Anaerobic oxidation of short-chain and cyclic alkanes by sulfate-reducing bacteria

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Zusammenfassung

Lange Zeit wurde angenommen, dass hauptsächlich die anaerobe Oxidation von Methan (AOM) für den Kohlenstoff- und Schwefelkreislauf in anaeroben Sedimentschichten kalter, geologischer Kohlenwasserstoffquellen verantwortlich ist. Neuere Untersuchungen an marinen Öl- und Gasquellen haben gezeigt, dass neben Methan auch der anaerobe biologische Abbau von anderen Kohlenwasserstoffen, wie z.B. Propan und *n*-Butan, durchaus eine wichtige Rolle für diese Stoffkreisläufe in der Tiefsee spielen kann.

Der erste und bislang einzige isolierte, anaerob Propan- und *n*-Butan-abbauende Sulfatreduzierer ist Stamm BuS5. Weiterhin sind mehrere Anreicherungskulturen bekannt, welche Ethan, Propan, *n*-Butan oder *n*-Pentan unter sulfatreduzierenden Bedingungen abbauen. Metabolitanalysen verschiedener Propan- und *n*-Butan-abbauender Kulturen zeigten, dass die anaerobe Aktivierung dieser *n*-Alkane über eine Fumarat-Addition am sekundären Kohlenstoffatom erfolgt. Zusätzlich wurde für Propan eine Aktivierung am primären Kohlenstoffatom beschrieben.

(1) Die vorliegende Arbeit beschreibt neue Propan- und *n*-Butan-abbauende Anreicherungskulturen aus Sedimenten kalter Kohlenwasserstoffquellen. Eine Propan-abbauende Anreicherungskultur entstammt dabei dem Golf von Mexiko (Prop12-GMe) und eine *n*-Butan-abbauende Anreicherungskultur dem Hydratrücken (But12-HyR). Beide Anreicherungskulturen waren in der Lage, Propan und *n*-Butan abzubauen, wohingegen Methan, Ethan, *iso*-Butan oder *n*-Pentan nicht als Substrate verwendet wurden. Die an den Abbau von Propan und *n*-Butan gekoppelte Sulfatreduktion war am höchsten zwischen 16 °C und 20 °C. Bei Temperaturen über 28 °C fand keine Sulfatreduktion mehr statt. In beiden Anreicherungskulturen wurden große Aggregate beobachtet, welche scheinbar hauptsächlich aus großen, ovalen Zellen zusammengesetzt waren. Es gelang, Teilsequenzen der großen Untereinheit der (1-Methyl)Alkylsuccinat-Synthase (*masD)* aus beiden Anreicherungskulturen und Stamm BuS5 zu amplifizieren und sequenzieren. Die phylogenetische Analyse dieser *masD*-Teilsequenzen zeigte zum einen, dass diese Sequenzen am ähnlichsten zueinander waren, und zum anderen eine große Ähnlichkeit zu *masD* Sequenzen des

thermophilen Stammes TD3 aufwiesen, welcher längerkettige *n*-Alkane anaerob abbauen kann. Die weitere phylogenetische Charakterisierung mittels molekularbiologischer Methoden ergab, dass beide Anreicherungskulturen hauptsächlich aus Bakterien bestanden, welche zu jeweils neuen und distinkten Phylotypen innerhalb der *Desulfosarcina/Desulfococcus*-Gruppe der *Deltaproteobakterien* gehören. Um die Funktion dieser Phylotypen in den beiden Anreicherungskulturen und in einer weiteren *n*-Butan-abbauenden Anreicherungskultur (But12-GMe) zu untersuchen, wurden diese mit ^{13}C-markiertem Propan oder *n*-Butan inkubiert und anschließend mittels nanoSIMS (nanoscale secondary ion mass spectrometer) analysiert. Die primäre Anreicherung von ^{13}C in den dominanten Phylotypen ließ darauf schließen, dass diese für den Abbau von Propan oder *n*-Butan verantwortlich sind.

(2) Der anaerobe Aktivierungsmechanismus von Propan und *n*-Butan wurde mithilfe von ″Compound-specific stable isotope analysis″ (CSIA) in Stamm BuS5 und drei Anreicherungskulturen untersucht. Während des biologischen Abbaus von Propan oder *n*-Butan wurden zweidimensionale Fraktionierungsfaktoren(Λ_{rp}) bestimmt, welche sich aus der Korrelation der reaktiven, positionsspezifischen Kohlenstoff- und Wasserstoff-Fraktionierungen ergaben. Unterschiedliche Kohlenstoff- und Wasserstoff-Fraktionierungen wurden nicht nur in Abhängigkeit des Substrats (Propan oder *n*-Butan), sondern auch in Abhängigkeit der Kultivierungsbedingungen (geschüttelt, nicht geschüttelt) beobachtet. Dieser Effekt war weniger ausgeprägt in Kulturen, welche Aggregate bildeten. Dies deutet darauf hin, dass die Diffusion der beiden gasförmigen Substrate aus der Gas- in die Flüssigphase, bei einer statischen Inkubation oder bei hohen Zelldichten, zur Substratlimitation führt, welche die Isotopenfraktionierung maskiert. Die ermittelten Λ_{rp} Werte aus geschüttelten, planktonisch wachsenden (nicht-diffusionslimitierten) Kulturen waren denen für längere *n*-Alkane, welche durch Fumarat-Addition aktiviert werden, sehr ähnlich. Jedoch waren sie während des Abbaus von Propan höher, als während des Abbaus von *n*-Butan. Experimente mit deuterierten Alkanen (D_2-, D_4-Propan; D_2-,D_6-Butan) und Stamm BuS5 ergaben, dass an beiden Alkanen eine Fumarat-Addition am sekundären Kohlenstoffatom stattfindet und zusätzlich am primären Kohlenstoffatom von Propan. Dies könnte die beobachteten höheren Λ_{rp} Werte für den anaeroben Abbau von Propan erklären.

(3) Der anaerobe Aktivierungsmechanismus von Ethan und die Phylogenie der ethanabbauenden Mikroorganismen sind nicht bekannt. In dieser Arbeit wurde die bisher einzige, anaerob Ethan-abbauende Anreicherungskultur auf ihre phylogenetische Zusammensetzung untersucht. Ganzzellhybridisierungen mit Archaeen- und Bakterien-spezifischen Gensonden ergab, dass sich diese Anreicherungskultur zu etwa 10% aus Archaeen und 90% Bakterien zusammensetzt. Eine 16S rDNS-Klonbank dieser Anreicherungskultur ergab, dass die meisten Sequenzen zu Phylotypen innerhalb der *Desulfosarcina/Desulfococcus*-Gruppe der *Deltaproteobakterien* clustern. Ganzzellhybridisierung mit einer *Desulfosarcina/Desulfococcus*-spezifischen Gensonde zeigte, dass etwas 60% aller Zellen der Anreicherungskultur zu dieser Gruppe gehören.

(4) Cycloalkane sind wichtige Bestandteile von Rohöl und biologisch schwer abbaubar. Es gibt nur wenige Studien über den aeroben Abbau von Cycloalkanen und noch weniger ist über deren anaeroben Abbau bekannt. Einige dieser Studien haben gezeigt, dass die Aktivierung durch eine Fumarat-Addition stattfindet und Cycloalkylsuccinate entstehen. In dieser Arbeit wurden anaerob Cyclohexan-abbauende Bakterien aus marinen, stark Kohlenwasserstoff-belasteten Sedimenten angereichert. Quantitative Wachstumsexperimente zeigten, dass Cyclohexan komplett zu CO_2 oxidiert wurde. Die Anreicherungskultur bestand zu 80% aus einem neuen Phylotypen, welcher zur *Desulfosarcina/Desulfococcus*-Gruppe der *Deltaproteobakterien* gehört. Experimente mit dichten Zellsuspensionen zeigten, dass die Anreicherungskultur nicht nur verschiedene Methyl-substituierte und unsubstituierte Cycloalkane abbauen kann, sondern auch C_4–C_6 *n*-Alkane. Die relative Abundanz des dominanten Phylotyps blieb während des Experiments stabil, was darauf hindeutete, dass dieser Phylotyp für den Abbau der verschiedenen Alkane verantworlich sein könnte. Die Bildung von Cyclohexylsuccinat in Cyclohexan-gewachsenen Kulturen zeigt, dass die Aktivierung von Cyclohexan in Analogie zum Abbau von *n*-Alkanen und einigen aromatischen Verbindungen über Fumarat-Addition erfolgt. Weitere gemessene Metabolite wie 3-Cyclohexylpropansäure und Cyclohexancarbonsäure deuten darauf hin, dass auch der weitere Abbau ähnlich dem der *n*-Alkane erfolgt.

Summary

The anaerobic oxidation of methane (AOM) has been assumed for a long time to be the dominant process at marine cold hydrocarbon seeps and therefore to mainly be responsible for the carbon and sulfur cycles in anaerobic sediments at these sites. Evidence from marine oil and gas seepages recently indicated that anaerobic biodegradation of non-methane hydrocarbons, such as propane and *n*-butane, may also be an important process influencing the cycling of carbon and sulfur at these sites.

To date, little is known about the microorganisms able to degrade C_2–C_4 *n*-alkanes under anaerobic conditions, and hitherto, strain BuS5 is the only anaerobic propane- and *n*-butane-degrading, sulfate-reducing isolate existing in pure culture. Some sulfate-reducing enrichment cultures were recently reported to degrade ethane, propane, *n*-butane or *n*-pentane. Analysis of metabolites revealed that propane and *n*-butane are activated by fumarate addition to the secondary carbon atom. Notably, unprecedented activation at a primary carbon atom has been described for propane.

(1) New propane- and *n*-butane degrading, sulfate-reducing enrichment cultures were obtained from sediments of cold marine hydrocarbon seeps. A propane-degrading enrichment culture (Prop12-GMe) was obtained from the Gulf of Mexico and an *n*-butane-degrading enrichment culture (But12-HyR) from Hydrate Ridge. Both enrichment cultures were able to degrade propane and *n*-butane, but were unable to utilize methane, ethane, *iso*-butane or *n*-pentane as substrates. The reduction of sulfate coupled to anaerobic degradation of propane and *n*-butane was fastest between 16 °C and 20 °C, whereas above 28 °C sulfate reduction ceased. In both enrichment cultures conspicuous aggregates were observed, apparently dominated by large, oval cells. Parts of the gene encoding the large subunit of (1-methylalkyl)succinate synthase (*masD*) have been amplified and sequenced from the cold-adapted enrichments. Phylogenetic analysis of *masD* partial sequences showed that they apparently formed a cluster with *masD* sequences from strain BuS5 and the thermophilic, long-chain alkane-degrading strain TD3. Characterization by molecular methods showed that each enrichment culture was dominated by a unique phylotype affiliated with the *Desulfosarcina/Desulfococcus*-cluster within the *Deltaproteobacteria*. To determine the role of the dominant microorganisms in the degradation of propane and *n*-butane, the newly obtained enrichment cultures, as well

as the previously described enrichment culture utilizing *n*-butane (But12-GMe; Kniemeyer *et al*., 2007) were incubated with ^{13}C-labeled propane or *n*-butane and analyzed by nanoSIMS (nanoscale secondary ion mass spectrometer). The analysis at single cell level showed that, in each enrichment culture, the dominant cells were the first to become enriched in ^{13}C, demonstrating that they were directly involved in alkane degradation.

(2) The anaerobic activation mechanisms of propane and *n*-butane by strain BuS5 and three sulfate-reducing enrichment cultures from marine hydrocarbon seeps were investigated by compound-specific stable isotope analysis (CSIA). Two-dimensional isotope fractionation factors, given by the correlation of reactive position-specific hydrogen and carbon fractionation (Λ_{rp}), were obtained during biodegradation of propane and *n*-butane in these cultures. Λ_{rp} values obtained were higher for propane than for *n*-butane. Differences in Λ_{rp} values were observed depending on the incubation conditions, i.e. in non-aggregate forming cultures, bulk carbon and hydrogen enrichment factors were significantly higher when cultures were shaken. This effect was less pronounced in aggregate-forming cultures, indicating that isotope fractionation was partially masked by substrate limitation, due to impaired headspace-liquid diffusion rates of the short-chain alkanes and high cell densities in aggregates. Obtained Λ_{rp} values from shaking incubations of cultures not forming aggregates, which were assumed to be not substrate-limited, were in the same range as those reported for other *n*-alkanes activated by fumarate addition. Experiments with deuterium-labeled alkanes (D_2-,D_6-propane; D_4-,D_6-butane) indicated that strain BuS5 activates *n*-butane by adding fumarate solely to the secondary carbon, while propane could be activated at the primary and secondary carbon atom. This could explain the observed higher Λ_{rp} values for propane.

(3) Anaerobic degradation of ethane was shown in one sulfate-reducing enrichment culture, obtained from sediments at cold hydrocarbon seeps in the Gulf of Mexico. To date, the activation mechanism of ethane and involved microorganisms are unknown. In this study, the phylogenetic composition of this enrichment culture was investigated. Whole-cell hybridization with kingdom- and group-specific oligonucleotide probes revealed that the enrichment culture contains ~ 10% archaea and ~ 90% of bacteria. The majority of sequences generated from a 16S rDNA clone library was affiliated with the *Desulfosarcina*/*Desulfococcus*-group within the

Deltaproteobacteria. Whole-cell hybridization with a *Desulfosarcina*/*Desulfococcus*-specific oligonucleotide probe revealed that ~ 60% of all cells in the enrichment culture belong to this group.

(4) Cycloalkanes are major constituents of crude oil and they are rather recalcitrant to biological degradation. Only few studies have reported the aerobic degradation of cycloalkanes and even less is known about their anaerobic biodegradation. Some of these studies showed that activation takes place through an addition to fumarate, yielding cycloalkylsuccinates. In this study, cyclohexane-degrading, sulfate-reducing bacteria were enriched from hydrocarbon-contaminated intertidal marine sediments. Quantitative growth experiments showed that cyclohexane was completely oxidized to CO_2. The obtained enrichment culture was dominated by a *Desulfosarcina/Desulfococcus*-group affiliated phylotype, which comprised ~ 80% of all cells. Incubations of dense cell suspensions showed degradation of C_4–C_6 *n*-alkanes and methyl-substituted and unsubstituted C_5- and C_6- cycloalkanes. During these incubations the abundance of the dominant phylotype remained constant, suggesting that this phylotype is capable of degrading a rather diverse spectrum of hydrocarbon compounds. GC-MS analysis of culture extracts during growth on cyclohexane detected cyclohexylsuccinate as a metabolite, indicating that the activation involves a carbon-carbon addition to fumarate, analogous to the activation of *n*-alkanes and some aromatic compounds. Other metabolites detected were cyclohexylpropionic acid and cyclohexylcarboxylic acid, suggesting that cyclohexylsuccinate is further degraded through enzymatic reactions similar to those of *n*-alkanes.

Part I. Anaerobic oxidation of short-chain and cyclic alkanes by sulfate-reducing bacteria

A. Introduction

A.1 Hydrocarbons

Hydrocarbons are composed solely of carbon and hydrogen atoms, which are connected through covalent bonds. Due to a similar electronegativity of carbon and hydrogen, none of the bonding atoms bear a significant charge, making the overall molecule rather inert. Hydrocarbons can occur in many different variations, i.e. numbers of carbon and hydrogen atoms and structural conformations. Generally, hydrocarbons can be divided into the three major compound classes of saturated, unsaturated and aromatic hydrocarbons. Since the main focus of this thesis is on *n*-alkanes and cycloalkanes, this introductory section focuses only on the saturated hydrocarbons. In saturated hydrocarbons, the carbon atoms are connected exclusively by σ-bonds, while the unsaturated hydrocarbons contain conjugated double or triple bonds.

A.1.1 *n*-Alkanes

Saturated hydrocarbons are commonly referred to as alkanes and they have the general sum formula C_nH_{2n+2}. They are very unreactive, due to the absence of functional or polar groups and the strong σ-bonds between the bonding carbon and hydrogen atoms. Since carbon atoms can form four covalent bonds and hydrogen only one, there is only one possible structure for alkanes with less than four carbon atoms (methane, ethane and propane). These alkanes can only occur as one straight chain of carbon atoms without branches, commonly referred to as *n*-alkanes. Alkanes with four or more carbon atoms can occur in different constitutional conformers. Aside from occurring as *n*-alkanes, their carbon atoms may be connected in a different order, leading to the formation of branched alkanes. The number of constitutional isomers of alkanes increases exponentially with the number of carbon atoms in the alkane molecule. There are only two isomers of the molecular formula C_4H_{10}, the *n*-alkane

butane and the one possible branched isomer *iso*-butane (2-methyl-propane). There are already three isomers of the molecular formula C_5H_{12} - the *n*-alkane pentane, the isomer *iso*-pentane and the isomer *neo*-pentane, containing a quaternary carbon atom (Fig.1). Overall, the degree of branches in one molecule will affect the physical properties of the isomer, i.e. the more branches occur in one alkane molecule, the more compact the shape will be, thus decreasing the susceptibility of branched alkanes to enzyme attacks.

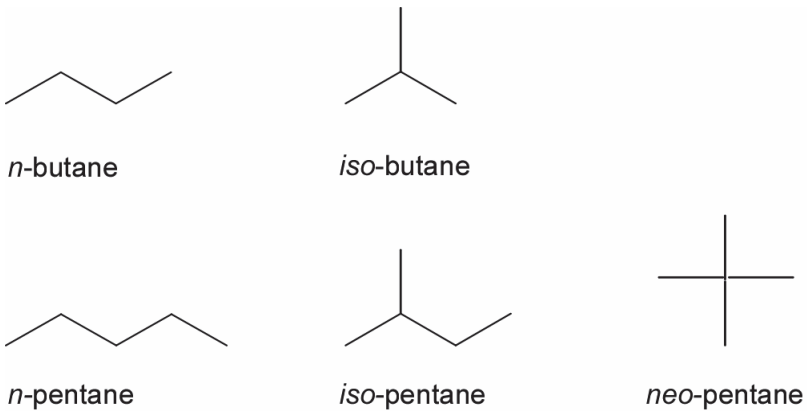

Fig.1 Structures of linear and branched C_4- and C_5- alkanes

Aside from branched isomers, alkanes consisting of three or more carbon atoms can also occur as cycloalkanes, where the carbon atoms are arranged in a ring. Consequently, cycloalkanes have two fewer hydrogen atoms, thus their general sum formula is C_nH_{2n}. Cycloalkanes with alkyl substitutents are referred to as alkylcycloalkanes (Wilkes and Schwarzbauer, 2010). Ideally, the tetrahedral bonds of cycloalkanes should, as in any alkane molecule, be arranged in angles of 109.5°, due to their sp^3-hybridized carbon atoms (Bruice, 2004). However, this angle can be smaller due to the fact that cycloalkanes are, with the exception of cyclopropane, not planar molecules but twist and bend in order to attain a structure where angle strain, torsional strain and steric strain are minimal (Bruice, 2004). It is assumed that the stability of cycloalkanes is dependent on the total strain energy (angle strain + torsional strain + steric strain). The low total strain energy of cyclohexane (0 kJ mol^{-1}) and also that of cyclopentane (6.2 kJ mol^{-1}) indicates their rather high stability and this is also reflected in the high natural abundance in crude oils (Tissot and Welte, 1984). On the other hand, cycloalkanes with less ideal bond angles, such as cyclobutane (90°) and cyclopropane (60°) for example have much higher total strain

energies (cyclobutane: 110 kJ mol^{-1}; cyclopropane: 114 kJ mol^{-1}) and are thus less stable.

A.1.2 Reactions of alkanes

Since alkanes are lacking polar groups, reactions with nucleophiles or electrophiles involving heterolytic C-H or C-C bond cleavage are rather unlikely. Thus, most of the known reactions of alkanes are mediated by radical species (unpaired electrons) via homolytic C-H cleavage, leading to the formation of alkyl radicals. Such homolytic bond cleavage requires the input of a significant amount of energy, also referred to as the homolytic C-H bond dissociation energy (ΔH^0). The C-H bond dissociation energy for a homolytic cleavage depends on the stability of the formed radical (Wilkes and Schwarzbauer, 2010). Thus, more energy is required for homolytic cleavage of a C-H bond at a primary carbon atom than it would be the case at a secondary carbon atom or a tertiary carbon atom. For example, the ΔH^0 to cleave one of the four equal C-H bonds of methane is 440 kJ mol^{-1} and that for one of the six equal C-H bonds of ethane is 411 kJ mol^{-1}. The ΔH^0 to cleave one of the three equal C-H bonds at each of the two primary carbon atoms of propane is 410 kJ mol^{-1}, whereas a homolytic cleavage of one of the two C-H bonds of the secondary carbon atoms of propane is 398 kJ mol^{-1} (McMillen and Golden, 1982). The radical reactions involving alkanes include pyrolysis (fragmentation of large hydrocarbon compounds to lower molecular weight hydrocarbon during catagenesis), combustions (complete oxidation to CO_2), substitutions (e.g. halogenation), addition to double bonds (polymer synthesis) and intramolecular rearrangements (biosynthesis of RNA) (Bruice, 2004).

A.1.3 Physical properties of alkanes

The physico-chemical properties of alkanes, as it is the case for all hydrocarbons, are directly related to the molecular structures, such as size and shape. Very generally spoken, the density, viscosity, melting points, boiling points, vapour pressure and solubility in water depend on the number of carbon atoms in the alkane molecules. The density, as well as the viscosity of alkanes, increases with increasing carbon numbers. With increasing temperatures, the viscosity decreases, which is the reason

why some heavy crude oils become more viscous or even solid upon retrieval from the original, deep, hot reservoir (Wilkes and Schwarzenbauer, 2010). As shown in Fig.2 A, the boiling points of *n*-alkanes increase with increasing number of carbon atoms. Alkanes with < 4 carbon atoms will be in a gaseous state at atmospheric pressure and room temperature, due to their low boiling points.

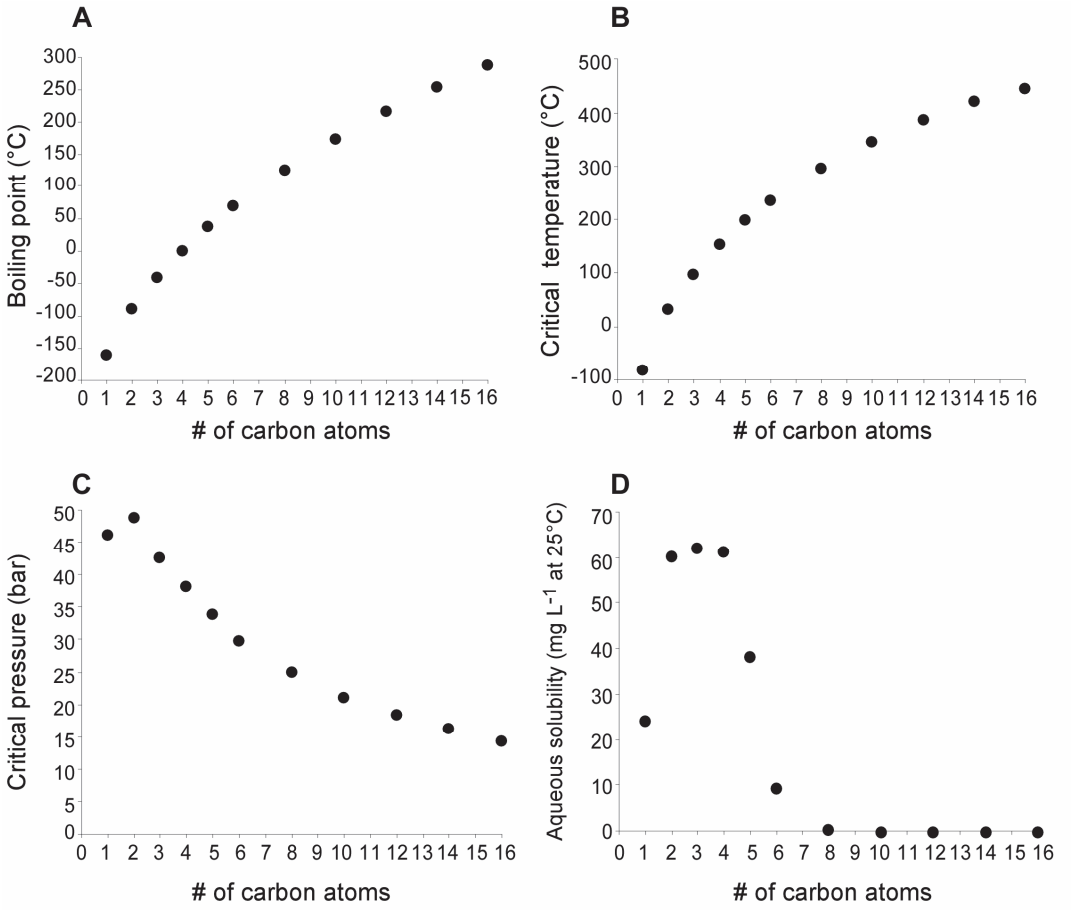

Fig.2 Compilation of boiling points (A) at atmospheric pressure, critical temperatures (B), critical pressures (C) and aqueous solubilities of *n*-alkanes with up to 16 carbon atoms at 25 °C (D). Data for boiling points, critical temperature and pressure are taken from Lide, 1993. Data for aqueous solutions are from Bell, 1973. Note that the aqueous solutions for gaseous *n*-alkanes in panel D were calculated by the authors as if the pressure was equal to the vapour pressure (solubility at 1 atm × vapour pressure)

The graphs depicting the critical temperatures (Fig.2 B) and pressures (Fig.2 C) for *n*-alkanes show that, if temperature is lowered and pressure increased, the *n*-alkanes with < 4 carbon atoms transform into the liquid state. The critical temperature is the temperature at and above which vapour of a substance cannot be

liquefied, no matter how much pressure is applied. The vapour pressure of a substance at the critical temperature is the critical pressure. It should be noted that in the case of methane the temperature would have to be lowered to –82.7 °C in order to truly liquefy the compound at all and that at the same time a pressure of at least 46.1 bar would have to exist. The aqueous solubility of *n*-alkanes > C_6 is negligible at 25 °C, whereas the gaseous alkanes are still relatively soluble (Fig.2 D). The solubility of gaseous *n*-alkanes (< C_4) increases at lower water temperature and elevated pressure. The solubility of liquid *n*-alkanes (> C_5) increases with increasing temperature. Overall, the aqueous solubility of hydrocarbons decreases with increasing salinity. It should be kept in mind that the compound-specific solubility values and also phase behaviours are furthermore altered, if mixtures of hydrocarbons occur, such as it is naturally the case in oil and gas reservoirs (Katz, 1959; Dhima *et al.,* 1998).

A.1.4 Formation of natural oil and gas

Cycloalkanes and *n*-alkanes occur as constituents of crude oil, accounting for up to 60% of total hydrocarbons (Tissot and Welte (1984) and they are also constituents of natural gas (C_1–C_4). The formation of crude oil and natural gas from organic matter is mainly a geological process, which happens over long time scales (5−100 million years, Tissot and Welte, 1984). In the marine environment, it starts already in the photic zone of the ocean at coastal upwelling areas, where nutrient concentrations are high. Due to relatively high sedimentation rates of organic matter from these areas down to the seabed, the formation of large, organic rich source beds in the sediments is supported. The highest accumulations of organic matter in source beds will occur in places where the water depth and oxygen concentrations are low, conditions which at the same time also promote a fast burial of the organic matter (Libes, 2009). During diagenesis, which takes place at temperatures below 50 °C, microorganisms transform the bulk organic matter into kerogen, a highly complex mixture of organic molecules. This breakdown of complex biomolecules into kerogen is mainly coupled to denitrification (removal of N), sulfate-reduction (removal of S) and methanogenesis, since most of the deeper sediments are anoxic. The latter process leads to the formation of methane (termed ″dry gas″), which becomes entrapped in ice cages of structure I gas hydrates (Sloan *et al.,* 2003) or, if the production occurred below the

zone of methane hydrate stability, seeps upwards through the sediments to enter the ocean. During the process of catagenesis, the source rock becomes further buried due to the accumulation of more organic matter onto the seabed, and the organic matter in the deep buried source rock undergoes abiotic reactions under the influence of elevated temperature (60−160 °C) and pressure, transforming the kerogen into bitumen and later oil. Upon further burial, resulting in even higher temperature (160 °C−200 °C) and pressure, oil is further broken down by ″thermal cracking″ into low molecular mass alkanes (methane, ethane, propane and *n*-butane). The non-methane, short-chain alkanes can make up to 20% of the formed natural gas (Tissot and Welte, 1984), which is thus termed ″wet gas″.

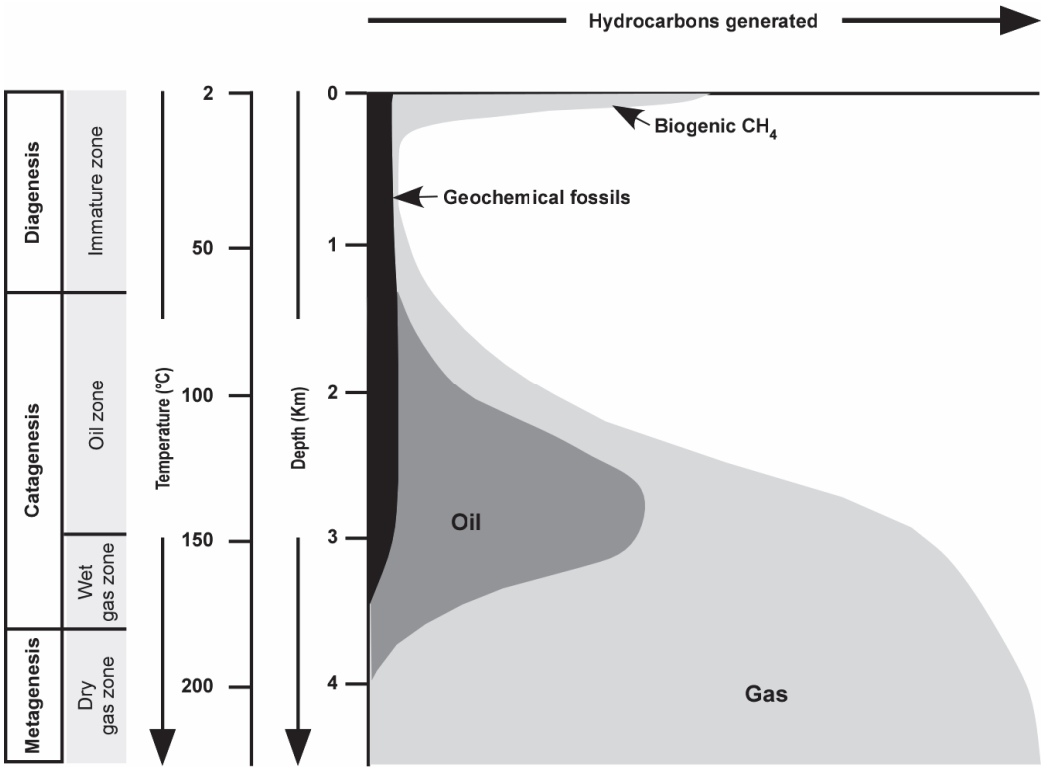

Fig.3 The relative quantities of hydrocarbons formed in masses of carbon in fine-grained sediments, indicated by the area under the curves. The depths and temperature intervals are estimates and can vary according to geological settings. Wet gas is comprised of methane, ethane, propane and butane. Dry gas is composed of only methane. The image is adapted and modified from Tissot and Welte (1984).

because they now take up more volume than solids, and the resulting fractures will facilitate the migration of the oil and gas compounds into adjacent, permeable rocks until an impermeable barrier is reached, usually a gas hydrate or salt dome. Here, the oil and gas pools in a trap, where the wet gas accumulates usually on top of the oil,

due to the lower molecular mass. The temperatures, at which oil and gas traps or reservoirs form, can be considerable lower than at the site of formation during catagensis, sometimes enabling microbial activity and thus breakdown and alteration of the oil and gas compounds.

A.2 Sources of hydrocarbons in the marine environment

A.2.1 Cold seeps and gas hydrates

As a result of salt tectonics at passive continental margins, fractures can occur in the impermeable rocks, gas hydrates or salt pillars, which seal oil and gas traps. The oil and gas components can leak out of the reservoir and migrate upwards through the sediments, finally entering the biosphere through cold hydrocarbon seeps on the ocean floor.

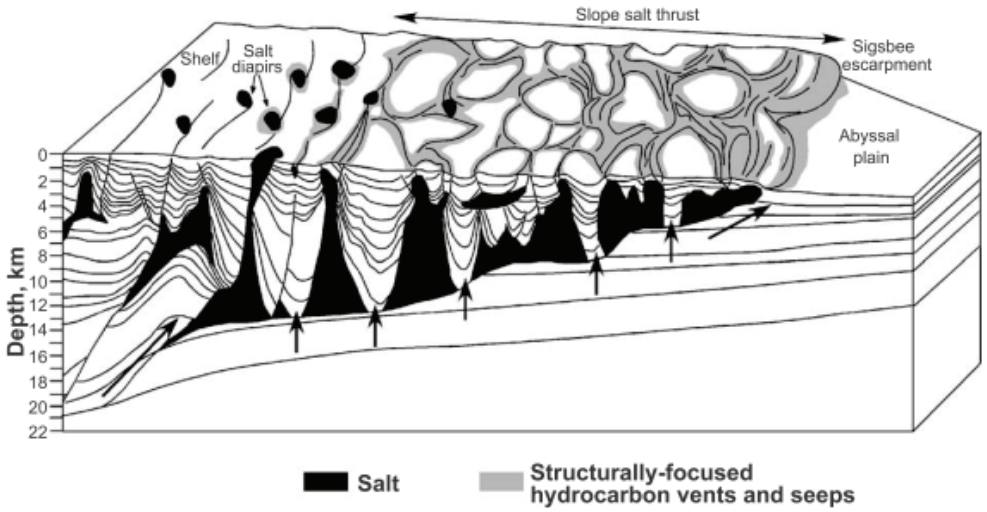

Fig.4 North-south cross section of the central Gulf of Mexico slope from the shelf to the abyssal plain. Within basins, salt and related faults provide conduits for vertical migration of fluids to reservoirs and to the seafloor. Hydrocarbon vents and seeps are focused by the structure near the rims of salt. Image adapted from Sassen *et al.* (2004).

The term ″cold seep″ is commonly used to describe a rather slow fluid flow and moderate temperatures, in contrast to the rapid venting at much higher temperatures of hydrothermal vents (smokers), which will be discussed in the next section. The Gulf of Mexico is a site where over 200 cold seeps have been discovered

(Mac Donald *et al.,* 2002), which is mainly attributed to the fact that the large petroleum and gas reservoirs present in this area are affected by massive salt domes and salt diapirs from evaporate-deposits, that underlie the petroleum reservoirs (Roberst *et al.,* 1997; MacDonald *et al.,* 2004). The presence of these salt diapirs creates a growth-fault network that provides conduits for the transport of fluids and hydrocarbons to the ocean floor (see Fig.4).

The hydrocarbons that reach the ocean floor can be free hydrocarbon gas that seeps out of the sediment or liquid hydrocarbons that form pertinacious slicks on the sea floor (Fig.5 A). Free hydrocarbon gas can become entrapped in ice cages, forming gas hydrates (solid) if the pressure is high (> 60 bar) and the temperatures are low (< 4 °C) (Fig.5 B). Cold seeps, venting mainly gaseous hydrocarbons are often referred to as ″gas seeps″ or ″mud volcanoes″, whereas cold seeps venting more heavy hydrocarbons are referred to as ″hydrocarbon seeps″ (Aharon, 1994) or ″asphalt volcanoes″ (Fig.5 C), if solidified asphalt is present (MacDonald *et al.,* 2004). It has been shown in many studies that cold gas seeps (Anderson *et al.,* 1983; Sassen *et al.,* 2004; Orcutt *et al.,* 2004) and also gas hydrates (Sassen *et al.,* 2004; Orcutt *et al.,* 2004; Hester & Brewer, 2009) in the Gulf of Mexico can contain significant amounts of non-methane, short-chain alkanes such as ethane (up to 30%), propane (13%) and *n*-butane (3.7%), indicating a thermogenic origin of these hydrocarbon gases (Sassen *et al*., 2004). Cold seeps can also form along active plate margins, where oceanic and continental plates slide atop and subduct each other, which in turn leads to an abiotic transformation of co-subducted organic matter into hydrocarbon compounds due to

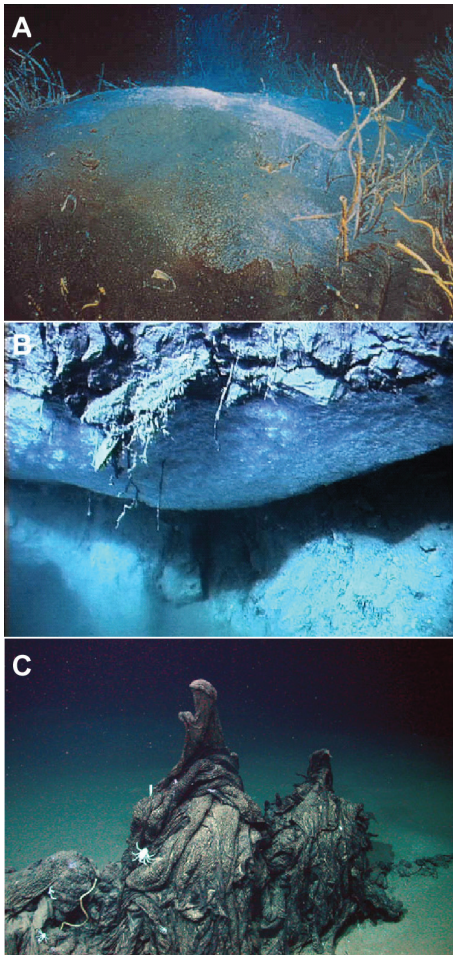

Fig.5 Examples of cold seep sites in the Gulf of Mexico. Panels show a Hydrocarbon gas seep (A) (Mac Donald), Gas hydrate (B) (NOAA) and Asphalt volcano (C) (European Geosciences Union).

evolving high temperatures and pressures (Suess, 2010). At such sites, the hydrocarbons seeping through the ocean floor are of thermogenic origin and as such also contain significant amounts of non-methane short-chain alkanes. At other cold seep sites, frequently found along sediment margins, organic rich sediments favour methanogenesis as part of the early microbial diagenesis. Thus, mainly biogenic methane migrates upwards through the shallow sediments and forms either methane hydrates or leaves the ocean floor through cold methane seeps or methane mud volcanoes (Suess, 2010). The possibility of a biological formation of ethane and propane from acetate and hydrogen in cold, deep marine sediments has recently been postulated (Hinrichs *et al.,* 2006), providing a hypothetical biogenic source of these short-chain alkanes.

At many cold gas and hydrocarbon seep sites, especially those that have been active over long time scales, large authigenic carbonates are visible (Suess, 2010). These structures are a result of microbial degradation of hydrocarbons to CO_2, leading to an increase in pore water alkalinity due to the production of bicarbonate and thus favouring the precipitation of authigenic carbonate minerals (Baker and Burns, 1985). The isotopic composition of the formed carbonates can be used to infer the dominant hydrocarbon sources for microbial degradation at seep sites. The stable carbon isotope composition of carbonates at some hydrocarbon seep sites, such as for example at Hydrate Ridge, indicates that mainly biogenic methane is fuelling the present microorganisms (Boetius and Suess, 2004). In the Gulf of Mexico, where mainly thermogenic hydrocarbons are fuelling microbial activity, the authigenic carbonates differ from those found at biogenic methane seeps (Formolo *et al.,* 2004).

Hydrocarbon seep sites usually host a large variety of micro- and macrofauna. Hydrocarbon-degrading microorganisms using oxygen and, in deeper anoxic sediments, sulfate as the terminal electron acceptor (see section A.3) are providing the basis of a hydrocarbon-fuelled food web. The production of hydrogen sulfide provides the basis for the development of large microbial mats, consisting of CO_2-fixing, giant sulfur-oxidizing bacteria, such as *Beggiatoa* and *Thiomargarita*. Usually, chemosynthetic bivalves and tubeworms, which bioturbate the seep-associated sediments can be found as well (Jørgensen and Boetius, 2007).

A.2.2 Hydrothermal vents

Although the world's largest oil and gas resources are formed through slow geothermal processes, which take place over 5−100 million years (Tissot and Welte, 1984), oil and gas hydrocarbon compounds are formed as well on much faster time scales (< 5000 years) at hydrothermal vent sites, such as those in the Guaymas Basin (Simoneit *et al.,* 1988; Whelan *et al.,* 1988). Hydrothermal vents usually occur along active spreading zones, where hydrothermal fluids, which are acidic and have high sulfide, metal and hydrogen concentrations, vent at fast flow rates and with high temperatures (300−400 °C; Jørgensen and Boetius, 2007) into the deep sea ocean water (Fig.6). In places where newly formed crust intrudes into the organic matter-rich, overlying sediments, hydrothermal alterations of these immature sediments (pyrolysis) at up to 1000 °C (Jørgensen and Boetius, 2007) results in the generation of petroleum products (Simoneit *et al.,* 1992). These formed petroleum products share some similarities with normal crude oils, but apparently have undergone only a mild thermal cracking (Didyk and Simoneit, 1990). Due to the thermogenic origin of the hydrocarbon gases, C_1−C_5 short-chain alkanes can be present here in relatively high concentrations (Simoneit *et al.,* 1988).

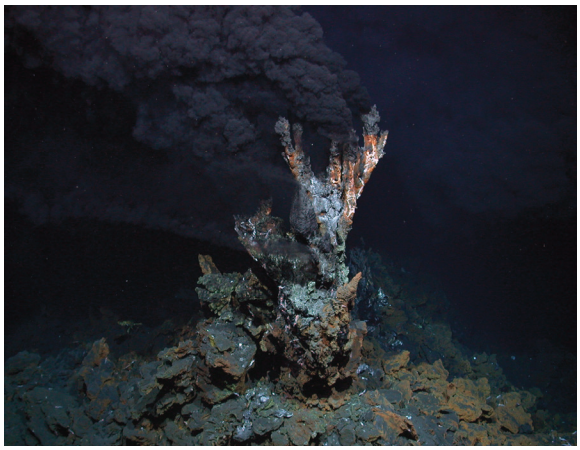

Fig.6 Hydrothermal vent in the Guaymas Basin (the image is courtesy of the University of Bremen).

A.3 Hydrocarbon-degrading microorganisms

A.3.1 Bioavailability of hydrocarbons for microorganisms

Some hydrocarbons, such as cyclic alkanes, aromatic compounds and alkanols have toxic effects, since they impair membrane fluidity (Sikkema *et al.,* 1994), permeabilize biological membranes (Sikkema *et al.,* 1995) and thus lower the energetic level of the cell (Neumann *et al.,* 2006). Nevertheless, many microorganisms can use hydrocarbons as substrates for growth (for reviews see Widdel and Rabus, 2001; Head *et al*., 2006; Widdel and Musat, 2010 and references therein). Due to the hydrophobicity of hydrocarbons, solubility is low and therefore they are only poorly bioavailable. Generally, the degree of hydrophobicity of a hydrocarbon will increase with the degree of saturation of the compound and the molecular mass. Alkanes are less soluble than aromatic compounds and within the alkanes, short-chain alkanes are better soluble than longer alkanes (see section A.1). In saline environments, the bioavailability of hydrocarbons will be even more reduced, due to a lower solubility. Another factor to consider with regard to bioavailability is the state in which the hydrocarbon compound is, i.e. hydrocarbons can be either in the gaseous state (gas phase or water dissolved), the liquid state (water dissolved, surface adsorbed or a bulk phase atop a water phase) or the solid state (Harms *et al.,* 2010). The state of the hydrocarbon compound can bring about an impaired bioavailability, which microorganisms have to overcome in order to use them as substrates for growth. Many microorganisms able to degrade hydrocarbons have developed mechanisms to overcome the low hydrocarbon bioavailability, such as direct adhesion to the hydrocarbon phase (Rosenberg, 1991, Wick *et al.*, 2002) or the secretion of biosurfactants/bioemulsifiers (Ron and Rosenberg, 2002, Walzer *et al.,* 2006). Another factor often limiting the rate of biodegradation in sediments is the low concentration of available hydrocarbon substrate due to, for example, impaired substrate diffusion through soils with low degrees of porosity (Kristensen *et al.,* 2010). The hydrocarbon diffusion rate towards the microbial cell (mass transfer) and the rate at which the microbial cell can degrade the compound (enzyme kinetics) will determine whether pr not the supply of the hydrocarbon compound becomes limiting (low mass transfer), reaches a steady state or if the compound even accumulates in the

cell due to passive inflow (high mass transfer). The capacity of the habitat to compensate for hydrocarbon substrate degradation will determine the hydrocarbon substrate bioavailability (Harms *et al.,* 2010).

A.3.2 A brief introduction to microbial degradation of hydrocarbons

The ability of aerobic microorganisms to degrade hydrocarbons has been known since the beginning of the 20th century (Söhngen, 1913). For a long time it was believed that in the absence of oxygen, microbial degradation of hydrocarbon compounds would be rather unlikely, due to their chemical inertness. Aerobic bacteria can make use of oxygen as both terminal electron acceptor for respiration and the formation of reactive oxygen species that are inserted into the hydrocarbons through the actions of oxygenases (Harayama *et al.,* 1992). In the absence of molecular oxygen, anaerobic microorganisms obviously have to make use of other mechanisms for the activation of hydrocarbons. The possibility for the existence of microorganisms, able to grow on hydrocarbon compounds under anoxic conditions, has been discussed since the 1930s. Although the production of hydrogen sulfide in oil reservoirs (Bastin, 1926) and the depletion of long-chain *n*-alkanes in crude oil were early indications for the existence of active, hydrocarbon-degrading microorganisms in anoxic oil reservoirs, it was not before the 1990s that the first hydrocarbon degrading, strictly anaerobic bacterium was isolated. Strain Hxd3 (meanwhile named *Desulfococcus oleovorans* strain Hxd3) was isolated from an oil-water separator and could grow on hexadecane with sulfate as the terminal electron acceptor (Aeckersberg *et al.,* 1991). Since then, many other anaerobic, hydrocarbon-degrading microorganisms have been reported, which can grow on short-, middle- and long-chain *n*-alkanes, cyclic alkanes, alkylsubstituted and unsubstituted mono- or polycyclic aromatic compounds. These processes can be coupled to nitrate-, iron- and sulfate-reduction, methanogenesis or anoxygenic photosynthesis (for a review see Widdel *et al.,* 2010).

A.3.3 Degradation of alkanes in the marine environment

The aerobic biodegradation of non-methane *n*-alkanes has been reported for marine sediments, which were subject to severe hydrocarbon contamination after oil spillage (Atlas and Bartha, 1972). Furthermore, degradation of *n*-hexadecane was reported with pure cultures obtained from deep sea sediments off the coast of Florida (Schwarz *et al.,* 1974) and from sediments associated with a hydrothermal vent in the Guaymas Basin (Bazylinski *et al.,* 1989). The short-chain alkanes ethane, propane and *n*-butane were also reported to be biodegraded by microorganisms present in sediment from the Coal Point hydrocarbon seep site (Kinnaman *et al.,* 2007; Redmond *et al.,* 2010).

Due to the high organic matter content at such hydrocarbon seep sites, including biogenic and thermogenic methane, thermogenic short-chain, long-chain and aromatic hydrocarbon compounds but also simple volatile fatty acids, the oxygen content in the associated sediments is often depleted already below the top few centimetres. As sulfate is an abundant electron acceptor in marine sediments (~ 28 mM), it is available to support anaerobic growth of microorganisms.

For a long time, the intensively studied anaerobic oxidation of methane (AOM; stoichiometric equation: $CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O$) was thought to be the dominant process at marine cold-seep ecosystems (Reeburgh, 2007; Jørgensen and Boetius, 2007), since very often the observed sulfate reduction rates (SRR) matched the observed anaerobic methane oxidation (AOM) rates in a nearly 1:1 ratio. A dominance of AOM as the main process accounting for the observable SRR may be the case at seep sites that are mainly fuelled by biogenic methane and at gas hydrates (of type-1 structure), which consist mainly of methane. However, recent biogeochemical studies at hydrocarbon seep sites, fuelled by complex hydrocarbon mixtures, such as in the Gulf of Mexico (Joye *et al.,* 2004; Orcutt *et al.,* 2004; Orcutt *et al.,* 2010; Bowles *et al.,* 2011) and the Gulf of Cadiz (Niemann *et al.,* 2006) have observed SRR which exceed AOM rates. A global compilation of available SRR and AOM rates from different types of hydrocarbon seeps shows that SRR exceed the AOM rates at cold seep sites by one order of magnitude (on average). The site-specific SRR/AOM ratio depends on the type of hydrocarbon seep, i.e. if biogenic methane is the dominant carbon source or thermogenic oil and gas co-occur (Bowles *et al.,* 2011). Based on these estimates it has become more obvious that at marine

hydrocarbon seep sites, which are fuelled by non-methane hydrocarbon compounds, AOM is not the dominant process fuelling SR.

