

ORIGINAL ARTICLE

Diverse sulfate-reducing bacteria of the *Desulfosarcina/Desulfococcus* clade are the key alkane degraders at marine seeps

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Biogeochemical and microbiological data indicate that the anaerobic oxidation of non-methane hydrocarbons by sulfate-reducing bacteria (SRB) has an important role in carbon and sulfur cycling at marine seeps. Yet, little is known about the bacterial hydrocarbon degraders active *in situ*. Here, we provide the link between previous biogeochemical measurements and the cultivation of degraders by direct identification of SRB responsible for butane and dodecane degradation in complex on-site microbiota. Two contrasting seep sediments from Mediterranean Amon mud volcano and Guaymas Basin (Gulf of California) were incubated with ¹³C-labeled butane or dodecane under sulfate-reducing conditions and analyzed via complementary stable isotope probing (SIP) techniques. Using DNA- and rRNA-SIP, we identified four specialized clades of alkane oxidizers within *Desulfobacteraceae* to be distinctively active in oxidation of short- and long-chain alkanes. All clades belong to the *Desulfosarcina/Desulfococcus* (DSS) clade, substantiating the crucial role of these bacteria in anaerobic hydrocarbon degradation at marine seeps. The identification of key enzymes of anaerobic alkane degradation, subsequent β -oxidation and the reverse Wood–Ljungdahl pathway for complete substrate oxidation by protein-SIP further corroborated the importance of the DSS clade and indicated that biochemical pathways, analog to those discovered in the laboratory, are of great relevance for natural settings. The high diversity within identified subclades together with their capability to initiate alkane degradation and growth within days to weeks after substrate amendment suggest an overlooked potential of marine benthic microbiota to react to natural changes in seepage, as well as to massive hydrocarbon input, for example, as encountered during anthropogenic oil spills.

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Introduction

Hydrocarbons are widespread on Earth and can be oxidized by a variety of aerobic and anaerobic microorganisms (e.g. reviewed by Head *et al.*, 2006; Widdel *et al.*, 2010). Natural sources of

hydrocarbons in marine systems are gas or oil seeps differing in hydrocarbon concentrations and composition. Marine gas seeps are, in general, dominated by high concentrations of methane (μM – mM range), whereas propane and butane are commonly found only in traces (μM range). In contrast, other marine hydrocarbon seeps contain a broad range of alkanes, alkenes and aromatic compounds (e.g. Anderson *et al.*, 1983; Bazylnski *et al.*, 1988). While degradation of hydrocarbons is well studied under oxic conditions (Head *et al.*, 2006; Kinnaman *et al.*, 2007; Prince *et al.*, 2010; Redmond *et al.*, 2010; Gutierrez *et al.*, 2013) and numerous bacterial and fungal degraders have been described (Shennan, 2006), knowledge about anaerobic hydrocarbon degradation and responsible organisms is still limited. The anaerobic hydrocarbon degradation has been reported to occur under

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nitrate-reducing, sulfate-reducing, iron-reducing and methanogenic conditions (for a review see Widdel *et al.*, 2010). In marine systems, degradation under sulfate-reducing conditions is of great importance owing to the high availability of sulfate as an electron acceptor. A large fraction of sulfate reduction (SR) at gas and hydrocarbon seeps is fueled by the anaerobic oxidation of methane (AOM; for a review see Reeburgh, 2007). However, the major part of total SR is likely to be fueled by the oxidation of non-methane hydrocarbons (for an overview see Bowles *et al.*, 2011) with a considerable impact on local carbon and sulfur cycling. The oxidation of non-methane alkanes with sulfate has recently been reported to occur across a range of temperatures in diverse marine and terrestrial seeps or vents (e.g. Joye *et al.*, 2004; Kniemeyer *et al.*, 2007; Orcutt *et al.*, 2010; Savage *et al.*, 2010; Quistad and Valentine, 2011; Jaekel *et al.*, 2012; Adams *et al.*, 2013; Bose *et al.*, 2013). Measured rates of alkane degradation and SR closely followed stoichiometric predictions that assume complete oxidation of alkanes to CO₂ (e.g. see Quistad and Valentine, 2011; Bose *et al.*, 2013). Reported rates for anaerobic short-chain alkane (SCA) oxidation were high in alkane-amended slurries of Gulf of Mexico or Middle Valley sediments (34–354 nmol C₂–C₄ ml⁻¹ per day; Adams *et al.*, 2013; Bose *et al.*, 2013). *In situ* anaerobic propane oxidation rates in fresh sediments from a gas seep located in the Santa Barbara Channel even exceeded rates from the batch cultures with up to 2100 nmol propane cm⁻³ per day (Quistad and Valentine, 2011). The rates were comparably high to those reported for AOM in similar environments (e.g. Joye *et al.*, 2004, Knittel and Boetius, 2009) suggesting the anaerobic oxidation of SCAs as an important process at marine gas seeps (Quistad and Valentine, 2011; Bose *et al.*, 2013), despite C₂–C₅ alkanes typically present at much lower concentrations than methane.

Most cultivated or enriched sulfate-reducing hydrocarbon degraders are widespread within the class *Deltaproteobacteria* (Figure 1). Yet, the only pure culture shown to degrade SCAs is strain BuS5 (Knemeyer *et al.*, 2007), capable of growth on propane and butane. It is phylogenetically affiliated with the *Desulfosarcina/Desulfococcus* (DSS) clade belonging to the family *Desulfobacteraceae*. The DSS clade was defined based on the coverage of probe DSS658 (Manz *et al.*, 1998) that is widely used for *in situ* detection and quantification of these organisms. Besides strain BuS5, DSS comprises organisms that have been shown to dominate propane- and *n*-pentane-degrading enrichments from a terrestrial hydrocarbon seep (Savage *et al.*, 2010), or enrichments from Gulf of Mexico or Hydrate Ridge seep sediments degrading C₂–C₄ alkanes (Jaekel *et al.*, 2012; Bose *et al.*, 2013). In addition to SCA degraders, DSS comprises degraders of mid-chain or long-chain alkanes (LCAs),

alkenes or aromatic compounds, such as toluene or naphthalene (e.g. Aeckersberg *et al.*, 1998; Harms *et al.*, 1999; So and Young, 1999; Meckenstock *et al.*, 2000). Further hydrocarbon-degrading sulfate-reducing bacteria (SRB) can be found within non-DSS *Desulfobacteraceae*, *Desulfobulbaceae*, *Syntrophobacteraceae* and *Desulfurellales* (Widdel *et al.*, 2010; Figure 1). Of these, *Desulfurellales* might be of particular interest as it comprises a broad diversity of organisms dominating anaerobic batch reactor incubations of sediments from the Middle Valley hydrothermal vent field (Adams *et al.*, 2013), as well as the sulfate-reducing partner bacteria of ANME-1-mediated thermophilic AOM in Guaymas Basin (GB) sediments (Holler *et al.*, 2011). Only few non-deltaproteobacterial SRB capable of degrading hydrocarbons are known and belong to the phylum *Firmicutes*, that is, *Desulfosporosinus* sp. strain Y5 (Liu *et al.*, 2004) and *Desulfotomaculum* sp. strain OX39 (Morasch *et al.*, 2004). All strains available in pure culture can usually degrade only a restricted range of hydrocarbons, for example, few aliphatic hydrocarbons within a narrow range of chain length (e.g. C₃–C₄ or C₆–C₁₀ or C₁₂–C₂₀) or few aromatic compounds.

In situ analysis of SRB communities at numerous marine gas and oil seeps indicated a high abundance and diversity of DSS with 10% to >60% of total cells (e.g. Orphan *et al.*, 2001; Orcutt *et al.*, 2010; Schreiber *et al.*, 2010; Kleindienst *et al.*, 2012). Potentially, the DSS clade is one of the most relevant lineages for hydrocarbon degradation in marine sediments, and could thus be of utmost relevance not only at natural hydrocarbon seeps but also upon catastrophic events. Still, the ecology and niche partitioning between these hydrocarbon degraders is quite poorly understood *in situ*, and studies directly linking functional information from both the lab and the field data are not available. Here, we aim at closing the gap between previous cultivation-dependent studies (i.e. isolates) and biogeochemical rate measurements *in situ* (i.e. hydrocarbon turnover). We studied two types of anoxic sediments from contrasting natural hydrocarbon seep environments: the GB hydrocarbon seep sediment was selected as a representative for a habitat with changing natural conditions characterized by the presence of steep gradients of temperature and electron acceptors, as well as seepage of a broad spectrum of hydrocarbons including alkanes <C₂₀. In contrast, the Amon mud volcano (AMV) located in the Mediterranean Sea was selected as a representative for a more stable natural seep environment that is characterized by constant temperatures and a narrow range of hydrocarbons (gaseous alkanes C₁–C₅).

