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Date: 15 June 1988

PROGRESS REPORT ON CONTRACT N00014-88-K-0177 R&T CODE 4412029

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PRINCIPLE INVESTIGATORS: Drs. Charles Fisher and James Childress

CONTRACTOR: Marine Science Institute, University of California, Santa Barbara

CONTRACT TITLE: Host-Symbiont Interactions Between a Marine Mussel and Methanotrophic Bacterial Endosymbionts

START DATE: 1 October 1987

RESEARCH OBJECTIVE: To delineate the interactions between a newly discovered mussel and its methanotrophic symbionts in order to reach a more complete understanding of the intact symbiosis.

PROGRESS (Year 1): Since last October we have made considerable progress in determining the importance of various food and energy sources to this symbiosis. Work begun in July of last year has demonstrated that the mussels can grow with methane as the sole carbon and energy source (see enclosed reprint). However, in a another study we have found that although the shell is growing in response to methane, the mussels' soft tissue is being depleted under these experimental conditions and this is reflected in a lower "condition index" of the mussels, when compared to freshly collected animals. This second study also demonstrates that neither unicellular algae (as a food source) nor thiosulfate (as a symbiont energy source) is sufficient for either shell or soft tissue growth in this mussel (Thiosulfate was suggested as an additional possible energy source for the symbionts, by some of our earlier work, Fisher et al 1987). We have, in a separate study, been directly testing the mussels' ability to filter feed on particulates (both algae and bacteria). Our data indicates that these mussels can clear both algae and bacteria from the surrounding water (at relatively low rates) and assimilate a significant portion of the particulates they clear.

We have also begun investigations into other possible sources of nitrogen for this symbiotic association. Our preliminary whole animal N_2 flux data supports some preliminary results of J. Stein's (J. Stein is a graduate student in Horst Felbeck's laboratory at Scripps.

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whom we have invited to collaborate on this aspect of the project) which indicate that the symbionts contain a Nif gene and are therefore capable of fixing nitrogen.

We have begun to develop a bacterial purification protocol for these symbionts. Using a combination of filtration through a graded series of Nitex screens and differential centrifugation we can purify these symbionts several fold with high recovery. We are also experimenting with density gradient centrifugation and alternative grinding methods to optimize purity, and yield of symbionts.

WORK PLAN (Year 2): Our objectives for next year are several, and will be accomplished largely using animals we will collect this fall (we have been awarded submersible time by NOAA to pursue these and other investigations of hydrocarbon seep animals):

1. To complete the laboratory analyses and prepare for publication the 2 food and energy source studies outlined above. These studies are largely complete and should require only a few weeks of laboratory work before we will be ready to write up the study.

2. To complete our investigations on the role of nitrogen fixation in this symbiosis. This will include some acetylene reduction experiments planned for this fall and further quantitative analysis of NH_4 and N_2 uptake by live animals.

3. We will began studies of nutrient transfer between the symbiont and host using live animals, 14-C methane, and both autoradiographic and dissection techniques. Some of these incubations will be done on board ship with fresh animals this fall, and the rest will be accomplished in the laboratory with animals we will maintain. (We still have mussels alive which were collected last July).

4. To perfect our symbiont purification protocol and conduct the carbon assimilation and flux studies necessary for our investigations of genetic autonomy of the symbionts. These same purified symbiont preparations will be used to investigate the release of organic products by the symbionts.

5. To measure rates of CH_4 and CO_2 flux in whole mussels and isolated symbionts under various concentrations of methane and oxygen to determine the dependency of the intact symbiosis, and the effects of the animal tissue on the uptake of these materials by the symbionts.

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1. We have yet to publish any work stemming directly from this grant but the following was done as a part of this study, although the work was completed before the award arrived (work conducted between July and October, 1987):

Cary, S. C., C. R. Fisher, and H. Felbeck (1988) Mussel growth supported by methane as sole carbon and energy source. Science, 240: 78-80. (reprint enclosed)

2. A paper detailing some of our studies with this mussel has been accepted for the 5th International Deep-Sea Biology Symposium in Brest, France and will be presented there later this month :

Childress, J. J. and C. R. Fisher (1988) The methanotrophic symbiosis in a hydrocarbon seep mussel. (abstract enclosed)

RELATED GRANT: We were awarded ten days of submersible time (20 dives) by NOAA's Office of Undersea Research for this fall. ("Studies of chemosythetic communities, oil seepage, and gas hydrates on the upper Gulf of Mexico continental slope"). We will collect mussels (and other symbiont-containing clams and tube-worms) from the Louisiana Slope during this cruise for studies on board ship as well as for shipment back to UCSB for laboratory studies over the next year. The exact dates of the cruise are uncertain at this time, we are tentively scheduled for early August.

