The use of small subunit rRNA sequences to unravel the relationships between anaerobic ciliates and their methanogen endosymbionts

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Anaerobic habitats and distribution of the anaerobic phenotype among eukaryotes

Anaerobic environments are widespread and common: they include marine and freshwater sediments, waterlogged soils, sewage and the gastro-intestinal tracts of animals. The interior of organic aggregates with diameters greater than about 1 mm may also be anaerobic if diffusion is the only means by which O₂ can penetrate (Fenchel & Finlay, 1991a). Anaerobic environments often contain large numbers of prokaryotes and these in turn support a variety of anaerobic protozoa which feed on them (Fenchel & Finlay, 1990). Protozoa which live exclusively in anaerobic environments and which lack the enzymes necessary for oxidative phosphorylation can be divided into two groups. The first includes protozoa which are probably 'primitively' anaerobic, principally the trichomonads, diplomonads and microsporidia; these taxa form the core of the phylum Archezoa (Cavalier-Smith, 1987). On the basis of their small subunit (SSU) ribosomal (r)RNA sequences, the few Archezoa sequenced so far form the basal lineages in the eukaryotic tree (Vossbrinck et al., 1987; Sogin et al., 1989; Chakrabarti et al., 1992; Van Keulen et al., 1993; Leipe et al., 1993). All Archezoa lack mitochondria and *Varionomorpha neutrix* has prokaryote-sized (70S) ribosomes (Vossbrinck et al., 1987). The Archezoa are living 'relics' of the earliest phase of anaerobic eukaryotic evolution (Cavalier-Smith, 1987) which occurred when free oxygen was scarce in the atmosphere.

The production of an oxygen-rich atmosphere by oxygenic photosynthetic prokaryotes around 2 billion years ago (Schopf, 1992) is one of the pivotal events in the history of the Earth. It allowed the development of high-energy-yielding pathways in bacteria based on the use of O₂ as terminal electron acceptor. In aerobic eukaryotes the final stages of oxidative phosphorylation are carried out in mitochondria which are themselves the descendants of endosymbiotic alpha-proteobacteria which possessed this ability (Yang et al., 1985; Cedergren et al., 1988; Gray et al., 1989). The earliest branching eukaryotes which clearly contain mitochondria and from which rRNA sequences have been analysed are members of the lineage containing *Euglena* and *Trypanosoma* (Euglenozoa; Sogin, 1991; Gray, 1992). If one invokes a monophyletic origin for all mitochondria [but see Gray (1992) for a detailed discussion of evidence for and against this hypothesis] then anaerobic eukaryotes which branch off from the eukaryotic tree later than the Euglenozoa, and which lack mitochondria, are probably secondarily adapted for the colonization of anaerobic habitats: these are the second group of anaerobic protozoa. Anaerobic habitats contain a diversity of anaerobic flagellates and amoebae (Fenchel & Finlay, 1990; Milnikov, 1991) many of which are probably primitively anaerobic. Eukaryotes which lack mitochondria and have clearly diverged (see Sogin, 1991) after the Euglenozoa include rumen chytrid fungi and ciliates, and a small number of free-living ciliates (Fenchel et al., 1977; Müller, 1988; Fenchel & Finlay, 1991a). The biology and ecology of free-living anaerobic ciliates have recently been reviewed (Fenchel & Finlay, 1991a). It is the evolutionary relationships of free-living anaerobic ciliates and their endosymbiotic methanogens with which we are concerned in the present review.

