Anaerobic Degradation of Hydrocarbons: Mechanisms of Hydrocarbon Activation in the Absence of Oxygen

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Abstract

Hydrocarbons are highly abundant in nature and are formed either via geochemical or biological processes. Their high C–H bond dissociation energies are responsible for low chemical reactivities. Due to the toxicity of many

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hydrocarbons, their biological degradation is of environmental concern. In the presence of oxygen, the C–H bond is activated by oxygenases involving enzyme-bound reactive oxygen species in exergonic reactions. In contrast, anaerobic hydrocarbon-degrading bacteria use a number of alternative enzymatic reactions for the mechanistically sophisticated C–H bond activation. Some of these reactions are only known from anaerobic hydrocarbon degradation pathways, and some follow unprecedented biochemical mechanisms. The known oxygen-independent activation reactions of hydrocarbons comprise (1) hydroxylation with water by enzymes containing molybdenum or flavin cofactors, (2) addition to fumarate by glycyl-radical enzymes, (3) carboxylation, (4) water addition at multiple bonds, and (5) reverse methanogenesis. Our current knowledge of these enzymes varies greatly. Whereas an ethylbenzene hydroxylating molybdenum enzyme, a glycyl-radical enzyme adding alkyl groups to fumarate, and different types of enzymes adding water to C=C double and triple bonds have structurally and functionally been characterized, less is known about enzyme(s) involved in naphthalene carboxylation and methane degradation via reverse methanogenesis. The initial mode of benzene activation is still at issue (carboxylation vs. hydroxylation).

1 Introduction

The ability of many bacteria or fungi to fully degrade aliphatic or aromatic hydrocarbons to CO_2 has been recognized in the 1940s, and the enzymatic reactions involved have extensively been studied for several decades. However, hydrocarbon degradation has long been considered as an exclusive feature of aerobic microorganisms, because the initial attack at hydrocarbons is always dependent on dioxygen as a cosubstrate for mono- or dioxygenase reactions in these species. These enzymes usually bind and activate O_2 molecules to highly reactive metal-bound oxo- or dioxo-complexes that enable the abstraction of hydrogen atoms from the highly inert C–H bonds of hydrocarbons, resulting in the hydroxylation of aliphatic alkane side chains or aromatic rings via radical-based mechanisms (Harayama et al. 1992; McLeod and Eltis 2008; Fuchs et al. 2011). For example, with alkylated aromatic hydrocarbons, typical products of these initial reactions are alcohols with terminal or subterminal hydroxyl groups in their side chains or phenolic compounds with one or two hydroxyl functions in their aromatic rings.

It has only been established in 1990 that aliphatic or aromatic hydrocarbons are also degraded under completely anaerobic conditions and that these processes play an important role in nature. Typical natural environments for these processes are hydrocarbon-contaminated marine sediments or aquifers, but also deep subsurface environments, where the limited amount of oxygen as terminal electron acceptor is used up and where more easily degradable substrates (e.g., sugars, fatty acids, alcohols) are depleted. Although the use of oxygenases at such sites is no more an option, hydrocarbon degradation is still going on, indicating the need for alternative initial reactions attacking these highly inert compounds (Rabus et al. 2016a). After

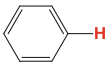
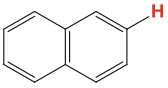
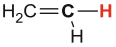
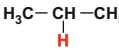
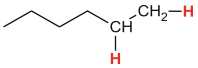
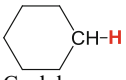
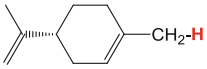
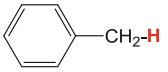
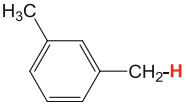
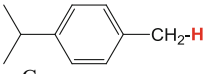
some of these alternative initial mechanisms have been identified during the last decades, very interesting additional tools for assessing the amount of anaerobic hydrocarbon degradation and the principal pathways involved have been developed in recent years, such as stable isotope fractionation analysis or sampling for functional marker genes (Musat et al. 2016; von Netzer et al. 2016). In the last three decades, the number of known anaerobic hydrocarbon-degrading bacteria and of enzymatic mechanisms replacing those of oxygenases for the initial reactions has increased continuously. A number of genome sequences of hydrocarbon-degrading anaerobic bacteria and archaea became available, enabling easier access to the genes and enzymes involved in their degradation pathways. Studies on anaerobic hydrocarbon metabolism have revealed unprecedented enzymatic mechanisms involved in C–H bond activation, which are already well understood in a few cases (e.g., ethylbenzene hydroxylation or benzylsuccinate formation) but still are at issue in others (e.g., anaerobic benzene degradation).

A number of reviews focusing on different aspects of anaerobic hydrocarbon metabolism have been published in recent years (Heider et al. 1998;Widdel and Rabus 2001; Boll et al. 2002; Heider 2007; Heider and Rabus 2008; Thauer and Shima 2008; Caldwell et al. 2008; Boll and Heider 2009; Carmona et al. 2009; Knittel and Boetius 2009; Fuchs et al. 2011; Thauer 2010, 2011; Meckenstock and Mouttaki 2011; Heider and Schühle 2013; Cui et al. 2015; Meckenstock et al. 2015, 2016; Heider et al. 2016a, b; Rabus et al. 2016a, b). This report is intended to present an overview of the known enzyme reactions involved in initial attack on hydrocarbons under anaerobic conditions. Note that some cases of anaerobic hydrocarbon-degrading bacteria have been discovered which appear to produce their own O_2 (or equivalent chemically reactive molecules) from anaerobic electron acceptors such as chlorate (Weelink et al. 2007; Salinero et al. 2009) or even nitrate or nitrite (Ettwig et al. 2010; Zedelius et al. 2011). These so-called "intra-aerobic" organisms are apparently capable of generating enough O_2 to fuel standard mono- or dioxygenases for hydrocarbon degradation, even if it does not suffice for aerobic respiration. We will not include these organisms into the topics of this chapter but rather concentrate on presenting the biochemical principles of the characterized or proposed mechanisms of the truly oxygen-independent metabolic enzymes. We also include some general considerations on the activation energies of C–H bonds of different hydrocarbons and correlate these to the types of individual enzymatic reactions involved.

2 Energetics of Hydrocarbon C–H Bonds

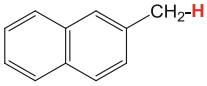
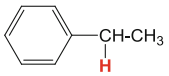
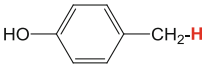
Table 1 shows the relatively high C–H bond dissociation energies of some selected aliphatic and aromatic hydrocarbons, as well as phenolic compounds. The energies range from ca. 350 kJ/mol to more than 550 kJ/mol, which suggests that not all of these compounds can be activated via the same mechanism. It is obvious that the enzymes catalyzing the initial attack on the hydrocarbons must use different strategies, depending on the amount of energy required for C–H bond cleavage.

