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KENTROPHOROS: A MOUTHLESS CILIATE WITH A SYMBIOTIC KITCHEN GARDEN

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ABSTRACT

The sand ciliate *Kentrophoros* carries on its dorsal surface a dense layer of sulphur bacteria which accounts for about half the biomass of the symbiotic consortium. It has previously been shown that the ciliate phagocytises the symbionts through its cell surface. Here we confirm this observation. Using S-35 labelled sulphide and a cytochemical method we demonstrate that the bacteria are capable of oxidising sulphide and of storing elemental sulphur in the cells. Using C-14 labelled bicarbonate we show that the bacteria are autotrophs; quantitative autoradiography yielded a maximum value of carbon specific uptake of 0.13 h^{-1} for the bacteria; this is sufficient to explain a ciliate generation time of about 18 hours. The relatively low growth rate may reflect sub-optimal incubation conditions. The ciliates show a chemosensory response to oxygen tension and tend to accumulate at a pO_2 of somewhat less than 5% atm sat; this may optimise bacterial metabolism in sediments where the chemical gradients are not too steep. The ciliates do not show a chemosensory response to sulphide. While many other types of ciliates harbour ecto- and endosymbiotic bacteria and many are specialised for life in microaerobic habitats, *Kentrophoros* seems to be unique among ciliates through its dependence on symbiotic chemolithotrophic bacteria as food. The symbiotic bacterium divides longitudinally, but in other respects it most closely resembles members of the genus *Thiobacterium*.

INTRODUCTION

The discovery of the fauna associated with hydrothermal vents and of the role of chemolithotrophy and symbiosis between invertebrates and chemolithotrophic bacteria in these habitats has recently inspired the search for similar phenomena in more shallow and accessible marine habitats (Southward 1987). Nevertheless, the significance of chemolithotrophy to the carbon flow within sediments has been known for some time (e.g. Fenchel 1969) and symbioses between chemolithotrophs and eukaryotes have periodically been described (the significance of these relationships was not always clearly understood). In this paper we discuss one such example: the ciliated protozoon *Kentrophoros*.

This organism was first described by Sauerbrey (1928). Kahl (1935) observed that the peculiar structures covering one side of the organism are bacterial rods

containing sulphur granules. Fauré-Fremiet (1950a, b) studied the bacteria in more detail; he noted their peculiar longitudinal fission and characterised them using various cytochemical staining methods. Finally, Raikov (1971, 1974), using electron microscopy, showed that the bacteria are phagocytised along the entire dorsal side of the mouthless ciliate, which apparently depends on the symbionts for food. Raikov (op. cit.) also suggested that the bacteria are chemolithotrophic sulphide oxidisers. Raikov then, should appropriately be credited for being the first to recognise an example of the symbiosis between chemolithotrophic bacteria and eukaryote marine organisms.

Here we demonstrate experimentally that the bacteria are autotrophic and that they oxidise reduced sulphur compounds. We offer some quantitative estimates of the rate of carbon fixation by the bacteria and the possible growth rate of the ciliate. Furthermore, we describe the behavioural properties of the ciliate which allow it to optimise the activity of the symbionts. Using transmission electron microscopy we are able to support Raikov's principal observation (that the ciliate phagocytises its symbionts over a large part of its cell surface) and to supplement his description of the bacteria.

We acknowledge support from the Danish Natural Science Research Council (grants nos 11-6480 and 11-6926) and from the Carlsberg Foundation (grant no 87-0119/40). We are grateful to Ms Harriet H. Hansen for preparative work for TEM, to Ms Jeanne Johansen for technical assistance and to Mr. Holger Knudsen for photographic work.

MATERIAL AND METHODS

Kentrophoros species occur in fine, well sorted sands with a reduced, sulphide layer at least some centimetres beneath the sediment surface. They are rare or absent where the reduced layer is so close to the sediment surface that the latter is covered by sulphur bacteria. We collected sand samples in Nivå Bay (a shallow sandy bay about 15 km south of Helsingør, see Fenchel 1969) during spring and summer of 1988. In the laboratory ciliates were picked individually from petri dishes with 2-3 ml of sand and seawater or they were extracted using the Uhlig seawater ice method (see Fenchel 1969).

Altogether eight species of *Kentrophoros* have been described (Dragesco 1960, Raikov 1962) but the species systematics is not entirely satisfactory and their identification is difficult. Most of the specimens we studied were probably *K. fasciolata* Sauerbrey, but it is possible that we were dealing with two species: one measuring 160-200 μm and the other, around 500 μm (Figs 1-2, 4). According to Dragesco (1960), *K. fasciolata* is very variable in length and the large and small forms we studied seem to have a similar number of kineties (about 12). The other form, found together with the above mentioned ciliates, has a characteristic dorsal "keel" and conforms closely to *K. latum* Raikov (Fig. 3).

Morphometry

Estimates of surface area and volume of ciliates were based on cells squeezed between a microscopic slide and a cover slip supported by petroleum jelly so that the cell had plane parallel sides; the thickness of the preparations was measured with the scale of the focusing adjustment of the microscope. The dimensions of the bacteria were obtained from light microscopy and from TEM photographs. In all cases, estimates of areas and linear dimensions were made with a semi-automatic image analyser (Mop Videoplan, Kontron).

Autoradiography

For the demonstration of CO_2 fixation we incubated ciliates (usually around ten cells) in a test tube with 12 ml of seawater (salinity: 16‰, a typical value for the sampling locality) with phosphate buffer (0.05 M, pH 7.5) and 4 μCi of ^{14}C-bicarbonate (C^{14}-Agency, Denmark) for exactly one hour in the dark. The following conditions were tried: pO_2 5% (atm. sat.) with either 1 μM $S^=$, 10 μM $S_2O_3^=$ or no substrate and complete anoxia with or without additional sulphide. Anoxia was obtained by bubbling the medium with N_2 (< 5 ppm of O_2) and with nitrogen in the head space during incubation (the tubes were closed with vinyl stoppers). Total carbonate of the anoxic medium was calculated from carbonate alkalinity and pH. For experiments with a pO_2 of 5%, the oxygen was included by adding air saturated buffer solution to the de-oxygenated medium. Like all other experiments, these were carried out at room temperature (20-22°C). All cells were extracted from sediment samples immediately before the experiments.

