Microbiology, Vol. 71, No. 5, 2002, pp. 587–594. Translated from Mikrobiologiya, Vol. 71, No. 5, 2002, pp. 681–689.

EXPERIMENTAL ARTICLES

Utilization of Methane and Carbon Dioxide by Symbiotrophic Bacteria in Gills of Mytilidae (*Bathymodiolus*) from the Rainbow and Logachev Hydrothermal Fields on the Mid-Atlantic Ridge

N. V. Pimenov*, M. G. Kalyuzhnaya, V. N. Khmelenina**, L. L. Mityushina*, and Yu. A. Trotsenko****

*** Institute of Microbiology, Russian Academy of Sciences, pr. 60-letiya Oktyabrya 7, k. 2., Moscow, 117312 Russia*

***Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushchino, Moscow oblast, 142292 Russia*

Received April 2, 2002

Abstract—Bivalve mollusks *Bathymodiolus asoricus* and *Bathymodiolus puteoserpentis* collected from the Rainbow and Logachev hydrothermal fields during dives of the *Mir 1* and *Mir 2* deep-sea manned submersibles were studied. Rates of methane oxidation and carbon dioxide assimilation in mussel gill tissue were determined by radiolabel analysis. During oxidation of $^{14}CH_4$, radiocarbon was detected in significant quantities not only in carbon dioxide but also in dissolved organic matter, most notably ^{14}C-formate and ^{14}C-acetate, occurring in a 2 : 1 ratio. Activities of hexulose-phosphate synthase, phosphoribulokinase, and ribulose 1,5-bisphosphate carboxylase were shown in the soluble fraction of gill tissue cells. At the same time, no activity of hydroxypyruvate reductase—the key enzyme of the serine pathway of C_1-assimilation—was detected. The results of PCR amplification using genetic probes for membrane-bound methane monooxygenase (pmoA) and methanol dehydrogenase (mxaF) attest to the presence of the genes of these enzymes in the total DNA extracted from gill samples. However, no appropriate PCR responses were obtained with the mmoX primer system, which is a marker for soluble methane monooxygenase. All samples studied showed amplification with primers for the genera *Methylobacter* and *Methylosphaera.* At the same time, no genes specific to the genera *Methylomonas, Methylococcus, Methylomicrobium,* or *Methylosinus* and *Methylocystis* were detected. Electron microscopic examinations revealed the presence of two groups of endosymbiotic bacteria in the mussel gill tissue. The first group was represented by large cells possessing a complex system of cytoplasmic membranes, typical of methanotrophs of morphotype I. The other type of endosymbionts, having much smaller cells and lacking intracellular membrane structures, is likely to be constituted by sulfur bacteria.

Key words: Mytilidae, endosymbiosis, methanotrophic bacteria, Mid-Atlantic Ridge.

In 1977, abundant populations of mussels were discovered on a deep-sea hydrothermal field in the Galapagos Rift, East Pacific [1]. Somewhat later, these mussels were assigned to a new genus *Bathymodiolus* with the type species *B. thermophilus* [2]. Since then, *Bathymodiolus* bivalves were found in deep-sea communities of many hydrothermal vents and cold-water hydrocarbon–sulfide seeps [3–6] both in the Atlantic and Pacific Oceans.

The gill tissue of all currently known species of *Bathymodiolus* was shown to contain symbiotic intracellular bacteria. In some of these organisms, intracellular symbionts are represented by sulfur bacteria alone [7], which autotrophically synthesize organic matter directly in the gill tissue by utilizing the energy of reduced sulfur compounds derived from hydrothermal liquid. Other *Bathymodiolus* were found to contain both sulfur and methanotrophic bacteria in their gill tissue. By virtue of such double symbiosis, these animals are able to utilize both the energy of reduced sulfur compounds and methane consumed by methanotrophic endosymbionts residing in their gill tissue [4, 7, 8]. The concurrent occurrence of sulfur and methanotrophic symbiotic bacteria was shown not only in hydrothermal *Bathymodiolus* but also in other bivalve mollusks and pogonophores that are members of communities inhabiting hydrothermal fields and cold methane seeps [9–11].

According to recent studies, many hydrothermal communities of the Mid-Atlantic Ridge are dominated not only by shrimp of the family *Bresilidae* but also by bivalves of the subfamily *Bathymodiolinae* [6, 12]. By morphological and genomic features, *Bathymodiolus* from hydrothermal communities of the Mid-Atlantic Ridge are divided into several species, occurring in different hydrothermal regions. Specifically, mussels from the Rainbow, Lucky Strike and Menez Gven hydrother-

0026-2617/02/7105-0587$27.00 © 2002 MAIK “Nauka / Interperiodica”

mal fields belong to the species *Bathymodiolus asoricus*, whereas the Snake Pit and Logachev regions, which are to the south, are inhabited by a different species, *Bathymodiolus puteoserpentis* [13].

