FEMS Microbiology Letters 117 (1994) 157–162

Published by Elsevier

FEMSLE 05875

Some rumen ciliates have endosymbiotic methanogens

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(Received 7 December 1993; revision received and accepted 11 January 1994)

Abstract: Most of the small ciliate protozoa, including *Dasytricha ruminantium* and *Entodinium* spp. living in the rumen of sheep, were found to have intracellular bacteria. These bacteria were not present in digestive vacuoles. They showed characteristic coenzyme F_{420} autofluorescence and they were detected with a rhodamine-labelled Archaea-specific oligonucleotide probe. The measured volume percent of autofluorescing bacteria (1%) was close to the total volume of intracellular bacteria estimated from TEM stereology. Thus it is likely that all of the bacteria living in the cytoplasm of these ciliates were endosymbiotic methanogens, using H_2 evolved by the host ciliate to form methane. Intracellular methanogens appear to be much more numerous than those attached to the external cell surface of ciliates.

Key words: Rumen ciliates; Endosymbiotic methanogens; Methane; Greenhouse gas; *Dasytricha; Entodinium*

Introduction

Methane is the most abundant hydrocarbon in the troposphere; it is an important greenhouse gas, and much effort is directed at identifying and quantifying the principal sinks and sources [1,2]. The emission from ruminants is one of the largest biogenic sources; it is also economically important as it can represent up to 15% of the animals' total energy intake [3]. We have recently made some observations which cause us to question where this methane comes from.

The rumen is the modified forestomach of cattle, sheep and some other big herbivores. It contains very large numbers of anaerobic microorganisms [4], especially bacteria and ciliate protozoa (approx. 10^{10} and 10^6 ml^{-1}, respectively), and these together with chytrid fungi degrade plant polymers to volatile fatty acids (the ruminants' main source of carbon and energy), H_2 and CO_2. The H_2 is used by methanogenic bacteria for the energy-yielding reduction of CO_2 to CH_4. The methane, which is useless to the ruminant, is lost to the atmosphere. It is commonly assumed that the methanogens live freely in the rumen and that their functional role is simply to carry out the final stage of anaerobic decomposition. Methanogens have previously

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SSDI 0378-1097(94)00020-R

been shown to be associated with the external surfaces of ciliates [11,12]. Here we provide the first documented case of methanogens living inside rumen ciliates.

Materials and Methods

Samples of rumen contents were obtained on two occasions during the summer of 1993 from six rumen-fistulated sheep. The rumen contents of these sheep had been defaunated initially by treatment with manoxol (dioctyl ester of sodium sulphosuccinate) [20], and the three following protozoal populations had been established in paired donors by re-inoculation with *Dasytricha ruminantium / Entodinium* spp., *Polyplastron multivesiculatum / Entodinium* spp. and a B-type population. The sheep were fed once daily with a pelleted cereal and cereal by-product based concentrate (250 g) that contained 35% barley and 18% crude protein; hay and water were available ad libitum. Samples of rumen contents (500 ml) were withdrawn before the sheep received the daily feed ration and the ciliate populations were isolated, cleaned and concentrated by differential filtration [5,20]. Methanogenic ecto- and endosymbionts were detected microscopically from coenzyme F_{420} autofluorescence in response to UV excitation [6,7]. A tetramethylrhodamine-

Figs. 1–4. Transmission electron micrographs. **Fig. 1.** *Dasytricha ruminantium*. The small electron-dense bodies in the ciliate's cytoplasm are the methanogens; the large circular body is the macronucleus of the ciliate, and there is a circumferential ring of hydrogenosomes (h); scale bar represents 2 μm. **Fig. 2.** The two morphotypes of methanogens (arrows) found in *D. ruminantium*. Scale bar represents 2 μm (Figs. 2 and 3). **Fig. 3.** Methanogens (electron-dense bodies) in *Entodinium* sp. They appear similar to those in *D. ruminantium* (Fig. 2). **Fig. 4.** Higher magnification of the two morphotypes of symbiotic methanogens found in both types of rumen ciliate. Scale bar represents 1 μm.

