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Co-existence of methane- and sulphur-based endosymbioses between bacteria and invertebrates at a site in the Skagerrak

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ABSTRACT: A small pogonophore from the Skagerrak, *Siboglinum poseidoni*, lives in symbiosis with methane-oxidizing bacteria. This was confirmed by the presence of 2 enzymes characteristic of bacterial C1-metabolism, methanol dehydrogenase and hexulosephosphate synthetase, in the bacteria-containing tissues. Specific activities were comparable to those found in free-living methane-oxidizing bacteria and in bacteria-containing tissues from other invertebrates with symbiotic methanotrophs. Stable carbon isotope ratios for tissues of pogonophores and other animals from the habitat were compared with those for organic matter in the sediment and for free and sorbed hydrocarbon gases. Free, biogenic methane ($\delta^{13}C = -80.3$ ‰ PDB) was the carbon source for *S. poseidoni* ($\delta^{13}C = -78.3$ ‰ in its bacteria-containing posterior parts). Methane sorbed to the sediment is probably not accessible to the organism. Tissue of the bivalve mollusc *Thyasira sarsi* from the same habitat was less ^{13}C-depleted ($\delta^{13}C = -37.4$ to -39.5 ‰), although more depleted than specimens found in sites with negligible methane. The value for the bivalve can be explained by available bicarbonate depletion and selection for ^{12}C by chemoautotrophic sulphur-oxidizing symbiotic bacteria in the gills. No methylotrophic enzymes were found in gill extracts. A heterotrophic polychaete apparently not influenced by symbiotic methanotrophs, *Leanira* sp., has a $\delta^{13}C$-value (-20.0 ‰) comparable to phytoplankton or organic matter from the sediment (-25 ‰).

INTRODUCTION

The small pogonophore *Siboglinum poseidoni*, which occurs in the central Skagerrak, is known to harbour methylotrophic endosymbiotic bacteria (Schmaljohann & Flügel 1987). This was confirmed by TEM-micrographs, culture experiments and $^{14}CH_4$-uptake data. This organism is ideal for studies on this type of symbiosis since it has only one type of endosymbiont and does not possess a digestive tract. Therefore results can be more easily interpreted than in species which have a mixed nutrition either by different types of symbionts, or by a functional digestive tract, as in some symbiotic bivalves.

Methane-oxidizing bacteria endosymbiotic in bivalves have been confirmed from 2 sites in the Gulf of Mexico. Cavanaugh et al. (1987) investigated such a symbiosis in the gills of a mytilid bivalve from the Florida escarpment, while Childress et al. (1986) and Fisher et al. (1987) described a similar association at the Louisiana hydrocarbon seep sites. In both cases TEM descriptions of the bacterial morphology showed large coccoid cells with the typical Type I intracytoplasmic membrane stacks. These are different from the rod-like bacteria in *Siboglinum poseidoni*. Enzyme assays for methanol dehydrogenase or hexulosephosphate synthetase gave further evidence for the methylotrophic nature of the gill symbionts.

A variety of other organisms from different hydrocarbon seep sites are possible candidates for symbiosis with methylotrophs. All these animals, which include tube worms, bivalves and gastropods, are unusually depleted in ^{13}C, showing $\delta^{13}C$-values < -50 ‰ but have not yet been investigated more closely (Paull et al. 1985, Kulm et al. 1986, Brooks et al. 1987). Caution is needed in attributing methanotrophy solely from carbon isotope data. The first suggestion of methanotrophic nutrition of Pogonophora (Southward et al.

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1981) was based on the great depletion of ^{13}C (−36 to −46 ‰) in species of *Siboglinum* which are now known to be dependent on sulphur-oxidizing bacteria (Southward et al. 1986).

Siboglinum poseidoni has been identified in only 2 very restricted areas in the Skagerrak (Flügel & Langhof 1983). In these areas the pogonophores have a very patchy occurrence. Box corer samples showed that there must be small spots, not larger than a few m^2, where a dense population of this species lives and the sediment is more reduced than in the surrounding areas. To elucidate this phenomenon and to learn more about the origin of the methane, an analysis of sediment gases, including hydrocarbons, was carried out. We also investigated the *S. poseidoni* symbiosis in respect of its enzymatic capabilities and carbon isotope composition to assess the extent to which methane may contribute to the tissue carbon of the pogonophore.