Comprehensive investigations of the Rainbow and Logachev hydrothermal fields were undertaken in 1998 and 1999 aboard the research vessel *Academician Mstislav Keldysh*. Using deep-sea manned submersibles *Mir 1* and *Mir 2*, samples of mussels prevailing, along with shrimp, among the hydrothermal fauna were collected during dives to active vents. The goal of the present work was to determine rates of methane oxidation and carbon assimilation by endosymbionts populating the gill tissue of these organisms. The measurements were carried out by the radiocarbon (^{14}C) method aboard the ship. The activities of the key enzymes implicated in methane conversion and carbon dioxide assimilation were also determined. The presence of the genes of membrane-bound (pmoA) and soluble (mmoX) methane monooxygenases and methanol dehydrogenase (mxaF) in the total DNA extracted from gill samples was studied using genetic probes.

MATERIALS AND METHODS

Samples of mussels were collected from the Rainbow (36° N) and Logachev (14° N) hydrothermal fields in the course of the 41st and 42nd cruises of the research vessel *Academician Mstislav Keldysh* using *Mir 1* and *Mir 2* manned deep-sea submersibles.

Mussels were picked up at different distances from an active hot spring, which means that they lived under different concentrations of methane and hydrogen sulfide released with hydrothermal effluents.

Experiments with Radioactive Compounds

Immediately upon being lifted aboard, mussels were washed many times with seawater filtered through Millipore membrane filters (0.2 μm). Their gills were separated and disintegrated in a porcelain mortar with quartz sand to a homogeneous state. The resultant tissue homogenate (0.1–0.2 ml) was incubated with sterile seawater (4.6 ml) and $^{14}CH_4$ (0.2 ml; 1 μCi) in penicillin bottles (10 ml). After incubation, the samples were acidified by adding 1 ml of 1% H_3PO_4. The evolving carbon dioxide was captured with 2-phenylethylamine present in the scintillation mixture. The tissue homogenate was trapped on membrane filters and washed; and the rate of ^{14}C-methane incorporation into the acid-resistant fraction was then determined. The overall radioactivity of the filtrate remaining after tissue homogenate separation was measured after removing residual radioactive methane by means of filtrate degassing in an ultrasonic bath (30 min at 55°C). This fraction was represented by exometabolites produced by methanotrophic endosymbionts during oxidation of ^{14}C-methane. The composition of the exometabolite fraction was studied on a Biotronik ion chromatograph (Germany). For this purpose, every 0.5 min 0.1 ml of the liquid flowing out of the column was dispensed into scintillation vials to measure radioactivity on the scintillation counter. In this way, the distribution of radiocarbon between the C_1–C_3 fractions of organic acids was quantitatively determined.

The assimilation of CO_2 by mussel gill tissue was determined in a manner similar to methane oxidation except that the tissue was not disintegrated in a porcelain mortar with quartz sand, but rather cut into relatively equal parts with scissors, each bit with a wet weight of about 2 mg. The tissue bits (4–6) were then put into penicillin bottles (10 ml) containing filtered seawater (4.8 ml) and $NaH^{14}CO_3$ (0.2 ml; 4 μCi). Upon incubation, the tissue bits were dipped into 10% H_3PO_4 and left to dry in the air. Then the bits were put into scintillation vials, and their radioactivity was measured on a Rakbeta liquid scintillation counter (LKB, Sweden).

The controls in experiments with $^{14}CH_4$ and $H^{14}CO_3^-$ were variants to which 25% glutaraldehyde was added to a final concentration of 2.5% prior to the addition of labeled substrate.

Electron Microscopic Examinations

Specimens for electron microscopy were prepared aboard ship. Gills of mussels were washed in filtered seawater and fixed in 2.5% glutaraldehyde for 8–12 h at 5–7°C. The gill tissue was then placed in a 2% solution of osmium tetroxide (OsO_4) for 24 h. The fixed material was dehydrated in a graded series of ethanol solutions and in absolute acetone and then embedded in a mixture of Epon and araldite. Ultrathin sections of the gill tissue were obtained using an LKB-III ultramicrotome and examined under a JEM-100C microscope (Japan) at an accelerating voltage of 80 kV.

Obtaining Cell Extracts and Determining Activities of Enzymes

Assays of enzymatic activities were carried out using gills of mussels frozen immediately upon lifting aboard and brought to the mainland laboratory without thawing. Bits of gills were placed in 0.05 M Tris-HCl buffer (pH 7.4) containing 1 μM of phenylmethylsulfonyl fluoride—an inhibitor of proteolytic enzymes—and disrupted at 0°C on an MSE ultrasonic disintegrator (England) operated at a power of 150 W at 20 kHz (3 times by 1 min with 1 min breaks). Cells that failed to disintegrate and large cell fragments were separated by centrifugation at 15 000 g for 30 min. Enzymatic activities were determined in the resultant supernatant, employing temperature-controlled cuvettes.

Hydroxypyruvate reductase was determined by spectrophotometric analysis at 340 nm from the oxidation of NAD(P)H in a reaction mixture (1 ml) composed of Tris-HCl buffer, 50 μmol; NADH or NADPH,

0. 25 μmol; lithium hydroxypyruvate, 5 μmol; and cell extract (1 mg protein).

