LETTERS

Anaerobic oxidation of short-chain hydrocarbons by marine sulphate-reducing bacteria

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The short-chain hydrocarbons ethane, propane and butane are constituents of natural gas. They are usually assumed to be of thermochemical origin[1], but biological formation of ethane and propane has been also observed[2]. Microbial utilization of short-chain hydrocarbons has been shown in some aerobic species[3,4] but not in anaerobic species of bacteria. On the other hand, anaerobic utilization of short-chain hydrocarbons would in principle be expected because various anaerobic bacteria grow with higher homologues ($\geq C_6$)[5]. Indeed, chemical analyses of hydrocarbon-rich habitats with limited or no access of oxygen indicated *in situ* biodegradation of short-chain hydrocarbons[6–10]. Here we report the enrichment of sulphate-reducing bacteria (SRB) with such capacity from marine hydrocarbon seep areas. Propane or *n*-butane as the sole growth substrate led to sediment-free sulphate-reducing enrichment cultures growing at 12, 28 or 60 °C. With ethane, a slower enrichment with residual sediment was obtained at 12 °C. Isolation experiments resulted in a mesophilic pure culture (strain BuS5) that used only propane and *n*-butane (methane, isobutane, alcohols or carboxylic acids did not support growth). Complete hydrocarbon oxidation to CO_2 and the preferential oxidation of ^{12}C-enriched alkanes were observed with strain BuS5 and other cultures. Metabolites of propane included iso- and *n*-propylsuccinate, indicating a subterminal as well as an unprecedented terminal alkane activation with involvement of fumarate. According to 16S ribosomal RNA analyses, strain BuS5 affiliates with *Desulfosarcina/Desulfococcus*, a cluster of widespread marine SRB. An enrichment culture with propane growing at 60 °C was dominated by *Desulfotomaculum*-like SRB. Our results suggest that diverse SRB are able to thrive in seep areas and gas reservoirs on propane and butane, thus altering the gas composition and contributing to sulphide production.

Saturated hydrocarbons (alkanes) belong to the chemically least reactive organic compounds, but are nevertheless used by diverse microorganisms. Whereas biodegradation of alkanes with oxygen has been well-known for a century, their anaerobic biodegradation[5] still awaits a deeper understanding with respect to biochemical reactions and the range of utilizable chain lengths. An intensely investigated process in this respect is the anaerobic oxidation of methane with sulphate in marine sediments[5]. On the other hand, several anaerobic bacteria have been isolated that utilize saturated hydrocarbons with six or more carbon atoms[5]. Hence, there is a gap in our knowledge—are there microorganisms that can anaerobically degrade hydrocarbons with shorter chain lengths, in particular ethane, propane and *n*-butane? We therefore attempted to find representatives of sulphate-reducing microorganisms with such capacity by way of enrichment from marine hydrocarbon seeps. Enrichments were set up in defined anoxic media, using sediment from the Gulf of Mexico[11,12] for potentially cold-adapted SRB, and sediment from the Guaymas basin (Gulf of California)[13,14] for mesophilic and thermophilic SRB.

Slow, yet clearly hydrocarbon-dependent, sulphide production was observed in all incubations with propane or butane, and in an incubation with ethane at 12 °C (Table 1). The thermophilic enrichment culture with propane exhibited a substantial sulphide (>12 mM) production within three months, whereas other cultures with propane or butane reached similar concentrations within approximately six months. In subcultures, the thermophilic enrichment with propane and the mesophilic enrichment with butane attained the fastest growth. Ethane-dependent sulphate reduction remained very slow (Supplementary Fig. 1). Sulphate reduction with isobutane has not been observed thus far.

