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Geomicrobiology Journal, 20:403–419, 2003
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ISSN: 0149-0451 print / 1362-3087 online
DOI: 10.1080/01490450390241071

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Characterization of Specific Membrane Fatty Acids as Chemotaxonomic Markers for Sulfate-Reducing Bacteria Involved in Anaerobic Oxidation of Methane

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Membrane fatty acids were extracted from a sediment core above marine gas hydrates at Hydrate Ridge, NE Pacific. Anaerobic sediments from this environment are characterized by high sulfate reduction rates driven by the anaerobic oxidation of methane (AOM). The assimilation of methane carbon into bacterial biomass is indicated by carbon isotope values of specific fatty acids as low as −103‰. Specific fatty acids released from bacterial membranes include $C_{16:1\omega5c}$, $C_{17:1\omega6c}$, *and* $cyC_{17:0\omega5,6}$, *all of which have*

Received 2 February 2002; accepted 7 April 2003.

We thank the officers, crew, and shipboard scientific party for excellent support during RV SONNE cruise SO-148/1. We are especially indebted to Dirk Rickert (GEOMAR, Kiel, Germany) for his help with sample handling and transport during and after the cruise. We also thank André Lipski (Universität Osnabrück, Germany) who provided literature data about $C_{16:1\omega5c}$ fatty acid-producing bacteria and specific taxa information on SRB. Beth Orcutt, Jutta Niggemann, and Solveig Bühring provided fruitful comments on an earlier version of the manuscript. Constructive criticism by two anonymous reviewers was highly appreciated. This study was part of the programs TECFLUX II (TECtonically induced FLUXes, FN 03G0148A) and MUMM (Mikrobielle UMsatzraten von Methan in gashydrathaltigen Sedimenten, FN 03G0554A) supported by the Bundesministerium für Bildung und Forschung (BMBF, Germany). Further support was provided from the Max-Planck-Gesellschaft (Germany). This is publication GEOTECH-16 of the program GEOTECHNOLOGIEN of the BMBF and the Deutsche Forschungsgemeinschaft (Germany).

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been fully characterized by mass spectrometry. These unusual fatty acids continuously display the lowest $\delta^{13}C$ values in all sediment horizons and two of them are detected in high abundance (i.e., $C_{16:1\omega 5c}$ and $cyC_{17:0\omega 5,6}$). Combined with microscopic examination by fluorescence in situ hybridization specifically targeting sulfate-reducing bacteria (SRB) of the Desulfosarcina/Desulfococcus *group, which are present in the aggregates of AOM consortia in extremely high numbers, these specific fatty acids appear to provide a phenotypic fingerprint indicative for SRB of this group. Correlating depth profiles of specific fatty acid content and aggregate number in combination with pore water sulfate data provide further evidence of this finding. Using mass balance calculations we present a cell-specific fatty acid pattern most likely displaying a very close resemblance to the still uncultured* Desulfosarcina/Desulfococcus *species involved in AOM.*

Keywords anaerobic oxidation of methane, cyclopropane fatty acid, dimethyl disulfide adducts, dimethyloxazoline derivatives, fatty acid methyl esters, methanotrophic archaea, stable carbon isotopes, sulfate-reducing bacteria, syntrophic consortia

Introduction

Abundant information now exists about lipid biomarkers associated with the process of anaerobic oxidation of methane (AOM) in the marine environment (e.g., Elvert et al. 2000; Hinrichs et al. 2000; Pancost et al. 2000; Thiel et al. 2001). Lipids derived from AOM are evident by their highly ^{13}C-depleted carbon isotopic compositions due to the unequivocally low and characteristic isotopic signal of the substrate methane (−50 to −110‰; Whiticar 1999). Therefore, methane carbon incorporation into microbial biomass can be regarded as a natural ^{13}C-depleted tracer experiment. Recent studies provided evidence that consortia of archaea and sulfate-reducing bacteria (SRB) mediate the process of AOM in various methane seep environments (Boetius et al. 2000; Orphan et al. 2001; Michaelis et al. 2002). Most of the studies on lipid biomarkers associated with AOM primarily focused on the presence and carbon isotopic compositions of a set of biomarkers derived from archaea anaerobically oxidizing methane: crocetane, pentamethylicosane, archaeol, hydroxyarchaeol, and glycerol tetraethers (Elvert et al. 1999; Hinrichs et al. 1999; Thiel et al. 1999; Pancost et al. 2001b; Schouten et al. 2001). In contrast, existing data on lipid biomarkers proposed to derive from the SRB partners involved in AOM suggest that SRB biomarkers are highly diverse and include straight chain and methyl-branched fatty acids and alcohols (Thiel et al. 1999; Hinrichs et al. 2000; Pancost et al. 2000; Elvert et al. 2001; Zhang et al. 2002) as well as mono- and dialkyglycerolethers (Hinrichs et al. 2000; Orphan et al. 2001; Pancost et al. 2001a; Werne et al. 2002).

To date, most of the studies on AOM-associated lipid biomarkers have been limited to single samples and only some of the published data provided total fatty acid patterns (Hinrichs et al. 2000; Zhang et al. 2002). Therefore, the present study aims to extend the inventory of membrane fatty acids usable to monitor SRB involvement in AOM in the modern and the fossil record. We investigated a sediment core with high AOM activity from southern Hydrate Ridge (Cascadia convergent margin, NE Pacific), off the coast of Oregon. Hydrate Ridge is a cold seep environment accompanied by methane hydrates that often occur just a few centimeters beneath the sediment surface (Suess et al. 1999, 2001). The environment is characterized by high fluid flow (Torres et al. 2002), steep gradients of pore water sulfate (Boetius et al. 2000; Elvert et al. 2001), high rates of AOM and sulfate reduction (Boetius et al. 2000; Treude et al. submitted), ^{13}C-depleted lipid biomarkers of archaeal and bacterial origin (Elvert et al. 1999; Boetius et al. 2000; Elvert et al. 2001), and ^{13}C-depleted authigenic carbonates (Kulm et al. 1986; Ritger et al. 1987; Bohrmann et al. 1998; Greinert et al. 2001). These cold seep sediments harbor aggregates of AOM consortia composed of archaea related to the order *Methanosarcinales* and SRB representing close relatives of *Desulfosarcina/Desulfococcus* species of the δ-proteobacteria (Boetius et al.