Table 1 C–H bond dissociation energies of selected hydrocarbons and types of reactions/enzymes/cofactors involved in their activation in anaerobic bacteria. Reactions/cofactors with questions marks have not been confirmed biochemically or genetically so far. Values for C–H bond dissociation energies were taken from Blanksby and Ellison (2003), Luo (2003), and Thauer and Shima (2008). Note that several different values of dissociation energies are found in the literature for most of these compounds. The numbers given here represent representative values, which may vary by 5–10 kJ/mol in different original analyses (Luo 2003)

Compound	Dissociation energy (kJ mol^{-1})	Mechanism of attack	Enzyme	Cofactors
$HC{\equiv}C{-}H$ Acetylene	556	Water addition	Acetylene hydratase	W-cofactor, FeS cluster
 Benzene	473	Carboxylation?	Unknown	Unknown
		Hydroxylation?	Unknown	Unknown
 Naphthalene	469 (C-1) 468 (C-2)	Unknown Carboxylation	Unknown UbiD-like Carboxylase	Unknown Unknown
 Ethylene	464	Unknown	Unknown	Unknown
$H_3C{-}H$ Methane	439	Reverse methanogenesis	Methyl-CoM reductase	F_{430}/Ni
$H_3C{-}CH_2{-}CH_2{-}H$ Propane (C-1)	423	Addition to fumarate	Glycyl-radical enzyme?	Glycyl-radical/FeS?
 Propane (C-2)	405	Addition to fumarate	Glycyl-radical enzyme?	Glycyl-radical/ FeS?
 Hexane	417 (C-1) 414 (C-2)	Unknown Addition to fumarate	Unknown Glycyl-radical enzyme	Unknown Glycyl-radical/FeS
 Cyclohexane	400	Addition to fumarate	Glycyl-radical enzyme?	Glycyl-radical/FeS?
 Limonene	384	Hydroxylation by water	Flavin enzyme	FAD?
 Toluene	376	Addition to fumarate	Benzylsuccinate synthase	Glycyl-radical/FeS
 *m*-Xylene	367	Addition to fumarate	Benzylsuccinate synthase	Glycyl-radical/FeS
 *p*-Cymene	363	Addition to fumarate	Glycyl-radical enzyme	Glycyl-radical/FeS?
		Hydroxylation by water	Ethylbenzene dehydrogenase-like	Mo-cofactor FeS Heme b

(*continued*)

Table 1 (continued)

Compound	Dissociation energy (kJ mol^{-1})	Mechanism of attack	Enzyme	Cofactors
 2-Methylnaphthalene	358	Addition to fumarate	Glycyl-radical enzyme	Glycyl-radical/FeS
 Ethylbenzene	355	Addition to fumarate	Glycyl-radical enzyme	Glycyl-radical/FeS?
		Hydroxylation by water	Ethylbenzene dehydrogenase	Mo-Cofactor FeS Heme b
 *p*-Cresol	335	Addition to fumarate	Glycyl-radical enzyme	Glycyl-radical/FeS?
		Hydroxylation by water	*p*-Cresol methylhydroxylase	FAD Heme c

The C–H bond dissociation energies of unsaturated C atoms range around 460–475 kJ/mol in aliphatic compounds with double bonds or in aromatic compounds like benzene or naphthalene and even at 556 kJ/mol in case of the alkyne acetylene. These compounds exhibit by far the highest C–H dissociation energies among all hydrocarbons (Table 1). They are followed by the C–H dissociation energies of saturated alkanes, which start at a relatively high value of 439 kJ/mol for methane and sharply drop to values between 400 and 417 kJ/mol for open-chain or cyclic alkanes of longer chain lengths. It is also clear that dissociation of the terminal methyl groups of the alkanes requires more energy than that of subterminal or internal methylene groups (Table 1). Because of the inductive effects of aromatic rings, alkyl-substituted aromatic compounds exhibit even lower values of C–H bond dissociation at the C atom directly attached to the aromatic ring, mostly ranging between 350 and 370 kJ/mol (Table 1).

In aerobic organisms, the low chemical reactivity of hydrocarbons expressed by their high C–H dissociation energies is always overcome by using highly reactive metal-bound oxygen species for the initial reactions. In case of monooxygenases, a bound dioxygen molecule is partially reduced to water and an enzyme-bound oxoferryl species, which initiates a radical-based hydroxylation mechanism (Lieberman and Rosenzweig 2004; Thauer and Shima 2008). Note that the dissociation energy of H_2O to form a free hydroxyl radical is at 497 kJ/mol, indicating the potential of this type of reactive oxygen radical to cleave any C–H bond (except for that in acetylene) in an exergonic reaction. However, even the most stable hydrocarbons, such as methane, benzene, or acetylene, have been observed to be oxidized and degraded under anoxic environments in a widely distributed manner in natural habitats. It is especially hard to reconcile the very high energies of C–H bond dissociation of benzene, naphthalene, methane, or unsaturated hydrocarbons with their apparently abundant natural turnover, although these compounds usually exhibit high recalcitrance under anoxic conditions. A small number of so-called "intra-aerobic" anaerobic bacteria generate O_2 from anaerobic electron acceptors, enabling a lifestyle with

hydrocarbons degraded via standard mono- or dioxygenases, but examples of truly anaerobic degradation pathways are known for all types of hydrocarbons, even those with the highest C–H dissociation energies. The topic of this review is these truly anaerobic enzyme reactions used by hydrocarbon-degrading bacteria that cannot generate reactive oxygen species and therefore need to use alternative mechanisms for hydrocarbon activation.

The current view on anaerobic hydrocarbon metabolism indicates five different general strategies for oxygen-independent initial reactions. These involve (1) oxygen-independent hydroxylation reactions of the alkyl chains of alkylbenzenes or secondary or tertiary C atoms of aliphatic hydrocarbons, which are catalyzed by periplasmic molybdenum enzymes or flavin-containing hydroxylases. Typically, these enzymes utilize hydrocarbons or phenolic compounds with lower C–H bond dissociation energies in the range of 350–400 kJ/mol (*p*-cresol, *p*-ethylbenzene, ethylbenzene, propylbenzene, *p*-cymene, isoprenoid side chains of sterols, aliphatic alkanes with chain lengths > C10). (2) Addition of hydrocarbons to the fumarate cosubstrate that has been recognized as a second major initial reaction in many anaerobic bacteria. This results in the formation of succinate adducts of the respective hydrocarbon via the action of specialized glycyl-radical-containing, fumarate-adding enzymes. The prototype of these reactions is the conversion of toluene to (*R*)-benzylsuccinate, but analogous reactions have been reported for substituted toluenes, 2-methylnaphthalene, and even alkanes of C3 and longer chain lengths, including compounds with bond dissociation energies up to 417 kJ/mol. (3) Compounds like benzene or naphthalene with very high bound very high bond dissociation energies are proposed to be directly carboxylated by unique carboxylases of the UbiD enzyme family. (4) Acetylene or alkenes exhibit the highest C–H bond dissociation energies and seem to be degraded via water addition to the C=C double bond by different types of enzymes, avoiding the need to break the highly stable C–H bonds of the substrate. Finally, (5) methane is anaerobically degraded by a unique pathway of "reverse methanogenesis" that is established in specialized Archaea, mostly in syntrophic associations with sulfate-reducing bacteria.

