic hosts, allowing the comparison among the different evolutionary innovations carried out by these bacteria on their way from free-living to varied stages of integration with their respective hosts. To detangle the changes involved in each stage, over the next paragraphs we will follow the path from facultative symbiosis to early obligate endosymbiosis, as it has been revealed by molecular studies and comparative genomics over the past years.

3.1 Facultative Symbionts

Many different types of facultative or S-symbionts have been described in arthropodes, and have been extensively studied in several lineages of aphid, psyllids, whiteflies, leafhoppers, tsetse flies, fruit flies and mosquitoes (Table 1). They can be maternally transmitted between host generations but, unlike P-endosymbionts, they can also be horizontally transferred among host individuals and species and, therefore they do not share long evolutionary histories with their hosts. S-symbionts do not reside exclusively in specialized cells and organs, and can also be found in gut tissues, glands or body fluids, and when a P-endosymbiont is also present, they can occupy cells surrounding the P-bacteriocytes, or even invade them. Phylogenetic studies indicate that facultative symbionts have established relatively recent associations with their hosts (Dale and Moran 2006). Thus, their genomes may resemble those in the early stages of a transition from a free-living lifestyle to an obligate mutualism.

Their uneven presence among species and individuals of the same species indicates that S-symbionts are not necessary for host survival, but their influence on host fitness is variable. A range of effects, from negative to beneficial, have been described. Some described S-symbionts have negative effects on growth and reproduction to the host or may establish neutral or parasitic associations. Heritable S-symbionts can spread among lineages by manipulating host reproduction to enhance matrilineal transmission through parthenogenesis, male killing and feminization of genetic males or cytoplasmic incompatibility. This is the case of *Wolbachia* infecting arthropods, where it undergoes transfer among host lineages (McGraw and O'Neill 2004). Remarkably, *Wolbachia* appears as a typical P-endosymbiont in filarial nematodes, where it is required for normal development. The complete genomes of four different *Wolbachia* strains are already available, allowing unraveling the molecular basis of their interaction with their respective hosts by comparative genomics. Three of them are reproductive parasites of arthropods, *Wolbachia pipientis w*Mel strain, found in *Drosophila melanogaster* (Wu et al. 2004); *w*Ri strain, from *Drosophila simulans* (Klasson et al. 2009), and *w*Pip strain, from the mosquitoes of the *Culex pipiens* group (Klasson et al. 2008); the last one, belongs to the *w*Bm strain, the obligate mutualist of the nematode

Brugia malayi (Foster et al. 2005). When the genomes of the parasitic strains were compared, a high degree of rearrangements was observed, revealing the most highly recombining obligate intracellular bacterial community examined to date (Klasson et al. 2009). The presence of abundant copies of transposable elements and prophages, that provide numerous sites for homologous recombination, can explain that. Most of the genome size differences are due to the presence of repeated elements, especially to the amplification of the WO prophage. Furthermore, the WO elements can experience intragenic recombination (Bordenstein and Wernegreen 2004). They present a conserved core of structural genes plus a variable fraction of genes that encode for ankyrin repeats, which correlate with the effects of the bacterial strain as reproductive parasite.

The first completely sequenced genome of a S-symbiont with no clear negative or positive effect corresponded to *Sodalis glossinidius* (Toh et al. 2006), the S-symbiot of the tsetse fly. It has been proposed to play a role in the acquisition of trypanosome infections (Welburn and Maudlin 1999). Its genome size (4.2 Mb) is close to that of free-living bacteria, but its coding capacity is highly diminished by the presence of a big amount of pseudogenes, only similar to what has been observed in some parasites such as *Mycobacterium leprae* (Cole et al. 2001; Gomez-Valero et al. 2007). The genome also contains certain amounts of repetitive and mobile DNA, such as transposable elements and bacteriophages, which could promote recombination. Therefore, it appears that this bacterium is at the early stages in the reductive process affecting symbiont genomes. *S. glossinidius* coexists in the gut lumen of tsetse flies with the P-endosymbiont, *W. glossinidia*, but occupying different portions of the insect gut, and it can be found both intra- and extracellularly (Toh et al. 2006). Moreover, it can be cultured in vitro (Dale and Maudlin 1999), an indication that the association with its host is not yet irreversible.

