A Methanotrophic Marine Molluscan (Bivalvia, Mytilidae) Symbiosis: Mussels Fueled by Gas

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An undescribed mussel (family Mytilidae), which lives in the vicinity of hydrocarbon seeps in the Gulf of Mexico, consumes methane (the principal component of natural gas) at a high rate. The methane consumption is limited to the gills of these animals and is apparently due to the abundant intracellular bacteria found there. This demonstrates a methane-based symbiosis between an animal and intracellular bacteria. Methane consumption is dependent on the availability of oxygen and is inhibited by acetylene. The consumption of methane by these mussels is associated with a dramatic increase in oxygen consumption and carbon dioxide production. As the methane consumption of the bivalve can exceed its carbon dioxide production, the symbiosis may be able to entirely satisfy its carbon needs from methane uptake. The very light ($\delta^{13}C = -51$ to -57 per mil) stable carbon isotope ratios found in this animal support methane ($\delta^{13}C = -45$ per mil at this site) as the primary carbon source for both the mussels and their symbionts.

BACTERIAL ENDOSYMBIONTS USING reduced sulfur compounds as energy sources and fixing CO_2 by means of the Calvin-Benson cycle have been implicated as the major source of primary production around the deep-sea hydrothermal vents (*1–3*). These bacteria are found in the gills of the clams and within the trunk of the vestimentiferan tubeworms that live near the vents. Since the initial discovery, such symbioses have been found in a variety of other taxa (*2, 4*) as well as a variety of other habitats (*5–7*) characterized by the availability of both reduced sulfur compounds and O_2. Shortly after the initial discovery of these symbioses, investigators began looking for symbioses based on other reduced compounds found in some of these environments. We present here evidence of a methane-based symbiosis between an animal and intracellular bacteria. Methane is the principal component of natural gas.

Childress *et al.* have shown CH_4 consumption by the bacteria-containing tissue of the vent tubeworm *Riftia pachyptila* (*8*), but the intact animal does not take up CH_4 (*9*). Other researchers have suggested on the basis of the internal membranes seen in some pogonophoran and mussel symbionts that some symbioses consume CH_4 (*2, 10*); however, there has been no demonstration of CH_4 uptake by these symbioses. On the basis of their observations of unusually light stable carbon isotope ratios, Kulm *et al.* have suggested that the clams and worms of the Oregon subduction zone consume CH_4 (*11*). However, the absence of any supporting data on the Oregon organisms and the fact that the same species found elsewhere have sulfur-based symbioses (*2*) make this suggestion highly speculative at best. Arp *et al.* have also failed to demonstrate significant CH_4 uptake in the vent clam *Calyptogena magnifica* (*12*). Thus, the results described in this report were unexpected. The very first measurements and all subsequent ones have shown that these mussels consume CH_4 at a high rate. Whole animal experiments have now been conducted on nine individual mussels. In addition, 28 separate experiments have been carried out on excised gill tissue from eight different individual mussels. In every case where O_2 was not limiting and no inhibitor was being used, the rate of CH_4 consumption was high, generally approaching that of O_2 consumption.

The mussels used in this study (*13*) were collected in two trawls near hydrocarbon seep sites on the Louisiana slope of the Gulf of Mexico (*6*) (27°41′N, 91°32′W) at bottom depths of 600 to 700 m. The same trawls also retrieved vestimentiferan tubeworms (*14*) and pogonophorans. Immediately after capture, the blood gas contents of seven vestimentiferans, which were collected in a clump at the first site, were analyzed by gas chromatography (*9*). Five of these animals contained H_2S (113, 42 and 27 μmol/liter and two with trace amounts), three contained CH_4 (142 and 110 μmol/liter and one with a trace amount), and two contained CO (24 and 16 μmol/liter). Thus, this appears to be a habitat with CH_4 as well as sulfide available at high concentrations. As confirmation, a piston core taken along the trawl track (27°40.5′N, 90°31.6′W) showed visible oil staining and H_2S. Methane concentrations in the sediments can be very high since gas hydrates are present in this region (*15*).