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SCIENCE

Mussel Growth Supported by Methane as Sole Carbon and Energy Source

S. CRAIG CARY, CHARLES R. FISHER, AND HORST FELBECK

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Mussel Growth Supported by Methane as Sole Carbon and Energy Source

S. CRAIG CARY, CHARLES R. FISHER, HORST FELBECK

Symbioses between chemoautotrophic bacteria and several specialized marine invertebrates are well documented. However, none of these symbioses have been demonstrated to provide sufficient energy and carbon to the host to enable it to grow. Growth rates of seep mussels collected from hydrocarbon seeps off the coast of Louisiana were measured in a controlled environment where methane was the sole carbon and energy source. The growth rates increased to a maximum of 17.2 micrometers per day in response to methane and approached zero in the absence of methane. These mussels contain methanotrophic symbiotic bacteria in their gills, which suggests that these bacteria provide their hosts with a net carbon flux originating from methane.

THE OCCURRENCE OF SYMBIOTIC chemoautotrophic bacteria that reside in highly specialized tissues of certain marine invertebrates was first described as occurring in hydrothermal vent communities and since then in many other diverse reducing marine environments (*1*). These bacteria typically oxidize reduced sulfur compounds from their environment and use the energy obtained to fix carbon dioxide from the surrounding seawater (*2*, *3*). These chemicals can be formed either geothermally from seawater sulfate, as at the hydrothermal vents, or biologically through sulfate-reducing bacteria in the sediments, as in mudflats or hypoxic deep-sea basins (*4*, *5*). Another source of energy and carbon has recently been demonstrated for mussels associated with hydrocarbon seepage sites on the Louisiana slope in the Gulf of Mexico (*5–7*) at the base of the Florida Escarpment (*8*) and for a small pogonophoran from the Skagerrak (*9*). These animals contain methanotrophic symbionts. The symbionts in the gill cells of the mytilids (*6*, *8*) and the trophosome cells of the pogonophorans (*9*) contain stacked internal membranes characteristic of type 1 methanotrophs. Furthermore, enzymatic tests, stable isotope determinations, net methane uptake studies, and the incorporation of ^{14}C-labeled methane indicate that these symbioses are methanotrophic (*6–8*). The methane necessary for the support of this metabolism in the mussels in situ originates either from hydrocarbon sources at the oil seeps or from biological processes at the Florida Escarpment communities (*4*, *5*).

For both chemolithoautotrophic and methanotrophic associations, researchers have proposed (*2*, *3*, *6*) that at least part of the nutritional requirements of the respective hosts is supplied by the bacteria. Some host animals have entirely lost the ability to take up and digest external food. The vestimentiferan tube worms, the pogonophorans, many oligochaetes of the subfamily Phallodrilinae, and several bivalves of the Solemyidae have lost their digestive systems and must depend on an alternative nutritional source—most likely the symbiotic bacteria (*10*). Similarly, most other bivalves known to contain chemoautotrophic symbiotic bacteria are characterized by a reduced digestive system (*10*, *11*). Other indirect evidence for the importance of bacterial carbon to the hosts includes studies in which stable isotope ratios (^{13}C/^{12}C) in symbiont-containing animals were measured. Comparisons of paired tissues from individual animals of a variety of symbiont-containing species, including the mussel species used in our study, showed little variation, indicating the importance of symbiont carbon to the host (*5*, *6*, *12*). This finding is especially convincing in the case of animals containing methanotrophic symbionts because of the negative ^{13}C/^{12}C associated with methane. Transfer of symbiont carbon to the host can be accomplished in several ways: through digestion of the bacteria by host lysosomes or through the translocation of part of the fixed carbon from the bacteria to the host. The first strategy has been proposed in the symbioses of the hydrothermal vent tube worm *Riftia pachyptila* by Bosch and Grasse (*13*), who document an intracellular degradation of symbiotic bacteria. Hand (*14*) and Giere and Langheld (*15*) observed similar phenomena in the bacteriocytes of *Riftia pachyptila* and in the oligochaete *Phallodrilus leukodermatus*, respectively. The second proposed strategy of nutrient transfer is the translocation of reduced organic material from the bacteria to the host, as was shown with radiolabeled bicarbonate in *Solemya reidi* (*16*). However, it has not been demonstrated that the hosts can grow when provided *only* with an inorganic chemical as a bacterial energy source. We report here that mussels harboring methanotrophic bacteria as symbionts (*17*) grow when supplied only with methane in the seawater.