Many other S-symbionts described in aphids confer beneficial effects on the survival and reproduction rates of their hosts. They can rescue the host from heat damage (Chen et al. 2000; Montllor et al. 2002), provide resistance to natural enemies (Ferrari et al. 2004; Guay et al. 2009; Oliver et al. 2005; Scarborough et al. 2005) or stress (Russell and Moran 2006), are involved in host plant specialization and reproduction (Simon et al. 2003; Ferrari et al. 2004; Tsuchida et al. 2004), and even compensate the loss of the essential endosymbiont, as it was experimentally proven (Koga et al. 2003). Recently, the genome of one strain of *Candidatus* Hamiltonella defensa (from now on *H. defensa*), S-symbiont of the pea aphid *Acyrtosiphon pisum*, also became available (Degnan et al. 2009b). *H. defensa* can be found in aphids and other sap-feeding insects, where it has been proposed to play a beneficial role by protecting its host from attack by parasitoid wasps. Genes that encode for toxins, effector proteins, and two type-III secretion systems have been identified in the sequenced genome and seem to be involved in this function. The 2.1-Mb sequenced genome has undergone significant reduction in size relative to its closest free-living relatives, and important gene losses have been detected (it relies on the the P-endosymbiont *B. aphidicola* for the synthesis of 8 of the 10 essential amino acids), which indicates that the reductive process affecting endosymbiont genomes is already advanced. Nevertheless, the genome contains

considerable amounts of genes devoted to regulatory functions involved in regulation of virulence factors and quorum-sensing genes, which indicates that it still retains at least a partial ability to deal with changing environments and invasion of new host species. This genome also contains important amounts of repetitive DNA (21% of the genome), including insertion sequences, group II introns, prophages and plasmids. APSE, a lysogenic phage that infects many *H. defensa* populations, has been involved in the protective role of this bacterium against parasitic wasps, since the different variants of APSE identified all encode toxins that target eukaryotic tissues (Oliver et al. 2009). Therefore, the beneficial role of the phage toxins for the insect host fitness is contributing to the spread and maintenance of *H. defensa* in host populations. This is another evidence of the direct implication of virulence factors on the basis of a mutualistic symbiosis. Furthermore, the APSE lysis region is a hot spot for non-homologous recombination of novel virulence cassettes, allowing gene exchange among S-symbionts by horizontal transmission (Degnan and Moran 2008).

Candidatus Regiella insecticola (from now, *R. insecticola*) is another common facultative symbiont in aphids. Similar to *H. defensa*, it is not only involved in resistance to parasitoid wasps but also to fungal pathogens (Scarborough et al. 2005). Most of its genome (about 2.07 Mb) has been sequenced and compared with the close relative *H. defensa* (Degnan et al. 2009a). The complete genome assembly was not performed, because it was hampered by the presence of high amounts of repetitive DNA, mostly insertions sequence (IS) elements, representing up to 14% of the genes and pseudogenes. Similar to what has been found in the parasitic *W. pipientis* strains, the genomic architecture of these two genomes is highly divergent, as a consequence of recombination and gene inactivation facilitated by the presence of mobile DNA. In contrast, core genes reveal clonal evolution in *H. defensa* and *R. insecticola*, and the nucleotide divergence in this case is similar to what has been found in obligate mutualists. No intact prophages have been found in the already sequenced part of the genome of *R. insecticola*.

The genomes of two *Serratia symbiotica* strains, from the cedar and tuja aphids, are also being sequenced. Although some strains of *S. symbiotica* appear as typical facultative symbionts, this is not the case of the SCc strain, which has become essential for its host, the cedar aphid *Cinara cedri* (Gosalbes et al. 2008). Preliminary results of its genome project indicate that it has established a permanent and stable cooperative consortium with the host and the P-endosymbiont, *B. aphidicola* BCc, thus becoming essential for the maintenance of the fitness of all three partners (see Sect. 6).