Our initial measurements of O_2 and CH_4 consumption by whole mussels showed extraordinarily rapid rates of initial CH_4 and O_2 consumption, making experimentation difficult. Therefore, we focused our efforts at sea on studying the metabolism of gill pieces (Fig. 1, A, B, and C, and Table 1). The gills were rapidly dissected free of the animals, rinsed in 0.45-μm membrane-filtered seawater (MFSW), cut into 0.4- to 0.5-g pieces, and kept at 7.5°C in MFSW until used. The gills' ciliary activity continued for more than 12 hours after dissection under these conditions; however, we completed all experiments within 7 hours. The separated pieces of gill were incubated at 7.5°C in 20-ml glass syringes closed with plastic valves. The syringes were filled with MFSW that had been partially decarbonated (total $[CO_2] \approx 600$ μmol/liter), adjusted to *p*H 8.3, and equilibrated with appropriate gases. Imme-

Table 1. Gas exchange rates in tissue pieces from intact mussels of an undescribed species from a hydrocarbon seep off Louisiana. The tissue pieces were incubated in 20-ml glass syringes in membrane-filtered (0.45 μm) seawater. The whole animals were measured in a flowing stream of water. All gases were analyzed by gas chromatography (*9*). All measurements were made at 7.5°C. Gill protein content averaged 15.3% of wet weight by the Lowry method with bovine serum albumin as a standard. Numbers in parentheses are the 95% confidence intervals.

Conditions	Tissue	*n*	O_2 range (μmol/liter)	CH_4 range (μmol/liter)	Gas exchange rates (μmol/g wet weight per hour)		
					CO_2	O_2	CH_4
Aerobic	Gill	6	90–200		+1.44(±0.35)	−1.35(±0.39)	
Aerobic, CH_4	Gill	10	90–200	44–190	+2.09(±0.38)	−2.10(±0.22)	−1.36(±0.23)
Aerobic, CH_4, acetylene	Gill	3	100–210	58–169	+1.36(±0.20)	−1.33(±0.43)	−0.10(±0.11)
Hypoxic, CH_4	Gill	4	5–30	200–435	+1.05(±0.47)	−0.15(±0.08)	−0.17(±0.25)
Aerobic, CH_4	Foot	2	130–200	110–207	+0.45, +0.94	−0.86, −0.43	+0.05, −0.11
Aerobic, CH_4	Mantle	2	140–200	84–226	+0.65, +0.38	−0.54, −0.53	−0.14, +0.07
Aerobic	Whole animal	2	120–200		+0.40, +0.34	−0.29, −0.24	
Aerobic, CH_4	Whole animal	2	50–150	20–205	+0.50, +0.37	−1.19, −1.20	−0.74, −0.90

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diately after introducing the gill piece, a sample of water taken from the syringe was analyzed for CO_2, O_2, N_2, CH_4, and acetylene by gas chromatography (*9*). In each sampling a total of 2 ml was removed from each incubation syringe to provide the 0.5-ml sample for analysis and to flush the sampling syringe. Each incubation syringe was sampled four times at 40- to 50-minute intervals. The rates declined with time, apparently because of mixing problems associated with the high metabolic rates, since changing the incubation media restored the original rates. The rates presented in Table 1 are based on the average rates over the first two sampling intervals (that is, the first three analyses). Control samplings demonstrated that the seawater medium showed no significant consumption of CH_4 or other gases and that the consumption rates were insignificant when the gills were removed after 2 hours and the incubation was continued with the same water. Measurements were also made on gill pieces from *Pseudomiltha* sp., *Vesicomya cordata*, *Solemya reidi*, and *Calyptogena elongata*, as well as on trophosome preparations from two vestimentiferan tubeworms [*Lamellibrachia* sp. and an unidentified vestimentiferan belonging to an undescribed genus (*14*)]. All of these species have either been demonstrated to harbor symbiotic sulfur-based chemoautotrophic bacteria or to have close relatives for which this mode of life has been demonstrated (*2, 4*). Although all of these other preparations from symbiont-containing tissues showed significant O_2 consumption and CO_2 production, none showed significant CH_4 consumption, supporting the contention that CH_4 consumption is occurring within the seep mussel gills and is not due to contaminating bacteria or an unknown artifact. Figure 1 shows the concentration changes observed in the syringes during experiments with tissues from the seep mussel and shows the typical range of conditions used and analytical precision achieved. This figure is not directly convertible to rates because the volumes in the syringes changed after each analysis.

