Identification of Methanotrophic Lipid Biomarkers in Cold-Seep Mussel Gills: Chemical and Isotopic Analysis

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A lipid analysis of the tissues of a cold-seep mytilid mussel collected from the Louisiana slope of the Gulf of Mexico was used in conjunction with a compound-specific isotope analysis to demonstrate the presence of methanotrophic symbionts in the mussel gill tissue and to demonstrate the host's dependence on bacterially synthesized metabolic intermediates. The gill tissue contained large amounts of group-specific methanotrophic biomarkers, bacteriohopanoids, 4-methylsterols, lipopolysaccharide-associated hydroxy fatty acids, and type I-specific 16:1 fatty acid isomers with bond positions at $\Delta 8$, $\Delta 10$, and $\Delta 11$. Only small amounts of these compounds were detected in the mantle or other tissues of the host animal. A variety of cholesterol and 4-methylsterol isomers were identified as both free and steryl esters, and the sterol double bond positions suggested that the major bacterially derived gill sterol [11.0% 4α-methyl-cholesta-8(14),24-dien-3β-ol] was converted to host cholesterol (64.2% of the gill sterol was cholest-5-en-3β-ol). The stable carbon isotope values for gill and mantle preparations were, respectively, −59.0 and −60.4‰ for total tissue, −60.6 and −62.4‰ for total lipids, −60.2 and −63.9‰ for phospholipid fatty acids, and −71.8 and −73.8‰ for sterols. These stable carbon isotope values revealed that the relative fractionation pattern was similar to the patterns obtained in pure culture experiments with methanotrophic bacteria (R. E. Summons, L. L. Jahnke, and Z. Roksandic, Geochim. Cosmochim. Acta 58:2853–2863, 1994) further supporting the conversion of the bacterial methylsterol pool.

It is now recognized that bacterial symbioses with eukaryotic organisms are widespread (16). Symbiotic associations between methanotrophic bacteria and bivalves have been reported to occur at a number of deep-sea hydrocarbon seep sites in the Gulf of Mexico (17). It is apparent from the results of stable carbon isotope studies that methane is the primary carbon source for these bivalves (43). Extensive enzymatic assays, stable carbon isotope determinations, [14C]methane consumption studies, and mussel growth studies (6–9, 13, 19) have provided considerable evidence that a methane-based symbiosis exists. Electron microscopy has revealed that symbionts with the stacked internal membranes characteristic of type I methanotrophs are present in the gill cells of seep mytilid mussels (8, 9), and indeed, the results of a recent analysis of a 16S RNA symbiont gene have confirmed that the symbionts are phylogenetically related to free-living type I methanotrophs (12).

The use of fatty acids as group-specific signature lipids or biomarkers has provided valuable information on the structure of microbial communities (51), and these compounds have been used to identify and quantify methanotrophic bacteria in natural systems (37, 39, 40). Such studies have relied principally on analyzing the unique monounsaturated fatty acid positional isomers synthesized by methanotrophs (27, 33, 40). Methanotrophs are also known to contain some distinctive cyclic triterpenes, hopanoids, and methylsterols (41), and the hopanoids have the added advantage that they have been isolated only from prokaryotic sources. Identification of meth-

anotrophic biomarker molecules in eukaryotic host tissue, particularly if it is used with a compound-specific carbon isotope analysis (48), could provide valuable information on the symbiont-host relationship. The association of isoprenoid biomarkers with source organisms has been particularly important for geochemical interpretation of sedimentary organic carbon (49) and can be used similarly in microbial ecology to interpret community structure (39). In this paper we describe a study in which we used organism-specific biomarkers and their isotopic compositions to identify bacterial symbionts in the gill tissue of a cold-seep mytilid mussel.

MATERIALS AND METHODS

Mussel. The mussel which we used, a *Bathymodiolus*-like mytilid mussel designated species Ia (17), was collected by C. R. Fisher from a shallow brine pool (depth, ~600 m) on the Louisiana slope of the Gulf of Mexico between 14 and 27 September 1991. This mussel was placed in chilled seawater and was maintained by bubbling methane through the water for approximately 10 min twice daily while the animal was aboard ship and four times daily after it arrived at Pennsylvania State University. The mussel was dissected on 2 October 1991 into gill, mantle, and remaining (foot, gut, etc.) tissues, frozen, and shipped to the Ames Research Center. The samples were maintained at −44°C until lipids were extracted.

Tissue extraction and analysis. To minimize lipid degradation, the weighed frozen tissues were thawed in methanol-chloroform-water (1:0.5:0.4, adjusted to include the wet weight of tissue) and then homogenized immediately with an Omni mixer (Omni International, Gainesville, Va.) for 2 min at 4°C (31). Each homogenate was centrifuged, the supernatant was removed, and the residue was reextracted in a similar fashion. After centrifugation, the supernatants were combined, and enough chloroform and water were added to the methanol-chloroform-water mixture (final ratio, 1:1:0.9) so that phase separation occurred. The lower chloroform layer (lipid material) was withdrawn and dried under N_2, and nonlipid contaminants were removed by the method of Folch (20). The total lipid was dried in vacuo and weighed. The extracted residues were frozen, lyophilized, and weighed.