2000). The observed aggregates account for more than 90% of all detected archaea and SRB in the sediments and therefore sediments of this setting can be considered as a natural enrichment culture of AOM consortia. Our special emphasis was the characterization of membrane fatty acids as reproducible and stable chemotaxonomic markers for the yet uncultured *Desulfosarcina*/*Desulfococcus* species in AOM consortia in methane-rich environments. Their specific fingerprint may be used in the future to examine the presence and activity of these and closely related species both in field studies and laboratory experiments.

Materials and Methods

Sample Collection and Storage

Sediment samples were obtained during the RV SONNE cruise SO-148/1 in August 2000 at the crest of southern Hydrate Ridge (44°34′N, 125°09′W; 780 m water depth) from an area of active methane venting using a video-guided multiple corer (station 19-2). Upon recovery, the sediment core, covered by a white mat of giant sulfide-oxidizing bacteria (*Beggiatoa*), was sliced into depth intervals of 2 cm (0–10 cm) and 3 cm (10–19 cm). Each sediment section was transferred into a pre-cleaned 20 ml glass vial and stored frozen at −30°C until lipid extraction. Sediment samples for the determination of AOM aggregates in the upper 10 cm were obtained from a replicate sediment core at station 19-2 subsampled in 1-cm intervals. The samples for cell numbers were processed and analyzed by microscopic examination using fluorescence in situ hybridization (FISH) as described previously (Boetius et al. 2000; Knittel et al. 2003, this issue).

Extraction of Sediment Samples and Preparation of Fatty Acid Methyl Esters (FAMES)

Lipids were extracted by ultrasonication of sediment samples (8 to 10 g of wet sediment) at 0°C (ice-cooling) using (I) 25 ml of methanol/dichloromethane (2:1, v/v), (II) 25 ml of methanol/dichloromethane (1:2, v/v), (III) and (IV) 25 ml of dichloromethane. Internal standards representing different compound classes (5α(H)-cholestane, *n*-C_{36}, 2-nonadecanon, nonadecanol, nonadecanoic acid) were added at known concentrations and $\delta^{13}C$ values prior to extraction. The extracts were pooled and partitioned against pre-extracted 0.05 M KCl-solution (25 ml) in a separatory funnel. The lower organic phase was collected and the solvent evaporated, yielding a total lipid extract (TLE). An aliquot of the TLE was transferred to a 10-ml glass vial, evaporated under a stream of nitrogen close to dryness and saponified with 2 ml 6% KOH in MeOH. The reaction was maintained at 80°C for 3 h. After cooling, the mixture was diluted with 2 ml 0.05 M KCl-solution. Neutral lipids were released from the basic mixture by extracting four times with 2 ml *n*-hexane. The remaining aqueous reaction mixture was treated with 25% HCl to pH 1 and free fatty acids were extracted four times with *n*-hexane (2 ml each). The combined extracts were dried under a stream of nitrogen and free fatty acids were reacted with 1 ml 14% BF_3 in MeOH at 70°C for 1 h to form FAMES. After cooling, the mixture was extracted four times with 2 ml *n*-hexane in the presence of 1 ml 0.05 M KCl-solution. Combined extracts were evaporated under a stream of nitrogen, redissolved in 100 μl *n*-hexane, and stored at −20°C until further analysis.

Preparation of Dimethyl Disulfide (DMDS) Adducts

The double bond positions in FAMES were determined by analysis as their DMDS adducts according to the method of Nichols et al. (1986). Briefly, 1/2 aliquot of a selected sample

dissolved in 50 μl *n*-hexane was treated with 100 μl DMDS (Aldrich Chemicals, Steinheim, Germany) and 20 μl of iodine solution (6% w/v in diethyl ether). The mixture was flushed with nitrogen and formation of DMDS adducts was carried out in a 2-ml screw-cap (teflon-lined) glass vial at 50°C for 48 h. After 48 h, the mixture was cooled and diluted with 500 μl of *n*-hexane. The excess of iodine was reduced by addition of 500 μl sodium thiosulfate (5% w/v in water). The organic phase was removed and the aqueous phase extracted twice with 500 μl of *n*-hexane. Combined organic phases were evaporated under a gentle stream of nitrogen and diluted with 50 μl of *n*-hexane prior to GC-MS analysis.

Preparation of 4,4-Dimethyloxazoline (DMOX) Derivatives

Ring positions in FAMES were determined by analysis of DMOX derivatives according to the method of Fay and Richli (1991). Briefly, 500 μl of 2-amino-2-methyl-1-propanol were added to a dried FAMES fraction (1/2 aliquot of a selected sample) in a 5-ml screw-cap (teflon-lined) reaction vial. The vial was flushed with nitrogen, tightly closed, and placed in a heating block at 190°C overnight. On the next day, the vial was cooled to room temperature. Then 2 ml diethyl ether-*n*-hexane (1:1, v/v) and 2 ml of water were added to the reaction mixture. The vial was vigorously shaken and the organic phase removed. The extraction step was repeated twice with 2 ml of fresh solvent. Combined organic phases were washed with water (2 mL), dried with anhydrous sodium sulfate, and evaporated to dryness in a gentle stream of nitrogen. Finally, DMOX derivatives were dissolved in 100 μl *n*-hexane prior to GC-MS analysis.

Gas Chromatography (GC)

Concentrations of FAMES were determined by GC analysis using a 50-m apolar HP-5 fused silica capillary column (0.32 mm internal diameter [ID], film thickness 0.17 μm; Hewlett Packard) in a HP 5890 Series II GC equipped with a split/splitless injector operated in splitless mode and a flame ionization detector (FID). Initial oven temperature was 60°C, held for 1 min, subsequently increased to 150°C at a rate of 10°C/min, then raised to 310°C at a rate of 4°C/min and held at 310°C for 25 min. The carrier gas was He at a constant flow rate of 2.0 ml/min. The injector temperature was set at 290°C and the detector at 300°C. Concentration for each compound was calculated relative to the internal standard present within the respective FAME fraction.