Sediments were incubated under sulfate-reducing conditions with ¹³C-labeled butane as a model substrate for a gas leakage and ¹³C-labeled dodecane as a model substrate for an oil spill. We hypothesized (i) that the DSS clade indeed dominates hydrocarbon degradation *in situ*, and (ii) that niche

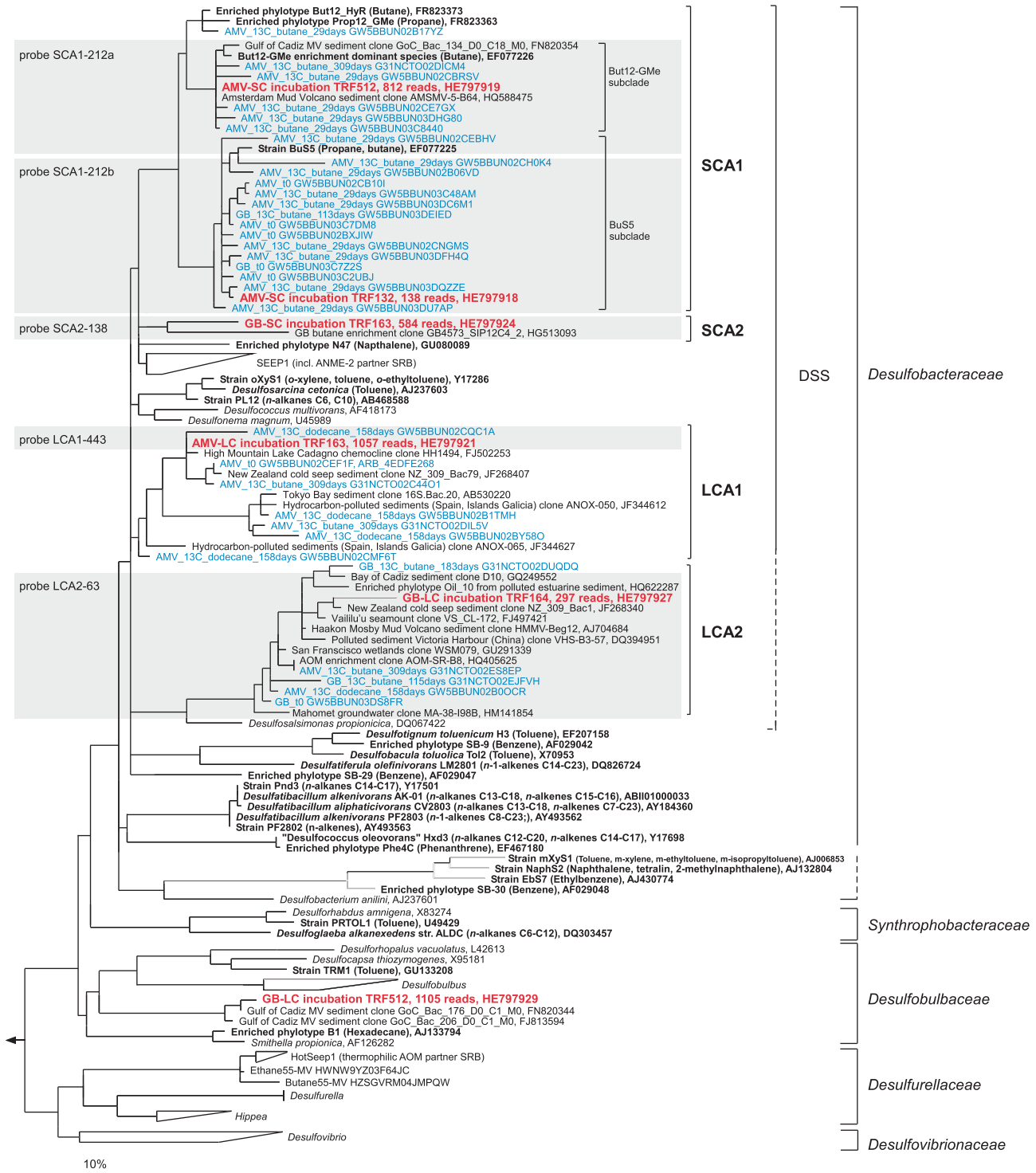


Figure 1 Phylogenetic tree showing the affiliations of 16S rRNA gene sequences retrieved from incubations of AMV and GB sediments with ¹³C-labeled butane or dodecane with selected reference sequences of the *Deltaproteobacteria*. A consensus tree based on the obtained maximum-likelihood (PhyML) tree is shown. A selection of gammaproteobacterial sequences was used as outgroup. Partial sequences were inserted into the reconstructed tree by using parsimony criteria. Entries corresponding to T-RFs of identified ¹³C-labeled alkane degraders are shown in red, and other sequences from the incubations are shown in blue. Sequences of cultivated anaerobic hydrocarbon degraders (black, boldface type) were included as additional reference; their substrate usage is given within parentheses. Probe coverage is indicated by gray-colored boxes. Scale bar represents 10% estimated sequence divergence.

partitioning of DSS subclades occurs based on alkane chain length. A combination of DNA-, RNA- and protein-based stable isotope probing

(SIP) enabled us to identify distinct clades of active SRB, respectively, involved in alkane degradation. These specialized degraders may catalyze a major

part of the SR-dependent non-methane hydrocarbon degradation in marine sediments.

Materials and methods

Study sites and sampling

Anoxic sediments were collected from the active AMV and a hydrocarbon seep at GB during the cruises MSM13-3 (*RV Maria S. Merian*) and AT15-56 (*RV Atlantis*) in 2009. AMV is located in the Nile deep-sea fan and comprises large subsurface gas reservoirs (Mastalerz *et al.*, 2009). Sampling was carried out with the remotely operated vehicle Quest 4000 during dive 240 at station 929 (PANGAEA database event MSM13/3_929-1; water depth 1122 m, 32°20.1321N, 31°42.6543E). The sediments derived from an area surrounding the central dome and were covered by a microbial mat. For details see Felden *et al.* (2013). GB is located in the central Gulf of California and harbors petroleum-rich hydrothermal sediments, covered with layers of buried sedimentary organic matter. Hydrothermal fluids contain elevated concentrations of hydrocarbons, including alkanes and aromatic hydrocarbons (Bazylnski *et al.*, 1988; Didyk and Simoneit, 1989). Fine-grained sediments below a white *Beggiatoa* mat were collected during dive 4573 (water depth 2010 m, 27°0.696N, 111°24.265W) with the submersible *Alvin*.