Lipid analysis. Samples of the total lipid were removed for isotopic analysis (see below), and the remaining material was separated by acetone precipitation of the phosphatides and bacteriohopanepolyol (BHP) from the neutral lipids

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(sterols, diplopterol, sterol esters, hydrocarbons) (47). The precipitate was dissolved in chloroform-methanol (1:1). Samples were removed and used for oxidation of BHP to its hopanol derivatives (28, 45) and for preparation of methylated fatty acids (fatty acid methyl esters [FAME]) by both the mild-alkaline methanolysis (52) and boron trifluoride-methanol (35) methods. For quantitation, duplicate FAME analyses were performed with and without an internal standard (diarachidoyl phosphatidylcholine). As an aid to identification, the FAME were separated by argentation thin-layer chromatography (TLC) into saturated, *trans* and *cis* monunsaturated, and polyunsaturated methyl esters. Adsorbosil Plus-1 (Alltech) plates were sprayed with 1% AgNO$_3$, dried at 110°C for 60 min, and cooled in a desiccator in the dark. The FAME were separated on these Ag-containing plates by developing the plates to 15 cm twice with methylene chloride. After the plates were sprayed with rhodamine 6G, the bands were viewed under UV light, their positions were related to the positions of standard compounds, and the corresponding zones were scraped and eluted with chloroform.

The neutral lipids in the acetone supernatant were separated by TLC on Silica Gel G plates (Merck) by using methylene chloride as the mobile phase (27). Under these conditions, the desmethylsterols, such as cholesterol and compounds with methyl or ethyl groups in their side chains at C-24, migrated together (R_f, 0.17), while 4-methylsterol (R_f, 0.23), 4,4-dimethyl- and 4,4,14-trimethylsterols (R_f, 0.26), and diplopterol (R_f, 0.32) could be separated. The compounds were eluted from the silica gel by the Bligh-Dyer extraction method (2). The steryl esters and hydrocarbons (R_f, >0.9) were recovered together, and the material was saponified by refluxing it with 60% KOH (2 ml) and 95% ethanol (10 ml) at 90°C for 1 h. The sterols and hydrocarbons were recovered by extracting the preparation with hexane-chloroform (4:1) and then were separated by TLC as described above.

As an aid for identifying the individual sterols, acetate derivatives were prepared (see below) and were separated by argentation TLC as described above. The migration patterns of the mussel sterols were compared with the migration patterns of standard compounds, including cholestan-3β-ol (cholestanol), cholest-5-en-3β-ol (cholesterol), cholesta-5,24-dien-3β-ol (demosterol), 24-ethyl-cholest-5-en-3-ol (sitosterol), 24-methyl-cholesta-5,7,22-trien-3β-ol (ergosterol), and 4,4,14-methyl-cholesta-8(9),24-dien-3β-ol (lanosterol), and the 4-methylsterols isolated from *Methylococcus capsulatus* (4, 25).

The lipopolysaccharide (LPS)-associated, β-hydroxy fatty acids were isolated from the lyophilized residue of the mussel tissues by using the method of Nichols et al. (39), modified by performing hydrolysis with 6 N HCl for 4 h at 100°C. For quantitation, samples were analyzed after α-OH-14:0 was added as an internal standard. Methyl esters were prepared and isolated by TLC as previously described (28).

Gas chromatography. Normal and hydroxy FAME were analyzed by using a Perkin-Elmer model Sigma 3B gas chromatograph equipped with a flame ionization detector and a 30-m DB-5 fused silica megabore column (J & W Scientific). For normal FAME, the oven temperature was programmed to increase from 165 to 225°C at a rate of 3°C/min and then was kept at 225°C for 15 min. For hydroxy FAME, the gas chromatograph was operated isothermally at 195°C.

Sterols and BHP-hopanols were acetylated as previously described (27) and were separated by using the DB-5 column described above operated at 290°C. Sterol and hopanol contents were determined by using internal injection standards (cholestane and cholestanol, respectively).

Gas chromatography-mass spectrometry. The bond positions of the *cis* monounsaturated FAME were determined by analyzing their dimethyl disulfide adducts (36), using a Hewlett-Packard model 5890 gas chromatograph equipped with a DB-5 capillary column and a model 5971 mass detector connected to a Vectra model 386S/20 data acquisition system. The oven temperature was kept at 195°C for 12 min, then increased at a rate of 4°C/min to 275°C, and finally kept at 275°C for 15 min.