Following incubation, the ciliates were rinsed twice in clean seawater, fixed with a 4% formaldehyde solution, washed, and affixed to slides with albumin which was subsequently hardened with ethanol. The slides were then washed in distilled water and coated in the dark with Kodak Nuclear Track emulsion (NTB2), then dried and stored in the dark at 5°C for exactly 72 hours. They were then developed (Kodak D19), fixed, dehydrated and mounted. Silver grains per unit area were counted with the image analysing system. Three sets of experiments were carried out. For calculating the assimilation rate we used the following expression: $U = 2n[C]/(R \times 3.7 \times 10^4 \times 3600 \times T \times I)$ where U is the assimilation rate (mg C per unit cell surface area per hour), [C] is total carbonate carbon (0.18 mg in 12 ml of incubation medium), n is the number of silver grains per μm^2, R is the added radioactivity (4 μCi), T is exposure time (72 h) and I is incubation time (1 h). The factor 2 in the enumerator compensates for the fact that only about half of the β-particles will pass the photographic emulsion while the other half will be directed towards the glass slide. This is likely to underestimate the C-14 uptake somewhat since some radiation may have escaped sideways or passed the emulsion without affecting it. Some labelled carbon may also have been dissolved by the fixative.

Oxidation of sulphide and the accumulation of elemental sulphur were demonstrated by adding 40 μCi of $^{35}S^{=}$ to about 2 ml of interstitial water with *Kentrophoros* (and other ciliates) in a glass dish and incubating for one hour. The isotope (Amersham) was provided as 9.3 mg Na_2S with a total activity of 1 mCi; it was dissolved with 5 ml of oxygen-free water and 0.25 ml was added to each dish. The procedure otherwise followed that described above for the bicarbonate assimilation experiment except what we used formaldehyde (30%) rather than ethanol for coagulating the albumen so as not to dissolve the elemental sulphur of the bacteria. Since we did not measure the sulphide concentration of the incubation medium these experiments cannot be used for quantifying sulphide oxidation rates.

Cytochemistry

The redox dye, benzyl viologen, has previously been used for the direct demonstration of sulphide oxidases (Powell & Somero 1985). The dye will act as an electron acceptor for the oxidation of sulphide, but this process is relatively slow in the absence of a catalyst. Reduced benzyl viologen has an intense purple colour. The assay must be carried out in the absence of oxygen because otherwise the dye is rapidly re-oxidised to the colourless form. We basically followed the procedure of Powell & Somero (1985) by adding an oxygen-free solution: 5 μM $S^{=}$ and 2 mM benzyl viologen in a glycine buffer (pH 9) to living cells in a drop of seawater between a slide and coverslip.

Electron microscopy

Attempts to fix *Kentrophoros* with buffered glutaraldehyde were unsuccessful (the cells lysed) so we used a 2% solution of OsO_4 in seawater. After washing and dehydration, cells were embedded in epon and sectioned on an LKB-ultramicrotome. Sections were stained with uranyl acetate and lead citrate and observed with a Zeiss EM9 electron microscope.

Behaviour in oxygen gradients

In order to study the ability of *Kentrophoros* to orient itself in chemical gradients we used micropipettes to insert cells in 3 cm long "microslide" capillaries (CamLab, Cambridge, UK; internal width 4 mm, depth 0.5 mm) containing oxygen-free seawater. One end was sealed with petroleum jelly or with an agar plug containing 10 mmoles l^{-1} of sulphide (pH: 7.5), while the other end had free access to the air (or to selected mixtures of air and nitrogen in a stoppered test tube). In this way oxygen or/and sulphide gradients formed inside the capillary; the position of the gradients could be changed by manipulating the composition of the gas phase.

In some cases we added a dense culture of an unidentified *Euplotes* sp. which we had previously shown (unpublished results) to accumulate at oxygen tensions around 5 % atm sat. This enabled us to locate this oxygen tension within the capillaries.

RESULTS AND DISCUSSION

General morphology and morphometry

Kentrophoros species are extraordinarily flat organisms. The side which is usually in contact with the substratum is ciliated and is here referred to as the ventral side. (Since the creature does not have a mouth this is an arbitrary decision. *Kentrophoros* is probably related to the loxodid ciliates. Therefore, it would perhaps be more correct to refer to the ciliated side as the right side since in loxodids the mouth is situated on the margin of the cell with the ciliated side lying to the right of the mouth.) The dorsal side of *Kentrophoros* is not ciliated except for two marginal kineties (Figs 1-4, 11, 14). Instead, the surface is almost entirely covered by a single layer of bacterial rods arranged perpendicularly to the cell surface (Figs 3-4, 11-16). Sometimes bacteria are absent from the anterior and posterior extremities of the dorsal side.

In the microscope the ciliates appear pigmented; different individuals have colours ranging from light or orange brown to almost black. Higher magnification discloses that this is due to the presence of sulphur droplets in the bacteria; the different shades reflect different amounts of elemental sulphur in the bacteria and if the sulphur is dissolved with pyridine the ciliate-bacterial consortium becomes colourless.

Figure 5 shows the relationship between cell length and total surface area and volume, respectively; the volume includes that of the bacteria. The large variance in the data reflects the variable ratio between length and width. Estimates of bacterial dimensions were different according to whether they were based on light microscopical observations or on electron micrographs; the former yielded an average length and width of 3.6 and 0.8 μm, respectively while the latter method provided a length of 3.4 and a width of 0.6 μm. The corresponding cell volumes are 1.8 and 1 μm^3, respectively. Measurements based on light microscopy of linear dimensions smaller than one micron are inaccurate. On the other hand, it is likely that a considerable shrinkage took place during fixation. Since it was not possible to evaluate this effect we will assume an average bacterial volume of 1.7 μm^3. There are on the average 0.75 bacteria per μm^2 of ciliate dorsal surface. If we consider a 170 μm long *Kentrophoros,* we see from Fig. 5 that the area of its dorsal side is about 6000 μm^2 and that the total volume (ciliate + bacteria) is about 15 000 μm^3. The ciliate then carries about 4500 bacteria with a total volume of 7650 μm^3; that is, the bacteria make up about half of the entire volume of the symbiotic consortium.