3.2 Insertion Sequences, Shaping the First Steps Towards an Obligate Endosymbiosis

It has been postulated that soon after the establishment of obligate symbiosis, a massive gene loss must occur, probably by means of large deletion events that cause

the elimination of series of contiguous genes (Moran and Mira 2001). Later on, as it has been shown by comparative genomics, genome shrinkage proceeds through a process of gradual pseudogenization and gene loss scattered throughout the genome (Gomez-Valero et al. 2004; Silva et al. 2001). However, the mechanism involved in the large deletion events was unknown at that time. The identification of the genome changes that occur in these initial stages of the adaptation towards endosymbiosis requires the genome analysis of clades of bacteria that have recently established such associations. For this purpose, our group selected SOPE, the P-endosymbiont of the rice weevil *Sitophilus oryzae*. With an estimated 3.0-Mb genome (Charles et al. 1997), within the range of many free-living bacteria, this γ-proteobacterium maintains a typical obligate mutualistic endosymbiosis with its host. The bacteria live inside bacteriocytes organized in an organ called bacteriome surrounding the midgut of the insect and near the female ovaries. The bacterium cannot be cultured outside the host, and it provides at least amino acids and vitamins to the insect, which has recognizable effects on fertility, development and the flying ability of adult insects (Heddi et al. 1999). SOPE is closely related with *S. glossinidius* (Dale and Welburn 2001; Heddi et al. 1998), which is still able to grow in laboratory culture conditions. Although SOPE and *S. glossinidius* are respectively P- and S-symbionts of hosts belonging to different insect orders (Coleoptera and Diptera), which feed on very different diets (storage grain and blood, respectively), their close phylogenetical position indicates a relative recent divergence. Therefore, the analysis of the similarities and differences between these two genomes will help to achieve a better understanding of the differences between the primary and secondary forms of endosymbiosis and what molecular events are implied in the establishment of an obligatory endosymbiosis.

The association of insects of the genus *Sitophilus* and their present endosymbionts is not antique. Some data indicate a recent endosymbiont replacement of an ancestral endosymbiont in the family Dryophtoridae to which the rice and maize weevils belong (Lefevre et al. 2004). During the first stages of the SOPE genome sequencing project (in progress), big amounts of repetitive DNA, mainly IS elements, were identified (Gil et al. 2008). It has been estimated that IS elements occupy about one third of its genome, and a similar situation has been observed in its close relative SZPE, the P-endosymbiont of the maize weevil (Plague et al. 2008). This impressive amount of repetitive DNA was not expected in an obligate mutualistic endosymbiont. Repetitive DNA is common in free-living bacteria, and its presence increases in bacteria that have recently evolved as specialized pathogens (e.g., the enteric bacteria *Shigella* and *Salmonella enterica* Typhi) (Jin et al. 2002; Wei et al. 2003), intracellular parasites (e.g. *W. pipientis* strains, reproductive parasite of arthropods) (Klasson et al. 2008, 2009; Wu et al. 2004), or facultative insect symbionts (e.g. *H. defensa*, *Candidatus* Arsenophonus arthropodicus and *R. insecticola*) (Dale and Moran 2006; Degnan et al. 2009a,b). Thus, the increase in transposable elements is a common trait among bacteria that have recently established mutualistic relationships with their hosts, and must have subsequent effects on the outcome of the symbiotic process (Bordenstein and Reznikoff 2005; Moran and Plague 2004). However, it was assumed that after the establishment of an

obligate endosymbiont lifestyle, repetitive DNA tends to diminish until its total disappearance. Several observations support this conclusion, from total absence of phages or transposable elements in bacterial endosymbionts with a long-established obligatory relationship with their hosts, to the identification of only 5.4% of repetitive DNA, mostly composed of inactivated IS, in the mutualistic *W. pipientis* *w*Bm (Foster et al. 2005).

The IS are the most abundant and simplest transposable elements in nature (Touchon and Rocha 2007). Habitually they only include the elements needed in its own mobilization: short terminal inverted repetitive sequences (IR) define the ends of the IS and flank the ORF(s) that encodes the transposase activity that mediates the transposition events after the recognition and processing of the IR sequences. The IS are able to move between replicons of a certain genome and can also be transferred between genomes of different organisms by horizontal gene transfer. Its persistence is usually explained by an intense ability for intergenomic mobilization and to its more or less efficient infecting capacity. Four IS types have been identified in SOPE (Gil et al. 2008). At least two of them (IS*sope*1 and IS*sope*2) are present in large copy numbers in SZPE (Plague et al. 2008), and IS*sope*1 has also been identified in *S. glossinidius*, but representing just 2.5% of the total genome (Toh et al. 2006), is an indication that this element must have been present in a common ancestor of these bacteria.

