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Intracellular Prokaryotes in Rumen Ciliate Protozoa: Detection by Confocal Laser Scanning Microscopy after in situ Hybridization with Fluorescent 16S rRNA probes

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SUMMARY

In situ hybridization of rumen ciliate protozoa with 16S ribosomal RNA fluorescent oligonucleotide probes specific for Archaea and Bacteria provided semi-quantitative indication of the location, type and extent of prokaryotic colonization of various protozoal species. The isotrichid holotrich ciliates generally carried a smaller load of intracellular microorganisms than did the entodiniomorphid species. Thus, the vast majority of the *Dasytricha ruminantium* population had neither bacterial not archaeal endosymbionts, although a very small minority of these ciliates (only 11 out of 447: < 3%), contained numerous Bacteria cells. Their food vacoules contained only Bacteria. Thirty per cent of *Isotricha* spp. were without endosymbionts. *Polyplastron multivesiculatum* invariably had large numbers (> 250 cells per ciliate) of intracellular Bacteria, but no Archaea. Only some *Epidinium* spp. had intracellular prokaryotes, whereas *Entodinium* spp. and *Ent. simplex* almost always had. Many of the larger entodiniomorphid genera were heavily colonized by both bacterial and archaeal species. *Eudiplodinium maggii* had no obvious bacterial associates, although the autofluorescence of ingested plant material made difficult the detection of possible endosymbionts.

Introduction

The rumen ciliate protozoa constitute about 50% of the biomass of the rumen microbial population [43, 44]. They perform many functions and contribute to the nutrition of the host, as their metabolites and the protozoal cells represent an important source of nutrients. The protozoal population influences the physicochemical characteristics of the ruminal ecosystem and modulates the population size, composition and activities of the other microorganisms present [1, 22, 41, 42, 44]. Interactions between rumen microbes have been demonstrated; these are of many kinds and include those which involve relationships of the protozoa with Archaea and Bacteria. The protozoa participate both in predator-prey and metabolic interactions [31, 46]. Interspecies H_2 transport from the hydrogenogenic protozoa to what have been assumed to be largely free-living methanogens has been studied at several levels using crude rumen liquor [11], or rumen simulation

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techniques [6, 7, 21]. Cocultures have also demonstrated the effective cooperation between pairs of species in the generation of CH_4 [4, 24].

Studies on the biochemistry of rumen protozoa are complicated by their association with ecto-and/or endobiotic prokaryotes [44] and various methods have been adopted to allow for the prokaryotic contribution. Attempts to culture putative symbionts have generally been unsuccessful [44], and although there are reports [5, 28, 40] of the isolation and cultivation of intracellular bacteria, it was not conclusively established that the cultured organisms were identical with the "endosymbionts" (i.e., by re-infection or by in situ hybridization techniques). The possibility exists therefore that the cultured organisms were from food vacuoles, ectosymbionts, protozoal pathogens or even adventitious contaminants. It is well established however that ectosymbiotic methanogens attach to the surfaces of certain rumen protozoa [25, 29, 39]. Electron micrographs of thin sections of rumen protozoa sometimes reveal the presence of prokaryotic cells. The best substantiated incidence of endosymbionts exists for *Entodinium* spp., where numerous "bacterial" profiles are a consistent feature of the protozoal ultrastructure [44]. The isotrichid *Dasytricha ruminantium* also occasionally shows numerous intracellular prokaryotes [35, 36], although the great majority of individuals appear to be free of endosymbionts. Quantitative estimations of the cytoplasmic volume occupied by endosymbionts require serial sections, although approximations by transmission electron microscope stereology and light microscopy have recently been attempted [20]. These investigators tentatively identified some of the "endosymbionts" of *D. ruminantium* and *Entodinium* spp. as Archaea using a specific rhodamine-labelled oligonucleotide probe, and as methanogens by their F-420 fluorescence.