3 Mechanisms of Oxygen-Independent Biochemical Hydrocarbon Activation

3.1 Anaerobic Hydroxylation

The first case of an oxygen-independent hydroxylation of a hydrocarbon has been observed in the anaerobic degradation of ethylbenzene and propylbenzene by denitrifying bacteria. This reaction is catalyzed by ethylbenzene dehydrogenase (EBDH), a soluble periplasmic molybdenum enzyme hydroxylating ethylbenzene stereospecifically with water to (*S*)-1-phenylethanol and two electron equivalents (Fig. 1a; Ball et al. 1996; Rabus and Heider 1998; Johnson and Spormann 1999; Johnson et al. 2001; Kniemeyer and Heider 2001). EBDH belongs to the dimethyl sulfoxide reductase family of molybdenum enzymes, and its structure has been

solved at 1.88 Å (Kloer et al. 2006). It consists of three subunits: the α-subunit carries a molybdenum-*bis*-molybdopterin guanine dinucleotide (MGD) cofactor and a [4Fe-4S] cluster, the β-subunit carries four further FeS clusters, and the γ-subunit contains an unusually ligated b-type cytochrome. Next to ethylbenzene, EBDH was found to hydroxylate more than 30 further substrates with exquisite stereospecificity (Szaleniec et al. 2007; Knack et al. 2012). However, the enzyme seems to be limited to substrates with side chains of two or more C atoms, since all tested analogs with methyl side chains act as inhibitors (Knack et al. 2012). A reaction mechanism (Fig. 1e) was proposed from structural and kinetic data (Kloer et al. 2006; Szaleniec et al. 2007) and further assessed by quantum mechanical (QM) and molecular mechanical modeling (QM/MM), which predicts the stepwise transfer of two

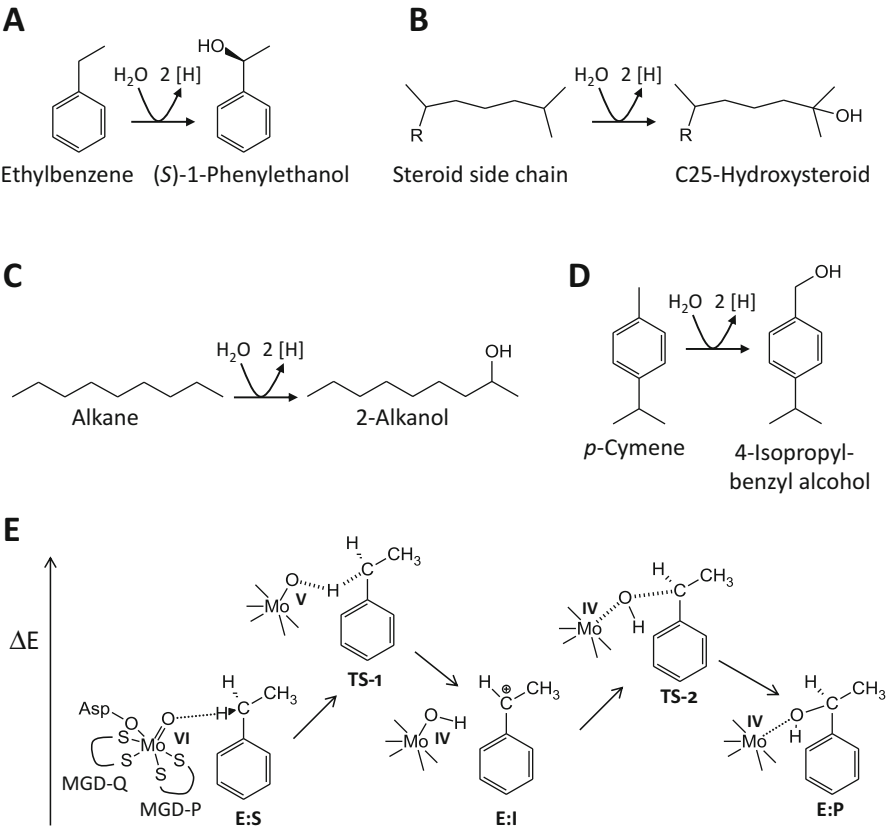


Fig. 1 Hydrocarbon hydroxylation reactions catalyzed by EBDH-like molybdenum enzymes. (**a**) Ethylbenzene hydroxylation by EBDH. (**b**) Hydroxylation of steroid side chains by cholesterol-C25-hydroxylase. (**c**) Putative alkane hydroxylation to iso-alcohols. (**d**) Hydroxylation of *p*-cymene. (**e**) Mechanistic model of ethylbenzene hydroxylation: E:S, substrate bound state; TS-1, transition state 1; E:I, bound intermediate in active site; TS-2, transition state 2; E:P, product bound state. ΔE indicates the relative energetic positions of the various states

electrons and one proton from the substrate to the MGD cofactor, reducing a Mo^{VI}-oxo starting state to a Mo^{IV}-hydroxy species with a liganded carbocation intermediate of the substrate. The last step of the mechanism then consists of a rebound reaction of the hydroxyl ligand to the carbocation intermediate, generating the alcohol products (Szaleniec et al. 2010, 2014). The model of the reaction mechanism also allowed to predict that the observed strict stereospecificity of the reaction is caused by structural prerequisites of the active site that allow a much faster reaction rate for removing the pro-(*S*) hydrogen from C1 of ethylbenzene, compared to the pro-(*R*) hydrogen (Szaleniec et al. 2014). Finally, the Mo^{VI} state of the cofactor is regenerated by the transfer of single electrons through the enzyme via the FeS clusters to the heme b-cofactor, which acts as exit site for further electron transfer to external carriers such as cytochrome c (Heider et al. 2016a).

Very recently, similar reactions have been discovered in the anaerobic degradation of the aromatic terpenoid hydrocarbon *p*-cymene, which is attacked at the methyl group to produce 4-isopropylbenzyl alcohol in *Aromatoleum aromaticum* (Fig. 1d; Strijkstra et al. 2014; Rabus et al. 2016b), and in anaerobic cholesterol metabolism in the related denitrifying bacterium *Sterolibacterium denitrificans*. In the latter case, a tertiary carbon atom of the isoprenoid side chain is anaerobically hydroxylated to a tertiary alcohol (Fig. 1b; Chiang et al. 2007; Dermer and Fuchs 2012; Heider et al. 2016a). Moreover, an EBDH-like enzyme may even be involved in an alternative pathway of anaerobic alkane degradation by the sulfate-reducing bacterium *Desulfococcus oleovorans* which does not utilize the more common alkane activation reaction via fumarate addition but rather hydroxylates alkanes at the subterminal methylene group to *iso*-alcohols (Fig. 1c; Heider and Schühle 2013; Heider et al. 2016a; Sünwoldt and Heider unpublished results).