From the mesophilic enrichment with butane, a pure culture, strain BuS5, was isolated (Fig. 1a, left). Strain BuS5 and enrichment cultures with propane and butane were phylogenetically analysed on the basis of 16S rRNA gene sequences (Fig. 1b). Retrieved sequences were used to design 16S rRNA-specific fluorescent probes for visualization of abundant phylotypes in the enrichment cultures via cell hybridization (Supplementary Table 1; Fig. 1a; Supplementary Fig. 3). Strain BuS5 was a member of the *Desulfosarcina/Desulfococcus* cluster within the Deltaproteobacteria. This cluster comprises widespread bacteria, many of which have been detected in seep areas. Strain BuS5 was a major component (~50% of cells) of the parental mesophilic enrichment culture (Supplementary Fig. 3). A close relative of strain BuS5 was dominant (75% of cells) in the cold-adapted enrichment culture with butane (Butane12-GMe; Fig. 1). The thermophilic enrichment culture with propane (Propane60-GuB) was dominated (92% of cells) by a Gram-positive (*Desulfotomaculum*) species (Fig. 1a). The very slow sulphate-reduction with ethane (Supplementary Fig. 1) has not so far yielded a sediment-free subculture for comparable hybridization studies.

Strain BuS5 was characterized in more detail. Its growth occurred between 10 and 33 °C (optimum, 28 °C) and pH 6.0 and 7.4 (optimum, pH 6.9). Growing cells partly attached to the glass walls. The guanine plus cytosine content of the DNA was 40.9 mol%. Of several single compounds (H_2; alkanes including isobutane; primary and secondary alcohols; alkanoates from C_1 through to C_6; lactate; fumarate; succinate) tested, strain BuS5 used only propane and *n*-butane. During incubation with a mixture of methane, ethane, propane and butane, strain BuS5 consumed only propane and butane (Fig. 2). Because there was no pronounced exponential growth phase,

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Table 1 | Anaerobic sulphate-reducing enrichment cultures obtained with gaseous hydrocarbons

Substrate	Cultivation temperature (°C)	Inoculum (sediment)	Culture designation*	Characteristic morphotype(s)	Dominant phylotype†
Ethane	12	Gulf of Mexico	None	Not yet evident in early state	Not identified
Propane	12	Gulf of Mexico	None	Rod-shaped cells	Not identified
Propane	28	Guaymas basin	None	Rod-shaped cells	Not identified
Propane	60	Guaymas basin	Propane60-GuB	Spindle-shaped cells	*Desulfotomaculum*
n-Butane	12	Gulf of Mexico	Butane12-GMe	Oval and curved cells	DSS cluster‡
n-Butane	28	Guaymas basin	Butane28-GuB	Oval cells, like isolated strain BuS5	DSS cluster‡
n-Butane	60	Guaymas basin	None	Various rod-shaped cells	Novel cluster§

* For convenience, a designation was used for those cultures that appear in Fig. 1 and Supplementary Fig. 2.
† Identified via 16S rRNA-targeted whole cell hybridization with specifically designed probes (Supplementary Table 1).
‡ *Desulfosarcina/Desulfococcus* cluster, a group of widespread marine SRB.
§ All bacterial clones ($n = 38$) were closely related to a clone (a1b0202) from a 16S rRNA-based survey of sediment[30] that had been freshly retrieved from Guaymas basin during the same cruise; these clones may be the representatives of a novel bacterial lineage. On the other hand, archaeal clones were also detected that showed a higher diversity and affiliated with *Thermococcus mexicalis, Archaeoglobus profundus* and relatives of *Thermoplasma, Methanosaeta* and *Methanococcus*, which are unlikely to have a direct role in butane degradation. However, growth in this culture was accompanied by signs of lysis of a proportion of the cells and possible development of 'secondary feeders', so that hybridization results must be viewed critically.

a doubling time of 4–5 days could be estimated only roughly from an early phase of the sulphide production curve.