Setup of incubations for SIP

Sediments were stored for 4 months at 4 °C. During this time period, preincubations were carried out to identify sediments with highest alkane-degrading microbial activities based on sulfide and ¹³C₂ production. Slurries were prepared in an anoxic tent (N₂:CO₂, 9:1 v/v) by mixing sediments from 2 to 20 cm (AMV) and 0 to 10 cm (GB) depth in a 1:1 ratio with artificial sea water (Widdel and Bak, 1992), using a sterile metal spoon. During further processing and preparation of incubations, samples were always maintained under anoxic conditions at room temperature. Sediment slurries were further distributed in 15 ml aliquots in 22-ml tubes sealed with butyl rubber stoppers, under a headspace of N₂:CO₂. Either fully ¹³C-labeled or unlabeled butane were added to the headspace, using N₂-purged gastight syringes. ¹³C-labeled and unlabeled dodecane were injected into the tubes using N₂-flushed glass syringes. Hydrocarbon concentrations in our experiments (2.1 mM butane; 1.8 mM dodecane) exceeded the natural concentrations by >100-fold to prevent substrate availability limitations in SIP. Butane is a natural substrate at both seep types occurring in concentrations of 0.5–1.1 μM (AMV) and 6–16 μM (GB, at a site close by; MY Kellermann, personal communication). Dodecane has only been detected at hydrocarbon seeps in the GB (Bazylnski *et al.*, 1988). AMV sediments were incubated at 20 °C, which is slightly above the *in situ* temperature

of 14 °C, whereas for GB sediments, where the temperature range is wider (from 3 °C to 50 °C), 28 °C was chosen. For each site (AMV, GB) and substrate (unlabeled butane, ¹³C-labeled butane, unlabeled dodecane, ¹³C-labeled dodecane), six replicate tubes were incubated. Time points for DNA and RNA extraction were chosen based on sulfide production (Figure 2a). For these analyses, two of the replicate tubes were used for each time point. Sediments were centrifuged (10 min, 4000 g) and pellets stored at –80 °C. One tube each was used for nucleic acid-SIP and one for protein-SIP. For catalyzed reporter deposition-fluorescence *in situ* hybridization (CARD-FISH) analysis, 0.5 ml of incubated sediment slurries were fixed for 1 h at 4 °C with a final concentration of 1% paraformaldehyde, washed with 1 × phosphate-buffered saline (130 mM NaCl, 10 mM sodium phosphate; pH 7.2) and finally stored in phosphate-buffered saline-ethanol (1:1) at –20 °C.

Chemical analysis

Sulfide was quantified photometrically as colloidal CuS (Cord-Ruwisch, 1985). For each of the two replicate incubations, two technical replicates were measured. Butane concentrations were measured by gas-chromatographic headspace analysis (oven 110 °C, injector 150 °C, detector 280 °C, nitrogen carrier gas) as described before (Musat and Widdel, 2008). The C-isotopic composition of dissolved inorganic carbon (DIC) from sediment slurries was analyzed using gas chromatography combustion isotope ratio mass spectrometry (Fisons VG Optima) according to Assayag *et al.* (2006). Biological activity of the DIC controls was halted by the addition of 4% paraformaldehyde (final concentration).

CARD-FISH

In situ hybridization with horseradish peroxidase-labeled oligonucleotide probes followed by fluorescently labeled tyramide signal amplification (CARD) and microscopic counting was performed as described previously (Kleindienst *et al.*, 2012). Probes for alkane degraders were developed using the software package ARB and the implemented function ‘probe design’ (Ludwig *et al.*, 2004). The specificity of new probes was evaluated against reference strains having one or more mismatches. For probes SCA1-212a and b, we used strain BuS5 and GMe12, respectively, as a positive control. For probes SCA2, LCA1 and LCA2, stringent hybridization conditions were determined by Clone-FISH (Schramm *et al.*, 2002). Probe sequences and formamide concentrations required for specific hybridization are given in Supplementary Table S1.

DNA/RNA-SIP

Nucleic acids were directly extracted from 3 to 4 g of incubated sediments and stored at –80 °C.

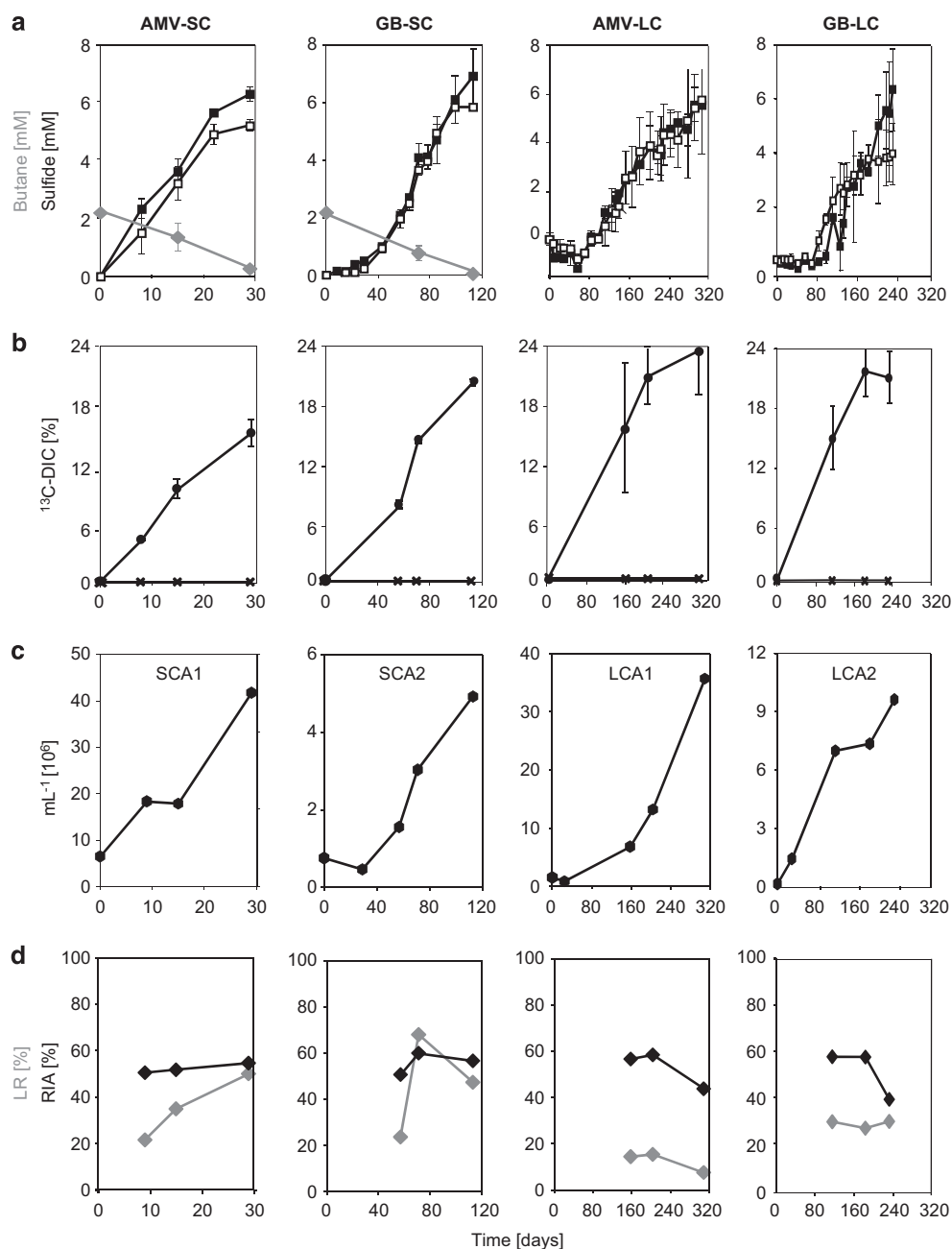


Figure 2 Measurements on incubations of AMV and GB sediments with ^{13}C -labeled butane (SC) or dodecane (LC) under sulfate-reducing conditions. AMV incubations were performed at 20°C , and GB incubations at 28°C . **(a)** Microbial butane- or dodecane-dependent sulfide production. Open symbols (\square), incubations with unlabeled alkanes; filled symbols (\blacksquare), incubations with ^{13}C -labeled alkanes. Average of two replicate samples is shown. Error bars give the standard deviation for the two replicates. Additionally, consumption of butane (\blacklozenge) is indicated for AMV-SC and GB-SC ^{13}C incubations. **(b)** Abundance of ^{13}C -DIC (at%) in incubations with (\bullet) ^{13}C -labeled and (\times) -unlabeled substrates in course of the incubation. Average of two replicate samples is shown. Error bars give the standard deviation for the two replicates. **(c)** Increase of specific cell concentrations of identified alkane degraders of clades SCA1, SCA2, LCA1 and LCA2 over the time course of the four incubations. **(d)** RIA in peptides (RIA, \blacklozenge) and labeling ratio of ^{13}C -labeled peptides to all peptide forms (LR, \blacklozenge).

From AMV sediment incubations, RNA was extracted according to McIlroy *et al.* (2008), whereas from GB sediments DNA was extracted according to Zhou *et al.* (1996), because RNA extraction did not result in the required amounts of high-quality rRNA.