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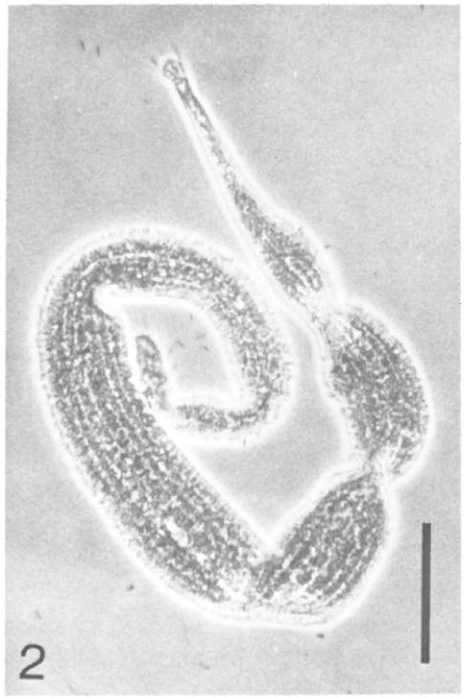
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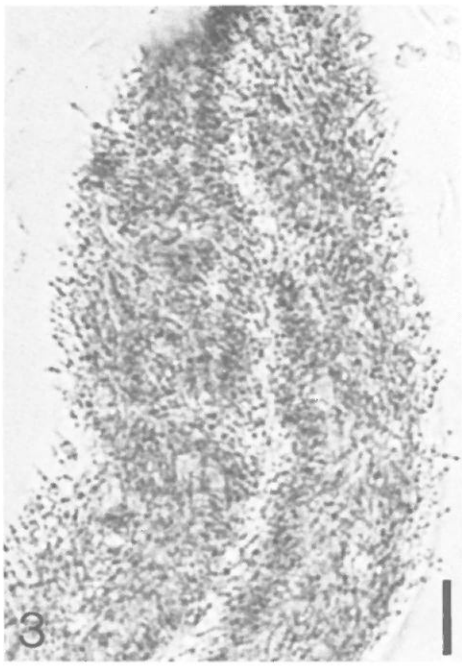
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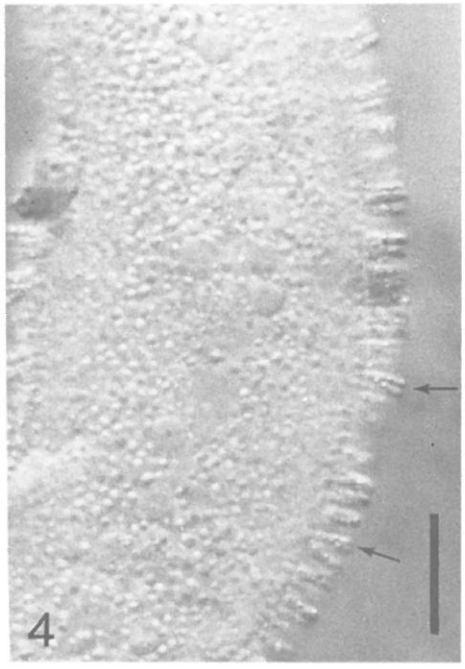
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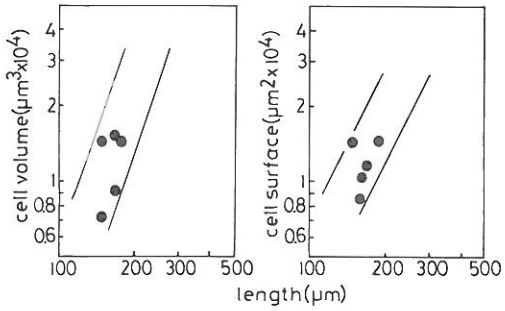

Fig. 5. Cell volume and surface area as function of length in the small version of *K. fasciolata.* The lines delimiting the data points have slopes of 3 and 2, respectively.

Since *Kentrophoros* depends on the bacteria as food (which are phagocytised along the dorsal side) and since the bacteria must have some finite rate of growth, the rate of food intake depends directly on the standing stock of its symbionts and this in turn depends on the surface area available for the bacteria, so the flattened shape may be understood as an adaptive trait for this type of bacteria "farming". To appreciate this, one can compare its surface to volume ratio with that of some other ciliates with a similar cell volume. The above mentioned figures yield a surface to volume ratio for *Kentrophoros* of 1.6 μm^{-1}. In comparison, ciliates like *Glaucoma scintillans, Colpoda cucullus,* or *Euplotes moebiusi,* with cell volumes within the range of 4 to 15×10^3 μm^3 have surface to volume ratios within a range of 0.22 to 0.28 μm^{-1} (Fenchel, unpublished data), that is, 5 to 7 times less than that of *Kentrophoros.* It is true that many ciliates found in the interstitial of marine sands are extraordinarily flattened or oblong (see, e.g. Dragesco 1960), but even so, *Kentrophoros,* with a thickness of only 2-3 μm (Fig. 11) is an extreme example which can be explained as an adaptation to provide space for a maximum number of ectosymbiotic bacteria.

Behaviour in gradients of oxygen

When *Kentrophoros* cells are placed in dishes with seawater they glide continuously along the bottom. When placed in an oxygen gradient in a capillary they tend to accumulate at oxygen tensions somewhat less than 5% atm. sat. where they then remain motionless for hours or days (Fig. 7). If the oxygen gradient is altered by changing the composition of the ambient atmosphere, the cells move accordingly. If the cells protrude into a region with a higher oxygen tension they display a phobic response. In contrast, the cells do not show a behavioural response to sul-

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Figs 1-4. Living specimens of *Kentrophoros.* Fig. 1. The small version of *K. fasciolata.* Scale bar: 50 μm. Fig. 2. The large version of *K. fasciolata.* Scale bar: 50 μm. Fig. 3. *K. latum;* the sulphur vesicles appear as black dots in the bacteria. Scale bar: 10 μm. Fig. 4. The bacteria of *K. fasciolata;* Sulphur vesicles and dividing cells (arrows) are visible; Scale bar: 10 μm.

phide; in a capillary with totally anoxic water they remain motile, but distributed randomly relative to the sulphide gradient. The behavioural response to pO_2 is probably solely responsible for the characteristic vertical distribution in sediments immediately above the anaerobic sulphide zone (Fenchel 1969, see also Fig. 6).