In this paper we show using fluorescent 16S RNA-targeted oligonucleotide probes [3, 9] specific for Archaea or Bacteria that there are interesting and important differences between different types of rumen protozoa with respect to the presence of intracellular bacteria. The isotrichid holotrichs (e.g. *D. ruminantium, Isotricha* spp.) usually do not harbour intracellular endosymbionts, whereas some of the entodiniomorphid ciliates may be colonized by either Bacteria (e.g. *Polyplastron multivesiculatum*), or Archaea (e.g. *Epidinium* spp.), or with both (*Entodinium* and the larger entodiniomorphids). A preliminary account of this work has already appeared [32].

Methods

Organisms

Samples of rumen contents were obtained from six rumen fistulated sheep on several occasions. These ruminants had been defaunated previously by administration of manoxol (dioctyl ester of Na-sulphosuccinate). Re-inoculation of animals was either with *Dasytricha ruminantium/Entodinium* spp., *Polyplastron multivesiculatum/Entodinium* spp., or a "B-type population" [10]. The paired sheep were kept in isolation from the others and fed once daily on a diet of hay and pelleted concentrate (250 g) that contained 35% barley and 18% protein; hay and water were available ad libitum. Samples of rumen contents (500 ml) (12 in all) were withdrawn before they were given this daily ration. Samples taken on two separate days showed similar results with respect to prokaryotic endosymbionts found in rumen ciliates. The ciliate population was isolated, cleaned and filtered to give different size fractions of protozoa as described in detail elsewhere [12, 45, 48]. Protozoa were washed several times in a salts buffer to remove non-adherent bacteria [12].

In situ hybridization with 16S rRNA-targeted fluorescent oligonucleotide probes.

Smears of protozoa on acid alcohol-cleaned slides were air-dried and then fixed (3 hours) in freshly prepared (4% w/v) formaldehyde in phosphate buffered saline solution (pH 7.2, PBS) [2]. Dehydration was in ethanol (3 minutes each of 50, 85 and 98% (v/v)) and was followed by air-drying.

We used two fluorescently labelled, 16S rRNA-targeted synthetic oligonucleotide probes, Eub 338, (5′-GCTGCCTCCCGTAGGAGT-3′ [3], a targeted fluorescein-label, complementary to a 16S rRNA region conserved for all Bacteria, and Arch 915 (tetramethylrhodamine-labelled) specific for Archaea, respectively [37]. Incubations were with 50 ng probe in hybridization buffer (HB) consisting of 0.9 M NaCl, 20 mM Tris HCl, 0.1% sodium dodecylsulphate (pH 7.2) at 46 °C for 2 h. Smears were rinsed sequentially in water and in HB for 20 min at 48 °C, before washing in distilled water. Air-dried smears were then mounted in 2% (w/v) DABCO (1,4 diazabicyclo (2,2,2,) octane) as a free radical scavenging "antibleach" agent [27].

Epifluorescence microscopy and photomicrography

Epifluorescence images were obtained with a Zeiss Axioplan microscope as previously described [34].

Confocal laser scanning microscopy

This was performed using a Molecular Dynamics Sarastro 2000 Confocal Scanning Microscope. Specimens were scanned using a 25 mW argon laser with appropriate excitation and emission filters for fluorescein (488/510 nm) or rhodamine (514/563 nm). To reduce photobleaching of fluorescence, the laser output was set at 15 mW and attenuated using a 3% transmission neutral density filter for fluorescein and a 30% filter for rhodamine. Specimens were examined using oil immersion objectives, magnification × 60 (50 μm confocal aperture) or × 100 (100 μm confocal aper-

ture). Series of optical sections (512 × 512 pixels: approximately 0.5 μm thick) were taken through entire organisms at a spacing of 0.7–1 μm. Three dimensional projections were prepared from the section series using Molecular Dynamics "Imagespace" volume rendering software running in Silicon Graphics UNIX workstations. Stereo projections were prepared either as stereo pairs or two colour overlays (anaglyphs; examined using red/green filtered spectacles) and printed using a Shinko CHC-S446i dye sublimation colour printer. Bacterial numbers were estimated using a 3D object count program within "Imagespace", whereby specified voxal (3D pixel) group sizes lying within preset maximum intensity thresholds are identified and counted.