Gas Chromatography-Mass Spectrometry (GC-MS)

FAMES were identified by GC-MS using a Thermoquest Trace GC interfaced to a Finnigan Trace MS. The GC-MS was operated in electron impact (EI+)-mode at 70 eV with a full scan mass range of m/z 40–900 (1.5 scans per second). The GC was equipped with a HP-5 MS fused silica capillary column (60 m, 0.32 mm ID; Hewlett Packard) coated with a cross-linked methyl silicone phase (film thickness 0.25 μm). The detector was set at 350 V, the interface was 300°C, and He was used as carrier gas (constant flow: 1.4 ml/min). The samples were injected in splitless mode (hot needle technique; injector temperature: 290°C) and subjected to the same temperature program given for GC measurements, with the exception of an elevated hold time of 50 min at 310°C.

Gas Chromatography-Isotope Ratio Mass Spectrometry (GC-IRMS)

Carbon isotope compositions of FAMES were determined by coupled GC-IRMS. The mass spectrometer (Finnigan Delta plus) was connected via a Finnigan Combustion Interface III to a HP 6890 Series GC equipped with a 50 m HP-5 (0.32 mm ID, 0.17 μm stationary

phase, Hewlett-Packard). The carrier gas was He at a constant flow rate of 1.5 ml/min. The samples were injected in splitless mode (injector temperature: 290°C) and subjected to the same temperature program given for GC-MS measurements. The oxidation oven of the combustion interface was operated at 940°C; the reduction oven at 640°C. Carbon isotope ratios are reported in the δ notation as per mil (‰) deviation from the Pee Dee Belemnite standard (PDB). Internal standards (nonadecanoic acid, n-C_{26}) within each run were used to monitor reproducibility and precision during this study. $\delta^{13}C$ values reported have an analytical error of less than ±1.0‰ and are corrected for the introduction of additional carbon atoms during derivatization with BF_3/MeOH.

Results

Fatty Acid Identification and Abundance

A typical fatty acid chromatogram of this study is shown in Figure 1. As of all other sediment horizons, the chromatogram is dominated by a cluster of monounsaturated $C_{16:1}$ fatty acids (i.e., $C_{16:1\omega7c}$, $C_{16:1\omega7t}$, and $C_{16:1\omega5c}$), accounting for approximately 50% of the total fatty acids present. The most abundant fatty acid in nearly all sediment horizons is $C_{16:1\omega5c}$. Other prominent fatty acids include $C_{14:0}$, ai-$C_{15:0}$, $C_{16:0}$, and $C_{18:1\omega7c}$ (Table 1).

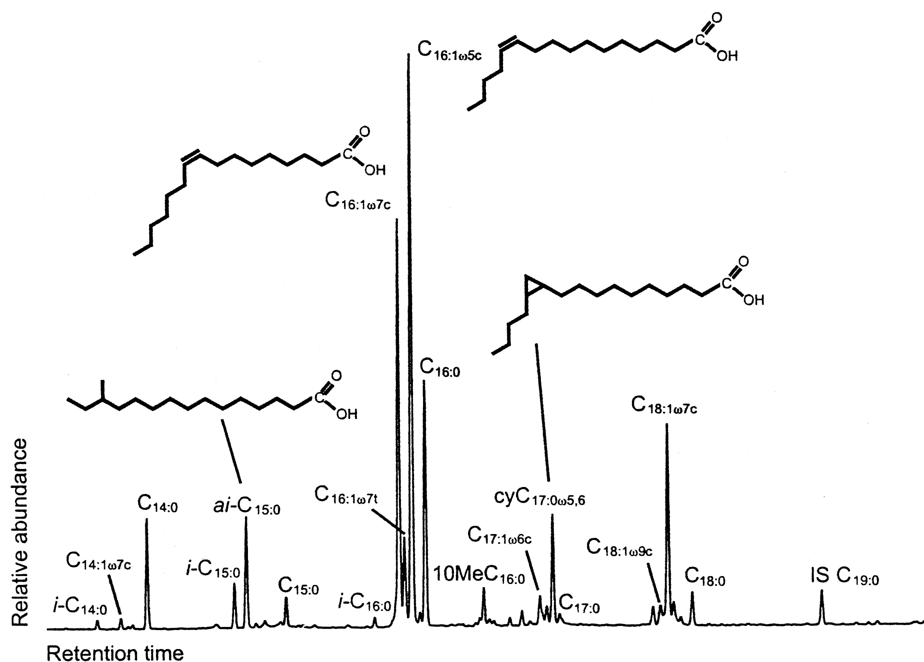

FIGURE 1 Partial gas chromatogram of fatty acids (as FAMES) obtained from an anaerobic sediment section (4–6 cm sediment depth) within a core covered by a white *Beggiatoa* mat. Structures of key fatty acids as naturally occurring in bacterial membranes are illustrated. Fatty acid abbreviations characterize carbon chain length, number of double bonds, and position of the double bond from the aliphatic (ω) end of the molecule. Configuration of the double bond is indicated by "c" for *cis* and "t" for *trans*. Thus, for example, $C_{16:1\omega5c}$ represents *cis*-11-hexadecenoic acid. *Iso* (i) and *anteiso* (ai) FAMES are methyl-branched one and two carbon atoms from the aliphatic end, respectively. The cyclopropane fatty acid (CFA) is designated as "cy", with the ring positioned at the two carbon atoms indicated.

