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Association of Methanogenic Bacteria with Rumen Ciliates

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In 11 species of rumen ciliates belonging to nine genera of the family *Ophryoscolecidae* (order *Entodiniomorphida*) an ectosymbiosis with methanogenic bacteria was found. The bacteria could be identified as methanogens on the basis of the presence of specific fluorescent coenzymes (F_{350} and F_{420}). This somatic interaction may reflect a metabolic interaction in which efficient interspecies hydrogen transfer benefits both partners.

Hydrogen and carbon dioxide are the main substrates for methanogenesis in the rumen (12, 13). Hydrogen can be delivered to the methanogenic bacteria by hydrogenic bacteria (4, 20) and anaerobic ciliates (2, 12, 17, 19). We now report data indicating that this metabolic interaction is accompanied by a somatic interaction between methanogenic bacteria and ciliates belonging to the family *Ophryoscolecidae* (order *Entodiniomorphida*).

MATERIALS AND METHODS

A total of 500 ml of rumen contents were collected from each cow immediately after slaughter in the slaughter house of Nijmegen. The samples were kept for 1 h at 37°C to allow sedimentation of ciliates and coarse material. Bright-field and epifluorescence microscopy was done with a Leitz Dialux microscope equipped with water immersion objectives and a Ploemopak 2.3 illuminator for incident light (filter systems A and D). Methanogenic bacteria were identified on the basis of the presence of the fluorescing coenzymes F_{350} and F_{420} in these organisms (7). The preparations were examined by interrupting the bright-field illumination under conditions of continuous epifluorescence, since no significant fading of fluorescence occurred. The ciliates were identified according to Dogiel (8); taxonomy was adopted from Corliss (6). Stainings were performed with methyl green and acridine orange. Association is defined as attachment of at least 20 bacteria to the surface of a ciliate cell. Attached bacteria could be easily distinguished from both engulfed and freely floating bacteria by focusing on the ciliate at different depths with a ×40 or ×100 objective and by producing a slight streaming in the preparation. Scanning electron microscopy was done according to Fenchel et al. (11) after freeze-drying (except one washing with water instead of three was used).

RESULTS

Ciliates present in the rumen. Table 1 presents 11 species of entodiniomorphid ciliates which were regularly found in a study of 25 rumens from healthy cows. Four other species (*Eudiplodinium rostratum, Entodinium vorax* subsp. *vorax* and *E. vorax* subsp. *bispinosum, Entodinium rostratum* subsp. *rostratum* and *E. rostratum* subsp. *bifidum, Entodinium triacum*) were occasionally found, and a few cells of *Epidinium ecaudatum* subsp. *bicaudatum, E. ecaudatum* subsp. *tricaudatum*, and *E. ecaudatum* subsp. *hamatum* were encountered. Because of their scarcity, these organisms could not be included in this study on the association with methanogens. Although Eadie (9), Abou Akkada et al. (1), and Clarke (5) reported a mutual exclusion of *Eudiplodinium maggii* and *Polyplastron multivesiculatum* in stable rumens, we found these two species living together in samples from four rumens. Possibly this coexistence reflects some instability of the rumen biotope. In two of the four cases, many of the *Eudiplodinium maggii* cells were much larger than those of this species from populations without *P. multivesiculatum*. Eadie (10) suggests that such an increase in size of the first organism might be a reaction on the predatory behavior of the latter.

In almost all rumens investigated three species of isotrich ciliates (*Dasytricha ruminantium, Isotricha intestinalis*, and *Isotricha prostoma*) were present in different amounts. These ciliates were excluded from this study since no externally associated methanogens could be observed.

Association of methanogens with rumen ciliates. From the 11 species listed in Table 1, at least 200 individuals were chosen at random and examined to determine the occurrence of association with methanogens, according to the procedures described in Materials and Methods. A comparison of bright-field and epifluorescence microscopy revealed that virtually all bacteria attached to the ciliate cell surface could be identified as methanogens on the basis of their specific fluorescence (Fig. 1). The attached cells are rods 0.9 to 3.8 μm long and 0.6 to 0.7 μm wide which occur as clusters or long chains. Whereas