Prior to compound-specific isotope analysis, sterols and hopanols were identified by gas chromatography-mass spectrometry by using acetate and trimethyl-silyl derivatives, as well as the underivatized compounds. Sterol and hopanol derivatives were prepared by using mixtures of acetic acid and pyridine (1:1) or bistrimethylsilyltrifluoroacetamide and trimethylchlorosilane (99:1), respectively, and authentic cholestanol, cholest-5-en-3β-ol, cholest-7-en-3β-ol, cholest-8(9)-en-3β-ol, cholest-8(14)-en-3β-ol, cholesta-5,24-dien-3β-ol, and lanosterol standards were included for comparison. Identification of 4α-methyl-cholesta-8(14),24-dien-3β-ol was based on comparison with the same compound present in *M. capsulatus*. Analyses were performed by using a Finnigan model Incos-50 instrument-equipped Varian model 3400 gas chromatograph with a septum programmable on-column injector and a DB-5 column (25 m by 0.3 mm; J & W Scientific); the temperature was programmed to increase from 60 to 320°C at a rate of 10°C min^{-1}. Some analyses were performed by using a VG 70E instrument equipped with a Hewlett-Packard Ultra-1 column as described by Summons and Jahnke (47).

Isotope analysis. The $^{13}C/^{12}C$ ratios (δ^{13}C) of total lipid and extracted residue preparations were determined by burning the preparations over CuO in sealed, evacuated vycor tubes (48). The resulting CO$_2$ was analyzed by using a Finnigan model MAT 252 mass spectrometer. The isotopic compositions of individual sterols, hopanols, and fatty acids were determined by compound-specific isotope techniques. Gas chromatography-combustion-isotope ratio mass spectrometry was performed by using a Finnigan model MAT 252 mass spectrometer equipped

TABLE 1. Carbon isotopic compositions of seep mussel tissues[a]

Component	Gill tissue		Mantle tissue		Remains	
	Dry wt (mg)	δ^{13}C (‰)	Dry wt (mg)	δ^{13}C (‰)	Dry wt (mg)	δ^{13}C (‰)
Total lipid	233	−60.6	80	−62.4	317	−63.3
Cell residue	1,080	−58.7	756	−60.2	3,030	−56.0
Total tissue	1,310	−59.0	836	−60.4	3,340	−56.7

[a] Total lipid was extracted and nonlipid cell residue was recovered as described in Materials and Methods. Carbon isotope compositions are reported as δ^{13}C values, which were calculated as follows: δ^{13}C = [(R_{sample} − $R_{standard}$)/$R_{standard}$] × 10^3, where R_{sample} is the $^{13}C/^{12}C$ ratio of the sample and $R_{standard}$ is the $^{13}C/^{12}C$ ratio of Peedee belemnite.

with a Varian model 1400 gas chromatograph and a CuO-Pt microvolume combustion furnace as previously described (21, 48).

RESULTS

Mussel tissue composition. The seep mussel was dissected into two distinct tissue types, gill tissue and mantle tissue, which accounted for 34 and 17% of the total tissue wet weight (50.6 g), respectively. A third tissue sample, which was designated as the remains, contained the remaining tissues, including the foot and the gut. The gill tissue contained the highest level of total lipids (17.8%, compared with only 9.6% for the mantle tissue and 9.5% for the remains) (Table 1). Both the total lipid and the extracted cell residue of all three tissue samples were highly depleted in ^{13}C. Using the recovered dry weights of the individual components and their δ^{13}C values, we calculated a δ^{13}C of −57.8‰ for the whole animal.

Fatty acids. We used both the boron trifluoride and mild-alkaline hydrolysis methods to prepare phospholipid FAME, and we obtained similar values for concentrations and compositions with the two methods (Table 2). Large amounts of polyunsaturated fatty acids, particularly those with 20- and 22-carbon chains, were present in all three seep mussel tissues; the concentrations of these compounds were 13.8, 9.7, and 11.5 mg g^{-1} (dry weight) in the gill, mantle, and remains tissue samples, respectively. The gill tissue contained the highest concentration of phospholipid fatty acids, which was due in part to the increased levels of several unique 16:1 isomers in this tissue. The concentration of the 16:1 isomers with the double bond at Δ8, Δ10, or Δ11 and the isomers with the *trans* configuration was 10.4 mg g^{-1} (dry weight) in the gill tissue, compared with only 0.1 and 0.3 mg g^{-1} in the mantle and remains, respectively. Differences in the 18:1 isomer contents between the gill and other tissues were also apparent; the Δ10, Δ12, and Δ13 isomers were somewhat enriched in the gill tissue, while the Δ9 and Δ11 isomers were more prevalent in the mantle and remains tissue samples.

The results of our compound-specific carbon isotope analysis of the FAME prepared by the BF$_3$-methanol method are shown in Table 3. The individual phospholipid fatty acids were all highly ^{13}C depleted. We calculated that the total isotopic compositions based on the FAME analyzed resulted in δ^{13}C values of −60.2, −63.9, and −63.7‰ for the gill, mantle, and remains tissue samples, respectively. These values suggest that the carbon isotopic compositions of the phospholipid and total fatty acid pools were similar, as reflected by the δ^{13}C values for the total lipid.