Labeled and unlabeled nucleic acids ($5\ \mu\text{g}$ Guaymas-DNA and $750\ \text{ng}$ AMV-rRNA) were loaded

onto a CsCl gradient (average density $1.71\ \text{g ml}^{-1}$; Calbiochem, Merck, Darmstadt, Germany) or cesium trifluoroacetate (CsTFA; average density $1.795\ \text{g ml}^{-1}$; Amersham Pharmacia Biotech, Freiburg, Germany) in gradient buffer ($0.1\ \text{M}$ Tris-HCl at pH 8, $0.1\ \text{M}$ KCl, $1\ \text{mM}$ EDTA). Fractionation of gradients, refractometric measurement of fraction buoyant density and

precipitation of nucleic acids was carried out as published before (Lueders, 2010). Distribution of Guaymas-DNA or AMV-RNA within the gradients was analyzed using bacterial 16S rRNA gene-targeted real-time polymerase chain reaction and real-time reverse-transcription polymerase chain reaction in an Mx3000P Cyclor (Stratagene, La Jolla, CA, USA) with the primers Ba519f/Ba907r as described in Lueders *et al.* (2004). FAM-labeled amplicons for terminal restriction fragment length polymorphism fingerprinting of selected density-resolved nucleic acids were generated with bacterial primers 27f/907r as described in detail elsewhere (Lueders *et al.*, 2004; Piloni *et al.*, 2011). Briefly, amplicons were digested with *MspI* (Promega, Madison, WI, USA) and size-separated on an ABI 3730 DNA sequencer (Applied Biosystems, Life Technologies, Grand Island, NY, USA). Further analysis was performed with T-REX (Culman *et al.*, 2009). Background noise filtering was on the default factor 1.2; the clustering threshold for aligning peaks across samples was set to 1.5 bp. Relative terminal restriction fragment (T-RF) abundance was inferred from peak heights. For reduction of data complexity, T-RFs that occurred in <10% of the samples were excluded from further analysis.

AMV sediment frozen directly after sampling was used as a control. The resulting T-RF pattern was similar to the one obtained from the SIP inoculum (Supplementary Figure S1), suggesting no pronounced bottle effect on sediment microbiota during the 4-month storage before setting up the SIP incubations.

Pyrotag sequencing was performed on unfractionated total DNA from sediments at the time point of SIP inoculation, as well as on 'heavy' fractions of rRNA and DNA from the respective gradients. Amplicon sequencing on a 454 GS FLX pyrosequencer (454 Life Sciences, a Roche company, Branford, CT, USA), data processing and read quality trimming were performed as described (Piloni *et al.*, 2012). Pyrotag reads were taxonomically classified according to the ARB SILVA taxonomy (SSURef_108, release September 2011) using the SILVA-based next-generation sequencing pipeline (Quast *et al.*, 2013). The taxonomic assignment of sequences to DSS subclades, that is, SEEP-SRB1a, SCA1, SCA2, LCA1 and LCA2 (Table 1), has been carried out using ARB (Ludwig *et al.*, 2004). One representative sequence of all operational taxonomic units (OTUs) (95% criterion) that have been assigned to *Desulfobacteraceae* based on the SILVA-based next-generation sequencing pipeline has been imported into ARB SILVA database SSURef_111. After manual optimization of alignments sequences have been added to the provided tree under parsimony criteria.

For T-RF prediction, forward and reverse sequences were assembled into contigs (Piloni *et al.*, 2012). Subsequently, dominating contigs were again taxonomically classified using the SILVA-based next-generation sequencing pipeline

and imported into ARB. T-RFs of contigs were predicted using TRiFle (Junier *et al.*, 2008) and search functions implemented in the ARB software package. Given OTU numbers were calculated based on a 95% criterion using the reverse sequences.

masD clone library

The delta subunit of the (1-methylalkyl)succinate synthase gene (*masD*) was amplified using primers 7757f-1 and 7757f-2/8546r (von Netzer *et al.*, 2013) or *ass/bssF* and *ass/bssR* (for GB-SC incubation; Callaghan *et al.*, 2010) as described previously. Cloning and sequencing was performed as described previously (Kleindienst *et al.*, 2012).

Phylogenetic analysis

Phylogenetic 16S rRNA-based trees were calculated by neighbor-joining and maximum-likelihood (RAxML and PhyML) analysis with a filter considering 1244 positions that are conserved in at least 50% of the selected deltaproteobacterial sequences. For tree calculation, only nearly full-length sequences were used. Unstable branching orders are visualized as a multifurcation. Partial sequences from this study were inserted into the reconstructed tree by parsimony criteria. *MasD*-based trees were calculated from deduced amino acids by maximum-likelihood (RAxML and PhyML) analysis with a filter considering 159-amino-acid positions that are conserved in at least 30% of the selected sequences.

Sequence accession numbers

16S rRNA gene sequences and *masD* sequences have been submitted to the EMBL/GenBank/DDBJ databases under the accession no. HE797917-HE797929 and HG764655-HG764728, respectively. Pyrotag sequencing raw data have been stored in the sequence read archive under SRA accession number ERP001437.

Protein-SIP

Two grams of incubated sediments were suspended in 5.4 ml extraction buffer (Benndorf *et al.*, 2009) and subjected to three cycles of freeze (in liquid nitrogen) and thaw (water bath at 65 °C) with the addition of 0.6 ml of sodium dodecyl sulfate (10%, w/v) before the last thawing step. After phenol extraction and acetate precipitation of the organic and aqueous phase, extracted proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Laemmli, 1970). Five sections of each lane were excised from the sodium dodecyl sulfate-polyacrylamide gel electrophoresis and treated according to Jehmlich *et al.* (2008). Eluted peptides from the aqueous-phase gel slices were combined. Purification was carried out using ZipTip_{C18} columns (Merck Millipore, Billerica, MA, USA), resulting in six fractions per sample.

Table 1 Overview of phylogenetic affiliations of bacterial 16S rRNA gene sequences retrieved from our four incubations, AMV-SC, AMC-LC, GB-SC and GB-LA, based on the output from the SILVA/NGS pipeline

Phylogenetic affiliation ^a	AMV			GB		
	Inoculum	AMV-SC	AMV-LC	Inoculum	GB-SC	GB-LC
		¹³ C-butane	¹³ C-dodecane		¹³ C-butane	¹³ C-dodecane
	t ₀	29 days	309 days	t ₀	113 days	183 days
No. of reads						
<i>Bacteria</i>	5139	6181	4927	5244	5782	3145
<i>Acidobacteria</i>	9	11	43	23	24	3
<i>Bacteroidetes</i>	64	21	31	431	393	33
BD2-2	13	1	0	66	11	1
Uncult. <i>Marinilabiaceae</i>	5	5	3	63	347	27
VC2.1 Bac22	6	1	5	218	34	3
Candidate division OP3	2	0	173	13	248	615
Candidate division OP9	137	20	276	43	376	92
Candidate division WS3	15	2	23	77	67	4
<i>Chloroflexi</i>	84	47	250	263	400	131
<i>Deferribacteres</i>	28	10	68	75	45	29
<i>Firmicutes</i>	10	1	7	93	4	1
<i>Fusobacteria</i>	55	13	99	78	260	51
<i>Proteobacteria</i>	4329	5894	3634	2514	2791	1870
<i>Deltaproteobacteria</i>	3646	5544	3443	1834	2494	1837
<i>Desulfarculaceae</i>	44	23	54	222	418	74
<i>Desulfobacteraceae</i>	3203	5333	2830	474	1432	378
LCA1	2	0	984	0	1	0
<i>Desulfobacterium</i> spp.	7	4	2	19	37	2
<i>Desulfobacula</i> spp.	30	13	6	143	4	4
<i>Desulfococcus</i> spp.	48	216	78	18	6	4
LCA2	5	0	302	28	26	328
<i>Desulfosarcina</i> spp.	59	25	24	6	22	1
SEEP-SRB1	2560	614	1081	39	144	11
SCA1	209	3246	177	37	213	8
SCA2	47	65	88	14	616	6
<i>Desulfobulbaceae</i>	181	59	280	93	120	1273
SEEP-SRB2	51	20	70	44	54	5
Uncult. <i>Desulfobulbaceae</i>	1	3	107	8	57	1268
<i>Desulfuromonadales</i>	128	40	46	195	79	12
<i>Myxococcales</i>	19	1	77	22	8	4
Sva0485	50	57	81	60	18	10
<i>Syntrophobacteriales</i>	8	24	15	10	108	1
HotSeep1	1	0	1	723	272	56
<i>Epsilonproteobacteria</i>	235	105	79	585	66	17
<i>Gammaproteobacteria</i>	420	227	95	72	213	10
<i>Methylococcales</i>	136	92	10	7	1	0
<i>Spirochaetes</i>	7	8	16	110	31	3
<i>Thermodesulfobacteriales</i>	0	0	0	71	33	7
Other Bacteria	116	95	140	451	343	58

Abbreviations: AMV, Amon Mud Volcano; GB, Guaymas Basin; LC, long chain; NGS, next-generation sequencing; SC, short chain; SIP, stable isotope probing; T-RF, terminal restriction fragment.

