A marine microbial consortium apparently mediating anaerobic oxidation of methane

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A large fraction of globally produced methane is converted to CO_2 by anaerobic oxidation in marine sediments[1]. Strong geochemical evidence for net methane consumption in anoxic sediments is based on methane profiles[2], radiotracer experiments[3] and stable carbon isotope data[4]. But the elusive microorganisms mediating this reaction have not yet been isolated, and the pathway of anaerobic oxidation of methane is insufficiently understood. Recent data suggest that certain archaea reverse the process of methanogenesis by interaction with sulphate-reducing bacteria[5–7]. Here we provide microscopic evidence for a structured consortium of archaea and sulphate-reducing bacteria, which we identified by fluorescence *in situ* hybridization using specific 16S rRNA-targeted oligonucleotide probes. In this example of a structured archaeal-bacterial symbiosis, the archaea grow in dense aggregates of about 100 cells and are surrounded by sulphate-reducing bacteria. These aggregates were abundant in gas-hydrate-rich sediments with extremely high rates of methane-based sulphate reduction, and apparently mediate anaerobic oxidation of methane.

At the Cascadia convergent margin off the coast of Oregon, discrete methane hydrate layers are exposed at the sea floor, at a water depth of 600–800 m that corresponds to the hydrate stability limit[8]. These hydrate layers are formed from gaseous methane that continuously ascends along faults generated by accretionary tectonics. The crest of the southern Hydrate Ridge (44° 34′ N, 125° 09′ W, 780 m water depth) is populated by large communities of clams of the genus *Calyptogena,* and by thick bacterial mats of the sulphide-oxidizing *Beggiatoa,* both of which indicate areas of active gas seeping[9]. Undisturbed sediment cores with *Beggiatoa* mats were obtained using a video-guided multiple corer during RV *SONNE* Cruise SO143-2 in August 1999 (ref. 10). These samples often released gas bubbles due to decompression during recovery.

Sulphate reduction rates (SRRs) were extremely high in sediments covered by *Beggiatoa* mats, reaching more than 5 μmol cm^{-3} d^{-1} in the surface sediments (Fig. 1). Integrated over the upper 15 cm, the resulting SRR is 140 mmol m^{-2} d^{-1}; this is, to our knowledge, the highest value ever measured in cold marine sediments. At a nearby reference station without gas hydrates and vent colonization, SRRs were below the detection limit (< 1 nmol cm^{-3} d^{-1}). Thus, at Hydrate Ridge, sulphate reduction is clearly fuelled by high methane fluxes from below, while organic deposition from surface waters is not a significant substrate source for sulphate-reducing bacteria (SRB). A similar phenomenon was observed at gas seeps in the Gulf of Mexico, where 600-fold higher SRRs were measured at methane seeps (up to 2.5 μmol cm^{-3} d^{-1}, calculated from sulphate concentration profiles) compared to reference stations[11]. The restriction of such high SRRs to sediments rich in methane is evidence for a direct link between the processes of methane and sulphate turnover. Sulphate has been proposed to be the terminal electron acceptor in the zone of anaerobic oxidation of methane[3], according to:

$$CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O \quad (1)$$

Assuming this stoichiometry, the turnover of methane can exceed 5 mM d^{-1} in the sediments of the Hydrate Ridge, where a dissolved methane concentration of 80 mM is reached above decomposing gas hydrates at *in situ* temperature (4 °C) and hydrostatic pressure (8 MPa). As one product of anaerobic oxidation of methane, sulphide accumulates to concentrations almost equivalent to those of sulphate consumed (Fig. 1). Intense sulphide production