Hexulose-phosphate synthase was determined by the loss of formaldehyde in the reaction mixture (0.5 ml) containing (μmol) sodium phosphate buffer (pH 7.0), 20; ribulose-5-phosphate, 2; $MgCl_2$, 2; and cell extract (1 mg protein). The reaction was initiated by the addition of 2 μmol of formaldehyde. The mixture was incubated for 10 min at 30°C; aliquot portions of the solution (0.1 ml) were taken every 2 min, supplemented with 1.4 ml of 0.6 N $HClO_4$ and 1.5 ml of Nash reagent, incubated for 5 min at 58°C, and cooled, after which the optical density was determined at 420 nm. The variants containing no ribulose-5-phosphate were used as controls.

Ribulose 1,5-bisphosphate carboxylase was determined from the rate of incorporation of $NaH^{14}CO_3$ radiocarbon into acid-resistant products. The reaction mixture (1 ml) was composed of (μmol) Tris-HCl buffer (pH 7.6), 100; $MgCl_2$, 2.5; reduced glutathione, 10; ribulose 1,5-bisphosphate (sodium salt), 2.5; $NaH^{14}CO_3$, 10 (10 μCi); and cell extract (1 mg). The reaction was carried out at 30°C. Aliquot samples (50 μl) were withdrawn every 1 min, placed on square patches of Whatman GF/F glass-fiber paper, fixed by the addition of 50 μl 6 N HCl, and dried, upon which their radioactivity was measured. Phosphoribulokinase was determined by spectrophotometry at 340 nm from the ribulose-5-phosphate-dependent formation of ADP from ATP in a reaction mixture (1 ml) composed of (μmol) ribulose-5-phosphate (sodium salt), 5; ATP, 5; $MgCl_2$, 5; phosphoenolpyruvate, 5; NADH, 0.25; pyruvate kinase from rabbit muscle, 2 units; and lactate dehydrogenase, 2 units. Spectrophotometric analysis was carried out on a UV VIS spectrophotometer (Germany) in temperature-controlled cuvettes.

PCR Amplification

In order to detect the presence of methanotrophic bacteria, samples of total DNA isolated from gill tissue of *Bathymodiolus* were studied. Thawed gill tissue (200 mg) was washed in 0.2 M sodium phosphate buffer (pH 8.0), and DNA was extracted as described elsewhere [14]. Group-specific oligonucleotide primers mxaF1003f/1561r, mmoX883f/1403r, pmoA189f/682r, and 16SrRNA 27f/Mb1007r, Mm1007r, Mc1005r, Ms1020r were used [15]. Based on the results of 16S RNA sequence analysis, two new primer systems 16SrRNA 27f/Mb854r (5'-ATGCGTTCTGCGCCACTA-3') and 16SrRNA 27f/Mh996r (5'-CACTCTACTATCTCTAACGG-3') targeting the genera *Methylobacter* and *Methylosphaera*, respectively, were developed [14].

The PCR reaction was performed on a PHC-2 DNA thermocycler (Techne, England) using the following schedule: 94°C, 4 min; 35 cycles: 94°C, 1 min; 52°C, 1 min; 72°C, 1 min; and the last cycle 72°C, 5 min. The reaction mixture (50 μl) contained 16 mM $(NH_4)_2SO_4$; 67 mM Tris-HCl (pH 8.8); 0.1% Tween 80; 2.5 mM $MgCl_2$; 0.2 mM of each dNTP; 0.5 μM of the corresponding primer; and 1.5 unit of *Taq*-polymerase. The reaction products were separated by electrophoresis in a 1% agarose gel.

RESULTS

Electron Microscopic Examinations

Two groups of symbiotic bacteria were found in the gill tissue of mussels from the Rainbow and Logachev hydrothermal fields (Figs. 1a and 1b). The first group, represented by larger bacteria, contained intracytoplasmic membranes, characteristic of methanotrophs of morphological type I. The second group lacked any membrane-like intracellular structures and had much smaller cells. Cells of both types typically occurred in clusters, and single cells were rare (Figs. 1c and 1d). Each cell was, apparently, surrounded by an electron-transparent "autolysis" zone, itself surrounded by a three-layered membrane of host tissue cells. The observed groups of bacterial cells (Fig. 1c) also resided in the electron-transparent "autolysis" zone behind a common membrane belonging to mussel gill tissue.

Both types of endosymbiotic bacteria were found in gill tissue cells of all mussels studied and collected from different parts of the Logachev and Rainbow hydrothermal sites.

$^{14}CH_4$ Oxidation in Gill Tissue

The obtained rates of ^{14}C-methane oxidation to $^{14}CO_2$ in gill tissue of mussels collected from the Rainbow and Logachev hydrothermal fields near active vents are shown in Fig. 2. No methane oxidation in the leg and mantle tissue was detected. The amount of carbon dioxide formed depended linearly on the incubation time in the range of 3–8 min. Therefore, the duration of tissue incubation with $^{14}CH_4$ in comparative experiments measuring rates of methane oxidation in mussels was limited to 8 min.

