The use of rRNA sequences and fluorescent probes to investigate the phylogenetic positions of the anaerobic ciliate *Metopus palaeformis* and its archaeobacterial endosymbiont

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The polymerase chain reaction (PCR) was used to amplify small-subunit ribosomal DNA from the anaerobic ciliated protozoan *Metopus palaeformis*, and from its uncultured endosymbiotic bacteria. This was accomplished directly from total DNA extracted from protozoa without prior isolation or enrichment for symbiont cells. The double-stranded amplification products were precipitated and directly sequenced using the linear PCR reaction. Fluorescent oligonucleotide probes were designed and used in whole-cell hybridizations to provide direct visual evidence that the sequences originated from the host ciliate and from the endosymbiont. Phylogenetic analysis of the *Metopus palaeformis* sequence consistently placed it as a deep-branching lineage near the root of the ciliate tree. However, the present data were insufficient to resolve the detailed relationship between *Blepharisma* and *Metopus* and thus to determine if the heterotrichs are mono- or paraphyletic. Phylogenetic analysis of the symbiont partial sequence clearly demonstrated that it is an archaeobacterium and that it is closely related to, but distinct from, *Methanobacterium formicicum*.

Introduction

Comparative sequencing of ribosomal RNA has revolutionized studies in microbial classification and provided a framework for interpreting natural diversity (Woese, 1987). Methods based on RNA analyses are increasingly being used in studies of environmental microbiology (Olsen *et al.*, 1986; Stahl *et al.*, 1988; DeLong *et al.*, 1989; Amman *et al.*, 1990, 1991). For example, the polymerase chain reaction (PCR) (Saiki *et al.*, 1988) and the development of methods for probing single cells (Giovannoni *et al.*, 1988; DeLong *et al.*, 1989) mean that it is now possible to investigate the evolutionary relationships of uncultured micro-organisms (Amman *et al.*, 1991; Fry *et al.*, 1991). The rRNA genes from eukaryotes, bacteria and archaeobacteria have been shown to vary significantly in their primary sequence (Woese, 1987; Neefs *et al.*, 1991). It is thus relatively simple to design PCR primers that will

specifically amplify RNA sequences from particular phylogenetic groups directly from heterogeneous mixtures of templates (Giovannoni *et al.*, 1990; Amman *et al.*, 1991; Fry *et al.*, 1991). Having obtained the sequences, fluorescent probes can be designed and used in whole-cell hybridization experiments, thereby linking the sequence to a defined morphotype (Edman *et al.*, 1988; Giovannoni *et al.*, 1988; DeLong *et al.*, 1989).

Metopus palaeformis is an anaerobic ciliate which is common in a variety of freshwater habitats and anaerobic municipal landfill (Finlay & Fenchel, 1991). Its phylogenetic position among eukaryotes is uncertain but it is currently classified as a member of the class Spirotrichea, subclass Heterotrichia, on the basis of its oral and somatic infraciliature (Lynn & Corliss, 1991). This group also contains *Blepharisma americanum*, which is currently the deepest-branching ciliate (Greenwood *et al.*, 1991a, b; Schlegel *et al.*, 1991). Like many of the free-living protozoa in anaerobic environments, members of the genus *Metopus* contain symbiotic methanogenic bacteria (Fenchel *et al.*, 1977; Van Bruggen *et al.*, 1984, 1986; Fenchel & Finlay, 1991a; Finlay & Fenchel, 1991). The symbionts of *Metopus striatus* and *Metopus contortus* have been isolated and tentatively identified (on the

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The nucleotide sequences determined in this work have been deposited in GenBank and have been assigned the accession numbers M86385 (*M. palaeformis*) and M86386 (endosymbiont).

basis of morphology and G+C ratios) as *Methanobacterium formicicum* and *Methanoplanus endosymbiosus*, respectively (Van Bruggen *et al.*, 1984, 1986). *Metopus palaeformis* contains a rod-shaped putative methanogen which has not been isolated and thus remains unidentified (Finlay & Fenchel, 1991).

