Handbook of Hydrocarbon and Lipid Microbiology

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Anaerobic Utilization of Hydrocarbons, Oils, and Lipids

Springer

Handbook of Hydrocarbon and Lipid Microbiology

This handbook is the unique and definitive resource of current knowledge on the diverse and multifaceted aspects of microbial interactions with hydrocarbons and lipids, the microbial players, the physiological mechanisms and adaptive strategies underlying microbial life and activities at hydrophobic material:aqueous liquid interfaces, and the multitude of health, environmental and biotechnological consequences of these activities.

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Anaerobic Utilization of Hydrocarbons, Oils, and Lipids

With 84 Figures and 14 Tables

Springer

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Preface

Hydrocarbons are characterized by their chemical inertness owing to the great stability of their C–H bonds and their low water solubility. For these reasons, hydrocarbons are generally classified as recalcitrant compounds. Until the 1990s, there was general consensus that biodegradation of hydrocarbons is essentially limited to aerobic bacteria and fungi. These microorganisms make intensive use of mono- or dioxygenases that use molecular oxygen as co-substrate for the oxidative attack of C–H bonds of aliphatic, alicyclic, or aromatic hydrocarbons. However, in the past decades the number of known anaerobic bacteria that completely degrade hydrocarbons to CO_2 coupled to the reduction of nitrate, sulfate, metal oxides, carbon dioxide, or protons has continuously been growing. These bacteria employ a fundamentally different enzyme inventory for the difficult attack of non activated C–H bonds or resonance-stabilized aromatic systems of hydrocarbons. Indeed, the anaerobic degradation of hydrocarbons has developed into a treasure trove of novel enzymology. Many of the only very recently identified key enzymes of anaerobic hydrocarbon degradation follow unprecedented principles and are often catalyzed by metal or radical enzymes. These key enzymes follow different mechanistic strategies to attack hydrocarbons without oxygen, depending on the nature of substrate and the strength of the C–H-bond to be cleaved. Major biocatalysts involved in anaerobic hydrocarbon degradation are (i) Mo-dependent dehydrogenases that hydroxylate secondary or tertiary carbons with water, (ii) the glycyl-radical alkyl-/arylsuccinate synthases that add alkyl groups to fumarate, (iii) (de)carboxylases of the UbiD-enzyme family, (iv) reversely operating alkyl-CoM reductases, or (v) metal containing enzymes catalyzing Birch-like dearomatization reactions.

The initial identification and biochemical characterization of these key players of anaerobic hydrocarbon degradation opened the door for multidisciplinary approaches to study anaerobic hydrocarbon degradation. The identification of their conserved encoding genes in genomes and metagenomes in a big variety of phylogenetically non-related bacteria served as starting point for a wealth of omics-based studies. They serve as valuable functional markers of anaerobic hydrocarbon degradation at environmental sites such as marine, freshwater sediments, aquifers, or oil reservoirs. Protein-based stable isotope probing has developed into a powerful tool to identify the key enzymes of anaerobic hydrocarbon degradation in environmental samples and to assign them to individual species. The mechanistic insights obtained

from key reactions of anaerobic hydrocarbon degradation represent a prerequisite for the application of compound-specific isotope fractionation, which is meanwhile widely used for the evaluation of anaerobic hydrocarbon degradation at contaminated sites.

This volume presents a compendium of current knowledge in the field of anaerobic hydrocarbon degradation from a biochemical, microbiological, and environmental perspective. The contributions span a bridge from basic research on the enzymology involved in anaerobic hydrocarbon degradation up to novel bioremediation strategies for future applications.

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Kenneth Timmis studied microbiology and obtained his Ph.D. at Bristol University. He undertook postdoctoral training at the Ruhr-University Bochum, Yale and Stanford, at the latter two as a Fellow of the Helen Hay Whitney Foundation. He was then appointed Head of an independent research group at the Max Planck Institute for Molecular Genetics in Berlin and subsequently Professor of Biochemistry in the University of Geneva, Faculty of Medicine. Thereafter, for almost 20 years, he was Director of the Division of Microbiology at the National Research Centre for Biotechnology (GBF)/now the Helmholtz Centre for Infection Research (HZI), and concomitantly Professor of Microbiology in the Institute of Microbiology of the Technical University Braunschweig. He is currently Emeritus Professor in this institute.

The Editor-in-Chief has worked for more than 30 years in the area of environmental microbiology and biotechnology, has published over 400 papers in international journals, and is an ISI Highly Cited Microbiology-100 researcher. His group has worked for many years, inter alia, on the biodegradation of oil hydrocarbons, especially the genetics and regulation of toluene degradation, and on the ecology of hydrocarbon-degrading microbial communities, discovered the new group of marine oil-degrading hydrocarbonoclastic bacteria, initiated genome sequencing

projects on bacteria that are paradigms of microbes that degrade organic compounds (*Pseudomonas putida* and *Alcanivorax borkumensis*), and pioneered the topic of experimental evolution of novel catabolic activities.

He is Fellow of the Royal Society, Member of the European Molecular Biology Organisation, Fellow of the American Academy of Microbiology, Member of the European Academy of Microbiology, and Recipient of the Erwin Schrödinger Prize. He is the founder and Editor-in-Chief of the journals *Environmental Microbiology*, *Environmental Microbiology Reports*, and *Microbial Biotechnology*.

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Matthias Boll studied Biology at the University of Freiburg and started his Ph.D. thesis in the group of Prof. Dr. Georg Fuchs at the University of Ulm and finished his thesis on dearomatizing benzoyl-CoA reductases at the University of Freiburg. He stayed as postdoctoral researcher in the group of Dr. David Lowe, John Innes Centre, Norwich, UK, where he performed spectroscopic studies on different metalloenzymes. Back at the University of Freiburg, he started his own research group and received his habilitation in the field of Microbiology. In 2006 he received a full professorship at the University of Leipzig in the Institute of Biochemistry where he studied basic and applied environmental aspects of anaerobic degradation of aromatic compounds. Since 2012 he has a full professorship at the University of Freiburg, where he was responsible for the newly established B.Sc. and M.Sc. studies. From 2008 to 2014 M. Boll was the principle coordinator of the Scientific Priority Program of the Germany Research Foundation (DFG) SPP1319: "Biological transformation of hydrocarbons without oxygen: from the molecular to the global scale." He is in an internationally leading position in the field of biochemistry and

metabolism of anaerobic degradation of aromatic compounds and steroids. M. Boll has authored around 100 articles in journals such as *Nature Chemical Biology*, *Nature Communications*, *PNAS*, *Journal of the American Chemical Society*, *Angewandte Chemie*, and *ISME Journal*.

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Part I

Biochemistry of Anaerobic Degradation of Hydrocarbons

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

1

Matthias Boll, Sebastian Estelmann, and Johann Heider

Contents

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

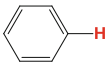
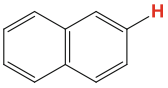
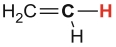
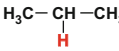
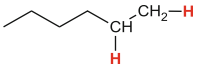
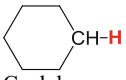
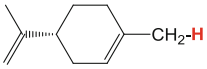
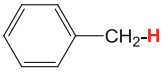
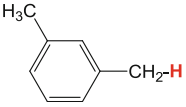
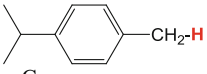
some of these alternative initial mechanisms have been identified during the last decades, very interesting additional tools for assessing the amount of anaerobic hydrocarbon degradation and the principal pathways involved have been developed in recent years, such as stable isotope fractionation analysis 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

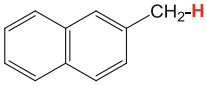
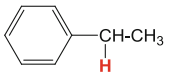
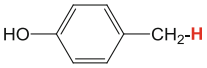
Table 1 shows the relatively high C–H bond dissociation energies of some selected aliphatic and aromatic hydrocarbons, as well as phenolic compounds. The energies range from ca. 350 kJ/mol to more than 550 kJ/mol, which suggests that not all of these compounds can be activated via the same mechanism. 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

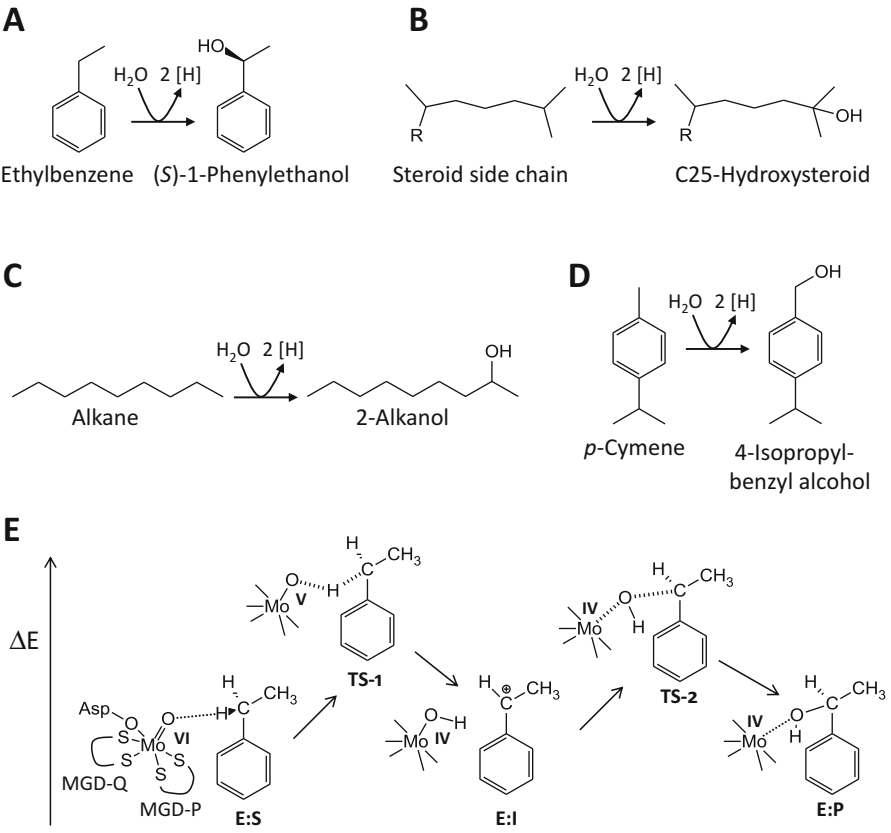


Fig. 1 Hydrocarbon hydroxylation reactions catalyzed by EBDH-like molybdenum enzymes. (**a**) Ethylbenzene hydroxylation by EBDH. (**b**) Hydroxylation of steroid side chains by cholesterol-C25-hydroxylase. (**c**) Putative alkane hydroxylation to iso-alcohols. (**d**) Hydroxylation of *p*-cymene. (**e**) 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

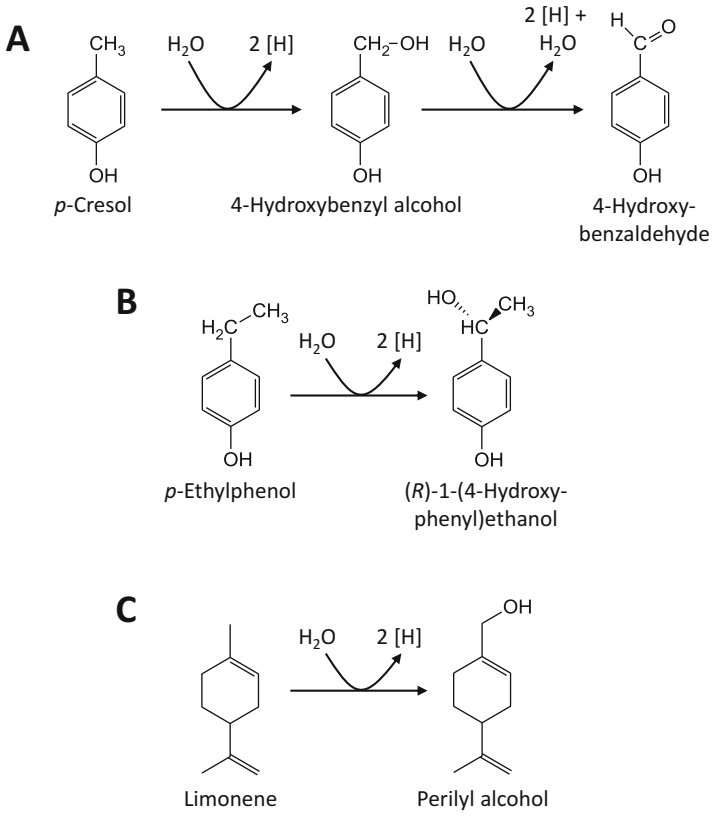


Fig. 2 Hydroxylation reactions catalyzed by flavin-containing hydroxylases. (**a**) *p*-Cresol methylhydroxylase catalyzing a two-step oxidation to 4-hydroxybenzaldehyde. (**b**) *p*-Ethylphenol methylenehydroxylase. (**c**) Limonene methylhydroxylase

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,

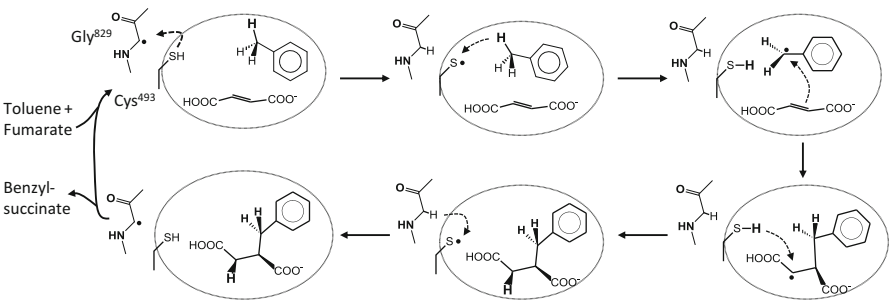


Fig. 3 Reaction mechanism of BSS. The active site cavity accessible for the substrates is indicated by the dotted ovals. Transfer steps of hydrogen atoms are indicated by broken arrows

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

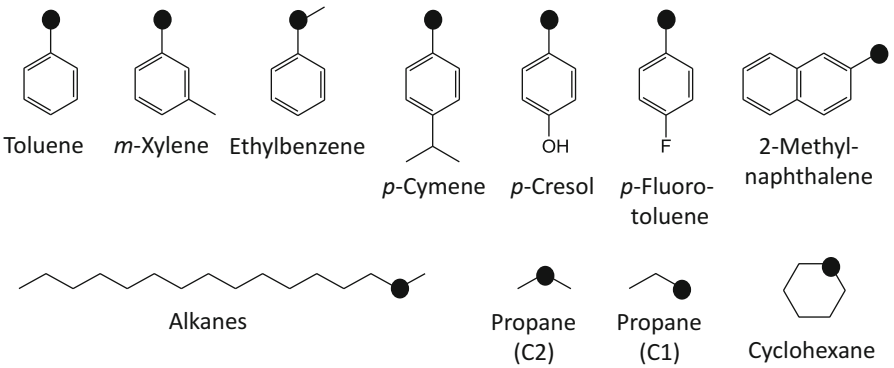


Fig. 4 Known substrates of fumarate-adding GRE. The C atom added to fumarate is labeled by a black dot

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

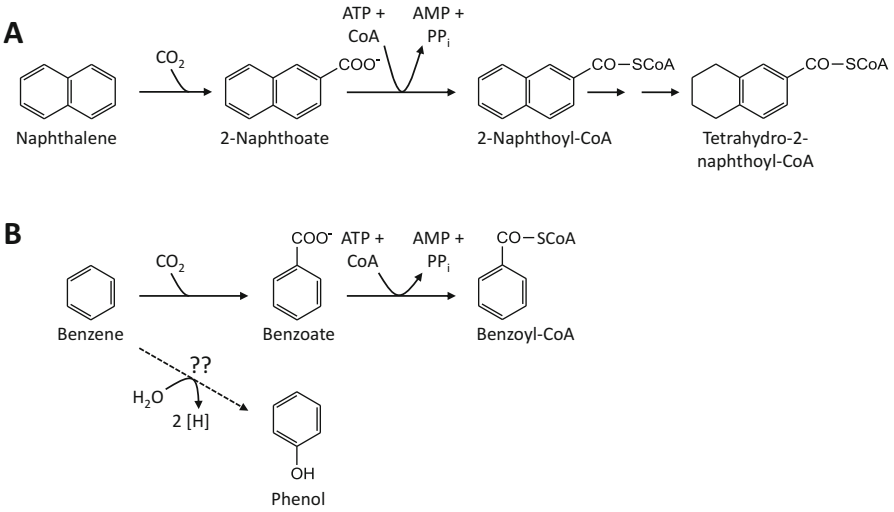


Fig. 5 Degradation pathways of naphthalene (**a**) and benzene (**b**). After an initial carboxylation, the generated organic acids are activated with CoA and subjected to ring reduction. A potential alternative pathway may involve benzene hydroxylation to phenol in some organisms

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,

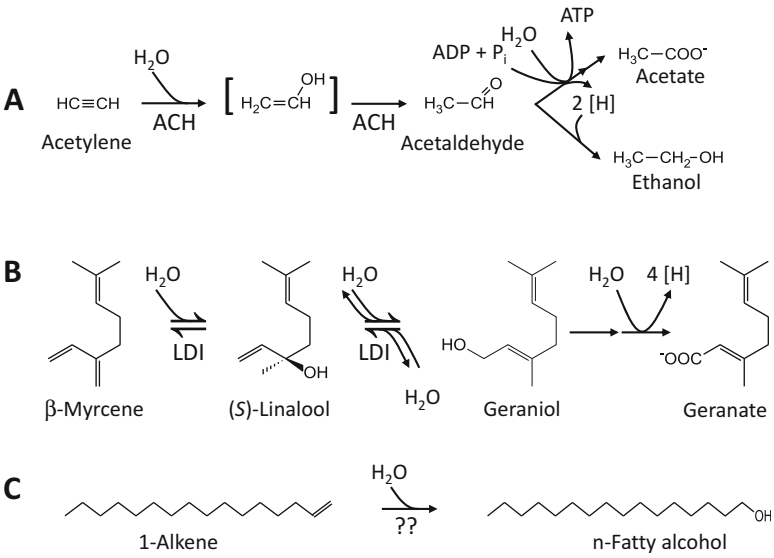


Fig. 6 Water addition reactions to unsaturated hydrocarbons. (**a**) Acetylene conversion to acetaldehyde catalyzed by ACH, followed by disproportionation to acetate and ethanol. (**b**) Degradation of the terpenoid hydrocarbon *β*-myrcene via hydration to linalool and isomerization to geraniol by LDI, 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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Catabolic Pathways and Enzymes Involved in Anaerobic Methane Oxidation

2

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Abstract

Microbes use two distinct catabolic pathways for life with the fuel methane: aerobic methane oxidation carried out by bacteria and anaerobic methane oxidation carried out by archaea. The archaea capable of anaerobic oxidation of methane, anaerobic methanotrophs (ANME), are phylogenetically related to methanogens. While the carbon metabolism in ANME follows the pathway of reverse methanogenesis, the mode of electron transfer from methane oxidation to the terminal oxidant is remarkably versatile.

This chapter discusses the catabolic pathways of methane oxidation coupled to the reduction of nitrate, sulfate, and metal oxides. Methane oxidation with sulfate and metal oxides is hypothesized to involve direct interspecies electron transfer and extracellular electron transfer. Cultivation of ANME, their mechanisms of energy conservation, and details about the electron transfer pathways to the ultimate oxidants are rather new and quickly developing research fields, which may reveal novel metabolisms and redox reactions. The second section focuses on the carbon catabolism from methane to CO_2 and the biochemistry in ANME with its unique enzymes containing Fe, Ni, Co, Mo, and W that are compared with their homologues found in methanogens.

1 Introduction

Life can be sustained with energy from only slightly exergonic chemical reactions. The anaerobic oxidation of methane (AOM) with sulfate is an extreme example, as the overall free energy yield allows for the production of less than one ATP per methane oxidized. In addition to this small thermodynamic driving force, the activation energy for the non-catalyzed reaction is particularly high, because methane and sulfate are inert compounds. Nevertheless, AOM with sulfate is the globally most important anaerobic sink of the potent greenhouse gas methane and thus relevant in the context of global climate changes.

AOM with sulfate as the oxidant is carried out syntrophically (syntrophy = Greek, "eating together"), which means that this catabolic process is split into two distinct organisms. Anaerobic methanotrophs (ANME archaea) carry out the oxidative (anodic) half-reaction from methane to CO_2 and sulfate-reducing bacteria carry out the reductive (cathodic) half-reaction from sulfate to hydrogen sulfide. As a consequence, the two partners are strictly dependent on each other and need to share the small energy yield derived from their overall catabolism. This chapter summarizes current knowledge about the syntrophy of AOM with sulfate and discusses methane oxidation pathways with the oxidants nitrate and metal oxides.

2 Catabolic Pathways Involved in Methane Oxidation

2.1 Geobiochemical Pathways of Methane Oxidation

Around 1.5 Gt per year of methane are produced via microbial and geochemical processes. Microbes consume most of it via oxidation with various electron acceptors in the subsurface, which limit input into the atmosphere. Figure 1 summarizes the global methane cycle with its most important sources and sinks, indicating different electron acceptors used for methane oxidation.

In the presence of molecular oxygen, Alphaproteobacteria, Gammaproteobacteria (Semrau et al. 2010), and Verrucomicrobia (Op den Camp et al. 2009) can thrive from methane oxidation using methane monooxygenases that utilize oxygen-derived radicals for the activation of methane. Methanol is obtained as the first intermediate that is further catabolized via formaldehyde and formate to CO_2 (Chistoserdova et al. 2005). The anaerobic organism *Methylomirabilis oxyfera* developed the ability to produce its own oxygen for the conversion of methane to methanol. It uses nitrite as the oxidant and converts it via nitric oxide to N_2 and O_2 that is utilized for radical methane activation (Ettwig et al. 2010). *M. oxyfera* may thus be classified as an

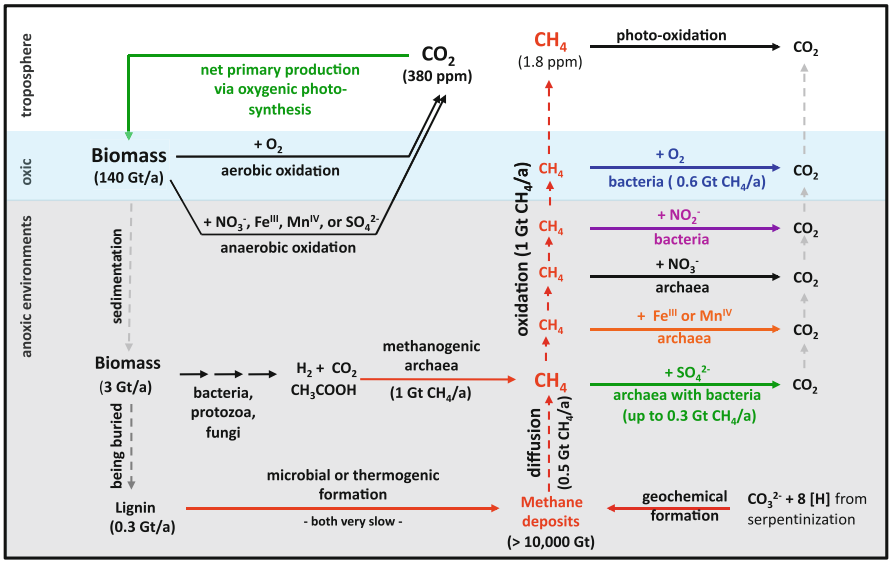

Fig. 1 Summary of global methane cycle. In oxic environments, methane is oxidized with molecular oxygen, a very exergonic reaction that provides ample energy for bacterial growth. In the anaerobic zones, the alternative oxidants nitrite, nitrate, metal oxides, and sulfate are respired for microbial methane catabolism (Modified from Thauer 2011)

obligate anaerobe (Luesken et al. 2012) that carries out an aerobic pathway of methane activation. Here we describe pathways and enzymes related to methane oxidation devoid of molecular oxygen.

2.2 Anaerobic Oxidation of Methane (AOM): The Reverse of Methanogenesis

A special class of archaea termed anaerobic methanotrophs (ANME archaea) that are phylogenetically related to methanogens carry out AOM. It has originally been speculated that they carry out the reverse of hydrogenotrophic methanogenesis according to Eq. 1 (Hoehler et al. 1994):

$$CH_4 + 2H_2O = CO_2 + 4H_2 \quad \Delta G^{\circ\prime} = +131 \text{ kJ mol}^{-1} \tag{1}$$

$$CH_4(80 \text{ bar}) + 2H_2O = CO_2(1 \text{ bar}) + 4H_2\left(10^{-6}\text{bar}\right) \quad \Delta G' = \sim 0 \text{ kJ mol}^{-1} \tag{1'}$$

Metagenomic analysis (Hallam et al. 2004) and enzymatic studies (Scheller et al. 2010) support a catabolic pathway of CH_4 oxidation to CO_2 via the reverse of methanogenesis. The reducing equivalents from AOM, however, are not released via hydrogen formation, because hydrogen concentrations would need to be kept below 1 nM (ca. 10^{-6} bar) to conserve energy from Eq. 1 ($\Delta G' < 0$, Eq. 1'). At such low hydrogen concentrations, the possible maximal diffusion rates would be distinctly smaller than the diffusion rates calculated for the observed methane oxidation rates (Orcutt and Meile 2008).

The pathway of reverse methanogenesis is initiated by the enzyme methyl-coenzyme M reductase (Mcr) that cleaves the C–H bond in methane using its nickel cofactor F430 (Jaun and Thauer 2007) and transfers the resulting methyl group to the thiolate moiety of coenzyme M. The sulfur-bound methyl-group in methyl-S-CoM is subsequently transferred to N5 of the C_1-carrier tetrahydromethanopterin (H_4MPT) and further oxidized to CO_2 as depicted in Fig. 2a.

The pathway from methyl-S-CoM to CO_2 is identical to the oxidative branch in methylotrophic methanogens, where methyl-S-CoM derived from methanol, methylamines, or methanethiol (X = O, N, or S) is disproportionated to CH_4 and CO_2 in a 3:1 stoichiometry (Fig. 2b). Therefore, only the reversal of the enzyme Mcr and the reversal of the heterodisulfide reductase (Hdr) are unique to AOM and unprecedented in methanogens.

For methylotrophs, the 6 electrons generated from the oxidation of the methyl group are internally re-cycled to reduce 3 heterodisulfides (CoM-S-S-CoB), which allows disposing these electrons via methane production (Fig. 2b). In the case of full methane oxidation, all 8 electrons obtained need to be disposed externally (Fig. 2a) via different transfer pathways to the ultimate oxidants as described below.

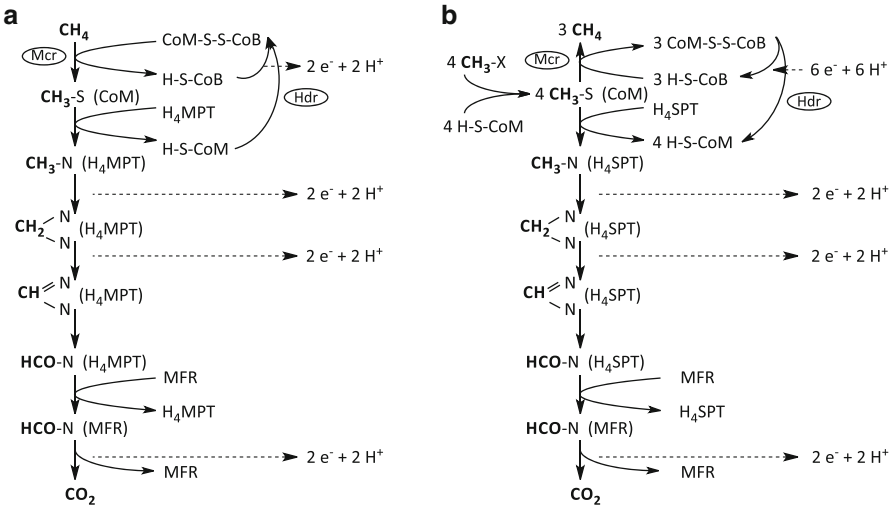

Fig. 2 Catabolic pathway of AOM and methanogenesis. *Mcr* methyl-S-CoM reductase. *Hdr* heterodisulfide reductase. C_1-carriers and enzymes are discussed in the second part of this chapter; methylotrophic methanogenic archaea contain tetrahydrosarcinapterin (H_4SPT) instead of the H_4MPT. (**a**) Complete reverse methanogenesis pathway from CH_4 to CO_2 as present in ANME archaea. (**b**) Metabolism in methylotrophic methanogens that disproportionate the substrates methanol, methylamines, or methanethiol (CH_3-X) to CH_4 and CO_2 for comparison

2.3 AOM Coupled to Nitrate Reduction

Anaerobic oxidation of methane with nitrate (Raghoebarsing et al. 2006) is currently the best-understood mode of anaerobic methane oxidation, because highly enriched co-cultures are available that yielded comprehensive genomic and transcriptomic data (Haroon et al. 2013). AOM is carried out by *Candidatus* "*Methanoperedens nitroreducens*," also known as ANME-2d, which uses a nitrate reductase to dispose methane-derived electrons via the formation of nitrite (Eq. 2):

$$CH_4 + 4NO_3^- = CO_2 + 4NO_2^- + 2H_2O \quad \Delta G^{\circ\prime} = -521 \text{ kJ mol}^{-1} \qquad (2)$$

Reported enrichments of ANME-2d require co-cultures with partner-bacteria that remove the toxic product nitrite efficiently. Incubations with methane and nitrate as the sole energy substrates resulted in a co-culture dominated by ANME-2d and *M. oxyfera*, whereby both organisms carry out methane oxidation (Ettwig et al. 2008). The nitrogen metabolism is partitioned, ANME-2d reduces nitrate to nitrite (Eq. 2), and the *M. oxyfera* partner reduces nitrite to molecular nitrogen (Fig. 3a).

By feeding equimolar amounts of ammonium and nitrate to the methanotrophic culture, ANME-2d are enriched with Kuenia partner bacteria instead of *M. oxyfera* (Haroon et al. 2013). In this case, ANME-2d are the sole species with methanotrophic capabilities. The *Kuenenia* bacteria remove the toxic product nitrite via the anammox

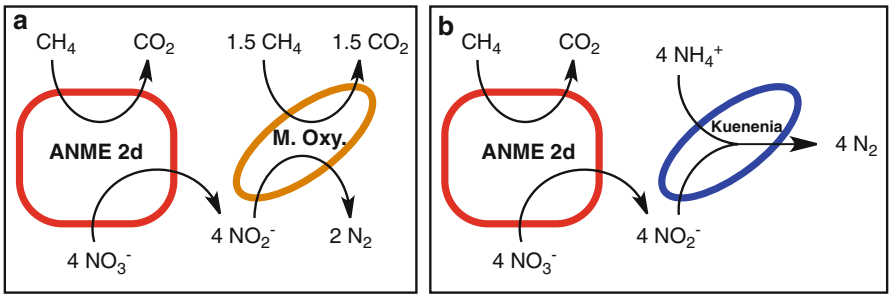

Fig. 3 Co-cultures of ANME-2d with partner-bacteria that remove the toxic product nitrite efficiently. (**a**) Combination of anaerobic and aerobic methanotrophy involving ANME-2d and *M. oxyfera* (Raghoebarsing et al. 2006). (**b**) Co-culture of ANME-2d with Kuenenia for nitrite removal (Haroon et al. 2013)

pathway, the comproportionation of nitrite and ammonium to molecular nitrogen (Fig. 3b). Evaluation of the substrate and product fluxes in the bioreactors experimentally verified the expected stoichiometry between the overall substrates methane, nitrate, and ammonium and the product nitrogen (Eq. 3) (Haroon et al. 2013):

$$CH_4 + 4NO_3^- + 4NH_4^+ = CO_2 + 4N_2 + 10H_2O \quad \Delta G^{\circ\prime} = -1952 \text{ kJ mol}^{-1} \quad (3)$$

ANME-2d may serve as the model ANME for physiological studies and for isolation of active enzymes, since highly enriched co-cultures are available. Genome sequencing confirmed the presence of all genes required for the pathway of reverse methanogenesis; most of them, in particular the key enzyme Mcr, are highly expressed (Haroon et al. 2013).

Metagenomic data of related organisms suggest that nitrate used as the oxidant for AOM might be fully reduced to ammonium by the ANME-2d alone (Arshad et al. 2015), but physiological details were not reported yet. Such studies highlight that we have just begun to gain control over the cultivation and physiology of some ANME members and that the microbial diversity is a treasure box filled with metabolic surprises.

2.4 AOM Coupled to Sulfate Reduction

Geochemists reported long before the discovery of ANME archaea that a pathway of methane oxidation coupled to sulfate reduction (Eq. 4) must be present at benthic methane seeps, based on inverse sulfate-methane concentration gradients measured (Iversen and Jorgensen 1985).

$$CH_4 + SO_4^{2-} + 2H^+ = CO_2 + H_2S_{(aq.)} + 2H_2O \quad \Delta G^{\circ\prime} = -21 \text{ kJ mol}^{-1} \quad (4)$$

Fluorescence in situ hybridization with oligonucleotide probes targeting the 16S rRNA allowed the visualization of microorganisms apparently mediating AOM coupled to sulfate reduction (Fig. 4a) (Boetius et al. 2000).

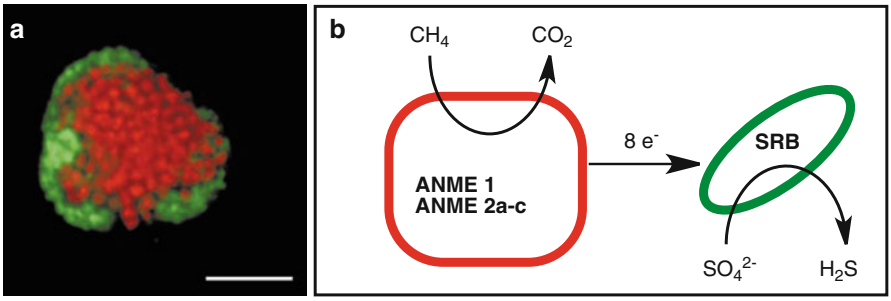

Fig. 4 (**a**) Microscopy image of ANME-SRB consortium apparently mediating AOM: *red* (*dark color*) = ANME, *green* (*light color*) = SRB; scale bar = 5 μm (transferred from Boetius et al. 2000). (**b**) Cartoon of the metabolism of AOM with sulfate and its compartmentalization into ANME archaea and SRB

The high diversity of ANME phylotypes involved in the AOM with sulfate can be dissected into two main groups with distinct morphology. ANME-1, which are related to methanogens of the order Methanomicrobiales, form loose associations with sulfate-reducing bacteria (SRB) and are often found as single cells (Orphan et al. 2002; Losekann et al. 2007). ANME-2a-c and ANME-3, which are related to Methanosarcinales (Knittel and Boetius 2009), form tight aggregates with SRB as exemplified in Fig. 4a.

The correlation of the phylogenetic identity with stable isotope analyses indicated that those archaea are carrying out methane oxidation (Orphan et al. 2001). AOM with sulfate is thus a syntrophic reaction requiring both ANME and SRB. This process can be considered as a single overall metabolism that is split into two distinct organisms, which requires transfer of reducing equivalents (Fig. 4b). ANME archaea are responsible for the carbon metabolism and SRB are responsible for the sulfur metabolism. A study involving sulfur stable isotope probing suggested that ANME may carry out both methane oxidation and sulfate reduction (Milucka et al. 2012), but the responsible archaeal genes for sulfate-reduction have not been assigned to date.

The mechanism of electron transfer from ANME to their SRB partners has been a mystery for a long time. Investigation of AOM communities is hampered by high genetic diversity, yielding only incomplete genomic data, and by the slow growth rates, with doubling times range between 2 and 9 months under laboratory conditions (Girguis et al. 2005; Nauhaus et al. 2007; Meulepas et al. 2009; Holler et al. 2011).

A major goal in the field is the decoupling of the syntrophy between ANME and SRB that would open the door to grow the individual organisms in pure cultures. Supplying the specific substrate (intermediate) for one partner only, or adding a compound that removes the specific product (intermediate) efficiently, should induce selective stimulation of only one type of organisms. Putative intermediates, such as hydrogen, formate, and acetate, have been extensively tested in laboratory studies (Nauhaus et al. 2005; Meulepas et al. 2010). For a thermophilic enrichment of ANME-1 and SRB, the bacterial partner of the ANME-1 could be stimulated by hydrogen addition (Wegener et al. 2015, 2016), which allowed to highly enrich (>95%) these

sulfate-reducing bacteria (Krukenberg et al. 2016). Hydrogen, however, appears to be an alternative electron donor for these particular SRB and not to be produced by the corresponding ANME during sulfate-driven AOM (Wegener et al. 2015, 2016). All attempts failed to detect diffusive intermediates consumed or produced by the ANME.

Single cell activity mapping via secondary-ion-mass-spectrometry at the nanometer scale of active AOM-SRB consortia in combination with metabolic modeling excluded diffusive intermediates as electron carriers and suggested that the syntrophy is based on direct interspecies electron transfer from the ANME to their bacterial partners (McGlynn et al. 2015).

2.5 AOM with Release of Single Electrons

Multiheme *c*-type cytochromes possibly involved in single electron transfer were discovered in metagenome sequence analyses (Meyerdierks et al. 2010; Kletzin et al. 2015). Later, the hypothesis of direct interspecies electron transfer (DIET) as the coupling mechanism between ANME and SRB could be derived from two independent studies using ANME-1 and ANME-2, respectively. In the study with ANME-1, evidence for methane oxidation via the release of single electrons was derived from putative nanowires visualized by electron-microscopy (Fig. 5a) and transcriptional studies (Wegener et al. 2015). In the study with ANME-2, the putative multiheme *c*-type cytochromes were stained in situ and localized by electron-microscopy (McGlynn et al. 2015).

Mechanistically, methane-derived electrons travel via electrical conductance to the bacterial partners of the ANME. The basis to achieve such an electron transfer is an "electron-hopping" mechanism following the array of aligned iron centers in the multiheme *c*-type cytochromes (Pirbadian and El-Naggar 2012). Catabolic pathways involving release of single electrons and DIET are known from the laboratory organisms *Shewanella* and *Geobacter* (Gorby et al. 2006; Gralnick and Newman 2007; Lovley 2012). According to this hypothesis of DIET in ANME-SRB consortia, electrons derived from reverse methanogenesis reduce the methanophenazine, which is present in the membrane of ANME, from which single electrons are transferred via multiheme *c*-type cytochromes to the surface of the cell, through the S-layer domain towards the bacterial partner that is tightly aggregated to the ANME (Fig. 5b).

Apparently both ANME-1 and ANME-2a-c simply carry out the half reaction of methane oxidation (Eq. 5), which suggests that the overall metabolism of methane oxidation with sulfate is equally split into the carbon and sulfur half reactions without transfer of molecular primary metabolites.

$$CH_4 + 2H_2O = CO_2 + 8H^+ + 8e^- \quad E^{\circ\prime} = -245\ \text{mV} \tag{5}$$

Following this proposal, it should be possible to decouple AOM from sulfate reduction via an auxiliary cathodic half-reaction that removes single electrons. Experiments with single electron acceptors, such as anthraquinone-disulfonate (AQDS), demonstrated methane oxidation by ANME-2a + c in the absence of sulfate (Scheller et al. 2016) according to Eq. 6.

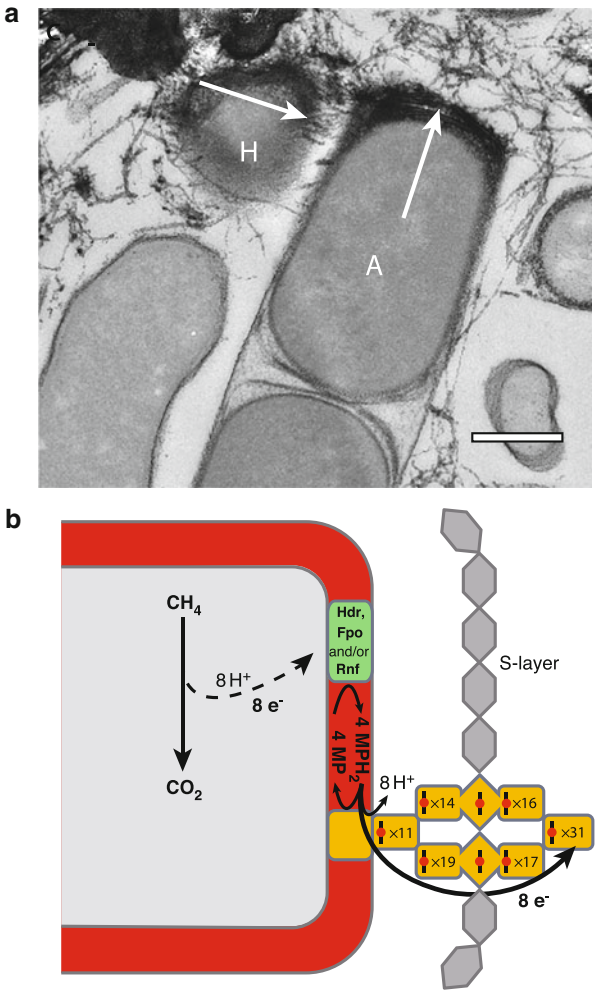

Fig. 5 Mechanism of electron transfer from ANME to SRB. (**a**) Putative microbial nanowires (*arrows*) between ANME-1 (*A*) and HotSeep-1 SRB (*H*) visualized via electron microscopy (scale bar = 300 nm; transferred from Wegener et al. 2015). (**b**) Simplified scheme for the electron transfer from ANME-2a-c to their SRB partners. Reducing equivalents from methane oxidation generate reduced methanophenazine (MPH_2) via different membrane-bound enzymes (putatively Hdr, Fpo, and Rnf). Multiheme *c*-type cytochromes (*orange*) transport the electrons from MPH_2 through the membrane and the S-layer domain to the outside of the cell to be taken up by the syntrophic partner (Modified from McGlynn et al. 2015)

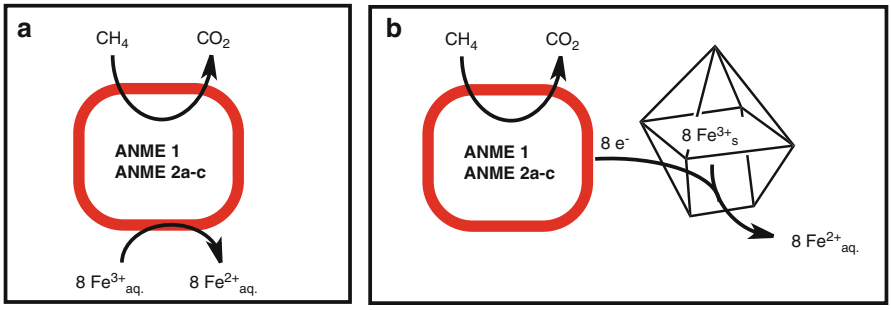

Fig. 6 Extracellular electron transfer from ANME to the environment. (**a**) Electron transfer to soluble iron(III)-complexes reported for ANME-2a + c (Scheller et al. 2016) (**b**) Putative respiratory metabolism allowing for AOM coupled to metal oxide reduction (Rotaru and Thamdrup 2016)

$$CH_4 + 4AQDS + 2H_2O = CO_2 + 4\ AQH_2DS \quad \Delta G^{\circ\prime} = -46\ \text{kJ mol}^{-1} \qquad (6)$$

With AQDS, the catabolic rate of AOM and biomass synthesis in ANME-2a + c cells was similar as with the native oxidant sulfate, but the bacterial partners remained completely inactive (Scheller et al. 2016). Similar results were obtained with a range of different single electron acceptors, such as soluble iron(III)-complexes or humic acids, which is expected according to the hypothesis of DIET as the syntrophic mechanism between ANME and their sulfate-reducing partners.

This metabolic separation of archaeal methane oxidation from their partner bacteria lays the foundation to grow ANME in pure cultures, because the bacterial partners may be outcompeted by the auxiliary electron acceptors.

2.6 AOM Coupled to Metal Oxide Reduction

Iron and manganese oxides have been reported to serve as oxidants for methane oxidation (Beal et al. 2009), but no specific organism could be directly attributed to carry out this catabolism. The apparent ability of multiple members of ANME to carry out AOM with release of single electrons may explain these findings on the basis of extracellular electron transfer from ANME to the metal oxide particles (Fig. 6), a proposal that currently lacks direct experimental support.

While highly specialized in their carbon metabolism, ANME seem to be promiscuous for the terminal oxidant, implying that one specific type of organism may use sulfate-reducing bacteria or metal oxides as the sink for methane-derived electrons. Aggregates of ANME archaea belonging to different clades have been found to be active in the absence of a bacterial partner (Orphan et al. 2002), which could be explained by a metabolism of electron transfer from these ANME to redox active components present in the corresponding environment, such as metal oxide particles.

Extracellular electron transfer to various oxidants may be an evolutionary transient solution of a methanogen on its way to a syntrophic partnership with sulfate-reducing bacteria. Perhaps methylotrophs evolved coupling of AOM to metal oxide reduction as a first and syntrophy with sulfate-reducing bacteria as a second evolutionary step (Scheller et al. 2016). However, further research is needed to substantiate such a speculation and to shed light on the process of AOM coupled to metal oxide reduction.

3 Enzymes Involved in AOM

3.1 General Aspects of AOM Biochemistry

Homologues of methanogenic enzymes catalyze the oxidation of methane to CO_2. This bi-directionality of the methanogenesis pathway is enabled via the relatively flat energy-profile of all intermediates involved (Thauer 1998, 2011; Hedderich and Whitman 2006; Thauer et al. 2008; Ferry 2010; Welte and Deppenmeier 2014;

Costa and Leigh 2014). The utilization of the same catabolic pathway in both directions highlights the role of enzymes to simply lower transition state energies between intermediates. The equilibria between the intermediates and thus the directionality of the overall pathway are determined by the substrate and product concentrations, coupling to redox-cofactors, and by ion-gradient-consuming or producing enzymes. The exact mechanism of energy conservation in ANME is still speculative and may vary between different phylotypes. Figure 7 presents the catabolic pathway of reverse methanogenesis with its corresponding enzymes and redox-cofactors.

Reverse methanogenesis implicates a different chemistry for the challenging step of alkane activation in comparison to the pathway present in most organisms carrying out anaerobic oxidation of long-chain alkanes. For long-chain alkanes, enzyme-bound radicals abstract a H atom of the alkane, which is then transferred to fumarate (Widdel and Grundmann 2010). The only exception currently known is the anaerobic oxidation of butane by archaea, which has just recently been discovered (Laso-Pérez et al. 2016). For methane, the particularly strong C–H bond is cleaved via organometallic chemistry rather than via a conventional radical abstraction. This reaction is carried out by methyl-coenzyme M reductase (Mcr) (Gunsalus and Wolfe 1980) that uses CoM-S-S-CoB as the oxidant. For the enzymatic part of this contribution, we discuss this unique reaction in detail. The Mcr-catalyzed reaction of methane activation and methyl-S-CoM formation is associated with a Gibbs free energy difference ($\Delta G^{\circ\prime}$) of +30 kJ mol^{-1} at standard conditions. With 8 bars of methane and 1 mM intracellular CoM-S-S-CoB, for example, net methane oxidation occurs if the intermediates methyl-S-CoM and HS-CoB are kept at concentrations below 0.2 mM each ($\Delta G' = 0$). The enzymes Mtr and Hdr (Fig. 7) probably keep the concentrations of methyl-S-CoM and HS-CoB low in order to drive methane oxidation. Hdr catalyzes formation of CoM-S-S-CoB from HS-CoM, HS-CoB and reduced ferredoxin; the electron acceptor is postulated to be two molecules of F_{420}, but not experimentally confirmed.

The endergonic methyl group transfer from methyl-S-CoM to methyl-H_4MPT ($\Delta G^{\circ\prime} = +30$ kJ/mol) is driven by coupling to the exergonic sodium ion influx through the cytoplasmic membrane ($\Delta G' = -30$ kJ/mol) (Gottschalk and Thauer 2001; Thauer et al. 2008). Methyl-H_4MPT is dehydrogenated to methylene-H_4MPT ($\Delta G^{\circ\prime} = +6.2$ kJ/mol). Methylene-H_4MPT is dehydrogenated to methenyl-H_4MPT^+ by F_{420}-dependent methylene-H_4MPT reductase reaction ($\Delta G^{\circ\prime} = -5.5$ kJ/mol). Methenyl-H_4MPT^+ is hydrolyzed to formyl-H_4MPT and then the formyl group is transferred to methanofuran. The last reaction in AOM is catalyzed by formylmethanofuran dehydrogenase (Fmd or Fwd), by which formylmethanofuran (formyl-MFR) is oxidized to CO_2 and methanofuran (MFR). This reaction is almost in equilibrium at standard conditions ($\Delta G^{\circ\prime} = 0$ kJ/mol) because the redox potential of ferredoxins ($E_0' = -500$ mV) and CO_2 + MFR/formyl-MFR couple ($E_0' = -530$ mV) are similar (Kaster et al. 2011).

The predicted metabolic pathway and the primary structure of the enzymes involved in reverse methanogenesis was deduced from metagenomic data of sulfate-dependent ANME-1 and ANME-2b and nitrate-dependent ANME-2d (Hallam et al. 2004; Meyerdierks et al. 2010; Stokke et al. 2012; Haroon et al. 2013; Arshad et al. 2015). Some ANME-1 enzymes have been heterologously

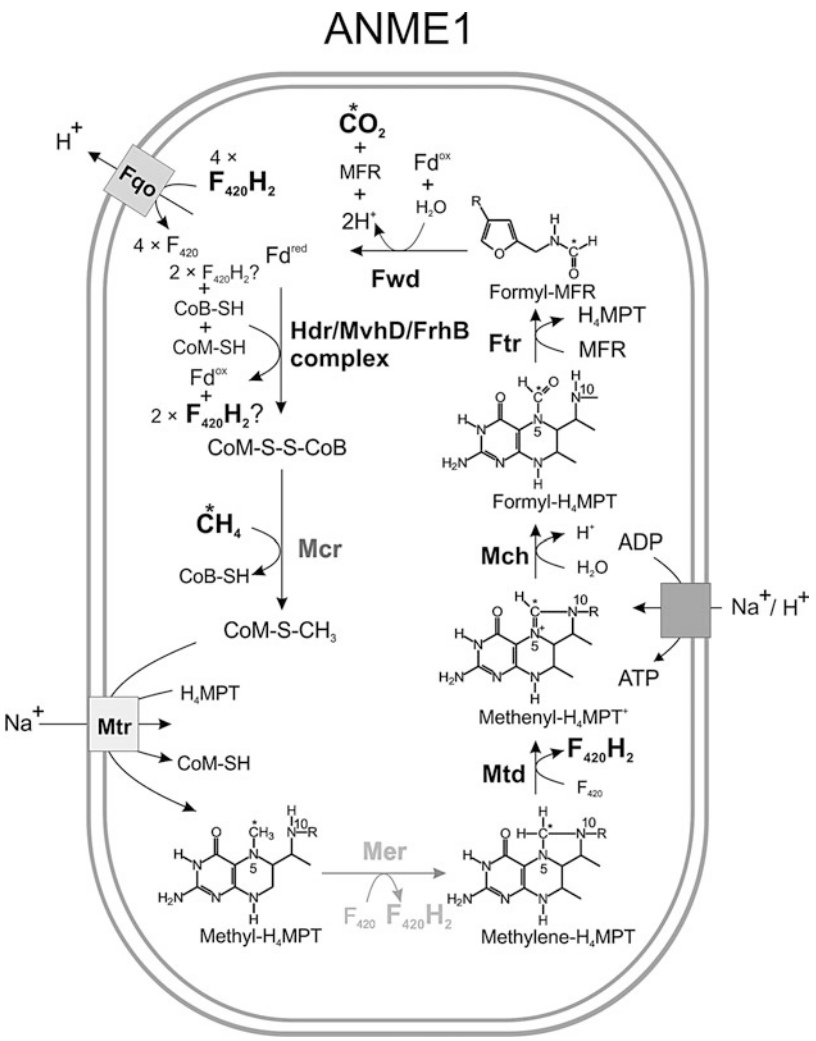

Fig. 7 Predicted reverse methanogenesis pathway operating in ANME-1 archaea, modified from (Shima 2014). The biochemistry for ANME-2 is discussed elsewhere (McGlynn 2017; Timmers et al. 2017). Genes encoding for the Mer enzyme (in gray) have not been found in the ANME-1 metagenome sequence, but they are present in ANME-2a and ANME-2d (see later in this document). Na^+/H^+ antiporters appear to be required for this pathway, but the genes are not annotated in the ANME-1 metagenome. *Mcr* methyl-S-CoM reductase. *Mtr* integral membrane methyltransferase. *Mer* F_{420} dependent methylene-H_4MPT reductase. *Mtd* F_{420} dependent methylene-H_4MPT dehydrogenase. *Mch* cyclohydrolase. *Ftr* formyltransferase. *Fwd* tungsten-containing formylmethanofuran dehydrogenase. *Hdr/MvhD/FrhB* hypothetical Hdr/F_{420} oxidoreductase complex

produced and characterized (Kojima et al. 2014), and ANME Mcr could even be crystallized from extracted ANME-1 biomass derived from microbial mats (Krüger et al. 2003) as described below.

3.2 Methyl-Coenzyme M Reductase (Mcr)

3.2.1 Primary Structure and Isolation of ANME Mcr

Metagenome sequencing of microbial mats performing AOM always revealed genes encoding for ANME-1, 2, and 3 Mcr according to the three branches found (Caldwell et al. 2008; Meyerdierks et al. 2010; Reeburgh 2007). Sequence identities between AMNE and methanogenic, ANME-1 and ANME-2, as well as ANME-2 and ANME-3 Mcr are around 50%, 45%, and 70%, respectively. Methane metabolism on the basis of the methanogenic pathways has been suggested for various Bathyarchaeota (Evans et al. 2015), with sequence identities between Bathyarchaeota and methanogenic/ANME Mcr of around 45%/40%. Profound biochemical studies on isolated Mcr to prove its function are difficult because Bathyarchaeota and methanotrophic Euryarchaea are currently not available in pure cultures. Fortunately, microbial mats collected from the northwestern Black sea shelf, provided sufficient biomass of multispecies ANME consortia containing abundant but heterogeneous ANME-1 and ANME-2 Mcr. Chromatographic purification of these mat-extracts yielded a higher Ni-protein I population hosting methylthio-F430 (Fig. 8b) and a lower Ni-protein II population with F430 bound (Krüger et al. 2003). The Ni-protein I elution peak is composed of at least six different ANME-1 Mcr populations (Shima et al. 2012). SDS-PAGE analysis revealed the three Mcr subunits α, β, and γ as expected (Krüger et al. 2003). The catalytic activity and the substrate specificity are not measurable because ANME Mcr could only be isolated in an inactive form, which could not be reactivated so far. The capability of the phylogenetically related methanogenic Mcr to oxidize methane to methyl-S-CoM (Fig. 8a) at specific rates sufficient to account for those observed *in vivo* for AOM (Scheller et al. 2010) supports the postulated function of ANME Mcr as a methane oxidase. Structural data would allow more profound information about the active site and the applied substrates.

3.2.2 Structure of ANME-I Mcr

The proportion of the Ni-protein I in the mat extracts was calculated to be ca. 7% of the total protein extracted which opened a door for structural analysis (Krüger et al. 2003). Despite the highly heterogeneous sample solution after purification, crystals grew. The resulting crystal structure (Fig. 8c) at 2.1 Å resolution revealed ANME-1 Mcr of a single organism (D1JBK2–4) confirmed by a mass-spectrometric analysis of the dissolved crystal used for structure determination (Shima et al. 2012).

AMNE-1 Mcr was found as a compact heterohexameric $(\alpha\beta\gamma)_2$ complex of 300 kDa (Fig. 8c) which is virtually identical to that of methanogenic Mcr (Ermler et al. 1997; Grabarse et al. 2001). The protein complex harbors two active sites built up of subunits α, α′, β, and γ that are ca. 50 Å apart from each other. Each of them is positioned at the bottom of a 30 Å long funnel-shaped channel. Methylthio-F430 sits at its base and is noncovalently fixed to the polypeptide scaffold by multiple mostly polar interactions to its propionate, acetate, and acetamide side chains (Fig. 8d). In addition to the four equatorial nitrogens of the tetrapyrrole ring, the nickel centers are axially coordinated to the side chain amide oxygen of Glnα′155 (Fig. 8d).

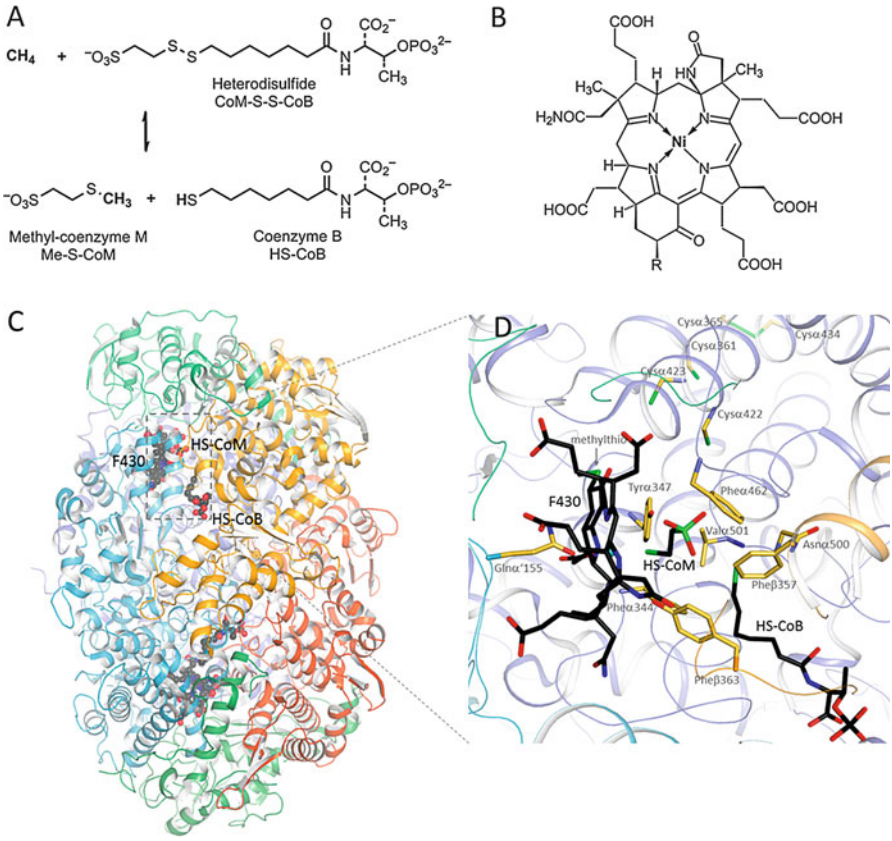

Fig. 8 (**a**) Mcr-catalyzed reaction of methane and CoM-S-S-CoB to methyl-S-CoM and coenzyme B, requiring anaerobic conditions under participation of the unique nickel hydrocorphin cofactor F430. (**b**) Structure of cofactor F430. ANME-1 Mcr contains 17″-methylthio-F430 (Mayr et al. 2008) with R = S-CH_3 and ANME-2 + 3 contain F430 as found in methanogens with R = H. (**c**) Overall architecture of AMNE-I Mcr. The heterohexameric Mcr complex (α in light blue, α′ in azure blue, β in orange red, β′ in bright red, γ in bright green, and γ′ in forest green) has a size of about 120 Å by 85 Å by 80 Å. Minor deviations in the overall structures between methanogenic and methylotrophic Mcr are found in a few loop regions and at the N- and C-terminal ends of the subunits only. Coenzyme F430 (C in *black*) sits at the channel bottom and HS-CoB (C in *black*) in its most narrow segment with the elongated thioheptanoyl pointing towards methylthio-F430 and the threoninephosphate group pointing towards its entrance. HS-CoM (C in *black*) sits in between methylthio-F430 and HS-CoB. (**d**) Active site. The active site state of ANME-1 Mcr is nearly congruent to that of the methanogenic $Mcr_{ox1\text{-}silent}$ state. The main difference between ANME-1 and methanogenic Mcr is five pronounced cysteine residues between methylthio-F430 and the protein surface. This striking cysteine-rich patch might be used as a redox-relay system for the reduction of ANME-1 Mcr from the inactive Ni(II) to the active Ni(I) oxidation state (Prakash et al. 2014). Another source of structural diversity is several post-translational modifications that differ between the structurally known ANME-1 and methanogenic Mcr, but also within methanogenic Mcr variants (Wagner et al. 2016a).

The X-ray structure of ANME-1 Mcr contains nickel of methylthio-F430 in an inactive Ni(II) oxidation state and HS-CoM and HS-CoB in the same positions as found in the methanogenic $Mcr_{ox1\text{-}silent}$ structure (Ermler et al. 1997). HS-CoM sits in front of its hydrocorphin plane of methylthio-F430. Its thiol group is coordinated to the sixth ligation site of the nickel and interacts with the hydroxyl groups of Tyrα347 and Tyrβ363. HS-CoB sits above HS-CoM in the narrowest segment of the channel (Fig. 8d). Two hydrogen donors (the amide and peptide nitrogens of Asnα500 and Valα501) contact the sulfur of HS-CoB and thus facilitate the release of the proton. The space in the active site between nickel and the thiol group of HS-CoB is completely inaccessible for bulk solvent after binding of HS-CoB and lined up by an annular arrangement of hydrophobic aromatic residues (Pheα344, Tyrα347, Pheα462, Pheβ357 and Tyrβ363) (Fig. 8d). No specific gas channel for methane could be detected in the ANME-1 structure, suggesting that methane enters via the substrate channel, but other possible routes via cavities adjacent to the active site cannot be excluded.

ANME-1 Mcr in complex with the substrates CoM-S-S-CoB and/or methane could not be structurally characterized, but the corresponding structure for the methanogenic (Mcr_{silent} state) is available that contains CoM-S-S-CoB (Ermler et al. 1997), albeit not in a catalytically competent position. The S-CoM moiety is shifted more than 4 Å apart from its binding site in the ANME-1 Mcr structure such that one oxygen atom of the sulfonate group becomes axially coordinated to Ni(II). Different enzyme states can be characterized via EPR spectroscopy, which depend on the redox state of the nickel center and on the nature of substrates, products, intermediates, artificial substrate analogs, or inhibitors bound. ^{19}F–ENDOR data on active methanogenic Mcr in the presence of HS-CoM and CF_3-S-CoB indicated a shift of the 7-thioheptanoyl chain towards nickel by more than 2 Å relative to its position in the structurally determined inactive form (Ebner et al. 2010), which reflects the potential of Mcr to undergo a major conformational change of the protein during catalysis. In the active conformation, the sulfonate tail of HS-CoM and the threonine phosphate tail of HS-CoB presumably remain fixed in their binding sites. The structural data for ANME and methanogenic Mcr reveal virtually identical overall and active site structures, as well as the same substrates and products, which implicates the same catalytic mechanism.

3.2.3 Mechanistic Basis of Methane Oxidation by Mcr

Methane oxidation under mild conditions, in aqueous solution and, in particular, in the absence of O_2 is a considerable chemical challenge due to the strong C–H bond in methane with a dissociation energy of 439 kJ/mol. Mcr with its Nickel cofactor F430 (Fig. 8b) is able to catalyze such a reaction by converting methane and a disulfide (CoM-S-S-CoB) into a methylthioether (methyl-S-CoM) and a thiol (HS-CoB). The catalytic mechanism is still disputed because no intermediates with the native substrates could be characterized and no precedence from laboratory chemistry is available for such a reaction.

To become enzymatically active, F430 Ni(II), or 17″-methylthio-F430 Ni(II), of the resting state has to be reduced to the reactive Ni(I) oxidation state (Goubeau et al. 1997). *In vivo*, Ni(II) to Ni(I) reduction is achieved by an protein complex using iron/sulfur cluster containing proteins as electron donor whose redox potential is

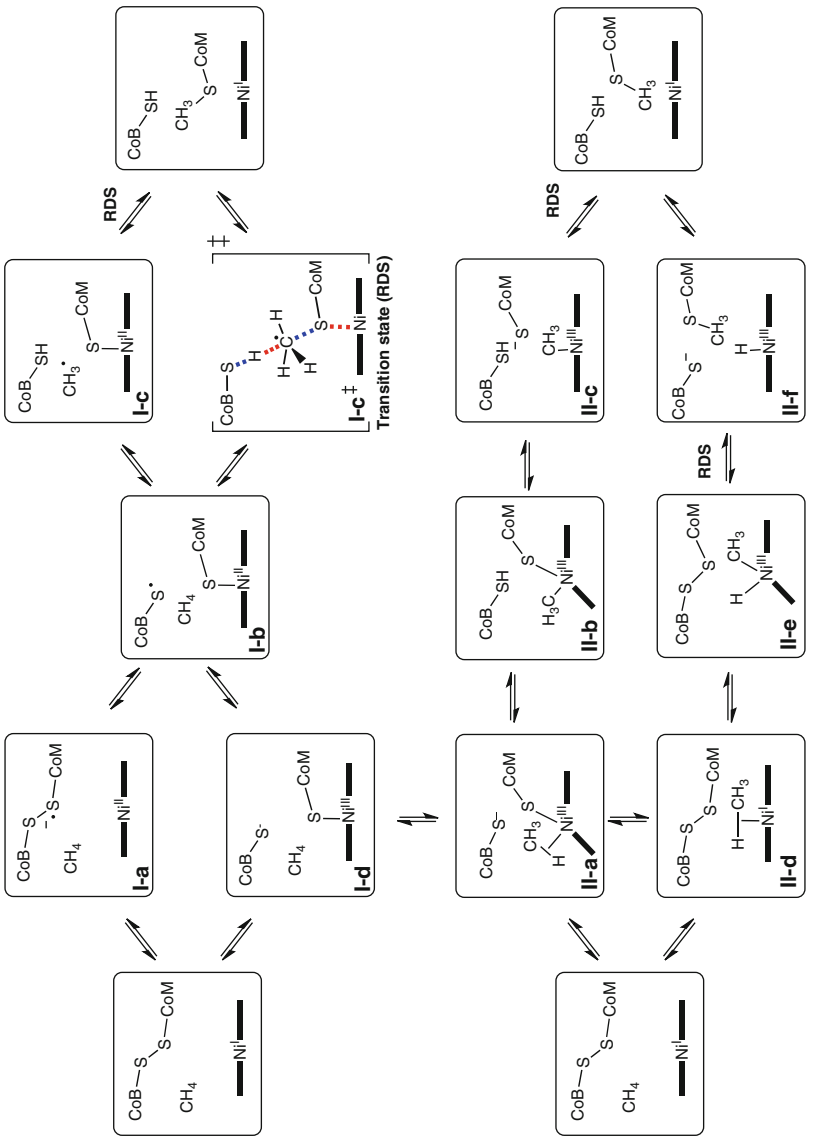

Fig. 9 (continued)

decreased via ATP hydrolysis (Prakash et al. 2014). Interestingly, nickel has suitable redox and ligation properties for catalysis only after integration into a special hydrocorphin ring system created by nature exclusively for Mcr that allows metal-centered reduction and oxidation to Ni(I) and Ni(III) oxidation states, respectively (Jaun and Pfaltz 1986; Jaun 1990). Mcr is quite specific for the substrates methane and methyl-S-CoM. For Mcr from *M. marburgensis*, ethane and ethyl-S-CoM serve as alternative substrates, but overall turnovers are slow; propyl-S-CoM and ally-S-CoM do not react (Scheller et al. 2013a) although they bind to the active site (Goenrich et al. 2004). A new family of enzymes related to Mcr has been described to be responsible for the conversion of butane to butyl-S-CoM (Laso-Pérez et al. 2016), thereby enlarging the substrate repertoire of this type of reaction.

According to the principle of microscopic reversibility, the reaction mechanism is identical for both directions of the catalyzed reaction. Substantial net turnover *in vitro* can only be performed in the methanogenic direction, because the equilibrium for the Mcr-catalyzed reaction is on the methane side under standard conditions ($\Delta G^{\circ\prime} = -30$ kJ mol^{-1} for methane formation). Methane oxidation *in vitro* could be monitored under equilibrium conditions with the use of ^{12}C and ^{13}C isotopes (Scheller et al. 2010).

Figure 9 provides an overview of reaction pathways with their corresponding intermediates that are derived from EPR studies, DFT calculations, and analogies to organometallic laboratory chemistry. A central question is whether H abstraction from methane proceeds radically or in a nonradical fashion, which leads to two distinct types of mechanisms that are discussed here.

Intermediates on the upper half (labeled I-a to I-d) correspond to radical mechanisms and involve interaction of the nickel center with the sulfur atom of coenzyme M as the first step in both directions. Intermediates drawn on the lower half (labeled II-a to II-f) correspond to organometallic 2-electron mechanisms and involve interaction of the nickel center with carbon, hydrogen, or methane as the first step.

Radical Mechanisms

Based on hybrid density functional theory calculations (B3LYP method), a radical mechanism via intermediates I-a and I-b and transition state I-c has been proposed

Fig. 9 Intermediates for conceivable reaction pathways from the ternary enzyme + methane + CoM-S-S-CoB complex to the ternary enzyme + methyl-S-CoM + HS-CoB complex. The large ^{13}C–kinetic isotope effect shows that C–S bond formation must be the rate-determining step (Scheller et al. 2013b), indicated "RDS." *Upper half*: Sequence involving radical intermediates and Ni(II), where the nickel center is interacting with the sulfur atom of methyl-S-CoM or CoM-S-S-CoB as a first step in both directions (Pelmenschikov and Siegbahn 2003). *Lower half*: Sequences involving nonradical intermediates highlighting alternative reaction pathways. In these sequences, the nickel center is interacting with methane, hydrogen, or carbon as a first step of catalysis. Methane activation may also be initiated via the Ni(III) complex I-d (reaction I-d to II-a). The bent in F430 symbolizes states where one tetrapyrrol ring is out of plane allowing for two adjacent coordination sites available for catalysis as inferred from EPR data (Harmer et al. 2008). Intermediates from the lower part may also be able to form species with nickel in the +2 oxidation state and sulfur-based radicals on the coenzymes

(Chen et al. 2012; Pelmenschikov and Siegbahn 2003), involving a highly endergonic hydrogen atom abstraction by a thiyl radical (Int I-b) from methane to form a -methyl-radical (Intermediate I-c, or transition state I-c), which then immediately combines with a nickel(II)-thiolate to form methyl-S-CoM and F430 in the Ni(I) oxidation state (Fig. 9, upper half). This mechanism has been calculated using the following components: F430 without methyl-, acetamide-, or carboxylate substituents, *p*-cresols to mimic Tyrα333 and Tyrβ367, acetamide to mimic Glnα'147, thioacetone to mimic HS-CoM, and ethane thiol to mimic HS-CoB. The methyl radical has originally been calculated to be an intermediate (int. I-c) (Pelmenschikov et al. 2002), which is not compatible with the experimental finding of net configurational inversion of the reaction (Ahn et al. 1991) because alkyl-radicals are expected to undergo rapid racemization. Therefore, the calculation was adjusted to a "transient methyl-radial" as a transition state (transition state I-c) that does not undergo racemization (Pelmenschikov and Siegbahn 2003). In this mechanistic proposal, homolytic cleavage of the C–H bond in methane and C–S bond formation occur in one step, which requires a linear array of five atoms (Ni, S, C, H, S) in the transition state (TS I-c), in which two bonds are broken and two bonds are formed simultaneously.

Using the substrate analog of HS-CoB that is one methylene-group shorter denoted "HS-CoB_6," the putative intermediate I-b has been characterized via MCD spectroscopy (Wongnate et al. 2016). Further, secondary deuterium isotope effects are in line with formation of a methyl radical (Scheller et al. 2013b). However, the thiyl radical in I-b could not be clearly identified via EPR spectroscopy (Wongnate et al. 2016).

Hydrogen atom abstraction from methane by a thiyl radical (int. I-b) is highly endergonic and has never been shown experimentally. It is unusual that the alkane is not interacting with the transition metal center directly, as this is the key feature to achieve alkane C–H bond activation in organometallic chemistry.

Organometallic Mechanisms

EPR spectroscopic data demonstrate that cofactor F430 is able to form Ni-C, Ni-H, and Ni–S bonds (Harmer et al. 2005, 2008; Hinderberger et al. 2006; Sarangi et al. 2009; Yang et al. 2007). We compiled conceivable intermediates that involve two-electron reactions (Fig. 9, lower half) as alternative pathways to the radical mechanism discussed above.

Nonradical C–H bond activation is initiated by a coordination of the methane to the nickel center of F430 to allow for an organometallic intermediate II-a or II-d, which is in analogy to a sigma-methane complex characterized for rhodium(I) (Bernskoetter et al. 2009). Such complexes are usually the first intermediates in alkane C–H bond cleavage reactions known from oxidative addition or Shilov reactions in laboratory chemistry.