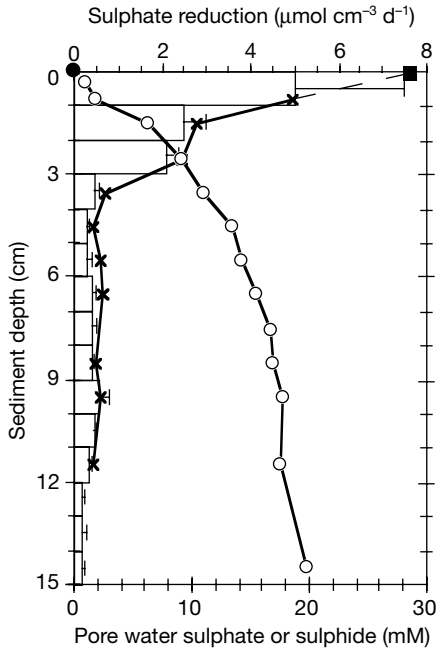

Figure 1 Depth profiles of various quantities from methane-rich sediments. Crosses, porewater sulphate concentrations (bottom axis); open circles, porewater dissolved sulphide concentrations (bottom axis); filled circle and filled square, concentrations in overlying bottom water of sulphate and sulphide, respectively. Columns with error bars show average values of sulphate reduction rate (SRR). The multiple corer SO143/173-1B-TVMC was positioned on a *Beggiatoa* mat[10]. One core was taken for porewater chemistry, and three replicate subcores were obtained for SRR. Error bars indicate the standard deviation between the subcores.

explains the occurrence of sulphide-based *Beggiatoa/Calyptogena* communities at the Hydrate Ridge. The methane-derived bicarbonate precipitates as calcium carbonate, and forms large structures exposed at the crest of the Hydrate Ridge[8].

In Hydrate Ridge sediments, the archaeal isoprenoids crocetane and pentamethylicosane were found to be highly depleted in ^{13}C ($\delta^{13}C$ = −124‰ vs PDB)[12]. Additionally, in *Beggiatoa*-covered sediments of 0–10 cm depth, we found archaeol and *sn*-2-hydroxyarchaeol at high concentrations (8 μg per g sediment dry mass) and similarly depleted in ^{13}C (−114‰ and −133‰, respectively). These lipids are common in archaea, and are particularly prominent in methanogens[13]. Such highly ^{13}C depleted lipid biomarkers are due to consumption of methane with a $\delta^{13}C$ of −62‰ to −72 ‰ (ref. 8) and subsequent fractionation. Very light iso- and anteiso-C_{15} fatty acids (−63‰ and −75‰, respectively) which occur abundantly in SRB were also detected at high concentrations (10 μg per g sediment dry weight). These values are similar to, or even lower than, values reported from methane-rich environments, such as a gas-hydrate-bearing seep[7], a Miocene limestone from an ancient vent system[14], and an active mud volcano[15].

In the *Beggiatoa*-covered sediments of the Hydrate Ridge, abundant cell aggregates were detected by fluorescence *in situ* hybridization (FISH) specific for the domain Archaea[16]. These cell aggregates were not found at the reference station without methane seepage. The archaeal cells in the aggregates were detected with probe EelMS932 targeting clone sequences which were retrieved from a similar methane-rich environment (Eel River basin, California)[7] and which are phylogenetically affiliated with the order Methanosarcinales. The aggregated archaea were poorly stained with 4′,6′-diamidino-2-phenylindole (DAPI; Fig. 2a, c, e), and were recognized as such only by the probe signal (Fig. 2b, d, f). Specific FISH analysis of the outer layer of DAPI-stained cells revealed that these are members of the domain Bacteria[17], and belong to the SRB of the delta-proteobacteria. The SRB surrounding the archaeal aggregates were targeted with probe DSS658 (Fig. 2g) specific for the branch *Desulfosarcina/Desulfococcus*[18], and with probe DSS225 which is highly specific for a new subgroup of that branch[19]. The closest cultivated relative of this subgroup is *Desulfosarcina variabilis* with up to 91.2% 16S rDNA sequence similarity.