In the present investigation we have used PCR to amplify and directly sequence the small-subunit ribosomal DNA from *Metopus palaeformis* and from its endosymbiotic bacteria. Fluorescent probes were designed and used in whole-cell hybridizations to confirm the origins of the sequences. The sequences were then analysed in order to determine the phylogenetic positions of *Metopus palaeformis* and its endosymbiont.

Methods

The ciliate and its symbionts. The anaerobic ciliate *Metopus palaeformis*, containing symbiotic bacteria, was isolated from anoxic municipal landfill (Finlay & Fenchel, 1991). The ciliates were maintained anaerobically, using the indigenous microbial flora as food (Fenchel & Finlay, 1990). Cells of *M. palaeformis* were purified from free-living archaeobacteria by electromigration. The electromigration device was identical to that described by Wagener *et al.* (1986) except for the insertion of a loose cotton wool plug in the capillary tube through which the ciliates were forced to swim. The isolated ciliates were suspended in anoxic (purged with N₂), dithionite-reduced mineral medium (Wagener & Pfennig, 1987; S. Wagener, personal communication) containing (g l⁻¹): KH₂PO₄ (0.15), NH₄Cl (0.035), NaCl (0.4), MgCl₂·6H₂O (0.2), KCl (0.15), CaCl₂·2H₂O (0.25), NaHCO₃ (1.0), and adjusted to pH 7.0.

DNA extraction. Approximately 10⁴ ciliates were centrifuged at 4000 g for 5 min and washed once with distilled water before resuspending in 50 µl sterile Millipore water. DNA was isolated using the rapid guanidium method devised by Pitcher *et al.* (1989), but omitting the Sarkosyl from the lysis reagent. The DNA was redissolved in 50 µl sterile Millipore water and stored in 10 µl aliquots at -70 °C.

Selection of PCR primers. PCR primers were designed to selectively amplify archaeobacterial (1.1 kb fragment) or eukaryotic (full-length), small-subunit ribosomal DNA. The sequences of the archaeobacterial primers were biased towards the methanogens and their close relatives (the *Euryarchaeota*; Woese *et al.*, 1990). The primers were 1A forward (f) 5' TCGKTTGATCCYGSCRGAG and 1100A reverse (r) 5' TGGGTCTCGCTCGTTG. The primers for the eukaryote small subunit rRNA sequences were forward primer 5' AYCTGGTTGAT-YYTGCCAG and reverse 5' TGATCCATCTGCAGGTTACCT. These primers are essentially the same as those devised by Medlin *et al.* (1988) but without the polylinkers. The primers amplify almost the entire small subunit RNA sequence of most eukaryotes (Neefs *et al.*, 1991). RNA sequences for the ciliate protozoa *Colpidium campylum*, *Glaucoma chattoni*, *Opisthionecta henneguyi*, *Onychodromus quadricornutus*, *Oxytricha granulifera*, *Blepharisma americanum* and *Colpoda inflata* were taken from published papers (Greenwood *et al.*, 1991a, b; Schlegel *et al.*, 1991).

PCR amplifications. Each amplification contained 2 µl of template DNA solution, 10 µl of PCR buffer (670 mM-Tris/HCl, 20 mM-MgCl₂, pH 8.8), 0.5 µl of stock NTPs (each at 25 mM), 20 pmol of each primer (either eukaryotic or archaeobacterial specific), 0.1 unit of Perfect Match Polymerase Enhancer (Promega) and sterile Millipore water to 98 µl. The tubes were heated to 95 °C in a Hybaid TR2 Intelligent

Heating Block and 2 µl (1 unit) of *Taq* polymerase (Cetus) was added to each tube, followed by 50 µl of sterile mineral oil. The thermal programme comprised 10 cycles of denaturation at 94 °C for 40 s, hybridization at 55 °C for 40 s and extension at 72 °C for 2 min. This was followed by 20 cycles of 92 °C for 30 s, 55 °C for 40 s and extension at 72 °C for 2.5 min. A 6 min extension at 72 °C completed the programme.