labelled Archaea-specific oligonucleotide probe was constructed [8,9] and used for whole-cell hybridisation experiments [9]. Ciliates were prepared for transmission electron microscopy by fixation for 1 h with 2% glutaraldehyde followed by 30 min in 2% OsO_4 (both fixatives prepared in 0.1 M Na-cacodylate buffer pH 7), dehydration in ethanol, and embedding in Spurr resin. The volume fraction of methanogens within the ciliate cytoplasm was determined by light microscopy after compressing formalin-fixed ciliates to a (measured) thickness of 5–20 μm (depending on species). The total volume of bacteria in the ciliate cytoplasm was estimated from electron micrographs, using stereological methods [10].

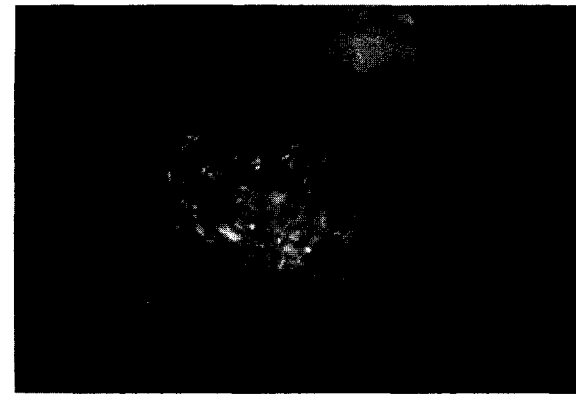


Fig. 5. Autofluorescence (following UV excitation) of the two endosymbiotic methanogen cell types inside *D. ruminantium*. The ciliate is approximately 70 μm in length.

Results and Discussion

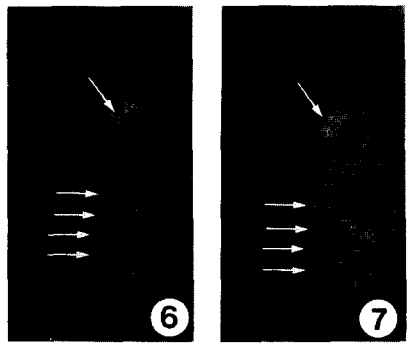
Almost all rumen ciliates supported some methanogens on their external surfaces. Numbers of these were usually less than 10, and never more than 20 per ciliate. These observations are consistent with previous work [12]. More importantly, the most abundant ciliate species (*Dasytricha ruminantium* and *Entodinium* spp., which together accounted for > 90% of all rumen ciliates) also contained intracellular bacteria free in the cytoplasm. Electron microscopy (Figs. 1–4) confirmed that these bacteria were enclosed, usually singly, within membrane-bounded vacuoles, and suggested that they were not digested by the ciliate (they did not appear in food vacuoles). In *Entodinium* spp. and *D. ruminantium*, respectively, there were on average 96 and 520 endosymbiotic methanogens per ciliate (> 1000 in some *D. ruminantium*; see Table 1). These bacteria had the characteristic F_{420} autofluorescence of methanogens [13] (Fig. 5), and they were readily detected (Figs. 6, 7) by in situ probing with a rhodamine-labelled Archaea-specific oligonucleotide [8]. Two independent measures were obtained of the percentage of the ciliate volume accounted for by endobionts (by direct measurement using light/autofluorescence microscopy, and by TEM stereology; Table 1). Bearing in mind that self-shading of superimposed fluorescing bacteria may lead to a slight understimate of their volume, and that both methods do have some inherent imprecision, the results obtained by the different methods are quite close to each other. Together, they point to the likelihood that all of the intracellular bacteria are methanogens and that they account for approximately 1–2% of host ciliate volume. Roughly the same figure has been determined on numerous occasions for the methanogens in free-living ciliates [13], but it would be premature to claim that the endosymbioses are functionally equivalent in free-living