Extraction of the tissue residues after acid hydrolysis resulted in the isolation of several C$_{16}$ hydroxy fatty acids which had presumably been released from an LPS-like compound. The results of duplicate analyses of the gill tissue residue

TABLE 2. Phospholipid fatty acid compositions of
seep mussel tissues

Fatty acid[a]	Amt (µg g^{-1} [dry wt]) in:		
	Gill tissue	Mantle tissue	Remains
14:0	36	36	107
15:0	181	18	21
16:1Δ8c	6,340	53	106
16:1Δ9c	4,100	1,410	3,190
16:1Δ10c	1,230	36	85
16:1Δ11c	218	18	43
16:1t	2,570	53	20
16:0	2,900	1,260	2,120
17:1c	217	160	64
17:0	326	445	150
18:3	2,360	1,650	2,270
18:2	1,340	356	599
18:1Δ9c	73	320	257
18:1Δ10c	399	71	65
18:1Δ11c	725	1,420	1,180
18:1Δ12c	181	70	64
18:1Δ13c	36	ND[b]	ND
18:1t	544	338	342
18:0	580	853	791
19:2	326	178	470
19:1c	217	231	66
20:2	7,070	4,620	5,580
20:1Δ7c	435	267	299
20:1Δ11c	399	463	535
20:1Δ12c	36	ND	ND
20:1Δ13c	508	249	278
20:1t	109	142	66
22:2	2,760	2,900	2,570
Other	109	72	43

[a] Fatty acids are designated as follows: total number of carbon atoms:number of double bonds. The number after Δ indicates the position of the double bond from the carboxylic (Δ) end of the molecule. The suffixes c and t indicate cis and trans geometry, respectively. No attempt was made to determine bond positions for trans monoenoics or polyenoics. The percentages of fatty acids in the gill, mantle, and remains samples were 11.1, 14.7, and 14.9%, respectively. The percentages of cis monounsaturated fatty acids in the gill, mantle, and remains samples were 42.1, 26.8, and 29.1%, respectively. The percentages of trans monounsaturated fatty acids in the gill, mantle, and remains samples were 8.9, 3.0, and 2.0%, respectively. The percentages of polyunsaturated fatty acids in the gill, mantle, and remains samples were 38.2, 54.6, and 53.7%, respectively. The concentrations of total fatty acids in the gill, mantle, and remains samples were 36.3, 17.8, and 21.4 mg g^{-1} (dry weight).

[b] ND, none detected.

agreed within 4%; the values obtained were 4,600 µg of β-OH-16:0 per g (dry weight) and 975 µg of α-OH-16:0 per g. Only minor amounts of these hydroxy FAME were detected in the mantle and remains tissue residues (200 and 141 µg g^{-1}, respectively).

Hopanoids and sterols. The mussel tissues contained large amounts of two distinct types of cyclic triterpenoids, the hopanoids and sterols (Table 4). The results of a compound-specific isotope analysis showed that all of the isoprenoid lipids were highly depleted in ^{13}C. Two hopanols, a C$_{31}$ hopanol and a C$_{32}$ hopanol, were isolated predominantly from the gill tissue. The different C$_{31}$ and C$_{32}$ hopanol molecules arose because various polyhydroxylated side chains could be associated with a C$_{30}$ pentacyclic hopane to make up BHP. Thus, cleavage of the BHP side chain during periodate oxidation resulted in hopanol derivatives having different numbers of carbon atoms (Fig. 1).

Major differences in sterol distribution were apparent in the different tissue types (Table 4). Although cholesterol was the most abundant sterol in all three types of tissue samples, eight

TABLE 3. Carbon isotopic compositions of phospholipid
fatty acids in seep mussel tissues[a]

Tissue	δ^{13}C (‰)[a]						
	16:1	16:0	18:1	18:0	20:2	20:1	22:2
Gill	−58.3	−62.9	NA[b]	−60.7	−61.5	−62.0	NA
Mantle	−64.0	−63.8	−65.0	−62.3	−63.2	−63.4	−64.5
Remains	−62.6	−64.1	−62.4	−61.6	−63.0	−63.6	−62.3

[a] Phospholipid FAME were prepared by the BF$_3$-methanol procedure and were analyzed by the compound-specific isotope techniques described in Materials and Methods. Data were corrected for methyl carbon (−39.0‰) addition. An internal quantitation standard, 20:0 FAME (nominal δ^{13}C, −29.4‰), had measured values within 1‰ for all FAME isotopic analyses.

[b] NA, not analyzed.

other compounds were detected; these compounds differed in the number and position of double bonds and in the presence of methyl groups at C-4 and C-14 (Fig. 1 shows the sterol structures and carbon numbers). The 4α-methylsterols and lanosterol accounted for 9.8% of the total sterols in the whole animal. The gills contained the highest levels of both free and esterified sterols, and methylsterols accounted for 17.2 and 43.9% of these free and esterified sterols, respectively. While in the gill tissue 4α-methyl-cholesta-8(14),24-dien-3β-ol was the major free methylsterol (11.0%), a 4-methyl-cholesta-dienol with bonds at position 7,24 or 8(9),24 accounted for 34.9% of the steryl ester fraction.