Results

(i) Isotrichid ciliates

Dasytricha ruminantium. Most *D. ruminantium* showed no intracellular symbionts when taken through the hybridization procedure either with the archaeal or the bacterial probe (Fig. 1). Many organisms showed food vacuoles occupied by Bacteria (but not by Archaea). Sections of unstained control organisms or those stained with the archaeal probe showed no autofluorescence in food vacuoles and this suggests that no chloroplast products were present. These observations also suggest that this raptorial-feeding gymnostome ciliate does not extensively phagocytose plant-derived particles, and furthermore discriminates between different types of prokaryotic cells. It thus appears that only Bacteria are ingested. A small minority of the population (11 out of 447) were heavily infested with Bacteria. Published electron micrographs of unwashed organisms [26, 35, 44] confirmed the morphology and diversity of prokaryotes on the surface of organisms. In some cells they noted prokaryotes entangled in the cilia, in the cytoproct, and in food vacuoles. A possible explanation for low incidence is that the association may only be transient rather than permanent, or alternatively that those individuals with completely internalized Bacteria free in the endoplasm observed previously in electron micrographs and in the present study have become infected by opportunistic microorganisms, perhaps subsequently to physical injury or damage.

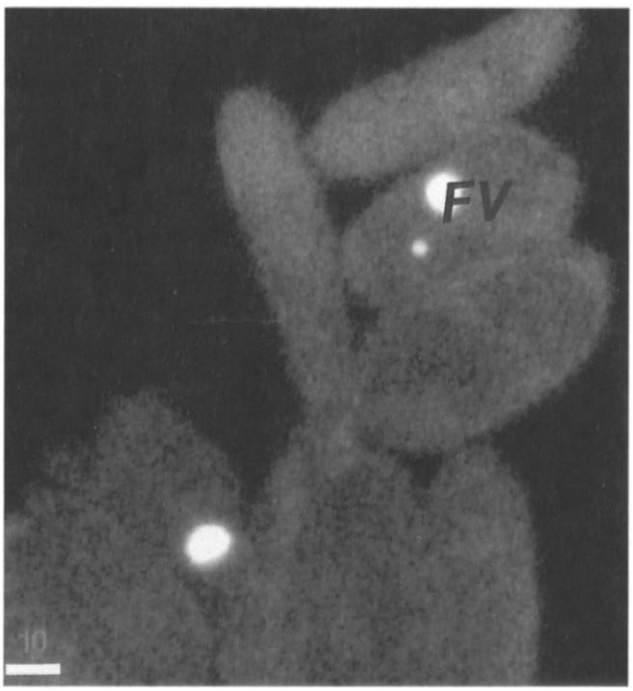
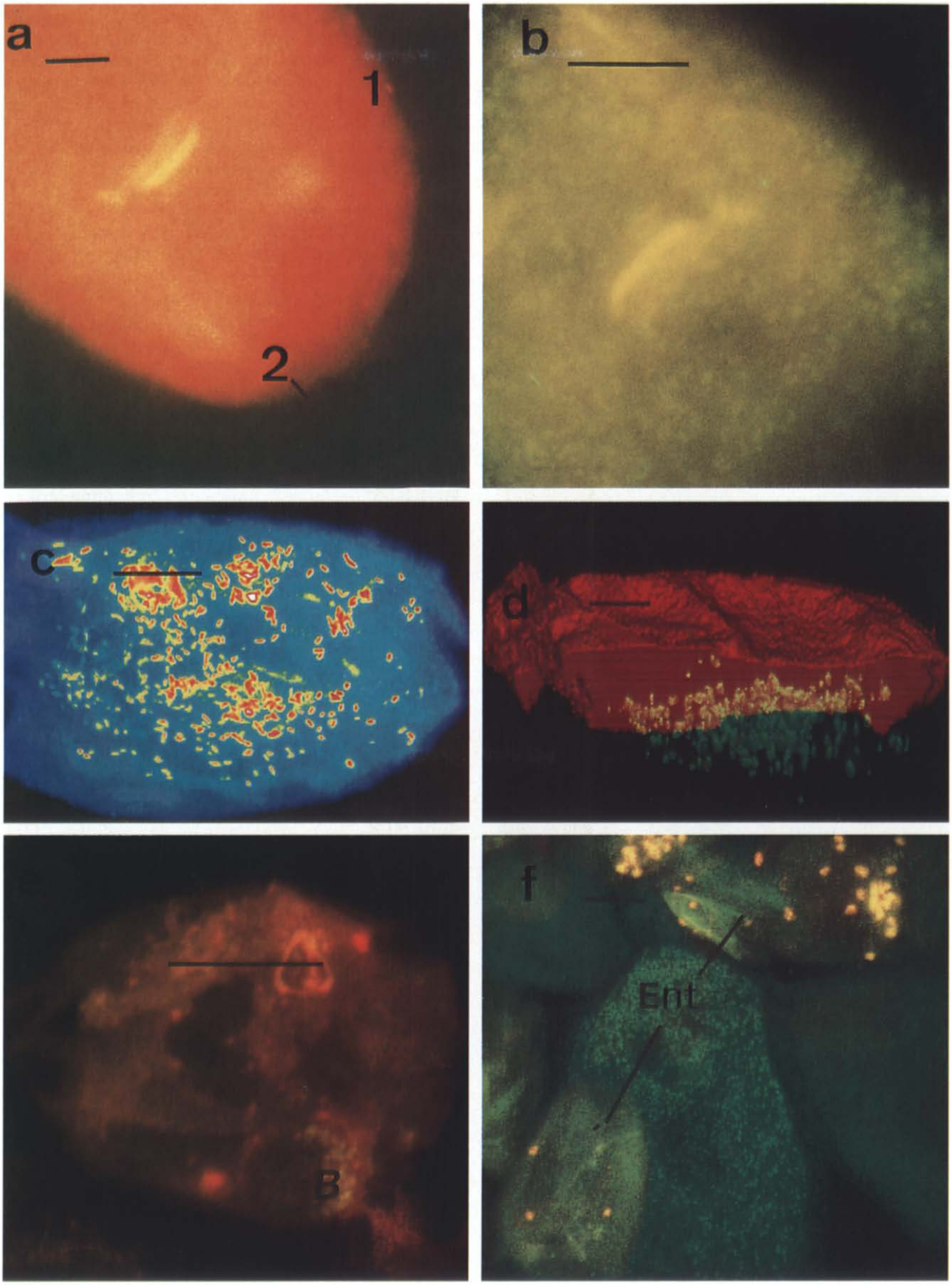