TABLE 1. *Association between methanogenic bacteria and rumen ciliates*

Species	No. of rumens in which species was found (n = 25)	Cells exhibiting association[a] (% of total)	Range of association[b] (%)
Diplodinium dentatum (syn. *Diplodinium denticulatum*)	16	30	20–100
Diploplastron affine (syn. *Eudiplodinium affine*)	3	40	35–45
Enoploplastron triloricatum (syn. *Ostracodinium triloricatum*)	7	30	25–35
Entodinium simplex	25	15	10–20
Entodinium caudatum	16	40	20–50
Entodinium longinucleatum	14	8	5–10
Epidinium ecaudatum	18	20	0–40
Eremoplastron bovis (syn. *Eudiplodinium neglectum*)	12	40	10–100
Eudiplodinium maggii	20	55	30–90
Ostracodinium obtusum	25	55	20–95
Polyplastron multivesiculatum	10	50	20–100

[a] Frequency of association among the cells of the indicated species as found in counts on various rumens. From each species at least 200 individuals were checked for association. Not all species were investigated simultaneously and in the same rumen.

[b] The range indicates the lowest and highest percentages of association found in counts on various rumens.

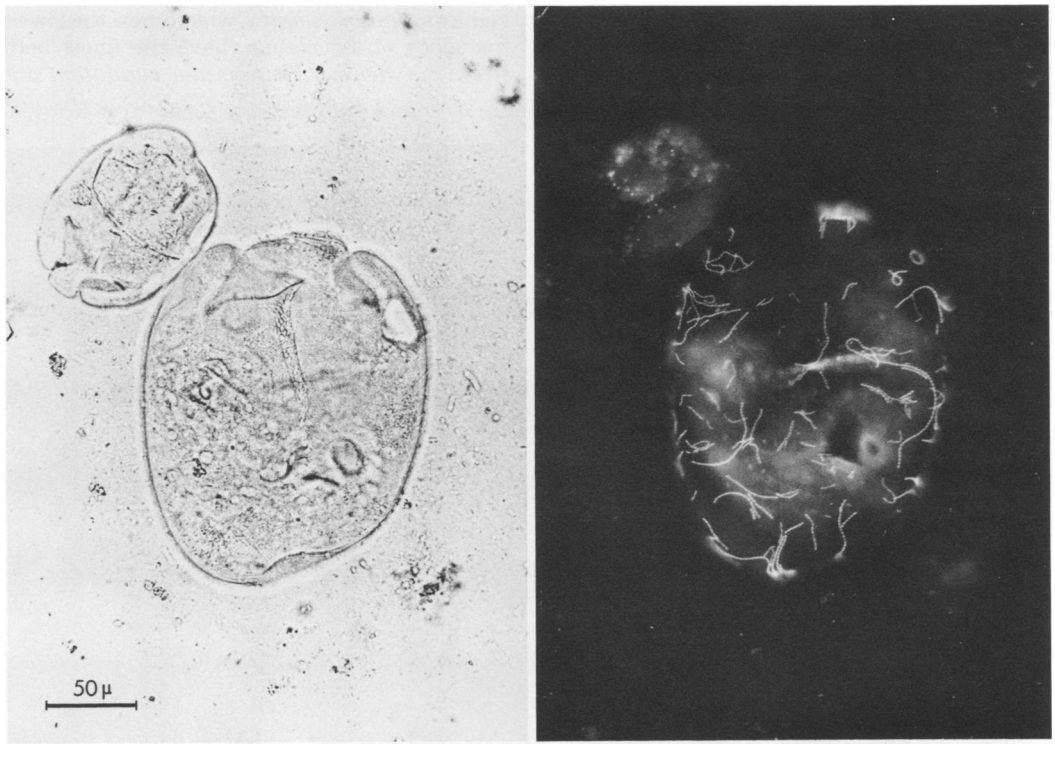

FIG. 1. *Cell of Eudiplodinium maggii (large ciliate) and Eremoplastron bovis (small ciliate) in bright-field (left) and epifluorescence (right) illumination. The methane bacteria are visible as bright rods attached to the Eudiplodinium cell surface. The fluorescent particles not in focus are engulfed plant material and methane bacteria.*

single cells of methanogens were abundant in the rumen fluid, chains of these organisms were seldom found floating freely or attached to substances other than ciliates. Probably the attachment of the bacteria to the ciliate pellicle favors the growth of the cells in chains.