In the methanogenic direction, S_N2 reaction via nucleophilic attack of the nickel center on the methyl group of CH_3-S-CoM yielding intermediate II-c appears to be a reasonable mechanism analogous to the chemistry of Co(I) in cobalamine-dependent enzymes. Such a mechanism is supported by the methyl-Ni(III) formation observed

via CH_3-I addition to active enzyme as a replacement for methyl-S-CoM (Sarangi et al. 2009; Yang et al. 2007), but secondary deuterium isotope effects (1.19 per deuterium in the methyl-group) exclude an S_N2 reaction as the rate-determining step (Scheller et al. 2013b). In addition, the putative intermediate characterized recently with the alternative substrate HS-CoB_6 contains nickel in the +2 oxidation state and not in the expected +3 state (Wongnate et al. 2016).

Involvement of nickel-hydrides (intermediates II-e or II-f) is conceivable based on Ni(III) hydrides characterized via EPR spectroscopy (Harmer et al. 2008). Furthermore, nickel-hydride formation before the rate-limiting step in the methanogenic direction would account for the large deuterium equilibrium isotope effect observed when enzymatic methane formation is carried out in deuterated medium; an equimolar mixture of H_2O and D_2O in the reaction buffer yields 21% CH_3D and 79% CH_4 without a significant kinetic isotope effect (Mayr 2009).

The existence of a rhombic nickel hydride in Mcr (Harmer et al. 2008) implies the availability of 2 adjacent coordination sites at F430 during catalysis that would enable formation of the methyl-hydrido-Ni(III) complex II-e that is prerequisite for a mechanism of reductive elimination/oxidative addition. However, the exact reaction pathway, in particular the reaction from intermediate II-e to II-f, is speculative and would possibly involve additional steps.

Mechanistic Conclusions

While the calculated radical mechanism (Pelmenschikov and Siegbahn 2003) is supported by recent spectroscopic evidence (Wongnate et al. 2016) and matches measured kinetic isotope effects (Scheller et al. 2013a, b), many experimental findings remain unexplained. The substrate analog allyl-S-CoM binds to the active site (Goenrich et al. 2004) but does not react at all (Scheller et al. 2013a), even though allyl-radical formation is expected to proceed much more readily than formation of methyl-radicals. The ability of Mcr to provide two adjacent coordination sites on F430 for catalysis (Harmer et al. 2008) and to undergo major conformational changes in the protein (Ebner et al. 2010) are likely relevant for enzyme performance, but are not accounted for in the calculated radical mechanism as currently formulated.

In summary, the catalytic mechanism of Mcr is still nebulous and further experimental data are required to unravel the exact chemistry behind this amazing transformation designed by nature that lacks precedent to manmade catalysts.

3.2.4 Optimization of Mcr Oxidation

The Mcr reaction is slow, $v_{max} = 100$ μmol min^{-1} mg^{-1} measured for the *M. marburgensis* enzyme (Goubeau et al. 1997), and considered rate-limiting in the methanogenesis and AOM catabolism. Therefore, it is conceivable that nature created solutions to optimize catalysis on the level of the enzyme, cell, and organism, probably without directly changing the above-discussed enzymatic mechanism.

Because the active site is already highly optimized as reflected in its strict conservation among different Mcr variants, we speculate that post-translational modifications fine-tune the active site geometry/electrostatics in order to lower

the activation barrier of the enzymatic process, if improvements by ordinary substitutions of amino acids are not possible anymore. Mcr from methanotrophic and methanogenic archaea contains several not strictly conserved post-translational modifications, which include 1-*N*-methylhistidine, *S*-methylcysteine, (*S*)-5-methylarginine, (*S*)-2-methylglutamine, 2,3-didehydroaspartate, 7-hydroxytryptophan, *S*-oxymethionine, and thiopeptide-bound thioglycine (Grabarse et al. 2000; Kahnt et al. 2007; Wagner et al. 2016a). All of them are close to the active site but do not appear to be directly involved in the catalytic cycle, perhaps except for the thioglycine.

Coupling of two active sites – 50 Å apart in Mcr (Fig. 8c) – in a manner that the energy produced in one active site is used to reduce the transition energy of the rate-limiting step in the second active site may further increase the catalytic efficiency. Structural studies suggest a connection between the two active sites via the tight associated subunits α and α' allowing the transfer of local conformational changes over long distances. Spectroscopic studies on Mcr of *M. marburgensis* substantiated this proposal as only 50% of the enzyme can be transformed between two active states (Mcr_{red1} into Mcr_{red2}) after addition of HS-CoM and HS-CoB (Goenrich et al. 2005). Conformational rearrangement of a glycine-rich loop in the active site and an increased flexibility over a wide protein area was found in the structure of the $Mcr_{red-silent}$ state devoid of substrates (Grabarse et al. 2001) and a movement of HS-CoB towards the nickel during the reaction cycle presumably accompanied by conformational changes was inferred from ^{19}F ENDOR studies (Ebner et al. 2010) and transient kinetic studies (Dey et al. 2010).

The rate of methane oxidation can also be increased by expressing larger amounts of ANME Mcr in the cell, which is realized by the ANME consortia in the Black sea where 7% and 3% of the total protein content is ANME-1 and 2 Mcr (Krüger et al. 2003). Many hydrogenotrophic methanogens synthesize two Mcr isoenzymes with a sequence identity of 60–70%, which is not found for methanotrophic archaea. Their differential expression in *M. marburgensis* is known to be regulated by the growth conditions – temperature and pH (Bonacker et al. 1992) – suggesting an adaptation of their respective active sites to specific environmental conditions. The microbial diversity of ANME consortia with slightly modified Mcr might serve the same purpose.

3.3 Heterodisulfide Reductase (Hdr)

The metagenome analysis indicated the presence of five copies of HdrABC in the genomes of ANME-1 archaea from the Black sea microbial mat (Meyerdierks et al. 2010). However, the genes encoding MvhABG, which is CoM-S-S-CoB reductase-associating hydrogenase found in methanogens, was not found in the genome of any ANME. The absence of genes encoding for MvhABG suggests that in ANME, the Hdr does not donate electrons to H^+ forming H_2, although H_2 is the electron donor of soluble Hdr system in methanogenic archaea. Interestingly, one of the *hdr* gene clusters contains genes encoding MvhD and FdhB. MvhD is proposed to be an

electronic connector of the HdrABC-MvhAGD complex. FdhB belongs to an F_{420} binding protein family, which are associated with F_{420}-reducing enzymes, such as F_{420}-reducing hydrogenase (FrhAGB), which suggests that electrons from ferredoxin, HS-CoM and HS-CoB may be transferred to F_{420} in the methanotrophic direction (Meyerdierks et al. 2010).

ANME-2a and ANME-2d archaea contain an integral membrane Hdr (HdrDE), which catalyzes the reduction of methanophenazin ($E_0' = -165$ mV (Tietze et al. 2003)) with the oxidation of HS-CoM and HS-CoB ($E_0' = -143$ mV (Tietze et al. 2003)) to CoB-S-S-CoB. This endergonic reaction ($\Delta G^{\circ\prime} = +4.2$ kJ/mol) (Bäumer et al. 1998) could be driven by a proton ion influx. HdrABC type enzyme was also found in the genome of ANME-2a and ANME-2d. In the genome of ANME-2d, the encoding genes for MvhD- and FrhB-homologues are found near the genes encoding HdrABC, suggesting that FrhB might be complexed with HdrABC and serves as an enzyme complex catalyzing electron confurcation from reduced ferredoxin, HS-CoM and HS-CoB to two molecules of F_{420} (Arshad et al. 2015). In the genome analysis of ANME-2a, presence of MvhD and the F_{420}-oxidoreductase subunit was not mentioned (Wang et al. 2014).

3.4 Methyl-Tetrahydromethanopterin: Coenzyme M Methyltransferase (Mtr)

In ANME archaea, the MtrA-H complex catalyzes methyl transfer from methyl-S-CoM to H_4MPT. This endergonic reaction is driven by a coupling to sodium-ion influx. There are two homologues *mtrA* genes encoded in methanogenic archaea from Methanomicrobiales and Methanococcales, one of which is fused with the *mtrG* and *mtrF* genes, respectively (Wang et al. 2015). In the *mtr* operon of ANME-1 archaea, only one mtrA gene is coded and fused with *mtrF* gene in an *mtrA-H* gene cluster (CBH38255). In ANME-2b archaea, two sets of *mtrA-H* genes are detected. One of the gene sets is expressed more strongly, suggesting that only this set is functional (Wang et al. 2014).

3.5 Methylene-Tetrahydromethanopterin Reductase (Mer)

In ANME-2, Mer catalyzes the reversible dehydrogenation of methyl-H_4MPT to methylene-H_4MPT with $F_{420}H_2$ as the electron acceptor. F_{420} is a deazaflavin derivative with a structure similar to flavin but with a redox potential of $E_0' = -360$ mV (Thauer 1998), like NAD(P)H ($E_0' = -320$ mV). The crystal structure of Mer from the methanogen *M. barkeri* complexed with F_{420} is available (Aufhammer et al. 2005). The Mer-encoding gene was found in the genomes of ANME-2a and ANME-2d but not in those of ANME-1 (Meyerdierks et al. 2010; Stokke et al. 2012). It has been proposed that a protein homologous to MetF, which is a methylene-tetrahydrofolate reductase structurally similar to Mer, could catalyze the Mer-reaction in ANME-1, but this hypothesis has not been tested yet

(Stokke et al. 2012). We tried to heterologously overexpress the *metF* gene homolog from ANME-1, but the protein formed inclusion bodies that disabled functional studies (Shima et al. unpublished result).

3.6 Methylene-Tetrahydromethanopterin Dehydrogenase (Mtd), Cyclohydrolase (Mch), and Formyltransferase (Ftr)

The genes from the metagenome sequences of ANME-1 were heterologously overexpressed and the enzymes Mtd, Mch, and Ftr were purified to homogeneity by column chromatography without tags (Kojima et al. 2014). The three enzymes revealed high activity at 10 °C, which is near the physiological temperature of the ANME-1 archaea harvested. The specific activity substantiated their predicted functions in the AOM pathway. Mtd is quite unstable in buffer solutions with low salt concentrations. Interestingly, the stability also decreases by lowering the temperature; the half-life of the enzymatic activity at 20 °C, 10 °C (near the in situ temperature of the Black Sea mat) and on ice were 10 h, 3 h, and 0.5 h, respectively. The Mtd enzyme from ANME-1 was stabilized by addition of 0.5 M potassium phosphate. The stability of Mch from ANME-1 is not dependent on the presence of salt, but the activity was found to be dependent on the presence of potassium phosphate, the maximum activity was observed at 0.6–0.8 M of potassium phosphate; at low salt concentrations, no activity could be detected.

It is known that the cytoplasm of the hyperthermophile *Methanopyrus kandleri* contains very high concentrations of salt, which is mainly composed of ~1 M cyclic 2,3-diphosphoglycerate, probably with potassium as the main cation. The activity and stability of some of the methanogenic enzymes are increased on the presence of lyotropic salts, i.e., with potassium phosphate, ammonium sulfate and potassium cyclic 2,3-diphosphoglycerate (Shima et al. 1998). Salt-dependent stabilization of Mtd and salt-dependent activation of Mch from ANME-1 archaea suggested that ANME-1 archaea contains high concentrations of lyotropic salts in the cells.

3.7 Formylmethanofuran Dehydrogenase (Fmd and Fwd)

The formyl group in formyl-methanofuran is oxidized and hydrolyzed to CO_2 by two enzyme variants, Fmd or Fwd. Ferredoxin serves as the natural electron acceptor. The two isoenzymes Fmd (ANME-2b and ANME-2d) and Fwd (ANME-1) contain molybdenum and tungsten in the pterin cofactor, respectively. The biochemically characterized Fwd complex from methanogenic archaea is composed of six subunits (FwdABCDFG) (Wagner et al. 2016b). FwdB contains the sequence motif for binding of the pterin cofactor. FwdA, an amidohydrolase, catalyzes the hydrolysis of formyl-methanofuran, and FwdF is a polyferredoxin and carries eight 4Fe-4S clusters. The genes-encoding Fwd proteins are found in ANME-1 archaea, but those

for FwdG and FwdF iron-sulfur cluster proteins are not detected in this genome (Meyerdierks et al. 2010). However, FmdG and FmdF are annotated in the ANME-2b and ANME-2d genomes.

4 Research Needs

4.1 Pathways

Knowledge about the physiologies of different ANME clades has substantially increased over the last decade due to metagenomic sequencing, the enrichment of ANME-2d co-cultures, and via the recently proposed hypothesis of direct interspecies electron transfer as a syntrophic coupling mechanism for some members of ANME.

Now, the focus on AOM needs to transition from –omics-driven investigations towards the isolation of ANME in pure cultures, and activity studies that include detection and quantification of the metabolic reactions, biochemical characterization of active ANME enzymes. Hypotheses of energy conservation in distinct ANME clades need to be probed experimentally, the minimal energy requirements for growth tested, and the exact stoichiometries between methane molecules oxidized, ion-gradients formed, and ATP-yields quantified. Once elucidated, the distinct physiologies of ANME and methanogens can then be compared to gain a deeper understanding of the putative evolution of ANME from methanogens.

Niche differentiation among various ANME clades apparently doing the same overall reaction is poorly understood. ANME-1, for example, apparently lack the *mer*-gene that seems required for the primary metabolism of AOM. The Mer-step may be replaced by an alternative enzyme that needs to be characterized to understand why ANME-1 are not utilizing Mer. The metagenomics-derived proposal that some members of the ANME-2d clade carry out full denitrification from nitrate to ammonium requires clear experimental verification and isolation of the corresponding species in pure cultures to study their physiology in depth. The presence of multiheme *c*-type cytochromes in ANME-2d may also allow for extracellular electron transfer to oxidants other than nitrate.

The hypothesis of direct interspecies electron transfer from ANME-1 and ANME-2a-c to their partner-bacteria needs profound analysis to enable growth of ANME in pure cultures by substituting the electron-"disposing" partner-bacteria with suitable laboratory settings. Cultivation of ANME on electrodes with defined redox potential may serve as a platform to probe their physiology. The electrode can mimic the metal oxide particles and thus simulate specific environmental conditions.

Pure cultures of ANME open the door for genetic engineering enabling ultimate control over the AOM-process, which may be relevant to apply the mechanism of methane oxidation with release of single electrons for electric power generation from natural gas at ambient temperatures.

4.2 Enzymes

As all enzymes in reverse methanogenesis operate close to the thermodynamic equilibrium, they may reveal catalytic strategies of how enzymes are optimized to carry out difficult chemical transformations in an energy-efficient manner that may be useful for bio-catalytic conversions in an industrial setting. Heterologous expression of all enzymes involved in reverse methanogenesis is advantageous, but some of them require elucidation of the genes required to biosynthesize special cofactors and install the posttranslational modifications present, in particular Mcr with its F430 cofactor and the 6 posttranslational modifications.

The mechanism of the Mcr-catalyzed reaction remains mysterious despite progress made via DFT calculations, characterization of intermediates with substrate analogs and measurements of isotope effects. The reason for the ability of Mcr to provide two adjacent sites for catalysis on F430 concomitant with a major conformational change in the protein, as well as a chemical involvement of the thioglycine residue during catalysis needs to be elucidated. Understanding the nickel-chemistry of F430-driven methane activation may lead to an integration of the Mcr-reaction into the toolbox of organometallic chemistry to selectively functionalize alkanes with the cheap transition metal nickel. The recent discovery of nickel-catalyzed conversion of butane to butyl-S-CoM broadens the scope for this type of catalysis.

Finally, the study of multiheme *c*-type cytochromes as a tool in biology to conduct electrical current between microbes is in its infancy, but may find industrial utility as a tool to convert fuel-derived electrons directly to electricity. These processes require substantial empirical and theoretical research for a thorough understanding.

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3 Catabolic Pathways Involved in the Anaerobic Degradation of Saturated Hydrocarbons

Heinz Wilkes and Ralf Rabus

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;

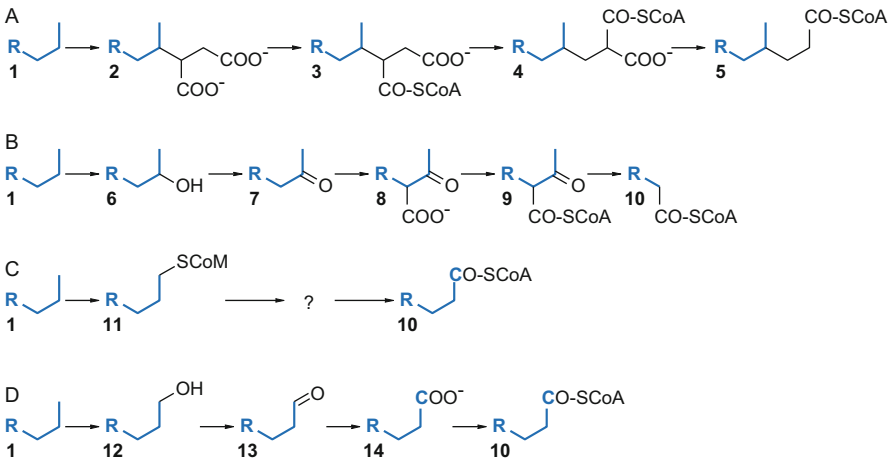

Fig. 1 Overview of anaerobic enzymatic activation reactions and subsequent catabolic pathways of *n*-alkanes until the level of coenzyme-A-esters suitable for further degradation via β-oxidation; (**a**) addition to fumarate; (**b**) dehydrogenation/anaerobic hydroxylation; (**c**) formation of alkyl-coenzyme-M; (**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

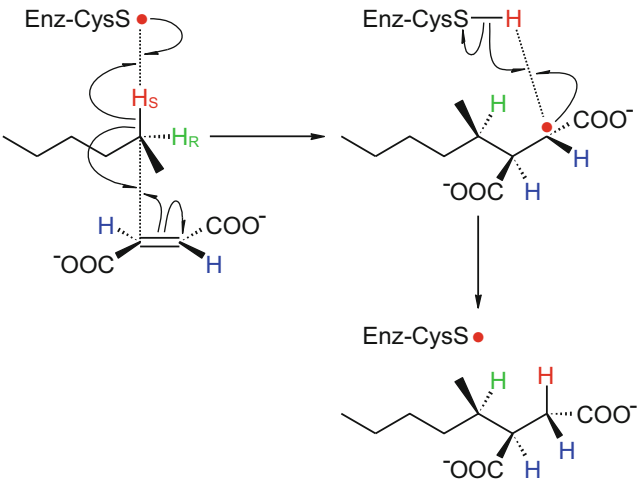

Fig. 2 Suggested mechanism for (1-methylalkyl)succinate formation via a concerted reaction. (Reprinted from Jarling et al. (2012), with permission from John Wiley and Sons)

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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Catabolic Pathways and Enzymes Involved in the Anaerobic Degradation of Monocyclic Aromatic Compounds

4

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Abstract

Monocyclic aromatic compounds (MAC) comprise the second most abundant class of natural compounds, many of which are hazardous for the environment and human health. MAC can readily be degraded by many aerobic microorganisms by the extensive using of oxygenases for aromatic ring hydroxylation and cleavage. However, under anoxic conditions, this strategy is not an option and MAC degrading anaerobic prokaryotes employ a totally different enzyme inventory for attacking the resonance-stabilized aromatic ring system or the C–H bond of alky chains from aromatic hydrocarbons. The anaerobic degradation of MAC has become a treasure trove for the discovery of unprecedented enzymatic principles; many involve metalloenzymes catalyzing radical-based reactions. Characteristic enzymatic reactions involved in anaerobic MAC degradation comprise: (i) the addition of alkylated aromatics to fumarate by glycyl-radical enzymes, (ii) the water-dependent hydroxylation or transhydroxylation of MAC by Mo- or flavin-dependent enzymes, (iii) the carboxylation/decarboxylation of aromatic rings by UbiD-/UbiX-like enzyme systems, and (iv) the dearomatization of aromatics rings by ATP-dependent FeS-enzymes or ATP-independent W-enzymes. The multitude of MAC is converted via peripheral channeling pathways to only a few central intermediates that serve as substrates for dearomatizing ring reductases. Depending on the nature of these central intermediates, we divide the anaerobic MAC degradation pathways into five subgroups and highlight the individual characteristic enzymatic steps involved.

1 Introduction

Only surpassed by the glycosyl moiety, the aromatic benzene nucleus is the second most abundant biogenic structural unit in nature. Aromatic rings mainly derive from plant secondary metabolism (e.g., lignin) where they are synthesized via the shikimate pathway, but they are also abundant in all other living beings in the form of aromatic amino acids or quinones. Aromatic hydrocarbons represent a significant portion of fossil oil reservoirs and serve as source for solvents, dyes, resins, plasticizers, polymers, flame retardants, pesticides, insecticides, and many other synthetic chemicals. Many aromatic hydrocarbons such as BTEX (benzene, toluene, ethylbenzene, xylenes) or polycyclic aromatic hydrocarbons (PAH) are toxic and/or cancerogenic and affect human health and the environment. Due to the chemical inertness of the resonance stabilized ring

system, and, in case of aromatic hydrocarbons, low solubility, many aromatic compounds are considered recalcitrant.

The complete degradation of aromatic compounds to CO_2 is predominantly performed by microorganisms and has been studied in aerobic bacteria since more than 50 years (Stanier and Ornston 1973; Harayama et al. 1992; Harwood and Parales 1996; Díaz et al. 2013). Aromatic compound degrading aerobes attack aromatic ring systems by the aid of ring-hydroxylating mono- or dioxygenases. A few dihydroxylated central aromatic intermediates (e.g., catechol, protocatechuate, or gentisate) are then dearomatized by ring-cleaving oxygenases. Considering the massive use of the dioxygen molecule in the degradation pathways of aromatic compounds in aerobic microorganisms, their degradation at anoxic sites such as marine/freshwater sediments, oils reservoirs, or aquifers remained obscure until the early 1990s.

Meanwhile, the number of anaerobic microorganisms that are known to use aromatic compounds as growth substrates is steadily growing. They comprise facultative anaerobes, such as denitrifiers or bacteria with anoxygenic photosynthesis (α-, β-Proteobacteria), and obligate anaerobes including sulfate- and metal oxide-reducing or fermenting bacteria in methanogenic co-culture (δ-Proteobacteria and Firmicutes) (Boll et al. 2014). To date, the only known archaeon and hyperthermophilic organism capable of completely degrading aromatic growth substrates to CO_2 is the Fe(III)-respiring, strictly anaerobic *Ferroglobus placidus* (Tor and Lovley 2001).

Today, most of the anaerobic degradation pathways of monocyclic aromatic compounds (MAC) have been elucidated. Characteristic key reactions such as C–H bond activation of aromatic hydrocarbons or the dearomatization of the benzene ring system are typically catalyzed by metalloenzymes, many of which catalyze unprecedented radical-based reactions. In analogy to the aerobic degradation pathways, anaerobic microorganisms channel the multitude of monocyclic aromatic compounds into only a few central intermediates that still contain the aromatic moiety such as benzoyl-CoA and analogues of it, or di-/trihydroxybenzenes with hydroxyl groups in *meta*-positions (Fuchs et al. 2011). They serve as substrates for dearomatizing ring reductases that, depending on the stability of the aromatic ring, use different electron donors and may couple ring reduction to an exergonic reaction. Dearomatizing ring reductases acting on aryl-CoA-thioesters are also employed in the anaerobic degradation of PAH. However, the pathways of anaerobic PAH degradation differ fundamentally from those of anaerobic MAC degradation. For this reason, anaerobic PAH degradation is the topic of a separate chapter of this volume (▶ Chap. 5, "Catabolic Pathways and Enzymes Involved in the Anaerobic Degradation of Polycyclic Aromatic Hydrocarbons").

In this contribution, the current knowledge of experimentally verified degradation pathways of monocyclic aromatic compounds with a focus on the function of characteristic key enzymes is summarized and discussed. There are many excellent recent reviews about anaerobic MAC degradation with other focuses than this compendium (Carmona et al. 2009; Fuchs et al. 2011; Philipp and Schink 2012; Heider and Schühle 2013; Boll et al. 2014; Rabus et al. 2016b). Here, we divide anaerobic MAC degradation pathways into five groups with respect to the nature of the substrates for central dearomatizing reductases: (1) benzoyl-CoA, (2–4) *ortho*-, *meta*-, or *para*-substituted benzoyl-CoA derivatives, and (5) di-/trihydroxybenzenes

with *meta*-positioned hydroxy substituents. In all cases dearomatization is accomplished by transferring two electrons to the aromatic ring system yielding cyclic diene products; the latter may undergo tautomerization and/or other spontaneous reactions.

2 Degradation via Dearomatization of Benzoyl-CoA

In anaerobic microorganisms, most MAC are channeled into benzoyl-CoA, including the abundant BTEX, phenol, benzoates, phthalates, phenylpropane-derived compounds, phenylacetic acid, phenylalanine, tyrosine, and many more (Fig. 1).

2.1 General Benzoyl-CoA Degradation Pathway

The so-called benzoyl-CoA degradation pathway is initiated by the key reaction of MAC degradation, the reductive dearomatization of the benzene moiety of benzoyl-CoA. There are two nonrelated classes of dearomatizing benzoyl-CoA reductases (BCRs, class I and II), both yielding the same cyclohexa-1,5-diene-1-carboxyl-CoA (1,5-dienoyl-CoA) product (Fig. 2) (Boll and Fuchs 1995; Kung et al. 2009). An exception was found during benzoate degradation in *Rhodopseudomonas palustris*, where the 1,5-dienoyl-CoA formed by a class I BCR is further reduced to a cyclic monoenoyl-CoA – probably by the same BCR (Koch et al. 1993; Egland et al. 1997). The redox potential of the benzoyl-CoA/1,5-dienoyl-CoA redox couple is $E^{\circ\prime} = -622$ mV, more negative than that of any conventional cellular electron donor (Kung et al. 2010). Consequently, BCRs have to couple benzene ring reduction to an exergonic reaction, which is accomplished differently in the two BCR classes. Consequently, the architectures and cofactor contents of both BCR classes differ fundamentally.

The further catabolism of 1,5-dienoyl-CoA involves a series of β-oxidation-like reactions (Fig. 2). The 1,5-dienoyl-CoA is first hydrated by a specific enoyl-CoA hydratase catalyzing a rather unusual 3,6-addition instead of the typical 2,3-addition of water with respect to the C1-carbonyl of the thioester (Laempe et al. 1998; Peters et al. 2007). In the next step, the hydroxyl group is dehydrogenated to a ketone, yielding 6-oxocyclohex-1-ene-1-carboxyl-CoA that serves as substrate for a ring-cleaving, 3-hydroxypimeloyl-CoA forming hydrolase (Laempe et al. 1999; Kuntze et al. 2008). The encoding gene of this enzyme, referred to as *bamA* (*Geobacter metallireducens* notification), is highly conserved and serves as a specific functional marker for monitoring MAC degradation by PCR-, microarray-, or sequencing-based tools (Kuntze et al. 2011a; Porter and Young 2013). The subsequent degradation of the aliphatic 3-hydroxypimeloyl-CoA proceeds again via β-oxidation reactions and yields acetyl-CoA and glutaryl-CoA. After decarboxylation of the latter by a soluble glutaryl-CoA dehydrogenase or by a membrane-bound, sodium-pumping glutaconyl-CoA decarboxylase, crotonyl-CoA, and finally two acetyl-CoA are formed. The benzoyl-CoA degradation pathway can be summarized by the following equation (without considering ATP hydrolysis by class I BCR):

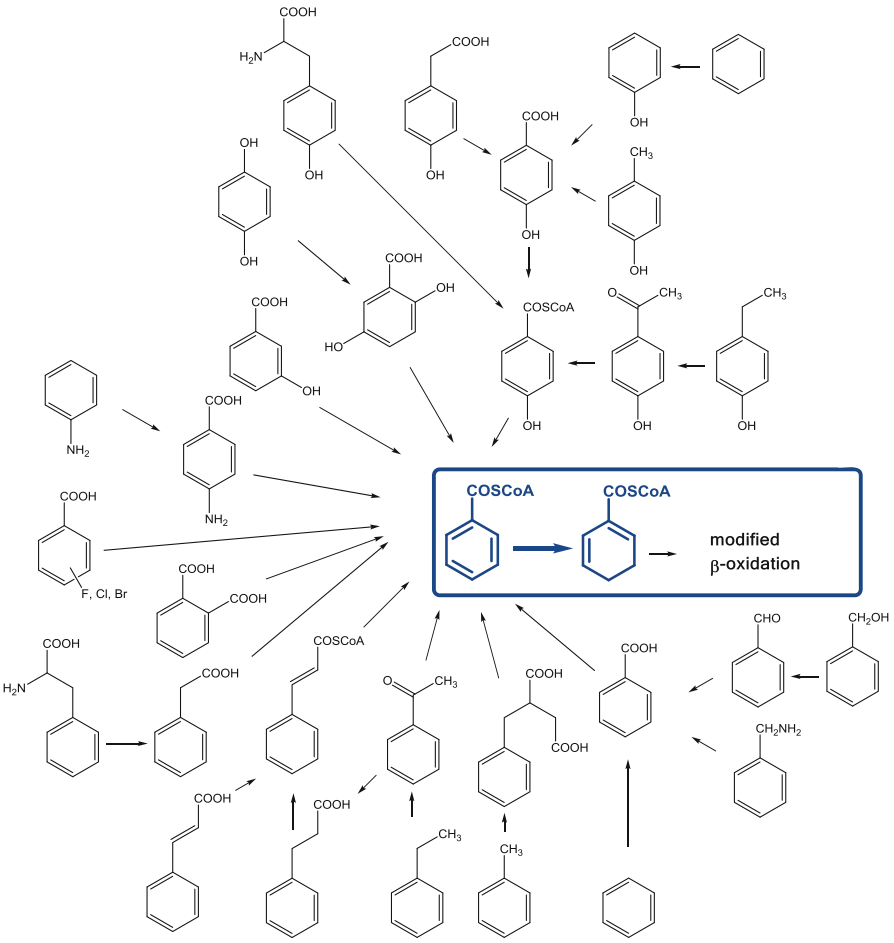

Fig. 1 Degradation of selected MAC via benzoyl-CoA. The central intermediate is dearomatized by different classes of benzoyl-CoA reductases yielding the same cyclohexa-1,5-diene-1-carboxyl-CoA (1,5-dienoyl-CoA) product

$$\text{Benzoyl-CoA} + 2\ \text{Fd}_{\text{red}}^{-} + 2\ \text{CoA} + 4\ \text{H}_2\text{O} + 3\ \text{NAD}^{+} + \text{ETF}_{\text{ox}} \rightarrow 3\ \text{Acetyl-CoA} + \text{CO}_2 + 2\ \text{Fd}_{\text{ox}} + 3\ \text{NADH} + \text{H}^{+} + \text{ETF}_{\text{red}}$$

2.2 Benzoyl-CoA Reduction

2.2.1 Class I BCRs

The first BCR was isolated and characterized from the denitrifying β-Proteobacterium *Thauera aromatica*; to date, it still represents the only characterized enzyme of the class I BCR (Boll and Fuchs 1995). The enzyme uses a reduced ferredoxin as electron donor and couples endergonic transfer of two single electrons

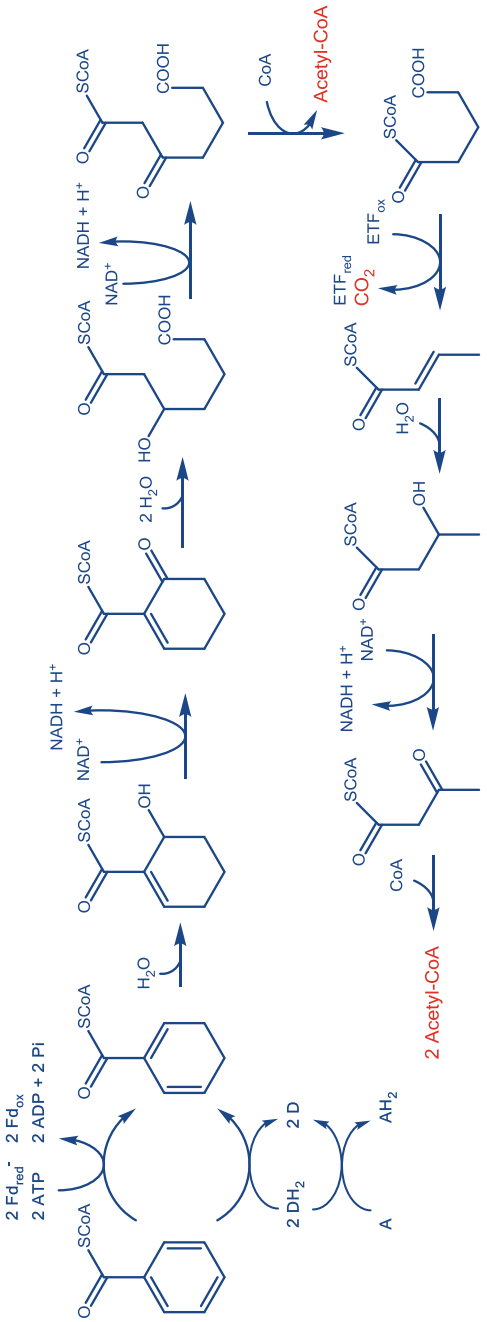

Fig. 2 Enzymatic reactions involved in the benzoyl-CoA degradation pathway. Benzoyl-CoA is reduced to 1,5-dienoyl-CoA either by ATP-dependent class I (upper reaction) or ATP-independent class II BCR (lower reaction); the latter may drive endergonic electron transfer by electron bifurcation, using an electron donor of insufficient redox potential to reduce the aromatic ring directly (DH_2) by coupling its oxidation to the exergonic reduction of a second acceptor (A)

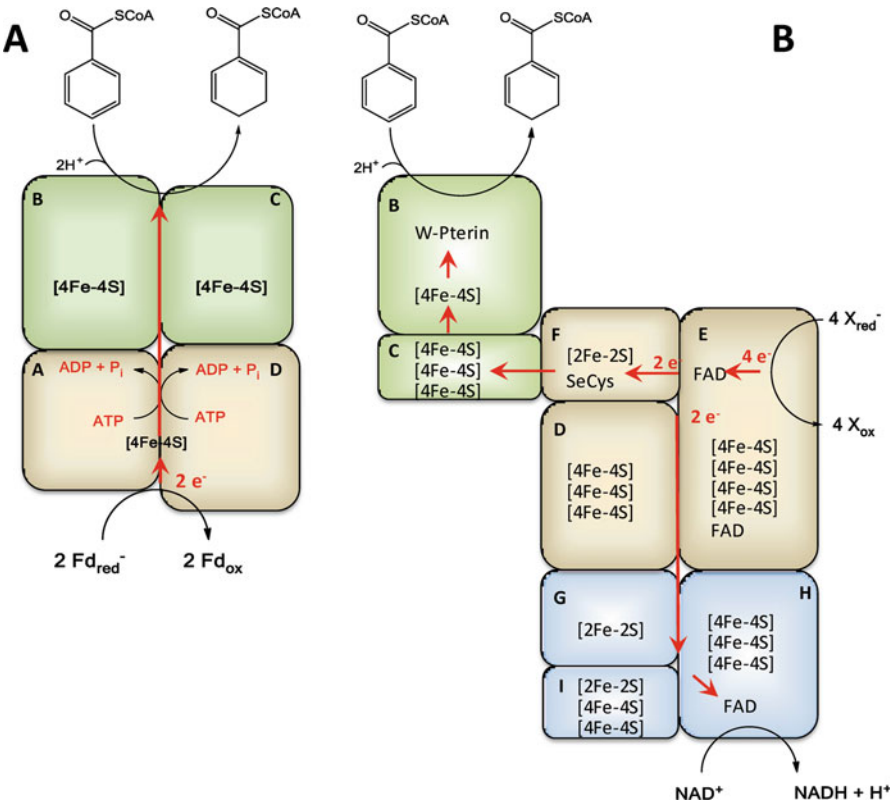

Fig. 3 Molecular architectures of class I (**a**) and class II BCRs (**b**). In class I BCR (BcrABCD subunits) endergonic electron transfer from reduced ferredoxin ($E' \approx -500$ mV) to benzoyl-CoA ($E^{\circ\prime} = -622$ mV) is driven by stoichiometric ATP hydrolysis. In class II BCR (BamBCDEFGHI), a flavin-based electron bifurcation is hypothesized in which endergonic electron transfer from an unknown donor (X_{red}^-) to benzoyl-CoA is driven by the exergonic electron transfer from the same donor to a high-potential acceptor. Due to similarities of BamH with NAD-binding components of NADH:quinone oxidoreductase, NAD^+ ($E^{\circ\prime} = -320$ mV) has been proposed as a potential second electron acceptor

to the aromatic ring to a stoichiometric hydrolysis of 2 ATP to 2 ADP + 2 P_i (Fig. 3a) (Boll et al. 1997; Boll and Fuchs 1998). A Birch-like mechanism via reactive radical species has been proposed, which proceeds via alternate single electron transfer and protonation steps; some indirect evidence for such a scenario has been obtained (Möbitz and Boll 2002; Thiele et al. 2008).

The 170 kDa class I BCR from *T. aromatica* binds three $[4Fe\text{-}4S]^{+1/+2}$ clusters and has a BcrABCD-architecture (Fig. 3a) (Buckel et al. 2014). The BcrAD subunits bind a [4Fe-4S] cluster and two ATP molecules; ATP hydrolysis causes conformational changes promoting the electron transfer to the two other [4Fe-4S] clusters of the BcrBC subunits. Class I BCRs are present in aromatic compound degrading facultative anaerobes belonging to genera of the α-Proteobacteria (e.g., *Magnetospirillum*, *Rhodopseudomonas*,

Rhodomicrobium) or β-Proteobacteria (e.g., *Aromatoleum*, *Thauera*, *Azoarcus*, *Georgfuchsia*). Surprisingly, a class I BCR has also been identified in the strictly anaerobic archaeon *Ferroglobus placidus* (Schmid et al. 2015). Based on the amino acid sequence similarities and subunit sizes of the BcrAD-modules, two subclasses of ATP-dependent BCRs have been identified: the *Thauera*- and the *Azoarcus*-type. A BCR of the latter subclass has not been isolated and characterized, yet (Buckel et al. 2014). A third BCR subclass recently identified in a 4-methylbenzoate degrading *Magnetospirillum* species may be specifically involved in the reduction of benzoyl-CoA analogues (Rabus et al. 2016a) (see Sect. 5.1).

2.2.2 Class II BCRs

The active site BamBC components (bam = benzoic acid metabolism) of a class II BCR were isolated and characterized from the Fe(III)-respiring δ-Proteobacterium*G. metallireducens* (Kung et al. 2009). The BamB subunit contains an active site W-*bis*-tungstopterin (*bis*WPT) cofactor and shows similarities to the W-containing aldehyde:ferredoxin oxidoreductases. The crystal structures of a $Bam(BC)_2$ complex in the presence of the substrate, product, and inhibitor revealed that the tungsten atom is coordinated by four sulfur atoms from the dithiolene groups of the cofactor, by a cysteine-sulfhydryl group and a sixth inorganic ligand, probably a sulfido or cyano group (Fig. 4) (Weinert et al. 2015). Remarkably, the aromatic ring of benzoyl-CoA does not bind directly to the cofactor; instead electron transfer from the reduced

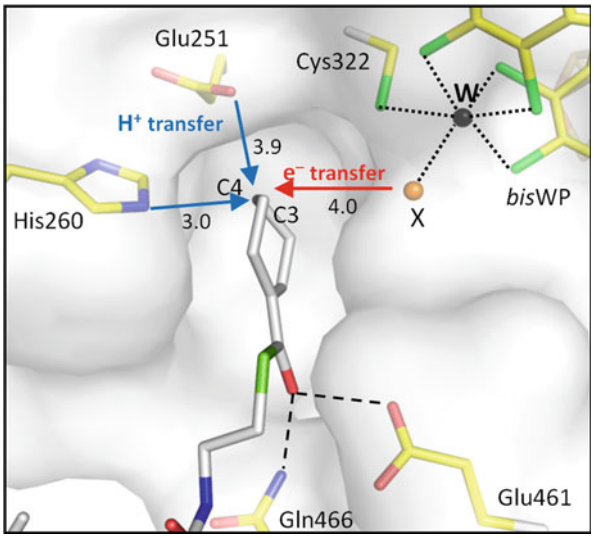

Fig. 4 Crystal structure of the active site BamB component from*G. metallireducens* class II BCR. The ring of a benzoyl-CoA analogue (a cyclic monoenoyl-CoA) is bound in an aprotic cavity. Electrons for aromatic ring reduction are transferred from the reduced W-atom, coordinated by five sulfur ligands (green), via an unknown inorganic ligand (X). Protons are transferred from His260 and probably Glu251. The structure promotes a Birch-like mechanism via spatially separated single electron transfer and protonation steps; modified from Weinert et al. (2015)

*bis*WPT to the aromatic ring appears to proceed via the sixth inorganic ligand, probably via an outer shell electron transfer mechanism.

The putative other components of the class II BCR complex are encoded by the *bamDEFGHI* genes that share similarities to genes encoding soluble heterodisulfide reductase and NADH-binding components of NADH:quinone oxidoreductases (Wischgoll et al. 2005). In silico analysis and preliminary experimental work suggest that class II BCRs form a large BamBCDEFGHI complex harboring bisWPT, >20 FeS clusters, three FAD, and one selenocysteine as cofactors. The BamBCDEGHI complex is considered to drive endergonic benzoyl-CoA reduction via a flavin-based electron bifurcation: the endergonic electron transfer from an unknown donor, probably a reduced ferredoxin, to the aromatic ring is coupled to electron transfer from the same donor to a high-potential second acceptor, probably NAD^+. Notably, such an electron bifurcation process has previously been demonstrated for a soluble heterodisulfide reductase from methanogenic archaea which exhibits significant similarities to the BamDE components (Kaster et al. 2011).

The *bamBCDEFGHI* genes encoding class II BCRs are found in all obligately anaerobic bacteria with the capacity to degrade MAC, but never in any facultatively anaerobic MAC-degrading organism (Löffler et al. 2011). The occurrence of class I BCRs in facultative anaerobes and class II BCRs in obligate anaerobes may be rationalized by the energetic costs for benzoyl-CoA dearomatization. The energy yield of facultatively anaerobic bacteria (e.g., denitrifiers) is relatively high which allows the use of an ATP-dependent, essentially irreversibly operating BCR. A flavin-based electron bifurcation, however, is considered to operate closer to equilibrium and consequently seems to be less energy demanding (Buckel and Thauer 2013). However, it has to be taken into account that the synthesis of a class II BCR depends on efficient metal uptake systems and cofactor synthesis/insertion machineries (tungstopterin, flavins, selenocysteine, FeS clusters).

2.3 Degradation of Phenolic Compounds

2.3.1 Phenol Carboxylation

The pathway of anaerobic phenol degradation has mostly been studied in facultative anaerobes, particularly in the denitrifying bacterium *Thauera aromatica*. This bacterial species metabolizes phenol via a two-step, ATP-dependent carboxylation of the aromatic ring in *para*-position to the phenolic hydroxyl group, catalyzed by two distinct enzymes (Fig. 5) (Lack and Fuchs 1994; Breinig et al. 2000; Schühle and Fuchs 2004). In the initial step, phenylphosphate is formed from phenol as a stable activated intermediate (Lack and Fuchs 1994; Narmandakh et al. 2006). This reaction is catalyzed by a phenylphosphate synthase complex (PPS) in an ATP-dependent reaction. Subsequently, phenylphosphate is dephosphorylated and simultaneously carboxylated to 4-hydroxybenzoate by a second multisubunit enzyme complex, phenylphosphate carboxylase (PPC) (Breinig et al. 2000; Schühle and Fuchs 2004).

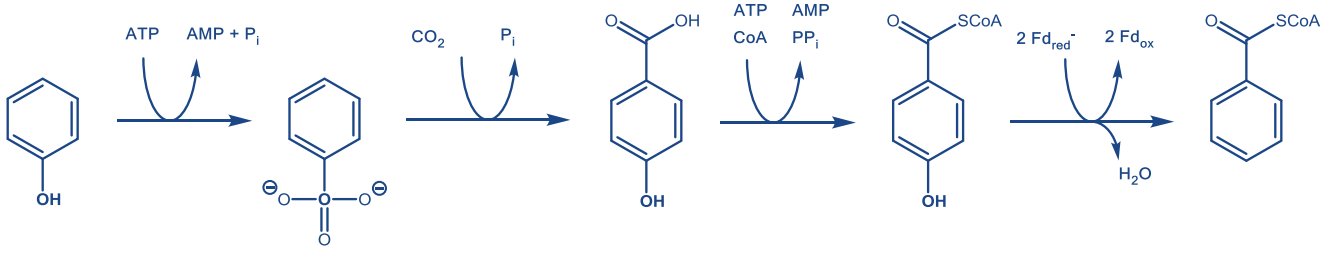

Fig. 5 Enzymatic reactions involved in the conversion of phenol to benzoyl-CoA. Phenylphosphate synthase catalyzes the first step, followed by a carboxylation reaction by phenylphosphate carboxylase. The generated product 4-hydroxybenzoate is then activated to 4-hydroxybenzoyl-CoA, which is reductively dehydroxylated to benzoyl-CoA

Both PPS and PPC have been purified and biochemically characterized (Schühle and Fuchs 2004; Schmeling et al. 2004; Narmandakh et al. 2006), and putative reaction schemes have been derived for both reactions from the biochemical properties of the enzymes. PPS consists of three different subunits, two of which are sufficient to catalyze the reaction. These subunits, PpsA and PpsB, show significant sequence similarity to phospho-*enol*-pyruvate (PEP) synthetases (Breinig et al. 2000; Schühle and Fuchs 2004; Schmeling et al. 2004), while the third subunit increases the enzyme activity severalfold via an unknown mechanism. The proposed reaction mechanism of PPS involves the transfer of pyrophosphate to a conserved histidine, hydrolysis of the bound pyrophosphate, and the transfer of the remaining phosphoryl group to phenol, yielding phenylphosphate (Schmeling et al. 2004; Schmeling and Fuchs 2009).

The PPC complex consists of four subunits, one of which is proposed to be involved in dephosphorylation of phenylphosphate. This generates phenol (or phenolate) in a tightly enzyme-bound state which seems to be more capable of being carboxylated than free phenol. The second partial reaction consists of the actual carboxylation of the enzyme-bound "phenolate" by subunits affiliated to the UbiD/UbiX family of carboxylases/decarboxylases, constituting a biochemical analog of the chemical Kolbe-Schmitt process (Payne et al. 2015). This partial reaction is freely reversible, causing the extensive exchange between free CO2 and the carboxyl group of 4-hydroxybenzoate as side reaction. In accordance with this proposal, the phosphatase subunit of PPC is only necessary for phenylphosphate carboxylation, but not for CO_2 exchange (Schmeling and Fuchs 2009). In contrast to PPS, PPC is highly oxygen sensitive. Oxygen-mediated inactivation of the enzyme is reversible and PPC activity can (at least partially) be restored by removal of oxygen and addition of reducing agents (Schmeling and Fuchs 2009).

Remarkably, strictly anaerobic bacteria, for example, Fe(III)-reducing *Geobacter* species or several species of sulfate-reducing bacteria, appear to use slightly different biochemical strategies for phenol degradation (Schleinitz et al. 2009; Ahn et al. 2009; Wöhlbrand et al. 2013). Phenol metabolism in these organisms is proposed to proceed via a similar pathway as outlined for *T. aromatica*, and cell extracts of *G. metallireducens* grown anaerobically on phenol actually exhibit low activities of both PPS and PPC (Schleinitz et al. 2009). Strictly anaerobic phenol-degrading species contains genes for orthologs of all three PPS subunits, but genes coding for two of the four subunits of PPC are lacking. Further details on the biochemistry of these processes are as yet unknown.

2.3.2 para-Cresol Hydroxylation with Water

The initial steps in the degradation of *para*-cresol (*p*-cresol, 4-methylphenol) are identical in aerobic and anaerobic bacteria and comprise the hydroxylation of the methyl functionality to 4-hydroxybenzoyl-CoA via 4-hydroxybenzyl alcohol/aldehyde (Fig. 6) (Hopper and Taylor 1977; Hopper et al. 1991; Rudolphi et al. 1991). The initial hydroxylation with water is catalyzed by *p*-cresol methylhydroxylase, a flavocytochrome *c*. The enzyme is composed of the active site subunit in which FAD is covalently attached to a tyrosine residue, and an electron transferring cytochrome

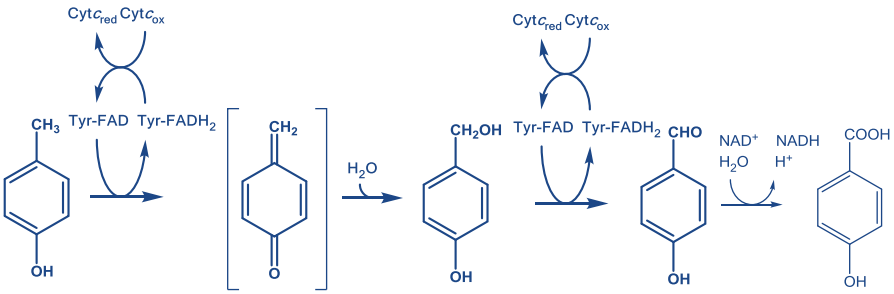

Fig. 6 Enzymatic reactions involved in the conversion of *p*-cresol to 4-hydroxybenzoate in aerobic and facultatively anaerobic bacteria. The *p*-cresol methylhydroxylase catalyzes hydride abstraction from *p*-cresol by the covalently bound FAD cofactor, yielding an enzyme-bound quinone methide; addition of water then yields the product 4-hydroxybenzyl alcohol. A similar reaction mechanism is proposed for the analogous conversion of 4-ethylphenol to 4-hydroxyphenylethanol. The alcohol may be further oxidized to the aldehyde by the same enzyme or by a NAD^+-dependent, separate dehydrogenase. Soluble *p*-cresol methylhydroxylase from aerobic and facultatively organisms transfers electrons to cytochrome *c*, whereas the membrane-bound enzyme from the obligately anaerobic *G. metallireducens* is suggested to transfer electrons to menaquinone (not shown)

c subunit (McIntire et al. 1985; Cunane et al. 2000). The relatively low C–H bond dissociation energy of *p*-cresol (335 kJ mol^{-1}) allows a hydride abstraction from the methyl group by the FAD cofactor generating a relatively stable quinone-methide intermediate; addition of water yields then the product 4-hydroxybenzyl alcohol, which may be further oxidized to the aldehyde by the same enzyme. In aerobic and facultatively anaerobic *p*-cresol degrading organisms, electrons are transferred to cytochrome *c*. In contrast, *p*-cresol methylhydroxylase was found to be strongly membrane bound in the obligately anaerobic *G. metallireducens*, probably due to complex formation with cytochrome *bc*1 complex-like components that contain two heme b and a 2[Fe-2S] cluster (Johannes et al. 2008). In such a complex, electrons derived from *p*-cresol oxidation may not be transferred to cytochrome *c* but rather to menaquinone. During the oxidation of 4-hydroxybenzyl alcohol to the corresponding aldehyde ($E^{\circ\prime} \approx -200$ mV) by *p*-cresol methylhydroxylase, electron transfer to menaquinone ($E^{\circ\prime} \approx -75$ mV) would be highly exergonic and may be coupled to proton transport across the membrane. Experimental evidence for such an energy conserving process is still lacking.

In sulfate-reducing bacteria, the redox potential of the terminal electron acceptors adenosine-5′-phosphosulfate ($E^{\circ\prime} = -60$ mV) or sulfite ($E^{\circ\prime} = -116$ mV) are too negative to accept electrons from *p*-cresol hydroxylation to 4-hydroxybenzyl alcohol ($E^{\circ\prime} = +80$ mV). For this reason, a different strategy exists for complete *p*-cresol oxidation coupled to sulfate reduction. In the *p*-cresol-degrading bacterium *Desulfobacterium cetonicum*, in vitro evidence for an addition of *p*-cresol to fumarate was provided, yielding 4-hydroxybenzylsuccinate as intermediate (Müller et al. 2001). The latter was then further degraded via 4-hydroxybenzoyl-CoA by a reaction sequence similar to that involved in toluene degradation (Fig. 7) (see Sect. 2.8.1).

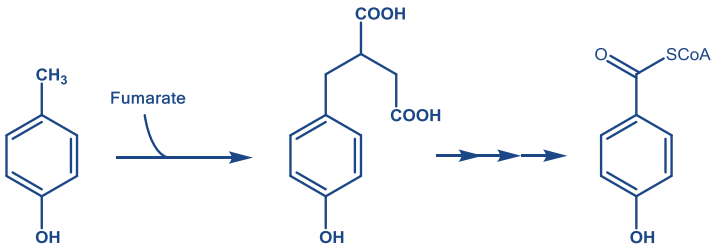

Fig. 7 Enzymatic reactions involved in the conversion of *p*-cresol to 4-hydroxybenzoyl-CoA in sulfate-reducing bacteria. The reactions leading from 4-hydroxybenzylsucinate to 4-hydroxybenzoyl-CoA are similar to those of benzylsuccinate to benzoyl-CoA involved in toluene degradation (Sect. 2.8.1)

2.3.3 4-Hydroxybenzoyl-CoA Dehydroxylation

Phenol and *p*-cresol are both metabolized via 4-hydroxybenzoate, which is subsequently activated by a specific AMP-forming CoA ligase to 4-hydroxybenzoyl-CoA (Biegert et al. 1993). This activation is essential for the following reductive dehydroxylation to benzoyl-CoA and water, using reduced ferredoxin as electron donor (Fig. 5) (Brackmann and Fuchs 1993; Breese and Fuchs 1998). The reaction is catalyzed by a 4-hydroxybenzoyl-CoA reductase that belongs to the xanthine oxidase family of molybdenum-cofactor (MPT) containing enzymes. It has so far only been isolated and studied in the denitrifying *T. aromatica*. The 275 kDa enzyme has an Hcr $(ABC)_2$ composition with the large HcrA-subunit harboring the MPT cofactor, while the HcrB-subunit contains two [2Fe-2S] clusters, and the C-subunit contains an FAD cofactor and an unusual [4Fe-4S] cluster. The latter are usually absent from members of the xanthine oxidase family of Mo enzymes, so this cofactor represents a distinguishing feature of 4-hydroxybenzyol-CoA reductases (Unciuleac et al. 2004). The redox potentials of the Mo cofactor of 4-hydroxybenzoyl-CoA reductase are unusually low ($E^{\circ\prime} = -500$ mV for the MoV/MoIV transition) reflecting the mechanistically demanding reaction of the enzyme (Boll et al. 2001). A radical-based mechanism has been proposed in which the first electron transfer yields a radical anion that is largely stabilized by the CoA thioester functionality. It also explains why 4-hydroxybenzoate needs to be activated prior to dehydroxylation.

Homologs of the encoding *hcrABC* genes are present in all phenol, *p*-cresol or 4-hydroxybenzoate degrading organisms. Due to the relatively high amino acid sequence similarities to other xanthine oxidase-related enzymes, an unambiguous assignment based on the amino acid sequence is not always feasible, although the reaction mechanism of 4-hydroxybenzoyl-CoA reductase fundamentally differs from all other MPT-containing enzymes.

2.3.4 Degradation of Other Para-Hydroxylated Compounds

The degradation of 4-ethylphenol is initiated by hydroxylation to 4-hydroxyphenylethanol with water. The enzyme catalyzing this reaction in *A. aromaticum* and most probably other 4-ethyphenol degrading organisms appears to be highly similar to

p-cresol methylhydroxylase, and identical mechanisms via a quinone methide have been proposed (Fig. 6) (Wöhlbrand et al. 2008; Muhr et al. 2015). The proposed subsequent steps resemble those of the ethylbenzene degradation pathway, involving dehydrogenation of the alcohol to 4-hydroxyacetophenone, and carboxylation of the latter followed by CoA-ester formation. Thiolytic cleavage yields acetyl-CoA and 4-hydroxybenzoyl-CoA. These reactions are discussed in more detail below (see Sect. 2.8.2 ethylbenzene degradation).

Tyrosine and 4-hydroxyphenylacetate are both converted to 4-hydroxybenzoyl-CoA by enzymes similar to those involved in the conversion of the non-phenolic analogues phenylalanine and phenylacetate and will be discussed in Sect. 2.6. Hydroquinone degradation in the fermenting strain HQGö1 is initiated by *ortho*-carboxylation to gentisate, followed by activation to gentisyl-CoA by an AMP forming CoA ligase (Gorny and Schink 1994a, c). In vitro assays demonstrated the subsequent reductive dehydroxylation to benzoyl-CoA without the accumulation of a mono-hydroxylated intermediate. The genes and enzymes involved in hydroquinone degradation are still unknown.

2.4 Reductive Dehalogenation by Class I BCRs

The major strategy for the anaerobic dehalogenation of haloaromatics is usually associated with organohalide respiration involving vitamin B12-dependent reductive dehalogenases. In this process, halogenated MAC serve only as terminal electron acceptors and the dehalogenated aromatic intermediates are generally not further degraded (Hug et al. 2013). However, there are a number of facultative and obligate anaerobes that completely degrade halobenzoates to CO_2 plus the halide anion as only carbon sources (Schennen et al. 1985; Kazumi et al. 1995; Song et al. 2000, 2001; Egland et al. 2001). The position and elemental nature of the halogen substituents in particular halobenzoates seems to determine whether degradation is possible or not. For example, the complete degradation of 3-Cl-benzoate is frequently observed in anaerobic bacteria, whereas anaerobic 3-F-benzoate degradation has never been reported. In contrast, a number of 2- and 4-F-benzoate degrading cultures are known, whereas 2-Cl-/4-Cl-benzoate degradation appears to be rather rare under anoxic conditions.

The complete degradation of 3-Cl-, 3-Br-, and 4-F-benzoates has recently been studied in detail in halobenzoate degrading *Thauera* strains (Kuntze et al. 2011b; Tiedt et al. 2016). The halobenzoates are first activated by AMP-forming CoA ligases followed by reductive, ATP-dependent dehalogenation by a class I BCR (Fig. 8). The transfer of two single electrons and a proton to 3-Cl-benzoyl-CoA or 4-F-benzoyl-CoA leads to the formation of an anionic transition state, as also proposed for benzoyl-CoA reduction. In case of 3-Cl- or 3-Br-benzoyl-CoA, the anion is irreversibly protonated yielding a 3-Cl-/3-Br-1,5-dienoyl-CoA intermediate, which spontaneously eliminates HCl/HBr in an E2 mechanism. In the case of 4-F-benzoyl-CoA, protonation of the anionic transition state is less favored than fluoride release; the latter event results in re-aromatization to benzoyl-CoA (Tiedt et al. 2016).

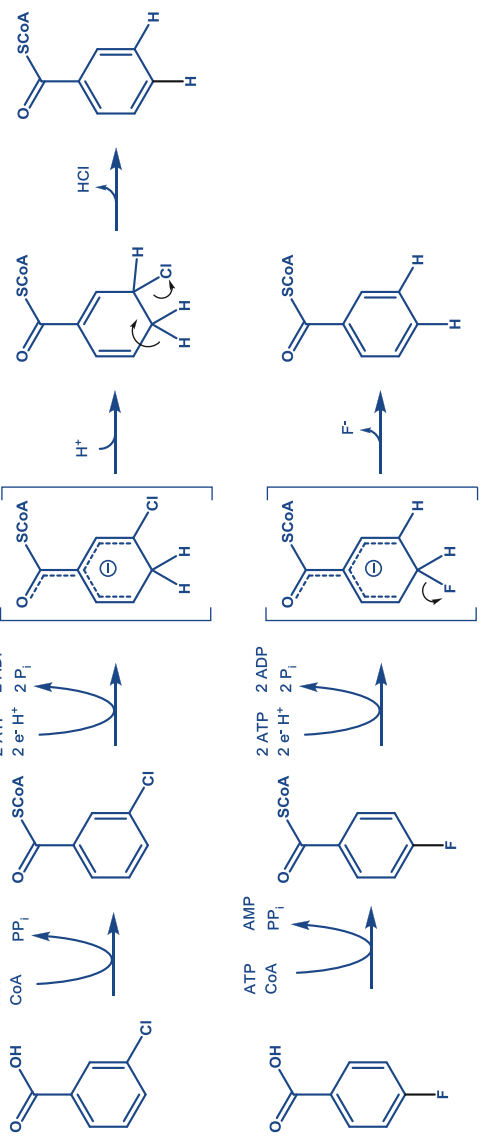

Fig. 8 Enzymatic reactions involved in the conversion of halobenzoates to benzoyl-CoA. After activation of 3-Cl- or 4-F-benzoate to the corresponding CoA-thioesters by AMP-forming CoA ligases, the 3-Cl-/4-F-benzoyl-CoA formed are converted by promiscuous class I BCR to the common, enzyme-bound anionic transition states (in brackets). In the case of 3-Cl-benzoyl-CoA, irreversible protonation yields 3-Cl-1,5-dienoyl-CoA that spontaneously eliminates HCl. In the case of 4-F-benzoyl-CoA conversion, fluoride release from the anionic transition state is favored over protonation, resulting in a formal nucleophilic aromatic substitution at C4

Notably, this ATP-dependent defluorination of 4-F-benzoyl-CoA to date is the only known oxygen-independent C–F-bond cleavage reaction of any fluoroaromatic in biology (C-F dissociation energy 530 kJ mol^{-1}). In summary, 4-F-benzoyl-CoA defluorination formally represents a nucleophilic aromatic substitution in contrast to the elimination of HCl/HBr from the 3-Cl-/3-Br-1,5-dienoyl-CoA product. Whether complete degradation of halogenated MAC is restricted to halobenzoates needs to be further studied. It is likely that at least some halogenated benzenes, toluenes, phenols, or other MAC may be degraded by promiscuous enzymes to the corresponding halogenated benzoyl-CoA intermediates.

2.5 Decarboxylation of Phthalates

Phthalate (1,2-dicarboxybenzene) and its isomers isophthalate (1,3-dicarboxybenzene) and terephthalate (1,4-dicarboxybenzene) are annually produced in the million ton scale due to their massive use of their esters as plasticizers or as precursors for polymers. In this context, it is surprising that the degradation pathways of phthalates remained obscure for a long time. In recent studies with phthalate-degrading species of the genera *Aromatoleum*, *Azoarcus*, and *Thauera*, phthalate degradation was shown to be initiated by a highly specific phthaloyl-CoA forming, succinyl-CoA-dependent CoA transferase (Ebenau-Jehle et al. 2016; Junghare et al. 2016). The phthaloyl-CoA formed is then decarboxylated by an oxygen-sensitive enzyme belonging to the UbiD-family of decarboxylases (*ubiD* encodes 3-octaprenyl-4-hydroxybenzoate carboxy-lyase involved in ubiquinone biosynthesis) (Fig. 9). The UbiD-like protein involved in phthaloyl-CoA decarboxylation shows significant similarities to phenylphosphate carboxylase or putative carboxylases involved in aromatic hydrocarbon carboxylation. Thioesterification is considered as an essential step for stabilizing an anionic transition state during phthaloyl-CoA decarboxylation. The extreme instability of phthaloyl-CoA requires

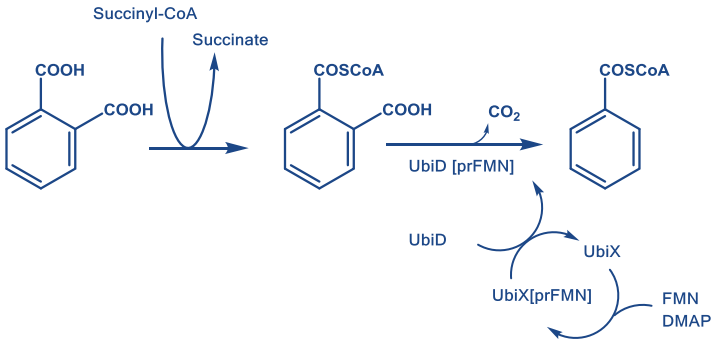

Fig. 9 Enzymatic reactions involved in the conversion of phthalate to benzoyl-CoA. The phthaloyl-CoA formed by a specific, succinyl-CoA dependent CoA transferase is decarboxylated by the oxygen-sensitive UbiD-like phthaloyl-CoA decarboxylase possibly carrying a prenylated FMN cofactor (prFMN). The prenylated cofactor is suggested to be formed by the UbiX-like prenyl transferase using dimethylallyl monophosphate (DMAP) as co-substrate

highly balanced phthaloyl-CoA forming/decarboxylating enzyme activities to avoid its cellular accumulation that would result in spontaneous hydrolysis.

In the genomes of all investigated phthalate-degrading organisms, the *ubiD*-like genes coding for phthaloyl-CoA decarboxylase are flanked by *ubiX*-like genes (Nobu et al. 2015; Ebenau-Jehle et al. 2016; Junghare et al. 2016). A UbiX-like protein was recently reported to act as a prenyltransferase that forms a UbiX-bound prenylated FMN cofactor. The latter is then transferred to the apo-form of an UbiD-like enzyme catalyzing the decarboxylation of cinnamate to styrene; dimethylallyl monophosphate serves as prenyl-donor for UbiX (Payne et al. 2015; White et al. 2015). In analogy, phthaloyl-CoA decarboxylase and other UbiD-like carboxylases/decarboxylases, whose genes are often flanked by *ubiX*-like genes, are all suggested to contain a prenylated flavin cofactor that is formed by an UbiX-like flavin prenyltransferase (Fig. 9).

Only little is known about the anaerobic degradation of isophthalate and terephthalate, though the complete degradation has been reported in strictly anaerobic *Pelotomaculum* and *Syntrophorhabdus* species (Qiu et al. 2004, 2006, 2008). In case of terephthalate, a similar strategy as for phthalate degradation via thioesterification followed by UbiX/UbiD-dependent decarboxylation is conceivable, and UbiD-like candidate genes have been identified by genomic/proteomic studies (Nobu et al. 2015). Nothing is known about the genes and enzymes involved in isophthalate degradation.

2.6 Degradation of Phenylalanine, Tyrosine, Phenylacetates, and Phenylpropionates

All three aromatic amino acids, phenylalanine (Phe), tyrosine (Tyr), and tryptophan (Trp), are readily degraded in the absence of oxygen (Heider and Fuchs 1997a, b). Many organisms capable of anaerobic respiration mineralize these compounds completely in pure or mixed cultures, and many fermentative bacteria or archaea are transforming aromatic amino acids to simpler aromatic or even aliphatic fermentation products (Barker 1981).

Fermentative transformation of aromatic amino acids is well known via the pathway of Stickland fermentation in strictly anaerobic bacteria affiliated to the Clostridia or related families of Firmicutes (Barker 1981; Schink and Stams 2013). Stickland fermentation involves the transformation of amino acid pairs, one of which is oxidized and decarboxylated to yield an organic acid lacking one C-atom, whereas the other is reduced to the organic acid of the same size as the amino acid. The oxidative pathway can be coupled to energy conservation via substrate-level phosphorylation from the acyl-CoA intermediate, while the reductive pathway is usually only required for equilibrating the redox balance and does not lead to energy conservation (with the exception of glycine reduction). All three aromatic amino acids can principally be channeled either into oxidative or reductive pathways. Both are initiated by the conversion of the amino acids to the respective arylpyruvates via transaminases, using 2-oxoglutararate as acceptor molecule. The respective

arylpyruvate intermediates are then either oxidized to the arylacetates plus CO_2 or reduced to the arylpropionates.

In the case of phenylpyruvate (Fig. 10), the reductive pathway involves an initial reduction to phenyllactate, which is then activated to the CoA-thioester by a specific enzyme affiliated to class III of CoA-transferases (Heider 2001) and dehydrated to cinnamoyl-CoA by a complex phenyllactyl-CoA dehydratase complex in a radical-type reaction mechanism (Dickert et al. 2002; Buckel et al. 2012). Cinnamoyl-CoA serves as CoA-donor for phenyllactate activation, generating cinnamate, which is finally reduced to phenylpropionate by an enoate reductase (Buckel et al. 2012). Because of the involvement of a CoA-transferase, the pathway proceeds without energy consumption despite the required activation of phenyllactate for the mechanistically difficult elimination of the α-hydroxy group (Dickert et al. 2002; Buckel et al. 2012).

The oxidative part of arylpyruvate metabolism is common to all known degradation pathways, but occurs in two variants. The first variant involves simultaneous oxidation and decarboxylation of the arylpyruvates by a ferredoxin-dependent oxidoreductase. The best characterized of these enzymes is indolepyruvate oxidoreductase from the hyperthermophilic archaeum *Pyrococcus furiosus*, which converts any arylpyruvate derived from the aromatic amino acids to the corresponding acylacetyl-CoA derivative (Mai and Adams 1994). The same type of enzyme is known from other archaea degrading aromatic amino acids (Parthasarathy et al. 2013; Aklujkar et al. 2014), and genes coding for this type of enzymes regularly occur in anaerobic amino-acid-degrading bacteria, although there are no reports on their expression patterns available. The thioesters generated by this reaction provide a means of energy conservation via substrate level phosphorylation (Fig. 10), either by CoA transfer to succinate and succinyl-CoA synthetase or by ADP-coupled acetyl-CoA synthetases with broad substrate specificities that include the arylacetyl-CoAs (Mai and Adams 1996; Bräsen and Schönheit 2004). Therefore, this variant of the pathway appears to be particularly required for fermentative microorganisms which excrete the corresponding arylacetates as fermentation products and use these reactions as their main energy conserving pathway during growth on aromatic amino acids.

The second variant of arylpyruvate oxidation has mostly been documented in denitrifying microorganisms capable of fully degrading phenylalanine. It involves an initial decarboxylation of phenylpyruvate to phenylacetaldehyde, which is then oxidized to phenylacetate (Heider and Fuchs 1997a, b; Debnar-Daumler et al. 2014). Phenylpyruvate decarboxylases are very common enzymes in microorganisms and have been identified in the phenylalanine degradation pathways of denitrifying bacteria affiliated to the β-Proteobacteria (Heider and Fuchs 1997a, b; Debnar-Daumler et al. 2014). The subsequent oxidation of phenylacetaldehyde to phenylacetate is then either catalyzed by NAD- or NADP-coupled aldehyde oxidoreductases or by aldehyde:ferredoxin oxidoreductases containing a tungsten cofactor (Debnar-Daumler et al. 2014). The organic acids generated from the aromatic amino acids are further degraded via separate metabolic modules. We describe here the pathways of anaerobic phenylacetate and phenylpropionate metabolism, which are probably shared with the 4-hydroxyphenyl acids derived from tyrosine (Fig. 1). For indoleacetate degradation see Sect. 3.3.

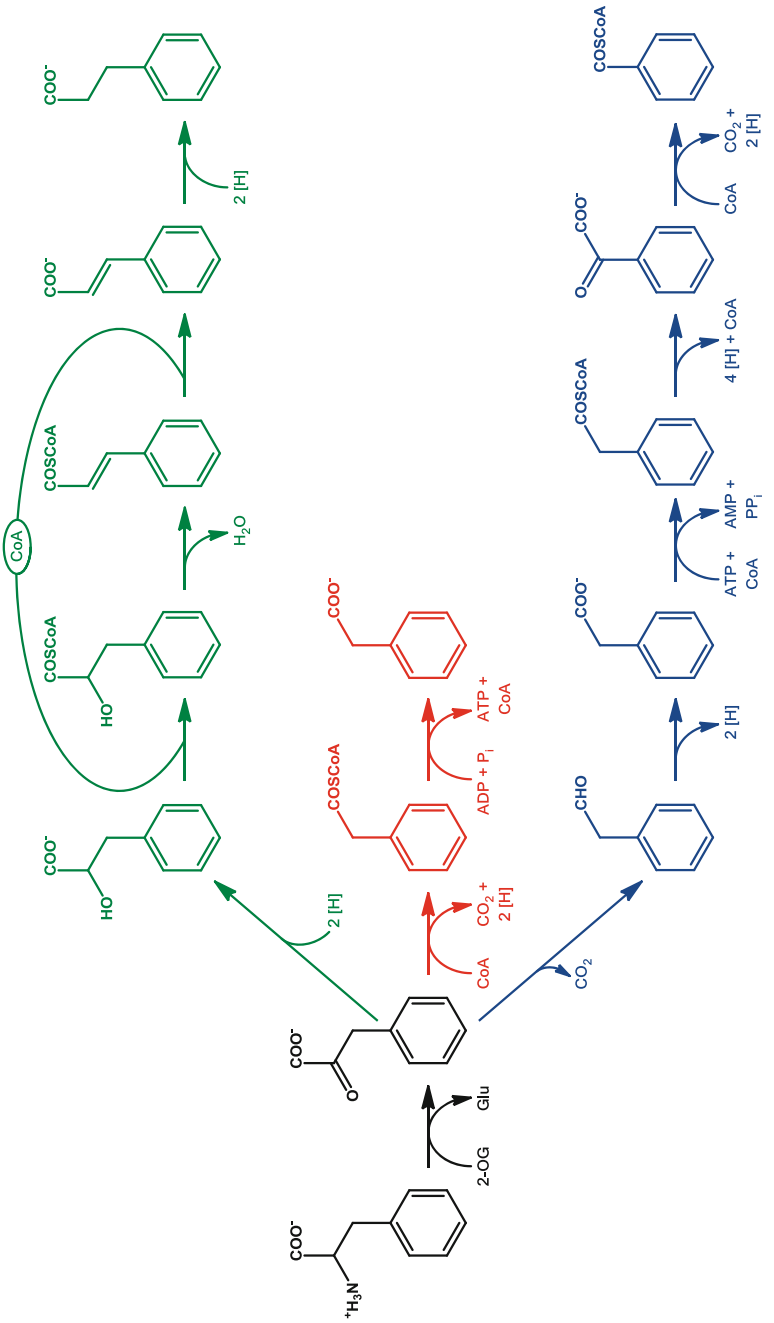

Fig. 10 Anaerobic degradation of aromatic amino acids. Reactions in green: reductive branch during fermentative transformations via Stickland fermentation; reactions in red: oxidative branch during fermentative Stickland reactions or during oxidative transformations in hyperthermophilic archaea; reactions in blue: complete degradation in denitrifying bacteria

Phenylacetate metabolism is initiated by its activation to the CoA-thioester by an AMP-generating CoA ligase, followed by a four-electron oxidation to phenylgloxylate, as catalyzed by a membrane-bound molybdenum enzyme, phenylacetyl-CoA:acceptor oxidoreductase (Rhee and Fuchs 1999). Finally, phenylgloxylate is oxidized and decarboxylated by phenylgloxylate:ferredoxin oxidoreductase, yielding the common intermediate benzoyl-CoA (Hirsch et al. 1998) (Fig. 10). In contrast, anaerobic phenylpropionate degradation follows a classical β-oxidation pathway, as inferred by a proteomic analysis in the denitrifying species *Aromatoleum aromaticum* (Trautwein et al. 2012) and recent biochemical characterization of some of the enzymes (J. Heider, unpublished data). Phenylpropionate is activated by a AMP-producing CoA ligase which also accepts several substrate analogs such as cinnamate or several hydroxyphenylpropionates (J. Heider, unpublished data), indicating that the same enzymes are involved in metabolizing intermediates of tyrosine or lignin degradation. Phenylpropionyl-CoA then seems to be oxidized to cinnamoyl-CoA, hydrated to 3′-hydroxyphenylpropionyl-CoA, further oxidized to benzoylacetyl-CoA and finally cleaved to benzoyl-CoA and acetyl-CoA by a specific thiolase. The genes for all proteins involved in this pathway appear to form a common substrate-induced operon in *A. aromaticum* (Trautwein et al. 2012).