Growth has been considered an excellent

S. C. Cary and H. Felbeck, Scripps Institution of Oceanography, Marine Biology Research Division, University of California San Diego, La Jolla, CA 92093.
C. R. Fisher, Marine Science Institute and Department of Biological Science, University of California Santa Barbara, Santa Barbara, CA 93106.

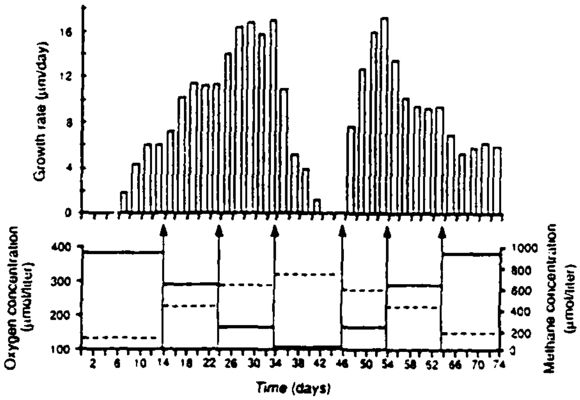

Fig. 1. Growth rates of a symbiont-containing mytilid bivalve species from oil seeps off the coast of Louisiana at various concentrations of methane and oxygen. The vertical bars illustrate the mean growth rate ($n = 8$) averaged over 2 days. The standard deviations for each growth period never exceeded ±1 µm/day. The horizontal solid lines in the lower section indicate the methane, and dotted lines are the oxygen concentrations during the respective periods. The arrows show the times when experimental conditions were changed.

indicator of overall health and vitality of a bivalve (*18*). Under stress, shell growth is one of the first factors to change. To avoid the typically long-term growth measurements, that is, measurement of length, width, weight, volume, or others in which it usually takes months to demonstrate significant changes, we chose a more sensitive technique. By means of laser diffraction, we determined the growth rate by measuring the change of the width of a gap created by the edge of the mussels and a fixed plastic tab. The projected pattern was sensed by a photodiode array, the output of which was fed into a computer for analysis. This technique has been used to measure short-term changes in the growth rate of the shallow water mytilid *Mytilus edulis* that occurred as a result of varying phytoplankton concentrations (*19*). Width changes of the gap as small as 3 µm can be observed. The growth of the mussels was determined every 2 days until a plateau was reached (*20*). The ratio of methane to air bubbled through the incubation water was then altered (*21*).

The growth rates demonstrate a clear dependence on the availability of methane in the water (Fig. 1). After an initial period of no growth when no methane was supplied, the growth rates increased to a maximum of 17.2 µm/day in the presence of methane. Without methane, growth stopped after a short period and quickly increased again when the methane supply was resumed. A response could be measured in each case after an initial lag of 2 days. This response time is comparable to that of *M. edulis* when fed different concentrations of cultured phytoplankton. To ensure that the growth was actually dependent on methane, the stepwise changes were repeated in reverse order after the period without methane and without growth. The growth rates increased again after methane was added to the seawater to approximately the same level as recorded earlier in the presence of methane.

The mussel growth rates also show a correlation to the methane and oxygen concentrations. The highest growth rates were obtained at the lowest measured methane concentration (245 µmol/liter) and a high oxygen concentration (290 µmol/liter). The lowest growth rates were observed at the highest methane (980 µmol/liter) and lowest oxygen concentration (140 µmol/liter). Methanotrophic bacteria grown in culture normally show increased yields at higher methane levels, limited only by very low oxygen concentrations (*22*). The different response reported here for an intact symbiotic association could be due to several factors. First, at high levels of methane the oxygen concentrations could be limiting for the host. Mussels in general are poor regulators of oxygen consumption, and their metabolic rates are dependent on the oxygen concentration. The lower growth rates could be due to the lower metabolic rates of the host. Second, the symbiotic bacteria in the presence of methane could be limited by the oxygen diffusing into the bacteriocytes; therefore, the bacteria respond to any changes of the oxygen concentration with an altered supply of organic carbon to the host. Third, the reduced growth could be due to an unidentified growth inhibitor in natural gas. However, the possibility that the trace hydrocarbon contaminants present in natural gas (*21*) would be deleterious to a mussel collected from a site characterized by crude oil and natural gas seeping from the sediments (*23*) is unlikely.

