Anaerobic Ciliates and Their Methanogenic Endosymbionts

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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 epi-symbiotic 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 endosym-bionts 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 methano-gens, 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 hydrogeno-somes (Embley and Finlay 1994; Embley et al. 1995; Fenchel and Finlay 1995).
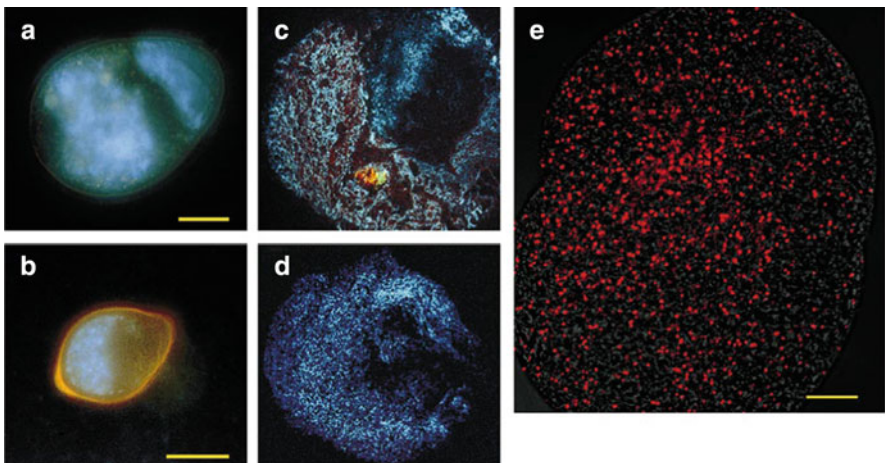

Fig. 1 Endosymbiotic methanogens of *Nyctotherus ovalis*. (**a–d**) F_{420} autofluorescence. (**e**) In situ hybridization. (**a**) *Nyctotherus ovalis* from *Blaberus* sp. var. Amsterdam. (**b**) Cyst of *N. ovalis* from the same isolate. (**c**) Squash preparation of *N. ovalis* from *Blaberus* sp. 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
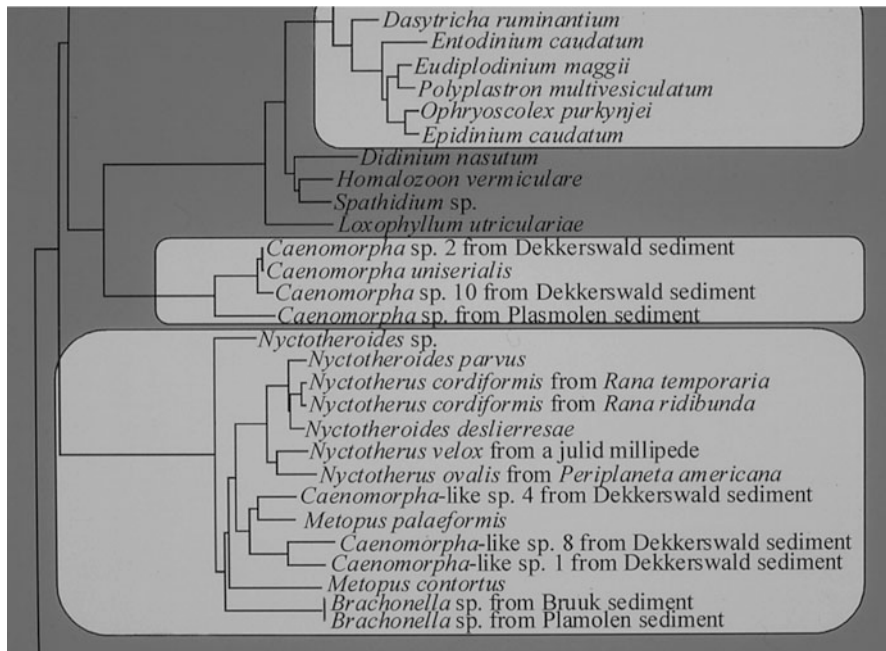
Fig. 2 Detail of a phylogenetic tree (Molphy Star Decomposition, Adachi and Hasegawa 1996) demonstrating the evolution of the relevant ciliate taxa on the basis of their 18S rDNA. *Boxed* species are anaerobes and possess hydrogenosomes. The *unboxed* species are aerobes with mitochondria. The *Nyctotherus/Nyctotheroides* 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
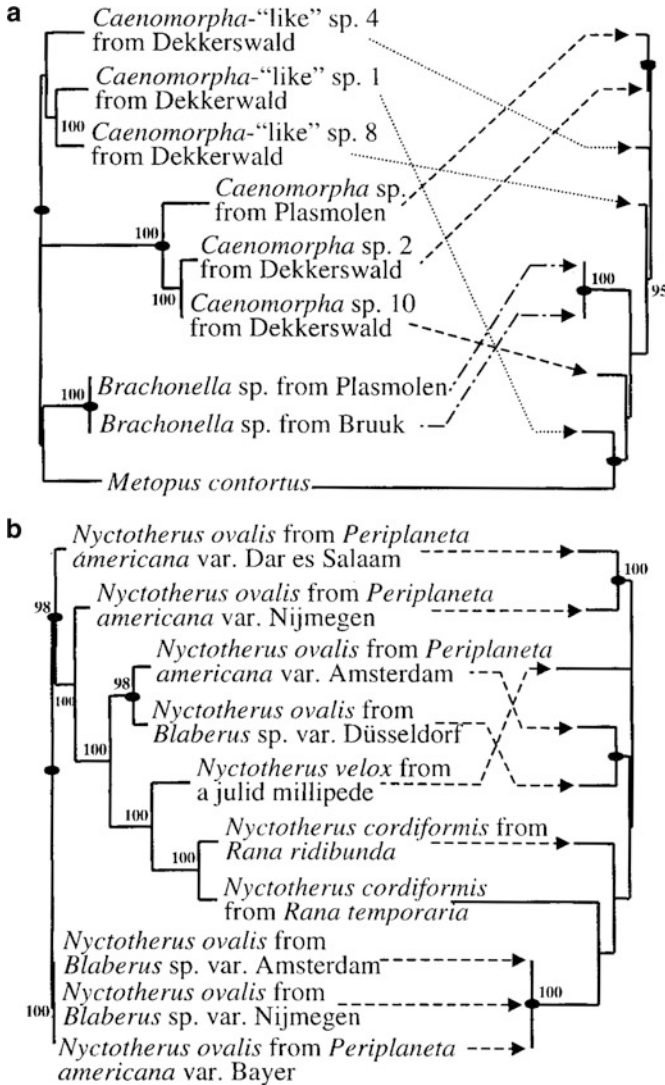

