3 Catabolic Pathways Involved in the Anaerobic Degradation of Saturated Hydrocarbons

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Contents

Abstract

Structurally diverse saturated hydrocarbons (*n*-alkanes, branched alkanes, cycloalkanes) occur frequently and abundantly in microbial habitats. A diversity of enrichment and pure cultures of microorganisms which originate from such environments and degrade saturated hydrocarbons under strictly anoxic conditions have been characterized physiologically and phylogenetically. Typically, *n*-alkane-degrading anaerobic microorganisms exhibit more or less pronounced substrate specificities with respect to chain length range of utilizable *n*-alkanes; notably, very limited knowledge exists regarding anaerobic degradation of

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ethane. Currently, four different metabolic strategies are known to be employed by such organisms when growing anaerobically with *n*-alkanes. Best characterized is the pathway initiated by the addition of the hydrocarbon substrate to the co-substrate fumarate catalyzed by a glycyl radical enzyme. Other enzyme reactions apparently used for activation of the highly inert substrates include dehydrogenation/anaerobic hydroxylation, transformation to alkyl-coenzyme M, and "intra-aerobic" oxidation. Subsequent catabolic pathways necessarily differ depending on the chemical nature of the initial activation product. Branched alkanes and cycloalkanes appear to be metabolized through analogous activation reactions and catabolic pathways; however, their degradation by anaerobic microorganisms is less well understood.

1 Introduction

Hydrocarbons make up a quantitatively important portion of organic matter on Earth. They are believed to derive almost entirely from biomass, either directly as natural products or indirectly as products of heat-driven transformation reactions. The greatest portion of hydrocarbons occurs in deposits of fossil organic matter such as petroleum reservoirs. It has been recognized rather early that these hydrocarbon accumulations may be subject to biological alteration processes. This has remained enigmatic for long time since it was also clear that these habitats are devoid of oxygen which, however, had been believed to be essential as oxygen-independent enzyme reactions for the activation of hydrocarbon substrates were still unknown. In other words, the characteristic patterns of compositional alteration of crude oil recognized in petroleum reservoirs (Evans et al. 1971; Peters et al. 2005; Elias et al. 2007) could have been regarded as a clear evidence for the existence of a significant anaerobic biosphere capable of degrading hydrocarbons without oxygen. Today it is clear that the metabolic activity of this anaerobic biosphere is pivotal to the global carbon cycle, preventing that the deposition of fossil organic matter in sedimentary rocks may function as a permanent sink of carbon.

Hydrocarbons which by definition consist exclusively of atoms of the elements carbon and hydrogen are subdivided into several subclasses according to their structures and properties (Wilkes et al. 2019). This review deals with anaerobic degradation of fully saturated aliphatic hydrocarbons. Counterpart is the aromatic hydrocarbons whose anaerobic degradation is discussed in separate chapters of this volume (Boll et al. 2018; Boll and Estelmann 2018). Among the saturated hydrocarbons, *n*-alkanes are the subclass which so far has been studied most extensively with respect to their degradation by anaerobic microorganisms. This is easy to understand since *n*-alkanes do not only represent the major constituents of natural gas and pristine crude oil but do also occur in significant amounts as natural products, in particular as constituents of plant cuticular waxes. Anaerobic degradation of *n*-alkanes has been documented for a considerable chain length range from C_3 up to (ca.) C_{50}. While anaerobic oxidation of methane (C_1) is a key topic in microbiological and biogeochemical research

(Scheller et al. 2017), only very limited information is available about the degradability of ethane under anoxic conditions. This is despite existing evidence from in situ studies that ethane may be degraded under anoxic conditions (for overview see Musat 2015). In contrast to *n*-alkanes, much less is known about the anaerobic degradation of other types of saturated hydrocarbons, i.e., branched alkanes and cycloalkanes.

Considering the widespread occurrence of hydrocarbons in the environment and the technosphere, it is not surprising that anaerobic microorganisms which are capable of degrading saturated hydrocarbons have been successfully enriched and isolated from a very wide variety of habitats (Table 1). Many of these habitats are man-shaped with respect to the presence of hydrocarbons, e.g., contaminated aquifers and sediments or petroleum production plants. It should, however, not be overlooked that hydrocarbons have been present in anoxic environments over geological timescales, e.g., in petroleum reservoirs or at natural hydrocarbon seeps, implying that the preconditions for the evolution of anaerobic microorganisms capable of degrading hydrocarbons have existed most likely throughout most of the Earth's history. The greatest variety of alkane-degrading enrichment or pure cultures is currently available among the sulfate reducers, which obviously is linked to the specific relevance of hydrocarbon occurrences in sulfate-rich marine ecosystems. A slightly lesser number of nitrate-reducing cultures have been described while reports on chlorate- and arsenate-reducing bacteria are scarce (Kaiya et al. 2012; Mehboob et al. 2009, 2016; Mohapatra et al. 2017). Up to now, no metal ion-reducing bacteria have been described that are capable of utilizing saturated hydrocarbons. Very recently, it has been shown that anaerobic degradation of *n*-alkanes may take place under conditions of electrophototrophy/electrode reduction (Venkidusamy and Megharaj 2016a, b). An emerging topic is the understanding of syntrophic communities, which utilize *n*-alkanes anaerobically (Gieg et al. 2014). Current evidence suggests that both bacteria and archaea may be responsible for initial activation of the hydrocarbon substrate as well as for consuming the reducing equivalents produced by their respective syntrophic partners in such communities. Obviously, the degradation of hydrocarbons by methanogenic communities attracts rapidly increasing attention, since anaerobic degradation of hydrocarbons in the quantitatively most significant hydrocarbon accumulations, i.e., the petroleum reservoirs, is known to yield huge amounts of secondary microbial methane (Milkov 2018) due to the fact that these habitats are typically devoid of any electron acceptor, not only oxygen.