Sequencing of amplified ribosomal DNA. The amplified material was sequenced using the linear PCR procedure described by Embley (1991). The archaeobacterial sequencing primers were 519 reverse (r) GWATTACCGCGCKGCTG, 380r TTTCGCGCCTGCTGC and 1A. The eukaryote sequencing primers were taken from Elwood *et al.* (1985) and comprised 377r TCAGGCTTCYTCTCCGG, 557r ACCGCGGCKGCTGGC, 892r AGAATTCACCTCTG, 1262r CGGCCATGCACCACC, 1705r ACGGGCGGTGTGTRC and 1705f GYACACACCGCCCGT. The products of the sequencing reactions were analysed on standard wedge-shaped sequencing gels.

Data analysis. The primary sequence data for the symbiont and for *Metopus palaeformis* have been deposited in GenBank (M86386 and M86385).

The sequence for the endosymbiont was aligned by eye against sequences for reference archaeobacteria and corrected for known secondary structure (Neefs *et al.*, 1991). Positions which could not be aligned unambiguously were omitted; the final data comprised 333 bases. Distance values were calculated using the method of Jukes & Cantor (1969) contained in the DNADIST program (PHYLIP 3.4, J. Felsenstein, University of Washington, USA). A phylogenetic tree was generated by the neighbour-joining method (Saitou & Nei, 1987), using *Thermococcus celer* as an outgroup (Woese *et al.*, 1990).

The sequence for *Metopus palaeformis* was aligned by eye against 13 published sequences for ciliates, and the sequence for the dinoflagellate *Prorocentrum micans*, as an outgroup (Greenwood *et al.*, 1991a; Neefs *et al.*, 1991). The small-subunit RNA sequences of eukaryotes may show considerable variation in the length and sequence of certain domains such as region V4 (Neefs *et al.*, 1991). In order to align the sequences from this region the secondary-structure models proposed by Nickrent & Sargent (1991) and Neefs & De Wachter (1990) were used. Sites where it was uncertain that truly homologous positions were being compared (approx. 200 bases), were omitted. A total of 1419 bases comprised the final alignment; this is available from the authors on request. In initial analyses, pairwise distance values between sequences were obtained using the Jukes & Cantor (1969) correction for multiple substitutions (contained in DNADIST, version 3.4). The values obtained were generally less than 0.2 (the matrix is available on request). Since values in this range are unlikely to be significantly affected by the use of different substitution corrections (Jin & Nei, 1990) only the Jukes & Cantor (1969) correction was used. Phylogenetic trees were constructed from the distance matrix using either the neighbour-joining (Saitou & Nei, 1987), or Fitch & Margoliash (1967) methods contained in PHYLIP 3.4. In order to evaluate the robustness of the results, 100 bootstrap resamplings of the data were taken (SEQBOOT 3.4, PHYLIP) and neighbour joining performed on each sample. A consensus tree was produced using CONSENSE 3.4 (PHYLIP). Bootstrapped (100 times) maximum parsimony (PAUP 3.0q, Swofford, 1991) was also employed to analyse the data. In an effort to resolve the deeper internal branches of the ciliate phylogenetic tree, the linear invariants method of Lake (1987) was used (PAUP 3.0q). Since all methods of phylogenetic reconstruction are potentially biased by variation in base frequencies (Gillespie, 1986), a modification of Lake's method (Sidow & Wilson, 1990), which attempts to correct for base compositional differences, was also used.