Oxygen-independent hydroxylation reactions of alkyl substituents are also known to initiate the degradation pathways of hydrocarbon-like phenolic compounds such as *p*-Cresol (*p*-methylphenol) or *p*-ethylphenol to the corresponding alcohols or aldehydes, as demonstrated in aerobic, denitrifying, and Fe(III)-reducing bacteria (Fig. 2; Peters et al. 2007; Wöhlbrand et al. 2008). The corresponding methyl- or methylenehydroxylases have been intensively studied and appear to be similar to each other, albeit completely different from EBDH (Reeve et al. 1989; Cunane et al. 2000, 2005; Efimov et al. 2004). They are localized in the periplasm either as soluble enzymes (Reeve et al. 1989; Cunane et al. 2005) or as parts of larger membrane-bound complexes (Johannes et al. 2008) and do not contain a molybdenum cofactor. Rather, they are flavocytochromes with a covalently attached FAD cofactor at the active site, which abstract a hydride equivalent from the C1 atom of the respective alkyl group, yielding relatively stable neutral quinone methide intermediates. Water addition to the C=C double bond then results in the production of the respective alcohol. In the case of *p*-cresol methylhydroxylase a subsequent slower hydroxylation of the intermediate, 4-hydroxybenzyl alcohol, followed by water elimination from the resulting geminal alcohol, produces 4-hydroxybenzaldehyde as main product (Fig. 2a; Efimov et al. 2004; Peters et al. 2007). The reaction mechanism implies that hydroxylation by these enzymes is only possible for

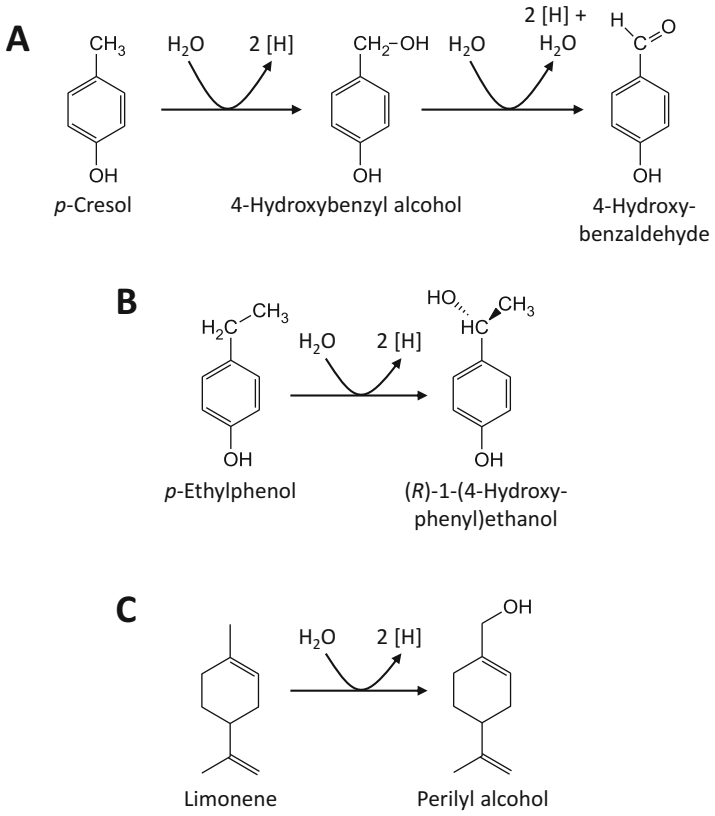


Fig. 2 Hydroxylation reactions catalyzed by flavin-containing hydroxylases. (**a**) *p*-Cresol methylhydroxylase catalyzing a two-step oxidation to 4-hydroxybenzaldehyde. (**b**) *p*-Ethylphenol methylenehydroxylase. (**c**) Limonene methylhydroxylase

substrates which can produce stabilized quinoid intermediates, e.g., via the 4-hydroxy substituent. Stabilization of the quinoid intermediate is also the main factor for the significantly lower energy of C–H bond dissociation for *p*-cresol, compared to hydrocarbon analogs (Table 1). A recent study with cell extracts of *A. aromaticum* grown either with ethylbenzene or *p*-ethylphenol showed indeed that the former exhibit hydroxylation activity with both substrates (via EBDH), but the latter are active only with *p*-ethylphenol (via methylenehydroxylase), not with ethylbenzene (Muhr et al. 2015).

Finally, a recent report described oxygen-independent hydroxylation as the initial step of anaerobic degradation of the terpenoid hydrocarbon limonene in the denitrifying betaproteobacterium *Castellaniella defragrans*, generating the corresponding perillyl alcohol (Fig. 2c; Petasch et al. 2014). The enzyme responsible has been identified on the basis of transposon mutants in the respective genes and is predicted to be a FAD-containing enzyme related to phytoene dehydrogenase, which

introduces additional double bonds during the conversion of phytoene to carotenoids (Petasch et al. 2014). While this reaction represents a methyl hydroxylation of an actual hydrocarbon substrate, the reactivity seems to depend on the presence of an adjacent C=C double bond in the substrate (Fig. 2c). Although the formal C–H dissociation energy of the methyl group is quite high (Table 1), the presence of the additional double bond is expected to stabilize partially oxidized intermediates (e.g., a carbocation intermediate after abstraction of a hydride equivalent by the flavin cofactor). The energy required for C–H bond cleavage at the methyl group should be considerably decreased by this effect, analogous to the effect of quinoid intermediates in *p*-cresol or *p*-ethylphenol hydroxylation. These "facilitated" hydroxylation reactions appear to be feasible with flavin cofactors, which are able to abstract hydride equivalents in one step, whereas the hydroxylation reactions of alkyl side chains or even alkanes in molecules without stabilizing substituents may need a molybdenum cofactor-based one-electron transfer mechanism to proceed.

3.2 Fumarate Addition

The discovery of benzylsuccinate as excreted metabolite in toluene-degrading cultures (Beller et al. 1992; Evans et al. 1992) and later as the actual initial intermediate of anaerobic toluene degradation (Biegert et al. 1996) led to the identification of a novel biochemical reaction, namely, the addition of nonactivated alkyl chains to the double bond of a fumarate cosubstrate. The enzymes involved form the subbranch of fumarate-adding enzymes (FAE) within the glycyl-radical enzymes (GRE), which also include pyruvate formate lyases or anaerobic ribonucleotide reductases (Selmer et al. 2005). The FAE are involved in anaerobic C–H bond activation for many substrates including toluene, xylenes, ethylbenzene, cresols, methylnaphthalene, cyclohexane, and *n*-alkanes reaching from propane to chain lengths of >16 C atoms. Increasing numbers of reports have been coming out in recent years on FAE, of which we selected some important original descriptions and reviews here (Biegert et al. 1996; Müller et al. 2001; Rabus et al. 2001; Wilkes et al. 2002; Kniemeyer et al. 2003, 2007; Morasch et al. 2004; Safinowski and Meckenstock 2004; Selmer et al. 2005; Heider 2007). A similar reaction has recently been discussed as alternative possible activation pathway for anaerobic methane oxidation coupled to denitrification (Thauer and Shima 2008), but disproven since, in accordance with the much higher C–H dissociation energy of methane compared to those of the known substrates of FAE (Table 1). In the following the principles of C–H bond activation by addition to fumarate are presented for benzylsuccinate synthase (BSS), the prototype of this class of enzymes, and the principal differences of FAE activating other substrates are pointed out.