This behaviour may, of course, seem adaptive in terms of the requirements of the symbionts for the simultaneous presence of oxygen and reduced sulphur compounds. However, it is noteworthy that while the symbiosis with chemolithotrophic bacteria seems to be a unique feature among ciliated protozoa, microaerophilic forms are common. Microaerophily has so far been best studied in the freshwater genus *Loxodes,* the results of which also apply to the marine genus *Remanella* (Fenchel & Finlay 1984, 1986a, b, Finlay et al. 1986, Finlay & Fenchel 1986). Both of these are relatives of *Kentrophorus.* They prefer oxygen tensions of around 5%, they show various behavioural responses which result in an accumulation of the cells in their preferred habitat, and oxygen seems to be the only chemical species by which the cells orient themselves in the chemical gradients of sediments. *Kentrophoros* differs from these other genera in the absence of geotaxis for orientation in vertical oxygen gradients and in being indifferent to light, which in *Loxodes* decreases the preferred value of pO_2. It may therefore be conjectured that microaerophilic behaviour was a characteristic of the ancestors of *Kentrophoros* and a prerequisite for the evolution of the symbiotic relationship with sulphur bacteria. Since *Loxodes* and *Remanella* seem unaffected by the presence of sulphide (and representatives of the latter co-occur with *Kentrophoros* in the same sediment strata) it seems unlikely that the adaptive nature of the symbiotic bacteria was primarily one of sulphide detoxification for the ciliate.

If *Kentrophoros* orients itself using only the oxygen tension, then the ciliate can be effective only in sediments where the gradients of oxygen and sulphide are not too steep. In sulphide-rich environments, where transport of the chemical species occurs only by molecular diffusion, a bacterial plate will form. This will consume all the sulphide coming from below and the oxygen tension in the upper part of the bacterial plate may be considerably lower than 5% atm. sat. (Nelson et al. 1986). In sandy and usually less productive sediments, however, dense bacterial plates do not develop, the chemical gradients are less steep and sulphide occurs at higher oxygen tensions. With regard to the symbiotic bacteria, the adaptive nature of the association is probably that of being transported to a suitable chemical environment by the ciliate. In sands, the vertical position of the sulphide layer changes seasonally and over shorter time periods due to wave exposure, input of organic matter, worm burrows, and local concentrations of decaying organic material may create complex patterns (Fenchel 1969). The relatively high motility of the ciliate will enable the bacteria to exploit this heterogeneity more efficiently.

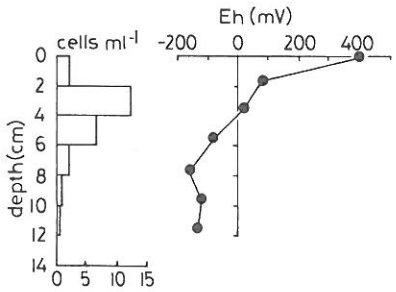


Fig. 6. The vertical distribution of *Kentrophoros* spp. and the redox profile in a sandy sediment in Nivå Bay (May, 1967) showing the concentration of cells around the oxic-anoxic boundary layer (data from Fenchel 1969).

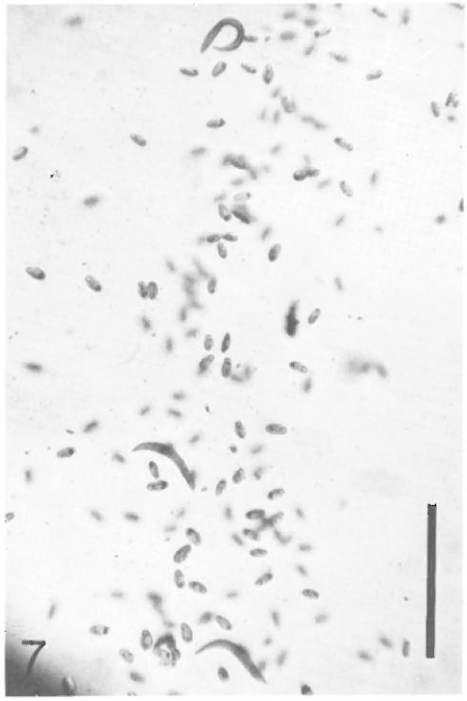
Sulphide oxidation

The symbiotic bacteria are capable of reducing benzyl viologen in the presence of sulphide (Fig. 8). Since this reaction will take place eventually in the absence of catalysis, and since a variety of unspecified organic materials also catalyse sulphide oxidation, results from this method should be interpreted with some caution. However, the bacteria clearly stained much faster and more intensely than the cytoplasm of the ciliate. Various other ciliates and a heterotrophic bacterium (*Pseudomonas* sp.) also stained very slowly and weakly as compared to the bacteria of *Kentrophoros*. This does indicate that the symbiotic bacteria contain a high sulphide oxidase activity.

Autoradiography shows that the symbiotic bacteria readily accumulate S-35 (presumably as elemental sulphur) when exposed to $^{35}S^{=}$ (Fig. 9). The experiment could not be used for a quantitative evaluation, but again, a number of other "control" ciliates included in the incubation (*Parablepharisma, Remanella, Condylostoma, Tracheloraphis*) did not show a radioactivity which deviated significantly from the background of the preparations.

Carbon dioxide fixation

The results of one set of the C-14 incubation experiments are shown in Table 1. The two other experiments gave similar results. The calculation of the carbon uptake as a percentage of the carbon content of the bacteria is based on the assumption that carbon constitutes 5% of the wet weight after deducting the volume of sulphur vesicles. We also assume that 5% of the wet weight of ciliates is carbon. When evaluating the results it must be kept in mind that the autoradiographic method probably underestimated the C-14 fixation. On the other hand, some CO_2 is fixed in the Krebs cycle and some apparent uptake may be due to metabolic exchange reactions. However, considering the measured uptake rates, such effects can only account for a small fraction of the uptake since otherwise the cells would have had an unrealistically high carbon turnover. Also other species of ciliates (without bacterial symbionts) incubated together with *Kentrophoros* did not show a radioactivity which was significantly above the background.