This review summarizes our current understanding of activation mechanisms and catabolic pathways employed by these anaerobic microorganisms when utilizing saturated hydrocarbons as sole source of carbon and/or energy; it is structured according to compound types and eventually activation mechanisms. Various aspects of the topic have also been addressed by other recent review articles (Callaghan 2013a; Gieg et al. 2014; Heider and Schühle 2013; Abbasian et al. 2015; Musat 2015; Wilkes et al. 2016). Methane and terpenoid hydrocarbons have been excluded as they are covered by separate reviews in this volume (Scheller et al. 2017; Harder and Marmulla 2017).

Table 1 Known enrichment and pure cultures utilizing saturated hydrocarbons under strictly anoxic conditions

Electron acceptor	Culture	Origin	Phylogenetic affiliation	Substrates	References
Nitrate	Enrichment	Diesel fuel-contaminated aquifer material	Not reported	2,6,10,14-Tetramethylpentadecane (Pristane)	Bregnard et al. (1997)
Nitrate	Strain HxN1	Homogenized mixture of sediment samples from ditches	Betaproteobacteria	*n*-Alkanes C_6–C_8	Ehrenreich et al. (2000)
Nitrate	Strain OcN1	Homogenized mixture of sediment samples from ditches	Betaproteobacteria	*n*-Alkanes C_8–C_{12}	Ehrenreich et al. (2000)
Nitrate	Strain HdN1	Homogenized mixture of sediment samples from ditches	Gammaproteobacteria	*n*-Alkanes C_{14}–C_{20}	Ehrenreich et al. (2000)
Nitrate	*Marinobacter* sp. (BC36, BC38, BP42)	Not available	Gammaproteobacteria	*n*-Octadecane	Bonin et al. (2004)
Nitrate	*Pseudomonas balearica* (BerOc6)	Brackish lagoon	Gammaproteobacteria	*n*-Alkanes C_{15}–C_{18}	Grossi et al. (2008)
Nitrate	Enrichment	Lake sediment	Alphaproteobacteria, Betaproteobacteria	*n*-Hexadecane	Callaghan et al. (2009)
Nitrate	Enrichment	Activated sludge from a wastewater treatment plant	Bacteroidetes, Betaproteobacteria, Gammaproteobacteria	Pristane, 2,6,10,14-Tetramethylhexadecane (Phytane)	Dawson et al. (2013)
Chlorate	*Pseudomonas chloritidismutans* (AW-1)	Biomass of a chlorate-reducing bioreactor	Gammaproteobacteria	*n*-Alkanes C_7–C_{12}	Mehboob et al. (2009)

Arsenate	*Rhizobium naphthalenivorans* (TSY 03b)	Laboratory-scale semi-anaerobic PCDD/F-transforming microcosm	Alphaproteobacteria	*n*-Pentadecane, *n*-Nonadecane	Kaiya et al. (2012), Mohapatra et al. (2017)
Arsenate	*Rhizobium arsenicireducens* (KAs 5–22)	High As content groundwater	Alphaproteobacteria	*n*-Dodecane, *n*-Pentadecane, *n*-Nonadecane	Mohapatra et al. (2017)
Sulfate	*Desulfococcus oleovorans* (Hxd3)	Precipitates from an oil water separator in an oil production plant	Deltaproteobacteria	*n*-Alkanes C_{12}–C_{20}	Aeckersberg et al. (1991)
Sulfate	*Desulfothermus naphthae* (TD3, thermophilic, 60 °C)	Marine sediment	Deltaproteobacteria	*n*-Alkanes C_6–C_{16}	Rueter et al. (1994)
Sulfate	Strain Pnd3	Marine sediment	Deltaproteobacteria	*n*-Alkanes C_{14}–C_{17}	Aeckersberg et al. (1998)
Sulfate	*Desulfatibacillum alkenivorans* (AK-01)	Estuarine sediment with a history of chronic petroleum contamination	Deltaproteobacteria	*n*-Alkanes C_{13}–C_{18}	So and Young (1999)
Sulfate	Enrichment	Gas condensate-contaminated aquifer	Deltaproteobacteria, Firmicutes	Ethylcyclopentane	Rios-Hernandez et al. (2003)
Sulfate	*Desulfatibacillum aliphaticivorans* (CV2803)	Marine sediment	Deltaproteobacteria	*n*-Alkanes C_{13}–C_{18}	Cravo-Laureau et al. (2004)
Sulfate	*Desulfoglaeba alkanexedens* (ALDC)	Sludge from a naval, oily wastewater-storage facility	Deltaproteobacteria	*n*-Alkanes C_6–C_{12}	Davidova and Suflita (2005), Davidova et al. (2006)
Sulfate	*Desulfoglaeba alkanexedens* (Lake)	Produced water from an oilfield	Deltaproteobacteria	*n*-Alkanes C_6–C_{10}	Davidova and Suflita (2005), Davidova et al. (2006)
Sulfate	Strain BuS5	Marine sediment	Deltaproteobacteria	Propane, *n*-Butane	Kniemeyer et al. (2007)

(*continued*)

Table 1 (continued)