Substrate and product quantification (Supplementary Table 2) in growth experiments with the thermophilic enrichment with propane (Propane60-GuB) and the mesophilic strain BuS5 with butane suggested a complete oxidation according to following respective equations:

$$2C_3H_8 + 5SO_4^{2-} + 4H^+ \rightarrow 6HCO_3^- + 5H_2S + 2H_2O \quad (1)$$

$$\Delta G^\circ{}_{pH7} = -102\ \text{kJ per mol propane or } -41\ \text{kJ per mol sulphate}$$

$$4C_4H_{10} + 13SO_4^{2-} + 10H^+ \rightarrow 16HCO_3^- + 13H_2S + 4H_2O \quad (2)$$

$$\Delta G^\circ{}_{pH7} = -138\ \text{kJ per mol } n\text{-butane or } -42\ \text{kJ per mol sulphate}$$

The indicated energy yields are far less than in the case of an oxidation with O_2, which would yield −2,094 kJ per mol propane and −2,727 kJ per mol *n*-butane.

Metabolites potentially indicative of the mode of activation of the short-chain hydrocarbons were searched for in the thermophilic enrichment culture with propane (Propane60-GuB) and strain BuS5. Anaerobic bacteria growing with higher alkanes are assumed to activate their substrates through homolytic C–H-bond cleavage at a subterminal carbon atom and addition to fumarate, yielding (1-methylalkyl)succinates[5,6,15]. Upon growth with propane, however,

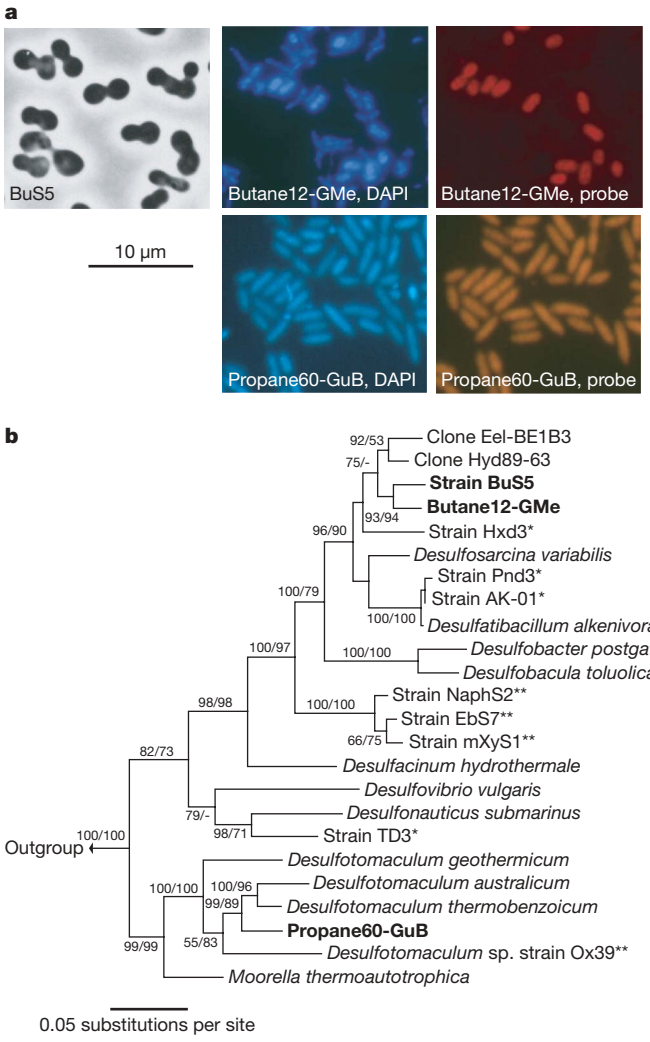

Figure 1 | Microscopy and phylogenetic analysis. **a**, Left, strain BuS5 grown with butane at 28 °C, phase contrast; middle, enrichments grown with butane at 12 °C (Butane12-GMe, top) and with propane at 60 °C (Propane60-GuB, bottom), with DAPI fluorescence; right, the same preparations as in the middle, but with fluorescence of 16S rRNA-targeted probes. **b**, Maximum-likelihood tree (16S rRNA-based) with present cultures (in bold), other bacteria and uncultured environmental phylotypes. Degraders of higher alkanes (*) and aromatic hydrocarbons (**) have been marked. Numbers indicate bootstrap values (>50%) from distance (first number) and parsimony (second number) analyses. See also Supplementary Fig. 2.