Although some studies have indirectly shown that presence of hydrocarbons other than methane and short-chain alkanes correlates with elevated SRR (Orcutt *et al*., 2010), most of the direct evidence for microbial degradation of non-methane hydrocarbons comes from studies where short-chain alkanes were analyzed. Evidence from a study at a cold seep in the Gulf of Mexico indicated microbial degradation of propane, *n*-butane, *n*-pentane and to a lesser extent also ethane (Sassen *et al.,* 2004). A study on sediments at a mud volcano in the Gulf of Cadiz reported the disappearance of ethane, propane and *n*-butane along with methane (Niemann *et al.,* 2006). Furthermore, the disappearance of propane and *n*-butane coupled to an isotopic enrichment in ^{13}C and D of the residual pool indicated the presence of propane and *n*-butane degrading, anaerobic microorganisms in sediments from the Isis and Amon mud volcanoes in the Nile deep sea fan (Mastalerz *et al.,* 2009). Microbial degradation rates for the anaerobic oxidation of propane at the Coal Point hydrocarbon seep were determined to be in the same range as those reported for AOM in similar environments (Quistad and Valentine, 2011). These recent findings suggest that the anaerobic oxidation of short-chain alkanes at marine hydrocarbon seeps may play a significant role in the carbon cycling at these sites.

The degradation of short-chain alkanes coupled to sulfate-reduction should also have an influence on the sulfur cycle at these seep sites. The sulfate pool in anoxic sediments at seep sites depends on the downwards advection of sulfate-replenishing fluids from sulfide-oxidizing *Beggiatoa*-mats or *Epsilonproteobacteria* atop the sediments (Bowles *et al.,* 2011) or the upwards advection of sulfate which originates from the dissolution of barite ($BaSO_4$) deposits in deeper sediments (Greinert *et al*., 2002; Orcutt *et al.*, 2005). The availability of sulfate is thus limiting the SRR at seeps. Thermodynamically, the anaerobic degradation of propane ($2\ C_3H_8 + 5\ SO_4^{2-} + 4\ H^+ \rightarrow 6\ HCO_3^- + 5\ H_2S + 2\ H_2O$; $\Delta G°' = -41.0$ kJ per mol sulfide) and *n*-butane coupled to the reduction of sulfate to sulfide ($4\ C_4H_{10} + 13\ SO_4^{2-} + 10\ H^+ \rightarrow 16\ HCO_3^- + 13\ H_2S + 4\ H_2O$; $\Delta G°' = -42.5$ kJ per mol sulfide) are more efficient than AOM ($\Delta G°' = -16$ kJ per mol sulfide) and thus can be expected to proceed faster. Due to these stoichiometries, the degradation of propane and *n*-butane by sulfate-reducers would lead to more sulfate reduced per mol of propane (2.5 mol sulfate) or *n*-butane (3.25 mol sulfate) consumed than per mol of

methane (1 mol sulfate) consumed. Taking these into consideration, it is likely that propane- and *n*-butane-degrading sulfate-reducing bacteria are competing with AOM consortia for available sulfate and may thus indirectly limit the AOM rates at such seep sites. The recently published rates for the anaerobic degradation of propane (Quistad and Valentine, 2011) furthermore suggest that the activity of anaerobic microorganisms degrading short-chain alkanes might be important to prevent propane from entering the ocean and subsequently the atmosphere. The latter point could have important implications for global photochemical pollution, tropospheric ozone production, and tropospheric chemistry (Katzenstein *et al.,* 2003). Given that the global propane emissions from geological sources, such as hydrocarbon seep sites, account for nearly one fourth of the total global propane emissions (Etiope and Ciccioli, 2009), the activity of propane degrading microorganisms might be relevant for the Earth's climate.

A.3.4 Anaerobic alkane degrading microorganisms

Anaerobic degradation of alkanes is energetically feasible when coupled to various electron acceptors, such as O_2, NO_3^-, ClO_3^-, Fe^{3+}, $S_2O_3^{2-}$, SO_4^{2-} and HCO_3^- (recently reviewed in Mbadinga *et al.,* 2011). Most of the anaerobic, alkane-degrading pure cultures isolated to date are either sulfate-reducing bacteria belonging to the *Deltaproteobacteria*, which were isolated either from oil fields, petroleum-contaminated waste water or deep-sea hydrocarbon seeps, or nitrate-reducing bacteria, belonging either to the *Beta-* or *Gammaproteobacteria* and were isolated from activated sludge, ditch sediment, brackish lagoons or lake sediments. The majority of the known strains can degrade long-chain *n*-alkanes ($\geq C_6$) (Table 1).

Only few studies reported the isolation, or enrichment of anaerobic, short-chain *n*-alkane (C_2–C_5) degrading microorganisms. These included sulfate-reducing bacteria from either marine deep-sea hydrocarbon seep sites (Kniemeyer *et al.,* 2007), or from terrestrial hydrocarbon rich sediments (Savage *et al.,* 2010). The hitherto only pure culture able to degrade short-chain *n*-alkanes (strain BuS5, Kniemeyer *et al.,* 2007) was isolated from sediments associated with hydrothermal vents in the Guaymas Basin. This mesophilic strain is very specialized with regard to substrate range, since it apparently can solely degrade propane and *n*-butane. The same study

reported also the enrichment of cold-adapted, sulfate-reducing bacteria from sediments at hydrocarbon seep sites in the Gulf of Mexico, which degrade *n*-butane. Just like strain BuS5, the dominant phylotype in this enrichment culture belongs to the *Desulfosarcina*/*Desulfococcus*-cluster within the *Deltaproteobacteria*. A thermophilic (growth at 60 °C), propane-degrading enrichment culture, dominated by a *Desulfotomaculum* sp., has been obtained from hydrothermal vent sites in the Guaymas Basin (Kniemeyer *et al.,* 2007). Interestingly, none of the isolates known to date, which can grow on *n*-alkanes is able to utilize any other hydrocarbon substrate, such as cyclic alkanes or aromatic compounds. It was reported that the denitrifying strain HxN1 could co-activate, but not grow on cyclopentane and ethylcyclopentane, as well as *n*-butane and *n*-pentane (Wilkes *et al.,* 2003). Very recent findings showed that some nitrate-reducing and sulfate-reducing, *n*-alkane-degrading strains can co-activate aromatic compounds and further metabolize the derived metabolites down to the level of benzoyl-CoA. However, none of the investigated strains was able to grow on any of the tested aromatic compounds. The same study showed that activation of *n*-alkanes by aromatic hydrocarbon degrading strains seems impossible (Rabus *et al.,* 2011).

Biodegradation of cycloalkanes was reported under sulfate-reducing (Rios-Hernandez *et al.,* 2003; Townsend *et al.,* 2004), methanogenic (Townsend *et al.,* 2004) or nitrate- reducing conditions (Musat *et al.,* 2010). To date, not much is known about the types of microorganisms that can degrade cycloalkanes. A cyclohexane degrading, denitrifying enrichment culture was shown to be dominated by *Geobacter* spp., the likely cyclohexane degrader (Musat *et al.,* 2010). Based on DGGE analysis, it was proposed that a strain related to *Desulfotomaculum* was responsible for the degradation of ethylcyclopentane in a sulfate-reducing enrichment culture (Rios-Hernandez *et al.,* 2003).

The biodegradation of *n*-alkanes under methanogenic conditions has been reported for the first time for an enrichment culture, which was growing on *n*-hexadecane and produced methane and CO_2 (Zengler *et al.,* 1999). It is believed that a syntrophic association of three partners is decomposing the *n*-alkane at first to acetate and H_2 (by a *Syntrophus* sp.), which is then consumed by acetoclastic methanogenic archaea to form methane and CO_2. Another group of methanogenic archaea then converts CO_2 and H_2 to methane (Zengler *et al.,* 1999; Dolfing *et al.,* 2008).

Table 1. List of anaerobic, *n*-alkane-degrading strains and enrichment cultures (adapted from Mbadinga *et al.*, 2011)

Organism	e^- acceptor	Phylogenetic affiliation	Substrate range	Activation mechanism	Temperature	Source	References
Clone Propane60-GuB	SO_4^{2-}	*Firmicutes*[a]	C_3	Fumarate add.	60°C	Marine hydrocarbon seep (Guaymas basin)	Kniemeyer *et al*. 2007
Enrichment culture	SO_4^{2-}	*Deltaproteobacteria*[c]	C_3	Fumarate add.	22°C	Terrestrial hydrocarbon seep	Savage *et al*. 2010
Strain BuS5	SO_4^{2-}	*Deltaproteobacteria*	C_3 - C_4	Fumarate add.	10 - 33°C	Marine hydrocarbon seep (Guaymas basin)	Kniemeyer *et al*. 2007
Clone Butane12-GMe	SO_4^{2-}	*Deltaproteobacteria*[a]	C_4	n.d.	12°C	Marine hydrocarbon seep (Gulf of Mexico)	Kniemeyer *et al*. 2007
Enrichment culture	SO_4^{2-}	*Deltaproteobacteria*[c]	C_5	n.d.	22°C	Terrestrial hydrocarbon seep	Savage *et al*. 2010
Strain PL12	SO_4^{2-}	*Deltaproteobacteria*	C_6, C_{10}	n.d.	30 - 34°C	Petroleum cont. sediment	Higashioka *et al*. 2009
Strain Lake	SO_4^{2-}	*Deltaproteobacteria*	C_6 - C_{10}	Fumarate add.	20 - 55°C	Oilfield production water	Davidova *et al*. 2006
Strain ALDC	SO_4^{2-}	*Deltaproteobacteria*	C_6 - C_{12}	Fumarate add.	31 - 37°C	Oily sludge	Davidova *et al*. 2006
Enrichment culture	SO_4^{2-}	n.d.	C_{12}	Fumarate add.	32°C	Oily sludge	Kropp *et al*. 2000
StrainTD3	SO_4^{2-}	*Deltaproteobacteria*	C_6 - C_{16}	Fumarate add.	55 - 65°C	Guaymas Basin sediment	Rueter et al. 1994
Strain Hxd3	SO_4^{2-}	*Deltaproteobacteria*	C_{12} - C_{20}	Carboxylation	28 - 30°C	Oil-water separator	Aeckersberg *et al*. 1991
Strain AK-01	SO_4^{2-}	*Deltaproteobacteria*	C_{13} - C_{18}	Fumarate add.	26 - 28°C	Petroleum-cont. estuarine sediments	So and Young 1999
Strain CV2803	SO_4^{2-}	*Deltaproteobacteria*	C_{13} - C_{18}	Fumarate add.	28 - 35°C	Hydrocarbon-cont. marine sediments	Cravo-Laureau *et al*. 2004, 2005
Strain Pnd3	SO_4^{2-}	*Deltaproteobacteria*	C_{14} - C_{17}	Fumarate add.	28 - 30°C	Marine sediments	Aeckersberg *et al*. 1998
Enrichment culture	SO_4^{2-}	*Deltaproteobacteria*[b]	Ethylcyclopentane	Fumarate add.	30°C	Gas-condensate-cont. aquifer	Rios-Hernandez *et al*. 2003
Several Enrichment cultures	SO_4^{2-}	n.d.	Diff. cycloalkanes	Fumarate add.	RT	Gas-condensate-cont. aquifer	Townsend *et al*. 2004
Strain HxN1	NO_3^-	*Betaproteobacteria*	C_6 - C_8	Fumarate add.	28°C	Ditch sediments	Ehrenreich *et al*. 2000; Rabus *et al*. 2001
Strain HdN1	NO_3^-	*Gammaproteobacteria*	C_6 - C_{30}	"Intracellular O_2 generation" [?]	28°C	Activated sludge	Ehrenreich *et al*. 2000; Zedelius *et al*. 2010
Strain OcN1	NO_3^-	*Betaproteobacteria*	C_8 - C_{12}	Fumarate add.	28°C	Ditch sediments	Ehrenreich *et al*. 2000
Pseudomonas balearica strain BerOc6	NO_3^-	*Gammaproteobacteria*	C_{15} - C_{18}	n.d.	n.d.	Brakish lagoon	Grossi *et al*. 2008
Enrichment culture	NO_3^-	n.d.	C_{16}	Carboxylation	30°C	Lake sediments	Callaghan *et al*. 2009
Marinobacter sp. BC36	NO_3^-	*Gammaproteobacteria*	C_{18}	n.d.	n.d.	Lagoon mats	Bonin *et al*. 2004
Marinobacter sp. BC42	NO_3^-	*Gammaproteobacteria*	C_{18}	n.d.	n.d.	Lagoon mats	Bonin *et al*. 2004
Enrichment culture	NO_3^-	n.d.	Pristane	n.d.	25°C	Diesel-fuel cont. sediment	Bregnard *et al*. 1997
Enrichment culture	NO_3^-	*Deltaproteobacteria*	Cyclohexane	Fumarate add.	n.d.	Lake sediments	Musat *et al*. 2010
Pseudomonas chloritidismutans AW1^T	ClO_3^-	*Gammaproteobacteria*	C_7 - C_{12}	"Intracellular O_2 generation"	30°C	Chlorate-reducing sludge	Mehboob *et al*. 2009

a = as determined by relative abundance with phylotype specific probes b= as determined by DGGE c= as determined by relative abundance of clone sequences in clone library

A.4 Mechanisms of alkane degradation by microorganisms

From their chemical properties it is assumed that alkanes diffuse freely through biological membranes and towards the activating enzyme. Therefore, an uptake system is not required.

Aerobic microorganisms use molecular oxygen for the activation of alkanes. Reactive oxygen species are formed by partial reduction at metal centers of monooxygenases (Groh and Nelsen, 1990). The reactive oxygen then attacks an apolar C-H bond at the primary carbon atom of the alkane molecule (Fig.7 A) (see van Beilen and Funhoff (2007) for a review). The resulting primary *n*-alcanol is oxidized to the corresponding aldehyde and carboxylic acid, further metabolized via β-oxidation to acetyl-CoA units, which may enter the citric acid cycle.

Anaerobic microorganisms have developed unprecedented reaction mechanisms to overcome the chemical inertness of the C-H bond of the alkane molecules. The first attempts to elucidate the activation of *n*-alkanes under anaerobic conditions were performed with strain Hxd3 (Aeckersberg *et al.,* 1998). During growth on the even-carbon numbered (C-even) *n*-hexadecane, the formation of C-odd fatty acids was observed and during growth on the C-odd *n*-heptadecane, the formation of C-even fatty acids was observed. This observation led to the hypothesis that the anaerobic activation of *n*-alkanes involved the terminal addition of a C_1-carbon unit (Aeckersberg *et al.,* 1998). Incubations of the strain in the presence of $H^{13}CO_3^-$ however resulted in the detection of isotopically labeled fatty acids, which bared the ^{13}C-label at the carboxyl group, indicating that $H^{13}CO_3^-$ was directly involved in the activation reaction. It was suggested that the *n*-alkane is carboxylated, most likely at the C_3-atom and that thereafter two carbon atom are removed from the formed fatty acid (Fig.7 D), since the formed fatty acids are by one carbon atoms shorter than the initial *n*-alkane (So *et al.,* 2003). However, the immediate activation product (2-ethylalkanoate) was never detected and there is an ongoing debate as to whether this reaction is indeed taking place and if it is overall thermodynamically feasible (Thauer and Shima, 2008).

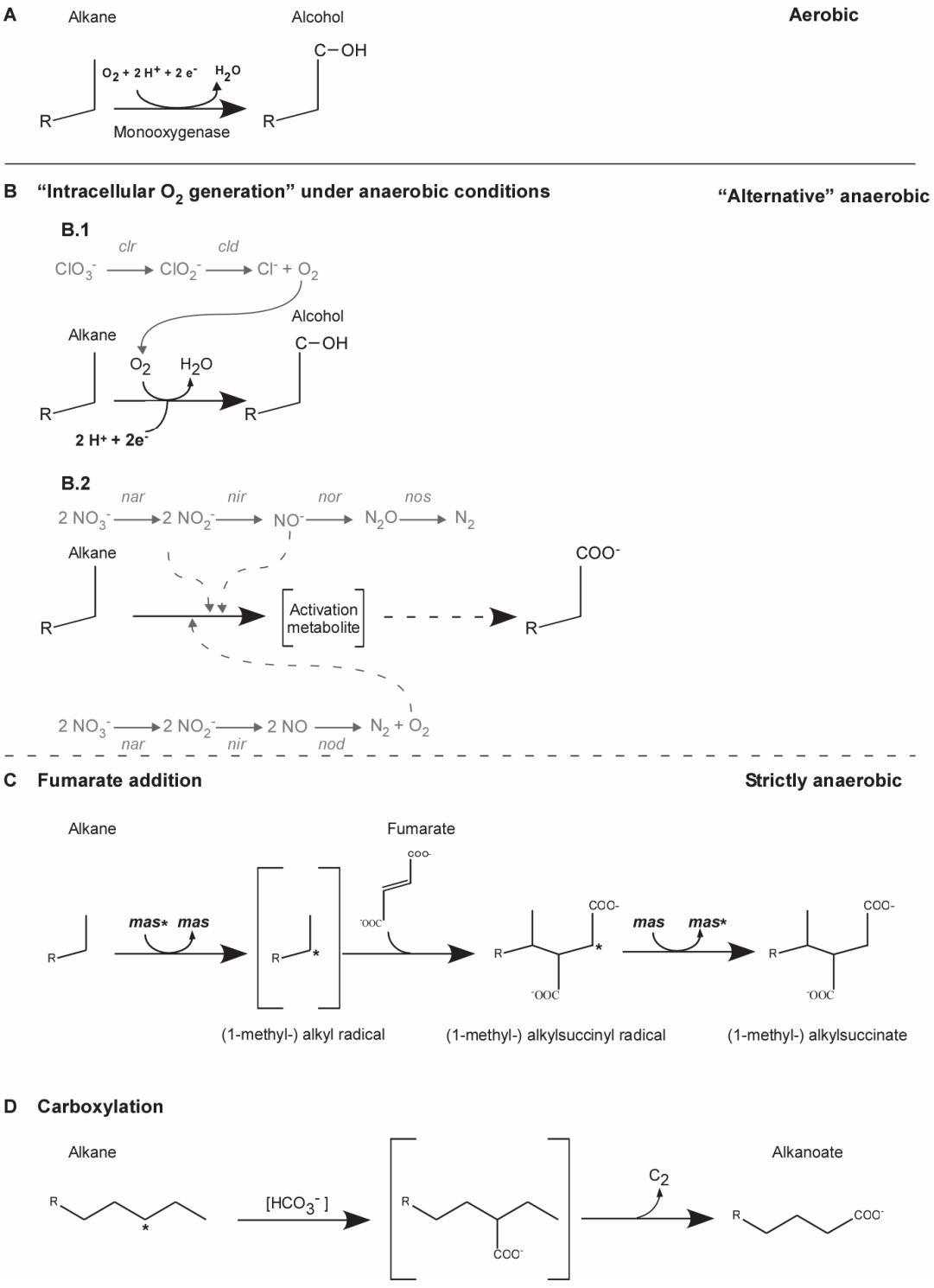

Fig.7 General activation mechanisms for *n*-alkanes under aerobic (A) and anaerobic conditions (B, C, D). Abbreviations stand for the following: *clr* = chlorate reductase, *cld* = chlorite dismutase, *nar* = nitrate reductase, *nor* = nitric oxide reductase, *nos* = nitric oxide synthase, *nod* = nitric oxide dismutase, *mas* = (1methyl)alkylsuccinate synthase. For detailed explanations, see the text.

A more ubiquitous activation mechanism of *n*-alkanes in anaerobic bacteria appears to be a carbon-carbon addition to fumarate, yielding alkylsuccinates (Fig.7 C). This reaction is to some degree analogous to the well-studied activation of alkyl-substituted aromatic hydrocarbons, which involves the formation of an organic radical by the enzyme benzylsuccinate synthase (Bss), which is a glycyl-radical enzyme (for a review, see Heider, 2007). Formation of (1-methyl)alkylsuccinates by *n*-alkane-grown, anaerobic bacteria indicated an addition to fumarate at the secondary carbon atom (Kropp *et al.,* 2000; Rabus *et al.,* 2001; Callaghan *et al.,* 2006). The proposed mechanism is illustrated in Fig.8.

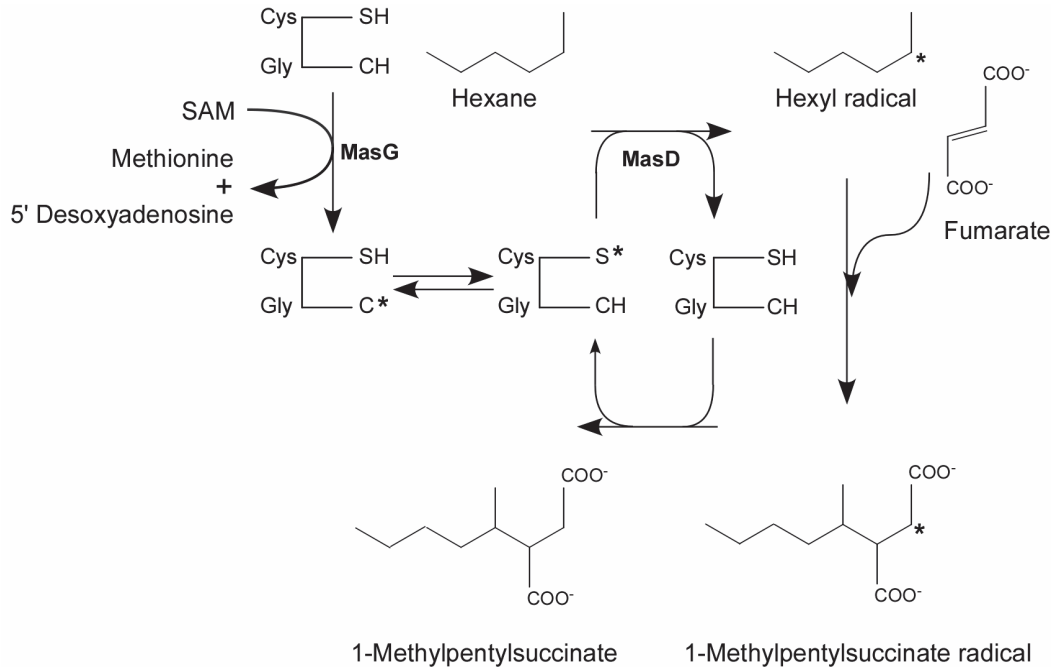

Fig.8 Proposed mechanism for activation of the *n*-alkane hexane, via the activating enzyme (1-methyl)alkylsuccinate synthase (Mas). A radical is generated at the large subunit of the (1-methyl)alkylsuccinate synthase (MasD). The radical is derived from S-adenosyl-L-methionine (SAM) through an activase (MasG). Activated MasD catalyzes the addition of hexane to fumarate, via a transfer of the radical to hexane. The radical attacks the double bond of fumarate, leading to a carbon-carbon addition of fumarate to hexane, forming 1-methyl-pentylsuccinate (redrawn from Werner, 2009).

The enzyme and its encoding proteins were investigated in the denitrifying strain HxN1 (Grundmann *et al.,* 2008) and in the sulfate-reducing strain AK-01 (Callaghan *et al.,* 2008). The *n*-alkane activating enzyme is abbreviated either as Mas ((1-methyl)alkylsuccinate synthase; Grundmann *et al.,* 2008) or Ass (alkylsuccinate synthase; Callaghan *et al.,* 2008). The proteins, formed during growth on *n*-alkanes in strain HxN1 showed a high similarity to the large subunit of the Bss (BssA) and (2-naphthylmethyl)succinate synthase (NmsA), the glycyl radical enzymes mediating the anaerobic activation of toluene and 2-methylnaphthalene, respectively (Leuthner *et al.,* 1998; Musat *et al.,* 2009). Mas appears to be a heterotrimer (MasCDE) and the catalytical subunit (MasD) contains a conserved glycine motif near the C-terminus and conserved cystein residues in the middle of the polypeptide chain (Grundmann *et al.,* 2008), which are involved in transferring a radical from the glycine residue to a cysteine residue. In the genome of strain AK-01, two loci have been identified to encode genes that may be involved in the reactions catalyzed by Ass. Whereas one of the catalytic subunits of the two gene loci encoding Ass (AssA1) has been shown to be produced when AK-01 is grown on hexadecane, the role of the AssA2 has still not been determined (Callaghan *et al*., 2008; Callaghan *et al.*, 2011).

The further degradation of (1-methyl)alkylsuccinates was investigated by analysis of metabolites, which were generated during incubations of strain HxN1 with deuterium-labelled hexane (Wilkes *et al.,* 2002). It was found that strain HxN1 formed diastereomers of the activation product (methylpentylsuccinate) indicating that the activating enzyme exhibits a rather relaxed stereo-specificity. The toluene-activating enzyme Bss however, appears to be stereo-selective, since only *R*(+)-benzylsuccinate is formed (Beller and Spormann, 1998). Formed methylpentylsuccinate is most likely ligated to CoenzymeA and thereafter undergoes a carbon-skeleton rearrangement to form 2-methylhexyl-malonyl-CoA, which is then decarboxylated to form 4-octanoyl-CoA. The resulting fatty acid can then undergo β-oxidation through several intermediates, yielding CO_2 (Fig.9)

In the case of the short-chain alkanes propane and *n*-butane, metabolites were detected, which suggest that activation also commences via addition to fumarate. The downstream degradation pathway appears to be analogous to that reported for *n*-hexane in strain HxN1 (Kniemeyer *et al.,* 2007). Interestingly, in the case of propane, metabolites were identified, suggesting that activation takes place at both, the secondary and at one of the primary carbon atoms (Kniemeyer *et al.,* 2007,

Savage *et al.,* 2010). For such activation at a primary carbon atom, a higher C-H bond dissociation energy has to be overcome. It was speculated that this activation reaction at a primary carbon atom could, in principle, be a feasible route for activation of ethane, the degradation of which has been observed to proceed at an extremely slow pace (Kniemeyer *et al.,* 2007). Nevertheless, to date the involved activation mechanism for ethane remains unresolved.

Activation and degradation mechanisms similar to those for *n*-alkanes were also observed in studies with cycloalkane-degrading, sulfate- and nitrate-reducing cultures (Rios-Hernandez *et al.,* 2003; Musat *et al.,* 2010). In the case of the tentative glycyl-radical-enzymes activating cycloalkanes, nothing is hitherto known with regard to the phylogenetic relatedness to other hydrocarbon-activating glycyl-radical-enzymes, the structures of the encoding operons or their stereospecificity.

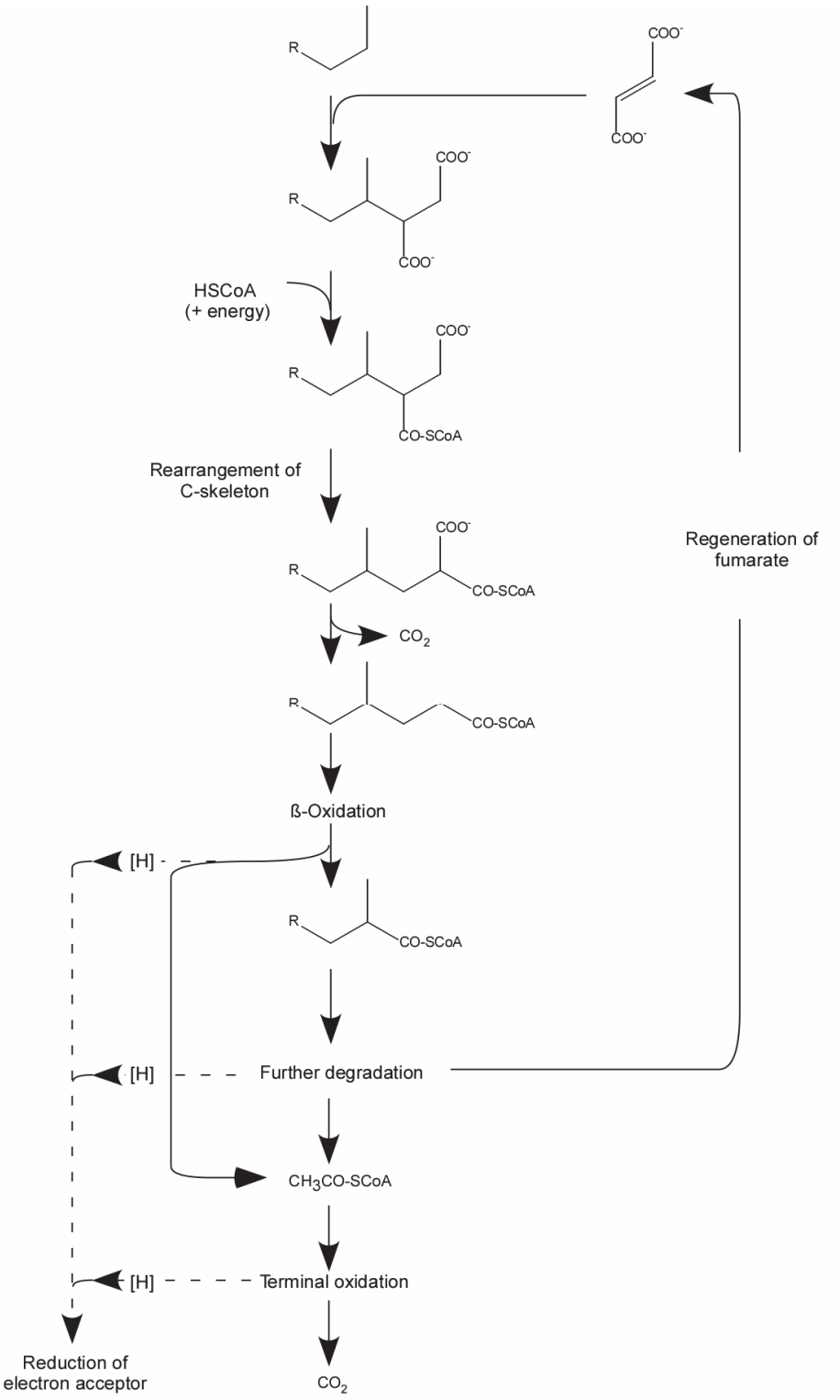

Fig.9 Postulated generalized pathway for the anaerobic degradation of *n*-alkanes (redrawn from Wilkes *et al.,* 2003).

In addition to the above described mechanisms for activations of alkanes, some facultative anaerobic microorganisms have developed remarkable mechanisms to activate *n*-alkanes under strictly anaerobic conditions. For example, the generation of ″intracellular oxygen″ by strain *Pseudomonas chloritidismutans* AW-1^T is hypothesized to proceed through the reduction of chlorate to chlorite, which is dismutated to oxygen and a chloride anion (Fig.7 B.1). Reactive oxygen species derived from oxygen are thought to enable the active involvement of alkane oxygenases (Mehboob *et al*., 2009). A similar mechanism has been suggested for the denitrifying and *n*-alkane degrading strain HdN1 (Zedelius *et al.*, 2010). Two different working hypotheses suggest either the direct involvement of NO_2^- or NO (generated during the reduction of nitrate) as a co-reactant for the activation of alkanes, or the generation of minimal amounts of O_2 by a so far unprecedented dismutation reaction of 2 NO to N_2 and O_2 (Fig.7 B.2). The latter hypothesis was recently described for a nitrate-reducing methanotrophic enrichment culture (Ettwig *et al*., 2010). However, in case of strain HdN1, this hypothetical pathway still awaits further confirmatory experiments, since so far the formation of O_2 by the strain could not be demonstrated.

A.4 Stable isotope fractionation

As microorganisms degrade a compound, the isotopic composition of the residual portion of the compound is altered, because in nearly all cases the involved enzymes discriminate against the heavier isotopes. This kinetic isotope effect (KIE) is due to a higher activation energy required to break a chemical bond between a heavy and a light isotopologue, compared to a bond containing only light isotopologues (Elsner *et al.*, 2010). This effect, in turn, leads to higher enzymatic turnover rates for molecules containing only lighter isotopologues at bonds which are broken or formed during an enzymatic transformation of a compound. Consequently, heavy isotopologues are enriched in the residual portion of the compound pool as an enzymatic reaction cascade proceeds and the original compound pool is degraded. Studying mechanisms of chemical reactions by substituting, i.e. a H or C atom with the heavy isotope (deuterium, tritium, ^{13}C or ^{14}C), have long been popular, due to the effects these isotope cause on the rates and equilibrium constants (for a review see

Cleland, 2007). In defined microbial cultures, where the loss of the growth substrate is only due to microbial degradation, a characteristic bulk stable isotope enrichment factor (ε_{bulk}) can be obtained for the biodegradation of a given compound over time *in vitro*. These ε_{bulk} values can be applied to determine the *in situ* biodegradation rates of the compound as a result of microbial activity (Meckenstock *et al.,* 2004; Hofstetter *et al.,* 2008). The KIE can be masked by limitations of reaction rates due to non-fractionating reaction steps preceding the initial bond cleavage (Northrup *et al*., 1981; Elsner *et al*., 2005; Thullner *et al*.; 2008, Kampara *et al.*, 2008). Thus, the observed apparent kinetic isotope effect (AKIE) can be much lower than the actual KIE. Therefore, two dimensional compound specific stable isotope analyses (2D-CSIA) has been suggested as a tool to overcome such masking effects (Elsner *et al*., 2005; Zwank *et al*., 2005; Fischer *et al*., 2007; 2008). Two elements, which are directly involved in a bond cleavage during the first enzymatic reaction, are analyzed with regard to their bond-specific AKIEs. The bond-specific AKIE takes into account the number of total atoms of each element per molecule and the number of atoms of each molecule in reactive and competing positions. Assuming that both elements will be equally affected by masking effects, correlating the stable isotope fractionation effect of both elements can be used as a marker for a given biochemical reaction. The slope of the linear regression line between the two elements involved in the bond, i.e. carbon ($\Delta\delta^{13}C_{reactive\ position}$) *versus* hydrogen ($\Delta\delta^{2}H_{reactive\ position}$), gives the so-called ″reactive position-specific Lamda″- value ($\lambda_{reactive\ position}$). This value can be regarded as a fingerprint for the initial bond cleaving reaction of a certain biochemical degradation pathway.

Combination of known, reaction-specific $\lambda_{reactive\ position}$ values for biochemical transformation pathways obtained from defined laboratory culture experiments, with CSIA data of organic compounds from field site studies, allows detection of *in situ* transformation pathways of these compounds (for critical reviews, see Elsner *et al*., 2005 and 2010).

B. Objectives of the present work

(I) Recent studies have indicated that at marine hydrocarbon seep sites, where thermogenic hydrocarbons occur, the microbial degradation of hydrocarbons other than methane may be important for carbon and sulfur cycling. The anaerobic degradation of short-chain alkanes has only recently been reported for the first time and to date only little is known about the microorganisms able to degrade C_2–C_5 *n*-alkanes. Until today, there exists only one pure culture of a propane- and *n*-butane-degrading strain (Kniemeyer *et al*., 2007). In few cases, enriched cultures degrading propane, *n*-butane or pentane were reported as well (Kniemeyer *et al*., 2007; Savage *et al*., 2010). All of these cultures were rather slow growing, with doubling times of ~ 5 days. Also, a sulfate-reducing, ethane-degrading enrichment culture was reported to produce sulfide at an extremely slow pace (Kniemeyer *et al*., 2007). In the beginning of the study, there was no information about the types of microorganisms able to degrade ethane or about the degradation mechanism.

The aim of this study was to enrich for novel short-chain alkane-degrading bacteria from marine hydrocarbon seeps. The types of microorganisms able to degrade C_2–C_5 *n*-alkanes were investigated in more detail, as their diversity is not adequately represented in the few available cultures.

In previous studies it was shown that propane and *n*-butane are activated by addition to fumarate at the secondary carbon atom under sulfate-reducing conditions (Kniemeyer *et al*., 2007; Savage *et al.*, 2010). This activation reaction is analogous to that of longer *n*-alkanes in anaerobic sulfate- and nitrate-reducing bacteria and has been shown to be mediated by glycyl-radical enzymes. In the case of propane, a metabolite analysis has previously indicated that activation takes place both at the secondary and the primary carbon atom. However, because the activation at a primary carbon atom is more difficult, it was not clear whether this unprecedented route of activation represents just a side reaction or a major route of activation. Carbon and hydrogen stable isotope fractionation analysis was used to characterize the mechanism of activation in newly obtained propane- and *n*-butane-degrading enrichment cultures. At the beginning of this study, no datasets that combine the analysis of both carbon and hydrogen fractionation during degradation of *n*-alkanes were available.

In addition, genes encoding the large subunit of the activating enzyme involved in the anaerobic degradation of propane and *n*-butane should be amplified to investigate

their phylogenetic relatedness to other known hydrocarbon-activating glycyl radical enzymes.

(II) Cycloalkanes are major constituents of crude oil and they are rather recalcitrant to biological degradation. Only few studies have reported the anaerobic biodegradation of cycloalkanes by microorganisms in enrichment cultures under sulfate- or nitrate-reducing and methanogenic conditions. Some of these studies showed that activation takes place through an addition to fumarate (Rios-Hernandez *et al.*, 2003; Musat *et al.*, 2010), yielding cycloalkylsuccinate derivatives. There was only little information at the beginning of this study concerning the types of bacteria able to degrade cycloalkanes under anaerobic conditions or their versatility with regard to the degradable spectrum of hydrocarbon compounds.

In this study, cyclohexane-degrading, sulfate-reducing bacteria were enriched from marine hydrocarbon seeps and from hydrocarbon-contaminated intertidal sediments. The obtained enrichment cultures were analyzed with respect to the types of sulfate-reducing bacteria involved in the cycloalkane degradation, the range of hydrocarbons used as substrates and the mechanism of cycloalkane activation.

C. Results and Discussion

The following section is intended to provide a comprehensive overview of the most important results, which were obtained during this study. Therefore, the reader is referred to the manuscripts in part II for more extensive explanations of the results and detailed descriptions of the materials and methods used in this study.

C.1 Enrichment of short-chain alkane-degrading microorganisms from hydrocarbon seeps in the Guaymas Basin

Due to the scarce reports about the types of microorganisms capable to degrade non-methane short-chain alkanes, one of the objectives of this study was to enrich further microorganisms that can grow on ethane, propane, *n*-butane, *iso*-butane and *n*-pentane. The sediments for an enrichment of such microorganisms were obtained from hydrocarbon seeps in the Guaymas Basin. At some of these sites, the concentrations of short-chain alkanes decreases towards the sediment surface, which is an indicator for biodegradation by microorganisms in anoxic sediments (Simoneit *et al.*, 1992).

C.1.2 Sediment samples used as starting material

The sediments used for the set-up of enrichment cultures in this study were obtained from the Guaymas Basin hydrothermal vent site, which is located at ~ 2000 m water depth. The vents are located several hundred meters beneath sediment, which is very rich in organic matter. The vent fluids migrate upwards through the sediment and alter the organic matter due to the high temperatures and pressures into petroleum and thermogenic gas. The sediment cores taken during a cruise in 2008 on the RV Atlantis originate from two different hydrocarbon seep sites: ″Oil Town″ and ″Theme Park″. The sediment from ″Oil Town″ was muddy and had a moderate temperature (~ 30 °C). It was loaded with heavy hydrocarbons, a dark brown oil slick was visible in the sediment and a strong ″smell of oil″ was noticeable. The sediment from “Theme park” was hard, stony and had a much higher temperature (80 °C–200 °C; only sediment from 80 °C–100 °C was used for enrichments). The hydrocarbon load was lighter- a yellow-brownish watery film and the ″smell of diesel fuel″ were noticeable. Upon retrieval to the ship, the cores were sliced and sediment layers of interest were transferred into pre-flushed glass bottles (N_2),

completely filled with bicarbonate-buffered, anoxic, sulfide-reduced (1 mM) artificial sea water (Widdel and Bak, 1992) and sealed with butyl stoppers. The bottles were stored at 4 °C until set-up of enrichment cultures.

C.1.2 Enrichment and growth of sulfate-reducing microorganisms

To enrich for sulfate-reducing microorganisms, hydrocarbon rich sediments from the two sites in the Guaymas Basin were mixed in a 1:1 ratio in an anaerobic chamber and were used to inoculate (5% volume/volume;v/v) anoxic artificial sea water. In order to cover a wide temperature spectrum, enrichments were incubated at 12 °C, 28 °C, 60 °C and 80 °C. The enrichments at 12 °C and 28 °C were set-up in butyl-stoppered, 100 ml flat glass bottles and enrichments at 60 °C and 80 °C were set-up in teflon-stoppered 120 ml serum bottles. Enrichments were amended with ethane, propane, *n*-butane, *iso*-butane or other hydrocarbons of interest (see Table 2). Negative controls without additional hydrocarbon amendment were also included.

Table 2. Hydrocarbons used as growth substrates for enrichment cultures

Substrate	12 °C	28 °C	60 °C	80 °C
Ethane[1]	10^5 Pa	10^5 Pa	0.5×10^5 Pa	0.5×10^5 Pa
Propane[1]	10^5 Pa	10^5 Pa	0.5×10^5 Pa	0.5×10^5 Pa
n-Butane[1]	10^5 Pa	10^5 Pa	0.5×10^5 Pa	0.5×10^5 Pa
iso-Butane[1]	10^5 Pa	10^5 Pa	0.5×10^5 Pa	0.5×10^5 Pa
n-Pentane	1% in 2.5 ml HMN	1% in 2.5 ml HMN	0.5% in 5 ml HMN	0.5% in 5 ml HMN
n-Hexadecane	0.4 ml	0.4 ml	0.2 ml	0.2 ml
Cyclohexane	0.5% in 2.5 ml HMN	0.5% in 2.5 ml HMN	0.2% in 5 ml HMN	0.2% in 5 ml HMN
Cyclopentane	0.5% in 2.5 ml HMN	0.5% in 2.5 ml HMN	0.2% in 5 ml HMN	0.2% in 5 ml HMN
Benzene	0.5% in 2.5 ml HMN	0.5% in 2.5 ml HMN	0.2% in 5 ml HMN	0.2% in 5 ml HMN

[1]for gaseous hydrocarbons (C_2–C_4) the partial pressure is indicated

Most of the enrichment cultures at 12 °C, 28 °C and 60 °C produced up to ~ 15 mM sulfide within the first 100 days (data not shown). The negative controls also produced sulfide up to the same concentration, indicating that the sulfate-reducing microorganisms present degraded the endogenous hydrocarbons rather than the added ones. The enrichments at 80 °C showed sulfide production of up to ~ 8 mM as well, but only in bottles with *n*-pentane, cyclopentane, cyclohexane and benzene. Most of the enrichment cultures ceased to produce sulfide in subsequent transfers, for which 10% of the previous enrichment culture was used as inoculum. Enrichment cultures showing hydrocarbon-dependent sulfate-reduction in subsequent transfers were those with *n*-butane and

n-pentane (12 °C), ethane and benzene (28 °C), and propane (60 °C) (Fig.10). The negative control at 28 °C still produced sulfide when this work was written, although at lower concentration than in incubations with ethane or benzene. This indicates that these enrichment cultures still contain organic matter, which has not yet been consumed or "out-diluted". Further transfers leading to sediment-free enrichment cultures with higher cell densities are required for future studies and isolation attempts.

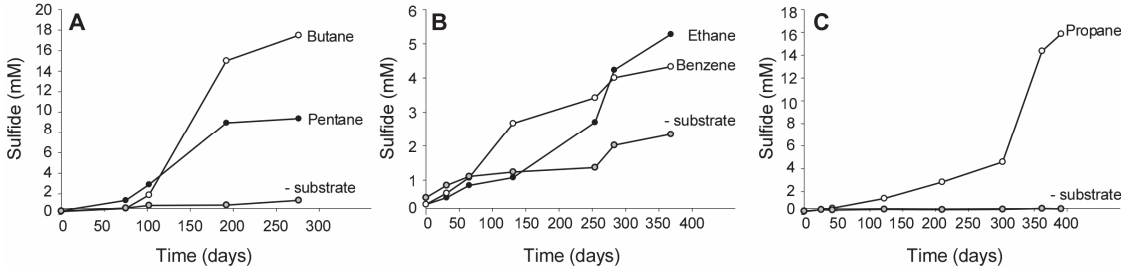

Fig.10 Sulfide production in successful anaerobic enrichment cultures from hydrocarbon seeps in the Guaymas Basin with different hydrocarbon substrates at 12 °C (A), 28 °C (B) and 60 °C (D), relative to the negative control, which did not receive an additional hydrocarbon source.

Due to the slow development of these cultures, the degradation of short-chain alkanes by sulfate-reducing bacteria was investigated in detail with dense, sediment-free enrichment cultures previously obtained from the Gulf of Mexico and Hydrate Ridge. This will be discusses in the following sections

C.2 Propane and *n*-butane degrading sulfate-reducing bacteria from cold marine hydrocarbon seeps

C. 2. 1 Enrichment of propane- and *n*-butane-degrading sulfate-reducing bacteria from cold marine hydrocarbon seeps and determination of the optimum temperature

Enrichment of propane- and *n*-butane-degrading sulfate-reducing bacteria from cold marine hydrocarbon seeps was attempted with sediments from the Gulf of Mexico and with sediment associated with structure II gas hydrates at Hydrate Ridge. The sediment samples from the Gulf of Mexico were collected during a cruise on the RV Seward Johnson I in July 2001 from a brine-influenced site at 650 m water depth (a detailed site description can be found in Joye *et al*., 2004). The sediment samples from Hydrate Ridge were collected during a cruise on the RV SONNE in July 2000 at 777 m water depth (Treude *et al.*, 2003).

Incubations were started at 12 °C, which is close to the rather low *in situ* temperatures (< 10 °C), and also at 28 °C to cover a greater range of temperature-adapted microorganisms. After 6 months at 12 °C, incubations with propane and Gulf of Mexico sediments (Prop12-GMe) and incubations with *n*-butane and Hydrate Ridge sediments (But12-HyR) produced up to 18 mM sulfide vs. controls lacking additional substrates, which produced only 3 mM sulfide (data not shown). Within the same incubation time, the sulfide production in bottles incubated with propane or *n*-butane at 28 °C did not exceeded that of substrate-free controls. Sediment-free enrichment cultures obtained by repeated transfers (20% v/v inoculum) in fresh medium formed approximately 15 mM sulfide within 5–6 weeks. The successful enrichment at 12 °C but not at 28 °C suggested that these short-chain alkane-degrading bacteria are rather cold-adapted, reflecting the *in situ* temperature conditions.

Temperature-dependent sulfate reduction profiles of these new enrichment cultures, another previously described *n*-butane degrading enrichment culture obtained from a cold seep in the Gulf of Mexico (But12-GMe) and for the mesophilic strain BuS5 (Kniemeyer *et al.*, 2007), show that the propane- and *n*-butane-degrading microorganisms in the enrichment cultures from cold seeps are true psychrotrophs (Fig.11), since their minimum temperature for sulfate reduction is > 0 °C, the optimum is < 25 °C and the

maximum is < 35 °C (Isaksen and Jørgensen, 1996). These observations suggest that the *in situ* respiration, and consequently the growth rates of microorganisms as those described here are sub-optimal, amounting to about 50% of the maximum attainable rates.

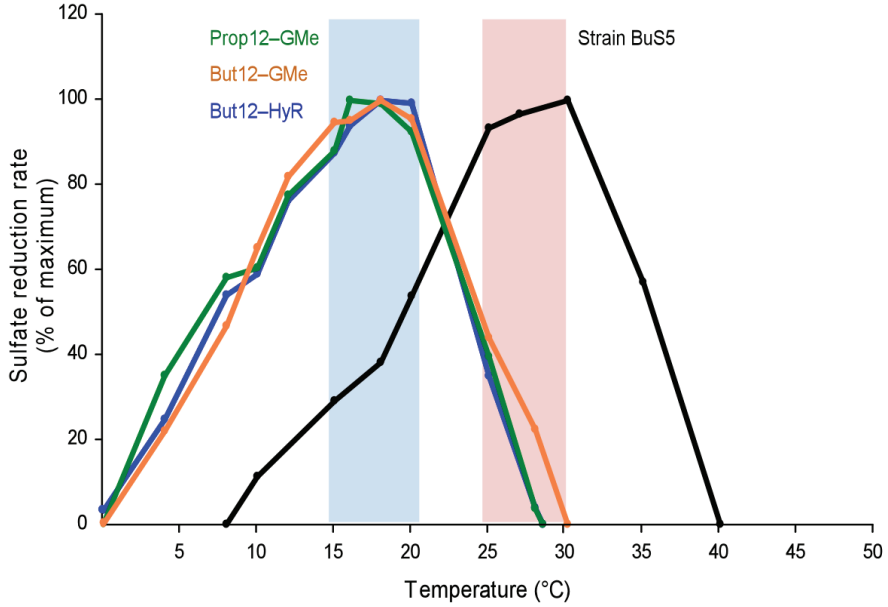

Fig.11 Temperature-dependent sulfate-reduction rates (SRR) of the propane and butane-degrading enrichment cultures from marine hydrocarbon seeps. Incubations were done for up to 11 days. The SRR are expressed as percent of the highest rate determined. The maximum sulfate-reduction rates (mM H_2S day^{-1}) were 0.62 (Prop12-GMe), 0.82 (But12-GMe), 0.9 (But12-HyR) and 1.12 (strain BuS5).

C.2.2 Quantitative degradation experiments, growth tests with other hydrocarbons and amplification of *masD* genes

Quantitative growth experiments showed that the enrichment cultures degraded propane and *n*-butane completely to CO_2, with stoichiometric reduction of sulfate to sulfide, as described for other propane- and *n*-butane-degrading sulfate-reducing bacteria (Kniemeyer *et al.*, 2007; Savage *et al.*, 2010). The enrichment cultures were tested for their ability to degrade methane, the dominant hydrocarbon at gas seeps, and other alkanes found in relatively high concentrations in such environments, e.g. ethane, *iso*-butane and *n*-pentane. Incubations with mixtures of methane, ethane, propane and *n*-butane showed that the enrichment cultures Prop12-GMe and But12-HyR degraded propane and *n*-butane simultaneously, while methane and ethane were not degraded

(Fig.12). In similar incubations, the culture But12-GMe degraded only *n*-butane. None of the enrichment cultures degraded *iso*-butane or *n*-pentane when supplied as single substrates or together with propane or *n*-butane.