TABLE 1 Concentrations of major fatty acids (in μg/g dw) in the *Beggiatoa* covered sediment core

Depth	Fatty acid (μg/g dw)													
(cm)	$C_{14:0}$	i-$C_{15:0}$	ai-$C_{15:0}$	$C_{15:0}$	$C_{16:1\omega7c}$	$C_{16:1\omega7t}$	$C_{16:1\omega5c}$	$C_{16:0}$	$10MeC_{16:0}$	$C_{17:1\omega6c}$	$cyC_{17:0\omega5,6}$	$C_{18:1\omega9c}$	$C_{18:1\omega7c}$	$C_{18:0}$
0–2	3.40	1.50	2.82	0.75	19.86	3.75	12.52	8.53	0.76	0.99	1.47	2.41	11.87	1.15
2–4	5.60	2.71	5.35	1.17	30.08	6.90	26.52	10.40	1.33	1.81	4.10	2.08	15.89	1.22
4–6	2.52	1.11	2.53	0.71	8.37	2.05	11.01	4.48	0.88	0.95	2.32	0.91	4.26	0.75
6–8	1.79	0.77	1.67	0.49	5.44	1.36	6.47	3.41	0.66	0.65	1.19	0.68	3.08	0.67
8–10	2.40	1.10	2.16	0.67	5.74	1.74	10.10	3.46	0.61	0.98	2.07	0.68	3.36	0.59
10–13	1.71	0.79	1.66	0.54	4.12	1.19	6.35	2.80	0.44	0.77	1.48	0.55	2.73	0.59
13–16	0.84	0.33	0.80	0.31	1.69	0.40	2.17	1.43	0.16	0.44	0.63	0.29	1.40	0.36
16–19	0.51	0.20	0.54	0.20	0.96	0.17	1.01	1.06	0.08	0.24	0.26	0.19	0.82	0.26

FIGURE 2 Electron-impact mass spectrum of the DMOX derivative of 11,12-methylene-hexadecanoic acid ($cyC_{17:0\omega5,6}$).

Interestingly, all fatty acid fractions also contain relatively high amounts of an unknown fatty acid eluting slightly before $C_{17:0}$ with an equivalent chain length (ECL) of 16.95 (HP-5). Although this particular fatty acid showed similar FAME mass spectral information as obtained for an unsaturated $C_{17:1}$ fatty acid, a double bond was not revealed by the addition of DMDS. This indicated the presence of a cyclopropane ring in the fatty acid molecule. Indeed, the unknown fatty acid present in the anaerobic methane-rich sediments was identified as 11,12-methylene-hexadecanoic acid ($cyC_{17:0\omega5,6}$) by its specific retention time and its mass spectral data of the DMOX derivative (Figure 2). Prominent peaks of the DMOX derivative are at $m/z = 113$ as a product of McLafferty rearrangement and at $m/z = 126$ probably formed by cyclization-displacement (see Spitzer 1997 for a review). The cyclopropane ring at position $\omega5,6$ is indicated by a mass difference of 12 amu instead of 14 amu between $m/z = 224$ and $m/z = 236$ accompanied by an intense ion fragment at $m/z = 278$. The same characteristic ion fragments of $m/z = 224$ and $m/z = 236$ were observed for the DMOX derivative of $C_{16:1\omega5c}$, additionally confirming the cyclopropane ring position at $\omega5,6$.

Stable Carbon Isotopic Compositions

$\delta^{13}C$ measurements of fatty acids examined from the anaerobic sediment horizons revealed a repetitive carbon isotopic pattern that is shown in Figure 3. The fatty acid profiles of all samples analyzed display the lowest, most depleted $\delta^{13}C$ values for the fatty acids $C_{16:1\omega5c}$, $cyC_{17:0\omega5,6}$, and $C_{17:1\omega6c}$ (in order of abundance). $\delta^{13}C$ values for these fatty acids in the sediment core range from −75 to −96‰ for $C_{16:1\omega5c}$ (Average: −90‰), from −94 to −103‰ for $cyC_{17:0\omega5,6}$ (Average: −100‰), and from −65 to −88‰ for $C_{17:1\omega6c}$ (Average: −80‰) (Table 2). In these three fatty acids the lowest $\delta^{13}C$ value of −103‰ was measured for $cyC_{17:0\omega5,6}$ in the 4–6-cm sediment section, whereas the highest $\delta^{13}C$ value of −65‰ was obtained for $C_{17:1\omega6c}$ in the uppermost depth interval (0–2 cm). All other fatty acids also show ^{13}C-depleted carbon isotopic compositions but they do not exhibit as strong ^{13}C-depletions

TABLE 2 Carbon isotopic composition of major fatty acids in the *Beggiatoa* covered sediment core

Depth	Fatty acid ($\delta^{13}C$ in ‰)													
(cm)	$C_{14:0}$	*i*-$C_{15:0}$	*ai*-$C_{15:0}$	$C_{15:0}$	$C_{16:1\omega7c}$	$C_{16:1\omega7t}$	$C_{16:1\omega5c}$	$C_{16:0}$	10Me$C_{16:0}$	$C_{17:1\omega6c}$	cy$C_{17:0\omega5,6}$	$C_{18:1\omega9c}$	$C_{18:1\omega7c}$	$C_{18:0}$
0–2	−46	−58	−63	−54	−31	−44	−75	−41	−52	−65	−94	−38	−37	−38
2–4	−59	−69	−74	−72	−32	−46	−80	−50	−57	−76	−101	−44	−34	−38
4–6	−66	−67	−67	−73	−48	−62	−92	−51	−49	−83	−103	−44	−46	−38
6–8	−57	−63	−63	−65	−48	−63	−91	−47	−48	−76	−100	−43	−48	−37
8–10	−73	−78	−76	−79	−56	−70	−96	−56	−56	−83	−101	−51	−57	−43
10–13	−70	−73	−73	−77	−57	−70	−96	−51	−57	−84	−101	−50	−60	−40
13–16	−59	−75	−79	−74	−69	−77	−95	−51	−61	−87	−99	−49	−74	−38
16–19	−46	−80	−87	−67	−75	−81	−96	−44	−64	−88	−99	−51	−80	−36
Average	−59	−70	−73	−70	−52	−64	−90	−49	−56	−80	−100	−46	−54	−39