Fig. 1. *Dasytricha ruminantium.* Confocal scanning microscopy optical section after in situ hybridization with the bacterial oligonucleotide probe. FV = food vacuoles. The food vacuoles are the only sites of positive reaction to the 16S rRNA probe. Bar = 10 μm.

Isotricha spp. *Isotricha intestinalis* and other unidentified large isotrichs, like *D. ruminantium* often bear no internalized bacteria. In individuals containing prokaryotes, small numbers of both Archaea and Bacteria were present.

(ii) Entodiniomorphid ciliates

Polyplastron multivesiculatum: Large numbers > 250 Bacteria were invariably observed in this organism, they were widely distributed through all parts of the cytoplasmic space (Figs. 2, 3). Thus Fig. 2 (a, b) shows *Polyplastron multivesiculatum* stained with the archaeal and bacterial probes as revealed by epifluorescence microscopy; autofluorescence is a major problem especially at the excitation/emission wavelengths used for rhodamine (Fig. 2 a). Enumeration of the intracellular Bacteria is not possible due to the dense packing and overlapping evident in the deep focal plane of the microscope and blurring from superimposed out-of-focus images. It is also not possible to be unequivocal about the location of the Archaea present; two prokaryotes are clearly adherent to the outside of the protozoon, but others could be inside. In Fig. 2 b numerous Bacteria are seen as small green fluorescent objects widely scattered and thus extensively overlapping throughout the organism and again it is not possible to be sure that these are not on the outside surface of the organism. Larger green autofluorescent structures are also seen in unstained controls. Confocal optical sectioning (0.5 μm sections with 1.0 μm space between sections) provides clearly resolved images not obtainable by epifluorescence. A total of 40 images were obtained, and we only present two (Fig. 3 a). Here it is clear that the intracellular Bacteria are widely distributed within the cytosolic space and we can be sure that in this specimen no adherent Bacteria were present. Automated counting of Bacteria is rapid and checks for overlaps in their images between sections are routine (the procedure incorporated in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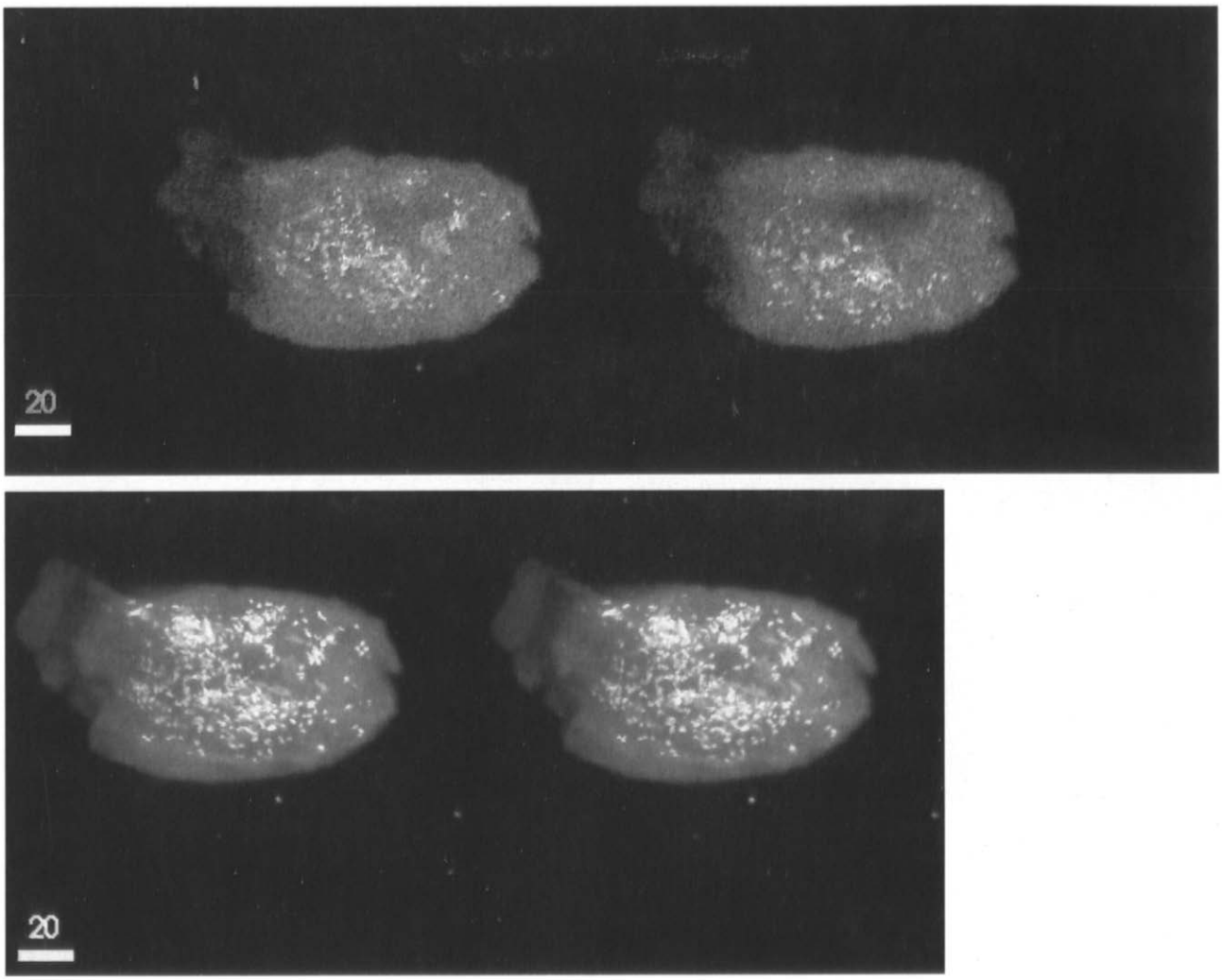