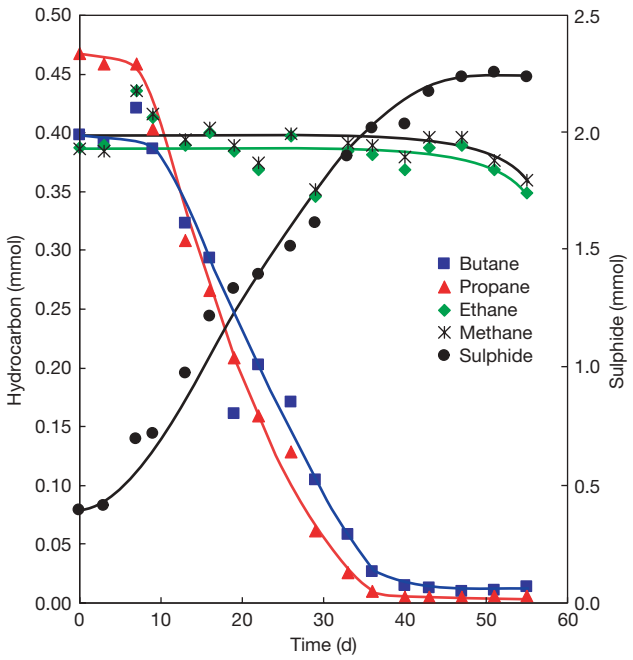

Figure 2 | Time course of the anaerobic consumption of propane and butane by a mesophilic strain. Strain BuS5 (200 ml culture, 52 ml gas space) consumes propane and *n*-butane simultaneously from a gas mixture and forms sulphide from sulphate. Methane and ethane were not degraded, but show a slight abiotic loss also observed in sterile controls. (For measurements with other cultures, see Supplementary Figs 4 and 5.)

we identified *n*-propylsuccinate in both cultures in addition to the expected isopropylsuccinate (Supplementary Fig. 6). This finding suggests two routes for propane, one starting with the common activation at the secondary carbon atom and another with the unprecedented activation at the primary carbon atom (Fig. 3). So the microorganisms in the slow, early-state enrichment with ethane (which has only primary carbon atoms) could in principle make use of the reaction principle involving fumarate. Activation at a primary carbon atom has to overcome a higher energetic barrier than activation at a secondary carbon (Supplementary Table 3), such that the former reaction may be the slower one. This assumption would offer an explanation for the observed very slow development of the more 'difficult' ethane oxidation in comparison to propane and butane oxidation. If strain BuS5 was grown with *n*-butane, only (1-methylpropyl)succinate was detected as activation product; it apparently occurred in diastereomers (data not shown), as the activation products of higher alkanes[5,15].

As the synthesis of cellular fatty acids in many alkane-degrading bacteria starts with a building block derived from the alkane