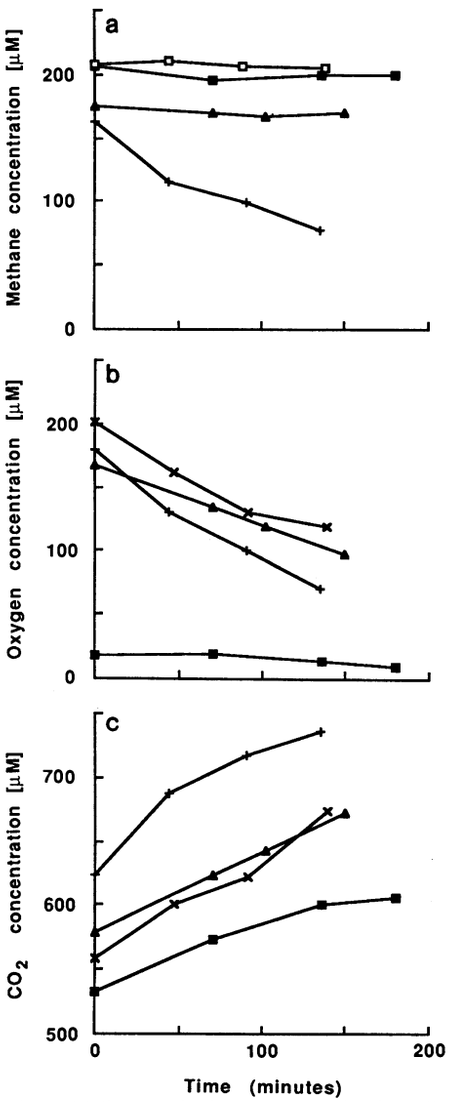

Fig. 1. (a) Changes in CH_4 concentration in a 20-ml syringe during the incubation of a piece of mussel flesh in membrane-filtered (0.45 μm) seawater. Two milliliters was withdrawn at each time period, and 0.5 ml was analyzed by gas chromatography (*9*). +, Mussel gill (0.445 g) under aerobic conditions with CH_4 present; ■, mussel gill (0.462 g) hypoxic in presence of CH_4; ▲, mussel gill (0.418 g) under aerobic conditions with CH_4 and 38 μmol/liter acetylene present; □, mussel foot (0.667 g) under aerobic conditions with CH_4. (b) Changes in O_2 concentration under same conditions as (a). Same symbols as in (a) with the addition of x, mussel gill (0.436 g) aerobic with no CH_4. (c) Changes in CO_2 concentration under same conditions as in (b) with the same symbols.

Table 1 and Fig. 1 show significant consumption of CH_4 by the mussel gills in the presence of O_2. The rates of O_2 consumption and CO_2 production are also significantly elevated (Mann-Whitney U test, $P = 0.01$ for CO_2 and $0.01 > P > 0.005$ for O_2) when CH_4 is being consumed. These data indicate that CH_4 is being oxidized by O_2 but that much of the carbon is also being retained within the organism, presumably as organic carbon. The observed CH_4 consumption is abolished by low O_2 or by acetylene (20 to 40 μmol/liter), as is typical of methanotrophic bacteria (*16*). Neither foot nor mantle tissue of the mussels show significant consumption of CH_4, indicating that it is limited to the bacteria-containing gills of the mussel and is not caused by bacteria living on the surface of the mussel tissues.