Fig. 3. (a) *Polyplastron multivesiculatum*. Confocal laser scanning microscopy; two optical sections (bacterial probe); the whole series consisted of 40 × 0.5 μm thick sections. Blurring due to overlap of out-of-focus information in Fig. 2 b is obviated and details at successive levels are resolved. (b) 3D reconstruction presented as a stereo pair. Bars = 20 μm.

software). The three dimensional reconstruction (Fig. 3 b) facilitates a complete view of the intracellular features of the protozoa. Digital image enhancement (Fig. 2 c) enables quantitation of the fluorescence of each bacterium and the imaging software enables the production of a fluorescence frequency distribution histogram of the population. Fluorescence intensity is an indication of the numbers of ribosomes per bacterium [3] and hence it may be possible to measure the growth rates of individual prokaryotes. Figure 2 d shows both the surface of the ciliate and its intracellular Bacteria. Computation of occupancy of voxels (assigned a volume of 50 μm^3, as this is approximately the space occupied by a single

◀ Fig. 2 a, 2b. *Polyplastron multivesiculatum*. Organisms were photographed under epifluorescent illumination after in situ hybridization with fluorescent archaeal (rhodamine) (a) or bacterial (fluorescein) (b) probes. Diffuse autofluorescence in (a), from out of focus material in these large protozoa makes the imaging of specifically stained prokaryotes impossible except those adherent around the periphery (indicated at 1 and 2); (b) shows the presence of a large number of small yellowish-green fluorescently-stained Bacteria. (c) Digitally enhanced image of a single section: colours of the artificial scale (black, blue, green, yellow, red to white) represent increasing fluorescence intensities. (d) Image as in (c), but reconstructed after latitudinal rotation and cut away to show surface features (in red) and internalized Bacteria (in green). (e) *Entodinium* spp. dual-probed for Archaea and Bacteria; both archeal (orange-red) and bacterial (green) endosymbionts are evident. In (e) the cluster of Bacteria (B) resembles the "cyst-like structure" previously described [33, 44]. In (f) *Entodinium* spp. (Ent) are indicated amongst other unidentified larger ciliates. Archaeal (yellow) and bacterial (green) endosymbionts are evident. Bars = 20 μm.

bacterium and clusters are rare) enables exact and rapid quantification. The protozoon shown has about 250 intracellular Bacteria. Hybridization with the Archaea-specific probe occasionally revealed externally adherent organisms (not shown), but no intracellular Archaea were observed.

Epidinium spp. In these organisms, intracellular Bacteria were usually not detected, whereas a few individuals contained Archaea. Occasionally a ciliate containing prokaryotes was noted, but these were a small percentage (about 20%) of those examined. One highly atypical individual contained approximately 160 Archaea and 16 Bacteria as determined from serial optical sections as explained and illustrated for *Polyplastron multivesiculatum.*

Other large entodinomorphid genera. Both probes gave positive results with these organisms. Some of the other large species (e.g. *Ostracodinium* spp., *Eremoplastron* spp. and *Diplodinium* spp.) always showed the presence of numerous Archaea and Bacteria.

Entodinium spp. these organisms showed the presence of both intracellular Archaea and Bacteria (Fig. 2e, f). The Bacteria-containing cyst-like structure (Fig. 2e) has been previously described as "parasitizing" its host [33, 35, 46].

Eudiplodinium maggii. No intracellular symbionts were usually observed in this species, although their apparent absence may be due to difficulties in detection associated with the presence of large amounts of highly autofluorescent particulate material of plant origin (Fig. 4). This autofluorescence arises from partially digested chlorophyll and its breakdown products. Out of 50 individuals, only 4 definitely contained intracellular Archaea.

Table 1 summarizes these data.

Discussion

Advantages of confocal laser scanning microscopy

Comparison of fluorescent images produced by a conventional epifluorescence microscope with the various presentations of a confocal laser scanning microscope illustrates the enormous advantages of the new technique, especially when studying large (optically deep) organisms, as is the case here.

Rumen protozoa and their endosymbionts

The isotrichid (holotrich) protozoa are responsible for the assimilation of soluble sugars as well as heterophagic ingestion of bacteria into their food vacuoles, whereas the entodiniomorphid ciliates are large particle feeders with a preference for comminuted plant particulates, starch and bacteria [43]. In general, the isotrichid rumen ciliates from the ovine rumen appear to be less colonized with intracellular symbionts than are their entodiniomorphid counterparts.