Alkanes. Gill tissues contained significant amounts of saturated hydrocarbons and hop-22(29)-ene (diploptene) (Fig. 2). The saturated compounds included a suite of n-alkanes, with even-over-odd carbon number preference for C$_{20}$ to C$_{24}$ compounds and slight odd carbon number preference for C$_{25}$ to C$_{33}$ compounds, monomethylalkanes, and alkylcyclohexanes. The n-alkanes were separated from the branched and cyclic compounds by using silicalite, a shape-selective molecular sieve (23), and then analyzed for their individual ^{13}C contents. The resulting values were between −28.4 and −30.9‰, and the average was −30.4‰ (Fig. 2). Hop-22(29)-ene in the branched and cyclic fraction had a δ^{13}C value of −70.6‰ and clearly had a different origin than the n-alkanes.

DISCUSSION

While the C$_{20}$ and C$_{22}$ polyunsaturated fatty acids characteristic of marine mytilid mussels (14, 53) were present in all three tissue samples, only gill tissue contained specific methanotrophic signature FAME. The phospholipid fatty acids of type I and type X methanotrophs normally contain cis and trans 16:1 with the double bond at Δ8, Δ9, Δ10, or Δ11, while the type II methanotrophs contain cis and trans 18:1 that is unsaturated at Δ10, Δ11, or Δ12 (5, 27, 33, 40). While 16:1Δ9c, 18:1Δ9c, and 18:1Δ11c are common monounsaturated compounds in many organisms, 16:1 molecules with the double bond at Δ8, Δ10, and Δ11 and the trans isomers are diagnostic for type I or X methanotrophic bacteria. A comparison of the fatty acid composition of the symbiont-containing gill tissue with the fatty acid compositions of the symbiont-free mantle tissue and a type X methanotroph (Fig. 3) clearly demonstrated that methanotrophic bacteria were present in the gill tissue. The elevated level of 16:1Δ8 in the gill tissue may reflect the water temperature (6°C) at which these animals live, since it has been shown that decreasing the growth temperature results in increased levels of 16:1Δ8 in M. capsulatus (25). The 18:1 isomers in the gill tissue are probably due to the active

TABLE 4. Tissue distribution and $\delta^{13}C$ values for seep mussel cyclic triterpenoids

Compound[a]	Gill tissue		Mantle tissue		Remains	
	Total concn ($\mu g\ g^{-1}$)[b]	$\delta^{13}C$ (‰)[c]	Total concn ($\mu g\ g^{-1}$)[b]	$\delta^{13}C$ (‰)[c]	Total concn ($\mu g\ g^{-1}$)[b]	$\delta^{13}C$ (‰)[c]
Free sterols	3,460		2,750		1,950	
Cholest-5-en-3β-ol	2,220	−70.9	2,130	−72.2	1,520	−71.0
Cholestan-3β-ol	90	−72.8	228	−71.5	135	−72.9
Cholesta-5,24-dien-3β-ol	387	−69.8	124	−72.6	150	−71.0
Cholest-7-en-3β-ol	166	−72.8	132	−69.5	52	−73.7
4-Methyl-cholesta-dien-3β-ol	11	NA[d]	ND[e]		ND	
4-Methyl-cholesta-8(14),24-dien-3β-ol	381	−67.3	53	NA	31	−72.6
4-Methyl-cholest-7-en-3β-ol	83	−74.2	57	−72.3	47	−75.1
4-Methyl-cholesta-7,24-dien-3β-ol	45	−71.6	25	NA	18	NA
4,4,14-Trimethyl-cholesta-8(9),24-dien-3β-ol	73	−77.4	ND		4	NA
Steryl esters	132		82		8	
Cholest-5-en-3β-ol	68	−75.4	61	NA	5	NA
Cholestan-3β-ol	ND		ND		ND	
Cholesta-5,24-dien-3β-ol	6	NA	4	NA	1	NA
Cholest-7-en-3β-ol	ND		4	NA	1	NA
4-Methyl-cholesta-8(14),24-dien-3β-ol	5	NA	6	NA	1	NA
4-Methyl-cholest-7-en-3β-ol	5	NA	4	NA	<1	NA
4-Methyl-cholesta-7,24-dien-3β-ol	46	−74.9	3	NA	<1	NA
4,4,14-Trimethyl-cholesta-8(9),24-dien-3β-ol	2	NA	ND		ND	
Hopanepolyols	1,040		14		130	
C_{31} hopanol	828	−70.7	12	NA	106	−73.3
C_{32} hopanol	212	−68.5	2	NA	24	−71.7

[a] Sterols and hopanols are listed in relative order of elution from DB-5 columns and were identified by a combination of TLC mobility on conventional and silver-impregated plates, relative retention times on DB-5 columns, and the mass spectra of trimethylsilyl and acetate derivatives. Trace amounts of cholest-8(9)-en-3β-ol and a 4,4-dimethyl monene were also detected in gill tissue. The 4-methyl-cholesta-7,24-dien-3β-ol may have an internal double bond at either C-7(8) or C-8(9). The C_{31} and C_{32} hopanols are products of cleavage of the polyhydroxylated side chains of the various BHP molecules (Fig. 1) (45).