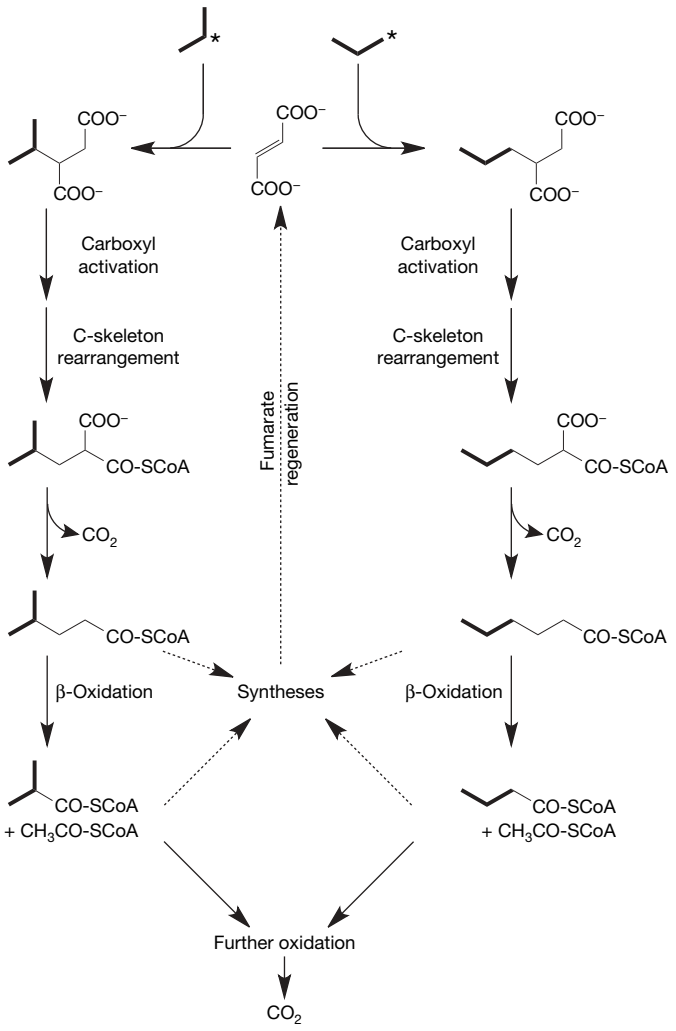

Figure 3 | Anaerobic activation reactions of propane suggested by identified metabolites. Whereas previous studies on the anaerobic degradation of alkanes suggested their activation (by homolytic C−H-bond cleavage) exclusively at a secondary carbon atom[5,6,15], the new cultures obviously activate propane at both the secondary and the primary carbon (marked with asterisks). The products from reaction with fumarate, isopropylsuccinate and *n*-propylsuccinate were identified by chemical analyses including authentic standards (Supplementary Fig. 6). Subsequent reactions are suggested according to degradation schemes of other alkanes (for details see ref. 5 and Supplementary Fig. 7) and identified fatty acids (see text and Supplementary Table 4).

metabolism[5], we also analysed the lipid fraction of strain BuS5 to further substantiate the proposed activation mechanisms (Supplementary Table 4). Strain BuS5 indeed synthesized from propane mainly *iso*-tetradecanoic acid (*i*-14:0) and *iso*-hexadecanoic acid (*i*-16:0) and from butane mainly *anteiso*-pentadecanoic acid (*ai*-15:0). The structure of these fatty acids is in accordance with the proposed further metabolism of the activation products of propane and butane, isopropylsuccinate and (1-methylpropyl) succinate, respectively, leading to 4-methylpentanoyl-CoA and 4-methylhexanoyl-CoA, respectively, as starting molecules for fatty acid biosynthesis (Supplementary Fig. 7; CoA, coenzyme A). Signatures of the additional terminal propane activation are less clear in the fatty acid patterns because the resulting C-even *n*-fatty acid (Supplementary Fig. 7) would add to the regular (substrate-independent) background of these common types of fatty acids.

Finally, we also examined whether anaerobic alkane oxidation in the obtained cultures is associated with stable carbon isotope (^{12}C versus ^{13}C) fractionation; such fractionation *in situ* is regarded as an important indicator of biodegradation. Indeed, the cultures analysed so far preferentially used the isotopically lighter hydrocarbons. The isotope ratio of propane and butane in sterile controls remained unaffected. During propane degradation by the cold-adapted enrichment culture (no designation), the mesophilic strain BuS5, and the thermophilic enrichment culture (Propane60-GuB), we observed fractionation factors (α_C) of 1.0059, 1.0052 and 1.0059, respectively. During butane degradation, the cold-adapted enrichment culture (Butane12-GMe) and the mesophilic strain BuS5 both exhibited a fractionation factor of 1.0016 (Supplementary Table 5). The fractionation factor for the anaerobic oxidation of methane in marine sediments is between 1.0088 and 1.011 (refs 16−18). Obviously, the extent of isotopic discrimination decreases with the molecular size of the alkane substrate, as also observed during their aerobic degradation[19].