The frequency of association among the various rumen samples varied over a broad range

(Table 1), probably due to large differences in diet and time of last feeding of the animals. All species examined appeared to exhibit the association, albeit to various extents: only 8% of the cells of *Entodinium longinucleatum* bore attached methanogens, whereas more than half of the cells of *Ostracodinium obtusum* and *Eudiplodinium maggii* did so. Even between the subspecies of one species (not shown in Table 1) such differences may be present: *Epidinium ecaudatum* subsp. *caudatum* and *Epidinium ecaudatum* subsp. *ecaudatum* showed 5 and 35% association, respectively. In *Epidinium ecaudatum* subsp. *caudatum* the associated bacteria were frequently located parallel to the oral syncilia (Fig. 2), whereas in the other species the bacteria were scattered over the ciliate cell surface. Creases and folds in the ciliate pellicle were locations frequently colonized by methanogens. Association also may occur with ciliates which are apparently in a state of decay; whether the methanogens had colonized the decaying cell or had remained attached from the time the ciliate was living is yet to be determined.

Scanning electron microscopy shows the attached bacterial cells, singly and in chains (Fig. 3).

DISCUSSION

Somatic association between ciliates and methanogenic bacteria could be observed in all species of the genera *Diplodinium*, *Epidinium*, *Diploplastron*, *Enoploplastron*, *Entodinium*, *Eremoplastron*, *Ostracodinium*, *Eudiplodinium*, and *Polyplastron* commonly found in the rumen. The differences in frequency of association found with the same ciliates in the various rumen samples might reflect dietary differences in the cows; similarly, the abundancy of rumen ciliates is a reflection of availability of energy and nitrogen and of dietary quality (5).

The differences found in frequency of association between the taxonomic groups of ciliates are still poorly understood. The physical structure of the pellicles as expressed in the body striation of the different groups (15, 18) may play a role, since *Entodinium longinucleatum* and *Entodinium simplex*, which show the lowest frequency of association, have the finest body striation, whereas *Entodinium caudatum* and

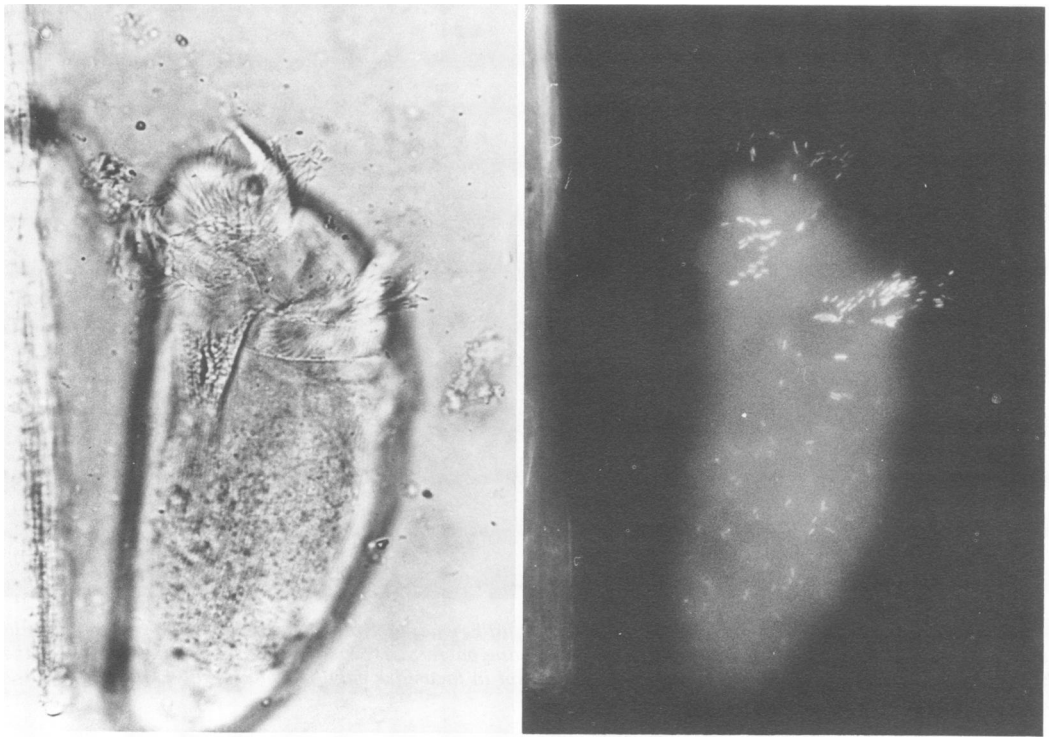

FIG. 2. *Cell of Epidinium caudatum subsp. ecaudatum in bright-field (left) and epifluorescence (right) illumination. The methane bacteria form a moustache-like configuration parallel to the syncilia. Magnification as in Fig. 1.*