Design of probes. The sequence for the endosymbiont was compared to the sequence for *Metopus palaeformis* and to the published rRNA data base for archaeobacteria (Neefs *et al.*, 1991). Two oligonucleotide

probes were constructed, SYM1 5' AACCCGTACAGATCAAAGG and SYM2 5' GACCATTCCAGGAATCTCTA, to bind specifically to rRNA from the symbiont. The probes recognized sequences within helices 12 and 13, and helix 10, respectively, by reference to the *Halobacterium cutirubrum* secondary structure (Neefs *et al.*, 1991). A probe (5' TTTACGTTTGCCCATTC) was designed to bind exclusively to rRNA sequences (within helix 18; Neefs *et al.*, 1991) of *Metopus palaeformis*. Control probes designed (Stahl & Amman, 1991) to bind selectively to either archaeobacterial or eukaryote rRNA were also prepared. All of the probes were synthesized using Amino-link 2 (Applied Biosystems) and coupled to tetramethyl-6-carboxyrhodamine or 6-carboxyfluorescein (Applied Biosystems) using the manufacturer's protocols. Hybridization experiments were carried out at 48 °C for 4–6 h using procedures described by Stahl & Amman (1991) with only slight modifications. The preparations were not prehybridized. Each hybridization was carried out on a standard 'subbed' microscope slide to which 20 µl of hybridization solution containing 50 ng of specific probe was added. A cover slip was applied and sealed to the slide using clear nail-varnish to retard evaporation.

Epifluorescence microscopy and photomicrography. After hybridization each slide was mounted using Citifluor antifadent (AF1, Citifluor Ltd, London) and examined using a Leica Aristoplan epifluorescence microscope fitted with filter sets specific for rhodamine or fluorescein. Photographs were taken on Kodak Ektachrome 400 ASA black and white film; exposures were controlled via a photoautomat MPS-46 attached to the microscope. Living ciliates were fixed with 4% (v/v) formaldehyde solution prior to preparation for observation of autofluorescing endosymbiotic methanogens (Finlay & Fenchel, 1989).

Ciliates were prepared for electron microscopy as described by Finlay & Fenchel (1991).

Results and Discussion

The products of the PCR reactions were examined using agarose gel electrophoresis. The reactions using the archaeobacterial primers gave a single weak band of about 1.1 kb. The reactions with the eukaryote primers produced a single strong band of approximately 1.7 kb. The latter is in the size range which is frequently reported for ciliate small subunit rRNA genes (Greenwood *et al.*, 1991a, b; Schlegel *et al.*, 1991). No bands were observed in reactions lacking template. The amplified material was precipitated and directly sequenced (Embley, 1991). Each of the two sequences was unambiguous in regions of rRNA known to be highly variable (e.g. regions V2 and V4; Neefs *et al.*, 1991). This strongly suggests that the eukaryote and archaeobacterial sequences were each derived from a single species.

The final number of bases of sequence from the product amplified using the eukaryote primers was 1624. The sequence stretched from two bases downstream of the 3' terminus of each PCR primer, with a small gap of 50 bases near to the base of helix 47 (Neefs *et al.*, 1991). The PCR primers used in this investigation can bind to rRNA genes from most eukaryotes (including humans) and it is possible that PCR products could arise from contaminants. Furthermore, since unicellular eukaryotes are often classified solely on the basis of morphology

(Margulis *et al.*, 1989) it is useful to be able to link a sequence to a cell type. Like most protozoa, *Metopus palaeformis* can demonstrate a variety of forms depending on its growth phase and nutritional status, and this can lead to difficulties in its identification (Finlay & Fenchel, 1991). To prove that our sequence originated from *Metopus palaeformis*, and to demonstrate the utility of whole-cell probing for the identification of protozoa, a fluorescent probe was designed. Fig. 1(a) shows a phase-contrast image of a mixed field containing *Metopus palaeformis* and a selection of eukaryotes and prokaryotes. This preparation was simultaneously probed with a fluorescein-conjugated eukaryote specific oligonucleotide (Stahl & Amman, 1991) and a *Metopus palaeformis*-specific oligonucleotide labelled with tetramethylrhodamine. Fig. 1(b) shows the same field under blue excitation for the fluorescein-labelled probe. All of the eukaryotic cells in the field, but none of the prokaryotes, show fluorescence. Fig. 1(c) shows the same field excited for tetramethylrhodamine, when only the single cell of *Metopus palaeformis* gives a signal. This clearly demonstrates that the eukaryotic sequence is from *Metopus palaeformis*. It also supports the use of fluorescent probes for the identification and enumeration of unicellular eukaryotes in mixed populations. The fluorescence is evenly distributed over the cell, allowing cell shape to be observed.