Our present findings offer an explanation at the organism level of several *in situ* phenomena previously attributed to an assumed biological oxidation of propane and butane. In the marine environment, such phenomena are, for instance: the obvious disappearance of ethane, propane and butane together with methane in mud volcanoes (Gulf of Cadiz)[10]; the ^{13}C-enriched propane and butane in sediment interstitial water in comparison to propane and butane in parental gas hydrates[9]; the carbonate alkalinity around gas hydrates (Gulf of Mexico) largely attributed to oxidation of propane and butane[20]; the insufficient explanation of bulk sulphate reduction at gas seeps (Gulf of Mexico, Guaymas basin) solely by methane oxidation, and postulation of other hydrocarbons serving as electron donors[11,21]; the formation of so-called dry gas[8] (methane-enriched) caps above biodegraded oil rims due to biodegradation of short-chain (non-methane) hydrocarbons, in particular propane and butane[22]; and the suspected nourishment by short-chain hydrocarbons of microbial communities in serpentinite-hosted hydrothermal systems, such as the Lost City hydrothermal field[23]. The activities of such organisms could potentially also lead to destabilization of structure II gas hydrates[20]. On a global scale, these microbial activities may influence the emission of non-methane hydrocarbons from the oceans[24]. Our culture data also substantiate findings in terrestrial environments, such as detection of metabolites of short-chain hydrocarbons in polluted aquifers[6] and the supposed support by short-chain hydrocarbons of a deep subsurface community that included molecular signatures of *Desulfotomaculum*[25]. A growing number of studies also indicates the preferential degradation of propane and butane in subsurface gas reservoirs, a process that would render the geochemical composition-based evaluation of the gas origin and migration more difficult[7,8]. According to our cultures, phylogenetically and phenotypically diverse SRB belonging to the Deltaproteobacteria and Gram-positive bacteria are able to utilize propane and butane. In view of the limitations of cultivation techniques, an even greater diversity can be

expected to be responsible for oxidation of short-chain hydrocarbons *in situ*.

METHODS SUMMARY

Anoxic sediment samples were collected at hydrocarbon seep areas in the Gulf of Mexico at 550 m water depth and the Guaymas basin (Gulf of California) at 2,000 m water depth.

Cultures were enriched and grown at 12, 28 or 60 °C anaerobically in defined synthetic seawater medium[5] with 28 mM sulphate in stoppered bottles (usually 100 or 200 ml) or tubes (20 ml) under an anoxic atmosphere (one-third of the bottle volume) containing the hydrocarbon gas, nitrogen and 10% CO_2. For subcultivation, 10% of the culture was transferred to fresh medium of the same composition. The pure culture of strain BuS5 was obtained via dilution in anoxic agar tubes with butane in the head space.

Isolation of DNA and sequencing of amplified 16S rRNA genes (~1,500 bp) was performed according to standard protocols. Sequences were phylogenetically analysed using the ARB software[26–28]. The phylogenetic tree was inferred from maximum likelihood analysis.

Fluorescence whole-cell hybridization with specifically designed Cy3-labelled oligonucleotide probes (Supplementary Table 1) and determination of total cell counts using DAPI was carried out as described[29].