Sequencing was carried on DNA from inoculum sediments (t₀) and on ¹³C-enriched RNA (AMV) or DNA (GB) from our incubations with ¹³C-labeled butane or ¹³C-labeled dodecane after density gradient centrifugation. Number of reads give the sum of retrieved forward (primer 27F) and reverse (primer 519r) sequences. Characteristic T-RFs were predicted via assembled amplicon contigs and are given as T-RFs actually observed in the electropherograms as verified according to Winderl et al. (2010).

Percentages of T-RFs give the portion of sequences of the individual clades assigned to the respective T-RF.

^aBased on ARB SILVA taxonomy.

Mass spectrometric measurement was carried out using the nanoAcquity UPLC (Waters, Milford, MA, USA) coupled LTQ-Orbitrap XL (Thermo Fisher Scientific, Waltham, MA, USA) (Herbst *et al.*, 2013). Trapping was performed for 6 min. To elute the peptides the acetonitrile solvent gradient was set in the first 54 min from 2% to 20% and subsequently 28 min from 20% to 85%.

For peptide identification, first a survey search using Mascot (Perkins *et al.*, 1999) against all prokaryotic entries of NCBI nr (28 April 2013) was performed. These unfiltered results and a six-frame translation of the genome of strain BuS5 (Stefan M Sievert, Woods Hole Oceanographic Institution, Woods Hole, MA, USA; JGI project number 403224; <http://img.jgi.doe.gov/cgi-bin/w/main.cgi>) were used as database for peptide identification. The translation was made with MaxQuant 1.2.0.18 (Cox and Mann, 2008) and a minimum of 50 amino acids per ORF. Furthermore, contaminants and reverse entries as decoys were added to the database. The main search used OMSSA (Geer *et al.*, 2004) in an OpenMS pipeline (Sturm *et al.*, 2008). Carbamidomethylation of cysteines was treated as fixed modification and oxidation of methionines as variable modification. Mass deviation tolerances were 5 p.p.m. for precursor masses and 0.5 Da for product ions. The maximal false discovery rate for peptides was set to 1%.

Protein relative isotope abundance (RIA) and protein labeling ratio

RIA and the labeling ratio were determined using OpenMS and the MetaproSIP tool (<http://openms.de/metaprosip>) with a minimum correlation of 0.7 and a mass window of 10 p.p.m.

Results and discussion

Bacterial diversity in AMV and GB sediments

We initially characterized the bacterial diversity in contrasting sediments from a gas seep at AMV emitting high fluxes of methane and other SCA and in sediments from a hydrocarbon seep in the GB releasing a complex hydrocarbon mixture similar to crude oil with various *n*-alkanes and aromatic hydrocarbons. AMV gas seep sediments used as inoculum (t_0) for later SIP experiments were clearly dominated by *Deltaproteobacteria* contributing almost three-quarters of the obtained 5139 pyrosequencing reads (Table 1). The diversity within the *Deltaproteobacteria* was high with 579 OTUs on the genus level (95% cutoff, Yarza *et al.*, 2010) from overall 1151 bacterial OTUs. About 50% of total AMV sequences (=70% of *deltaproteobacterial* sequences) were affiliated with the DSS subclade SEEP-SRB1. Of these, 65% affiliated with SEEP-SRB1a, the sulfate-reducing partner of ANME-2a and ANME-2c in AOM habitats (Schreiber *et al.*, 2010). Sequences found at lower frequency were affiliated

with *Gammaproteobacteria* (8%), *Epsilonproteobacteria* (5%) and candidate division OP9 (3%).

In GB hydrocarbon seep sediments, a total of 546 bacterial OTUs were detected. Of these, 159 OTUs (=29% of bacterial OTUs) have been assigned to *Deltaproteobacteria*, suggesting a lower diversity in GB sediments compared with AMV. About 14% of total GB sequences (=40% of *deltaproteobacterial* sequences) were assigned to the deep-branching group Hot-Seep1 that was shown to be involved in thermophilic AOM (Holler *et al.*, 2011; Table 1). Other sequences frequently found in GB sediments were affiliated with *Epsilonproteobacteria* (12%), *Bacteroidetes* (8%) and *Chloroflexi* (5%). The detected phyla match well with the current picture of bacterial diversity in sediments from similar sites at AMV (Omeregíe *et al.*, 2009; Pachiadaki *et al.*, 2011) and GB (Teske *et al.*, 2002; Dhillon *et al.*, 2003; Biddle *et al.*, 2011) or more geographic distant seep sites (e.g. Orphan *et al.*, 2001; Knittel *et al.*, 2003; Lloyd *et al.*, 2010; Orcutt *et al.*, 2010; Nunoura *et al.*, 2012; Adams *et al.*, 2013; Rubin-Blum *et al.*, 2014). This congruency also confirms that SIP experiments were carried out with benthic communities that typically occur at these seeps.

Alkane-dependent sulfide production

Incubations with ^{13}C -labeled butane (SC) or dodecane (LC) were started in replicates under sulfate-reducing conditions, resulting in four types of incubations: AMV-SC, AMV-LC, GB-SC and GB-LC.

Alkane-dependent microbial activity differed among the four incubations. The incubations of AMV sediments with butane (AMV-SC) had the fastest response. Butane degradation started without lag phase, as determined based on butane-dependent sulfide production, butane oxidation and production of $^{13}\text{CO}_2$ (Figures 2a and b). After 29 days, the selected end point of AMV-SC incubation, almost complete removal of butane occurred, which coincided with 6 mM sulfide production and a strong labeling of resulting CO_2 (15% ^{13}C -DIC). In contrast, a pronounced lag phase of 20 days was observed for incubations of GB sediments with butane (GB-SC). By day 113, butane was consumed resulting in 21% ^{13}C -DIC.

Dodecane-dependent SR was delayed in both incubations (AMV-LC and GB-LC). Significant sulfide production started only after 80 days (Figure 2a) and 6 mM sulfide was produced after 232 days (GB) and 309 days (AMV), leading to an enrichment of 24% and 21% ^{13}C -DIC, respectively (Figure 2b). Our data show that both sediments are able to react within weeks to months on the addition of butane or dodecane.

Four DSS clades are key degraders of butane and dodecane

An early time point and the end point of SIP experiments were selected for DNA and RNA extraction on each of the duplicate ^{13}C -labeled alkane

incubations for subsequent ultracentrifugation. Except for GB-LC, no considerable relative increase in total cell counts was observed between t_0 and the individual first sampling points (Supplementary Figure S2). The absence of significant cell growth is the prerequisite for a reliable identification of primary degraders by SIP experiments whereas cross-feeding could therefore be neglected at t_1 . All density-resolved nucleic acid fractions were first subjected to terminal restriction fragment length polymorphism fingerprinting, to identify the T-RFs of ^{13}C -labeled populations, which were subsequently identified by pyrotag sequencing of selected gradient fractions.