Ciliate phylogeny has traditionally been interpreted in terms of structural features of the cortex and of somatic and oral ciliature (Lynn & Corliss, 1991). However, the complexities of ciliate architecture and the difficulties in distinguishing between ancestral and derived morphologies have produced a number of conflicting phylogenies (Lynn & Corliss, 1991). The analysis of molecular sequence data can provide an independent test of conflicting hypotheses. Fig. 3 shows the relationships between *Metopus palaeformis* and reference ciliates based on small-subunit rRNA sequences. The dinoflagellate *Prorocentrum micans* was used as an outgroup. The tree illustrates the consensus topology (100 bootstrap replicates) obtained using the Jukes & Cantor (1969) correction and the neighbour-joining method (Saitou & Nei, 1987). The topology of the tree is very similar (for common species) to previously published distance trees based on SSrRNA (Greenwood *et al.*, 1991a, b; Schlegel *et al.*, 1991). Thus the clades containing the stichotrichs and the hymenostomes, respectively, were strongly supported using the distance (Fig. 2) and parsimony methods (not shown). There was also support in all analyses for a relationship between *Colpoda inflata*, *Paramecium tetraurelia*, *Opisthonecta henneguyi* and the hymenostomes. *Euplotes aediculatus* was recovered on the branch leading to the stichotrichs in the distance and parsimony analyses and this is in agreement with

Fig. 2. Consensus phylogenetic tree showing the relationships of *Metopus palaeformis* to other ciliates and an outgroup, the dinoflagellate *Prorocentrum micans*. The tree is based on 100 bootstrap samplings of the data, each analysed using the Jukes & Cantor (1969) correction and neighbour-joining (Saitou & Nei, 1987) method. Figures reflect the number of times the group consisting of the species to the right of that node occurred among the trees. Names in parentheses are those currently in common use to denote higher taxa, i.e. class or subclass (Lynn & Corliss, 1991). The scale represents 0.02 substitutions per sequence position (K_{nuc}).

previous work (Sogin *et al.*, 1986; Schlegel *et al.*, 1991). Interestingly, the linear invariants method (Lake, 1987) and its modification (Sidow & Wilson, 1990) placed *Euplotes* significantly with the outgroup *Prorocentrum micans*. However, close examination of the *Euplotes* sequence suggested a substantial transversion bias towards uracil. This is a violation of the assumptions of the linear invariants method (and all other methods to some extent) and thus the results of these analyses should be treated with caution. The *Euplotes* sequence is also unusual in that it contains a number of insertions which are absent in the other ciliates (Sogin *et al.*, 1986).

The heterotrichs are currently considered to be a monophyletic group (Lynn & Corliss, 1991). The results of the present investigation provided only limited support for this hypothesis. All of the methods agreed that the heterotrichs *Metopus palaeformis* and *Blepharisma americanum* are deep-branching lineages. However,

they appeared as sister taxa, with modest support (71% of bootstrap replicates), in only the maximum parsimony analysis. The distance methods consistently placed *Blepharisma americanum* as a separate lineage at the root of the ciliates. This finding is in agreement with previous studies which reported *Blepharisma* as the deepest-branching ciliate (Greenwood *et al.*, 1991a). *Metopus palaeformis* appeared with low to moderate support (58% in neighbour joining Fig. 2, 70% in the Fitch–Margoliash consensus tree – not shown) as a deep branch on the lineage leading to the stichotrichs (Fig. 2). The invariants methods were unable to resolve the relationship between the two heterotrichs with any confidence. It was thus impossible on the basis of the present data to decide if the heterotrichs form a mono- or paraphyletic group. Two approaches may help to resolve the relationships between these taxa. One is to sample additional species, especially among the heterotrichs and hypotrichs. The