An average archaea/SRB consortium consisted of an inner sphere of 2.3 ± 1.3 μm diameter containing about 100 coccoid archaeal cells, each 0.5 μm in diameter. These were partially or fully surrounded by about 200 cells of SRB (0.3–0.5 μm in diameter), which formed an outer shell of mostly 1–2 cell layers. The size spectrum of 100 archaea/SRB consortia ranged from 1 to 11 μm in diameter with an average of 3.2 ± 1.5 μm. The smallest aggregates consisted of only 1–3 archaeal cells and 1–3 cells of SRB, and may represent early stages of the consortium development, while the largest contained about 10,000 cells. The consortia were highly abundant in surface sediments at sulphide concentrations < 10 mM (Fig. 1), with a maximum of 7×10^7 aggregates cm^{-3} at 1–2 cm depth (Fig. 3). The average number in the upper 5 cm was 3×10^7 aggregates cm^{-3}, which is equivalent to about 3×10^9 archaeal cells cm^{-3} and 6×10^9 cells of SRB cm^{-3}. Hence, the consortia comprised 94% of all archaea detected with domain-specific probe ARCH915[16] and 96%

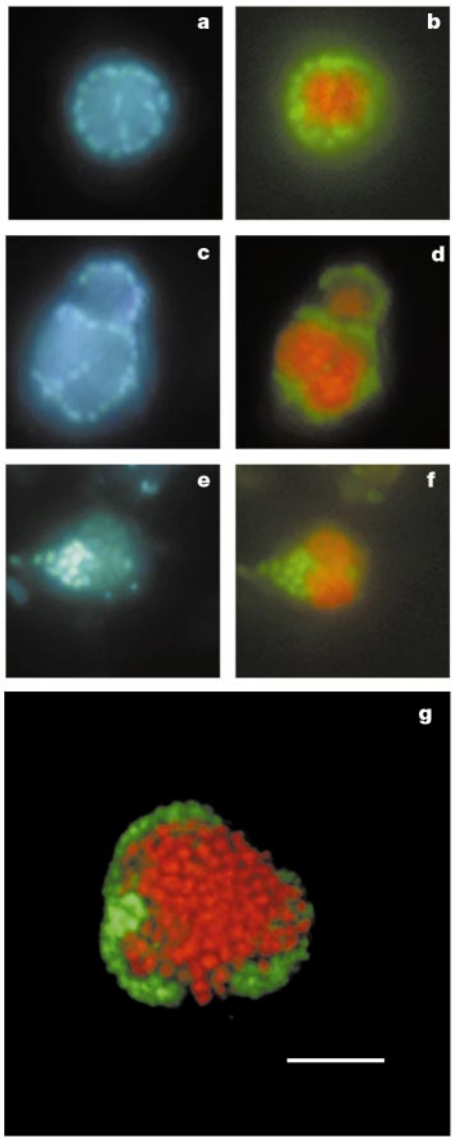

Figure 2 *In situ* identification of archaea/SRB aggregates with fluorescently labelled rRNA-targeted oligonucleotide probes. The archaea are shown in red, and the SRB in green. The aggregates were visualized using filter sets specific for DAPI, CY3 and FLUOS for identical microscopic fields. Single *xy* images of the same optical section were combined. **a/b**, **c/d**, **e/f**, Epifluorescence micrographs of different aggregates stained with DAPI (left panel) and hybridized with the CY3-labelled probe EelMS932 (5′-AGCT CCACCCGTTGTAGT-3′) and the FLUOS-labelled probe DSS658 (5′-TCCACTTCCCTCT CCCAT-3′)[19] (right panel). After testing for their specificity at ~60% v/v formamide, both probes were hybridized at 40% formamide to give optimal brightness. Scale bar, 5 μm. **g**, Confocal laser scanning micrograph of the hybridization with the CY3-labelled probe EelMS932 (archaea) and the FLUOS-labelled probe DSS658 (SRB). Details of probes are available, see Supplementary Information. Scale bar, 5 μm.