Sulphide was quantified colorimetrically in a reaction yielding methylene blue. Gaseous hydrocarbons were quantified using a gas chromatograph with a flame ionization detector. Metabolites were extracted, converted to methyl esters and analysed by gas chromatography–mass spectrometry[15]. The included standards of *n*-propylsuccinic and isopropylsuccinic acid methyl esters were synthesized using the respective alkylmercuric acetates, fumaric acid dimethyl ester and sodium borohydride. Bacterial fatty acids were analysed as methyl esters by gas chromatography–mass spectrometry. Carbon isotope ratios of propane and butane were analysed through coupling of gas chromatography, combustion and isotope ratio mass spectrometry.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Information The nucleotide sequences have been deposited at EMBL, GenBank and DDBJ under accession numbers EF077225 (strain BuS5), EF077226 (enrichment culture 'Butane12-GMe'), EF077227 (enrichment culture 'Propane60-GuB') and EF077228 (enrichment culture with butane at 60 °C). Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to F.W. (fwiddel@mpi-bremen.de).

METHODS

Sediment samples. Anoxic sediment samples from marine hydrocarbon seep areas were collected with research submersibles. Samples from the Gulf of Mexico were collected in the Green Canyon area at 550 m depth at sites GC232 (27° 44.4566′ N, 91° 18.981′ W) and GC234 (27° 44.7003′ N, 91° 13.3093′ W) during a cruise in July 2002 on board the RV *Seward Johnson II* (Harbor Branch Oceanographic Institution). Samples from Guaymas basin (Gulf of California) were collected during *Alvin* dive 3203 at 2,000 m depth at station 1 (27° 00.762′ N, 111° 24.656′ W) during a cruise in April/May 1998 on board the RV *Atlantis* (Woods Hole Oceanographic Institution; detailed information about the sampling site is available elsewhere[31]). Samples were stored anoxically under N_2 at 6 °C until use.

Enrichment, isolation and cultivation. Cultures were grown at 12, 28 or 60 °C in anoxic synthetic seawater medium[5,32] (CO_2/bicarbonate as buffer; sodium sulphide as reductant) with 28 mM sulphate in butyl-rubber-stoppered bottles (100–200 ml) or tubes (20 ml) under an anoxic atmosphere (one-third of bottle volume) containing the hydrocarbon gas, N_2 and 10 vol.% CO_2. For subculturing, 10% of the culture liquid was transferred. A pure culture of strain BuS5 was obtained via dilution and colony isolation using anoxic agar tubes[32] with butane in the head space.

Analyses of 16S rRNA genes. Isolation of DNA and sequencing of amplified 16S rRNA genes (~1,500 bp) was performed according to standard protocols. Sequences were aligned with those of the database of the Technical University Munich using the ARB program package[26]. Aligned sequences were imported into PAUP version 4.0b10 for phylogenetic analysis[27]. Models for minimum evolution and maximum likelihood searches were chosen using the likelihood ratio test in the MODELTEST program version 3.5 (ref. 28), with application of the GTR+I+G model. The tree was inferred from maximum likelihood analysis by heuristic searches using five random addition replicates with TBR branch swapping. Bootstrapping of minimum evolution and maximum parsimony analyses included 1,000 bootstrap replicates, each with 10 random additions.

Fluorescence hybridization. Fluorescence whole-cell hybridization with Cy3-labelled oligonucleotide probes (Biomers.net) and determination of total cell counts using DAPI (4′,6-diamidino-2-phenylindole) were carried out as previously described[29]

Probe But5-620 developed for the parental enrichment culture of the mesophilic strain BuS5 (Supplementary Fig. 3) has at least one mismatch to available 16S rRNA sequences. *Desulfococcus biacutus*, which has one mismatch, yielded only a weak signal with this probe at 50−60% formamide. *Desulfosarcina variabilis*, *Desulfofaba gelida*, and the dominant phylotype in enrichment with butane at 12 °C (Butane12-GMe), which have two mismatches, did not show signals at >10% formamide. A second probe, But5-476, substantiated the hybridization of strain BuS5 in the parental enrichment culture (not shown). Probe But5-476 is specific for strain BuS5 and its closest relative, an environmental sequence from the Gulf of Mexico (GenBank accession number AY324498; 98.3% sequence similarity). *Desulfovibrio longus*, which has two mismatches, did not show a signal with probe But5-476 at >10% formamide.