Whether or not the intracellular bacteria observed on in situ hybridization with fluorescent probes represent

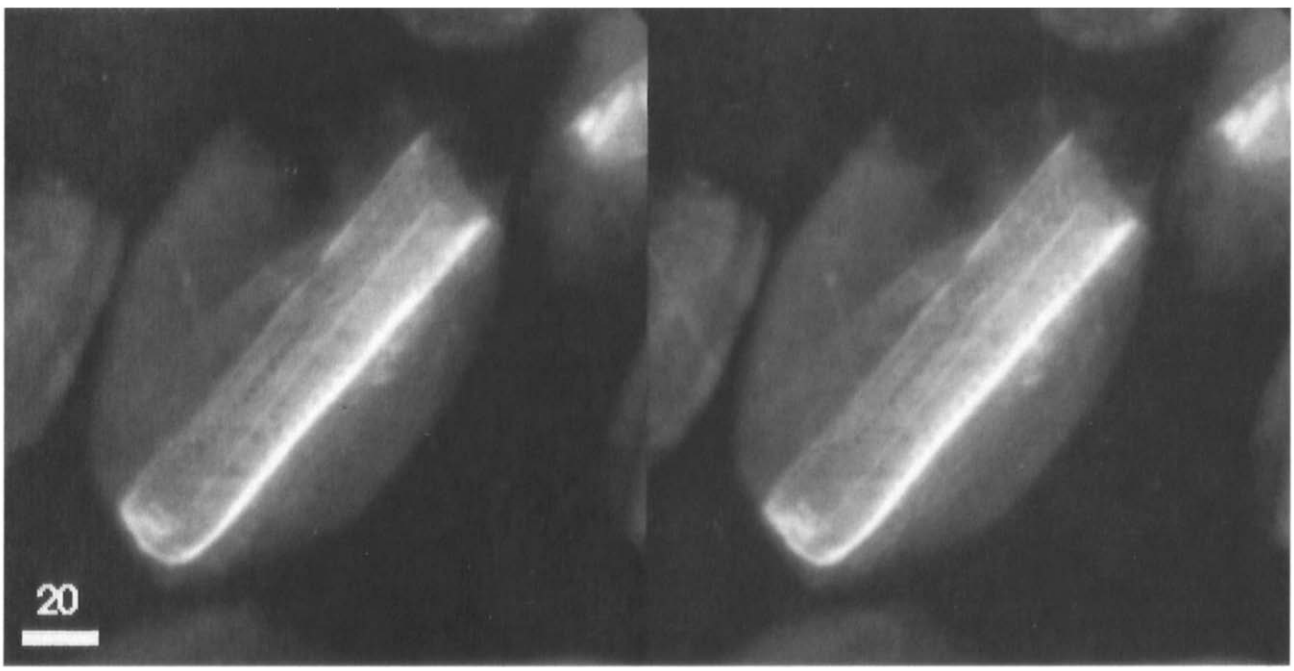

Fig. 4. *Eudiplodinium* spp., organisms after in situ hybridization with the archaeal probe (stereo pair). The large internalized brightly autofluorescent structure is a tracheid. No Archaea were observed within this organism. Bar = 20 μm.

Table 1. Presence of intracellular bacteria in rumen ciliate protozoa

Rumen Ciliate	Length (μm)	Protozoa observed	Hybridization with 16S rRNA probe specific for		Prokaryote negative
			Archaea	Bacteria	
Dasytricha ruminantium[a]	35–75	447	0	11	436
Isotricha spp.[b]	80–200	48	18	12	18
Polyplastron multivesiculatum	123–205	20	0	20	0
Epidinium spp.	105–150	43	7	2	34
Eudiplodinium maggii[c]	105–198	50	4	0	46
Entodinium spp.	22–95	243	104	86	53

a Occasional occurrence of individuals with large numbers of intracellular Bacteria. Food vacoules contain Bacteria but no Archaea.
b Only small number of intracellular bacteria of both types in some individuals.
c No intracellular bacteria observed; large volumes of autofluorescent plant-derived particles invariably present in the endoplasm.