In whole animal experiments (Table 1), individuals were placed in chambers in a running stream of filtered (0.2 μm), ultraviolet-sterilized, antibiotic-treated (penicillin G and streptomycin sulfate at concentrations of 50 mg/liter each) seawater at 7.5°C and of controlled gas content. The gas concentrations in the seawater before entering and after leaving the chamber were measured by gas chromatography (*9*) and compared to those from a control chamber. The measurements support the gill studies, showing very high rates of CH_4 consumption, four- to fivefold stimulation of O_2 consumption in the presence of CH_4, and CH_4 stimulation of CO_2 production. Rates of CH_4 consumption were sustained for more than 24 hours, supporting the concept that these mussels are oxidizing CH_4. The fact that CH_4 consumption exceeds CO_2 production at these CH_4 levels (50 to 200 μmol/liter) suggests that this symbiosis can potentially derive its carbon needs entirely from methane consumption.

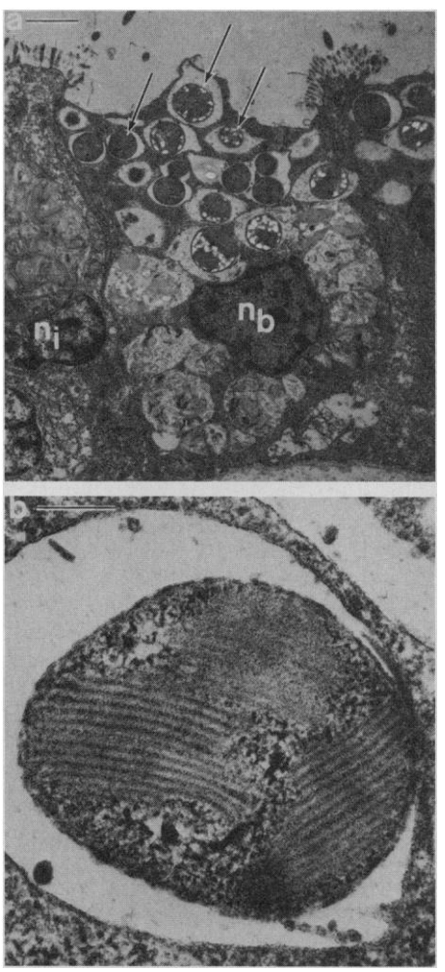

Fig. 2. (a) Electron micrograph of a cross section through a mussel gill filament. Central bacteriocyte is flanked by an intercalary cell with microvilli on the left, and an intercalary cell and bacteriocyte on the right. Some of the symbiotic bacteria are indicated by arrows; n_b: nucleus of bacteriocyte; n_i: nucleus of intercalary cell (×2550). Scale bar, 2 μm. (b) Electron micrograph of a bacterium within a mussel gill cell. Stacked internal membranes are typical of type I methanotrophs (×38,250). Scale bar, 0.2 μm.

The gill tissues of three mussels of this species had carbon isotope ratios ($\delta^{13}C$) of −51.8, −51.6, and −52.1 per mil, and the mantles of the same mussels had $\delta^{13}C$ values of −57.3, −52.1, and −52.3 per mil, respectively. Such extremely light numbers separate the seep mussels from the other (apparently sulfur-based) animal bacterial symbioses around these seeps, which have $\delta^{13}C$ values between −27 and −35 per mil (*6*). Since, as indicated above, the other symbioses tested do not appear to consume CH_4, the $\delta^{13}C$ values in the mussel may reflect the carbon isotopic composition of thermogenic CH_4 (−45 per mil) in its environment (*16*). The apparent homogeneity of the $\delta^{13}C$ values throughout the seep mussel tissues indicates that the carbon derived from CH_4 is distributed throughout the animal. Since CH_4 oxidation is taking place only in the gills, this implies the translocation of organic carbon derived from CH_4 from the gills to other tissues throughout the animal. The degree to which the $\delta^{13}C$ differs from that of the other organisms from the same environment also suggests that oxidation and incorporation of CH_4 carbon is a major nutritional input for this mussel. The stable carbon isotope data suggest that the $\delta^{13}C$ of animal tissue on the Louisiana slope may be useful for differentiating CH_4-based symbioses from sulfur-based symbioses. However, extrapolation of isotopic ranges between chemosynthetic systems (hydrothermal vent, subduction zones, brine seeps) is highly speculative, since a variety of processes can affect carbon isotope ratios, and CH_4 stable isotope ratios vary widely (*17*).