[b] Total amount of material recovered per gram (dry weight) based on the results of an analysis of isolated tissues obtained from a single dissected mussel.

[c] $\delta^{13}C$ values were determined by performing a compound-specific analysis for the acetate derivatives of individual hopanol and sterol compounds that were large enough to analyze. The reported $\delta^{13}C$ values are the values for the free hydroxyl and were calculated after we determined empirically the isotope effect due to acetate derivative formation.

[d] NA, not analyzed.

[e] ND, compound not detected.

fatty acid C_2 elongation mechanism that has been reported for mytilid mussels (53).

In a previous fatty acid analysis of seep mussels Fang et al. (14) did not detect any of the methanotrophic signature FAME which we observed. However, Fang et al. reported the total fatty acid composition for whole animals only. In our analysis the 16:1 signature FAME made up only 14% of the total phospholipid fatty acid pool. It is important to consider the fact that large amounts of fatty acids are also esterified as glycerides in marine molluscs (10), so depending on the numbers of bacteria present in gill tissue, a methanotrophic biomarker input could be masked.

The bacterial LPS-associated hydroxy fatty acids were also clearly associated with the symbiont-containing gill tissue. Acid hydrolysis of lipid-depleted bacterial or tissue residue is necessary for detection of LPS-associated hydroxy fatty acids (40). The LPS of methanotrophs normally contains relatively long-chain hydroxy fatty acids, and β-OH-16:0 or β-OH-18:0 generally predominates (5, 39, 40). The amount of β-OH-16:0 recovered in our analysis (5,670 $\mu g\ g^{-1}$ [dry weight]) from the gill tissue suggests that a considerable number of type I methanotrophs was present in this tissue.

Carbon isotope effects associated with carbon cycling in natural environments are valuable for characterizing food sources. The light $\delta^{13}C$ value obtained for this animal (−57.8‰) is consistent with values for other seep mussels obtained from this area of the western Gulf of Mexico (9, 17) and clearly reflects the composition of the thermogenic methane ($\delta^{13}C$,
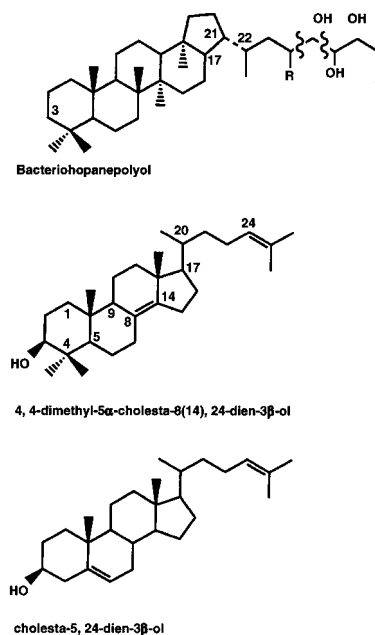

FIG. 1. Structures of diagnostic terpenoids obtained from mussel tissues. In BHP, X represents a variety of possible side chain structures, and R = OH and R = H yield C_{31} and C_{32} hopanols, respectively, as a result of the sodium periodate-sodium borohydride cleavage procedure (45).
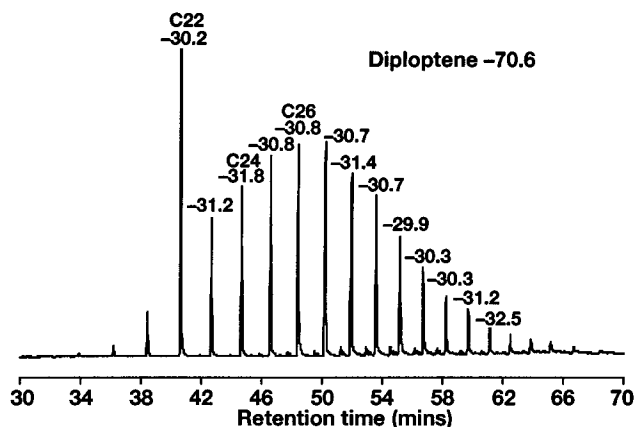
FIG. 2. Gas chromatographic profile of the hydrocarbons obtained from mussel gill tissue, showing the presence of petroleum-derived *n*-alkanes and the carbon isotopic composition of the *n*-alkanes. The profile for nonadducted silicalite, branched, and cyclic hydrocarbons with bacterially derived diploptene at −70.6‰ is not shown.

isomers that are isotopically heavier than the corresponding saturated 16:0 isomers (26, 48).