2.7 Fermentative Formation of Toluene, *p*-Cresol, or Skatole

Some anaerobic fermentative organisms affiliated to the Clostridia are able to decarboxylate the arylacetates formed from the three aromatic amino acids to produce toluene, p-cresol, or skatole as final fermentation products. In the case of 4-hydroxyphenylacetate conversion to *p*-cresol, the enzyme responsible has been intensively studied and biochemically characterized. 4-Hydroxyphenylacetate decarboxylase (4Hpad) from the pathogenic gut bacterium *Clostridium difficile* has been identified as a glycyl radical enzyme and its structure and mechanism have been identified (Selmer and Andrei 2001; Selmer et al. 2005; Selvaraj et al. 2016). While *C. difficile* is resistant against very high concentrations of *p*-cresol, the presence of 4Hpad allows it to accumulate *p*-cresol at toxic concentrations for most other bacteria or the cells of the gut wall. Therefore, this metabolic pathway may be considered as one of the pathogenicity factors of this bacterium. Very similar enzymes seem to be involved in producing skatole from indoleacetate (Yu et al. 2006) or even toluene from phenylacetate (Zargar et al. 2016).

2.8 Degradation of Alkylbenzenes

2.8.1 Fumarate Addition to Toluene

Toluene is one of the most widely occurring aromatic hydrocarbons and appears to be readily degraded under anaerobic conditions. Anaerobic toluene degraders are known among different phylogenetic groups of denitrifying, metal-ion or sulfate-reducing bacteria, as well as among anoxygenic phototrophic bacteria and syntrophic

proton-reducing bacteria in co-culture with methanogenic archaea or other hydrogen-consuming anaerobes (Evans et al. 1992; Heider et al. 1998; Heider and Schühle 2013; Rabus et al. 2016a, b). The most prevalent genera of anaerobic toluene degraders are affiliated to the betaproteobacterial genera *Thauera* and *Aromatoleum/Azoarcus* or the deltaproteobacterial genera *Geobacter*, *Desulfobacula*, and *Desulfobulbus*. Despite the wide range of phylogenetic distribution, the pathway of anaerobic toluene degradation is completely conserved in all known cases, starting with the formation of (*R*)-benzylsuccinate from toluene and the fumarate co-substrate by the glycyl-radical enzyme benzylsuccinate synthase (BSS; Leuthner et al. 1998; Heider et al. 2016b). BSS exhibits a heterohexameric $(\alpha\beta\gamma)_2$ structure with two large α subunits of ca. 100 kDa harboring a single glycyl radical at the active center of one of the subunits and two very small β- and γ- subunits containing unusual Fe_4S_4 clusters (Leuthner et al. 1998; Funk et al. 2015; Heider et al. 2016b). As known for all glycyl radical enzymes, BSS needs to be activated to the active radical-containing form by an activating enzyme, which belongs to the family of "S-adenosylmethione radical" enzymes. In the activated state, BSS is proposed to catalyze the reaction by binding both substrates into a shielded active site pocket, followed by abstracting a hydrogen from the methyl group of toluene, radical addition of the transiently formed benzyl radical to the double bond of fumarate, and donating the hydrogen back to the generated benzylsuccinyl (product) radical, forming the product benzylsuccinate and regenerating the radical form of BSS (Heider et al. 2016b; Szaleniec and Heider 2016).

The intermediate (*R*)-benzylsuccinate is further degraded by β-oxidation via a conserved specific pathway consisting of a benzylsuccinate CoA-transferase, benzylsuccinyl-CoA dehydrogenase, phenylitaconyl-CoA hydratase, (hydroxybenzyl) succinyl-CoA dehydrogenase, and benzoylsuccinyl-CoA thiolase (Leuthner and Heider 2000; Heider et al. 2016b; Fig. 11). In all investigated bacteria capable of anaerobic toluene degradation, all enzymes needed for benzylsuccinate formation and β-oxidation are encoded in two separate but coordinately induced genetic units, namely the *bss* and *bbs* operons (Heider et al. 2016b).

The strategy of adding fumarate to activate nonreactive alkyl groups is used for a surprisingly large number of other substrates in addition to toluene. The enzymes involved in these reactions are all highly similar to BSS and apply the same apparent mechanism starting with a glycyl radical in their active sites. For example, fumarate addition has been reported in anaerobic degradation pathways of *m*-xylene (Rabus et al. 2016; see Sect. 4.1), *m*- or *p*-cresol (see Sects. 4.2 and 2.3.2), 2-methylnaphthalene (see ▶ Chap. 5, "Catabolic Pathways and Enzymes Involved in the Anaerobic Degradation of Polycyclic Aromatic Hydrocarbons" about PAH degradation of this volume on the degradation of polycyclic aromatic hydrocarbons in this volume) *p*-cymene (see Sect. 5.2), ethylbenzene (see Sect. 2.8.2), or even alkanes or cycloalkanes (Wilkes et al. 2016; see ▶ Chap. 3, "Catabolic Pathways Involved in the Anaerobic Degradation of Saturated Hydrocarbons" on the degradation alkanes in this volume). In the case of *p*-cresol, *p*-cymene, ethylbenzene, and possibly long-chain alkanes, the degradation pathways initiated by fumarate addition are not unique, and alternative pathways initiated by oxygen-independent hydroxylation are known. At least in some of these cases, the distribution of these different pathways appears to be dependent on the physiological

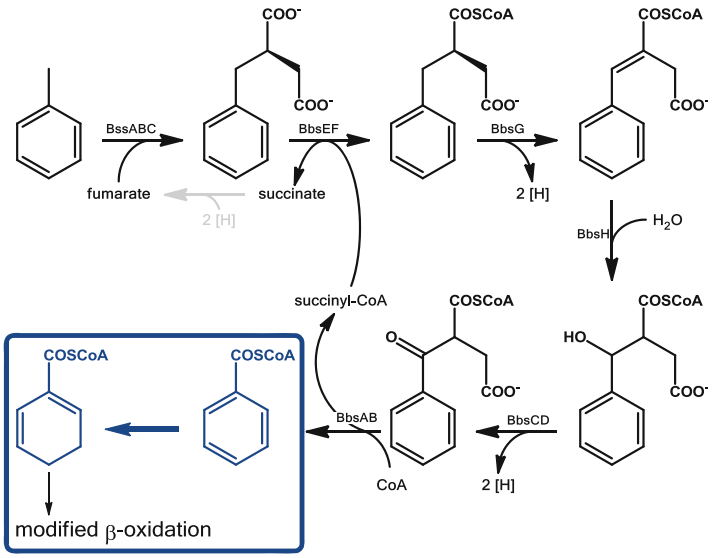

Fig. 11 Anaerobic degradation of toluene. Reactions are shown that are catalyzed by enzymes encoded by genes of the *bss* and *bbs* operons

type of the respective bacterial strains: *p*-cresol or ethylbenzene seem to be preferentially degraded via hydroxylation in facultatively anaerobic denitrifying bacteria and via fumarate addition in strictly anaerobic sulfate-reducers. However, both types of initial reactions have been observed for *p*-cymene in closely related species of denitrifying bacteria (Strijkstra et al. 2014; Rabus et al. 2016a), and alkanes seem to be degraded by either initial reaction in different sulfate-reducing bacteria (Heider and Schühle 2013).

2.8.2 Hydroxylation of Ethylbenzene or Propylbenzene by Water

The first indications of an oxygen-independent hydroxylation reaction initiating anaerobic hydrocarbon degradation arose with the isolation of denitrifying bacteria capable of degrading ethylbenzene or propylbenzene (Rabus and Widdel 1995; Rabus and Heider 1998). The initial reaction is catalyzed by ethylbenzene dehydrogenase (EBDH), a new type of soluble periplasmic molybdenum enzyme, which hydroxylates ethylbenzene or propylbenzene stereospecifically to the respective 1-(*S*)-alcohols (Kniemeyer and Heider 2001a). EBDH belongs to subfamily 2 of the dimethylsulfoxide reductase enzyme family of Mo-enzymes. It is a trimer comprising a large catalytic subunit, which contains a Mo-*bis*-molybdopterin guanine dinucleotide (MGD) cofactor and an Fe_4S_4 cluster, a medium subunit containing four additional FeS-clusters, and a small subunit containing a heme b cofactor (Kloer et al. 2006). Hydroxylation of the substrate occurs at the Mo cofactor with water as hydroxyl donor (Ball et al. 1996), and the probable mechanism of this reaction has been modeled by computational methods (Szaleniec et al. 2010, 2014). The electrons released from oxidation of the substrate are then transferred through the enzyme from the Mo-*bis*-MGD cofactor via the FeS-clusters toward the heme b,

where they are transferred to an external acceptor such as cytochrome c (Kloer et al. 2006).

The generated alcohol enters the cytoplasm and is further oxidized to the ketone (acetophenone or propiophenone, respectively) by a short-chain alcohol dehydrogenase (Kniemeyer and Heider 2001b). The genes for EBDH and the alcohol dehydrogenase are encoded in a common substrate-induced operon (Rabus et al. 2005). The further metabolism of the aromatic ketones is initiated by an ATP-dependent carboxylation to benzoylacetate or 2-benzoylpropionate, respectively, by acetophenone carboxylase, which belongs to a special family of carboxylases together with acetone carboxylases from many microorganisms (Jobst et al. 2010; Heider et al. 2016a). Benzoylacetate is finally activated to the CoA-thioester by a specific CoA ligase, which is then cleaved to benzoyl-CoA and acetyl-CoA by a thiolase (Fig. 12). The genes coding for the subunits of acetophenone carboxylase and benzoylacetate-CoA ligase form a second operon, which is specifically induced by the presence of acetophenone or a few very similar substrates (Heider et al. 2016a; Muhr et al. 2016).

Molybdenum enzymes with high similarities to EBDH have also been identified in the anaerobic degradation of the aromatic hydrocarbon *p*-cymene (see Sect. 5.2) and the isoprenoid side chains of cholesterol or other steroids (Dermer and Fuchs 2012; Heider et al. 2016c, see ▶ Chap. 7, "Anaerobic Biodegradation of Steroids" on the degradation of steroids in this volume) and may also be involved in an alternative pathway of anaerobic alkane degradation (Heider et al. 2016c).

2.9 Degradation of Benzene

The C–H bond dissociation energy of benzene is the strongest among all hydrocarbons (473 kJ mol^{-1}), and the oxygen-independent attack on the benzene ring has been considered to afford an unprecedented mechanism. To date numerous benzene-degrading pure/enrichment cultures have been obtained under denitrifying, sulfate-reducing, Fe(III)-reducing conditions and in methanogenic consortia [for a recent review see (Meckenstock et al. 2016)]. Recently, even the genetically tractable *G. metallireducens* was reported to completely degrade benzene (Zhang et al. 2013). Considering the number of well-characterized benzene-degrading cultures available, it is surprising that benzene degradation is still controversially discussed. The identification of benzene-induced UbiD-like enzymes in conjunction with a number of ^{13}C-labeling studies in benzene degrading Fe(III)-reducing or methanogenic enrichment cultures (Kunapuli et al. 2008; Abu Laban et al. 2009, 2010; Luo et al. 2014), as well as in the hyperthermophilic, benzene-degrading *Ferroglobus placidus* (Holmes et al. 2011), convincingly suggested that benzene is initially carboxylated to benzoate (Fig. 13). However, a recent study with *G. metallireducens* reported the anoxic hydroxylation of benzene to phenol with water (Zhang et al. 2013) (Fig. 13). In this study, $H_2{}^{18}O$ labeling experiments indicated that the hydroxyl functionality of phenol derived from water and not from reactive oxygen species that may be formed by reduced media components during sample preparation. In contrast to the

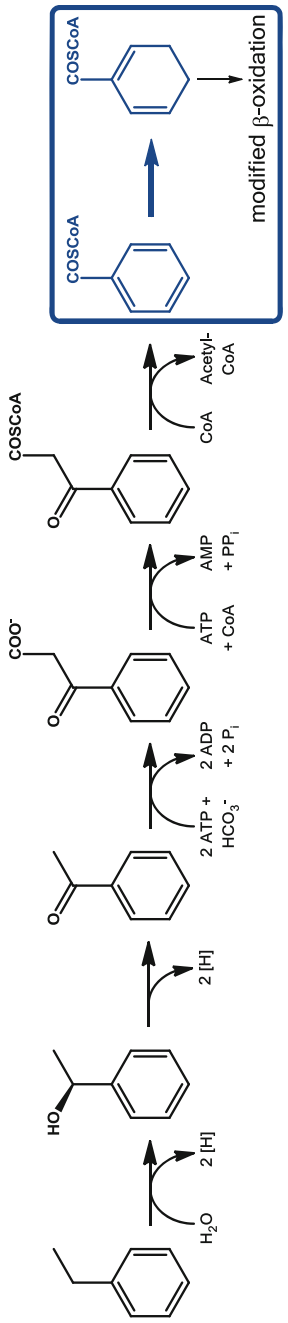

Fig. 12 Anaerobic degradation of ethylbenzene. The degradation of p-ethylphenol (4-hydroxyethylbenzene) proceeds in analogous reactions in an induced pathway to 4-hydroxybenzoyl-CoA

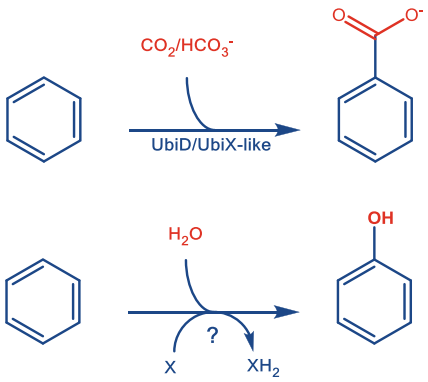

Fig. 13 Scenarios for the initial conversion of benzene without oxygen. The two alternative reactions in benzene degrading Fe(III)-reducing/methanogenic enrichment cultures (carboxylation) or *G. metallireducens* (hydroxylation) are shown. In the case of benzene carboxylation, the induction of *ubiD*-like genes putatively coding for a benzene carboxylase is in favor for the carboxylation scenario

carboxylation scenario, no plausible genes were identified that could be assigned to water-dependent benzene hydroxylation (Zhang et al. 2014). In summary, anaerobic benzene degradation still remains obscure, but recent work suggested that anaerobic benzene activation may be accomplished by different strategies in different organisms thriving at different habitats.

3 Degradation via Dearomatization of *ortho*-Substituted Benzoyl-CoAs

A number of aromatic compounds are anaerobically degraded via *ortho*-substituted benzoyl-CoA analogues including (acetyl-)salicylate, anthranilate, indoleacetate, ortho-xylene, or 2-F-/2-benzoates. *Ortho*-positioned substituents cannot be removed by specific reductases, but hydroxy- or amino-substituented benzoyl-CoA analogues may be directly converted to intermediates of the benzoyl-CoA degradation pathway after dearomatization by benzoyl-CoA reductases (Fig. 14).

3.1 Degradation of Salicylate and Anthranilate

Growth with salicylate (2-hydroxybenzoate) and anthranilate (2-aminobenzoate) has been reported for denitrifying bacteria and was always initiated by the activation to the corresponding CoA esters by specific and growth substrate-induced AMP forming CoA ligases (Bonting and Fuchs 1996; Lochmeyer et al. 1992). The anthranoyl-CoA and salicyl-CoA formed serve both as direct substrates for class I BCRs that form the corresponding instable *ortho*-substituted 1,5-dienoyl-CoAs. In the case of 2-hydroxy-1,5-dienoyl-CoA, tautomerization yields 2-oxocylohex-1-

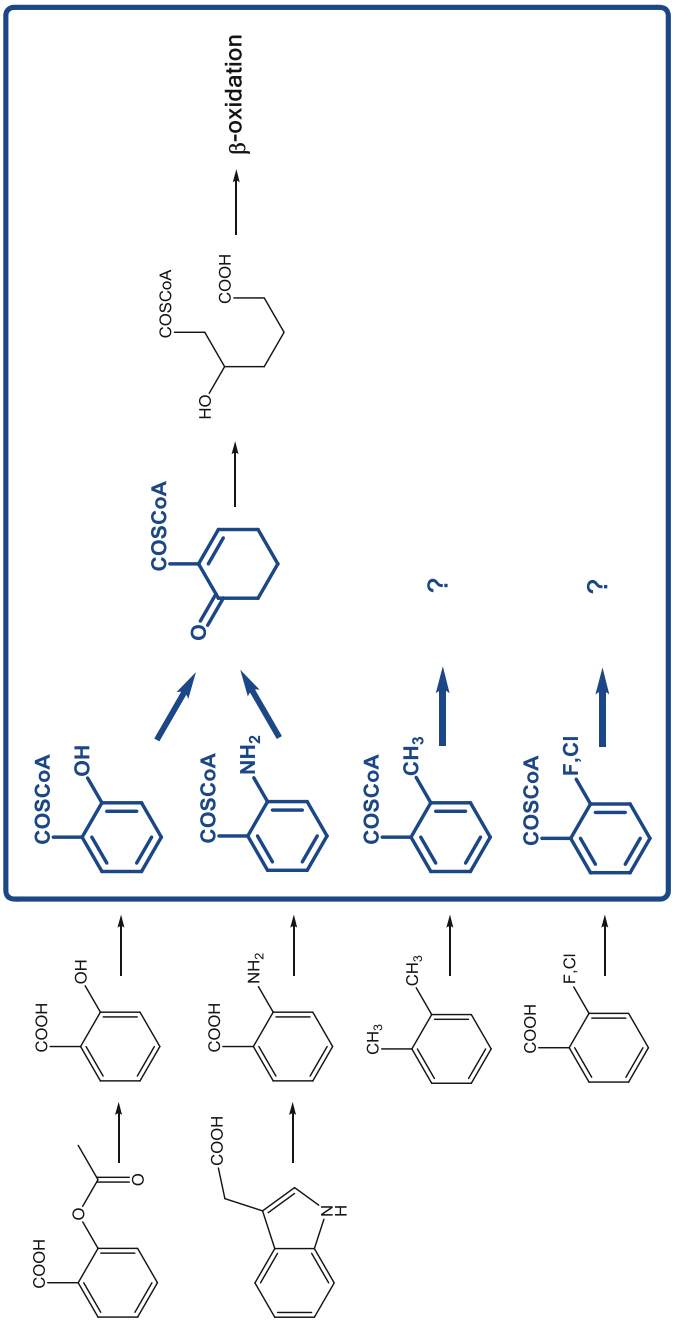

Fig. 14 Degradation of MAC via *ortho*-substituted benzoyl-CoA intermediates

ene-1-carboxyl-CoA, which is the substrate for the ring-cleaving hydrolase of the general benzoyl-CoA degradation pathway (Fig. 14). In case of 2-amino-1,5-dienoyl-CoA, the imine-tautomer will spontaneously hydrolyze to 2-oxocylohex-1-en-1-carboxyl-CoA. In summary, both salicylate and anthranilate degradation requires only a specific CoA ligase for channeling the growth substrates into the benzoyl-CoA degradation pathway.

3.2 Degradation of *o*-Xylene, 2-Methyl-, and 2-Fluorobenzoate

The anaerobic degradation of *o*-xylene has been demonstrated for several anaerobic cultures and is considered to be initiated by addition to fumarate followed by the oxidation to 2-methylbenzoyl-CoA in analogy to the benzylsuccinate degradation pathway involved in toluene degradation. Reaction of BSS isoenzymes with all three xylene isomers has previously been demonstrated, although it is unclear how the generated methylbenzylsuccinates are further metabolized to the corresponding methylbenzoyl-CoAs (Verfürth et al. 2004). The further degradation of 2-methylbenzoyl-CoA via a modified benzoyl-CoA degradation pathways has not been studied yet. In case of 2-F-benzoate, complete degradation has frequently been reported for denitrifying bacteria that are capable of growing with benzoate. Though standard benzoate-CoA ligase and class I BCRs generally show a high activity with the 2-F-analogues, the enzymes involved in C–F-bond cleavage are unknown. Mechanisms as reported for C-halide cleavage during 3-Cl- or 4-F-benzoyl-CoA reduction by class I BCR are not applicable in case of 2-F-benzoyl-CoA. Thus, C–F-bond cleave has to occur in so far unknown downstream reactions of the benzoyl-CoA degradation pathway.

3.3 Degradation of Indoleacetate via Anthranoyl-CoA

Indoleacetate is a common degradation intermediate of the amino acid tryptophan and serves as an important plant hormone, auxin. Therefore, it occurs in large amounts and serves as growth substrate for bacteria such as *Aromatoleum* or *Azoarcus* species, even under anaerobic conditions. Recently, the pathway of anaerobic indoleacetate metabolism in these bacteria was discovered and the enzymes and genes involved were identified (Fig. 15; Ebenau-Jehle et al. 2012; Schühle et al. 2016). The pathway consists of ten successive enzymatic reactions, starting with uptake and activation of indoleacetate to the CoA-thioester using a highly specific CoA ligase (Schühle et al. 2016), followed by hydroxylation at the pyrrole ring to 2-oxoindoleactyl-CoA by a molybdenum enzyme of the xanthine dehydrogenase family and hydrolytic ring opening to yield (2-aminophenyl)succinyl-CoA (Fig. 15). The CoA moiety is then intramolecularly transferred to the other carboxy group by a CoA-transferase (Schühle et al. 2016), allowing the reconfiguration of the molecule to (2-aminobenzyl)malonyl-CoA via a coenzyme B12-dependent mutase (Fig. 15). Finally, the remaining pathway consists of a simultaneous oxidation and

decarboxylation to 2-aminocinnamoyl-CoA, followed by β-oxidation steps generating anthranoyl-CoA and acetyl-CoA (Fig. 15).

4 Degradation via Dearomatization of *meta*-Substituted Benzoyl-CoAs

A number of MAC containing *meta*-positioned functionalities are converted to the corresponding *meta*-substituted benzoyl-CoA analogues (Fig. 16). Non-halogenic substituents in *meta*-position generally cannot be easily removed during reductive dearomatization, and stable *meta*-substituted 1,5-dienoyl-CoA analogues are formed by BCRs. For this reason the degradation via *meta*-substituted benzoates often requires modifications of the benzoyl-CoA degradation pathway which include the synthesis of isoenzymes acting on the *meta*-substituted benzoyl-CoA analogues and their metabolites in a modified benzoyl-CoA degradation pathway. An exception of this rule was reported for 3-hydroxybenzoate degradation in the fermenting *Sporotomaculum hydroxybenzoicum* where a reductive dehalogenation of 3-OH-benzoyl-CoA to benzoyl-CoA was reported (Müller and Schink 2000).

4.1 Degradation via Dearomatization of 3-Methylbenzoyl-CoA

3-Methylbenzoyl-CoA is suggested to be an intermediate during the degradation of 3-methlybenzoate, *m*-xylene and *o*-cresol (Fig. 16). During 3-methylbenzoate degradation in the denitrifying *Azoarcus* sp. CIB, a specific AMP-forming CoA ligase forms 3-methylbenzoyl-CoA (Juárez et al. 2013). The anaerobic degradation of *m*-xylene has been demonstrated in several denitrifying and sulfate-reducing pure/enrichment cultures. The initial conversion of *m*-xylene to 3-methylbenzylsuccinate was reported either by metabolite analyses of the culture medium or by in vitro assays (Krieger et al. 1999; Elshahed et al. 2001; Morasch et al. 2004; Verfürth et al. 2004); 3-methylbenzylsuccinate is then expected to be converted to 3-methylbenzoyl-CoA via a modified benzylsuccinate degradation pathway (Juárez et al. 2013). In the case of *o*-cresol, carboxylation to 3-methyl-4-hydroxybenzoyl-CoA is suggested to be catalyzed by a phenyl-phosphate carboxylase-like enzyme system (Bisaillon et al. 1991; Rudolphi et al. 1991) (Fig. 16). Reductive dehydroxylation by a 4-hydroxybenzoyl-CoA reductase-like Mo-enzyme then results in 3-methylbenzoyl-CoA formation. In analogy to *p*-cresol oxidation to 4-hydroxybenzoate, *o*-cresol could also be oxidized to 2-hydroxybenzoate; however, experimental evidence for such a degradation pathway is lacking so far (Schink et al. 2000).

The genome of *Azoarcus* sp. CIB contains the 3-methylbenzoate-induced *mbd* gene cluster comprising the genes encoding a 3-methylbenzoate-inducible class I BCR. The gene product is specifically involved in the reduction of 3-methylbenzoyl-CoA to a methylated 1,5-dienoyl-CoA (Fig. 17) (Juárez et al. 2013). Other 3-methylbenzoate-induced gene products are involved in modified beta-oxidation of CoA ester substrates carrying the additional methyl group (e.g., the methylated

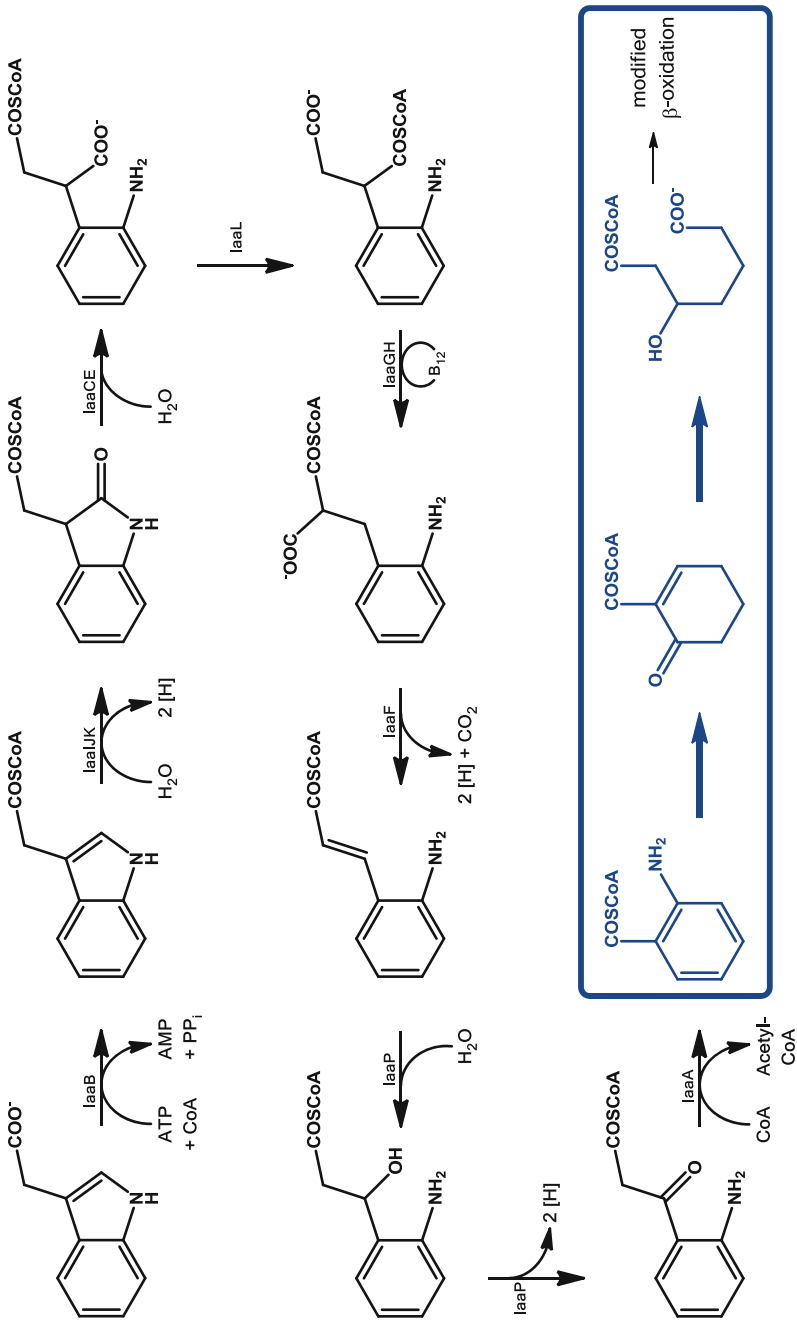

Fig. 15 Conversion of indoleacetate to 2-aminobenzoyl-CoA. The reactions catalyzed by the Iaa-gene products are indicated

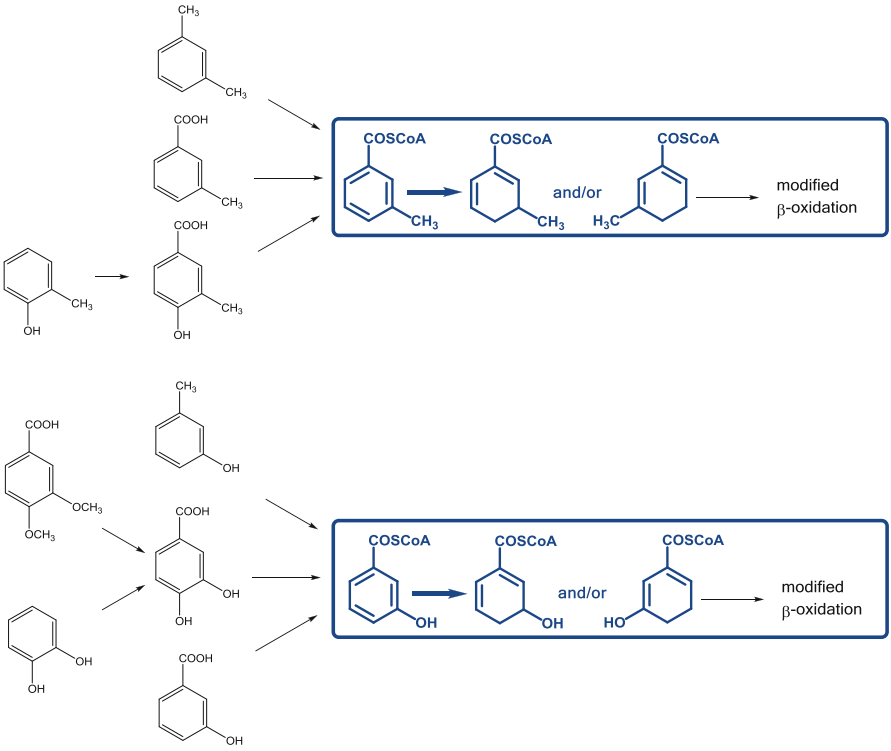

Fig. 16 Degradation of MAC via *meta*-substituted benzoyl-CoA intermediates

1,5-dienoyl-CoA) (Fig. 17). It is unknown whether 3-methylbenzoyl-CoA reductase from *Azoarcus* sp. CIB forms the 3-methyl-1,5-dienoyl-CoA or 5-methyl-1,5-dienoyl-CoA isomer or both. Depending on the regioselectivity of the 3-methylbenzoyl-CoA reductase, 2-methylcrotonyl-CoA and/or 2,3-dehydrovaleryl-CoA (2-pentenoyl-CoA) intermediates would be formed by isoenzymes or promiscuous enzymes of the lower benzoyl-CoA degradation pathway. In any case, 3-methylbenzoyl-CoA is expected to be converted to two acetyl-CoA, propionyl-CoA and CO_2 (Juárez et al. 2013).

4.2 Degradation via Dearomatization of 3-Hydroxybenzoyl-CoA

The degradation of 3-hydroxybenzoate via dearomatization of 3-hydroxybenzoyl-CoA was studied in the denitrifying *T. aromatica*, and shown to be initiated by an AMP-forming, specific 3-hydroxybenzoate CoA ligase (Laempe et al. 2001). The activated thioester was then reduced by ATP-dependent class I benzoyl-CoA reductase to either 3-hydroxy-1,5-dienoyl-CoA or 5-hydroxy-1,5-dienoyl-CoA; the latter is expected to tautomerize to the more stable keto-form (Fig. 18). However, the metabolites

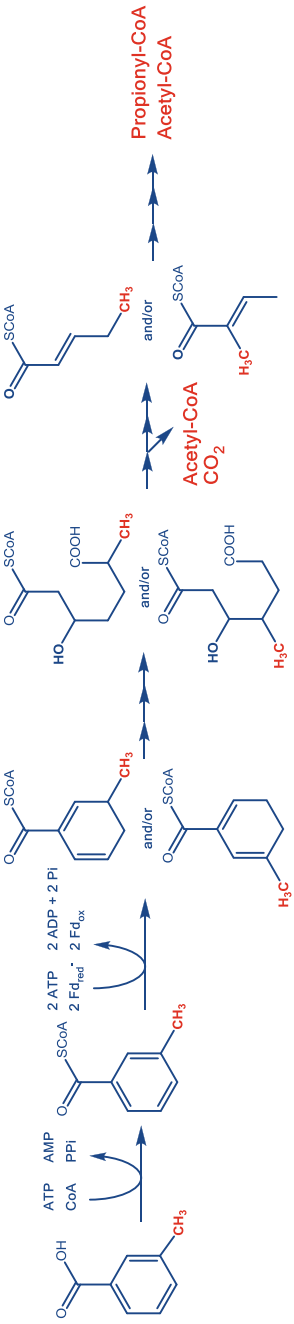

Fig. 17 Enzymatic reactions involved in the degradation of 3-methylbenzoyl-CoA to acetyl-CoA, propionyl-CoA, and CO_2. The degradation has been studied in *Azoarcus* sp. CIB

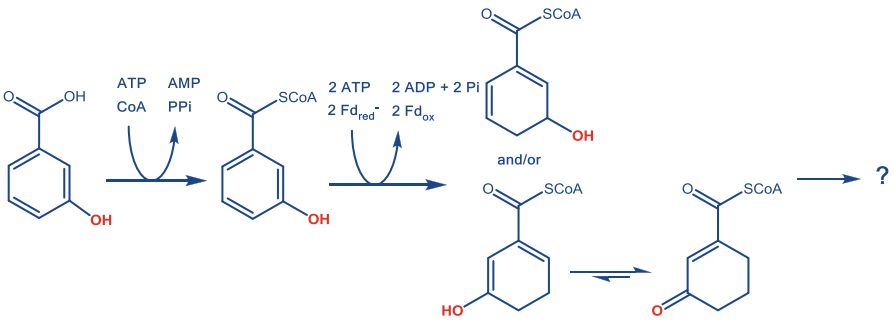

Fig. 18 Initial enzymatic reaction involved in the degradation of 3-hydroxybenzoate via 3-hydroxybenzoyl-CoA dearomatization. The degradation has been studied in *T. aromatica*; the steps involved in modified β-oxidation after ring reduction are still unknown

and enzymes involved in the further degradation of the products of 3-hydroxybenzoyl-CoA reduction are still unknown. It is obvious that the degradation pathway of 3-hydroxybenzoyl-CoA has to differ substantially from that of benzoyl-CoA.

In the sulfate-reducing *Desulfosarcina cetonica*, the formation of 3-hydroxybenzylsuccinate from *meta*-cresol is catalyzed by a fumarate-adding glycyl-radical enzyme (Müller et al. 1999). The further degradation is then similar to that of toluene and results in the formation of 3-hydroxybenzoyl-CoA.

Protocatechuate (3,4-dihydroxybenzoate) is a central intermediate in the aerobic degradation of many aromatic compounds, but also one in the anaerobic degradation of catechol and 3,4-methoxylated aromatic carboxylic acids that may derive from lignin degradation (vanillate, isovanillate, or veratrate). Anaerobic catechol degradation was studied in *Desulfobacterium* sp. strain Cat2 and in *T. aromatica*. It appears to be initiated by promiscuous enzymes of anaerobic phenol degradation and involves the phosphorylation to catechuylphosphate followed by carboxylation to protocatechuate (Gorny and Schink 1994b; Ding et al. 2008). Under anoxic conditions, methoxylated aromatic compounds are substrates for methylotrophic acetogens that use corrinoid-dependent *O*-demethylases to cleave the phenyl methyl ether bond and to transfer the methyl group to tetrahydrofolate (Engelmann et al. 2001). As a result, protocatechuate may be formed, for example, from vanillate. Complete protocatechuate degradation is initiated by the activation to protocatechuyl-CoA by a promiscuous 3-hydroxybenzoate-CoA ligase, followed by reductive dehydroxylation to 3-hydroxybenzoyl-CoA, possibly catalyzed by a promiscuous 4-hydroxybenzoyl-CoA reductase (Gorny and Schink 1994b; Ding et al. 2008).

5 Degradation via Dearomatization of *para*-Substituted Benzoyl-CoA Derivatives

Insights into the degradation of MAC via dearomatization of non-halogenic/non-hydroxylated *para*-substituted benzoyl-CoA derivatives have only recently been obtained in studies of 4-methylbenzoate and *p*-cymene degradation in

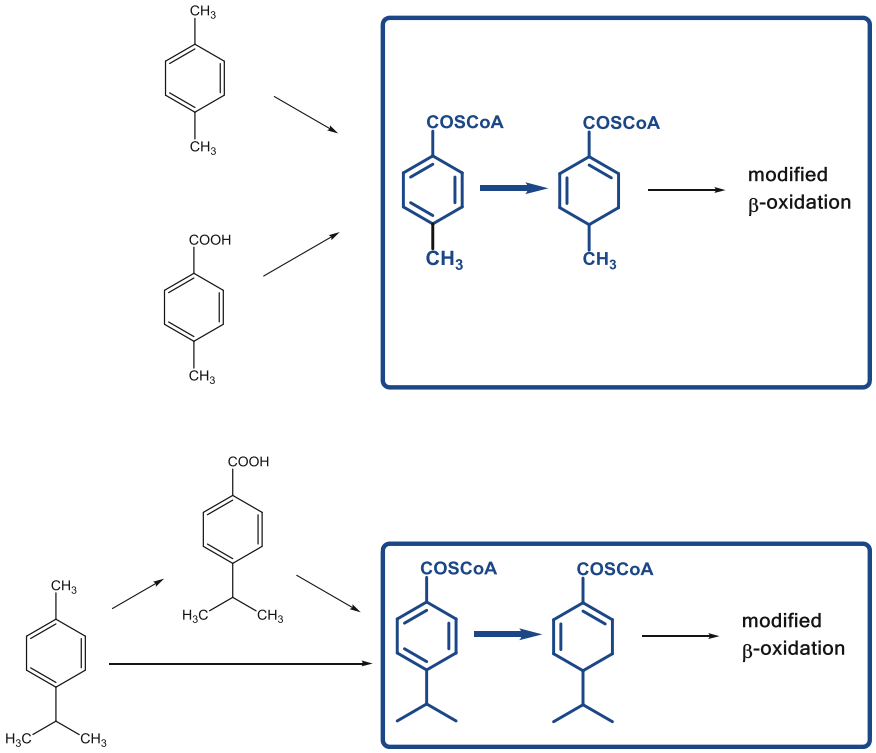

Fig. 19 Degradation of MAC via *para*-substituted benzoyl-CoA intermediates

Magnetospirillum, *Aromatoleum*, and *Thauera* strains (Lahme et al. 2012; Strijkstra et al. 2014) (Fig. 19).

In *Magnetospirillum* sp. pMbN1, an ATP-dependent class I BCR catalyzed the dearomatization of 4-methylbenzoyl-CoA to 4-methyl-1,5-dienoyl-CoA (Lahme et al. 2012) (Fig. 19). This finding is remarkable as conventional *Thauera*- and *Azoarcus*-type BCRs have not been reported to accept *para*-substituted benzoyl-CoA analogues other than 4-F-benzoyl-CoA (see Sect. 2.4). The 4-methylbenzoyl-CoA converting BCR differs from the typical class I BCRs with respect to substrate preference and amino acid sequence similarity, and it has been suggested that it represents a member of a new subclass of class I BCRs.

A 4-methylbenzoyl-CoA reductase is also expected to be involved in anaerobic *p*-xylene degradation (Fig. 19). Metabolite analyses in *p*-xylene-degrading, sulfate-reducing, and denitrifying enrichment cultures revealed that 4-methylbenzylsuccinate is an intermediate during *p*-xylene degradation (Morasch and Meckenstock 2005; Rotaru et al. 2010). This finding suggests that the degradation pathway of *p*-xylene is similar to that of toluene: after initial addition of *p*-xylene to fumarate by a glycyl-radical enzyme homologous to benzylsuccinate synthase, the 4-methylbenylsuccinate formed is then oxidized to 4-methylbenzoyl-CoA by a set

of enzymes catalyzing β-oxidation like reactions similar to those involved in benzylsuccinate conversion to benzoyl-CoA.

5.1 Enzymatic Reactions Involved in 4-Methylbenzoate Degradation

The catabolism of 4-methylbenzoate has been elucidated in recent studies with *Magnetospirillum* pMbN1 by proteogenomic analyses, metabolite analyses, and in vitro enzyme assays (Lahme et al. 2012) (Fig. 20). After activation of 4-methylbenzoate by an AMP-forming 4-methylbenzoyl-CoA synthetase, a specific 4-methylbenzoyl-CoA reductase (class I BCR) formed 4-methyl-1,5-dienoyl-CoA in an ATP- and electron donor-dependent manner. In the following, a series of β-oxidation-like reactions similar to those of the standard benzoyl-CoA degradation pathway converted 4-methyl-1,5-dienoyl-CoA to central intermediates. Similar to 3-methylbenzoyl-CoA the methyl group is retained during the entire pathway. However, due to the different positions of the methyl-group in the ring, the 4-methylbenzoyl-CoA degradation pathway is suggested to yield acetoacetate and acetyl-CoA, whereas propionyl-CoA, acetyl-CoA, and CO_2 are proposed to be formed from 3-methylbenzoyl-CoA.

5.2 Enzymatic Reactions Involved in *p*-Cymene Degradation

The degradation of *p*-cymene (4-isopropyltoluene) was studied in a denitrifying *A. aromaticum* and a *Thauera* sp. strain related to *T. terpenica* (Strijkstra et al. 2014). Surprisingly, both use different strategies for initiation of complete *p*-cymene degradation (Fig. 21).

In the *A. aromaticum* strain pCyN1, a Mo-enzyme similar to ethylbenzene dehydrogenase catalyzed the hydroxylation of *p*-cymene to 4-isopropylbenzyl alcohol with water. The presumptive *p*-cymene dehydrogenase is encoded by the *cmdABC* genes, and a mechanism similar to that established for ethylbenzene dehydrogenase has been proposed. The further conversion of 4-isopropylbenzyl alcohol is then likely to be accomplished by two dehydrogenases that oxidize the alcohol stepwise to the carboxylic acid; finally, an AMP-forming CoA ligase activates 4-isopropylbenzoate to a thioester (Fig. 21).

An alternative pathway has been identified in the *p*-cymene degrading *Thauera* strain pCyN2, where *p*-cymene was initially converted to 4-isopropylbenzylsuccinate; most likely by addition to fumarate catalyzed by a glycyl radical enzyme. Proteogenomic analyses suggested a pathway in which 4-isopropylbenzylsuccinate is oxidized to 4-isopropylbenzoyl-CoA, similar to the benzylsuccinate degradation pathway involved in toluene degradation. The further degradation of the branched chain containing 4-isopropylbenzoyl-CoA remains to be studied; the presence of a novel modification of the benzoyl-CoA degradation pathway is anticipated (Fig. 21).

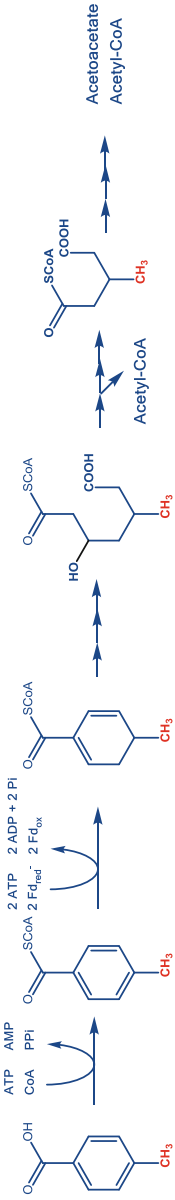

Fig. 20 Enzymatic reactions involved in the anaerobic degradation of 4-methylbenzoate. The degradation has been studied in *Magnetospirillum* pMbN1

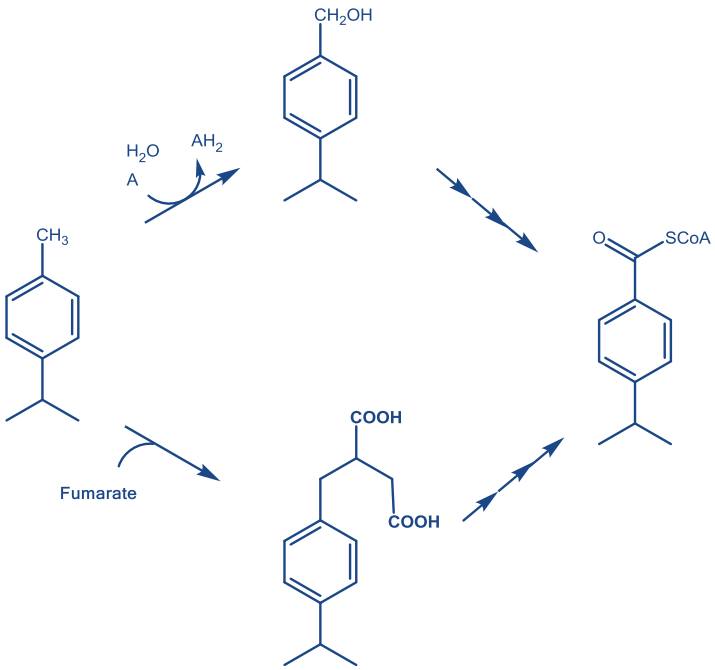

Fig. 21 Enzymatic reactions involved in the conversion of *p*-cymene to 4-isopropylbenzoyl-CoA. The upper pathway was identified in the *p*-cymene-degrading *A. aromaticum* strain pCyN1 and proceeds via anoxic hydroxylation to 4-isopropylbenzyl alcohol by a Mo-enzyme related to ethylbenzene dehydrogenase. The alternative lower route is initiated by addition of *p*-cymene to fumarate similar to addition of toluene to fumarate by benzylsuccinate synthase; it was found in the *p*-cymene-degrading *Thauera* strain pCyN2. The enzymes involved have not been studied, yet, and the further degradation of 4-isopropylbenzoyl-CoA is unknown

6 Degradation via Dearomatization of Di- and Trihydroxybenzenes

Next to benzoyl-CoA and derivatives of it, three di- and trihydroxybenzenes with at least two *meta*-positioned phenolic hydroxy-functionalities serve as substrates for dearomatizing enzymes: resorcinol, hydroxyhydroquinone, and phloroglucinol. The possibility of these intermediates to tautomerize to 1,3-diketones strongly weakens the aromatic character of these compounds and, consequently, common biological electron donors such as reduced ferredoxin or NAD(P)H serve directly as electron donors without the need for coupling the ring reduction to an exergonic reaction (Schink et al. 2000; Fuchs et al. 2011). In case of hydroxyhydroquinone degradation, even an oxidative dearomatization of the aromatic ring is possible – the only such example under anoxic conditions. The dearomatized cyclic 1,3-diketones can then be cleaved hydrolytically, and the acyclic products are converted to central intermediates (Fig. 22).

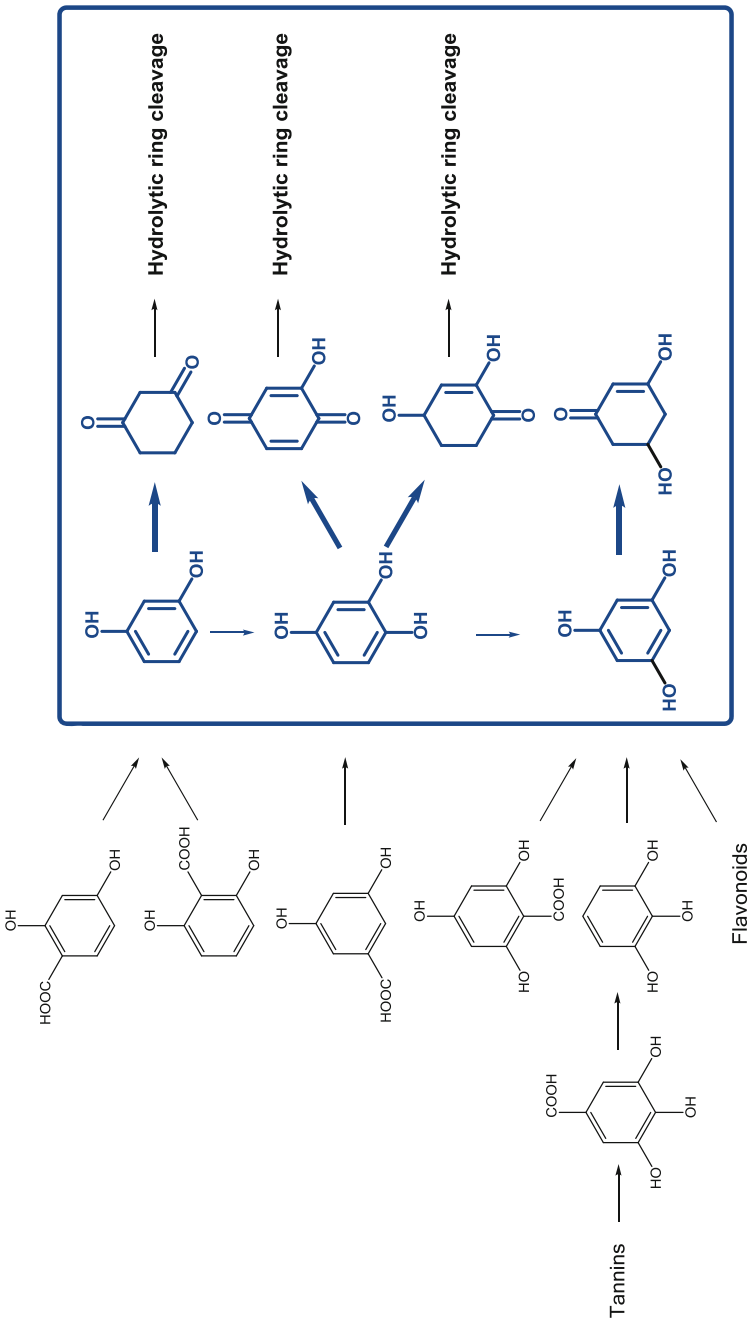

Fig. 22 Degradation of MAC via resorcinol, hydroxyhydroquinone, and phloroglucinol

6.1 Degradation via Dearomatization of Resorcinol

The anaerobic degradation of 2,4- and 2,6-resorcyclic acids (β- and γ-resorcyclic acids) has initially been studied in a fermenting *Clostridium* co-culture (Tschech and Schink 1985; Kluge et al. 1990). After decarboxylation by specific enzymes, the resorcinol (1,3-dihydroxybenzene) formed was found to be reduced to 1,3-cyclohexadione by resorcinol reductase (Fig. 22). The purified enzyme contains a FAD cofactor and probably uses a reduced ferredoxin as electron donor (Schink et al. 2000). The 1,3-cyclohexadione product is finally hydrolytically cleaved to 2-oxocaproate, which is then finally converted to the fermentation products acetate and butyrate. As an alternative, resorcinol can be converted via hydroxyhydroquinone (see Sect. 6.2).

6.2 Degradation via Dearomatization of Hydroxyhydroquinone

Hydroxyhydroquinone (1,2,4-trihydroxybenzene) serves as a substrate for different dearomatizing enzymes. It may be formed via anoxic hydroxylation of resorcinol (Philipp and Schink 1998), or by hydroxylation of 3,5-dihydroxybenzoate (α−resorcylate) to a trihydroxybenzoate, followed by decarboxylation (Gallus and Schink 1998). Resorcinol hydroxylase was studied in the denitrifying *Azoarcus anaerobius* strain LuFRes1, and the genes encoding a Mo-enzyme with similarities to pyrogallol-phloroglucinol transhydroxylase of *Pelobacter acidigallici* were identified (Darley et al. 2007) (see Sect. 6.3).

There are three strategies for hydroxyhydroquinone dearomatization that have been identified in different physiological classes of anaerobic bacteria. The oxidative pathway was identified in the denitrifying *Azoarcus anaerobius* and involves a membrane bound hydroxybenzoquinone-forming dehydrogenase. Finally, a series of noncharacterized reactions yield acetate and malate (Philipp and Schink 1998) (Fig. 23).

Alternatively, hydroxyhydroquinone is dearomatized by reduction to dihydrohydroxyhydroquinone in the sulfate-reducing *Desulfovibrio inopinatus* (Reichenbecher et al. 2000). The product is then further oxidized to acetate and to a second nonidentified compound. Finally, a series of at least three consecutive transhydroxylation reactions were suggested to be involved to form phloroglucinol from hydroxyhydroquinone in *Pelobacter massiliensis*. Mo-containing enzymes,

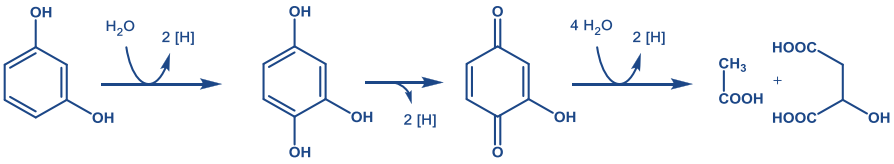

Fig. 23 Catabolism of resorcinol via the oxidative hydroxyhydroquinone degradation pathway

similar to pyrogallol transhydroxylase (see Sect. 6.3), were suggested to be involved (Brune et al. 1992). The phloroglucinol formed is then oxidized to three acetate.

6.3 Degradation via Dearomatization of Phloroglucinol

Phloroglucinol is an important intermediate during the anaerobic degradation of tannins and flavonoids derived from plants. For example, degradation of hydrolysable tannins yields gallate (3,4,5-trihydroxybenzoate), which can easily be decarboxylated to pyrogallol (1,2,3-trihydroxybenzene) (Schink and Pfennig 1982). This compound is not feasible for direct reduction but can be converted to phloroglucinol catalyzed by the molybdenum enzyme pyrogallol transhydroxylase (Fig. 24). Phloroglucinol is then dearomatized by NADPH-dependent reduction to dihydrophloroglucinol. The hydrolytic cleavage of the latter results in the formation of 3-hydroxy-5-oxohexanoic acid; in fermenting bacteria, the latter is converted to three acetate molecules as fermentation end products (Schink et al. 2000).

The isomerization of pyrogallol to phloroglucinol is catalyzed by the Mo-enzyme transhydroxylase that has been isolated and studied in detail in the fermenting *Pelobacter acidigallici* (Reichenbecher et al. 1994). The transhydroxylase belongs to the dimethylsulfoxid reductase family of Mo-enzymes and requires 1,3,4,5-tetrahydroxybenzene as a co-substrate (Fig. 24). The crystal structure in conjunction with the chemical synthesis of reaction intermediates gave rise to a hexahydroxydiphenyl ether intermediate in the course of catalysis (Messerschmidt et al. 2004; Paizs et al. 2007). The ether is formed by covalently linking the substrate phloroglucinol and the cofactor 1,3,4,5-tetrahydroxybenzene. Its cleavage releases the initial co-substrate as phloroglucinol, whereas the pyrogallol is converted to the co-substrate. In summary, the transhydroxylase catalyzes an intermolecular hydroxyl transfer between two phenolic compounds.

Phloroglucinol reductase has been isolated and characterized in some detail from the fermenting rumen bacterium *Eubacterium oxidoreducens* (Haddock and Ferry 1989). The monomeric 78 kDa enzyme specifically uses NADPH as electron donor, but does not depend on any other cofactor.

7 Research Needs

In the past 20 years, our knowledge in the field of anaerobic MAC degradation has increased tremendously, and the function of some of the previously enigmatic key enzymes is now understood on the molecular level. The unique, but meanwhile well-characterized processes comprise the anaerobic hydroxylation of alkyl chains from aromatic hydrocarbons with water by Mo- or flavo-enzymes, or the addition of alkyl chains to fumarate by glycyl radical enzymes. In addition, class I BCRs emerged as biocatalysts with a much broader function than previously anticipated, and a number of MAC degradation pathway depend on the catalytic versatility of class I BCRs.

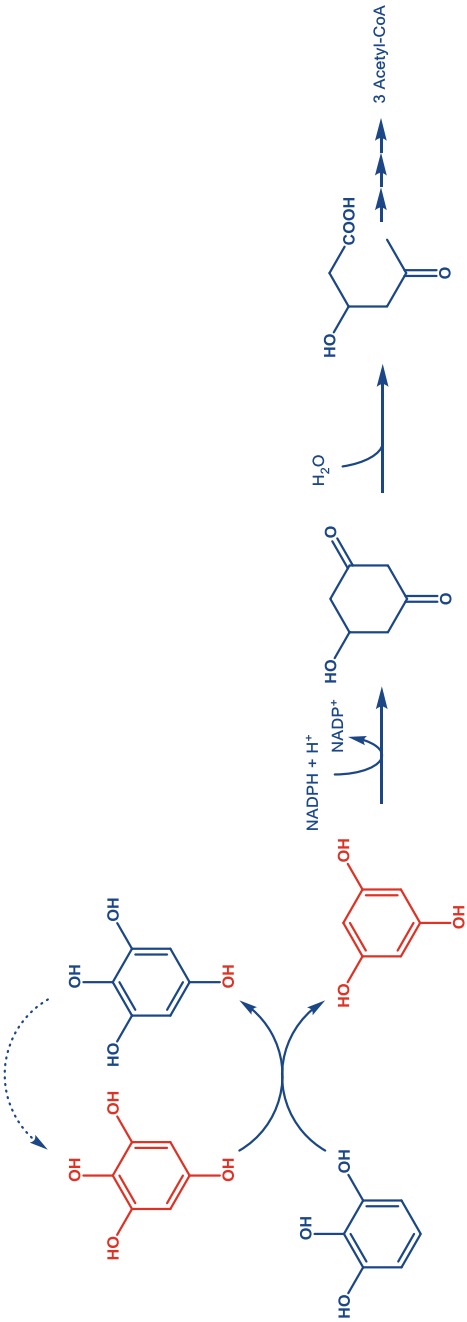

Fig. 24 Degradation of pyrogallol via the phloroglucinol degradation pathway. Pyrogallol degradation is initiated by intermolecular hydroxyl transfer catalyzed by the Mo-enzyme transhydroxylase. An enzyme-bound 1,3,4,5-tetrahydroxybenzene (in red) serves as cofactor, which after hydroxyl transfer to pyrogallol is released as product. The cofactor is regenerated by the hydroxyl accepting pyrogallol

Next to benzoyl-CoA, these enzymes accept numerous analogues as substrates for reductive dearomatization, and depending whether substituents can be removed or not by class I BCRs, modified dienoyl-CoA degradation pathways are induced. Moreover, the capacity to dehalogenate fluorinated or chlorinated benzoyl-CoA analogues appears to be a key process for the complete degradation of haloaromatics in anaerobic bacteria. The lack of a crystal structure, however, still hampers detailed knowledge of the function of class I BCRs. Although valuable insights into W-cofactor containing active site of class II BCRs have been obtained, novel crucial questions about these enzymes have emerged. One of the most intriguing questions is the energetic coupling of endergonic benzoyl-CoA reduction in the ATP-independent class II BCRs; experimental evidence for the proposed and plausible flavin-based electron bifurcation is still lacking.

The probably least understood process in anaerobic MAC degradation is the initial reaction involved in anaerobic benzene degradation. While the previously favored methylation is meanwhile rather considered as unlikely, there is evidence for two completely different scenarios for the initial attack of the extremely stable C–H bond of benzene: the carboxylation by an UbiD-like enzyme and the hydroxylation with water by an unknown enzyme. The former scenario appears to be plausible due to the identification of induced UbiD-like genes during anaerobic growth with benzene. In this context, the previously obtained insights obtained into the role of UbiD/UbiX-enzyme system in decarboxylation of aromatic carboxylic acids could open the door to understand the carboxylation of nonsubstituted aromatic compounds by related enzymes. It is possible that either initiation reaction, benzene carboxylation or hydroxylation may occur in different anaerobic benzene degrading organisms, dependent on their phylogenetic positions.

In addition to the importance of anaerobic MAC degradation for the biological removal of environmentally hazardous compounds, a number of the enzymatic reactions involved may be useful for biotechnological applications. These comprise the formation of chiral alcohols, either via stereospecific hydroxylation of alkyl side chains or by reduction of the ketones formed, the carboxylation of phenol and related reactions, enzymatic Birch reductions, and many other reactions. Thus, anaerobic MAC degradation has indeed emerged as a treasure trove for the discovery of new biocatalysts.

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Catabolic Pathways and Enzymes Involved in the Anaerobic Degradation of Polycyclic Aromatic Hydrocarbons

5

Matthias Boll and Sebastian Estelmann

Contents

Abstract

Polycyclic aromatic hydrocarbons (PAHs) are hazardous environmental pollutants that can be degraded exclusively by microorganisms. Whereas the enzymes involved in aerobic PAH degradation have been studied intensively since decades, the degradation of PAH in anaerobic bacteria is sparsely characterized. Only a few anaerobic strains and enrichment cultures degrading PAHs have been described. Today only the anaerobic naphthalene and methylnaphthalene

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degradation pathways have been elucidated. A few key reactions of these pathways have been studied to some detail and are presented in this chapter. The initial activation of PAHs without oxygen represents a major challenge and is accomplished by direct carboxylation of non-substituted PAHs or by addition to fumarate in case of methylnaphathlene. In the case of naphthalene degradation, CoA-thioesterification yields 2-naphthoyl-CoA, which undergoes dearomatization by three consecutive two-electron transfer steps to the napthyl ring system. The non-substituted ring of 2-naphthoyl-CoA is dearomatized first by a member of the flavin-containing old yellow enzyme (OYE) family. After another reduction step by a second OYE-like enzyme, the dearomatization of the second ring is catalyzed by an ATP-dependent enzyme homologous to dearomatizing class I benzoyl-CoA reductases. The nonaromatic hexahydronaphthoyl-CoA ring system formed is then cleaved by modified β-oxidation reactions yielding cyclohexane ring containing intermediates. Though little is known about the anaerobic degradation of PAHs with more than two rings, similar principles as those identified for naphthalene degradation are proposed to be involved. The expected common enzymatic processes comprise: (i) initial carboxylation, (ii) activation to a CoA ester, (iii) the reductive dearomatization of the polycyclic ring systems by OYE-like enzymes and/or homologues of benzoyl-CoA reductases, and (iv) oxidation to acetyl-CoA by modified β-oxidation reactions via a *cis*-carboxycyclohexylacetyl-CoA intermediate.

1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are widespread pollutants that impair human health due to their persistence, toxicity, and carcinogenicity. They are natural constituents of crude oil and coal and are mainly released into the environment by anthropogenic activities. PAHs consist of two or more fused benzene rings and are characterized by nonactivated C–C and C–H bonds and low solubility in water. In spite of their low bioavailability, PAHs can be degraded by microorganisms, and this constitutes the major mechanism for their removal from contaminated sites (Haritash and Kaushik 2009).

Aerobic bacteria and fungi use molecular oxygen to overcome the chemical inertness of PAHs. Ring-hydroxylating and ring-cleaving mono- and dioxygenases are key enzymes of aerobic PAH degradation pathways and have been studied intensively (Peng et al. 2008). In contrast, anaerobic bacteria that degrade PAHs need to use alternative strategies. Due to the low solubility of oxygen in water (258 μM at 25 °C (Wagner et al. 2011)), PAH-contaminated sites frequently turn anoxic. Several enrichment cultures were obtained from soils, sediments, aquifers, or sewage sludge that have the capability of degrading naphthalene, methylnapthalene, or phenanthrene coupled to the reduction of sulfate, and in a few cases, to nitrate, metal oxides, or CO_2. These cultures grow very slowly and produce little biomass which hampered the investigation of degradation pathways and enzymes. In the past 10 years, our knowledge of anaerobic PAH degradation was greatly enhanced in studies with naphthalene degrading pure and/or enrichment cultures. There are excellent reviews dealing with anaerobic PAH degradation with a different focus

than this contribution (Meckenstock et al. 2004, 2016; Heider 2007; Meckenstock and Mouttaki 2011; Boll et al. 2014). The present chapter provides an overview of anaerobic PAH degradation with a focus on the key enzymatic steps involved. Techniques for detecting/monitoring anaerobic PAH degradation in the environment (e.g., isotope fractionation, detection of functional or phylogenetic marker genes, metagenomics, marker metabolite analyses) are described in detail in other chapters of this volume.

2 Model Organisms for Studying Anaerobic PAH Degradation

The utilization of naphthalene, methylnaphthalene, and phenanthrene as sole carbon and energy source has frequently been reported under sulfidogenic conditions (Coates et al. 1996; Zhang and Young 1997; Galushko et al. 1999; Meckenstock et al. 2000; Sullivan et al. 2001; Davidova et al. 2007; Kümmel et al. 2015). This finding indicates a major role of sulfate reducing bacteria in natural PAH decontamination. In most reports on anaerobic naphthalene degradation, bacteria belonging to the *Desulfobacteriaceae* within the *Deltaproteobacteria* were identified. Several phylogenetically closely related marine strains were obtained in pure culture and genomic information became available for the NaphS2 pure culture (DiDonato et al. 2010). This strain not only uses naphthalene, 2-methylnaphthalene, and 2-naphthoic acid but also benzoate, pyruvate, and acetate, making it valuable for comparative gene expression analysis. (Meta)genomic information is also available for the culture N47 (Selesi et al. 2010), which was enriched from a contaminated aquifer. Growth of the N47 enrichment culture is supported by naphthalene and 2-methylnaphthalene, and the degradation pathways of both PAHs were studied intensively in this culture.

Anaerobic naphthalene degradation was also detected in non-sulfidogenic bacteria. For example, the enrichment culture N49 couples naphthalene degradation to Fe(III)-respiration (Kleemann and Meckenstock 2011). Bacteria of the N49 enrichment culture are phylogenetically affiliated to the *Peptococcaceae* within the Gram-positive bacteria. Evidence has also been provided for anaerobic naphthalene degradation under methanogenic and nitrate reducing conditions (Chang et al. 2006; Rockne et al. 2000), but cultures reducing nitrate are currently not available.

3 Metabolite Analyses of Anaerobic PAH Degradation Pathways

Metabolite analyses of culture supernatants by GC/MS or LC/MS techniques have been successfully applied to gain initial insights into the anaerobic degradation pathways of PAH. In PAH contaminated sediment samples (Zhang and Young 1997; Phelps et al. 2002), and cultures of NaphS2, N47 and N49 (Musat et al. 2009; Meckenstock et al. 2000; Kleemann and Meckenstock 2011) grown with naphthalene, 2-naphthoic acid was identified as a signature metabolite suggesting that carboxylation is the initial activation of PAH. Using stable isotope labeling,

2-phenanthroic acid was identified as derivative of phenanthrene (Zhang and Young 1997; Davidova et al. 2007). In contrast, substituted PAHs like 2-methylnaphthalene were shown to be activated by fumarate addition yielding arylsuccinates, similar to the anaerobic degradation of toluene and *n*-alkanes (Annweiler et al. 2000).

Reduced 2-naphthoic acid derivatives like 5,6,7,8-tetrahydro-2-naphthoic acid were detected in PAH-contaminated sediments (Zhang et al. 2000) and culture supernatants of N47 grown with naphthalene (Annweiler et al. 2002). In addition, cyclohexanedicarboxylic acid–derived compounds were found. From these data it was proposed that degradation of the 2-naphthoic acid intermediate proceeds by reduction of the non-substituted ring followed by reduction and opening of the substituted ring (Annweiler et al. 2002). Reactions similar to the β-oxidation of fatty acids were assumed to act on cyclohexanedicarboxylic acids finally leading to central metabolites (Meckenstock et al. 2016). Although valuable information about PAH degradation were obtained by metabolite analysis, many of the proposed intermediates could not be detected; finally such studies can never unambiguously distinguish between true metabolites or excreted dead-end products.

4 Initiation of Anaerobic Naphthalene Degradation

The carboxylation of naphthalene to 2-naphthoic acid was demonstrated in cell extracts of the N47 enrichment culture at a specific activity of 0.12 mU/mg protein by following the incorporation of ^{13}C-labeled bicarbonate (Mouttaki et al. 2012). These extracts also catalyzed a much faster exchange reaction of ^{13}C-labeled bicarbonate with the carboxyl group of unlabeled 2-naphthoic acid at a specific activity of 3.2 mU/mg protein. Both reactions were attributed to a 2-naphthoic acid forming naphthalene carboxylase (Fig. 1); both activities were oxygen-sensitive and did not appear to depend on the presence of ATP or divalent metal ions.

In the N47 and NaphS2 strains, naphthalene-induced gene clusters were identified that contain putative genes that are homologous to subunits of phenylphosphate carboxylase (Bergmann et al. 2011; DiDonato et al. 2010). The latter is a key enzyme in anaerobic phenol degradation and converts phenylphosphate and CO_2 to 4-hydroxybenzoate and inorganic phosphate; it belongs to the UbiD-like family of (de)carboxylases (Schühle and Fuchs 2004). The prototypical UbiD gene product was identified in *Escherichia coli*, and it decarboxylates 3-octaprenyl-4-hydroxybenzoate during ubiquinone biosynthesis (Cox et al. 1969). Recently, a UbiD-like decarboxylase acting on cinnamic acid was reported to depend on a prenylated flavin mononucleotide (FMN) cofactor (Payne et al. 2015), which is synthesized by a UbiX-like prenyltransferase (White et al. 2015). In N47 a *ubiX*-like gene is not part of the naphthalene carboxylase genes containing cluster, but *ubiX*-like candidates are located elsewhere in the genome. Genes encoding UbiD and UbiX were also found to be upregulated in enrichment cultures anaerobically degrading benzene, which is most likely carboxylated to benzoate in a first activation step (Abu Laban et al. 2010).