It should be noted that methane oxidation in mussel gill tissue was observed only when the samples were used in radioisotopic experiments immediately upon their arrival aboard ship. Storing the tissue in a deep-freezer for just one day at –12 to –17°C led to total suppression of methane oxidation.

A considerable share of carbon of methane oxidized by methanotrophic bacteria is known to be incorporated into organic compounds, constituted by bacterial biomass (the insoluble fraction) and exometabolites (soluble fraction). The proportions between major methane oxidation products formed when mussel tissue was incubated with ^{14}C-methane are given in Table 1. Over the first 8 min of incubation, more than 70% of radiocarbon was detected in the fraction of dissolved organic matter. With longer incubation intervals, the amount of meth-

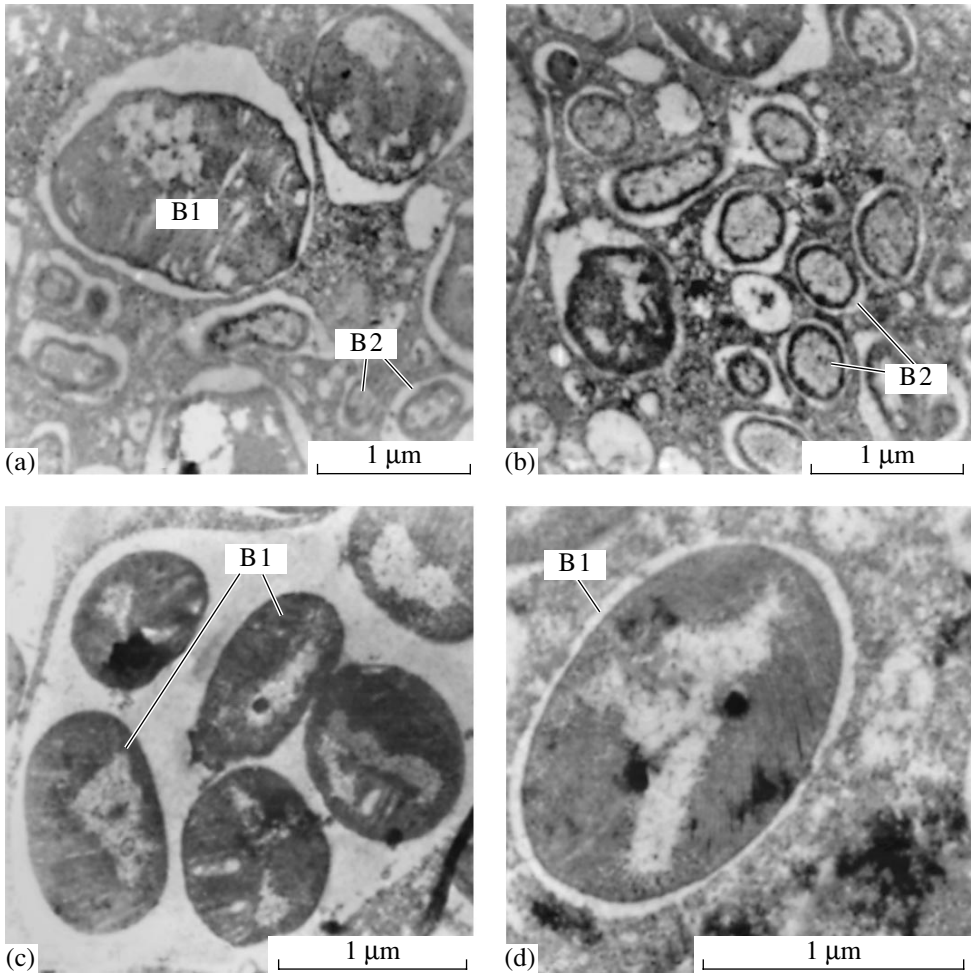

Fig. 1. Micrographs of sections of mussel gill tissue: (a) and (b) show gill tissue containing bacterial endosymbionts; B1 are bacteria with a complex system of intracytoplasmic membranes (ICPM) and B2 are bacteria with no discernible ICPM; (c) and (d) show bacteria with ICPM at different magnifications.

ane oxidized to CO_2 increased. As revealed by ion chromatography, the dissolved organics was mostly represented by ^{14}C- formate and ^{14}C-acetate, occurring in a ratio of 2 : 1 (Fig. 3). These compounds, along with endosymbiotic bacteria per se, may be actively implicated in host cell metabolism.

Table 1. Shares of oxidation products formed in incubation of mussel tissue with $^{14}CH_4$

Time of incubation with $^{14}CH_4$, min	Methane carbon oxidized to CO_2, %	Methane carbon incorporated into soluble OC fraction, %	Methane carbon incorporated into insoluble OC fraction, %
3	20	76.5	3.5
8	17.5	78.5	4.0
12	43	52.7	4.3
40	49	46.0	5.0

Note: OC denotes organic compounds.

The utilization of methane by mussel gill tissue was observed over a wide range of temperatures, from 1 to 45°C (Fig. 1), with the maximum between 15 and 40°C. It should also be noted that the oxidation of methane in mussel gills did not cease even at 1–2°C, although its rate was much lower than at the optimal temperature.