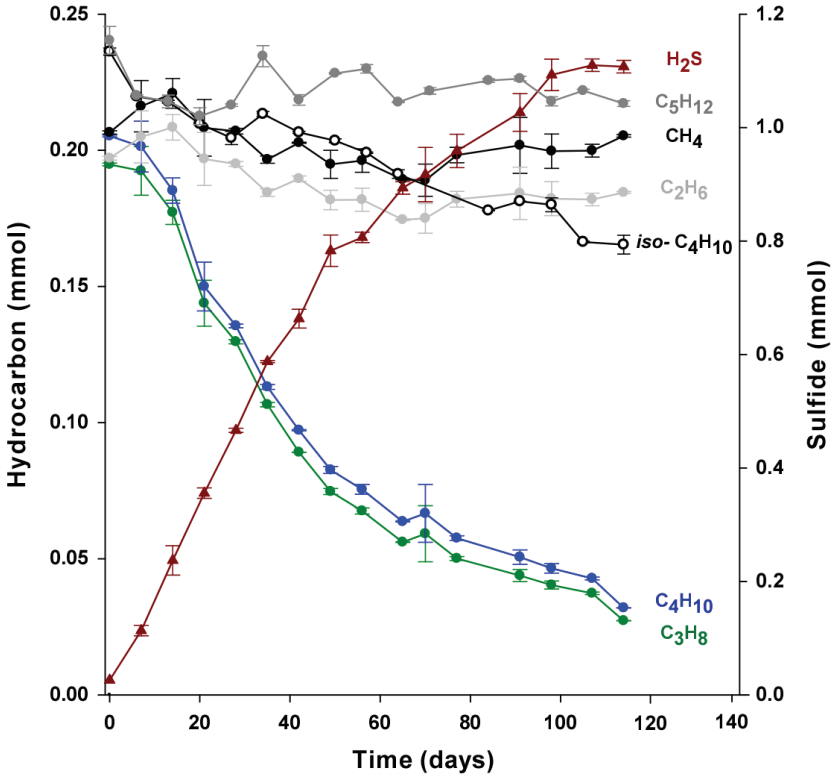

Fig.12 Consumption of propane (●) and *n*-butane (●) coupled to the production of sulfide (●) by the enrichment culture Prop12-GMe. Methane (●), ethane (●), *iso*-pentane (●) and *iso*-butane (○) were not degraded if supplied as single substrates or in mixtures with propane or *n*-butane. Cultures were set up in 120 ml serum bottles supplied with 79 ml artificial sea water medium and 1 ml inoculum from a dense cell suspension (10× concentrated), and incubated at 16 °C with slow shaking (40 rpm). Measurements of gas concentrations and sulfide were done in triplicate.

During the incubations with mixtures of alkanes, the microbial community structure of the enrichment cultures was stable, as demonstrated by hybridizations with fluorescently labeled, sequence-specific oligonucleotide probes (see ″Identification of dominant phylotypes″). The ability of the enrichment cultures Prop12-GMe and But12-HyR and also of strain BuS5 (Kniemeyer *et al*., 2007) to degrade propane and *n*-butane, but not shorter or longer alkanes, suggests rather specialized activating enzymes with respect to substrate spectrum. Moreover, the simultaneous degradation of propane and *n*-butane suggests the presence of a single activating enzyme.

Genes coding for alkane activating enzymes have been recently described in the nitrate-reducing strain HxN1, degrading C_6–C_8 *n*-alkanes ((1-methylalkyl)succinate synthase, *mas*; Grundmann *et al.*, 2008) and in the sulfate-reducing bacterium *Desulfatibacillum alkenivorans* AK-01, utilizing C_{13}–C_{18} *n*-alkanes (Alkylsuccinate synthase, *ass*; Callaghan *et al*., 2008). Also, partial sequences encoding the gene for the large subunit of the Mas (or Ass) enzymes have been retrieved from hydrocarbon-contaminated sediments and an enrichment culture with propane (Callaghan *et al*., 2010). Nevertheless, genes coding for Mas or Ass enzymes in propane- and *n*-butane-degrading bacteria are largely unknown. We attempted to amplify the *masD* gene from the enrichment cultures Prop12-GMe, But12-GMe and But12-HyR, and also from the alkane degrading, sulfate-reducing strains BuS5 (C_3–C_4), Pnd3 (C_{14}–C_{17}) and TD3 (C_6–C_{16}). A full-length *mas*D sequence was obtained from strain Pnd3 (2505 bp), an almost full-length *mas*D sequence from strain TD3 (2380 bp), and only partial sequences from strain BuS5 (483 bp) and the enrichment cultures (429 bp). For each enrichment culture, identical *masD* sequences were obtained by cloning and sequencing of the PCR products ($n \geq 30$ clones). Phylogenetic analysis of deduced amino acid sequences showed that MasD from the psychrotrophic enrichment cultures clustered together, and were most closely related to MasD of strain BuS5 and of the thermophilic, long-chain alkane degrader TD3 (Fig.13).

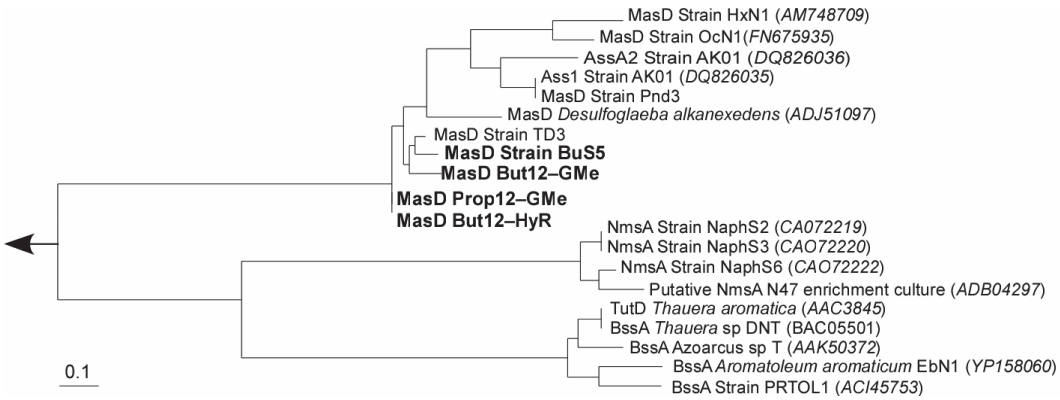

Fig.13 Maximum likelihood phylogenetic tree of MasD, BssA and NmsA amino acid sequences. The tree was calculated in ARB using only partial sequences shared by all species considered. MasD sequences of propane and butane degraders are indicated in boldface. Scale bar indicates 0.1 substitutions per site.

C.2.3 Identification of dominant phylotypes

The sediment-free enrichments Prop12-GMe and But12-HyR formed macroscopic, dark-red aggregates with a diameter of up to 1 mm. Microscopic analysis showed that aggregates of both enrichment cultures consisted of densely packed, mainly oval, slightly curved cells (Fig.14 A, B). Further description of the apparently dominant morphotypes was attempted using 16S rRNA-based methods. Construction and analysis of 16S rRNA gene libraries showed a high diversity of sulfate-reducing bacteria in both enrichment cultures (not shown). Sequences were closely related to those of *Desulfobacula, Desulfococcus and Desulfosarcina* species, including strain BuS5 and the dominant phylotype in the *n*-butane-degrading enrichment culture, But12-GMe (Kniemeyer *et al*., 2007). Sequences obtained from an increasingly diluted (10^{-2}) gDNA template of both enrichment cultures were identical with a clone in the library of Prop12-GMe (clone 235) and in the library of But12-HyR (clone 303), both affiliated with the genus *Desulfosarcina*, suggesting that these phylotypes were dominant in the Prop12-GMe and But12-HyR enrichment culture, respectively.

To verify this hypothesis, a nested FISH approach was used. Hybridizations of aggregate sections with the group-specific probe DSS658 showed that phylotypes affiliated with the *Desulfosarcina*/*Desulfococcus*- group of the *Deltaproteobacteria* represented approx. 80% of the Prop12-GMe and approx. 95% of the But12-HyR enrichment culture (not shown). Furthermore, hybridizations of homogenized aggregate samples with specifically designed oligonucleotide probes showed that Prop12-GMe clone 235 accounted for up to 74% of the total cell number of the Prop12-GMe enrichment culture, and But12-HyR clone 303 for up to 69% of the total cell number of the But12-HyR enrichment culture (Fig.14 C, D).

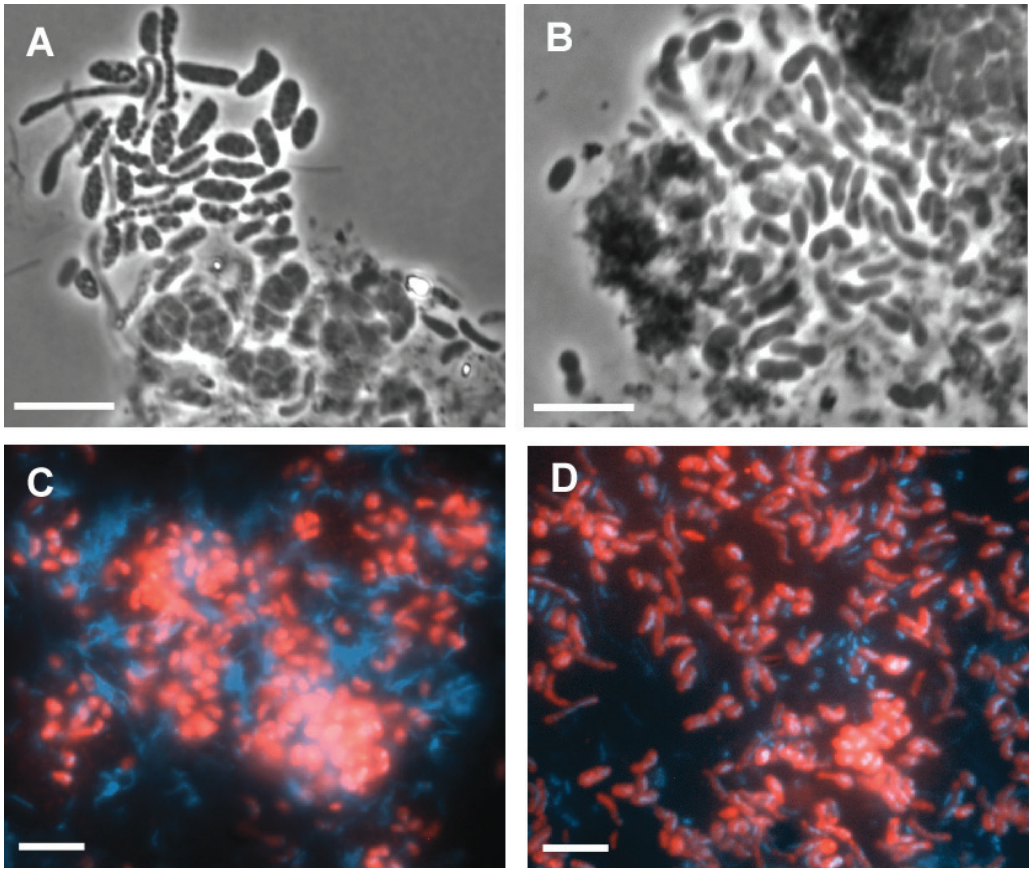

Fig.14 Microscopic images of the enrichment cultures Prop12-GMe (A, C), and But12-HyR (B, D). Phase-contrast images show densely packed, mostly oval-shaped cells in both enrichment cultures (A, B). Hybridizations of partly homogenized aggregates with sequence-specific oligonucleotide probes further indicated that each enrichment culture was dominated by unique phylotypes (C, D). The hybridization images (C, D) show overlays of probe (orange) and DAPI (blue) signals. Scale bars indicate 5 μm.

The high abundance of a distinct phylotype in each of the psychrotrophic enrichment cultures (Prop12-GMe, But12-GMe and But12-HyR) suggests that the corresponding microorganisms were most likely responsible for the degradation of propane or *n*-butane. In order to test this hypothesis, the enrichment cultures were incubated with ^{13}C-labeled propane or *n*-butane, the cells were hybridized with HRP-labeled sequence-specific oligonucleotide probes, followed by deposition of Fluorine (F)-containing tyramides, and analyzed by nanoSIMS.

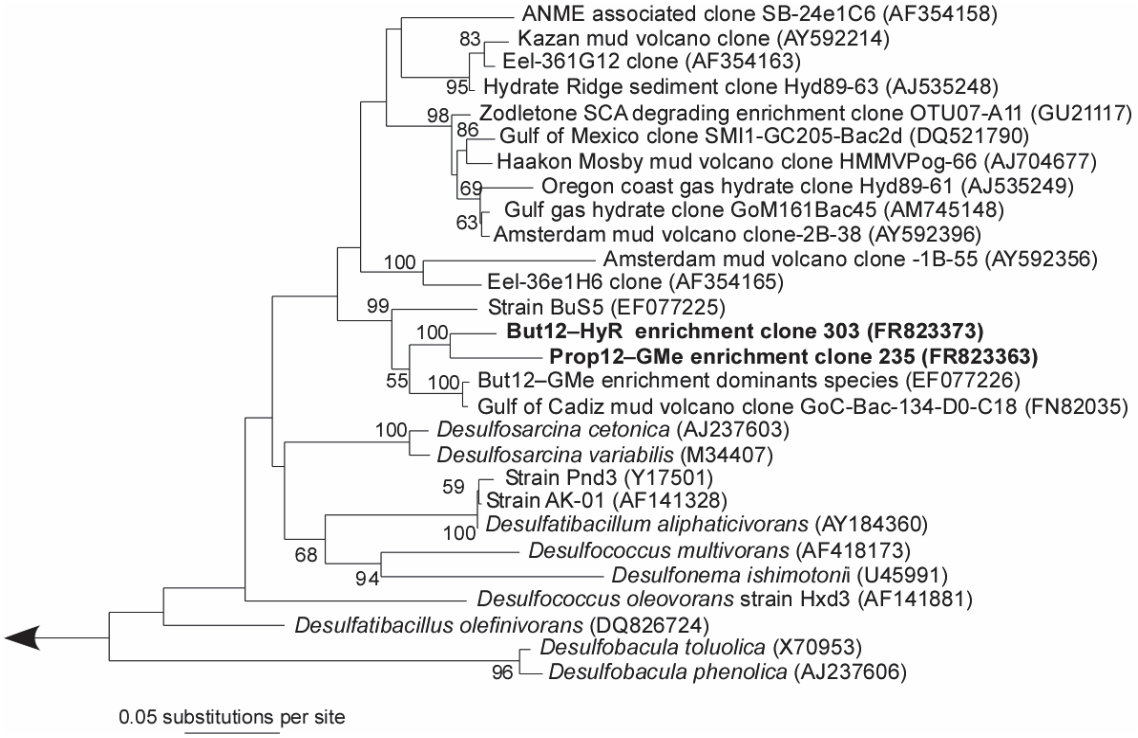

Fig.15 Phylogenetic affiliation of the dominant phylotypes in the enrichment cultures Prop12-GMe and But12-HyR (marked in boldface). The phylogenetic tree was calculated by maximum likelihood, using only nearly full length sequences (> 1400 bp). The numbers next to the nodes indicate bootstrap values; only values larger than 50% are shown.

C.2.4 Incubations with ^{13}C-labeled hydrocarbons and nanoSIMS analysis

Enrichment cultures incubated with labeled propane or *n*-butane became increasingly enriched in ^{13}C as revealed by mass spectrometric (MS) analysis of the bulk biomass. Based on the bulk MS analysis, samples were further selected for single cell analysis by nanoSIMS (0, 3 and 5 days of incubation for Prop12-GMe; 0, 5 and 10 days for But12-GMe; 0, 3 and 6 days for But12-HyR). For each sample, secondary ion images of ^{12}C (measured as $^{12}C^-$) ^{14}N (measured as $^{12}C^{14}N^-$), ^{13}C ($^{13}C^-$) and ^{19}F ($^{19}F^-$) were recorded simultaneously. Using the $^{12}C^{14}N^-$ ion images (total biomass), regions of interest (ROI) were drawn around individual cells and used to determine $^{13}C/^{12}C$ ratios for the dominant and accompanying cells (Fig.16). The cells of the dominant phylotype were identified by the presence of ^{19}F. In all enrichment cultures, they were the first to assimilate ^{13}C from the labeled substrate, while the accompanying cells showed no or only minor label incorporation (Fig.17). With longer incubation time, increasing ^{13}C-labeling was observed

in the accompanying cells (Fig.17). These results demonstrate that the main phylotypes were responsible for the degradation of propane or *n*-butane, while the accompanying bacteria were most probably growing at the expense of excreted degradation intermediates or cellular constituents from lysed dominant cells.

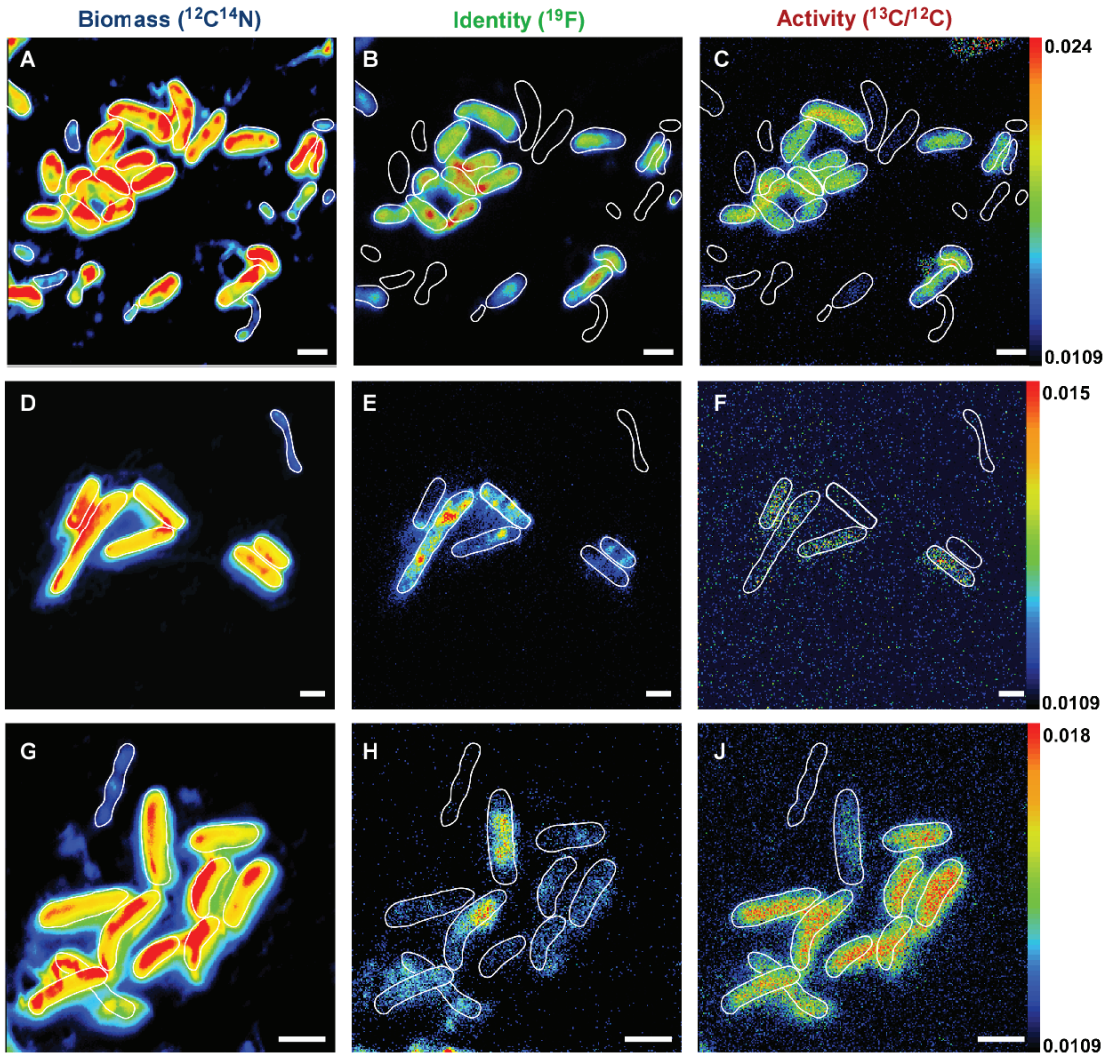

Fig.16 NanoSIMS images of the enrichment cultures Prop12-GMe (A–C), But12-GMe (D–F), and But12-HyR (G–I) after incubation with ^{13}C-labeled propane or butane for 3, 5 and 3 days, respectively. The total biomass ($^{12}C^{14}N$) images (A, D, G) were used to draw regions of interest around individual cells. Identification was done based on detection of ^{19}F (B, E, H) introduced in the cells by deposition of F-containing tyramides after hybridization with HRP-labelled oligonucleotide probes. The ratio images of $^{13}C/^{12}C$ show incorporation of label by individual cells (C, F, I). Scale bars indicate 1 µm.

Average assimilation rates were calculated for cells showing an enrichment in ^{13}C vs. cells at t_0. The average values obtained ranged from 10 ± 9 amol C cell^{-1} d^{-1} (But12-GMe) to 60 ± 28 amol C cell^{-1} d^{-1} (Prop12-GMe). Using the C assimilation rates, hydrocarbon assimilation rates were calculated, and assuming the typical 1:9 ratio of substrate assimilation vs. dissimilation for sulfate-reducing bacteria, the hydrocarbon dissimilation rates were determined. Using the latter, and considering the complete oxidation of propane and *n*-butane (Kniemeyer *et al.*, 2007), the cellular sulfate-reduction rates were calculated (page 117, Table 1). To our knowledge this is the first study reporting hydrocarbon-degradation and sulfate-reduction rates of single cells for propane and *n*-butane degrading microorganisms. These rates could be useful in future studies to estimate the contribution of propane and *n*-butane degraders to the total sulfate reduction rates, provided the abundance of such microorganisms will be quantified.

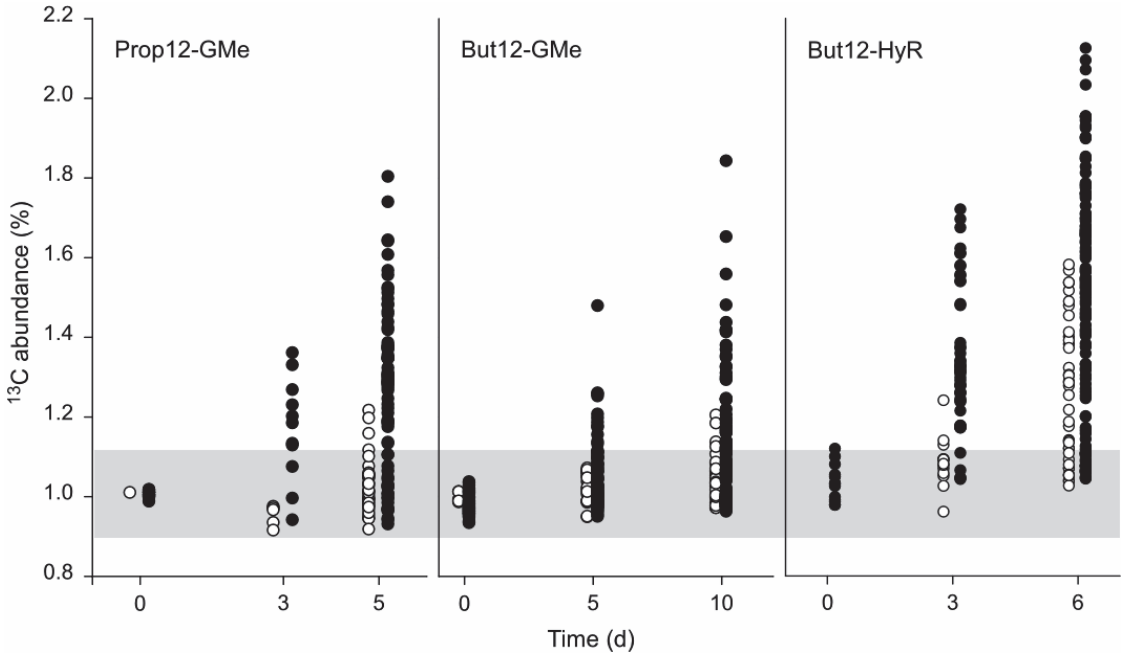

Fig.17 Abundance of ^{13}C in single cells of the enrichment cultures Prop12-GMe, But12-GMe, and But12-HyR incubated with 13C-labeled propane (Prop12-GMe) or butane (But12-GMe and But12-HyR). The ^{13}C abundance was calculated from the $^{13}C/^{12}C$ ratios determined for individual cells of the dominant phylotype (●) or accompanying cells (○). The dominant cells were identified based on the presence of ^{19}F signals. For clarity, accompanying and dominant cells at each time point were plotted with an off scale of ± 0.2. Shaded area indicates the range of ^{13}C-abundance values obtained for cells at 0 days of incubation (no enrichment vs. natural abundance of ^{13}C).

C.2.5 Conclusion

In the present study, new propane and *n*-butane-degrading sulfate-reducing bacteria have been enriched from deep-sea sediments collected around hydrocarbon seeps. Using hybridizations with sequence-specific oligonucleotide probes, incubations with ^{13}C-labeled substrates and nanoSIMS analysis, it could be demonstrated that each enrichment culture was dominated by unique microorganisms, which were responsible for the degradation of the hydrocarbon substrate. These microorganisms were apparently specialized, capable of degrading only propane and *n*-butane, but not longer or shorter-chain alkanes. This supports *in situ* observations reporting that propane and *n*-butane appear to be biologically degraded in oil and gas reservoirs (James and Burns, 1984; Boreham *et al*., 2001; Head *et al.*, 2003; Larter *et al.*, 2005) and also at marine hydrocarbon-rich sites (Orcutt *et al*., 2010; Quistad and Valentine 2011), whereas ethane, *iso*-butane and *n*-pentane appeared rather recalcitrant.

The cold-adapted propane and *n*-butane degraders described here were closely related to strain BuS5, forming an obvious phylogenetic cluster of short-chain alkane-degrading, marine sulfate-reducing bacteria within the *Desulfosarcina/Desulfococcus* group of the *Deltaproteobacteria.* Members of this group have been found in relatively high abundance at gas and oil hydrocarbon seeps, reaching up to 10% of the total free cells (about 5×10^8 cells cm^{-3}; e.g. Orcutt *et al*., 2005, 2010). Sulfate reduction rates (SRR) determined at such sites often exceeded SRR that could be explained by anaerobic oxidation of methane by several fold, and have been proposed to be due to oxidation of hydrocarbons other than methane, including short-chain alkanes (Kallmeyer and Boetius, 2004; Joye *et al.*, 2004; Niemann *et al.*, 2006; Orcutt *et al.*, 2004, 2010). Propane and *n*-butane-degrading sulfate reducers as those described here could thus have an important role for the *in situ* carbon and sulfur cycling, contributing significantly to the observed excess of sulfate-reduction. Future studies, making use of incubations with stable isotope-labeled substrates, hybridizations with specifically-designed genetic probes followed by nanoSIMS analyzes, or quantification of specific *masD* genes, on naturally-occurring populations could determine the abundance of such bacteria and their contribution to the short-chain alkane degradation and sulfate-reduction directly in the environment.

C.3 Carbon and hydrogen stable isotope fractionation effects during the degradation of propane and *n*-butane by sulfate-reducing bacteria

Microbial degradation of hydrocarbons is associated with an enzymatic discrimination against substrate molecules containing the heavy, stable isotopes ^{13}C and ^{2}H (D). This is essentially due to the stronger chemical bonds of these isotopes with their bonding atoms. This effect leads to higher enzymatic turnover of the lighter isotopologues and consequently to an enrichment of the residual substrate in heavy isotopologues. The resulting kinetic isotope effect (KIE) is characteristic for the degradation of a given compound. Isotope fractionation factors can be accurately determined *in vitro*, with enriched or pure cultures, where loss of substrate is only due to microbial degradation, and applied in the environment to estimate *in situ* degradation rates (Meckenstock *et al*., 2004; Hofstetter *et al*., 2008). The extent of isotope fractionation can be masked by non-fractionating steps, preceding the initial irreversible reaction itself, e.g. reduced substrate availability due to poor solubility and diffusion (Thullner *et al*., 2008, Kampara *et al.*, 2008) or high cell densities (Templeton *et al.*, 2006; Kampara *et al*., 2009), slow uptake of the substrate into the cell or binding to the enzyme (Elsner *et al*., 2005). Thus, the observed apparent kinetic isotope effect (AKIE) can be much lower than the actual KIE. Therefore, two dimensional (2D) compound-specific isotope analyses (CSIA) has become an established method to obtain information on the mechanism of activation for a certain compound *in situ* (Elsner *et al.,* 2005; Zwank *et al.,* 2005; Fischer *et al.*, 2007, 2008). The underlying principle is that both elements, directly involved in the initial activation (the bond cleavage) are analyzed with respect to their stable isotope fractionation effect. Assuming that both elements will be equally affected by masking effects, a correlation of fractionation of both elements will cancel out such effects and can be used as a biochemical marker for a given reaction.

2D-CSIA has been successfully applied to at first characterize the activation mechanisms of different aromatic compounds during their anaerobic biodegradation by defined cultures and thereafter to detect these values in undefined enrichment cultures and the environment (Vogt *et al*., 2008; Elsner *et al*. 2010).

Currently, the only 2D-CSIA data available for cultures of anaerobic *n*-alkane degrading bacteria is for *n*-hexane and *n*-octane degradation by strain *Desulfoglaeba alkanexedens* (Morris *et al.,* submitted). We have determined carbon and hydrogen

fractionation for strain BuS5 during growth with propane or *n*-butane, which is known to activate both substrates by addition to fumarate and for the enrichment cultures Prop12-GMe (propane), But12-GMe (*n*-butane) and But12-HyR (*n*-butane) for which it was hitherto not known what the involved activation mechanism is. If the activation mechanism by the enrichment cultures was the same as for strain BuS5, the 2D-CSIA data should be similar.

C.3.1 Bulk enrichment factors of anaerobic propane and *n*-butane degradation-masking effects.

Enrichment factors (ε) were determined for cultures incubated with shaking, to ensure optimal mass transfer of the substrate, and for standing cultures, which might be affected by substrate diffusion limitations and likely better represent marine diffusive sediment environments. The bulk carbon and hydrogen fractionation during propane and *n*-butane degradation was lower in static incubations, but also for actively mixed cultures which form aggregates (see Table 1 on page 155 for all results obtained in this study). This indicated isotope fractionation masking effects due to (in the first case) impaired substrate diffusion into the medium, and (in the second case) to diffusion limitations for cells on the inside of the aggregates.

C.3.2 Apparent kinetic isotope effects (AKIEs) of anaerobic propane and *n*-butane degradation − mechanistic aspects.

For comparing the reaction mechanisms of propane and *n*-butane degradation, bulk enrichment factors were converted to position specific enrichment factors (ε_{rp}) and apparent kinetic isotope effects (AKIEs), which considers the number of total, reactive and competing C or H atoms of a molecule in a given reaction mechanism (Elsner *et al*., 2005; see materials and methods on page 136 for a detailed description) (Fig.18 A, B). These values can thus be used to compare activation mechanisms for similar compounds. For shaken planktonic cultures, AKIE values for both carbon (1.02–1.026) and hydrogen (1.35–2.14) were in a range expected for isotope effects linked to the cleavage of a C-H-bond (Elsner *et al*., 2005). The highest AKIE values for carbon and hydrogen were observed for propane degradation by strain BuS5 (Fig.18 A and B; Table 1 on page 155).

AKIE values for *n*-butane might be on the one hand slightly lower due to a (small) kinetic masking effect, still existing in shaken cultures caused by the lower solubility of *n*-butane in water (Oswald coefficient (*L*) = 0.03702 at 28 °C and 0.05154 at 12 °C) than for propane (*L* = 0.04376 bat 28 °C and 0.05825 at 12 °C) (Dean, 1992). In addition, these higher values during degradation of propane could be due to a second route of activation for propane at both the secondary and the primary carbon atom. Previous studies (Kniemeyer *et al.*, 2007; Savage *et al.,* 2010) have reported metabolites, which are indicative for an additional activation of propane at the primary carbon atom via carbon-carbon addition to fumarate, by sulfate-reducing bacteria.

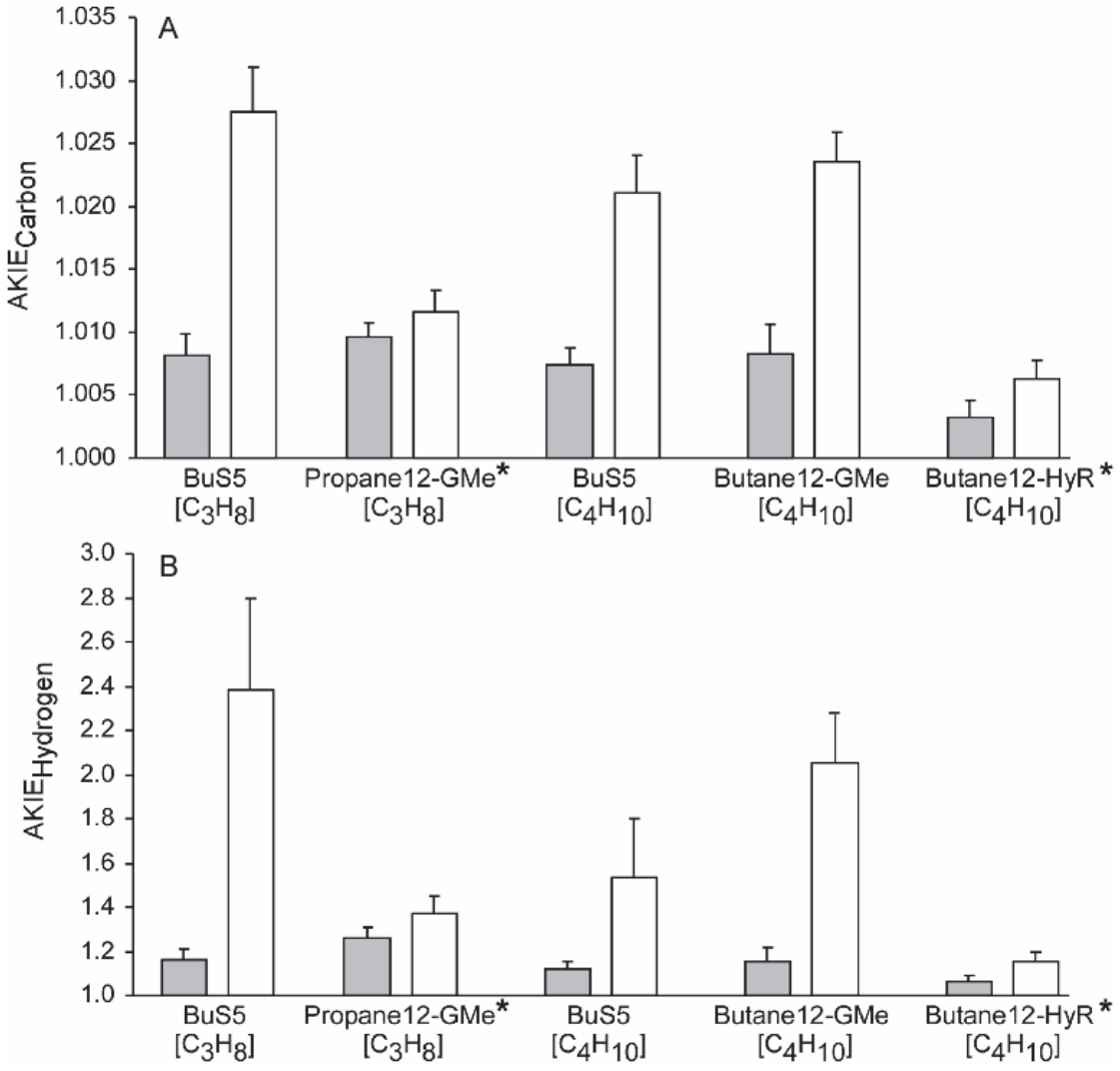

Fig.18 Overview of AKIE values for carbon (A) and hydrogen (B) during degradation of propane (C_3H_8) or *n*-butane (C_4H_{10}) by strain BuS5 and the enrichment cultures. Grey bars represent static incubations and white bars actively mixed cultures. Enrichment cultures forming aggregates are marked (*).

C.3.3 Investigation of the activation mechanisms by two-dimensional isotope fractionation analysis

By plotting the shifts of hydrogen and carbon isotope signatures, a slope develops (lambda value (Λ), Fischer *et al.*, 2008), which can be used to characterize distinct substrate activation mechanisms, as shown e.g. for the activation of toluene (Vogt *et al*., 2008; Tobler *et al*., 2008) or benzene (Mancini *et al*., 2008; Fischer *et al*., 2008). In our study, Λ values of shaken cultures were always and in most cases significantly higher than Λ values of standing cultures (Fig.19). To our knowledge, this effect of substrate limitation on the extent of the two dimensional isotope fractionation effect has not been investigated before. Previous studies, which have investigated the effect of bioavailability restrictions due to substrate limitations (Kampara *et al.*, 2008) or high cell densities (Staal *et al*., 2007; Templeton *et al.*, 2006; Kampara *et al*., 2009), have only investigated the effect on carbon isotope fractionation alone. Thus, the assumption that masking effects will affect the two elements involved in the biochemical cleavage or formation of a bond equally (Elsner *et al.,* 2005; Zwank *et al.,* 2005; Fischer *et al.*, 2007, 2008) may not always be true.

Possible factors causing a shift in Λ values during substrate limiting vs. non-limiting conditions could be a change of the transition state of the activating enzyme during substrate-limiting conditions. Briefly, when an atom is transferred from one molecule to another, the D or ^{13}C isotope effects will be at maximum when the transition state is symmetrical. This means that the force constants of the bonds being made and broken are equal. However, in cases where the two atoms do not form equally strong bonds, the maximum isotope effect may not correspond to this situation (Botting, 1994). Opposed effects for carbon and hydrogen isotope fractionation have been observed, i.e. for the formate dehydrogenase, provided with four different coenzymes (nucleotides), having different redox potentials. This was explained by changing transition states upon each reaction due to change in redox potentials of the reaction partners (Botting, 1994).

Alternatively, isoenzymes might be induced, with higher substrate affinities during conditions of low substrate availability and which therefore probably also exhibit different transition states. This may lead to altered two-dimensional isotope fractionation patterns during substrate-limiting conditions. For example, the C_{13}–C_{18} alkane degrading sulfate-reducing bacterium *Desulfatibacillum alkenivorans* (strain AK-01) contains two

highly similar, but not identical copies of the operon coding for the activating enzyme alkylsuccinate synthase (Ass) (Callaghan *et al.*, 2008). The role of the second operon is still not clear (Callaghan *et al.*, 2011) and one could speculate that a second enzyme with higher substrate affinities may be induced when substrate concentrations are low. However, the reason for the observed shift in Λ values during substrate limiting vs. non-limiting conditions remains unknown and will be addressed in future studies.

For comparing the degradation pattern of propane and *n*-butane, we considered the Λ values of shaken, non-limited cultures. For this purpose, we calculated Λ values of the reactive position (Λ_{rp}). The Λ_{rp} values were in the same range for *n*-butane (7.8–9.9) or propane (10.5–11.8), respectively, indicating that each alkane was activated by an identical mechanism in the tested cultures. Notably, Λ_{rp} values for propane activation were significantly higher than values for *n*-butane activation in strain BuS5 and enrichment culture Butane12-GMe (page 155, Table 1), indicating that propane and *n*-butane were activated by slightly different activation mechanisms in these cultures.

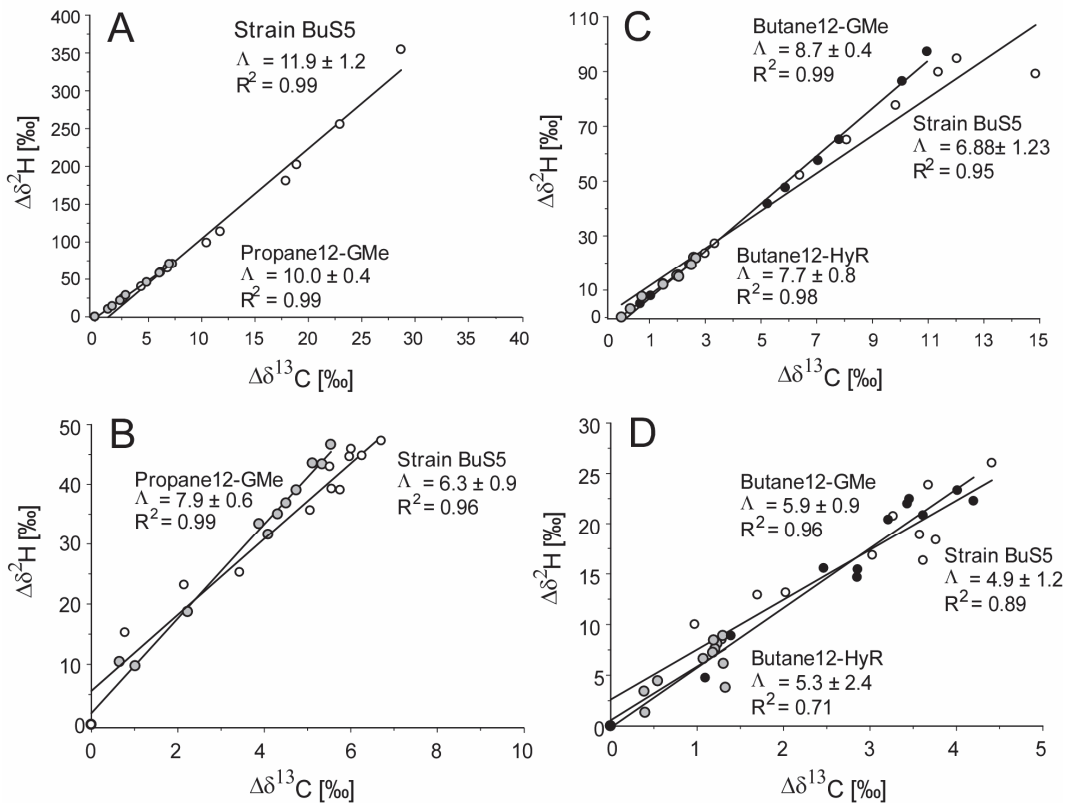

Fig.19 Carbon vs. hydrogen fractionation during growth of strain BuS5 and Prop12-GMe with propane (A, B), and of strain BuS5, But12-GMe and But12-HyR with *n*-butane (C, D). Fractionation factors of shaken incubations (A, C) are compared with those determined for static incubations (B, D).

For the sulfate-reducer *D. alkanexedens*, Λ_{rp} values of 9.1 and 6 were reported for anaerobic *n*-hexane and *n*-octane degradation, respectively (Morris *et al*., submitted). Notably, this organism is known to activate *n*-hexane and *n*-octane by fumarate addition at the secondary carbon atom (Kropp *et al*., 2000; Davidova *et al.*, 2005), allowing a direct comparison with the values gained in our study. The values were similar to those found for *n*-butane activation, indicating an analogous activation mechanism.

We furthermore calculated a Λ_{rp} value of 12 for anaerobic propane degradation at a marine mud volcano by taking ε_C and ε_H values reported by Mastalerz *et al.* (2009) and assuming that propane is activated at the secondary carbon atom by fumarate addition (as we did in our calculations). The determined value is nearly identical to what we observed for the degradation of propane by our tested cultures. This indicates that propane is indeed activated by fumarate addition at the mud volcano site, showing also the strength of the two-dimensional approach.

The reported ε_C and ε_H values for the aerobic degradation of propane by microorganisms in marine sediment from a hydrocarbon seep (Kinnaman *et al*., 2007) on the contrary, result in a λ_{bulk} factor of 3.1, which is lower than our λ_{bulk} values for the anaerobic degradation of propane (10.0–11.9). The resulting Λ_{rp} value of 2.7, assuming activation of propane via insertion of molecular oxygen by a monooxygenase at a primary carbon atom during aerobic degradation (Shennan *et al.*, 2006), is also much lower than our values for anaerobic degradation of propane by fumarate addition (10.5–11.8). This result shows that activation mechanisms for propane under aerobic and anaerobic conditions result in different Λ_{rp} values, reflecting the differences in stable carbon and hydrogen isotope fractionation during different activation mechanisms, which are mediated by different classes of enzymes.

C.3.4 Experiments with deuterated substrates – is activation of propane at the primary carbon atom a significant route of activation?

To investigate whether the observed higher λ_{rp} values for propane, relative to those observed for *n*-butane, could be due to an additional activation of propane at one of the primary carbon atoms, we incubated strain BuS5 with mixtures of equal amounts of natural propane and propane, which was deuterated either at the primary carbon atoms (propane-D_6) or at the secondary carbon atoms (propane-D_4). For reference, strain BuS5

was also grown with mixtures of equal amounts of natural *n*-butane and either *n*-butane, which was deuterated at the primary carbon atoms (butane-D_6) or at the secondary carbon atoms (butane-D_4). The degradation rates were monitored by GC, using a newly developed method for separation of the isotopologues, and used to calculate fractionation factors.

During growth with butane-D_6 and natural butane, strain BuS5 degraded natural butane as fast as butane-D_6. Thus, the hydrogen isotope ratios (R_t) of butane-D_6 to natural butane stayed constant during the course of degradation and no significant D/H fractionation (enrichment factors of −25.2 ‰ ± 22.6) was observed. In contrast, when strain BuS5 was grown with butane-D_4 and natural butane, it degraded natural butane faster, leading to a D/H enrichment factor of −331.9 ‰ ± 46.7. These results strongly indicate that butane was solely activated at the secondary carbon atom, as suggested before by metabolite analysis (Kniemeyer *et al*., 2007).

During growth with propane-D_6 and natural propane, strain BuS5 degraded natural propane faster than propane-D_6. The resulting D/H fractionation resulted in an enrichment factor of −167.6 ‰ ± 46.6, which is significantly higher than the one observed for degradation of butane with deuterium label at the primary carbon atom. Still, the enrichment factor obtained from the degradation of propane-D_2 and natural propane was higher (−436.7 ‰ ± 81.4) than that for primary carbon atom deuterated propane and natural propane.

The results (Fig.20) indicate that in contrast to the activation of *n*-butane, which seems to take place solely at the secondary carbon atom, a second route for activation of propane at the primary carbon atom took place. The fact that the enrichment factor during degradation of a mix of butane-D_4 and natural *n*-butane is lower than that observed for the mix of propane-D_2 and natural propane can be due to masking effects caused by a lower solubility of butane compared to that of propane. The additional activation of propane at the primary carbon atom might explain the above discussed higher Λ_{rp} values for the anaerobic degradation of propane, compared to *n*-butane, during non substrate limiting conditions. Future experiments, using assays with crude extracts or a purified enzyme and quantification of metabolites, may reveal the true frequency of this so far unprecedented activation reaction.

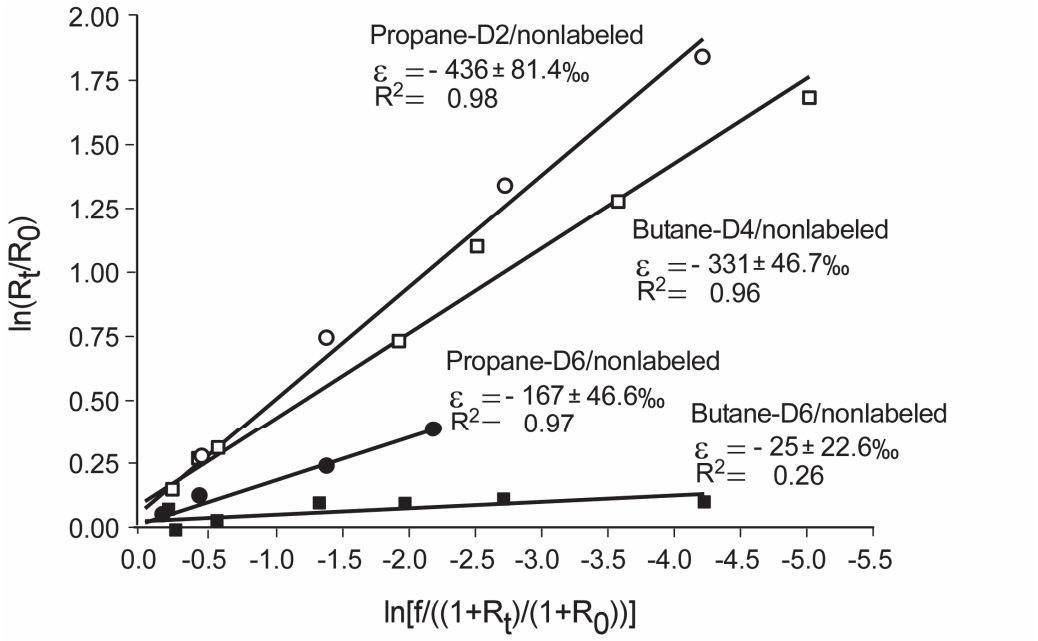

Fig.20 D/H isotope fractionation during the degradation of heavy or light isotopologues of propane and butane by strain BuS5: degradation of equal mixtures of propane and propane-D_2 (○), propane and propane-D_6 (●), butane and butane-D_4 (□), and butane and butane-D_6 (■)

C.3.5 Conclusion

To our knowledge, this study reports for the first time that substrate limitations can lead to significantly decreased two dimensional isotope fractionation factors (Λ values). This observation contradicts the assumption that masking effects will always equally affect the two elements involved in the biochemical cleavage or formation of a bond. This effect should be further investigated in future studies, since it may have an influence on interpretations of 2D-CSIA data from field sites, where limitations in substrate bioavailability can be assumed to occur.