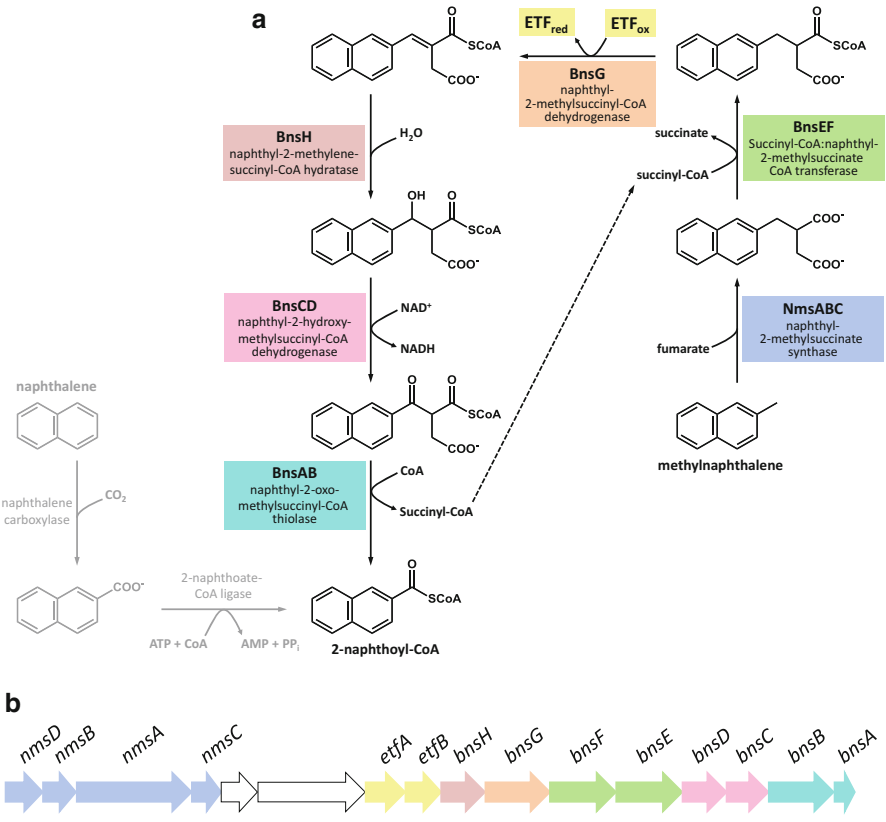

Fig. 1 Proposed initial steps involved in anaerobic naphthalene and 2-methylnaphthalene degradation. (**a**) Formation of 2-naphthoyl-CoA from naphthalene (gray) and 2-methylnaphthalene (black). (**b**) Section of the gene cluster proposed to encode enzymes of anaerobic 2-methylnaphthalene degradation in N47. *etf*, electron transferring flavoprotein; *nmsD*, activating enzyme of naphthyl-2-methylsuccinate synthase; *bns*, β-oxidation of naphthyl-2-methylsuccinate. Enzymes shown in (**a**) and corresponding genes in (**b**) are depicted by the same color code

In summary, UbiD-like-carboxylases appear to be generally involved in the initial activation of non-substituted aromatic hydrocarbons. In case of naphthalene carboxylase, a large multisubunit enzyme complex is predicted with a UbiD-like active site subunit (Meckenstock et al. 2016).

The 2-naphthoic acid formed is proposed to be activated to 2-naphthoyl-coenzyme A (NCoA), an important intermediate in the anaerobic naphthalene degradation pathway (Fig. 1). Transcriptomic and proteomic studies suggested candidate genes encoding an AMP-forming 2-naphthoic acid CoA ligase (Bergmann et al. 2011; DiDonato et al. 2010). Though such an activity was measured in cell extracts of N47 (S. Estelmann and M. Boll, unpublished results), the corresponding enzyme has not been isolated and characterized, yet.

5 Initiation of Anaerobic 2-Methylnaphthalene Degradation

A well-established mechanism of oxygen-independent C–H bond activation is the addition of alkyl chains to fumarate (Heider 2007). For example, toluene degradation is initiated by the glycyl radical enzyme benzylsuccinate synthase (Biegert et al. 1996), and the *bssA* gene encoding the active site subunit was established as a functional marker gene to detect anaerobic toluene and related hydrocarbon degradation at contaminated sites (Winderl et al. 2007). In case of 2-methylnaphthalene degradation, an addition to fumarate catalyzed by a benzylsuccinate synthase-like enzyme was anticipated.

In the N47 culture, genes encoding putative enzymes involved in the conversion of 2-methylnaphthalene to NCoA are organized in a gene cluster (Fig. 1); their expression is induced during growth with 2-methylnaphthalene (Selesi et al. 2010). Using cell suspensions, the fumarate-dependent conversion of 2-methylnaphthalene to naphthyl-2-methyl-succinate was measured at a rate of 0.02 μU/mg protein, which was comparable to benzylsuccinate synthase assayed under similar conditions (Annweiler et al. 2000). Based on sequence similarity to benzylsuccinate synthase, the putative α-, β- and γ-subunits of naphthyl-2-methyl-succinate synthase were identified in the gene cluster involved in 2-methylnaphthalene degradation in N47 (Selesi et al. 2010). Due to the high similarities of the corresponding gene clusters, the β-oxidation of the side chain of naphthyl-2-methyl-succinate has been proposed to proceed via analogous enzymatic steps as in toluene degradation (Leuthner and Heider 2000). Notably, naphthyl-2-methylene-succinate was identified in culture extracts indicating a CoA thioesterification of naphthyl-2-methyl-succinate followed by an acyl-CoA dehydrogenase reaction (Annweiler et al. 2000). Genes encoding a putative CoA transferase and an FAD-containing acyl-CoA dehydrogenase along with its interaction partner electron transferring flavoprotein (ETF) are present in the 2-methylnaphthalene degradation clusters in N47 and NaphS2. These clusters contain additional genes encoding a putative enoyl-CoA hydratase, a 3-hydroxyacyl-CoA dehydrogenase, and a thiolase; they are required for further β-oxidation like reactions. The thiolase is predicted to remove succinyl-CoA from a naphthyl-2-oxomethyl-succinyl-CoA intermediate, thus producing NCoA which is further metabolized in the central NCoA degradation pathway.

6 The Central 2-Naphthoyl-CoA Degradation Pathway

In the anaerobic degradation of monocyclic aromatic compounds, the central intermediate benzoyl-CoA is dearomatized by a two-electron reduction to cyclohexa-1,5-diene-1-carboxyl-CoA (dienoyl-CoA) by two different classes of benzoyl-CoA reductases (BCRs) (Boll et al. 2014). In facultative anaerobes, class I BCRs couple reductive dearomatization to a stoichiometric hydrolysis of ATP to ADP and P_i. Class I BCRs consist of four subunits and coordinate three FeS clusters. In contrast, obligate anaerobes use ATP-independent class II BCRs which are large enzyme

complexes of eight subunits that coordinate numerous FeS clusters and flavins and have an unusual tungsten cofactor at the active site (Weinert et al. 2015). In class II BCRs, the endergonic reduction of benzoyl-CoA is most likely driven by coupling to an exergonic reduction, this process is known as electron bifurcation and allows dearomatization with less energy demand as in class I BCRs (Boll et al. 2014; Buckel and Thauer 2013). Despite these remarkable differences, class I and II BCRs share a common feature and this is their extreme sensitivity to molecular oxygen.

In cell extracts of N47, the dearomatization of NCoA to 5,6,7,8-tetrahydro-2-naphthoyl-CoA (THNCoA) was measured via HPLC analysis at a rate of 5.1 mU/mg protein when using sodium dithionite as artificial electron donor. The reaction was neither oxygen-sensitive nor ATP-dependent. The reduction of the THNCoA intermediate to a hexahydro-2-naphthoyl-CoA (HHNCoA) was measured at a rate of 0.7 mU/mg protein and was dependent on the presence of ATP and NADH (Eberlein et al. 2013b). In contrast to NCoA reduction, this reaction was strictly ATP-dependent and highly sensitive to oxygen. These findings indicated that reduction of THNCoA is catalyzed by a class I BCR-like enzyme, whereas dearomatization of the non-substituted ring of NCoA is catalyzed by a novel aryl-CoA reductase.

6.1 Reduction of 2-Naphthoyl-CoA to 5,6,7,8-Tetrahydro-2-naphthoyl-CoA

A NCoA reductase (NCR) was initially purified from extracts of N47 grown with naphthalene under sulfate-reducing conditions (Eberlein et al. 2013a). The enzyme consists of a single 76.5 kDa subunit and apparently catalyzed the four-electron reduction of NCoA to THNCoA at a rate of 39 mU/mg protein using dithionite as artificial electron donor. The encoding gene was identified and NCR was found to be a novel member of the flavin-containing old yellow enzyme (OYE) family; it represents the prototype of a previously unknown class of dearomatizing reductases, also referred to as class III aryl-CoA reductases. In both N47 and NaphS2, the encoding *ncr* genes are part of a naphthalene-induced gene cluster and are located adjacent to a second gene encoding an OYE. Further genes of this cluster encode a putative product with similarities to NAD(P)H-binding modules of hydrogenases and NADH:quinone oxidoreductases (Eberlein et al. 2013a).

Surprisingly, heterologously produced NCRs from both N47 and NaphS2 catalyzed only the two-electron reduction of NCoA to 5,6-dihydro-2-naphthoyl-CoA (DHNCoA) at rates between 22 and 31 mU/mg protein (Estelmann et al. 2015). Heterologous expression of the OYE genes located next to *ncr* genes identified the product as a specific 5,6-dihydro-2-naphthoyl-CoA reductase (DHNCR) catalyzing the two-electron reduction of the substrate to THNCoA at rates of 625 mU/mg protein (DHNCR from N47) and 95 mU/mg protein (DHNCR from NaphS2). Thus, NCoA is reduced by two consecutive two-electron reduction steps catalyzed by NCR and DHNCR (Fig. 2) (Estelmann et al. 2015). The two OYEs have almost the same molecular mass and show 34% amino acid sequence identity to each other.

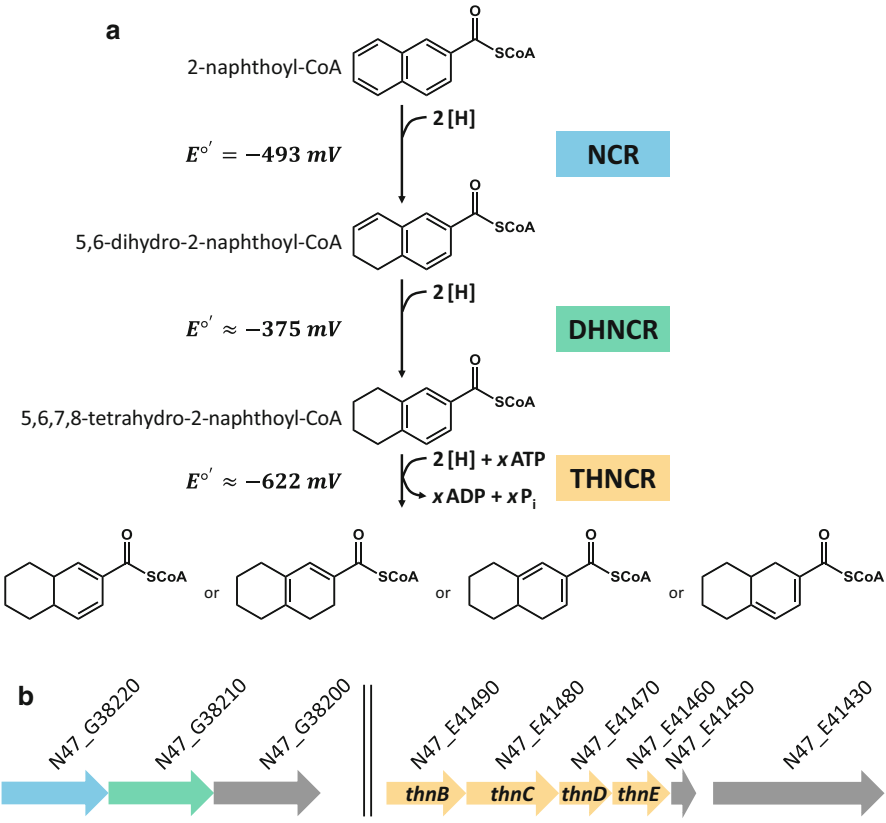

Fig. 2 Enzymatic steps involved in the stepwise reduction of 2-naphthoyl-CoA during anaerobic naphthalene degradation. (**a**) Enzymatic reactions catalyzed by ATP-independent NCR, ATP-independent DHNCR, and ATP-dependent THNCR. The exact redox potentials of the reactions catalyzed by DHNCR and THNCR are not known, the one given for the DHNCR reaction was estimated (Estelmann et al. 2015) and the one given for the THNCR reaction refers to the benzoyl-CoA/dienoyl-CoA redox couple (Kung et al. 2010). It is unknown, which of the four possible isomers is the product of the THNCR reaction. (**b**) Section of gene clusters encoding dearomatizing reductases involved in anaerobic naphthalene degradation in strain N47. Genes encoding enzymes potentially involved in the electron transfer to dearomatizing reductases are highlighted in gray: N47_G38200, putative NAD(P)H:quinone oxidoreductase; N47_E41450, ferredoxin; N47_E41430, putative NADPH:ferredoxin oxidoreductase. Enzymes shown in (**a**) and corresponding genes in (**b**) are depicted by the same color code

Their binding properties to chromatography materials are virtually identical, thus explaining the initial oversight of the much less abundant DHNCR in NCR preparations.

OYEs generally catalyze the NAD(P)H-dependent reduction of C=C double bonds of activated alkenes (Toogood et al. 2010). Yeast OYE was the first flavoenzyme that was discovered and consists of a single FMN-binding triosephosphate isomerase (TIM) barrel domain. The catalytic mechanism is a ping pong mechanism with NAD(P)H binding and reduction of FMN followed by substrate binding and reduction at the same

binding site. In contrast, NCR and DHNCR are composed of three domains: in addition to the FMN-binding TIM barrel domain, an FAD binding flavdoxin and a [4Fe-4S] cluster binding domain were identified. In agreement, stoichiometric amounts of FMN, FAD, and Fe were found in all heterologously produced NCRs (Estelmann et al. 2015). Some phylogenetically related OYEs show the same domain architecture as NCR and DHNCR. The 2,4-dienoyl-CoA reductase is an auxiliary enzyme involved in β-oxidation of fatty acids. The structural characterization of the enzyme from *E. coli* revealed that the reaction is initiated by a hydride transfer from NADPH to FAD which then transfers single electrons to the active site FMN via the 4Fe-4S-cluster (Hubbard et al. 2003). The fully reduced FMN then delivers a hydride to the substrate, and two conserved tyrosine and histidine residues form a catalytic dyad involved in protonation. Notably, Tyr-167 and His-252 are conserved in NCRs and DHNCRs suggesting a similar reaction mechanism. In case of the NCR, a hydride transfer to the C6-position of the naphthyl ring system results in an anionic transition state that can optimally be stabilized over the entire naphthyl ring system (Estelmann et al. 2015, Fig. 3). Thus, dearomatization of aromatic systems fundamentally differs in NCRs and BCRs since catalysis of the latter proceeds via radical intermediates by alternate electron and proton transfer to the aromatic ring (Thiele et al. 2008).

The different mechanisms of NCRs and BCRs may be rationalized by the differing redox potentials of the individual substrate/product couples. The redox potential of the bicyclic NCoA/DHNCoA redox couple ($E^{\circ\prime} = -493$ mV (Estelmann et al. 2015)) is substantially more positive than that of the monocyclic benzoyl-CoA/dienoyl-CoA redox couple ($E^{\circ\prime} = -622$ mV (Kung et al. 2010)). For this reason a hydride transfer is considered to be feasible for NCoA but not for benzoyl-CoA dearaomatization. However, the physiological electron donors of NCR and DHNCR are so far unknown. NAD(P)H ($E^{\circ\prime} = -320$ mV) cannot serve as direct electron donor for NCoA reduction, but the coupling to an exergonic reaction could drive endergonic electron transfer from NAD(P)H to NCoA. For example, the putative, FMN and FeS cluster containing product of an open reading frame located next to *ncr* genes (Fig. 2) could catalyze a flavin-based electron bifurcation. In such a process, endergonic reduction of NCoA by NADPH could be driven by the reduction of a second acceptor with a redox potential > -280 mV. Alternatively, reduced ferredoxin ($E' \approx -500$ mV) could serve as direct electron donor for NCoA reduction; however, no gene encoding a ferredoxin-like component has been found in the gene cluster containing the *ncr* genes.

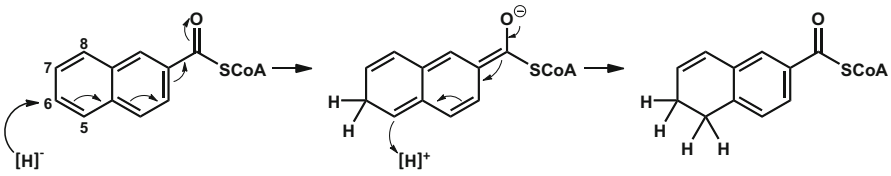

Fig. 3 Proposed mechanism for 2-naphthoyl-CoA reductase. A hydride transfer to C6 generates an anionic state that is stabilized as enolate; protonation at C5 yields the product 5,6-dihydro-2-naphthoyl-CoA

DHNCR production in *E. coli* resulted in low yields and low degrees of purity which did not allow a detailed enzyme characterization. In contrast to NCRs, DHNCRs did not catalyze the reverse reaction, and for this reason the redox potential of the DHNCoA/THNCoA redox couple could only be estimated with $E^{\circ\prime} \approx -375$ mV (Estelmann et al. 2015). NADH is considered to serve as natural electron donor of DHNCR; using this donor in excess, cell extracts of N47 fully converted DHNCoA to THNCoA indicating no need for a coupling to an exergonic reaction as suggested for NCoA reduction (S. Estelmann and M. Boll, unpubl. results).

6.2 Reduction of 5,6,7,8-Tetrahydro-2-naphthoyl-CoA

Cell extracts of N47 catalyzed the MgATP- and electron donor-dependent two-electron reduction of THNCoA to a conjugated HHNCoA in a highly oxygen-sensitive reaction (Eberlein et al. 2013b). It is unknown, which of the four possible HHNCoA isomers is formed (Fig. 2). THNCoA reductase (THNCR) shows typical properties of class I BCRs and the corresponding genes in NaphS2 were upregulated during growth on naphthalene (DiDonato et al. 2010). In both N47 and NaphS2, these genes designated as *thnBCDE* are part of an operon presumably encoding enzymes involved in the downstream HHNCoA degradation pathway (Meckenstock et al. 2016). The deduced ThnBCDE subunits show similarities to the four subunits of class I benzoyl-CoA reductases. Thus, it is very likely that the ATP-dependent dearomatization of THNCoA is catalyzed by ThnBCDE. Notably, the highest rate of THNCoA conversion by cell extracts of N47 was observed with NADH as reductant (Eberlein et al. 2013b) whereas class I BCRs usually depend on reduced ferredoxin provided by either a 2-oxoglutarate:ferredoxin oxidoreductase or an NADPH:ferredoxin oxidoreductase (Dörner and Boll 2002; Ebenau-Jehle et al. 2003). Assuming a redox potential of THNCoA/HHNCoA comparable to the benzoyl-CoA/dienoyl-CoA redox couple ($E^{\circ\prime} = -622$ mV (Kung et al. 2010)) an additional electron activation mechanism has to be involved in THNCoA reduction if electrons are indeed provided by NADH ($E' \approx -280$ mV).

In summary, two different types of dearomatizing reductases are involved in anaerobic PAH-degradation. NCR and THNCR show remarkable differences in architecture, cofactor composition, and biochemical properties (Fig. 4). NCR is the prototype of a previously unknown class of dearomatizing aryl-CoA reductases, whereas THNCR shows similarities to class I BCRs. According to the current knowledge, class II BCRs are not involved in anaerobic PAH degradation.

7 Downstream Degradation of Hexahydro-2-naphthoyl-CoA

Naphthalene and 2-methylnaphthalene are fully degraded to CO_2 in the sulfate-reducing strains N47 and NaphS2. Based on initial metabolite analyses, HHNCoA degradation appears to be initiated by reactions similar to those involved in the well-established benzoyl-CoA degradation pathway (Meckenstock et al. 2016). The enzymes involved in

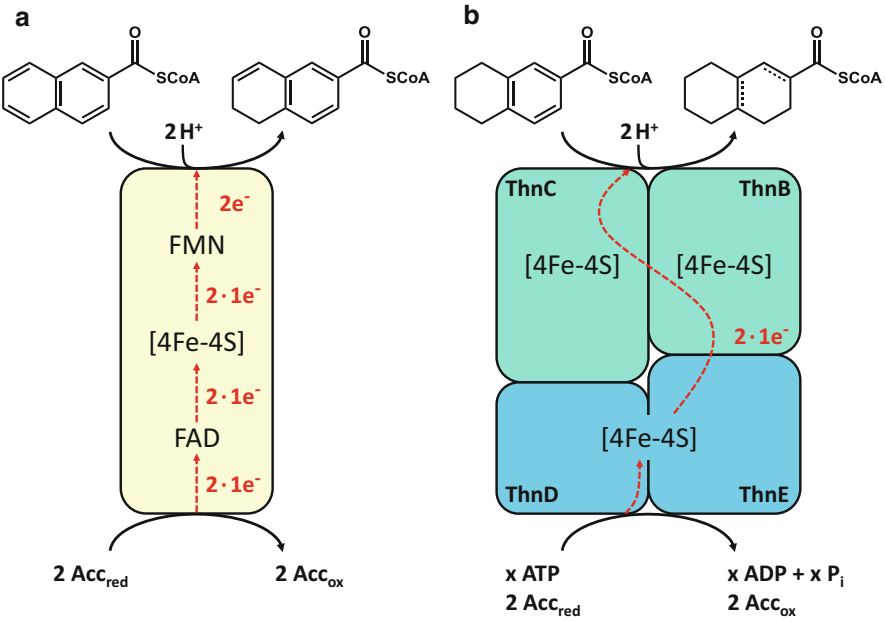

Fig. 4 Architecture of dearomatizing reductases involved in anaerobic PAH degradation. (**a**) ATP-independent 2-naphthoyl-CoA reductase from sulfate-reducing strains N47 and NaphS2. The arrangement of cofactors is based on the closely related 2,4-dienoyl-CoA reductase from *Escherichia coli* (Hubbard et al. 2003). The natural electron donor of NCR is not known. (**b**) ATP-dependent 5,6,7,8-tetrahydro-2-naphthoyl-CoA reductase from sulfate-reducing strains N47 and NaphS2. Based on homology to class I benzoyl-CoA reductases from *Thauera aromatica* and *Azoarcus* sp. THNCR is assumed to be composed of two different modules: (i) the electron activation module comprised of the ThnDE subunits harboring ATP-binding sites and a bridged [4Fe-4S] cluster (blue), and (ii) the ring reduction module consisting of the ThnBC subunits with one THNCoA-binding site and two additional [4Fe-4S] clusters (green) (Buckel et al. 2014). The natural electron donor of THNCR is not known

the conversion of HHNCoA to central metabolites are putatively encoded by the *thn* operon (Fig. 5). Although in vitro evidence for any of the downstream reactions is lacking, a plausible pathway has been postulated (Meckenstock et al. 2016). Dearomatization of THNCoA to HHNCoA is most likely followed by a 1,4-water addition to the cyclic dienoyl-CoA system catalyzed by an enoyl-CoA hydratase (Peters et al. 2007). In the next step, the resulting hydroxy group is oxidized to a ketone by a 3-hydroxyacyl-CoA dehydrogenase. Hydrolytic ring cleavage and a second hydration of a double bond would finally result in the formation of an intermediate containing a cyclohexanecarboxylate moiety linked to a 3-hydroxyacyl-CoA side chain. The β-oxidation of this side chain with concomitant thiolytic release of acetyl-CoA would yield a CoA-thioester of 2-carboxycyclohexylacetic acid. This acid was identified during initial metabolite studies with culture supernatants of N47 which is supportive for the proposed pathway (Annweiler et al. 2002). However, the CoA ester functionality could

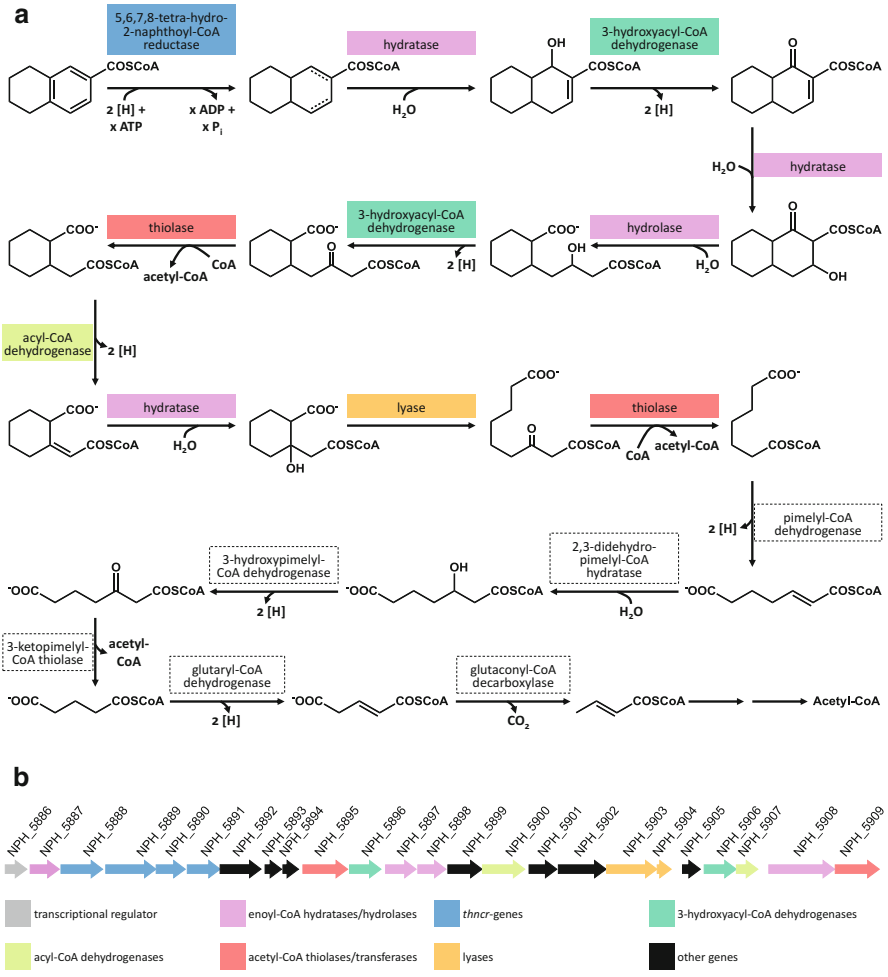

Fig. 5 Putative downstream degradation of hexahydro-2-naphthoyl-CoA (**a**) and genes of the *thn* operon in NaphS2 (**b**). Enzymes of the *thn* operon are assumed to be significantly involved in the further degradation of hexahydro-2-naphthoyl-CoA. Putative functions of the enzymes encoded in the *thn* operon are indicated by the same color code

be located at either of the two carboxy functionalities of 2-carboxycyclohexylacetic acid. If the ring opening hydrolase cleaves between C1 and C2 of the naphthyl ring system, the CoA moiety would be at the acetate moiety whereas it would be at the carboxy group in case of a cleavage between C2 and C3. A CoA transferase could interconvert both isomers. The introduction of a double bond into the CoA-activated acetyl group by an acyl-CoA dehydrogenase followed by an enoyl-CoA hydratase and a lyase reaction results in the cleavage of the second ring (Fig. 5). The ring-cleaving lyase was shown to be specific for the *cis*-isomer of 2-carboxycyclohexylacetyl-CoA (Weyrauch et al. 2017). Pimeloyl-CoA would be formed after thiolytic removal of a further acetyl-CoA

unit. The further degradation then proceeds via known reactions of the benzoyl-CoA degradation pathway (Fuchs et al. 2011) finally forming three additional acetyl-CoA units that can be fully oxidized in the citric acid cycle or assimilated via the glyoxylate shunt. In total, the 11 carbon atoms of NCoA are converted to 5 acetyl-CoA + CO_2.

8 Anaerobic Degradation of Phenanthrene

The knowledge of anaerobes degrading PAH with more than two rings is so far limited to a few studies with phenanthrene degrading, sulfate-reducing cultures. The initial evidence for carboxylation as the initial activation of phenanthrene was obtained in studies with enriched sediment samples in which the label of [^{13}C] bicarbonate was identified in phenanthrene carboxylic acid (Zhang and Young 1997). In agreement, the formation of 2-phenanthrene carboxylic acid from phenanthrene was convincingly demonstrated by labeling studies in a sediment-free, sulfate-reducing enrichment culture (Davidova et al. 2007). Members of the *Desulfobacteriaceae* were detected in this enrichment culture obtained from a hydrocarbon-contaminated marine sediment. Nothing is known about the further steps involved in anaerobic phenanthrene degradation. It is likely, that after activation to phenanthroyl-CoA a series of consecutive dearomatization reactions catalyzed by NCR-like OYEs and modified β-oxidation result in a stepwise degradation of the three-ringed system to acetyl-CoA units.

9 Research Needs

Although significant progress has been made in the past 10 years, knowledge about anaerobic PAH degradation is still very preliminary. Plausible naphthalene and 2-methylnaphthalene degradation pathways have been partially elucidated, but for most of the proposed reactions in vitro evidence is lacking. Though there is little doubt that anaerobic PAH degradation is initiated by carboxylation, very little is known about the enzyme involved and the mechanism of carboxylation. The very recently obtained insights into the function of the UbiD/UbiX enzyme systems will be valuable for studying the UbiD-like enzymes involved in PAH carboxylation. The only isolated enzyme of anaerobic PAH degradation is NCR; in addition, a DHNCR has been enriched. Future studies of NCR will focus on the elucidation of the structural basis of NCoA reduction. A particularly interesting question will be the nature of the electron donor for NCoA reduction. Preliminary results point to the involvement of a flavin-based electron bifurcation scenario, it would probably be the first one without involving a ferredoxin.

The degradation pathways of PAHs other than naphthalene are still obscure. Research is generally hampered by the slow growth and the low biomass yields of cultures in combination with the instability and oxygen sensitivity of many of the enzymes involved. However, strategies coping with these challenges involve metagenomic analyses which in turn will help to identify key enzymes in the degradation

pathways. The successful heterologous expression of *ncr* and *dhncr* genes in *E. coli* demonstrated that OYE-like enzymes involved in aryl-CoA dearomatization can be produced in an active form. Similar progress could be obtained with enzymes involved in β-oxidation. Most important will be the application of high-resolution mass spectrometric analysis of metabolites. These should not be limited to carboxylic acids excreted to the medium but should also involve the analysis of cellular CoA ester pools, as the major part of anaerobic PAH degradation proceeds via thioesterified carboxylic acids.

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Catabolic Pathways and Enzymes Involved in the Anaerobic Degradation of Terpenes

6

Jens Harder and Robert Marmulla

Contents

Abstract

Monoterpenes are ubiquitous and, in contrast to other terpenes, their anaerobic mineralization has been studied, especially in denitrifying betaproteobacteria. *Castellaniella defragrans* has a degradation pathway for cyclic monoterpenes, with a limonene dehydrogenase and a ring-cleavage reaction known from anoxygenic phototrophic bacteria. Toxic monoterpene alcohols are transformed in the periplasm by the linalool dehydratase/isomerase (Ldi) to the less toxic myrcene. *Thauera linaloolentis* degrades linalool with a membrane-anchored linalool isomerase and the enzymes of the Atu/Liu pathways for acyclic mono-

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terpenes. The development of a genetic system for *Castellaniella defragrans* together with physiological and biochemical studies have provided an excellent toolbox to study the monoterpene metabolism. On the horizon, *Pseudomonas aeruginosa* and other gammaproteobacterial pseudomonads are waiting for a thorough exploration of their monoterpene metabolism.

1 Introduction

Isoprenoids are derived from isoprene (2-methyl-buta-1,3-diene) and comprise an extreme variety of natural compounds. A large number of reference books describe the chemical features of these compounds (reviewed in Harder 2009a). The biosynthesis of isoprenoids occurs via the mevalonate or the 2-*C*-methyl-D-erythritol 4-phosphate pathway and provides molecular structures like quinones, hopanoids, steroids, and ether-type membrane lipids, as well as photosynthetic pigments (rhodopsins, carotenoids, and chlorophylls), and a large variety of secondary metabolites (reviewed in Boronat and Rodríguez-Concepción 2015; Schmidt-Dannert 2015; Tholl 2015). The biological mineralization of all these diverse structures has been studied only to a limited extent. Most studies so far reported the transformation of monoterpenes and sequiterpenes by aerobic organisms (Noma and Asakawa 2016; Asakawa and Noma 2016). The biochemical and genetic characterization of pseudomonads and Gram-positive bacteria has elucidated degradation pathways for a number of monoterpenes, including limonene, pinene, geraniol, citronellol, myrcene, and camphor (reviewed in Marmulla and Harder 2014; for chemical structures, see Fig. 1). In this contribution, recent insights into the anaerobic degradation of monoterpenes are reviewed, updating the earlier reviews of Hylemon and Harder (1998) and Harder (2009b). This chapter is complemented by the next ▶ Chap. 7, "Anaerobic Biodegradation of Steroids" (Chiang and Ismail 2017).

Isoprenoids as electron donor and carbon source for anaerobic bacteria were first studied under oxygen-free, nitrate-reducing conditions with monoterpenes (Harder and Probian 1995) and cholesterol (Harder and Probian 1997). Betaproteobacterial strains of *Castellaniella* (ex *Alcaligenes*) *defragrans* were obtained from enrichment cultures grown on single monoterpenes as sole organic carbon source: the strains 51Men, 54Pin^T, 63Car, and 65Phen were isolated on the monocyclic monoterpenes menthene, α-pinene, 2-carene, and α-phellandrene, respectively (Foß et al. 1998) (Fig. 1). The complete genome of strain 65Phen has 3,952,282 bp and includes a genetic island for monoterpene utilization (Petasch et al. 2014). In contrast, oxygen-functionalized monoterpenes as substrate in enrichment cultures yielded denitrifying strains of the betaproteobacterial genus *Thauera*: *T. linaloolentis* 47Lol^T on linalool, *T. terpenica* 58Eu^T on eucalyptol, and *T. terpenica* 21Mol on menthol (Foß and Harder 1998). Draft genomes of *T. linaloolentis* 47Lol^T and *T. terpenica* 58Eu^T are available (Liu et al. 2013).

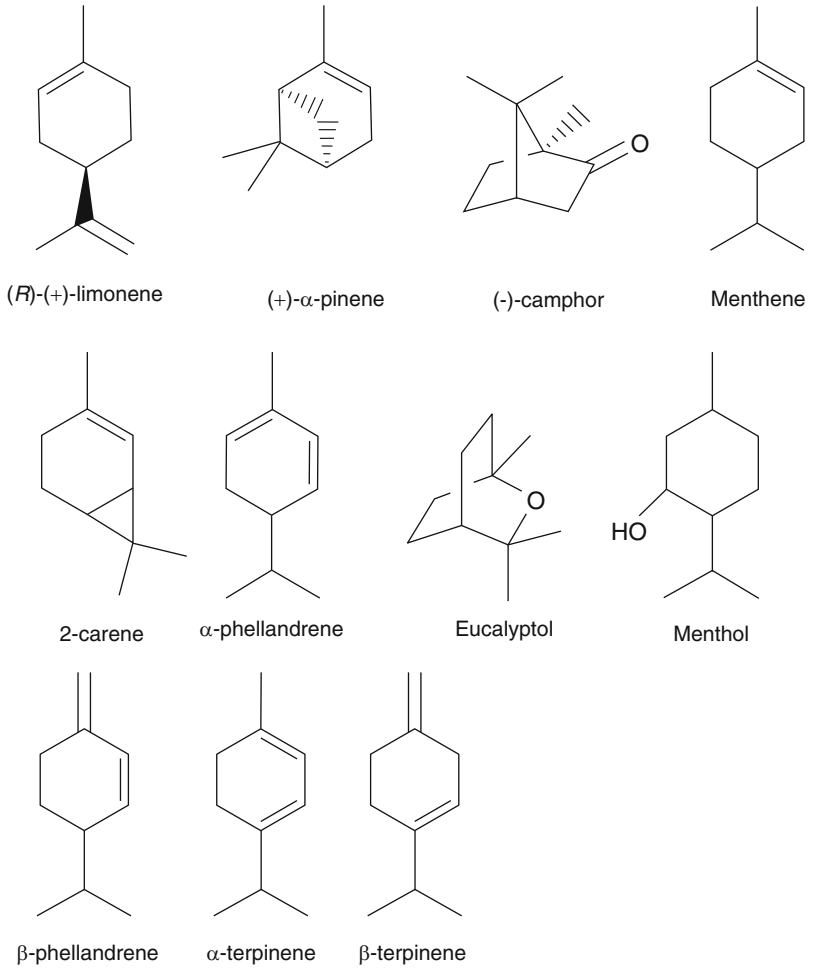

Fig. 1 Monoterpene structures

2 Degradation of Acyclic Monoterpenes: From Myrcene to Geranic Acid

β-myrcene (7-methyl-3-methylene-1,6-octadiene) is an acyclic monoterpene with a buta-1,3-diene structural motif. Some strains of the betaproteobacterium *C. defragrans* use myrcene as growth substrate (Heyen and Harder 2000). The detection of geranic acid as metabolite in cells and culture medium guided the identification of myrcene as growth substrate and precursor of geranic acid (Heyen and Harder 2000). The transformation is catalyzed by a linalool dehydratase/

isomerase (Ldi) yielding (*S*)-linalool ((*S*)-3,7-dimethyl-octa-1,6-dien-3-ol) and geraniol ((*trans*)-3,7-dimethyl-octa-2,6-dien-1-ol) (Brodkorb et al. 2010; Lüddeke and Harder 2011) and by two dehydrogenases acting on geraniol and geranial ((*trans*)-3,7-dimethyl-octa-2,6-dienal) (GeoA and GeoB) (Lüddeke et al. 2012b).

2.1 Linalool Dehydratase/Isomerase (Ldi) of *C. defragrans* 65Phen

In the thermodynamically favorable direction, the periplasmic enzyme reversibly catalyzes the isomerization from the primary alkenol geraniol into the tertiary alkenol (*S*)-linalool and its dehydration to β-myrcene (Fig. 2). Activity occurs *in vitro* in oxygen-free conditions in the presence of a reductant (dithiothreitol). Two years after the first publication (Brodkorb et al. 2010), the first patent applications reported the use of the dehydration reaction of Ldi or genetically engineered variants thereof for the production of butadiene and isoprene (reviewed in Weidenweber et al. 2015). The enzyme may become a key enzyme in the biotechnological production of these precursors for many polymers (e.g., nylon, polyester, polyisoprene).

The gene *ldi* of *C. defragrans* 65Phen encodes a preprotein with an N-terminal signal peptide for transport into the periplasmatic space. Expression of the gene in *E. coli* yielded a protein lacking the predicted signal peptide. Crystals of this Ldi preparation contained a homopentameric holoenzyme (PDB:5HLR), and incubation experiments with linalool as substrate yielded crystals with either myrcene or geraniol in the active site of the monomers (PDB:5HSS) (Weidenweber et al. 2015). A third crystal structure of the mature Ldi was published 2 months later, in June 2016 (PDB:5I3T).

Ldi is a homopentameric protein complex in the form of a planar rosette with a central hole. The monomers have a classical $(\alpha,\alpha)_6$ barrel fold (Fig. 3). This basket structure is well known from many monomeric terpene-transforming enzymes and glycosyl epimerases and hydrolases. An unprecedented structure is the pentameric complex with narrow channels between two adjacent monomers that allows the hydrophobic substrate to enter the active site. The neighboring monomer provides a loop that closes the in $(\alpha,\alpha)_6$ barrel fold-enzymes usually polar and accessible binding site. The peptide segment 36′–52′ presents a stable ram build on three legs by the peptide backbone of the loop and an essential intrasubunit disulfide bond between Cys48′ and Cys101′. The electron density in the linalool-soaked crystals

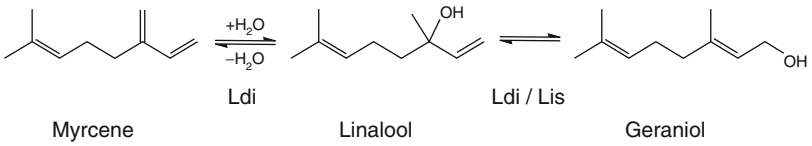

Fig. 2 Myrcene to geraniol conversion

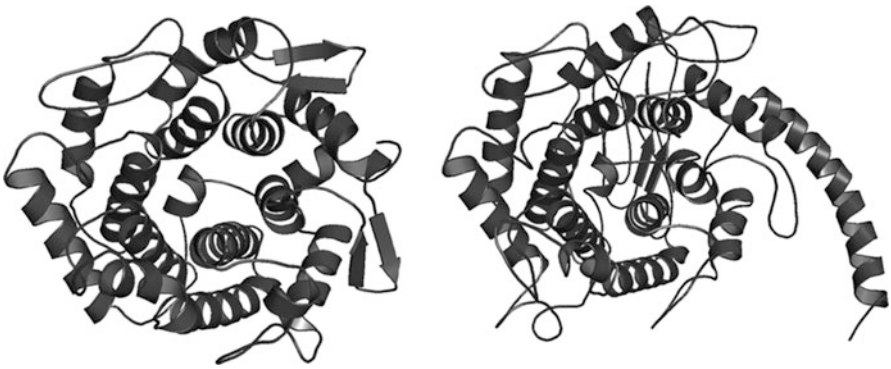

Fig. 3 The linalool dehydratase/isomerase structure (*left*) and the linalool isomerase model (*right*)

was best fitted with β-myrcene or geraniol, thus suggesting that linalool binding was less preferred and the enzymes in the crystals were catalytically active.

The unusual pentameric structure has led to a reaction hypothesis in which the pentamer plays its own role. Overall, the LDI structure evokes associations with a high pressure press. Linalool is less favorably bound in what can be considered to be an anvil at the bottom of a channel. In it, a ram press is moving up and down which is the adjacent loop with the amino acid residues Asp38′ and Tyr44′ on top that interact with the substrate. Thus, the movement of one monomer into the active site of the adjacent monomer is enforcing the catalysis. If no linalool is present, the collision of the monomers invokes an energy transfer and sends the "anvil" monomer as ram press in the direction of the next monomer in the ring. Thus, all monomers are active one after the other orbiting around the center of the complex. The system can be seen as molecular pentagonal Klemperer rosette – a gravitational system of equal bodies rotating around a barycenter (Klemperer 1962).

Ldi of *C. defragrans* 65Phen is still a unique protein as bioinformatic searches currently reveal no related protein with an *E*-value below 10^{-20}. However, linalool is also the growth substrate of *Thauera linaloolentis* 47Lol^T, and we identified in this bacterium a linalool isomerase (Lis) that shares in the catalytic domain structural similarities to Ldi.

2.2 Linalool Isomerase (Lis) of *Thauera linaloolentis* 47Lol^T

Metabolite formation in cultures of *Thauera linaloolentis* 47Lol^T with an excess of the electron donor and carbon source linalool over the electron acceptor nitrate suggested the presence of a 3,1-hydroxyl-Δ^1-Δ^2-mutase activity transforming linalool into geraniol (Foß and Harder 1997). The corresponding protein was enriched as geraniol isomerase yielding linalool and was located in the inner membrane (Marmulla et al. 2016b). Subcellular fractionation and a twofold sucrose gradient centrifugation yielded a highly enriched geraniol isomerase fraction containing

NCBI:ENO87364 as dominant protein. This protein (644 aa) has a predicted N-terminal membrane domain consisting of four transmembrane helices within the first 139 aa and a C-terminal, cytosolic domain of 505 aa that aligned with the Ldi (371 aa) with 62 identical and totally 132 positive amino acids (blastp). The secondary structure prediction using JPred (Drozdetskiy et al. 2015) matched the helices of the Ldi crystal structure and predicted a similar number of helices in the Lis. This information was incorporated in a sequence alignment using the Clustal Omega tool (Sievers et al. 2011) and with I-Tasser (Yang et al. 2015) the soluble domain of Lis was modeled into the Ldi structure. The resulting model showed that the Lis protein can fold into a highly similar $(\alpha,\alpha)_6$ structure (Fig. 3). The active site is in both enzymes a hydrophobic cavity with four conserved amino acids (Ldi: Y65, M124, C170, E171, C179; Lis: Y220, M287, C349, E350, C358) and three similar amino acids (Ldi: F69, H128, F176; Lis: H224, W246, Y355) that matched in the sequence alignment and in the superimposed structures (Fig. 4). Several other amino acids are conserved in the same structural position (Marmulla 2015).

The superimposition of the Ldi structure and the Lis model suggests similarities in the isomerase reaction. The lack of the dehydratase reaction in the Lis is likely due to the missing loop of an adjacent monomer. It may also explain why the Ldi turns over only (*S*)-linalool, whereas the Lis acts on both (*R*) and (*S*) isomers of linalool.

The cellular location of Lis inside of the inner membrane offers the product geraniol to cytosolic enzymes that oxidizes the alcohol with NAD. In contrast, the periplasmatic location of Ldi suggests a contribution to the defense: monoterpene alcohols arriving at the periplasm are to a large extent transformed into the less toxic monoterpene myrcene. Still, a small pool of monoterpene alcohols is present and enters the cytoplasm either by passive diffusion or by active transport.

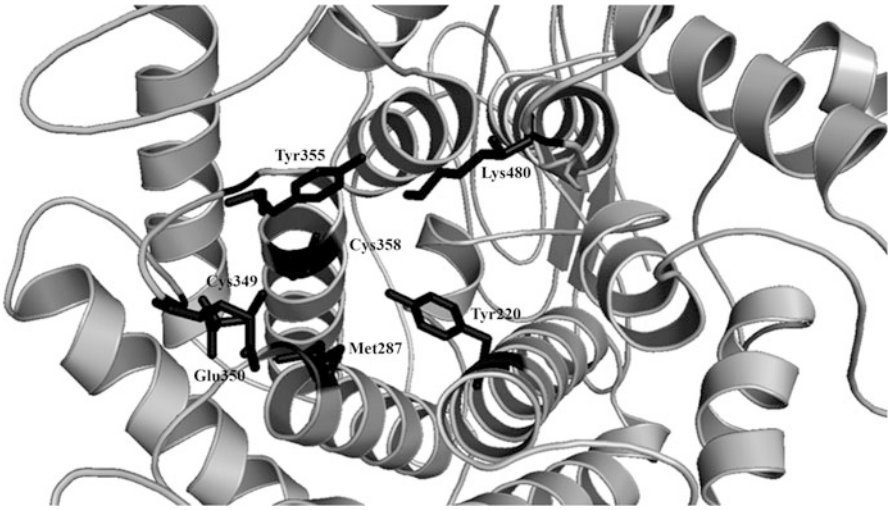

Fig. 4 Active site of the structure model of the linalool isomerase, with the conserved Tyr220, Met287, Cys349, Glu350, Tyr355, Cys358, and Lys480

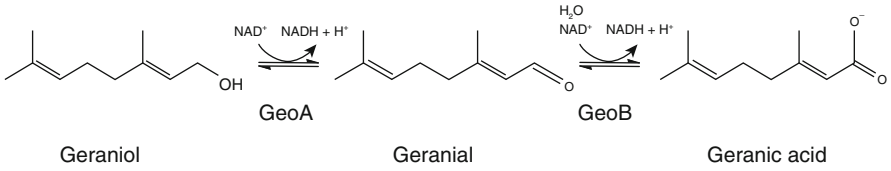

Fig. 5 Enzymatic reaction sequence from geraniol to geranic acid

2.3 Geraniol and Geranial Dehydrogenases

Allyl and benzyl alcohols both have sp^2-hybridized C2- and C3-atoms next to the carbon carrying the alcohol group. Hence, allyl alcohol dehydrogenases have an activity on benzyl alcohol and vice versa. The geraniol dehydrogenase GeoA was purified from *C. defragrans* 65Phen grown on monocyclic monoterpenes (limonene or α-phellandrene) (Lüddeke et al. 2012b). The homodimeric enzyme affiliating with zinc-containing benzyl alcohol dehydrogenases in the medium-chain dehydrogenases/reductases (MDR) superfamily was highly expressed (Petasch et al. 2014) and catalyzed the oxidation of perillyl alcohol more efficient than the one of geraniol (Lüddeke et al. 2012b). The oxidation product geranial is further oxidized by the highly expressed GeoB protein, a member of the aldehyde dehydrogenase superfamily (Lüddeke et al. 2012b) (Fig. 5).

The physiological relevance of GeoA was studied in a *geoA*-deletion mutant of *C. defragrans* 65Phen: growth rate and biomass yield were reduced on limonene, α-phellandrene, or β-myrcene as sole carbon and energy source in the culture, indicating a role of the enzyme in the acyclic and in the monocyclic monoterpene degradation (Lüddeke et al. 2012a). Extracts still contained geraniol dehydrogenase activity (Lüddeke et al. 2012a), and the purification of the enzyme identified the protein NCBI:CDM24151: an aryl alcohol dehydrogenase of the cinnamyl alcohol dehydrogenase family (Koska et al. unpublished) that was not among the proteins induced by α-phellandrene as substrate in the wild-type strain (Petasch et al. 2014).

Two proteins with a high similarity to GeoA and GeoB (>70% sequence identities) were found to be expressed during the mineralization of *p*-cymene in the betaproteobacterial strain pCyN1: their suggested function is the oxidation of 4-isopropylbenzyl alcohol to the corresponding aldehyde and acid, yielding 4-isopropylbenzoate (Strijkstra et al. 2014). Future studies on these enzymes and on GeoA and GeoB may provide an understanding on the substrate specialization for allylic and benzylic substrates.

3 The Acyclic Terpene Utilization Pathway (Atu/Liu)

In aerobic pseudomonads, Atu enzymes together with the leucine degradation enzymes (*liu*) channel the carbon atoms of the acyclic monoterpene alcohols and acids into the tricarboxylic acid cycle (reviewed in Marmulla and Harder 2014; Poudel et al. 2015) (Fig. 6).

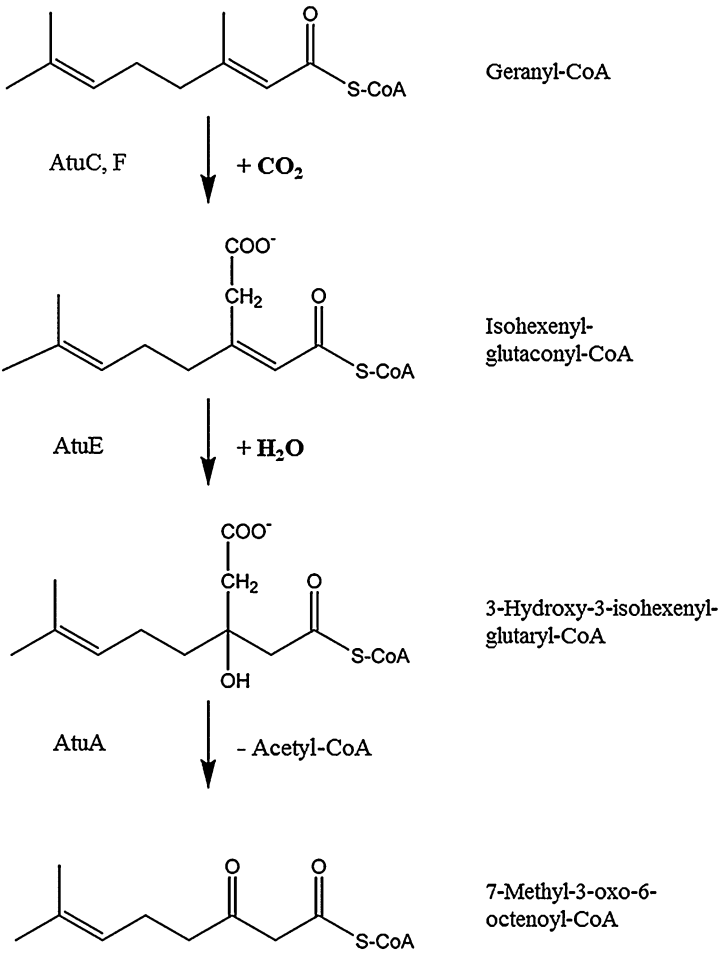

Fig. 6 Removal of a methyl group in β-position via carboxylation as demonstrated for geranyl-CoA

The presence of Atu and Liu enzymes was recently demonstrated in denitrifying *Thauera linaloolentis* 47Lol^T cells grown on linalool (Marmulla et al. 2016a). All subunits of the geranyl-CoA carboxylase (AtuCF) and 3-methylcrotonyl-CoA carboxylase (LiuBD) were identified by Maldi-Tof, together with Lis, GeoA, and the 3-hydroxy-3-isohexenylglutaryl-CoA:acetate lyase (AtuA). The corresponding genes were present in two contigs, generated from the available draft genomes (Marmulla et al. 2016a). *AtuCFE* and *lis* seem to be one transcriptional unit, and the *liu* cluster has been enlarged by a membrane protein with at least four transmembrane helices (NCBI:ENO88224, annotated as DUF4216) and a protein with a periplasmatic N-terminal domain of 200 aa, a membrane-spanning linker and a C-terminal fatty acid-CoA ligase (NCBI:ENO88222). These findings demonstrate

the presence of the Atu/Liu pathway for the degradation of acyclic monoterpenes in anaerobic bacteria. Key enzymes are the carboxylases that transform the methyl group in β-position to a CoA-thioester into a removable carboxymethylene group. This principle of branched-chain degradation pathway may also be operative for larger acyclic terpenes.

Although *C. defragrans* 65Phen grows on or metabolizes the acyclic compounds myrcene, linalool, geraniol, nerol, geranial, neral, and geranic acid, the closed genome of *C. defragrans* 65Phen did not contain the expected pathway for further mineralization for the acyclic monoterpene utilization, the *atu* genes. Transposon mutants in the degradation pathway of monocyclic monoterpenes also lacked the capacity to grow on β-myrcene (Petasch et al. 2014). This is evidence for an enzymatic formation of monocyclic monoterpenes from acyclic monoterpenes, although the genome does not code for classical monoterpene synthases.

4 Degradation of Cyclic Monoterpenes

Our knowledge on the degradation of cyclic monoterpenes originates from the genome of *C. defragrans* 65Phen and the corresponding proteome of cells grown on α-phellandrene in comparison to the proteome of cells grown on acetate (Petasch et al. 2014). Cyclic monoterpenes have a water solubility of ~50 μM, and growth experiments showed the simultaneous consumption of several monoterpenes (Harder et al. unpublished; Foß et al. 1998; Harder et al. 2000). Initial transformations may involve a ring-opening reaction of bicyclic monoterpenes to monocyclic monoterpenes, as suggested by the formation of metabolites in cultures growing on 2-carene, 3-carene, eucalyptol, or α-pinene (Harder and Probian 1995). The initial enzyme of the monocyclic monoterpenes seems to be a limonene dehydrogenase (Fig. 7), and the product perillyl alcohol is oxidized by GeoA and GeoB to perillic acid. A ligase seems to synthesize the perillyl-CoA thioester that undergoes ring cleavage reactions similar to the ring opening reactions of cyclohexanecarboxyl- and benzoyl-CoA thioesters (Fig. 8). The gene clusters involved were named *ctm* for cyclic terpene metabolism and *mrc* for monoterpene ring cleavage-associated genes (Petasch et al. 2014).

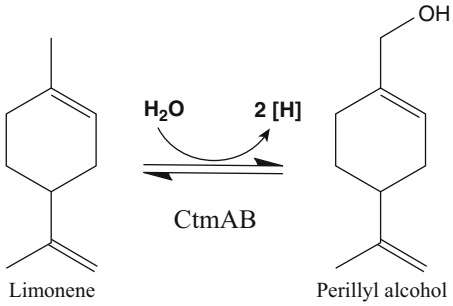

Fig. 7 Limonene oxidation to perillyl alcohol

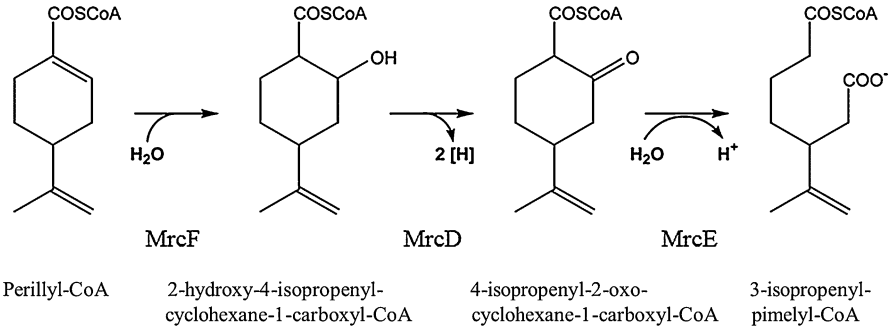

Fig. 8 From perillyl-CoA to 3-isopropenyl-pimelyl-CoA

4.1 Limonene Dehydrogenase

A collection of transposon mutants of *C. defragrans* 65Phen with a loss of denitrifying growth on limonene was dominated by mutants with an inactivation of *ctmA*: 45 of 72 mutant strains had insertions of the transposon in 22 different positions (Petasch et al. 2014). The *ctm* gene cluster was also inactivated in three mutants in *ctmB* and two mutants in *ctmE*. Transposon mutants in *ctmA*, *ctmB*, or *ctmE* did grow on perillyl alcohol. Growth studies with the in-frame deletion mutant *C. defragrans* 65Phen *ΔgeoB* revealed the *in vivo* formation of perillyl alcohol from limonene. These experiments assigned a function as limonene dehydrogenase to the *ctm* gene cluster. The proteomic study identified only the two FAD-dependent oxidoreductases (CtmA and CtmB) and an electron transfer system consisting of a 2Fe-2S ferredoxin (CtmE) and a NADH:ferredoxin oxidoreductase (CtmF) as proteins induced by α-phellandrene, but not the other genes in the *ctm* gene cluster: three hypothetical genes (*ctmCD*, *ctmG*) and a putative transcriptional regulator of the luxR family.

CtmA and CtmB belong to COG1233, a group of proteins related to phytoene dehydrogenase. In the biosynthesis of conjugated systems in vitamins and carotenoids, these enzymes oxidize a hexa-1,5-diene into a hexa-1,3,5-triene moiety, oxidizing the two allylic methylene groups located between the isolated alkene bonds. The use of this protein class for the oxidative introduction of an alcohol group is unprecedented (Petasch et al. 2014). The fate of the electrons is likely a participation in the reduction of nitrate. Electrons coming from NADH via the NADH:ferredoxin oxidoreductase CtmF may eventually join the electrons coming from CtmAB on the ferredoxin CtmE and together may be channeled into the respiratory chain at a specific complex. A candidate for this is an electron transfer flavoprotein:ubiquinone oxidoreductase (NCBI: CDM23589) that was identified as essential for the cyclic monoterpene degradation by transposon mutagenesis (Petasch et al. 2014).

4.2 Perillyl Alcohol Oxidation

The proteomic study (Petasch et al. 2014) identified the aforementioned GeoA and GeoB as candidates for the oxidation of perillyl alcohol to perillic acid. This is supported by the verified broad substrate spectrum of GeoA (Lüddeke et al. 2012b) and the cometabolic formation of perillyl alcohol and perillyl aldehyde *in vivo* by the in-frame deletion mutant *C. defragrans* 65Phen *ΔgeoB*. An induced protein annotated as ATP-dependent ligase (NCBI:CDM25265) with a gene location next but one to *geoA* is currently considered as perillic acid-CoA ligase preparing the substrate for the ring cleavage pathway (Petasch et al. 2014). The protein shares conserved domains with long-chain-fatty-acid-CoA and benzoate-CoA ligases.

4.3 Ring Opening Reactions of Cyclic Terpenes

The degradation of perillic acid-CoA seems to resemble the degradation pathways of cyclohexane carboxylate or benzoate in the phototrophic *Rhodopseudomonas palustris* (Pelletier and Harwood 2000), with cyclohex-1-ene-carboxyl-CoA as intermediate. In all other facultative and obligate anaerobes, an alternative benzoyl-CoA pathway exists with 6-hydroxy-cyclohex-1-ene-carboxyl-CoA as intermediate and *bamA* as hydrolase. The latter was suggested to be widely used as functional marker in environmental analyses (Kuntze et al. 2008). A copy of *bamA* is absent in *C. defragrans* 65Phen.

The monoterpene ring cleavage-associated gene cluster *mrcABCDEFGH* is part of the genetic island in *C. defragrans* 65Phen that contains most genes for the monoterpene metabolism (Petasch et al. 2014). The proteins MrcABCDEF were highly expressed during growth on α-phellandrene. Like the transposon mutants in *ctmA*, *ctmB*, and *ctmE*, transposon mutants in *mrcC* and *mrcF* are unable to grow on limonene, α-phellandrene, and β-myrcene (Petasch et al. 2014). The degradation of perillyl-CoA likely starts with a hydration catalyzed by MrcF. Oxidation of the product 2-hydroxy-4-isopropenylcyclohexane-1-carboxyl-CoA by MrcD may form 4-isopropenyl-2-oxocyclohexane-1-carboxyl-CoA. A hydrolysis by MrcE is assumed to form 4-isopropenylpimelyl-CoA. This is paralogous to the BadK, BadH, and BadI enzymes of the anaerobic benzoate catabolism in *Rhodopseudomonas palustris* strains that catalyze the oxidation of cyclohexenecarboxyl-CoA to pimelyl-CoA. *MrcC* codes for a 2,4-dienoyl reductase and presumably the substrates are the cyclohexadienyl CoA esters that may result from the methyl group-oxidation of the monocyclic menthadienes. The product of the ring cleavage reactions, 4-isopropenylpimelyl-CoA, is suitable for β-oxidation. Besides acetyl-CoA, methacrylyl-CoA may be produced and further undergoes decarboxylation to propionyl-CoA which may be metabolized via the methylcitrate cycle fueling the citric acid cycle. This suggestion is supported by the increased expression of several enzymes of the valine degradation pathway in cells grown on α-phellandrene. Two transposon

mutants in 2-methylcitrate synthase and 2-methylcitrate dehydratase supported the participation of the methyl citrate cycle in anaerobic monoterpene mineralization (Petasch et al. 2014).

5 Difficulties to Use Monoterpenes as Only Carbon Source

The application of transposon mutagenesis for the identification of catabolic genes involved in the monoterpene degradation has yielded a large number of noncatabolic genes that are essential for growth on monoterpenes, but not on acetate (Petasch et al. 2014; Marmulla et al. 2016a). This included transport systems and biosynthetic pathways for membrane components, an indication for a change in the composition of the cellular membranes in response to the presence of monoterpenes. Additional experimental evidence for an adaption to the toxicity of monoterpenes came from the analysis of membrane fatty acids and from proteomic studies (Foß et al. 1998; Petasch et al. 2014).

The toxicity of hydrocarbons is well understood (Sikkema 1995). Monoterpenes have a low absolute solubility in water – below 100 μM, resulting in a low diffusive flux – and a partition coefficient of logP(octanol/water) of 4.46 (pinene, limonene); thus in equilibrium, one molecule stays in the aqueous phase and 16,893 molecules are located in the hydrophobic solvent octanol (as proxy for the hydrophobic membrane interior). In contrast, monoterpenoids (oxygen-functionalized monoterpenes like geraniol and geranial) are soluble in millimolar amounts and have a lower logP value, e.g., 3.56 for geraniol. *C. defragrans* 65Phen grows in the presence of a pure limonene phase (Heyen and Harder 2000), but already 5 μM geraniol in the aqueous phase are toxic for the cells (Lüddeke et al. 2012b). As a consequence, biomass on monoterpene alcohols is only obtained either in the presence of a large organic carrier phase (2,2,4,4,6,8,8-heptamethylnonane) or in multi-fed batch cultures. Also, growth failure of cultures on monoterpenes can often be attributed to aging of monoterpenes by air, likely the accumulation of monoterpenoids. The diffusive flux of volatile hydrocarbon substrates through the gas phase to a colony on a plate is more efficient than the diffusive flux through an aqueous phase (Harms 1996). This coincides with our observation that several transposon mutants isolated on plates did not show the phenotype “lack of growth” in liquid cultures (Petasch et al. 2014; Marmulla et al. 2016a).

6 Research Needs

This review on the anaerobic degradation of terpenes focuses on monoterpenes because knowledge on isoprene and larger terpenes is nonexistent. The last decade has envisioned the first enzymes in the anaerobic monoterpene metabolism. The linalool dehydratase/isomerase or genetic variants thereof are already in consideration for the biotechnological production of butadiene. The characterization of the limonene dehydrogenase will provide access to the hydroxylation of allylic methylene groups. Physiological observations depicted the existence of more unusual enzymes. For *Castellaniella*, genetic methods like transposon mutagenesis and in-frame deletion mutant constructions were established and allowed together with

well-designed physiological studies of in-frame deletion mutants insights into the *in vivo* metabolism. In a broader view, little research has been performed on the aerobic monoterpene metabolism of the opportunistic pathogen *Pseudomonas aeruginosa*, a facultative anaerobe capable of denitrification. Nowadays, over 100 complete and over 2000 draft genomes of *Pseudomonas* strains await an exploration for aerobic and anaerobic pathways of monoterpene mineralization and this may contribute to a better understanding of the health benefits of essential oils.

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Anaerobic Biodegradation of Steroids

7

Yin-Ru Chiang and Wael Ismail

Contents

Abstract

Steroids, comprising sterols, steroid hormones, and bile acids, are ubiquitous and abundant in nature. Steroids are largely produced by eukaryotes; however, their complete degradation is only accomplished by microorganisms. Steroids degradation is challenging because of their low aqueous solubility and complicated sterane structures. Aerobic steroids catabolism depends on molecular oxygen as a cosubstrate of oxygenases to activate and cleave the recalcitrant sterane. Consequently, steroids degradation in oxygen-limited environments (e.g., freshwater and marine sediments) is generally slow. Obviously, anaerobic biodegradation

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needs to substitute all oxygenase-mediated reactions by an oxygen-independent strategy to overcome the chemical recalcitrance of steroids. This article presents an overview of current knowledge on anaerobic steroids biodegradation. In the past two decades, several denitrifying proteobacteria capable of utilizing steroids as sole carbon and energy sources were described. The denitrifiers adopt a common metabolic pathway, the steroid 2,3-*seco* pathway, to degrade cholesterol and testosterone under anoxic conditions. The anaerobic steroid catabolic pathways include various unprecedented catabolic enzymes (e.g., the molybdoenzymes steroid C25 dehydrogenase and 1-testosterone hydratase/dehydrogenase), which introduce the hydroxyl groups in the aliphatic side-chain and sterane of steroid substrates, and the oxygen atoms originate from water. The corresponding genes were found in genomes of steroid-degrading, denitrifying bacteria. Culture-independent investigations indicated that the proposed anaerobic catabolic pathways of steroids are widespread in the environment.

1 Introduction

Steroids constitute a class of terpenoid lipids represented by cholesterol, which is characterized by a planar and relatively rigid carbon skeleton composed of four fused alicyclic rings. More than 1000 different steroid structures are found in nature (Myant 1981). Examples of these steroids include sterols (e.g., cholesterol, phytosterols, and ergosterols), steroid hormones (e.g., 17β-estradiol, progesterone, and testosterone), and bile acids (e.g., cholic acid; see Fig. 1 for their structures). The chemical structure of steroids was mainly elucidated by two German chemists, Adolf Otto Reinhold Windaus (1876–1959) and Heinrich Otto Wieland (1877–1957). In steroids, the three cyclohexane rings are designated A, B, and C, whereas the fourth ring, a cyclopentane, is designated D (Fig. 1). The A/B rings of most naturally occurring steroids, including sterols and steroid hormones, are fused in a *trans* configuration, which yields a nearly planar structure. Moreover, the *trans*-fusion of the cycloalkane rings creates a minimally strained all-chair configuration. By contrast, the *cis*-fusion of steroid A/B rings is rare. Examples of steroids having the *cis*-fused A/B rings are bile acids, which therefore possess buckled structures (e.g., cholic acid in Fig. 1).

A remarkable property of steroids is their extremely low aqueous solubility. Cholesterol has a maximum solubility of 4.7 μM (=1.8 mg/L) in aqueous solutions (Haberland and Reynolds 1973). Therefore, in the bloodstream of animals, cholesterol is transported bound to lipoproteins; the same applies for steroid hormones. The aqueous solubility of steroid hormones is also extremely low. The solubility of natural estrogens (17β-estradiol and estrone) in neutral aqueous solutions is approximately 1.3 ~ 1.5 mg/L (Shareef et al. 2006) at room temperature, whereas the experimental aqueous solubility of testosterone at 25 °C is approximately 23.4 mg/L (Barry and El Eini 1976).

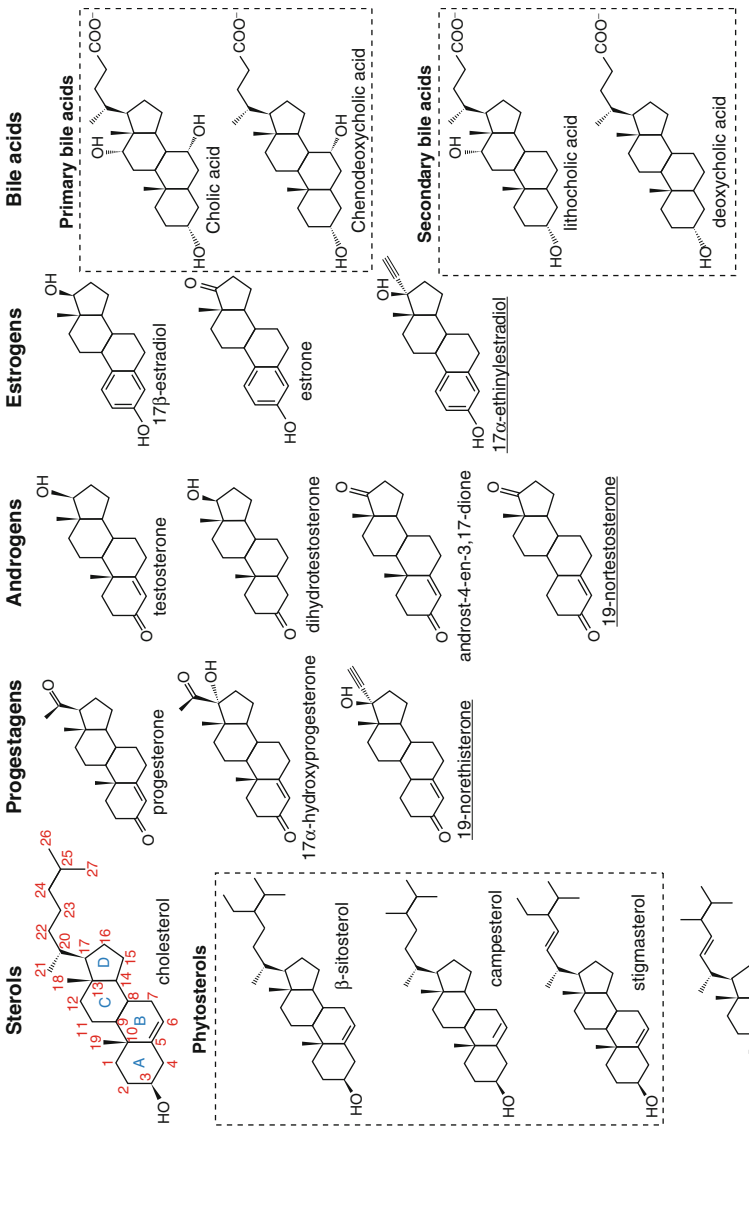

Fig. 1 The chemical structures of prevalent natural and synthetic steroids. The ring identification (A–D) and carbon numbering systems (1–27) of steroids are shown on cholesterol. Underlined compounds are synthetic steroids

In eukaryotic membranes, cholesterol and the related sterols such as phytosterols and ergosterols are crucial constituents modulating membrane fluidity and permeability (Edwards and Davis 1996). The rigid sterane structure and amphipathic properties make sterols key regulators of membrane fluidity in eukaryotic cells. The sterols restrain cell membrane fluidity (Yeagle 1985). Notably, most prokaryotes lack steroids. In bacteria, the function of steroids is compensated by pentacyclic hopanoids. Nevertheless, *Methylococcus capsulatus* and *Nannocystis exedens* contain sterols in amounts comparable to those in eukaryotes (Bird et al. 1971; Kohl et al. 1983). A gene cluster encoding homologs of essential eukaryotic sterol biosynthetic enzymes, including squalene monooxygenase and oxidosqualene cyclase, has been identified in the genome of *M. capsulatus* (Lamb et al. 2007). Hopanoids and sterols, present in the organic matter of all sediments, are likely the most abundant natural products on earth (Ourisson and Albrecht 1992).

Animal and plant physiology is regulated by various steroid hormones. Therefore, numerous steroids are used in medicine for treating cancer, arthritis, and allergies as well as for birth control (Peter et al. 1994; Haubrick and Assmann 2006). In animals, cholesterol is the precursor of all classes of steroid hormones, namely glucocorticoids (e.g., cortisol), mineralocorticoids (e.g., aldosterone), and sex hormones (androgens, estrogens, and progestogens). In plants, brassinosteroids control a broad range of physiological activities, including seed germination, stem and root elongation, vascular differentiation, leaf expansion, and apical dominance (Halliday 2004).

In animals, steroid hormone synthesis and secretion occur in several tissues, such as the adrenal cortex, ovaries, placenta, and testes. The biosynthetic pathways of steroid hormones involve the elimination of the cholesterol side-chain and hydroxylation of the steroid nucleus. Because of their hydrophobicity, the blood transport of steroid hormones to target organs is facilitated through their complexation with plasma protein carriers (Chen and Farese 1999). These carriers can be either nonspecific (e.g., albumin) or specific (e.g., corticosteroid-binding globulin). In the liver, steroid hormones undergo structural modifications and are converted into inactive excretion products. The solubility of the resulting products is promoted through conjugation with glucuronic acid or sulfate. Approximately 20 ~ 30% of these conjugates are secreted into the bile and eventually excreted in feces. The rest is excreted in urine after being filtered from blood plasma in the kidneys (Hadd and Blickenstaff 1969; Harvey and Ferrier 2011).