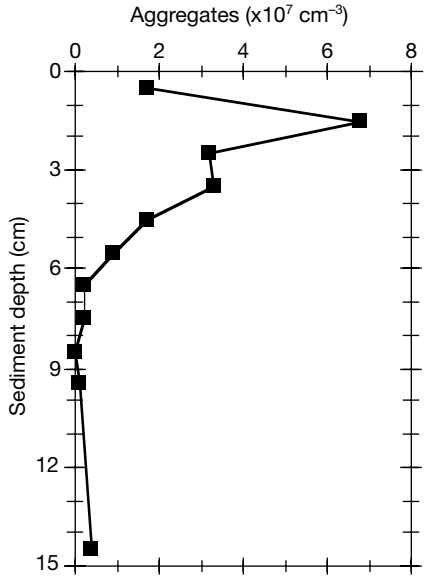

Figure 3 Abundance of archaea/SRB consortia in a sediment core from a *Beggiatoa* mat.

of all SRB detected with eight different genus-specific probes[18,20,21]. From the abundance and biovolume of cells in the consortia, a biomass of SRB of 0.12 mg cell dry mass per cm^3 is calculated. Hence, the SRR of 5 μmol cm^{-3} d^{-1} would yield a specific rate of 42 μmol per mg cell dry mass per day. This value is within the range of specific rates in cultures of SRB grown under optimal conditions in the laboratory (15–430 μmol per mg cell dry mass per day)[22].

To identify other organisms potentially consuming methane in the Hydrate Ridge sediments, we developed specific probes for FISH of other archaea and methylotrophic bacteria. Four different probes for the newly described phylogenetic ANME-1 cluster, suspected of consuming methane anaerobically in Eel River basin sediments[7], did not hybridize with the aggregated archaea. Hybridization of non-aggregated archaea with ANME-1 probes was far below 1% of the total DAPI cell counts at lowest stringency (0% formamide). Furthermore, no other methanogenic archaea of the orders Methanosarcinales and Methanobacteriales were detected by FISH using specific 16S rRNA probes tested with appropriate reference organisms. FISH counts of aerobic methylotrophs of the alpha- and gamma-proteobacteria were also below detection limits at all stations. Thus, aerobic oxidation of methane by relatives of known methanotrophs does not appear to be an important process in the methane-rich sediments of the Hydrate ridge. Rather, the abundant, strongly ^{13}C depleted, consortia of methanogenic archaea and SRB are mediating the anaerobic oxidation of methane. We assume that this process is a reversal of methane formation, involving methanogens and a sulphate reducing partner which effectively scavenges intermediates such as H_2 or acetate[5,6,26].

So far, only few examples of prokaryotic symbioses based on metabolic interaction in direct cell contact have been identified; for example, the microbial consortium "Chlorochromatium"[23] and the cluster of the nitrifying bacteria *Nitrosomonas* and *Nitrobacter*[24]. The advantage of the archaea/SRB consortium compared with free-living cells would be a highly efficient transfer of intermediates by molecular diffusion[25]. Members of the Methanosarcinales, as observed in the consortia, encompass metabolically diverse methanogens. They may form methane not only from CO_2 and H_2, but also from simple methyl-group-containing compounds such as acetate, methanol, methylamines and methyl sulphide (but not from formate which is used by different methanogens). Members of the *Desulfosarcina/Desulfococcus* branch include nutritionally versatile SRB that oxidize organic compounds (including acetate) completely to CO_2, and several species can grow autotrophically with CO_2, H_2 and sulphate.