Butane degraders. Two subclades of DSS were identified in AMV sediments: after only 9 days, a T-RF of 132 bp length became dominant in the heavy rRNA fraction (Figure 3) and was identified as a close relative of the butane-degrading strain BuS5 (93% 16S rRNA gene sequence similarity; Figure 1). The second subclade was identified after 29 days based on a T-RF of 512 bp length, representing butane degraders closely related (99.6%) to phylo-type ‘But12_GMe’ that dominated an enrichment culture on butane (Kniemeyer *et al.*, 2007; Jaekel *et al.*, 2012). A neighbored subclade contains two phylotypes dominant in enrichments with propane

(Prop12_GMe) or butane (But12_HR; Jaekel *et al.*, 2012). We name these three neighboring clusters of propane and butane degraders ‘SCA1’. In addition to the identified populations represented by these major labeled T-RFs, several further SCA1-OTUs based on a 95% similarity criterion were identified in AMV sediments, resulting in a total of 5 OTUs for subclade But12_GMe and 14 OTUs for subclade BuS5, suggesting the presence of multiple closely related genera capable of butane degradation. In support of our hypothesis, enrichments with sediments from Garden Banks mud volcano (Northern Gulf of Mexico) supplemented with C_2 – C_4 alkanes were dominated by members of SCA1 (Bose *et al.*, 2013). Furthermore, public database analysis shows that members of SCA1 are indeed globally distributed. They have been retrieved from marine seep sites in the Mediterranean (Pachiadaki *et al.*, 2011), at Hydrate Ridge (Jaekel *et al.*, 2012), Gulf of Mexico or GB (Kniemeyer *et al.*, 2007; Jaekel *et al.*, 2012). With this functional characterization of members of SCA1, we substantiate that this group is likely of relevance in the environment and that the isolate BuS5 is a relevant model organism.

In GB sediments, butane stimulated a different DSS subclade: after 57 days, a 163 bp T-RF (Figure 3) was detected, which we named ‘SCA2’ (Figure 1).

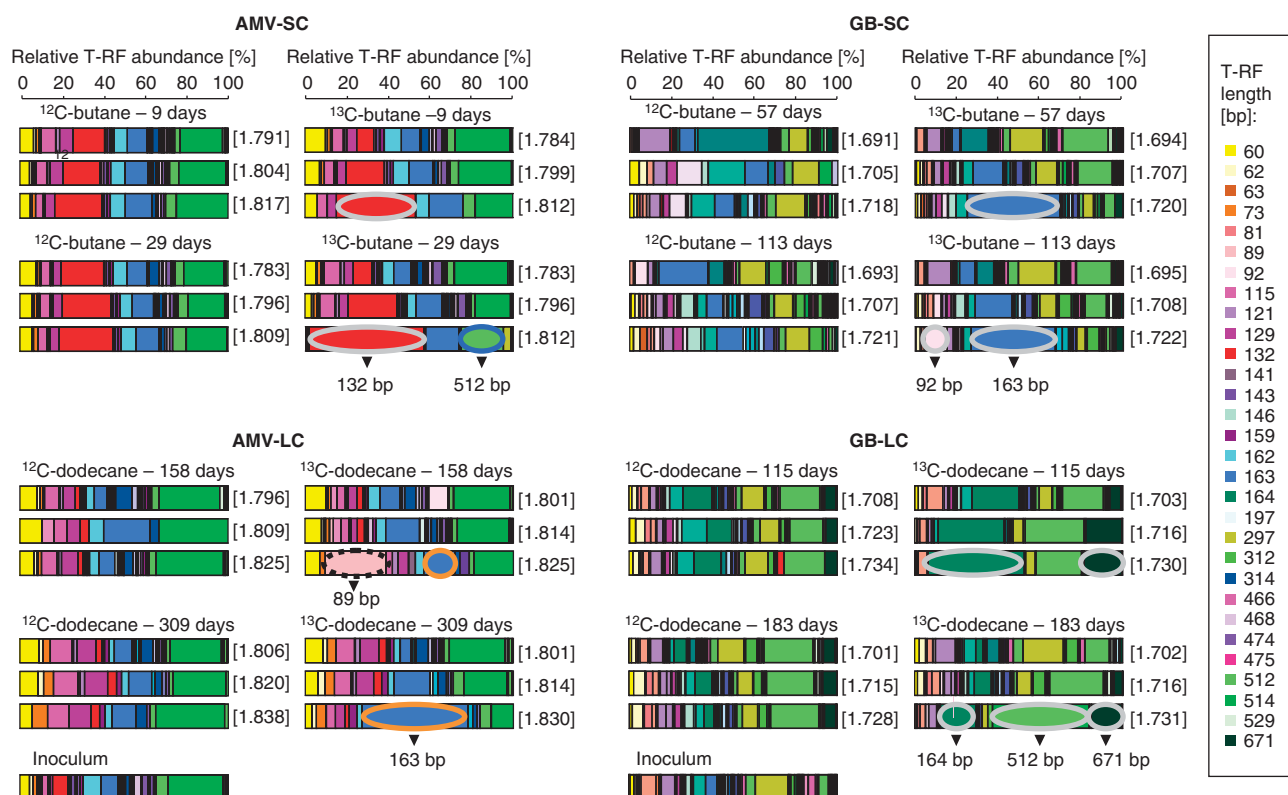


Figure 3 Bacterial community structure as shown by terminal restriction fragment length polymorphism (T-RFLP) fingerprinting of density-resolved rRNA (AMV) and DNA (GB) fractions after density centrifugation of sediments incubated with ^{13}C -labeled butane (SC) or dodecane (LC) under sulfate-reducing conditions. Fingerprints of representative ‘light’, ‘intermediate’ and ‘heavy’ gradient fractions are shown for all gradients of respective ^{13}C -labeled and -unlabeled alkane incubations, for the first and the last sampling time point of each SIP treatment, respectively. The buoyant densities (g ml^{-1}) for each fraction are given in brackets. T-RFs corresponding to taxa identified to be involved in the degradation of butane and dodecane are highlighted with circles. Fingerprints for sediment inocula are shown as reference.

SCA2 is phylogenetically closely related to SCA1 with up to 95% sequence similarity, but appears to be restricted to GB. After 113 days, an additional labeled T-RF of 92 bp length emerged and was identified as uncultured *Marinilabiaceae* of the phylum *Bacteroidetes*. In general, *Bacteroidetes* are known to degrade high-molecular-weight compounds (e.g. polysaccharides, proteins) and to have a preference for growth attached to particles (Fernández-Gómez *et al.*, 2013). We therefore assume that members of this clade were secondarily labeled, by using dead biomass of or other trophic interaction with the primary hydrocarbon consumers, rather than degrading butane on its own. Yet, it should be noted that related sequences originate from several seeps in the Mediterranean, Gulf of Mexico and Pacific Ocean (e.g. Omeregíe *et al.*, 2008; Ruff *et al.*, 2013). Therefore, it would be worthwhile to investigate the role of this clade in marine hydrocarbon catabolism more specifically.

Dodecane degraders. Another 163 bp T-RF became apparent in heavy rRNA of AMV-LC. It was assigned to organisms of another DSS subclade, which we named 'LCA1'. LCA1 comprises eight OTUs from AMV sediments and shows an intragroup 16S rRNA similarity of as low as 88.5%. It is phylogenetically clearly distinct from SCA1 and SCA2 (Figure 1). It comprises not only sequences from marine seep sites or hydrocarbon-polluted sediments (Zhang *et al.*, 2010; Elsaied *et al.*, 2011; Acosta-González *et al.*, 2013; Ruff *et al.*, 2013) but also sequences retrieved from the chemocline in the meromictic freshwater lake Cadagno (Halm *et al.*, 2009). Moreover, a second ¹³C-labeled T-RF (89 bp) was detected in the AMV-LC incubation only for the first time point (Figure 3). However, phylogenetic assignment of this T-RF failed, as no sequences with a matching restriction site were found in the corresponding 454 data set. In GB-LC incubations, a dominating 164 bp T-RF was identified in heavy rRNA after 115 days of incubation with ¹³C-dodecane. This T-RF represents a novel clade of *Desulfobacteraceae* that is most closely related to *Desulfosalsimona propionica*. We propose to name this clade as 'LCA2' and regard it as another subclade of DSS, even though members of clade LCA2 hold one mismatch to probe DSS658 (position 14, C–T). Two and four OTUs of LCA2 were identified in AMV and GB sediments, respectively. Other sequences from marine seeps or oil-contaminated sites (Figure 1; e.g. Lösekann *et al.*, 2007; Abed *et al.*, 2011; Zhang *et al.*, 2011; Ruff *et al.*, 2013) also belong to the LCA2 clade, and could potentially also be involved in dodecane degradation.