The massive presence of IS must be related with some of the syndromes that appear at the beginning of the intracellular life (Fig. 2). IS elements are widespread in free-living bacteria, but their transposition is tightly controlled, so that only a few copies of a limited number of categories appear in each genome. The dramatic increase of these elements in intracellular bacteria must reflect an enhanced replicative transposition of elements that were already present at the onset of symbiosis, and can then act as a source of gene inactivation and chromosomal rearrangements. After the establishment of the symbiosis, the decrease in the selective pressure caused by functional conditions and population dynamics in the new environment, can favor the uncontrolled proliferation of such elements, which could be involved in the inactivation of non-essential genes. The high abundance of very similar (or even identical) repetitive elements in direct orientation can then serve as a substrate for unequal recombination, which would lead to a loss of the region between two elements, thus promoting genome size reduction in early stages. Additionally, the presence of these elements in opposite orientation, will lead to genome rearrangements. Comparative genomics analyses between several *B. aphidicola* strains form different aphids, *B. floridanus*, and close free-living relatives indicate that the massive gene loss that took place in the process towards the last common symbiotic ancestor (LCSA) of both species was accompanied by many chromosomal rearrangements. The former presence of repetitive elements, already disappeared in the present genomes, might explain such genome reorganizations, while the current lack of repetitive sequences, with a great potential as recombination sites, as well as the loss of *loci* needed for the catalysis of such recombination events in later stages of the symbiosis (see next section), appears to be in the origin of the high genomic-architecture stability levels in old endosymbionts, quite unusual among the

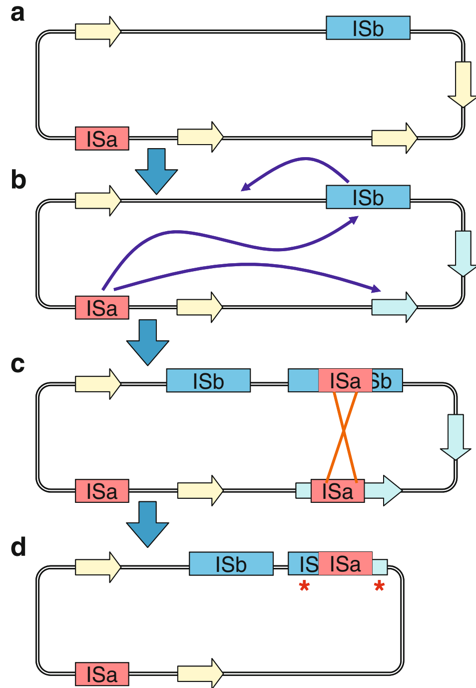

Fig. 2 An evolutionary scenario for the implication of IS in gene inactivation, genome reduction and chromosomal rearrangements. (**a**) Free living cell. (**b**) Beginning endosymbiosis: Many genes become superfluous or redundant. Massive transposition. (**c**) IS can be a source of genomic recombination. (**d**) Interrupted genes and IS degenerate by mutation

prokaryotes (Silva et al. 2003). This is an indication that most of genomic modeling, including chromosomal rearrangements and the loss of many functionally dispensable genes, must take place at an early stage of the process of genomic adaptation to intracellular life (Dougherty and Plague 2008; Touchon and Rocha 2007). Genes needed for DNA repair and recombination are also among the first losses detected, thus contributing to genomic stasis in further steps in the endosymbiotic evolutionary path. The loss of the genes coding for the enzymes RecA and RecF in SOPE, SZPE, and *S. glossinidius* (Dale et al. 2003) supports this idea.

4 Long-Established P-Endosymbioses

Most bacterial insect P-endosymbionts that have been analyzed belong to the γ-proteobacteria (Table 1). However, more recently, the genomes of several endosymbionts belonging to other groups of proteobacteria and to the phylum Bacteroidetes have also been analyzed (McCutcheon and Moran 2007; Lopez-Sanchez et al. 2008; Tokuda et al. 2008; Sabree et al. 2009), revealing convergent evolution among endosymbionts belonging to different phyla (López-Sánchez et al. 2009). In general, endosymbionts with a long-established relationship with their hosts have