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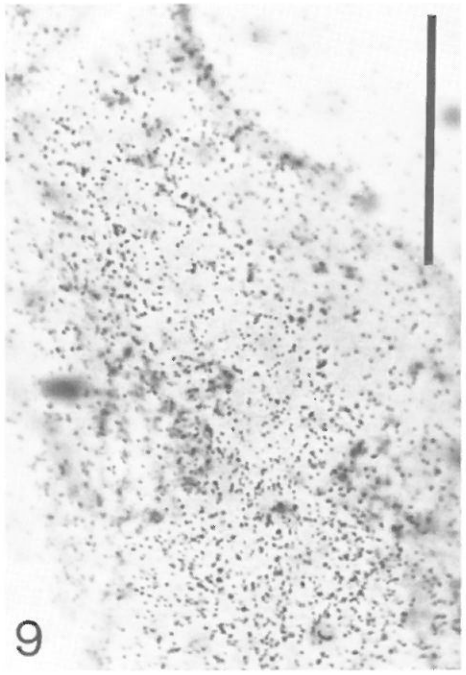

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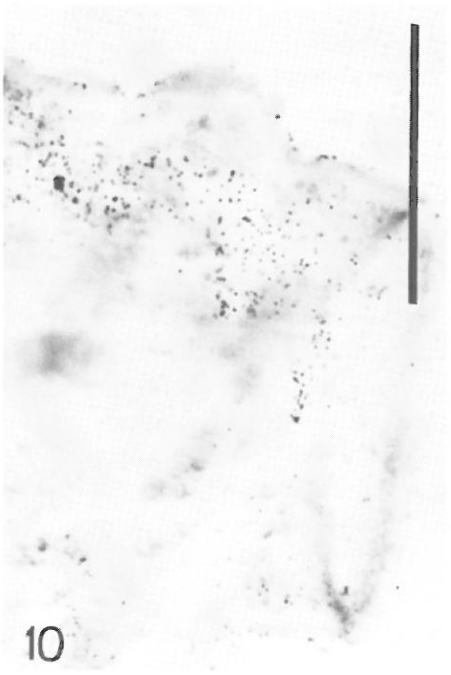

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Table 1. Uptake of C-14 labelled bicarbonate. Average values for 3-5 cells.

treatment	Ag-grains μm^{-2} (SD, %)	ng C $\times 10^{-7}$ bacterium^{-1} h^{-1}	carbon specific uptake by bacteria, h^{-1}
pO_2: 5% no substr.	0.35 (27)	66.0	0.117
pO_2: 5% $S^=$	0.11 (38)	21.1	0.037
pO_2: 5% $S_2O_3^=$	0.07 (27)	12.6	0.013
anoxic	0.01 (40)	2.2	0.002

As expected, carbon fixation was very low under anoxia (about 2% of the maximum values observed for aerobically incubated cells after correcting for the background density of silver grains). It could be expected that the addition of sulphide and thiosulphate (another potential substrate for the bacteria) will not stimulate carbon fixation due to the storage of elemental sulphur in the cells. Using planimetry of the sulphur vesicles on TEM-micrographs we calculated an average sulphur content of 32%, a figure which accords with the maximum values found in *Beggiatoa* (Nelson & Castenholz 1981). Nelson et al. (1986) calculated that the molar yield of *Beggiatoa* growing on the basis of the oxidation of elemental sulphur is 3.2 g C per mole S°. If this applies to the bacteria of *Kentrophoros* and if the bacteria assimilated 6.6×10^{-6} ng C per cell per h (the highest average values found) then the bacteria can maintain this rate for about ten hours on the basis of the stored sulphur). The failure of sulphide to stimulate growth has also been found for some sulphur bacteria symbiotic with invertebrates (Southward 1987). In all our experiments, however, the presence of sulphide suppressed C-14 uptake somewhat and thiosulphate suppressed the uptake substantially. The concentration of sulphide was insufficient to deplete oxygen in the incubation medium and this explanation would not apply to the thiosulphate experiments. We are not able to explain this effect.

In order to relate the production rate of symbiotic bacteria with the growth rate of the host we apply the concept of "carbon specific carbon uptake", that is, the uptake of carbon per unit time as a fraction of cell carbon. Let μ_b be the carbon

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Fig. 7. Three *Kentrophoros* cells (and many *Euplotes* cells) in a capillary with an oxygen gradient. The meniscus (not visible) and hence higher oxygen concentrations is situated to the right. Scale bar: 0.5 mm. Fig. 8. A cell treated with benzyl viologen and sulphide; the bacteria are densely coloured. Scale bar: 10 μm. Figs 9-10. Autoradiography of *Kentrophoros*. Fig. 9. *K. fasciolata* incubated with S-35 sulphide. Fig. 10. *K. latum* incubated with C-14 labelled bicarbonate, sulphide and oxygen. *Kentrophoros* cells tend to roll up when fixed. Scale bars: 50 μm.

specific carbon uptake of the bacteria (= the growth rate constant of the bacterial population in the absence of ciliate predation) and μ_c be the growth rate constant of the ciliate (= the population growth rate of the bacteria since their density remains constant). The bacterial production left over for ciliate consumption then equals $(\mu_b - \mu_c)\ B_b$ where B_b is the carbon content of the bacteria. The carbon specific carbon uptake of the ciliate is therefore given as $(\mu_b-\mu_c)\ B_b/B_c$ where B_c is the carbon content of the ciliate. The growth rate constant of the ciliate is then given by $\mu_c = (\mu_b-\mu_c)\ YB_b/B_c$, where Y is the yield or gross growth efficiency of the ciliate. Solving the equation we find:

$$\mu_c = \mu_b Y/(B_c/B_b + Y) \qquad [1]$$

The equation underscores the importance of the biomass ratio between the symbionts and the host for this type of relationship. If B_c/B_b significantly exceeds unity, the host can only maintain a very low growth rate relative to the symbiont food organism.