Electron acceptor	Culture	Origin	Phylogenetic affiliation	Substrates	References
Sulfate	Thermophilic (60 °C) enrichment (Propane60-GuB)	Marine sediment	Firmicutes	Propane	Kniemeyer et al. (2007)
Sulfate	Enrichment (Butane 12-GMe)	Marine sediment	Deltaproteobacteria	*n*-Butane	Kniemeyer et al. (2007)
Sulfate	Strain PL12	Marine sediment	Deltaproteobacteria	*n*-Hexane, *n*-Decane	Higashioka et al. (2009)
Sulfate	Enrichment	Hydrocarbon seep in a spring	Deltaproteobacteria	Propane, *n*-Pentane	Savage et al. (2010)
Sulfate, Thiosulfate	*Archaeoglobus fulgidus* (VC-16)	Marine hydrothermal system	Archaea	*n*-Alkanes C_{10}–C_{21}	Khelifi et al. (2010, 2014)
Sulfate	Enrichment (Prop12-GMe)	Marine sediment associated with cold hydrocarbon seeps	Deltaproteobacteria	Propane, *n*-Butane	Jaekel et al. (2013)
Sulfate	Enrichment (But12-HyR)	Marine sediment associated with structure II gas hydrates	Deltaproteobacteria	Propane, *n*-Butane	Jaekel et al. (2013)
Sulfate	Syntrophic enrichment	Marine sediment	Archaea and Bacteria	*n*-Butane	Laso-Pérez et al. (2016)
Thiosulfate	*Abyssivirga alkaniphila* L81^T	Deep sea hydrothermal vent system	Firmicutes	*n*-Alkanes C_5–C_{25}, Pristane, Phytane	Schouw et al. (2016)
Methanogenesis	Enrichment	Ditch sediment	Deltaproteobacteria, Archaea	*n*-Hexadecane	Zengler et al. (1999)
Methanogenesis	Anaerobic sediment slurries	Marine sediment	not reported	Pristane	Grossi et al. (2000)
Methanogenesis	Enrichment	Oil sands tailings	Diverse Bacteria and Archaea	*n*-Alkanes C_6–C_8, C_{10}, C_{14}, C_{16}, C_{18}	Siddique et al. (2006, 2011, 2012)

Methanogenesis	Enrichment	Brackish river sediment	Deltaproteobacteria, Archaea	*n*-Hexadecane	Jones et al. (2008)
Methanogenesis	Enrichment	Gas-condensate-contaminated subsurface sediments	Diverse Bacteria and Archaea	*n*-Alkanes (chain length not specified)	Gieg et al. (2008)
Methanogenesis	Enrichment	Oil reservoir	Diverse Bacteria and Archaea	*n*-Alkanes	Wang et al. (2011)
Methanogenesis	Enrichment	Production water of a high-temperature petroleum reservoir	Diverse Bacteria and Archaea	*n*-Alkanes C_{15}–C_{20}	Mbadinga et al. (2012)
Methanogenesis	Enrichment	Disposal plant of an oilfield	Diverse Bacteria and Archaea	*n*-Hexadecane	Cheng et al. (2013), Ding et al. (2015)
Methanogenesis	Enrichment	Oil sands tailings	Diverse Bacteria and Archaea	*n*-Alkanes C_6–C_{10}, 2-Methylpentane, Methylcyclopentane	Tan et al. (2013, 2015b)
Methanogenesis	Enrichment	Produced water from an oilfield	Diverse Bacteria and Archaea	*n*-Alkanes C_{15}–C_{20}	Liang et al. (2016)
Methanogenesis	Enrichment	Marine sediment	Diverse Bacteria and Archaea	*n*-Alkanes C_{28}–C_{50}	Wawrik et al. (2016)
Electrode respiration	*Stenotrophomonas maltophilia* (MK2)	Hydrocarbon-fed microbial electrochemical remediation system	Gammaproteobacteria	*n*-Alkanes C_8–C_{36}	Venkidusamy and Megharaj (2016a)
Electrophototrophy	*Rhodopseudomonas palustris* (RP2)	Hydrocarbon-fed microbial electrochemical remediation system	Alphaproteobacteria	*n*-Alkanes (chain length not specified)	Venkidusamy and Megharaj (2016b)

2 *n*-Alkanes

Saturated hydrocarbons are energy-rich substrates which, however, are highly inert. This is due to the fact that they contain exclusively apolar C–C and C–H σ-bonds but no functional groups. Therefore, their transformation to catabolizable, i.e., functionalized, compounds requires specific enzymatic reactions enabling energetically and/or mechanistically demanding C–H bond activation and eventually homolytic cleavage. Notably, the stability of alkyl radicals formed by homolytic bond cleavage differs significantly depending on the substitution pattern of the carbon atom from which a hydrogen atom is removed. Stabilization of carbon-centered radicals by alkyl substituents occurs through hyperconjugation resulting in C–H bond-dissociation energies which decrease in the order primary > secondary > tertiary. This general pattern may explain why activation of terminal C–H bonds apparently is less common compared to subterminal C–H bonds. Currently, evidence is available for the existence of four independent strategies employed by anaerobic microorganisms to activate *n*-alkanes for subsequent catabolism. Among these, the addition of the hydrocarbon substrate to fumarate has been most extensively studied and may be regarded as the currently by far best understood mechanism. The consecutive catabolic pathways differ to accommodate the different chemical nature of the initial activation products.

2.1 Addition to Fumarate

First evidence that *n*-alkanes may be activated by addition to fumarate – in analogy to the at that time already established activation of toluene via transformation to benzylsuccinate catalyzed by the gylcycl radical enzyme benzylsuccinate synthase (Bss) (Leuthner et al. 1998) – was provided for *n*-dodecane in a sulfate-reducing enrichment culture via tentative identification of the substituted succinate formed (Kropp et al. 2000). A concurrent study with denitrifying strain HxN1 and *n*-hexane provided the following important insights into the nature of this type of activation reaction (Rabus et al. 2001): (i) using synthetic reference standards, it was shown that two isomers of (1-methylpentyl)succinate (MPS) were formed during anaerobic growth with *n*-hexane; (ii) experiments with stable isotope-labeled *n*-hexane and fumarate proved fumarate dependency of MPS formation from *n*-hexane; and (iii) electron paramagnetic resonance spectroscopy revealed the presence of an organic radical in *n*-hexane-grown cells, which was absent in *n*-hexanoate-grown cells and whose signal was in agreement with a glycyl radical. Based on this evidence, it was suggested that *n*-hexane is activated at carbon atom 2 by a radical reaction and added to fumarate (co-substrate), yielding MPS as the first stable product (Fig. 1a) (Rabus et al. 2001). The postulated enzyme (1-methylalkyl)succinate synthase (Mas, also termed alkylsuccinate synthase, Ass) was identified by means of proteogenomics (Grundmann et al. 2008). Subsequently, it has been shown that (1-methylalkyl) succinates are formed by various denitrifying and sulfate-reducing bacteria during anaerobic growth with *n*-alkanes of different chain length (e.g. Callaghan et al. 2006;