BSS isoenzymes have initially been isolated and characterized from the denitrifying bacteria *Thauera aromatica* (Leuthner et al. 1998) and *Azoarcus* strain T (Beller and Spormann 1999). BSS catalyzes the first step in anaerobic toluene catabolism, the stereospecific addition of toluene to fumarate yielding (*R*)-benzylsuccinate (Biegert et al. 1996; Beller and Spormann 1998; Leutwein

and Heider 1999). The reaction has also been shown to involve a *syn*-addition of the benzyl portion and the initially abstracted hydrogen from toluene to fumarate and to result in an inversion of the configuration of the methyl group of toluene (Qiao and Marsh 2005; Seyhan et al. 2016). Like all members of the GRE, BSS needs to be activated by an *S*-adenosylmethionine-dependent radical-generating (SAM-radical) enzyme and is extremely oxygen labile in the activated state due to the presence of the glycyl-radical in the peptide chain. The presence of a glycyl-radical is indicated by a typical electron paramagnetic resonance spectrum (Krieger et al. 2001; Duboc-Toia et al. 2003; Verfürth et al. 2004) and results in the irreversible peptide chain cleavage at the position of the radical species after exposure to oxygen (Leuthner et al. 1998). The glycyl-radical is not considered to be directly involved in catalysis; it rather represents a relatively stable form of an enzyme radical that initiates the catalytic cycle by abstracting a proton from a nearby cysteine residue forming a much more reactive thiyl "working" radical (Boll et al. 2002; Himo 2005; Fig. 3). BSS is composed of three subunits with an $(\alpha\beta\gamma)_2$ heterohexamer architecture, and its structure has recently been solved (Funk et al. 2015): the large α-subunit carries the glycyl-radical and the active site, and both smaller subunits carry unusual FeS clusters which structurally resemble the clusters from high-potential iron proteins (HIPIP) but exhibit extremely low redox potentials (Funk et al. 2015; Hilberg et al. 2012). The small subunits are positioned at the outside of the complex, and their role for BSS activity is not clear (Funk et al. 2015).

Mechanistic models of the BSS reaction have been calculated by quantum mechanics methods in gas phase systems (Himo 2005), and recently an initial QM model was also based on the actual enzyme structure (Szaleniec and Heider 2016). These models support the initially proposed catalytic mechanism (Heider et al. 1998) to be initiated by the generation of thiyl radical which abstracts a hydrogen atom from the methyl group of toluene, yielding a benzyl radical. The benzyl radical then adds to the distal atom of the C=C double bond of fumarate (relative to the conserved cysteine), yielding an (*R*)-benzylsuccinyl radical, which re-abstracts a hydrogen atom from the conserved cysteine of the enzyme at the proximal C atom (Szaleniec and Heider 2016). This reaction sequence yields benzylsuccinate in the active site, while the reaction cycle is closed by regeneration of the glycyl-radical,

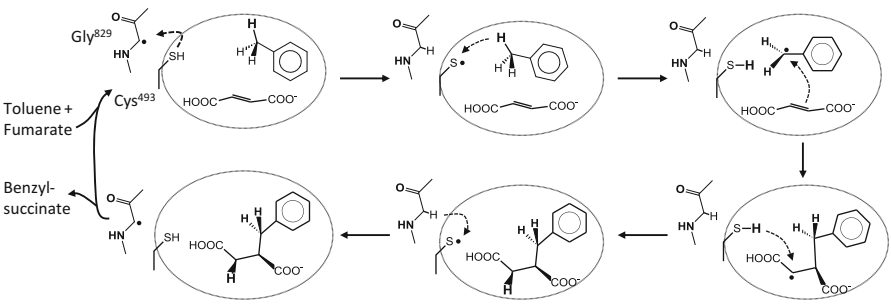


Fig. 3 Reaction mechanism of BSS. The active site cavity accessible for the substrates is indicated by the dotted ovals. Transfer steps of hydrogen atoms are indicated by broken arrows

followed by the release of the product and binding of two new substrates (Fig. 3). The modeled mechanism explains the strict stereospecificity of the reaction and also predicts the observed *syn*-addition mechanism and stereochemical inversion of the configuration of the methyl group (Szaleniec and Heider 2016). The energetics calculation of the BSS reaction predicts a clearly exergonic overall reaction as expected for the formation of a stable new C–C σ–bond at the expense of the weaker π–bond of fumarate ($\Delta G^{\circ\prime}$ of -56 kJ mol^{-1} for gas-state models and of -32 kJ mol^{-1} for enzyme-bound substrates and product in the thiyl radical state of BSS). Therefore, the reaction should be considered to be essentially irreversible, although one study reported a very slow apparent backward reaction, albeit under harsh experimental conditions (Li and Marsh 2006). The QM calculation of an active site model predicts the hydrogen transfer from toluene to the conserved cysteine as rate-limiting reaction with the highest activation energy requirement, which is consistent with the rather high observed kinetic isotope effects of BSS with deuterated toluenes (Seyhan et al. 2016; Li and Marsh 2006).

Fumarate addition is still the only known initiation mechanism for anaerobic toluene degradation so far, but not limited to this substrate. The compounds activated by FAE have steadily been increasing in number and are shown in Fig. 4. It has been noticed quite early that BSS from toluene-degrading organisms is quite flexible in converting other substrates, such as cresols, fluorotoluenes, or xylenes (Biegert et al. 1996; Beller and Spormann 1999; Verfürth et al. 2004). It appears that different strain-specific BSS isoenzymes vary in their substrate recognition patterns: BSS from the toluene and *m*-xylene-degrading *Azoarcus* strain T is apparently involved in either pathway and converts all three xylene isomers in addition to toluene, whereas BSS from *T. aromatica* strain K172, which only degrades toluene, does not convert any xylene isomer (Verfürth et al. 2004). The enzymes classified as BSS from different phylogenetic groups of anaerobic toluene degraders form a broad clade with several branches in a phylogenetic tree analysis (Heider et al. 2016b), consistent with the observation of subtle biochemical differences, not only in their substrate

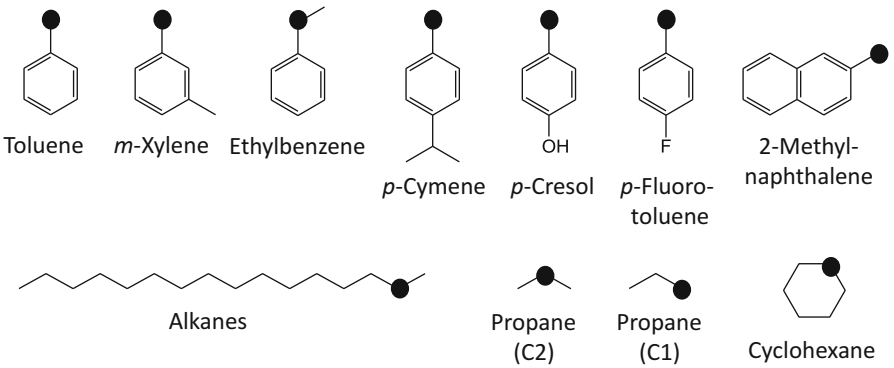


Fig. 4 Known substrates of fumarate-adding GRE. The C atom added to fumarate is labeled by a black dot