Data on the higher hydrocarbons isolated from gill tissues, together with measured fractionation factors for *M. capsulatus*, can be used to construct a model to describe the expected isotopic compositions of mussel metabolites. The resulting data also provide convincing evidence concerning the isotopic composition of the methane used as the carbon source by the bacterial symbionts. Figure 2 shows a petroleum-like distribution of higher *n*-alkanes likely to have been associated with the seep gas. The average $\delta^{13}C$ for these C_{22} to C_{30} compounds was −30.1‰ Peedee belemnite, and thermogenic methane associated with alkanes having this composition would normally be further depleted by approximately 14‰ (29) (that is, with a $\delta^{13}C$ value of −44‰ and close to measured values for methane associated with Gulf of Mexico petroleum). If this value were further adjusted for the 16‰ fractionation measured for the $\delta^{13}C$ of *M. capsulatus* soluble methane monooxygenase (48), the bacterial biomass would be approximately −60‰. In *M. capsulatus*, sterols and hopanols can be further ^{13}C depleted by 10‰ compared with total biomass. The bacterial hopanoid diploptene that co-occurs with the *n*-alkanes described above had a $\delta^{13}C$ value of −70.6‰, which is very close to the calculated value. The $\delta^{13}C$ value for the predominant hopanols and sterols isolated from the gill and mantle tissues was also close to this value (−72‰). Fatty acids of *M. capsulatus* are depleted by 2 to 3‰ compared with biomass (48), and again, except for the anomalous 16:1 isolated from the gill tissue, the $\delta^{13}C$ values for the fatty acids in the mussel tissue were −62 to −65‰ and thus were depleted relative to biomass.

All methanotrophic bacteria that have been analyzed contain large amounts of various types of polyhydroxylated hopanoids referred to collectively as BHP (45). BHP have never been identified in a eukaryotic organism and, therefore, are an

−45‰) found in this environment rather than a more ^{13}C-enriched heterotrophic food source (6). Although the mussel which we used was maintained for a short period of time with commercial methane (18), the laboratory conditions were such that net carbon incorporation would have been no greater than 0.1% per day (32), which would have resulted in only a minor effect on the total carbon isotopic composition. This fact is supported by the slight variation in $\delta^{13}C$ values observed for phospholipid FAME (Table 3). While the gill tissue contained somewhat heavier 16:1 molecules, it is important to note that in methanotrophic bacteria unsaturated fatty acid synthesis occurs by a novel anaerobic mechanism and results in 16:1
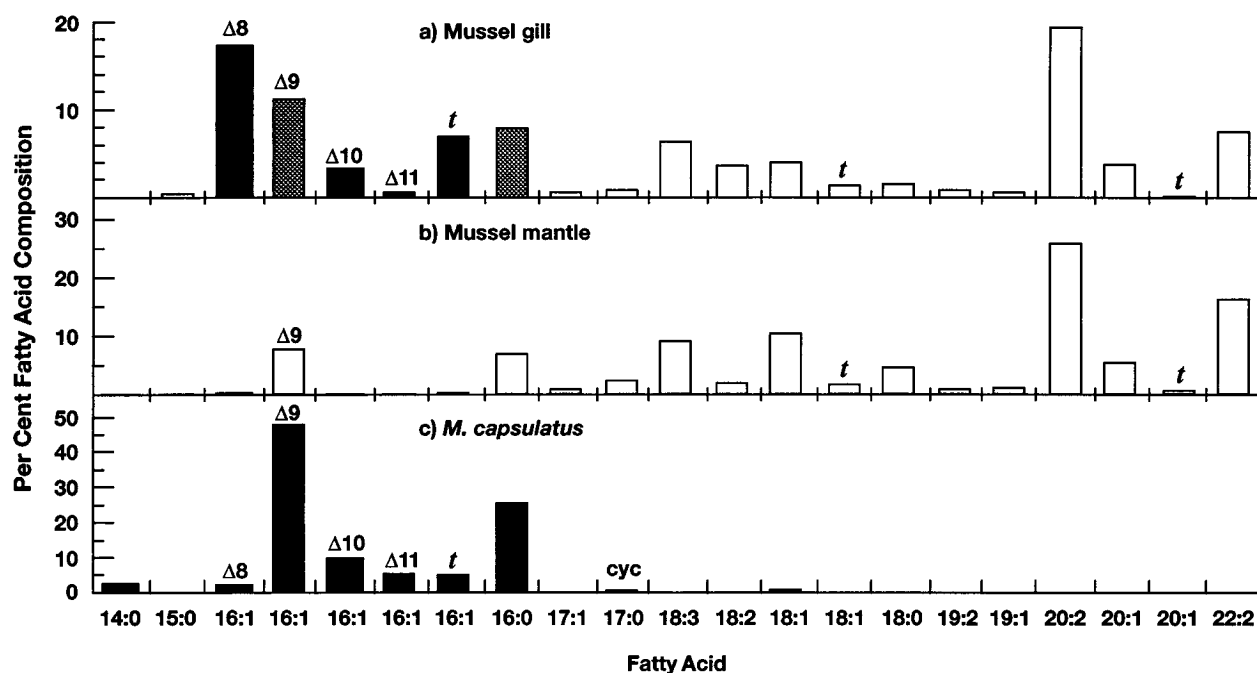

FIG. 3. Comparison of the phospholipid fatty acid compositions of seep mussel gill (a) and mantle tissues (b) with the composition of the methanotrophic bacterium *M. capsulatus* (25, 27) (c). The graphs show the fatty acids in mussel gill tissue associated principally with the host tissue (open bars), with the methanotrophic symbiont (solid bars), and with both organisms (shaded bars). All monounsaturated FAME were *cis* unless indicated otherwise. *t*, *trans*; cyc, cyclopropane C_{17}.

excellent indication that bacteria are present. The large amount of highly ^{13}C-depleted BHP isolated from the gill tissue clearly indicates that a methanotrophic bacterium was present (48). Almost no hopanol was detected in the mantle tissue, and the low levels observed in the remains tissue were probably the result of the presence of free-living bacteria in the gut portion of the sample.