If we consider the highest carbon specific uptake rates of the bacteria observed: 13.3% h^{-1} (the highest average value found was 11.7% h^{-1}) this corresponds to a doubling time of 5.3 h. Assuming a total carbon content of the bacteria of 0.255 ng, 0.37 ng ciliate carbon (a 170 μm long ciliate) and a ciliate yield of 40% (a figure typical of aerobic protozoa) then eq. [1] gives $\mu_c = 0.038\ h^{-1}$ or a generation time of about 18 hours. This is a possible if somewhat low growth rate for such a small ciliate as *Kentrophoros*; most studied ciliates in this size range have doubling times closer to five hours at room temperature. However, the experiments do indicate that the organism is capable of growing on the basis of the autotrophic symbionts.

Comparisons with the rate of carbon fixation of metazoan symbionts are not easy because most published accounts do not allow the calculation of the specific carbon uptake of the bacteria; rather, they are based on the uptake by the complete animal-bacteria consortium or by isolated organs harbouring the symbionts. Giere et al. (1988) found for the gutless oligochaete, *Phallodrilus,* a maximum uptake of 3.7 μ mole CO_2 per g wet weight per hour, equivalent to a specific carbon uptake of $8.9 \times 10^{-3}\ h^{-1}$(corresponding to a production of the total biomass of the worm + bacteria in 112 h if we do not take the respiration and other losses of the worm into consideration). This result is about 10% of the maximum carbon specific uptake rates we found for the *Kentrophoros* consortium. That the small ciliate should have a faster growth rate than the much larger worm is very likely. Southward (1987) reviewed a number of other studies in which the carbon uptake is usually expressed per unit weight of the animal organ or tissue harbouring the bacteria. The gill of the clam *Solemya* showed an uptake which is about 10% of that found for *Phallodrilus,* and estimates of most other sulphur bacteria harbouring organisms were considerably lower still, so that if they apply in situ they would

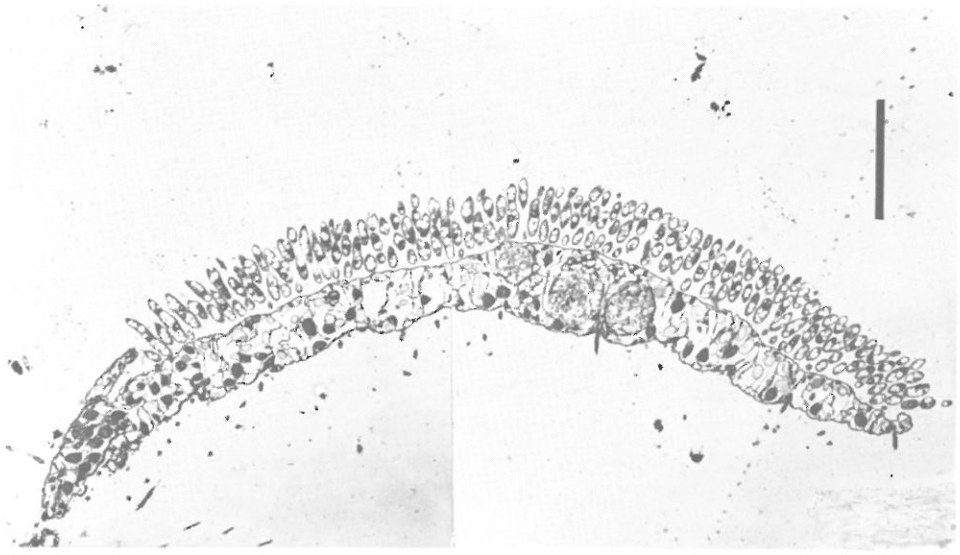


Fig. 11. Transverse section of a *K. fasciolata* cell. Scale bar: 5 μm.

seem to be hardly able to sustain growth of the hosts. In all probability, these experiments suffered from the same failing as ours, in not being carried out under conditions which were optimal for the bacteria.

We share Raikov's assumption that *Kentrophoros* is entirely dependent on the symbionts for its food. This is supported by the fact that the ciliate does not have a mouth. However, there are a few reports in the literature (for references see Fenchel 1968) that small diatoms and other ingested particles have been observed within *Kentrophoros*. During the present study we never observed this but it is not impossible that the ciliates may occasionally ingest particles other than the bacteria (e.g. particles adhering to the bacteria) through the cell surface.

The role of Kentrophoros in the sulphur cycle

The sulphur cycle is of paramount importance for the mineralisation of organic matter and for the carbon cycle in sediments. Bacterial re-oxidation of the reduced sulphur compounds deriving from dissimilatory sulphate reduction drives a substantial part of the production of biomass in the seabottom. We might pause to question the role of symbiotic sulphide oxidisers in this process. As far as *Kentrophoros* is concerned, this role is vanishingly small. The ciliate is widely distributed in shallow water and subtidal sands, but extensive sampling (Fenchel 1969) shows that their numbers never exceed about 50 cells per cm^2 and usually they are much rarer. This maximum density represents a bacterial biomass of 3.7×10^{-4} g m^{-2}. As a comparison, Jørgensen (1977) found for a variety of sedi-

ments that one type of sulphide oxidising bacteria, the filamentous *Beggiatoa,* accounts for between 5 and 20 g m^{-2}. From a biogeochemical point of view, the *Kentrophoros* symbiosis is an exotic phenomenon which makes only a symbolic contribution to the oxidation of the copious amounts of sulphide produced in sediments.