Anaerobic free-living ciliates

The existence of free-living strictly anaerobic ciliates was established by Fenchel et al. (1977). They were able to show that ciliates isolated from sulphide-rich sands, including members of the genera *Caenomorpha*, *Metopus*,
Parablepharisma, Plagiopyla, Saprodinium and Sonderia, lacked mitochondria and cytochrome oxidase. Subsequent work on these and other free-living ciliates (Finlay & Fenchel, 1989; Broers et al., 1991; Esteban et al., 1993) demonstrated that although they lacked mitochondria, they did contain specialized redox organelles called hydrogenosomes (Fig. 1a–c). Hydrogenosomes are not confined to ciliates; they were first described in trichomonads (Lindmark & Müller, 1973; Müller, 1988) and have been detected in species of the rumen chytrid Neocallimastix (Yarlett et al., 1986; Marvin-Sikkema et al., 1992). The biochemistry of hydrogenosomes in ciliates has been studied mainly in the rumen ciliates Daytricba, Isotricha and Polyplastron (Yarlett et al., 1981, 1983, 1984) but the biochemistry is probably the same or similar in those free-living ciliates which produce H₂ (Fenchel & Finlay, 1992). The hydrogenosomes in Dasytricha ruminantium oxidize pyruvate with the production of ATP, CO₂, H₂ and acetate (Yarlett et al., 1981, 1982). They contain a pyruvate:ferredoxin oxidoreductase which reduces a ferredoxin and an hydrogenase which catalyses the re-oxidation of the ferredoxin with the production of H₂. It is this H₂-evolving fermentation which is thought to underpin the symbiosis between anaerobic ciliates and endosymbiotic methanogens.

Symbioses between anaerobic free-living ciliates and methanogenic Archaea

The earliest evidence that some of the intracellular bacteria observed in anaerobic ciliates (Fenchel et al., 1977) might be methanogens came from observations of their UV-induced autofluorescence (Vogels et al., 1980). It was subsequently shown (Van Bruggen et al., 1983) that the fluorescing compound was coenzyme F₄₂₀, which is found mainly in methanogenic Archaea (Jones et al., 1987). Cells
Fig. 2. For legend see facing page.
of *Metopus palaformis, Metopus contortus* and *Plagiopyla frontata* which contain fluorescing bacteria (Fig. 2a–f) also produce methane (Finlay & Finlay, 1992). Anaerobic protozoa are the only eukaryotes which are known to contain endosymbiotic Archaea, although symbioses between eukaryotes and Bacteria are common and well documented (Margulis, 1993). The intracellular methanogens probably gain energy by reducing CO₂ with the waste H₂ from host hydrogenosomes (Van Bruggen et al., 1983; Jones et al., 1987). The benefits to the host are less obvious and in most ciliates they are unknown. However, some large ciliates including *Metopus contortus* and *Plagiopyla frontata* do benefit, since their growth rate and yield are reduced if the methanogens are specifically inhibited (Finlay & Finlay, 1991b). The physiology and ecology of ciliate–methanogen consortia have recently been reviewed by Finlay & Fenchel (1992b).

**Ultrastructural aspects of host/endosymbiont interactions**

Electron microscopy has revealed that the symbioses between different free-living anaerobic ciliates and their endosymbiotic methanogens present different levels of structural integration. In *Metopus palaformis* the symbionts are rod-shaped (Fig. 3a) and apparently unattached to host hydrogenosomes (Finlay & Fenchel, 1991a). However, growth and division of the symbiont are closely coupled to host growth and division (Finlay & Fenchel, 1992a). This suggests a tight dependence of the symbiont on host metabolism but a comoncentric benefit for the host which has not been demonstrated (Finlay & Fenchel, 1991a). A strain of *Metopus contortus* isolated from marine sand contained a variety of shapes and sizes of endosymbiotic methanogens (Finlay & Fenchel, 1991b). These were interpreted as representing the morphological transformation of a single symbiont (Fig. 3b–d) rather than a mixture of different endosymbionts. A plausible transformation series could be assembled from electron micrographs ranging from small rods/plates to large thinned discs closely attached to hydrogenosomes. It has been suggested that the close juxtaposition of symbiont and hydrogenosome facilitates H₂ transfer (Finlay & Fenchel, 1991b). *Metopus contortus* benefits from its endosymbionts since its growth rate and yield are reduced when they are treated with the methanogen inhibitor bromoethanesulphonic acid (Fenchel & Finlay, 1991b).

The only other ciliate for which a morphological transformation of the endosymbiont has been suggested is an isolate of the freshwater ciliate *Trimyema* (Finlay et al., 1993). Thin sections of this ciliate revealed electron-dense bodies (Fig. 4), of various shapes and sizes, which could be arranged into a series of stages in a morphological transformation. Some of the electron-dense bodies are considerably larger in section than others and they show indentation of their outline. Each of these large morphotypes is enclosed by a cell wall and each is surrounded by hydrogenosomes.