MATERIAL AND METHODS

Sampling. Specimens of *Siboglinum poseidoni* (Pogonophora), *Thyasira sarsi* (Bivalvia), and *Leanira* sp. (Sigalionidae, Polychaeta) were collected with a beam trawl and a Van Veen grab in the Skagerrak (58°02.85′ N; 9°40.04′ E) at 280 to 340 m water depth (Flügel & Langhof 1983).

Sediment samples (0 to 10 cm depth) for gas analysis were taken from a Van Veen grab containing a dense population of *Siboglinum poseidoni*. Material from this grab was also used for the analysis of carbon isotopes in organic matter. Sediment from deeper layers (60 to 70 cm) was sampled from a small gravity corer fitted with a 75 mm i. d. PVC-tube. The samples for the analysis of stable isotopes were stored at −18 °C before preparation.

Enzyme assays. For enzyme assays individual *Siboglinum poseidoni* were removed from their tubes immediately after collection and divided into the anterior part and the posterior part which included the girdle region. Approximately 50 individuals were used for each preparation. The combined anterior or posterior parts were gently homogenized in 200 µl ice-cold, filtered seawater in an all glass Potter-Elvehjem homogenizer. The homogenate was centrifuged for 2 min at 6700 × *g*. The pellet which contained the bacteria was re-homogenized in 200 µl distilled water to rupture the bacteria and this second homogenate was used for enzyme assays.

The protein content of the homogenates was determined by the method of Bradford (1976).

Hexulosephosphate synthetase activity was assayed on 10 to 50 µl aliquots of tissue homogenate using ^{14}C-labelled formaldehyde as substrate (Levering et al. 1981). The formaldehyde concentration was 2 mM, unless otherwise stated, and the incubation time varied between 10 and 60 min at a temperature of 20 °C. The enzyme activity was stopped by the addition of 300 µl of ethanol and 200 µl of 5 % barium chloride solution to the 200 µl reaction mixture in 2 ml Eppendorf microcentrifuge tubes. The tubes were stored at 4 °C overnight, centrifuged and washed 3 times with a mixture of 100 mM formaldehyde : ethanol : 5 % barium chloride solution (1 : 1 : 1), with centrifuging after each wash. Some control tubes were stopped at zero time and other controls were incubated for the assay period in the absence of added ribose-5-phosphate. Radioactivity in the final precipitate was measured in a liquid scintillation counter after the addition of 10 ml Instagel (Packard) scintillation fluid.

Methanol dehydrogenase (E. C. 1.1.99.8) was determined according to Anthony & Zatman (1965) using 50 µl aliquots of the homogenate.

Determination of stable carbon isotopes. Organic matter was extracted from the sediments by dichloromethane in a standard soxhlet apparatus. A possible isotope fractionation towards lighter isotopes by preferential extraction of lipids cannot be excluded but seems negligible considering the strong ^{13}C-depletion in methane-influenced samples. After extraction and evaporation of the solvent the residue was heated at 850 °C and oxidized with cupric oxide in a quartz vessel. Dried animal tissues were similarly combusted to form CO_2.

Free gases were removed (and then collected) from the sediments in a vacuum (water vapour pressure) while stirring the wet, unsieved samples. After sieving the sediments (washing in water) sorbed gases were released from the fraction < 63 µm by adding phosphoric acid to the stirred samples in the vacuum system (Faber & Stahl 1983, Faber et al. in press). Gas concentrations in the sediments and compositions were determined in a gas chromatograph fitted with FID (flame ionization detector) and TCD (temperature conductivity detector). Sediment methane concentration (yield) is given as weight of methane per weight of wet sediment × 10^9 (ppb). For stable carbon isotope determination the hydrocarbons were separated in a gas chromatographic column and online-combusted to CO_2 (Dumke et al. 1989).

Isotope ratios were determined on the CO_2 in a Finnigan MAT 250 mass spectrometer. Carbon isotope ratios are given in the δ-notation with reference to the PDB standard:

$$\delta = \left(\left(R_{sample} / R_{standard} \right) - 1 \right) \times 1000 \text{ (‰)} \quad \text{where} \quad R = {}^{13}C/{}^{12}C.$$

The accuracy of the carbon isotope data is 0.2 ‰ for organic matter and better than 1 ‰ for the hydrocarbon gases.