The low $\delta^{13}C$ signature of SRB lipids is best explained if reverse methanogenesis leads to an organic intermediate that serves not only as electron donor for sulphate reduction, but also as a cellular carbon source. A hypothesized bimolecular reaction of methane, in which both carbon atoms of acetate are derived from methane coupled to simultaneous H_2 formation[26], would offer the most favourable explanation for a strong ^{13}C-depletion in SRB. But with any organic intermediate, the cellular carbon of SRB would still be partly derived from CO_2 via some biosynthetic reactions. However, at the high methane oxidation rate estimated from our SRR measurements, the densely packed consortia may maintain a methane-derived isotopically light CO_2/HCO_3^- pool (equation (1)) that is not fully equilibrated with the surrounding heavier porewater pool. Furthermore, autotrophic growth of SRB using the carbon monoxide dehydrogenase pathway (present in members of the *Desulfosarcina* branch) for CO_2 fixation is associated with pronounced ^{13}C discrimination[27]. Hence, reverse methanogenesis via CO_2 and H_2 may remain relevant as a model.

The free energy change by anaerobic oxidation of methane (equation (1)) at the pressure (about 8 MPa) in the zone with the highest numbers of aggregates is $\Delta G = -40$ kJ per mol methane ($\Delta G = -30$ kJ at the methane pressure of 0.1 MPa prevailing after sampling). The intermediate concentrations required to keep both reverse methanogenesis and sulphate reduction energetically feasible are 10^{-10} to 10^{-9} M (0.01 to 0.1 Pa) for hydrogen and 3×10^{-12} to 3×10^{-8} M for acetate. This requirement cannot easily be reconciled with the observed high SRR. Due to the lack of reliable half-saturation constants (K_M values) of SRB and mass transfer rates within densely packed consortia, the kinetics of this system, involving extremely low intermediate concentrations, remain uncertain. The maintenance of a microenvironment strongly depleted in hydrogen, acetate or other possible intermediates is probably a prerequisite for the anaerobic oxidation of methane via reversed methanogenesis in the consortia. □

Methods

Sulphate reduction rates

Sediment cores were immediately transferred to a cold room (4 °C), and $^{35}SO_4^{2-}$ was injected horizontally into the intact sediment cores at 1-cm depth intervals. The cores were incubated for 24 h at *in situ* temperature before the reaction was stopped by mixing the sediments with 20% zinc acetate. The samples were stored frozen until the single-step acidic distillation of the Cr(II)-reduced sulphur compounds was performed as described in ref. 28. SRR was calculated from the ratio of radioactive sulphide to the total radioactive sulphate added. Measurements of porewater sulphate and sulphide were performed as described previously[8].

Lipid analysis

Freeze-dried and gently ground sub-samples were extracted by successive sonication and centrifugation in methanol, methanol:methylene chloride (1:1) and methylene chloride. After saponification (6% KOH), the neutral fraction was extracted with hexane, and derivatized with bis-(trimethylsilyl)-trifluoroacetamide (BSTFA; Sigma) before injection onto a HP5 chromatographic column (30 m length, 0.32 mm i.d., 0.17 μm film thickness). Column temperature was programmed from 90 to 180 °C at a rate of 10 °C min^{-1} and then at a rate of 6 °C min^{-1} to 320 °C (30 min isothermal). The acid fraction was recovered after adding HCl and fatty acids were transferred to fatty acid methyl esters with BF_3-methanol. Chromatographic conditions were the same as for the neutral fraction. Individual compounds were identified on a Finnigan MAT GCQ ion trap. Stable carbon isotopes were determined under the same chromatographic conditions with a HP6890 gas chromatograph coupled to a Finnigan Delta Plus isotope mass spectrometer. Reported δ values are corrected for the introduction of additional carbon atoms by derivatization with either BSTFA or BF_3-MeOH.

FISH

Sediment cores from methane-rich sites and from a reference site not enriched in methane were sliced into 1-cm sections. Samples were fixed for 2–3 h with 4% formaldehyde, washed twice with PBS (10 mM sodium phosphate; 130 mM NaCl) and finally stored in PBS/EtOH (1:1) at −20 °C. Stored samples were diluted and treated by mild sonication for 20 s with a MS73 probe (Sonopuls HD70, Bandelin) at an amplitude of 42 μm and a power of < 10 W. An aliquot was filtered on 0.2-μm polycarbonate filters (GTTP, Millipore). Hybridization and microscopy counts of hybridized and DAPI-stained cells were performed as described previously[29]. CY3- and carboxyfluorescein- (FLUOS-) labelled oligonucleotides were obtained from Interactiva (Germany).