Transmission electron micrographs reveal that the symbionts in *Plagiopyla frontata* are closely integrated into the host life-cycle (Fenchel & Finlay, 1991c). The symbiotic methanogens and host hydrogenosomes form aggregates arranged like stacks of coins in which the two partners alternate for position (Fig. 5a). Similar structures have been observed in *Plagiopyla minuta* and *Leichnmya mystax* (Berger & Lynn, 1992). In *Plagiopyla frontata* the timing of the reproduction of the symbionts is closely integrated with the host cell-cycle and the host obtains an energetic advantage from the symbiosis (Finlay & Finlay, 1991b). In *Plagiopyla nasuta*, the endosymbionts appear to be quite different and they do not form stacks with the hydrogenosomes. They are long rods (Fig. 5b) which are always orientated perpendicular to the ciliate cell surface.

A new type of symbiotic interaction was recently discovered and described by Esteban et al. (1993) in the small (20–30 µm long) anaerobic unicellular algae *Cyclidium poratum*. Each ciliate contains a tightly organized complex (ca. 8 µm) of three independent components: about 15 hydrogenosomes, and two different sized prokaryotes (Fig. 5c). There are approximately five large, thick rods and about 40 small, slim rods associated with the complex in each ciliate. The small rods show the characteristic autofluorescence of methanogenic Archaea but the larger rods resemble *Bacteria* and do not autofluoresce.

**Identification of the endosymbiotic methanogens from anaerobic ciliates**

The accurate identification and classification of endosymbionts and their hosts can reveal important information about the diversity and evolution of the symbioses. For example, it can reveal how many different organisms are involved in an association and how widespread is the ability to establish symbioses. Furthermore, by comparing host and symbiont taxonomies, it is possible to gain an insight into the stability and specificity of the associations. The first attempts to identify the endosymbiotic methanogens from anaerobic protozoa relied on traditional methods of isolation and cultivation. Rod-shaped symbionts were isolated from washed cells of the ciliate *Metopus striatus* (Van Bruggen et al., 1984) and from individuals of the giant amoeba *Pelomyxa palustris* (Van Bruggen et al., 1988). Both methanogens were classified as strains of *Methanobacterium formicicum*. A polymorphic methanogen classified as *Methanoplanus endosymbioticus* was subsequently isolated from a strain of *Metopus contortus* (Van Bruggen et al., 1986). The results of these culture-based studies have important implications for the specificity of the symbiotic associations. Strains of *Methanobacterium formicicum* appear to be able to colonize very different hosts and the congeners *Metopus striatus* and *Metopus contortus* contain methanogens from two different orders; *M. formicicum* in the *Methanobacteriales* and *Methanoplanus endosymbioticus* from the *Methanomicrobiales*. However, two things should be noted about these studies; it was not demonstrated that the cultures actually were the endosymbionts (e.g. by reinfection or in situ probing) rather than food bacteria or contaminants, and secondly, only a small number of characters were used to identify and classify the isolates.
Anaerobic ciliates and their methanogen endosymbionts

Fig. 3. (a) The permanently rod-shaped methanogen in *Metopus palaeformis*. The specimen shown has reached maximum length (6.6 μm) before division. The methanogen is closely associated with, but apparently not attached to, the hydrogenosomes (H) of the ciliate. (b, c, d) Sequential stages in the polymorphic transformation of the symbiotic methanogens in *Metopus contortus*. The vacuolar membrane (arrow in b) is always obvious. The cell wall of the methanogen is stripped off (arrow in c) and the ‘plasticized’ wall-less methanogen, still enclosed in a ciliate vacuolar membrane (arrows in d) is attached to hydrogenosomes. All adapted from Finlay & Fenchel (1991b). Scale bars: 1 μm in (a); 0.5 μm in (b)–(d).

Over the past few years molecular tools have been developed which allow the identities of uncultured bacteria such as endosymbionts to be precisely determined. Fundamental to this approach is the comparative analysis of rRNA sequences to classify and identify microorganisms (Woese, 1987; Sogin, 1991) and the ease by which rRNA sequences can be recovered from uncultured microbes using the polymerase chain reaction (PCR) and specific primers (Saiki et al., 1988).