The question remains: what is the agent responsible for the oxidation? Optical and transmission electron microscopy (*18*) reveal the presence of abundant intracellular coccoid bacteria in vacuoles within the gills (Fig. 2a). Stacks of intracytoplasmic membranes, typical of type I methanotrophs (*16*), are visible in many of these bacteria (Fig. 2b). Type I methanotrophs have the ribulose monophosphate cycle and incorporate carbon from CH_4 into organic compounds (*16*). These symbionts are very close to the gill surface, which would facilitate CH_4 uptake from the seawater.

This symbiosis between a methanotrophic bacteria and an animal host is potentially able to derive a large fraction of its energetic and carbon needs from the consumption of the reduced single carbon compound CH_4 (*19*). This form of symbiosis may well be found in other vent and seep mussel species.

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13. This is an apparently undescribed species of mussel related to *Bathymodiolus*. Specimens have been provided to R. Turner, Museum of Comparative Zoology, Harvard University.
14. Specimens of these tubeworms have been provided to M. L. Jones, U.S. National Museum of Natural History, Smithsonian Institution, Washington, DC.
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18. Tissues were removed from living animals and fixed (on board ship) within hours of collection in 3% glutaraldehyde in 0.1*M* phosphate-buffered 0.35*M* sucrose (*p*H 7.35) and stored at 4°C until returned to the laboratory (2 weeks). The tissues were then postfixed in 1% osmium in buffered sucrose, dehydrated through a graded ethanol series, and embedded in Spurr's resin. Sections (80 to 90 Å in width) were stained with uranyl acetate and lead citrate, and then viewed with a Siemens 1A electron microscope.
19. Although many sulfur-fueled symbioses (lucinid clams excepted) are reputed to be rather repulsive food items because of their high sulfide contents, one of us (J.J.C.) has sampled this mussel and finds its flesh to be quite sweet and delicious.

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Effects of Alcohol on the Generation and Migration of Cerebral Cortical Neurons

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Prenatal exposure to alcohol produces many developmental defects of the central nervous system, such as microcephaly, mental retardation, motor dysfunction, and cognitive deficiencies. Therefore, the generation of neurons in the cerebral cortex was examined in the offspring of female rats fed a diet containing ethanol. Prenatal exposure to ethanol delayed and extended the period during which cortical neurons were generated, reduced the number of neurons in the mature cortex with the same time of origin, and altered the distribution of neurons generated on a particular day. Thus, the proliferation and migration of cortical neurons are profoundly affected by in utero exposure to ethanol.

ALCOHOL IS A POTENT TERATOGENIC substance, and as such it is the prime cause of mental retardation in the Western world (*1*). It is estimated that as many as 2% of all babies born alive are afflicted with fetal alcohol effects (*2*). Prenatal exposure to alcohol affects the development of various systems, including the musculoskeletal, cardiovascular, and peripheral and central nervous systems. Many of these defects are neurological and indicate abnormal development of the cerebral cortex, particularly motor areas. Such abnormalities include hypotonia, language and coordinative difficulties, and deficiencies in cognitive and fine motor skills (*1, 3*). Moreover, anatomical studies of humans and rodents prenatally exposed to ethanol show that their brains are smaller and contain ectopic cell clusters (*1, 4, 5*), both of which suggest that neuronal development is altered by ethanol. My study provides evidence that in the earliest stages of neuronal ontogeny—that is, neuronal proliferation and migration—cortical neurons are affected profoundly by prenatal exposure to ethanol.

The time of origin of cortical neurons was determined with [^{3}H]thymidine autoradiography in rats prenatally exposed to ethanol and in control rats. Pregnant hooded rats were fed one of two protein-enriched liquid diets from gestational day (GD) 6 to the day of birth, which was GD 23 for ethanol-exposed rats and GD 22 for control

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