The sensitivity of the trace label method employed was sufficient to reliably detect methane oxidation in mussel gill tissue even at $^{14}CH_4$ concentrations in the reaction medium as low as 1–2 μl/l, which is equal to the background methane concentration in the near-bottom ocean layer.

On the Rainbow and Logachev hydrothermal fields, large bunches of mussels were observed not only in the immediate vicinity of high-temperature vents but also in the discharge zone of low-temperature sources ($t < 10$°C) (“moires”) and at basements of inactive chimneys. In this regard, it was of interest to compare rates of methane oxidation in mussels picked in methane-rich zones with those in mussels collected near footings of inactive chimneys, where, as our data show, the concentration of

methane was not different from the background level (up to 2 μl/l) [16]. The measured dynamics of methane oxidation in gill tissue of mussels originating from different parts of the Rainbow and Logachev hydrothermal fields are shown in Fig. 5. The oxidation of methane was most active in the tissue of mussels inhabiting the zone close to hot vents and moire seeps. By contrast, methane oxidation in gills of mussels collected from an inactive chimney located at a large distance from the hot vent was very weak. It is also worth mentioning that mussels from the inactive chimney persisted under much less favorable conditions than those near the active vent and in the zone of moire seeps. Thus, the net weight of soft tissue of such mussels with a valve size of 14 cm amounted to only 40% of the corresponding weight of mussels with the same valve size originating from the immediate vicinity of the active vent.

$^{14}CO_2$ Assimilation in Mussel Gills

The dynamics of CO_2 carbon incorporation in the gill tissue of mussels is shown in Fig. 6. Carbon dioxide was actively fixed in uncrushed bits of gill tissue during the first 2.5 h of incubation with labeled bicarbonate. Grinding of tissue samples with quartz sand resulted in a considerable reduction of the strength of this process.

Like in the case of methane oxidation, freezing of tissue samples was found to cause an almost total suppression of carbon dioxide assimilation. The addition to the reaction mixture of 10 or 100 μM of thiosulfate or sulfide failed to stimulate carbon dioxide assimilation in mussel gill tissue.

Enzyme and PCR Assays

The activity of hexulose-phosphate synthase (HPS), which is the key enzyme of the ribulose monophosphate (RMP) cycle of formaldehyde fixation, was found in the soluble fraction of cell extracts from the gill tissue disintegrated by ultrasonic treatment. We also found relatively low activities of phosphoribulokinase and ribulose 1,5-bisphosphate carboxylase (RuBPC), the key enzymes of the Calvin cycle. No activity of hydroxypyruvate reductase, which is an indicator enzyme of the serine pathway of C_1-assimilation, was detected (Table 2).

The PCR reactions accomplished with the use of genetic probes for membrane-bound methane monooxygenase (*pmoA*) and methanol dehydrogenase (*mxaF*) revealed the presence of these genes in the total DNA purified from gill samples (Table 3). However, no corresponding PCR responses were obtained with the *mmoX* primer system, a marker for soluble MMO. All samples studied amplified with primers for the genera *Methylobacter* and *Methylosphaera*. No genes specific to the genera *Methylomonas*, *Methylococcus*, *Methylomicrobium* or *Methylosinus* and *Methylocystis* were detected.

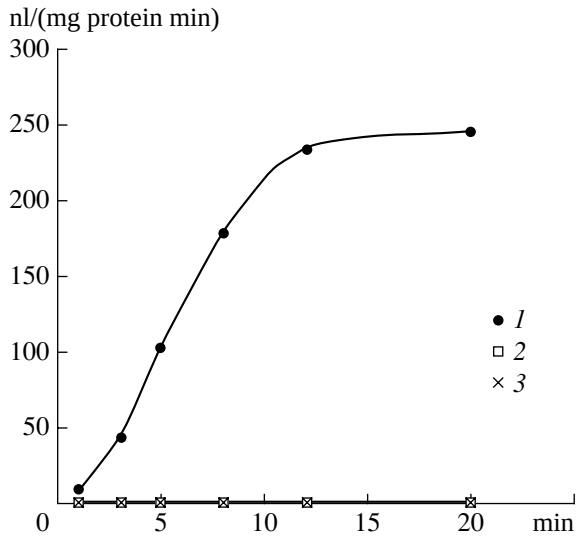

Fig. 2. Dynamics of methane oxidation in mussel tissue. The incubation mixture contained 0.2 ml of crushed tissue; 0.2 ml of $^{14}CH_4$ with the activity 4.1×10^6 counts/min; and 4.6 ml of filtered seawater: (*1*) is gill tissue; (*2*) is muscle and leg tissue; and (*3*) is gill tissue treated with glutaraldehyde.