specificities but also in their properties related to stable isotope fractionations (Kümmel et al. 2013). In addition to the BSS isoenzymes with primary specificity toward toluene, a number of even further divergent FAE have been described that exhibit different substrate specificities (Heider et al. 2016b; see Fig. 4 for an overview). Among these are a *p*-cymene-activating enzyme adding fumarate to the methyl group of this substrate to generate 4-isopropylbenzylsuccinate (Strijkstra et al. 2014), a specific substrate-induced *p*-cresol-converting enzyme, which is encoded in the genome of the sulfate-reducing bacterium *Desulfobacula toluolica* as a second FAE copy next to toluene-converting BSS (Wöhlbrand et al. 2013), and an enzyme adding fumarate to 2-methylnaphthalene (Selesi et al. 2010). Finally, a last branch of FAE has been implicated in anaerobic alkane degradation (Wilkes et al. 2016; Heider et al. 2016b). This has first been shown for anaerobic alkane degradation by a denitrifying bacterium (Rabus et al. 2001; Grundmann et al. 2008) but since been demonstrated for degradation of long-chain alkanes (Herath et al. 2016; Wawrik et al. 2016) as well as for short gaseous alkanes like butane or propane (Kniemeyer et al. 2007). Fumarate addition to alkanes appears to occur generally at the subterminal methylene atom of the alkane chain, except for propane which is either activated at C2 or C1 (Kniemeyer et al. 2007; Wilkes et al. 2016). This is probably caused by the lower required energy to dissociate the C–H bond of a secondary C atom, compared to a methyl group (Table 1). All known FAE seem to share the same principal mechanism with BSS, as evident form analyzing the stereochemistry of hexane activation, which showed inversion of the configuration at the corresponding methylene carbon (Jarling et al. 2012). Subtle mechanistic differences between the isoenzymes are indicated by different patterns of deuterium exchange during the reactions, but there are not enough data available to point out their molecular basis (Rabus et al. 2011; Kümmel et al. 2013; Jarling et al. 2015). Further cases of fumarate addition to secondary methylene groups of hydrocarbons have been reported for sulfate-reducing bacteria degrading ethylbenzene (Kniemeyer et al. 2003) or cyclohexane (Jaekel et al. 2015) or for methanogenic consortia degrading isoalkanes (Abu Laban et al. 2015), but no further information on the type of enzymes is available. The different clades of FAE activating different types of substrates also seem to vary widely in their cometabolic substrate preferences. For example, the alkane-activating FAE of a denitrifying bacterium was shown to slowly cometabolize toluene, whereas toluene-activating BSS isoenzymes have never been observed to activate an alkane (Rabus et al. 2011; Jarling et al. 2015).

3.3 Carboxylation

The initial reactions involved in anaerobic degradation of benzene and naphthalene were difficult to identify because of the lack of well-growing model organisms and their slow growth rates and low yields. Most of the current knowledge has been obtained with a handful of existing naphthalene-degrading pure or highly enriched cultures and only two or three benzene-degrading enrichment cultures, which consist mostly of sulfate-reducing bacteria (Meckenstock et al. 2016). A direct carboxylation

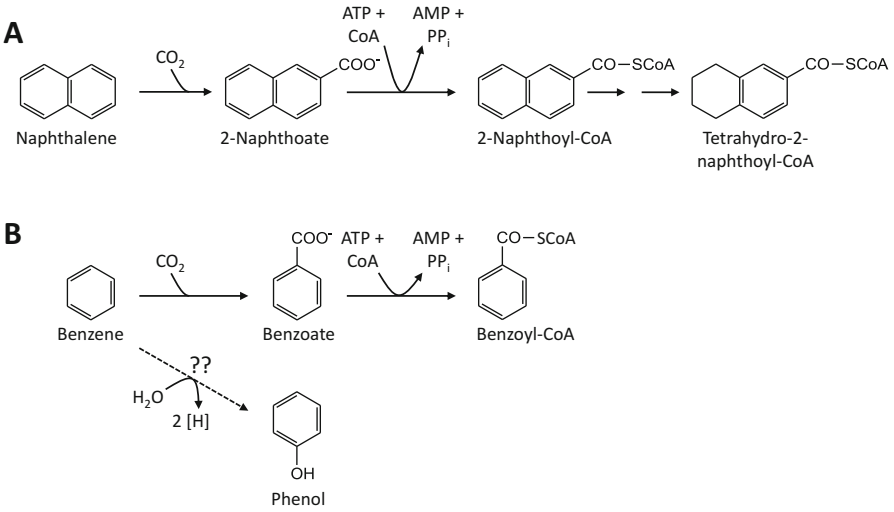


Fig. 5 Degradation pathways of naphthalene (**a**) and benzene (**b**). After an initial carboxylation, the generated organic acids are activated with CoA and subjected to ring reduction. A potential alternative pathway may involve benzene hydroxylation to phenol in some organisms

of naphthalene to 2-naphthoic acid (Fig. 5a) has been discussed as initial reaction based on labeling studies with $^{13/14}$C-bicarbonate (Zhang and Young 1997; Zhang et al. 2000). Additional evidence for an initial activation of naphthalene by carboxylation has been provided in studies with different marine sulfate-reducing and naphthalene-degrading bacteria (Musat et al. 2009; Bergmann et al. 2011a, b) and the biochemical identification of a highly complex naphthalene carboxylase (Mouttaki et al. 2012; see also ▶ Chap. 5, "Catabolic Pathways and Enzymes Involved in the Anaerobic Degradation of Polycyclic Aromatic Hydrocarbons" of this handbook). Previous hypotheses on the initiation of naphthalene metabolism by methylation to 2-methylnaphthalene (Annweiler et al. 2002; Safinowski and Meckenstock 2006) were disproven in recent years, leaving carboxylation as the only feasible initiation reaction (Meckenstock and Mouttaki 2011; Meckenstock et al. 2016). In contrast, 2-methylnaphthalene is degraded to 2-naphthoic acid independently from naphthalene by addition to fumarate and β-oxidation in analogy to anaerobic toluene degradation (Safinowski and Meckenstock 2004; Fig. 4). When grown on naphthalene, these organisms metabolized 2-methylnaphthalene only after a lag phase and a BSS α-subunit-like protein probably involved in methyl group activation was only present during growth on 2-methylnaphthalene, but not during growth on naphthalene.

Anaerobic degradation of benzene has been demonstrated in sulfate-reducing, Fe(III)-reducing, and nitrate-reducing enrichment cultures and even in methanogenic consortia (Ulrich et al. 2005; Kunapuli et al. 2008; Musat and Widdel 2008; Abu Laban et al. 2009; Sakai et al. 2009; Luo et al. 2015, and references therein). Recently, denitrifying *Azoarcus* and iron(III)-reducing *Geobacter* strains were

described as benzene-degrading pure cultures, but the evidence remains circumstantial, and no initial activation reaction of benzene has been reported (Kasai et al. 2006, 2007; Zhang et al. 2012, 2013). The dissociation energy of the C–H bond of benzene is even higher than that of methane (Table 1), which implies that a yet-unknown enzymatic reaction is involved. The mechanisms currently proposed are carboxylation to benzoate or hydroxylation to phenol, while methylation to toluene has been disfavored (Meckenstock and Mouttaki 2011).