Table 1

Characteristics of the small rumen ciliates and their intracellular bacteria

	Entodinium spp.	*Dasytricha ruminantium*
Ciliate cell volume ($\mu m^3 \times 10^3$)	6.0 (2.5–15)	25 (13–38)
Number of autofluorescing bacteria inside each ciliate	96 (0–283)	520 (145–1035)
Autofluorescing bacteria/ciliate (volume %)	0.93	0.97
Intracellular bacteria/ciliate (volume %; TEM stereology)	1.2	1.6

Values are arithmetic means and total ranges ($n = 30$ and 25 for *Entodinium* spp. and *Dasytricha ruminantium*, respectively). Seven of the 30 cells of *Entodinium* spp. did not have intracellular bacteria. Ciliate volumes were determined by measuring the three axes of formalin-fixed partially flattened cells.



Figs. 6, 7. Fluorescence images of a cell of *Entodinium* sp. The cell has been probed with a rhodamine-conjugated Archaea-specific probe. On the left (**Fig. 6**) is the (rapidly fading) residual F_{420} autofluorescence, marking the location of the methanogens. **Fig. 7** shows the fluorescence from the Archaea-probe which highlights the same particles (e.g. arrows) showing autofluorescence (**Fig. 6**). The wavelength bands for excitation of autofluorescence and rhodamine fluorescence were mutually exclusive, and excitation of a probe-free rhodamine control gave a negative result.

and in rumen ciliates. Two differences are worth mentioning. Firstly, some representatives of the *Entodinium* spp. were free of methanogens: coexistence of congeneric ciliates with and without endosymbionts is unknown for free-living ciliates, but it is possible that more than one *Entodinium* species was present. Secondly, the same two morphotypes of methanogens were simultaneously present in the *Entodinium* spp. and in *D. ruminantium*. It is unknown if these morphotypes belong to the same species. In cases where polymorphic transformation of methanogens takes place in free-living ciliates, it is possible to detect intermediate stages in transmission electron micrographs [9]. We were unable to do this in the case of the rumen ciliates. There is no established case of more than one type of endosymbiotic methanogen coexisting in an anaerobic ciliate.

Rumen entodiniomorphid and isotrichid holotrich ciliates have been shown to produce about 20 pmol H_2 h^{-1} [14,15,20,21,22]. If this is consumed by endosymbiotic methanogens, they will produce 5 pmol CH_4/ciliate/h [16]. It is therefore of interest that enriched fractions of rumen ciliates have actually been shown to produce on average 5.3 pmol CH_4/ciliate/h [11]. For a typical sheep with 5 l of rumen liquor [17], producing 19 l CH_4 per day [18], methanogenic ciliates (5×10^5 per ml) could account for 37% of total methane production. This figure will undoubtedly vary greatly in relation to such factors as host diet and ciliate community structure.

It is not surprising that methanogens have found this intracellular habitat. Rumen ciliates depend on H_2-evolving fermentation which is inhibited by high pH_2, so an interspecies H_2 transfer will benefit both the ciliate and its symbionts [13,16,19]. What is surprising is the absence of any other published reference to intracellular methanogens in rumen ciliates, forcing the conclusion that the phenomenon may not be ubiquitous in ruminants. We have observed that the methanogen content of rumen ciliates is variable. Methane production by ruminants is also variable [18].

Methane emission from ruminants reduces their productivity [23] and contributes to the rising level of methane in the atmosphere [1], so it is important to identify precisely the sources and understand the processes by which it is produced. The elimination of ciliate protozoa from the rumen has been proposed as a means of increasing the productivity of ruminants [3,20]. As defaunation also reduces ruminal methanogenesis [20,24], this treatment may have the additional benefit of reducing the adverse environmental impact of farmed ruminants.

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