Fine structure of Kentrophoros and its symbionts

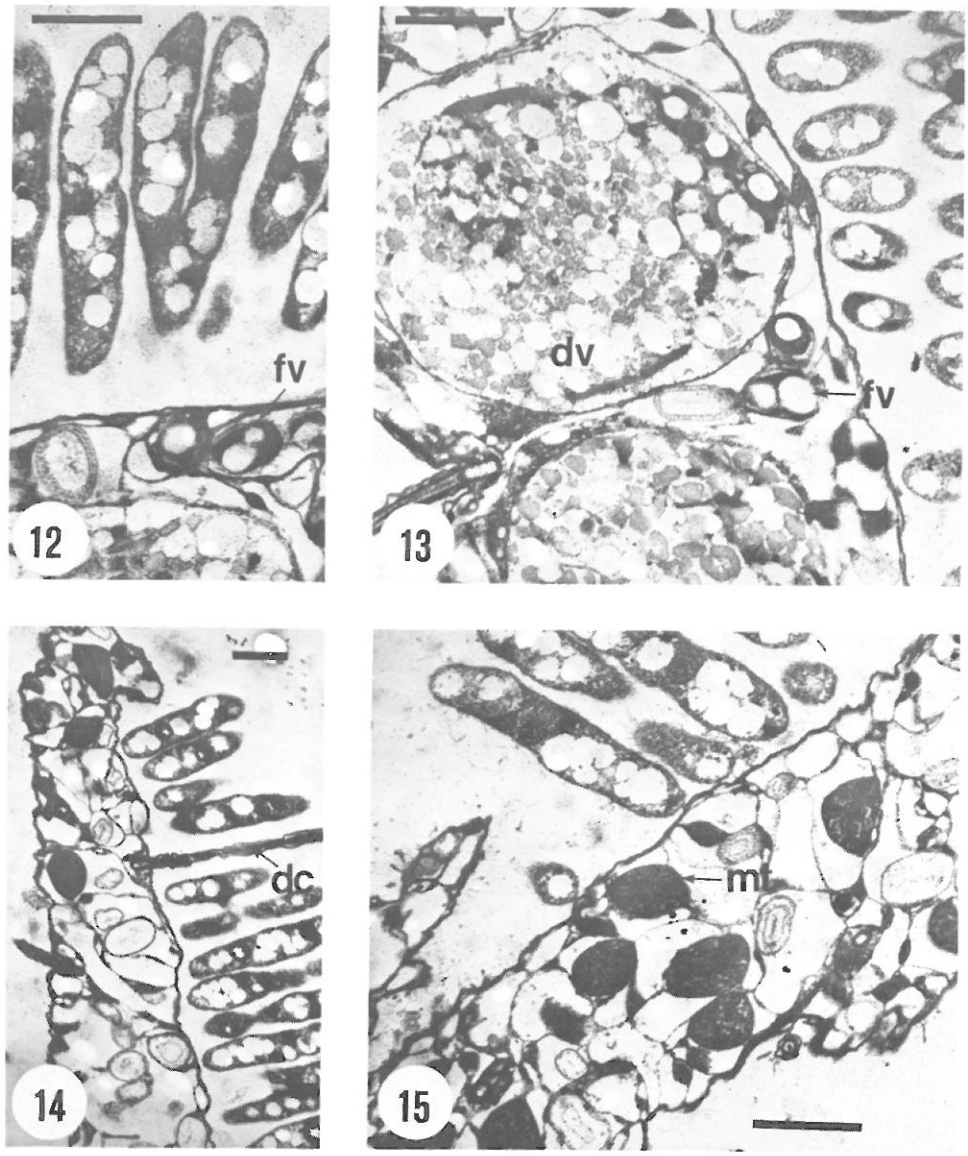
Raikov (1971, 1974) described the fine structure of *K. fistulosum* and *K. latum* and in many respects we can confirm his observations for *K. fasciolata* (Figs 11-16). The ciliate is covered by a single unit membrane although the extensive vacuolisation of the cell may sometimes give the impression of a double membrane (Fig. 16). In this respect it resembles loxodid ciliates (Fenchel & Finlay 1986a). Mitochondria are found mainly along the ventral side and along the margins of the cell; they measure between 0.5 and 1 μm in diameter. They contain a very electron dense matrix, but few tubular cristae (Figs 11, 15-16). Newly-formed feeding vacuoles contain single symbiont cells (Figs 12-13) and seem to form all over the dorsal side. Eventually these fuse to form large digestive vacuoles (Figs 11, 13) containing many more-or-less digested bacteria, but the sulphur droplets remain intact. These vacuoles are mainly found along the longitudinal axis of the cell.

Like *K. fistulosum, K. fasciolata* harbours only one type of bacterium, while in *K. latum,* ectosymbiotic spirochaetes were found among the sulphur bacteria. The latter species also harbours intracellular prokaryote symbionts. The functional role of these organisms is unknown (Raikov 1974). The sulphur bacteria of all *Kentrophoros* species are peculiar in having a longitudinal fission (Figs 4, 12, 16).

In our preparations the bacterial layer is in most places not in direct contact with the cell membrane of the ciliate; this is undoubtedly a fixation artefact. In some places, however, such contact is seen (Fig. 15), but no specialized thickening of the bacterial cell wall was obvious as reported by Raikov (op. cit.). How the bacteria are kept in place in vivo is not understood. It is possible that they are embedded in a layer of mucus that covers the dorsal surface.

In certain respects, and especially concerning the fine structure of the sulphur bacteria, our findings are at variance with those of Raikov (1971, 1974). Raikov (op. cit.) could not find a cell wall; this is clearly visible in our micrographs (Fig. 16). Its outer layer has an undulating appearance resembling that of the genus *Thiobacillus* (Shively et al. 1970). It is likely that the "double cell membrane" described for the symbionts of *K. latum* by Raikov (1974) is in fact the cell wall. The sulphur vesicles are membrane-bound and dispersed throughout the cytoplasm proper (Fig. 16). We could not find any invaginations connecting the outer cell membrane with the vesicles. There are no internal membrane systems comparable to those found in nitrifying or photosynthetic bacteria.

Among the cell inclusions are ribosomes and other electron dense material,



Figs 12-15. Electron micrographs of *K. fasciolata.* Fig. 12. A dividing bacterium and a food vacuole containing a symbiont cell (fv.). Fig. 13. A food vacuole and a digestive vacuole (dv). Fig. 14. A dorsal cilium. Fig. 15. Mitochondria (m). All scale bars: 1 μm.

possibly storage carbohydrates. Characteristic of the cells is the presence of several membrane-bound, nearly spherical organelles containing a granular, electron dense material ("c" in Fig. 16). They resemble and may be carboxysomes; organelles found in certain autotrophic bacteria (Shively et al. 1973). These or-

ganelles contain the enzyme, ribolose-1,5-bisphosphate carboxylase, the pivotal enzyme for CO_2 fixation within the Calvin-Benson cycle.