Use of 16S rRNA sequences to classify and identify the methanogen endosymbionts of anaerobic ciliates

The advantages of rRNAs for studying microbial phylogeny have been extensively discussed (Woese, 1987). Of particular relevance to the present discussion is that, on the basis of rRNA sequence comparisons, all cellular life can be divided into three domains; the *Bacteria*, *Archaea* and *Eucarya* (Woese et al., 1990; Winker & Woese, 1991).

Each domain contains some conserved sequence motifs which are absent from the other two. It is thus relatively simple to design PCR primers that will specifically amplify rRNA sequences from one phylogenetic group in the presence of competing templates from another (Giovannoni et al., 1990; Amann et al., 1991; Fry et al., 1991). On present data the methanogen phenotype appears to be restricted (with one exception *Methanopyrus kandleri*; Burggraf et al., 1991) to a phylogenetically coherent subdomain of the *Archaea* called the Euryarchaeota (Woese et al., 1990). PCR primers which amplify rRNA genes from the Euryarchaeota without concomitant amplification of ciliate rRNA genes have been designed (Embley et al., 1992a) using published methanogen sequences (Larsen et al., 1993). Using these primers it is not necessary to purify symbiont DNA prior to amplification and PCR can be done directly on crude DNA preparations or small numbers of heat-lysed ciliates (Embley et al., 1992a, b, 1994; Finlay et al., 1993). Using PCR primers specific for eukaryote SSU rRNA genes (Embley et al., 1992a) it is also possible to recover host sequences from the same crude DNA preparations.
Once a sequence has been obtained, the simplest way to demonstrate that it belongs to an endosymbiont or to a specific host is to carry out an in situ hybridization experiment (e.g. Fig. 2g-i). Oligonucleotide probes to rRNA (which can be designed from the sequence of a symbiont or host PCR product) can be labelled with fluorescent dyes (DeLong et al., 1989) and used to probe intact protozoa which have been made permeable by treatment with paraformaldehyde (Amann et al., 1991; Embley et al., 1992a). rRNA sequences are mosaics of conserved, semi-conserved and very variable sequences (Woese et al., 1983; Woese, 1987) and it is relatively simple to design highly specific probes by targeting an appropriate region (Stahl & Amann, 1991). Probes to rRNA are very sensitive, as ribosomes are abundant in actively growing cells (DeLong et al., 1989).

The first symbiosis to be examined in detail using molecular methods (Embley et al., 1992a) was that between Metopus palaformis and its rod-shaped endosymbiont (Finlay & Fenchel, 1991a). A single endosymbiont sequence was obtained using PCR which, when analysed, demonstrated that the symbiont was a member of the genus Methanobacterium (Fig. 6). Two fluorescent oligonucleotide probes were designed to bind to the symbiont rRNA sequence and used to probe intact Metopus palaformis and demonstrate that the sequence originated from intracellular bacteria. The symbiont sequence was different to published sequences from free-living methanogens including Methanobacterium formicicum (Lechner et al., 1985). The 16S rRNA gene from the strain of Methanobacterium formicicum previously isolated from Metopus striatus (Van Bruggen et al., 1984) has also been sequenced (Embley et al., 1994). Phylogenetic analysis revealed that it too is different from Methanobacterium formicicum, but it is also different to the Metopus palaformis endosymbiont and it is probably a new species (Fig. 6).

Molecular analyses (Embley et al., 1992b) have confirmed that the polymorphic methanogens in an isolate of Metopus contortus represent stages in the morphological transformation of a single endosymbiont (Finlay & Fenchel, 1991b), rather than a mixture of different methanogens. Experiments designed to selectively amplify symbiont sequences recovered only a single methanogen sequence from heat-lysed ciliates. When a specific fluorescent probe was designed to this sequence and used to probe Metopus contortus it hybridized to a range of sizes and shapes of fluorescing intracellular bacteria (Fig. 2j). The same intracellular bacteria also gave positive signals with a control Archaebacteria-specific probe (Stahl & Amann, 1991). Phylogenetic analysis of the symbiont sequence (Embley et al., 1994) demonstrated that it is closely related (but different) to the 16S rRNA sequence from the free-living methanogen Methanocorpusculum parvum.
As previously mentioned, *Trimyema* sp. (Finlay et al., 1993) is the other ciliate in which a single endosymbiont appears to undergo morphological transformation. rRNA sequences recovered from this consortium confirm, once again, that a single symbiont is morphologically transformed (Fig. 2k, l). When the symbiont sequence was analysed (Embley et al., 1994) its nearest relatives were the *Metopus contortus* endosymbiont and *Methanocorpusculum parvum* (Fig. 6). It is fascinating (and may be significant to understanding the phenomenon) that in the two cases where a morphological transformation has been documented (in taxonomically unrelated marine and freshwater ciliates) the symbionts are very closely related to each other.