cytoplasmically internalized organisms, or whether they are still enclosed in enveloping vacuole membranes cannot be decided without evidence from electron microscopy. Thus, for instance, in electron micrographs of *D. ruminantium* and in some entodiniomorphid species, many of the individual bacteria are observed within small single membrane vacuoles [28, 36]. As such, they may still be physically separated from the cytoplasmic space. Some or all of these organisms may represent ingested bacteria that are resistant to digestion by protozoal enzymes or a population of pathogens that have not breached the protozoal defences. Large and obviously mixed cell populations may indicate opportunistic invasion of physically or nutritionally stressed, or dead ciliates. However, where low numbers of intracellular prokaryotes are only occasionally observed (e.g. in the isotrichid holotrichs), these may represent transient occupancy.

In the entodiniomorphid species, interesting and specific differences suggest more permanent associations which probably do represent endosymbiotic relationships of the type proposed for the methanogens of the anaerobic free-living ciliates (e.g. *Metopus* spp.) that inhabit freshwater and sediments [18, 19]. The proposal that the archaeal intracellular populations in rumen ciliates also represent methanogens [20] is reasonable and probably correct, although it should be noted that autofluorescence due to F 420 is not *uniquely* associated with methanogens and the cofactor has also been isolated from other Archaea, *Streptomyces* spp., *Nocardia* spp. and *Mycobacterium* spp. [8].

It is also not sufficient to claim that a UV excitable endogenous fluorochrome in bacteria is necessarily F 420 unless extraction and partial purification is attempted. Other coenzymes (e.g. oxidized flavins) have similar but not identical characteristics to those of F 420, and thus in vivo characterization may not be unequivocal. Further work is necessary to identify the intracellular Archaea and Bacteria described here before functional relationships can be elucidated. Attempts to cultivate intracellular endosymbionts from *P. multivesiculatum* were unsuccessful [28]. Even in the case of *Methanobacterium* spp., in the free living anaerobic ciliates *Metopus striatus, M. contortus* and *M. palaeoformis*, the lack of co-evolutionary sequence adaptation of the kind expected in long-term endosymbionts [16, 17, 38] raises questions about the permanence of the observed associations.

Variations in the reported presence of "endosymbionts" in rumen ciliates have several possible underlying sources: (i) variations between ruminant hosts; (ii) variations with diet; (iii) variations as a consequence of protozoal isolation procedures. The rumen ciliates differ in their aerotolerance [30], and it is possible that the more aerotolerant rumen protozoa and methanogenic endosymbionts may not be compatible. The virtual absence of Archaea from *D. ruminantium* may be a result of the protocol adopted. In this study we used fistulated sheep fed once daily and employed an extensive washing and starvation procedure designed to minimize both the occurrence of externally attached prokaryotes and those internalized within food vacuoles. This method contrasts to that used previously [20], in which a minimal washing procedure was used purposely so as to produce *D. ruminantium* with detectable endosymbionts. We have also previously shown that the present procedure produces *D. ruminantium* suspension without detectable prokaryotic markers [48] and able to actively produce H_2 [23]. If methanogens were present in the numbers shown in the unwashed, non-starved suspensions [20] it is probable that CH4 would be the major metabolite to accumulate.

A number of biochemical studies [12, 23, 49], have been carried out on extracts of well-washed *D. ruminantium* populations where no bacterial marker (diaminopimelic acid) could be found, and no reaction detected to *Limulus polyphemus* antibody [47]. Observations presented in this paper confirm that biochemical activities measured in those studies are not due to prokaryotes. However, for *P. multivesiculatum* and *Eudiplodinium maggii* [13, 14], excretion of fermentative products (butyric, acetic and lactic acids,

H_2, and CO_2) is probably modulated by the interaction with intracellular Bacteria. Further work is necessary to isolate and characterise fully these intracellular prokaryotes in order to elucidate the nature of the protozoal – bacterial interactions. These new insights into the important differences between different species of rumen ciliates are crucial in establishing the metabolic input of protozoa in the rumen and identifying the role of the ciliates in ruminal methanogensis which has both economic and environmental implications.

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