Table 1. *Siboglinum poseidoni*. Activity of hexulosephosphate synthetase and methanol dehydrogenase in tissues. Values in parenthesis are based on whole homogenate protein, i.e. including supernatant before osmotic rupture of bacteria. Preparation 4 (a): untreated; 4 (b): + toluene; 4 (c): + lysozyme. Preparation 5 was measured at a lower formaldehyde concentration (0.5 mM) than the others. nd: Not determined

Preparation no.	Section of pogonophore	Hexulosephosphate synthetase	Methanol dehydrogenase
		(μmol g^{-1} protein min^{-1})	
1	Posterior	103.5 (8.04)	45.9 (3.6)
1	Anterior	5.1 (1.0)	4.1 (0.8)
2	Posterior	286.7 (28.2)	20.3 (2.0)
2	Anterior	1.3 (0.24)	nd
3	Posterior	165.9 (14.5)	5.8 (0.5)
4 (a)	Posterior	nd	4.3 (1.3)
(b)	Posterior	nd	8.8 (2.8)
(c)	Posterior	nd	10.9 (3.4)
5	Posterior	36.2 (3.6)	6.2 (0.6)
5	Anterior	0 (0)	1.5 (0.3)

RESULTS

Enzyme assays

Table 1 lists the activities of hexulosephosphate synthetase and methanol dehydrogenase in preparations of anterior and posterior sections of *Siboglinum poseidoni*. Figures are given for the specific activity in the bacteria-containing pellet and also for the activity based on the total protein content of the preparation. Most of the protein was contained in the first supernatant. It is probable that not all the bacteria were lysed by a single homogenization with distilled water (Southward et al. 1986), and an attempt was made to increase the enzyme activity by adding a drop of toluene and also by preincubating the homogenate in the presence of lysozyme. These treatments increased the amount of methanol dehydrogenase activity in Preparation 4. The time-course of the hexulosephosphate synthetase assay was found to be linear over a 40 min period.

Carbon isotope analysis

Results for benthic organisms and organic matter in the sediment are given in Table 2. The tissues of *Siboglinum poseidoni* are extremely depleted in ^{13}C. Their $\delta^{13}C$-values range from −78.3 ‰ (bacteria-containing posterior parts) to −73.6 ‰ (bacteria-free anterior parts), while the tubes belonging to this species show a $\delta^{13}C$ of −62.2‰, probably because of contamination with heterotrophic bacteria growing on the tube.

These ratios contrast with those found in *Thyasira sarsi* from the same habitat. This bivalve contains sulphur-oxidizing bacteria (Dando & Southward 1986). The bacteria-containing gills ($\delta^{13}C$ = −39.5 ‰) are slightly more depleted than the rest of the body ($\delta^{13}C$ = −37.4 ‰). Both values reflect the different carbon source compared to *S. poseidoni*.

Leanira sp., a polychaete feeding on organic matter in the sediment, had a $\delta^{13}C$-value of −20.0 ‰, similar to that of extracts from the organic matter of the sediment (−25.3 ‰ to 24.5 ‰).

The free gases (Table 3a) mainly consist of nitrogen and oxygen (air contamination), with traces of carbon dioxide and methane. Higher hydrocarbon gases were not detected. The carbon isotope data ($\delta^{13}C$ = −72.9 ‰ and −80.3 ‰) clearly show the bacterial origin of the methane (Rice 1980) which is supported by the absence of higher hydrocarbons (C_{2+}-compounds were not detected, therefore the exact C_1/C_2+C_3-ratios cannot be calculated). The isotope ratio of the associated carbon dioxide was $-19.4‰ < \delta^{13}CO_2 < 17.7‰$. The concentration of free methane was 24 ppb in the upper layer (0 to 10 cm). In the 60 to 70 cm layer (from a separate gravity corer) a concentration of only 4 ppb was found. This sample may not have been taken in a pogonophore zone.

Table 2. Carbon isotope data from organisms and organic matter in the sediment

BGR-No.	Sample	$\delta^{13}C$ (‰)
109155	*Siboglinum poseidoni*, anterior part	−73.6
109156	*Siboglinum poseidoni*, posterior part	−78.3
109157	*Siboglinum poseidoni*, tube	−62.2
110486	*Thyasira sarsi*, gills	−39.5
110487	*Thyasira sarsi*, rest of body	−37.4
109283	*Leanira* sp. (polychaete)	−20.0
109158	Organic matter in sediment, 0–10 cm	−25.3
109281	Organic matter in sediment, 0–10 cm	−24.5
109282	Organic matter in sediment, 60–70 cm	−24.6

Table 3 (a). Concentrations and carbon isotope data of free gases in sediment samples