It is difficult to identify the bacterial symbionts with certainty. The genus *Thiobacillus* accommodates many rod-shaped chemolithotrophic sulphide oxidisers with carboxysomes and rippled cell envelopes, but these organisms also have polar flagella and they deposit sulphur extracellularly. The symbionts in question have certain characteristics, notably their large size, internal sulphur deposition, absence of flagella and the possible secretion of mucus, which allow them to be placed more easily in the genus *Thiobacterium* (see Buchanan & Gibbons 1974 and Fjerdingstad 1979). Lackey & Lackey (1961) described an organism which they called *Thiodendron mucosum* and which is now usually placed within the genus *Thiobacterium.* The bacterium forms colonies embedded in a jelly-like matrix. These colonies are three-dimensional, branching, and attached to various surfaces including the stalk of the ciliate *Vorticella.* But *Vorticella* is unlikely to form a nutritional symbiosis with this "dendritic" bacterium. The capacity for longitudinal division in the *Kentrophoros* symbiont is, however, a unique characteristic among chemolithotrophic sulphur bacteria.

Symbiosis with chemolithotrophs in ciliates and in animals

In recent years a large number of associations between chemolithotrophs (oxidising reduced sulphur compounds or methane) has been recorded for a variety of metazoa including molluscs, annelids and pogonophora (reviewed by Southward 1987) and nematodes (the genus *Leptonemella,* see Gerlach 1978 and Jensen 1987). It seems very likely that this list will grow in the coming years. Regarding ciliates, there are a large number of species which harbour intracellular or extracellular prokaryote symbionts; in most cases the exact nature of the relationship is unknown. A special case is the symbionts of anaerobic ciliates. Many of these harbour intracellular bacteria and some have been shown to be methanogens, which sequester the hydrogen derived from the fermentative processes of the ciliates (Van Bruggen et al. 1984). Other forms may in addition (or instead) carry ectosymbionts; their functional role is still not entirely understood, but it is reasonable to assume that they depend somehow on the products of fermentation (volatile fatty acids, hydrogen) produced by the host (Fenchel et al. 1977). In at least one case (*Parablepharisma*), the ciliate phagocytises its ectosymbionts through the cell surface, in a manner similar to *Kentrophoros,* although it has a mouth and it also feeds on ordinary prey, especially photosynthetic bacteria (Fenchel & Finlay, unpublished observations).

It is strange that *Kentrophoros* is the only ciliate known which has a symbiotic relationship with chemolithotrophs. As discussed above, many ciliates are microaerophiles and they live in environments such as the chemocline between oxidised and anaerobic reducing layers, where the evolution of such relationships

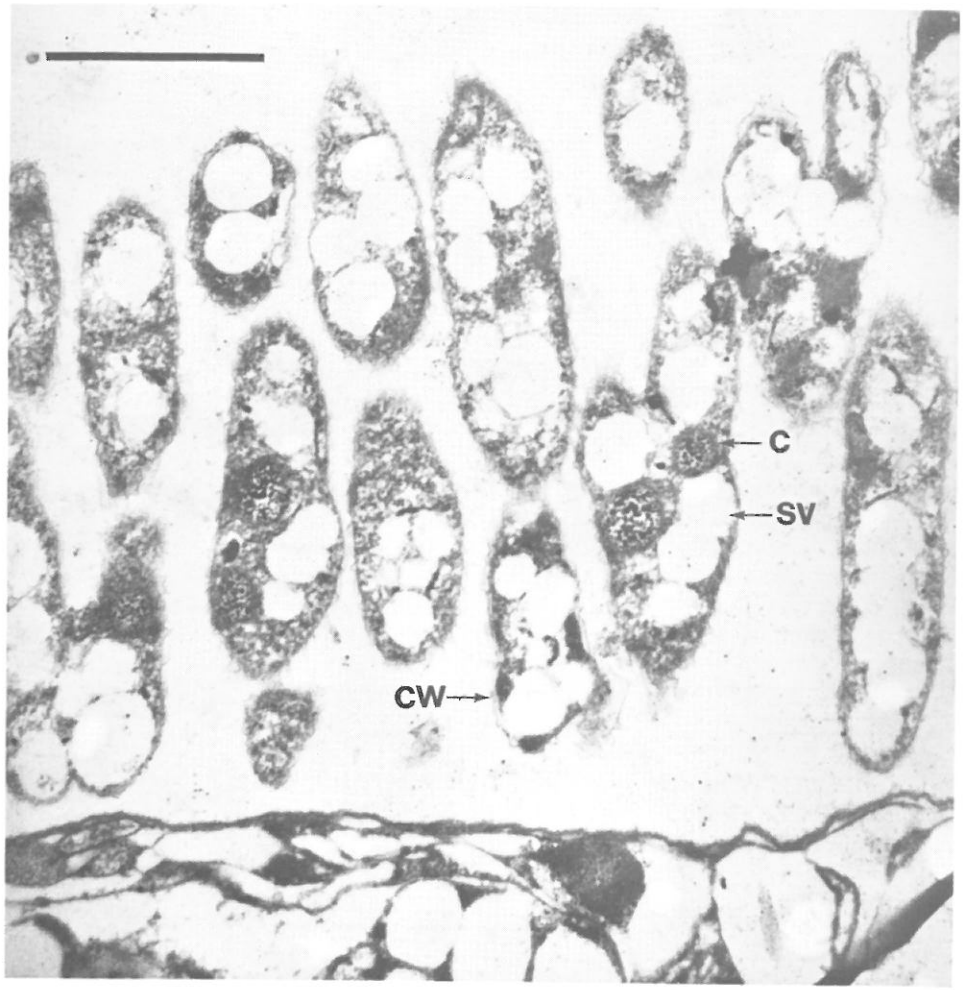


Fig. 16. Symbiont bacteria showing sulphur vesicles (sv), cell wall (cw) and assumed carboxysomes (c). Scale bar: 1 μm.

would be favoured. However, and as expressed by eq. [1], the biomass of symbionts required to sustain a reasonably high growth rate may constrain the evolution of this type of symbiosis in small organisms such as ciliates with a relatively high weight specific food uptake. Larger animals have lower weight specific food requirements and so the requirements for maintaining a relatively large biomass of symbionts are less demanding.

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