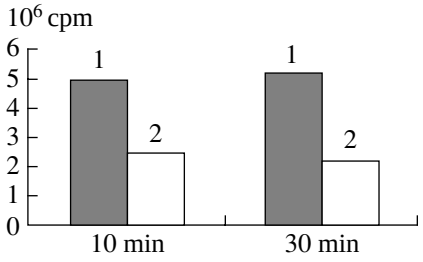

Fig. 3. Production of (1) formate and (2) acetate in the soluble fraction during incubation of mussel gill tissue with $^{14}CH_4$. The incubation times were 10 and 30 min.

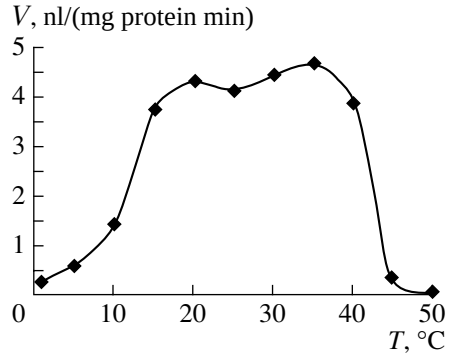

Fig. 4. Rate of methane oxidation (*V*) in mussel gill tissue as a function of temperature (*T*).

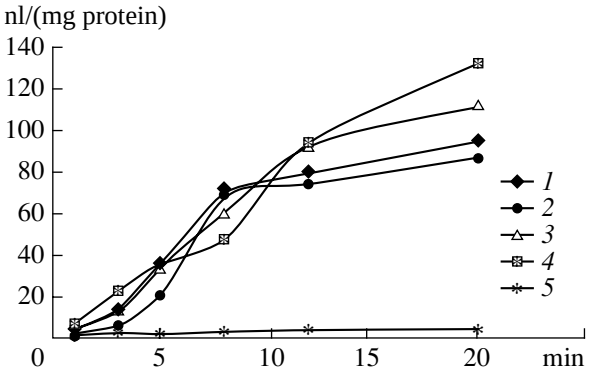

Fig. 5. Methane oxidation by the gill tissue of mussels picked from different parts of the hydrothermal field: (*1*) and (*2*) are mussels collected close to the hot vent; (*3*) and (*4*) are mussels from the moire zone; and (*5*) are mussels collected from an inactive chimney.

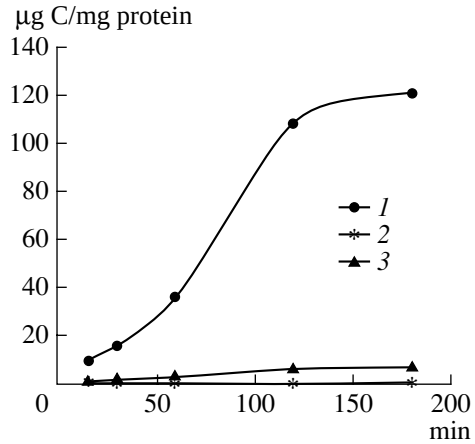

Fig. 6. Incorporation of ^{14}C carbon dioxide into acid-insoluble fraction: (*1*) bits of mussel gill tissue; (*2*) gill tissue suspension obtained by grinding with quartz sand; and (*3*) controls (bits and suspension of gill tissue fixed in glutaraldehyde to a final concentration of 2.5%).

DISCUSSION

On the grounds of morphological and genetic features, mussels inhabiting the Logachev and Rainbow hydrothermal fields were assigned to different species [12]. Meanwhile, our integrated study of endosymbiotic bacteria residing in mussel gill tissue failed to reveal any significant distinctions between mussels of different species collected on the Logachev and Rainbow hydrothermal fields. The gill tissue of these mussels was shown to contain bacterial endosymbionts of two morphological types. The larger cells, possessing a complex system of intracytoplasmic membranes, can be referred to methanotrophs of morphotype I, which is also suggested by the observed high activity of HPS.

The results of PCR assays are in agreement with electron microscopy and enzymological studies. The detected DNA sequences are characteristic of methanotrophs of the genera *Methylobacter* and *Methylosphaera*, which depend on the RMP cycle and, as a rule, contain membrane-bound MMO.

The uptake of ^{14}C-methane in short-term experiments with homogenate of gill tissue is unmistakable evidence of the high activity of endosymbiotic methanotrophic bacteria. The oxidation of methane was also observed even when the concentration of methane in the incubated mixture was close to its background level (about 2 μl/l). This gas, therefore, could play the role of an additional source of carbon in metabolism of hydrothermal mussels even when they develop at large distances from the active vent in zones with a low concentration of dissolved methane.