genomes eight to ten times smaller than those of their free-living relatives. In bacteria, whose genomes are highly compact, gene content correlates quite well with genome size (Casjens 1998). Therefore, the reduced size of endosymbiont genomes reflects the presence of a smaller number of genes than those of free-living bacteria. Several additional characteristic genome features have traditionally been associated with the degenerative syndrome affecting endosymbiotic bacteria. These include almost total absence of recombination, increased rate of nucleotide substitution, high A + T content (although as it will be discussed later, this no longer can be considered a general trait), accumulation of deleterious mutations by random genetic drift, loss of codon bias towards A or T, and accelerated sequence evolution (Andersson and Kurland 1998; Clark et al. 1999; Moya et al. 2002; Wernegreen 2005). Most of these characteristics are linked with the above mentioned informational and demographic factors affecting bacteria that live in close association with eukaryotic cells, although the accommodation to symbiotic life varies according to the age of the association, the host lifestyle, and the way of living within the host.

The analysis of gene order in the first completely sequenced endosymbiont genomes lead to interesting observations regarding the evolution of these genomes. The availability of complete genome sequences from four different strains of *B. aphidicola* clonally evolving in their aphid hosts revealed that, after a short period of large genome rearrangements at the beginning of the symbiotic process, there were large periods of evolutionary stasis. All these strains present a nearly perfect gene-order conservation (Perez-Brocal et al. 2006; Shigenobu et al. 2000; Tamas et al. 2002; van Ham et al. 2003), which suggests that *B. aphidicola* can be considered as a "gene-order fossil", and that the onset of genomic stasis coincided with the establishment of the obligate symbiosis with aphids, 80–150 MY ago (von Dohlen and Moran 2000). As mentioned in the previous section, this astonishing genome stasis can be explained by the total absence of repetitive DNA in these genomes, as well as the loss of genes involved in DNA repair and recombination in early stages of the symbiotic integration. Repetitive DNA is quite abundant at the beginning of the obligate endosymbiosis, but these elements tend to disappear in the later stages of the relationship and are absolutely absent in endosymbionts that share long evolutionary histories with their hosts (Fig. 2). The progressive loss of transposable elements might have been favored by the energetic benefit of decreasing transposase activity and avoiding the increase in genome size derived from the proliferation of these elements or by the need to control the mutagenizing effect of its mobilization. It is presumable that, at some point, IS elements expansion will be deleterious and these elements would be also affected by the process of genome degradation that these genomes suffer. The sexual isolation of P-endosymbionts and the loss of recombination genes must also have participated in the process, since horizontal gene transfer is the way of entrance of these elements in prokaryotic genomes (Touchon and Rocha 2007). The reduced genomes of endosymbiotic bacteria and some pathogens have lost most (if not all) genes involved in recombination processes and, consequently, the genome size cannot be increased by acquisition of foreign DNA (Silva et al. 2003). Nevertheless, some recombination events can still take place in these reduced genomes, probably involving the RecBCD

system, which in the absence of RecA might serve as a general exonuclease repair enzyme (Sabater-Munoz et al. 2004), as revealed by the great plasticity of the plasmids involved in the biosynthesis of leucine in different lineages of *B. aphidicola*, showing that several events of insertion from a plasmid to the main chromosome have occurred since the divergence of these strains (Latorre et al. 2005).

In general, smaller genomes correlate with longer obligate associations. The differences in host lifestyle are also introducing changes in this degenerative process among strains of the same endosymbiont species. The small genomes of *B. aphidicola* are still suffering this reductive process, as evidenced by the fact that *B. aphidicola* strains from several aphid subfamilies showed differences up to 200 Kb (Gil et al. 2002), and the presence of pseudogenes in the *B. aphidicola* genomes that have been sequenced (Perez-Brocal et al. 2006; Shigenobu et al. 2000; Tamas et al. 2002; van Ham et al. 2003). In addition, the degenerative process is randomly affecting different genes in each genome, conditioning the essentiality of the rest of the genes that are present in these reduced genomes. Therefore, although we can hypothesize that the LCSA of *B. aphidicola* suffered a drastic genome reduction at the beginning of the symbiotic integration, since then, the different strains of the bacteria have undergone a reductive process in a way that correlates with their hosts.