A second labeled T-RF (512 bp) was identified in heavy DNA of the GB-LC incubation. The corresponding organisms form a cluster within *Desulfobulbaceae*, together with sequences retrieved

from a mud volcano in the Gulf of Cadiz (e.g. clone GoC_Bac_176, FN820344; Figure 1). If members of this cluster are capable of dodecane oxidation or have been labeled through cross-feeding needs further investigation. Comparative 16S rRNA analysis (Table 1) suggests a low abundance in GB sediments.

Additionally, members of the candidate phylum *Omnitrophica* (OP3; Rinke *et al.*, 2013), belonging to the *Planctomycetes/Verrucomicrobia/Chlamydiae* superphylum, were found labeled in GB-LC incubations based on a T-RF of 671 bp length (Figure 3). The OP3 lineage seems to be ubiquitous in anoxic environments, such as marine habitats. Recent studies reported the genomic potential for anaerobic respiration (Glöckner *et al.*, 2010) or the syntrophic involvement of OP3 in anaerobic monoterpene degradation (Rotaru *et al.*, 2012). Further studies are clearly required to unravel whether these microbes were ¹³C labeled in our incubations owing to syntrophy or cross-feeding. In summary, all identified clades from our study and suggested carbon flow in the distinct sediments are depicted in Figure 4.

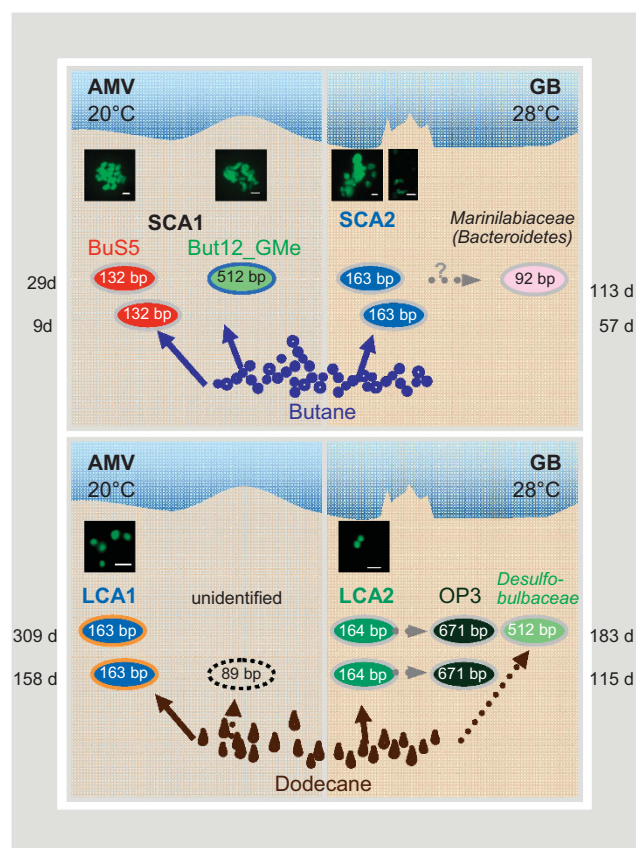


Figure 4 Suggested ¹³C carbon flow for alkane degradation in AMV and GB sediments. The role of active DSS subclades is based on the results of nucleic acid-SIP as visualized by the corresponding T-RFs. Fluorescent images show CARD-FISH signals as obtained after hybridization with oligonucleotide probes specific for SCA1, SCA2, LCA1 and LCA2. Scale bars represent 2 μm.

Detection of SCA- and LCA-degrading DSS subclades *in situ*

A set of CARD-FISH probes was developed for the specific detection of butane- and dodecane-degrading DSS subclades (Figure 1 and Supplementary Table S1). After careful adjustment of specific hybridization conditions, the new probes were applied on our SIP incubations. Cell size and morphology differed for SCA1 subclades (Figure 4): cells belonging to subclade 'BuS5' have an almost coccoid morphology of $1.8\ \mu\text{m} \times 2\ \mu\text{m}$, whereas cells of subclade 'But12_GMe' are curved rods of $1.5\ \mu\text{m} \times 2.1\ \mu\text{m}$. Cells of SCA2 were either short rods ($1\ \mu\text{m} \times 1.3\ \mu\text{m}$) or vibrio-like ($2.5\ \mu\text{m} \times 1.5\ \mu\text{m}$). LCA1 and LCA2 cells were both cocci with a diameter of approximately $1\ \mu\text{m}$.

We observed a strong specific cell increase for all four clades in course of the experiment (Figure 2c). SCA1 and SCA2 cell numbers increased by a factor of approximately 6 within 29 and 113 days, respectively. LCA1 and LCA2 cell numbers increased by a factor of 23 and 59 during incubation. The detected growth of these clades strongly supports the correct taxonomic assignment of the identified T-RFs by polymerase chain reaction-independent means, and thus the capability of these organisms for alkane degradation.

The *in situ* presence and relevance of SCA1, SCA2 and LCA2 was confirmed by CARD-FISH on nearby sediments with relative abundances between 0.2% and 2% of total single cells (max 4.4×10^7 cells ml^{-1} ; Supplementary Table S2). LCA1 cells could not be detected in these samples, possibly because of a too high selectivity of probe LCA1-443, which covers only the specific organisms represented by the 163 bp T-RF, plus one additional sequence.

This evidence for the presence of three out of four of the identified DSS subclades in low abundances at the two investigated seep sites is compelling. From this seed bank, different SC and LC alkane degraders were apparently capable of growing, over relatively short time frames from days to weeks, and initiate alkane degradation.

Diversity of *masD* genes in SC and LC incubations

MasD gene libraries constructed from the four incubations revealed a high diversity of organisms associated with alkane degradation: of a total of 247 *masD* sequences, 22 OTUs (based on 93% amino acid identity) were detected in AMV-SC, 25 OTUs in AMV-LC, 11 OTUs in GB-SC, and 16 OTUs in GB-LC. Several habitat-specific clusters became obvious in the phylogenetic tree comprising sequences either from AMV or from GB (Supplementary Figure S3). Substrate-specific clades were detected only for GB-SC and GB-LC incubations. The high *masD* diversity supported our findings based on 16S rRNA, that is, the presence of multiple closely related genera capable of alkane degradation.

Furthermore, this diversity suggests a great potential of marine benthic communities to respond to hydrocarbon inputs and natural changes in seepage. Gas hydrate decay, gas leakage or oil spills are only some of the possible events able to cause an increased hydrocarbon input on the sediments.

Proteins involved in SC and LC alkane degradation under sulfate-reducing conditions

We directly identified enzymes and catabolic pathways involved in butane and dodecane oxidation in these sediments using protein-SIP. In AMV-SC, AMV-LC, GB-SC and GB-LC incubations, the functional metaproteomic approach identified between 511 (1143 peptide species) and 1493 (3869 peptide species) proteins (see Supplementary Table S3). ^{13}C -labeled peptides showed mostly hits to proteins of the *Desulfobacteraceae*, thus further substantiating our finding that active alkane degraders in AMV and GB sediments belong to this family.

A crucial step in the anaerobic degradation of alkanes is their activation. A common activation reaction is the addition of alkanes to fumarate yielding alkylsuccinates (Figure 5; Rabus *et al.*, 2001). The genes responsible for the initial alkylsuccinate synthase reaction have been identified and named *masD* (1-methylalkyl)succinate synthase; Grundmann *et al.*, 2008) or *assA* (alkylsuccinate synthase; Callaghan *et al.*, 2008). Based on the available BuS5 genome (SCAFFOLD 2:TRUE:69872), ^{13}C -labeled *MasD* peptides could be identified within the AMV-SC (28 peptide species), AMV-LC (2 peptide species) and GB-SC incubations (2 peptide species; Supplementary Table S3). Fumarate addition is followed by a carbon skeleton rearrangement before β -oxidation. The β -oxidation has been proposed as an analog to the degradation of LC fatty acids (Wilkes *et al.*, 2002). The detection of ^{13}C -labeled peptides in AMV-SC, AMV-LC and GB-SC incubations involved in β -oxidation corroborate this pathway. These peptides were assigned to β -oxidation enzymes responsible for dehydrogenation (i.e. acyl-coenzyme A (CoA) dehydrogenase), hydration (i.e. enoyl-CoA hydratase/isomerase) and oxidation (3-hydroxyl-CoA dehydrogenase) (Supplementary Tables S3 and S4).