Fig. 4 TreeMap trees of hosts and symbionts (Page 1995) based on 460 positions of the 18S rDNA sequences of the ciliate hosts and 770 positions of the 16S rDNA sequences of the methanogenic endosymbionts. (**a**) Freshwater ciliates (*left tree*) and their methanogenic endosymbionts (*right tree*). (**b**) 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
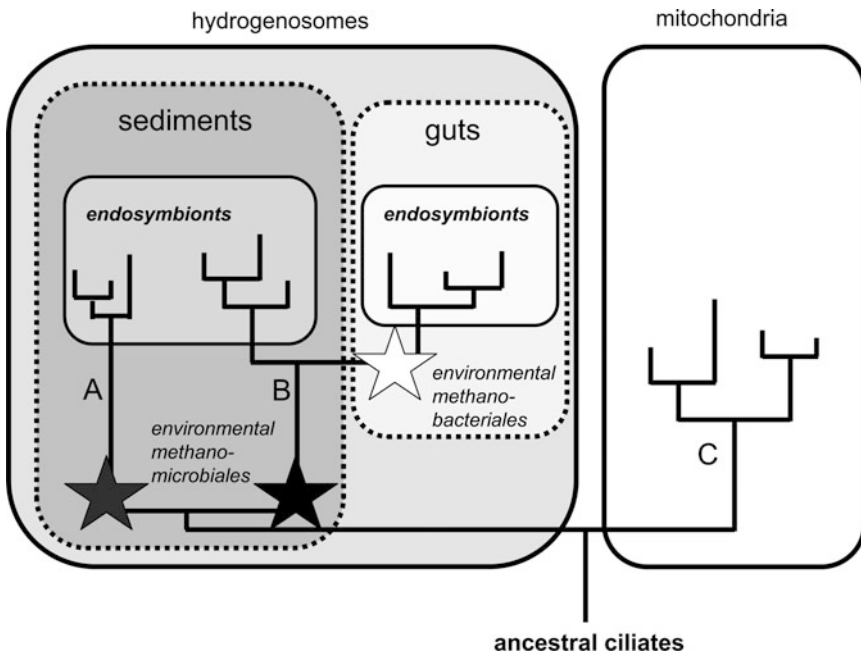

Fig. 5 Cartoon summarizing the evolution of anaerobic heterotrichous ciliates [(**a**) Caenomorphidae, (**b**) Armophoridae and Clevelandellids] and their endosymbiotic methanogens. Ancestral ciliates diverged into aerobic, mitochondria-bearing ciliates [most likely the Stichotrichs (**c**)] and anaerobic, 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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