In addition to changes in genome size, obligate and facultative endosymbionts of different insect hosts also differ in nucleotide composition. P-endosymbionts with an old association with their hosts have in general small genomes, and an A + T content higher than 70%, while P-endosymbionts with a younger association and S-symbionts have a genome size and an A + T percentage intermediate with respect to older P-endosymbionts and free-living relatives (Dale and Maudlin 1999; Heddi et al. 1998; McCutcheon and Moran 2007; Moya et al. 2002; Nakabachi et al. 2006). The loss of the bias in codon usage in these obligate intracellular bacteria, highly mitigated in P-endosymbionts with larger genomes and in S-symbionts and almost absent in *B. aphidicola*, is considered to be a consequence of this base composition bias (Moya et al. 2002; Rispe et al. 2004). This notable enrichment in A + T has been related to the loss of DNA repair enzymes, since the most common chemical changes in DNA (cytosine deamination and guanosine oxidation) led to changes in GC pairs leading to AT. However, several cases that do not follow this nucleotide composition rule have been described. The partial genome sequences available from *Candidatus* Tremblaya princeps, the P-endosymbiont of the mealybug *Planococcus citri*, indicated that this genome has a 57% G + C content, much higher than expected for an endosymbiont (Baumann et al. 2002). Recently, a remarkable small genome with a high G + C content has also been reported (McCutcheon et al. 2009) (see next section). *Candidatus* Hogkinia cicadicola (from now on *H. cicadicola*), P-endosymbiont of the cicada *Dieroprocta semicincta*, presents a 144-Kb genome with a 58.4% G + C content. Therefore, it has been proposed that, while gene loss associated with genome reduction is a critical step in endosymbiont genome evolution, mutational pressure favoring A + T is not.

There is only one case of advanced symbiosis described in archaea: *Nanoarchaeum equitans*, a tiny coccus living attached to the outside of the cells of its host, the

Crenarcheote *Ignicoccus hospitalis*. The study of this association, including the sequencing of the genome of both species (Waters et al. 2003; Podar et al. 2008), shows a highly specialized relationship, which so far cannot be assigned to any classical symbiosis type (mutualism, commensalism or parasitism). With a highly reduced genome (491 Kb), *N. equitans* was initially suggested to be a representative of a novel phylum within the domain archaea, Nanoarchaoeta. However, further genomic analyses indicate that it is likely to be a highly derived Euryarchaeon, possibly related to the Thermococcales that has evolved through a unique pathway of genome degradation (Brochier et al. 2005; Makarova and Koonin 2005). Features such as the extreme *N. equitans* genome reduction, bias in codon usage, and evolutionary acceleration, are common to those observed in bacterial endosymbionts, probably an indication of the generality of the reductive mechanisms among prokaryotes. Interestingly, this reductive process has affected simultaneously both genomes, since the *I. hospitalis* genome is only 1.3 Mb in length, one of the smallest among free-living organisms. Further analyses will be necessary to understand the implication of this dual reductive genome process (Forterre et al. 2009).

5 Final Stages in Endosymbiotic Relationships

As the endosymbiotic integration progresses, genes that are rendered unnecessary experience a random process of gradual pseudogenization and gene loss scattered throughout the genome (Gomez-Valero et al. 2004; Silva et al. 2001). The final step of this minimization process might, in theory, lead to the loss of all genes except those that are essential for keeping the host-bacterial interaction reproducing. Therefore, even the most reduced genome must retain those genes involved in the symbiotic relationship, as well as a reduced repertoire of genes necessary to maintain the three essential functions that define a living cell: maintenance, reproduction and evolution (Luisi et al. 2002). One of the most comprehensive efforts to define the minimal core of essential genes was that presented by Gil et al. (2004b). This study can be a good starting point to identify essential genes involved in informational processes that must be present in any living cell, while the essential genes devoted to the symbiotic association can be deduced by the knowledge of the host needs for survival and reproduction. However, most extremely reduced genomes that have been described have lost part of such essential functions. In most cases, as it will be discussed in the next section, genome degradation can proceed over the expected limit because of the implication of a second endosymbiont on the relationship. But there is an intriguing case: *Candidatus* Carsonella ruddii (from now on, *C. ruddii*), considered the P-endosymbiont of the psylid *Pachpsylla venusta*. Although a second bacterial symbiont has not been found in the psyllid, *C. ruddii* does not fulfil the conditions to be considered as a mutualistic endosymbiont, not even as a living organism. Its genome consists of a circular chromosome of 160 Kb, averaging 83.5% A + T content (Nakabachi et al. 2006). It also presents a high coding density (97%), and 182 described open reading frames, many of

