Some rumen ciliates have endosymbiotic methanogens

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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 F420 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 H2 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), H2 and CO2. The H2 is used by methanogenic bacteria for the energy-yielding reduction of CO2 to CH4. 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
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 sulphasuccinate) [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 F₄₂₀ autofluorescence in response to UV excitation [6,7]. A tetramethylrhodamine-
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% OsO₄ (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].

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 F₄₂₀ 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 underestimate 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

<table>
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<tr>
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<th>Entodinium spp.</th>
<th>Dasytricha ruminantium</th>
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<tbody>
<tr>
<td>Ciliate cell volume (μm³ x 10⁻³)</td>
<td>6.0 (2.5–15)</td>
<td>25 (13–38)</td>
</tr>
<tr>
<td>Number of autofluorescing bacteria inside each ciliate</td>
<td>96</td>
<td>520</td>
</tr>
<tr>
<td>Autofluorescing bacteria/ciliate (volume %)</td>
<td>0.93 (0–283)</td>
<td>0.97 (145–1035)</td>
</tr>
<tr>
<td>Intracellular bacteria/ciliate (volume %; TEM stereology)</td>
<td>1.2</td>
<td>1.6</td>
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</table>

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 F420 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 H₂ h⁻¹ [14,15,20,21,22]. If this is consumed by endosymbiotic methanogens, they will produce 5 pmol CH₄/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 CH₄/ciliate/h [11]. For a typical sheep with 5 l of rumen liquor [17], producing 19 l CH₄ per day [18], methanogenic ciliates (5 × 10⁵ 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 H₂-evolving fermentation which is inhibited by high pH₂, so an interspecies H₂ 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.

References