The other type of endosymbiotic bacteria was characterized by much smaller cells lacking any intracellular membrane structures. Most researchers agree that these cells belong to sulfur bacteria, which are responsible for the capacity of many hydrothermal animals to utilize the energy of reduced sulfur compounds carried up with hydrothermal fluid [8, 17, 18]. Our tests with labeled bicarbonate also showed active incorporation of $H^{14}CO_3^-$ carbon into the acid-insoluble fraction of mussel gill tissue. However, we failed to reliably establish a stimulating influence of thiosulfate and sulfide on the rate of carbon dioxide assimilation in mussel gill tissue, as is the case with hydrothermal gastropods of the backarc Manus and Lau Basin [10]. The activity of RuBPC in extracts of gill tissue of gastropods was also fairly low (Table 2). RuBPC is known to occur in methanotrophs of the genus *Methylococcus.* However, the

Table 2. Activities of enzymes of C_1-assimilation in mussel gill tissue (nmol/(min mg protein))

Enzyme	Cofactor	Gill tissue sample, no.	
		5 (hot vent)	7 (moire seep)
Hexulose-phosphate synthase		60	47
Phosphoribulokinase	ATP	8.8	7.3
Ribulose bisphosphate carboxylase		1.2	1.5
Hydroxypyruvate reductase	NADH	0	0
	NADPH	0	0

Table 3. PCR-amplification of fragments of group-specific structure genes from samples of total DNA obtained from mussel gill tissue

Detected gene	Total DNA samples		
	M01*	M02*	M04**
pmoA (mMMO)	+	+	+
mmoX (sMMO)	–	–	–
mxaF (methanol dehydrogenase)	+	+	+
Mb 854 (Methylobacter)	+	+	+
Mm 1007 (Methylomonas)	–	–	–
Mb1007 (Methylomicrobium)	–	–	–
Mc1005 (Methylococcus)	–	–	–
Ms1020 (Methylosinus)	–	–	–
Mh996 (Methylosphaera)	–	–	–

Note: mMMO is membrane-bound methane monooxygenase; sMMO is soluble methane monooxygenase.
* Gills of mussels collected on the Logachev hydrothermal field.
** Gills of mussels collected on the Rainbow hydrothermal field.

fact that we found neither hydroxypyruvate reductase nor 16S rRNA sequences specific to representatives of the genus *Methylococcus*, in which the RMP cycle functions along with the serine pathway and the minor Calvin cycle, suggests that low activities of phosphoribulokinase and RuBPC in gill samples most likely belong to chemoautotrophic rather than to methanotrophic bacteria.

Electron microscopic examination and the analysis the isotopic tissue composition ($\delta^{13}C$) of mussels collected not only from different hydrothermal fields of the Mid-Atlantic Ridge but also within the same field showed different roles of methanotrophic and sulfur endosymbiotic bacteria in the nutrition of these animals [8, 18, 19]. The number of methanotrophic endosymbionts in the gill tissue of mussels inhabiting methane-rich zones was significantly higher than in mussels developing at large distances from the active vent under methane concentrations that are essentially at the background level. By using ^{14}C-methane, we were able to show (Fig. 5) that the oxidation of methane was most active in the gill tissue of mussels picked in the immediate vicinity of the hot vent and in the zone of moire seeps.

The results of our investigations, therefore, confirm the previously made contention that methanotrophic endosymbionts play a leading role in the nutrition of hydrothermal *Bathymodiolus* mussels proliferating on hydrothermal vent fields of the Mid-Atlantic Ridge. One can also claim that despite the division of Mid-Atlantic Ridge *Bathymodiolus* mussels into different species, the nutrition strategy of these animals is governed by the presence in their gill tissue of two types of endosymbionts—methanotrophic and sulfur bacteria.

ACKNOWLEDGMENTS

We are grateful to the crew of the *Mir* deep-sea submersibles and the crew of the research vessel *Academician Mstislav Keldysh*. We are especially grateful to A.M. Sagalevich, the research manager of all recent cruises of the ship and Commander of the *Mir* submersible. It is due to his personal activity that a unique collection of deep-sea mussels used in this work was gathered. We are also grateful to M.V. Ivanov and V.F. Gal'chenko for valuable comments on the manuscript.

This work was supported by the Russian Foundation for Basic Research (project no. 00-15-97897) and by a grant for junior scientists from the Russian Academy of Sciences.