Sterols are ubiquitous and abundant in the environment; however, knowledge regarding their ecophysiological impact on wildlife is limited. In general, sterols are considered less bioactive compared with steroid hormones; however, it is documented that phytosterols may adversely affect animal physiology (Gilman et al. 2003; Orrego et al. 2009, 2011). Steroid hormones have been considered major endocrine disruptors (Wise et al. 2011). Both biogenic and anthropogenic steroid hormones are frequently detected in the environment (Ternes et al. 1999; Shore and Shemesh 2003; Fan et al. 2007, 2011; Wise et al. 2011). For instance, surface water in American and Dutch rivers has been reported to contain androgens and estrogens in a nanogram per liter range (Belfroid et al. 1999; Baronti et al. 2000; Kolodziej et al. 2003; Huang and Sedlak 2001). Examples of steroid hormones from anthropogenic

sources include 17α-ethinylestradiol, 19-norethisterone, and 19-nortestosterone (see Fig. 1 for their structures) (Loose and Stancel 2006; Zeilinger et al. 2009; Kugathas and Sumpter 2011). Steroid hormones can be discharged into the environment through various routes; major sources include human urine and livestock manure (Shore and Shemesh 2003). For instance, human estrogen excretion can be as high as 7 mg/day in pregnant women (Johnson et al. 2000). In the United States, livestock excretion is estimated to be responsible for a total estrogenic activity of 48.5 metric tons 17β-estradiol equivalent per year (Lange et al. 2002). Steroid hormones can also be discharged into the environment through agricultural applications of livestock manure and municipal biosolids as fertilizers (Lorenzen et al. 2004). Moreover, phytosterols in pulp and paper mill effluents can be transformed to androgens in river sediments (Jenkins et al. 2003).

The occurrence and persistence of steroid sex hormones in surface water ecosystems has become a major concern in environmental research and policy because long-term exposure to these compounds, even at extremely low concentrations, may adversely affect animal physiology and behavior (Teles et al. 2004; Fan et al. 2007; Ghayee and Auchus 2007). Young et al. (2002) proposed the predicted-no-effect-concentration values for 17β-estradiol (1 ng/L) and estrone (3 ng/L). In Europe, intersex fish were observed near sewage treatment plants (Jobling et al. 2006). Morthorst et al. (2014) reported that exposure to 53.6 and 133 ng/L 17β-estradiol elicited abnormal development among eelpout embryos. Furthermore, Lambert et al. (2015) reported that offspring sex ratios among the wild frog population in the United States are affected by estrogens released into suburban ponds. Estrogens are also suspected carcinogens (Yager and Davidson 2006). Masculinization can occur in freshwater wildlife exposed to androgens in polluted rivers (Parks et al. 2001; Orlando et al. 2004). In addition to endocrine disruption, Kolodziej et al. (2003) identified steroids as pheromones, which can adversely affect fish behavior.

Synthetic glucocorticoids (e.g., dexamethasone and prednisolone) are used in large amounts as anti-inflammatory drugs, which are commonly prescribed in human and veterinary medicine (LaLone et al. 2012). These endocrine disruptors reach the aquatic environment in quantities that may induce adverse physiological effects (e.g., increased plasma glucose level) in aquatic organisms (Kugathas and Sumpter 2011; LaLone et al. 2012). In a water sample assessment that employed a chemically activated luciferase gene expression (CALUX) bioassay, glucocorticoid-like activity levels of 0.39 ~ 1.3 and 11 ~ 243 ng dexamethasone equivalent/L were detected in surface water and sewage treatment plant effluents, respectively (Van der Linden et al. 2008). Gilbert (2011) detected 10 μg/L dexamethasone in a river downstream of a French pharmaceutical factory.

2 Microorganisms Capable of Steroid Degradation

The interest in and significance of investigating steroid-microorganism interactions has increased because of four main reasons. First, steroids are carbon-rich, highly reduced compounds that are abundant and ubiquitous in the environment.

Consequently, they are important carbon and energy sources for microorganisms, and their degradation contributes to the biogeochemical cycling of carbon and other elements on a global scale (Mackenzie et al. 1982; Wakeham 1989). Second, microbial transformation or degradation of steroids has been exploited in the pharmaceutical industry for producing high-value steroid drugs through biotechnology processes (Fernandes et al. 2003; Donova 2007; García et al. 2012). Third, in pathogens (e.g., *Mycobacterium tuberculosis*), cholesterol degradation is essential for their virulence and persistence in host cells (Pandey and Sassetti 2008). Fourth, microbial degradation is crucial for removing steroid hormones from polluted ecosystems (Johnson and Sumpter 2001; Andersen et al. 2003; Khanal et al. 2006).

Microorganisms that can use, mineralize, and thus completely degrade steroids may be suitable for environmental bioremediation. By contrast, microorganisms that can only structurally modify steroid substrates (biotransformation) may be useful for the biotechnological production of steroid drugs. Although steroid biotransformation can be mediated by various types of microorganisms, including bacteria (Fernandes et al. 2003), yeast (Liu et al. 2016), fungi (Banerjee et al. 1993), and microalgae (Pollio et al. 1994), complete degradation of natural steroids has been exclusively documented in bacteria (Ismail and Chiang 2011; Bergstrand et al. 2016). Most of the characterized steroid-degrading bacteria belong to the phyla Actinobacteria (Donova 2007) and Proteobacteria (Horinouchi et al. 2012). The capability of aerobic steroid catabolism appears to be ubiquitously present in most actinobacteria, including many genera in the suborder Corynebacterineae (e.g., *Corynebacterium*, *Dietzia*, *Gordonia*, *Mycobacterium*, *Nocardia*, and *Rhodococcus*) (Kieslich 1985; Donova 2007; Drzyzga et al. 2011; Donova and Egorova 2012) as well as the genera *Arthrobacter*, *Bacillus*, and *Streptomyces* (Liu et al. 1980; Lartillot and Kedziora 1990; Kim et al. 2002). In general, actinobacteria prefer using sterols as growth substrates; in addition, some actinobacteria can grow with bile acids and androgens (Bergstrand et al. 2016). However, few actinobacteria can degrade estrogens (Ke et al. 2007).

The occurrence of steroid degradation capabilities among proteobacteria is generally patchy, with this metabolic trait often observed in only a few species of the proteobacterial taxa (Bergstrand et al. 2016). For instance, among the 11 *Comamonas* species validated thus far (Young et al. 2008; Narayan et al. 2010; Liu et al. 2015), only *C. testosteroni* could use steroids as the sole carbon source (Tamaoka et al. 1987). Notably, various marine bacteria have been characterized as steroid degraders, including OM60 and SAR86 members as well as *Endozoicomonas montiporae* (Dupont et al. 2012; Ding et al. 2016). Although the metabolic capabilities of the aerobic degradation of androgens, bile acids, and sterols are widely distributed among bacterial taxa, few bacteria can degrade estrogens completely; most of these belong to the classes Alphaproteobacteria and Gammaproteobacteria (Yu et al. 2013).

Thus far, information regarding microorganisms that can metabolize steroids under anoxic conditions is insufficient. Nevertheless, anaerobic steroid transformation has been extensively studied. In animal intestines, the double bond at C-5/C-6 of cholesterol can be reduced to coprostanol by fermentative bacteria (Freier et al. 1994) either directly by using cholesterol reductase or through an alternative indirect

pathway (Björkhem and Gustafsson 1971; Li et al. 1995; Fig. 2). Several strictly anaerobic cholesterol-reducing bacteria belong to the genus *Eubacterium* (Eyssen et al. 1973; Sadzikowski et al. 1977; Brinkley et al. 1982). The enzyme responsible for the reduction is cholesterol reductase (Dehal et al. 1991). Depending on the diet composition, coprostanol can constitute more than 50% of total fecal sterols of humans. Coprostanol, unlike cholesterol, is absorbed poorly by the human intestine, and any increase in formation of coprostanol could decrease absorption of cholesterol (Bhattacharyya 1986). In addition to cholesterol transformation, intestinal bacteria can transform cholic acid and chenodeoxycholic acid into approximately 20 different secondary bile acids (Hayakawa 1973; Ridlon et al. 2006; Philipp 2011). Some of these transformations include deconjugation of bile acids, dehydrogenation of hydroxyl groups at C-7, reduction of oxo bile acids, and dehydroxylation reactions. In all cases, the sterane rings of the transformed steroids remain intact. The physiological significance of the deconjugation of bile salts is not sufficiently understood. Some hypotheses implicate deconjugation as a survival and colonization mechanism. For instance, De Smet et al. (1995) hypothesized that deconjugation of bile salts is a detoxification mechanism, enabling bile salt tolerance and thus bacterial survival in host intestines. Furthermore, Tanaka et al. (2000) proposed that amino acids released from bile salts may serve as nutrients and energy sources for bacteria. Moreover, Dambekodi and Gilliland (1998) proposed that deconjugation facilitates incorporation of the steroid moiety into bacterial membranes, likely altering membrane fluidity and permeability, which can improve colonization and survival in the gut.

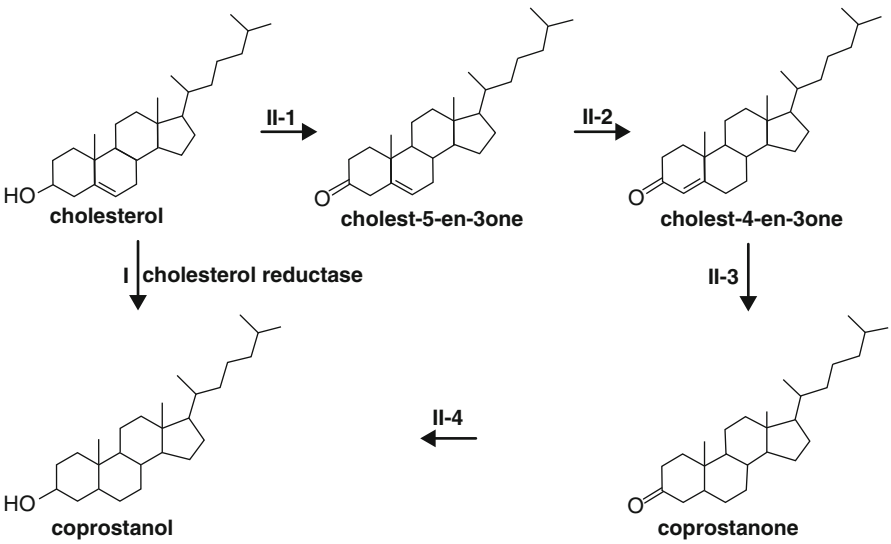

Fig. 2 Established pathways for microbial transformation of cholesterol into coprostanol. *I* direct pathway, *II* indirect pathway

In general, without oxygen, microorganisms cannot degrade steroids efficiently; thus, anoxic sediments and soil are considered reservoirs for steroids (Mackenzie et al. 1982; Hanselman et al. 2003). All steroid-degrading anaerobes characterized thus far are betaproteobacteria or gammaproteobacteria, both of which can use only nitrate or oxygen as electron acceptors (Table 1). Because only a few growth media have been developed for steroid-degrading anaerobes, these anaerobes have been isolated and characterized from very-narrow-spectrum bacterial taxa. Moreover, most denitrifiers are facultative anaerobes and can be handled easily under oxic conditions. *Sterolibacterium* (*Stl.*) *denitrificans* strain Chol-1S (=DSMZ 13999) (Tarlera and Denner 2003) and *Sterolibacterium* sp. strain Chol72 (=DSMZ 12783) (Harder and Probian 1997) are the only known organisms that can mineralize cholesterol to carbon dioxide under anoxic conditions. Both of these can grow only on a few hydrophobic substrates (sterols and long-chain fatty acids) and are closely associated with the betaproteobacterial genera *Thauera* and *Azoarcus* (Tarlera and Denner 2003). *Stl. denitrificans* can also grow anaerobically with androgens when cyclodextrin is added to the growth medium as a carrier molecule (Wang et al. 2014). Fahrbach et al. (2006) isolated and characterized the denitrifying bacterium *Denitratisoma oestradiolicum*, which can anaerobically grow on natural

Table 1 Characterized bacterial strains that are capable of degrading steroids under anoxic conditions

Phylum	Strain	Genome information		Steroid substrates	Electron acceptors (growth with steroids)
		G + C content (mol%)	Accession number		
Beta-proteobacteria	*Denitratisoma oestradiolicum* AcBE2-1 (=DSM 16959)	61.4	NA	17β-estradiol, Estrone	Nitrate
	Sterolibacterium denitrificans Chol-1S (=DSM 13999)	65.3	LFZK00000000 (draft genome)	Androst-4-en-3,17-dione, Cholesterol, Testosterone,	Nitrate, Oxygen
	Sterolibacterium sp. 72Chol (=DSM 12783)	ND	NA	Cholesterol	Nitrate, Oxygen
	Thauera terpenica 58Eu (=DSM 12139)	64.2	ATJV01000070 (draft genome)	Testosterone[a]	Nitrate
Gamma-proteobacteria	*Steroidobacter denitrificans* FS (=DSM 18526)	61.7	CP011971 (complete genome)	Androst-4-en-3,17-dione, 17β-estradiol, Estrone, Testosterone	Nitrate, Oxygen

[a]*T. terpenica* can anaerobically degrade testosterone, but cannot use testosterone as the sole carbon source (Yang et al. 2016) Abbreviations: *NA* not available, *ND* not determined

estrogens, but not androgens or cholesterol; however, this bacterium failed to aerobically grow on estrogens. Phylogenetic analysis of its 16S rRNA gene sequence revealed that the closest relatives of *D. oestradiolicum* are *Stl. denitrificans* and *Sterolibacterium* sp. 72Chol, with <93.9% sequence similarity. *Steroidobacter* (*Sdo*.) *denitrificans*, a gammaproteobacterium, can completely degrade natural androgens or estrogens under denitrifying conditions (Fahrbach et al. 2008). *Sdo. denitrificans* can also anaerobically grow on acetate, heptanoic acid, or glutamate, but not cholesterol. Recently, Yang et al. (2016) reported that a denitrifying terpenoid-degrader, *Thauera terpenica*, can anaerobically degrade androgens in the presence of acetate.

3 Anaerobic Catabolic Pathways of Bacterial Steroid Degradation

3.1 Overview of Aerobic Biodegradation of Steroids

The aerobic catabolic pathways of steroids, including sterols, androgens, estrogens, progestagens, and bile acids, have been elucidated in various bacteria. Of these, the biochemical mechanisms and catabolic enzymes of aerobic cholesterol degradation have been studied in detail. This is mainly because of the biotechnological applications of actinobacteria in industrial production of steroid drugs (Fernandes et al. 2003; Donova and Egorova 2012) as well as the essential role of cholesterol catabolism in mycobacterial persistence and infection (Pandey and Sassetti 2008; VanderVen et al. 2015). Although aerobic cholesterol catabolism has not been completely elucidated in any cholesterol-degrading bacteria, its catabolic pathways can be postulated by combining the biochemical and genetic studies performed in different organisms (García et al. 2012). The complete aerobic mineralization of cholesterol by various bacterial genera, such as *Arthrobacter*, *Corynebacterium*, *Mycobacterium*, *Nocardia*, *Pseudomonas*, and *Rhodococcus* was studied extensively during the 1960s–1980s. These studies focused on the production and chemical characterization of cholesterol catabolic intermediates, but overlooked genetic and biochemical aspects. According to these chemical studies, Kieslich (1985) proposed a general scheme of aerobic cholesterol degradation. This aerobic pathway is widely abundant in actinobacteria (Bergstrand et al. 2016), suggesting this metabolic trait is crucial for actinobacterial survival in the environment. The genomic analysis revealed that the cholesterol catabolic pathway is encoded by a cluster of over 80 catabolic genes in *tuberculosis* (Van der Geize et al. 2007). The regulation of catabolic genes has been investigated (Crowe et al. 2015; Kendall et al. 2010). Under oxic conditions, the bacterial degradation of cholesterol involves two major processes: the elimination of the C-17 side-chain and degradation of the sterane structure. Notably, *M. tuberculosis* appears to completely catabolize the sterane to CO_2 while incorporating the side-chain carbons into their lipid pools (Pandey and

Sassetti 2008). The order of the aforementioned two processes in vivo may differ among microorganisms (Rosłoniec et al. 2009). In some actinobacteria, side-chain degradation and sterane cleavage appear to occur concurrently, producing ring-cleaved intermediates with partially degraded side-chains (Capyk et al. 2011; Casabon et al. 2013). The catabolic mechanisms and enzymes involved in the side-chain degradation (Capyk et al. 2009; Rosłoniec et al. 2009; Ouellet et al. 2010) and the oxygenolytic cleavage of A/B-rings (Van der Geize et al. 2002; Yam et al. 2009; Dresen et al. 2010; Lack et al. 2010; Petrusma et al. 2009, 2011, 2014) of cholesterol have been extensively studied in actinobacterial species. The identification and characterization of actinobacterial enzymes initiating steroid C/D-ring degradation is still ongoing. As shown in Fig. 3, several reactions in this common aerobic pathway are catalyzed by monooxygenases (e.g., CYP 125, KshAB, and HsaAB) and dioxygenases (e.g., HsaC), both of which require molecular oxygen as a cosubstrate. In actinobacteria, cholesterol is taken up by the ATP-dependent Mce4 transporter located in cytoplasmic membrane (Casali and Riley 2007; Mohn et al. 2008). For further information on the aerobic catabolism of cholesterol, see the recent reviews by García et al. (2012) and Wipperman et al. (2014). The aerobic catabolic pathways of other sterols (e.g., β-sitosterol) in actinobacteria were also established (Wilbrink et al. 2011), all of which require additional catabolic enzymes for the activation and elimination of the branched C-17 side-chain.

The aerobic pathway of androgen degradation has been elucidated in the betaproteobacterium *Comamonas testosteroni* (see the review by Horinouchi et al. (2012)). Horinouchi et al. (2001, 2003, 2004) have conducted a series of gene disruption experiments to identify androgen catabolism-related genes, and various catabolic intermediates were identified from the *C. testosteroni* mutants lacking androgen degradation genes. However, most corresponding enzymes of *C. testosteroni* have not been biochemically characterized. In addition, detailed studies have been conducted on the regulation of genes involved in testosterone degradation. In *C. testosteroni*, the gene product of *teiR* (a testosterone-inducible regulator) positively regulates the transcription of genes involved in the initial steps of steroid degradation (Horinouchi et al. 2004). A *teiR*-disrupted mutant strain cannot use testosterone as its sole carbon and energy source (Pruneda-Paz et al. 2004). TetR, a repressor protein isolated and characterized from *C. testosteroni* (Wu et al. 2015), may be specifically responsible for the expression of the 3β,17β-hydroxysteroid dehydrogenase gene (Pan et al. 2015).

Bile salts are biosurfactants derived from cholesterol. Bile salts are toxic to numerous bacteria at concentrations above 10 mM (Philipp et al. 2006). Various groups of bacteria such as actinobacteria and proteobacteria can completely degrade bile salts in oxic environments (Hayakawa and Fujiwara 1977; Philipp 2011; Holert et al. 2015). Based on the structures of degradation intermediates, the aerobic pathway for bacterial bile salt degradation was established (for a recent review, see Philipp (2011)). In summary, the aerobic catabolic pathways of sterols, androgens, and bile acids all proceed through an oxygen-dependent 9,10-*seco* pathway (Fig. 3), with the secosteroid, 3-hydroxy-9,10-*seco*-androsta-1,3,5(10)-triene-9,17-dione or related structures as characteristic intermediates. Although the androgen catabolic

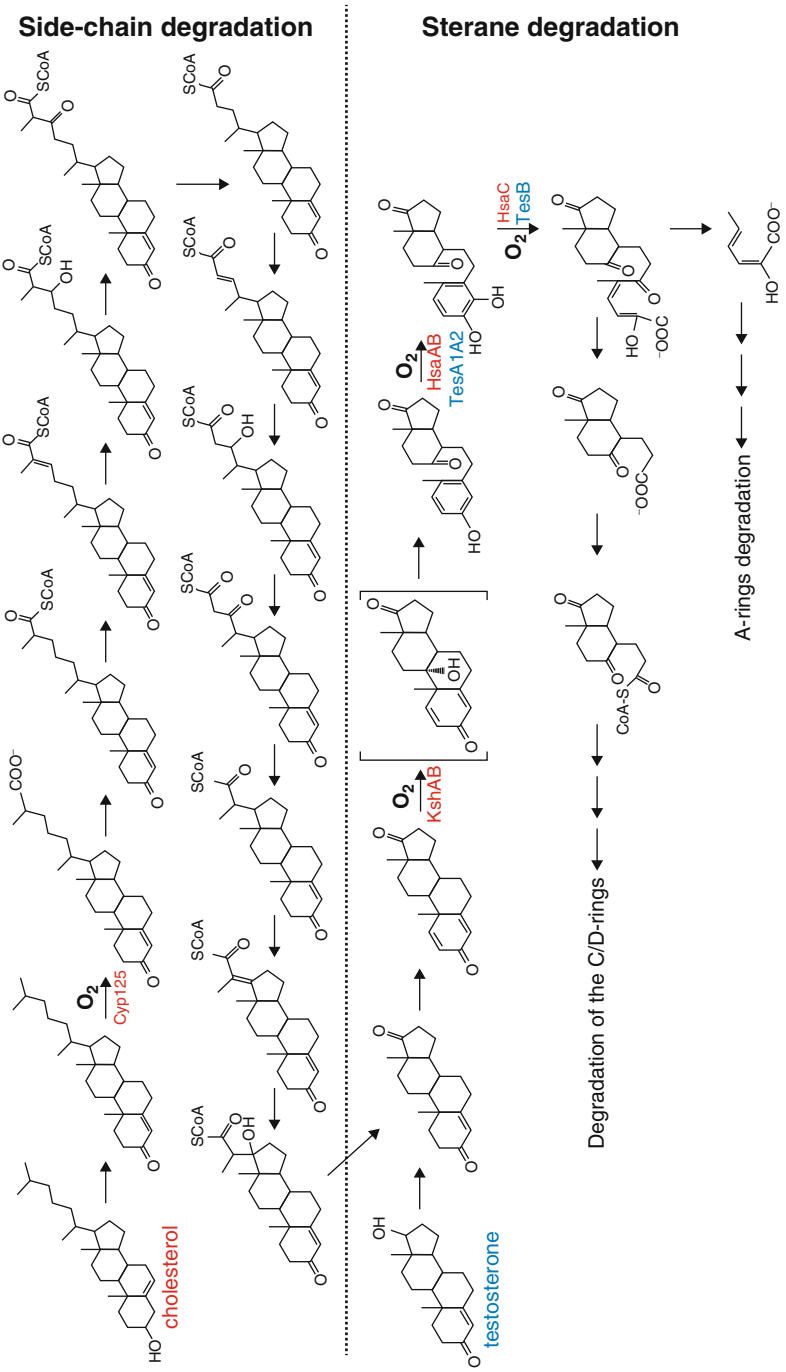

Fig. 3 The steroid 9,10-*seco* pathway for aerobic degradation of cholesterol and testosterone. Characterized oxygenases involved in aerobic cholesterol catabolism by *Mycobacterium tuberculosis* H37Rv are marked *red*, while those involved in aerobic testosterone catabolism by *Comamonas testosteroni* TA441 are marked *blue*. The structure in the bracket is very unstable and has been never detected. A highly similar pathway was proposed for aerobic degradation of cholate (Holert et al. 2015)

pathway of proteobacteria is highly similar to the metabolic pathway for mycobacterial cholesterol degradation, the steroid catabolic genes of proteobacteria and actinobacteria appear to have evolved independently and show low sequence similarity (Chen et al. 2016). By contrast, Bergstrand et al. (2016) suggested that the steroid catabolic genes of proteobacteria might have originated from actinobacteria through horizontal gene transfer. Nevertheless, a more comprehensive phylogenetic analysis is required to sufficiently establish the evolution of aerobic steroid degradation pathways.

Among steroids, only estrogens contain a phenolic A-ring, which makes them less biodegradable compared with other steroids. Estrogens are the most concerned endocrine disruptors (Ghayee and Auchus 2007). Moreover, estrogens are also suspected carcinogens (Yager and Davidson 2006). Microorganisms belonging to various bacterial taxa can degrade estrogens aerobically. However, compared with the extensive information available on aerobic catabolism of sterols, androgens, and bile acids, considerably less is known regarding the biochemical mechanisms involved in aerobic estrogen biodegradation. Thus far, the aerobic pathways of estrogen biodegradation have been reported to be sporadic (Yu et al. 2013), but no information regarding catabolic genes and enzymes is available. Literature on aerobic biodegradation of progestagens is also very limited. Liu et al. (2013) proposed the initial catabolic pathway of progesterone degradation. The metabolite analysis suggested that androgens (e.g., androst-4-en-3,17-dione and androsta-1,4-diene-3,17-dione) are the catabolic intermediates of aerobic progesterone biodegradation.

3.2 Anaerobic Degradation Pathways of Sterols

In contrast to the well-documented metabolic mechanisms and machineries of aerobic steroid biodegradation, less is known regarding the bacterial mechanisms for the import and metabolism of steroids under oxygen-limited or fluctuating conditions. Steroid-degrading anaerobes have been isolated for two decades, but only the recent emergence of omics techniques has enabled the collection of information on their catabolic genes and metabolites. *Stl. denitrificans* has been adopted as the model organism for anaerobic cholesterol catabolism. Catabolic intermediates involved in the side-chain degradation and the A-ring cleavage of cholesterol have been purified and characterized in *Stl. denitrificans* (Chiang et al. 2007; Wang et al. 2014; Lin et al. 2015). According to steroid metabolite profiles, an oxygenase-independent pathway for cholesterol catabolism has been proposed in the model organism (Fig. 4). This anaerobic pathway begins with cholesterol oxidation to produce cholest-4-en-3-one, followed by a hydroxylation at the tertiary C-25 atom of the side-chain (Chiang et al. 2007). The tertiary alcohol is then moved to the C-26 atom through an isomerization reaction, and the subsequent oxidation reactions produce cholest-4-en-3-one-26-oic acid, enabling incorporation with coenzyme A (CoA) to form a hypothetical CoA-thioester. The activated side-chain is then

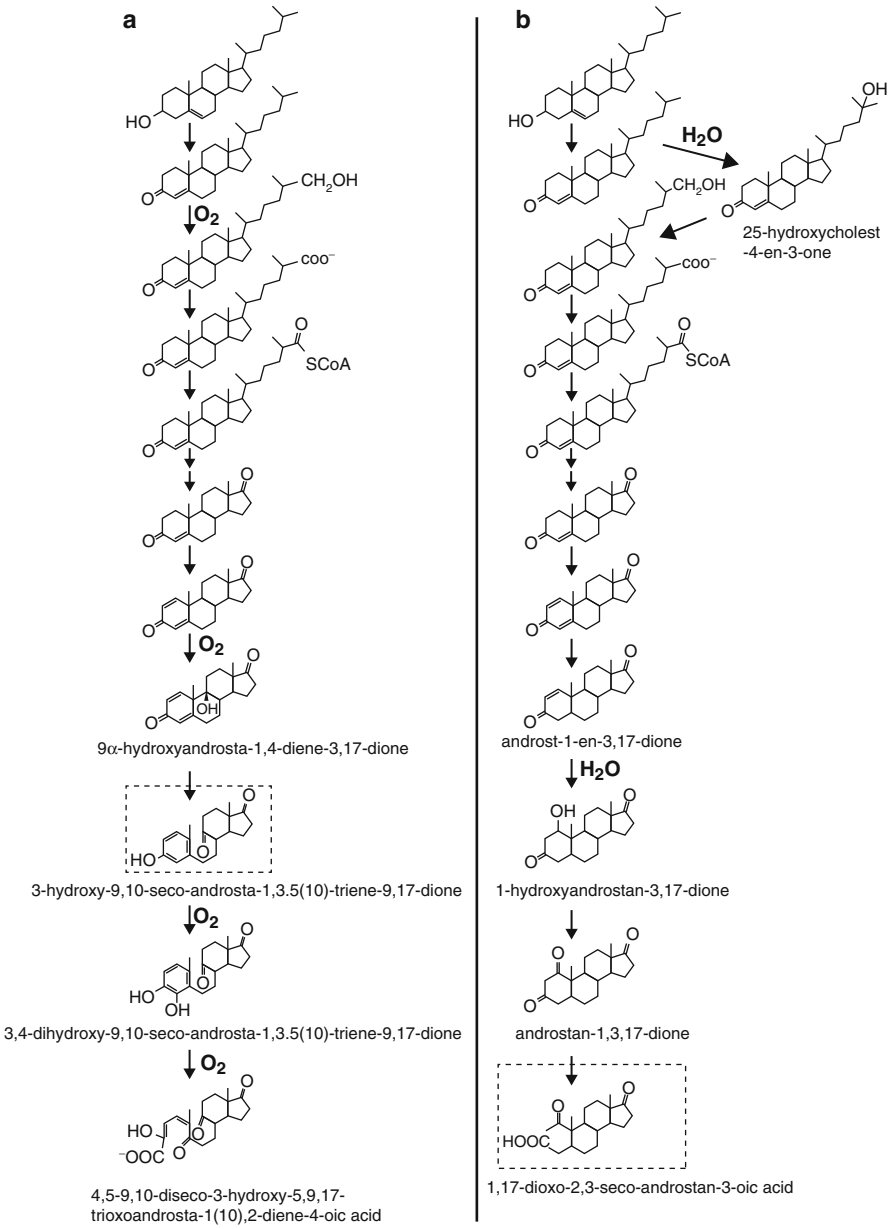

Fig. 4 The aerobic (**a**) and anaerobic (**b**) pathways of bacterial cholesterol catabolism. The aerobic 9,10-*seco* pathway is widely present in actinobacteria. The anaerobic 2,3-*seco* pathway was established in the denitrifying *Stl. denitrificans*. First ring-cleaved intermediates appearing in the catabolic pathways are enclosed in *boxes*. The common names of intermediates specifically present in the aerobic and anaerobic pathways are shown

degraded through a series of retro-aldol and β-oxidation reactions to form C_{24} and C_{22} acidic intermediates as well as C_{19} androgens. The sterane of androgen intermediates is activated through a hydration reaction at C-1/C-2. After the subsequent dehydrogenation reaction, the resulting 1,3-dioxo structure is cleaved through a hydrolytic mechanism.

This anaerobic pathway includes some common but several fundamentally different intermediates compared with the aerobic 9,10-*seco* pathway (Fig. 4). In *Stl. denitrificans*, degradation of the C-17 side-chain precedes sterane cleavage. Therefore, androgens are common intermediates in both the aerobic and anaerobic pathways. However, *Stl. denitrificans* activated the aliphatic side-chain of cholesterol through an anaerobic hydroxylation reaction, in which the oxygen atom originates from water (Chiang et al. 2007). Moreover, aerobic cholesterol catabolism of actinobacteria depends on an extradiol dioxygenase for the oxygenolytic cleavage of the steroid A-ring, whereas *Stl. denitrificans* cleaves the A-ring of steroid metabolites through a hydrolysis reaction (Wang et al. 2014; Lin et al. 2015).

The microbial degradation of hydrophobic substrates such as cholesterol is typically hindered by the low solubility (poor bioavailability) of the substrates. Some hydrocarbon degraders overcome this obstacle by producing solubilizing or emulsifying agents such as biosurfactants, which enhance the aqueous solubility of the substrates (Mnif et al. 2011). However, *Stl. denitrificans* does not produce and excrete biosurfactants into the extracellular medium; it possibly interacts directly with undissolved cholesterol aggregate through adhesion to facilitate the uptake process (Lin et al. 2015). The additional outer membrane complicates steroid uptake in proteobacteria. The lipopolysaccharide leaflet on the outer surface of the outer membrane prevents steroids from passively diffusing through the outer membrane bilayer (Plésiat and Nikaido 1992). In addition, the lack of ATP in the periplasmic space excludes the possibility that a Mec4-similar transporter functions in the proteobacterial outer membrane. The uptake of hydrophobic molecules through the outer membrane of proteobacteria can be mediated by three different machineries: porins, TonB-dependent outer membrane receptors (TBDRs), and long-chain fatty acid transporters (FadL family) (Lepore et al. 2011). Lin et al. (2015) investigated the substrate uptake mechanism in *Stl. denitrificans* by using steroid uptake assays for bacterial cells and spheroplasts. Their results suggested that this bacterium may adopt a FadL-like transporter to import cholesterol through facilitated diffusion (Fig. 5). However, the cholesterol transporter of *S. denitrificans* awaits further investigations at the molecular level.

According to the results of substrate uptake assays for whole cells and spheroplasts as well as steroid transformation assays for fractionated proteins, the subcellular compartmentation of anaerobic cholesterol catabolism was elucidated in *Stl. denitrificans*. Moreover, the initial catabolic enzymes were purified and characterized from this model organism. The anaerobic pathway is initiated through the oxidation of the 3-hydroxyl group and the subsequent isomerization at C-5/C-4 to produce cholest-4-en-3-one. These two reactions are catalyzed by a bifunctional enzyme, cholesterol dehydrogenase/isomerase (AcmA) (Chiang et al. 2008b). This enzyme belongs to the short-chain dehydrogenase/reductase (SDR) superfamily that

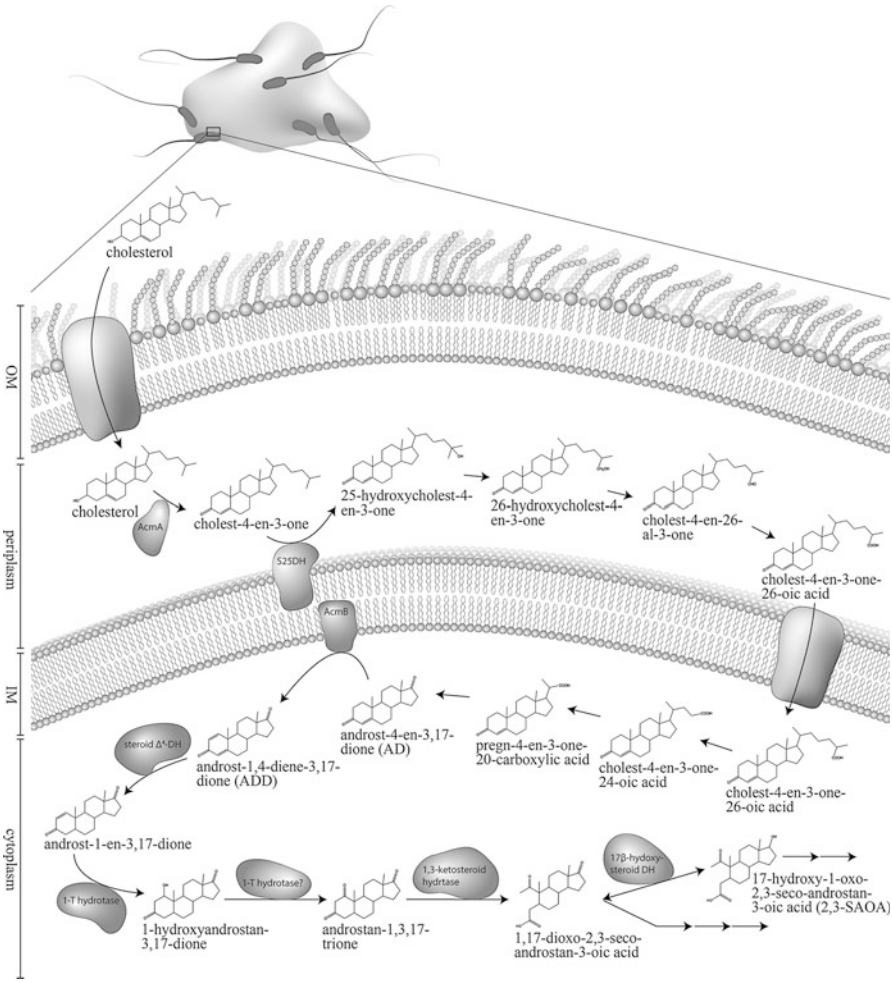

Fig. 5 The proposed model of substrate uptake and subcellular compartmentation of anaerobic cholesterol catabolism by *Stl. denitrificans* cells (Lin et al. 2015)

comprises an increasing number of NAD(P)-dependent nonmetallo-oxidoreductases, which bind NAD(P)(H) with a Rossmann fold motif (Jörnvall et al. 1995). Both the aerobic and anaerobic catabolic pathways are initiated by the oxidation of cholesterol A-ring. Aerobic bacteria use the highly similar NAD^+-dependent enzymes or FAD-dependent cholesterol oxidases to transform cholesterol to cholest-4-en-3-one; some of these enzymes are extracellular (Kreit and Sampson 2009). Thus far, no extracellular enzymes have been reported to be involved in anaerobic cholesterol catabolism by *Stl. denitrificans*.

The anaerobic degradation of the cholesterol side-chain involves anaerobic hydroxylation at the tertiary C-25 of steroid substrates, resulting in the formation of a tertiary alcohol (Chiang et al. 2007). This reaction fundamentally differs from

the oxygen-dependent hydroxylations, which typically occur during aerobic steroid metabolism. In anaerobic catabolism of hydrocarbons, molybdenum-containing hydroxylases replace the oxygenases in aerobic catabolism (Hille 2005). These molybdoenzymes use water as a source of oxygen atoms to incorporate it into the product requiring an electron acceptor. By contrast, oxygenases use molecular oxygen as the source of oxygen and many of these require an electron donor (Rosłoniec et al. 2009). A well-known example for such an anaerobic hydroxylase acting on the hydrocarbon side-chain is ethylbenzene dehydrogenase (Kniemeyer and Heider 2001). The corresponding enzyme steroid C25 dehydrogenase was purified and characterized as a heterotrimer, containing one molybdenum cofactor, five [Fe-S] clusters, and one heme b (Dermer and Fuchs 2012). The molybdoenzyme may be associated with the periplasmic side of the cytoplasmic membrane. Genes (*S25dA*, *S25 dB*, and *S25dC*) encoding the heterotrimeric enzyme were identified in the genome of *Stl. denitrificans* (Dermer and Fuchs 2012). The deduced amino acid sequence of the large subunit of steroid C25 dehydrogenase demonstrates high similarity to the molybdopterin-containing subunit of members of the DMSO reductase types II family. A cysteine-rich motif (GTHTRAN**C**IGA**C**SWDVNPRG**C**QK) was identified in the N-terminus of the large subunit, and is characteristic for type II enzymes with an aspartate molybdenum ligand in the active site (Jormakka et al. 2004). Moreover, the N-terminal part of the large subunit contains a conserved twin arginine motif (MQI**SRRQFI**V) for the Tat-dependent translocation (Berks 1996), supporting the location of steroid C25 dehydrogenase in the periplasmic space. Further analysis of this genome revealed the presence of seven other gene clusters encoding proteins with high sequence similarity to steroid C25 dehydrogenase (Dermer and Fuchs 2012). Yet it remains unclear why *Stl. denitrificans* possesses a set of eight steroid C25 dehydrogenase-like enzymes. Some of these proteins may be functionally redundant. 25-Hydroxycholesterol and the related structures have an important role in the innate immunity of mammals (Cyster et al. 2014; Singaravelu et al. 2015). Steroid C25 dehydrogenase has potential applications in the biotechnological productions of 25-hydroxysteroids (Warnke et al. 2016).

The detection of 26-hydroxycholest-4-en-3-one in *Stl. denitrificans* implies the transformation of 25-hydroxycholest-4-en-3-one to a 26-hydroxyl structure through an isomerization reaction (Wang et al. 2013a; Lin et al. 2015); however, the corresponding protein has not been identified. Various acidic intermediates, including cholest-4-en-3-one-26-oic acid, cholest-4-en-3-one-24-oic acid, and pregn-4-en-3-one-20-carboxylic acid, as well as androgen metabolites, have been observed in *Stl. denitrificans* cultures grown with cholesterol, suggesting the anaerobic degradation of cholesterol side-chain through β-oxidation-like reactions. This hypothesis is supported by the presence of various β-oxidation enzymes in a partial genome sequence of *Stl. denitrificans* (accession No.: LFZK00000000). The hypothetical CoA-thioester intermediates have not been detected in the *Stl. denitrificans* cultures or cell extracts, mainly because (i) the extraction procedures used in the relevant studies focused on hydrophobic metabolites, and hydrophilic CoA-thioester compounds tend to remain in aqueous fractions; (ii) the mass spectrometric analyses in these studies focused on the metabolite ions with a molecular mass of less than 500;

and (iii) mass spectrometry depends on atmospheric pressure chemical ionization (APCI), which prefers to ionize hydrophobic metabolites. To detect the CoA-thioester intermediates of anaerobic cholesterol catabolism, the development of alternative metabolite extraction procedures and mass spectrometric analyses is necessary. Recently, Holert et al. (2015) detected the CoA-thioester structures involved in aerobic biodegradation of bile acids using liquid chromatography-electrospray ionization-mass spectrometry. Similar approaches could be used to identify CoA-thioester intermediates produced by the cholesterol-grown *Stl. denitrificans*.

According to the results of in vitro assays using the cell extracts of *Stl. denitrificans* to transform steroid substrates, an FAD-dependent enzyme, cholest-4-en-3-one-Δ^1-dehydrogenase (AcmB), used cholest-4-en-3-one as its physiological substrate (Chiang et al. 2008a). However, a recent investigation concerning subcellular localization of the cholesterol catabolic enzymes indicated that AcmB is located at the cytoplasmic side of the inner membrane of *Stl. denitrificans*; thus, AcmB does not encounter cholest-4-en-3-one, a periplasmic intermediate (Fig. 5). According to the subcellular localization and substrate preference of AcmB as well as the metabolite analysis of *Stl. denitrificans* cultures grown with cholesterol, androst-4-en-3,17-dione should be the physiological substrate of AcmB (Lin et al. 2015). Thus, AcmB was renamed to 3-ketosteroid-Δ^1-dehydrogenase (KSTD). Dichlorophenolindophenol can be an efficient artificial electron acceptor for AcmB. This flavoprotein may use quinones as physiological electron acceptors. Redox enzymes with sequences highly similar to AcmA and AcmB are widely involved in aerobic steroid biodegradation (García et al. 2012; Horinouchi et al. 2012). Moreover, genes encoding various steroid redox enzymes were identified in the genome of *Stl. denitrificans* (Dermer and Fuchs 2012). Therefore, bacteria likely use the same redox enzymes (e.g., cholesterol dehydrogenase, KSTD, 3-ketosteroid-Δ^4-dehydrogenase, and 3β,17β-hydroxysteroid dehydrogenase) to transform steroids, regardless of oxygen availability.

In the aerobic catabolic pathway of cholesterol, sterane degradation begins at C-9/C-10 of the B-ring, which is initiated through a monooxygenase-catalyzed hydroxylation reaction at C-9 to produce a phenolic A-ring. By contrast, in the anaerobic cholesterol catabolism of *Stl. denitrificans*, the sterane cleavage first occurs in the A-ring, and metabolites with an aromatic structure are not produced. As shown in Fig. 5, after side-chain degradation, the resulting intermediate, androst-4-en-3,17-dione is transformed to androst-1-en-3,17-dione through a series of redox reactions. Through a hydration reaction at the unsaturated C-1/C-2, a hydroxyl group forms at C-1. Although the corresponding genes for this metabolic step were recently identified (Yang et al. 2016), the involved enzyme requires characterization. The subsequent reactions include the oxidation of the 1-hydroxyl group to form a 1,3-dioxo structure and the subsequent ring cleavage at C-2/C-3 of the steroid substrate, producing the characteristic intermediate, 17-hydroxy-1-oxo-2,3-*seco*-androstan-3-oic acid (2,3-SAOA) (Wang et al. 2013a; Lin et al. 2015). Thus far, most *Stl. denitrificans* enzymes involved in the degradation of side-chain and steroid A-ring remain unknown (Lin et al. 2015).

Bacterial cholesterol catabolism was studied under either oxic or denitrifying conditions. Bacteria are considered to use the oxygenase-dependent 9,10-*seco* pathway to degrade cholesterol in the presence of oxygen (Bergstrand et al. 2016), whereas denitrifiers are considered to use the oxygenase-independent 2,3-*seco* pathway to anaerobically grow on this compound. However, *Stl. denitrificans* uses the 2,3-*seco* pathway to degrade cholesterol even under oxic conditions (Wang et al. 2013a). Aerobic bacteria appear to be dependent on multiple catabolic strategies to degrade sterols. Moreover, in estuaries or marine ecosystems, where sterols are abundant because of the input from terrestrial area or in situ production by microalgae, nitrate is often limited, whereas sulfate is abundant. Sulfate-reducing bacteria, which are primarily obligate anaerobes, may use a different strategy to degrade sterols under strictly anoxic conditions. Sterols, which are considered fossil molecules (Mackenzie et al. 1982), have been present on earth since microalgae evolved. These compounds are ubiquitous and abundant in the environment and exist in various terrestrial and marine ecosystems where oxygen conditions and electron acceptor availability are extremely different. Current knowledge about the metabolic and phylogenetic diversity associated with microbial degradation of sterols, particularly that in anoxic environments, is limited.

3.3 Anaerobic Degradation of Androgens Through the 2,3-*seco* Pathway

The anaerobic catabolic pathway of androgens has been established in *Steroidobacter* (*Sdo.*) *denitrificans* DSMZ 18526 (Chiang et al. 2010; Fahrbach et al. 2010; Leu et al. 2011; Wang et al. 2013b). This pathway shares many common intermediates, particularly those involved in the activation and degradation of steroid A-ring with metabolites for anaerobic cholesterol catabolism. For instance, both pathways include 2,3-SAOA as the first ring-cleaved metabolite (Wang et al. 2013b; Lin et al. 2015). The catabolic genes associated with anaerobic androgen degradation in the genome of *Sdo. denitrificans* (accession No.: CP011971) were recently reported (Yang et al. 2016). As shown in Fig. 6, testosterone is first transformed to 1-testosterone by the 3-ketosteroid Δ^1-dehydrogenase/reductase and 3-ketosteroid Δ^4-dehydrogenase/reductase. A bifunctional molybdoenzyme, 1-testosterone hydratase/dehydrogenase (AtcABC), then catalyzes the hydration reaction at C-1/C-2 of 1-testosterone and the subsequent oxidation of the resulting 1-hydroxyl group. AtcABC was purified from anaerobically grown *Sdo. denitrificans* and characterized as a heterotrimer. The phylogenetic analysis of the sequences of AtcABC suggested that this enzyme belongs to the xanthine oxidase family containing molybdopterin, FAD, and iron-sulfur clusters (Yang et al. 2016). The genes (*atcA*, *atcB*, and *atcC*) were clustered in the *Sdo. denitrificans* genome. Moreover, transcriptomic analysis revealed specific expression of these genes during anaerobic growth of *Sdo. denitrificans* on testosterone. Amino acid sequences from individual subunits of AtcABC show 38 ~ 64% sequence identity with those of the 3-hydroxycyclohexanone dehydrogenase

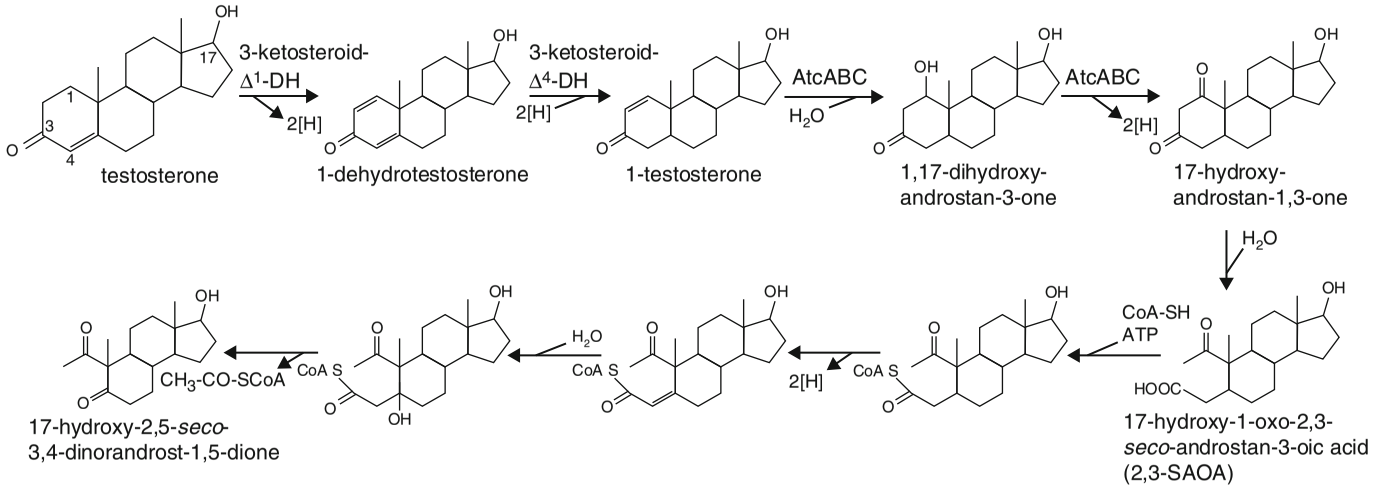

Fig. 6 The proposed pathway for anaerobic testosterone catabolism by the denitrifying bacteria (Wang et al. 2013b, 2014). The relevant carbons of testosterone are marked according to the numbering system of steroids. The CoA-thioester intermediates are hypothetical and are not detected in bacterial cultures. Abbreviations: *AtcABC* 1-testosterone hydratase/dehydrogenase, *CoA* coenzyme A, *DH* dehydrogenase

(MhyADH) of the cyclohexanol-degrading *Alicycliphilus denitrificans* (Jin et al. 2011). However, in contrast to MhyADH, substrate preference experiments indicated that AtcABC can hardly metabolize monocyclic 2-cyclohexenone. The hydrolase catalyzing the transformation of 17-hydroxyl-androstan-1,3-dione to 2,3-SAOA needs to be identified, which is largely impeded by a lack of the commercially available substrate.

In addition to *Sdo. denitrificans*, the *atcABC*-like genes are present in various betaproteobacteria, including *Azoarcus toluclasticus*, *Stl. denitrificans*, and *Thauera terpenica*. *T. terpenica* can degrade androgens under denitrifying conditions (Yang et al. 2016). The androgen degradation capacity of *A. toluclasticus* requires experimental confirmation. The phylogenetic analysis of AtcABC-like proteins suggested that the anaerobic steroid degradation capability is conserved in proteobacteria. Nevertheless, a more comprehensive comparative genomic analysis and more steroid catabolic gene sequences are required to gain a more detailed insight into the evolution of this pathway.

The genomes of several androgen-degrading anaerobes (*Sdo. denitrificans*, *Stl. denitrificans*, and *T. terpenica*) have been sequenced. To search for steroid catabolic genes commonly present in these genomes, an orthologous cluster analysis was performed using OrthoVenn (Wang et al. 2015) with the default setting (e-value = 1e-5 and inflation value = 1.5). The results showed the presence of 357 orthologous clusters, including 89% of single-copy genes, among the four genomes (Fig. 7a). After excluding the primary metabolic genes on the basis of the BlastKOALA results with *Sdo. denitrificans* genes as the query, a total of 41 genes were proposed to be involved in anaerobic steroid degradation, including those encoding steroid C25 dehydrogenase-like molybdoenzyme, β-oxidation enzymes involved in steroid C/D-rings degradation, and 1-testosterone hydratase/dehydrogenase (Fig. 7b, c, and d) (Yang et al. 2016).

3.4 Anaerobic Degradation of Estrogens and Other Steroids

Anoxic river sediments and soil are considered major reservoirs for estrogens, and microbial activity is crucial for the removal of estrogens from these contaminated ecosystems (Hanselman et al. 2003). Two estrogen-degrading denitrifiers, *Denitratisoma oestradiolicum* and *Sdo. denitrificans*, were isolated from activated sludge and anoxic digested sludge, respectively (Fahrbach et al. 2006, 2008). These denitrifiers can completely degrade natural estrogens, including estrone and 17β-estradiol. However, none of the related catabolic intermediates have been reported thus far, and the catabolic mechanisms involved in anaerobic estrogen biodegradation remain unknown. The elucidation of the biochemical mechanisms and catabolic machineries involved in anaerobic degradation of the phenolic A-ring of estrogens is of particular interest. To our knowledge, microorganisms capable of degrading progestagens and corticosteroids under anoxic conditions have not been isolated thus far.

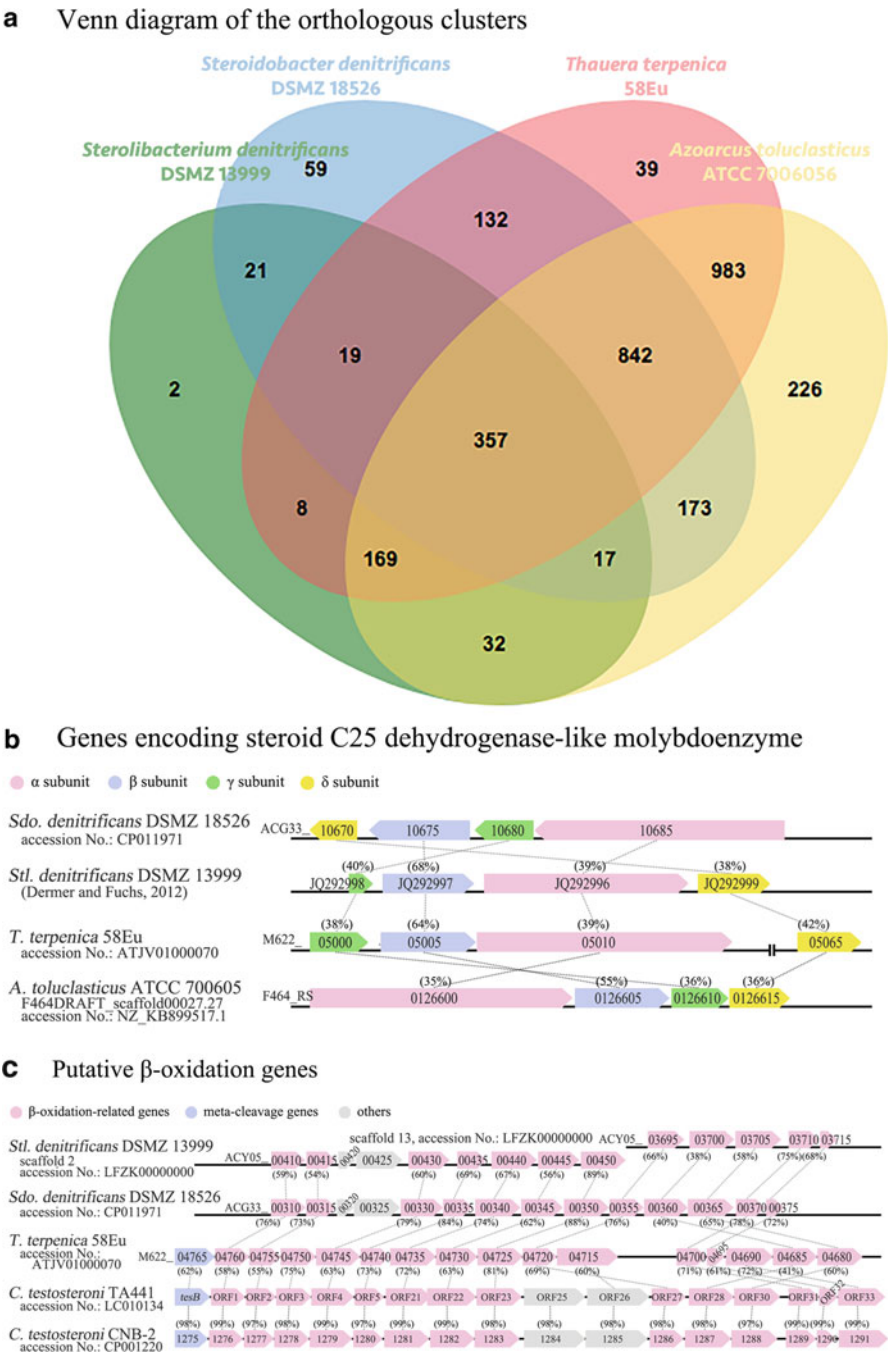

Fig. 7 (continued)

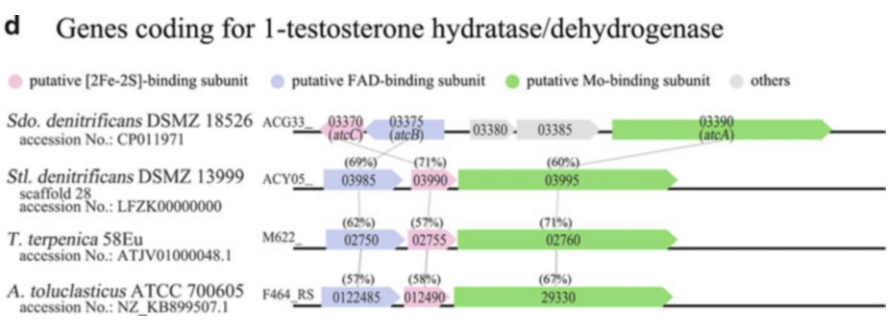

Fig. 7 Comparative genomic analysis of four androgen-degrading anaerobes. (**a**) Venn diagram of the orthologous clusters. The numbers indicate the orthologous genes found to be shared among or within different genomes. (**b**) Genes coding for a steroid C25 dehydrogenase-like molybdoenzyme are found in the genomes of *A. toluclasticus*, *Sdo. denitrificans*, *Stl. denitrificans*, and *T. terpenica*. Homologous ORFs (*colored arrows*) between different bacterial strains are connected with *dotted lines*. Numbers (%) indicate the identity of the deduced amino acid sequences with those of the genes of *Sdo. denitrificans*. (**c**) Genes encoding β-oxidation enzymes are conserved among androgen-degrading anaerobes and aerobes (e.g., *C. testosteroni*). The numbers (%) indicate the identity of the amino acid sequences with those of the functionally confirmed genes of *C. testosteroni* TA441. (**d**) Genes coding for the 1-testosterone hydratase/dehydrogenase. The numbers (%) indicate the identity of deduced amino acid sequences with those of the corresponding genes of *Sdo. denitrificans*

4 Culture-Independent Studies of Anaerobic Steroid Biodegradation in the Environment

Most natural steroids can be degraded through microbial activities under oxygen-limited conditions. Taylor et al. (1981) enriched denitrifying bacterial cultures capable of mineralizing cholesterol and identified catabolic products that included 5α-cholestan-3β-ol, cholest-4-en-3-one, 5α-androstan-3,17-dione, and androst-4-en-3-17-dione. Dykstra et al. (2014) reported the anaerobic biodegradation of phytosterols (e.g., campesterol, stigmasterol, and β-sitosterol) under denitrifying and sulfate-reducing conditions and identified stigmast-4-en-3-one and the related structures as initial metabolites.

Anaerobic biodegradation of natural estrogens and androgens in the denitrifying tanks of sewage treatment plants was observed (Andersen et al. 2003; Joss et al. 2004). In addition, Fan et al. (2007) investigated the anaerobic degradation of estrogens and androgens via microbial activity in agricultural soils. Because the conditions in sediments can often be anoxic, studies in sediments can be exploited to assess the differences in steroid degradation under oxic and anoxic conditions. Jürgens et al. (2002) investigated river sediments and revealed that degradation of 17β-estradiol and estrone was faster in oxic sediments than in anoxic sediments. In oxic marine sediments, the half-life of 17β-estradiol degradation was 4.4 days, whereas in anoxic sediments, it was 70 days (Ying et al. 2003). Czajka and Londry (2006) investigated the anaerobic degradation of natural and synthetic estrogens in

lake sediments under methanogenic as well as sulfate-, iron-, and nitrate-reducing conditions. Their data suggested that natural estrogens can be efficiently degraded under all tested conditions; however, synthetic 17α-ethynylestradiol was not degraded by the microorganisms. In addition, anaerobic transformation of 17β-estradiol to 17α-estradiol and estrone was observed in activated sludge (Lee and Liu 2002) and lake sediments (Czajka and Londry 2006). These reports indicated that 17β-estradiol was degraded through microbial activity under anoxic conditions. Various anaerobes (e.g., denitrifiers, sulfate-reducing and iron-reducing bacteria) may be involved in the anaerobic degradation of steroids, but neither sterane-cleaving mechanisms nor phylogenetic affiliation of anaerobic steroid degraders have been investigated. The metabolic and phylogenetic information is scant, mainly because of the lack of suitable anoxic media for the isolation and cultivation of steroid-degrading anaerobes (e.g., iron- or sulfate-reducing bacteria). In addition, suitable culture-independent tools for bacterial steroid degradation are insufficient.

Zang et al. (2008) studied the in situ [2,4,6,7-^{3}H(*N*)]estrone uptake by major bacterial groups in activated sludge using microautoradiography–fluorescence in situ hybridization (MAR–FISH) and suggested that betaproteobacteria and gammaproteobacteria are the main estrogen degraders. Their study estimated that aerobic estrogen degraders account for approximately 1 ~ 2% of the total bacterial community. Furthermore, Kurisu et al. (2015) used more specific probes and suggested that *Sphaerotilus-Leptothrix*-related betaproteobacteria are the major estrone degraders in aerobic sludge. Conventional culture-independent approaches, such as stable-isotope probing (SIP) and MAR-FISH, are rarely used for studying microbial steroid degradation because (i) completely ^{13}C-labeled steroids are not commercially available and (ii) hydrophobic compounds such as steroids easily attach to or pass through bacterial membranes. Therefore, distinguishing the metabolic activities (e.g., passive diffusion or active uptake, and redox transformation or complete degradation) of radiolabeled bacterial cells is difficult. Moreover, the catabolic intermediates and genes involved in anaerobic steroid biodegradation remain largely unknown. Taken together, the lack of molecular markers and suitable culture-independent techniques has impeded in situ investigations of microbial steroid degradation. Yang et al. (2016) investigated anaerobic androgen degradation in the denitrifying tank of a municipal sewage treatment plant by using the following approaches: (i) ultra-performance liquid chromatography - tandem mass spectrometry (UPLC-MS/MS) identification of signature metabolites, (ii) identification of major catabolic players through next-generation sequencing techniques, and (iii) PCR-based identification of functional genes. Through metabolite analysis, the authors revealed that denitrifying bacteria in the anoxic sewage use the 2,3-*seco* pathway to degrade androgens. The subsequent metagenomic analysis and PCR-based functional assays demonstrated that androgen degradation in the anoxic sewage was performed by *Thauera* spp. (mainly *T. terpenica*) through the action of 1-testosterone hydratase/dehydrogenase. Before this study, *T. terpenica* was not recognized as a steroid degrader. Thus, this integrative omics approach can be used for culture-independent investigations of the biodegradation of organic compounds when isotope-labeled substrates are not accessible.

5 Research Needs

Steroids, with more than 1000 chemical structures, are ubiquitous and abundant in the biosphere. Thus, steroid-degrading microorganisms can survive in aerobic, microaerobic, and anaerobic environments. However, literature on the anaerobic steroid biodegradation remains limited. Thus far, only five steroid-degrading anaerobes belonging to the classes Betaproteobacteria and Gammaproteobacteria have been isolated and characterized. All of the isolates have been reported as denitrifiers, which were cultivated using highly similar growth media. The development of novel growth techniques and media is critical for exploring the microbial diversity of anaerobic steroid catabolism. For instance, literature has suggested that sulfate-reducing bacteria can anaerobically degrade steroids. Strictly anaerobic growth media should be developed for isolating and cultivating such bacteria from marine and brackish samples.

Few genomes of steroid-degrading anaerobes have been sequenced. The genomes of additional relevant microorganisms are required for elucidating the evolution of anaerobic steroid catabolic pathways. Moreover, all studied species do not grow on solid media; this impedes conventional genetic engineering and molecular biology investigations such as mutagenesis, gene overexpression, and cloning. Consequently, the catabolic genes have been identified mainly through the isolation and characterization of the corresponding enzymes (reverse genetics). However, without gene disruption experiments, the physiological roles of these identified catabolic genes cannot be ascertained. Moreover, the purification and characterization of steroid-transforming enzymes is hindered by the lack of commercially available steroid substrates. Consequently, most steroid substrates are produced by the researchers themselves, which is labor and time intensive. The isolation of suitable bacterial strains for molecular biological studies is crucial for identifying genes encoding steroid transporters and those involved in the regulation of anaerobic steroid catabolism.

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Part II

Functional Genomics of Anaerobic Degradation of Hydrocarbons

8 Functional Genomics of Anaerobic Degradation of Hydrocarbons: An Introduction

Ralf Rabus and Heinz Wilkes

Abstract

Anaerobic biodegradation of hydrocarbons is typically studied with newly isolated microorganisms not yet accessible to genetic manipulation. Functional genomics, viz., genome-based differential proteomics in conjunction with targeted metabolite analysis, has been instrumental for discovering a multitude of novel reactions and pathways involved in the biochemically challenging process of anaerobic hydrocarbon degradation.

The rapid advancements in genome sequencing technologies subjected life sciences to fundamental methodological and conceptual expansions during the last two decades. The technological development starting with the first-generation sequencing (Sanger) proceeded via massively parallel sequencing by means of second-generation instruments (e.g., Illumina), providing huge amounts of short read length (150 bp) with high quality, to the third-generation sequencing that analyzes single DNA molecules in real time, generating long read lengths (median ~6 kbp), but with quality still in need for improvement (e.g., PacBio and MinION) (Shendure et al. 2017; Reuter et al. 2015). The second- and third-generation sequencing technologies do not only provide low cost access to high quality genome sequences of any pure culture of interest but will also open up new avenues for profiling microbial communities and for microbiome research (Kerkhof et al. 2017; Michas et al. 2017). This dramatic increase in access to sequencing data is met by open bioinformatics platforms for automated data processing and analysis, e.g., IMG/M (Chen et al. 2016)

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and MG-RAST (Wilke et al. 2016). Notwithstanding, manual expert annotation is still very much required in the context of newly discovered metabolic capacities or reconstruction of metabolic networks that are not covered by well-studied standard organisms. Despite the affluent information provided by genomics, these data are restricted to the current state of knowledge present in the database and only allow for a theoretical view on an organism's metabolism, cellular properties, and potential interactions. Considering that proteins are the active entities that bring a cell to life, proteomics plays a key role in postgenomic investigations of a study organism. Owing to the immense diversity of proteins (amino acid composition, size, polarity, subcellular localization, complex formation, abundance dynamics, etc.), there is not a single methodological approach that covers a given proteome. Rather, a whole suite of methods ranging from sample preparation via (quantitative) protein/peptide separation to mass spectrometry-based analysis and bioinformatics-based identification have to be integrated according to the demands of a given project (Wöhlbrand et al. 2013). Proteomics standards are required for cross-project comparisons and particularly for metaproteomics; this challenge is particularly met by the initiatives within the framework of the Human Proteome Project (e.g., Deutsch et al. 2017). The combination of genomics and proteomics, viz., proteogenomics, is a particularly promising and growing field, as it allows refining the genome annotation and at the same time unraveling the dynamics and complexity of protein formation/abundance (e.g., Menschaert and Fenyö 2017).

The benefit of applying proteogenomics to the field of anaerobic microbial hydrocarbon degradation was early on substantiated by enabling the identification of the complete gene cluster for the anaerobic degradation of ethylbenzene in the denitrifying bacterium *Aromatoleum aromaticum* EbN1 (Rabus et al. 2002, 2019). The subsequently reported genome of strain EbN1 (Rabus et al. 2005) together with a comprehensive proteome profiling of substrate-adapted cells (Wöhlbrand et al. 2007) yielded new reaction/pathway discoveries and first global insights into the regulatory capacities of such an anaerobic degradation specialist. Meanwhile, several proteogenomic studies concerned with a variety of facultatively and strictly anaerobic microorganisms have further expanded our knowledge on the genes, enzymes, and pathways involved in anaerobic degradation of aromatic compounds (e.g., Heintz et al. 2009; Bergmann et al. 2011; Nobu et al. 2015).

A further instrumental approach supplementing proteogenomics in pathway discovery is targeted metabolite analysis, e.g., with isotope labeled substrates. Unequivocal identification of hitherto unknown metabolites strongly corroborates the predictions from differential proteogenomics and likewise provides initial hints on new reactions to be further elaborated by proteogenomics. This is exemplified by the identification of *p*-hydroxyacetophenone as a key intermediate of the newly discovered anaerobic degradation pathway for *p*-ethylphenol in *A. aromaticum* EbN1 (Wöhlbrand et al. 2008). Further integration of a global metabolomics approach will be an important leverage to eventually promote research on anaerobic degradation of hydrocarbons to the systems biology level.

This section on anaerobic degradation of hydrocarbons is subdivided into three chapters, reflecting the mode of energy generation: denitrification, sulfate reduction, and metal reduction.

The chapter on denitrifying bacteria by Rabus and Wilkes (n.d.-a) first summarizes the state of knowledge on *A. aromaticum* EbN1, which represents the proteogenomically best studied anaerobic degrader of aromatic compounds (including hydrocarbons), covering insights into pathways, the architecture and regulation of the catabolic network, and adaptation to habitat-relevant conditions such as solvent stress, substrate mixtures, and slow growth. The second major part of this chapter revolves around *Azoarcus* sp. strain CIB, which is best studied with respect to the mechanisms of transcriptional control, e.g., the *bzd* and *mbd* operons for anaerobic degradation of benzoate and 3-methylbenzoate, respectively; furthermore, a new strategy to achieve solvent tolerance involving c-di-GMP is mentioned. The chapter progresses by presenting insights into the anaerobic degradation of *para*-alkylated benzoate and toluenes in *A. aromaticum* EbN1, *Thauera* sp. strain pCyN2, and *Magnetosprillum* sp. strain pMbN1. Finally, genome-based analyses of *Azoarcus* sp. strain PA01, *Azoarcus anaerobius* and *Thauera aromatica* are summarized.

The chapter on sulfate-reducing bacteria by Rabus and Wilkes (n.d.-b) is first concerned with toluene-degrading *Desulfobacula toluolica* Tol2, which represents the aromatic compound-degrading sulfate reducer to be first comprehensively studied by integrating genomics, proteomics, and targeted metabolite analyses. A second emphasis is on naphthalene-degrading SRB, involving the pure cultures NaphS2, NaphS3, and NaphS6, as well as the intensively studied enrichment culture N47. Finally, genome-based studies on *Desulfobacula* sp. TS, *Desulfococcus multivorans*, and *Desulfotomaculum gibsoniae* Groll^{T} are presented.

The chapter on metal-reducing bacteria capable of anaerobic degradation of aromatic compounds by Tremblay and Zhang (n.d.) first summarizes available cultures and then proceeds according to compound type, from benzene, via alkylbenzenes to polyaromatic hydrocarbons. A large part of the presented studies uses the iron-reducing deltaproteobacterium *Geobacter metallireducens* as a model system and comprises biochemical and/or functional genomic approaches. Particular emphasis is on the proposed anaerobic hydroxylation of benzene and the ATP-independent reduction of the central intermediate benzoyl-CoA.

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Functional Genomics of Denitrifying Bacteria Degrading Hydrocarbons

9

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Abstract

The betaproteobacterial *Azoarcus*/"*Aromatoleum*"/*Thauera* cluster harbors a large variety of denitrifiers capable of anaerobic degradation of aromatic compounds and hydrocarbons. The application of genomics and proteomics (proteogenomics) in conjunction with targeted metabolite analysis has proven instrumental to the discovery of a wide variety of novel reactions and pathways, the reconstruction of complex catabolic networks, and first steps into understanding how the latter are tuned to adapt to environmental changes. Over the past two to three decades, major advances have been achieved along three major organism-/approach-specific directions: (i) biochemistry of novel enzymatic reactions with *Thauera aromatica* K172 and "*Aromatoleum aromaticum*" EbN1, (ii) physiology and proteogenomics with "*A. aromaticum*" EbN1, and (iii) molecular mechanisms of transcriptional regulation with *Azoarcus* sp. strain CIB.

1 Introduction

Aromatic compounds belong to the most abundant chemical structures in the biosphere and the geosphere. Firstly, they constitute the building blocks of the second most abundant biopolymer on earth, namely, the plant cell wall component lignin. Its phenylpropanoid monomers are synthesized via the cinnamate/monolignol pathways (Umezawa 2010) and then to the most part linked by β-aryl ethers or biphenyl bonds to form the complex and highly irregular polymer (Bugg et al. 2011). Secondly, aromatic compounds are major constituents of fossil organic matter including kerogen, coal, and crude oil (Overton et al. 2016) which harbor a major share of the global carbon pool. The monoaromatic hydrocarbons benzene, toluene, ethylbenzene, and the three xylene isomers (BTEX) are extensively used in gasoline and synthetic chemical industry and are of particular concern due to their health impacting properties (Bolden et al. 2015). Against this background, microbial biodegradation of aromatic compounds is highly relevant for maintaining the global carbon cycle as well as from the perspective of environmental protection. However, aromatic hydrocarbons (e.g., toluene) represent difficult-to-handle substrates due to their low chemical reactivity resulting from the delocalized π-electron system and the exclusive presence of C–C and C–H bonds; even aromatic compounds carrying functional groups (e.g., benzoate) remain challenging. Aerobic degradation, viz., in the presence of molecular oxygen (O_2) is long known, with oxygenase-catalyzed reactions performing initial activation as well as subsequent ring cleavage reactions (Gibson and Parales 2000). Considering that large parts of the biosphere are devoid of O_2 (anoxic conditions), also the anaerobic degradation of aromatic hydrocarbons is of eminent significance. During the past three decades, a multitude of bacteria have been newly isolated and demonstrated to degrade aromatic compounds under anoxic conditions, employing a variety of novel and intriguing reactions (for overview see, e.g., Widdel and Rabus 2001; Carmona et al. 2009; Fuchs et al. 2011; Rabus et al. 2016a).