Since we have observed particulates in the guts of freshly collected seep mussels, it appears that additional assimilation through normal feeding may be possible. At this time, it is uncertain to what extent this feeding mode contributes to the overall nutritional requirements of the host. Methane can supply only carbon and energy to the bacteria; the essential elements phosphorus, nitrogen, and sulfur must be derived from another source. Since the natural growth rate of the mussels is unknown, it cannot be excluded that the maximal growth rate reported here is lower than the one found in situ. When fed natural concentrations of phytoplankton, *M. edulis* grew an order of magnitude faster than reported here for the seep mussels (*19*). Methane may account for only part of the growth of these animals, the remainder being supported by particulate or dissolved material. However, the experiment described here shows that mussels, known to contain methane-oxidizing bacteria, can grow with methane as their sole carbon and energy source.

REFERENCES AND NOTES

1. C. M. Cavanaugh, *Bull. Biol. Soc. Wash.* 6, 373 (1985); H. Felbeck, M. A. Powell, S. C. Hand, G. N. Somero, *ibid.*, p. 261.
2. H. Felbeck, *Physiol. Zool.* 58, 272 (1985).
3. A. E. Anderson, J. J. Childress, J. A. Favuzzi, *J. Exp. Biol.* 131, 1 (1987).
4. C. K. Paull *et al.*, *Nature (London)* 317, 709 (1985); C. K. Paull *et al.*, *Science* 226, 965 (1984).
5. J. M. Brooks *et al.*, *Science* 238, 1138 (1987).
6. J. J. Childress *et al.*, *ibid.* 233, 1306 (1986).
7. C. R. Fisher, J. J. Childress, R. S. Oremland, R. R. Bidigare, *Mar. Biol.* 96, 59 (1987).
8. C. M. Cavanaugh, P. R. Levering, J. S. Maki, R. Mitchell, M. E. Lidstrom, *Nature (London)* 325, 346 (1987).
9. R. Schmaljohann and H. J. Flügel, *Sarsia* 72, 91 (1987).
10. M. L. Jones, *Bull. Biol. Soc. Wash.* 6, 117 (1985); C. Erseus, *Zool. Scr.* 13, 239 (1984); E. C. Southward, *J. Mar. Biol. Assoc. U.K.* 62, 889 (1982).
11. R. G. B. Reid and D. G. Brand, *Veliger* 29, 3 (1986); M. Schweimanns and H. Felbeck, *Mar. Ecol. Prog. Ser.* 24, 113 (1985).
12. G. H. Rau, *Science* 213, 338 (1981); B. Spiro, P. B. Greenwood, A. J. Southward, P. R. Dando, *Mar. Ecol. Prog. Ser.* 28, 233 (1986).
13. *C. Bosch and P. P. Grasse, C. R. Acad. Sci.* 299, 413 (1984); *ibid.* 301, 1 (1984).
14. S. C. Hand, *Biol. Bull. (Woods Hole)* 173, 260 (1987).
15. O. Giere and C. Langheld, *Mar. Biol.* 93, 641 (1987).
16. H. Felbeck, *J. Comp. Physiol.* 152, 3 (1983); C. R. Fisher and J. J. Childress, *Mar. Biol.* 93, 59 (1986).
17. The animals were collected at a depth of 700 m with the submersible *Johnson Sealink* on a cruise to the Louisiana oil seeps (27°41′N, 91°32′W) in the spring of 1987. They were brought to the surface in a temperature-insulated container and maintained in flowing seawater bubbled with methane at in situ temperatures.
18. C. B. Jörgensen, *Ophelia* 15, 175 (1976); R. L. Thompson and B. L. Bayne, *Mar. Biol.* 27, 317 (1974).
19. T. Strömgren, *Limnol. Oceanogr.* 20, 845 (1975); ______ and S. C. Cary, *J. Exp. Mar. Biol. Ecol.* 76, 23 (1984).
20. Intact small mussels were fixed to Lucite panels by gluing one valve to the support. A fixed gap was then created by attaching plastic tabs near the valve edge. A laser beam was projected through the gap to create a characteristic diffraction pattern. The distance between the light maxima in this diffraction pattern is directly proportional to the gap width. A panel with eight mussels was incubated in a 7-liter water tank at in situ temperature (9.0° ± 0.5°C). The incubation chamber was held in a secondary water bath to maintain constant temperature. The filtered seawater (0.45-µm mesh) was changed daily to avoid growth and accumulation of methylotrophic bacteria that could have served as an additional food source. Different ratios of natural gas and air, monitored by gas flow meters, were bubbled continuously through the water to keep methane concentrations at constant levels. The ratio was changed stepwise from 10 to 50% methane. The rate of