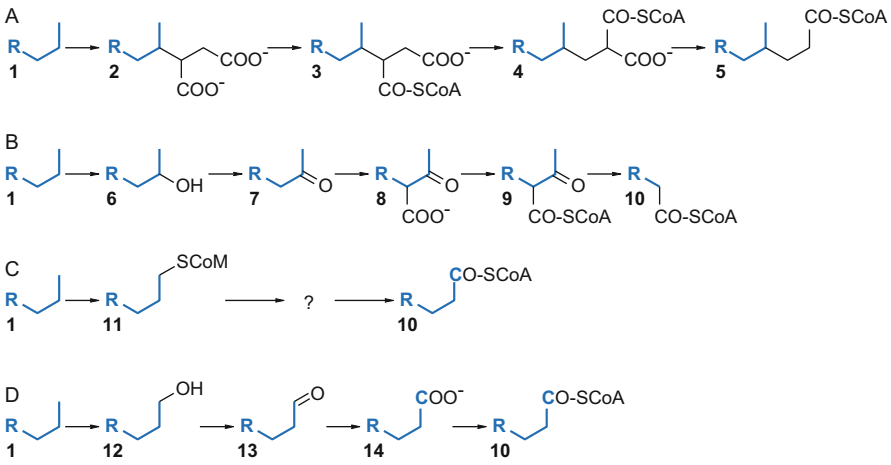

Fig. 1 Overview of anaerobic enzymatic activation reactions and subsequent catabolic pathways of *n*-alkanes until the level of coenzyme-A-esters suitable for further degradation via β-oxidation; (**a**) addition to fumarate; (**b**) dehydrogenation/anaerobic hydroxylation; (**c**) formation of alkyl-coenzyme-M; (**d**) "intra aerobic" oxidation; **1**, *n*-alkane; **2**, (1-methylalkyl)succinate; **3**, (1-methylalkyl)succinyl-CoA; **4**, (2-methylhexyl)malonyl-CoA; **5**, 4-methyloctanoyl-CoA; **6**, alkan-2-ol; **7**, alkan-2-one; **8**, 2-acetylalkanoate; **9**, 2-acetylalkanoyl-CoA, **10**, alkanoyl-CoA; **11**, 2-(butylsulfanyl)ethane-1-sulfonate (1-butyl-coenzyme M); **12**, alkan-1-ol; **13**, alkanal; **14**, alkanoate

Cravo-Laureau et al. 2005; Davidova et al. 2005; Kniemeyer et al. 2007; Savage et al. 2010; Zedelius et al. 2011).

A detailed study of the stereochemistry of *n*-hexane addition to fumarate in strain HxN1 provided unprecedented insights into the putative reaction mechanism (Jarling et al. 2012). It was shown that (i) the (2*R*,1'*R*)- and (2*S*,1'*R*)-diastereoisomers of MPS are formed, (ii) exclusively the pro-*S* hydrogen atom is abstracted from the *n*-alkane, (iii) the reaction thus proceeds with inversion of configuration at the carbon atom of *n*-hexane that forms the new C–C bond, and (iv) the reaction is associated with a significant kinetic isotope effect for hydrogen >3, indicating that the cleavage of the C–H bond must be involved in the first irreversible step of the reaction mechanism. Based on these observations, it has been suggested that the addition of *n*-hexane to fumarate may proceed in a concerted reaction mechanism (Fig. 2), which would avoid the formation of a highly energetic free alkyl radical and could explain how the difference in bond-dissociation energy between a thiol and a C–H bond in an alkane of $\approx$ 40 kJ/mol could be overcome. It has been hypothesized that even methane could be added to fumarate (Thauer and Shima 2008) although the difference in the bond-dissociation energies would be as high as $\approx$ 70 kJ/mol; however, evidence for such a reaction has never been reported. Based on results of quantum chemical calculations, it has been suggested that the addition of methane to fumarate itself is exothermic and has an overall energy change between 410 and 470 kJ/mol (Beasley and Nanny 2012). A gas-phase ab initio study provided

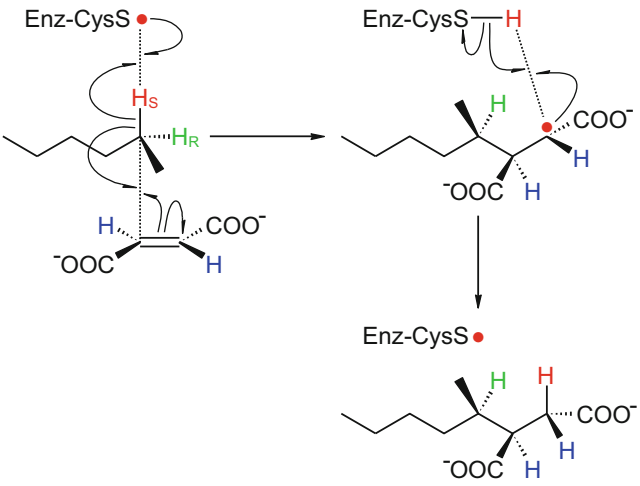

Fig. 2 Suggested mechanism for (1-methylalkyl)succinate formation via a concerted reaction. (Reprinted from Jarling et al. (2012), with permission from John Wiley and Sons)

evidence that the rate of *n*-alkane addition to fumarate is about 100 times slower than of toluene at 298 K and that the step of hydrogen abstraction is kinetically significant (Bharadwaj et al. 2015), which is in agreement with the hydrogen isotope effect mentioned above. Furthermore, it has been suggested that the stereochemical course of the reaction is controlled by the differences in the radical addition rate constants for the various isomers.