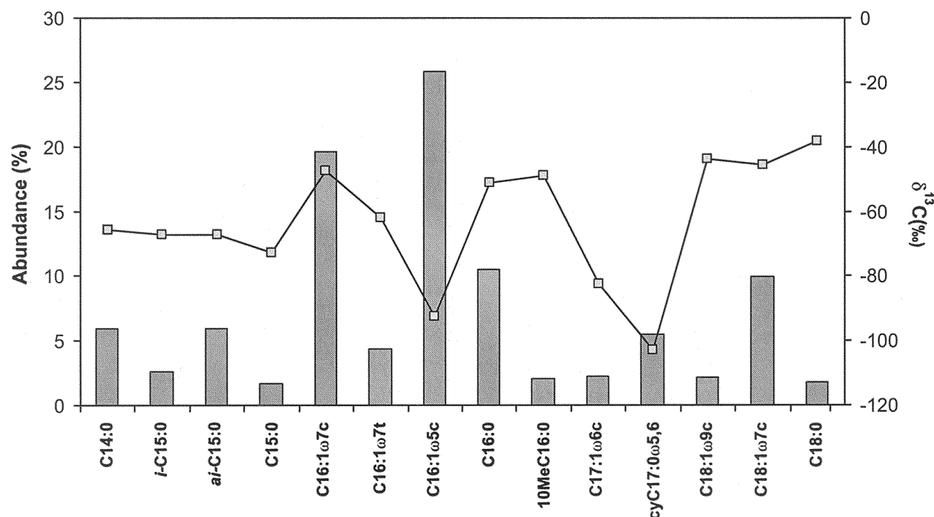

FIGURE 3 Relative abundance (dark gray bars) and carbon isotopic composition (light gray squares) of major fatty acids obtained in the 4–6 cm sediment section.

as observed for $C_{16:1\omega 5c}$, $cyC_{17:0\omega 5,6}$, and $C_{17:1\omega 6c}$ in a respective sediment section. In contrast to the above, the two major fatty acids $C_{16:1\omega 7c}$ and $C_{18:1\omega 7c}$ show relatively enriched $\delta^{13}C$ values between −31 and −37‰ in the upper 4 cm of the sediment core. This probably indicates strong contributions to the content of $C_{16:1\omega 7c}$ and $C_{18:1\omega 7c}$ from the white mat of sulfide-oxidizing *Beggiatoa*, which use pore water CO_2 strongly mixed with marine seawater as carbon source and that have been shown to contain high amounts of these two fatty acids (Carsten Schubert, unpublished data).

Discussion

Fatty Acid Fingerprint of **Desulfosarcina/Desulfococcus** *Species Involved in AOM*

Our fatty acid data clearly show an enrichment of unusual fatty acids (i.e., $C_{16:1\omega 5c}$, $C_{17:1\omega 6c}$, and $cyC_{17:0\omega 5,6}$) above marine gas hydrates in the fluid flow-impacted sediments of Hydrate Ridge (Figure 1). The high abundance of the $C_{16:1\omega 5c}$ fatty acid is an exceptional finding because the anaerobic fatty acid synthesis desaturase pathway in bacteria preferentially leads to the production of $\omega 7$ isomers (Alexandrino et al. 2001). Additionally, the unusual fatty acids reveal very low $\delta^{13}C$ values throughout the sediment core (Table 2, Figure 3), indicating a bacterial source involved in AOM at this setting. Indeed, Hydrate Ridge sediments are characterized by a predominance of SRB of the *Desulfosarcina/Desulfococcus* group as part of anaerobic methane-oxidizing consortia (Boetius et al. 2000), also recently described as Seep SRB1 group (see Knittel et al. 2003, this issue). Accordingly, depth profiles of specific fatty acid contents (i.e., $C_{16:1\omega 5c}$ and $cyC_{17:0\omega 5,6}$) and AOM aggregate number in the samples are in very good agreement (Figure 4), permitting for the first time a positive correlation between these parameters (r^2 [$C_{16:1\omega 5c}$] = 0.66; r^2 [$cyC_{17:0\omega 5,6}$] = 0.62). Fatty acid abundance and aggregate number increase from the sediment surface to the 2–4 cm sediment horizon and decrease simultaneously in the deeper part of the sediment core. Highest fatty acid contents obtained are 26.52 and 4.10 μg/g sediment dry weight (dw) for $C_{16:1\omega 5c}$ and $cyC_{17:0\omega 5,6}$, respectively (Table 1), whereas aggregate abundance reaches a number of up to 11.4×10^7 per cm^{-3} in the samples that is similar to counts obtained in

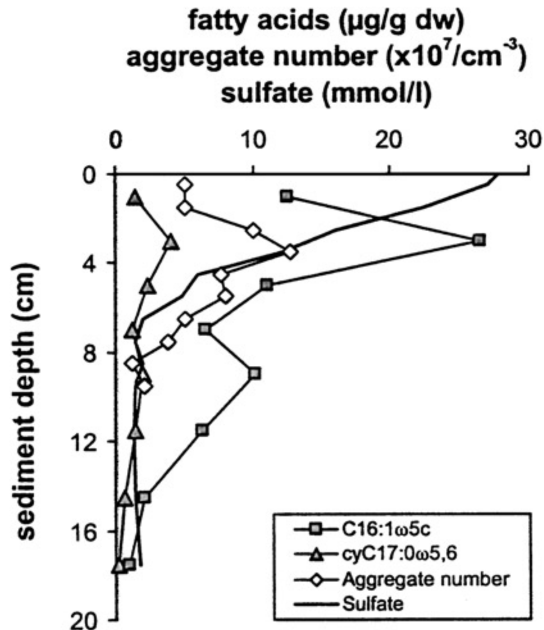

FIGURE 4 Depth profiles of specific membrane fatty acids derived from SRB (dark gray squares and triangles), aggregate number (white diamonds), and pore water sulfate (black line) within a core covered by a *Beggiatoa* mat. Pore water sulfate data have been kindly provided by Dirk Rickert (GEOMAR, Kiel, Germany).

previous studies (Boetius et al. 2000; Nauhaus et al. 2002). The maximum peaks co-occur in sediment sections where pore water sulfate is rapidly decreasing with depth and, thus, high sulfate reduction rates have been measured (Treude et al. submitted), which again suggests that both trends are strongly associated with AOM activity.