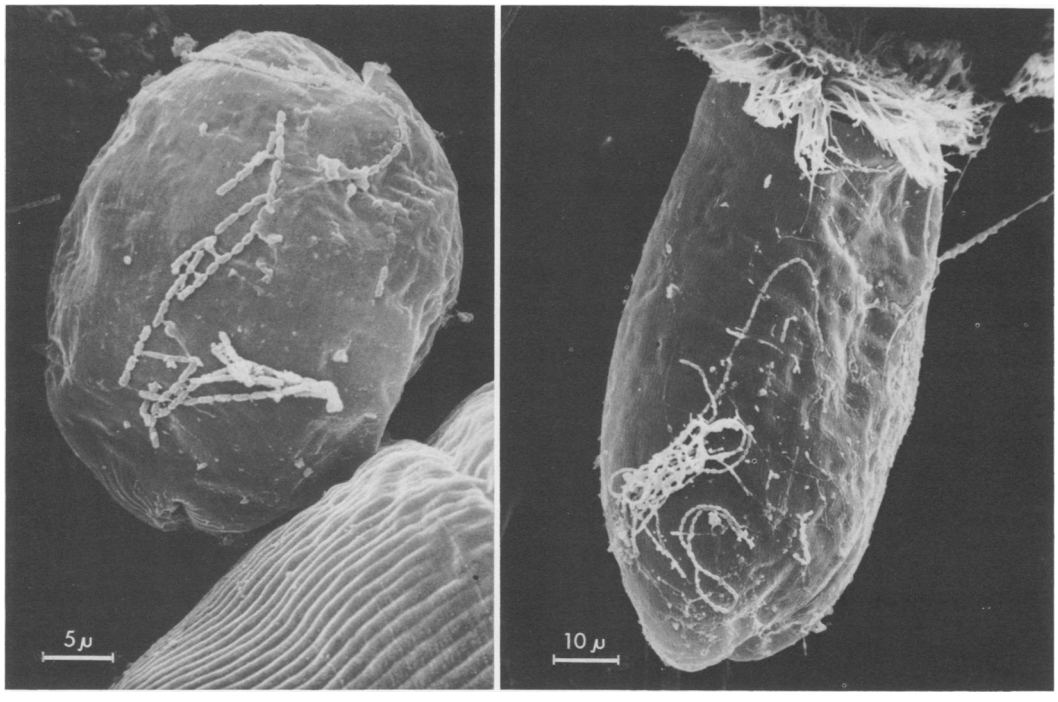

FIG. 3. *Scanning electron micrograph of putative methane bacteria attached to rumen ciliates: (left) probably Eremoplastron bovis; (right) Diplodinium dentatum.*

P. multivesiculatum, which both have a high frequency of association, exhibit a coarse surface structure. Also, the complicated interior structure of the entodiniomorphid cell cortex, with its differentiation in four cortical layers (16), may influence association. It appears attractive to correlate association of methanogens with transport of metabolites through the cell cortex, since close attachment of methanogenic bacteria occurs preferably to ciliate cells. Production of hydrogen by rumen ciliates is described by Abou Akkada and Howard (2), Hungate (12), Prins and Prast (17), and Williams and Harfoot (19); hydrogen is inhibitory to ciliate metabolism and is, together with carbon dioxide, the main substrate for methane synthesis in the rumen (12, 13). Attachment of methanogenic bacteria to rumen ciliates would be of selective value to both organisms since it enables an effective interspecies hydrogen transfer by which both can grow faster (20).

Bacteria attached to ciliates from the ovine rumen were previously described by Imai and Ogimoto (14), who demonstrated the presence of gram-positive, spherical or ovoid cells which reacted with antisera against *Streptococcus bovis* and *Ruminococcus albus*. Furthermore, ectosymbiotic bacteria were described for the sand-dwelling ciliates, intestinal flagellates, a few free-living flagellates (3), and ciliates from marine, sulfide-containing sediments (11). The latter organisms, which live (like the rumen ciliates) in a biotope free of oxygen, are covered with relatively high numbers of bacteria as compared with the rumen ciliates. This may reflect differences in the quantities of substrate flow and utilization or a better morphological adaptation of sulfide ciliate species to the attachment of bacteria.

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