The only reported case of terminal activation of an *n*-alkane for addition to fumarate is that of propane, which is converted to a mixture of iso- and *n*-propylsuccinate by sulfate-reducing bacteria (Kniemeyer et al. 2007). Based on experiments with deuterium-labeled propane, it has been estimated that 70% of the propane activation events occur at the subterminal and 30% at the terminal carbon atoms (Jaekel et al. 2014). It has been argued that *n*-propylsuccinate formation from propane might be due to incomplete regioselectivity of the alkylsuccinate synthase involved (Jarling et al. 2015) similar to formation of (1-ethylbutyl)succinate as an apparent byproduct formed during growth of strain HxN1 with *n*-hexane (Rabus et al. 2001). It is currently unclear whether *n*-propylsuccinate is an intermediate of an energy-conserving catabolic pathway in the propane-utilizing, sulfate-reducing bacteria studied or not. Formation of short-chain *n*-alkylsuccinates (alkyl = C_1–C_5) has also been observed during growth of sulfate-reducing strain TD3 with C_7–C_{12} *n*-alkanes (Jarling et al. 2015). The pattern was such that *n*-alkylsuccinates with a C-odd alkyl group were specifically formed from C-even *n*-alkanes and vice versa. Thus, terminal activation of short-chain *n*-alkanes could be excluded in this case. It has been suggested that this metabolism might play a role in coping with solvent stress, particularly at elevated temperature. In line with this suggestion, Jarling et al. (2015) documented that the capability for co-metabolic addition of non growth supporting diverse hydrocarbons to fumarate was most pronounced in thermophilic

strain TD3 among the denitrifying and sulfate-reducing bacteria compared. Previously, it has been shown that anaerobic *n*-alkane-degrading bacteria are capable of transforming toluene co-metabolically to benzylsuccinate, while by contrast anaerobic alkylbenzene-degrading bacteria apparently are incapable of transforming *n*-alkanes to alkylsuccinates (Rabus et al. 2011).

Genomic studies concerning the enzymatic addition of *n*-alkanes to fumarate have provided considerable insight into the relevance of this environmentally significant type of biochemical transformation. Grundmann et al. (2008) identified a gene cluster in strain HxN1 whose deduced proteins (Mas) are similar to benzylsuccinate synthases. Mas was characterized as a presumable heterotrimer (MasDEC), which like Bss, contains a motif (in MasD, the large subunit) characteristic for glycyl radical-bearing sites. Furthermore, Mas has been suggested to represent a separate line of descent within the glycyl radical enzymes. Likewise, the genome of sulfate-reducing *Desulfatibacillum alkenivorans* strain AK-01 contains two genes (*assA1* and *assA2*) in different operons, which encode catalytic subunits of glycyl radical-type enzymes (Callaghan et al. 2008). A 95-kDa protein detected in *n*-hexadecane-grown cells of strain AK-01 which was absent in hexadecanoate-grown cells matched the deduced amino acid sequence encoded by *assA1*, based on which the involvement of AssA1 in *n*-alkane metabolism has been suggested. In line with this, the use of RT-PCR revealed induction of *assA1* during growth with all tested *n*-alkane substrates, while induction of *assA2* was not observed (Herath et al. 2016). Alkylsuccinate synthases may also be encoded by genes of the *n*-alkane-degrading, arsenate-reducing bacterium *Rhizobium arsenicireducens*, which was isolated from arsenic-rich groundwater (Mohapatra et al. 2017). The capability to enzymatically add *n*-alkanes to fumarate is not restricted to the domain of Bacteria, according to recent findings with the *n*-alkane-utilizing thermophilic sulfate- and thiosulfate-reducing archaeon *Archaeoglobus fulgidus* strain VC-16 (DSM 4304). Its genome contains a gene previously annotated as pyruvate-formate lyase which based on three dimensional modeling of the corresponding protein and molecular dynamics simulations might rather encode an alkylsuccinate synthase (Khelifi et al. 2014). However, according to phylogenetic analysis, this gene is of bacterial origin and was likely acquired by horizontal gene transfer. First evidence for the presence of genes encoding methylpentylsuccinate viz. alkylsuccinate synthases in methanogenic consortia came from studies with thermophilic cultures derived from production waters of a high-temperature petroleum reservoir (Mbadinga et al. 2012). Metagenomic studies have revealed that genes encoding such enzymes occur widespread in methanogenic consortia of diverse origin (Liang et al. 2016; Tan et al. 2013, 2015a). Moreover, RT-PCR confirmed their expression in *n*-alkane-degrading methanogenic cultures (Tan et al. 2015b; Wawrik et al. 2016). Evidence has been provided that the genome of *Smithella* as a dominant bacterial member of such consortia may contain genes encoding such enzymes, and thus, that *Smithella* may play an important role in methanogenic degradation of *n*-alkanes (Tan et al. 2014); furthermore, genomic insights have been achieved on the multiple metabolic interactions between *Smithella* and its methanogenic partners (Embree et al. 2015). Two genes encoding

glycyl radical enzymes related to the alkylsuccinate synthases of *D. alkenivorans* AK-01 have also been identified in the genome of the recently described strictly anaerobic, mesophilic, syntrophic, alkane-degrading strain, L81^T, which was isolated from a biofilm sampled from a black smoker chimney (Schouw et al. 2016). Following from these studies, PCR primers targeting genes for (1-methylpentyl) succinate viz. alkylsuccinate synthases are increasingly used to assess the occurrence and distribution of *n*-alkane-degrading anaerobic microorganisms in their natural habitats, particularly in marine sediments representing diverse biogeochemical regimes (Callaghan et al. 2010; Kleindienst et al. 2014; Gittel et al. 2015; Stagars et al. 2016). With certain exceptions, the succinate-derivatives formed by the enzymes targeted by these probes are highly specific products of the biochemical transformation of hydrocarbons under anoxic conditions and therefore have a high potential to function as signature metabolites for targeted metametabolomics in environmental studies (Agrawal and Gieg 2013; Callaghan 2013b).