which overlap and present a reduced gene length. A detailed analysis of the coding capacities of *C. ruddii*, revealed that the extensive degradation of the genome is affecting vital and symbiotic functions (Tamames et al. 2007). Most genes for DNA replication, transcription and translation are completely absent, and gene shortening causes, in some cases, the loss of essential domains and functional residues needed to fulfil these and other vital functions. In addition to the essential functions that define life, as a mutualistic endosymbiont, *C. ruddii* should provide its host all essential complements to its nutritionally deficient diet, limited to phloem sap, rich in sugars but relatively poor in nitrogenated compounds, especially essential amino acids. However, the genomic analysis revealed that the pathways for the synthesis of three essential amino acids (i.e. histidine, phenylalanine and tryptophan) are lost. Since this strain of *C. ruddii* is not able to sustain the requirements of its host, neither to sustain its own vital functions, it can be viewed as a further step towards the degeneration of the former P-endosymbiont, and its transformation in a subcellular new entity between living cells and organelles, which might be taking advantage of mitochondrial functions encoded by the nucleus, especially for basic informational processes needed for maintenance and multiplication. It might even be possible that some *C. ruddii* genes have been transferred to the host nuclear DNA, as it has been proved for present organelles. If confirmed, this would be the first example of such a scenario in animal cells.

6 Replacement or Complementation, and the Establishment of Microbial Consortia

Eventually, after the establishment of a permanent symbiotic association between a bacterium and an animal host, a second bacterial species can join the association. Although initially this new association can be facultative (as seen in Sect. 3.1), if the second bacterium provides benefits to the organization, with time, it can become essential for host fitness. The involvement of two bacteria in the fitness of an insect host adds one extra element to the evolutionary scenario that explains the reductive evolution of endosymbiont genomes, but there is no need to invoke any supplementary reductive factor in addition to the informational and population dynamis factors already indicated. Subsequently, all three components of the association will co-evolve, and the evolutionary process of genome shrinkage will now affect both bacteria. New genes will become unnecessary due to redundancy, but which one of the two bacterial genomes loses them will be a matter of chance. Depending on which genome is affected by the loss of genes needed for the synthesis of essential molecules, either both bacteria will become indispensable to keep a healthy consortium (complementation) or one of them can enter an extreme degenerative process, which may end with its extinction (replacement), and the retained bacteria will continue the degenerative process alone (Moya et al. 2009). Replacement has already been reported, for example, in the Family Dryophthoridae, where a former

endosymbiont *Candidatus* Nardonella was replaced by the ancestor of the *Sitophilus* P-endosymbionts (Lefevre et al. 2004). However, there are many more described cases in which both bacteria loose part of the gene complement necessary for their host fitness, so that both of them become indispensable and a stable consortium is established. Several symbiotic consortia have already been reported and sequenced, using metagenomics approaches, and many more will surely be available in the near future thanks to the use of new massive sequencing technologies.

One of the first described consortia involves strains of *B. aphidicola* and *S. symbiotica* living inside the cedar aphid. *S. symbiotica* appears as a facultative symbiont in many aphid species. However, it was always found in cedar aphids, coexisting with *B. aphidicola* BCc in the insect bacteriome, so that *S. symbiotica* strain SCc cannot be considered as a facultative symbiont. Comparative, functional and evolutionary genomic analysis, plus microscopic observations, led Perez-Brocal et al. (2006) to conclude that *S. symbiotica* SCc might be replacing *B. aphidicola* BCc. Contrary to other sequenced *B. aphidicola* strains, BCc has partially lost its symbiotic role, as it cannot synthesize tryptophan. Genes involved in the biosynthesis of this essential molecule were found in the genome of *S. symbiotica* SCc (Gosalbes et al. 2008), but included an additional surprise: the pathway to synthesize tryptophan is distributed between both genomes: *B. aphidicola* BCc produces a metabolic intermediate that is then provided to *S. symbiotica* SCc to synthesize the final product. Therefore, coexistence of both bacteria is needed to keep a healthy consortium due to metabolic complementation, and both of them keep an intracellular obligatory mutualistic association with their host.