Still, we were able to show that by using 2D-CSIA, it is possible to distinguish between aerobic (insertion of oxygen by a monooxygenase) and anaerobic (fumarate addition by (1-methyl)alkylsuccinate synthase) activation of propane, based on the as yet observable carbon and hydrogen stable isotope fractionation (Kinnaman *et al*., 2007; this study). The enrichment factors for carbon and hydrogen obtained for the aggregate forming and thus substrate-limited propane degrading enrichment culture (Propane12-GMe) are similar to what has been reported for anaerobic propane degradation at marine deep sea mud volcanoes under sulfate-reducing conditions (Mastalerz *et al*., 2009).

Considering the rather low *in situ* concentration of propane and *n*-butane at natural hydrocarbons seep sites (Claypool and Kvenvolden, 1983) and the reduced diffusion of gases due to the particles (Millington, 1959; Kristensen *et al.*, 2010) that make up marine sediments, it seems likely that microorganisms degrading propane and *n*-butane at natural hydrocarbon seeps mighty actually be limited in substrate, even if they were only present at low abundances. Thus, masking effects due to low substrate bioavailability can be expected and should be considered when investigating stable isotope fractionation associated with propane and *n*-butane degradation *in situ*.

When comparing the obtained Λ_{bulk}-values for the anaerobic degradation of propane by the aggregate forming enrichment culture Propane12-GMe and strain BuS5 (this study) during actively mixed incubations to the Λ_{bulk}-values resulting from the reported enrichment factors for carbon and hydrogen by Mastalerz *et al*. (2009), the values are nearly identical. This clearly shows that the correlation of measured carbon and hydrogen fractionation can be useful in identifying a specific reaction mechanism *in situ*. Our obtained Λ_{rp}-values for *n*-butane degradation during non-substrate limiting conditions are furthermore in the same range as those for *n*-hexane and *n*-octane degradation under sulfate-reducing conditions (Morris *et al.*, submitted), indicating that Λ_{rp}-values are good indicators for detecting the degradation of several alkanes by the fumarate addition pathway in the environment.

Degradation experiments performed with strain BuS5 and deuterium-labeled alkanes (D_2- and D_4-labelled propane; D_2- and D_6-labeled butane) indicated that fumarate was added solely at the secondary carbon atom of *n*-butane, but at both the primary and secondary carbon atoms of propane.

C.4 Phylogenetic analysis of ethane-degrading sulfate-reducing bacteria from cold marine hydrocarbon seeps

Ethane is a major constituent of natural thermogenic gas and can be present at natural hydrocarbon seeps at concentrations of up to 30% of the total hydrocarbon gas. Whereas there is evidence that propane and *n*-butane are being biodegraded in deep thermogenic gas reservoirs and in anoxic sediments at natural hydrocarbon seeps, ethane appears to be rather recalcitrant. Degradation of ethane under anaerobic conditions was shown so far only with one enrichment culture obtained from sediments around cold hydrocarbon

seeps in the Gulf of Mexico, albeit the degradation proceeded at an extremely slow rate (Kniemeyer *et al*., 2007). To date, nothing is known about the involved activation mechanism or the microorganisms capable to degrade ethane under anoxic conditions.

C.4.1 Enrichment of ethane-degrading sulfate-reducing bacteria from cold marine hydrocarbon seeps

Enrichment of ethane-degrading sulfate-reducing bacteria from cold marine hydrocarbon seeps was attempted with sediments from the Gulf of Mexico, which were incubated at 12 °C. The observed ethane-dependent sulfate-reduction was very slow and the enrichment culture had produced ~ 15mM sulfide within the first ~ 400 days of incubation (Kniemeyer *et al*., 2007). Microscopic investigations of samples taken from the enrichment culture showed a relatively high abundance of small, rod-shaped cells, which were on average 1.4 µm in length and 0.6 µm in width. Since this enrichment culture has been maintained over a long period, during which it showed a very slow, yet clearly ethane-dependent production of sulfide, it can be assumed that such an abundant morphotype likely represents the ethane-degrading species.

C.4.2 Dominance of *Desulfosarcina/Desulfococcus*-cluster affiliated bacteria

Hybridization with kingdom specific oligonucleotide probes revealed the presence of ~ 10% archaeal cells and ~ 90% bacterial cells in the enrichment culture (Fig.21 A and B). The analysis of a 16S rRNA clone library revealed that 45% of all obtained sequences affiliated with the *Deltaproteobacteria*. These clone sequences were of particular interest and therefore fully sequenced, since many of the so far known hydrocarbon degrading, sulfate-reducing bacteria affiliate with this phylogenetic group, including propane- and *n*-butane-degrading bacteria (Kniemeyer *et al.*, 2007; Jaekel *et al*., in prep). Amplification of the 16S rRNA gene, using a 10^{-3} diluted gDNA sample as template, revealed a phylotype closely related to Ethane12-GMe clones 5, 11, 13 (> 97.8% sequence similarity) and more distantly also to Ethane12-GMe clone 18, suggesting that these phylotypes could represent the abundant rod-shaped cells.

Interestingly, these clone sequences were closest related to clone sequences within the *Desulfosarcina*/*Desulfococcus*-cluster, which were obtained from marine deep sea

hydrocarbon seep sites, such as in the Gulf of Mexico (Llyod *et al*., 2006), the Eel river basin (Orphan *et al.*, 2001), gas hydrates (Knittel *et al*., 2003) and mud volcanoes (Lösekann *et al*., 2007). In order to verify the PCR based result, fixed cells from this enrichment culture were hybridized with the probe DSS658, which targets members of *Desulfosarcina*/*Desulfococcus*-cluster (Fig.21 C). Counts of cells targeted by this probe revealed that ~ 60% of all cells in this enrichment culture belong to this cluster, including the abundant, small, rod-shaped cells. Based on this finding, a phylotype-specific oligonucleotide probe was designed (Ethane12-GMe_1017), targeting the Ethane12-GMe clone sequences 5, 11 and 13. Hybridizations of cells with this probe, followed by cell counts showed that, surprisingly, these phylotypes accounted for only ~ 5% of all cells in the enrichment culture and based on morphology also does not resemble the small rods but rather large, oval cells (Fig.21 D). It is possible that due to a PCR bias, a phylotype other than the dominant one was amplified.

Since hybridization with probe DSS658 showed that the abundant, small-rod shaped morphotype belongs to *Desulfosarcina*/*Desulfococcus*-cluster, the Ethane12-GMe12 clone sequences 18 or 29 should be investigated in more detail, since the DSS658 probe targets them as well (Fig.22). Hybridization with oligonucleotide probes targeting each one for these two phylotypes could reveal whether one of them perhaps represents the abundant phylotype and, thus, the likely ethane-degrading strain.

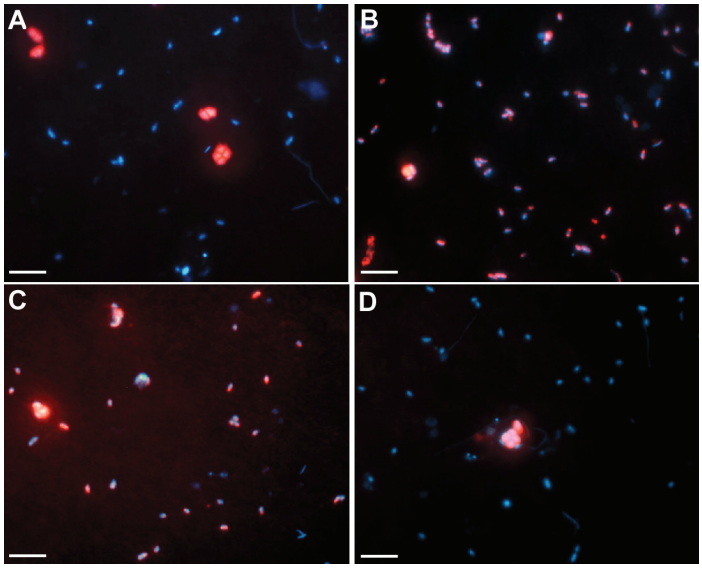

Fig.21 Whole cell hybridization with probes targeting *Archaea* (A), *Bacteria* (B), members of the *Desulfosarcina*/*Desulfococcus* cluster within the *Deltaproteobacteria* (C) and the phyloytpe represented by the Ethane12-GMe clones 5, 11, and 13 (D). Hybridization was followed by CARD-signal amplification (red) and DAPI staining (blue) of fixed cells in enrichment culture Ethane12-GMe. Scale bars represent 5 µm.

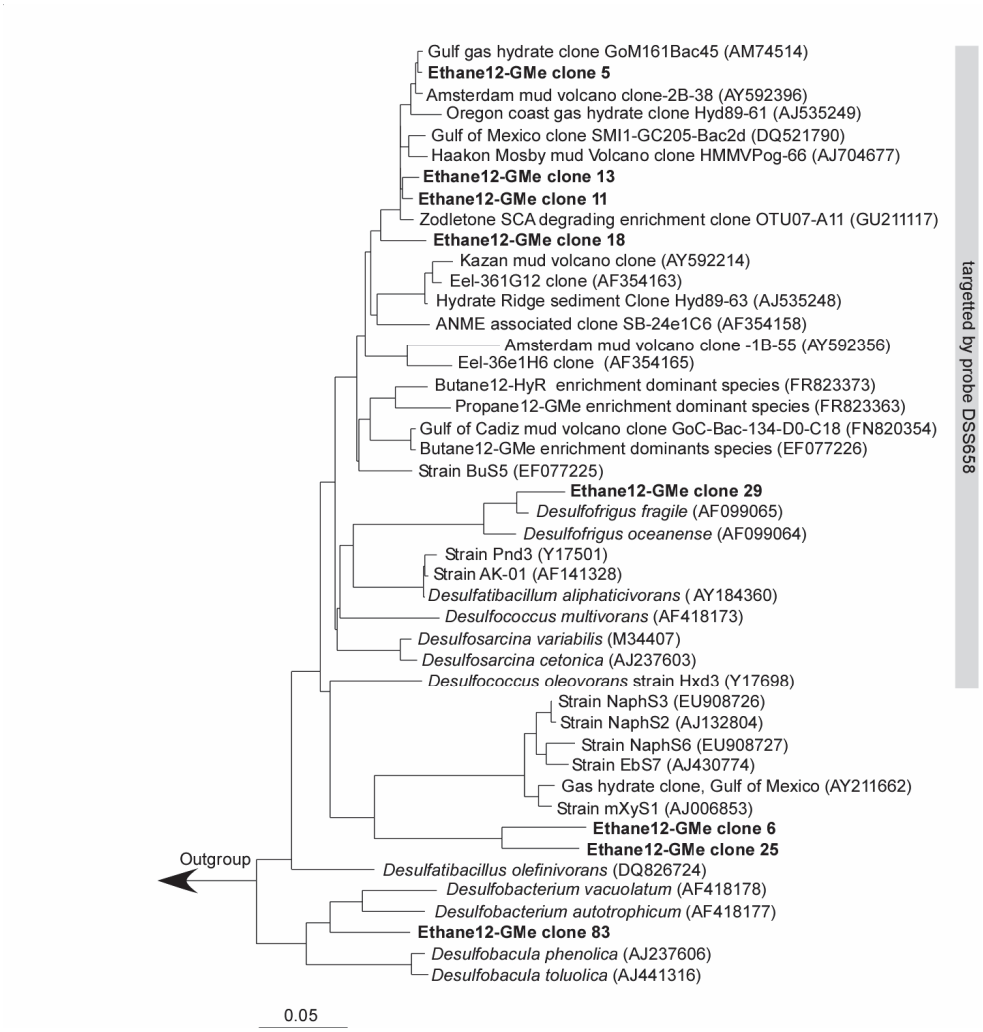

Fig.22 Phylogenetic affiliation of the *Deltaproteobacteria*–related 16S rRNA sequences from the ethane–degrading enrichment culture (marked in bold face). The tree was calculated in ARB by maximum likelihood and using different sets of filters. 16S rRNA sequences from *Escherichia coli* strains were used as outgroup. Scale bar indicates 5% sequence divergence.

C.5 A cyclohexane-degrading sulfate-reducing enrichment culture

Cycloalkanes are major constituents of crude oil and they are rather recalcitrant to biological degradation. Only few studies have reported the aerobic biodegradation of cycloalkanes by microorganisms and even less is known about the microorganisms capable to degrade these compounds anaerobically. The degradation of cycloalkanes under aerobic conditions commences through the insertion of reactive oxygen species into the molecule by a monooxygenase, yielding cycloalkanols (Cheng *et al.*, 2002). Anaerobic degradation of cycloalkanes was shown under sulfate-reducing (Rios-

Hernandez *et al.*, 2003; Townsend *et al.*, 2004), methanogenic (Townsend *et al.*, 2004) or nitrate-reducing conditions (Musat *et al.*, 2010). Detection of cycloalkylsuccinates by analysis of metabolites suggested that cycloalkanes are activated by addition to fumarate (Rios-Hernandez *et al.*, 2003; Musat *et al.*, 2010), yielding cycloalkylsuccinate derivatives. Another study showed that the nitrate-reducing Strain HxN1 co-activated cyclopentane and methylcyclopentane during growth on *n*-hexane by addition to fumarate (Wilkes *et al.*, 2003).

C.5.1 Enrichment of cyclohexane-degrading sulfate-reducing bacteria from marine hydrocarbon-contaminated intertidal sediments

Enrichment of cyclohexane-degrading bacteria with sulfate as the terminal electron acceptor was attempted with intertidal sediment from a hydrocarbon-contaminated lagoon in the Mediterranean. Enrichments were set up anaerobically in 100 ml flat glass bottles, sealed with butyl rubber stoppers and containing 50 ml of bicarbonate-buffered artificial sea-water medium (Widdel and Bak, 1992) under a headspace of N_2:CO_2 (90:10, v/v). The bottles were inoculated with 5 ml of homogenized sediment and amended with 2.5 ml of an inert carrier phase (2,2,4,4,6,8,8-Heptamethylnonane; HMN), containing 0.5% (v/v) cyclohexane. Control bottles without the addition of hydrocarbon substrate were set up in the same way. All bottles were incubated at 28 °C, lying nearly horizontally.

After approximately 5 months, the incubation with cyclohexane (0.5% v/v in HMN as a carrier phase) had formed ~ 15 mM sulfide, while control incubations (without addition of hydrocarbons) formed only ~ 8 mM sulfide. The relatively fast cyclohexane-dependant sulfate-reduction suggests that the sulfate-reducing microbial community in these sediments was adapted to the presence of hydrocarbon compounds. Microscopic analysis of the cells in the enrichment culture showed many oval, rod-shaped cells of an average of 1.5 µm length and 0.8 µm width. Quantitative growth experiments (Fig.23) and the calculation of a net electron balance for the degradation of cyclohexane (see Table 1, p. 190) showed that the electrons derived from cyclohexane consumption were nearly entirely used for reduction of sulfate to sulfide, indicating a complete degradation of cyclohexane to CO_2 according to the theoretical stoichiometric equation (Eq. 1).

$$2\ C_6H_{12} + 9\ SO_4^{2-} \rightarrow 12\ HCO_3^- + 9\ HS^- + 3\ H^+ \qquad [1]$$

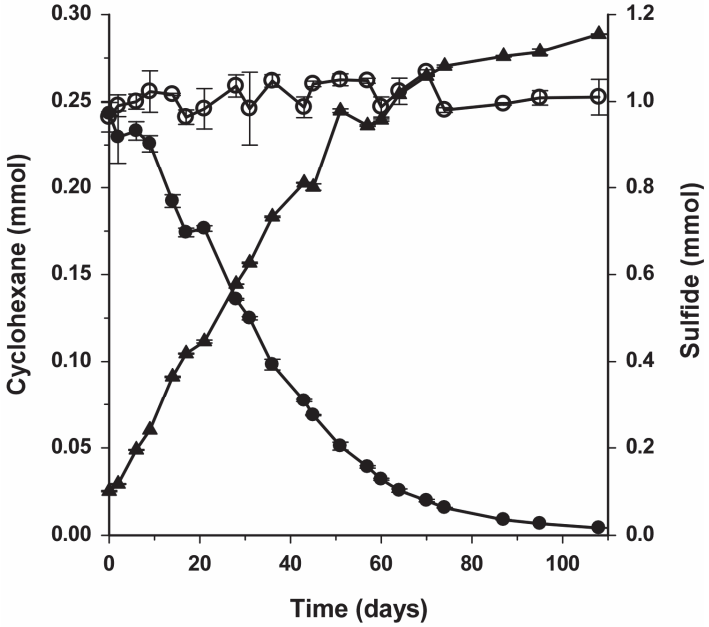

Fig.23 Degradation of cyclohexane by the sulfate-reducing enrichment culture Cyclohexane28-EdB. The consumption of cyclohexane (●) was coupled to the reduction of sulfate to sulfide (▲). No cyclohexane was lost in an abiotic control (○). Incubations were done in 120 ml round glass bottles with a culture volume of 100 ml and 5 ml of an inert carrier phase (HMN), containing 0.5% (v/v) cyclohexane. Measurements of cyclohexane and sulfide were done in triplicates.

C.5.2 Dominance of *Desulfosarcina/Desulfococcus*-cluster affiliated bacteria

A 16S rRNA gene library, which was constructed with primers targeting *Bacteria*, was dominated by sequences affiliated with the *Deltaproteobacteria*, with highest sequence similarity to *Desulfosarcina cetonica* (> 94%). A ″dilution-PCR″, using increasingly diluted gDNA template was used for amplification of the 16S rRNA gene. The sequence obtained with the highest diluted template that still yielded an amplification product (10^{-2} diluted gDNA template), which was identical to a clone sequence from the clone library (Cyclohexane28-EdB Clone63; Fig.24), which affiliated with the *Desulfosarcina*/*Desulfococcu*s cluster. This suggests that this phylotype might represent the tentative cyclohexane degrader in the enrichment culture.

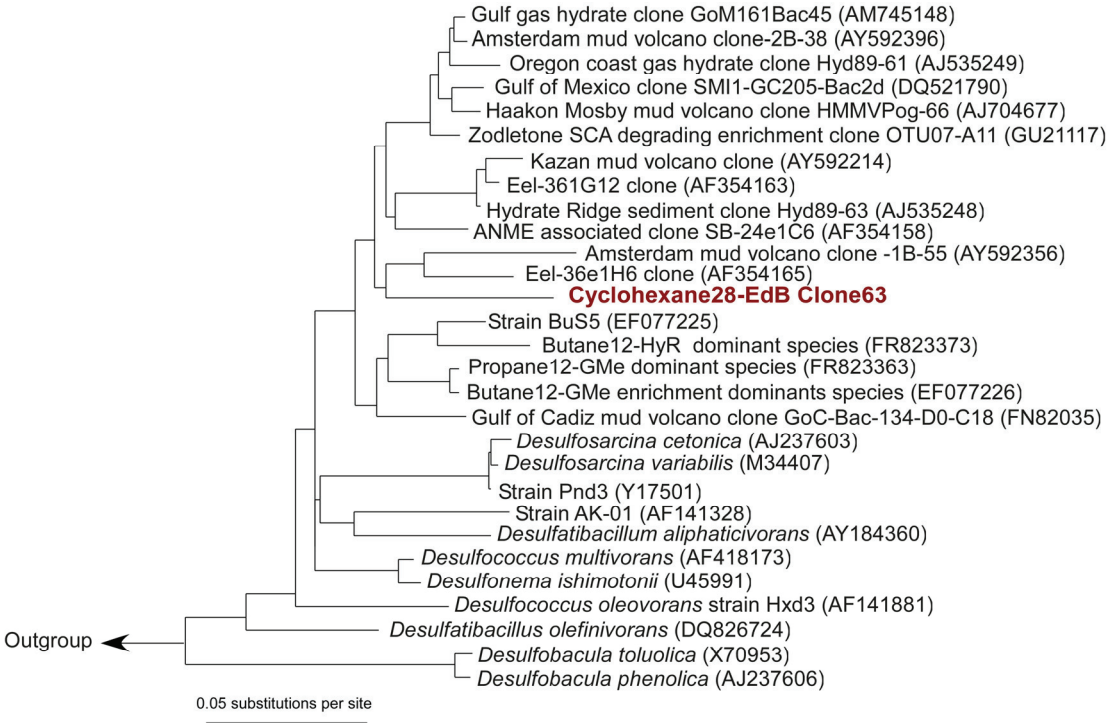

Fig.24 The phylogenetic affiliation of the most abundant phylotype in the enrichment culture Cyclohexane28-EdB (in red). The phylogenetic tree was calculated in ARB by maximum-likelihood, with application of different sets of filters, and by using only nearly full-length sequences (> 1300 nt). The scale bar indicates 5% sequence divergence.

In order to verify the results of the ″dilution-PCR″, fixed cells were hybridized with a group specific oligonucleotide probe (DSS658). Indeed, 84.3% of all cells (determined by DAPI staining) were targeted by this probe, thus confirming that the dominant phylotype belongs to the *Desulfosarcina/Desulfococcus*-cluster. Hybridization with a specifically designed oligonucleotide probe (CycS28-EdB_152) showed that Cyclohexane28-EdB-clone 63 constitutes 80.2% of all cells in this enrichment culture (Fig.25) and thus is most probably responsible for the degradation of cyclohexane. Cyclohexane28-EdB clone 63 is closest related to a 16S rRNA clone sequence obtained from a sulfate-reducing enrichment culture originating from Hydrate Ridge, which anaerobically oxidizes methane (AOM) (clone LARHR_58-01 B01, Schreiber *et al*., 2010). Other closely related clone sequences were obtained from marine hydrocarbon seep sites, such as the Eel River Basin, and marine deep sea mud volcanoes. The next closest cultivated species of Cyclohexane28-EdB clone 63 are strain BuS5 (92.7%, sequence identity), a sulfate-reducing bacterium affiliated with the *Desulfosarcina/Desulfococcus*-clusters, degrading

solely propane and *n*-butane (Kniemeyer *et al.*, 2007) and two dominant phylotypes, Butane12-GMe (Kniemeyer *et al.*, 2007) and Butane12-HyR (Jaekel *et al.*, in prep), which are dominant in two sulfate-reducing enrichment cultures from the Gulf of Mexico and Hydrate Ridge. These were shown to be responsible for the observed degradation of *n*-butane in the two cultures (Jaekel *et al.*, in prep). The new phylotype Cyclohexane28-EdB-clone 63 represents the first member of the *Desulfosarcina/Desulfococcus*-group that shows the ability to degrade cyclic alkanes.

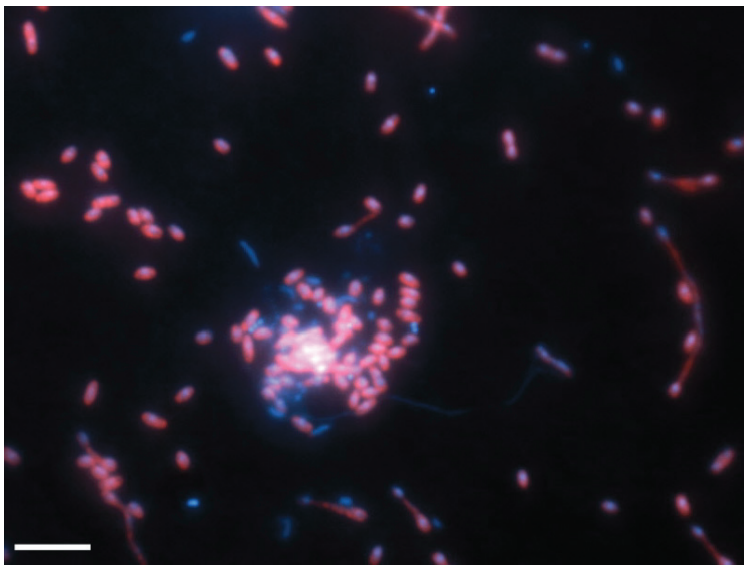

Fig.25 Whole cell hybridization (CARD-FISH) with the sequence specific probe CycS28-EdB_152 (red) and DAPI staining (blue) of fixed cells in the enrichment culture Cyclohexane28-EdB. Scale bar represents 5 µm.

C.5.3 Analysis of the activation mechanism for cyclohexane

The GC-MS analysis of metabolites from cultures grown with cyclohexane showed the presence of organic acids, which were absent in controls without added cyclohexane and in sterile controls (not shown). The presence of metabolites with specific fragments (see p. 184) suggested that the degradation of cyclohexane was initiated by addition to fumarate, yielding cyclohexylsuccinic acid (Fig.26 A), which was subsequently ligated to coenzyme A (CoA) and transformed via carbon skeleton rearrangement to cyclohexylmalonyl-CoA. Cyclohexylmalonyl-CoA is decarboxylated to cyclohexylpropionyl-CoA (Fig.26 B), which is further metabolized by β-oxidation through 3-oxo-3-cyclohexylpropionyl-CoA to cyclohexylcarboxylic acid (Fig.26 C).

From there, ring cleavage of the cyclohexyl-moiety may regenerate fumarate and yield acetyl-CoA, which can be channeled into the activation reaction or the central metabolic pathways.

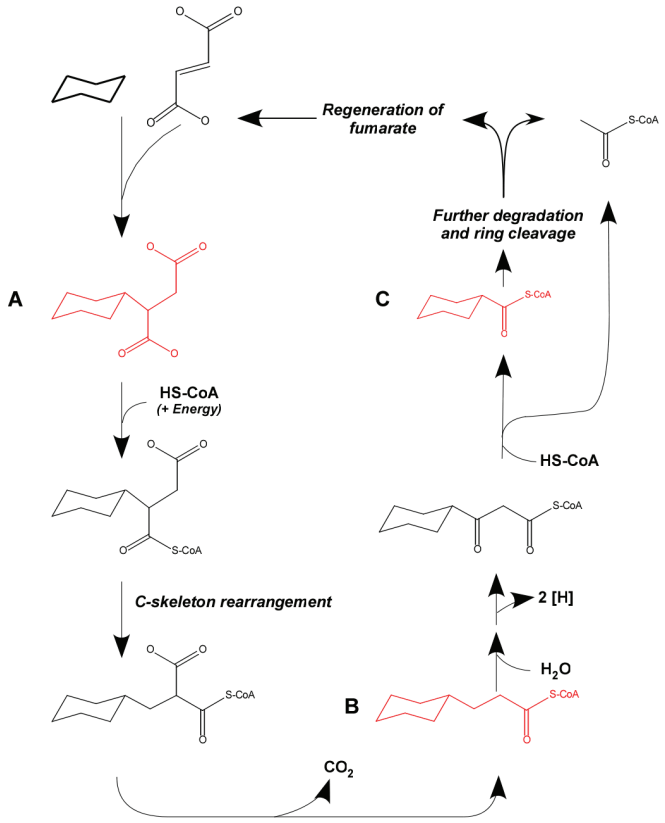

Fig.26 The proposed mechanism for the degradation of cyclohexane by the sulfate-reducing enrichment culture Cyclohexane28-EdB based on detected metabolites (red). See text for explanations. Metabolites detected by GC-MS analysis are marked in red.

The proposed scheme for the degradation of cyclohexane by enrichment culture Cyclohexane28-EdB would be analogous to the proposed degradation pathway of *n*-alkanes (Wilkes *et al.,* 2002), ethylcyclopentane in a sulfate-reducing enrichment culture (Rios-Hernandez *et al*., 2003) and cyclohexane by a nitrate-reducing enrichment culture (Musat *et al.*, 2010).

C.5.4 Substrate tests with other hydrocarbons

The enrichment culture Cyclohexane28-EdB was tested for its ability to degrade other hydrocarbons than cyclohexane. To prevent the growth of other microorganisms in the enrichment culture as a response to addition of new substrates, all experiments were performed with active, dense cell suspensions and incubated for short time periods. In addition, the abundance of the main phylotype was further investigated at the end of the incubation time. Sulfide production started without a lag phase in incubations with *n*-pentane, *n*-hexane, cyclohexane, cyclopentane, methylcyclohexane and methylcyclopentane, suggesting that the enzymes expressed during cyclohexane degradation were also able to activate and degrade these alkanes. In the incubation with *n*-butane, substantial sulfide production was observed as well, without a change in abundance of the phyloytpe Cyclohexane28-EdB clone 63. However, because sulfide production started only after a lag phase of 4 days, it is likely that different types of enzymes had to be induced in order to activate and degrade *n*-butane. To date, all reports about microorgansims capable of degrading cycloalkanes under anaerobic conditions have focused on the ability of these microorganisms to degrade single cycloalkanes (Rios-Hernandez *et al*., 2003; Townsend *et al*., 2004; Musat *et al.*, 2010).

C.5.5 Conclusion

The new, dominant phylotype is the first member of the *Desulfosarcina/Desulfococcus* group, able to degrade cycloalkanes and also C_4–C_6- *n*-alkanes. With the present study, we show that sulfate-reducing microorganisms, capable to degrade *n*-butane, can also be found in intertidal, hydrocarbon-rich sediments. Furthermore, the ability of the new phylotype to degrade a rather versatile spectrum of hydrocarbons, sheds further light on the metabolic capabilities of the members of the *Desulfosarcina*/*Desulfococcus*-group. These bacteria are often found at high abundances in marine, organic matter-rich, intertidal (Llobret-Brossa *et al*., 2002) and Arctic sediments (Ravenschlag *et al.*, 2000) or at hydrocarbon seep sites (Orcutt *et al*., 2010, Hamdan *et al.*, 2011), where they could play an important role in the overall carbon cycling through degradation of hydrocarbons, which are present at these sites either through natural seepage or as environmental pollutants.

D. Conclusions

D.1 Phylogeny

The new sulfate-reducing bacterial phylotypes, shown in by this study to degrade propane and *n*-butane at low temperatures (optima at ~ 16 °C), form an obvious phylogenetic cluster within the *Desulfosarcina*/*Desulfococcus*-group of the *Deltaproteobacteria* (Fig.27).

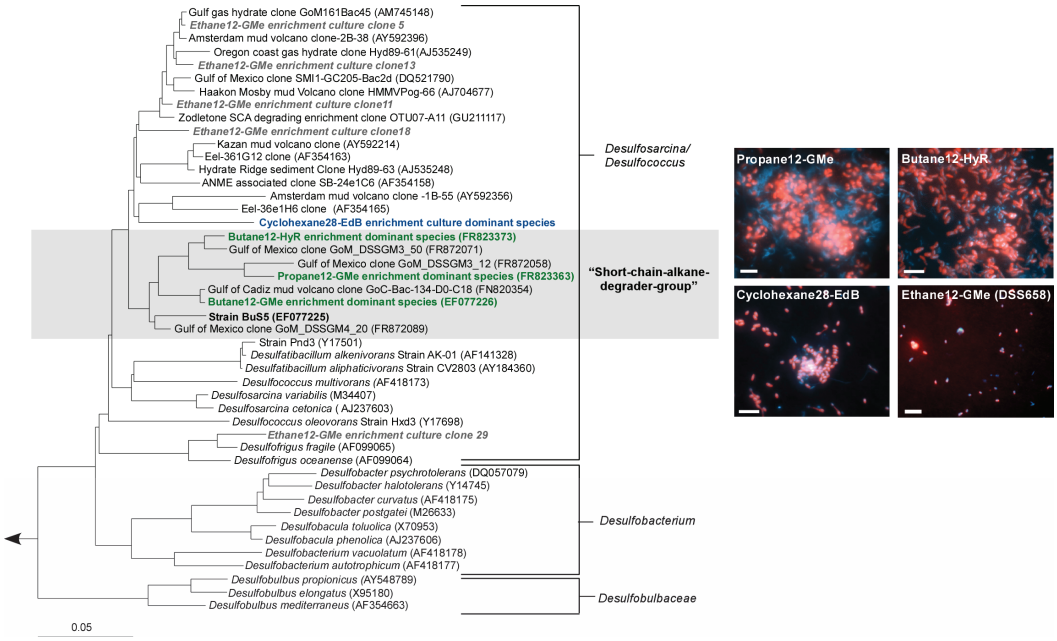

Fig.27 16S rRNA phylogeny of sulfate-reducing bacteria, which were shown in this study to degrade propane and *n*-butane in enrichment cultures from cold marine hydrocarbon seeps (green). An ethane-degrading enrichment culture from cold marine hydrocarbon seeps was shown to be dominanted by a morphotype, which belongs to the *Desulfosarcina/Desulfococcus*-group, as well. A cyclohexane-degrading enrichment culture from hydrocarbon-contaminated marine sediments was shown to be dominanted by a single phylotype (blue), which also affiliates with this group. Scale bar represents 5% sequence divergence.

The only pure culture of another propane and *n*-butane degrading sulfate-reducer known to date, strain BuS5 (Kniemeyer *et al*., 2007), grows at higher temperatures (optimum at ~ 28 °C) and also affiliates with this phylogenetic cluster. All of these cold-adapted and mesophilic sulfate-reducing bacteria were enriched from marine hydrocarbon seep sites in the Guaymas Basin, the Gulf of Mexico or Hydrate Ridge.

The next closest related phylotypes were found in clone libraries generated from hydrocarbon seep samples in the Gulf of Mexico (Kleindienst *et al.*, in prep.). Based on the metabolic capabilities of these phylogenetically related microorganisms, one might call this subgroup within the *Desulfosarcina/Desulfococcus*-group a ″short-chain-alkane-degrader group″. Other propane- and *n*-butane-degrading, thermophilic (growth at 60 °C) sulfate-reducing bacteria were shown to affiliate with *Desulfotomaculum* spp. (Kniemeyer *et al*., 2007). This study has furthermore provided evidence that a cold-adapted, ethane-degrading, sulfate-reducing bacterium may be affiliated with the same ″short-chain-alkane-degrader group″. However, the latter point still waits to be proven in future experiments. Other 16S rRNA sequences, similar to the mesophilic and cold-adapted propane- and *n*-butane-degrading phylotypes have been found at marine mud volcanoes (Heijs *et al*., 2007; Heijs *et al*., 2008; Lösekann *et al*., 2007), hydrocarbon seep-associated sediments (Lloyd *et al.*, 2001; Orphan *et al*., 2001) or marine sediments associated with gas hydrates (Knittel *et al.*, 2003; Orcutt *et al*., 2010). Sulfate reduction rates (SRR) determined at such sites often exceeded SRR that could be explained by anaerobic oxidation of methane several fold, and have been proposed to be due to oxidation of hydrocarbons other than methane, including short-chain alkanes (Kallmeyer and Boetius, 2004; Joye *et al*., 2004; Niemann *et al*., 2006; Orcutt *et al*., 2010; Bowles *et al*., 2011). The present study has contributed to the understanding of the of short-chain alkane-degrading, sulfate-reducing bacteria from marine hydrocarbon seeps, which may be contributing to the observed excess of sulfate reduction and thus to the overall carbon and sulfur cycling at such seep sites.

Another phylotype (Cyclohexane28-EdB) was shown to be dominant in a cyclohexane-degrading enrichment culture obtained from marine, hydrocarbon-contaminated intertidal sediment and also affiliates with the *Desulfosarcina*/*Desulfococcus*-cluster. This phylotype therefore likely represents the cyclohexane degrader. The enrichment culture could, aside from different cycloalkanes, also degrade C_4–C_6- *n*-alkanes. The new phylotype (Cyclohexane28-EdB clone 63) represents the first member of the *Desulfosarcina,* which is able to degrade cycloalkanes and also C_4–C_6- *n*-alkanes. The ability of the new phylotype to degrade a rather broad spectrum of hydrocarbons sheds further light on the metabolic capabilities of the members of the *Desulfosarcina*/*Desulfococcus*-group.

D. 2 Activation mechanisms

D.2.1 Propane and *n*-butane

The new propane- and *n*-butane-degrading enrichment cultures investigated in this study were shown to be, just like strain BuS5, very specialized with respect to their substrate spectrum and could degrade propane and *n*-butane simultaneously but not shorter or longer alkanes. At the beginning of the study, strain BuS5 was known to degrade propane and *n*-butane by addition to fumarate to the secondary carbon atom (Kniemeyer *et al.*, 2007). Interestingly, strain BuS5 was shown to also activate propane at the primary carbon atom but since these results were based on analysis of metabolites only, it was not clear whether this is a significant route of activation at the beginning of this study.

We used two dimensional (2D) carbon and hydrogen compound specific stable isotope fractionation analysis (CSIA) of strain BuS5 grown with either propane or *n*-butane and several other sulfate-reducing, propane- and *n*-butane-degrading enrichment cultures from marine hydrocarbon seeps to compare the activation mechanisms for these alkanes by the different cultures. The specific correlation of carbon vs. hydrogen fractionation indicates that all of the *n*-butane-degrading cultures show the same pattern of carbon and hydrogen fractionation (Fig.28, in blue).

The carbon and hydrogen isotope fractionation during *n*-butane degradation by these cultures was in the same range as during *n*-hexane and *n*-octane degradation by another sulfate-reducer, strain *Desulfoglaeba alkanexedens*, which activates these alkanes also by addition to fumarate (Fig.27, green and grey) (Morris *et al*., submitted). The carbon and hydrogen isotope fractionation during degradation of propane (Fig.28, red) by the sulfate-reducing cultures in this study was in the same range as values reported for propane degradation under sulfate-reducing conditions in marine sediments from a hydrocarbon seep at the deep sea Nile fan (Fig.28, orange). The correlation between carbon and hydrogen fractionation, which was reported for the degradation of propane in aerobic sediments from a hydrocarbon seep site ″Coal point″ were on the other hand significantly lower (Fig.28, pink). This is most likely due to the fact that activation of propane by aerobic microorganisms commences through a different mechanism, involving the insertion of reactive oxygen species at

the primary carbon atom by a monooxygenase. These results show (*i*) that 2D-CSIA can be used to distinguish between different mechanisms of activation (i.e. aerobic vs. anaerobic propane degradation) and (*ii*) to detect a certain activation mechanism in the environment or in enrichment cultures where the involved activation mechanism is not known.

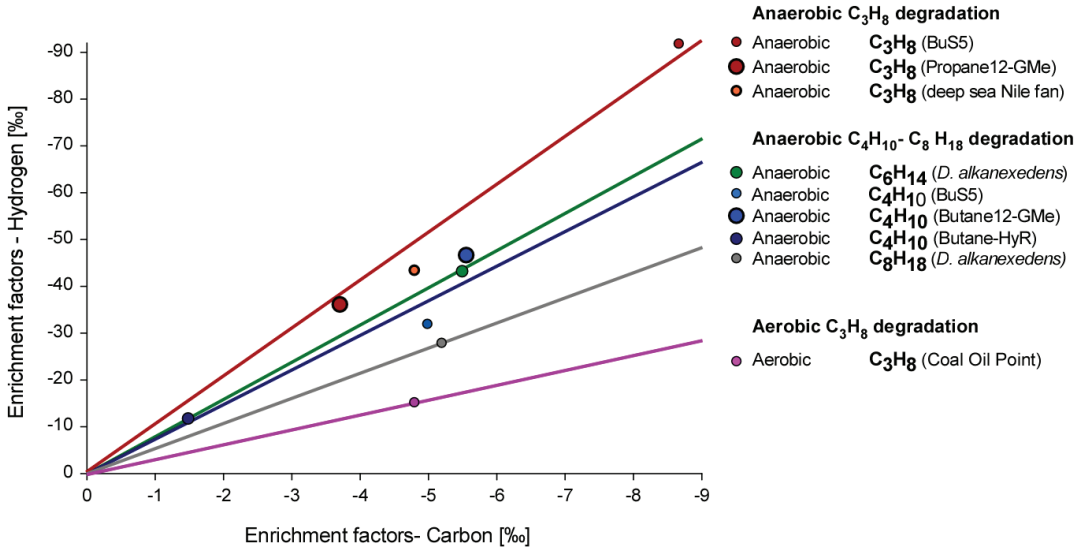

Fig.28 Correlation between enrichment factors for carbon and hydrogen during degradation of propane and *n*-butane by sulfate-reducing cultures (this study), for propane during aerobic degradation in marine sediments (Kinnaman *et al.,* 2007) and for *n*-hexane and *n*-octane during degradation by the sulfate reducing strain *Desulfoglaeba alkanexedens* (Morris *et al.*, submitted). Strain BuS5 and *Desulfoglaeba alkanexedens* are known to activate the alkanes by addition to fumarate to the secondary carbon atom. Strain BuS5 also activates propane at the primary carbon atom (Kniemeyer *et al.*, 2007). At the beginning of this study, it was not clear whether this is a major route of activation or only a side reaction.

The correlation of carbon vs. hydrogen isotope fractionation for propane degradation by the sulfate-reducing cultures and also that for propane degradation in marine sediments under sulfate-reducing conditions was always significantly higher than for *n*-butane, *n*-hexane or *n*-octane. Degradation experiments performed with strain BuS5 and deuterium-labeled alkanes (D_2- and D_4-propane; D_2- and D_6-butane) indicated that fumarate was added solely at the secondary carbon atom of *n*-butane, but at both primary and secondary carbon atoms of propane (Fig.29).

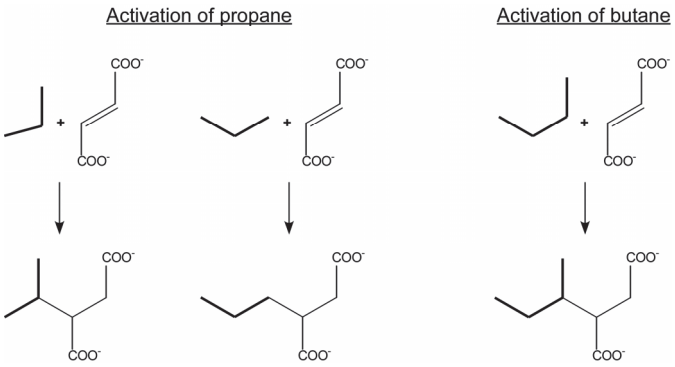

Fig.29 Propane and butane are activated at the secondary carbon atom. Previous metabolite studies have indicated that propane is also activated at the primary carbon atom. Incubations with specifically deuterated propane and butane have revealed that the activation of propane at the primary carbon atom is a significant route for activation.

D.2.2 Ethane

No physiological experiments were conducted in this study that would provide a direct evidence for the possible activation mechanism of ethane by the ethane degrading sulfate-reducing enrichment culture from cold hydrocarbon seeps (in the Gulf of Mexico). However, several indications were obtained, allowing at least speculation about tentative activation mechanisms for this alkane.

On the one hand, the demonstration that activation of propane by addition to fumarate at the primary carbon atom appears to be a significant route of activation, makes it a possible route of activation for ethane as well. The bond dissociation energy, which would have to be overcome to homolytically cleave a hydrogen from the primary carbon atoms of ethane (411 kJ mol^{-1}) is very similar to that of a primary carbon atom of propane (410 kJ mol^{-1}). The tentative activation of ethane by addition to fumarate would at first yield ethylsuccinate, which could then be (in analogy to the degradation of other *n*-alkanes) further degraded as depicted in Fig.30, yielding regenerated fumarate and acetyl-CoA. Activation of propane and *n*-butane by addition to fumarate has been shown to be performed by other members of the *Desulfosarcina/Desulfococcus* cluster and if this was the route for activation of ethane in this enrichment culture, it is imaginable that the dominant phylotype, which was shown to belong to the same cluster, is performing the activation and degradation of ethane.

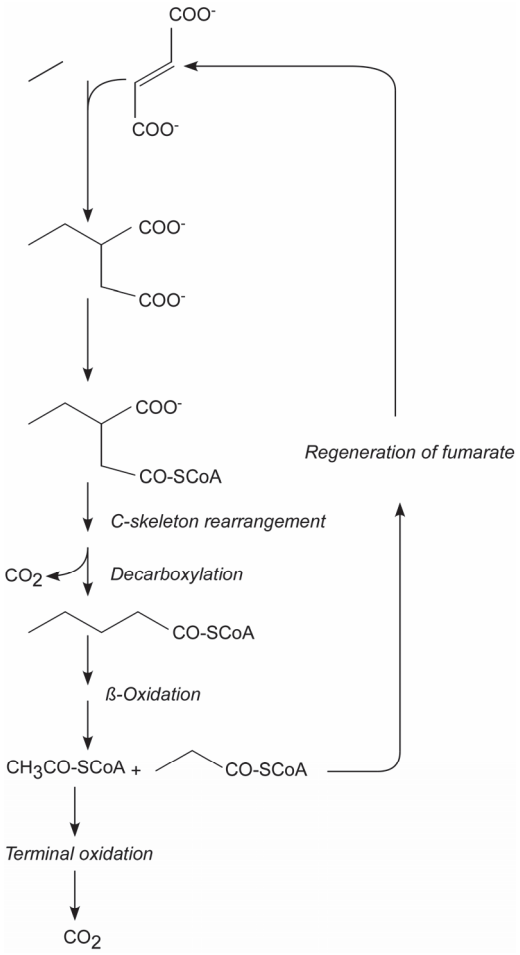

Fig.30 Proposed scheme for a possible activation and degradation of ethane by a sulfate-reducing enrichment culture.

However, the relatively high number of archaea (~ 10% of all cells) in this enrichment culture would have to be living of some intermediate from degradation of ethane by the sulfate-reducing bacteria or dead biomass. An alternative way of activation and degradation of ethane by this enrichment culture could be imagined, where ethane is activated through ″reverse ethanogenesis″. In this hypothetical pathway, methanogenic archaea would activate ethane to ethyl-coenzyme M by the enzyme methyl-coenzyme M reductase (MCR). *Methanosarcina barkeri* has been shown to produce small amounts of ethane, if grown on ethanol and H_2 (Belay and Daniels, 1988). In enzyme assays using cell extracts, the MCR in *M. barkeri* has been shown to catalyze the formation of ethane from ethyl-coenzyme M (Gunsalus *et al*., 1978; Ahn *et al*., 1991). Recent studies with the purified MCR from *M. barkeri* have shown that this enzyme also catalyzes the endergonic back-reaction from methane to

methyl-coenzyme M (Scheller *et al*., 2010 a) and from ethane to ethyl-coenzyme M (Scheller *et al.*, 2010 b). The latter reaction is regarded as a side-reaction of the enzyme. If both methane and ethane are present in equal amounts, the enzyme converts much more methane into methyl-coenzyme M than ethane into ethyl-coenzyme M (Scheller e*t al.*, 2010 b).

One could imagine that, in analogy to the anaerobic oxidation of methane, ethanogenesis is working in reverse in methanogenic archaea in the enrichment culture, leading to the formation of ethanol from ethane. Ethanol could be further degraded by the methanogen to acetate, leading to the net reaction shown in Eq. 2:

$$4\ C_2H_6 + 8\ H_2O \rightarrow 4\ C_2H_3O_2^- + 4H^+ + 12H_2 \qquad [2]$$
$$\Delta G^0 = +\ 548\ \text{kJ mol}^{-1}$$
$$\Delta G = -\ 23\ \text{kJ mol}^{-1}$$

As can be seen from this, the reaction would not be exergonic under standard conditions. Only if the concentrations of acetate and hydrogen were kept low enough (0.1 mM acetate and 10^{-7} atm hydrogen), the reaction becomes exergonic (considering 1 atm for ethane, as performed in the incubations). It could be imagined that the sulfate-reducing bacteria in this enrichment culture would ″remove″ the formed and excreted acetate and hydrogen to further degrade the acetate to CO_2 (Eq. 3) and reduce sulfate to sulfide (Eq. 4).

$$4\ C_2H_3O_2^- + 4\ SO_4^{2-} \rightarrow 8\ HCO_3^- + 4\ HS^- \qquad [3]$$
$$\Delta G^0 = -\ 184\ \text{kJ mol}^{-1}$$
$$\Delta G = -\ 271\ \text{kJ mol}^{-1}$$

$$12H_2 + 3\ SO_4^{2-} + 3\ H^+ \rightarrow 3\ HS^- + 12\ H_2O \qquad [4]$$
$$\Delta G^0 = -\ 573\ \text{kJ mol}^{-1}$$
$$\Delta G = -\ 175\ \text{kJ mol}^{-1}$$

Net reaction:
$$4\ C_2H_6 + 7\ SO_4^{2-} \rightarrow 8\ HCO_3^- + 7\ HS^- + 4\ H_2O + H^+ \qquad [5]$$
$$\Delta G^0 = -\ 209\ \text{kJ mol}^{-1}$$
$$\Delta G = -\ 469\ \text{kJ mol}^{-1}$$

Both the degradation of acetate and the oxidation of hydrogen with reduction of sulfate would still be feasible at the considered concentrations for acetate (0.1 mM), hydrogen (10^{-7} atm), bicarbonate (30 mM), sulfate (25 mM) sulfide (3 mM)). In this model (Fig.31), the tentative methanogenic archaea (~ 10% of all cells) are degrading ethane to acetate (gaining six electrons for growth) through a ″reverse ethanogenesis″ in a reaction that is only exergonic if the produced acetate and hydrogen are consumed by the sulfate-reducing bacteria (~ 60% of all cells). The ability of sulfate-reducing bacteria affiliated with the *Desulfosarcina* (as they have been detected in this enrichment culture) to utilize both hydrogen and also acetate has been documented (Rabus *et al.*, 2006). The production of acids from alcohols by methanogenic archaea has also been documented (Widdel, 1986). Such a degradation of ethane would have syntrophic character, since both partners are dependent on each other.