Molecular analysis of the endosymbionts in *Plagiopyla frontata* and *Plagiopyla nasuta* (Embley et al., 1994) confirmed ultrastructural evidence (Fig. 5a, b) that they are different methanogens. The symbiont in *Plagiopyla frontata* is closely related to the free-living *Methanoborus tindarius* which was originally isolated from marine sediment (König & Stetter, 1982). The sequence recovered from lysed cells of *Plagiopyla nasuta* forms a deep branching lineage within the radiation of the *Methanomicrobiales* group (Fig. 6). This level of sequence divergence is similar to that between some of the genera of free-living methanogens in Fig. 6. The exact position of the *Plagiopyla nasuta* endosymbiont could not be resolved, as it shifted according to the method of analysis and the composition of reference sequences. Cells of *Plagiopyla frontata* and *Plagiopyla nasuta* exhibit high levels of background autofluorescence when fixed with paraformaldehyde and it has not yet been possible to confirm the origin of symbiont sequences from these two hosts by in situ probing. However, identical sequences were recovered from three independent preparations of micromanipulated ciliates for which fluorescence microscopy was used to check for the absence of free-living methanogens. Furthermore, electron micrographs of the *Plagiopyla frontata* endosymbiont (Fig. 5a, and B. J. Finlay, unpublished) show a close resemblance to published micrographs of *Methanoborus tindarius*, which has a very thin cell wall (Stetter, 1989).

The precise identities of the prokaryote endosymbionts detected in *Cyclidium porcatum* have not yet been determined but their higher-level affiliations have been investigated using domain-specific fluorescent probes.
Fig. 6. Phylogenetic trees for anaerobic ciliates and endosymbiotic methanogens based on SSU rRNA sequences. The tree was constructed from corrected distances (Jukes & Cantor, 1969) using the Neighbour-Joining method (Saitou & Nei, 1987). Reference sequences for methanogens and ciliates were obtained from the Ribosomal RNA Database Project (Larsen et al., 1993). The symbiont tree is based on 762 base positions and the host tree is based upon 1372 positions. The scale bar represents 10% estimated substitutions. Bootstrapping (100 replicates) was used to assess the support for groups (Felsenstein, 1985) and bootstrap proportions for both methods are displayed on the relevant nodes (D, distance; P, parsimony). Adapted from Embley et al. (1994).
(Esteban et al., 1993). Fig. 2(m) demonstrates the simultaneous application of the Archaea-specific (red) and Bacteria-specific (green) fluorescent oligonucleotide probes (Stahl & Amann, 1991). The five large prokaryotes clearly fluoresce green and are thus Bacteria whereas the others fluoresce red and are therefore Archaea. In considering the current fascination with biodiversity it is sobering to encounter a eukaryote which is less than 25 μm long but which, with its endosymbionts, encompasses the three domains of life!

**Phylogenetic diversity of endosymbiotic methanogens and anaerobic ciliates**

Fig. 6 summarizes the phylogenetic relationships of the sequences obtained so far from the endosymbiotic methanogens of anaerobic ciliates. Each sequence is novel, paralleling recent molecular studies which have demonstrated that most environmental micro-organisms represent previously unrecorded lineages (Giovannonni et al., 1990; DeLong, 1992; Furhman et al., 1992). The endosymbiont sequences are distributed throughout the methanogen tree and some endosymbionts are closely related to free-living methanogens. It is clear from these data that symbioses have formed repeatedly and independently. There are currently three major methanogen lineages which have been recognized by analysing 16S rRNA sequences, with the taxonomic distribution of their endosymbionts within the Methanomicrobiales. The distribution of endosymbiont sequences suggests that the ability to form associations with anaerobic ciliates probably predates the divergence of these two major clades. However, the absence of an endosymbiont sequence within the Methanoococcales may simply reflect the small sample of sequences available and the trait may eventually prove to be much older.