Calculation of biomass and rates

The biovolume and biomass of SRB were calculated assuming a spherical cell shape with a diameter of 0.5 μm. Consequently, 6×10^9 cells would have a biovolume of 0.39 mm^3. With a wet mass/volume ratio of approximately 1 mg mm^{-3} and a conversion coefficient of 0.3 mg dry mass per mg wet mass, the cell dry mass per cm^3 of sediment was 0.12 mg.

Calculation of free energy

Free energy changes (ΔG values) were calculated from $G°_f$ data[30] via $\Delta G°$ values. Calculations were done for the indicated methane pressures, a temperature of 4 °C, a pH of 7.5 (if H^+ ions are involved), and average concentrations of SO_4^{2-}, HCO_3^- and HS^- of 2×10^{-2}, 1×10^{-2} and 2×10^{-3} M, respectively (as prevailing at the sediment depth with the highest number of aggregates). For SO_4^{2-}, HCO_3^- and HS^- in sea water, activity coefficients of 0.1, 0.5 and 0.5, respectively, were estimated[30]. The influence of temperature on $\Delta G°$ (for example, for equation (1): $\Delta G°_{277K} = -16.2$ kJ versus $\Delta G°_{298K} = -16.6$ kJ per mol methane oxidized) was calculated by the integrated Gibbs–Helmholtz equation including enthalpy ($\Delta H°$) values[30]. H_2 pressures and acetate concentrations were calculated that allow a free energy threshold of approximately −10 kJ per mol methane for each partner involved in the overall reaction (equation (1)).

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Supplementary information is available on *Nature*'s World-Wide Web site (http://www.nature.com) or as paper copy from the London editorial office of *Nature*.

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Modern freshwater microbialite analogues for ancient dendritic reef structures

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Microbialites are organosedimentary structures that can be constructed by a variety of metabolically distinct taxa[1]. Consequently, microbialite structures abound in the fossil record, although the exact nature of the biogeochemical processes that produced them is often unknown[2]. One such class of ancient calcareous structures[3–5], *Epiphyton* and *Girvanella*, appear in great abundance during the Early Cambrian. Together with *Archeocyathids*, stromatolites and thrombolites, they formed major Cambrian reef belts. To a large extent, Middle to Late Cambrian reefs are similar to Precambrian reefs[6], with the exception that the latter, including terminal Proterozoic reefs[7], do not contain *Epiphyton* or *Girvanella*. Here we report the discovery in Pavilion Lake, British Columbia, Canada, of a distinctive assemblage of freshwater calcite microbialites, some of which display microstructures similar to the fabrics displayed by *Epiphyton* and *Girvanella*. The morphologies of the modern microbialites vary with depth, and dendritic microstructures of the deep water (>30 m) mounds indicate that they may be modern analogues for the ancient calcareous structures. These microbialites thus provide an opportunity to study the biogeochemical interactions that produce fabrics similar to those of some enigmatic Early Cambrian reef structures.

Pavilion Lake is nestled in a steep-walled limestone valley known as Marble Canyon[8]. It is a small (5.7 × 0.8 km, Fig. 1), deep (maximum recorded depth 65 m), slightly alkaline (pH 8) freshwater lake. The lake water is very clear with Secchi depths of more than 15 m. Surface streams do not enter the lake and the predominance of limestone canyon walls indicates that karst hydrology dominates. Multi-beam and side-scan sonar profiles indicate that the microbialite assemblages that are distributed along the walls of the lake basins and surrounded by white silt are orientated roughly perpendicular to the shoreline (Fig. 1, inset). A decrease with depth in both the friability and porosity of the Pavilion Lake microbialites accompanies the distinct changes in their morphologies and

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