Fig. 1. Photographs of the same field of whole fixed cells simultaneously probed with fluorescently-labelled oligonucleotides specific for either eukaryotes or *Metopus palaeformis*. (a) Phase-contrast image showing a single cell of *Metopus palaeformis* (M) among a variety of different eukaryotes, bacteria and archaeobacteria. (b) Epifluorescence image following specific excitation for the fluorescein-labelled eukaryote-specific probe. (c) Epifluorescence image following specific excitation for the tetramethylrhodamine-labelled *M. palaeformis* probe.

second approach would be to obtain more sequence data from the taxa already sampled. In this study the number of variable sites in the alignment of ciliate sequences (out of 1419 sites aligned and omitting *Euplotes* and *Prorocentrum*) was 417, of which 261 were informative in the parsimony sense (Swofford, 1991). These data gave internal branches separating the heterotrichs and other taxa which were very short. It may thus prove impossible to resolve tree topology at this level using only small-subunit sequences, and molecules which show a greater degree of variation (e.g. 28S rRNA or protein-coding genes) may be required. A relationship between the heterotrichs, hypotrichs and stichotrichs in the class *Spirotrichea* was proposed by Lynn & Corliss (1991) on the basis of similarities in oral and somatic kinetids. Our data give qualified support for a clade containing *Metopus*, *Euplotes* (a hypotrich) and the stichotrichs.

The sequence for *Metopus* is the first to be published for an anaerobic ciliate. All of the analyses place *Metopus* close to aerobic ciliates and the distance methods consistently place it internal to the aerobe *Blepharisma*. The proximity of *Metopus* to aerobic ciliates is consistent with recent ultrastructural evidence which indicates that the hydrogenosomes of *Metopus* and other anaerobic ciliates are derived from mitochondria (Finlay & Fenchel, 1989). The accumulating evidence thus suggests that anaerobic ciliates are derived from aerobic species.

The observation that two out of three of the deepest lineages in Fig. 2 are heterotrichs gives further support for the hypothesis that they branched off early in ciliate evolution (Greenwood *et al.*, 1991a). Karyorelictan ciliates such as *Loxodes* and *Protocruzia* are the other candidates for the deepest-branching ciliates (Corliss, 1974; Lynn & Corliss, 1991). An unpublished ciliate phylogeny based on partial large subunit sequences (cited in Schlegel, 1991) recovered *Loxodes* and *Blepharisma* as sister taxa. Further data from a representative sample of both groups are needed to fully resolve the association between the karyorelictids and the heterotrichs and to provide a clearer insight into ciliate evolution.

The amount of amplified template from the endosymbiont was sufficient to determine 416 bases of sequence from the base of stem 3 to the distal base of helix 18 on the *Halobacterium cutirubrum* secondary-structure model (Neefs *et al.*, 1991). This covered one of the regions known to be most variable in prokaryotes, i.e. region V2 (Neefs *et al.*, 1991). The symbiont sequence was 92% similar to the sequence of *Methanobacterium formicicum* for the same region (Lechner *et al.*, 1985). The base differences comprised 15 transitions, 15 transversions and three insertions/deletions. All of the base differences occurred in regions known to be variable in archaeobacteria, and a plausible secondary structure (using

Fig. 3. Phylogenetic tree showing the relationships of the endosymbiont to representative archaeobacteria. The tree was generated using the Jukes & Cantor (1969) correction and neighbour-joining method (Saitou & Nei, 1987). The scale bar represents 0.02 substitutions per sequence position (K_{nuc}).

models in Neefs *et al.*, 1991) could be constructed. The endosymbiont sequence was compared to sequences from the same region for representatives of the *Euryarchaeota*. Fig. 3 shows a phylogenetic tree constructed using the Jukes & Cantor (1969) correction and the neighbour-joining method (Saitou & Nei, 1987). The symbiont is a member of the genus *Methanobacterium* and it is closely related to *Methanobacterium formicicum*. The assignment of the symbiont to *Methanobacterium* is supported by its morphology (Fig. 4) of electron-dense rod-shaped cells with conically pointed ends (Boone & Mah, 1989). In the absence of phenetic data it is difficult to assign a taxonomic rank to the symbiont. However, the depth of branching between the symbiont and *Methanobacterium formicicum* suggests that they are closely related species.