Probe K2-3-190 developed for the enrichment culture with propane at 60 °C (propane60-GuB) has at least two mismatches to available sequences. The sequences AB088961 and AF287791 (GenBank accession numbers), which have two mismatches, are from uncultivated phylotypes. *Desulfotomaculum thermobenzoicum*, which has three mismatches, did not show a signal.

Probe But12-1275 developed for the enrichment cultures with butane at 12 °C, (butane12-GMe) has at least two mismatches to available sequences. Use of the probe for this culture at 0%, 10% and 20% formamide revealed the same proportion of hybridizing cells, indicating that there were no other organisms with highly similar target regions.

The antisense probe EUB338 never yielded a signal with the presently investigated cultures, thus verifying the absence of unspecific probe binding.

Chemical analyses. Sulphide was quantified colorimetrically via reaction with N,N-dimethylphenylenediamine and subsequent oxidation yielding methylene blue[33] in a miniaturized assay[34].

Gaseous hydrocarbons were quantified on a GC14 gas chromatograph (Shimadzu) equipped with a Supel-Q PLOT (Sigma-Aldrich) fused silica capillary column (30 m × 0.53 mm) and a flame ionization detector. The carrier gas was N_2. Temperatures: column, 110 °C (isothermal); detector, 280 °C; injector, 150 °C. Standards were prepared in glass vials sealed with butyl stoppers. Volumes of 0.1 ml were withdrawn from the culture head space or the standard vials using an anoxic, gas-tight syringe (with a needle lock). Amounts of gases dissolved in the aqueous medium were taken into consideration. The partial-pressure-dependent (minor, in our experiments) dissolved proportions were calculated from determined partial pressures using molar solubility constants ($M\,atm^{-1}$): propane, 1.3×10^{-3} (30 °C) and 0.74×10^{-3} (60 °C); butane, 2.3×10^{-3} (10 °C), 1.0×10^{-3} (30 °C) and 0.52×10^{-3} (60 °C).

Metabolites were extracted with dichloromethane, converted to methyl esters and analysed by gas chromatography–mass spectrometry as described previously[16]. The included standards of *n*-propylsuccinic and isopropylsuccinic acid methyl esters were synthesized using the respective alkylmercuric acetates, fumaric acid dimethyl ester and sodium borohydride[35–37].

Cellular fatty acids were analysed as methyl esters by gas chromatography–mass spectrometry. Fatty acid methyl esters were obtained from ~20 mg cell dry mass by extraction and transesterification with trimethylchlorosilane/methanol (1/8, vol./vol.; 2 h, 70 °C), and re-extraction with *n*-hexane versus H_2O. Compounds were identified by comparison of the gas chromatographic equivalent chain lengths with those of an authentic fatty acid methyl ester mixture (37 components FAME mix; Sigma-Aldrich) as standard.

Carbon isotope ratios of propane and butane were analysed by coupling of gas chromatography, combustion, and isotope ratio mass spectrometry. Hydrocarbon gases were injected into a cooled injection system 4 (CIS4; Gerstel) operated in splitless mode. The injection system, which was packed with 10 mg PoraPak Q (80–100 mesh; Varian) was cooled to −100 °C for trapping and subsequently heated to 100 °C at 12 °C s^{-1} for desorption. The HP6890 gas chromatograph (Hewlett Packard) was equipped with a CP PoraPLOT Q capillary column (25 m × 0.32 mm, 10 μm film thickness; Varian). The temperature program was 30 °C (8 min), 5 °C min^{-1} to 150 °C (0 min), and 15 °C min^{-1} to 200 °C (5 min). The gas chromatograph was interfaced to a Delta^{Plus}XL mass spectrometer (Finnigan) equipped with a CuO/Ni/Pt combustion furnace operated at 940 °C. Fractionation factors (α_C) were calculated as described previously[18].

The DNA base ratio was determined at the DSMZ (Braunschweig, Germany) using a standard chromatographic technique.

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