supply of the mixture never exceeded 100 cm^3/min. The growth resulting from the different methane levels was then measured every 2 days. The methane concentrations in the water were measured by gas chromatography with a Porapak Q column and a flame ionization detector. The oxygen concentrations were monitored with a polarographic oxygen electrode (Strathkelvin, U.K.). During the entire duration of this study the mussels appeared to be in good health, that is, none of the animals died, the siphons were extended, and extensive byssal threads were produced.

21. Monthly Gas Quality Report, Ehrenberg meter station, no. 32002, sampling date, 30 July 1987 (San Diego Gas and Electric, San Diego, August 1987).
22. C. Anthony, *The Biochemistry of Methylotrophs* (Academic Press, New York, 1982).
23. M. C. Kennicutt II *et al.*, *Nature (London)* 317, 351 (1985).

24. Supported by NSF grants OCE86-10512 (H.F.) and OCE86-10514 (J. J. Childress and C.R.F.). The National Undersea Research Program of the National Oceanic and Atmospheric Administration supported the collecting cruise and the dive time with the RV *Seward Johnson* and the DSRV *Johnson Sealink*, and *NR1* submersible support was provided by the Office of Naval Research. We thank the crew and pilots of the RV *Seward Johnson* and the DSRV *Johnson Sealink* for their assistance in collecting animals, B. Ward for the use of the gas chromatograph, and J. J. Childress for critically reviewing the manuscript.

16 October 1987; accepted 4 February 1988

Fifth Deep-Sea Biology Symposium
Brest - June 26th/July 1st 1988

THE METHANOTROPHIC SYMBIOSIS IN A HYDROCARBON SEEP MUSSEL

JAMES J. CHILDRESS and CHARLES R. FISHER (Marine Science Institute, University of California, Santa Barbara, CA 93106 USA)

A mussel found around hydrocarbon seeps on the continental slope off Texas and Louisiana has been shown to have methanotrophic bacteria as symbionts. These bacteria are located within vacuoles in cells at the surface of the gill filaments. The gills in this mussel are greatly enlarged, accounting for about 20% of the wet tissue weight of the animal. Both gill pieces and intact mussels consume methane at high rates showing a simultaneous increase in carbon dioxide production and oxygen consumption. This indicates that methane is being oxidized in this symbiosis. In the intact animals oxygen consumption can increase more than 5-fold in the presence of methane, however the increase in oxygen consumption is not sufficient to completely oxidize the methane to carbon dioxide. Similarly the increase in carbon dioxide production in the presence of methane is not sufficient to account for all of the methane consumed, therefore an appreciable fraction of the methane carbon being consumed is being incorporated. We have also shown a low rate of consumption of nitrogen gas in the intact symbiosis in the presence of methane suggesting that these symbionts may also fix nitrogen.

The symbiotic bacteria have the internal membrane structure typical of Type 1 methanotrophic bacteria, which incorporate carbon as formaldehyde into organic compounds. Gill pieces from the mussels can oxidize C14 -methane to carbon dioxide with appreciable incorporation of labelled carbon into organic compounds. The second enzyme in the pathway for the oxidation of methane is found in the gills and its activity is proportional to the methane consumption rate of gill pieces from the same gills. A variety of additional data suggest that this symbiosis can live with methane as the primary source of energy and carbon.

We believe that the high oxygen demand of methanotrophic symbioses may well limit their occurrence in low oxygen habitats resulting in this being a reatively rare type of symbiosis.