In concert with the widespread occurrence of anoxic habitats, a huge diversity of anaerobic bacteria exists that can be classified amongst others by their mode of energy conservation, with the redox potential of the terminal electron acceptors defining the energetic output of catabolism (Decker et al. 1970). Bacteria reducing nitrate (NO_3^-) to molecular nitrogen (N_2) for energy conservation are termed denitrifiers and achieve a high energy yield, second only to oxygen respiration (Zumft 1997). Denitrification is widespread among bacteria of unrelated phylogenetic affiliation (Shapleigh 2013) and represents a widely recognized key process in the global nitrogen cycle responsible for replenishing the atmospheric N_2 pool (Fowler et al. 2013). Denitrifying bacteria are also long known to be widespread in nature (e.g., Tiedje et al. 1982), ranging from up/wetland soils (Conrad 1996), tropical forest soils (Pajares and Bohannan 2016), and marine sediments (Devol 2015) to water-flooded oil reservoirs (Nazina et al. 2017).

Among the first pure cultures of denitrifying bacteria reported to be capable of anaerobic degradation of aromatic hydrocarbons were toluene-degrading *Thauera aromatica* K172 (Anders et al. 1995) and ethylbenzene-degrading "*Aromatoleum aromaticum*" EbN1 (Rabus and Widdel 1995), both belonging to the β-subclass of *Proteobacteria*. Meanwhile a considerable collection of denitrifying bacteria with corresponding degradative capacities has become available (for overview see, e.g., Widdel et al. 2010). This chapter summarizes genome-based studies with aromatic compound-degrading denitrifiers, putting particular emphasis on genome-enabled discoveries, holistic perspectives on metabolic adaptation, and the integration of proteomics (proteogenomics) and metabolite analysis. This review is structured according to organisms.

2 "*Aromatoleum aromaticum*" EbN1

The study organism "*Aromatoleum aromaticum*" EbN1 was originally isolated with ethylbenzene as sole source of organic carbon and energy and nitrate as electron acceptor (Rabus and Widdel 1995) and shown to utilize its two alkylbenzene substrates (toluene and ethylbenzene) also directly from crude oil (Rabus and Widdel 1996). "*A. aromaticum*" EbN1 can at present be regarded as the anaerobic aromatic compound degrader best characterized on the proteogenomic level (Rabus 2005; Rabus et al. 2014) and as a promising model to study the adaptation to environmental changes and stressors on a systems biology level. In the first instance, proteogenomic research focused on the comprehensive reconstruction of the catabolic network and its substrate-specific regulation. More recently, emphasis has shifted toward mimicking habitat-relevant conditions, such as the presence of substrate mixtures, growth-limiting substrate concentrations, and solvent stress.

2.1 Genome

Prior to obtaining the complete genome sequence of "*A. aromaticum*" EbN1, proteomics-derived protein sequence data were used to assemble for the first time

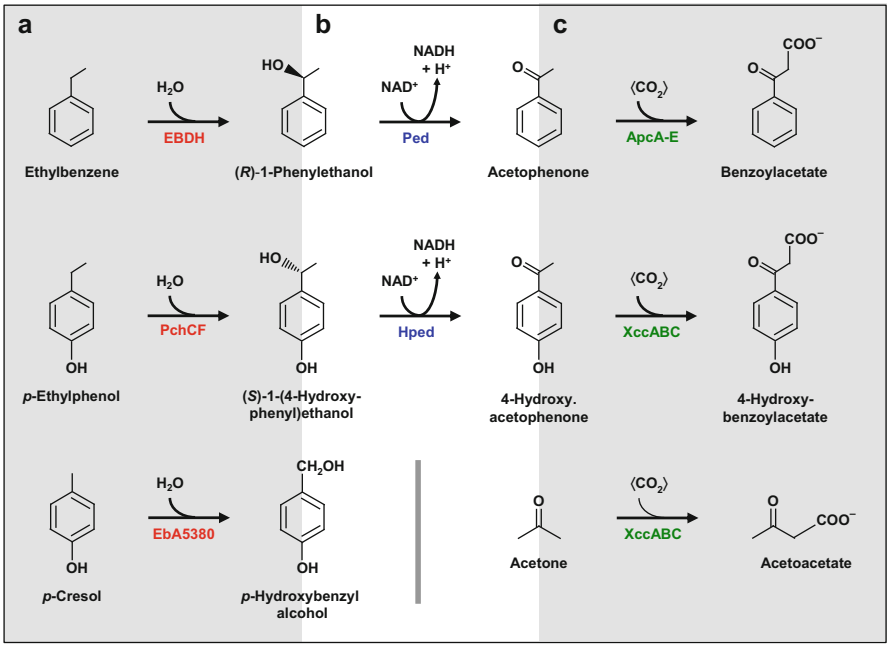

Fig. 1 Different enzymes for the same reaction type discovered in "*Aromatoleum aromaticum*" EbN1. (**a**) C-H bond activation by O_2-independent hydroxylation: ethylbenzene dehydrogenase (EBDH) which contains a *bis*-molybdopterin guanine dinucleotide cofactor, five Fe-S clusters and a heme b cofactor (Kniemeyer and Heider 2001a; Heider et al. 2016); putative *p*-ethylphenol methylenehydroxylase (PchCF) (Wöhlbrand et al. 2008) and *p*-cresol methylhydroxylase (EbA5380) (Wöhlbrand et al. 2007) both of which should by sequence similarity represent flavocytochrome enzymes (Reeve et al. 1989). (**b**) Stereospecific dehydrogenases for secondary aromatic alcohols: (*S*)-1-phenylethanol dehydrogenase (Ped) (Kniemeyer and Heider 2001b; Höffken et al. 2006) and (*R*)-1-(4-hydroxyphenyl)ethanol dehydrogenase (Hped) (Büsing et al. 2015a). (**c**) Carboxylation (ATP-dependent) of aromatic and aliphatic ketones: acetophenone carboxylase (ApcA-E) (Kühner et al. 2005; Jobst et al. 2010), 4-hydroxyacetophenone carboxylase (XccABC) (Wöhlbrand et al. 2008), and acetone carboxylase. (Wöhlbrand et al. 2007; Schühle and Heider 2012)

the complete gene clusters required for the anaerobic degradation of ethylbenzene (Rabus et al. 2002) and toluene (Kube et al. 2004) from a genomic shotgun dataset. In the case of ethylbenzene (Fig. 1), the major findings were: (i) a presumptive periplasmic localization of the heterotrimeric ethylbenzene dehydrogenase, (ii) the genetic organization of the peripheral pathway into an upper (from ethylbenzene to acetophenone) and a lower part (from acetophenone to benzoyl-CoA), (iii) the presence of a presumably five subunit acetophenone carboxylase, and (iv) two predicted two-component sensory/regulatory systems for a possibly sequential transcriptional control of the two pathway modules. Analysis of the "toluene" gene cluster revealed that the genes for the initial activation of toluene to benzylsuccinate and for its subsequent β-oxidation-like conversion to the central intermediate

benzoyl-CoA colocalize as two neighboring operon-like structures. Moreover, a single adjacently encoded two-component sensory/regulatory system has been suggested to control the transcription of both operons in a concerted manner. These regulatory predictions were then tested and verified on the RNA (RT-PCR and DNA microarray) and protein (two-dimensional difference gel electrophoresis (2D DIGE) combined with MALDI-TOF-MS) level, using substrate-adapted cells, i.e., anaerobic growth with benzoate, toluene, ethylbenzene, or a mixture of the two alkylbenzenes (Kühner et al. 2005). Recently, a fluorescent reporter strain of "*A. aromaticum*" EbN1 was constructed, by fusing the chromosomal *apcA* gene (related to acetophenone carboxylation) with the gene for the fluorescent protein mCherry, allowing for acetophenone quantification in the range of 50–250 μM (Muhr et al. 2016). The novelty of the described pathway for anaerobic ethylbenzene degradation motivated the determination of crystal structures for the first three enzymes: ethylbenzene dehydrogenase (EBDH, Kloer et al. 2006), (*S*)-1-phenylethanol dehydrogenase (Ped, Höffken et al. 2006), and acetophenone carboxylase (Apc, Weidenweber et al. 2017). In turn, this research provided access to a novel biocatalyst (Ped) for the biotechnologically relevant enantioselective reduction of ketones (Breuer et al. 2008; Dudzik et al. 2015).

The complete genome of "*A. aromaticum*" EbN1 was the first to be determined for an anaerobic hydrocarbon-degrading prokaryote. It consists of a circular chromosome (4.3 Mb) and two plasmids (0.21 and 0.22 Mb) and codes for ~4600 proteins (Rabus et al. 2005). Manual annotation of the genome allowed for the following major insights: (i) genes for the anaerobic and aerobic degradation of aromatic compounds (10 and 4, respectively) mostly form substrate-specific operon-like clusters, which are widely distributed across the chromosome; (ii) a high genome plasticity was suggested from the high number of paralogous genes and frequent mobile genetic elements; (iii) the presence of multiple respiratory complexes has been proposed to enable optimal adaptation during oxic-to-anoxic transitions; (iv) the presence of genes of multiple sensory/regulatory proteins suggested the capacity to finely tune the metabolic network according to available substrate and changing environmental conditions; (v) the absence of genes for N_2-fixation or specific interactions, and the lack of genomic synteny clearly separated strain EbN1 as a soil-inhabiting degradation specialist from the phylogenetically closely related *Azoarcus* sp. strain BH72, which is a mutualistic, N_2-fixing grass endophyte (Krause et al. 2006). Notably, recent studies with *Azoarcus* sp. strain CIB demonstrated that a denitrifier anaerobically degrading a variety of aromatic compounds can also pursue a N_2-fixing endophytic life style (Fernández et al. 2014).

2.2 Catabolic Network and Substrate-Specific Regulation

The genome inspired hypothesis that "*A. aromaticum*" EbN1 possesses finely tuned regulatory capacities to adapt its catabolic network in a substrate-specific manner was tested for 22 different substrate and redox conditions. For this purpose, the substrate-specific proteome profiles were analyzed by a combination of 2D DIGE and MALDI-TOF-MS/MS, generating a unique matrix of the 18 different anaerobic

substrate conditions against the respective abundances of 66 involved proteins (Wöhlbrand et al. 2007). Overall, the proteins of peripheral degradation routes channeling the individual aromatic and aliphatic substrates into the central metabolism displayed highly substrate-specific abundance profiles. In contrast, proteins for central metabolic modules, e.g., TCA cycle, amino acid biosynthesis, or for genetic information processing showed essentially constant profiles under all tested conditions. Thus, this study provided a comprehensive understanding of regulated versus constitutive subproteomes in "*A. aromaticum*" EbN1. Furthermore, it allowed for a refined annotation of the catabolic network, including the discovery of novel hitherto unpredicted catabolic capacities (e.g., anaerobic degradation of *o*-aminobenzoate), some of which have subsequently been confirmed by biochemical studies (see below). At present the proteogenomically reconstructed anaerobic catabolic network encompasses 18 different aromatic compounds and is composed of 85 proteins, 65 of which have been identified on the proteomic level corresponding to a 76.5% coverage (Rabus et al. 2014).

2.3 Proteogenomics-Driven Reaction and Pathway Discoveries

As already indicated in the above paragraph, differential proteomics in conjunction with metabolite studies and iterative genome analysis allowed the discovery of degradative capacities and pathways previously not known for "*A. aromaticum*" EbN1 (Fig. 1) and other anaerobic degraders.

***p*-Cresol** Based on the comprehensive proteome profiling across multiple substrate adaptation conditions (Wöhlbrand et al. 2007), the presumptive enzyme for initial methyl group activation of *p*-cresol was reassigned. Instead of the genomically predicted *p*-cresol methylhydroxylase, the *p*-cresol-specific abundance profile suggested involvement of a FAD-dependent oxidase (EbA5380).

***p*-Ethylphenol** The anaerobic degradation of *p*-ethylphenol proceeds in analogy to the pathway established for ethylbenzene; however, fundamentally different enzymes are apparently involved (Wöhlbrand et al. 2008). The initial hydroxylation of *p*-ethylphenol to 1-(4-hydroxyphenyl)ethanol involves a *p*-cresol methylhydroxylase-like protein instead of an analog of the Mo-dependent ethylbenzene dehydrogenase (Kniemeyer and Heider 2001a). The second reaction, a dehydrogenation forming *p*-hydroxyacetophenone may involve either of two predicted alcohol dehydrogenases, which are distinct from (*S*)-1-phenylethanol dehydrogenase (Ped) (Kniemeyer and Heider 2001b). Subsequent carboxylation should involve a biotin-dependent enzyme instead of an analog of ATP-dependent acetophenone carboxylase (Jobst et al. 2010). Quantitative differential proteomics by 2D DIGE clearly demonstrated the degradation pathways for ethylbenzene and *p*-ethylphenol to be specifically regulated despite the structural similarity of the two compounds (Wöhlbrand et al. 2008). In accord, subsequent studies measuring specific enzyme activities of the two pathways yielded essentially the same specific induction pattern (Muhr et al. 2015). Using the newly developed genetic system for "*A. aromaticum*" EbN1 (Wöhlbrand and Rabus 2009), two

unresolved questions were tackled. By means of unmarked *in-frame* deletion, 1-(4-hydroxyphenyl)ethanol dehydrogenase (Hped) could be unambiguously assigned to the second reaction step in anaerobic degradation of *p*-ethylphenol. Moreover, the X-ray structure of Hped revealed its (*R*)-specificity, contrasting the (*S*)-specificity of Ped from the anaerobic ethylbenzene degradation pathway (Büsing et al. 2015a). The proteogenomic analysis had predicted a σ^{54}-dependent regulator (EtpR) to mediate the substrate-specific expression of the genes for anaerobic degradation of *p*-ethylphenol. This hypothesis was confirmed by the generation of an unmarked *in-frame* deletion mutation of the *etpR* gene and its *in trans* complementation (Büsing et al. 2015b).

3-Phenylpropanoids Differential proteogenomics combined with detailed metabolite analyses provided evidence for the capacity of "*A. aromaticum*" EbN1 to degrade plant-derived 3-phenylpropanoids (Trautwein et al. 2012c). Differential proteomics suggested that a gene cluster of hitherto undefined β-oxidation functionality should be responsible for the peripheral degradation of cinnamate, hydrocinnamate, *p*-coumarate, and 3-(4-hydroxyphenyl)propanoate. Metabolite analysis confirmed this pathway and, furthermore, indicated a high substrate specificity of the enzymes, as nongrowth supporting 3-phenylpropanoids were not transformed cometabolically.

Phenylalanine While proteomics essentially confirmed the genome-predicted first two reaction steps in the degradation of phenylalanine, i.e., deamination by phenylalanine aminotransferase (Pat) and decarboxylation by phenylpyruvate decarboxylase (Pdc), a specifically upregulated aldehyde:ferredoxin oxidoreductase (Aor) was suggested to substitute the predicted dehydrogenase (Pdh) (Wöhlbrand et al. 2007). Subsequent biochemical studies demonstrated that, in fact, both dehydrogenases can be operative in the peripheral degradation of phenylalanine (Debnar-Daumler et al. 2014) and that in their absence aldehyde dehydrogenase from benzaldehyde degradation can substitute this functionality (Schmitt et al. 2017).

Indoleacetate The plant hormone auxin (indoleacetate, IAA) is an *N*-heterocyclic compound and also represents an intermediate of tryptophane degradation. Using a differential proteomic approach the hitherto functionally unassigned *iaa* gene cluster was identified, harboring the genes required to degrade the *N*-heterocyclic pyrrole ring yielding 2-aminobenzoyl-CoA. The reaction sequence is proposed to be initiated by activation of IAA to the respective CoA-thioester (specific CoA ligase) (Schühle et al. 2016) followed by an anaerobic hydroxylation (molybdenum cofactor-containing dehydrogenase) and a hydrolytic ring opening (hydantoinase-like enzyme) (Ebenau-Jehle et al. 2012).

***o*-Phthalate** Differential proteomics revealed a gene cluster for anaerobic degradation of *o*-phthalate (1,2-dihydroxybenzene), comprising a putative TRAP transporter for uptake, a succinyl-CoA:phthalate CoA transferase for activation to phthaloyl-CoA, and an UbiD-like (de)carboxylase for conversion to the central intermediate benzoyl-CoA. Genome comparisons indicated this proposed pathway to also be present in *Thauera chlorobenzoica* 3CB-1, *Azoarcus* sp. strain PA01, and *Azoarcus toluclasticus* ATCC

700605 (Ebenau-Jehle et al. 2017). Subsequently, the key enzyme phthaloyl-CoA decarboxylase was purified from *T. chlorobenzoica* and shown to be a hexameric enzyme containing the cofactors prenylated FMN, K^+, and Fe^{2+} (Mergelsberg et al. 2017).

2.4 Adaptation to Solvent Stress Conditions

Aromatic hydrocarbons represent in a way Janus-faced compounds, as they are covetable substrates due to their energy richness and at the same time problematic due to their membrane toxic properties (Sikkema et al. 1995). In particular, in habitat settings characterized by elevated hydrocarbon exposure arising from natural seeps or anthropogenic contamination, alkylbenzene concentrations are known to reach levels exerting solvent stress on the microbial flora. To study the adaptation of the model organism "*A. aromaticum*" EbN1 to such conditions, anaerobic growth experiments were conducted with ethylbenzene, toluene, *p*-cresol, and phenol amended at semi-inhibitory concentrations. "*A. aromaticum*" EbN1 was found to pursue a complex adaptation strategy on several physiological levels: (i) the central metabolism was rerouted from the TCA-cycle to poly(3-hydroxybutyrate) formation to keep degradation of the aromatic substrates operative while denitrification was already impaired; (ii) the abundance of specific presumptive solvent efflux systems was increased; and (iii) the membrane lipid composition (relative abundance of different phospholipid head groups as well as the relative content of unsaturated versus saturated fatty acid side chains) was modified to counteract the membrane macerating effect of the solvents (Trautwein et al. 2008; Zink and Rabus 2010). While the rerouting of the intracellular C-flux appears to be a hitherto unknown strategy, the observed involvement of solvent efflux systems and the effects on membrane lipid composition are in accord with findings for other solvent-stressed anaerobic or aerobic bacteria (Duldhardt et al. 2010; Heipieper et al. 2007; Ramos et al. 2002).

2.5 Unusual Diauxie

While the single substrate approach proved straightforward for the above described research questions, it however did not represent the natural situation which is typically characterized by the presence of complex mixtures of potential substrates. The early study by Kühner et al. (2005) on a mixture of toluene and ethylbenzene demonstrated that both respective pathways were concomitantly operational in "*A. aromaticum*" EbN1. This indicated that a regulatory process such as catabolite repression to discriminate for a preferential substrate was not effective under the conditions applied. In a subsequent experiment, C_4-dicarboxylate-adapted cultures of "*A. aromaticum*" EbN1 displayed a diauxic growth with a mixture of the respective C_4-dicarboxylate (succinate, fumarate, or malate) and benzoate. Surprisingly, benzoate was first used instead of the C_4-dicarboxylate representing the adaptation substrate and yielding better growth (Trautwein et al. 2012a), which contrasted the utilization pattern in the aerobic benzoate degrader *Pseudomonas putida* (Morales et al. 2004) or anaerobic benzoate degrader *Azoarcus* sp. strain CIB (López Barragán et al. 2004).

Comprehensive differential protein profiling (2D DIGE, shotgun, membrane protein enriched fraction) combined with targeted quantitative transcript analyses (Northern) revealed that during benzoate utilization (viz. non C_4-dicarboxylate utilization), expression of the genes for the presumptive succinate uptake system (DctPQM, a predicted tripartite ATP-independent periplasmic (TRAP) transporter) was repressed. It only started to resume C_4-dicarboxylate-specific levels upon depletion of benzoate. The molecular mechanism underlying the observed diauxie and repression of *dctPQM* transcription in "*A. aromaticum*" EbN1 is at present unclear. However, one may speculate that, e.g., benzoate or its activation product benzoyl-CoA may interfere with the two-component sensory/regulatory system DctSR, which is suggested to control the transcription of the *dctPQM* operon.

2.6 Adaptation to Slow Growth

Many natural habitats are characterized by limited availability of substrates, i.e., the concentration of an individual compound does not exceed a few μg L^{-1} (Egli 2010). To study the adaptation of microorganisms to substrate limitation, the long-known continuous cultivation in chemostats is still the method of choice (Novick and Szilard 1950; Ferenci 2008). Cultivation in chemostats was performed with "*A. aromaticum*" EbN1 using benzoate as the sole and limiting organic substrate under nitrate-reducing conditions (Trautwein et al. 2012b). Strain EbN1 displayed a complex physiological and proteomic response to slow ($\mu_{low} = 0.036\ h^{-1}$) in comparison to rapid ($\mu_{high} = 0.180\ h^{-1}$) growth: (i) growth rate positively correlated with cell size, the DNA/RNA content, and other cellular parameters as well-known, e.g., from *Escherichia coli* (Schaechter et al. 1958); (ii) during slow growth, the highest energy requirement for maintenance was determined using a thermodynamics approach (Tijhuis et al. 1993); (iii) at μ_{low}, benzoate uptake probably occurs via an ABC-type transporter, whereas at μ_{high} a benzoate:H^+ symporter is likely to participate; (iv) not unexpectedly, the proteins involved in anaerobic benzoate catabolism showed decreased abundance during slow growth; (v) remarkably, at μ_{low}, proteins of uptake systems and peripheral routes for the aerobic or anaerobic degradation of various aromatic compounds were formed, despite benzoate being the only present organic substrate. These findings are in strong contrast to the high specificity of regulation when individual aromatic growth substrates are provided at high concentrations (see above Sect. 2.2). One may speculate, that "*A. aromaticum*" EbN1 derepresses and/or passively induces this variety of catabolic capacity as a preparatory means for future substrate availabilities. The "unproductive" preparedness could be one contributor to the observed high maintenance energy requirements at slow growth.

3 "*A. aromaticum*" pCyN1 and *Thauera* sp. Strain pCyN2

The two betaproteobacteria "*A. aromaticum*" pCyN1 and *Thauera* sp. strain pCyN2 represent the first pure cultures reported to be able to anaerobically degrade a *para*-alkylated toluene, i.e., the aromatic plant hydrocarbon *p*-cymene (4-isopropyltoluene)

(Harms et al. 1999). *para*-Alkylated toluenes had until then been considered as particularly recalcitrant. Combined differential proteogenomics and metabolite analysis revealed that the two strains employed fundamentally different peripheral pathways for the anaerobic degradation of *p*-cymene converging at the level of 4-isopropylbenzoyl-CoA (Strijkstra et al. 2014). "*A. aromaticum*" pCyN1 activates *p*-cymene by means of anaerobic hydroxylation to 4-isopropylbenzyl alcohol, which is subsequently converted to 4-isopropylbenzoyl-CoA via two dehydrogenation reactions. In contrast, *Thauera* sp. strain pCyN2 activates *p*-cymene by addition to fumarate forming (4-isopropylbenzyl) succinate, which is subjected to a modified β-oxidation also yielding 4-isopropylbenzoyl-CoA. Notably, the catalytic subunits (CmdA and IbsA) of the two initial enzymes, i.e., *p*-cymene dehydrogenase (CmdABC) and (4-isopropylbenzyl) succinate synthase (IbsA-F), form new substrate-specific branches in the phylogenetic trees of O_2-independent alkyl chain hydroxylases as well as alkyl- and arylalkylsuccinate synthases. These proteogenomics-derived phylogenetic trees shed new light on the substrate-specificity of these two classes of anaerobically hydrocarbon-activating enzymes and provide a new functional resolution power for habitat studies at hydrocarbon/oil exposed sites.

4 *Azoarcus* sp. Strain CIB

Azoarcus sp. strain CIB is a betaproteobacterium capable of anaerobic degradation of a variety of aromatic compounds under nitrate-reducing conditions (López Barragán et al. 2004) and originated from a diesel fuel-contaminated laboratory aquifer column (Hess et al. 1997). In contrast to "*A. aromaticum*" EbN1, *Azoarcus* sp. strain CIB according to its genomic repertoire is capable of fixing nitrogen (Fernández et al. 2014; Martín-Moldes et al. 2015) and can also colonize intracellular spaces of rice roots. Shared properties of the genomes of both biodegraders include the high degree of plasticity, e.g., resulting from the high number of mobile genetic elements, and the rather random distribution of genes for the anaerobic and aerobic degradation of aromatic compounds. A major research focus with *Azoarcus* sp. strain CIB was on elucidating the molecular mechanisms of transcriptional control, in particular, of anaerobic/aerobic degradation of benzoate (Valderrama et al. 2012), anaerobic degradation of 3-methylbenzoate (Juárez et al. 2015), and anaerobic degradation of toluene/*m*-xylene (Blázquez et al. 2018).

4.1 Regulation of the *bzd* Operon for Anaerobic Degradation of Benzoate

A chromosomal DNA fragment (~20 kbp) was cloned from *Azoarcus* sp. strain CIB containing the *bzd* operon for anaerobic benzoate degradation as well as the neighboring *bzdR* gene coding for a transcriptional regulator. Enzyme activity measurements and transcriptional analysis indicated that benzoate degradation in strain CIB was repressed by the aliphatic acids succinate, acetate, and malate, and that this

regulation requires the involvement of BzdR (López Barragán et al. 2004; Durante-Rodríguez et al. 2008). This catabolite repression contrasts the behavior of "*A. aromaticum*" EbN1 (see above Sect. 2.5). The structure of homodimeric repressor BzdR suggests it as the prototype of a new subfamily of transcriptional regulators (Barragán et al. 2005). BzdR possesses a *N*-terminal helix-turn-helix (HTH) motif for cooperative binding to the P_N promotor of the *bzd* operon, repressing its expression. Binding of benzoyl-CoA to the *C*-terminal domain of BzdR induces a conformational change transmitted via the linker region to the HTH-motif which then releases from the P_N promotor allowing transcription of the *bzd* operon (Barragán et al. 2005; Durante-Rodríguez et al. 2010).

The succinate-mediated repression of the anaerobic degradation of benzoate is proposed to be mediated by the AccR response regulator. Phosphorylated AccR dimerizes and binds to conserved inverted repeats within the P_N promotor, thereby inhibiting expression of the *bzd* operon (Valderrama et al. 2014).

Considering the requirement for strictly anoxic conditions of benzoyl-CoA reductase (Koch and Fuchs 1992), oxygen (O_2) is expected to play a central role in the transcriptional regulation of anaerobic benzoate degradation. Indeed, the genome of *Azoarcus* sp. strain CIB contains the *acpR* gene, a homolog of the well-known FNR transcriptional regulator responsive to anoxic conditions. The functionality of AcpR is evident from the incapacity of an *acpR* mutant of strain CIB to grow anaerobically with aromatic compounds (Durante-Rodríguez et al. 2006).

4.2 Regulation of the *mbd* Operons for Anaerobic Degradation of 3-Methylbenzoate

Azoarcus sp. strain CIB employs the products of the neighboring but divergent *mbd* operons for the anaerobic degradation of 3-methylbenzoate, with the horizontally acquired *mbd* genes being paralogous to the *bzd* genes. Expression of the *mbd* operons from specific promotors was suggested to be mediated by the transcriptional regulator MbdR and overimposed by the O_2-dependent activator AcpR (Juárez et al. 2013) and the catabolite control responding AccR regulator (Juárez et al. 2015). Subsequent studies characterized the three promotors (P_O, P_{B1}, and P_{3R}) and the operator regions of the transcriptional repressor MbdR and demonstrated that 3-methylbenzoyl-CoA functions as inducer (Juárez et al. 2015).

4.3 Regulation of *gcdH* Encoding Glutaryl-CoA Dehydrogenase

Glutaryl-CoA is a well-known intermediate in the metabolism of various organic substrates, including the anaerobic degradation of benzoate downstream of pimelyl-CoA (Wischgoll et al. 2009). Further degradation of glutaryl-CoA to crotonyl-CoA involves glutaryl-CoA dehydrogenase (Gcd), a bifunctional flavoenzyme which catalyzes dehydrogenation as well as decarboxylation (Fu et al. 2004). In *Azoarcus* sp. strain CIB, the *gcdH* gene could be demonstrated to encode the glutaryl-CoA

dehydrogenase required for anaerobic degradation of aromatic compounds. Transcription of the *gcdH* gene was found to be controlled by the LysR-type transcriptional activator GcdR, which is encoded in close proximity to the *gcdH* gene (Blázquez et al. 2008).

4.4 The ICE_{XTD} Element

Integrative and conjugative elements (ICEs) can spread horizontally between different bacterial strains and stably integrate into a given host chromosome, and are thus important players in the horizontal transfer of genomic islands (Bellanger et al. 2014). Notably, the ICE_{XTD} of *Azoarcus* sp. strain CIB carries the genetic information for the aerobic (*tod* genes) and anaerobic (*bss*-*bbs* and *mbd* genes) degradation of alkylbenzenes and can confer this capacity also to other non-alkylbenzene degraders. As similar ICEs are also present in other strains related to *Azoarcus* sp. strain CIB, the ICE_{XTD} may have contributed to shaping the degradation capacities of betaproteobacterial denitrifiers (Zamarro et al. 2016).

4.5 Solvent Stress Tolerance Involving c-di-GMP

Typical and new strategies of "*A. aromaticum*" EbN1 for solvent stress tolerance have been summarized above (Sect. 2.4). A recent study by Martín-Moldes et al. (2016) with *Azoarcus* sp. strain CIB unraveled a new strategy involving the second messenger cyclic diguanosine monophosphate (c-di-GMP). The latter is known to control a diversity of cellular functions (Römling et al. 2013). In *Azoarcus* sp. strain CIB, the toluene-sensing hybrid two-component system TolR mediates degradation of c-di-GMP in response to aromatic hydrocarbons. Apparently, modulation of the intracellular c-di-GMP concentrations facilitates tolerance toward aromatic hydrocarbons under anoxic conditions.

5 *Azoarcus* sp. Strain PA01

The denitrifying betaproteobacterium *Azoarcus* sp. strain PA01 was isolated from a wastewater treatment plant and shown to degrade the priority pollutant *o*-phthalate (benzene-1,2-dicarboxylate) and various other aromatic compounds under anoxic conditions. Its draft genome has a size of 3.9 Mb and revealed the presence of genes for the classical benzoyl-CoA degradation pathway (Junghare et al. 2015). Subsequent proteomic analysis suggested the following degradation pathway: (i) uptake of *o*-phthalate might involve a TRAP-transporter; (ii) a putative succinyl-CoA:*o*-phthalate CoA-transferase should activate *o*-phthalate to the respective CoA-ester; and (iii) *o*-phthaloyl-CoA could be decarboxylated by an UbiD-like decarboxylase yielding the central intermediate benzoyl-CoA (Junghare et al. 2016).

6 *Azoarcus anaerobius*

Azoarcus anaerobius LuFres1^T was originally isolated from anoxic sewage sludge and reported to completely degrade resorcinol (benzene-1,3-diol) to CO_2 under nitrate-reducing conditions (Springer et al. 1998). Using heterologous expression in *Thauera aromatica*, genes involved in anaerobic resorcinol degradation were identified, supporting a degradation pathway via hydroxylation and dehydrogenation to 2-hydroxy-1,4-benzoquinone, which is then cleaved hydrolytically (Darley et al. 2007). A subsequent study revealed that these genes are organized in five transcriptional units, three of which are controlled by σ^{54}-dependent promotors and a heterodimeric regulator (Pacheco-Sánchez et al. 2017).

7 *Magnetospirillum* sp. Strain pMbN1

Benzoyl-CoA, the central intermediate of anaerobic degradation of aromatic compounds, is reductively dearomatized by benzoyl-CoA reductase, which cannot handle substituents at the *para*-position (Rabus et al. 2016b), providing a biochemical explanation for the hitherto observed recalcitrance of *para*-alkylated aromatic compounds. The *Magnetospirillum* sp. strain pMbN1 was the first pure culture of a denitrifying bacterium demonstrated to degrade *p*-methylbenzoate completely to CO_2 under anoxic conditions (Lahme et al. 2012a). Even though the other strains covered in this review all affiliate with the *Betaproteobacteria*, the alphaproteobacterial strain pMbN1 is mentioned here for its specific degradative capacity. Differential proteomics combined with targeted metabolite analysis revealed that strain pMbN1 metabolized this *para*-alkylated compound via a specific 4-methylbenzoyl-CoA pathway with the methyl substituent conserved along the entire pathway. For the anaerobic degradation of benzoate, strain pMbN1 is equipped to use the conventional benzoyl-CoA pathway (Lahme et al. 2012b). *Magnetospirillum* sp. strain pMbN1 displayed diauxic growth on binary or ternary mixtures of succinate, 4-methylbenzoate and benzoate, with the latter being always preferred. Repression of succinate and/or 4-methylbenzoate was apparently mediated by inhibition of their uptake as well as of succinate conversion (Lahme et al. 2014), contrasting the behavior of "*A. aromaticum*" EbN1 with respect to succinate (see above Sect. 2.5).

8 *Thauera aromatica* and *T. chlorobenzoica*

Members of the genus *Thauera* can degrade a variety of aliphatic and aromatic compounds under oxic as well as anoxic (viz. nitrate-reducing) conditions (Heider and Fuchs 2005). Biochemical studies over the last 3 decades with *Thauera aromatica* K172 have been instrumental for elucidating many novel reactions and their enzymatic mechanisms involved in the anaerobic degradation of aromatic compounds (Heider and Fuchs 1997; Boll et al. 2002; Fuchs et al. 2011). Studies

with *T. chlorobenzoica* provided novel insights into the enzymatical conversion of the exceptionally stable carbon-halogen bonds (e.g., Tiedt et al. 2016). Complete genome sequences have been reported for *T. aminoaromatica* MZ1^T (Jiang et al. 2012) and *T. phenolivorans* ZV1C^T (Yin et al. 2017). Using among others differential proteomics, several new degradation pathways were detected in *Thauera* spp. as described in the following:

Catechol *T. aromatica* anaerobically degrades catechol (benzene-1,2-diol) via a modular route charged by enzymatic elements from other known catabolic pathways. Initial activation to catechylphosphate followed by carboxylation to protocatechuate (3,4-dihydroxybenzoate) is analogous to the anaerobic degradation of phenol. Subsequent activation to the CoA-ester and reductive dehydroxylation to 3-hydroxybenzoyl-CoA are reminiscent of the degradation pathways for 3- and 4-hydroxybenzoate. Finally, a devoted 3-hydroxybenzoyl-CoA pathway is suggested. It is hypothesized that promiscuity of enzymes and regulators enable this modular route (Ding et al. 2008).

α-Resorcylate *T. aromatica* AR-1 has previously been reported to anaerobically degrade α-resorcylate (3,5-dihydroxybenzoate) via oxidation and decarboxylation to hydroxyhydroquinone (Gallus and Schink 1998). Coding genes were identified based on their homology to those of *A. anaerobius* (see above Sect. 6), and their relevance was corroborated by knockout mutagenesis. This included also a putative cytoplasmic quinone oxidoreductase and a TRAP transport system. Expression analysis revealed five resorcinol-inducible operons with their genetic organization structurally differing from that observed in *A. anaerobius* (Molina-Fuentes et al. 2015).

Halogenated Aromatic Compounds *Thauera chlorobenzoica* is capable of utilizing certain halogenated benzoates under nitrate-reducing conditions (Song et al. 2001). *meta*-Substituted 3-chloro- and 3-bromobenzoate as well as *para*-substituted 4-chloro- and 4-bromobenzoate are utilized by *T. chlorobenzoica* employing the classical benzoyl-CoA pathway upon removal of the halogen atom involving the additional capacity of class I benzoyl-CoA reductase (BCR) for reductive dehalogenation, while transformation of 3-fluorobenzoate which does not support growth results in fluorinated dead end products formed via a fluorinated dienoyl-CoA (Kuntze et al. 2011; Tiedt et al. 2018). In contrast, *T. aromatica* utilizes 4-fluorotoluene and 4-fluorobenzoate implying the capability to enzymatically cleave the extraordinarily stable arylic C–F bond (Tiedt et al. 2016). It has been suggested that removal of the fluorine atom proceeds in a formal nucleophilic substitution catalyzed by BCR. The significantly decreased pK_a of the assumed 4-F-dienoyl-CoA compared to that of other dienoyl-CoA esters thus would favor essentially irreversible fluoride release in an E1$_{cb}$-type elimination driven by rearomatization and directly yielding benzoyl-CoA but not 4-F-dienoyl-CoA formation. In case of anaerobic 2-fluorobenzoate degradation, a hitherto unknown mode of C–F bond cleavage was discovered in *T. aromatica*, with defluorination

accomplished by promiscuous enoyl-CoA hydratases/hydrolyases of the classical benzoyl-CoA pathway (Tiedt et al. 2017).

9 Research Needs

Apparent research needs can be exemplified as follows. (i) More complete genomes with manual expert annotation from the *Azoarcus*/"*Aromatoleum*"/*Thauera* group are needed to address by comparative genomics the evolution and horizontal dissemination of genetic modules for the anaerobic degradation of aromatic compounds and hydrocarbons in this interesting group of degradation specialists. (ii) The past decades have impressively demonstrated what treasure chests in particular "*A. aromaticum*" EbN1 and *T. aromatica* K172 have been. Still many genes with potential catabolic functions remain to be studied by combined physiological/genetic/biochemical efforts. (iii) The postgenomic experimental studies should embark on understanding the adaptation of these degradation specialists to their natural habitats with a holistic perspective by integrating habitat-guided physiological experiments with systems biology approaches.

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Functional Genomics of Sulfate-Reducing Bacteria Degrading Hydrocarbons

10

Ralf Rabus and Heinz Wilkes

Contents

Abstract

Sulfate-reducing bacteria (SRB) are well known for their significance in the carbon and sulfur cycles of marine sediments. Despite their general energetic restriction, some SRB are capable of anaerobic degradation of aromatic hydrocarbons in the marine environments, such as deep sea sediments characterized by recent oil formation. Proteogenomics has allowed hitherto unknown insights into the physiology of SRB from single catabolic reactions to complex metabolic

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networks. Best studied aromatic compound-degrading SRB at present are toluene-degrading *Desulfobacula toluolica* Tol2 and the naphthalene-degrading enrichment culture N47.

1 Introduction

Sulfate-reducing bacteria (SRB) are anaerobic microorganisms that thrive by coupling the oxidation of organic substrates to the respiratory reduction of sulfate (SO_4^{2-}) to sulfide (S^{2-}). Owing to the high concentrations (28 mM) of sulfate in sea water, SRB belong to the pivotal drivers of the biogeochemical cycles of carbon and sulfur in the marine seafloor (Jørgensen 1982). Most recently, the global rates of sulfate-reduction in marine sediments were predicted to account for 12–29% of the organic carbon mineralization in these environments (Bowles et al. 2014). Bacterial sulfate reduction has previously been reported to occur at elevated temperatures (80 to above 100 °C) in the production fluids of oil reservoirs in the North Sea (Stetter et al. 1993) and deep-sea hydrothermal vent sediments of the Guaymas Basin in the Gulf of California (Jørgensen et al. 1992). In fact SRB enriched/isolated from these two environments were shown to anaerobically grow with crude oil as sole source of organic carbon and energy by depleting *n*-alkanes or alkylbenzenes directly from the crude oil (Rueter et al. 1994; Harms et al. 1999; Wilkes et al. 2000). This capacity of SRB is of great interest for reservoir geochemistry, albeit an undesired process in oil industry as it deteriorates the quality of the oil by compositional alterations and by souring due to H_2S generation (Head et al. 2014). Formed H_2S can also contribute to the corrosion of iron in technical installations, e.g., oil rigs and pipelines, and some SRB can even use metallic iron as electron donor for sulfate reduction (Enning and Garrelfs 2014). Then again, anaerobic degradation of hydrocarbons represents a beneficial process for bioremediation efforts at hydrocarbon contaminated sites, such as aquifers (e.g., Townsend et al. 2003; Griebler et al. 2004) or marine/estuarine systems (e.g., Miralles et al. 2007; Kimes et al. 2014). For a more general overview on SRB and their role in global biogeochemical cycles, technical processes, biomedicine, and fundamental microbiology, refer to other reviews (e.g., Muyzer and Stams 2008; Rabus et al. 2015). In the following, an overview on aromatic compound-degrading, sulfate-reducing bacteria that have been studied by functional genomics is provided.

2 *Desulfobacula toluolica* Tol2

Desulfobacula toluolica Tol2 represents the first pure culture of a sulfate-reducing bacterium that was demonstrated to completely oxidize toluene to CO_2, with this capacity found to be inducible in whole cell cultures (Rabus et al. 1993). Subsequent experiments indicated initial activation of toluene not to proceed via benzyl alcohol, but via benzylsuccinate (Rabus and Widdel 1995; Rabus and Heider 1998). The occurrence of *Desulfobacula* phylotypes has been reported for a tidal sand flat in the German Wadden Sea (Gittel et al. 2008), for a reduced organic-rich surface sediment in the Baltic

Sea (Sinkko et al. 2013), and for a sediment core from the Caspian Sea subjected to simulated petroleum seepage in laboratory experiments (Stagars et al. 2017).

The complete 5.2 Mbp genome of *D. toluolica* Tol2 was the first to be determined for an aromatic compound-degrading, marine SRB, and functional predictions were refined by comprehensive differential proteomics and targeted metabolite analyses uses of substrate-adapted cells (Wöhlbrand et al. 2013). The high genome plasticity is reminiscent of the genome from the denitrifying degradation specialist "*Aromatoleum aromaticum*" EbN1 (Rabus et al. 2005). Anaerobic degradation of toluene and *p*-cresol was found to be initiated by addition to fumarate followed by modified β-oxidation of the formed succinate derivative to benzoyl-CoA and 4-hydroxybenzoyl-CoA, respectively. Notably, separated gene clusters could be assigned to these two substrates, with the *p*-cresol-specific initial enzyme ((4-hydroxybenzyl)succinate synthase, HbsA catalytic subunit) forming an own branch in the phylogenetic tree of alkyl-/arylalkylsuccinate synthases. Interestingly, the dehydrogenation reactions in both β-oxidation branches are apparently linked via the activity of dedicated electron transfer proteins directly to the membrane redox pool. Anaerobic degradation of phenylalanine involves nonoxidative deamination to cinnamate. Dearomatization of the central intermediate benzoyl-CoA expectedly involves an ATP-independent class II benzoyl-CoA reductase (BCR) as known from *Geobacter metallireducens* GS-15 (Kung et al. 2009). Complete oxidation of acetyl-CoA to CO_2 proceeds via the Wood-Ljungdahl pathway as widespread in the *Desulfobacteraceae* family which strain Tol2 is affiliating with (Rabus et al. 2015). Detailed analysis by 1D blue native-PAGE complexome profiling and 2D blue native-/SDS-PAGE demonstrated an elaborated system of membrane protein complexes for electron transport, including, e.g., dimer and quadruple formation of the central DsrMKJOP complex and the potential capacity for Na^+-based bioenergetics via the RnfABCDEG complex (Wöhlbrand et al. 2016).

3 *Desulfobacula toluolica* TS

A microcosm established from oil-contaminated tidal flat sediment was demonstrated to degrade toluene coupled to sulfate reduction by determining the formation of $^{13}CO_2$ from ^{13}C-labeled toluene. A metagenomics approach was applied to this microcosm yielding a draft genome of 125 scaffolds that showed highest similarity to the genome of *D. toluolica* Tol2. Accordingly, the degradation pathways for several aromatic compounds could be reconstructed, mirroring the network established before for strain Tol2 and underpinning its model character (Kim et al. 2014).

4 Strains NaphS2, NaphS3, and NaphS6

Strain NaphS2 originates from sulfidic marine sediment, represents the first pure culture of an anaerobic bacterium demonstrated to degrade naphthalene completely to CO_2 (Galushko et al. 1999), and is closely related to sulfate-reducing strain EbS7 anaerobically degrading ethylbenzene via addition to fumarate (Kniemeyer et al.

2003). Differential proteogenomics revealed that strain NaphS2 and two further naphthalene-degrading SRBs (strains NaphS3 and NaphS6) activated 2-methylnaphthalene via addition to fumarate and that the presumptive (2-naphthylmethyl)succinate synthase was absent in naphthalene-adapted cells of all three strains. This finding indicated that the three studied marine strains did not employ a methylation as initial activation of anaerobic naphthalene degradation (Musat et al. 2009), as previously suggested for the sulfate-reducing enrichment culture N47 (Safinowski and Meckenstock 2006). Instead, carboxylation of naphthalene is discussed as a possible activation reaction (DiDonato et al. 2010; Musat et al. 2009).

5 Enrichment Culture N47

The sulfate-reducing enrichment culture N47 was obtained from soil material of a contaminated aquifer and by labeling studies (with [^{13}C]bicarbonate) suggested to anaerobically degrade naphthalene via a possible carboxylation to 2-naphthoic acid (Meckenstock et al. 2000). Anaerobic degradation of 2-methylnaphthalene was proposed to proceed in analogy to the anaerobic degradation of toluene, i.e., via addition to fumarate followed by β-oxidation of the succinate moiety (Annweiler et al. 2000). The interim hypothesis of direct methylation of naphthalene to connect with the 2-methylnaphthalene pathway (Safinowski and Meckenstock 2006) was not sustained, as proteomic and enzymatic evidence for the possible involvement of a putative naphthalene carboxylase showing sequence similarity to phenylphosphate carboxylase emerged (Bergmann et al. 2011a; Mouttaki et al. 2012). Subsequently, a draft 4.7 Mbp genome comprised of 17 contigs was obtained for the enrichment culture N47 (Bergmann et al. 2011b), which formed the basis for a proteomics investigation. The latter allowed identification of all genes required for anaerobic degradation of 2-methylnaphthalene as well as a class I benzoyl-CoA reductase (Selesi et al. 2010). Initial biochemical studies suggested that the four-electron reduction of naphthoyl-CoA to 5,6,7,8-tetrahydro-2-naphthoyl-CoA is catalyzed by a novel type of dearomatizing reductase containing FAD, FMN, and an Fe-S cluster as cofactors (Eberlein et al. 2013). Meanwhile, it could be shown that actually two distinct enzymes are involved in the anaerobic reduction of the naphthyl ring, each catalyzing a two-electron reduction (Estelmann et al. 2015).

6 *Desulfococcus multivorans*

Desulfococcus multivorans strain 1be1 is a nutritionally versatile sulfate-reducing bacterium originally isolated from a sewage digester (Widdel 1980). *D. multivorans* is a representative of the *Desulfosarcina-Desulfococcus* clade within *Desulfobacteraceae* that has been repeatedly reported to dominate the SRB community in diverse marine sediments (e.g., Ravenschlag et al. 2000; Mußmann et al. 2005) and represent the bacterial partner in anaerobically methane-oxidizing

consortia (Boetius et al. 2000). The complete 4.46 Mbp genome of *D. multivorans* contains a single chromosome and represents the first to be reported for a member of the *Desulfosarcina-Desulfococcus* clade (Dörries et al. 2016). The metabolic reconstruction was fostered by detailed differential proteome profiling across 17 different substrate conditions, revealing a catabolic network comprising 170 proteins (154 detected; ~91% coverage). Peripheral degradation of aromatic compounds feeds via the benzoyl-CoA pathway (class II benzoyl-CoA reductase) into the Wood-Ljungdahl (WL) pathway for terminal oxidation to CO_2. While peripheral degradation pathways (including benzoyl-CoA pathway) displayed high substrate-specific formation, the central catabolic modules (WL and methylmalonyl-CoA pathways) as well as the membrane protein complexes involved in electron transfer were constitutively formed. This pattern appears to be a common property of to date proteogenomically studied members of the *Desulfobacteraceae*.

7 *Desulfotomaculum gibsoniae* GrollT

The mesophilic sulfate-reducing bacterium strain Groll was isolated from a black mud sample from a small freshwater ditch and shown to anaerobically grow with several aromatic compounds, including phenol, cresols, and catechol (Kuever et al. 1993). The bacterium was subsequently classified as *Desulfotomaculum gibsoniae* Groll^T (Kuever et al. 1999). The ~4.9 Mbp genome allowed for a metabolic reconstruction (Kuever et al. 2014). The anaerobic degradation of cresols is suggested to follow the route previously suggested for *D. toluolica* Tol2 (see above Sect. 2), i.e., initial activation via addition to fumarate is followed by modified β-oxidation to feed via class II BCR into the central benzoyl-CoA pathway and from there to the WL-pathway for terminal oxidation to CO_2. Catechol degradation is proposed to involve initial activation by phosphorylation followed by carboxylation as previously proposed for the denitrifying bacterium *Thauera aromatica* K172 (Ding et al. 2008).

8 Research Needs

While the biogeographical significance and biogeochemical imprint of SRB on a global scale are well established, the understanding of the catabolic properties that form the basis of an organism's functional role in the habitat is far less advanced. Thus, more complete expert-annotated genomes of model SRBs being relevant for an intriguing degradation ability and/or abundance in the habitat are required. The integration of genomics, proteomics, and metabolite analysis has proven particularly rewarding for elucidating novel reactions and reconstructing complete catabolic networks and needs to be further developed. A broader functional genomics understanding of aromatic compound-degrading SRB promises new catabolic discoveries, insights into how these bacteria operate when degrading recalcitrant compounds at the thermodynamic limit, and to what extent the catabolic networks revealed from

pure cultures of SRB can be actually found in the natural habitat (e.g., Michas et al. 2017). In this respect, metaOMICS investigations of sulfidic sites rich in aromatic compounds will be highly interesting.

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Functional Genomics of Metal-Reducing Microbes Degrading Hydrocarbons

11

Pier-Luc Tremblay and Tian Zhang

Contents

Abstract

The anaerobic oxidation of petroleum hydrocarbons can be coupled to the reduction of metals. At contaminated subsurface sites, this phenomenon will accelerate the removal of pollutants and will have an important influence on biogeochemical cycles. Due to its abundance, iron is the most prominent metallic terminal electron acceptor involved in hydrocarbon degradation followed by manganese. Dissimilatory metal-reducing microbes (DMRM) capable of oxidizing either monocyclic aromatic or polycyclic hydrocarbons are phylogenetically diverse with representatives from bacteria as well as from archaea. It has

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been shown that the monocyclic aromatic hydrocarbons benzene, toluene, ethylbenzene, and xylene and the polyaromatic hydrocarbons naphthalene, 1-methylnaphthalene, and 2-methylnaphtalene can be degraded by metal-reducing enrichment cultures or pure cultures. In recent years, significant breakthroughs have been made in the field of functional genomics for the characterization of the metabolic pathways, enzymes, and genes participating to hydrocarbons degradation by metal-reducing microbes. Here, we present an updated portrait of the monocyclic aromatic and polycyclic hydrocarbons metabolism of DMRM.

1 Introduction

Contamination of sediments, soil, aquifer, and groundwater with petroleum hydrocarbons is a widespread concern posing a threat to human health and ecosystems. Due to the fast depletion of oxygen, polluted sites often become anaerobic and under these circumstances, the degradation of hydrocarbons requires the metabolic activity of microbes using other terminal electron acceptors (TEA) (Aburto-Medina and Ball 2015). This includes metals abundant in soil and sediments such as iron and manganese. Dissimilatory metal-reducing microbes (DMRM) couple the oxidation of organic molecules such as hydrocarbons with the reduction of metals as their sole TEA for energy conservation (Foght 2008). Because metals in soil and sediments are usually in a solid state, DMRM have developed unique extracellular electron transfer mechanisms. This includes direct electron transfer *via* outer membrane c-type cytochromes, conductive nanowires and pili as well as the synthesis of soluble electron shuttles (Lovley 2012; White et al. 2016).

The anaerobic oxidation of monocyclic aromatic hydrocarbons including benzene, toluene, ethylbenzene, and xylene has been demonstrated with DMRM-containing consortia as well as with DMRM pure cultures either with Fe(III) or Mn(IV) as the TEA (Lovley et al. 2011; Meckenstock and Mouttaki 2011; Meckenstock et al. 2016; Lovley and Lonergan 1990; Heider et al. 1998; Chakraborty and Coates 2004; Botton and Parsons 2006; Rooney-Varga et al. 1999; Jahn et al. 2005; Langenhoff et al. 1997a, b; Villatoro-Monzón et al. 2003). Furthermore, several studies have reported the microbial degradation of polycyclic hydrocarbons such as naphthalene and its derivatives with Fe(III) as TEA (Anderson and Lovley 1999; Kleemann and Meckenstock 2011). Until now, the microbial degradation of aliphatic hydrocarbons including alkane, alkene, and cycloalkane coupled with metal reduction by DMRM has never been reported.

Metabolic pathways responsible for the anaerobic oxidation of aromatic hydrocarbons by DMRM are complex and comprise multiple reactions with unique chemistry. Currently, many of those reactions are still poorly understood. This chapter will focus on the ongoing research effort aiming at elucidating the anaerobic hydrocarbon oxidation metabolism of DMRM and at characterizing the genes and enzymes involved.

2 Dissimilatory Metal-Reducing Microbes Involved in Anaerobic Hydrocarbons Degradation

Key Fe(III)-reducing microorganisms involved in the complete oxidation of monocyclic aromatic hydrocarbons at anoxic contaminated environments are often members of the *Geobacteraceae* family (Rooney-Varga et al. 1999) (Table 1). From this group, only pure cultures *Geobacter metallireducens* and *Geobacter* strain Ben have been shown to degrade both benzene and toluene (Lovley and Lonergan 1990; Lovley et al. 1993; Zhang et al. 2012). Two other *Geobacter* species, *Geobacter grbiciae* and *Geobacter toluenoxydans*, only degrade toluene (Coates et al. 2001; Kunapuli et al. 2010). Other Fe(III)-reducing bacteria capable of oxidizing toluene in pure culture include *Georgfuchsia toluolica* and *Desulfitobacterium aromaticivorans*, which are members of the Betaproteobacteria and Clostridia, respectively (Kunapuli et al. 2010; Weelink et al. 2009). *D. aromaticivorans* can also oxidize *o*-xylene with Fe(III) as TEA. When manganese is the TEA, *G. toluolica* and an enrichment culture dominated by an *Azoarcus* species can

Table 1 DMRM coupling aromatic hydrocarbon oxidation with metal reduction

Species	Classification	Degraded aromatic hydrocarbons with reduced metals	Reference
Geobacter metallireducens	Gram - *Deltaproteobacteria Geobacteraceae*	Benzene-Fe(III) Toluene-Fe(III)	Lovley and Lonergan 1990; Lovley et al. 1993; Zhang et al. 2012
Geobacter strain Ben		Benzene-Fe(III) Toluene-Fe(III)	Zhang et al. 2012
Geobacter grbiciae		Toluene-Fe(III)	Coates et al. 2001
Geobacter toluenoxydans		Toluene-Fe(III)	Kunapuli et al. 2010
Georgfuchsia toluolica	Gram - *Betaproteobacteria Rhodocyclaceae*	Toluene-Fe(III) or Mn(IV) Ethylbenzene-Mn(IV)	Weelink et al. 2009; Dorer et al. 2016
Azoarcus sp.-dominated microbial consortium		Toluene-Mn(IV) Ethylbenzene-Mn(IV)	Dorer et al. 2016
Desulfitobacterium aromaticivorans	Gram + *Clostridiales Peptococcaceae*	Toluene-Fe(III) *o*-xylene-Fe(III)	Kunapuli et al. 2010
Culture N49[a]		Naphtalene-Fe(III) 1-methylnaphthalene-Fe(III) 2-methylnaphthalene-Fe(III)	Kleemann and Meckenstock 2011
Ferroglobus placidus	Archaea *Archaeoglobales Archaeoglobaceae*	Benzene-Fe(III)	Holmes et al. 2011

[a]Culture N49 is composed of 90% of a bacteria species from the *Peptococcaceae* family

degrade both toluene and ethylbenzene (Dorer et al. 2016). Besides mesophilic bacteria, the hyperthermophilic archaeon *Ferroglobus placidus* can also degrade benzene and transfer electrons to Fe(III) (Holmes et al. 2011).

Polycyclic aromatic hydrocarbons degradation by metal-reducing microbes was demonstrated initially *via* the anaerobic oxidation of naphthalene with Fe(III) as the TEA (Anderson and Lovley 1999). Community composition involved in polycyclic aromatic hydrocarbons degradation with metal as TEA has not been investigated as much as for BTEX degradation. The only study to our knowledge on bacterial population in Fe(III)-reducing enrichment degrading naphthalene identified a Gram-positive spore-forming bacterium of the *Peptococcaceae* family as the dominating species (Kleemann and Meckenstock 2011). This enrichment could also degrade 1-methylnaphthalene and 2-methylnaphthalene.

3 Degradation of Monocyclic Aromatic Compounds

Genes, enzymes, and metabolic pathways involved in monocyclic aromatic hydrocarbons degradation by metal-reducing bacteria have mainly been studied in *G. metallireducens*, which is genetically tractable and had its genome sequenced in 2005 (Tremblay et al. 2012; Oberender et al. 2012; Butler et al. 2007; Lovley and Lonergan 1990; Lovley et al. 1993; Zhang et al. 2012). Genome survey of *G. metallireducens* have led to the identification of genes participating in the anaerobic oxidation of monocyclic aromatic hydrocarbons and of other monocyclic aromatic compounds including phenol, *para*-cresol, benzyl alcohol, benzaldehyde, and 4-hydroxybenzoate (Butler et al. 2007; Heinnickel et al. 2010). Additionally, *G. metallireducens* has been used as a model bacterium to study extracellular electron transfer to insoluble TEA such as Fe(III) oxide or the electrode of a bioelectrochemical system for electricity production (Lovley 2012; Bond et al. 2002; Smith et al. 2013).

3.1 Degradation of Benzene to Benzoyl-CoA

Because of its structural stability and high solubility in water, benzene is the most persistent monocyclic aromatic hydrocarbon in the environment (Vogt et al. 2011). Benzene is harmful for humans and other animals as it can cause cancer and aplastic anemia and impairs the reproductive system (Aburto-Medina and Ball 2015). Anaerobic bacteria such as *G. metallireducens* convert monocyclic aromatic compounds including benzene into the central intermediate benzoyl-CoA before subsequent dearomatization (Heinnickel et al. 2010; Zhang et al. 2012) (Fig. 1). The metabolic pathway from benzene to benzoyl-CoA in *G. metallireducens* has not been completely characterized yet. Several activation mechanisms with specific thermodynamic constraints have been proposed as the first step of anaerobic benzene oxidation including hydroxylation to phenol, carboxylation to benzoate or methylation to toluene (Vogel and Grbìc-Galìc 1986; Grbić-Galić and Vogel 1987;

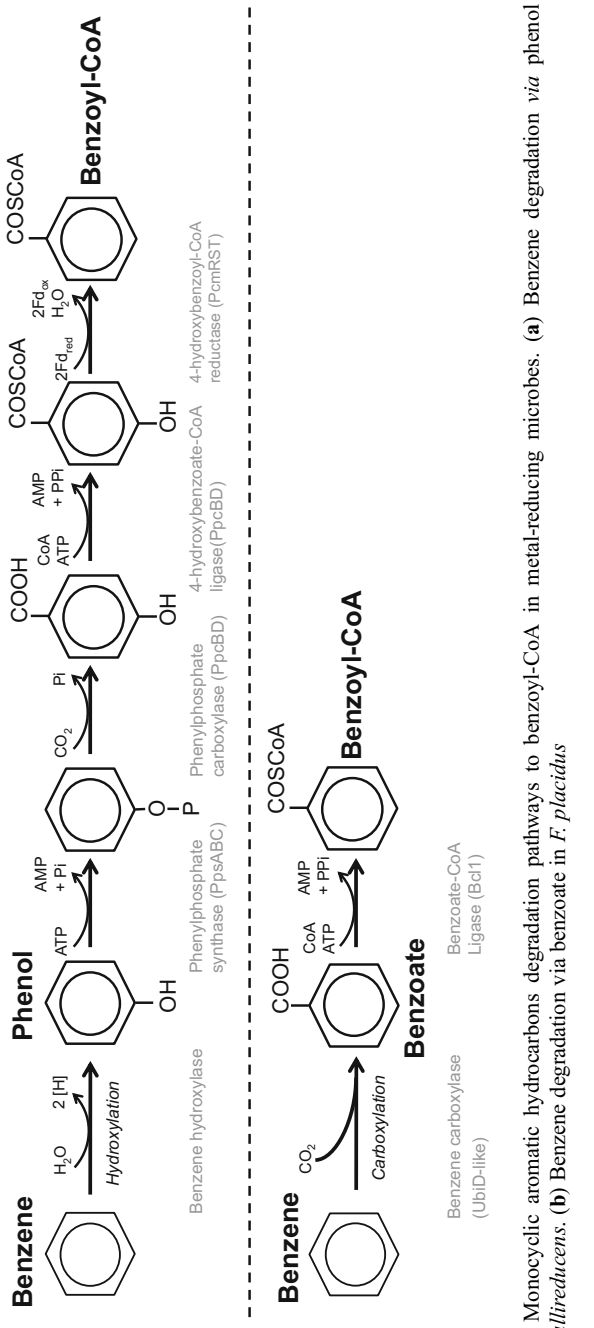

Fig. 1 Monocyclic aromatic hydrocarbons degradation pathways to benzoyl-CoA in metal-reducing microbes. (**a**) Benzene degradation *via* phenol in *G. metallireducens*. (**b**) Benzene degradation via benzoate in *F. placidus*

Caldwell and Suflita 2000; Chakraborty and Coates 2005; Abu Laban et al. 2009, 2010; Phelps et al. 2001; Kunapuli et al. 2008; Ulrich et al. 2005). In 2013, Zhang et al. presented evidences indicating that benzene is degraded *via* hydroxylation to phenol by *G. metallireducens*. In this study, phenol was labeled with ^{18}O when $H_2{}^{18}O$ was added to benzene-oxidizing cell suspension and complete benzene oxidation to CO_2 was inhibited when genes essential for the phenol metabolism were deleted (Zhang et al. 2013). A recent functional genetic study led to the identification of two adjacent genes (Gmet_0231 and Gmet_0232) coding for a zinc-containing oxidoreductase and a hypothetical protein required for benzene oxidation but not for phenol oxidation that may be involved in the first step of benzene degradation (Zhang et al. 2014). Still, the identity of the enzyme responsible for benzene hydroxylation and its mechanism of action are still unclear.

The suggested product of benzene hydroxylation, phenol, is degraded in *G. metallireducens via* a metabolic pathway analogous to the one found in the denitrifying bacterium *Thauera aromatica* (Butler et al. 2007; Breinig et al. 2000) (Fig. 1a). Phenol is first converted to phenylphosphate by an ATP-dependent phenylphosphate synthase composed of three different subunits (PpsABC) (Schmeling et al. 2004). Phenylphosphate is then carboxylated by a phenylphosphate carboxylase (PpcBD) to 4-hydroxybenzoate. In *T. aromatica*, the phenylphosphate carboxylase has four subunits. The δ-subunit is thought to catalyze the exergonic dephosphorylation of phenylphosphate resulting in a phenolate anion that will be carboxylated with CO_2 by the subunits α, β, and γ (Schühle and Fuchs 2004). Phenylphosphate carboxylase activity has been detected in *G. metallireducens* but the enzyme structure and mechanism of action may be different from *T. aromatica* since only two subunits are encoded on the genome (Schleinitz et al. 2009; Lovley et al. 2011). Subsequently, 4-hydroxybenzoate is converted to 4-hydroxybenzoyl-CoA by a 4-hydroxybenzoate-CoA ligase. Activity of this enzyme has been detected in *G. metallireducens* but its exact identity is still unknown (Peters et al. 2007; Wischgoll et al. 2005). Finally, 4-hydroxybenzoyl-CoA is reduced to benzoyl-CoA by a 4-hydroxybenzoyl-CoA reductase composed of three subunits ($\alpha_2\beta_2\gamma_2$) (PcmRST) (Butler et al. 2007; Breese and Fuchs 1998).

Other studies suggested that carboxylation is also an important benzene activation reaction in hydrocarbons contaminated environments. Proteome analysis of a benzene-degrading mixed community dominated by *Peptococcaceae*-related Gram-positive bacteria led to the identification of a putative anaerobic benzene carboxylase (Abu Laban et al. 2010). This enzyme is probably formed by at least two subunits (AbcDA) homologous to proteins comprised in two families of carboxylases: phenylphosphate carboxylase and 3-octaprenyl-4-hydroxybenzoate carboxy-lyase. Furthermore, benzoate is an important intermediate of benzene degradation in the Fe(III)-reducing archaeon *F. placidus* (Holmes et al. 2011) (Fig. 1b). Accumulation of trace amounts of benzoate as well as higher transcript abundance of genes thought to be involved in the conversion of benzene to benzoate during benzene metabolism are evidences supporting a carboxylation-dependent pathway in *F. placidus*. One upregulated gene in particular is thought to code for a putative benzene carboxylase homologous to proteins of the 3-octaprenyl-4-hydroxybenzoate carboxy-lyase (UbiD-like) family.

After benzene carboxylation, benzoate will be converted to benzoyl-CoA at the expense of ATP via a benzoate-CoA ligase (Bcl1/BamY) formed by a single protein (Holmes et al. 2011; Butler et al. 2007; Schühle et al. 2003).

3.2 Degradation of Toluene, Ethylbenzene, and Xylene

Degradation of toluene by DMRM such as *G. metallireducens* involves the addition of fumarate to toluene via glycyl radical mediated C-C bond formation by a benzylsuccinate synthase yielding benzylsuccinate (Butler et al. 2007; Rabus et al. 2005; Rabus 2005) (Fig. 2). The benzylsuccinate synthase comprises three subunits with a $(\alpha\beta\gamma)_2$ structure (BssABC) (Leuthner et al. 1998). The catalytic α-subunit harbors a glycyl radical enzyme fold surrounding the glycyl radical cofactor and the active site (Funk et al. 2014). The glycyl radical cofactor will be formed from a conserved glycine residue by an activating enzyme (BssD) of the S-adenosyl-L-methionine radical enzymes superfamily (Leuthner et al. 1998). The function of subunits β and γ that both possess a FeS cluster is still unclear (Li et al. 2009; Hilberg et al. 2012). Recently, it has been suggested that they may be involved in modulating the conformational dynamics of the benzylsuccinate synthase enzyme (Funk et al. 2014).

Subsequently, a succinyl-CoA:benzylsuccinate CoA transferase will transfer coenzyme A from succinyl-CoA to benzylsuccinate generating benzylsuccinyl-CoA and succinate (Heider et al. 1998). This enzyme comprises two subunits (BbsEF) arranged in a $\alpha_2\beta_2$ structure (Leutwein and Heider 2001). Benzylsuccinyl-CoA will then be oxidized to (E)-phenylitaconyl-CoA by a benzylsuccinyl-CoA dehydrogenase (BbsG) (Leutwein and Heider 2002). Deletion of the gene *bbsG* coding for this homotetrameric enzyme in *G. metallireducens* eliminates its capacity to degrade toluene further confirming the importance of this enzyme (Chaurasia et al. 2015). E-phenylitaconyl-CoA will then be converted by a (E)-phenylitaconyl-CoA hydratase (BbsH) into 2-carboxymethyl-3-hydroxy-phenylpropionyl-CoA which will be oxidized into benzoyl-succinyl-CoA by a 3-hydroxyacyl-CoA dehydrogenase (BbsCD). Finally, the succinyl-CoA moiety is replaced by CoA in a reaction catalyzed by a benzoylsuccinyl-CoA thiolase (BbsB) to generate the central metabolite benzoyl-CoA (Butler et al. 2007; Leuthner and Heider 2000).

Metabolic pathways involved in ethylbenzene and *o*-xylene oxidation by iron-reducing bacteria including *G. toluolica* and *D. aromaticivorans* are poorly characterized. A mixed culture dominated by an *Azoarcus* species has been shown to couple the degradation of ethylbenzene with manganese reduction (Dorer et al. 2016). In this study, it is not clear if the *Azoarcus* species is directly coupling ethylbenzene oxidation with manganese reduction or if a syntrophic interaction with another microbial species is required. In the closely related denitrifying bacterium *Azoarcus* sp. EbN1, degradation of ethylbenzene is initiated by the hydroxylation of the C1-methylene carbon of the sidechain yielding (*S*)-1-phenylethanol, which is catalyzed by an ethylbenzene dehydrogenase (EbdABC) (Fuchs et al. 2011; Ball et al. 1996; Kniemeyer and Heider 2001a) (Fig. 3). The ethylbenzene dehydrogenase is a heterotrimeric molybdoenzyme located in the periplasmic space of

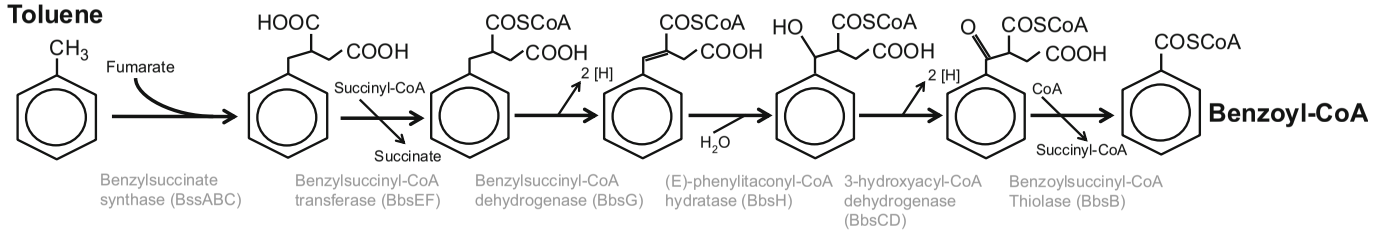

Fig. 2 Toluene degradation pathway in *G. metallireducens*

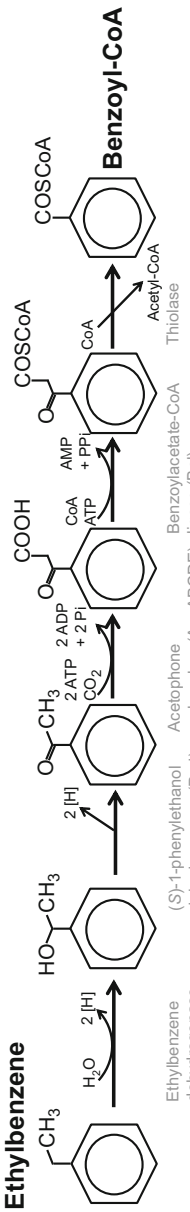

Fig. 3 Ethylbenzene degradation pathway in *Azoarcus* sp. EbN1

microbial cells (Heider et al. 2016; Kloer et al. 2006). (*S*)-1-phenylethanol is then oxidized to acetophenone by a (*S*)-1-phenylethanol dehydrogenase (Ped) (Kniemeyer and Heider 2001b). Later, acetophenone is carboxylated into benzoylacetate in an ATP-dependent manner by a complex acetophenone carboxylase comprising five different subunits (ApcABCDE) (Jobst et al. 2010). Subsequently, benzoylacetate is activated to benzoylacetate-CoA by a putative benzoylacetate-CoA ligase (Bal). In the last step, benzoylacetate-CoA is cleaved into benzoyl-CoA and acetyl-CoA by an unknown thiolase (Rabus 2005; Muhr et al. 2015). In the case of *o*-xylene, a lot less is known about its degradation pathway, but the initial reaction is possibly the addition of a fumarate molecule to the alkyl side-chain by a glycyl radical enzyme (Heider 2007).

3.3 Benzoyl-CoA Reduction

As described above, benzene, toluene, ethylbenzene, and *o*-xylene are converted to the central metabolite benzoyl-CoA in DMRM. The next step in the degradation of monocyclic aromatic hydrocarbons is the two electrons reduction of benzoyl-CoA to cyclohex-1,5-diene-1-carboxyl-CoA, a reaction comparable to the chemical Birch reduction catalyzed by the benzoyl-CoA reductases (Boll et al. 2014) (Fig. 4). Two different classes of benzoyl-CoA reductases are active in DMRM. In the strictly anaerobic archeon *F. placidus*, an ATP-consuming class I benzoyl-CoA reductase is responsible for the dearomatization of benzoyl-CoA during benzene metabolism (Holmes et al. 2011) (Fig. 4a). This is unusual since class I benzoyl-CoA reductases are normally found in facultative anaerobes, while strict anaerobes possess class II benzoyl-CoA reductases (Boll et al. 2014). The four genes (*bzdNOPQ*) coding for the class I benzoyl-CoA reductase found in the genome of *F. placidus* had higher transcript abundance in benzene-grown cells compared to acetate-grown cells. Recently, in vitro assays with purified BzdNOPQ showed that these proteins catalyze the ATP-dependent reduction of benzoyl-CoA with two electrons to cyclohex-1,5-diene-1-carboxyl-CoA (Schmid et al. 2015, 2016). The four subunits of the class I benzoyl-CoA reductases are organized in two different modules (Boll et al. 2014). The first module composed of subunits α (BzdQ) and δ (BzdP) harbors two ATP-binding sites and a single bridging [4Fe-4S] cluster. Its function is to transfer electrons from reduced ferredoxins in an ATP-dependent manner to the second modules catalyzing the benzoyl-CoA ring reduction (Boll 2005). The second module formed by subunits β (BzdO) and γ (BzdN) includes the benzoyl-CoA binding site as well as two [4Fe-4S] clusters involved in electron transport to the substrate.