To demonstrate that the new sequence was derived from the *Metopus* endosymbiont, two fluorescent probes were designed. The rRNA content of micro-organisms is proportional to growth rate and metabolic activity (DeLong *et al.*, 1989). Since the physiological status and ribosome content of the symbiont were unknown, two probes were designed to bind to different regions of the symbiont rRNA and thus increase the signal. The probes

Fig. 4. Transmission electron micrograph of a dividing endosymbiont (E) and a hydrogenosome (H) in the ciliate *Metopus palaeformis*. Bar, 1 μm .

Fig. 5(a-c). Photographs of the same fixed whole cell of *Metopus palaeformis* simultaneously probed with archaeobacterial and symbiont-specific fluorescent oligonucleotide probes. (a) Phase contrast image; 'a' and 'p' indicate the anterior and posterior limits of the ciliate and 'N' the macronucleus. The ciliate is surrounded by large numbers of unidentified micro-organisms and debris. (b) Epifluorescence image of the same ciliate following specific excitation of the fluorescein-labelled archaeobacterial probe. (c) Epifluorescence image of the same ciliate following specific excitation of the tetramethylrhodamine-labelled symbiont-specific probes. (d) F_{420} autofluorescence in methanogens contained in another ciliate. Bar, 10 μm (magnification is the same for parts a-d).

were targeted to sequences where there were significant differences between the endosymbiont, *Methanobacterium formicicum* and *Metopus palaeformis*. Fig. 5(a) shows a phase-contrast image of a single cell of *Metopus palaeformis* surrounded by large numbers of unidentified

micro-organisms and debris. This cell was simultaneously probed with the fluorescein-labelled archaeobacterial probe (Fig. 5b) and the tetramethylrhodamine-labelled symbiont probes (Fig. 5c). All three probes clearly bind to the same bacterial-sized particulate

material within the ciliate. Whereas the archaeobacterial probe gives faint signals from a small amount of the material surrounding the ciliate, the symbiont probes only bind to particles within the *Metopus* cell. The fluorescing particles have similar dimensions to the autofluorescing bacteria shown in another separately prepared ciliate (Fig. 5d). These results confirm that the amplified sequence is from the *Metopus palaeformis* endosymbiont. As far as we are aware this is the first direct evidence for an archaeobacterial endosymbiont. As expected, the combined fluorescence from the two symbiont probes exceeded the signal from the single archaeobacterial control probe. However, in both cases the high intensity of the fluorescence suggests that the symbionts are viable and metabolically active. This is supported by the fact that a dividing symbiont can be observed in the electron micrograph (Fig. 4). In contrast to the bacteria shown in Figs 4 and 5(d), it was not possible to discern clear rod-shaped particles using the fluorescent probes. This may have been due to glare caused by the high intensity of the probe fluorescence.

The functional significance of the *Metopus palaeformis* symbiosis is incompletely understood (Finlay & Fenchel, 1991). Experimental inhibition of the methanogens did not lead to a detectable reduction in ciliate growth rate or yield (Finlay & Fenchel, 1991). This is in contrast to the situation in *Metopus contortus*, where loss of its symbiont reduces the growth rate of the host (Fenchel & Finlay, 1991a, b). However, the bacteria in *Metopus contortus* are different from those in *M. palaeformis* since they do not react with the symbiont-specific probes (unpublished data). The potential benefits of the association for the *Metopus palaeformis* endosymbiont are clear. The symbiont is a member of the genus *Methanobacterium* and can probably (like cultured methanobacteria) obtain energy from coupling the oxidation of H_2 to the reduction of carbonate (Jones *et al.*, 1987; Boone & Mah, 1989). *Metopus palaeformis* contains microbodies (Fig. 4) which are hydrogenase-positive (hydrogenosomes) and which release hydrogen during energy generation. It therefore seems plausible that the symbiont is benefiting from the H_2 produced as an excretory product by the ciliate (Muller, 1988; Finlay, 1990).

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