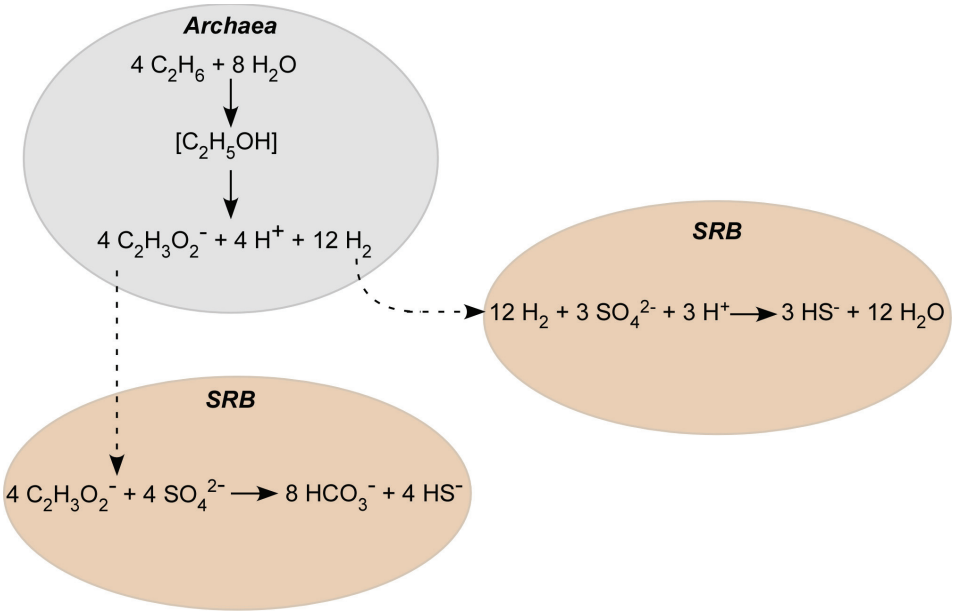

Fig.31 Scheme of a (speculative) alternative degradation mechanism for ethane by a sulfate-reducing enrichment culture. The proposed route of degradation involves a syntrophic association between the archaea (~ 10% of all cells) in the enrichment culture, which activate ethane and degrade the formed ethanol to acetate and the sulfate-reducing bacteria (~ 60% of all cells), which further degrade the excreted acetate to CO_2 and oxidize the hydrogen, coupled to reduction of sulfate to sulfide. The degradation of ethane to acetate by the archaea is only exergonic, if the formed acetate and hydrogen concentrations are kept low.

D.2.3 Cyclohexane

In this study, we also report the enrichment of cyclohexane-degrading, sulfate-reducing bacteria from hydrocarbon-contaminated intertidal marine sediments. Quantitative growth experiments showed that cyclohexane was completely oxidized to CO_2. GC-MS analysis of culture extracts during growth on cyclohexane showed cyclohexylsuccinate as a metabolite, indicating that the activation involves a carbon-carbon addition to fumarate, analogous to the activation of *n*-alkanes and some aromatic compounds. Other metabolites detected were cyclohexylpropionic acid and cyclohexylcarboxylic acid, suggesting that cyclohexylsuccinate is further degraded through enzymatic reactions similar to those of *n*-alkanes, involving carbon-skeleton rearrangement, decarboxylation and β-oxidation.

E. Perspectives

E.1 Global abundance, distribution and activity of short-chain alkane-degrading sulfate-reducing bacteria

Using incubations with ^{13}C-labeled hydrocarbons and nanoSIMS analysis of single cells, we were able to determine rates of carbon assimilation, from which we then derived hydrocarbon degradation- and sulfate-reduction rates of anaerobic propane and *n*-butane degrading bacteria.

However, these rates were determined under conditions which do not resemble *in situ* conditions at deep sea hydrocarbon seep sites. Considering that pressure, temperature, redox conditions and the presence of other organic compounds have an influence not only on the solubility of the short-chain alkanes but also on the functioning of the involved enzymes, it can be assumed that the propane and *n*-butane degradation rates and thus also the corresponding sulfate-reduction rates are different under *in situ* conditions. Incubations with ^{13}C-labeled ethane, propane and *n*-butane as growth substrates, which are conducted in high-pressure incubators that can be operated at near *in situ* temperatures and redox potentials, like the anaerobic methane incubation system (AMIS; Girguis *et al*., 2003) would provide a set-up to determine more realistic growth rates. Incubations using such set-ups, in combination with the use of sediments from such sites, which were sampled using a high-pressure recovery vessel (HPRV), should result in the enrichment of microorganisms, with activities that more closely resemble their *in situ* activity levels. The sediments in these incubations should be investigated by nanoSIMS analysis of single cells, which are targeted by probes based on the cluster of short-chain alkane-degrading, sulfate-reducing bacteria as described in this study. In addition, analysis of 16S rRNA genes, which are enriched in ^{13}C from the labeled growth substrates (DNA stable isotope probing; SIP) could reveal further microorganisms, involved in the anaerobic degradation of these short-chain alkanes and which are hitherto not known because they may have not been cultivated under the right conditions. Especially with regard to ethane-degrading microorganisms this could reveal new types of bacteria, since to date only one report of an enrichment of such bacteria exists (Kniemeyer *et al.,* 2007).

Using primers, which target the gene for the *n*-alkane activating enzyme ((1-methyl)alkylsuccinate synthase; Mas) in propane and *n*-butane-degrading bacteria, quantitative PCR could be used to estimate the abundance of bacteria which contain the *masD* gene *in situ*. This approach in combination with FISH counts, using probes specific for the propane and *n*-butane degrading phylogenetic cluster on sediment samples from hydrocarbon seeps, could provide insights into the abundance of non methane, short-chain alkane degrading bacteria at different hydrocarbon seep sites and also their spatial distribution in these sediments along sulfate and hydrocarbon gradients.

E.2 Activation mechanisms for short-chain alkanes and cycloalkanes under anaerobic conditions

Using stable carbon and hydrogen isotope fractionation analysis, we were able to see that activation of propane at both the primary and the secondary carbon atom by addition to fumarate, is a significant route of activation. This is in contrast to *n*-butane and the longer *n*-alkanes (Morris *et al*., submitted), which are activated only at the secondary carbon atom. Future experiments, using assays with crude cell extracts or even a purified activating enzyme, could reveal the true frequency of this unprecedented activation of propane at the primary carbon atom, relative to activation at the secondary carbon atom. We have furthermore observed that growth under substrate-limiting (static, high cell densities) and substrate non-limiting conditions (actively mixed, planctonic growing cultures), leads to a shift in Λ values (carbon fractionation vs. hydrogen fractionation) during degradation of the same substrate by one culture. Future studies should furthermore investigate the effect of substrate-limiting conditions on the two dimensional stable isotope fractionation effects. It is imaginable that the observed shift in Λ values is due to the expression of a different activating enzyme with higher substrate affinity during substrate-limiting conditions. Sequencing and analysis of the genome of strain BuS5 could provide information about how many operons, coding for the propane- and *n*-butane-activating enzyme are present and whether they differ from each other.

Mainly due to the slow growth of the so far unique ethane-degrading enrichment culture, we still do not know whether ethane, a molecule containing only

primary carbon atoms, is activated by the same mechanism. Future studies should at first aim to improve the growth of this enrichment culture. This could be achieved by using culture bottles, which allow a large surface to volume ratio of the liquid phase and the headspace. The bottles should be incubated at optimized horizontal shaking speed, an optimized incubation temperature, a headspace which contains a mix of mainly ethane (ethane/CO_2 (90:10) instead of N_2/CO_2 (90:10)) and at elevated pressure (2–3 bar instead of 1 bar). If growth rates are faster, incubations of the enrichment culture with ^{13}C-labeled ethane and analysis of formed metabolites could reveal whether ethane is activated by fumarate addition. Amplification with primers, which were successfully used to amplify partial sequences of the *masD* gene in the propane and *n*-butane degrading enrichment cultures, would be another indication for the presence of this mode of activation in the ethane-degrading strain.

Alternatively, another theory, ″reversed ethanogenesis″ (Fig.31), could be tested in incubations, where the tentative (m-)ethanogen in this enrichment culture is inhibited. Bromoethane sulfonate has been shown to successfully inhibit ethanogenesis in *M. barkeri* (Belay and Daniels, 1988). This should lead to a stop in ethane consumption from the headspace. In another experiment, using ^{13}C-labeled ethane, the transfer of heavy carbon from the archaea (using an oligonucleotide probe targeting *Archaea*) to the sulfate-reducing bacteria (using an oligonucleotide probe, which targets members of the *Desulfosarcina/Desulfococcus*-group) could be monitored by nanoSIMS analysis. Furthermore, the use of ^{14}C-labeled ethane could reveal, whether the tentative intermediate (acetate) is formed and excreted into the medium. A sensitive HPCL set-up, connected to a radioactivity detector, could detect ^{14}C-labeled acetate. The enrichment culture should also be further investigated with regard to the phylogenetic identity of the archaea present in this culture (~ 10% of total cells), by clone libraries generated with primers targeting *Archaea* and whole-cell hybridization using specifically designed, phylotype-specific oligonucleotide probes. In the end, this alternative mechanism of ethane degradation by the sulfate-reducing enrichment culture remains speculative.

Another interesting enrichment culture was shown to degrade both linear and cyclic alkanes, coupled to the reduction of sulfate to sulfide. Since no other anaerobic, *n*-alkane degrading strain has been shown hitherto to also degrade cycloalkanes (only to activate them, Wilkes *et al.*, 2003), it would be interesting to further investigate, whether the same enzymes, which activate cyclohexane by fumarate addition and

further degrade the resulting activation product by mechanisms similar to that of *n*-alkanes, are able to degrade both cycloalkanes and *n*-alkanes. Isolation of the cyclohexane-degrading strain from the sulfate-reducing enrichment culture could be useful to prove that all of the tested substrates are indeed activated and degraded by the same strain. If so, it would be interesting to further analyze the activating glycyl-radical enzyme with respect to how it differs from other *n*-alkane activating enzymes, which have a much narrower substrate spectrum. Furthermore, it would be interesting to compare the enzymes of the downstream degradation pathway of this strain to that of the *n*-alkane degrading strain HxN1, which can activate but not degrade cycloalkanes. Such an approach would most likely involve the sequencing of the genome after the strain has been isolated and brought into pure culture. This would also reveal whether the strain has one or multiple versions of Mas-like enzymes. Substrate tests with *n*-butane have shown that the enrichment culture is able to degrade it but only after a lag phase, which indicates that induction of another enzyme is required.

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Part II. Manuscripts

List of manuscripts and explanation of contributions

1. Anaerobic degradation of propane and *n*-butane by sulfate-reducing bacteria from marine hydrocarbon cold seeps

Ulrike Jaekel, Niculina Musat, Birgit Adam, Marcel Kuypers, Olav Grundmann and Florin Musat

Concept development and manuscript preparation was done by Ulrike Jaekel and Florin Musat. The initial enrichment cultures were started by Florin Musat. All microbiological experiments, generation of clone libraries, design of oligonucleotide probes and optimization of hybridization protocols were done by Ulrike Jaekel. NanoSIMS analysis and interpretation was performed in collaboration with Niculina Musat, Birgit Adam and Marcel Kuypers. Olav Grundmann provided the partial *masD* gene sequence from strain BuS5 and full length *masD* gene sequences from strain TD3 and Pnd3.

2. Carbon and hydrogen stable isotope fractionation associated with the degradation of propane and *n*-butane by sulfate-reducing bacteria: mechanistic aspects and environmental implications

Ulrike Jaekel, Carsten Vogt, Anko Fischer, Hans-Hermann Richnow, Florin Musat

The development of the concept and manuscript preparation was done by Ulrike Jaekel, Florin Musat, Carsten Vogt, Anko Fischer and Hans-Hermann Richnow. Ulrike Jaekel performed all microbiological experiments, GC-IRMS measurements and the development of a GC-method to separate deuterated from natural propane and butane.

3. Ethane-degrading sulfate-reducing bacteria from marine hydrocarbon seeps

Ulrike Jaekel, Friedrich Widdel and Florin Musat

The development of the concept of the study and manuscript preparation was done in collaboration with Friedrich Widdel and Florin Musat. The generation of the clone library, design of an oligonucleotide probe and optimization of the hybridization protocol were performed by Ulrike Jaekel.

4. Degradation of cyclohexane by a sulfate-reducing enrichment culture from hydrocarbon-contaminated intertidal sediments

Ulrike Jaekel, Johannes Zedelius, Heinz Wilkes, Friedrich Widdel and Florin Musat

The development of the concept of the study and manuscript preparation was done by Ulrike Jaekel and Florin Musat. Johannes Zedelius performed the construction of 16S rRNA gene clone libraries. Florin Musat performed the measurements for the quantitative growth experiments. Heinz Wilkes analyzed metabolites.

1

Anaerobic degradation of propane and *n*-butane by sulfate-reducing bacteria from marine hydrocarbon cold seeps

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Manuscript in preparation

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Abstract

Novel propane and *n*-butane-degrading sulfate-reducing bacteria have been enriched from marine sediments collected around hydrocarbon cold seeps. A propane-degrading enrichment culture was obtained from Gulf of Mexico sediments (Prop12-GMe), and an *n*-butane-degrading enrichment culture from Hydrate Ridge sediments (But12-HyR). The enrichment cultures were able to degrade simultaneously propane and *n*-butane, but not ethane, *iso*-butane or *n*-pentane. They were cold-adapted, showing highest sulfate-reduction rates between 16 °C and 20 °C, and no sulfate reduction at temperatures higher than 28 °C. Both enrichment cultures formed conspicuous aggregates, apparently dominated by large, oval cells of 3×1 µm (Prop12-GMe) or 2.5×0.8 µm (But12-HyR). Partial fragments of the gene coding for the large subunit of the (1-methylalkyl) succinate synthase (*masD*) have been amplified and sequenced. Phylogenetic analysis showed that the *masD* genes of the cold-adapted enrichment cultures formed an apparent cluster with *masD* sequences from strain BuS5 and the thermophilic long-chain alkane degrader strain TD3. Construction and analysis of 16S rRNA gene libraries, followed by whole-cell hybridizations with group and sequence-specific oligonucleotide probes showed that each enrichment culture was dominated by a unique phylotype affiliated with the *Desulfosarcina/Desulfococcus*-cluster within the *Deltaproteobacteria*. To determine the role of the dominant microorganisms in the degradation of propane or *n*-butane, the newly obtained enrichment cultures, as well as a previously-described enrichment culture with *n*-butane (But12-GMe, Kniemeyer *et al*., 2007) were incubated with ^{13}C-labeled propane or *n*-butane. Samples withdrawn at defined time intervals were chemically fixed, hybridized with HRP-labeled, sequence-specific genetic probes, followed by the deposition of Fluorine-containing tyramides and nanoSIMS analysis. The analysis at single cell level showed that, in each enrichment culture, the dominant cells were the first to become enriched in ^{13}C, demonstrating that they were directly involved in hydrocarbon degradation. The present study further expands our knowledge on the diversity of marine, propane and *n*-butane-degrading sulphate reducers, and provides tools that could be used to tackle the abundance and environmental role of such microorganisms.

Introduction

Propane and *n*-butane are short-chain alkanes, which occur as constituents of natural gas and are also found, in smaller amounts, dissolved in crude oil (Tissot and Welte, 1984). Propane and *n*-butane are gaseous under normal standard temperature and pressure conditions (NTP, 20 °C, 0.1 MPa), and liquid at low temperatures (at 0.1 MPa, the boiling point of propane is –42 °C and that of butane –0.5 °C) or at high pressure (at 20 °C, ≥ 0.75 MPa for propane, and ≥ 0.22 MPa for *n*-butane) (Schwarzenbach *et al*., 2003). In places where tectonism causes formation of fractures in deep rocks, gas and oil from deep-seated reservoirs are migrating upwards through the sediment as free gas or as dissolved components of geothermal fluids, coming into contact with the biosphere (Hovland 1993; Sassen *et al*., 2001). Propane and *n*-butane are moderatly soluble in water (saturation at 20 °C, 1.76 mM propane, 1.46 mM *n*-butane), with solubility increasing at lower temperatures and higher pressures (Dean, 1992; Chapoy *et al*., 2004), conditions often prevailing at deep-sea environments. Seepage in such environments may lead to entrapping of significant amounts of short-chain alkanes in structure II gas hydrates, such as those found in the Gulf of Mexico, containing up to 36% propane and up to 4% *n*-butane (Sassen *et al*., 2004; Orcutt *et al*., 2004).

Recent studies have shown that propane is degraded *in situ* along with methane and ethane in aerobic, hydrocarbon-contaminated marine sediments (Kinnaman *et al*., 2007; Redmond *et al*., 2010) and in the water column after an oil spill (Valentine *et al*., 2010). The aerobic degradation of propane and *n*-butane has been studied in detail and numerous strains of propane- or *n*-butane-degrading bacteria and fungi have been described (Shennan *et al*., 2006 and references therein). Under oxic conditions, these alkanes are typically activated at the primary carbon atom by reactive oxygen species derived from O_2, leading to primary alcohols, reactions catalyzed by monooxygenases.

Geochemical studies of anoxic deep-sea sediments provided the first indications that propane and *n*-butane are degraded under anaerobic conditions. The ^{13}C-enrichment of propane and *n*-butane in sediment interstitial water atop gas hydrates (Sassen *et al*., 2004) or at deep sea mud volcanoes (Mastalerz *et al*, 2009), sulfate-reduction rates much higher than could be accounted for by anaerobic oxidation of methane in hydrothermal sediments or at cold seeps (Kallmeyer and

Boetius, 2004; Orcutt *et al.* 2004; Joye *et al*., 2004; Orcutt *et al*., 2010), or the apparent consumption of short-chain alkanes in mud volcanoes (Niemann *et al.*, 2006), suggested that the anaerobic degradation of propane and *n*-butane may play an important role in the carbon and sulfur cycling in these environments. In addition, *in situ* rates of anaerobic oxidation of propane have been determined recently in sediments collected from marine gas seeps (Quistad and Valentine, 2011). Anaerobic degradation of short-chain alkanes was demonstrated with a pure culture of a marine sulfate-reducing bacterium, strain BuS5 (Kniemeyer *et al.*, 2007), and with several enrichment cultures obtained from sediments around marine and terrestrial hydrocarbon seeps (Kniemeyer *et al.*, 2007; Savage *et al*., 2010). Strain BuS5 can degrade both propane and *n*-butane and is phylogenetically affiliated with the *Desulfosarcina*/*Desulfococcus*-cluster of the *Deltaproteobacteria*. Analysis of metabolites showed that propane and *n*-butane are activated similar to higher *n*-alkanes by addition to fumarate (Kropp *et al*., 2000; Rabus *et al*., 2001) primarily at the secondary carbon atom, yielding *iso*-propyl- and (1-methylpropyl)-succinate, respectively (Kniemeyer *et al*., 2007; Savage *et al*., 2010). The genes coding for the activating glycyl-radical enzyme have been so far identified in a nitrate-reducing bacterium, strain HxN1 (Grundmann *et al*., 2008) and in the sulfate-reducing strain AK-01 (Callaghan *et al*., 2008).

In the present study, novel propane- and *n*-butane-degrading sulfate-reducing bacteria were enriched from cold marine hydrocarbon seeps. Physiological investigations of the enrichment cultures with respect to their hydrocarbon substrate spectrum and optimum growth temperature suggested rather stringent environmental niches. The propane- and *n*-butane-degrading bacteria were identified in the enrichment cultures using 16S rRNA gene-targeted oligonucleotide probes and incubations with ^{13}C-labeled substrates, followed by nanoSIMS analysis. Hydrocarbon assimilation rates of single cells were calculated for the propane or *n*-butane-degrading bacteria in each enrichment culture.

Materials and methods

Source of organisms, media and cultivation techniques

Enrichment of novel propane and *n*-butane degrading sulfate-reducing bacteria was attempted with sediment collected around marine hydrocarbon seeps at Hydrate Ridge and Gulf of Mexico. The sediment samples from Hydrate Ridge were collected during the RV SONNE cruise, leg 148-1, in July 2000 at 777 m water depth from Station 28 and 29 *Beggiatoa* field (44° 34' N, 125° 09' W; Treude *et al.*, 2003). The sediment samples from the Gulf of Mexico were collected using the RV Seward Johnson I and the Johnson Sea Link submersible in July 2001 from the brine-influenced site GC233 (650 m water depth, 27° 44' N, 92° 17' W; Joye *et al.*, 2004). The cultures were set up in 100 ml flat bottles sealed with butyl rubber stoppers, containing 50 ml of bicarbonate-buffered artificial sea-water medium (Widdel and Bak, 1992; Widdel, 2010), under a headspace of N_2:CO_2 (9:1, v/v). Propane and butane (Air Liquide, Düsseldorf-Germany) were added as 50% v/v of the headspace. The bottles were inoculated with 5 ml homogenized sediment, and incubated at 12 °C and 28 °C nearly horizontally in order to increase the hydrocarbon diffusion surface; orifices were kept below the medium surface to avoid direct contact between the hydrocarbon and the stopper (Rabus and Widdel, 1995). Control incubations without the addition of hydrocarbons were set up in the same conditions. Sediment-containing cultures were incubated without shaking, while sediment-free cultures were incubated with slow (40–50 rpm) horizontal shaking. For subcultivation, 10% v/v of an active culture was used as inoculum. Sediment-free enrichment cultures were amended with 3 ml l^{-1} trace element solution (Widdel & Bak, 1992). Cultivation of strain BuS5 and of the butane-degrading enrichment culture But12-GMe was described before (Kniemeyer *et al.*, 2007). The optimum temperature of growth was determined with duplicate 10 ml cultures incubated in a temperature gradient block, at temperatures ranging from 0 to 40 °C.

To test the ability of the enrichment cultures to degrade other hydrocarbons, cultures were set up in 120 ml serum bottles containing 79 ml artificial sea-water medium. The bottles were provided with a mixture of methane, ethane, propane and *n*-butane (6 ml each), *iso*-butane (6 ml), or *n*-pentane (1% v/v in 2,2,4,4,6,8,8-heptamethylnonane, HMN, as an inert carrier phase). Sterile bottles with

hydrocarbons and inoculated bottles lacking hydrocarbons were used as controls. The bottles were equilibrated for 48 h with slow shaking (40 rpm) at the incubation temperature (12 °C or 17 °C), and inoculated with 1 ml of a 10× concentrated cell suspension. To prepare concentrated cell suspensions 150 ml grown cultures were collected under anoxic conditions, and centrifuged for 20 min at 14300 g (Beckman Coulter Avanti® J-26 XP, Krefeld-Germany). The cell pellets were homogeneously suspended in fresh culture medium; cultures forming aggregates were in addition homogenized by repeatedly passing the aggregates through a hypodermic needle (gauge 0.6 mm) attached to an N_2-flushed syringe.

For halogen *in situ* hybridization-secondary ion mass spectroscopy (HISH-SIMS) analysis, 15 ml cultures in 22-ml cultivation tubes were supplied with propane and $^{13}C_1$-propane (yielding 11.7 atomic percent ^{13}C) or *n*-butane and $^{13}C_1$-butane (11.7 atomic percent ^{13}C for But12-HyR enrichment culture and 25 atomic percent for But12-GMe enrichment culture). To facilitate further analyzes, the aggregate-building cultures were partly homogenized before incubation started under anoxic conditions, using a glass mortar (0.1 ml, Wheaton, Millville, USA). $^{13}C_1$-propane and $^{13}C_1$-butane (99 atom %) were purchased from ISOTEC (Miamisburg-USA). Samples (0.5 ml) for mass spectrometry analysis of the bulk ^{13}C-incorporation were withdrawn every 24 h and added onto pre-combusted glass-fiber filters. In addition, 0.5 ml samples were fixed for whole cell hybridization (see Fluorescence *in situ* hybridization section).

Analytical techniques

Sulfide was quantified photometrically as colloidal CuS (Cord-Ruwisch, 1985). Methane, ethane, propane, *n*-butane, *iso*-butane and *n*-pentane concentrations in the headspace were determined by gas chromatography using a Shimadzu GC-14B gas chromatograph equipped with a 30 m Supel-Q PLOT column (0.53 mm inner diameter, film thickness 30 µm; Supelco, Bellefonte-USA) and a FID. The carrier gas was N_2 at a flow rate of 3 ml min^{-1}. The detector was operated at 280 °C and the injector at 150 °C. For quantification of single hydrocarbons, the oven was operated isocratic at 140 °C. For analysis of mixtures of alkanes, the oven was maintained for 1 min at 35 °C, then heat with a rate of 20 °C min^{-1} to 140 °C where it was maintained for 1 min. Headspace samples (0.1 ml) were withdrawn with a N_2-flushed gastight glass syringe and injected without split. Measurements of hydrocarbon and

sulfide concentrations were done in triplicates. The glass fiber filters from incubations with ^{13}C-labeled propane or butane were analyzed for bulk abundance of ^{13}C and ^{12}C. They were freeze-dried, decalcified by HCl aerosol and packed into tin-cups. Subsequently, bulk biomass enriched in ^{13}C-labeled propane or butane was analyzed with an automated elemental analyzer (Thermo Flash EA, 1112 Series, Thermo Fischer, Dreieich-Germany) and a Finnigan Delta Plus Advantage mass spectrometer (Thermo Fischer), using CO_2 released by flash combustion in excess oxygen at 1050 °C.

Determination of cellular carbon content

The carbon content μm^{-3} was determined for strain BuS5 as a model organism using the carbon content and the cell number ml^{-1} culture, and an average cellular volume. Defined volumes (5, 10 and 15 ml) of a grown (13 mM H_2S, $OD_{660\,nm}$ = 0.2) culture of strain BuS5 were transferred in triplicate onto pre-combusted glass fiber filters, air dried for 24 h at room temperature and analyzed for total carbon content using a Carlo Erba NA-1500 CNS analyzer (Thermo Fischer), and sulfonilamid as standard. For the same culture the total cell number ml^{-1} was determined by counting of 4,6-diamidino-2-phenylindole (DAPI) stained cells. Average cellular volumes of strain BuS5 and of the dominant bacteria in the enrichment cultures were calculated by measuring the length and width of at least 30 cells and considering the volume formula of a rod with hemispherical ends (Musat *et al*., 2010). The cells in the enrichment cultures were measured from epifluorescence microscope images after hybridization with sequence-specific oligonucleotide probes and DAPI staining.

Clone libraries, sequencing and phylogenetic analysis

Genomic DNA extracted from the enrichment cultures (Zhou *et al.*, 2006) was used to amplify almost full-length 16SrRNA gene using bacteria-specific primers 8fw (Hicks *et al.*, 1992) and 1496r (Kane *et al.*, 1993), and partial *masD* gene using primers BP-MasD18f (TGATGACTATGTTGATGAAAT) and BP-MasD426rev (GCTGCTCTTAGAGTTCGATCA, annealing at 55 °C). The PCR products were purified using the QIAquick Gel Extraction kit (Qiagen, Hilden-Germany), cloned into the pCR4 vector (TOPO-TA cloning kit Invitrogen, Groningen-The Netherlands)

and transformed into *E. coli* Top 10 competent cells (Invitrogen). Positive clones were sequenced using the ABI Prism BigDye Terminator v 3.0 cycle sequencing kit and an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Darmstadt-Germany). Sequences were assembled with the DNA Baser software (www.dnabaser.com). The 16S rRNA gene sequences were aligned to those of the Silva database (Pruesse *et al.*, 2007). Phylogenetic trees were constructed using the ARB software (Ludwig *et al.*, 2004) by neighbor joining and maximum likelihood with application of different sets of filters, and in addition by maximum likelihood using the RAxML algorithm (Stamatakis *et al.*, 2008; http://phylobench.vital-it.ch/raxml-bb). The deduced aminoacid sequences of *masD* were imported into ARB and aligned with aminoacid sequences of other glycyl-radical enzymes. Phylogenetic trees were constructed using ARB by maximum likelihood using the Jones-Taylor-Thornton model.

Fluorescence *in situ* hybridization

For fluorescence *in situ* hybridization (FISH) 0.5 ml samples were fixed with 0.5 ml 2% paraformaldehyde in 1× phosphate-buffered saline (PBS; 10 mM sodium phosphate pH 7.2, 130 mM NaCl) for 1 hour at room temperature, washed with 1× PBS, and stored in 1× PBS - ethanol (1:1) at –20 °C. Aliquots of fixed cells were filtered onto 0.2 µm pore GTTP polycarbonate filters (Millipore, Eschborn-Germany). Fixed samples of aggregate-building cultures were in addition homogenized using a glass mortar (0.1 ml, Wheaton) before filtration. Alternatively, aggregates were double embedded in agar and paraffin as follows: single aggregates were first embedded in 3% agar containing 3.7% formaldehyde, dehydrated through a series of ethanol baths (50%, 80% and 96%, 30 min each), treated with xylol (3 baths, 40 min each) and finally embedded in paraffin (3 × 30 min in 60 °C paraffin-filled molds) (Musat *et al*., 2007). The paraffin blocks were left to solidify for 24 h at room temperature, and 4 µm thick cross sections were cut using a Leica RM 2165 microtome (Leica, Nussloch-Germany). The sections were mounted onto agarose-coated glass slides. The paraffin was removed by passing the glass slides through 3 xylol baths (10 min each), the cells were re-hydrated in a declining ethanol series (96%, 80% and 70%, 5 min each) permeabilized (0.2 M HCl, 10 min and 20 mM TrisHCl, 10 min) and air-dried. Cells on filters or cross sections were hybridized with Cy3-labeled 16SrRNA-targeted oligonucleotide probes, additionally stained with

DAPI and microscopically counted as previously described (Snaidr *et al*., 1997). The hybridized aggregate sections were analyzed with a Zeiss Axioskop 2 mot plus fluorescence microscope (Zeiss, Oberkochen-Germany), and the number of hybridized cells estimated using the PHLIP software (Müller *et al*., 2006). The oligonucleotide probes used in this study, Prop12-GMe-133 (TATCCCGATTTCCAGGGT, 50% formamide (FA), this study), But12-HyR-193 (GAGGCCACCTTTAATCTG, 40% FA, this study), But12-1275 (Kniemeyer *et al.*, 2007), DSS658 (Manz *et al.*, 1998), EUB338 (Amann *et al.*, 1990) and NON338 (Amann *et al*., 1990) were purchased from Biomers GmbH (Ulm-Germany). The sequence-specific probes designed in this study (in ARB, after Hugenholtz *et al.*, 2001), were evaluated for specificity in hybridization assays with increasing FA concentrations (0-60%, with 10% increment). *Desulfovibrio aespoensis* (2 mismatches vs. Prop12-GMe12-133) and strain BuS5 (1 mismatch vs. But12-HyR-193) were used as negative controls.

Halogen *in situ* hybridization-secondary ion mass spectroscopy and calculation of assimilation rates

Aliquots of fixed samples from the incubations with ^{13}C-labeled alkanes were filtered onto Au/Pd-coated GTTP filters and hybridized with HRP-labeled oligonucleotide probes (Musat *et al.*, 2008). The HRP molecule catalyzed the deposition of ^{19}F-containing tyramides (Oregon Green®). Hybridized samples were analyzed with a NanoSIMS 50L (Cameca, Gennevilliers Cedex-France). The filters were pre-sputtered with a Cs+ primary ion beam (average FCp current = 30 nA) in order to achieve a sputtering equilibrium. Secondary ion images of $^{12}C^-$, $^{13}C^-$, $^{12}C^{14}N^-$, and $^{19}F^-$ were simultaneously recorded with an average mass resolution of 7000, 50-100nm beam size, and a raster size of 20×20 µm or 10×10 µm with a beam dwelling time of 1 ms pixel^{-1}. For each region analyzed, 15 to 150 scans (planes) were acquired. The data was analyzed with the Look@NanoSIMS software (Polerecky *et al*., in revision). For each field analyzed, individual scans were simultaneously accumulated and drift corrected. Regions of interest (ROIs) were drawn around individual cells based on the biomass ($^{12}C^{14}N^-$) images, and ROIs were then used to calculate cell specific $^{13}C/^{12}C$ ratios and to identify ^{19}F-labeled cells. On average, 70 cells were analyzed for every time point. From the $^{13}C/^{12}C$ ratio of each cell an average $^{13}C/^{12}C$ ratio of cells at t_0

was subtracted in order to calculate the excess ratio of $^{13}C/^{12}C$ (R_{exs}). The rate of carbon assimilation (C_{asm}) for individual cells was calculated using the $^{13}C/^{12}C$ excess, the percent label (L), the cellular carbon content (C_{cell}; see Determination of cellular carbon content section) and the incubation time (T) according to:

$$C_{asm} = \frac{R_{exc} \times C_{cell}}{1 + C_{cell}} \times L^{-1} \times T^{-1} (fmolC \times cell^{-1} \times day^{-1})$$

Results and discussion

Enrichment and determination of the optimum temperature

Enrichment of propane- and *n*-butane-degrading sulfate-reducing bacteria from cold marine hydrocarbon seeps was attempted with sediments from the Gulf of Mexico and with sediment associated with structure II gas hydrates at Hydrate Ridge. Incubations were started at 12 °C, which is close to the rather low *in situ* temperatures (< 10 °C), and also at 28 °C to cover a higher range of temperature-adapted microorganisms. After 6 months at 12 °C, incubations with propane and Gulf of Mexico sediments (Prop12-GMe) and incubations with butane and Hydrate Ridge sediments (But12-HyR) produced up to 18 mM sulfide vs. controls lacking addition of substrate, which did not exceeded 3 mM (not shown). Within the same incubation time, the sulfide production in bottles incubated with propane or *n*-butane at 28 °C did not exceeded that of substrate-free controls. Sediment-free enrichment cultures obtained by repeated transfers (20% v/v inoculum) in fresh medium formed approximately 15 mM sulfide within 5–6 weeks. The successful enrichment at 12 °C but not at 28 °C suggested that these short-chain alkane-degrading bacteria are rather cold-adapted, reflecting the *in situ* temperature conditions. To test this hypothesis, sulfate-reduction rates over a wide range of temperatures (0 °C–60 °C) were determined for the enrichment cultures Prop12-GMe and But12-HyR, and for a previously described enrichment culture obtained with butane from Gulf of Mexico sediments (But12-GMe; Kniemeyer *et al.*, 2007). For comparison, temperature-dependent sulfate-reduction rates were determined for the mesophilic strain BuS5, isolated from hydrothermal vent-associated sediments (Kniemeyer *et al.*, 2007). All enrichment cultures displayed significant substrate-dependent sulfate-reduction rates between 4 °C and 25 °C, with maximum (cardinal) temperatures between 16 °C and 20 °C (Fig.1). The sulfate-

reduction rates at 28 °C dropped below 5% of the maximum, excepting the enrichment culture But12-GMe, which retained about 20% of the maximum sulfate-reduction rate. No sulfate-reduction rates could be measured at temperatures ≥ 30 °C. In contrast, strain BuS5 showed *n*-butane-dependent sulfate-reduction rates between 10 °C and 35 °C, with highest rates at 27 °C–30 °C (Fig.1).

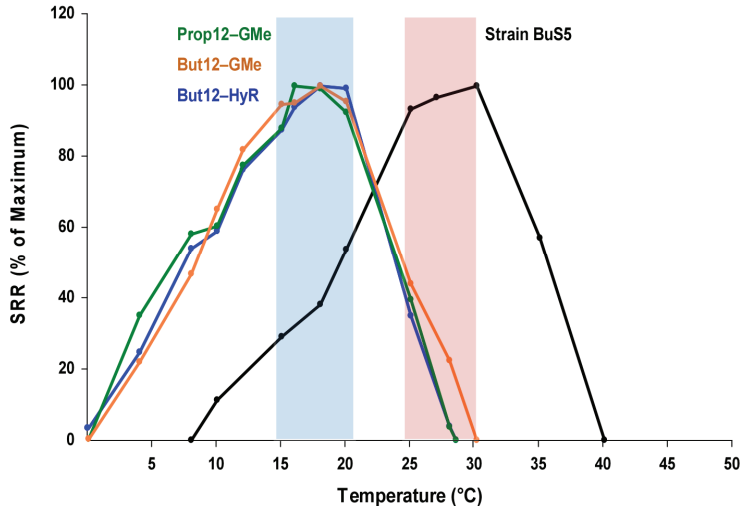

Fig.1 Temperature-dependent sulfate reduction rates (SRR) of the propane and *n*-butane-degrading enrichment cultures from marine hydrocarbon seeps. Incubations were done for up to 11 days. The SRR are expressed as percent of the highest rate determined. The maximum sulfate reduction rates (mM H_2S day^{-1}) were 0.62 (Prop12-GMe), 0.82 (But12-GMe), 0.9 (But12-HyR) and 1.12 (strain BuS5).

The temperature profiles show that the propane- and *n*-butane-degrading microorganisms in these enrichment cultures are true psychrotrophs, since their minimum temperature for sulfate reduction is > 0 °C, the optimum is < 25 °C and the maximum is < 35 °C (Isaksen and Jørgensen, 1996). The determined optimum temperature is significantly higher than the reported *in situ* temperature at cold seeps and gas hydrates (4 °C–8 °C; Orcutt *et al*., 2004; Suess *et al.*, 1999). Similar findings were reported in previous studies (Li and Dickies, 1987; Isaksen and Jørgensen, 1996; Finke and Jørgensen, 2008, Hubert *et al.*, 2009). It was commonly observed that microorganisms living in arctic environments displayed optimum growth or respiration rates at temperatures significantly higher than those encountered *in situ*, whereas microorganisms from hot environments exhibit optimum temperatures closer to the actual *in situ* temperatures (Stetter *et al.*, 1990; Jørgensen *et al*., 1992). The

reason for these observations likely involve lowered enzymatic affinities at low temperatures, an impaired ability of enzymes to undergo conformational changes, or impaired fluidity of cellular membranes affecting the transport of substrates and products (Wiegel, 1990). These observations suggest that the *in situ* respiration, and consequently growth rates of microorganisms as those described here is sub-optimal, amounting to about 50% of the maximum attainable rates.

Quantitative degradation experiments, growth tests with other hydrocarbons and amplification of *masD* genes

Quantitative growth experiments showed that the enrichment cultures degraded propane or *n*-butane completely to CO_2, with stoichiometric reduction of sulfate to sulfide, as described for other propane- and *n*-butane-degrading sulfate-reducing bacteria (Kniemeyer *et al.*, 2007; Savage *et al.*, 2010). The enrichment cultures were tested for their ability to degrade methane, the dominant hydrocarbon at gas seeps, and other alkanes found in relatively high concentrations in such environments, e.g. ethane, *iso*-butane and *n*-pentane. Incubations with mixtures of methane, ethane, propane and *n*-butane showed that the enrichment cultures Prop12-GMe and But12-HyR degraded propane and *n*-butane simultaneously, while methane and ethane were not degraded (Fig.2). In similar incubations, the culture But12-GMe degraded only *n*-butane. None of the enrichment cultures degraded *iso*-butane or *n*-pentane when supplied as single substrates or together with propane or *n*-butane (Fig.2). During the incubations with mixtures of alkanes the microbial community structure of the enrichment cultures was constant, as demonstrated by hybridizations with fluorescently-labeled, sequence-specific oligonucleotide probes (see Identification of dominant phylotypes section). The ability of the enrichment cultures Prop12-GMe and But12-HyR and also of strain BuS5 (Kniemeyer *et al.*, 2007) to degrade propane and *n*-butane, but not shorter or longer alkanes suggest rather specialized activating enzymes with respect to substrate spectrum. Moreover, the simultaneous degradation of propane and *n*-butane indicate the presence of a single activating enzyme.

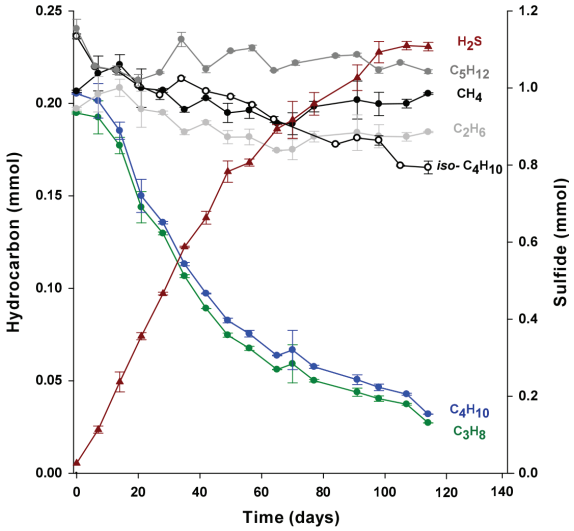

Fig.2 Consumption of propane (●) and *n*-butane (●) coupled to the production of sulfide (●) by the enrichment culture Prop12-GMe. Methane (●), ethane (●), *n*-pentane (●) and *iso*-butane (○) were not degraded if supplied as single substrates or in mixtures with propane or *n*-butane. Cultures were set up in 120 ml serum bottles supplied with 79 ml artificial sea water medium and 1 ml inoculum from a dense-cell suspension (10× concentrated), and incubated at 16 °C with slow shaking (40 rpm). Measurements of gas concentrations and sulfide were done in triplicate.

Genes coding for alkane activating enzymes have been recently described in the nitrate-reducing strain HxN1 degrading C_6–C_8 *n*-alkanes ([1-methylalkyl]succinate synthase, *mas*; Grundmann *et al*., 2008) and in the sulfate-reducing bacterium *Desulfatibacillum alkenivorans* AK-01, utilizing C_{13}–C_{18} *n*-alkanes (alkylsuccinate synthase, *ass*; Callaghan *et al*., 2008). Also, partial sequences of the large subunit of the Mas or Ass enzymes have been retrieved from hydrocarbon-contaminated sediments and an enrichment culture with propane (Callaghan *et al*., 2010). Nevertheless, genes coding for Mas or Ass enzymes in propane- and *n*-butane-degrading bacteria are largely unknown. We attempted to amplify the *masD* gene from the enrichment cultures Prop12-GMe, But12-GMe and But12-HyR, and also from the alkane-degrading sulfate-reducing strains BuS5, Pnd3 (C_{14}–C_{17}) and TD3 (C_6–C_{16}). Almost full-length masD sequences were obtained from strains Pnd3 (2502 bp) and TD3 (2380 bp), and only partial sequences from strain BuS5 (483 bp) and the enrichment cultures (429 bp). For each enrichment culture, identical *masD* sequences were obtained by cloning and sequencing of the PCR products ($n \geq 30$ clones). Phylogenetic analysis of deduced aminoacid sequences

showed that MasD from the psychrotrophic enrichment cultures clustered together, and were most closely related to MasD of strain BuS5 and of the thermophilic, long-chain alkane degrader TD3 (Fig.3).

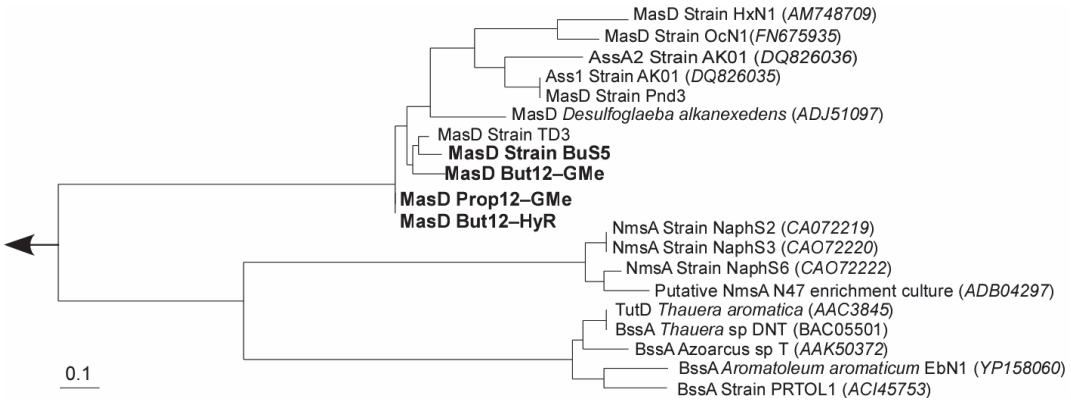

Fig.3 Maximum likelihood phylogenetic tree of MasD, BssA and NmsA amino acid sequences. The tree was calculated in ARB using only partial sequences shared by all species considered. MasD sequences of propane and butane degraders are indicated in boldface.

Identification of dominant phylotypes

The sediment-free enrichments Prop12-GMe and But12-HyR formed macroscopic, dark-red aggregates with a diameter of up to ~ 1 mm. Microscopic analysis showed that aggregates of both enrichment cultures consisted of densely packed, mainly oval, slightly curved cells, of 3 µm length and 1 µm diameter (on average; Prop12-GMe) or 2.5 µm length and 0.8 µm diameter (But12-HyR) (Fig.4 A, B). Further description of the apparently dominant morphotypes was attempted using 16S rRNA-based methods. Construction and analysis of 16S rRNA gene libraries showed a relatively high diversity of sulfate-reducing bacteria in both enrichment cultures (Supplementary Fig.1). Sequences were closely related to those of *Desulfobacula*, *Desulfococcus* and *Desulfosarcina* species, including strain BuS5 and the dominant phylotype in the *n*-butane-degrading enrichment culture, But12-GMe (Kniemeyer *et al.*, 2007). Sequences obtained from increasingly diluted (10^{-2}) DNA template were identical with Prop12-GMe clone 235 and with But12-HyR clone 303 (Fig.5), both affiliated with the genus *Desulfosarcina*, suggesting that these phylotypes were dominant in the

Prop12-GMe and But12-HyR enrichment culture, respectively. To verify this hypothesis, a nested FISH approach was used. Hybridizations of aggregate sections with the group-specific probe DSS658 showed that phylotypes affiliated with the *Desulfosarcina*/*Desulfococcus* group of the *Deltaproteobacteria* represented approx. 80% of the Prop12-GMe and approx. 95% of the But12-HyR enrichment culture (Fig.4 C, D). Furthermore, hybridizations of homogenized aggregate samples with specifically designed oligonucleotide probes showed that Prop12-GMe clone 235 accounted for up to 74% of the total cell number of the Prop12-GMe enrichment culture, and But12-HyR clone 303 for up to 69% of the total cell number of the But12-HyR enrichment culture (Fig.4 E, F).

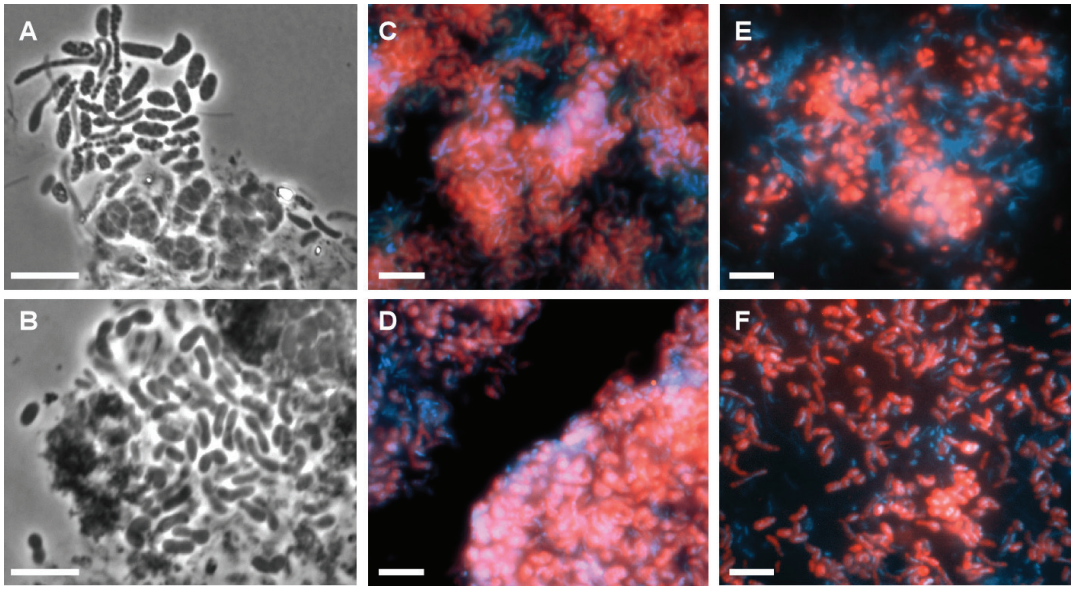

Fig.4 Microscopic images of the enrichment cultures Prop12-GMe (A, C, E), and But12-HyR (B, D, F). Phase-contrast images show densely packed, mostly oval-shaped cells in both enrichment cultures (A, B). These cells affiliated with the *Desulfosarcina*/*Desulfococcus* cluster of the *Deltaproteobacteria*, as demonstrated by hybridization of aggregate cross-sections with the probe DSS658 (C, D). Hybridizations of partly homogenized aggregates with sequence-specific oligonucleotide probes further indicated that each enrichment culture was dominated by unique phylotypes (E, F). The hybridization images (C–F) show overlays of probe (orange) and DAPI (blue) signals. Scale bars = 5 µm.

Prop12-GMe clone 235 and But12-HyR clone 303 shared 95.5% sequence identity and were closely related to strain BuS5 (93.1% and 94.5%, respectively) and to the dominant phylotype in the enrichment culture But12-GMe (94.3% and 95.3%, respectively; Kniemeyer *et al.*, 2007) (Fig.5). Other close relatives include phylotypes retrieved from marine mud volcanoes (Heijs *et al*., 2007; Heijs *et al*., 2008; Lösekann *et al*., 2007), hydrocarbon seep-associated sediments (Lloyd *et al*., 2001; Orphan *et al*., 2001) or marine sediments associated with gas hydrates (Knittel *et al*., 2003; Orcutt *et al.*, 2010) (Fig.5).