The further catabolic pathway of MPS to carbon dioxide has been proposed on the basis of metabolite studies performed with strain HxN1 anaerobically growing with *n*-hexane (Fig. 1a) (Wilkes et al. 2002). After activation as coenzyme-A-thioester, (1-methylpentyl)succinyl-CoA is rearranged to (2-methylhexyl)malonyl-CoA with the latter representing a suitable substrate for enzymatic decarboxylation yielding (*R*)-4-methyloctanoyl-CoA (Wilkes et al. 2002; Jarling et al. 2012). Key evidence for the proposed rearrangement was the 1,2-migration of a hydrogen atom observed in experiments with deuterium-labeled substrates in analogy to that occurring during transformation of succinyl-CoA to methylmalonyl-CoA (Rétey 1982). It has been suggested that the originally formed stereoisomer of MPS does not have the correct configuration for this rearrangement and thus requires an epimerization which would explain the observed occurrence of MPS as two stereoisomers and the exchange of the hydrogen atom at carbon atom two of MPS with external hydrogen (Rabus et al. 2001; Jarling et al. 2012). (*R*)-4-Methyloctanoyl-CoA is then subjected to β-oxidation yielding acetyl-CoA and (*R*)-2-methylhexanoyl-CoA. The latter requires another epimerization before it can be degraded via two further rounds of β-oxidation to give two acetyl-CoA and one propionyl-CoA (Wilkes et al. 2002, 2016). Thus, overall one molecule *n*-hexane is converted to three molecules acetyl-CoA, which may be channeled into the TCA cycle and biosynthesis of cell constituents. It has also been suggested that propionyl-CoA may be used to regenerate fumarate, possibly involving trans-carboxylation and coenzyme A transfer (Wilkes et al. 2002). Subsequently, it has been shown, also on the basis of metabolite studies, that an analogous pathway is employed by *n*-alkane-degrading sulfate-reducing bacteria (Callaghan et al. 2006; Davidova et al. 2005; Kniemeyer et al. 2007).

While complete genome sequences of three anaerobic *n*-alkane utilizers are publicly available (Callaghan 2013a), so far the annotation of the genome has only been published for *D. alkenivorans* AK-01 (Callaghan et al. 2012). The circular chromosome of this bacterium contains 5361 genes, 5296 of which encode proteins. Most importantly the annotated genome fully supports the catabolic pathway proposed on the basis of metabolite studies, including genes encoding the

glycyl radical enzyme activating the *n*-alkane (see above) as well as all enzymes required for further conversion of the activation product to acetyl-CoA and for the regeneration of fumarate from propionyl-CoA. *D. alkenivorans* AK-01 also has the necessary genes for completely oxidizing acetyl-CoA via the reverse Wood-Ljungdahl pathway. A transcriptomic analysis revealed upregulation of genes potentially involved in (1-methylalkyl)succinate metabolism, including methylmalonyl-CoA mutase and a putative carboxyl transferase during growth with *n*-alkanes (Herath et al. 2016).

2.2 Dehydrogenation/Anaerobic Hydroxylation

The first reported pure culture of an anaerobic bacterium capable of utilizing saturated hydrocarbons under strictly anoxic conditions was sulfate-reducing *Desulfococcus oleovorans* strain Hxd3 which had been isolated from an oil production plant and grows with C_{12}–C_{20} *n*-alkanes (Aeckersberg et al. 1991). It was found that this bacterium produces cellular fatty acids with a predominantly even number of carbon atoms when utilizing *n*-alkanes with an odd number of carbon atoms and vice versa (Aeckersberg et al. 1998). Such a pattern is in disagreement with activation of long-chain *n*-alkanes via addition to fumarate during which formation of C-even fatty acids from C-even *n*-alkanes and C-odd fatty acids from C-odd *n*-alkanes is expected. In line with this, evidence has been provided based on stable isotope-labeling experiments that formation of fatty acids in *D. oleovorans* strain Hxd3 goes along with removal of the terminal and subterminal carbon atoms from one end of the *n*-alkane while the carboxyl group (at the same end) originates from external inorganic carbon (So et al. 2003). Subsequently, similar patterns have been reported to occur during anaerobic biodegradation of *n*-hexadecane by a nitrate-reducing consortium (Callaghan et al. 2009). Key elements of a fitting catabolic pathway have been briefly discussed in review articles by Callaghan (2013a), Heider and Schühle (2013), and Heider et al. (2016). Crucial to it is the detection of genes in the publicly available genome sequence of *D. oleovorans*, which encode an ethylbenzene dehydrogenase-like protein, while genes encoding hydrocarbon-activating glycyl radical enzymes (alkylsuccinate synthases) apparently are absent. It thus has been suggested that long chain *n*-alkanes may be hydroxylated yielding alkan-2-ols, which after dehydrogenation to the corresponding alkan-2-ones would be carboxylated at carbon atom 3 (Fig. 1b). The resulting 2-acetylalkanoates, after transformation to the corresponding coenzyme A esters, may then be subject to further degradation by β-oxidation.