The establishment of an endosymbiotic bacterium consortium can be in the origin of big evolutionary changes in host lifestyle. This is the case of the consortium formed by *Baumannia cicadellinicola* and *Sulcia muelleri*, co-resident P-endosymbionts of the xylem-feeding sharpshooter *Homalodisca vitripennis*. Their whole genome analysis revealed that they have complementary sets of biosynthetic capabilities needed to provide to their host the nutrients that are lacking in the xylem sap (Wu et al. 2006). While *B. cicadellinicola* contains a large number of pathways for biosynthesis of vitamins, *S. muelleri* encodes the enzymes involved in the biosynthesis of most essential amino acids. Phylogenetic studies indicate that *S. muelleri* was ancestrally present in a host lineage that acquired *B. cicadellinicola* at the same approximate time that the host ancestor switched to a xylem-feeding lifestyle, consistent with the view that *Baumannia's* nutrient-provisioning capabilities were a requirement for the acquisition of this new feeding behavior.

A newly described consortium also involving *S. muelleri* is on the basis of the dramatic genome reduction experienced by *H. cicadicola*, the P-endosymbiont of the cicada *D. semicincta* (McCutcheon et al. 2009). *H. cicadicola* is an α-proteobacteria with the smallest described genome to date (144 Kb), an unusually high G + C content (58.4%), and a coding reassignement of UGA stop codon to Trp. It has been found in other cidadas, thus suggesting that this symbiont infected an ancestor of the cidadas and, since then, has been maternally transmitted.

Our group is also involved in the metagenomic study of another exceptional symbiotic consortium: the one established among the mealybug *P. citri* and their

two endosymbiotic bacteria: the P-endosymbiont *T. princeps*, a β-proteobacterium (Thao et al. 2002), which contains inside a γ-proteobacterium (von Dohlen et al. 2001), considered as an S-symbiont based on its polyphyletic origin (Thao et al. 2002). This is the first described case of a double-endosymbiosis, although the symbiotic relationship between the two bacteria (parasitic, commensal or mutualistic) has not been elucidated (Kono et al. 2008). As it has been mentioned in Sect. 4, *T. princeps* was the first endosimbiotic genome in which it was detected that the A + T bias rule does not apply (Baumann et al. 2002).

Some other consortia can involve more than two microorganisms. The marine oligochaete *Olavius algarvensis*, which lacks a digestive and excretory system, harbors four co-occurring symbionts essential for host survival (Woyke et al. 2006). The symbionts are located just below the worm cuticle, and they are essential to manage energy and waste needs of the host. The symbionts, γ1- and γ3-(sulphur-oxidizing chemolithoautotrophs), and δ1- and δ4-(sulphate reducer) proteobacteria, are engaged in an endosymbiotic sulphur cycle, fix $CO_{2,}$ provide nutrients to the host, and are also involved in host waste recycling. They can heterotrophycally feed the host by taking up dissolved organic carbon compounds from the environment, and can synthesize almost all amino acids and several vitamins. The host probably takes these nutrients by digesting the bacteria (Fiala-Médioni et al. 1994). This is another case in which, contrary to most cases of obligate host-associated bacteria, the available genomic sequences do not show A + T bias.

7 Concluding Remarks

Symbiosis between prokaryotes and eukaryotes is an expanding field, thanks to the advent of the metagenomics and high-throughput sequencing technologies. Systems biology approaches are also allowing the exploration of metabolic interdependences among the members of the symbiotic consortium. Now that endosymbiont genomes are accumulating, comparative analyses allow making predictions on the evolutionary paths followed by endosymbiotic bacteria in their adaptation to the intracellular environment provided by the host. Now, more clearly than ever before, the association and functional interaction of genomes from different species observed during symbiosis can be viewed as a power, like mutation, recombination and other genome rearrangements, able to generate genetic variation, acting as a fuel for evolution. The action of forces such as natural selection and/or random drift will be the responsible of transforming this variation in evolutionary novelties. However, as the number of the available genomes increases, new features are appearing and open new questions that need to be experimentally solved. We do not know what drives symbiotic associations to mutualism versus parasitism, since both types of associations derive from common mechanisms for symbiont-host interaction. We cannot anticipate when a facultative association will become essential for host fitness and, when two or more prokariotes are involved, we cannot determine which forces will lead it towards the establishment of a consortium or,

alternatively, will end up in a replacement. More recently, an additional question was opened about the nucleotide content bias, most of the times towards an increase of A + T, but also possible towards an increase in G + C content... For sure, we will learn a lot more about the molecular mechanisms and evolutionary forces acting on these systems once eukaryotic host genomes become available.

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