Recent studies with Fe(III)-respiring benzene-degrading enrichment cultures using ^{13}C-labeled compounds provided convincing evidence for a direct carboxylation of benzene to benzoic acid (Kunapuli et al. 2008; Abu Laban et al. 2010). The concept of benzene carboxylation was supported by the fact that the culture degraded benzene but neither toluene nor phenol, the expected intermediates in case of initial methylation or hydroxylation (Kunapuli et al. 2008; Musat and Widdel 2008). Subunits of the putative benzene carboxylase involved in benzene degradation were identified by proteomic analysis of substrate-induced proteins and show similarity to several subunits of naphthalene carboxylase (Abu Laban et al. 2010). Moreover, both alleged carboxylases for benzene and naphthalene appear to belong to the UbiD-family of enzymes, which also includes decarboxylases involved in ubiquinone biosynthesis and phenylphosphate carboxylase, which initiates anaerobic phenol metabolism and carboxylates its substrate to 4-hydroxybenzoate (Schühle and Fuchs 2004). In contrast to phenol, benzene and naphthalene cannot be activated by an ATP-dependent phosphorylation, which is necessary for phenol carboxylation. Thus, if carboxylation indeed represents the first step in benzene or naphthalene degradation, the putative carboxylases have to operate via a different, as-yet unknown mechanism. An alternative initiation reaction for anaerobic benzene metabolism may be hydroxylation to phenol, as reported in several cases (Caldwell and Suflita 2000; Kunapuli et al. 2008; Zhang et al. 2012, 2013). A recent study reports on several genes that seem to be required for benzene degradation via phenol in *G. metallireducens* (Zhang et al. 2014), but none of the corresponding gene products can be correlated to any probable function. Therefore, a possible alternative hydroxylation-based pathway of benzene degradation is still highly speculative, especially since it has been shown that benzene may easily be hydroxylated by chemical side reactions of hydroxyl radicals, which may be formed accidentally during the handling of samples (Kunapuli et al. 2008).

Anaerobic carboxylation reactions have also been reported as initial steps for degrading some other hydrocarbons, such as biphenyl, phenanthrene, or even alkanes (Zhang and Young 1997; So et al. 2003; Callaghan et al. 2006; Selesi and Meckenstock 2009), but such reports need to be interpreted cautiously as incorporation of labeled carbon dioxide into intermediates does not necessarily prove the assumed direct carboxylation reaction of the hydrocarbon substrates.

3.4 Hydration of Alkenes and Alkynes

Unsaturated hydrocarbons like alkenes and alkynes exhibit ever higher energies for C–H bond dissociation than saturated compounds (Table 1). Therefore, their

biological degradation is mostly initiated at the multiple bond. Still, aerobic alkene degradation requires molecular oxygen as cosubstrate for monooxygenases, e.g., to convert the alkene into an epoxide intermediate. Conversely, microbial degradation of these compounds under anaerobic conditions is initiated by enzymatic addition of water at the multiple bond. While this topic has not been studied in detail, there are at least two completely different types of enzymes known that catalyze water addition at unsaturated hydrocarbons and initiate their anaerobic degradation, the tungsten cofactor containing acetylene hydratase (Boll et al. 2016), and a cofactor-less linalool dehydratase/isomerase (Brodkorb et al. 2010).

Acetylene hydratase (ACH) was purified and characterized from the fermentative bacterium *Pelobacter acetylenicus* and is involved in fermentation of acetylene as only substrate by this species (Schink 1985). The enzyme belongs to the DMSO reductase family of molybdenum enzymes but contains tungsten in the form of a W-*bis*-MGD cofactor rather than molybdenum. It has been studied in great detail over the last 30 years, including its structure and biochemical and computational studies on its reaction mechanism (Boll et al. 2016). The W-cofactor in the active site is ligated by the two MGD cofactors, a cysteine side chain of the protein and a tightly coordinated water molecule, and appears to be permanently in the reduced W^{IV} form (Seiffert et al. 2007). As indicated by biochemical experiments with mutant variants and computational modeling, the mechanism of ACH is believed to involve the water ligand of the W-cofactor, which is supposed to be added to the triple bond of acetylene aided by a close-by aspartate, although the exact mechanistic details are still unclear (tenBrink et al. 2011; Liao and Thiel 2013; Boll et al. 2016). The initially formed product is vinyl alcohol, which tautomerizes spontaneously to acetaldehyde (Fig. 6a). After acetylene has been converted to acetaldehyde by ACH, the organisms disproportionate the aldehyde to acetate (via acetyl-CoA) and ethanol to fuel its energy metabolism, resulting in almost equal concentrations of acetate and ethanol as fermentation products (Fig. 6a). Energy conservation is possible by substrate level phosphorylation from acetyl-CoA, which is generated by a CoA-acylating aldehyde dehydrogenase (Schink 1985).

An enzyme involved in anaerobic degradation of an alkene hydrocarbon substrate has recently been characterized from the terpenoid-degrading denitrifying *Betaproteobacteria Castellaniella defragrans* and *Thauera linaloolentis* (Brodkorb et al. 2010; Lüddeke et al. 2012; Marmulla et al. 2016a, b). The enzyme from *C. defragrans* is located in the periplasm and has been classified as linalool dehydratase/isomerase (LDI, Fig. 6b). It was shown to catalyze the stereospecific reversible hydration of the acyclic terpenoid alkene β-myrcene to the terpenoid alcohol (*S*)-linalool (Brodkorb et al. 2010; Lüddeke and Harder 2011), as well as an exchange reaction of the hydroxyl groups of linalool and geraniol (Brodkorb et al. 2010). The structure of LDI has recently been solved and consists of an α_5 pentameric complex with active site cavities fitting well to the substrates to be turned over. In particular, the active site contains two tightly bound water ligands which are positioned exactly adjacent to the double bonds of β-myrcene to be hydrated (Weidenweber et al. 2016). The partial reactions of LDI comprising two hydrations, an isomerization and a dehydration are perfectly balanced within the active site,

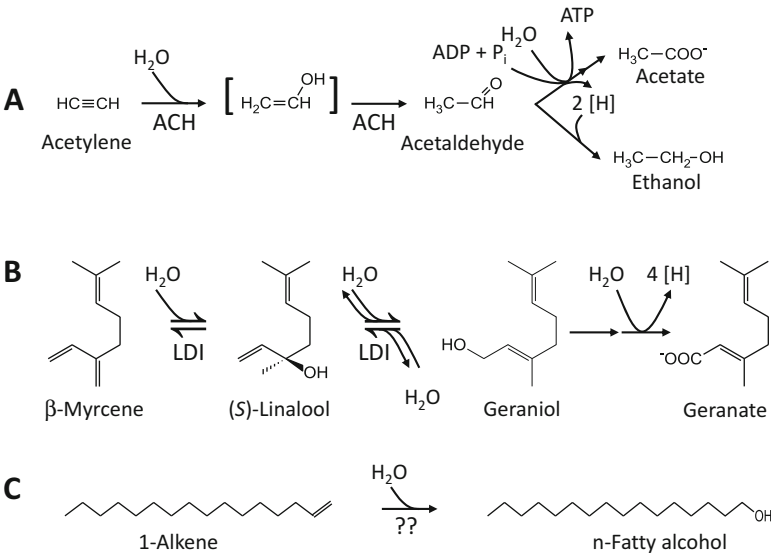


Fig. 6 Water addition reactions to unsaturated hydrocarbons. (**a**) Acetylene conversion to acetaldehyde catalyzed by ACH, followed by disproportionation to acetate and ethanol. (**b**) Degradation of the terpenoid hydrocarbon *β*-myrcene via hydration to linalool and isomerization to geraniol by LDI, followed by oxidation to geranic acid. (**c**). Proposed water addition as initial hydration reaction of long-chain alkene degradation

which explains (Brodkorb et al. 2010; Weidenweber et al. 2016). The observed preference for forming linalool over geraniol during β-myrcene hydration is probably due to the kinetically more favorable formation of the tertiary alcohol, compared to a primary alcohol. Further degradation involves a two-step oxidation of geraniol to geranic acid, which is apparently degraded by a modified β-oxidation pathway (Lüddeke and Harder 2011; Petasch et al. 2014).