In the model bacterium *G. metallireducens* that is a strict anaerobe, an ATP-independent class II benzoyl-CoA reductase is responsible for the dearomatization of benzoyl-CoA (Löffler et al. 2011; Boll et al. 2014) (Fig. 4a). Class II benzoyl-CoA reductases require significantly less energy than class I benzoyl-CoA reductases and this is probably the reason why class II enzymes are widespread in strict anaerobes. Class II benzoyl-CoA reductase of *G. metallireducens* is encoded by a cluster of eight genes, *bamBCDEFGHI* (Wischgoll et al. 2005).

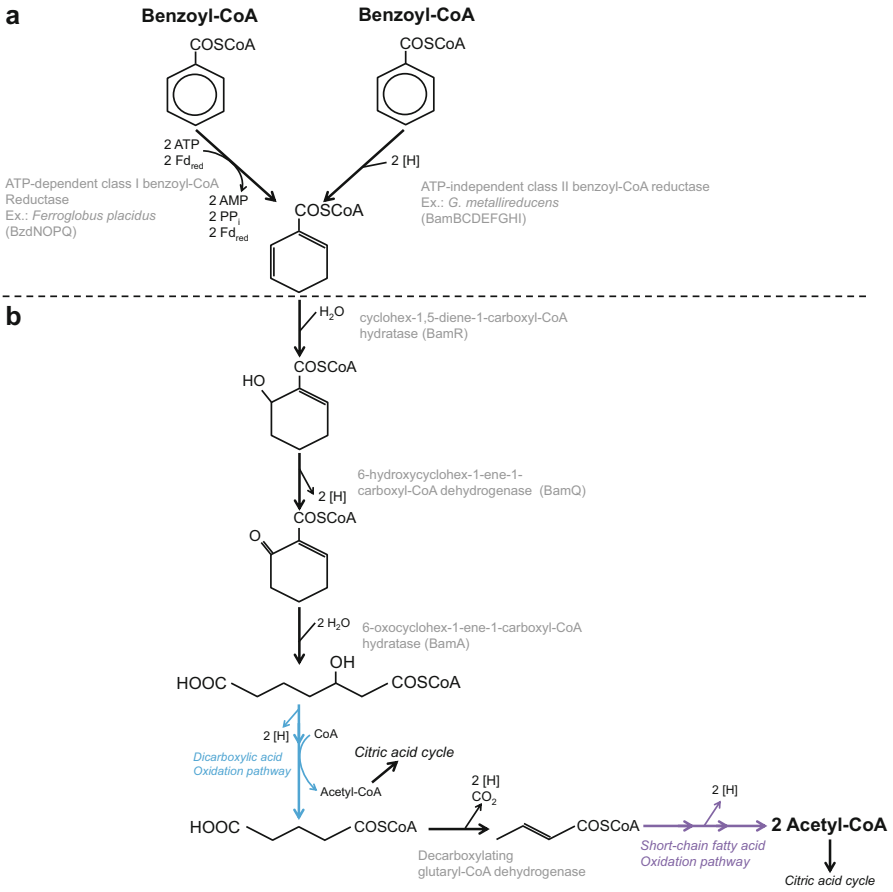

Fig. 4 Degradation pathways from benzoyl-CoA to acetyl-CoA. (**a**) Benzoyl-CoA reduction in *F. placidus* by a class I benzoyl-CoA reductase (BzdNOPQ) and in *G. metallireducens* by a class II benzoyl-CoA reductase (BamBCDEFGH). (**b**) Reduction of cyclohex-1,5-diene-1-carboxyl-CoA to acetyl-CoA. BamRQA enzymes are common to both *F. placidus* and *G. metallireducens*

A recent study showed that the enzyme is crystallized as a $Bam(BC)_2$ heterotetramer (Weinert et al. 2015). The substrate benzoyl-CoA binds to the subunit BamB (Kung et al. 2009). Reduction of the substrate occurs via electrons transferred from the tungsten-*bis*pyranopterin monophosphate cofactor of the BamB subunit. A single [4Fe-4S] cluster in BamB and three [4Fe-4S] clusters in BamC have been proposed to shuttle electrons from BamDEF to the tungsten-bispyranopterin monophosphate cofactor (Weinert et al. 2015). The exact function of the products of *bamDEFGHI* genes is still unknown, but the amino acids sequences and the presence of binding sites for flavin adenine dinucleotide cofactors suggest that they could participate in an energy conservation mechanism analogous to flavin-based electron bifurcation coupling endergonic and exergonic redox reactions (Boll et al. 2014; Buckel and Thauer

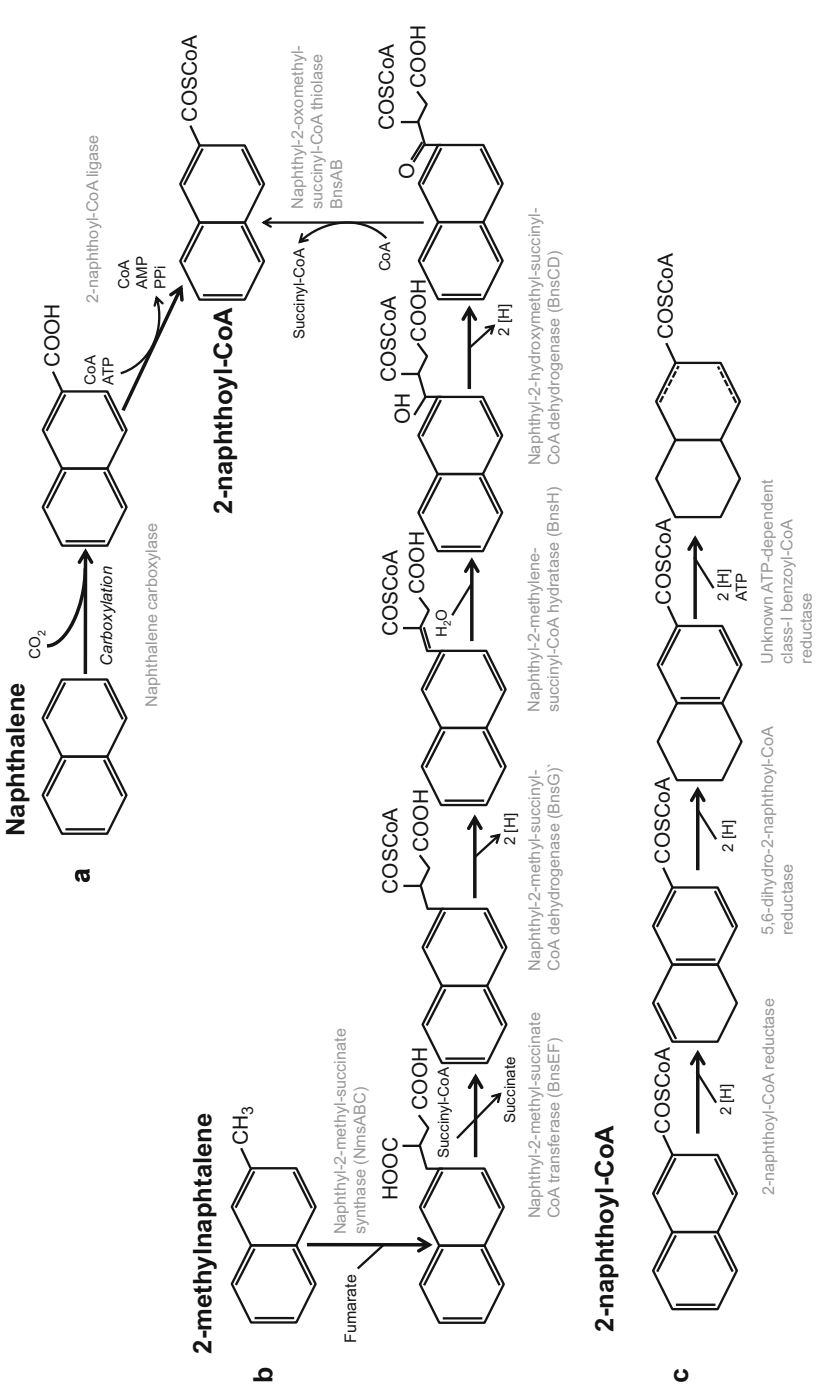

Fig. 5 (continued)

2013). It has been hypothesized that electrons from the oxidation of four reduced ferredoxins are bifurcated by the BamCDEF module for the endergonic reduction of benzoyl-CoA by BamB and the exergonic reduction of NAD^+, which has been predicted to bind to the BamGHI module (Boll et al. 2014; Fuchs et al. 2011).

3.4 Degradation of Cyclohex-1,5-Diene-1-Carboxyl-CoA to Central Carbon Metabolites

After benzoyl-CoA reduction, metal-reducing bacteria such as *G. metallireducens* as well as the archaeon *F. placidus* convert cyclohex-1,5-diene-1-carboxyl-CoA to 3-hydroxypimeloyl-CoA *via* a pathway widespread in microbes oxidizing monocyclic aromatic compounds that was initially characterized in *T. aromatica* (Butler et al. 2007) (Fig. 4b). This pathway comprises three reactions and starts with the addition of water to cyclohex-1,5-diene-1-carboxyl-CoA by a hydratase (BamR) yielding 6-hydroxycyclohex-1-ene-1-carboxyl-CoA (Peters et al. 2007; Laempe et al. 1998; Schmid et al. 2015). This product is then converted to 6-oxocyclohexa-1-ene-1-carboxyl-CoA by a NAD^+-dependent dehydrogenase (BamQ). The third reaction is catalyzed by a 6-oxocyclohex-1-ene-1-carboxyl-CoA hydratase (BamA) and consists in a hydration followed by ring cleavage to form 3-hydroxypimeloyl-CoA (Kuntze et al. 2008; Schmid et al. 2015; Laempe et al. 1998). This molecule will be further oxidized *via* three steps: (1) a dicarboxylic acid oxidation pathway, (2) followed by a decarboxylation and oxidation reaction catalyzed by a decarboxylating glutaryl-CoA dehydrogenase, and (3) a short-chain fatty acid oxidation pathway. In total, the oxidation of 3-hydroxypimeloyl-CoA requires six reactions and yields three acetyl-CoA molecules (Harwood and Gibson 1997; Carmona et al. 2009; Wischgoll et al. 2009).

4 Polycyclic Aromatic Hydrocarbons Degradation Pathway in Iron-Reducing Gram-Positive Bacteria

Two initial activation mechanisms have been proposed for the degradation of naphthalene by anaerobic microbes including DMRM (Fig. 5). The first mechanism proceeds *via* the carboxylation of naphthalene into 2-naphthoic acid. Experimental evidence supporting this pathway includes the accumulation of the metabolite 2-naphthoic acid during naphthalene degradation (Kleemann and Meckenstock 2011; Meckenstock et al.

Fig. 5 Naphthalene and 2-methylnaphthalene degradation pathway in anaerobic bacteria. (**a**) Carboxylation of naphthalene and degradation to 2-naphthoyl-CoA. (**b**) Addition of fumarate to 2-methylnaphthalene and degradation to 2-naphthoyl-CoA. (**c**) Reduction of 2-naphthoyl-CoA. 2-naphthoyl-CoA reductase and 5,6-dihydro-2-naphthoyl-CoA reductase may form an enzymatic complex in vivo. Identity of the enzyme responsible for the conversion of 5,6-dihydro-2-naphthoyl-CoA to hexahydro-naphthoyl-CoA is unknown. Downstream reactions are also poorly understood

2000; Zhang et al. 2000). Furthermore, growth with naphthalene in ^{13}C–bicarbonate-buffered medium resulted in the formation of ^{13}C–labelled 2-naphthoic acid (Zhang and Young 1997; Mouttaki et al. 2012). Alternatively, naphthalene degradation could be activated *via* a methylation reaction yielding 2-methyl-naphthalene (Safinowski and Meckenstock 2006). However, biochemical evidence supporting a naphthalene methylation pathway is lacking until now.

In recent years, the carboxylation pathway has mainly been investigated in naphthalene-degrading sulfate-reducing bacteria. The activation reaction in this pathway is mediated by an ATP-independent naphthalene carboxylase (Mouttaki et al. 2012) (Fig. 5a). Proteomic investigation and genome survey carried out in the sulfate-reducing enrichment culture N47 led to the identification of the α- and β-subunits of a putative carboxylase possibly catalyzing the conversion of naphthalene into 2-naphthoic acid (Bergmann et al. 2011). The genes coding for these two subunits are found in a cluster organized similarly in N47 as well as in the naphthalene-degrading sulfate-reducing Deltaproteobacterium NaphS2 (DiDonato et al. 2010).

Subsequently, 2-naphthoic acid is converted into 2-naphthoyl-CoA by an ATP-dependent 2-naphthoyl-CoA ligase (Eberlein et al. 2013b). 2-naphthoyl-CoA is then converted into 5,6-dihydro-2-naphthoyl-CoA *via* a two-electron reduction mediated by a 2-naphthoyl-CoA reductase, a member of the "old yellow enzyme family" of flavoproteins (Estelmann et al. 2015). The 5,6,-dihydro-2-naphthoyl-CoA formed is subsequently reduced with two electrons into 5,6,7,8-tetrahydro-2-naphthoyl-CoA by a 5,6-dihydro-2-naphthoyl-CoA reductase (Eberlein et al. 2013a; Estelmann et al. 2015). The 2-naphthoyl-CoA reductase and the 5,6-dihydro-2-naphthoyl-CoA reductase could not be completely separated *via* purification steps, and there is a significant possibility that both enzymes form a complex in vivo. In the next step, 5,6,7,8-tetrahydro-2-naphthoyl-CoA is possibly reduced to hexahydro-naphthoyl-CoA by an ATP-dependent class I benzoyl-CoA reductase with two electrons (Eberlein et al. 2013b; Estelmann et al. 2015). Until now, the natural electron donors of both the 2-naphtoyl-CoA reductase and the 5,6-dihydro-2-naphthoyl-CoA reductase are unknown, but genes located nearby as well as cofactors associated with the enzymes indicate that a flavin-based electron bifurcation energy conservation mechanism may be involved (Estelmann et al. 2015). After dearomatization, it has been suggested that the complete degradation of naphthalene to CO_2 is continued further *via* cyclohexane ring comprising CoA esters instead of monocyclic aromatic compounds (Annweiler et al. 2002).

Until now, the anaerobic degradation pathway of 1-methylnaphthalene remains unresolved (Kleemann and Meckenstock 2011). Like with naphthalene, the degradation pathway of 2-methyl-naphthalene has not been investigated in metal-reducing microbes but only in sulfate-reducing bacteria (Annweiler et al. 2000; Annweiler et al. 2002; Safinowski and Meckenstock 2004; Musat et al. 2009). Similar to anaerobic toluene oxidation, 2-methylnaphthalene is first converted to naphthyl-2-methyl-succinate by the addition of a fumarate molecule to the methyl group (Selesi et al. 2010) (Fig. 5b). This reaction is mediated by the glycyl radical enzyme naphthyl-2-methyl-succinate synthase (NmsABC). Subsequently, naphthyl-2-methyl-succinate is activated to yield naphthyl-2-methylene-succinyl-CoA by the naphthyl-2-methyl-succinate CoA transferase (BnsEF). The following four reactions catalyzed respectively by a naphthyl-2-methyl-succinyl-CoA dehydrogenase

(BnsG), a naphthyl-2-methylene-succinyl-CoA hydratase (BnsH), a naphthyl-2-hydroxymethyl-succinyl-CoA dehydrogenase (BnsCD), and a naphthyl-2-oxomethyl-succinyl-CoA thiolase (BnsAB) result in the β-oxidation of the side chain leading to the formation of 2-naphtoyl-CoA, which will then be dearomatized.

5 Research Needs

Exploring anaerobic hydrocarbon oxidation by DMRM could lead to several promising applications. For instance, many of the reactions involved in hydrocarbon oxidation have unusual chemistry that could be exploited for biotechnological purposes. Microbes capable of transferring electrons to solid metal oxides such as *Geobacter* spp. can also use a solid electrode as the TEA (Franks and Nevin 2010). This characteristic has conducted to the development of a bioremediation strategy where electrodes are buried in contaminated sediments to stimulate hydrocarbons degradation (Zhang et al. 2010; Morris and Jin 2012; Wang et al. 2012). Because electrodes are low-maintenance and can be installed permanently at polluted sites, electrobioremediation is a promising approach since it has the potential of being less expensive than competing technologies. Another possible application associated with DMRM, which usually generate magnetite from the Fe(III) oxide reduction, is the possibility of developing tools for the localization of petroleum deposit based on magnetic anomaly signals (Lovley et al. 2004).

Developing novel technologies related to hydrocarbons degradation with DMRM will require a better understanding of their metabolism. Although important progress has been made in the last 5 years, hydrocarbons degradation pathways in DMRM are still only partially characterized. Outstanding questions remaining include: (1) the identity of the enzymes responsible for benzene hydroxylation in *G. metallireducens* (Zhang et al. 2013) and benzene carboxylation in *F. placidus* (Holmes et al. 2011); (2) the nature of the electron transport route and energy conservation mechanism involved in benzoyl-CoA reductase, 2-naphthoyl-CoA reductase, and 5,6-dihydro-2-naphthoyl-CoA reductase activity (Boll et al. 2014; Estelmann et al. 2015); and (3) the nature in metal-reducing microbes of the polycyclic hydrocarbons degradation pathways described only in other phylogenetic classes of microbes until now. These examples are representing only a small fraction of the unexplored elements that will require extensive research work before achieving a complete portrait of the hydrocarbon degradation metabolism in metal-reducing microbes.

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Part III

Ecophysiology and Diversity of Anaerobic Hydrocarbon Degradation

Next-Generation Sequencing of Functional Marker Genes for Anaerobic Degraders of Petroleum Hydrocarbons in Contaminated Environments

12

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Abstract

The anaerobic degradation of petroleum hydrocarbons is an important ecosystem service provided by microbes in systems impacted by pollution. Research in recent years has resulted in substantial advances in our understanding of the diversity and ecology of natural populations of anaerobic hydrocarbon degraders, both in marine and terrestrial sedimentary and subsurface habitats. Part of this research has been fueled by the development and optimization of specific functional marker genes for anaerobic hydrocarbon degraders, i.e., qualitative and quantitative molecular assays, allowing for the detection of key catabolic genes or transcripts involved in degradation. These include the so-called "fumarate-adding enzymes" benzylsuccinate synthase (*bssA*) and alkylsuccinate synthase (*assA/masD*) involved in the primary activation of alkanes and alkylated aromatic hydrocarbons under anaerobic conditions. Here, we summarize the most important advances in the field and highlight the appeal of recent next-generation sequencing-based approaches toward catabolic marker genes for in-depth degrader community dissection. This contributes to a more routine and thorough inspection of intrinsic degrader populations responsible for key catabolic services in systems contaminated with petroleum hydrocarbons.

1 Introduction

Petroleum hydrocarbons, such as alkanes and aromatics, are widespread organic contaminants in the environment and of considerable concern to water quality, ecosystem status, and human health. While contaminations in terrestrial systems are mostly connected to anthropogenic activities (Lueders 2017), marine sediments also often feature natural hydrocarbon seeps (Scoma et al. 2017). Biodegradation by microbes is a key process reducing hydrocarbon loads in these systems, but the factors controlling the activity and efficiency of these populations are still poorly understood. Especially under anoxic conditions, which often prevail in polluted marine sediments or in the terrestrial subsurface, the detection and characterization of intrinsic degrader populations represent a lasting research challenge. To address this challenge, next to targeted cultivation strategies or non-targeted metagenomic approaches, the detection of catabolic marker genes involved in anaerobic degradation is an indispensable tool. The approach allows for a targeted detection, localization, quantification, identification, and often also respiratory classification of anaerobic hydrocarbon degraders. Such information can be vital for site monitoring or the conceiving of site-specific remediation strategies (Lueders 2017). In this chapter, we provide a synopsis of degradation pathways and catabolic gene markers which have actually been applied to trace anaerobic degrader populations in environmental systems. We also demonstrate the appeal of next-generation sequencing technologies as a high-throughput screening strategy for degrader diversity and community composition.

2 Anaerobic Degradation Pathways of Petroleum Hydrocarbons

The initial activation reaction is often the crucial step in the degradation of aliphatic or aromatic hydrocarbons by anaerobic degraders. Petroleum hydrocarbons are chemically rather stable and difficult to functionalize in the absence of oxygen as co-substrate for an oxidative attack (Rabus et al. 2016). For this reason, the capacity of microbes to degrade petroleum hydrocarbons under anaerobic conditions had been doubted until the mid-1980s (Grbić-Galić and Vogel 1987). However, a fair number of anaerobic degraders, degradation pathways, and activation mechanisms for petroleum hydrocarbons have been identified over the last decades (Rabus et al. 2016). Similar to aerobic hydrocarbon catabolism, the anaerobic degradation of petroleum hydrocarbons also proceeds via several initial activation and transformation mechanisms (peripheral degradation pathways). These funnel the compounds to central metabolites, which are then further degraded to assimilatory building blocks or fully oxidized to CO_2 (Callaghan 2013b; Fuchs et al. 2011). Three major strategies for the anaerobic activation of petroleum hydrocarbons are currently known: (i) for alkanes or alkylated aromatics the addition of a methyl or methylene group to fumarate (the so-called fumarate addition; Heider et al. 2016a); (ii) oxygen-independent hydroxylation known to be involved in the degradation of ethylbenzene and related substituted benzenes (Heider et al. 2016b); and (iii) carboxylation as proposed for alkanes, benzene, and polycyclic aromatic compounds (Callaghan 2013a; Meckenstock et al. 2016).

Among these distinct strategies, fumarate addition is considered as an archetype of anaerobic hydrocarbon degradation mechanisms. It was first discovered for the degradation of toluene by the denitrifying *Thauera aromatica* (Biegert et al. 1996) and is catalyzed by a glycyl radical enzyme named benzylsuccinate synthase (Bss). The enzyme forms a benzyl radical from toluene, adds it to the fumarate double bond, and finally releases benzylsuccinate as the first metabolite of toluene degradation (Fig. 1). Meanwhile, a role of benzylsuccinate synthase and related fumarate-adding enzymes (FAEs) has been reported for a wide phylogenetic diversity and respiratory variety of anaerobic degraders and also for the degradation of other alkylated mono- and polyaromatics, linear and cyclic alkanes, and even linear alkylbenzene sulfonate detergents, as summarized elsewhere (von Netzer et al. 2016; Wilkes et al. 2016). Depending on the nature of the substrate (Fig. 1), the FAEs involved are also named naphthylmethylsuccinate synthases (Nms; Annweiler et al. 2000; Musat et al. 2009) or (1-methyl)alkylsuccinate synthases (Ass/Mas; Callaghan et al. 2008; Grundmann et al. 2008).

Hydroxylation of alkyl chains by ethylbenzene dehydrogenase (EBDH) and related enzymes is a central mechanism in the activation of ethylbenzene and related substituted benzenes (Heider et al. 2016b). Although respective enzymes are currently only known from a few denitrifying strains, the mechanism seems to be involved in biodegradation also under a range of other redox conditions (Dorer et al. 2016). For non-substituted aromatics like benzene and naphthalene, the current understanding of anaerobic degradation pathways is still incomplete. Yet, a number of recent studies suggest that carboxylation may be a conserved mechanism for the activation of these

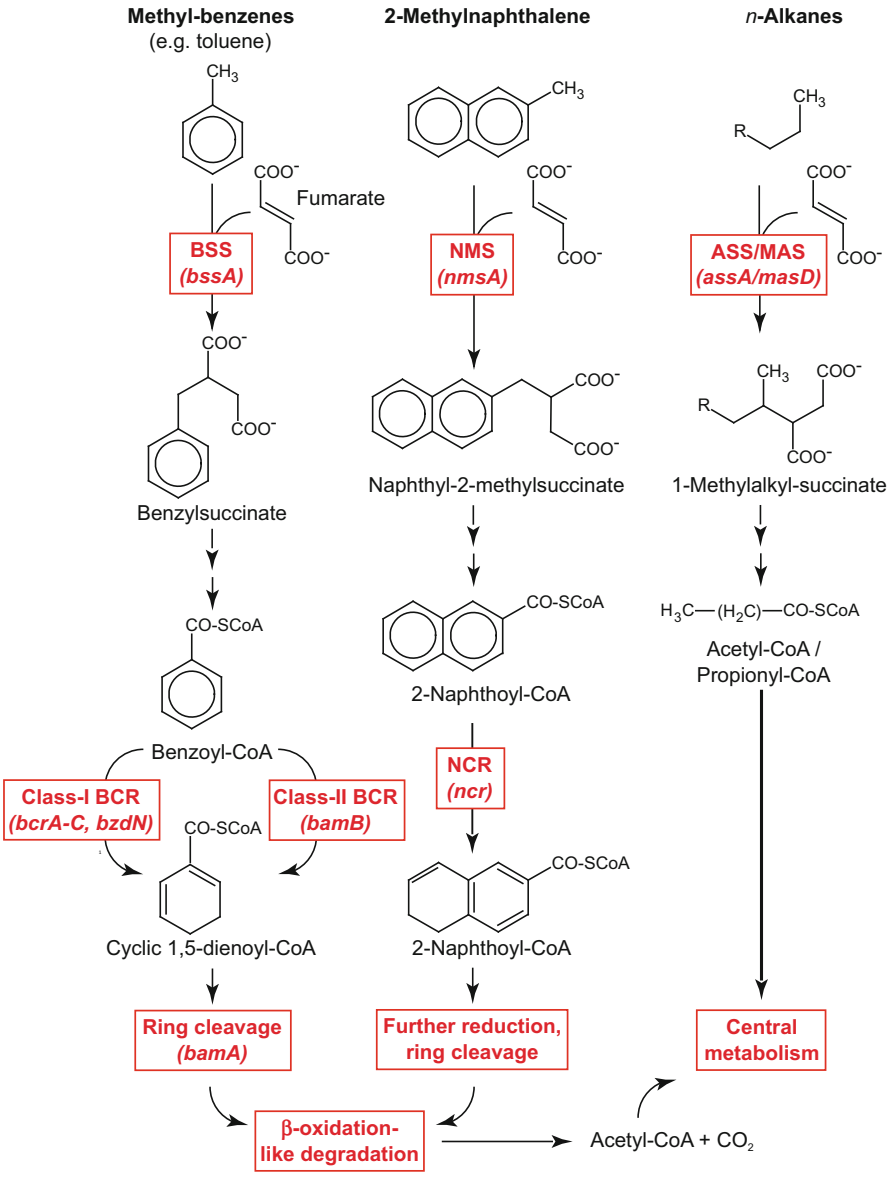

Fig. 1 Overview of important peripheral and central catabolic pathways in the anaerobic degradation of alkylated aromatic hydrocarbons and non-methane alkanes. Genes of key enzymes in use as catabolic marker genes for degraders in environmental systems are highlighted. *BSS* benzylsuccinate synthase, *NMS* naphthylmethylsuccinate synthase, *ASS/MAS* (1-methyl) alkylsuccinate synthase, *BCR* benzoyl-CoA reductase, *NCR* naphthoyl-CoA reductase, *BamA* ring-cleaving 6-oxocyclohex-1-ene-1-carbonyl-CoA hydrolase (Scheme is adapted from Lueders and von Netzer 2014; von Netzer et al. 2016)

substrates, catalyzed by the proposed anaerobic benzene carboxylase (ABC; Abu Laban et al. 2010; Luo et al. 2014) or by naphthalene carboxylase (Bergmann et al. 2011; Mouttaki et al. 2012). The resulting aromatic acids (benzoate, 2-naphthoate) can then be activated directly by CoA ligation and funneled into the central degradation pathways used also for alkylated aromatics (Meckenstock et al. 2016; von Netzer et al. 2016).

All of the above peripheral aromatic degradation pathways funnel their substrates to central metabolites, which are then subjected to further reduction, dearomatization and ring cleavage (Rabus et al. 2016). Benzoyl-CoA is the central metabolite of monoaromatic hydrocarbon degradation, and two enzyme systems are known to be involved in its dearomatization: either the ATP-dependent class-I benzoyl-CoA reductase (Bcr/Bzd) in facultative anaerobes like denitrifying *Thauera* or *Azoarcus* spp. or the ATP-independent class-II benzoyl-CoA reductase (BamB) in strict anaerobes like in iron-reducing *Geobacter* spp. or sulfate-reducing *Desulfobacterium* spp. (Boll et al. 2014; Porter and Young 2014). After ring reduction, a ring-cleaving hydrolase (BamA) transforms the former aromatic ring into linear CoA-fatty acids (Kuntze et al. 2008; Porter and Young 2013; Staats et al. 2011), which are then subject to a β-oxidation-like degradation to acetyl-CoA or complete oxidation to CO_2 (Fig. 1). Such linear CoA-fatty acids are also products of the anaerobic degradation of *n*-alkanes (Callaghan 2013a; Wilkes et al. 2016). The further sections of this chapter focus on the application of the catabolic genes involved in anaerobic degradation of petroleum hydrocarbons to dissect degrader communities in complex natural systems.

3 Catabolic Marker Genes for Anaerobic Hydrocarbon Degraders in Natural Systems

A wide diversity of bacterial cultures and enrichments is known to make use of the above catabolic reactions while degrading petroleum hydrocarbons and respiring different anaerobic electron acceptors (Callaghan 2013a; Heider and Schühle 2013; Rabus et al. 2016; Weelink et al. 2010; Widdel et al. 2010). Generally speaking, typical anaerobic hydrocarbon degraders can be found within the *Rhodocyclaceae* (*Betaproteobacteria*), the *Geobacteraceae*, *Desulfobacteraceae*, *Syntrophobacteraceae* (*Deltaproteobacteria*), and the *Peptococcaceae* (*Clostridia*). They either depend on typical anaerobic electron acceptors such as nitrate, ferric iron, or sulfate, while some are also capable of fermentative hydrocarbon degradation. These different guilds of anaerobic hydrocarbon degraders are, at best, functionally defined (via substrate usage and respiratory capacity) but phylogenetically diverse and often widely distributed throughout bacterial lineages. Therefore, anaerobic hydrocarbon degraders cannot be selectively targeted in environmental samples using ribosomal marker genes, at least not above the strain level. This is why soon after their initial discovery, researchers have realized the potential of genes for conserved key enzymes in anaerobic hydrocarbon degradation (so-called functional or catabolic

marker genes) to develop PCR assays allowing to specifically trace anaerobic hydrocarbon degraders in environmental systems.

3.1 Genes for Fumarate-Adding Enzymes

The benzylsuccinate synthase alpha subunit (*bssA*) and related FAE genes have been widely used as catabolic markers. Originally introduced for the detection of denitrifying toluene degraders within the *Betaproteobacteria* (Beller et al. 2002), increasing genomic and environmental sequence availability and continuous primer development have led to optimized detection systems for anaerobic alkylbenzene degraders applicable in iron-reducing, sulfate-reducing, and methanogenic systems. A comprehensive overview of published primers and assays is beyond the scope of this chapter but available elsewhere (von Netzer et al. 2016). Related to the sequence motifs realized in *bssA* detection assays, PCR primers capable of specifically recovering *assA* (Callaghan et al. 2010) and *nmsA* genes (von Netzer et al. 2013) in the environment have also been developed.

In samples taken directly from the terrestrial subsurface, *bssA* genes were first reported from a number of tar-oil-contaminated aquifer sediments in Germany (Winderl et al. 2007). Several as-of-then unidentified catabolic gene lineages were found, especially at sites dominated by sulfate reduction. The results of this study emphasized the possibility of yet unknown degrader populations to be important for bioremediation in situ. *BssA* gene-based diversity screenings of anaerobic degraders have since been conducted at a number of contaminated terrestrial systems, both in the USA (Callaghan et al. 2010; Yagi et al. 2010) and in Europe (Benedek et al. 2016; Osman et al. 2014; Staats et al. 2011), revealing locally dominating and apparently site-specific populations of either beta- or deltaproteobacterial degraders to be detectable at respective sites. Ongoing optimization of FAE-targeted primer sets has also demonstrated that *bssA* genes of clostridial affiliation and *nmsA* genes can be recovered from terrestrial samples (Martirani-Von Abercron et al. 2016; von Netzer et al. 2013). While the detection of non-proteobacterial FAE genes had been a challenge with initial primer pairs, adequate tools are now at hand, and the importance of clostridial hydrocarbon degraders in terrestrial systems of complex microcosms derived thereof is undisputed (Abu Laban et al. 2015; Aitken et al. 2013; Fowler et al. 2012; Sun et al. 2014; Winderl et al. 2010). Nevertheless, the optimization of assays for a more comprehensive recovery of FAE genes with primers less selective for known proteobacterial FAE gene sequences is still an ongoing process.

The potential of *bssA*-targeted qPCR to identify hot-spots of biodegradation by localizing and quantifying degrader populations across vertical plume transects was first demonstrated for a tar-oil-contaminated site in Germany (Winderl et al. 2008). Similarly, *bssA*-targeted qPCR was also used to compare distinct degrader abundances across longitudinal transects of a plume (Oka et al. 2011), revealing degrader enrichment in zones of highest contamination. A qPCR assay designed to detect *bssA* genes of specific sulfate-reducing hydrocarbon degraders was applied to monitor degrader abundance in groundwater samples from comparative bioremediation

galleries at a US Air Force base (Beller et al. 2008), revealing that alternative electron donor amendment (ethanol) could actually stimulate degrader abundance. Moreover, in water samples taken from an artificial toluene plume introduced to an indoor model aquifer, comparative *bssA* gene-to-transcript ratios were quantified to discriminate between actual active gene expression (mRNA) and inactive degrader populations transported downstream of biodegradation zones by groundwater flow (Brow et al. 2013). This elaborate example illustrates nicely how multiple levels of molecular data and spatial analyses can be combined to better elucidate processes in contaminated systems.

In marine systems, FAE gene-based degrader detection was first introduced for *assA* genes, revealing a remarkable diversity of yet-unknown catabolic gene lineages to be extant in sediments from several hydrocarbon-contaminated waterways in the USA (Callaghan et al. 2010) and also in sediments of the Chesapeake Bay (Johnson et al. 2015). Several novel clusters of *bssA* and *assA* genes were also reported for different marine sediments in Spain, contaminated either by accidental oil spills or by experimental hydrocarbon exposure (Acosta-González et al. 2013). Deltaproteobacterial FAE genes were especially frequent in these libraries, as expected for marine sediments that are typically rich in sulfate-reducing bacteria. Degrader diversity seemed to depend on both nature and severity of the contamination. Similarly, Kimes and colleagues used a simultaneous screening of *assA* and *bssA* genes to successfully demonstrate anaerobic catabolic potentials in sediments impacted by the Deepwater Horizon oil spill (Kimes et al. 2013). Very recently, *bssA*-targeted qPCR successfully revealed that anaerobic toluene degraders were quantitatively stimulated in polluted marine sediments subjected to anodic electroremediation (Daghio et al. 2016). These studies clearly demonstrate the ample potential of FAE gene-targeted detection assays in the monitoring and population-informed remediation of contaminated sites.

Also from marine systems exposed to natural oil seeps and mud volcanoes, FAE gene pools have been recovered (Gittel et al. 2015; Stagars et al. 2016; von Netzer et al. 2013). While the study of Gittel and colleagues revealed marked distinction in *masD* gene pools recovered from a variety of either pristine or seepage-impacted sediment samples off the Danish coast, Stagars et al. were the first to apply next-generation sequencing (NGS) to FAE gene amplicons. Thus, they were capable of revealing a remarkable diversity of no less than 420 different MasD species-level OTUs (at 96% amino acid similarity) from a global selection of marine methane, gas, and hydrocarbon seeps (Stagars et al. 2016). This study highlighted the still largely untapped potential of NGS approaches in functional marker gene screenings of anaerobic degraders of petroleum hydrocarbons.

3.2 Genes for Central Catabolic Markers

Marker genes from central catabolic pathways in anaerobic hydrocarbon degradation have also been used in environmental studies. Especially for degraders of non-substituted aromatic compounds, where peripheral activation mechanisms often

remain unknown, central pathways offer indispensable handles for degrader detection. The concept was introduced in 2005, when two independent groups reported notable diversities and partially unknown lineages of class-I benzoyl-CoA reductase genes (*bcrA* and *bzdN*) in contaminated groundwater and in estuarine sediment (Hosoda et al. 2005; Song and Ward 2005). As-of-then unidentified *bcrA* genes of deltaproteobacterial affiliation were also reported from crude oil-contaminated soils (Higashioka et al. 2009). Later, the quantification of class-I BCR genes has been used to localize and quantify anaerobic aromatics degraders in different wells of a crude oil-impacted US aquifer (Fahrenfeld et al. 2014) and in successive stages of a wastewater treatment system for dyeing effluents (Li et al. 2015). The applicability of the dearomatizing 2-naphthoyl-CoA reductase (NCR) for detecting degraders of polycyclic aromatic hydrocarbons has, in principle, also been demonstrated for a number of enrichment cultures (Morris et al. 2014), albeit not directly for environmental samples.

Class-II BCRs (*bamB*) and also the downstream ring-cleaving hydrolases (*bamA*) have also been employed to detect and characterize anaerobic degraders in contaminated systems. Most comprehensively, Kuntze et al. (2011) performed a comparative qualitative assessment of *bcrC*, *bamB,* and *bamA* gene pools in samples from two benzene-contaminated aquifers in Germany, revealing that mostly beta- or deltaproteobacterial degraders could be consistently recovered from both via the different markers. *BamA* gene pools were considerably more diverse than that of *bssA* in a landfill leachate plume in the Netherlands but surprisingly less abundant in highly contaminated zones than outside the plume (Staats et al. 2011). Crude oil-degrading enrichment cultures from marine sediments incubated at psychrophilic vs. mesophilic temperatures were shown to host diverse but clearly distinct *bamA* gene pools affiliated to sulfate-reducing degraders (Higashioka et al. 2011). Similarly, Sun and colleagues found that the phylogeny of *bamA* gene pools recovered from toluene-degrading enrichment cultures reflected the actual redox conditions across a whole range of different inocula, including contaminated soils and activated sludge (Sun et al. 2014). Most recently, the structure of anaerobic degrader communities as recovered via *bamA* genes from different Antarctic soils was shown to be distinct in highly contaminated samples vs. soils with intermediate or no contamination (Sampaio et al. 2017). It must be stated, however, that the detection of the central catabolic genes mentioned above need not always be strictly linked to the presence of anaerobic degraders of petroleum hydrocarbons. Also the anaerobic degradation of humic acids, lignins, and aromatic amino acids involves the respective catabolic routes and metabolites, as summarized recently by Porter and Young (2013, 2014). Therefore, an approach toward targeted anaerobic degraders involving multiple lines of evidence, including peripheral and central markers, is always highly commended.

4 NGS Approaches for Functional Marker Genes

The fact that only one of the studies summarized above has relied on a NGS-based marker gene query (Stagars et al. 2016) clearly illustrates the untapped potential of this approach for analyzing anaerobic degrader communities. The use of NGS-based

amplicon sequencing for functional marker genes is well-established, e.g., for genes of the particulate methane monooxygenase, *pmoA* (Lüke and Frenzel 2011); the ammonia monooxygenase, *amoA* (Pester et al. 2012); the nitric oxide reductase, *nosZ* (Philippot et al. 2013); or the alkane monooxygenase, *alkB* (Wallisch et al. 2014), to name only a few. In contrast to ribosomal amplicon sequencing, where prominent pipelines and databases are generally available (Caporaso et al. 2010; Schloss et al. 2009), functional gene amplicon sequencing still often relies on specific and manually curated sequence databases, i.e., as found in the FunGene repository (Fish et al. 2013), and on in-house data handling pipelines. The AnHyDeg repository, a specific and highly curated database for catabolic genes involved in anaerobic hydrocarbon degradation has only recently been released (Callaghan and Wawrik 2016).

Technically speaking, read length is clearly an issue in all marker gene sequencing approaches. In most applications of NGS-based amplicon sequencing, depending on the platform, sequencing has been limited to relatively short amplicons between ~300 and 500 bp in length (Luo et al. 2012). For certain platforms, additional problems with high numbers of frameshifts have been reported for functional marker genes (Zhang et al. 2015). For amplicons longer than the respective read lengths, it was either necessary to reduce the primer window, thus producing shorter amplicons, or to use a paired-end approach. However, recent technical developments in sequencing platforms have resulted in significant increases in sequencing length, to over several dozen of kb for the PacBio and MinION platforms (Benítez-Páez et al. 2016; Wagner et al. 2016). Together with other methodological innovations like the primer-free sequencing of full-length marker genes (Karst et al. 2016), or "epicPCR" allowing to link ribosomal and functional marker gene sequencing at the single-cell level (Spencer et al. 2016), a significant potential remains to be realized for catabolic marker gene sequencing.

Nevertheless, a few years before the discontinuation of the 454 GS FLX platform in 2016, Roche/454 had released a new long-read sequencing chemistry (~800–1000 bp) for its FLX+ sequencer. Due to the relatively short life span of the technology before becoming outdated, it has rarely been used for amplicon sequencing (D'Amore et al. 2016). The long-read sequencing chemistry perfectly matched the typical ~800 bp length of FAE gene amplicons necessary for an adequate diversity coverage of anaerobic degraders (von Netzer et al. 2016), which motivated us to test this approach in a proof-of-principle demonstration of long-read FAE gene amplicon sequencing. This was appealing, since FAE gene primers are typically highly degenerate, yielding notorious PCR by-products and resulting in classical cloning-and-sequencing approaches to be very laborious (von Netzer et al. 2013). In the following section of this chapter, the procedure and results of a long-read NGS approach are presented for the example of FAE gene amplicons generated from a tar-oil-contaminated aquifer in Germany. The main objective was to demonstrate that long (~800 bp) functional marker gene reads can actually be recovered from complex degrader communities and to query how NGS-based screenings of the degraders would go beyond previous Sanger sequencing-based characterization of the same degraders (von Netzer et al. 2013; Winderl et al. 2007).

Another goal was to test whether bidirectional amplicon sequencing would be necessary to recover full-length amplicon sequence information or whether unidirectional sequencing would suffice.

5 Long-Read Sequencing of FAE Gene Amplicons

5.1 Methodology

Sediment from the lower fringe (6.85 m below surface) and just below (7.15 m) a toluene plume in Flingern (Düsseldorf, Germany) was sampled in 2009, as previously described (Pilloni et al. 2011). The sediment was stored at −20 °C until nucleic acid extraction in 2012. DNA was extracted in three different replicates per depth using a phenol-chloroform extraction with bead beating as described (Pilloni et al. 2011). Two technically replicated sequencing libraries were generated per triplicate biological DNA extract from 6.85 m depth, while triplicate extracts were sequenced without further technical replication from 7.15 m depth.

The primer sets 7772f (Winderl et al. 2007)/8543r (von Netzer et al. 2013) for *bssA* libraries and 7768f / 8543r (von Netzer et al. 2013) for FAE-B libraries were used. The FAE-B primers were designed to recover a diversity of more deeply branching *bssA* homologues and also *nmsA* genes (von Netzer et al. 2013). Direct amplification of template DNA was not optimally effective with fully adapter- and identifier-tagged primers; thus pre-amplified FAE gene amplicons were first generated with untagged primers (~20–25 PCR cycles), as described by von Netzer et al. (2013). Afterward, these primary amplicons were re-amplified with a second round of PCR and fully tagged primers (five to ten PCR cycles). Two distinct sequencing approaches were used. For unidirectional reads, sequencing occurred from the forward primer alone. For the bidirectional approach, both forward and reverse primers were barcoded, and thus, sequencing was done from both ends of the amplicons. Tagging was done with either Lib-A and Lib-B (bidirectional) or Lib-L (unidirectional) adapters and multiplex identifiers (MID) attached to the primers as previously described (Pilloni et al. 2012; Zhang and Lueders 2017). Emulsion PCR, emulsion breaking, and sequencing on a 454 GS FLX+ sequencer were done with appropriate chemistry as recommended by the manufacturer (Roche Diagnostics, Penzberg, Germany).

Reads were de-multiplexed and quality-trimmed as previously described (Pilloni et al. 2012) but using the Greengenes trimming algorithm as implemented in Prinseq (version 0.20.4, http://prinseq.sourceforge.net). Reads below 250 bp length were excluded from further processing (Pilloni et al. 2012). FAE gene amplicons were classified using mothur version 1.33.3 (Schloss et al. 2009) with an in-house FAE gene alignment of 795 *bssA, nmsA*, and homologous genes generated in ARB (Ludwig et al. 2004). Our alignment excluded *assA* genes, as these were not to be expected at the Flingern site (von Netzer et al. 2013). All sequencing raw data have been deposited with the NCBI sequence read archive under the SRA accession number SRP131608. Diversities of detected FAE gene lineages were calculated in

R using the package "vegan" (Oksanen et al. 2017). For the comparison of read length and sequencing depth resulting from the different sequencing approaches, boxplot diagram were obtained in R with the script *boxplot,* with standard setting identifying outliers as measurements beyond the 1.5 * interquartile range.

5.2 FAE Gene Libraries from Flingern Sediments

Anaerobic hydrocarbon degraders at the Flingern site have been previously investigated via "classical" FAE gene sequencing, making it an ideal test site for the establishment of long-read FAE amplicon sequencing. A low diversity of FAE genes affiliated to the deltaproteobacterial "F1-cluster" *bssA* and also deltaproteobacterial *nmsA* genes have been previously reported to dominate clone libraries from the site, depending on which primers were utilized (von Netzer et al. 2013; Winderl et al. 2007).

Reasonable average yields of ~4000 reads across all *bssA* libraries (Table 1) were obtained. However, average read numbers were much lower for FAE-B amplicons, with only ~1400 reads and ~650 reads in average for the bidirectional and unidirectional libraries, respectively (Table 1). This finding was interpreted as a clear (and not unexpected) case of primer bias, as the 7768f primer used for FAE-B amplicons is highly degenerate, which is necessary to recover also more deeply branching *bssA* homologues. Resulting amplicons were more difficult to adequately purify from shorter and non-specific PCR by-products, which was reflected in lower numbers of total "good" reads and also a higher frequency of unidentified (non-FAE gene) sequences in these libraries. Still, a fair recovery of targeted FAE gene pools seemed to be possible with both primer sets. Overall median read lengths were between 640 and 760 bp for all libraries, but clearly highest in average (~750 bp) for unidirectional libraries (Fig. 2). However, unidirectional read libraries also contained a notable number of shorter reads. There was no significant decrease in median read length after trimming, which was taken as a general sign of high sequencing quality. There was also no significant difference between forward and reverse primer read lengths observed for separated bidirectional sequencing data sets (Fig. 2).

The composition of FAE gene pools as recovered from the two sediment depths and via the different sequencing approaches is illustrated in Fig. 3. Phylogenetic placement of the detected lineages is shown in Fig. 4. Libraries of *bssA* amplicons were always dominated by reads affiliated to the desulfobulbal F1-cluster *bssA*

Table 1 Average sequence read yield for different sequencing libraries generated with either bi- or unidirectional FLX+ sequencing in this study. Averages are given ± standard deviation of nine amplicon libraries per column

	bssA bidirectional		FAE-B bidirectional		*bssA* unidirectional		FAE-B unidirectional	
Raw	4300	±820	1423	±689	4252	±609	653	±154
Trimmed, >250 bp	4297	±821	1421	±690	4251	±609	652	±154

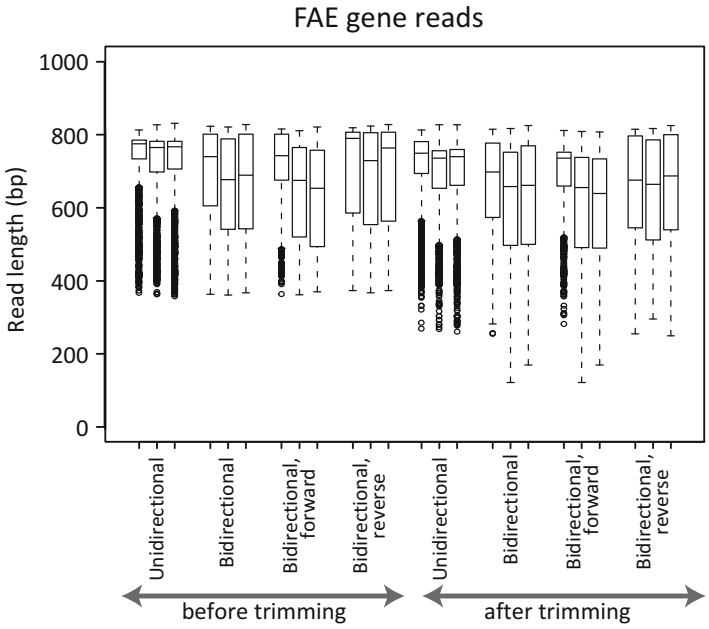

Fig. 2 Distribution of read length in FAE gene amplicon libraries before and after quality trimming. Three biological replicates were sequenced per sample in 6.85 m (technical replicate I), 6.85 m (technical replicate II), and 7.15 m. The dots represent outliers identified in R

(50–58% of all reads), irrespective of sequencing strategy or sediment depth. The second most abundant lineage, the clostridial F2-cluster *bssA*, was slightly more abundant at 7.15 m than at 6.85 m sediment depth (~42% vs. 25%). Further FAE gene lineages consistently recovered in *bssA* libraries from both depths were affiliated to the *Betaproteobacteria*, other *Deltaproteobacteria*, as well as the more deeply branching and as-yet unidentified T-cluster *bssA* homologues. These lineages were previously not detected in clone libraries of FAE genes generated for the Flingern site. T-cluster FAE genes and betaproteobacterial *bssA* were also recovered in sequencing libraries of FAE-B amplicons, where the T-cluster was even dominant (50–70%) at 6.85 m (Fig. 3b). However, deltaproteobacterial *nmsA* genes were also abundant in these libraries, especially at 7.15 m (74–77%). The Shannon diversity of recovered FAE-B lineages was slightly lower than that of *bssA* libraries (0.8 ± 0.18 vs. 1.06 ± 0.12, respectively).

Specific distinctions were detected in lineage abundances between uni- and bidirectional sequencing libraries, such as a consistent lower abundance of T-cluster *bssA* homologs in all bidirectional libraries. Still, overall degrader community patterns were consistently recovered for both depths and amplicons, irrespective of whether uni- or bidirectional sequencing was employed. The technical and biological reproducibility between sequencing libraries was very strong (Fig. 3a), as reported previously for 16S rRNA gene libraries from the same site (Pilloni et al.

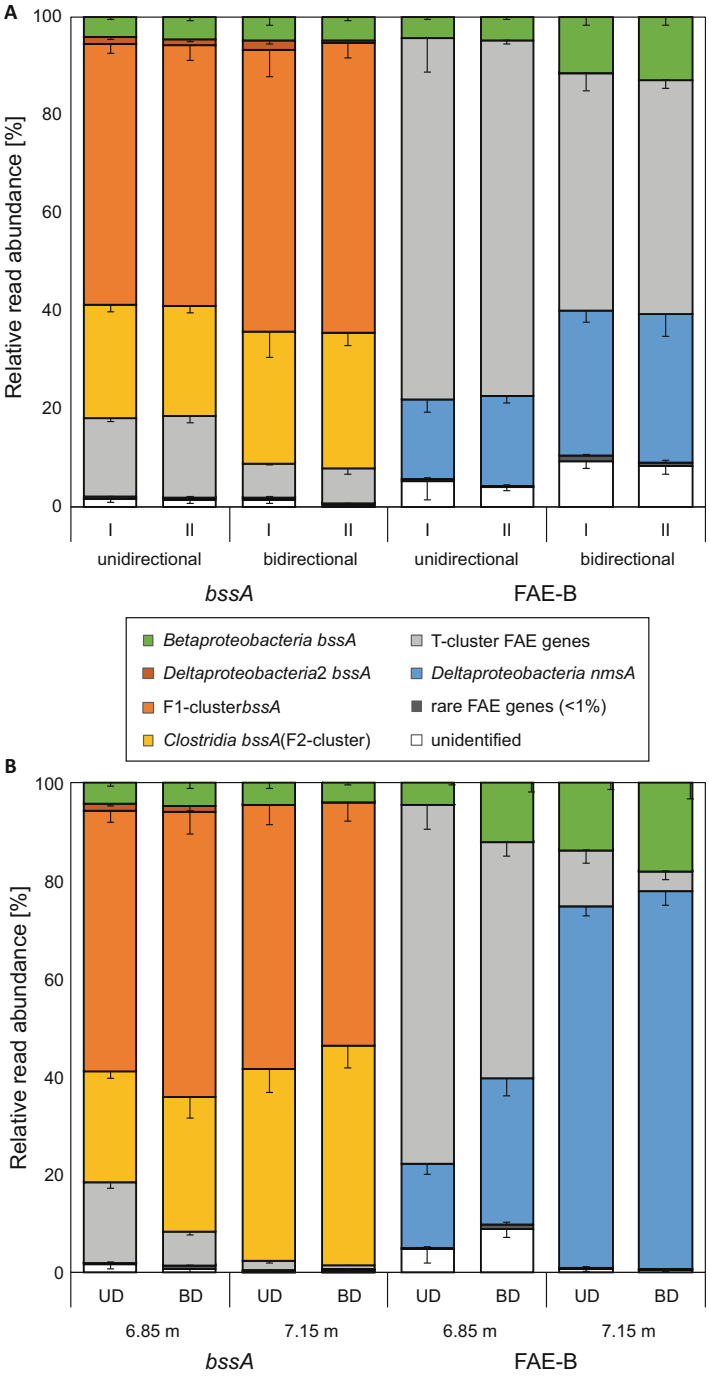

Fig. 3 Community structures of FAE gene pools as recovered from two different depths of a tar-oil-contaminated aquifer with different FAE primers. (**a**) Illustrates the technical reproducibility (I, II)

2012). This means that DNA extraction, amplification, and the NGS sequencing procedure were robust and that factors like primer selection, tagging, and sequencing strategy were much more important for outcomes on community structure.

In summary, this study demonstrates that long-read NGS of FAE gene amplicons is feasible and that it is capable of delivering reproducible screening results on anaerobic hydrocarbon degrader communities and degrader diversity. The much greater sequence yield compared to classical cloning-and-sequencing approaches (von Netzer et al. 2013; Winderl et al. 2007) delivers a greater diversity of detected lineages and more robust lineage abundances. In the present study, FAE gene OTUs were not resolved to species-level, i.e., as has been done for *masD* OTUs by Stagars et al. (2016) with shorter FLX titanium reads (~450 bp). Still, the much greater total number of NGS sequence reads and also the longer FAE gene information recovered in this study should allow for a meaningful dissection of degrader microdiversity via catabolic gene OTUs in successive studies.

6 Summary and Research Needs

This chapter summarizes the most recent state of the art in catabolic gene surveys for anaerobic degraders of petroleum hydrocarbons in environmental systems. Primer systems for a wide diversity of both peripheral and central catabolic marker genes in anaerobic degradation are now at hand. They have been widely employed in studies on terrestrial and marine systems either naturally or anthropogenically contaminated with alkanes and aromatic hydrocarbons. Thus, crucial insights on previously hidden lineages and diversities of anaerobic hydrocarbon degraders have been generated for many sites. Part of these catabolic gene lineages have now been securely associated with common degrader lineages within the *Betaproteobacteria*, the *Deltaproteobacteria*, and also the *Clostridia*, the latter being more frequently detected in the contaminated terrestrial subsurface than in marine systems. However, several prominent marker gene lineages, such as the T-cluster FAE gene homologues (Fig. 4), remain to be better integrated into current degrader taxonomy. Moreover, increasing experimental cues from enrichment cultures or (meta)genomic information on key enzymes and marker genes involved in activation mechanisms other than fumarate addition are currently becoming available. This will foster the development and application of assays capable of detecting degraders utilizing, e.g., oxygen-independent hydroxylation or carboxylation mechanisms for hydrocarbon activation.

Fig. 3 (continued) of biologically replicated sequencing libraries (all 6.85 m) generated with either uni- or bidirectional long-read 454 FLX+ amplicon sequencing. (**b**) Compares degrader community structure recovered with two different primer sets (*bssA*, FAE-B), from two different sediment depths and with either uni- or bidirectional amplicon sequencing. Read abundances are averaged over results from triplicate libraries per sample, error bars represent standard deviations and are shown as negative only. Color coding and naming of FAE gene lineages corresponds to that given in Fig. 4

The original research presented in the second part of this chapter shows that long-read NGS analysis of FAE gene amplicons is a powerful and reproducible tool to comprehensively screen degrader diversities in environmental systems. Recovery of FAE gene lineages was clearly higher via NGS, facilitating a unique "deep" access to degrader microbiota in contaminated systems. Although the utilized 454 GS FLX+ long-read sequencing platform is now already outdated, more modern long-read sequencing platforms such as the PacBio SMRT sequencing technology have recently become available for marker gene sequencing (Schloss et al. 2016; Wagner et al. 2016) and will surely also rapidly find their way into anaerobic degrader screening. Possibly, also other primer-independent NGS approaches can offer even more unbiased handles on degrader diversity, such as a recently introduced strategy involving sequence captured by hybridization and next-generation sequencing of FAE genes (Ranchou-Peyruse et al. 2016). In perspective, it remains to be emphasized that the vital information on intrinsic degrader assemblages accessible via marker gene sequencing must continue to find its way into an enhanced, population-based monitoring, management, and remediation strategies for contaminated sites.

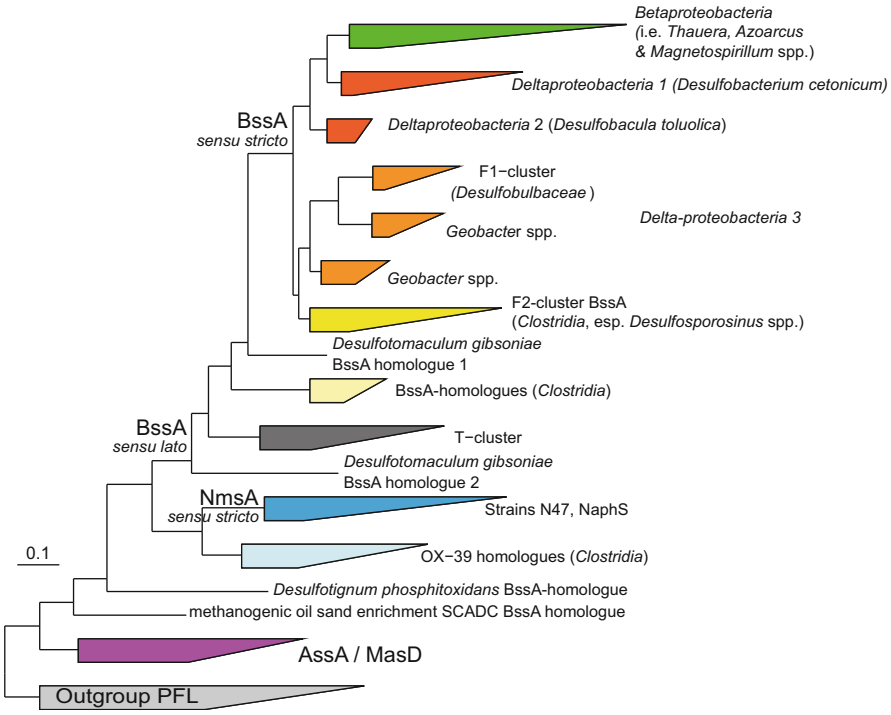

Fig. 4 Overview of the phylogeny of known pure culture and environmental FAE gene sequences as mentioned in the text. Several lineages are collapsed with only a few representatives named. Outgroup: related pyruvate formate lyase (PFL) genes. The scale bar represents 10%. Color coding and naming of FAE gene lineages corresponds to Fig. 3 (The tree has been adapted from Lueders and von Netzer 2014; von Netzer et al. 2016)

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Protein-Based Stable Isotope Probing (Protein-SIP): Applications for Studying Aromatic Hydrocarbon Degradation in Microbial Communities

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Nico Jehmlich and Martin von Bergen

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Abstract

Protein-based stable isotope probing (protein-SIP) was developed to link microbial-specific metabolic function to phylogenetic information. The principle of the SIP-concept is the supplement of stable isotope-labelled compounds resulting in the labelling of microorganisms that are capable of utilizing these substrates as carbon source. The sum of all proteins reflects the functional status of the living organisms, so that the degree of heavy isotope incorporation represents a proxy for substrate assimilation and their activity. The main focus of this chapter is on the application of protein-SIP to elucidate metabolic processes in general and in particular those involved in the anaerobic degradation of aromatic hydrocarbons. Thus, the application of protein-SIP is a useful method to investigate the composition and the functional state of microbial communities.

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1 Introduction

Meta-omics approaches allow a deep insight into the structure and function of microbial communities (Herbst et al. 2015). Metaproteomics also referred to as community proteomics or environmental proteomics has become over the last years metaproteomics, also referred to as community proteomics or environmental proteomics, has become an important tool in studying microbial ecology approaches (von Bergen et al. 2013) allowing to elucidate functional relationships between microbial community members (Bastida et al. 2015). However, metaproteome analysis alone cannot elucidate which protein and their corresponding species is currently active under specific conditions. For this reason, SIP techniques are required to solve this problem (Jehmlich et al. 2008a). During protein-SIP analysis, the metabolism of the labelled substrate can be detected with high precision on the peptide level. The identification of uniquely labelled peptides principally allows the assignment to phylotypes that express the encoding genes of the proteins providing a direct link of metabolic function to single members of the microbial community (Jehmlich et al. 2016). For these reasons, protein-SIP has developed into an important and widely used tool in microbial ecology. Since its development in 2008 (Jehmlich et al. 2008b), the spectrum of used stable isotopes in protein-SIP analyses has continuously broadened. By applying mass spectrometry (MS), the MS spectra provide biologically valuable information: (i) the presence/abundance of peptides/proteins, (ii) the primary amino acid sequence thereby revealing the proposed function, and (iii) the metabolic activity of the corresponding organism as a measure of incorporation of isotopes. Bioinformatics solutions for automatic data evaluation have been developed, and the range of application has been enlarged from microcosms over mesocosms up to in situ environmental sampling. This book chapter focuses on existing studies on protein-SIP and discusses potential trends of its application in environmental microbiology (e.g., labelled water) with a special focus on anaerobic degradation of aromatic hydrocarbons (Grob et al. 2015). Most technical aspects of protein-SIP applications have been reviewed previously (Taubert et al. 2011; von Bergen et al. 2013).

In the coming years, the development in the fields of mass spectrometry and bioinformatics will increase the resolution and specificity of protein-SIP applications. Further integration of comprehensive protein extraction procedures will promote the analysis of samples with lower amounts of protein, which opens the way to investigate natural microbial communities in more detail.

1.1 Protein-SIP Concept

Metabolic incorporation of heavy stable isotopes (usually ^{13}C or ^{15}N) into proteins has become a powerful technique for qualitative and quantitative proteome studies because the assessment of incorporation is a valuable parameter to investigate the general metabolic activity as well as protein turnover rates. In a time course

experiment, the accurate quantification of incorporation in protein-SIP enables also the identification of food webs in microbial consortia.

There are two ways of metabolic protein labelling: (i) the use of substrates or nutrients such as [$^{13}C_7$]-glucose or ^{15}N-ammonium which are biochemically incorporated into amino acids and (ii) the use of specifically labelled amino acids that are added to the medium (SILAC, for details see (Chen et al. 2015)). Stable isotope-labelled substrates (D, ^{13}C, ^{15}N, ^{18}O, $^{34/36}$S, or nutrients) have become attractive since the mass shift measured by mass spectrometry clearly allows the quantitative assessment of isotope incorporation, and this is an indirect measurement of metabolic activity in respect to a specific substrate or nutrient. Two important parameters can be retrieved from the mass spectrometry data. First, the relative isotope abundance (RIA) describes the number of labelled atoms in a peptide and gives information about the proportion of labelled substrate that was assimilated. The second parameter named labelling ratio (LR) describes the ratio of labelled to natural peptide and refers to protein turnover rates after addition of the labelled substrate. The greatest advantages of protein-SIP are the accurate and very sensitive quantification of incorporation and the physiological information that is obtained concomitantly.

1.2 Calculation of Stable Isotope Incorporation into Peptides

In contrast to the incorporation of isotopically defined labelled amino acids (SILAC) which results in fixed mass shift (e.g., +4 Dalton mass shift), metabolic labelling using growth substrates or nutrients leads to dynamic and unpredictable mass shifts in the MS spectrum. In environmental studies, protein-SIP incorporation is often difficult due to the low intense and complex MS spectra, thereby making the data processing time-consuming and demanding. The process in automatic evaluation tools increased the feasibility of protein-SIP applications. Exemplarily, Pan et al. (2011) developed the *Sipros* algorithm (open-source and available http://code.google.com/p/sipros) to identify peptide sequences and quantify their ^{15}N atom% composition. The abundance ratio between two isotopologues (heavy and light) of a protein was estimated in combination with *ProRata* (http://code.google.com/p/prorata/). Sachsenberg et al. (2015) developed *MetaProSIP* that calculates peptide relative isotope abundance for (i) ^{13}C, ^{15}N, deuterium (^{2}H), and oxygen (^{18}O), (ii) the labelling ratio (LR) between old and new synthesized proteins, and (iii) the shape of the isotopic distribution. Therefore, *MetaProSIP* provides high reliability and reproducibility which is combined with a quality reporting option (www.openms.de/MetaProSIP) (Sachsenberg et al. 2015). The *MetaProSIP* node was further implemented into Galaxy (homepage: https://galaxyproject.org, main public server: https://usegalaxy.org) which is a web-based scientific analysis platform to analyze genomics, proteomics, metabolomics, or imaging datasets (bioinformatics workflow in Galaxy to calculate incorporation Fig. 1) (Afgan et al. 2018). This allows a worldwide accessibility that will certainly encourage more groups to use labelled substrates to elucidate the functional behavior of microbial communities.

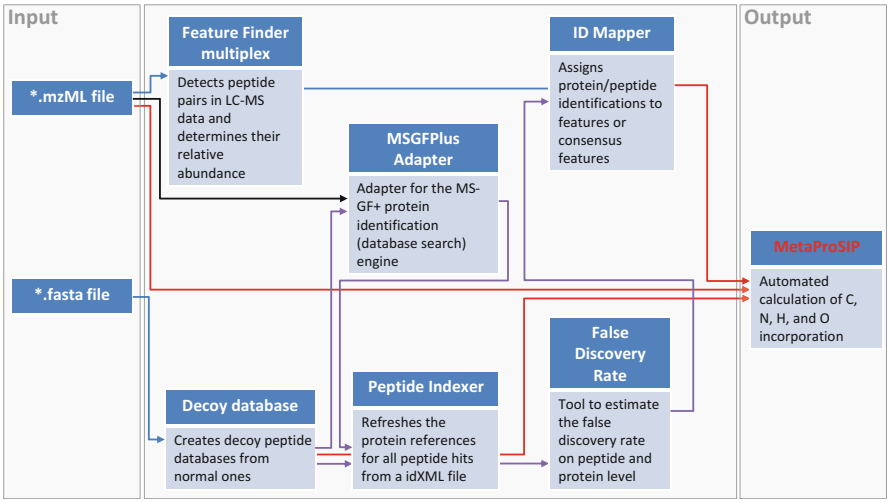

Fig. 1 Customized MetaProSIP workflow Galaxy that can be applied for the calculation of stable isotope incorporation into peptides/proteins

1.3 Protein-SIP Applications

There are several studies published that use protein-SIP in microbial ecology approaches. Exemplarily, we reviewed three applications that benchmark the field of protein-SIP, (i) a time-resolved protein-SIP, (ii) in situ protein-SIP studies, and (iii) labelled water to track general microbial activity.

A sulfate-reducing enrichment culture originating from a benzene-contaminated aquifer was investigated using time-resolved protein-SIP (Taubert et al. 2012). Benzene is a major contaminant in this aquifer, and the initial mechanisms behind its biodegradation under strictly anoxic conditions were not yet entirely clear. The carbon fluxes within the microbial community were investigated by addition of either ^{13}C-benzene or ^{13}C-carbonate. The utilization of the initial carbon source and the metabolic intermediates was relatively quantified, and the functional groups were affiliated to *Clostridiales*, *Deltaproteobacteria*, and *Bacteroidetes*/*Chlorobi*. Taubert et al. further observed that the *Clostridiales*-related organisms were involved in benzene degradation putatively by fermentation. This study revealed that protein-SIP can be applied in a time-dependent manner to obtain temporal and taxonomic information within a microbial community.

Polycyclic aromatic hydrocarbon (PAH) degrading bacteria often rely on laboratory enrichments and isolations. Herbst et al. performed the first in situ microcosms with ^{13}C-naphthalene (BACTRAP(R)s) in which the BACTRAPs were exposed to a PAH-contaminated aquifer (Herbst et al. 2013). Briefly, BACTRAPs are in situ microcosms that consist of perforated Teflon tubes of 5 cm length and 1 cm diameter with a perforation of 1 mm which are filled with 1 g of preheated (4 h at 300 °C) activated carbon pellets (Biocoal, Silcarbon Aktivkohle GmbH, Kirchhundem, Germany).

Following sterilization and hydration of the BACTRAPs by autoclaving at 121 °C, the BACTRAPs were each loaded with 25 mg [$^{13}C_6$]-naphthalene by dropping the compounds diluted in 1 mL *n*-hexane on the activated carbon pellets. As main result of this experiment, *Burkholderiales*, *Actinomycetales*, and *Rhizobiales* were the most active microorganisms in the groundwater communities; and the naphthalene degradation pathway showed high ^{13}C incorporation (about 50 atom%). This study convincingly demonstrated that a combination of in situ microcosms with protein-SIP is a suitable tool for the identification of metabolic key players as well as degradation pathways.

In another approach, the microbial processes in constructed wetlands (CWs) under controlled conditions were investigated. CWs are well-established treatment systems for the bioremediation of contaminated waste- and groundwaters (Schroder et al. 2007). While the systems provide high removal efficiencies for numerous organic contaminants, the microbial processes are not yet well known. Plant roots stimulate the microbial degradation activity within the CW by the exudation of organic compounds as well as oxygen (Lagos et al. 2015). Detailed knowledge about the microbial key players and degradation pathways can aid the design of CWs for improved and stable performances. A planted fixed bed reactor (PFR) was designed and operated in a greenhouse (Kappelmeyer et al. 2002). Toluene was added to the inflow to investigate the respective degradation processes. The microbial community composition was assessed by 16S rRNA gene sequencing and protein-SIP by pulsed addition of ^{13}C-labelled toluene and revealed fast degradation during 40 h inside the PFR (Lünsmann et al. 2015). After 20 h the ^{13}C label was detected in bacterial proteins; almost all labelled proteins could be assigned to the order of *Burkholderiales*, which constituted to only about 20% of the microbial community (Lünsmann et al. 2015). Among them, two bacterial families showed different proportions of ^{13}C-incorporation (RIA) in their proteins, leading to the conclusion that *Burkholderiaceae* derived more biomass from toluene (73% RIA), than the *Comamonadaceae* (64% RIA) (Lünsmann et al. 2015). The complete pathway of toluene degradation was retrieved by protein-SIP which demonstrated that toluene degradation was initiated by a monooxygenase yielding p-cresol, followed by a phenol hydroxylase introducing a second hydroxyl functionality group. It became evident that toluene-derived carbon might be fully oxidized via the citric acid cycle or could be stored by the microbes anabolically as polyhydroxyalkanoate (PHA) granules (Lünsmann et al. 2015, 2016). Toluene degradation is not limited by oxygen availability despite the applied concentrations in the PFR, and *Burkholderiales* may serve as indicators for effective hydrocarbon removal at low oxygen concentrations.

Micro-pollutants [herbicides, pesticides, and pharmaceuticals present in the environment at very low concentrations (ng-µg/L)] have emerged as an important topic in the field of environmental microbiology (Luo et al. 2014). The most important consequence of the low concentration is that these compounds are often co-metabolized (Fischer and Majewsky 2014; Kjeldal et al. 2016). By designing a protein-SIP experiment, the used concentration has to be remarkably exceeding the environmentally relevant concentration to obtain adequate stable isotope incorporation. If the

compounds are only co-metabolically transformed, the incorporation into the biomass of the microorganisms will not be enough, and a protein-SIP experiment using the labelled micro-pollutant is not suitable. Still, using labelled water as substrate-independent compound may be a promising approach.