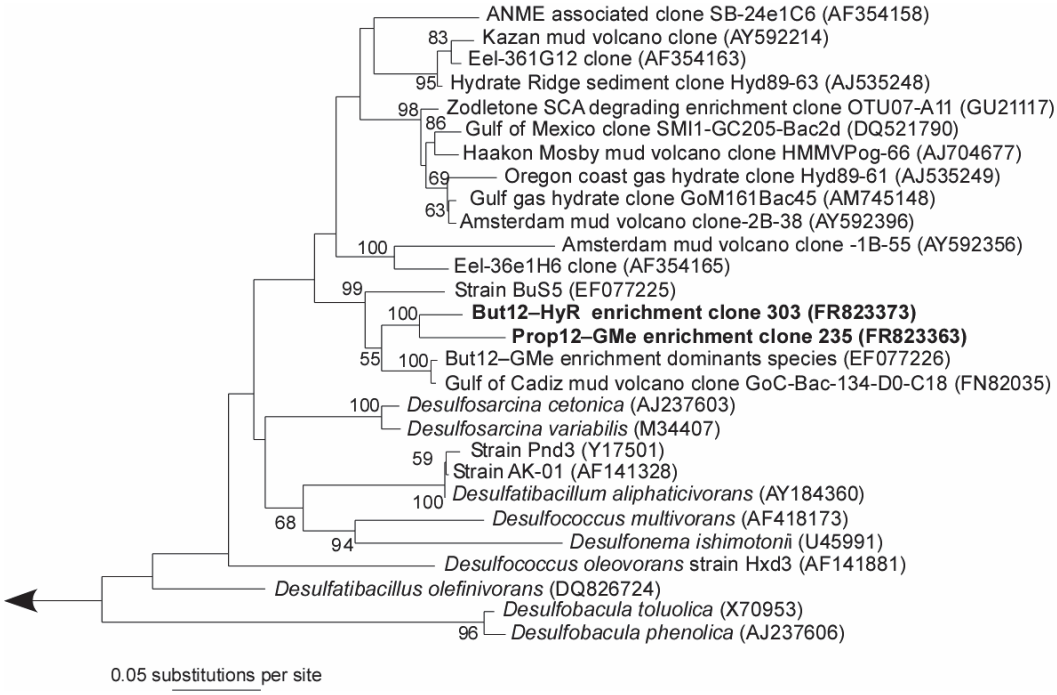

Fig.5 Phylogenetic affiliation of the dominant phylotypes in the enrichment cultures Prop12-GMe and But12-HyR (marked in boldface). The phylogenetic tree was calculated by Maximum Likelihood using only nearly full length sequences (> 1400 bp). The numbers next to the nodes indicate bootstrap values; only values larger than 50% are shown.

The high abundance of a distinct phylotype in each of the psychrotrophic enrichment cultures (Prop12-GMe, But12-GMe and But12-HyR) suggests that the corresponding microorganisms were most likely responsible for the degradation of propane or *n*-butane. In order to demonstrate this hypothesis, the enrichment cultures were incubated with ^{13}C-labeled propane or butane, the cells were hybridized with HRP-labeled sequence-specific oligonucleotide probes followed by deposition of F-containing tyramides, and analyzed by nanoSIMS.

Incubations with ^{13}C-labeled hydrocarbons and nanoSIMS analysis

The enrichment cultures incubated with labeled propane or *n*-butane became increasingly enriched in ^{13}C as revealed by MS analysis of the bulk biomass (Supplementary Fig.2). Based on the bulk MS analysis, samples were further selected for single cell analysis by nanoSIMS (0, 3 and 5 days of incubation for Prop12-GMe; 0, 5 and 10 days for But12-GMe; 0, 3 and 6 days for But12-HyR). For each sample, secondary ion images of ^{12}C (measured as $^{12}C^-$), ^{14}N (measured as $^{12}C^{14}N^-$), ^{13}C ($^{13}C^-$) and ^{19}F ($19F^-$) were recorded simultaneously. Using the $^{12}C^{14}N^-$ ion images (total biomass), regions of interest (ROI) were drawn around individual cells and used determine $^{13}C/^{12}C$ ratios for the dominant and accompanying cells (Fig.6).

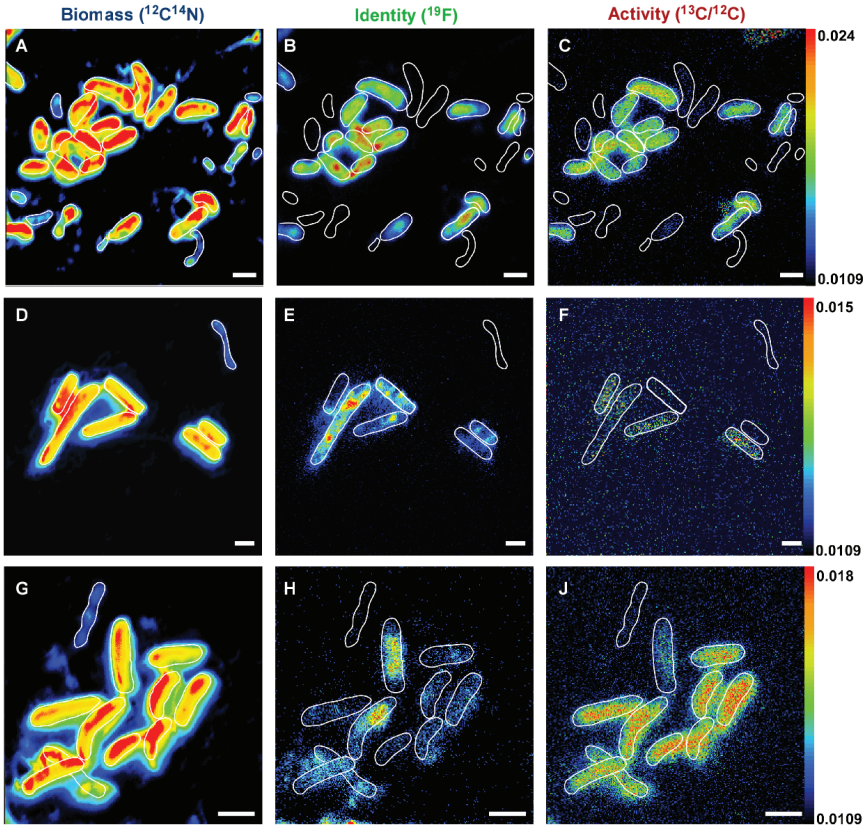

Fig.6 NanoSIMS images of the enrichment cultures Prop12-GMe (A–C), But12-GMe (D–F), and But12-HyR (G–I) after incubation with ^{13}C-labeled propane or butane for 3, 5 and 3 days, respectively. The total biomass ($^{12}C^{14}N^-$) images (A, D, G) were used to draw regions of interest around individual cells. Identification was done based on detection of $^{19}F^-$ (B, E, H) introduced in the cells by deposition of F-containing tyramides after hybridization with HRP-labelled oligonucleotide probes. The ratio images of $^{13}C/^{12}C$ show incorporation of label by individual cells (C, F, I). Scale bars = 1 µm.

The dominant phylotype cells were identified by the presence of $^{19}F^-$. In all enrichment cultures, the cells of the dominant phylotype were the first to assimilate ^{13}C from the labeled substrate, while the accompanying cells showed no or only minor label incorporation (Fig.6, 7). With longer incubation time, increasing ^{13}C-labeling was observed in the accompanying cells (Fig.7). These results demonstrate that the main phylotypes were responsible for the degradation of propane or *n*-butane, while the accompanying bacteria were most probably growing at the expense of excreted degradation intermediates or cellular constituents from lysing dominant cells.

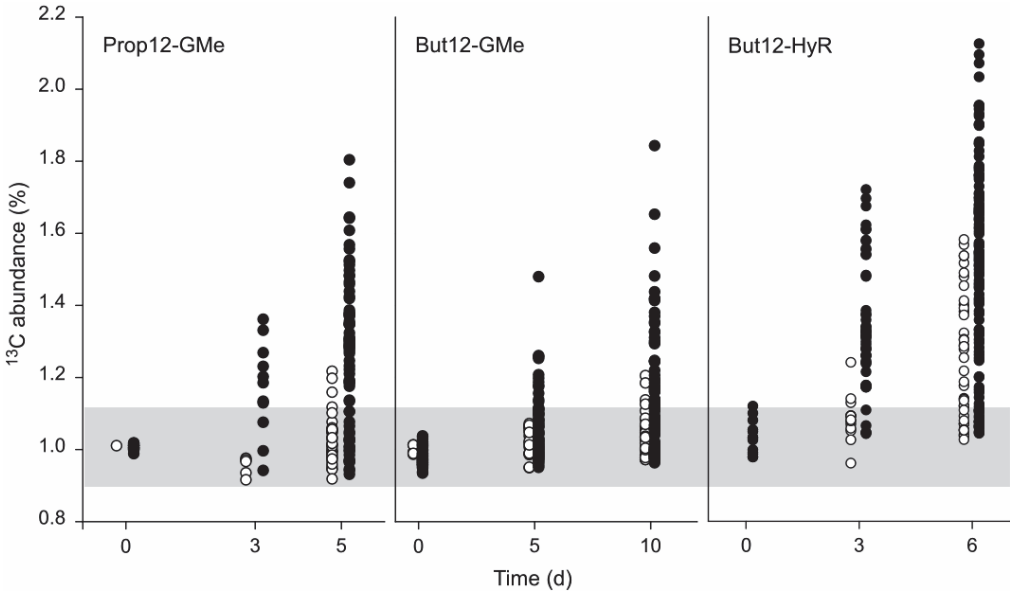

Fig.7 Abundance of ^{13}C in single cells of the enrichment cultures Prop12–GMe, But12–GMe, and But12–HyR incubated with ^{13}C-labeled propane (Prop12-GMe) or butane (But12-GMe and But12-HyR). The ^{13}C abundance was calculated from the $^{13}C/^{12}C$ ratios determined for individual cells of the dominant phylotype (●) or accompanying cells (○). The dominant cells were identified based on the presence of $^{19}F^-$ signals. For clarity, accompanying and dominant cells at each time point were plotted with an off scale of ± 0.2. Shaded area indicates the range of ^{13}C-abundance values obtained for cells at 0 days incubation (no enrichment vs. natural abundance of ^{13}C).

For each enrichment culture, the $^{13}C/^{12}C$ ratios of the dominant phylotype cells ranged from natural abundance (no incorporation of substrate) to relatively high values (Fig.7). This could be explained as population physiological heterogeneity, described also for other groups of microorganisms in natural environments (Musat *et al*., 2008). In addition, propane and *n*-butane as hydrocarbons have low water solubility, which could lead to diffusion and consequently substrate-availability limitations. For the

aggregate-forming enrichment cultures (Prop12-GMe and But12-HyR), the incubations were done with homogenized aggregates to facilitate single-cell analysis by nanoSIMS. The cells inside of these aggregates could have been strongly limited in both sulfate and substrate, and due to the relatively short incubation could have been still in a lag phase at the time of analysis.

Furthermore, using the $^{13}C/^{12}C$ ratios, substrate-carbon assimilation rates were calculated for the dominant cells in each enrichment culture (Table 1). For the calculation, the carbon content of the dominant phylotypes was considered the same as that determined for the phylogenetically and physiologically related strain BuS5 (13.6 fmol C $cell^{-1}$, 4.6 fmol C μm^{-3}). The average biovolumes of the dominant phylotype cells were 2.2 μm^3 (Prop12-GMe), 2.8 μm^3 (But12-GMe) and 1.1 μm^3 (But12-HyR). Average assimilation rates were calculated for cells showing enrichment in ^{13}C vs. cells at *t0*. The average values obtained ranged from 10 ± 9 amol C $cell^{-1}$ d^{-1} (But12-GMe) to 60 ± 28 amol C $cell^{-1}$ d^{-1} (Prop12-GMe), and were similar for the different incubation times of each enrichment culture (Table 1).

Table 1. Carbon and hydrocarbon-assimilation rates calculated from the ^{13}C abundance of individual cells as determined by nanoSIMS analysis. Sulfate reduction rates were calculated using the hydrocarbon dissimilation rates and the stoichiometry of complete oxidation of propane and *n*-butane by sulfate-reducing bacteria.

		Enrichment culture					
		Prop12-GMe (propane)		But12-GMe (*n*-butane)		But12-HyR (*n*-butane)	
	Incubation time (days)	3	5	5	10	3	6
Carbon assimilation (amol $cell^{-1}$ d^{-1})	Average [a]	60 ± 28 *n* = 9	55 ± 32 *n* = 64	10 ± 9 *n* = 65	10 ± 9 *n* = 89	43 ± 25 *n* = 51	33 ± 20 *n* = 123
	Maximum	104	141	52	45	96	76
Hydrocarbon assimilation [b] (amol $cell^{-1}$ d^{-1})	Average	20 ± 9	18 ± 11	2.5 ± 2.25	2.5 ± 2.25	11 ± 6	8 ± 5
	Maximum	35	47	13	11	24	19
Hydrocarbon dissimilated [c] (amol $cell^{-1}$ d^{-1})	Average	180 ± 81	162 ± 99	23 ± 20	23 ± 20	99 ± 54	72 ± 45
	Maximum	315	423	117	99	216	171
Sulfate reduction [d] (amol $cell^{-1}$ d^{-1})	Average	450 ± 202	413 ± 248	75 ± 65	75 ± 65	322 ± 176	234 ± 146
	Maximum	788	1058	380	322	702	556

a. Average rates of *n* cells analyzed
b. One mol C assimilated = 1/3 mol propane or 1/4 mol *n*-butane assimilated
c. Calculated considering the usual ratio of C assimilation/C dissimilation of 1:9, for sulfate-reducing bacteria
d. Calculated considering the complete oxidation of propane or *n*-butane (Kniemeyer *et al*., 2007)

Using the C assimilation rates, hydrocarbon assimilation rates were calculated, and assuming the typical 1:9 ratio of substrate assimilation vs. dissimilation for sulfate-reducing bacteria, the hydrocarbon dissimilation rates were determined. Using the latter, and considering the complete oxidation of propane and *n*-butane (Kniemeyer *et al.*, 2007), the cellular sulfate reduction rates were calculated (Table 1). To our knowledge this is the first study reporting hydrocarbon and sulfate-reduction rates of single cells for propane and *n*-butane degrading microorganisms. These rates could be useful in future studies to estimate the contribution of propane and *n*-butane degraders to the total sulfate reduction rates, provided the abundance of such microorganisms will be quantified.

Conclusion

In the present study, new propane and *n*-butane-degrading sulfate-reducing bacteria have been enriched from deep-sea sediments collected around hydrocarbon seeps. Using hybridizations with sequence-specific oligonucleotide probes, incubations with ^{13}C-labeled substrates and nanoSIMS analysis, it was demonstrated that each enrichment culture was dominated by unique microorganisms which were responsible for the degradation of the hydrocarbon substrate. These microorganisms were apparently specialized, capable of degrading only propane and *n*-butane, but not longer or shorter-chain alkanes. This supports *in situ* observations reporting that propane and *n*-butane appear to be biologically degraded in oil and gas reservoirs (James and Burns, 1984; Boreham *et al*., 2001; Head *et al*., 2003; Larter, 2005) and also at marine hydrocarbon-rich sites (Quistad and Valentine 2011; Orcutt *et al*., 2010), whereas ethane, *iso*-butane and *n*-pentane appeared rather recalcitrant.

The cold-adapted propane and *n*-butane degraders described here were closely related to strain BuS5, forming an obvious phylogenetic cluster of short-chain alkane-degrading, marine sulfate-reducing bacteria within the *Desulfosarcina/Desulfococcus*-group of the *Deltaproteobacteria* (Fig.5). Members of this group have been found in relatively high abundance at gas and oil hydrocarbon seeps, reaching up to 10% of the total free cells (about 5×10^8 cells cm^{-3}; e.g. Orcutt *et al*., 2005, 2010). Sulfate reduction rates (SRR) determined at such sites often exceeded SRR that could be explained by anaerobic oxidation of methane by several fold, and have been proposed to be due to oxidation of hydrocarbons other than methane, including short-chain

alkanes (Kallmeyer and Boetius, 2004; Joye *et al*., 2004; Niemann *et al*., 2006; Orcutt *et al.*, 2005, 2010, Bowles *et al.*, 2011). Propane and *n*-butane-degrading sulfate reducers as those described here could thus have an important role for the *in situ* carbon and sulfur cycling, contributing significantly to the observed excess of sulfate reduction. Future studies, making use of incubations with stable isotope-labeled substrates, hybridizations with specifically-designed genetic probes followed by nanoSIMS analyzes, or quantification of specific *masD* genes, on naturally-occurring populations could determine the abundance of such bacteria and their contribution to the short-chain alkane degradation and sulfate reduction directly in the environment.

Acknowledgements

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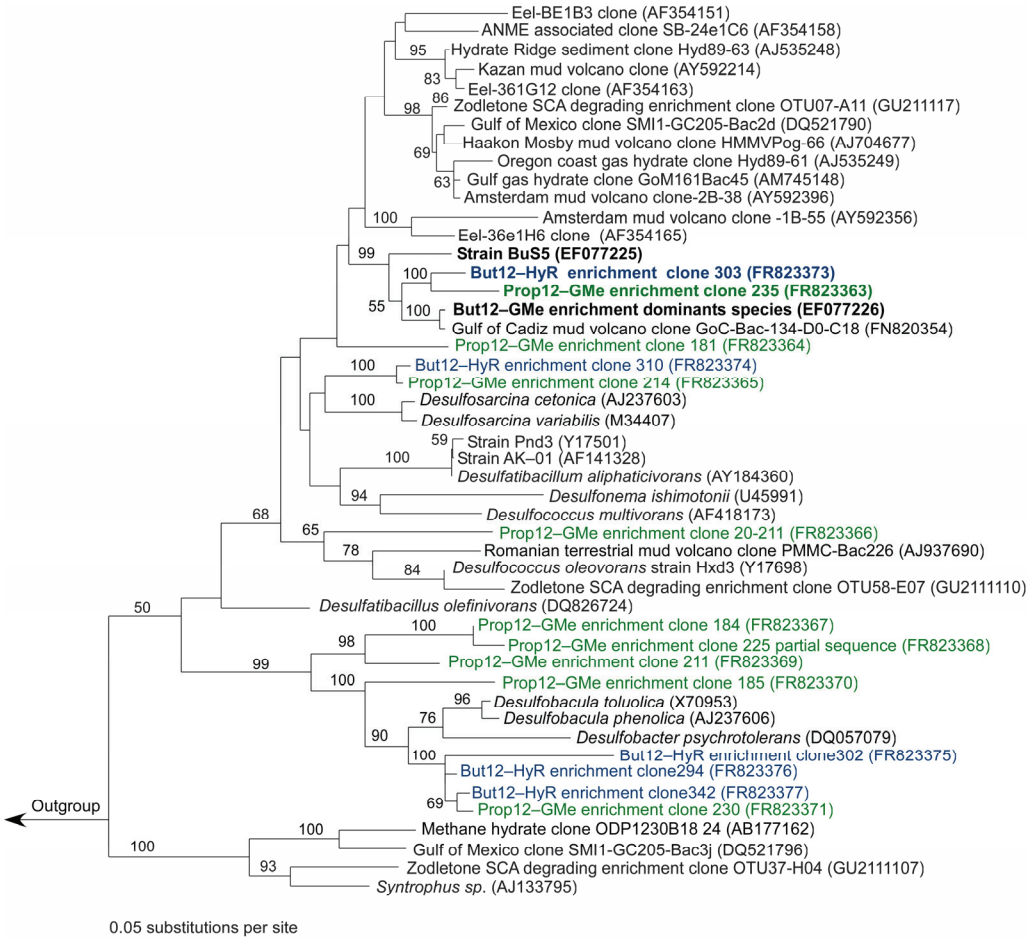
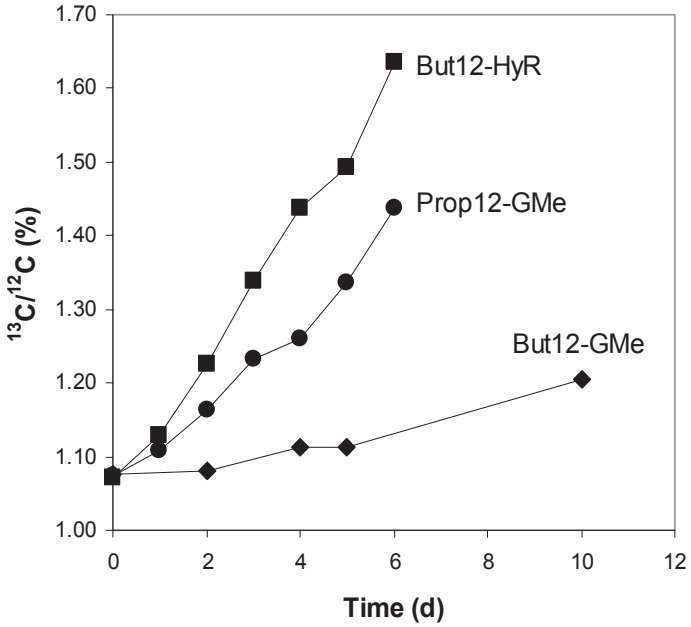
Supplementary Information



Supplementary Fig.1 Phylogenetic affiliation of the 16S rRNA gene sequences retrieved from the enrichment cultures Prop12-GMe (green) and But12-HyR (blue). Only sequences affiliating with the *Deltaproteobacteria* are shown. The gene libraries of both enrichment cultures also contained sequences affiliated with *Bacteroidetes*, *Firmicutes*, *Spirochaetes* and *Planctomycetes*. The phylogenetic tree was constructed by maximum likelihood using the RAxML algorithm. Sequences of *Escherichia coli* strains were used as outgroup. The numbers next to the nodes indicate bootstrap values; only values larger than 50% are shown.



Supplementary Fig.2 Enrichment in ^{13}C of the bulk biomass of the enrichment cultures Prop12-GMe, But12-GMe and But12-HyR during incubation with ^{13}C-labeled propane or *n*-butane. Culture samples (0.5 ml) were withdrawn from the cultures, added to pre-combusted glass-fiber filters, dried and analyzes with an elemental analyzer coupled with a mass spectrometer (see Materials and Methods).

2

Carbon and hydrogen stable isotope fractionation associated with the degradation of propane and *n*-butane by sulfate-reducing bacteria: mechanistic aspects and environmental implications

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Abstract

The mechanism of anaerobic activation of propane and *n*-butane by a pure culture (strain BuS5), which is known to activate both alkanes via addition to fumarate, and three sulfate-reducing enrichment cultures from marine hydrocarbon seeps, for which the activation mechanism is hitherto unknown, was investigated by compound specific stable isotope analysis (CSIA). Two-dimensional isotope fractionation factors, given by the correlation of reactive position specific hydrogen and carbon fractionation (Lambda $_{\text{reactive position}}$, Λ_{rp}) were compared between those obtained for the propane and *n*-butane degrading strain BuS5 and the propane and *n*-butane degrading enrichment cultures. Propane and *n*-butane degradation was always linked to carbon and hydrogen isotope fractionation. In non-aggregate forming cultures, bulk carbon and hydrogen enrichment factors were significantly higher when cultures were shaken. This effect was less pronounced for aggregate-forming cultures, demonstrating that isotope fractionation was partially masked by substrate limitations due to slow headspace-liquid diffusion rates or high cell densities. Although obtained Λ_{rp} values obtained during shaking incubations were in the same range with values obtained for other *n*-alkanes, which are activated by fumarate addition, they still differed significantly for the two alkanes, ranging from 7.8 to 9.4 for *n*-butane and 10.5 to 11.8 for propane, respectively. Degradation experiments performed with strain BuS5 and deuterium-labeled alkanes (D_2- and D_4-propane; D_2- and D_6-butane) indicated that fumarate was added solely at the secondary carbon atom of *n*-butane, but at both primary and secondary carbon atoms of propane. Our results demonstrate that CSIA is a suitable method for detecting anaerobic degradation of short-chain alkanes in the environment.

Introduction

Natural gas is composed of mainly methane and to smaller amounts also of ethane, propane, *n*-butane and *iso*-butane (Tissot and Welte, 1984). In thermogenic gas, these heavier hydrocarbons can make up 10% or more of the total hydrocarbon gas. In places where hydrocarbon gases seep upwards through the sediment, they may come into contact with the biosphere through seepages such as cold seeps, hydrothermal vents, mud volcanoes or become entrapped into ice cages of gas hydrates. Evidence from both deep subsurface natural gas and oil reservoirs (Welte, 1982; James and Burns, 1984; Wenger *et al*., 2002; Boreham *et al*., 2001; Head *et al*., 2003; Larter *et al*., 2005) and marine oil and gas seepages (Formolo *et al*., 2004; Joye *et al*., 2004; Orcutt *et al*., 2004; Sassen *et al*., 2004; Niemann *et al*., 2006; Mastalerz *et al*.; 2009, Orcutt *et al*.; 2010, Quistadt and Valentine, 2011) have indicated that short-chain, non-methane hydrocarbons are being biodegraded under anoxic conditions. From some of these studies it is evident that propane and *n*-butane are most susceptible to microbial biodegradation, whereas ethane and *iso*-butane seem to be rather recalcitrant.

Little is known about the microorganisms involved in the biodegradation of non-methane hydrocarbon gases under anoxic conditions, which are prevailing at most natural seepage sites and deep subsurface oil and gas reservoirs. Cultures of sulfate-reducing bacteria, which were obtained from deep sea hydrocarbon seeps (Kniemeyer *et al.*, 2007) and a terrestrial sulfidic hydrocarbon seep (Savage *et al*., 2010), were shown to activate propane and *n*-butane via fumarate addition, a well-known type of reaction by which several hydrocarbons are activated under anoxic conditons, e.g. alkylated aromatics (Boll and Heider, 2010), cyclic alkanes (Musat *et al*., 2010) or *n*-alkanes (Widdel and Grundmann, 2010). Butane and propane were shown to be activated at the secondary carbon atom, leading to the formation of 1-methyl-) alkylsuccinates (Kniemeyer *et al*., 2007). In case of propane degradation, metabolites were found, which suggest a so far unprecedented route of activation by addition of fumarate to the primary carbon atom (Kniemeyer *et al*., 2007; Savage *et al*., 2010). It is unclear, whether this route of activation is perhaps just a side reaction, or represents a major route of activation.

Compound specific stable isotope analysis (CSIA) is a method to elucidate reaction mechanisms and to quantify *in situ* degradation at contaminated field sites (for a review see Elsner, 2010). The method relays on the kinetic isotope effect (KIE) observed for many (bio)chemical reactions: due to the stronger bonds between heavy isotopes and their bonding atoms, higher enzymatic turnover rates for lighter isotopologues are usually observed. Consequently, the heavy isotopologue becomes enriched in the residual portion of the compound pool. Studying mechanisms of chemical reactions by substituting e.g., a H or C atom with a heavy isotope (deuterium (D), tritium or ^{13}C) have long been popular due to the effects these isotopes cause on rates and equilibrium constants (for a review see Cleland, 2007). The extent of a KIE depends generally on the rate limiting step of a biochemical reaction cascade. In defined microbial cultures, where it is known that a loss of the growth substrate is only due to microbial degradation, the characteristic bulk stable isotope enrichment factor (ε_{bulk}) can be obtained for the biodegradation of a given compound over time *in vitro*. These ε_{bulk} values can then be applied to quantify *in situ* biodegradation rates of the compound in the environment (Meckenstock *et al.,* 2004, Hofstetter *et al.*, 2008). The KIE can be masked by rate limitations due to non-fractionating reaction steps preceding the initial bond cleavage (Northrup, 1981; Elsner *et al.,* 2005; Thullner *et al.*, 2008; Kampara, *et al.*, 2008). Thus, the observed apparent isotope effect (AKIE) can be much lower than the actual KIE. Therefore, two dimensional compound specific stable isotope analyses (2D-CSIA) has been suggested as a tool to overcome masking effects (Elsner *et al.,* 2005; Zwank *et al.,* 2005; Fischer *et al.*, 2007, 2008). The underlying principle is that two elements, which are directly involved in a bond cleavage during the first enzymatic reaction, are analyzed with regard to their stable isotope fractionation effect. Assuming that both elements will be equally affected by masking effects, a correlation of the stable isotopes of both elements can be used as a marker for a given biochemical reaction.

In this study, carbon and hydrogen stable isotope fractionation was measured with cultures of propane and *n*-butane degrading, sulfate-reducing bacteria. Experimentally derived enrichment factors and apparent kinetic isotope effects (AKIEs) are compared between those obtained for the propane and *n*-butane degrading strain BuS5, which is known to activate both alkanes via addition to fumarate, and in addition several propane and *n*-butane degrading enrichment cultures from different marine hydrocarbon seeps, for which the activation mechanism is

hitherto unknown. Some of the investigated enrichment cultures are forming dense aggregates, whereas one of the enrichment cultures and the pure culture of strain BuS5 grow in homogeneous suspensions. The effects of substrate limitation on the observed stable isotope fractionation, due to mass transfer limitations into the dense aggregates and also the effect of low mass transfer into the surrounding medium, due to impaired substrate diffusion rates were investigated. Using specifically deuterated propane and *n*-butane, we also investigated whether the previously reported additional route of activation for propane at the primary carbon atom is a major route for activation. To test this, we used primary- or secondary carbon atom deuterated propane and *n*-butane. For this, we developed a new method to separate deuterated- and natural propane and *n*-butane and to quantify them via gas chromatography.

Materials and methods

Cultures and cultivation conditions

Strain BuS5 was isolated in our laboratory from sediments associated with a hydrothermal vent in the Guaymas basin (Kniemeyer *et al.,* 2007*)*. The propane and *n*-butane degrading enrichment culture Propane12-GMe (Jaekel *et al*.,in prep) and the butane degrading enrichment culture Butane12-GMe (Kniemeyer *et al.*, 2007) were obtained from sediment at cold seeps in the Gulf of Mexico. The propane and *n*-butane-degrading enrichment culture Butane12-HyR was obtained from sediment associated with a gas hydrate at Hydrate Ridge (Jaekel *et al*., in prep). All cultures were cultivated in anoxic and bicarbonate-buffered artificial seawater medium (Widdel and Bak, 1992) with 28 mM sulfate as primary electron acceptor and a headspace containing 10% CO_2 and 90% N_2. Cultures were incubated either statically or by slow shaking (50 rpm) at 28 °C (strain BuS5) or 12 °C (all enrichment cultures), lying in an angle to avoid contact of the headspace with the stopper. For the preparation of active and dense cell suspensions, 150 ml aggregate forming enrichment cultures (Propane12-GMe and Butane12-HyR) were concentrated by letting the aggregates settle to the bottom of the standing bottle and removing the overlaying medium with a glass pipette while flushing with a CO_2/N_2 gas mix. The aggregates were re-suspended in 15 ml anoxic medium and transferred into a 20 ml

Hungate tube with a black stopper (Wheaton, Millville-USA). For the non-aggregate forming (planktonic) enrichment culture (Butane12-GMe) and strain BuS5, dense cell suspensions were prepared by centrifuging 50 ml enrichment culture in anoxic plastic beakers at 14300 g for 20 minutes (Beckmann Coulter Avanti® J-26 XP, Krefeld-Germany), re-suspending the cell pellet in 15 ml anoxic medium and transferring the suspension into a 20 ml Hungate tube with a black stopper.

Isotope fractionation experiments

Isotope fractionation experiments with propane and *n*-butane having natural stable isotope abundance were performed in teflon-stoppered (Wheaton, USA) 120 ml serum bottles containing 79 ml medium. Defined amounts of propane (12 ml) or *n*-butane (9 ml) (purity 3.5, Air Liquide-Germany) were added with syringes through the stoppers and the bottles were left for equilibration of the gas at the corresponding temperature (12 °C or 28 °C) by either slow shaking (50 rpm) or statically for 2 days. The bottles were then inoculated with 1 ml of active and 10 × concentrated cell suspensions. For each culture, 10–12 parallel bottles were prepared. Individual cultures were inactivated after approximately 0, 10, 25, 50, 75, 80, 85, 90, 95 and 99% of the corresponding growth substrate had been consumed by adding 1 M NaOH until a pH of 12 was reached. As abiotic controls for each sampling point, ten serum bottles were prepared as described above, but inoculated with 1 ml autoclaved cells instead of living cells.

Isotope fractionation experiments with deuterium-labeled propane or butane were conducted only with strain BuS5. Teflon-stoppered 80 ml serum bottles were filled with 36 ml medium. Deuterium-labeled propane or *n*-butane (purity of 2.1, ≥ 98 atom % D; Campro Scientific, Berlin-Germany) was added together with equal volumes of natural propane or *n*-butane (purity 3.5, Air Liquide-Germany) to the bottles with syringes through the stoppers. The bottles were left for equilibration of the gas at 28 °C with slow shaking (50 rpm) for 2 days and subsequently inoculated with 4 ml of an active, 10× concentrated cell suspension of strain BuS5. Growth experiments were performed with mixtures of D-2,2-propane and natural propane (propane-D_2/natural propane), D-1,1,1,3,3,3-propane and natural propane (propane-D_6/natural propane), D-2,2,3,3-butane and natural butane (butane-D_4/natural butane)

and D-1,1,1,4,4,4-butane and natural butane (butane-D_6/natural butane). Autoclaved cells were used as inoculum in bottles with the above listed gas mixtures as abiotic controls.

Analytical methods

Sulfide concentrations were analyzed as described elsewhere (Cord-Ruwisch, 1985). Natural propane and *n*-butane concentrations were determined by gas chromatography using a Shimadzu GC-14B gas chromatograph, equipped with a Supel-Q PLOT column (30 m × 0.53 mm, 30 µm film thickness; Supelco-Bellefonte, USA) and a flame ionization detector. The column temperature was 140 °C and the injection and detection temperatures were 150 °C and 280 °C, respectively. The carrier gas was N_2 at a flow rate of 3 ml min^{-1}. Headspace samples (100 µl) were taken from the culture bottles using a gastight glass syringe and injected into the injector without split. Samples were analyzed in duplicates.

Natural abundance $^{13}C/^{12}C$ and D/H isotope ratios were determined by gas chromatography combustion isotope-ratio mass spectrometry (GC-IRMS). The system was composed of a GC (HP-5890; Hewlett-Packard) equipped with a CP-PoraBOND Q Fused Silica column (50 m length; inside diameter 0.32 mm; film thickness 5 µm; Varian, Middelburg-Netherlands); the carrier gas was helium with a flow rate of 1 ml min^{-1}. The column temperature was held at 140 °C for propane analysis and at 160 °C for *n*-butane analysis, respectively. The GC was connected to an isotope mass spectrometer (MAT 252 for $^{13}C/^{12}C$ analysis; MAT 253 for D/H analysis; Thermo Fischer Scientific). For $^{13}C/^{12}C$ analysis, samples were combusted to CO_2 prior mass spectrometric analysis in a GC-combustion interface. For D/H analysis, samples were pyrolysed to hydrogen prior mass spectrometric analysis in a pyrolysis reactor (GC-pyrolysis, Thermo Fischer Scientific). Samples were taken from the headspace in volumes ranging from 15–50 µl and injected with split ranging from 1:200–1:3 into the injection unit, which had a temperature of 250 °C. Each sample was measured at least three times. The total analytical uncertainty with respect to both accuracy and reproducibility was always better then ±0.5‰ for $\delta^{13}C$ and ±10‰ for $\delta^{2}H$, respectively.

The obtained isotope ratios were expressed in the delta notation (δ^{13C} and $\delta^{2}H$) in ‰ units, according to Eq.1.

$$\delta^{13}C_{sample} \text{ and } \delta^{2}H_{sample}\,[‰] = \left(\frac{R_{\text{sample}} - R_{\text{standard}}}{R_{\text{standard}}}\right) \cdot 1000 \qquad [1]$$

In Eq.1, R_{sample} and $R_{standard}$ refer to the $^{13}C/^{12}C$ or D/H-ratios of the measured sample and a standard, respectively. The standard for the carbon isotope measurements was Vienna Pee Dee Belemnite (V-PDB). The standard for hydrogen isotope measurements was Vienna Standard Mean Ocean Water (V-SMOW).

The D/H isotope fractionation in experiments with mixtures of deuterium-labeled and natural propane and *n*-butane was determined by separating the isotopologues by gas chromatography using a Shimadzu GC-14A gas chromatograph equipped with two connected (SGE SilTite^{TM} Mini-Union kit, Milton Keynes, UK) CP-PoraBOND Q Fused Silica columns (each had a length of 50 m; inside diameter 0.32 mm; film thickness 5 µm; Varian) and a flame ionization detector. The detection and injection temperatures were 150 °C and 130 °C, respectively. The carrier gas was hydrogen at a flow rate of 0.3 ml min^{-1}. The mixture of natural propane and propane-D_2 was injected at 30 °C column temperature. The retention time for propane-D_2 was 133.7 min and this compound was followed by natural propane at 136.8 min. The mixture of natural propane and propane-D_6 was injected at 60 °C column temperature. The retention times for propane-D_6 and natural propane were 48.1 min and 50.3 min, respectively. The mixtures of natural butane and either butane-D_4 or butane-D_6 were both injected at 75 °C column temperature. The retention times were 126.5 min for butane-D_6, 128.6 min for butane-D_4 and 132.8 for natural butane (see Fig.1 in the SI for chromatograms which illustrate the separation of isotopologues by GC). Headspace samples (100 µl) were taken from the culture bottles using a gastight glass syringe and injected into the injector without split. Samples were analyzed in duplicates.

Quantification of isotope fractionation with natural propane and *n*-butane

The Rayleigh equation for closed systems (Eq. 2) was used to describe the isotope fractionation due to the biological conversion of substrates having natural isotopic abundance.

$$\left(\frac{R_t}{R_0}\right) = \left(\frac{\frac{C_t}{C_0}}{\frac{R_t+1}{R_0+1}}\right)^{\frac{\varepsilon}{1000}} \qquad [2]$$

In natural systems, where heavy stable isotopes are present in low abundance and therefore $R_t + 1 \approx 1$, the Rayleigh equation can be expressed as:

$$\left(\frac{R_t}{R_0}\right) = \left(\frac{C_t}{C_0}\right)^{\frac{\varepsilon}{1000}} \qquad [3]$$

where C_t and C_0 are the concentration of a compound and R_t and R_0 are the ratios of heavy versus light isotopes at the time of sampling and the start of the experiment, respectively.

The enrichment factor ε (‰) is derived from the logarithmic form of the modified Raleigh equation (Eq. 4) by plotting the $\ln(C_t/C_0)$ versus $\ln[(\delta_t + 1000) / (\delta_0 + 1000)]$ and using the slope of the linear regression line (m) in Eq.5

$$\ln\left(\frac{\delta_t + 1000}{\delta_0 + 1000}\right) = \frac{\varepsilon}{1000} \ln\left(\frac{C_t}{C_0}\right) \qquad [4]$$

$$m = \frac{\varepsilon}{1000} \qquad [5]$$

For a general mechanistic interpretation of the degradation pathways, the non-reacting positions of the activation reaction have to be taken into account in order to

correct for a ″diluting effect″ of heavy isotopes in non-reacting positions, thus the bulk enrichment factors (ε_{bulk}) are converted to enrichment factors of the reacting position ($\varepsilon_{reactive\ position}$) according to Eq.6,

$$\ln\left(\frac{n/x\times\delta_t+1000}{\delta_0+1000}\right)=\frac{\varepsilon_{\text{reactive position}}}{1000}\ln\left(\frac{C_t}{C_0}\right) \qquad [6]$$

where *n* is the total number of atoms of a given element in the molecule and *x* the number of atoms in the reactive position (Elsner *et al*., 2005; Elsner *et al*., 2007). The enrichment factor of the reactive position, $\varepsilon_{reactive\ position}$ (‰), is derived from Eq.6 by plotting $\ln(C_t/C_0)$ versus $\ln[(n/x\times\delta_t+1000)/(\delta_0+1000)]$ and using the slope of the linear regression line (*m*) in Eq.7:

$$m=\frac{\varepsilon_{\text{reactive position}}}{1000} \qquad [7]$$

Since previous studies (Kniemeyer *et al.,* 2007, Savage *et al.*, 2010*)* indicated that both propane and *n*-butane are activated at the secondary carbon atom, we set for propane: *n*=3 and *x*=1 for carbon, and *n*=8, *x*=2 for hydrogen. For butane, we set *n*=4, *x*=2 for carbon and *n*=10, *x*=4 for hydrogen. The errors of the obtained enrichment factors are given as a 95% confidence interval (CI) and were calculated by regression analysis according to Elsner *et al*. (2007).

To compare the isotope fractionation factors obtained in this study to that of other substrates activated by fumarate addition, apparent kinetic isotope effects (AKIE) for both carbon and hydrogen were determined using Eq.8,

$$AKIE=\left(\frac{1}{1+z\cdot\frac{\varepsilon_{reactive\ position}}{1000}}\right) \qquad [8]$$

where *z* is the number of atoms, which are in identical position. In the case of propane we set *z*=1 for carbon and *z*=2 for hydrogen and in the case of *n*-butane *z*=2 for carbon and *z*=4 for hydrogen. The uncertainty of the AKIE was estimated by error propagation (Eq.9):

$$\text{error of AKIE} = \left| \frac{\partial \text{AKIE}}{\partial \varepsilon_{\text{reactive position}}} \right| \times \text{error of } \varepsilon_{\text{reactive position}} \qquad [9]$$

For the 2D-CSIA approach, the hydrogen vs. carbon isotope discrimination ($\Delta\delta^2H/\Delta\delta^{13}C$), given by $\Delta\delta = \delta_t - \delta_0$ was plotted and the slope of linear regression termed lambda value (Λ_{bulk}) (Fischer *et al*., 2008). Its uncertainty was given as 95% CI were derived from regression analysis according to Elsner *et al*. (2007).
For comparing the hydrogen vs. carbon discrimination of fumarate addition of alkanes of different chain lengths, we calculated the reactive-position specific lambda value ($\Lambda_{\text{reactive position}}$) by Eq.10:

$$\Lambda_{\text{reactive position}} = \frac{\Delta\delta^2\text{H}_{\text{reactive position}}}{\Delta\delta^{13}\text{C}_{\text{reactive position}}} \approx \frac{\varepsilon_{\text{H reactive position}}}{\varepsilon_{C\,\text{reactive position}}} \qquad [10]$$

For a general comparison of Λ values for different substrates and reaction types, we calculated Λ_{AKIE} given by $AKIE_H/AKIE_C$. The uncertainty of the Λ_{AKIE} was estimated by error propagation (Eq.11):

$$\text{error } \Lambda_{\text{AKIE}} = \left(\left| \frac{\partial \text{AKIE}_\text{H}}{\partial \Lambda_{\text{AKIE}}} \right| \times \text{error AKIE}_\text{H} \right) + \left(\left| \frac{\partial \text{AKIE}_\text{C}}{\partial \Lambda_{\text{AKIE}}} \right| \times \text{error AKIE}_\text{C} \right) \qquad [11]$$

Quantification of isotope fractionation with deuterium-labeled propane and *n*-butane.

For mixtures of deuterium-labeled and natural propane and *n*-butane, Eq.12 was used to calculate the reaction specific fractionation factor α (Hunkeler *et al*., 2002):

$$\ln \frac{\text{R}_\text{t}}{\text{R}_0} = \left(\frac{1}{\alpha} - 1 \right) \cdot \ln \frac{f}{(1 + R_t)/(1 + R_0)} \qquad [12]$$

where *f* is the fraction that has not reacted yet at time *t*, given by the Eq.13:

$$f = \frac{L_t + H_t}{L_0 + H_0} = \frac{L \cdot (1 + R_t)}{L_0 \cdot (1 + R_0)} \qquad [13]$$

where H and L are the concentrations of the substrate with heavy and light isotopes, respectively, at a given time. Using Eq.12, ε was determined by plotting $\ln(R_t/R_0)$ versus $\ln\{f/[(1+R_t)/(1+R_0)]\}$. The regression curve of this graph multiplied by 1000 gives the reactive position specific enrichment factor ε_{rp} (analogously to Eq. 7).

Results and discussion

Bulk enrichment factors of anaerobic propane and *n*-butane degradation − masking effects.

The degradation of propane and *n*-butane by the sulfate-reducing cultures investigated in this study was always linked to an enrichment of ^{13}C or D in the residual propane or butane. In abiotic controls, no loss of propane or *n*-butane and change in carbon or hydrogen isotopic composition was observed (data not shown), demonstrating that the carbon and hydrogen isotope fractionation effects in the biotic samples were due to microbiologically catalyzed transformations of the substrates. However, bulk enrichment factors (ε) differed significantly, ranging from −0.8 ± 0.3 ‰ to −8.7 ± 1.1‰ for carbon (ε_C) and −4.8 ± 2.1 ‰ to −91.7 ± 6.1 ‰ for hydrogen (ε_H). The ε_H values were overall about 10 times higher than the ε_C values which was expected due to the strong hydrogen kinetic isotope effect during C-H bond breakage (Elsner *et al*., 2005). All obtained values and subsequently derived factors are summarized in Table 1 in the supplementary information. The correlation between substrate concentration and isotope composition was always high ($R^2 \geq 0.82$), except for the aggregate-forming enrichment culture Butane12-HyR when incubated statically ($R^2 \geq 0.66$).

In statically incubated cultures, we always observed lower enrichment factors for carbon and hydrogen compared to cultures incubated by constant shaking. For the enrichment cultures Propane12-GMe and Butane12-HyR, which form dense

macroscopic aggregates, the differences in ε values for both carbon and hydrogen fractionation during static and shaking experiments were less pronounced than for strain BuS5 and enrichment culture Butane12-GMe, which grew in homogenous cell suspensions (planktonic) and did not form dense aggregates (Table 1 in the supplementary information). The degradation of propane or *n*-butane by cultures growing in homogeneous cell suspension was significantly faster during shaking experiments compared to static incubations (data not shown). These results indicate that during static incubations the actual carbon and hydrogen stable isotope fractionation effects were masked due to limiting propane and *n*-butane diffusion rates into the liquid medium and towards the cells, which had settled to the bottom of the incubation flasks, resulting in much smaller isotope fractionation for carbon and hydrogen. The dense cell aggregates of enrichment cultures Propane12-GMe and Butane12-HyR seem to have further impaired the substrate diffusion rates into the cells which make up the aggregates in a way that could not be overcome by increasing the amount of substrate diffusing into the medium through shaking. Such impact of bioavailability restrictions on observable stable isotope fractionation during microbial substrate degradation has been observed and described before (Thullner *et al*., 2008) and likely represent realistic environmental scenarios of mass transfer limitation due to low bioavailability of substrates (Kampara *et al.*, 2008) or high cell densities (Templeton *et al.*, 2006; Kampara *et al*., 2009) as it is the case within microbial aggregates or biofilms (Staal *et al*., 2007).

For the sulfate reducer *Desulfoglaeba alkanexedens*, ε_{bulk} values for *n*-hexane or *n*-octane degradation similar to those found in our study for propane and *n*-butane were recently reported: −5.5 ± 0.2 ‰ and −43.1 ± 6.3 ‰ for *n*-hexane degradation, and −5.2 ± 0.4 ‰ and −27.8 ± 4.2 ‰ for octane degradation, respectively (Morris *et al*., submitted). The ε_C and ε_H bulk values obtained from strain BuS5 for shaken incubations with propane (ε_C −8.7 ‰ ± 1.1; ε_H −91.7 ‰ ± 6.1) were higher than those reported for the anaerobic degradation of propane in marine sediments from mud volcanoes in the Nile deep-sea fan (Mastalerz *et al*., 2009), which were −4.8 ‰ for carbon and –43.3 ‰ for hydrogen. These values are most similar to those we observed during the anaerobic degradation of propane by the aggregate forming enrichment culture Propane12-GMe (−3.7 ‰ ± 0.05 for carbon and −36 ‰ ± 4.5) and imply that, given the same activation reaction was involved, these sediments were

limited in available propane. Notably, ε_C values for shaken propane and *n*-butane degrading cultures in our study were significantly higher than the previously reported ε_C bulk values for the anaerobic degradation of propane (−5.2 ‰) and *n*-butane (–1.6‰) by strain BuS5, and for the degradation of *n*-butane by the enrichment culture Butane12-GMe (–1.6‰) (Kniemeyer *et al*., 2007). It is possible that the values obtained by Kniemeyer *et al.* (2007) were obtained under substrate-limiting conditions, since their reported values are closer to those obtained during our study when cultures were incubated standing. For the aerobic degradation of propane and *n*-butane in sediments from a marine hydrocarbon seep site (Kinnaman *et al.*, 2007) and freshwater alluvial sediment (Bouchard *et al.*, 2008), ε_C values were reported to be −4.8‰ ± 0.9 (Kinnaman *et al.*, 2007) and −10.8‰ ± 0.7 (Bouchard *et al*., 2008) for propane and −2.9‰ ± 0.9 (Kinnaman *et al.*, 2007) and −5.6‰ ± 0.1 (Bouchard *et al*., 2008) for *n*-butane. The ε_C values from these studies show a large variation from each other and it was suggested that this might be due to different microbial populations degrading the compounds in the marine and freshwater sediments, possibly containing different types of monooxygenases (Bouchard *et al*., 2008). The ε_C values we have observed during the anaerobic degradation of propane and *n*-butane by strain BuS5 and *n*-butane by enrichment culture Butane12-GMe during shaking incubations are similar to the values reported by Bouchard *et al*. (2008) and higher than those reported by Kinnaman *et al.* (2007). In the study by Kinnaman *et al.* (2007), ε_H values for propane were determined to be −15.1‰ ± 1.9, whereas no ε_H values were reported for the degradation of *n*-butane.