Culture studies have shown that different species of SRB in the marine environment are distinguishable by their specific fatty acid pattern. For instance, the appearance (relative fatty acid content $>10\%$) of $10MeC_{16:0}$ accompanied by $cyC_{17:0\omega7,8}$ can be indicative for *Desulfobacter* species (Taylor and Parkes 1983; Dowling et al. 1986) although this same relation has recently also been encountered in *Desulfobacula* species (Kuever et al. 2001). Nevertheless, *Desulfobacter* species seem to be of minor importance in our samples since $10MeC_{16:0}$ is present in low abundance, does not display a significant ^{13}C-depleted carbon isotope signal, and is, perhaps more importantly, not accompanied by $cyC_{17:0\omega7,8}$. Other fatty acids with clear specificity inferred from cultures are i-$C_{17:1\omega7c}$ as indicator for *Desulfovibrio* species (Taylor and Parkes 1983; Vainshtein et al. 1992) and $C_{17:1\omega6c}$, a fatty acid indicating *Desulfobulbus* species (Taylor and Parkes 1983; Parkes and Calder 1985) but which has also been found in considerable amounts in *Desulforhabdus* species as well as *Desulforhopalus* species (Knoblauch et al. 1999; Rütters et al. 2001). Whereas the fatty acid i-$C_{17:1\omega7c}$ was only detected in trace amounts in our samples, $C_{17:1\omega6c}$ is clearly detectable (2.2% relative fatty acid content) and moreover carries a significant ^{13}C-depleted carbon isotope value (Table 2). *Desulfobulbus* or other related $C_{17:1\omega6c}$-producing species contain high amounts of this compound of up to 60% (Taylor and Parkes 1983; Parkes and Calder 1985; Knoblauch et al. 1999) but they contribute to less than 2% of all bacteria at Hydrate Ridge (Knittel et al. 2003, this issue). So far, they have not been found to be a partner in AOM consortia. Hence, it is either likely that free-living *Desulfobulbus* or other related $C_{17:1\omega6c}$-producing species

use a ^{13}C-depleted carbon substrate excreted from the AOM consortia or that $C_{17:1\omega6c}$ may be produced in small amounts by SRB of the genus *Desulfosarcina/Desulfococcus* involved in AOM.

The positive correlation of $C_{16:1\omega5c}$ and $cyC_{17:0\omega5,6}$ fatty acid content with aggregate number obtained by FISH is remarkable (Figure 4). ^{14}C-labeling studies have shown that formation of cyclopropane fatty acids (CFAs) is performed by the transfer of a methyl group from *S*-adenosylmethionine to the double bond of an unsaturated fatty acid of a phospholipid molecule (Grogan and Cronan 1997), so that the $C_{16:1\omega5c}$ fatty acid bound in the phospholipid membrane of *Desulfosarcina/Desulfococcus* species would be transformed *in situ* to $cyC_{17:0\omega5,6}$. Thus, the appearance of $C_{16:1\omega5c}$ accompanied by $cyC_{17:0\omega5,6}$ provides a highly specific fatty acid fingerprint of SRB of the genus *Desulfosarcina/Desulfococcus* involved in AOM. This finding would be new to the family *Desulfobacteraceae* to which the *Desulfosarcina/Desulfococcus* group belongs. In contrast, recent cultures of the relatives *Desulfococcus multivorans* and *Desulfosarcina variabilis* displayed no or insignificant contents of $C_{16:1\omega5c}$, respectively, and CFAs were almost below detection limit (Kohring et al. 1994; Rütters et al. 2001, 2002). The dominant monoenoic fatty acid $C_{16:1\omega5c}$ in methane-rich sediments of Hydrate Ridge has also been detected in significant amounts in three other genera of SRB, namely *Desulfotalea, Desulfobulbus*, and *Desulforhopalus* (Knoblauch et al. 1999; Rütters et al. 2001; Sass et al. 2002). But likewise, none of these culture studies revealed the presence of significant amounts of CFAs.

So far, only one report from a methane cold seep environment provided a total fatty acid pattern quite similar to the one shown in Figure 1 (Hinrichs et al. 2000). In combination with carbon isotope measurements of several fatty acids Hinrichs and co-workers showed that $C_{16:1\omega5c}$, $10MeC_{16:0}$, and an uncharacterized CFA were carrying the lowest $\delta^{13}C$ values in sediments of the Santa Barbara Basin. This pattern points to two groups of SRB involved in AOM in these sediments which are in part closely-related to *Desulfosarcina/Desulfococcus* species found in our setting ($C_{16:1\omega5c}$ and CFA) whereas others may be derived from *Desulfobacter*-like species ($10MeC_{16:0}$). Indeed, Orphan et al. (2001) were able to show that besides *Desulfosarcina/Desulfococcus* species other bacterial species, although rather peripherally related to known δ-proteobacteria, are also involved in AOM.

In contrast to the results obtained by Hinrichs et al. (2000) and our study, fatty acid patterns from Gulf of Mexico gas hydrate-bearing sediments are not enriched in the specific fatty acids $C_{16:1\omega5c}$, $10MeC_{16:0}$, $C_{17:1\omega6c}$, or $cyC_{17:0\omega5,6}$. Here *iso*- and *anteiso*-branched $C_{15:0}$ fatty acids predominantly carry the lowest $\delta^{13}C$ values and thus, different SRB species or other bacteria play an important role in AOM in this setting (Zhang et al. 2002). The results from the Gulf of Mexico are, on the other hand, in good agreement with fatty acid results from the Mediterranean Ridge (Pancost et al. 2000) and the Black Sea (Thiel et al. 2001). The latter finding is quite remarkable considering that SRB of the *Desulfosarcina/Desulfococcus* group in AOM aggregates determined by FISH from the Black Sea microbial mats belong to the same SRB taxon as found in Hydrate Ridge sediments (Michaelis et al. 2002). This most likely points to different *Desulfosarcina/Desulfococcus* species as part of AOM consortia in these two settings.