2.3 Transformation to Alkyl-Coenzyme M

Based on studies with a syntrophic enrichment culture (Butane50) originating from marine sediment from the Guaymas Basin in the Gulf of California, evidence has recently been provided for a further mode of activation of *n*-alkanes in anaerobic

microorganisms. In this syntrophic community, *n*-butane is initially activated and then completely oxidized by an archaeal member from whom reducing equivalents are channeled to a bacterial member (Laso-Pérez et al. 2016). The archaea (proposed genus "*Candidatus* Syntrophoarchaeum" which is closely related to *Methanosarcinales*) apparently transform *n*-butane to 2-(butylsulfanyl)ethane-1-sulfonate (1-butyl-coenzyme M) and 2-[(butan-2-yl)sulfanyl]ethane-1-sulfonate (2-butyl-coenzyme M) (Fig. 1c). Mechanistically, this resembles the well-known anaerobic oxidation of methane by archaea, which is initiated by enzymatic formation of methyl-coenzyme M catalyzed by methyl-coenzyme M reductase. This is supported by the detection of highly expressed genes encoding enzymes similar to methyl-coenzyme M reductase in "*Ca*. Syntrophoarchaeum," whereas genes encoding glycyl radical enzymes (*assA*/*masD*, *bssA*) that might be involved in anaerobic activation of hydrocarbons were not detected. The finding that "*Ca*. Syntrophoarchaeum" also expresses the genes encoding β-oxidation enzymes, carbon monoxide dehydrogenase, and reversible C_1 methanogenesis enzymes led to the suggestion of a pathway for complete oxidation of *n*-butane. Furthermore, it has been proposed that reducing equivalents are channeled to HotSeep-1, a thermophilic sulfate-reducing partner bacterium known from the anaerobic oxidation of methane. It has been discussed that 2-(butylsulfanyl)ethane-1-sulfonate and not 2-[(butan-2-yl)sulfanyl]ethane-1-sulfonate may more likely represent the genuine and directly metabolized activation product of *n*-butane, as it derives from C–H-bond activation at a terminal carbon atom which mechanistically is more similar to the corresponding transformation of methane by methyl-coenzyme-M reductase. Furthermore, evidence has been presented for propane-dependent formation of 2-(propylsulfanyl) ethane-1-sulfonate (propyl-coenzyme M) in the same enrichment culture.

2.4 "Intra Aerobic" Oxidation

The facultative anaerobic, gammaproteobacterial, denitrifying strain HdN1, which has been isolated from activated sludge from a sewage plant, utilizes C_6 – C_{30} *n*-alkanes under anoxic conditions with NO_3^- as electron acceptor (Ehrenreich et al. 2000). No hints for fumarate-dependent *n*-alkane activation in this bacterium could be deduced from analysis of genes in the complete genome sequence or from targeted analysis of metabolites in *n*-alkane-grown anaerobic cultures (Zedelius et al. 2011). In contrast to other *n*-alkane-utilizing denitrifying bacteria which grow with alkanes and NO_3^-, NO_2^- or N_2O added to the medium, strain HdN1 oxidizes *n*-alkanes only with the former two electron acceptors but not with added N_2O. However, N_2O serves as an electron acceptor during anaerobic growth with long-chain alcohols; furthermore, N_2O apparently does not inhibit growth with long-chain *n*-alkanes when NO_3^- is also present. This evidence suggests that NO_2^- – or a subsequently formed nitrogen compound other than N_2O – is needed for *n*-alkane activation according to a currently unknown mechanism. Noteworthy, a putatively related mode of activation of methane in nitrite-reducing "*Candidatus* Methylomirabilis oxyfera" apparently involves dismutation of NO to N_2 and O_2,

with the latter serving as the co-substrate of particulate methane monooxygenase (Ettwig et al. 2010). It has been suggested that enzymes related to quinol-dependent NO reductases (qNORs) might be involved in this formation of oxygen in "*Ca.* M. oxyfera" (Ettwig et al. 2012). Similar electron acceptor-dependent formation of O_2 might enable strain HdN1 to employ oxygenases for substrate activation in anaerobic oxidation of long-chain *n*-alkanes. In the genome of strain HdN1, various candidate monooxygenases have been predicted (Zedelius et al. 2011) although it is currently not known which of these might be formed in aerobic and denitrifying cultures.The bacterium *Pseudomonas chloritidismutans* AW-1^T grows with C_7–C_{12} *n*-alkanes both under oxic conditions as well as anaerobically with chlorate as electron acceptor (Mehboob et al. 2009). *P. chloritidismutans* is capable to produce oxygen along with chloride by dismutation of chlorite catalyzed by chlorate dismutase. The chlorite required for this reaction is generated by chlorate reductase. Both enzymes are constitutively present but most abundant under chlorate-reducing conditions (Mehboob et al. 2016). Proteomic analysis revealed the presence of all enzymes involved in aerobic oxidation of *n*-alkanes. Most importantly, an alkane monooxygenase was detected in *n*-decane-grown cells, but not in acetate-grown cells, both during aerobic respiration and chlorate reduction providing evidence that the same oxygen-dependent catabolic pathway is employed under oxic and anoxic conditions (Fig. 1d).

3 Branched Alkanes

Relatively limited information is available on the anaerobic degradation of branched alkanes. Low molecular weight branched alkanes are well known to occur in significant amounts in crude oil (Peters et al. 2005). Such hydrocarbons are important constituents of the so-called naphtha, the extraction solvent used in operations related to the exploitation of oil sands, and thus are of environmental concern for example with respect to processes occurring in oil sands tailings ponds (e.g., Siddique et al. 2015). A number of studies therefore have addressed their degradation under methanogenic conditions. Methanogenic enrichment cultures originating from oil sands tailings ponds degraded 2-methylbutane, 2- and 3-methylpentane, 2- and 3-methylhexane, and 2- and 4-methylheptane either as pure compounds or in mixture (Abu Laban et al. 2015; Siddique et al. 2015). Putative metabolites have been detected in such cultures which apparently indicate activation of these branched alkanes via addition to fumarate (Abu Laban et al. 2015; Tan et al. 2015b). Based on pyrotag sequencing, cloning and terminal restriction fragment length polymorphism of 16S rRNA genes, a novel member of the family *Peptococcaceae* (order *Clostridiales*), has been implicated to be responsible for this metabolism. Bacteria apparently have a limited potential to transform low molecular weight branched alkanes co-metabolically (Jarling et al. 2015). Three strains of *n*-alkane-utilizing anaerobic bacteria (two nitrate- and one sulfate-reducing) were found to only activate 2-methylpentane via addition to fumarate among the four branched C_6 alkane isomers.