BGR-No.	Depth (cm)	CO_2 (% of total gas)	CH_4	Yield-CH_4 (ppb)	$\delta^{13}C_1$ (‰)	$\delta^{13}C$-CO_2 (‰)
109281	0–10	0.05	0.50	24	−80.3	−19.4
109282	60–70	4.58	0.34	4	−72.9	−17.7

Table 3 (b). Concentrations and carbon isotope data of sorbed hydrocarbon gases in sediment samples

BGR-No.	Depth (cm)	CH_4	C_2H_6	C_3H_8	C_4	C_5	Yield-C_1 (ppb)	$\delta^{13}C_1$	$\delta^{13}C_2$ (‰)	$\delta^{13}C_3$
		(% of hydrocarbons)								
109281	0–10	86.7	7.1	2.8	1.9	1.5	100	−40.1	−33.5	−32.0
109282	60–70	85.3	7.6	3.1	2.0	1.9	106	−39.1	−31.3	−31.5

The sorbed gas data are summarized in Table 3b. The concentration of these gases in the 0 to 10 cm layer was very similar to that in the 60 to 70 cm layer. Methane was present at a concentration of ca 100 ppb and small amounts of higher hydrocarbons (C_2 to C_5) were also found. These sorbed gases are probably of thermal origin, considering the existence of C_{2+}-compounds ($C_1/C_2+C_3 \simeq 8$) and the methane carbon isotope ratios ($\delta^{13}C = -39$ ‰) which are typical for thermal gases.

DISCUSSION

Confirmation of the presence of methanotrophic bacteria in *Siboglinum poseidoni* is provided by the presence of hexulosephosphate synthetase and methanol dehydrogenase in extracts of the symbiont-containing post-annular tissues. Only low activities were found in the anterior sections of the pogonophores: probably because these preparations were slightly contaminated with trophosome tissue.

The activities of hexulosephosphate synthetase and methanol dehydrogenase in the bacteria-containing pellet from the post-annular tissues were comparable to those found in preparations from pure cultures of type I methanotrophic bacteria (Patel et al. 1978, Levering et al. 1981). Similar methanol dehydrogenase activities were reported from gill extracts of a mussel from a hydrocarbon seep on the Louisiana slope (Fisher et al. 1987). This mussel has endosymbiotic methanotrophic bacteria in the gills.

Activities of both enzymes in *Siboglinum poseidoni* were, however, lower than those reported by Cavanaugh et al. (1987) from the gills of another mussel with methanotrophic endosymbionts, from hypersaline seeps in the Gulf of Mexico. This latter mussel had enzyme activities ca 10-fold greater than those we report in *S. poseidoni*. Since the invertebrate tissues contain eukaryote organelles in addition to bacteria, it would be expected that higher enzyme activities would be found in bacterial cultures than in the tissue extracts. It is possible that the bacteriocytes may modify the bacteria and increase the enzymes of methane oxidation to enhance the assimilation pathway.

The concentration of free methane found in sediment surrounding the pogonophores (24 ppb) was very low. In several marine sediments the concentration of bacterial methane can be higher than 300 ppb (Faber & Stahl 1984). Low values are often found in sediments where bacterial oxidation converts methane to carbon dioxide (Whiticar & Faber 1986). This process generally increases the ^{13}C-concentration in the remaining methane due to the ^{12}C preference of the bacteria. If the free methane is a remnant of such an oxidation then the original methane should have a more negative $\delta^{13}C$ than the observed value of −80.3 ‰. However, the process of bacterial oxidation is thought to be unlikely because the methane and carbon dioxide isotope ratios fall within the zone of bacterial methane generation/CO_2-reduction (Whiticar & Faber 1986). The low free gas methane concentration may be explained by loss of methane during sampling or by sampling the sediment just within the zone of bacterial methane generation. In the latter case methane could have been consumed in sulphate- or oxygen-containing niches of this strongly bioturbated upper sediment layer.

Methanotrophy of the endosymbiotic bacteria of *Siboglinum poseidoni* is also confirmed by the carbon isotope analyses. The stable carbon isotope ratio of the bacteria-containing posterior parts of the pogonophores (−78.3 ‰) is very close to that of the free methane in the 0 to 10 cm layer ($\delta^{13}C = -80.3$ ‰). This suggests that the free methane but not the sorbed gas is consumed by the endosymbiotic methanotrophic bacteria. The sorbed methane has a different isotopic signature (cf. Table 3b) and is believed, due to its close association with the sediment grains, to be inaccessible to bacteria or small invertebrates (Faber 1987).