Mass Balance Calculation and Desulfosarcina/Desulfococcus *Specific Fatty Acid Pattern*

Stable carbon isotope labeling of lipid biomarkers can be used to obtain information about the functioning of specific bacteria and—if quantitatively analyzed—about the importance of various microbial populations, metabolic pathways, and biogeochemical processes in the natural environment (Boschker and Middelburg 2002). This approach has been used for

example in soils and sediments for the detection of methane-oxidizing bacterial populations (Boschker et al. 1998; Bull et al. 2000; Crossmann et al. 2001) and the degradation of aromatic hydrocarbons (Hanson et al. 1999; Johnson et al. 2002). Nevertheless, all of these approaches used artificially ^{13}C-labeled substrates. Biogenic methane as a substrate, on the other hand, with its characteristic low carbon isotopic composition can be regarded as a naturally available ^{13}C-depleted tracer (δ^{13}C of methane at Hydrate Ridge: −62 to −72‰; Elvert et al. 2001). This feature enables the evaluation of the activity of specific microbial populations involved in AOM and carbon flow further on into the benthic community (Levin and Michener 2002; Werne et al. 2002).

Mass balance calculations permit the determination of fatty acid patterns accounting for the dilution of the total fatty acid pool by ^{13}C-enriched components of sedimentary and other organisms not associated with AOM (Hinrichs et al. 2000). By using this approach we are able to present a corrected fatty acid pattern from the 4–6 cm sediment horizon where aggregate abundance was not biased by *Beggiatoa*-derived fatty acid signals (Figure 5). The determination of the AOM-dependent portion f in the total fatty acid pool was conducted using the following formula:

$$\delta^{13}C_{\text{measured fatty acid signal}} = f * \delta^{13}C_{\text{cyC}_{17:0\omega5,6}} + (1 - f) * \delta^{13}C_{\text{background fatty acid signal}}$$

with the carbon isotopic composition of $cyC_{17:0\omega5,6}$ in the particular sediment horizon as the maximum carbon isotopic fractionation associated with the carbon uptake of SRB during AOM on one hand (= 100% AOM-dependent SRB signal) and background δ^{13}C values of every single fatty acid obtained in a reference core without AOM activity from the uppermost sample (0–2 cm depth interval) on the other. Background fatty acid δ^{13}C values range from −25 to −31‰ ($C_{14:0}$ to $C_{18:0}$) with the exception of $C_{16:1\omega7c}$ (−35‰), $C_{16:1\omega7t}$ (−46‰), and $C_{16:1\omega5c}$ (−42‰); $cyC_{17:0\omega5,6}$ was not detected. The cell-specific pattern presented most likely displays a very close resemblance to the real pattern of *Desulfosarcina*/*Desulfococcus* species in AOM consortia. It is clearly dominated by the fatty acid $C_{16:1\omega5c}$ (43.8%) and

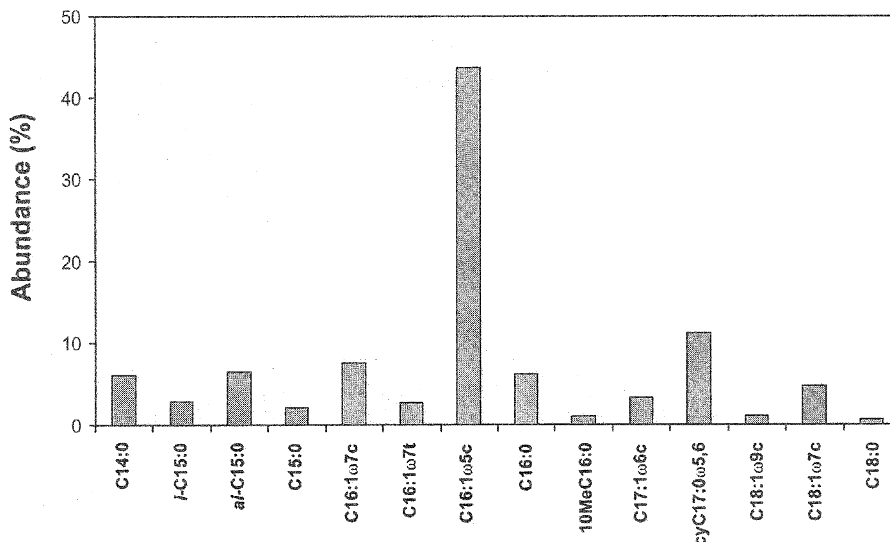

FIGURE 5 Fatty acid pattern of AOM-dependent SRB in the 4–6 cm sediment section. Relative abundance of fatty acids is based on mass balance calculations.

TABLE 3 Concentrations of major fatty acids (in μg/g dw) in the *Beggiatoa* covered sediment core after mass balance calculation