REFERENCES

1. Corliss, J.B., Dymond, J., Gordon, L., Edmond, J.M., von Herzen, R.P., Ballard, R.D., Green, K., Williams, D., Bainbridge, A., Crane, K., and van Andel, T.H., Submarine Thermal Springs on the Galapagos Rift, *Science*, 1979, vol. 203, pp. 1073–1083.
2. Kenk, V.C. and Wilson, B.R., A New Mussel (Bivalvia: Mytilidae) from Hydrothermal Vents in the Galapagos Rift Zone, *Malacologia*, 1985, vol. 26, pp. 253–271.
3. Cavanaugh, C.M., Levering, P.R., Maki, J.S., Mitchell, R., and Lindstrom, M.E., Symbiosis of Methylotrophic Bacteria and Deep-Sea Mussels, *Nature*, 1987, vol. 325, pp. 346–348.
4. Cavanaugh, C.M., Wirsen, C.O., and Jannasch, H.W., Evidence for Methylotrophic Symbionts in a Hydrothermal Vent Mussel (Bivalvia: Mytilidae) from the Mid-Atlantic Ridge, *Appl. Environ. Microbiol.*, 1992, vol. 58, no. 12. pp. 3799–3803.
5. Childress, J.J., Fisher, C.R., Brooks, J.M., Kennicutt, M.C., Bidigare, R., and Anderson, A., A Methanotrophic Marine Molluscan (Bivalvia, Mytilidae) Symbiosis: Mussels Fueled by Gas, *Science*, 1986, vol. 233, pp. 1306–1308.
6. Cosel, R., Metivier, B., and Hashimoto, J., Three New Species of Bathymodiolus (Bivalvia: Mytilidae) from Hydrothermal Vents in the Lau Basin and the Fiji Basin, Western Pacific, and Snake Pit Area, Mid-Atlantic Ridge, *The Veliger*, 1994, vol. 37, pp. 374–392.
7. Nelson, D.C., Hagen, D., and Edwards, D.B., The Gill Symbionts of the Hydrothermal Vent Mussel *Bathymodiolus thermophilus* Is a Psychrophilic, Chemoautotrophic, Sulfur Bacterium, *Marine Biology*, 1995, vol. 121, pp. 487–495.
8. Southward, E.C., Gebruk, A., Kennedy, H., Southward, A.J., and Chevaldonne, P., Different Energy Sources for Three Symbiont-Dependent Bivalve Mollusks at the Logachev Hydrothermal Site (Mid-Atlantic Ridge), *J. Mar. Biol. Ass. UK*, 2001, vol. 81, pp. 655–661.
9. Gal'chenko, V.F., Galkin, S.V., Lein, A.Yu., Moskalev, L.I., and Ivanov, M.V., The Role of Bacterial Symbionts in the Nutrition of Some Invertebrates from the Zones of Active Submarine Hydrotherms, *Okeanologiya* (Moscow), 1988, vol. 28(6), pp. 1020–1031.

10. Gal'chenko, V.F., Pimenov, N.V., Lein, A.Yu., Galkin, S.V., Miller, Yu.M., and Ivanov, M.V., Mixotrophic Type of Nutrition of *Olgaconcha tufari* Beck (Gastropoda: Prosobranchia) from Active Hydrothermal Field of the Manus Basin (Bismarck Sea), *Dokl. Akad. Nauk*, 1992, vol. 323, no. 3, pp. 776–781.

11. Pimenov, N.V., Savvichev, A.S., Rusanov, I.I., Lein, A.Yu., and Ivanov, M.V., Microbiological Processes of Carbon and Sulfur Cycles at Cold Methane Seeps of the North Atlantic, *Mikrobiologiya*, 2000, vol. 69, no. 6, pp. 831–843.

12. Maas, P.A.Y., O'Mullen, G.D., Lutz, R.A., and Vrijenhoek, R.C., Genetic and Morphometric Characterization of Mussels (Bivalvia: Mytilidae) from Mid-Atlantic Ridge Hydrothermal Vents, *Biological Bulletin, Marine Biological Laboratory, Woods Hole*, 1996, vol. 196, pp. 265–272.

13. Cosel, R., Comtet, T., and Krylova, E.M., *Bathymodiolus* (Bivalvia, Mytilidae) from Hydrothermal Vents on the Azores Triple Junction and the Logatchev Hydrothermal Field, Mid-Atlantic Ridge, *The Veliger*, 1999, vol. 42, pp. 18–48.

14. Kalyuzhnaya, M.G., Makutina, V.A., Rusakova, T.G., Nikitin, D.V., Khmelenina, V.N., Dmitriev, V.V., and Trotsenko, Yu.A., Methanotrophic Communities in the Soils of Russian Northern Taiga and Subarctic Tundra, *Mikrobiologiya*, 2002, vol. 71, no. 2, pp. 210–215.

15. Murrell, J.C., McDonald, I.R., and Bourne, D.G., Molecular Methods for the Study of Methanotroph Ecology, *FEMS Microbiol.*, 1998, vol. 27, no. 2, pp. 103–114.

16. Pimenov, N.V., Lein, A.Yu., Sagalevich, A.M., and Ivanov, M.V., Carbon Dioxide Assimilation and Methane Oxidation in Various Zones of the Rainbow Hydrothermal Field, *Mikrobiologiya*, 2000, vol. 69, no. 6, pp. 810–818.

17. Distel, D.L., Lee, H.K.-W., and Cavanaugh, C.M., Intracellular Coexistence of Methano- and Thioautotrophic Bacteria in a Hydrothermal Vent Mussel, *Proc. Natl. Acad. Sci. USA*, 1995, vol. 92, pp. 9598–9602.

18. Task, J.L. and Van Dover, C.L., Site-Specific and Ontogenetic Variations in Nutrition of Mussels (*Bathymodiolus* sp.) from the Lucky Strike Hydrothermal Vent Field, Mid-Atlantic Ridge, *Limnol. Oceanograph.*, 1999, vol. 44, pp. 334–343.

19. Lein, A.Yu., Pimenov, N.V., Gebruk, A.V., Moskalev, L.I., Bagirov, N.E., and Ivanov, M.V., Trophic Structure and Composition of the Hydrothermal Community of the Rainbow (36° N, Mid-Atlantic Ridge), *Dokl. Akad. Nauk*, 2001, vol. 376, pp. 120–123.