Alkane-degrading SRB within the *Desulfobacteraceae* have the capability for complete substrate oxidation, and thus they further metabolize acetyl-CoA. The most common pathway for acetyl-CoA/acetate dissimilation in SRB is the reverse Wood–Ljungdahl pathway, with carbon monoxide dehydrogenase (reverse acetyl-CoA synthetase) as a key enzyme (Schauder *et al.*, 1986). ^{13}C -labeled peptides corresponding to four enzymes of the Wood–Ljungdahl pathway were indeed identified: carbon monoxide dehydrogenase/acetyl-CoA synthase complex, methylenetetrahydrofolate reductase, formate-tetrahydrofolate ligase (also named formyltetrahydrofolate synthetase) and formate dehydrogenase. These findings strongly indicate

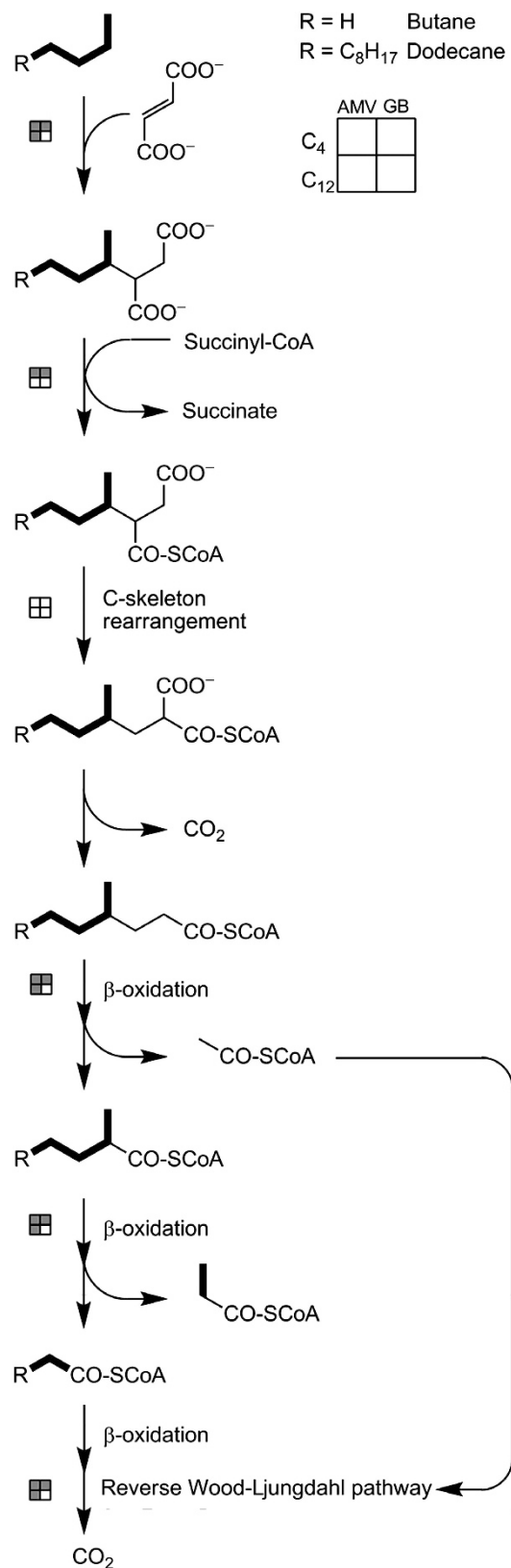


Figure 5 Reconstruction of the complete pathway of alkane activation and further degradation to CO₂ based on ¹³C-labeled peptides as identified via protein-SIP on AMV and GB incubations.

that alkane-degrading SRB use the Wood–Ljungdahl pathway for terminal oxidation and that acetyl-CoA is a central metabolite during alkane degradation. The presence of the carbon monoxide dehydrogenase/acetyl-CoA synthase complex suggests that these alkane degraders could, in principle, also grow on acetate as an alternative substrate. The identified labeled peptides allowed the reconstruction of the complete pathway of alkane activation and further degradation to CO₂ (Figure 5).

Furthermore, ¹³C-labeled peptides from AMV incubations could be assigned to the three key enzymes of dissimilatory SR, that is, sulfate adenylyltransferase, adenosine-5'-phosphosulfate reductase and dissimilatory sulfite reductase (DSR, subunits a and b; Supplementary Tables S3 and S4). This corroborates that alkane degradation is directly linked to SR. Labeled peptides involved in SR showed highest BLAST scores to proteins from *Desulfobacteraceae* supporting that the key butane and dodecane degraders belong to this family. The remaining labeled peptides were mainly derived from highly conserved proteins such as chaperones, ATP synthases and K⁺-insensitive pyrophosphate-energized proton pumps, again affiliating mainly with *Desulfobacteraceae*.

Protein-SIP also allowed quantitative interpretation of butane- and dodecane-derived carbon assimilation (Figure 2d). The RIA directly corresponds to the ¹³C content in newly synthesized proteins. Thus, it reflects the carbon source composition of newly synthesized biomass. As the ¹³C content of the provided alkane substrates was 100%, but the RIA only approximately 50%, roughly half of the biomass carbon did not derive from alkane degradation. The remaining half could be gained from either other non-labeled substrates or from fixation of inorganic carbon. Particularly in the AMV-SC samples, a good correlation between RIA and ¹³C-DIC increase could be observed. The increase in the average ¹³C content corresponded to 50% of the ¹³C-DIC increase (example shown in Supplementary Figure S4). These SRBs probably use about 50% alkanes and 50% DIC for biomass production. This is in accordance with current knowledge on SRBs, which are known to derive a large fraction of their carbon from CO₂ via carboxylation reactions (Rabus *et al.*, 2006). In AMV-SC and AMV-LC incubations, we found several ¹³C-labeled peptides assigned to pyruvate: ferredoxin/ flavodoxin oxidoreductase, an enzyme typically used by SRB for acetyl-CoA assimilation through reductive carboxylation to pyruvate.

Conclusions

The impact of non-methane-dependent SR was thoroughly described for various marine seep sites and has been suggested to be of global significance for the marine carbon and sulfur cycle. However, before our study, this concept has been based mostly

on rate measurements (e.g. Joye *et al.*, 2004; Kniemeyer *et al.*, 2007; Orcutt *et al.*, 2010; Bowles *et al.*, 2011; Quistad and Valentine, 2011; Bose *et al.*, 2013) and isolates or enrichment cultures of hydrocarbon-degrading SRB (e.g. Kniemeyer *et al.*, 2007; Jaekel *et al.*, 2012; Adams *et al.*, 2013; Bose *et al.*, 2013). Here, we provide the direct link between biogeochemical patterns and apparently specialized DSS subclades of key alkane degraders in marine sediments. Nucleic-acid-SIP and protein-SIP, which has been combined for the first time on a marine sediment sample, proved as a powerful approach for the investigation of the microbial community associated with alkane degradation.

Complementary to aerobic hydrocarbon-degrading bacterial populations in the marine realm (Hazen *et al.*, 2010; Abbriano *et al.*, 2011; Kostka *et al.*, 2011; Bælum *et al.*, 2012; Redmond and Valentine, 2012; Gutierrez *et al.*, 2013), this study corroborates the environmental importance of the DSS clade for the anaerobic degradation of alkanes at marine seeps. Moreover, the identification of diverse and substrate-specialized clades of sulfate-reducing bacteria initiating alkane degradation within days to weeks suggests an overlooked potential of natural marine benthic microbiota to react to massive hydrocarbon input, for example, as encountered during anthropogenic oil spills. Finally, we provided evidence that available isolates and their hydrocarbon activation mechanisms are of great relevance for natural settings.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

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