Depth	Fatty acid (μg/g dw)													
(cm)	$C_{14:0}$	i-$C_{15:0}$	ai-$C_{15:0}$	$C_{15:0}$	$C_{16:1\omega7c}$	$C_{16:1\omega7t}$	$C_{16:1\omega5c}$	$C_{16:0}$	$10MeC_{16:0}$	$C_{17:1\omega6c}$	$cyC_{17:0\omega5,6}$	$C_{18:1\omega9c}$	$C_{18:1\omega7c}$	$C_{18:0}$
0–2	0.96	0.71	1.52	0.32	0.00	0.00	8.05	1.44	0.26	0.55	1.47	0.44	1.53	0.21
2–4	2.38	1.57	3.40	0.72	0.00	0.00	17.31	2.90	0.49	1.19	4.10	0.51	1.24	0.20
4–6	1.27	0.60	1.36	0.43	1.58	0.58	9.10	1.31	0.22	0.69	2.32	0.21	0.98	0.12
6–8	0.74	0.38	0.82	0.26	1.12	0.44	5.43	0.83	0.17	0.43	1.19	0.16	0.86	0.11
8–10	1.49	0.77	1.43	0.48	1.86	0.78	9.37	1.28	0.22	0.74	2.07	0.22	1.32	0.14
10–13	0.98	0.50	1.04	0.36	1.35	0.53	5.78	0.85	0.16	0.59	1.48	0.18	1.17	0.11
13–16	0.38	0.22	0.59	0.20	0.89	0.24	2.02	0.45	0.07	0.37	0.63	0.09	0.89	0.06
16–19	0.13	0.15	0.43	0.11	0.59	0.12	0.96	0.22	0.04	0.20	0.26	0.09	0.60	0.04

accompanied by significant amounts of $cyC_{17:0\omega5,6}$ (11.2%). Other major fatty acids are $C_{16:1\omega7c}$ (7.6%), *i*- and *ai*-$C_{15:0}$ (2.9% and 6.5%, respectively), $C_{16:0}$ (6.3%), $C_{14:0}$ (6.1%), $C_{18:1\omega7c}$ (4.7%), $C_{17:1\omega6c}$ (3.3%), and $C_{16:1\omega7t}$ (2.8%). In combination with carbon isotope values this finding indicates a high specificity of the fatty acids $C_{16:1\omega5c}$ and $cyC_{17:0\omega5,6}$ whereas $C_{17:1\omega6c}$ seems to be of lesser specificity. Such a fatty acid pattern does not resemble any of the known cultured species of SRB. On the other hand, Peckmann et al. (1999) provided a quite similar fatty acid pattern obtained from a neoformed authigenic aragonite sample in the host rock of the Zechstein group (Harz Mountains, Germany), which was highly enriched in and dominated by the $C_{16:1\omega5c}$ fatty acid (16.78 μg/g aragonite cement). Although $cyC_{17:0\omega5,6}$ was missing and no carbon isotopic measurements have been carried out on the fatty acids, the $\delta^{13}C$ values of the aragonites of $\sim-10‰$ very probably point to a recent/subrecent activity of SRB, maybe related to *Desulfosarcina*/*Desulfococcus* species.

Based on the mass balance calculations, fatty acid compositions of AOM-dependent SRB of all sediment sections within the *Beggiatoa* covered sediment core are presented in Table 3. The calculations suggest that up to 63% of the total fatty acids are derived from SRB involved in AOM and more specifically, up to 95% of the $C_{16:1\omega5c}$ fatty acid signal is produced by species members of the genus *Desulfosarcina*/*Desulfococcus*. The latter estimate is in the similar range as obtained by FISH analyses, quantifying the amount of *Desulfosarcina*/*Desulfococcus* species in a replicate sediment core at station 19-2 (see Knittel et al. 2003, this issue). Assuming that the corrected depth profile of $C_{16:1\omega5c}$ represents a pure signal of viable cells in the sediment column, an estimate of the amount of specific fatty acid content per SRB cell is possible. Normalizing the amount of $C_{16:1\omega5c}$ in the upper 8 cm of the sediment core to the number of SRB cells in AOM aggregates, there are 0.46 to 0.58 $\times$ 10^{-15} g $C_{16:1\omega5c}$ per viable SRB cell. The amount of fatty acid $cyC_{17:0\omega5,6}$ (AOM-dependent SRB signal = 100%) ranges from 0.10 to 0.14 $\times$ 10^{-15} g per cell SRB. In this particular sediment zone correlation coefficients between the specific fatty acid contents—corrected ones used for $C_{16:1\omega5c}$, by definition uncorrected ones used for $cyC_{17:0\omega5,6}$—and aggregate number are significantly increased (r^2 [$C_{16:1\omega5c}$] = 0.93; r^2 [$cyC_{17:0\omega5,6}$] = 0.99). Between 8 and 10 cm sediment depth, the amount of $C_{16:1\omega5c}$ and $cyC_{17:0\omega5,6}$ fatty acid per SRB cell increases five times to 2.50 and 0.55 $\times$ 10^{-15} g, respectively, maybe indicating nonviable or starved cells because of depleted sulfate concentrations and, thus, of less favorable conditions for AOM aggregates in this sediment interval. The lesser correlation between specific fatty acid content and aggregate abundance in the 8–10 cm sediment section may also suggest that *Desulfosarcina*/*Desulfococcus* species here are only partially functioning as partners in AOM aggregates. Nevertheless, the cell-specific estimates may be used in the future to calculate the number of *Desulfosarcina*/*Desulfococcus* cells in AOM consortia and sediments from membrane fatty acid analysis alone.

Summary

Depth profiles of abundant specific fatty acids (i.e., $C_{16:1\omega5c}$, $cyC_{17:0\omega5,6}$) and numbers of AOM aggregates in combination with pore water sulfate show a very good correlation in anaerobic gas hydrate-bearing sediments. These unusual fatty acids can be used as chemotaxonomic markers of AOM-dependent *Desulfosarcina*/*Desulfococcus* species, both members of the family *Desulfobacteraceae*. Averaging ^{13}C-depleted carbon isotope values of $-90‰$ ($C_{16:1\omega5c}$) and $-100‰$ ($cyC_{17:0\omega5,6}$) are indicative of methane carbon flowing via methanotrophic archaea to the SRB. In the matching zone of specific fatty acids and aggregate number mass balance calculations enable not only the determination of a cell-specific fatty acid pattern, but they also allow the estimate of specific fatty acid content per viable *Desulfosarcina*/*Desulfococcus* cell. The values range between 0.46 to 0.58 $\times$ 10^{-15} g for

$C_{16:1\omega5c}$ and 0.10 to 0.14 × 10^{-15} g for $cyC_{17:0\omega5,6}$. These estimates may be used in the future to calculate the number of *Desulfosarcina*/*Desulfococcus* cells in AOM aggregates and sediments.

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