While the major sterol found in all tissues was cholesterol, 18% of the gill sterols were methylated at C-4. Methylsterols have not been found in mytilid mussels which are free of methanotrophic symbionts (10, 14, 30) and indeed, with the exception of dinoflagellates and some coccolithophorids (38, 50), are not found in eukaryotes except as intermediates in the biosynthesis of desmethylsterols, such as cholesterol (3, 22). Large amounts of methylsterols have been found in *M. capsulatus* (1), and both the 4,4-dimethyl and 4α-methyl forms of 5α-cholest-8(14)-en-3β-ol and 5α-cholest-8(14),24-dien-3β-ol are synthesized de novo by the demethylation of lanosterol in this bacterium (4, 44). In our seep mussel, the gill tissue contained a considerably higher level of 4-methylsterols (602 μg g^{-1} [dry weight]) than either the mantle tissue (135 μg g^{-1}) or the remains tissue (101 μg g^{-1}). However, it is interesting that a higher proportion of methylsterols than either bacteriohopanols or other bacterial biomarkers was present in the mantle tissue.

Several lines of evidence suggest that the animal host uses bacterial methylsterol as a metabolic precursor for biosynthesis of cholesterol. First, the steryl ester pool is largest in the gill tissue, and a major proportion of this pool is composed of methylsterol. Steryl esters are known to play important roles in both storage and intracellular translocation in eukaryotic cells (54). The gill steryl esters have high levels of the putative 4-methyl-cholesta-7,24-dien-3β-ol isomer (Table 4), an intermediate in the conversion pathway to cholest-5-ene-3β-ol. Second, the mantle and remains sterols are isotopically similar to the polyisoprenoid pool in the gill tissues. Third, the isotopic relationships between the sterols and fatty acids (see below) (the fatty acids are significantly enriched for ^{13}C compared with the sterols in the mantle and remains tissues) are not what is expected for the products of eukaryote biosynthesis. Fourth, as noted above, methylsterols are significant metabolites in the mantle and remains tissues, while bacteriohopanes and the bacterial 16:1 isomers are barely detected, indicating that the distribution of compounds does not reflect cross-contamination.

In our study, the ^{13}C content of the total lipid (−62.2‰) was less than the ^{13}C content of the total biomass (−57.8‰). This was an anticipated fractionation because of the synthesis of ^{13}C-depleted acetyl coenzyme A by the pyruvate decarboxylase (11). However, in our seep mussel, the isoprenoid lipids were more depleted than the total lipid (composed predominantly of esterified fatty acids) or the phospholipid fatty acids; this result was not expected. Monson and Hayes (34) have observed depletion of ^{13}C at the carboxyl carbon of acetyl coenzyme A compared with the methyl carbon. Keeping in mind that in eukaryotes biosynthetic mechanisms for isoprenoid synthesis result in incorporation of acetyl coenzyme A at a methyl/carboxyl ratio of 3:2 and that in fatty acid synthesis the ratio is 1:1, fatty acids should be more depleted than triterpenoids. This result (fatty acids more depleted in ^{13}C than sterols) has been reported in other bivalves (10). This was not the case with the lipids extracted from the mussel tissues in this study, nor is it the case in methanotrophic bacteria (48). Rohmer and his associates (46) have described a novel biosynthetic route which leads to isopentenyl diphosphate in prokaryotes and may result in these unusually light isoprenoids. The iso-

tope data for specific metabolites in this seep mussel strongly indicate that there is a common biosynthetic origin for the host and symbiont sterols.

Methylsterols have been identified in two other seep mussel species collected at a different site in the Gulf of Mexico (15). Although methylsterols are present in only trace amounts in most methanotrophic bacteria (24, 42), the presence of methylsterols in three mussel species, from different sites, clearly suggests that methylsterols play an important role in this host-symbiont relationship. It has been suggested that hopanoids are the functionally equivalent phylogenetic precursors of eukaryotic sterols (41). Both hopanoids and sterols are efficient membrane reinforcers (3, 41), and the 4-methylsterols of *M. capsulatus* have been shown to play an important role in modulating membrane fluidity at low growth temperatures (25). Both the methylsterols and BHP are highly enriched in the outer membrane of the gram-negative bacterium *M. capsulatus* (28). A very stable outer membrane is essential for maintenance of this host-symbiont relationship, and the presence of these isoprenoid lipids suggests that these compounds may play such a role.

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