A comparison of the small difference in isotopic ratios between the anterior and post-annular parts of the pogonophores indicates that most of the carbon of the anterior parts is contributed by metabolites assimilated by the methanotrophic symbionts in the posterior. If there was no transfer of carbon from the bacteria to the pogonophore tissues and if the uptake of soluble organic comounds via the epidermis played a major role there should be a larger difference in the isotopic ratio.

The carbon isotope ratios of *Siboglinum poseidoni* correspond to the most negative values from organic matter known. Only a mytilid mussel with methanotrophic symbionts from the Florida escarpment site showed a comparable low $\delta^{13}C$, −74.3‰ (Paull et al. 1985). Another mytilid mussel with methanotrophic symbionts from the Louisiana hydrocarbon seep sites was less depleted ($\delta^{13}C$ = −40.1 to −57.6‰), indicating a dependence on thermogenic methane (Brooks et al. 1987).

Other small pogonophores from the North Atlantic which contain sulphur-oxidizing symbionts have carbon isotope ratios between −35 and −45‰ (Spiro et al. 1986). These differ clearly from *Siboglinum poseidoni* and emphasize its special metabolic position. ^{13}C is also much less depleted in *Thyasira sarsi* from the Skagerrak site, $\delta^{13}C$ = −39.5‰, than in the associated *S. poseidoni*. It is, however, interesting to note that *T. sarsi* in the Skagerrak is much more depleted in ^{13}C than *T. sarsi* from a fjord near Bergen (Norway) (gills: $\delta^{13}C$ = −31.0‰, rest of body: −28.2‰; Spiro et al. 1986).

The shell carbonate in specimens of *Thyasira sarsi* from the *Siboglinum poseidoni* site has a $\delta^{13}C$ of −12‰ (B. Spiro pers. comm.)

The shell carbonate value probably represents the mixture of bicarbonate from the overlying water ($\delta^{13}C \simeq 0$‰) and the interstitial water from the sediment ($\delta^{13}C$ = −19.4‰). The mean temperature at 300 m in the Skagerrak is 6°C (Josefson 1986). At this temperature there is an enrichment in ^{12}C of +3.16‰ in the aragonite of the shell with respect to dissolved bicarbonate (Rubinson & Clayton 1969, Emrich et al. 1970). This implies that the bicarbonate incorporated into the shell has a mean $\delta^{13}C$ of −15.16‰, suggesting that the bivalve may be pumping 62% of its water from the tunnel network formed below it (Dando & Southward 1986) and the remainder through the inhalent tube from the surface. If we assume an average $\delta^{13}C$ of −12‰ in bicarbonate, from the mixed water reaching the gills of the Skagerrak *T. sarsi*, and a Δ = −25‰ ^{13}C depletion on fixation by sulphur-oxidizing bacteria (Ruby et al. 1987), then the organic carbon formed should have a $\delta^{13}C \simeq -40$‰. This is very close to the observed value of −39.5‰ for the entire gill (Table 2).

This isotope data for *Thyasira sarsi* from a methane seep confirms the conclusion from studies of other habitats (Dando & Southward 1986, Spiro et al. 1986) that this bivalve obtains most of its carbon from CO_2 fixed by ribulosebisphosphate carboxylase in the sulphur-oxidizing symbionts. Methanol dehydrogenase activity could not be detected in gill extracts from these *T sarsi*. These gills were not assayed for hexulosephosphate synthetase activity but the enzyme was not detected in gill extracts from specimens obtained from the Swedish coast at Lysekil. It is therefore unlikely that the methylotrophic bacteria isolated from the gills of *T. sarsi* by Wood & Kelly (1989) are the major symbiont species. *T. sarsi* is not a species usually found in the open Skagerrak, where the common species at 300 m are *T. equalis, T. obsoleta* and *T. ferruginea* (Josefson 1985). It is common in organic-rich sediments with a high sulphate-reduction rate. At the 300 m station it is found together with *Siboglinum poseidoni*, but is absent from the surrounding sediment areas. This suggests that the organic input driving the sulphate-reduction may be methane diffusing up towards the surface of the sediment. Iversen & Jørgensen (1985) showed that at a 200 m depth site in the Skagerrak there was a peak in methane oxidation at 100 cm sediment depth which coincided with a peak in sulphate reduction. Thus methane may provide the sulphide, via sulphate reduction, needed by the symbiotic sulphur-oxidizing bacteria in *T. sarsi* as well as the carbon and energy source for the methanotrophic bacteria in *S. poseidoni*.

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