Fig. 6 also compares the phylogenetic relationships of a sample of anaerobic ciliates based upon SSU rRNA sequences, with the taxonomic distribution of their endosymbionts (Embly et al., 1994). This comparison provides further evidence that the associations have formed repeatedly. There is no congruence between host and endosymbiont trees and thus no evidence of parallel speciation. Congeneric hosts in the genus Metopus and Plagiopyla contain endosymbionts which are more closely related to free-living methanogens than they are to each other. Conversely, polymorphic endosymbionts which are closely related to the free-living species Methanocorpusculum parvum occur in unrelated hosts; Metopus contortus and Trimyema. The pattern of relationships demonstrated between host and endosymbiont (Fig. 6) might be explained if each taxon is a generalist, capable of forming associations with taxonomically different partners. If this is true then the relationships in Fig. 6 simply reflect the associations formed in specific habitats at specific times. This could also explain why the sequence determined for the previously isolated (Van Bruggen et al., 1986) and cultured methanogen (Methanoplanus endosymbius) from Metopus contortus is quite different to the methanogen sequence in the M. contortus collected from another locality several years later. The latter is related to Methanocorpusculum parvum, and its identity was confirmed by in situ probing (Embly et al., 1992b). Further experiments to investigate symbionts in the same host from different locations should help to clarify precisely how specific the symbiotic relationships are.

On the basis of SSU rRNA sequences there is evidence for three separate lineages of ciliates possessing the anaerobic phenotype and hydrogenosomes (Embly et al., 1994). These are surrounded by ciliates which possess mitochondria and which are aerobic or microaerobic. However, close scrutiny of the ciliate tree (Fig. 6) reveals that some of the distances between internal nodes are short and that there is only low bootstrap support for some relationships. Three lines of evidence support the independent acquisition of anaerobiosis by the three lineages. There is strong support (Fig. 6) for a sister group relationship between the anaerobic metopids and the aerobic Spathidium sp. (P. Dyal unpublished sequence) and there is moderate support for a sister group relationship between Cyclidium porcatum (a scuticociliate) and the aerobic scuticociliates. Forcing the three anaerobic lineages to form a single lineage increases by 50 steps the length of the optimal tree that can be constructed from the ciliate SSU rRNA sequences using parsimony (Embly et al., 1994). Finally, there is no evidence from cell morphology that Plagiopyla and Trimyema, the metopid heterotrichs, and the scuticociliates, form a monophyletic group (Corliss, 1979).

If one accepts that the topology in Fig. 6 is an accurate reflection of anaerobic ciliate evolution, then the ability to live anaerobically has arisen at least three times within the ciliate radiation. As representatives in each of these three lineages have hydrogenosomes it is reasonable to assume that hydrogenosomes have also evolved on three independent occasions. The hydrogenosomes in Metopus sp., Trimyema sp. and Cyclidium porcatum (Finlay & Fenchel, 1989; Esteban et al., 1993) have double membranes and the inner membrane is sometimes folded to form cristae-like structures; in electron micrographs they strongly resemble mitochondria (Fig. 1a–c). There are currently two theories to explain the origins of hydrogenosomes; that they represent modified mitochondria (Cavalier-Smith, 1987; Finlay & Fenchel, 1989), or that they represent a separate endosymbiosis involving a pyruvate-oxidizing Clostridium-like bacterium (Müller, 1988). We accept that the origins of hydrogenosomes may be different in taxa such as trichomonads (Jindmark & Müller, 1973; Müller, 1988) which are probably primivitively anaerobic. However, the ciliate tree based upon SSU rRNA sequences strongly suggests that in the free-living ciliates at least, hydrogenosomes are derived from existing organelles and on the basis of ultrastructural evidence the most likely candidate is a mitochondrion. A key task for understanding the evolutionary development of anaerobiosis from aerobic eukaryotes is now to identify...
the origins and locations of the genes coding for the anaerobic physiology.

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