Isoprenoid hydrocarbons, in particular pristane (2,6,10,14-tetramethylpentadecane) and phytane (2,6,10,14-tetramethylhexadecane), are of high biogeochemical significance and well known to be susceptible to biodegradation, e.g., in petroleum reservoirs (Peters et al. 2005; Elias et al. 2007). Nitrate-reducing enrichment cultures originating from a diesel fuel-contaminated aquifer have been shown to efficiently degrade pristane (Bregnard et al. 1997). Also under nitrate-reducing conditions using incubations with samples obtained from an activated sludge from a wastewater treatment plant, not only pristane but also phytane were mineralized (Dawson et al. 2013). Pristane was also found to be degraded efficiently by a methanogenic consortium in anaerobic sediment slurry of marine origin under conditions limiting nitrate reduction (Grossi et al. 2000). The recently isolated *Abyssivirga alkaniphila* L81^T depleted pristane and phytane during growth with crude oil both during thiosulfate reduction and in methanogenic co-culture with *Methanothermococcus okinawensis* LG6 (Schouw et al. 2016). While these studies clearly indicate that multiple branched isoprenoid alkanes may be degraded under anoxic conditions, no insights have been reported into the putative activation reactions and catabolic pathways involved.

4 Cycloalkanes

Saturated hydrocarbons with five- and six-membered rings occur frequently in fossil organic matter, while smaller or larger rings are of much lesser relevance. This group of compounds does not only include monocyclic, but also polycyclic structures deriving from natural products with such carbon skeletons, e.g., steroids, hopanoids, and other triterpenoids. Yet so far only for certain monocyclic compounds, the utilization and degradation by anaerobic microorganisms has been reported. A sulfate-reducing enrichment culture obtained from a gas condensate-contaminated aquifer was capable of utilizing ethylcyclopentane which was completely oxidized (Rios-Hernandez et al. 2003). Gas chromatography-mass spectrometry provided evidence for the presence of (ethylpentyl)succinate, (ethylpentyl)propionate, ethylcyclopentylcarboxylate, and 2-ethylsuccinate in ethylcyclopentane-grown cultures. Based on this evidence it has been suggested that ethylcyclopentane, after addition to fumarate via a methylene group of the ring, is further degraded in analogy to the pathway used for the degradation of *n*-alkanes as described in Sect. 2.1. Unsubstituted cyclohexane is also degraded according to this pathway both in nitrate- and sulfate-reducing enrichment cultures, as evidenced by the identification of cyclohexylsuccinate and 3-cyclohexylpropionate as well as cyclohexanecarboxylate (Musat et al. 2010; Jaekel et al. 2015). The nitrate-reducing enrichment culture consisted of *Geobacteraceae* (75% of the bacterial cells) and "*Candidatus* Brocadia anammoxidans" (18% of the bacterial cells, anammox bacteria, member of the *Planctomycetales*). Reduction of nitrate to nitrite by the former through oxidation of cyclohexane is coupled to scavenging of nitrite and added ammonium by the latter to yield dinitrogen. The presence of long chain cyclohexyl-substituted fatty acids apparently indicated partial channeling of 3-cyclohexylpropionate into fatty acid

biosynthesis (Musat et al. 2010). The sulfate-reducing enrichment culture was also able to utilize cyclopentane, methylcyclopentane, methylcyclohexane, and even the C_4–C_6 *n*-alkanes (Jaekel et al. 2015). A methanogenic enrichment culture originating from oil sands tailings has been shown to deplete methylcyclopentane with concomitant formation of a putative succinate derivative (Tan et al. 2015b). Different *n*-alkane-utilizing, nitrate- and sulfate-reducing bacteria apparently are capable of co-metabolically activating cyclopentane, methylcyclopentane, ethylcyclopentane, and to a lesser extent, cyclohexane (Wilkes et al. 2003; Jarling et al. 2015).

5 Research Needs

Despite significant progress over the past three decades, anaerobic catabolism of *n*-alkanes is still an emerging topic. The only catabolic pathways that have by now been characterized relatively well are those initiated by addition of the inert substrates to fumarate catalyzed by glycyl radical-containing alkylsuccinate synthases. At least three other metabolic strategies exist, two of which have been discovered during the last decade. The associated catabolic pathways clearly require further research with respect to enzymes involved and their reaction mechanisms, proteogenomic characterization of the acting microorganisms, and the regulation of their catabolic networks. Furthermore, it should not be excluded that additional yet undiscovered modes of *n*-alkane transformation may exist. Overall, relatively little is known about anaerobic degradation of branched alkanes and cycloalkanes. With this in mind, the patterns of microbially driven compositional alteration of complex hydrocarbon assemblages in natural habitats, e.g., crude oil in petroleum reservoirs, should be considered as a very valuable source of information with respect to existing but as yet undiscovered degradative capabilities among anaerobic microorganisms. It is worth mentioning that the catabolic principles playing a role in anaerobic degradation of saturated hydrocarbons may be relevant for other compound classes as well. As an example, take the significant evidence that environmentally relevant linear alkylbenzene sulfonates apparently may be degraded under anoxic conditions via addition to fumarate and subsequently a pathway analogous to that of *n*-alkanes (Lara-Martín et al. 2010; Baena-Nogueras et al. 2014). To date not one single enzyme activating saturated hydrocarbons without oxygen has been characterized structurally. The recent studies into the structure of benzylsuccinate synthase (Funk et al. 2014, 2015) have commandingly underpinned the value of such research with respect to understanding mechanisms of unprecedented enzyme reactions. Activation of inert C–H bonds continues to be one of the most important research fields in chemistry, and investigations on the transformation of alkanes by anaerobic microorganisms have brought to light highly interesting enzymatic alternatives to the well-studied reactions catalyzed by oxygenases. In that perspective, the better understanding of biochemical principles of C–H bond activation may be an inspiring stimulation for the development of new biotechnological or biomimetic processes to convert inert saturated hydrocarbons to valuable components.

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