Apparent kinetic isotope effects (AKIEs) of anaerobic propane and *n*-butane degradation − mechanistic aspects

For comparing the reaction mechanisms of propane and *n*-butane degradation, bulk enrichment factors were converted to position specific enrichment factors (ε_{rp}) and apparent kinetic isotope effects (AKIEs) (Elsner *et al.*, 2005; see materials and methods section for a detailed description). It was assumed that propane and *n*-butane were activated in all cultures by addition of fumarate to the secondary carbon atom. Generally, fumarate addition is mediated by glycyl-radical enzymes, where a thiyl radical is formed from a cystein residue of the enzyme. This thiyl radical then

abstracts a hydrogen atom from a carbon atom of the hydrocarbon molecule, forming a transient radical which attacks the carbon-carbon bond of fumarate. Fumarate is then added to the carbon atom of the hydrocarbon, which formerly bared the radical via the formation of a new carbon-carbon bond (Heider, 2007). In contrast to the enzyme using alkylated aromatics as substrate, benzylsuccinate synthase, which has been characterized in detail (Li *et al*., 2009), nothing is known yet about (1-methyl-) alkylsucinate synthases. For shaken planktonic cultures, AKIE values for both carbon (1.02 – 1.026) and hydrogen (1.35 – 2.14) were in a range expected for isotope effects linked to the cleavage of a C-H-bond (Elsner *et al*., 2005). The highest AKIE values for carbon and hydrogen were observed for propane degradation by strain BuS5 (Fig.1 and Table 1, supplementary information). AKIE values for *n*-butane might be on the one hand slightly lower due to a (small) kinetic masking effect still existing in shaken cultures caused by the lower solubility of *n*-butane (Oswald coefficient (L) = 0.03702 at 28 °C and 0.05154 at 12 °C) than for propane (L = 0.04376 at 28 °C and 0.05825 at 12 °C) (Dean, 1992) in water.

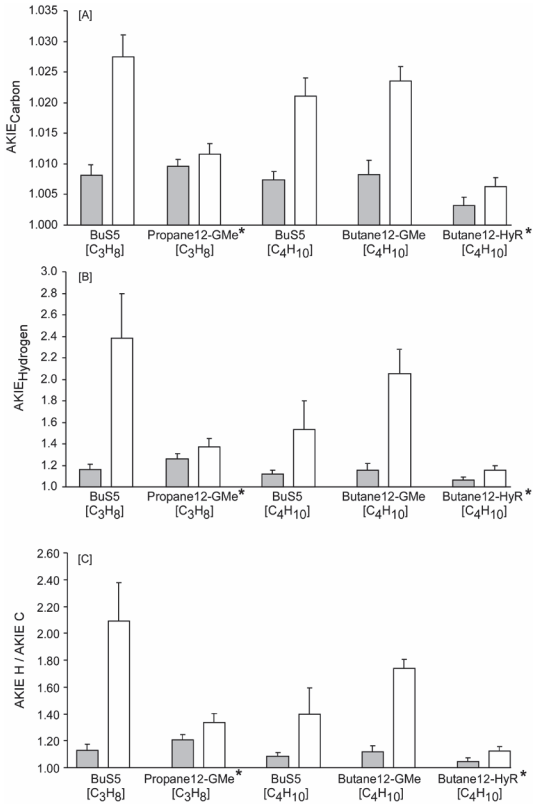

Fig.1 Overview of AKIE values for (A) Carbon, (B) Hydrogen and (C) AKIE H/AKIE C during propane and butane degradation. Grey bars represent values obtained during standing incubations and white bars represent values obtained during shaking incubations of cultures. Dense aggregate forming enrichment cultures are marked with a star (⋆).

On the other hand, an additional activation of propane at the primary carbon atom was observed for strain BuS5 (Kniemeyer *et al*., 2007), which could principally result in higher AKIEs for carbon and hydrogen during degradation of propane. Furthermore, secondary hydrogen isotope fractionation effects may play a larger role in propane activation due to the lower number of atoms in propane compared to butane, resulting in higher isotope fractionation.

Elucidation of the activation mechanisms by two-dimensional isotope fractionation analysis.

By plotting the shifts of hydrogen and carbon isotope signatures, a slope develops (lambda value (Λ), Fischer *et al.*, 2008) which can be used to characterize distinct substrate activation mechanisms, as shown e.g. for the activation of toluene (Vogt *et al*., 2008; Tobler *et al*., 2008) or benzene (Mancini *et al*., 2008; Fischer *et al*., 2008). In our study, Λ values of shaken cultures were always and in most cases significantly higher than Λ values of standing cultures (Fig.2 and Table 1 in the supplementary information).

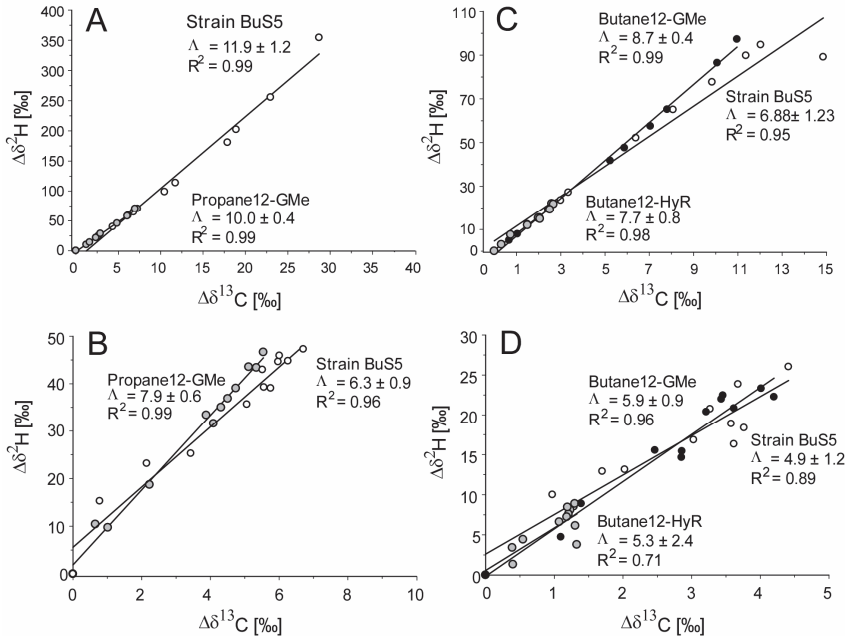

Fig.2 Plots of Δ δ^2H versus Δ δ^{13}C for each enrichment culture investigated in this study. The slope of the regression line gives the Λ values. Strain BuS5 (○) and enrichment culture Propane12-GMe (●), degradation of propane under shaking (A) and standing (B) conditions. Strain BuS5 (○), enrichment culture Butane12-GMe (●) and enrichment culture Butane12-HyR (●), degradation of *n*-butane under shaking (C) and standing (D) conditions.

To our knowledge, this effect of substrate limitation on the extent of the two dimensional isotope fractionation effect has not been investigated before. Previous studies, which have investigated the effect of bioavailability restrictions due to substrate limitations (Kampara *et al.*, 2008) or high cell densities (Staal *et al*., 2007; Templeton *et al.*, 2006; Kampara *et al*., 2009), have only investigated the effect on carbon isotope fractionation alone. Thus, the assumption that masking effects will affect the two elements involved in the biochemical cleavage or formation of a bond equally (Elsner *et al.,* 2005; Zwank *et al.,* 2005; Fischer *et al.*, 2007, 2008) may not always be true. Possible factors causing a shift in Λ values during substrate limiting vs. non-limiting conditions could be a change of the transition state of the activating enzyme during substrate-limiting conditions. Possible factors causing a shift in Λ values during substrate limiting vs. non-limiting conditions could be a change of the transition state of the activating enzyme during substrate-limiting conditions. Briefly, when an atom is transferred from one molecule to another, the D or ^{13}C isotope effects will be at maximum when the transition state is symmetrical. This means that the force constants of the bonds being made and broken are equal. However, in cases where the two atoms do not form equally strong bonds, the maximum isotope effect may not correspond to this situation (Botting, 1994). Opposed effects for carbon and hydrogen isotope fractionation have been observed, i.e. for the formate dehydrogenase, provided with four different coenzymes (nucleotides), having different redox potentials. This was explained by changing transition states upon each reaction due to change in redox potentials of the reaction partners (Botting, 1994). Alternatively, isoenzymes might be induced, with higher substrate affinities during conditions of low substrate availability and which therefore probably also exhibit different transition states. This may lead to altered two-dimensional isotope fractionation patterns during substrate-limiting conditions. For example, the C_{13}-C_{18} alkane degrading sulfate-reducing bacterium *Desulfatibacillum alkenivorans* (strain AK-01) contains two highly similar, but not identical copies of the operon coding for the activating enzyme alkylsuccinate synthase (Ass) (Callaghan *et al.*, 2008). The role of the second operon is still not clear (Callaghan *et al.*, 2011) and one could speculate that a second enzyme with higher substrate affinities may be induced when substrate concentrations are low. However, the reason for the observed shift in Λ values during

substrate limiting vs. non-limiting conditions remains unknown and will be addressed in future studies.

For comparing the degradation pattern of propane and *n*-butane, we used in the following the Λ values of shaken, non-limited cultures. For this purpose, we calculated Λ values of the reactive position (Λ_{rp}). Λ_{rp} values were in the same range for *n*-butane (7.8–9.9) or propane (10.5–11.8), respectively, indicating that each alkane was activated by an identical mechanism in the tested cultures. Notably, Λ_{rp} values for propane activation were significantly higher than values for *n*-butane activation in strain BuS5 and enrichment culture Butane12-GMe (Table 1 in the supplementary information), indicating that propane and *n*-butane were activated by slightly different activation mechanisms in these cultures.

We calculated a Λ_{rp} value of 12 for anaerobic propane degradation at a marine mud volcano by taking ε_C and ε_H values reported by Mastalerz *et al.* (2009) and assuming that propane is activated at the secondary carbon atom by fumarate addition (as we did in our calculations). The determined value is nearly identical to what we observed for the degradation of propane by our tested cultures. This indicates that propane is indeed activated by fumarate addition at the mud volcano site. As described above, isotope fractionation at the site might be masked, as indicated by rather low carbon and hydrogen enrichment factors for propane degradation.

For *D. alkanexedens*, Λ_{rp} values of 9.1 and 6 were reported for anaerobic *n*-hexane and *n*-octane degradation, respectively (Morris *et al.*, submitted). Notably, this organism is known to activate *n*-hexane and *n*-octane by fumarate addition at the secondary carbon atom (Kropp *et al*., 2000; Davidova *et al*., 2005), allowing a direct comparison with the values gained in our study. The values were similar to those found for butane activation, indicating an analogous activation mechanism.

The reported ε_C and ε_H values for the aerobic degradation of propane by microorganisms in marine sediment from a hydrocarbon seep (Kinnaman *et al*., 2007), result in a Λ_{bulk} factor of 3.1, which is significantly lower than our Λ_{bulk} values for the anaerobic activation of propane (10.0–11.9). The resulting Λ_{rp} value of 2.7, assuming a primary activation of propane via insertion of molecular oxygen by a monooxygenase (Shennan *et al.*, 2006), is also much lower than the Λ_{rp} values for the anaerobic activation of propane by fumarate addition (10.5–11.8). Hence, different

activation mechanisms for propane under aerobic and anaerobic conditions result in significantly different Λ_{rp} values.

Experiments with deuterated substrates − can propane be activated at the primary carbon atoms?

In order to further investigate the possibility for a second route of activation for propane at the primary carbon atom, we incubated strain BuS5 with mixes of equal amounts of natural propane and propane, which was deuterated either at the primary carbon atoms (propane-D_6) or at the secondary carbon atom (propane-D_2) as substrates for growth. For reference, strain BuS5 was also grown with mixes of equal amounts of natural *n*-butane and either *n*-butane which was deuterated at the primary carbon atoms (butane-D_6) or at the secondary carbon atoms (butane-D_4). During growth with butane-D_6 and natural butane, strain BuS5 degraded natural butane as fast as butane-D_6 (see supplementary information, Fig.2). Thus, the hydrogen isotope ratios (R_t) of butane-D_6 to natural butane stayed constant during the course of degradation and no significant D/H fractionation (enrichment factors of –25.2 ‰ ± 22.6) was observed. In contrast, when strain BuS5 was grown with butane-D_4 and natural butane, cells degraded natural butane faster, leading to a D/H enrichment factor of –331.9 ‰ ± 46.7 (Fig.3). These results strongly indicate that butane was solely activated at the second carbon atom, as suggested before by metabolite analysis (Kniemeyer *et al*., 2007).

Notably, during growth with propane-D_6 and natural propane, strain BuS5 degraded natural propane faster than propane-D_6 (see supplementary information, Fig.2). The resulting D/H fractionation resulted in an enrichment factor of –167.6‰ ± 46.6, which is significantly higher than the one observed for degradation of butane with deuterium label at the primary carbon atom. Still, the enrichment factor obtained from the degradation of propane-D_2 and natural propane was higher (–436.7 ‰ ± 81.4) than that for primary carbon atom deuterated propane and natural propane. These results (illustrated in Fig.3) indicate that in contrast to the activation of butane, which seems to take place solely at the secondary carbon atom, a second route for activation of propane at the primary carbon atom took place. The fact that the enrichment factor during degradation of a mix of butane-D_4 and natural butane is lower than that

observed for the mix of propane-D_2 and natural propane can be due to masking effects caused by a lower solubility of butane compared to that of propane. The enrichment factors obtained using deuterated alkanes were significantly higher than the enrichment factors obtained with non-labeled natural propane and butane (ε_H reactive position, Table 1 in the supplementary information), precluding a direct comparison of the results gained by the different experiments. The fact that deuterated substrates generate more pronounced isotope effects than their natural abundance counterparts has been observed before (Morasch *et al*., 2001) and this might be due to stronger secondary isotope effects occurring during reactions with multideuterated compounds.

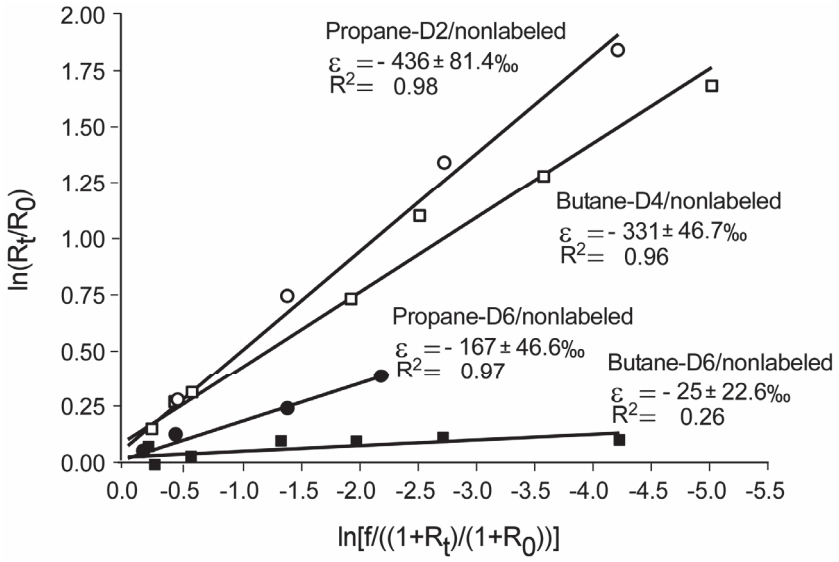

Fig.3 D/H isotope fractionation during the degradation of heavy or light isotopologues of propane and butane by strain BuS5: degradation of equal mixtures of propane and propane-D_2 (○), propane and propane-D_6 (●), butane and butane-D_4 (□), and butane and butane-D_6 (■).

The additional activation of propane at the primary carbon atom might explain the above discussed higher Λ_{rp} values for the anaerobic degradation of propane, compared to *n*-butane, during non substrate limiting conditions. Future experiments, using enzyme assays with crude extracts and quantification of metabolites may reveal the true frequency of this so far unprecedented activation reaction. These may then be used to re-calculate the AKIE values for carbon and hydrogen for the anaerobic degradation of propane.

Environmental implications

To our knowledge, this study reports for the first time that substrate limitations can lead to significantly decreased two dimensional isotope fractionation factors (Λ values). This observation contradicts the assumption that masking effects will always equally affect two (or more) elements involved in the biochemical cleavage or formation of a bond. This effect should be further investigated in future studies since it may have an influence on interpretations of 2D-CSIA data from field sites, where limitations in substrate bioavailability can be assumed to occur. Still, we were able to show that using 2D-CSIA it is possible to distinguish between aerobic (insertion of oxygen by a monooxygenase) and anaerobic (fumarate addition by (1-methyl-) alkylsuccinate synthase) activation of propane, based on the as yet reported carbon and hydrogen stable isotope fractionation (Kinnaman *et al*., 2007; this study). The enrichment factors for carbon and hydrogen obtained for the aggregate forming and thus substrate-limited propane degrading enrichment culture (Propane12-GMe) are similar to what has been reported for anaerobic propane degradation at marine deep sea mud volcanoes under sulfate-reducing conditions (Mastalerz *et al*., 2009). Considering the rather low *in situ* concentration of propane and butane at natural hydrocarbons seep sites (Claypool and Kvenvolden, 1983) and the reduced diffusion of gases due to the particles (Millington, 1959; Kristensen *et al.*, 2010) present in marine sediments, it seems likely that microorganisms degrading propane and *n*-butane at natural hydrocarbon seeps can be actually limited in substrate. Thus, masking effects based on low substrate bioavailability can be expected and should be considered when investigating stable isotope fractionation associated with propane and *n*-butane degradation *in situ*. When comparing the obtained Λ_{bulk} for the anaerobic degradation of propane by the aggregate forming enrichment culture Propane12-GMe and strain BuS5 (this study) during shaking incubations to the Λ_{bulk} resulting from the reported enrichment factors for carbon and hydrogen by Mastalerz *et al*. (2009), the values are nearly identical. This clearly shows that the correlation of measured carbon and hydrogen fractionation can be useful in identifying a specific reaction mechanism *in situ*, if the extend of substrate limitations are taken into account. Our obtained Λ_{rp} values for *n*-butane degradation during non-substrate limiting conditions are furthermore in the same range as those for *n*-hexane and *n*-octane degradation under

sulfate-reducing conditions (Morris *et al*., submitted), indicating that Λ_{rp}-values are good indicators for detecting the degradation of several alkanes by the fumarate addition pathway in the environment.

Acknowledgements

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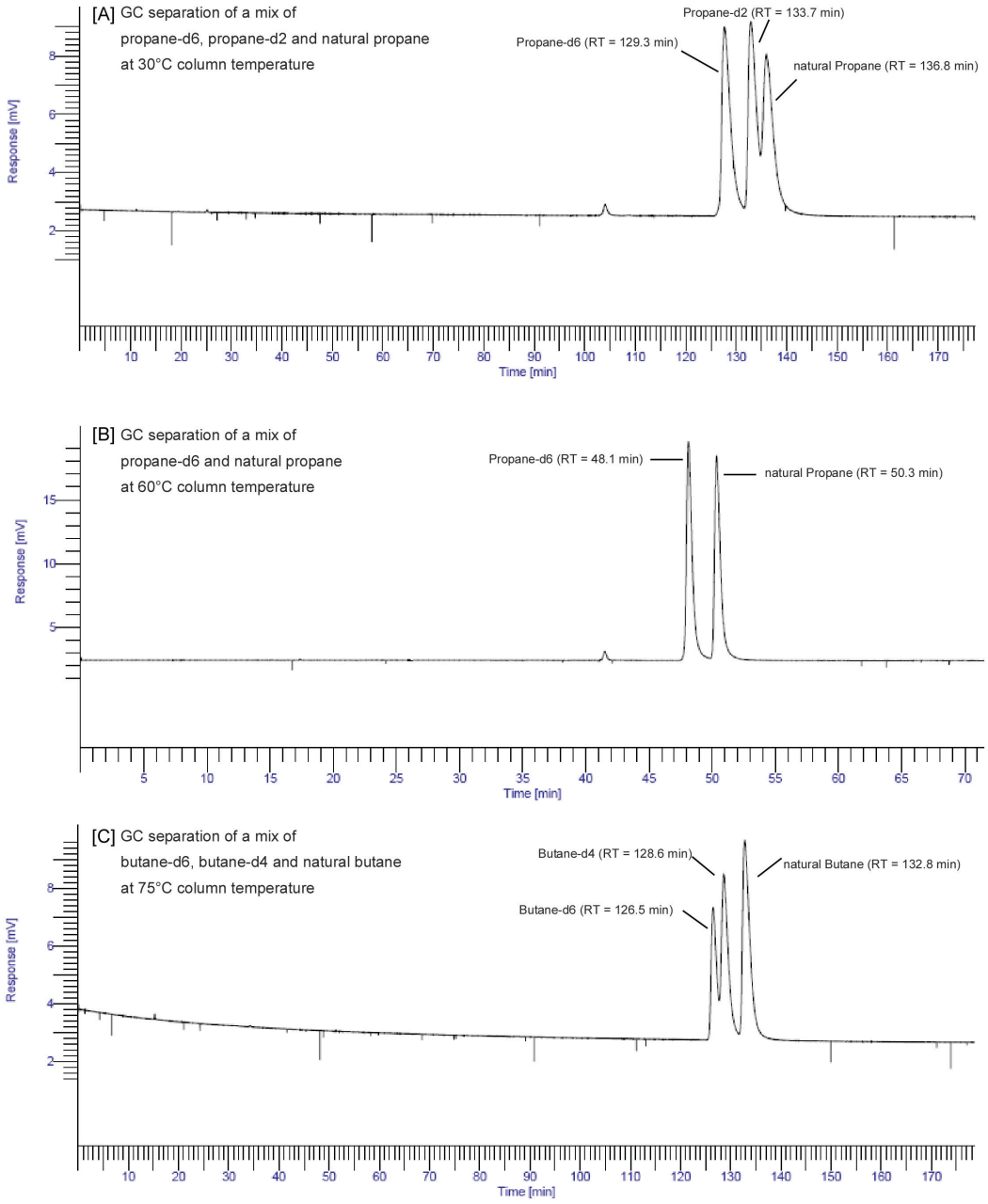
Table 1. Bulk ($_{bulk}$) and reactive position ($_{rp}$) specific enrichment factors for stable carbon (C) and hydrogen (H) fractionation, bulk and reactive positions specific Lamda values, apparent isotope effects (AKIE) and AKIE H / AKIE C for the anaerobic degradtion of propane and butane by marine sulfate-reducing enrichment cultures and strain BuS5. Numbers of total C and H atoms (n), those in reactive positions (x) and at intramolecular competing sites (z) for propane (a) and butane (b) are indicated below the table.

Culture	Substrate	Incubation conditions	Enrichment factor C_{bulk} (‰)	Enrichment factor H_{bulk} (‰)	Enrichment factor C_{rp} (‰)	Enrichment factor H_{rp} (‰)	Lambda$_{bulk}$	Lambda$_{rp}$	AKIE C	AKIE H	AKIE H /AKIE C
Strain BuS5	Propane[a]	static	-2.64 ± 0.56	-16.36 ± 4.13	-7.85 ± 1.66	-60.68 ± 15.99	6.30 ± 0.94	7.72 ± 1.20	1.0079 ± 0.0017	1.1381 ± 0.0414	1.1291 ± 0.0429
Strain BuS5	Propane[a]	shaking	-8.67 ± 1.07	-91.72 ± 6.07	-25.32 ± 3.25	-266.61 ± 31.17	11.90 ± 0.22	10.51 ± 0.22	1.0260 ± 0.0034	2.1432 ± 0.2861	2.0881 ± 0.2858
Propane 12-GMe*	Propane[a]	static	-3.11 ± 0.35	-23.84 ± 3.64	-9.29 ± 1.05	-89.21 ± 13.72	7.90 ± 0.57	9.65 ± 0.70	1.0094 ± 0.0011	1.2172 ± 0.0407	1.2058 ± 0.0415
Propane12-GMe*	Propane[a]	shaking	-3.71 ± 0.54	-36.00 ± 4.48	-11.04 ± 1.63	-130.15 ± 17.94	10.00 ± 0.40	11.76 ± 0.41	1.0112 ± 0.0017	1.3519 ± 0.0656	1.3369 ± 0.0670
Strain BuS5	Butane[b]	static	-1.78 ± 0.32	-8.89 ± 2.27	-3.54 ± 0.63	-21.36 ± 5.48	4.90 ± 1.20	5.88 ± 1.45	1.0071 ± 0.0013	1.0934 ± 0.0260	1.0857 ± 0.0274.
Strain BuS5	Butane[b]	shaking	-4.99 ± 0.70	-31.85 ± 10.18	-9.90 ± 1.39	-74.37 ± 24.15	6.88 ± 1.23	7.78 ± 1.42	1.0202 ± 0.0029	1.4234 ± 0.1957	1.3952 ± 0.1958
Butane12-GMe	Butane[b]	static	-1.99 ± 0.53	-11.48 ± 3.78	-3.96 ± 1.06	-28.15 ± 9.35	5.90 ± 0.90	7.19 ± 1.05	1.0080 ± 0.0022	1.1269 ± 0.0475	1.1179 ± 0.0415
Butane12-GMe	Butane[b]	shaking	-5.56 ± 0.54	-46.55 ± 4.72	-11.06 ± 1.07	-109.53 ± 10.64	8.70 ± 0.40	9.90 ± 0.22	1.0226 ± 0.0022	1.7797 ± 0.1348	1.7403 ± 0.0670
Butane12-HyR*	Butane[b]	static	-0.77 ± 0.34	-4.81 ± 2.11	-1.54 ± 0.68	-11.96 ± 5.23	5.30 ± 2.40	6.55 ± 2.91	1.0031 ± 0.0014	1.0502 ± 0.0231	1.0470 ± 0.0244
Butane12-HyR*	Butane[b]	shaking	-1.49 ± 0.37	-11.64 ± 2.47	-2.98 ± 0.74	-28.61 ± 0.74	7.70 ± 0.80	9.42 ± 0.99	1.0060 ± 0.0015	1.1292 ± 0.0313	1.1224 ± 0.0327

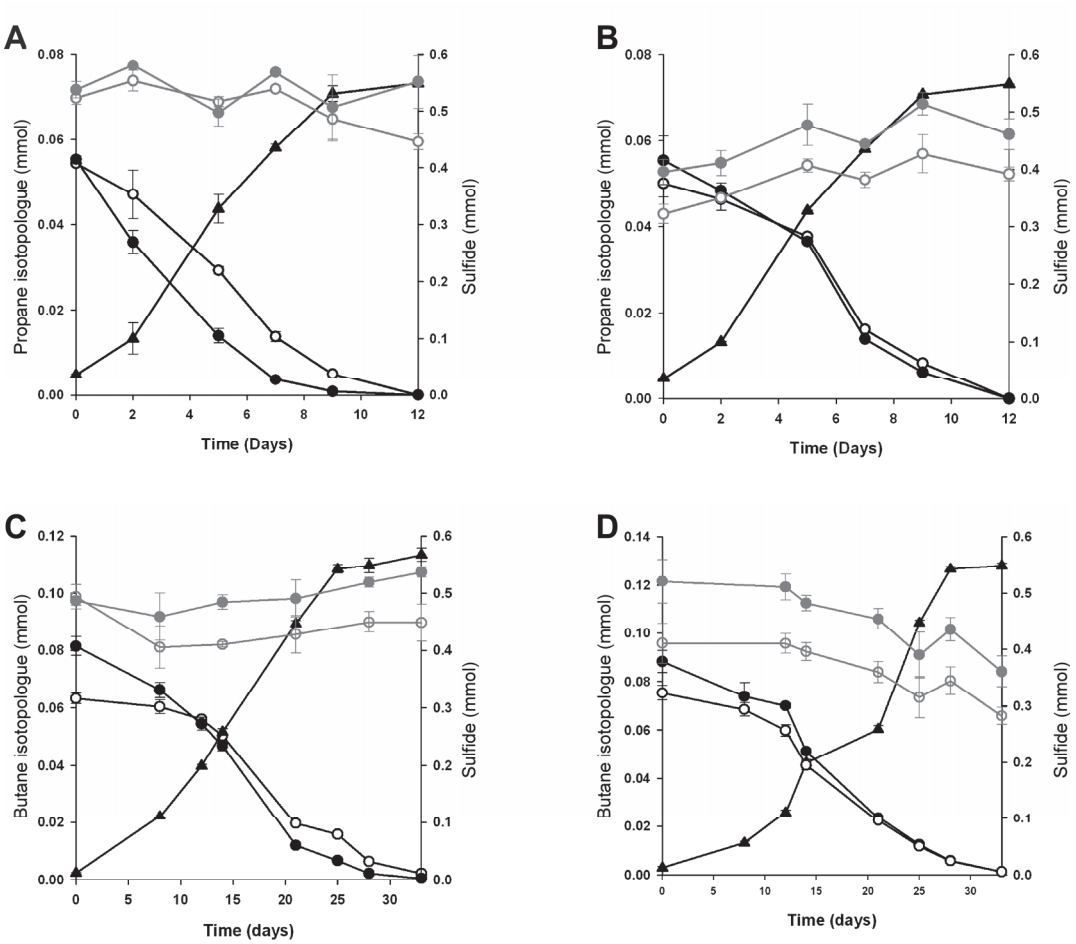
a. For carbon : $n=3$ $x=1$ $z=1$ For hydrogen: $n=8$ $x=2$ $z=2$

b. For carbon : $n=4$ $x=2$ $z=2$ For hydrogen: $n=10$ $x=4$ $z=4$

*. Cultures form dense macroscopic aggregates



Supplementary Fig.1 Gas chromatograms for the separation of different propane and butane isotopologues in mixes with natural propane or butane. Separation and quantification was done using two connected (SGE SilTite^{TM} Mini-Union kit) 50 m long CP-PoraBOND Q Fused Silica columns. (A) Separation of propane-D_6, propane-D_2 and natural propane was achieved at 30 °C column temperature. (B) Separation of propane-D_6 and natural propane was at 60 °C and (C) separation of butane-D_6, butane-D_4 and natural butane was at 75 °C column temperature.



Supplementary Fig.2 Substrate conversion during growth of strain BuS5 with (A) a mix of propane-D_2 (○) and nonlabeled propane (●), (B) propane-D_6 (○) and nonlabeled propane (●), (C) butane-D_4 (○) and nonlabeled butane (●) and (D) butane-D_6 (○) and nonlabeled butane (●). No loss of D-labeled (○) and nonlabeled substrates (●) was observed in abiotic controls. The production of sulfide (▲) coupled to substrate turnover is shown for each substrate mix.

3

Ethane-degrading sulfate-reducing bacteria from marine hydrocarbon seeps

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Abstract

Ethane is a major constituent of natural thermogenic gas and can be present at natural hydrocarbon seeps at concentrations of up to 30% of the total hydrocarbon gas. Whereas there is evidence that propane and *n*-butane are being biodegradation in deep thermogenic gas reservoirs and in anoxic sediments at natural hydrocarbon seeps, ethane appears to be rather recalcitrant. Degradation of ethane under anaerobic conditions was shown so far only with one enrichment culture obtained from sediments around cold hydrocarbon seeps in the Gulf of Mexico, albeit the degradation proceeded at an extremely slow rate (Kniemeyer *et al*., 2007). To date, nothing is known about the involved activation mechanism or the microorganisms capable to degrade ethane under anoxic conditions. Here, we report the phylogenetic composition of the so far only ethane-degrading, sulfate-reducing enrichment culture. Hybridization with kingdom and group-specific probes revealed that the enrichment culture contains ~ 10% archaea and ~ 90% of bacteria. Generation of a 16S rRNA gene clone library resulted in a majority of sequences affiliated with the *Desulfosarcina*/*Desulfococcus*-group within the *Deltaproteobacteria*. Hybridization of cells with an oligonucleotide probe targeting members of this cluster, revealed that ~ 60% of all cells belong the *Desulfosarcina*/*Desulfococcus*-group.

Introduction

The *n*-alkanes ethane, propane and *n*-butane are constituents of thermogenic natural gas. Although methane is always the dominant component, the short-chain, non-methane alkanes can collectively account for up to 40% of the total gas in thermogenic gas reservoirs (Claypool and Kvenvolden, 1983). Evidence from isotope studies in oil and gas reservoirs have shown that these alkanes are subject to biodegradation with a preference for propane > *n*-butane > *n*-pentane > *iso*-pentane > *iso*-butane > ethane (Boreham *et al.*, 2001, Larter *et al*., 2005), suggesting that ethane is rather recalcitrant towards anaerobic biodegradation. When fractures in the cap rocks of the oil or gas reservoir facilitate the upwards migration of geothermal fluids through deep sediments, short-chain alkanes may enter the biosphere through natural hydrocarbon seep sites, such as hydrothermal vents and cold seeps, or they may become entrapped in structure II gas hydrates if the right pressure and temperature conditions are met (Sloan, 2003). At such seepage sites, these alkanes can occur at concentrations as high as 30% ethane, 13% propane, 3.7% *n*-butane and 5.8% *iso*-butane (Sassen *et al*., 2004). Biogeochemical studies at hydrocarbon seepage sites have indicated that non-methane alkanes are most likely biodegraded by sulfate-reducing microorganisms in the anoxic sediment (Simoneit *et al*, 1992; Joye *et al*., 2004; Orcutt *et al*., 2004; Niemann *et al.*, 2006; Orcutt *et al.*, 2010). Some studies have in fact shown that propane and *n*-butane are being biodegraded by sulfate-reducing microorganisms in sediments obtained from hydrocarbon seep sites (Mastalerz *et al*., 2009; Quistad and Valentine, 2011).

The anaerobic degradation of propane and butane was recently reported with a sulfate-reducing pure culture (strain BuS5) and with several enrichment cultures from marine hydrocarbons seeps (Kniemeyer *et al.*, 2007; Jaekel *et al*., in prep) and also from a terrestrial hydrocarbon-contaminated site (Savage *et al.,* 2010). Strain BuS5 and the dominant microorganisms in the marine, cold-adapted enrichment cultures were shown to belong to the *Desulfosarcina*/*Desulfococcus*-cluster within the *Deltaproteobacteria* (Kniemeyer *et al.*, 2007; Jaekel *et al.*, in prep). A thermophilic enrichment culture degrading propane has been reported as well and was shown to be dominated by a *Desulfotomaculum* species (Kniemeyer *et al*., 2007). The microorganisms in the cold-adapted enrichment cultures and strain BuS5 were able to

degrade propane and butane but did not degrade ethane or longer alkanes, suggesting a rather specialized substrate spectrum (Kniemeyer *et al*., 2007; Jaekel *et al.*, in prep).

Interestingly, aside from an activation of *n*-butane and propane via addition to fumarate at the secondary carbon atom, an alternative route of activation was suggested for propane at the primary carbon atom, based on metabolite analysis (Kniemeyer *et al.*, 2007, Savage *et al.* 2010). Using deuterium-labeled propane for stable isotope fractionation studies, this unprecedented route of activation was shown to significantly take place (Jaekel *et al.*, in prep). Such activation of an alkane molecule at the primary carbon atom would, in theory, also represent a possible activation mechanism for ethane, a molecule containing exclusively primary carbon atoms. Still, an activation of an alkane by addition to fumarate at a primary carbon atom has to overcome a higher C-H-bond dissociation energy (411 kJ mol^{-1} for ethane, 410 kJ mol^{-1} for propane) than if it were to take place at a secondary carbon atom (398 kJ mol^{-1} for propane) (McMillen and Golden, 1982). Thus, an activation of ethane by addition to fumarate can be expected to proceed at a slow rate. The complete degradation of ethane to CO_2 under sulfate-reducing conditions (Eq. 1) yields furthermore less energy per mol sulfate reduced than during degradation of propane during sulfate-reducing conditions ($\Delta G^{0'} = -41$ kJ per mol SO_4^{2-}). These constraints could be a possible explanation for the observed low degree of biodegradation for ethane *in situ*.

$$4\ C_2H_6 + 7\ SO_4^{2-} + 6\ H^+ \rightarrow 8\ HCO_3^- + 7\ H_2S + 4\ H_2O \qquad [1]$$

$\Delta G^{0'} = -64$ kJ mol ethane^{-1} or –36.5 kJ per mol sulfate

This study was undertaken to enrich ethane-degrading anaerobic bacteria from marine cold hydrocarbon seeps and to identify their phylogentic affiliation using 16S rRNA gene-based methods.

Materials and methods

Enrichment and cultivation

Enrichment of ethane-degrading, sulfate-reducing bacteria was attempted with sediment collected around marine hydrocarbon seeps in the Gulf of Mexico. The sediment samples from the Gulf of Mexico were collected using the RV Seward Johnson I and the Johnson Sea Link submersible in July 2001 from the brine-influenced site GC233 (650 m water depth, 27° 44' N, 92° 17' W; Joye *et al*., 2004). The cultures were set up in 20 ml Hungate tubes, sealed with butyl rubber stoppers, containing 10 ml of bicarbonate-buffered artificial sea-water medium (Widdel and Bak, 1992; Widdel, 2010), under a headspace of N_2:CO_2 (9:1, v/v). Ethane (Air Liquide, Düsseldorf-Germany) was added as 50% v/v of the headspace. The bottles were inoculated with 2 ml sediment, and incubated at 12 °C. Control incubations without the addition of ethane were set up in the same conditions.

Phylogenetic characterization by 16S rRNA analysis

DNA was extracted according to Zhou *et al.* (1996) and the 16S rRNA genes were amplified by PCR using primers 8f (Hicks *et al*., 1992) and 1496r (Kane *et al*., 1993), which are targeting nearly all bacteria. The PCR products were purified using the QIAquick Gel Extraction kit (Qiagen, Hilden-Germany), cloned into the pCR4 vector (TOPO−TA cloning kit; Invitrogen, Groningen-Netherlands) and transformed into *E. coli* Top 10 competent cells (Invitrogen). Positive clones were sequenced using the ABI Prism BigDye Terminator v 3.0 cycle sequencing kit and an ABI Prism 3100 Genetic Analyzer (Applied Biosystems; Darmstadt-Germany). For a first screening of the phylogenetic affiliation of the clone library sequences, clones were sequenced with an internal primer for the 16S rRNA gene (517f in Muyzer *et al.*, 1993). The obtained partial 16S rRNA gene sequences were quality-checcked with the DNA Baser software (www.dnabaser.com) and analyzed using the BLAST(N) algorithm (Altschuld *et al*., 1990) and the NCBI GenBank database (http://www.ncbi.nml.nih.gov/blast/Blast.cgi). Clones containing 16S rRNA gene sequences with an affiliation to the *Deltaproteobacteria* were further sequenced using

the adjacent vector primers M13F and M13R (Invitrogen®) to obtain the full-length sequences which were assembled using the DNA Baser software. The obtained nearly full-length 16S rRNA gene sequences (> 1300 bp) were aligned to those of the Silva database (Pruesse *et al*., 2007). Phylogenetic trees were constructed in ARB (Ludwig *et al.*, 2004) by maximum likelihood, using different sets of filters.

Fluorescence *in situ* hybridization

For fluorescence *in situ* hybridization (FISH), cells in 1 ml of a 10× concentrated enrichment culture were fixed with 0.5 ml 2% paraformaldehyde in 1× phosphate-buffered saline (PBS; 10 mM sodium phosphate pH 7.2, 130 mM NaCl) for 1 hour at room temperature, washed with 1×PBS, and stored in 1× PBS:ethanol (1:1) at –20 °C. Aliquots of 100 µl fixed cells were filtered onto 0.2 µm pore GTTP polycarbonate filters (Millipore; Eschborn, Germany). Cells on filters were hybridized with HRP-labeled 16S rRNA-targeted oligonucleotide probes and the signal was amplified as described elsewhere (Pernthaler *et al*., 2002) using Alexa 488® tyramides (Invitrogen). The permeabilization step of the protocol before probe hybridization was modified, such that that the cells on the filters were at first permeabilized with Proteinase K (0.005 U µl $^{-1}$, Macherey-Nagel; Düren-Germany) in solution (0.05 M EDTA, 0.1 M Tris-HCl, at pH 8) for 30 minutes at 37 °C. After that, the filters were washed in 50 ml 1 × PBS at room temperature, followed by a second permeabilization treatment with Lysozyme (10^6 U ml^{-1}, Fluka; Steinheim-Germany) in solution (0.05 M EDTA, 0.1 M Tris-HCl, at pH 8) for 30 minutes at 37 °C. After signal amplification, all cells were counterstained with DAPI and microscopically counted as previously described (Snaidr *et al*., 1997). The hybridized cells were analyzed with a Zeiss Axioskop 2 mot plus fluorescence microscope (Zeiss, Oberkochen-Germany), using a HC F36–525 filter (AHF; Tübingen-Germany) for probe signal recording and a F81-360 filter (AHF; Tübingen-Germany) for DAPI signal recording. Recorded probe signals were attributed with orange color and DAPI signals with blue color during image processing, using the Zeiss Axio Vision 4.0 software program release 4.6.3 (Carl Zeiss Imaging Solutions; Hallbergmoos-Germany). The oligonucleotide probes used in this study, Ethane12-GMe_1017 (TTAGATGTGCCCTTCGGG; this study), DSS658 (Manz *et al.*, 1998), EUB338 (Amann *et al.*, 1990), ARCH915 (Stahl

and Amann, 1991) and NON338 (Amann *et al.*, 1990) were purchased from Biomers GmbH (Ulm-Germany). The sequence-specific probe designed in this study (in ARB, after Hugenholtz *et al*., 2001) was evaluated for specificity in hybridization assays with increasing formamide (FA) concentrations (0−60%, with 10% increment). 20% FA concentration in the hybridization buffer was determined to be the highest FA concentration, where strong fluorescent probe signals were still observed. No cultured strains were available as one or two mismatch controls.

Results and Discussion

Enrichment and cultivation

Enrichment of ethane-degrading sulfate-reducing bacteria from cold marine hydrocarbon seeps was attempted with sediments from the Gulf of Mexico. Incubations were started at 12 °C, which is close to the rather low *in situ* temperatures (< 10 °C). The observed ethane-dependent sulfate-reduction was very slow and the enrichment culture had produced ~ 15 mM sulfide after the first ~ 400 days of incubation (Fig.1). During subsequent transfers (20% of previous culture), the observed sulfide production by the enrichment culture remained slow (data not shown).

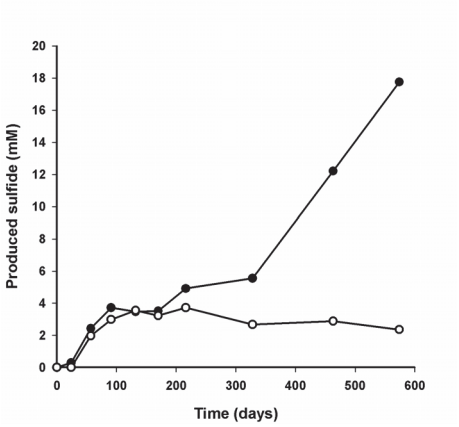

Fig.1 Ethane-dependent sulfide production (●) in incubations with sediment from around hydrocarbon cold seeps in the Gulf of Mexico. Sulfide production in similar incubations without added ethane ceased after approximately 150 days of incubation (○). The experiments were conducted in 20 ml culture tubes filled with 10 ml anoxic artificial seawater and 2 ml sediment. The tubes were incubated at 12 °C. Modified from Kniemeyer *et al.* (2007)

Microscopic investigations of samples taken from the enrichment culture (Fig.2) showed a relatively high abundance of small, rod-shaped cells, which were on average 1.4 µm in length and 0.6 µm in width. Since this enrichment culture has been maintained over a long period, during which it showed a very slow, yet clearly ethane-dependent production of sulfide, it can be assumed that such an abundant morphotype likely represents the ethane-degrading species.

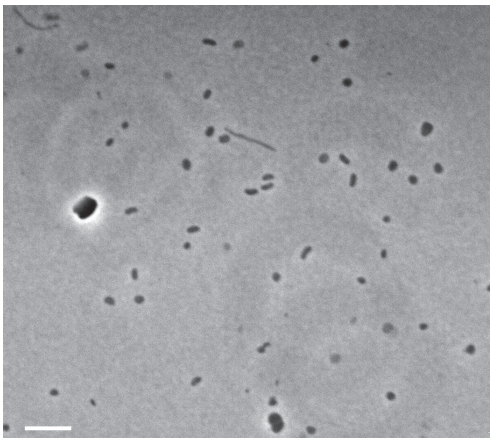

Fig.2 Phase contrast picture of viable cells in the enrichment culture Ethane12-GMe. Short rod-like cells (approximately 1 µm length and 0.5 µm width) appeared as the dominant morphotype. Scale bar represents 5 µm.

Phylogenetic analysis using kingdom specific oligonucleotide probes

In order to obtain first indications about whether the abundant morphotype belongs to the *Archaea* or *Bacteria*, fixed cells from the enrichment culture were hybridzed with probes targeting members of either kingdom (Fig.3 A and B). It should be noted that signals with the *Bacteria*-specific probe were only obtained for the abundant, small, rod-shaped morphotype if the cells were permeabilized with a combined treatment of proteinase K followed by lysozyme. Whether this could be due to a somewhat different cell wall or an ″encapsulation″ into a proteinaceous matrix of the cells, remains speculative. Furthermore, these cells exhibited a much lower signal intensity than some of the other cells in this enrichment culture (see Fig.3 B and C), indicating either a low ribosome content per cell or a still less efficient permeabilization of their cell walls. The enrichment culture was dominated by up to 90% bacterial cells and ~ 10% archeal cells (Fig.4). The presence of such relatively large amounts of archaea

in the enrichment culture suggests that these may be directly feeding on metabolic end-, or by-products, of the ethane-degrading cells. What exactly the metabolic capabilities and possible role in the overall carbon-cycling of these archaea in the enrichment culture is awaits further physiologic studies.

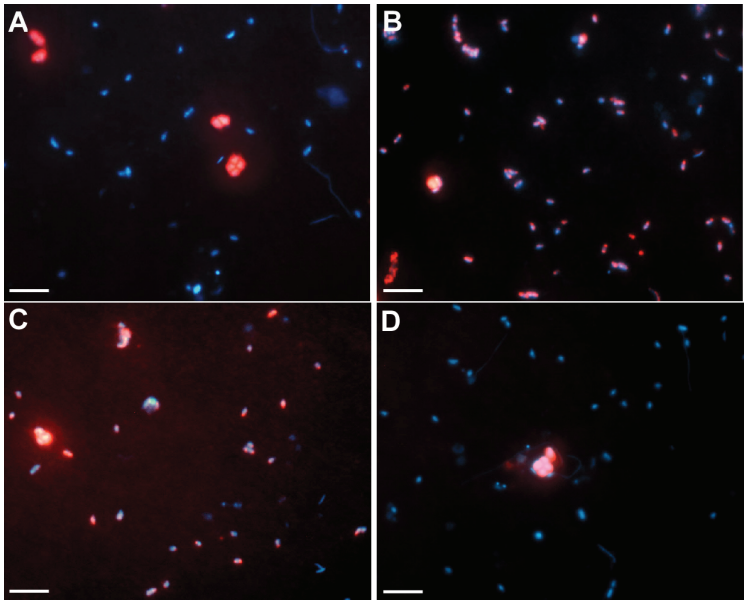

Fig.3 Whole cell hybridization with probes targeting *Archaea* (A), *Bacteria* (B), members of the *Desfulfosarcina*/*Desulfococcus*-cluster within the *Deltaproteobacteria* (C) and the phyloytpe represented by the Ethane12-GMe clones 5, 11, and 13 (D). Hybridization was followed by CARD-signal amplification (red) and DAPI staining (blue) of fixed cells in enrichment culture Ethane12-GMe. Scale bars represent 5 µm.

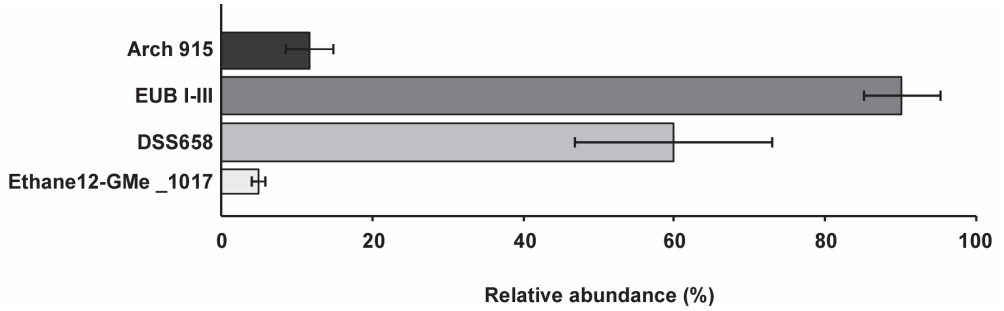

Fig.4 Relative abundance of cells hybridized with oligonucleotide probes targeting *Archaea* (Arch915), *Bacteria* (EUBI−III), members of the *Desulfosarcina*/*Desulfococcus*-cluster (DSS658) and Ethane12-GMe clones 5,11 and 13 (Ethane12-GMe_1017).

Survey of bacterial diversity using 16S rRNA clone libraries

In order to determine the phylogenetic affiliation of the dominant morphotype, a 16S rRNA bacterial clone library was constructed. A first screening of partial sequences from the 16S rRNA clone library revealed that 45% of all obtained sequences affiliated with the *Deltaproteobacteria*. These clone sequences were of particular interest and therefore fully sequenced, since many of the so far known hydrocarbon degrading, sulfate-reducing bacteria affiliate with this phylogenetic group, including propane- and *n*-butane-degrading bacteria (Kniemeyer *et al.*, 2007; Jaekel *et al*., in prep).