Finally, it has long been known that long-chain alkenes like 1-hexadecene are degraded by many species of sulfate-reducing bacteria. These organisms generally also degrade long-chain alkanes (see Sects. 3.1 and 3.2), but by completely different pathways. The degradation of 1-alkenes seems to be initiated by water addition to the C1atom, yielding the corresponding fatty alcohol (Fig. 6c), which is then further oxidized to the fatty acid and degraded via β-oxidation or incorporated into the cellular lipid pool (Aeckersberg et al. 1998; So et al. 2003; Callaghan et al. 2006). No information is available about the enzyme(s) catalyzing this alkene hydration event.

3.5 Reverse Methanogenesis

Only since 2000 clear evidence is available that anaerobic methane oxidation is of important ecological relevance (Boetius et al. 2000; Niemann et al. 2006;

Raghoebarsing et al. 2006; Thauer 2011; Haroon et al. 2013). The organisms capable of anaerobic methane oxidation were originally limited to archaea affiliated with the order Methanosarcinales that apparently form syntrophic associations with sulfate-reducing *Deltaproteobacteria* in different phylogenetic compositions, called ANME-1 to ANME-3 (Knittel et al. 2005; Nauhaus et al. 2005). The coupling of methane oxidation to sulfate reduction in these syntrophic associations is predicted to conserve just enough energy for survival and growth of both partner organisms at very slow growth rates (Boetius et al. 2000; Thauer and Shima 2008; Thauer 2011). Only recently, a new physiological type of anaerobic methane oxidation coupled to nitrate reduction was discovered (Raghoebarsing et al. 2006; Ettwig et al. 2008), which is apparently brought about by new types of Archaea affiliated to the methanogenic genus *Methanoperedens*, that should be less restrictive in terms of retrievable energy (Haroon et al. 2013; Arshad et al. 2015). Enormous progress has been made in recent years about the molecular basis of the process as well as the required interspecies electron transfer mechanisms driving the syntrophic metabolism (reviewed, e.g., in Caldwell et al. 2008; Knittel and Boetius 2009; Cui et al. 2015; Thauer 2010, 2011).

Biochemical analysis of anaerobic methane-oxidizing microbial mats from the bottom of the Black Sea revealed high concentrations (10% of total protein) of two Ni-containing methyl-coenzyme M reductases (MCR) (Krüger et al. 2003). MCR is usually involved in the last step of methanogenesis and catalyzes the formation of methane and a heterodisulfide from methyl-coenzyme M and coenzyme B (CoB). Surprisingly, one of the two types of MCR from the Black Sea mats contained a modified Ni-tetrapyrrole cofactor (F430) with an additional methylthio residue, which is missing in usual F430 cofactors (Thauer and Shima 2008), but this seems not to be universally conserved in all examples of ANME consortia (Thauer 2011). Detailed biochemical and biophysical studies together with an X-ray structure of MCRs showing preference for methane generation or oxidation (Shima et al. 2012; Scheller et al. 2010, 2013; Thauer 2011) revealed that either form of MCR is principally reversible, while the modified "reverse MCR" versions from methane-oxidizing mats appear to have indeed a greater propensity for methane oxidation to methyl-CoM with concomitant release of CoB from heterodisulfide than "standard" MCRs involved in methanogenesis (Harmer et al. 2008; Scheller et al. 2010, 2013). Final proof for the feasibility of the concept of reverse methanogenesis came recently from transferring the genes for a "reverse MCR" into cells of the "normal" methanogenic species *Methanosarcina acetivorans*, generating a synthetic methane-oxidizing organism (Soo et al. 2016).

The mechanism of the necessary transfer of redox equivalents from the methane-oxidizing Archaea to the sulfate-reducing bacteria in the ANME consortia has been an open question for over a decade. In some recent studies, it has been found that zero-valent sulfur species may be involved as redox carriers between the syntrophic partners (Milucka et al. 2012), while another study implies electron-conductive nanowires between the cells as means of redox equivalent transfer in a thermophilic anaerobic methane-oxidizing consortium (Wegener et al. 2015). Another recent study demonstrated the necessity of some kind of redox mediation by decoupling

methane oxidation and sulfate reduction with the addition of artificial electron acceptors (Scheller et al. 2016). Taken together, the new evidence shows clearly that all currently known examples of anaerobic methane oxidation can be explained by reverse methanogenesis. While some peripheral reactions, such as the mechanism of redox equivalent transfer, may differ between the different types of ANME consortia, the actual methane-oxidizing reaction seems to be always retained in the archaeal partner and catalyzed by special "reverse MCR" isoenzymes.

4 Research Needs

The anaerobic metabolism of hydrocarbons is still a treasure chamber of novel, only poorly understood enzymatic reactions. These comprise the initial reactions involved in benzene and naphthalene degradation as well as in the anaerobic methane oxidation. Furthermore, almost nothing is known about the enzymology involved in the degradation of polycyclic aromatic or alicyclic hydrocarbons. Among all of the many proposed glycyl-radical enzymes involved in the degradation of alkanes and aromatic hydrocarbons, only benzylsuccinate synthase has been studied in some detail, and likewise only ethylbenzene dehydrogenase and cholesterol-C25 hydroxylase have been studied in detail among the hydrocarbon-hydroxylating enzymes. Studying structure–function relationships of the enzymatic reactions involved in C–H bond activation reactions without oxygen will enable insights into novel biochemical processes but may also open a door for applications of these enzymes or their variants in biotechnology, bioremediation, and ecophysiology. First pilot studies on potential biotechnological applications have actually already been published in recent years: ethylbenzene dehydrogenase has been used as model system to assess biological effects of azaborine substrate analogs (a class of compounds promising potential new therapeutic agents; Knack et al. 2013) and tested as a new way for biotechnological generation of chiral alcohols (Tataruch et al. 2014), and a synthetic anaerobic methane-oxidizing organism may be useful in turning methane into biomass (Soo et al. 2016). Moreover, the known enzymes and genes involved in anaerobic hydrocarbon degradation have been used to develop new tools to be applied in the field for applications ranging from environmental monitoring to petroleum prospecting (von Netzer et al. 2016; Muhr et al. 2016).

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