Based on their phylogenetic affiliation (Fig.5), Ethane12-GMe clones 5, 13, 11 and 18 were closest related to clone sequences within the *Desulfosarcina*/*Desulfococcus*-cluster, which were obtained from mainly marine hydrocarbon seep sites, such as in the Gulf of Mexico (Llyod *et al*., 2006), the Eel river basin (Orphan *et al.*, 2001), gas hydrates (Knittel *et al*., 2003) and mud volcanoes (Lösekann *et al*., 2007). Another phyloytpe present in the clone library (Ethane12-GMe clone 29) was closest related to *Desulfofrigus fragile* (98% sequence similarity), a psychrophilic strain from arctic sediments, which utilizes fermentation products (Knoblauch *et al.*, 1999). Ethane12-GMe clones 6 and 25 were only distantly related (< 84% sequence similarity) to strains belonging to the family *Desulfobacteriaceae*, which are known to degrade aromatic hydrocarbon compounds, such as strain NaphS2 (Galushko *et al.,* 1999) or strain mXyS1 (Harms *et al*., 1999). Ethane12-GMe clone 83 was closest related to *Desulfobacterium autotrophicum*, which is growing lithotrophically on CO_2 and H_2 (Brysch *et al*, 1987). Amplification of the 16S rRNA gene using 10^{-3} diluted gDNA revealed a phylotype closely related to Ethane12-GMe clones 5, 11, 13 (> 97.8% sequence similarity) and more distantly also to Ethane12-GMe clone 18 (96.9 % sequence similarity), suggesting that these phylotypes could represent the abundant rod-shaped cells.

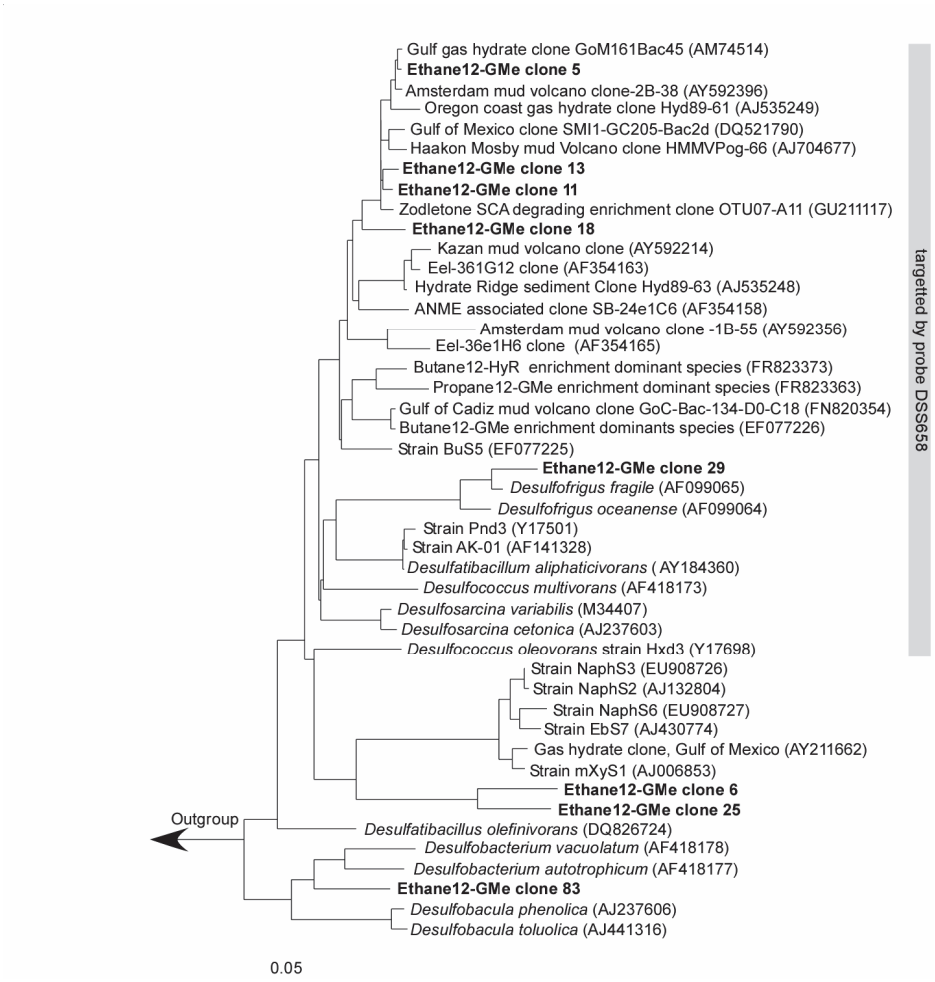

Fig.5 Phylogenetic affiliation of the *Deltaproteobacteria*-related 16S rRNA gene sequences from the ethane-degrading enrichment culture (marked in bold face). The tree was calculated in ARB by maximum likelihood and using different sets of filters. 16S rRNA gene sequences from *Escherichia coli* strains were used as outgroup. Scale bar indicates 5% sequence divergence.

Analysis of the most abundant phylotype using a nested FISH approach

In order to verify the PCR based result, fixed cells from this enrichment culture were hybridized with the probe DSS658, which targets members of *Desulfosarcina*/*Desulfococcus*-cluster (Fig.3 B). Counts of cells targeted by this probe revealed that ~ 60% of all cells in this enrichment culture belong to the *Desulfosarcina*/*Desulfococcus*-cluster (Fig.4), including the abundant, small, rod-shaped cells. Based on this finding, a phylotype-specific oligonucleotide probe was designed (Ethane12-GMe_1017), targeting the Ethane12-GMe clone sequences 5, 11 and 13. Hybridizations of cells with this probe, followed by cell counts showed that,

surprisingly, these phylotypes accounted for only ~ 5% of all cells in the enrichment culture and based on morphology also does not resemble the small rods but rather a large, roundish rod shaped morphotype (Fig.4 C). It is possible that due to a PCR bias, a phylotype other than the dominant was amplified in during the ″dilution PCR″. Since hybridization with probe DSS658 showed that the abundant, small-rod shaped morphotype belongs to the *Desulfosarcina*/*Desulfococcus*-cluster, the Ethane12-GMe12 clone sequences 18 or 29 should be investigated in more detail, since the DSS658 probe targets them as well (Fig.5). Hybridization with oligonucleotide probes targeting each one for these two phylotypes could reveal whether one of them perhaps represents the abundant phylotype and, thus, the likely ethane-degrading strain.

Conclusion

An extremely slow, yet clearly ethane-dependent sulfide production was observed in a cold-adapted enrichment culture obtained from a hydrocarbon seep in the Gulf of Mexico (Kniemeyer *et al*., 2007). Although ethane concentrations can be up to 30% of the total hydrocarbon gas at such seep sites (Sassen *et al.,* 2004), there is a lack of successful enrichments of microorganisms capable to degrade ethane under anaerobic conditions from such environments, such as the Guaymas Basin (Kniemeyer, personal communication) or Hydrate Ridge (Musat, personal communication). This suggests that a degradation of ethane without oxygen is a rather slow process but might, on long geological time scales, play a significant role for the overall carbon cycling at these hydrocarbon seep environments. It is also possible that faster sulfate-reducing, ethane-degrading microorganisms exist and are active at hydrocarbon seep sites but have so far not been cultured under the right conditions and were thus ″missed-out″ in the attempts to enrich them.

An interesting aspect of the anaerobic degradation of ethane is the involved activation mechanism. The possible underlying activation reaction by addition of ethane to the double bond of fumarate, assuming it to be at all analogous to that of longer-chain alkanes (Widdel and Grundmann, 2010), would have to involve a homolytic cleavage of a C-H bond from a primary carbon atom- a reaction shown to be taking place for propane in addition to activation at the secondary carbon atom (Kniemeyer *et al*., 2007; Jaekel *et al*., in prep). Whether or not this reaction is

employed by the ethane-degrading strain in this enrichment culture remains unknown. Future studies, making use of isotopically labeled ethane as a substrate for growth and subsequent extraction and analysis of metabolites will reveal if the fumarate-addition mechanism is involved in the activation of ethane under anaerobic conditions, or not. The high abundance of members of the *Desulfosarcina*/*Desulfococcus*-cluster, suggests that the ethane-degrading strain may be affiliated with this phylogentic group. Considering that all of the so far known mesophillic and cold-adapted, sulfate-reducing bacteria able to degrade short-chain alkanes belong to this group, it appears as if the ability for short-chain alkane degradation is common for these types of bacteria.

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4

Degradation of cyclohexane by a sulfate-reducing enrichment culture from hydrocarbon-contaminated intertidal sediments

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Abstract

Cycloalkanes are major constituents of crude oil and they are rather recalcitrant to biological degradation. Only few studies have reported the aerobic biodegradation of cycloalkanes by microorganisms and even less is known about the microorganisms capable to degrade these compounds anaerobically. We report here the enrichment of cyclohexane-degrading, sulfate-reducing bacteria from hydrocarbon-contaminated intertidal marine sediments. Quantitative growth experiments showed that cyclohexane was completely oxidized to CO_2. The obtained enrichment culture was dominated by a *Desulfosarcina/Desulfococcus*-cluster affiliated phylotype, which comprised ~ 80% of all cells. Incubations of dense cell suspensions showed degradation of C_4–C_6 *n*-alkanes and methyl-substituted and unsubstituted C_5- and C_6- cycloalkanes. During these incubations the abundance of the dominant phylotype did not change, suggesting that this strain is capable of degrading a rather diverse spectrum of hydrocarbon compounds. GC-MS analysis of culture extracts during growth on cyclohexane showed cyclohexylsuccinate as a metabolite, indicating that the activation involves a carbon-carbon addition to fumarate, analogous to the activation of *n*-alkanes and some aromatic compounds. Other metabolites detected were cyclohexylpropionic acid and cyclohexylcarboxylic acid, suggesting that cyclohexylsuccinate is further degraded through enzymatic reactions similar to those of *n*-alkanes, involving carbon-skeleton rearrangement, decarboxylation and β-oxidation.

Introduction

Cycloalkanes are major constituents of petroleum, accounting for as much as 60% of some crude oils (Tissot and Welte, 1984; Perry, 1984). Cycloalkanes are commonly refined from crude oil to be used as solvents and raw materials in the chemical industry, for example for the production of nylon (Perry, 1984). Due to their wide industrial applications, cycloalkanes are common environmental pollutants. Previous studies have shown that cycloalkanes are more recalcitrant towards biological degradation than *n*-alkanes, but are faster biodegraded than aromatic compounds (Leahy and Colwell, 1990). Environmental studies on the biodegradation dynamics in ocean surface waters after oil spills have shown that cycloalkanes are being less biodegraded than *n*-alkanes (Brown and Huffman, 1976). This is likely due to a higher toxicity of cycloalkanes than for *n*-alkanes, since these seem to disturb biological membrane functioning (Sikkema *et al*., 1994; Sikkema *et al*., 1995). Cyclohexane has the most stable structure of all cycloalkanes, due to the lowest overall strain energy on the molecule (Bruice, 2004). The recalcitrance of cyclohexane to biodegradation is also represented by the high abundance of this compound in crude oil (Atlas, 1981).

Studies investigating the biodegradability of cycloalkanes have often focused on cyclohexane as a representative cycloalkane (Stirling and Watkinson, 1977; Anderson *et al*., 1980; Trower *et al*., 1985; Rouviere and Chen, 2003). Even though the degradation of cycloalkanes by cultures of aerobic microorganisms has been studied in the past decades, there have only been a few reports about the anaerobic biodegradation of substituted and unsubstituted cycloalkanes. The degradation of cycloalkanes under aerobic conditions commences through the insertion of reactive oxygen species into the molecule by a monooxygenase (Cheng *et al*., 2002). The activation of cycloalkanes in the absence of oxygen thus requires other biochemical reactions. Some of the few studies reporting the biodegradation of cycloalkanes under sulfate-reducing (Rios-Hernandez *et al.*, 2003; Townsend *et al*., 2004), methanogenic (Townsend *et al*., 2004) or nitrate-reducing conditions (Musat *et al*., 2010), showed that activation takes place through an addition to fumarate (Rios-Hernandez *et al*., 2003, Musat *et al.*, 2010), yielding cycloalkylsuccinate derivatives. Another study showed that the nitrate-reducing strain HxN1 co-activated cyclopentane and

methylcyclopentane during growth on *n*-hexane by addition to fumarate (Wilkes *et al*., 2003).

Such activation is analogous to the more detailed studied activation reactions of *n*-alkanes and some aromatic compounds through the addition to fumarate, which are catalyzed by glycyl radical enzymes (Boll and Heider, 2010). To date, not much is known about the microorganisms degrading cycloalkanes under anaerobic conditions. The presence of bacteria closest related to sulfate-reducing *Desulfotomaculum* species in an ethylcyclopentane degrading sulfate-reducing enrichment culture (Rios-Hernandez *et al.*, 2003) and the dominance of a *Geobacter* species in a cyclohexane degrading, nitrate-reducing enrichment culture (Musat *et al*., 2010) are hitherto the only indications about which types of bacteria are capable to degrade cycloalkanes under anaerobic conditions.

In the present study we report the degradation of cyclohexane by a sulfate-reducing enrichment culture, which was obtained from intertidal sediment in a hydrocarbon-contaminated lagoon. We investigated the degradation of cyclohexane with quantitative growth experiments and analyzed metabolites via GC-MS to further investigate the activation and degradation pathway of cyclohexane by the enrichment culture. The dominant phylotype in the enrichment culture was determined using 16S rRNA clone libraries and Fluorescent *in situ* hybridization (FISH) with a phylotype-specific probe. Furthermore, the versatility of the enrichment culture with regard to other degradable hydrocarbon substrates was tested in incubation with dense cell suspensions.

Materials and Methods

Enrichment and cultivation of microorganisms

The sediment for the original enrichments was obtained from a hydrocarbon-contaminated lagoon in the Mediterranean. Enrichments were set up anaerobically in 100 ml flat glass bottles which were sealed with butyl rubber stoppers and contained 50 ml of bicarbonate-buffered artificial sea-water medium (Widdel and Bak, 1992) under a headspace of N_2:CO_2 (9:1, v/v). The bottles were inoculated with 5 ml of homogenized sediment and amended with 2.5 ml of an inert carrier phase (2,2,4,4,6,8,8-Heptamethylnonane; HMN; Aldrich, Steinheim-Germany), which contained 0.5% (v/v) cyclohexane (Merck, Damstadt-Germany). Control bottles without the addition of hydrocarbon substrate were set up in the same way. Bottles were incubated at 28 °C and lying nearly horizontally in order to increase the hydrocarbon diffusion surface; orifices were kept below the medium surface to avoid direct contact between the hydrocarbon and the stopper (Rabus and Widdel, 1995). Sediment-containing cultures were incubated without shaking, while sediment-free cultures were incubated with slow (60 rpm) horizontal shaking. Sediment-free enrichment cultures were amended with 3 ml l^{-1} trace element solution (Widdel & Bak, 1992). For subcultivation, 10% (v/v) of an active culture was used as inoculum. Quantitative growth experiments were set up in 120 round flat glass bottles, containing 90 ml medium, 10 ml inoculum and 5 ml HMN with 0.5% or 0.2% (v/v) cyclohexane.

Experiments to extract metabolites produced during the degradation of cyclohexane were performed in the same manner, but with the exception that the medium contained only 5 mM sulfate.

Substrate tests with other hydrocarbons were performed in 15 ml butyl stoppered Hungate tubes, containing 10 ml of a 15× concentrated cell suspension. All test tubes were amended with 50 µl of HMN which contained 0.5% (v/v) cyclohexane. The following substrates were tested: cyclopentane (0.2% v/v in HMN, 0.5 ml), methylcyclopentane (0.5% v/v in HMN, 0.5ml), methylcyclohexane (0.5% v/v in HMN, 0.5 ml), ethane (5 ml), propane (5 ml), *n*-butane (5 ml), *n*-pentane (1% v/v in HMN, 0.5 ml), *n*-hexane (1% v/v in HMN, 0.5 ml), benzene (0.5% v/v in HMN,

0.5 ml), toluene (0.5% v/v in HMN, 0.5 ml) and adamantan (5% v/v in HMN, 0.5ml). The gases ethane, propane and *n*-butane had a purity of 3.5 and were purchased from Air Liquide (Düsseldorf-Germany). All other chemicals, with the exception of adamantan, were purchased from Merck (Damstadt-Germany) and were of analytical grade. Adamantan was purchased from Alfa Aesar (Karlsruhe-Germany).

Analysis of metabolic products.

Sulfide concentrations were analyzed as described elsewhere (Cord-Ruwisch, 1985).

The concentration of cyclohexane in the headspace was measured with a Shimadzu GC-14B gas chromatograph, equipped with a Supel-Q PLOT column (30 m x 0.53 mm, 30 µm film thickness; Supelco-Bellefonte, USA) and a flame ionization detector. The column temperature was 140 °C and the injection and detection temperatures were 150 °C and 280 °C, respectively. The carrier phase was N_2 at a flow rate of 3 ml min^{-1}. Headspace samples (100 µl) were taken from the culture bottles anaerobically, using a gastight glass syringe and injected into the injector without split. Samples were analyzed in triplicates.

Prior to extraction of metabolites from cyclohexane degradation, the culture was inactivated at 85 °C for 30 minutes, cooled down to room temperature and acidified using HCl to a pH of 1−2. The culture medium was separated from the HMN phase in a glass funnel, previously extracted 3× with dichloromethane (Merck, Damstadt, Germany). Metabolites were extracted 3× from the acidified liquid medium with 1.5 volumes of dichloromethane and the extracts were dried over anhydrous Na_2SO_4 (AppliChem, Damstadt-Germany). The methylated metabolites were analyzed using a Trace GC-MS system (Thermo Scientific, Bremen-Germany). The gas chromatograph was equipped with a 5% phenyl polysilphenylene-siloxane capillary column (BPX-5, SGE; 50 m, 0.22 mm i.d., 0.25 µm film thickness). The column temperature was initially held at 50 °C for 1 min, then heated to 310 °C at a rate of 3 °C min^{-1} with a final hold time of 30 min. Helium was used as the carrier gas. The PTV injector temperature was programmed from 50 °C to 300 °C (10 min isothermal) at a rate of 10 °C sec^{-1}, and the injection volume was 1 µl in the splitless mode. Mass spectra were recorded from m/z 50 to 600. Identification of metabolites

(as methyl esters) was based on comparison of retention times and mass spectra with those of authentic standards.

Phylogenetic characterization by 16S rRNA analysis

Genomic DNA extracted from the enrichment culture (Zhou *et al.*, 1996) was used to amplify almost full-length 16SrRNA gene using bacteria-specific primers 8f (Hicks *et al.*, 1992) and 1496r (Kane *et al.*, 1993). The PCR products were purified using the QIAquick Gel Extraction kit (Qiagen, Hilden-Germany), cloned into the pCR4 vector (TOPO-TA cloning kit Invitrogen, Groningen-Netherlands) and transformed into *E. coli* Top 10 competent cells (Invitrogen). Positive clones were sequenced using the ABI Prism BigDye Terminator v 3.0 cycle sequencing kit and an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Darmstadt-Germany). For a first screening of the phylogenetic affiliation of the clone library sequences, clones were sequenced with an internal primer for the 16S rRNA gene (517f in Muyzer *et al*., 1993). The obtained partial 16S rRNA gene sequences were quality-checcked with the DNA Baser software (www.dnabaser.com) and analyzed using the BLAST(N) algorithm (Altschuld *et al.*, 1990) and the NCBI GenBank database (http://www.ncbi.nml.nih.gov/blast/Blast.cgi). Clones containing 16S rRNA gene sequences with an affiliation to the *Deltaproteobacteria* were further sequenced using the adjacent vector primers M13F and M13R (Invitrogen®) to obtain the full-length sequences which were assembled using the DNA Baser software. The obtained nearly full-length 16S rRNA gene sequences (> 1300 bp) were aligned to those of the Silva database (Pruesse *et al*., 2007). Phylogenetic trees were constructed in ARB (Ludwig *et al.*, 2004) by maximum likelihood, using different sets of filters.

Fluorescence *in situ* hybridization

For fluorescence *in situ* hybridization (FISH), cells in 1 ml enrichment culture were fixed with 0.5 ml 2% paraformaldehyde in 1× phosphate-buffered saline (PBS; 10 mM sodium phosphate pH 7.2, 130 mM NaCl) for 1 hour at room temperature, washed with 1× PBS, and stored in 1× PBS - ethanol (1:1) at –20 °C. Aliquots of fixed cells were filtered onto 0.2 µm pore GTTP polycarbonate filters (Millipore,

Eschborn-Germany). Cells on filters were hybridized with Horseradish peroxidase (HRP)-labeled 16S rRNA-targeted oligonucleotide probes and the signal was amplified as described elsewhere (Pernthaler *et al.*, 2002) using Alexa 488® tyramides (Invitrogen). Thereafter, all cells were additionally stained with DAPI and microscopically counted as previously described (Snaidr *et al.*, 1997). The probe-hybridized and DAPI stained cells were analyzed with a Zeiss Axioskop 2 mot plus fluorescence microscope (Zeiss; Oberkochen-Germany), using a HC F36-525 filter (AHF; Tübingen-Germany) for probe signal recording and a F81-360 filter (AHF; Tübingen, Germany) for DAPI signal recording. Recorded probe signals were attributed with orange color and DAPI signals with blue color during image processing, using the Zeiss Axio Vision 4.0 software program release 4.6.3 (Carl Zeiss Imaging Solutions; Hallbergmoos-Germany). The oligonucleotide probes used in this study, CycS28-EdB_152 (AGCAAGCCTTTCAGCATG; this study), DSS658 (Manz *et al.*, 1998), EUB338 (Amann *et al.*, 1990) and NON338 (Amann *et al.*, 1990) were purchased from Biomers GmbH (Ulm-Germany). The sequence-specific probe designed in this study (in ARB, after Hugenholtz *et al*., 2001), was evaluated for specificity in hybridization assays with increasing formamide (FA) concentrations (0−60%, with 10% increment). The highest FA concentration, where strong fluorescent probe signals were still observed was 20% FA. No cultured strains were available as one or two mismatch controls.

Results and discussion

Enrichment of microorganisms and electron balance of cyclohexane degradation

Enrichment of cyclohexane-degrading bacteria with sulfate as the terminal electron acceptor was attempted with intertidal sediment from a hydrocarbon hydrocarbon−contaminated lagoon in the Mediterranean. After approximately 5 months, the bottle with cyclohexane in the HMN carrier phase (0.5% v/v) had formed 15 mM sulfide. The bottle without additional hydrocarbon amendments had formed only 8 mM sulfide. The clear and promt cyclohexane-dependant production of sulfide suggests that the sulfate-reducing microbial community in these sediments was

adapted to the presence of hydrocarbon compounds. In subsequent, increasingly sediment–free transfers, the productions of sulfide accelerated, so that up to 15 mM sulfide was produced within roughly 2.5 months (data not shown). Microscopic analysis of the cells in the enrichment culture showed many oval, rod-shaped cells of on average 1.5 µm length and 0.8 µm width (Fig.1). Upon depletion of cyclohexane in the carrier phase or the formation of high concentrations of sulfide, cells became elongated and formed ″thread like″ structures.

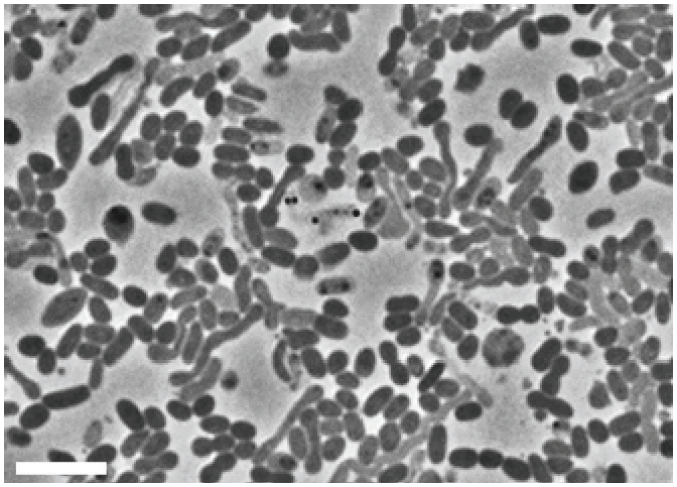

Fig.1 Phase contrast picture of viable cells in enrichment culture Cyclohexane28-EDB. Roundish rod-shaped cells (1.5 µm length, 0.8 µm width) appeared as the dominant morphotype. Scale bar = 5 µm.

Quantitative growth experiments (Fig.2) and the calculation of a net electron balance for the degradation of cyclohexane (0.24 and 0.10 mmol) (Table 1) showed that the electrons derived from cyclohexane consumption were nearly entirely used for reduction of sulfate to sulfide, indicating a complete degradation of cyclohexane to CO_2 according to the theoretical stoichiometric equation (Eq. 1).

$$2\ C_6H_{12} + 9\ SO_4^{2-} \rightarrow 12\ HCO_3^- + 9\ HS^- + 3\ H^+ \qquad [1]$$

Table 1. Electron balance of the observed degradation of cyclohexane coupled to the reduction of sulfate to sulfide.

Cyclohexane, Sulfate, Electrons(mmol)	Cyclohexane 28-EdB + 0.5% C_6H_{12} (v/v in HMN)	Cyclohexane 28-EdB + 0.2% C_6H_{12} (v/v in HMN)	Cyclohexane 28-EdB – C_6H_{12}	Abiotic control + 0.5% C_6H_{12} (v/v in HMN)
Supplied C_6H_{12}	0.24	0.10	-	0.24
Consumed C_6H_{12}	0.24[a]	0.10[a]	-	0
Electrons from C_6H_{12}[b]	8.60	3.60	-	-
Supplied SO_4^{2-}	2.80	2.80	2.80	-
Consumed SO_4^{2-}	1.03[c]	0.45[c]	0.02	-
Electrons for SO_4^{2-}[d]	8.24	3.60	-	-
Electron balance[e]	1.04	1.00	-	-

[a] The amount of C_6H_{12} consumed by enrichment culture was corrected for the loss of C_6H_{12} from the abiotic control

[b] Electrons from consumed C_6H_{12} were calculated considering complete oxidation: $C_6H_{12} + 12\ H_2O \rightarrow 6CO_2 + 36H^+ + \mathbf{36}e^-$

[c] The amount of consumed SO_4^{2-} was determined by quantification of produced H_2S (corrected for H_2S produced in C_6H_{12}-free bottles)

[d] Electrons for SO_4^{2-} reduction were calculated considering: $SO_4^{2-} + \mathbf{8}e^- + 9H^+ \rightarrow HS^- + 4H_2O$

[e] Electrons from dissimilation of C_6H_{12} divided by electrons for SO_4^{2-} reduction

No cyclohexane was consumed from the abiotic control bottle (Fig.2). In the control bottle which was not amended with cyclohexane only minor amounts of sulfate were reduced to sulfide (Table 1). This was most likely due to the consumption of a small amount of residual cyclohexane, transferred through inoculation.

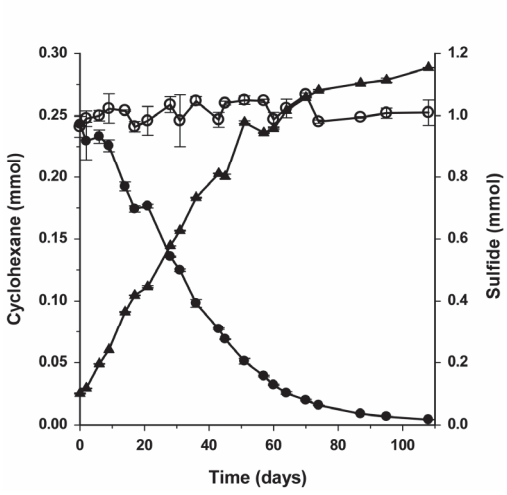

Fig.2 The consumption of cyclohexane (●) was coupled to the reduction of sulfate to sulfide (▲). No cyclohexane was lost in an abiotic control (○). Incubations were done in 120 ml round glass bottles with a culture volume of 100 ml and 5 ml of an inert carrier phase (HMN), containing 0.5% (v/v) cyclohexane. Measurements of cyclohexane and sulfide were done in triplicates.

Phylogenetic characterization of microorganisms in the enrichment culture

A first screening of the bacterial 16S rRNA gene library, using partial sequences, showed that 55% of all sequences were affiliated with the *Deltaproteobacteria*. Of these, most (43% of all sequences) were closest (94% sequence identity based on the best blast(N) hit) related to *Desulfosarcina cetonica*. Other *Deltaproteobacteria*-affiliated partial sequences were closest related to *Desulfobacterium anilini* (4% of all sequences) and *Desulfotignum balticum* (6% of all sequences). A large number of 16S rRNA genes in the clone library (41% of all sequences) were closest related to clone sequences belonging to the OP3-cluster, a group within the *Planctomycetes/Verrucomicrobia/Chlamydiae* (PVC) superphylum and from which hitherto no cultivated strains have been reported. A minor number of 16S rRNA genes (3% of all sequences) were closely related to *Cytophaga fermentans*. Since the hydrocarbon-degrading mesophillic and cold-adapted sulfate-reducing bacteria known so far affiliate with the *Deltaproteobacteria* (Teske, 2010), only the clones containing 16S rRNA gene sequences affiliated with this group were used for further full-length sequencing. Since microscopic investigations had already indicated a dominant morphotype among the cells in the enrichment culture, we were interested to find out if it belonged to one specific phylotype. Therefore, a ″dilution-PCR″, using increasingly diluted DNA template was used for amplification of the 16S rRNA gene. The sequence obtained with the highest diluted template that still yielded an amplification product (10^{-2} diluted DNA template) was identical to a clone sequence from the clone library (Cyclohexane28-EdB-Clone63; Fig.3, indicated in red).

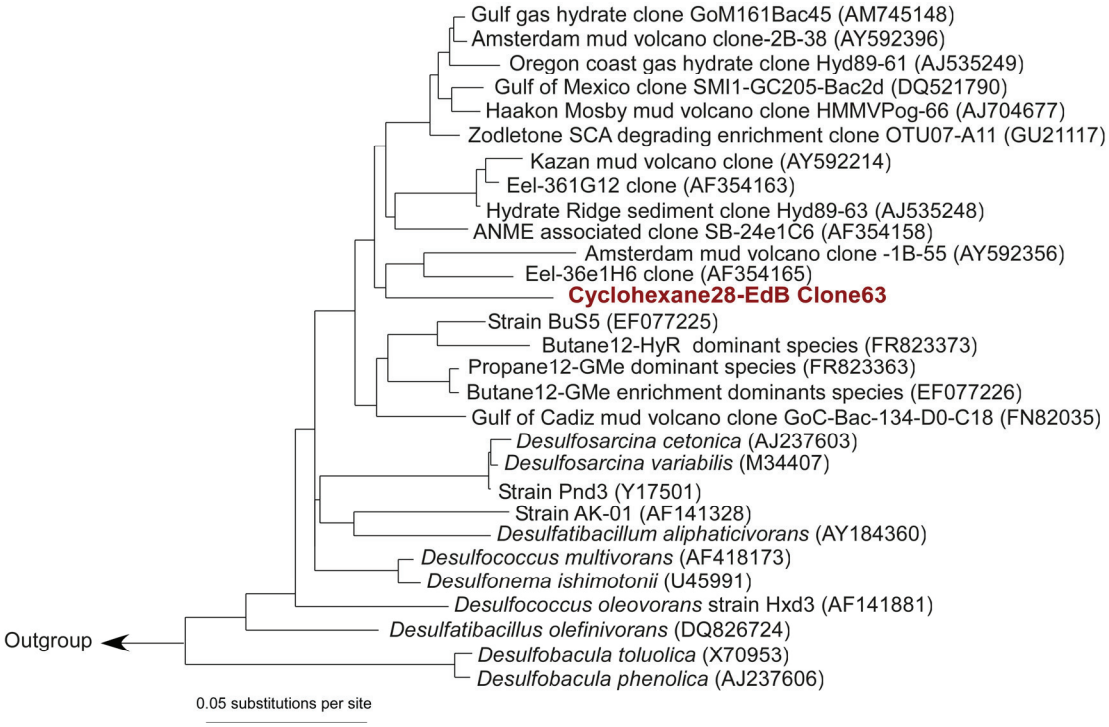

Fig.3 The phylogenetic affiliation of the most abundant phylotype in the enrichment culture Cyclohexane28-EdB (in red). The phylogenetic tree was calculated in ARB by maximum-likelihood, with application of different sets of filters, and by using only nearly full-length sequences (> 1300 nt). The scale bar indicates 5% sequence divergence.

In order to verify the results of the dilution-PCR, fixed cells were hybridized with a group specific oligonucleotide probe (DSS658) (Fig.4). Indeed, 84.3 % of all cells (determined by DAPI staining) were targeted by this probe, thus confirming that the dominant phylotype belongs to the *Desulfosarcina/Desulfococcus*-cluster within the *Deltaproteobacteria*. Hybridization with a specifically designed oligonucleotide probe (CycS28-EdB_152) showed that Cyclohexane28-EdB clone 63 constitutes 80.2% of all cells in this enrichment culture and thus is most probably responsible for the degradation of cyclohexane.

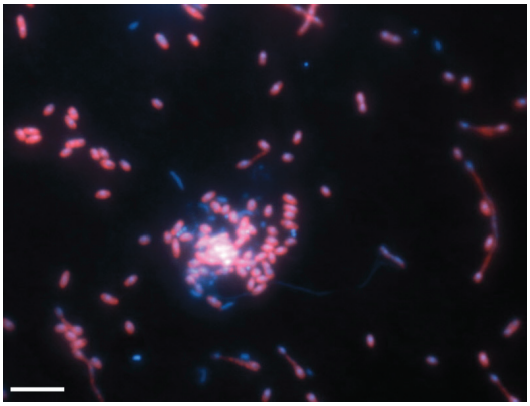

Fig.4 Whole cell hybridization (CARD-FISH) with the sequence specific probe CycS28-EdB_152 (red) and DAPI staining (blue) of fixed cells in the enrichment culture Cyclohexane28-EdB. Scale bar represents 5 µm.

Cyclohexane28-EdB clone 63 is closest related to a 16S rRNA gene clone sequence obtained from a sulfate-reducing enrichment culture originating from Hydrate Ridge, which anaerobically oxidizes methane (AOM) (clone LARHR_58-01 B01 in Schreiber *et al*., 2010). Other closely related clone sequences were obtained from marine hydrocarbon seep sites, such as the Eel River Basin, and marine deep sea mud volcanoes. The next closest cultivated species of Cyclohexane28-EdB clone 63 are strain BuS5 (92.7%, sequence identity), a sulfate-reducing bacterium affiliated with the *Desulfosarcina/Desulfococcus*-cluster*s,* which degrades solely propane and butane (Kniemeyer *et al.*, 2007), phylotypes Butane12-GMe (Kniemeyer *et al*., 2007) and phylotype Butane12-HyR (Jaekel *et al*., in prep), which are the dominant in two sulfate-reducing enrichment cultures from the Gulf of Mexico and Hydrate Ridge and which were shown to be responsible for the observed degradation of butane in the two cultures (Jaekel *et al.*, in prep). The next closest related species originate from marine deep sea hydrocarbon seeps (Hydrate Ridge and Guaymas Basin), whereas the original habitat of the enrichment culture in which Cyclohexane28-EdB clone 63 is most abundant, is intertidal marine sediment. The new phylotype Cyclohexane28-EdB clone 63 represents the first member of the *Desulfosarcina/Desulfococcus*-cluster, which can degrade cyclic alkanes.

Analysis of the activation mechanism for cyclohexane

The analysis of metabolites from cultures grown with cyclohexane in sulfate-limited medium by GC-MS showed the presence of organic acids which were absent in controls without added cyclohexane and in sterile controls (not shown). The presence of a metabolite with fragments at 114, 146, 155 and 197 was indicative for the formation of cyclohexylsuccinic acid dimethyl ester (Fig.5A). The presence of this metabolite indicates that in the sulfate-reducing enrichment culture cyclohexane is activated via addition to fumarate. Such an activation mechanism is analogue to the anaerobic activation of *n*-alkanes (see Widdel and Grundmann (2010) for a review and references herein). Activation of cycloalkanes by addition to fumarate was previously reported for ethylcyclopentane by a sulfate-reducing enrichment culture (Rios-Hernandez *et al*., 2003) and cyclohexane by a nitrate-reducing enrichment culture (Musat *et al.*, 2010). In addition, methyl-cyclopentane and cyclopentane were co-activated by addition to fumarate by the nitrate-reducing strain HxN1 during growth on *n*-hexane and crude oil (Wilkes *et al*., 2003). Two other metabolites were detected upon the GC-MS analysis of formed metabolites-cyclohexylpropionic acid (Fig.5B) and cyclohexylcarboxylic acid (Fig.5C). The formation of these acids suggests the further degradation of the activation product (cyclohexylsuccinic acid) by ligation to CoA, carbon-skeleton rearrangement to cyclohexylmethylmalonylCoA (not detected) and then decarboxylation to cyclohexylpropionyl-CoA (detected as cyclohexylpropionic acid). The detected cyclohexylcarboxylic acid is most likely formed from the cyclohexylpropionyl-CoA, which is further metabolized by β-oxidation through 3-oxo-3-cyclohexylpropiony-CoA (not detected).

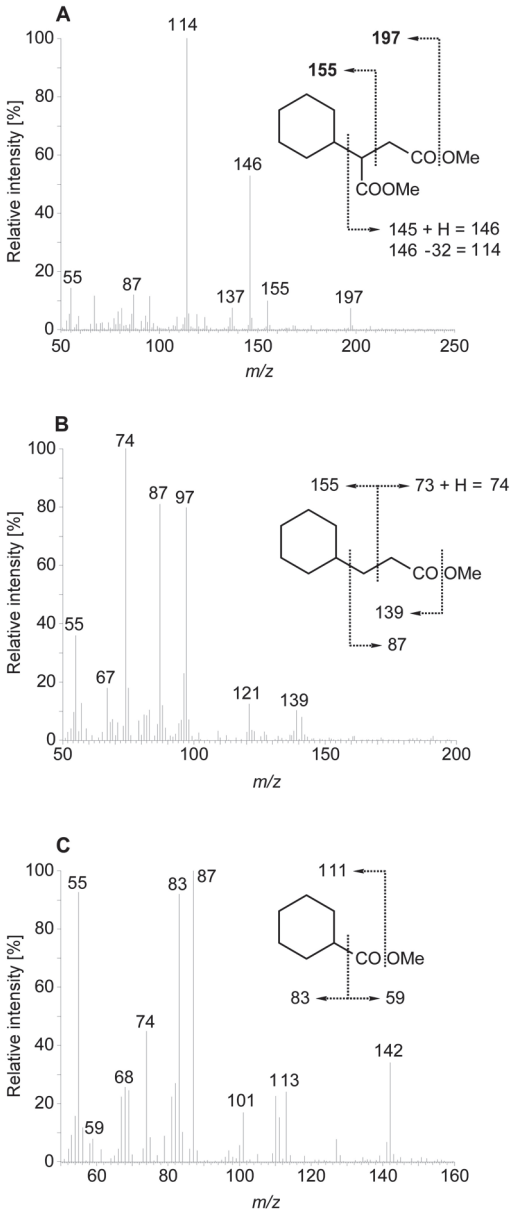

Fig.5 The mass spectra of (A) cyclohexylsuccinic acid dimethyl ester (B) cyclohexylpropionic acid methyl ester and (C) cyclohexylcarboxylic acid methyl ester, which were obtained from metabolite analysis of enrichment culture Cyclohexane28-EdB.

Thereafter, the cyclohexane ring may be cleaved, leading to the regeneration of fumarate and acetyl-CoA, which can be channeled back into the activation reaction cycle or into central metabolic pathways (Fig.6). The proposed scheme for the degradation of cyclohexane by enrichment culture Cyclohexane28-EdB would be analogous to the proposed degradation pathway of *n*-alkanes (Wilkes *et al,* 2002), ethylcyclopentane in a sulfate-reducing enrichment culture (Rios-Hernandez *et al*., 2003) and cyclohexane by a nitrate-reducing enrichment culture (Musat *et al.*, 2010).

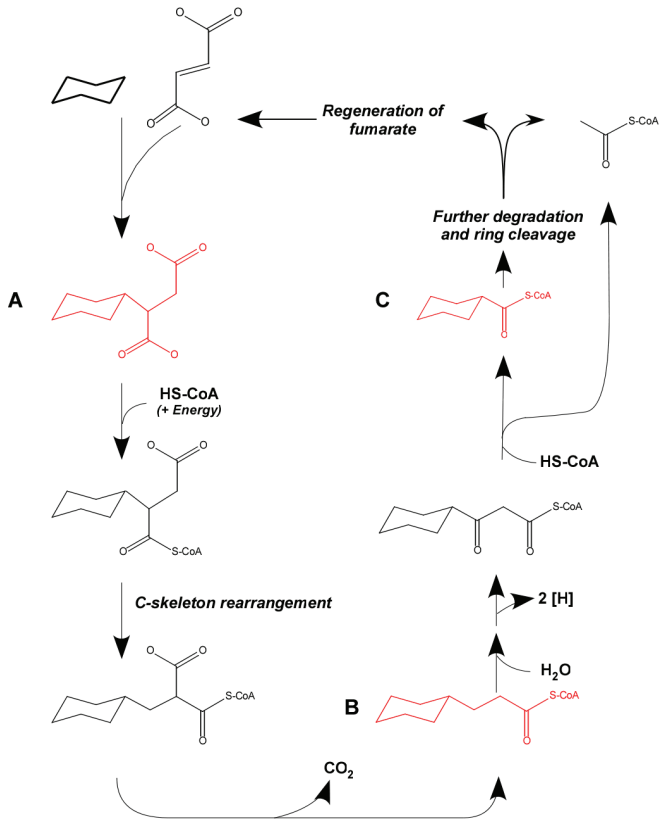

Fig.6 The proposed mechanism for the degradation of cyclohexane by the sulfate-reducing enrichment culture Cyclohexane28-EdB based on detected metabolites (red). See text for explanations. Metabolites detected by GC-MS analysis are marked in red.

Testing other hydrocarbon compounds as possible substrates for growth

The enrichment culture Cyclohexane28-EdB was tested for its ability to degrade other hydrocarbon substrates than cyclohexane. To prevent enrichment of microorganisms in the culture, due to an addition of new substrates, the experiments were performed with active, dense cell suspensions, which were only incubated for short time periods. Assuming that other enzymes might be required for the activation and degradation of the added substrates, all cultures received a background amount of cyclohexane. The substrates tested were structurally related cycloalkanes (cyclopentane, methylcyclopentane, methylcyclohexane and adamantan), *n*-alkanes (ethane, propane, *n*-butane, *n*-pentane, *n*-hexane) and aromatic hydrocarbons (benzene and toluene). Interestingly, during incubations of enrichment culture Cylohexane28-EdB with different *n*-alkanes, cyclic−alkanes, adamantan and aromatic compounds, some of

these tested substrates supported the formation of substantial amounts of sulfide. Hybridizations of fixed cells from incubations, which contained the tested hydrocarbon substrates and showed a substantial sulfide production with the probe CycS28-EdB_152, showed that the relative abundance of phyloytpe Cyclohexane28-EdB clone 63 remained stable throughout the incubation period. This finding supports the hypothesis that the same strain was able to grow on all tested hydrocarbon substrates which supported growth, i.e. no enrichment of other phylotypes occured. Sulfide production started without a lag phase (Fig.7) in incubations with *n*-pentane, *n*-hexane, cyclohexane, cyclopentane, methylcyclohexane and methylcyclopentane, indicating that the enzyme expressed during cyclohexane degradation were able to activate and degrade these *n*-alkanes and cycloalkanes. In the incubation with *n*-butane, substantial sulfide production was observed as well without a change in abundance of the phyloytpe Cyclohexane28-EdB clone 63, suggesting that the tentative cyclohexane degrading strain was able to degrade *n*-butane.

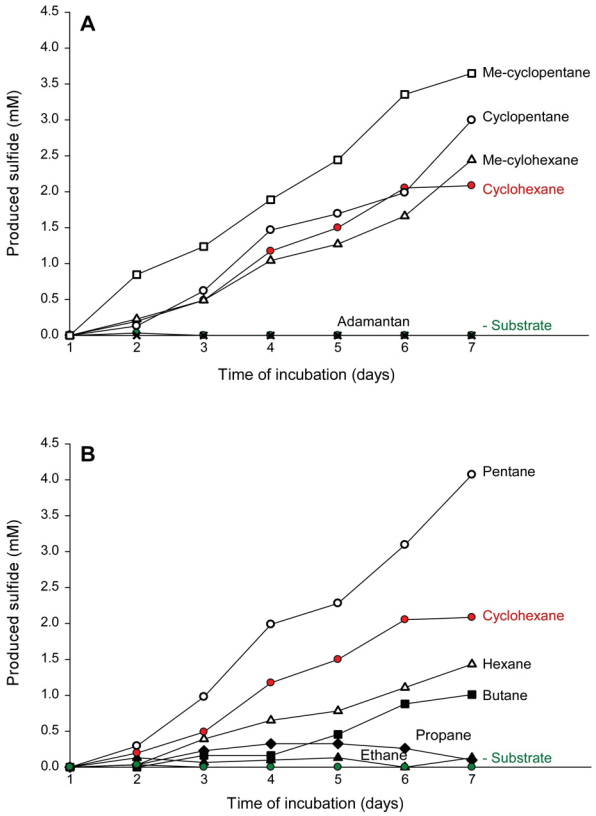

Fig.7 Ability of enrichment culture Cyclohexane28-EdB to degrade cyclic alkanes (A) and *n*-alkanes (B). Incubations were done in 15 ml Hungate tubes with 10 ml of 15× concentrated cell suspensions. All tubes were amended with 50 µl HMN which contained 0.5% (v/v) cyclohexane and the hydrocarbon substrate to be tested.

However, because sulfide production started only after a lag phase of 4 days, it is likely that a different type of enzyme had to be induced in order to activate *n*-butane. To date, all reports about microorgansims capable of degrading cycloalkanes under anaerobic conditions have focused on the ability of these microorganisms to degrade single cycloalkanes, such as cyclohexane (Musat *et al.*, 2010), ethylcyclopentane (Rios-Hernandez *et al*., 2003), cyclopentane, methyl-cyclopentane, methyl-cyclopentane, cyclohexane and methylcyclohexane individually by distinct sulfate-reducing enrichment cultures (Townsend *et al*., 2004). Thus, nothing is known about the versatility of the hydrocarbon substrate usage by cycloalkane degrading anerobic bacteria. For the nitrate reducing strain HxN1, it has been reported that it is able to co−activate, but not grow on cyclopentane and methyl-cyclopentane during growth of the strain on *n*-hexane or crude oil (Wilkes *et al*., 2003). The latter finding indicated that the same enzyme, which activates the *n*-alkane hexane, could also activate cyclopentane and methyl-cyclopentane, however the further degradation of the cycloalkylsuccinates could not be performed by the strain's expressed enzymes, which are at the same time catalyzing the further degradation of the (1-methyl-alkyl)succinate.

Our results indicate that bacteria able to degrade cycloalkanes may be versatile with regard to their degradable hydrocarbon substrate spectrum, comprising C_4−C_6 *n*-alkanes as well as methyl-substituted and unsubstituted C_5- and C_6- cycloalkanes. Future studies may reveal, if the enzyme activating the spectrum of positively tested cycloalkanes and *n*-alkanes in the tentative cyclohexane-degrading strain structurally differs from the glycy-radical enzymes known to activate *n*-alkanes in other anaerobic bacteria (Widdel and Grundmann, 2010 ; Callaghan *et al.*, 2010).

Conclusion

Using sediments from intertidal, hydrocarbon-contaminated sediment we were able to enrich a new deltaproteobacterial phylotype (Cyclohexane28-EdB clone 63) with cyclohexane as a substrate for growth. This strain affiliates with the *Desulfosarcina/Desulfococcus*-cluster and is closest related to other sulfate-reducing strains which degrade short-chain alkanes and originate from deep sea hydrocarbon seeps (Kniemeyer *et al.*, 2007; Jaekel *et al*., in prep). Interestingly, the new phylotype

(Cyclohexane28-EdB clone 63) represents not only the first member of the *Desulfosarcina/Desulfococcus*-cluster, which is able to degrade cycloalkanes but can also degrade C_4–C_6- *n*-alkanes. With the present study, we show that sulfate-reducing microorganisms capable to degrade butane can also be found in intertidal, hydrocarbon rich sediments. Furthermore, the ability of the new phylotype to degrade a rather versatile spectrum of hydrocarbons sheds further light on the metabolic capabilities of the members of the *Desulfosarcina*/*Desulfococcus*-group. These bacteria are often found at high abundances in marine, organic matter-rich, intertidal (Llobret-Brossa *et al*., 2002) and arctic sediments (Ravenschlag *et al.*, 2000) or at hydrocarbon seep sites (Orcutt *et al*., 2010; Hamdan *et al.*, 2011) and it is thus possible that the degradation of ubiquitous hydrocarbon contaminants, such as cycloalkanes and *n*-alkanes by these types of bacteria plays an important role in the overall carbon cycling in hydrocarbon rich, marine sediments.

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