Hydrogen has the highest abundance in proteins, but its application was limited due to a fast H/D-exchange, which will occur in living cells and during sample preparation. In addition, deuterium at high concentrations inhibits enzymes due to kinetic isotope effects. Furthermore, the chromatographic properties of deuterated compounds that originate from the higher hydrophilicity of CD-bonds compared to CH-bonds (Boersema et al. 2009), cause significant changes in the retention time (Zhang et al. 2002). However, the usage of heavy water, labelled with either deuterium or 18-oxygen, has advances and is promising as it assesses the global activity regardless of the used substrate. Some applications successfully demonstrated using D_2O in acid mine drainage biofilms (Justice et al. 2014) and soil communities using $H_2{}^{18}O$ in DNA (Blazewicz and Schwartz 2011; Schwartz 2009; Schwartz 2007) and RNA (Angel and Conrad 2013; Rettedal and Brozel 2015). Protein-SIP was applied to track the usage of $^{15}NH_4$ or deuterium oxide for different members of the community (Justice et al. 2014). There were relatively few ^{15}N-enriched archaeal proteins, and all showed low atom percent enrichment consistent with Archaea synthesizing protein using the predominantly ^{14}N biomass derived from recycled biomolecules (Justice et al. 2014). Deuterium oxide was used to detect general microbial activity in the samples. Interestingly, bacterial species showed only little protein synthesis using deuterium oxide which reflects that the exclusive ability of Archaea to synthesize proteins using ^2H_2O perhaps due to archaeal heterotrophy (Justice et al. 2014). In a combined Raman microspectroscopy, metaproteomics, and protein-SIP approach, the complex metabolic response using D_2O labelling allowed the monitoring of metabolic activity combined with a functional characterization of active populations isolates from groundwater (Taubert et al. 2018). In particular, 18-oxygen seems more promising than deuterium as the abiotic HD exchange of acidic hydrogens could cause a dilution of the label once the proteins are in contact with unlabelled water (Englander and Kallenbach 1983).

2 Conclusions

Metaproteomics of environmental samples is generally limited by three factors: first, the extraction of proteomes (with various biomass availabilities) from samples with background substances; second, the low genetic coverage in protein-coding databases; and third, the high complexity of the microbial community. For the last factor, protein-SIP contributes to an increase of the coverage because it points directly toward the detection of the active members within a community in respect to a specific substrate. Since the introduction of protein-SIP in 2008, all isotopes present in proteins have been used for studies. Thereby, a broad variety of studies based on energy flux (carbon), nitrogen utilization (nitrogen), or markers of general metabolic activity (deuterium and sulfur) have been performed. The latter examples underline the unique potential of protein-SIP for analyzing microbial communities in situ in

soil, sediments, aquifers, water bodies, and higher organisms. Still, one of the urgent needs is the development of new de novo approaches where the natural peptide information is not necessary. The identification of the amino acid composition from metabolically labelled isotopes in the MS spectra is generally performed by matching theoretical MS spectra to protein-coding sequence databases. Bioinformatics algorithms rely on monoisotopic precursor mass (natural abundance) for identification; likewise, *MetaProSIP* relies on the natural abundance precursor for identification and calculation of the dynamic mass shift starting from that.

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Compound-Specific Isotope Analysis for Studying the Biological Degradation of Hydrocarbons

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Carsten Vogt, Florin Musat, and Hans-Hermann Richnow

Contents

Abstract

Compound-specific isotope fractionation analysis (CSIA) has become a promising approach for studying biological degradation of hydrocarbons in the environment. The approach makes use of isotope fractionation processes taking place during enzymatic cleavage of carbon and hydrogen bonds formed by isotopologues due to rate limitations upon the first irreversible step of the reaction mechanism. The magnitude of isotope fractionation is usually expressed by the isotope enrichment factor ε for carbon (ε_C) and/or hydrogen (ε_H) using the Rayleigh equation, correlating isotope fractionation with concentration changes of the residual fraction of the substrate. For evaluating the magnitude of biodegradation at environmental sites, ε_C and/or ε_H determined from model cultures expressing known biochemical

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degradation pathways are used. By correlating the magnitude of carbon and hydrogen isotope fractionation (dual or multi-element compound-specific stable isotope analysis (ME-CSIA), resulting in lambda (Λ) values: $\Lambda = \Delta(\delta^2H)/\Delta(\delta^{13}C) \approx \varepsilon_H/\varepsilon_C$), distinct (bio)chemical reactions of degradation pathways can be further identified. In this review, we summarize ε_C, ε_H, and Λ values of currently known initial enzymatic reaction steps of aerobic and anaerobic hydrocarbon degradation pathways (dioxygenation, monooxygenation, hydroxylation with water, carboxylation, fumarate addition, and reactions by coenzyme M reductase) and discuss the opportunities for using them to identify degradation pathways and to quantify hydrocarbon degradation in environmental studies.

1 Introduction

1.1 Biogeochemistry and Abundance of Hydrocarbons

Hydrocarbons are organic compounds consisting entirely of carbon and hydrogen, exhibiting two stable isotopes with a natural abundance of 0.9893 and 0.0107 of the mole fraction of $^{12}C/^{13}C$ and 0.999885 and 0.000115 of the mole fraction of $^{1}H/^{2}H$, respectively (Audi et al. 2003).

The bulk hydrocarbons on Earth occur naturally as crude oils and natural gas. These consist predominantly of saturated hydrocarbons. Furthermore, plants synthesize large quantities of mostly unsaturated hydrocarbons, e.g., waxes, terpenes, caoutchouc, or carotenoids. Hydrocarbons can be also associated as minor compounds with kerogen of sediments and coals of different maturity and used as fossil fuel biomarkers for biogeochemical processes and origin and biodegradation of organic matter (Peters et al. 2007b). Petroleum-derived hydrocarbons are the most frequent environmental contaminants through spills, fossil fuel production, and processing. Non-methane hydrocarbons are furthermore dispersed in the environment as airborne solid and liquid particulates or gases often in association with soot from combustion processes.

Crude oils consist mostly of saturated (aliphatic) and unsaturated (aromatic) hydrocarbons (Tissot and Welte 1984). Saturated hydrocarbons (alkanes) contain exclusively single (sigma) bonds. Methane, the simplest and chemically most stable alkane, is the main constituent of natural gas. The most general form of saturated hydrocarbons is $C_nH_{2n+2(1-r)}$, where r is the number of rings forming the cycloalkanes. The carbon atoms in alkanes are sp^3 hybridized meaning the valence electrons are located in four equivalent orbitals derived from the combination of the 2s orbital and the three 2p orbitals. The C-H bonds are formed by the overlap of a carbon sp^3 orbital with the hydrogen 1s orbital, and the C-C single bonds are formed by the overlap of two sp^3 orbitals.

Unsaturated hydrocarbons contain one or more double (alkenes) or triple bonds (alkynes) between carbon atoms. Alkenes with one double bond have the formula C_nH_{2n}. A carbon double bond consists of one σ bond and one π bond. Alkynes containing triple bonds have one sigma bond and two π bond and the general formula C_nH_{2n-2}.

Aromatic hydrocarbons contain one or more aromatic rings; benzene is the simplest monocyclic compound. A single aromatic ring consists of six carbon atoms with σ bonds and delocalized π electrons between carbon atoms. Polycyclic aromatic hydrocarbons (PAHs) consist of fused aromatic rings; the simplest PAH is naphthalene with two fused rings. Aromatic hydrocarbons can be substituted by alkyl and allyl side chains or phenyl substituents leading to a large structural diversity. Aromatic hydrocarbons formed during geochemical processes are major constituents of oil, coal, and tars. Pyrolysis of organic material at elevated temperatures in the absence of oxygen typically forms aromatic hydrocarbons. They are also formed as by-products of incomplete combustion of fossil fuels or biomass.

Biodegradation is considered to be the main sink of hydrocarbons in the environment. Hydrocarbons are degradable under oxic and anoxic conditions (see below), but proving biodegradation in the environment is challenging due to several interfering physicochemical factors, e.g., dilution, volatilization, or sorption. Compound-specific stable isotope analysis (CSIA) is a relatively new concept for the analysis of hydrocarbon degradation (and other compounds) in the environment (Elsner et al. 2005). Hydrocarbon degradation pathways always start by an activation reaction of a C-H or C-C bond which is often associated with substantial carbon or hydrogen isotope fractionation, which will be described in the following. The mode of bond cleavage leading to isotope fractionation and the magnitude of isotope fractionation has the potential to detect and characterize the biodegradation reaction.

1.2 Stable Isotopes of Carbon and Hydrogen

The carbon and hydrogen isotopic compositions are typically reported as δ values (e.g., δ^2H and δ^{13}C) in parts per thousand (‰) relative to international standards (Pee Dee Belemnite (PDB) and Standard Mean Ocean Water (SMOW) for carbon and hydrogen, respectively) according to Eq. (1), where δE indicates the ^{2}H or ^{13}C isotope composition and R indicates the isotope ratio of ^{2}H/^{1}H or ^{13}C/^{12}C (Coplen 2011).

$$\left[\delta E_{sample}\right] = R_{sample}/R_{standard} - 1 \tag{1}$$

The stable isotope ratio (R) for hydrogen and carbon are calculated from the molar fractions (for H, ^{1}H 99.985, ^{2}H 0.015; for C, ^{12}C 98.88, ^{13}C 1.12), respectively. According to Eq. (1), the standards SMOW (hydrogen) and VPDB (carbon) have each a value of 0‰.

1.3 Characterization and Quantification of Isotope Fractionation

Biogeochemical processes in the environment can be associated with kinetic or equilibrium stable isotope fractionation. Equilibrium isotope fractionation is due to the partial separation of isotopologues of a substance at the chemical equilibrium in

two phases, for example, gas/liquid, dissolved/adsorbed, or dissolved/enzyme bound. Equilibrium isotope fractionation is mass- and temperature-dependent and usually increases at low temperatures (Swiderek and Paneth 2013). The extent of equilibrium isotope fractionation in an exchange reaction can be expressed as fractionation factor (α): α is the ratio of heavy isotopologues (subscript $_h$) to light isotopologues (subscript $_l$) and the concentration C and the isotope ratio R at the chemical equilibrium in phase A and B.

$$\alpha = C_R/C_R = \left[C_{h/l}\right]A/\left[C_{h/l}\right]B \tag{2}$$

Kinetic stable isotope fractionation is a fractionation process by which stable isotopologues are separated by their mass during unidirectional (bio)chemical reactions. Most biological, enzyme-catalyzed reactions such as hydrocarbon degradation are unidirectional with a kinetic component leading to kinetic isotope fractionation. The isotope fractionation due to bond changes in (bio)chemical unidirectional reactions can be described as kinetic isotope effect (KIE) (Bigeleisen and Wolfsberg 1958; Simon and Palm 1966; Van Hook 2011). KIEs depend on rate differences for the cleavage of chemical bonds formed by light (e.g., ^{1}H, ^{12}C) or heavy isotopes (e.g., ^{2}H, ^{13}C) of elements. Similarly, the isotope fractionation factor α has been introduced as the inverse of the KIE:

$$\alpha = \frac{1}{KIE} = \frac{^{heavy}r/^{heavy}C}{^{light}r/^{light}C} \tag{3}$$

where r and C are the reaction rates and concentrations of reactants containing the light or the heavy isotope. For the simplified case of first-order reaction kinetics, α and KIE can be expressed as:

$$\alpha = \frac{1}{KIE} = \frac{^{heavy}k}{^{light}k} \tag{4}$$

with *k* as the first-order rate parameters for the light and the heavy isotope fraction.

1.3.1 Calculation of Isotope Enrichment Factors

Stable isotope fractionation is quantified by the Rayleigh equation by which the changes in isotope composition and concentration occurring during a process are correlated (Eq. 5). Isotope fractionation factors describing the correlation between changes in concentration and isotope composition can be assessed applying the simplified Rayleigh equation for calculating the isotope enrichment factor (ε_{bulk}).

$$\frac{R_t}{R_0} = \frac{C_t^{\varepsilon_{bulk}}}{C_0} = f^{\varepsilon_{bulk}} \tag{5}$$

R_t and R_0 are the stable isotope ratios at time t and time 0, C is the concentration of the target compound at time t and time 0, the ratio is defined as the reaction

progress f, and ε is the enrichment factor, correlating changes in concentration to changes in the isotope composition (Dorer et al. 2014b). The isotope composition δ determined is used for calculation of the bulk isotope fractionation factor (ε) considering all atoms of an element (bulk) of a substrate:

$$\ln\left(\frac{\delta_t + 1}{\delta_0 + 1}\right) = \varepsilon_{bulk} * \ln\left(\frac{C_t}{C_0}\right) \tag{6}$$

δ represents the measured isotope ratio in ‰ and C the concentration of a substrate at the starting time of the degradation measurement (subscript 0) and at the following measuring points at time t (subscript t). In Box 1 is summarized how carbon and hydrogen enrichment factors and lambda plots (see Sect. 1.3.3) are calculated.

Box1: Calculation of C and H stable isotope fractionation and lambda (Λ) plots
Stable isotope fractionation is described by the Rayleigh equation:

$$\left(\frac{R_t}{R_0}\right) = \left(\frac{\frac{C_t}{C_0}}{\frac{R_t+1}{R_0+1}}\right)^{\varepsilon} \tag{11}$$

For low-abundance heavy stable isotopes is $R_t + 1 \sim 1$, therefore:

$$\left(\frac{R_t}{R_0}\right) = \left(\frac{C_t}{C_0}\right)^{\varepsilon} \tag{12}$$

Using the delta notation, the Rayleigh equation forms to:

$$\frac{(\delta_t + 1)}{(\delta_0 + 1)} = \left(\frac{C_t}{C_0}\right)^{\varepsilon} \tag{13}$$

Bulk enrichment factors (ε_{bulk}) are calculated by:

$$\ln\frac{(\delta_t + 1)}{(\delta_0 + 1)} = \varepsilon_{bulk}\ln\left(\frac{C_t}{C_0}\right) \tag{14}$$

and can be obtained by plotting $\ln(R_t/R_0)$ versus $\ln(C_t/C_0)$ shown in (B) for carbon and (C) for hydrogen.

In dual isotope analysis, the changes of carbon ($\Delta\delta^{13}C$) versus hydrogen ($\Delta\delta^{2}H$) isotope values are plotted resulting in the lambda (Λ) value as shown in (D).

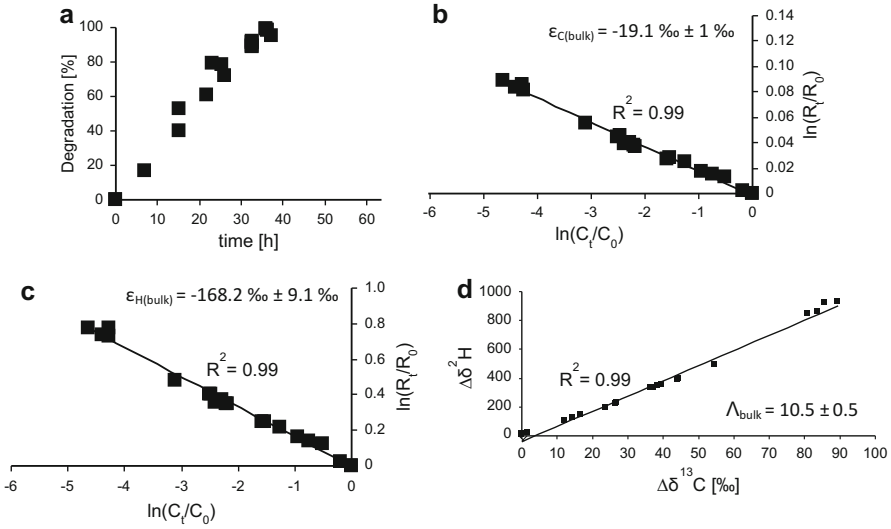
(*continued*)

Box1: Calculation of C and H stable isotope fractionation and lambda (Λ) plots (continued)

$$\Lambda_{\text{bulk}} = \frac{\Delta\delta^2\text{H}}{\Delta\delta^{13}\text{C}} \approx \frac{\varepsilon\text{H}}{\varepsilon\text{C}} \tag{15}$$

Exemplary data are shown for methane oxidation by *Methylocystis parvus* (modified from Feisthauer et al., 2011). The time course of methane oxidation is shown in (A).

Modified from Musat et al. (2016).



1.3.2 Calculation of the Apparent Kinetic Isotope Effect (AKIE)

For a mechanistic interpretation of isotope discrimination per reacting position, the enrichment factors have to be converted to apparent kinetic isotope effects (AKIEs) by which isotope effects of bond cleavage reactions can be compared. In a first step, the enrichment factor of the bond change at the reactive position is calculated (ε_{rp}). For normalizing the fractionation to bond changes at the reactive position, a correction for the total number of atoms (n) in nonreactive positions of an element is included assuming that these atoms do not change the isotope composition. x is the total number of atoms of an element in the reactive position.

$$\ln\left(\frac{\left(\delta_0 + \frac{\text{n}}{\text{x}}(\delta_\text{t} - \delta_0) + 1\right)}{\delta_0 + 1}\right) = \varepsilon_{\text{rp}} * \ln\left(\frac{\text{C}_\text{t}}{\text{C}_0}\right) \tag{7}$$

δ_0 is the isotope ratio in ‰ and C_0 the concentration of the target compound at the beginning of the measurement of the degradation process. δ_t and C_t describe the isotope ratio and the concentrations at certain time points during the reaction. ε_{rp} gives the enrichment factor at the reactive position. Whereas the KIE quantifies the intrinsic isotope effects of bond change reactions, the AKIE is calculated from experimental data and resembles the transition state of the first isotopically sensitive reaction step. Notably, AKIEs can be influenced by the rate limitation of preceding steps typical for biological reaction (see below). The AKIE is calculated using the ε_{rp} normalized for the number of atoms (z) of an element in identical reactive positions (Elsner et al. 2005):

$$\mathrm{AKIE} = \frac{1}{z * \varepsilon_{rp} + 1} \tag{8}$$

1.3.3 Calculation of Lambda (Λ)

The factor Λ is calculated by correlating the magnitude of carbon and hydrogen isotope fractionation; Λ is used diagnostically for characterizing the mechanism of C-H bond cleavage in the initial step of the hydrocarbon degradation pathway (Fischer et al. 2008; Fischer et al. 2007). Λ can be calculated via the slope of the linear correlation when plotting $\Delta\delta^2H$ versus $\Delta\delta^{13}C$ isotope values

$$\Lambda = \frac{\Delta\left(\delta^2\mathrm{H}\right)}{\Delta\left(\delta^{13}\mathrm{C}\right)} = \frac{\left(\delta_t^2\mathrm{H} - \delta_0^2\mathrm{H}\right)}{\left(\delta_t^{13}\mathrm{C} - \delta_0^{13}\mathrm{C}\right)} \tag{9}$$

or approximated by calculating the ratio of the isotope enrichment factors ε_H and ε_C (Eq. 10).

$$\Lambda \approx \frac{\varepsilon_{\mathrm{bulk,H}}}{\varepsilon_{\mathrm{bulk,C}}} \tag{10}$$

In the case of high magnitudes of isotope fractionation, which is often observed for hydrogen isotopes, Eqs. 9 and 10 should be regarded with caution. Recently, a concept was introduced for the calculation of hydrogen isotope fractionation factors and Λ values in case of very strong hydrogen isotope fractionation (Dorer et al. 2014a).

1.3.4 Definitions and Interpretation of Isotope Effects in Biological Reactions

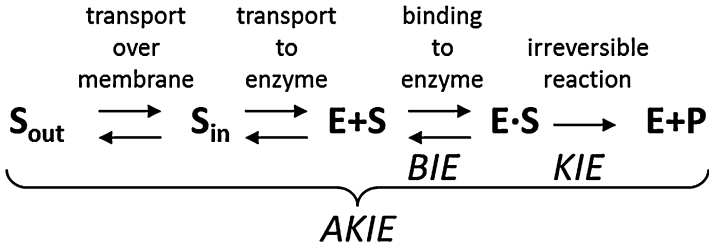
The definition of isotope effects and the theory of isotope fractionation in biological reactions have been summarized elsewhere (Swiderek and Paneth 2013). As discussed above, the KIE describes the differences in kinetic rates of bond changes of a chemical bond formed by isotopologues in an unidirectional reaction, thus characterizing the relative stability of bonds formed by heavy stable isotopes compared to light stable isotopes. The primary KIE is defined as the rate limitation of bond change of the cleavage or formation of an isotopologue substituted chemical

bond (Melander and Saunders 1980; Wolfsberg et al. 2009). A primary KIE can be substantial. Regarding the cleavage of C-H bonds in hydrocarbons, much higher KIEs can be reached for stable hydrogen isotopes ($^{2}H/^{1}H$) compared to stable carbon isotopes ($^{13}C/^{12}C$) due to the considerable larger mass difference of hydrogen vs. carbon isotopes (Elsner et al. 2005).

Secondary KIEs are observed when an adjacent bond to the atom of bond change is isotopically substituted and affects the rate of bond change in the rate-determining step of a reaction; the isotopic substitution is however not directly involved in bond change (Westaway 2006). Secondary KIEs are usually much smaller than primary KIEs except for hydrogen for which larger secondary KIEs effects can be observed due to the large mass difference between hydrogen and deuterium as discussed above (Elsner et al. 2005).

The concept for mechanistic interpretations of isotope effects in biological reactions was established making use of the transition state theory and considerations for rate limitations in biological reactions (Northrop 1981). The experimentally determined AKIEs in biological systems are considered to contain information regarding the isotope-sensitive mode of bond cleavage and all steps prior to commitment of catalysis which need to be taken into account when interpreting isotope fractionation pattern of biological reactions (Scheme 1).

In homogeneous chemical reactions, the AKIE characterizes the mode of bond change in the transition state. If kinetic rate limitation of preceding steps prior to the reaction can be neglected, the AKIE is similar to the KIE; in this case, the cleavage of the chemical bond is solely rate-limiting, and the stability of the isotopologue-substituted bond species determines the magnitude of rate limitation. In contrast, in complex biological systems, the AKIE can be affected by rate limitations prior to enzymatic catalysis, resulting in masking of the KIE and thus lowering of the AKIE (Northrop 1981). Rate-limiting steps can be uptake into the cell, transport within the cell, or binding to the enzyme (Scheme 1) (Nijenhuis and Richnow 2016). Hence, in a simplified model for biological reactions, the last step is the isotope-sensitive bond change after which the reaction becomes irreversible (commitment to catalysis), and all rate limitations of previous reaction steps modify (mostly lower) the KIE of the bond change. However, biological reactions may not always be completely



Scheme 1 Model for kinetic steps governing rate limitations in biological reactions. S_{out} substrate concentration outside the cell, S_{in} substrate concentration inside the cell, *E* enzyme, *P* product, *BIE* binding isotope effect, *KIE* kinetic isotope effect, *AKIE* apparent kinetic isotope effect. (Taken from Nijenhuis and Richnow 2016)

unidirectional in a strict sense (Northrop 1981). Reversal reactions are promoting a certain isotope equilibrium between chemical species of the forward and back reaction leading to an equilibrium isotope effect (EIE). EIEs are produced by physical processes or chemical reactions that reach an equilibrium. The EIE describes the equilibrium constants for isotopologues present in two phases, for example, the reversible binding of a ligand to a receptor (Swiderek and Paneth 2013), and could be thus relevant in reactions in which a ligand binds to the binding pocket of an enzyme. This isotope effect in enzyme reactions has been termed binding isotope effects (BIE) (Swiderek and Paneth 2013).

1.4 Compound-Specific Isotope Analysis (CSIA)

A GC connected to an isotope mass spectrometer via an interface for oxidation to CO_2 or thermal conversion to H_2 is typically used for CSIA for the analysis of ^2H and ^{13}C isotope composition of hydrocarbons in mixtures. For carbon isotope analysis, the interface consists of an oven in which the bound carbon is completely oxidized to CO_2; subsequently, the mass spectrometer monitors the isotope composition of the produced CO_2. The analytical methods for compound-specific ^2H and ^{13}C isotope analysis have been summarized elsewhere (Elsner et al. 2012). The sensitivity of modern CSIA is limited to about 30 nmol H and 0.8 nmol C for reliable isotope values. Compounds present in a mixture need to be separated before analysis, typically achieved by a baseline separation in a GC system. The compounds need to be completely oxidized to CO_2, and the oxidation reaction requires being free of any isotope fractionation. For carbon isotope analysis, the oxidation to CO_2 is achieved in a combustion interface typically consisting of a ceramic reactor tube filled with oxidized copper as oxygen source and/or nickel metal wires or platinum wires as catalyzer.

For hydrogen isotope analysis, hydrocarbons are converted to H_2 gas by high temperature conversion (1400–1450 °C) (Brenna et al. 1997). The interface for high temperature conversion consists of an empty ceramic tube or a ceramic tube filled with elementary chromium to support reduction. Chromium is sometimes used for reduction of compounds to release H_2 and to bind oxygen, sulfur, or halogens if present in mixtures containing the analyte (Gehre et al. 2015; Renpenning et al. 2015). Hydrogen is the only product leaving the chromium reactor; carbon is bound as elementary carbon or chromium carbides. For ^2H analysis of hydrocarbons, the conversion at high temperature is sufficient for determining reliable ^2H values even when low-concentrated hydrogen-containing side products are formed.

1.5 Isotope Fractionation Studies for Monitoring In Situ Degradation of Hydrocarbons

In the last decade, CSIA has been established as a crucial method for confirming and monitoring of hydrocarbon biodegradation at contaminated sites, due to the growing acceptance of monitored natural attenuation (MNA) and enhanced natural attenuation (ENA) as remediation technologies for the management of point pollutions

(US-EPA 2008; Vogt and Richnow 2014; Wiedemeier et al. 1999). MNA and ENA require the prediction of biodegradation over long time periods; thus the assessment of factors governing biodegradation is essential. Hydrocarbons present in surface water bodies, sediments, soils, or aquifers are affected by biochemical transformation processes (biodegradation) as well as physicochemical dispersal processes, e.g., dilution, sorption, or volatilization; the latter are generally not accompanied by the cleavage of chemical bonds and thus do not cause considerable isotope effects (Hunkeler and Elsner 2010; Kopinke et al. 2017; Kopinke et al. 2005). The carbon-hydrogen bond is relatively inert to purely chemical reactions (although advanced in situ oxidation techniques employing persulfate or OH radical reactions can also lead to destruction of hydrocarbons, resulting in isotope fractionation). Therefore, the magnitude of isotope fractionation can be usually used as a proxy for biodegradation. Recent reviews describe in detail the application of CSIA with regard to MNA and ENA, explaining the detection of distinct degradation pathways by multi-element compound-specific stable isotope analysis (ME-CSIA) (Vogt et al. 2016) or other stable isotope-based tools (Fischer et al. 2016), the quantification of in situ biodegradation of organic pollutants in contaminated aquifers using the Rayleigh equation (Thullner et al. 2012), and the effects of mass transfer-related processes on the bioavailability of hydrocarbons and thus on the rate of hydrocarbon degradation and magnitude of isotope fractionation (Thullner et al. 2013).

1.6 Hydrocarbon Transformation Reactions

Several different enzymatic reactions acting on hydrocarbons as substrates have been described. Most reactions include a cleavage of a C-H bond which is usually characterized by high bond dissociation energies (Blanksby and Ellison 2003). In case of a cleavage of a C-H bond, four different principal reaction mechanisms can be differentiated (Lewis et al. 2011): electrophilic aromatic substitution, electron abstraction followed by radical coupling, hydrogen abstraction followed by radical recombination, and hydrogen abstraction followed by radical reaction. For accomplishing these reactions, the responsible enzymes generally contain cofactors. The reactions take place either by using O_2 as co-substrate (aerobic reactions) or without involvement of O_2 (anaerobic reactions).

Aerobic degradation of hydrocarbons is known for more than a century (Söhngen 1913). Hydroxylation of aliphatic and aromatic hydrocarbons is carried out by intracellular mono- or dioxygenases introducing one or two hydroxyl groups to the hydrocarbon molecule, respectively (Fig. 1). Most oxygenases use O_2 as a source for substrate hydroxylation (Ullrich and Hofrichter 2007). Until the beginning of 1980, anaerobic biodegradation of hydrocarbons was regarded as negligible (Atlas 1981). Nowadays, four principally different hydrocarbon transformation reactions have been identified, acting either at aromatic or aliphatic hydrocarbons: carboxylation, fumarate addition, hydroxylation with water, and reactions with heterodisulfide (Fig. 2).

The reactions listed in Table 1 are the initial steps of different degradation pathways resulting in carbon assimilation or mineralization of hydrocarbons. As

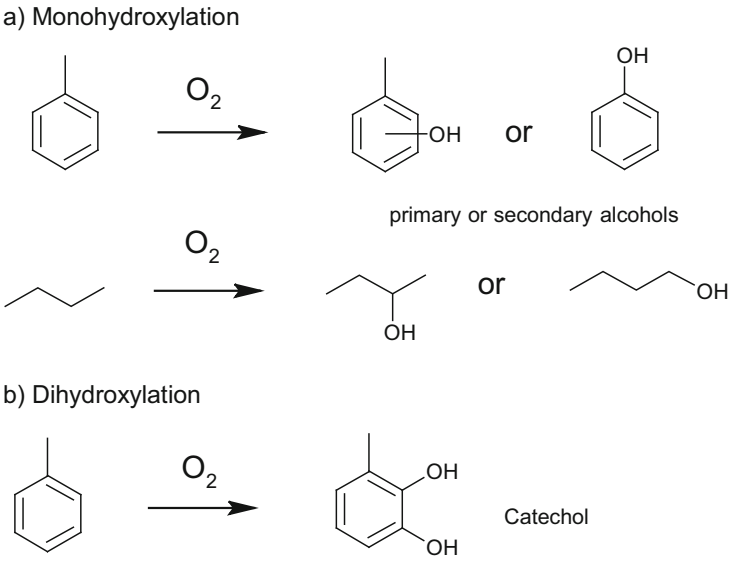


Fig. 1 Initial reactions for activation of hydrocarbons under aerobic conditions. (**a**) Monohydroxylations are catalyzed by monooxygenases incorporating dioxygen (O_2) into the substrate, acting on the aromatic ring, alkyl groups of aromatics, or alkanes of various chain lengths, predominantly at C1 or C2 position; examples are shown for toluene and butane as substrates. (**b**) Dihydroxylation of aromatic rings are catalyzed by dioxygenases leading to catechols as products; an example is shown for toluene

CSIA in environmental studies usually targets the isotope pattern of the residual hydrocarbon substrate, the isotope fractionation associated with these reactions is playing the crucial role for assessing the potential of CSIA to detect and quantify biodegradation as well as to identify specific biodegradation pathways (see Vogt et al. 2016 for a review). In the following chapters, the characteristics of the different reaction mechanisms are briefly described, and the corresponding carbon and hydrogen isotope enrichment factors and lambda values determined so far are given (Table 1).

2 Reactions and Fractionation Factors

2.1 Aerobic Reactions

Classifying aerobic transformation reactions of hydrocarbons is not straightforward as oxygenases are classified in several enzyme families due to the variety of reaction mechanisms and involved cofactors, prosthetic groups, and metal ions (Lewis et al. 2011; Ullrich and Hofrichter 2007). In this review, we discuss oxygenases according to the number of hydroxyl groups introduced, thus monooxygenases (introduction of a single hydroxyl group by O_2) and dioxygenases (introduction of two hydroxyl group by O_2).

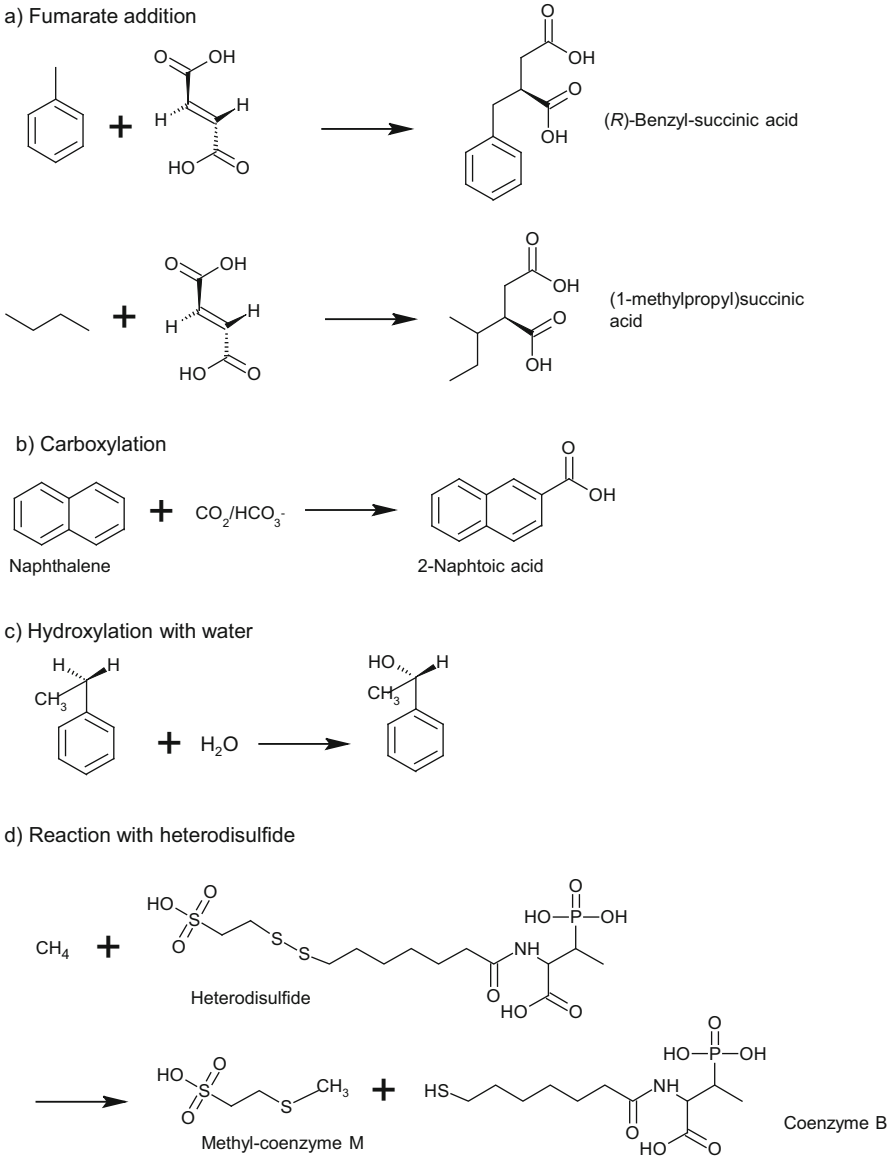


Fig. 2 Initial reactions for activation of hydrocarbons under anoxic conditions. (**a**) Fumarate addition is catalyzed by various sulfate reducers or syntrophic fermenters containing different fumarate-adding enzymes (FAEs) acting on alkylated aromatics (Heider et al. 2016a) or alkanes of various chain lengths (Heider et al. 2016a; Wilkes et al. 2016), predominantly at subterminal C2 position (Wilkes et al. 2016); examples are shown for toluene and butane as substrates. (**b**) Carboxylation of naphthalene has been biochemically verified for the sulfate-reducing strain N47 (Mouttaki et al. 2012). Metabolite studies indicate that phenanthrene is as well activated under sulfate-reducing conditions by carboxylation (Davidova et al. 2007; Zhang and Young 1997). (**c**) Hydroxylation of the ethyl side chain of ethylbenzene catalyzed by ethylbenzene dehydrogenase. (**d**) Activation of methane by reversed methanogenesis (Thauer and Shima 2008)

Table 1 Overview of carbon (ε_{bulkC}) and hydrogen (ε_{bulkH}) enrichment factors, apparent kinetic isotope effects ($AKIE_{C,\ H}$), and lambda values ($\Lambda \approx \varepsilon_{bulkH} / \varepsilon_{bulkC}$) for different hydrocarbon activation reactions

Reaction	Enzyme (organism)	Reference for enzymatic reaction	Substrate and electron acceptor conditions	ε_{bulkC}	$AKIE_c$	ε_{bulkH}	$AKIE_H$	Lambda (Λ)	Reference for isotope values
Dihydroxylation of the aromatic ring	Dioxygenase (*Rhodococcus opacus* B-4)	Na et al. (2005)	Benzene, aerobic	−1.3 ± 0.2	1.004	n.d.	0.985–1.015	± 5	Fischer et al. (2008)
	Dioxygenase (*Pseudomonas putida* ML2)	Axcell and Geary (1975)	Benzene, aerobic	−0.7 ± 0.1	1.002	n.d.	0.985–1.015	± 7	Fischer et al. (2008)
	Dioxygenase (*Rhodococcus opacus* B-4)	Na et al. (2005)	Toluene, aerobic	−1.8 ± 0.3	1.006	−2 ± 5	1.011 ± 0.032	1 ± 2	Vogt et al. (2008)
	Dioxygenase (*Pseudomonas putida* F1)	Yeh et al. (1977)	Toluene, aerobic	−0.4 ± 0.3	1.001	−28 ± 10[a]	1.029 ± 0.01	n.d.[b]	Morasch et al. (2002)
	Dioxygenase (*Pseudomonas putida* F1)[c]	Gibson et al. (1973)	Ethylbenzene, aerobic	−0.5 ± 0.1	1.002	4 ± 3	1.0	−7 ± 3	Dorer et al. (2014b)
	Naphthalene dioxygenase	Jeffrey et al. (1973)	Naphthalene, aerobic	−0.8 ± 0.1	1.004–1.008[d]	54 ± 4	0.699–0.832[d]	−18 ± 2	Kümmel et al. (2016)
	(*Pseudomonas putida* NCIB 9816)		Naphthalene, aerobic	−0.1 ± 0.2		−65 ± 13[a]		n.d.[b]	Morasch et al. (2002)
	Naphthalene dioxygenase (*Pseudomonas fluorescens* ATCC 17483)	Jeffrey et al. 1973	Naphthalene, aerobic	−1.6 ± 0.2	1.007–1.014[c]	21 ± 3	0.859-0.924[c]	9 ± 1	Kümmel et al. (2016)
	Naphthalene dioxygenase (*Pseudomonas putida* NCIB 9816)	Resnick et al. (1996)	2-Methylnaphthalene, aerobic	−1.3 ± 0.2	1.011–1.022[c]	71 ± 6	0.541–0.702[c]	−30 ± 5	Kümmel et al. (2016)

(*continued*)

Table 1 (continued)

Reaction	Enzyme (organism)	Reference for enzymatic reaction	Substrate and electron acceptor conditions	ε_{bulkC}	$AKIE_c$	ε_{bulkH}	$AKIE_H$	Lambda (Λ)	Reference for isotope values
	Naphthalene dioxygenase (*Pseudomonas fluorescens* ATCC 17483)	Lessner et al. (2002)	2-Methylnaphthalene, aerobic	−1.2 ± 0.2	1.007–1.014[c]	15 ± 2	0.813-0.897[c]	−15 ± 1	Kümmel et al. (2016)
	Nitrobenzene dioxygenase (from *Comamonas sp.* JS765 expressed in *E. coli*, Pati et al. (2016))		Naphthalene, aerobic	−0.4 ± 0.3	1.004	−15 ± 1	1.12	40 ± 28	Pati et al. (2016)
Monohydroxylation of the aromatic ring	Toluene 3-monooxygenase (*Ralstonia pickettii* PKO1, di-iron hydroxylase)	Tao et al. (2004)	Benzene, aerobic	−1.7 ± 0.2	1.005-1.01[c]	−11 ± 4	1.036–1.071[c]	3 ± 1	Fischer et al. (2008)
			Toluene, aerobic	−1.1 ± 0.2	1.008	−16 ± 5[a]	1.016	n.d.[b]	Morasch et al. (2002)
	Monooxygenase (*Cupriavidus necator* ATCC 17697)	Fischer et al. (2008)	Benzene, aerobic	−4.3 ± 0.4	1.013–1.026[d]	−17 ± 11	1.057–1.114[d]	11 ± 6	Fischer et al. (2008)
	Benzene monooxygenase (*Alicycliphilus denitrificans* BC)	Weelink et al. (2008)	Benzene, aerobic	−2.6 ± 0.8	1.008–1.016[d]	−16 ± 4	1.053–1.106[d]	5 ± 2	Fischer et al. (2008)
	Benzene monooxygenase (*Alicycliphilus denitrificans* BC)	Weelink et al. (2008)	Benzene, chlorate-reducing (intra-aerobic)	−1.5 ± 0.5	1.005 to 1.009[b]	−18 ± 6	1.061 ± 1.121[b]	10 ± 4	Fischer et al. (2008)
	Monooxygenase (enrichment culture[e])	Dorer et al. (2014b)	Ethylbenzene, aerobic	−0.4 ± 0.1	1.003	−2 ± 3	1.0	5 ± 6	Dorer et al. (2014b)
	Monooxygenase (*Pseudomonas fluorescens* SK1[e])	Dorer et al. (2014b)	Ethylbenzene, aerobic	−0.6 ± 0.1	1.005	−6 ± 1	1.1	9 ± 3	Dorer et al. (2014b)

Monohydroxylation of the alkyl group (reaction with molecular oxygen)	Phenol hydroxylase (*Acinetobacter calcoaceticus* NCIMB 8250)	Harayama et al. (1989)	Phenol, aerobic	−1.5 ± 0.1	1.009	n.a.	n.a.	–	Wei et al. (2016)
			p-Cresol, aerobic	−1.4 ± 0.1	1.01	n.a.	n.a.	–	Wei et al. (2016)
	Xylene monooxygenase (*Pseudomonas putida* mt-2, di-iron hydroxylase)		Toluene, aerobic	−2.8 ± 0.2	1.02	−140 ± 8	6.5	53 ± 5	Vogt et al. (2008)
			Toluene, aerobic	−1.8 ± 0.2 to −2.5 ± 0.3[e]	1.013–1.018[e]	−97 ± 5 to −159 ± 11[e]	n.a.	54 ± 4 to 68 ± 5[e]	Mancini et al. (2006)
			Toluene, aerobic	−3.3 ± 0.3	1.024	−905 to −956[a]	10.5–27.7	n.d.[b]	Morasch et al. (2002)
			m-Xylene, aerobic	−1.7 ± 0.04	1.014	n.a.	n.a.	–	Morasch et al. (2002)
			p-Xylene, aerobic	−2.3 ± 0.3	1.019	n.a.	n.a.	–	Morasch et al. (2002)
	Toluene monooxygenase (*Cladosporium sphaerospermum* T0, cytochrome P450)	Luykx et al. (2003)	Toluene, aerobic	−0.4 ± 0.2	1.0004	−8.6 ± 3.7	1.026	16 ± 6	Vogt et al. (2008)
	Particulate methane monooxygenase, pMMO (*Methylococcus capsulatus* Bath)	Zahn and DiSpirito (1996)	Methane, aerobic	−27.9 ± 1.7	1.029	−232 ± 30	13.5	8.7 ± 0.8	Feisthauer et al. (2011)
	pMMO (*Methylosinus sporium* 5)	Pilkington and Dalton (1991)	Methane, aerobic	−18.8 ± 1.4	1.019	−137 ± 20	2.2	7.7 ± 0.4	Feisthauer et al. (2011)
	pMMO (*Methylocystis parvus* OBBP)	Murrell et al. (2000)	Methane, aerobic	−19.1 ± 1	1.019	−168 ± 9	3.1	10.5 ± 0.5	Feisthauer et al. (2011)
	pMMO (*Methylomonas methanica* S1)	Colby et al. (1975)	Methane, aerobic	−27.7 ± 2.3	1.028	−226 ± 18	10.2	8 ± 0.3	Feisthauer et al. (2011)
	pMMO (*Methylocaldum gracile* 14L)	Bodrossy et al. (1997)	Methane, aerobic	−14.8 ± 0.9	1.015	−110 ± 12	1.8	7.3 ± 0.3	Feisthauer et al. (2011)
	pMMO (*Candidatus Methylomirabilis oxyfera*	Welte et al. (2016)	Methane, nitrate-reducing (intra-aerobic)	−29.2 ± 2.6		−227.6 ± 13.5		9.8	Rasigraf et al. (2012)

(*continued*)

Table 1 (continued)

Reaction	Enzyme (organism)	Reference for enzymatic reaction	Substrate and electron acceptor conditions	ε_{bulkC}	$AKIE_c$	ε_{bulkH}	$AKIE_H$	Lambda (Λ)	Reference for isotope values
	Soluble methane monooxygenase, sMMO (*Methylococcus capsulatus* Bath)	Colby and Dalton (1978)	Methane, aerobic	−22.9 ± 3.2	1.023	−192 ± 29	4.3	9.3 ± 0.5	Feisthauer et al. (2011)
	sMMO (*Methylosinus sporium* 5)	Pilkington and Dalton (1991)	Methane, aerobic	−21.5 ± 2.7	1.022	−183 ± 24	3.7	9.5 ± 0.7	Feisthauer et al. (2011)
Hydroxylation with water at the alkyl group	Ethylbenzene dehydrogenase (*Aromatoleum aromaticum* EbN1)	Kniemeyer and Heider (2001)	Ethylbenzene, nitrate-reducing	−3.8 ± 0.1	1.03	−111 ± 7	3.9	19.1 ± 1[f]	Dorer et al. (2014a, b)
			Ethylbenzene, manganese-reducing	−1.6 ± 0.1	1.013	−50 ± 4	1.6	23 ± 1[f]	Dorer et al. (2016)
	Ethylbenzene dehydrogenase (*Georgfuchsia toluolica* G5G6)	Dorer et al. (2014b)	Ethylbenzene, nitrate-reducing	−4.1 ± 0.2	1.033	−111 ± 12	4.4	18 ± 1[f]	Dorer et al. (2014b)
			Ethylbenzene, manganese-reducing	−1.7 ± 0.2	1.013	−53 ± 10	1.6	22 ± 2[f]	Dorer et al. (2016)
			Ethylbenzene, iron-reducing	−2.4 ± 0.2	1.02	−65 ± 13	2	23 ± 1[f]	Dorer et al. (2016)
	Ethylbenzene dehydrogenase (enrichment culture dominated by *Azoarcus*)	Dorer et al. (2014b)	Ethylbenzene, nitrate-reducing	−3.7 ± 0.1	1.029	−112 ± 6	4.2	22 ± 1[f]	Dorer et al. (2014b)
			Ethylbenzene, manganese-reducing	−1.3 ± 0.4	1.01	−78 ± 10	1.5	31 ± 9[f]	Dorer et al. (2016)
	p-Cresol methyl hydroxylase (*Pseudomonas pseudoalcaligenes* NCIMB 9867)	Hopper and Taylor (1975)	*p*-Cresol, aerobic	−2.3 ± 0.2	1.016	n.a.	n.a.	–	Wei et al. (2016)
								–	
	p-Cresol methyl hydroxylase (*Geobacter metallireducens* GS-15)	Peters et al. (2007a)	*p*-Cresol, nitrate-reducing	−3.6 ± 0.4	1.026	n.a.	n.a.	n.a.	Wei et al. (2016)

Fumarate addition	Benzylsuccinate synthase, BSS (*Thauera aromatica* DSM 6984)	Biegert et al. (1996)	Toluene, nitrate-reducing	−2.7 ± 0.1	1.019	−35 ± 14	1.4	11 ± 5	Vogt et al. (2008)
				−1.7 ± 0.1	1.012	n.a.	n.a.	n.a.	Meckenstock et al. 1999
	BSS (crude cell extract from *Thauera aromatic*a DSM 6984)	Biegert et al. (1996)	Toluene, enzyme assay	−2.9 ± 0.4	1.021	−35 ± 11	1.39	11 ± 3	Kümmel et al. (2013)
	BSS (*Azoarcus sp.* T	Beller and Spormann (1997)	Toluene, nitrate-reducing	−5.6 ± 0.3 to −6.2 ± 1.1	1.038–1.044	−78 ± 12 to −79 ± 21	2.2–2.4	11 ± 3 to 15 ± 2	Vogt et al. (2008)
	BSS (crude cell extract from *Azoarcus sp.* T)	Beller and Spormann (1997)	Toluene, enzyme assay	−2.8 ± 0.7	1.02	−38 ± 5	1.44	15 ± 2	Kümmel et al. (2013)
	BSS (*Aromatoleum aromaticum* EbN1)	Rabus and Heider (1998)	Toluene, nitrate-reducing	−2.9 ± 0.2 to 3.8 ± 0.5	1.02 to 1.027	−45 ± 15 to −58 ± 10	1.5–1.8	14 ± 1 to 14 ± 4	Vogt et al. (2008)
	BSS (*Geobacter metallireducens* GS-15)	Kane et al. (2002)	Toluene, iron-reducing	−1.8 ± 0.3	1.013	n.a.	n.a.	n.a.	Meckenstock et al. (1999)
				−1 ± 0.1	1.007	n.a.	n.a.	n.a.	Tobler et al. (2007)
				−1.3 ± 0.1 to −3.6 ± 0.7[g]	1.009–1.025[g]	−35 ± 0.9 to −98 ± 3[g]	1.35–1.96[g]	26.6–28.6[h]	Tobler et al. (2008)
	BSS (crude cell extract from *Geobacter metallireducens* DSM 7210)	Kane et al. (2002)	Toluene, enzyme assay	−1.8 ± 0.4	1.013	−55 ± 8		−26 ± 3	Kümmel et al. (2013)
	BSS (*Desulfosarcina cetonica* DSM 7267)	Morasch et al. (2001)	Toluene, sulfate-reducing	−2.3 ± 0.2 to −2.6 ± 0.2	1.016–1.018	−68 ± 5 to −80 ± 14	1.9–2.3	28 ± 4 to 30 ± 2	Vogt et al. (2008)
				−2.2	1.016	n.a.	n.a.	n.a.	Morasch et al. (2001)
	BSS (Strain TRM1)	Heider et al. (2016a)	Toluene, sulfate-reducing	−2 ± 0.2	1.014	−66 ± 19	1.8	31 ± 11	Vogt et al. (2008)
				−1.7	1.012	n.a.	n.a.	n.a.	Meckenstock et al. (1999)

(*continued*)

Table 1 (continued)

Reaction	Enzyme (organism)	Reference for enzymatic reaction	Substrate and electron acceptor conditions	ε_{bulkC}	$AKIE_c$	ε_{bulkH}	$AKIE_H$	Lambda (Λ)	Reference for isotope values
	Putative BSS (*Desulfosarcina ovata*)	Herrmann et al. (2009)	Toluene, sulfate-reducing	−2.5 ± 0.5	1.017	−107 ± 23	3.4	41 ± 8	Herrmann et al. (2009)
	Putative BSS (enrichment culture)	Vogt et al. (2008)	Toluene, sulfate-reducing	−2.8 ± 0.1	1.019	−87 ± 6	3.1	28 ± 2	Vogt et al. (2008)
	BSS (enrichment culture)	Herrmann et al. (2009)	Toluene, sulfate-reducing	−6.7 ± 0.4	1.046	−126 ± 9	4.7	20 ± 2	Herrmann et al. (2009)
	Putative BSS (enrichment culture)	Ahad et al. (2000)	Toluene, sulfate-reducing	−0.8	1.006	n.a.	n.a.	n.a.	Ahad et al. (2000)
	Putative BSS (soil column)	Richnow et al. (2003)	Toluene, sulfate-reducing	−1.5	1.011	n.a.	n.a.	n.a.	Richnow et al. (2003)
	BSS (enrichment culture)	Beller and Edwards (2000)	Toluene, methanogenic	−0.5	1.004	n.a.	n.a.	n.a.	Ahad et al. (2000)
	BSS (*Blastochloris sulfoviridis*)	Zengler et al. (1999)	Toluene, phototrophic	−4 ± 0.5	1.029	−58 ± 14	1.2	4 ± 3	Vogt et al. (2008)
	BSS (*Azoarcus sp.* T)	Beller and Spormann (1997)	*m*-Xylene, nitrate-reducing	−2.3 ± 0.1	1.018	−50 ± 6	1.93	21 ± 2	Herrmann et al. (2009)
	Fumarate-adding enzyme, FAE (enrichment culture)	Herrmann et al. (2009)	*m*-Xylene, sulfate-reducing	−2.7 ± 0.2	1.022	−48 ± 5	1.84	16 ± 2	Herrmann et al. (2009)
	FAE (strain OX39)	Morasch et al. (2004)	m-Xylene, sulfate-reducing	−1.8	1.015	n.a.	n.a.	n.a.	Morasch et al. (2004)
	Putative FAE (*Desulfosarcina ovata*)	Herrmann et al. (2009)	*o*-Xylene, sulfate-reducing	−2.3 ± 0.4	1.018	-41 ± 9	1.65	15 ± 4	Herrmann et al. (2009)
	FAE (enrichment culture)	Herrmann et al. (2009)	*o*-Xylene, sulfate-reducing	−0.7 ± 0.1	1.006	−25 ± 3	1.32	29 ± 5	Herrmann et al. (2009)

	FAE (strain OX39)	Morasch et al. (2004)	*o*-Xylene, sulfate-reducing	−1.5	1.012	n.a.	n.a.	n.a.	Morasch et al. (2004)
	Putative FAE (soil column)	Richnow et al. (2003)	*o*-Xylene, sulfate-reducing	−1.1	1.009	n.a.	n.a.	n.a.	Richnow et al. (2003)
	FAE (enrichment culture)	Herrmann et al. (2009)	*p*-Xylene, sulfate-reducing	−1.2 ± 0.3	1.01	−19 ± 3	1.23	12 ± 4	Herrmann et al. (2009)
	Putative FAE (enrichment culture)	Dorer et al. (2014b)	Ethylbenzene, sulfate-reducing	−0.7 ± 0.3	1.006	−96 ± 7	1.8	168 ± 19	Dorer et al. (2014b)
	Putative FAE (enrichment culture)	Dorer et al. 2014b	Ethylbenzene, anoxic	−0.6 ± 0.1	1.005	−76 ± 16	1.8	278 ± 123	Dorer et al. (2014b)
	FAE (*Desulfosarcina cetonica* DSM 7267)	Müller et al. 1999	*m*-Cresol, sulfate-reducing	−3.9	1.028	n.a.	n.a.	n.a.	Morasch et al. (2004)
	FAE (*Desulfosarcina cetonica* DSM 7267)	Müller et al. (1999)	*m*-Cresol, sulfate-reducing	−2.2 ± 0.3	1.016	n.a.	n.a.	n.a.	Wei et al. (2016)
	FAE (*Desulfosarcina cetonica* DSM 7267)	Müller et al. (2001)	*p*-Cresol, sulfate-reducing	−1.6	1.011	n.a.	n.a.	n.a.	Morasch et al. (2004)
	FAE (*Desulfosarcina cetonica* DSM 7267)	Müller et al. (2001)	*p*-Cresol, sulfate-reducing	−1.9 ± 0.2	1.014	n.a.	n.a.	n.a.	Wei et al. (2016)
	FAE (strain N47)	Griebler et al. (2004)	2-Methylnaphthalene, sulfate-reducing	−0.9 ± 0.1	1.01	n.a.	n.a.	n.a.	Griebler et al. (2004)
	FAE (strain BuS5)	Kniemeyer et al. (2007)	Propane, sulfate-reducing	−2.6 ± 0.6[i]	1.008[i]	−16 ± 4[i]	1.14[i]	6.3 ± 0.9[i]	Jaekel et al. (2014)
				−8.7 ± 1.1[j]	1.026[j]	−92 ± 6[j]	2.14[j]	11.9 ± 0.2[j]	Jaekel et al. (2014)
				−5.2[k]	1.016[k]	n.a.	n.a.	n.a.	Kniemeyer et al. (2007)
	FAE (enrichment culture)	Jaekel et al. (2014)	Propane, sulfate-reducing	−3.1 ± 0.4[i]	1.009[i]	−24 ± 4[i]	1.22[i]	7.9 ± 0.6[i]	Jaekel et al. (2014)
				−3.7 ± 0.5[j]	1.011[j]	−36 ± 4[j]	1.35[j]	10 ± 0.4[j]	Jaekel et al. 2014
				−5.9[k]	1.018[k]	n.a.	n.a.	n.a.	Kniemeyer et al. 2007
	FAE (enrichment culture)	Kniemeyer et al. (2007)	Propane, sulfate-reducing	−5.9[k]	1.018[k]	n.a.	n.a.	n.a.	Kniemeyer et al. (2007)

(*continued*)

Table 1 (continued)

Reaction	Enzyme (organism)	Reference for enzymatic reaction	Substrate and electron acceptor conditions	ε_{bulkC}	$AKIE_c$	ε_{bulkH}	$AKIE_H$	Lambda (Λ)	Reference for isotope values
	FAE (strain BuS5)	Jaekel et al. (2014)	*n*-Butane, sulfate-reducing	1.8 ± 0.3^{i}	1.007^{i}	-9 ± 2^{i}	1.09^{i}	4.9 ± 1.2	Jaekel et al. (2014)
				-5 ± 0.7^{j}	1.02^{j}	-32 ± 10^{j}	1.42^{j}	6.9 ± 1.2^{j}	Jaekel et al. (2014)
				-1.6^{k}	1.006^{k}	n.a.	n.a.	n.a.	Kniemeyer et al. (2007)
	FAE (enrichment culture)	Jaekel et al. (2014)	*n*-Butane, sulfate-reducing	-2 ± 0.5^{i}	1.008^{i}	-11 ± 4^{i}	1.13^{i}	5.9 ± 0.9^{i}	Jaekel et al. (2014)
				-5.6 ± 0.5^{j}	1.023^{j}	-47 ± 5^{j}	1.78^{j}	8.7 ± 0.4^{j}	Jaekel et al. (2014)
				-1.6^{k}	1.006^{k}	n.a.	n.a.	n.a.	Kniemeyer et al. (2007)
	FAE (enrichment culture)	Jaekel et al. (2014)	*n*-Butane, sulfate-reducing	0.8 ± 0.3^{i}	1.003^{i}	-5 ± 2^{i}	1.05^{i}	5.3 ± 2.4^{i}	Jaekel et al. (2014)
				-1.5 ± 0.4^{j}	1.006^{j}	-12 ± 2^{j}	1.13^{j}	7.7 ± 0.8^{j}	Jaekel et al. (2014)
	FAE (strain HxN1)	Rabus et al. (2001)	*n*-Hexane, nitrate-reducing	−2.3	1.014	n.a.	n.a.	n.a.	Vieth and Wilkes (2006)
Carboxylation	Carboxylase (enrichment culture N47)	Mouttaki et al. (2012)	Naphthalene, sulfate-reducing	-0.3 ± 0.1	1.003	-59 ± 8	1.67	29 ± 8	Bergmann et al. (2011)
	Putative carboxylase (strain NaphS2)	Musat et al. (2009)	Naphthalene, sulfate-reducing	-2 ± 0.4	1.02	-43 ± 6	1.41	107 ± 45	Bergmann et al. (2011)
	Putative carboxylase (strain NaphS6)	Musat et al. (2009)	Naphthalene, sulfate-reducing	-0.4 ± 0.3	n.d.	-11 ± 2 to -47 ± 4	1.1–1.57	n.d.	Kümmel et al. (2016)
	Putative carboxylase (enrichment culture)	Abu Laban et al. (2010)	Benzene, iron-reducing	-3 ± 0.5	1.018	-56 ± 8	1.51	17 ± 1	Bergmann et al. (2011)
	Putative carboxylase (enrichment culture)	Luo et al. (2014)	Benzene, nitrate-reducing	-2.8 ± 0.6	1.017	-47 ± 4	1.39	16 ± 2	Mancini et al. (2008)

	Phenylphosphate carboxylase (*Thauera aromatica* DSM 6984)	Schuhle and Fuchs (2004)	Phenol, nitrate-reducing	−0.7 ± 0.1	1.004	n.a.	n.a.	n.a.	Wei et al. (2016)
	Putative phenylphosphate carboxylase (*Desulfosarcina cetonica* DSM 7267)	Wei et al. (2016)	Phenol, sulfate-reducing	0.4 ± 0.1	0.998	n.a.	n.a.	n.a.	Wei et al. (2016)
Activation by coenzyme M	Reversed methanogenesis (enrichment culture)	Nauhaus et al. (2002)	Methane, sulfate-reducing	−11.9 ± 0	1.012	−100.7 ± 1.7	1.7	9.3 ± 0.2	Holler et al. (2009) and Feisthauer et al. (2011)
	Reversed methanogenesis (enrichment culture)	Nauhaus et al. (2002)	Methane, sulfate-reducing	−20.6 ± 1.3	1.021	−139.4 ± 9.4	2.3	7.7 ± 2.3	Holler et al. (2009) and Feisthauer et al. (2011)
	Reversed methanogenesis (enrichment culture)	Nauhaus et al. (2002)	Methane, sulfate-reducing	−35.7 ± 1.3	1.037	−229.6 ± 7.1	12.2	8.1 ± 0.6	Holler et al. (2009) and Feisthauer et al. (2011)

n.a. not analyzed
[a]Hydrogen isotope fractionation with mixtures (50:50) of per-deuterated and non-labeled substrate
[b]n.d. = not determined; due to the use of per-deuterated substrates
[c]Minor site reactions cannot be excluded (Dorer et al. 2014b)
[d]Depending on the reaction mechanism (stepwise or concerted)
[e]Depending on the concentration of iron ions in the growth medium (Mancini et al. 2006)
[f] calculated according to Dorer et al. (2014a)
[g]depending on the source of Fe(III)
[h]calculated by enrichment factors reported by Tobler et al. (2008)
[i]without agitation
[j]continuous agitation
[k]discontinuous agitation

The carbon and hydrogen isotope fractionation patterns differ between the two groups. Besides the classical aerobic degradation pathways, "intra-aerobic" degradation pathways have been recently reported to be active under nitrate-reducing or chlorate-reducing conditions, e.g., for the oxidation of methane (Ettwig et al. 2010; Welte et al. 2016), longer alkanes (Zedelius et al. 2011), or benzene (Weelink et al. 2008). "Intra-aerobic" means that molecular oxygen is produced by disproportionation of inorganic nitrate or chlorate species to form O_2 which is subsequently used for hydrocarbon activation. Intra-aerobic pathways are poorly investigated yet but might be of environmental relevance as a recent study suggests (Zhu et al. 2017).

2.1.1 Monooxygenation of Hydrocarbon Aromatics

Monooxygenases acting on aromatic compounds have been divided in four different types (Ullrich and Hofrichter 2007): heme-containing cytochrome P450 enzymes, di-iron hydroxylases, pterin-dependent monooxygenases, and metal-free flavin monooxygenases. The di-iron hydroxylases and the heme-containing cytochrome P450 enzymes are most relevant for the aerobic activation of aromatic hydrocarbons. Several heme-containing cytochrome P450 enzymes have been described; besides using aromatic hydrocarbons as substrates, these enzymes catalyze hydroxylations or epoxidations of a wide range of other substrates (Ullrich and Hofrichter 2007). The heme cofactor consists of a protoporphyrin IX-bound iron atom that binds and activates dioxygen which subsequently reacts with the substrate (Ullrich and Hofrichter 2007). Di-iron hydroxylases are bacterial multicomponent monooxygenases consisting of a hydroxylase, a NADH reductase, a small regulatory protein, and sometimes a Rieske-type ferredoxin protein (Lewis et al. 2011). The hydroxylase component contains the carboxylate-bridged di-iron site by which dioxygen is activated and subsequently inserted into a C-H bond of the hydrocarbon substrate (Ullrich and Hofrichter 2007). Principally, monooxygenases can hydroxylate aromatic rings or alkyl side chains.

For a number of ring-hydroxylating monooxygenases, carbon and hydrogen enrichment factors were determined. Carbon enrichment factors ranged considerably from -0.4 ± 0.1‰ to -4.3 ± 0.4‰, whereas hydrogen enrichment factors were rather small, ranging from -2 ± 3‰ to -18 ± 6‰ for toluene, benzene, ethylbenzene, phenol, or *p*-cresol as substrates (Table 1; Dorer et al. 2014b; Fischer et al. 2008; Morasch et al. 2002; Vogt et al. 2008; Wei et al. 2016). The wide range of carbon enrichment factors is probably due to the diversity of monooxygenases used in the fractionation experiments; they likely have different reaction mechanisms, resulting in either small or large carbon isotope fractionation upon the insertion of a C-OH bond into the aromatic ring system. However, the data indicate that the abstraction of the H atom during hydroxylation of the aromatic ring is only partially rate-determining due to the small hydrogen isotope effects observed ($AKIE_H \leq 1.12$). Thus, lambda values of monooxygenation reactions at the aromatic ring were generally low, ranging between 3 ± 1 and 11 ± 6 (Table 1). In contrast, monohydroxylation of the methyl group of toluene by xylene monooxygenase, a multicomponent di-iron hydroxylase, resulted in strong carbon and hydrogen isotope fractionation ($AKIE_H$ up to 27.7; Mancini et al. 2006; Morasch et al. 2002;

Vogt et al. 2008) leading to significant higher lambda values. This indicates that the cleavage of the C-H bond upon methyl group hydroxylation is indeed the rate-determining step of the reaction. Additional studies are needed to confirm whether this strong isotope effect is typical for alkyl group-hydroxylating monooxygenases of the di-iron Rieske-type. Methyl group hydroxylation of toluene by the fungal enzyme toluene monooxygenase, which is a cytochrome P450 enzyme (Luykx et al. 2003), was associated with much lower carbon and hydrogen isotope fractionation (Table 1, Vogt et al. 2008), indicating considerable masking of isotope fractionation.

2.1.2 Monooxygenation of Alkanes

Alkanes are hydroxylated by different monooxgygenases depending on their chain length. Methane, but also alkanes containing more than one carbon atom, are oxidized by methane monooxygenase (MMO) (Rojo 2009; van Beilen and Funhoff 2007). Two types of MMOs are known: a soluble methane monooxygenase (sMMO) expressed at low copper concentrations and a particulate membrane-bound methane monooxygenase (pMMO) more widely distributed (Hakemian and Rosenzweig 2007). sMMO belongs to the di-iron hydroxylases, whereas pMMO essentially contains mononuclear and dinuclear copper which explains the expression of sMMO under copper limitation. Carbon and hydrogen isotope enrichment factors were determined for several taxonomically different methanotrophic bacteria showing pMMOs or sMMO activity (Table 1; Feisthauer et al. 2011; Rasigraf et al. 2012); enrichment factors ranged from −14.8‰ to −29.2‰ for carbon and from −110‰ to −231.5‰ for hydrogen. Notably, lambda values were almost similar ranging between 7.3 and 10.5, indicating that methane oxidation catalyzed by sMMO and pMMO proceeds by a similar C-H bond cleavage mechanism. Thus, the ^{2}H and ^{13}C isotope fractionation patterns principally enable to identify and characterize methane biodegradation in aerobic environments by dual isotope analysis.

Other enzymes acting on short-chain alkanes (ethane, propane, butane) are similar to sMMO and pMMO (Rojo 2009; van Beilen and Funhoff 2007), e.g., butane monooxygenase of *Thauera butanivorans* (formerly *Pseudomonas butanovora*). Three types of enzymes have been described yet catalyzing the monooxygenation of alkanes containing 5–24 carbon atoms: cytochrome P450 alkane hydroxylases (Rojo 2009; van Beilen and Funhoff 2007), particulate non-heme di-iron monooxygenases termed AlkB (Rojo 2009; van Beilen and Funhoff 2007), and a Rieske-type monooxygenase (Li et al. 2013). The Alk system of *Pseudomonas putida* GPo1 catalyzing the oxidation of alkanes containing 5–12 carbon atoms is well-characterized: it consists of an alkane hydroxylase (AlkB), a membrane protein catalyzing the substrate oxidation, rubredoxin (AlkG), and rubredoxin reductase (AlkT) (Ji et al. 2013). The enzymes using alkanes of longer chain lengths (C_{24} to C_{30} and longer) as substrates are less good characterized; they are probably not related to the alkane hydroxylases mentioned above (Wang and Shao 2013). Generally, alkanes are oxidized either in terminal or subterminal position, leading to primary or secondary alcohols as reaction products (Rojo 2009). Remarkably, none of the mentioned alkane hydroxylase reactions have been investigated yet for carbon or hydrogen isotope fractionation. All carbon and hydrogen isotope

fractionation factors determined so far for aerobic alkane degradation have been done with microbial cultures in which the initial step of alkane degradation was not further characterized (Vogt et al. 2016). These results obtained with uncharacterized cultures demonstrate, however, that aerobic alkane degradation – probably generally initiated by monohydroxylation as discussed above – is principally linked to carbon and hydrogen isotope fractionation. Notably, the magnitude of carbon and hydrogen isotope fractionation of alkanes was considerably decreasing in these former studies as a function of chain length, indicating strong isotope masking effects for alkanes with increasing number of carbon atoms (Bouchard et al. 2008), possibly due to uptake of alkanes by the cell.

2.1.3 Dioxygenation of Hydrocarbon Aromatics

Dioxygenases introducing two hydroxyl groups in aromatic rings are named Rieske non-heme iron oxygenases and further classified by the names of the substrates degraded by specific organisms, e.g., the toluene/biphenyl family or the naphthalene family (Gibson and Parales 2000). A well-investigated model enzyme for Rieske non-heme iron oxygenases is naphthalene dioxygenase (NDO) containing a NADH oxidoreductase, a ferredoxin, and an oxygenase (termed NDO) comprising the active center. The active site contains a Rieske [2Fe−2S] center and a mononuclear non-heme iron attached which binds and activates molecular oxygen for subsequent naphthalene dihydroxylation. Oxygen is attracted by the π electrons of the aromatic ring resulting in the formation of cis-dihydrodiol; as this step is not associated with a σ bond cleavage or formation and also thought to be rate-limiting, dioxygenase reactions are generally not expected to be accompanied by considerable isotope fractionation.

Carbon and hydrogen isotope fractionation factors linked to the reactions of dioxygenases using benzene, toluene, ethylbenzene, naphthalene, or 2-methylnaphthalene as substrates are listed in Table 1. Carbon enrichment factors were low to moderate, ranging from insignificant (-0.1 ± 0.2‰) to -1.8 ± 0.3‰. Small carbon enrichment factors were also reported for dioxygenation of substituted benzenes, e.g., chlorobenzene by different dioxygenases (-0.1 ± 0.1‰ to -0.4 ± 0.1‰, Kaschl et al. 2005) or nitrobenzenes by 2-nitrotoluene dioxygenase (-0.1 ± 0.1‰ to -0.8 ± 0.2‰, Pati et al. 2016). Notably, carbon isotope enrichment factors during ring dioxygenation of nitrobenzene and different nitrotoluenes by another enzyme, nitrobenzene dioxygenase, varied considerably, showing values of -0.4 ± 0.2‰ to -1.4 ± 0.4‰ for nitrotoluenes and -3.7 ± 0.2‰ for nitrobenzene, respectively (Pati et al. 2016). The same trend was observed for hydrogen isotope fractionation (despite low magnitudes), resulting in similar lambda values and suggesting a common mechanism for dioxygenation of nitrobenzene and nitrotoluenes (Pati et al. 2016). The authors assume that isotope fractionation was controlled by the rate of enzymatic oxygen activation by which carbon (and hydrogen) isotope fractionation was masked. This type of isotope masking may be relevant also for other oxygenases and substrates. Low carbon and no or inverse hydrogen fractionation were observed for benzene derivatives which were attacked chemically by OH radicals at the ring (Zhang et al. 2016). This was also interpreted

as masking effect caused by pre-equilibrium between the substrate and OH radical preceding the rate-limiting step leading to reduced isotope fractionation.

Partly inconsistent results were observed for hydrogen isotope fractionation upon aromatics hydrocarbon dioxygenation (Table 1). In a number of studies, no hydrogen isotope fractionation was determined for different organisms and enzymes (Dorer et al. 2014b; Fischer et al. 2008; Vogt et al. 2008). Naphthalene and 2-methylnaphthalene dihydroxylation by naphthalene dioxygenase (Kümmel et al. 2016) and dihydroxylation of ethylbenzene (Dorer et al. 2014b) produced however an inverse hydrogen isotope effect, explicable by an inverse secondary stable hydrogen effect due to the conversion of a sp^2-hybridized π C-C bond into a sp^2-hybridized σ C-C bond (Kümmel et al. 2016). In one study, normal hydrogen isotope effects were observed for toluene and naphthalene dioxygenation (Morasch et al. 2002); the latter result is contradictory to the data observed by Kümmel et al. (2016) and might be related to the use of per-deuterated naphthalene by Morasch et al. (2002) (see also discussion in Kümmel et al. 2016).

2.1.4 Dioxygenation of Alkanes

Dihydroxylation of alkanes of the chain lengths C_{10} to C_{30} by an alkane dioxygenase was only reported for *Acinetobacter sp*. strain M-1 (Maeng et al. 1996); this reaction is probably not as usual as monooxygenase-catalyzed alkane hydroxylation. Carbon or hydrogen isotope fractionation factors have not been yet determined for alkane dihydroxylation.

2.2 Anaerobic Reactions

2.2.1 Anaerobic Hydroxylation of Aromatic Hydrocarbons

Oxygen-independent hydroxylation reactions were mainly reported for the hydroxylation of alkyl side chains of aromatics; two enzyme families can be differentiated. Flavocytochrome c hydroxylases have been described to hydroxylate the alkyl group of phenolic compounds; they are periplasmic or membrane-bound and contain a covalently bound flavin adenine dinucleotide (FAD) and an electron-transferring cytochrome c subunit (Boll et al. 2014). Due to the higher C-H bond dissociation energy of aromatic hydrocarbons lacking electronegative substituents, flavocytochrome c hydroxylases cannot catalyze hydroxylation reactions at the alkyl side chain of aromatic hydrocarbons (Boll et al. 2014). Instead, these reactions are catalyzed by hydroxylases containing a molybdenum cofactor, belonging to the DMSO reductase family (Boll et al. 2014). A well-investigated enzyme of this type is ethylbenzene dehydrogenase detected in nitrate-reducing strains (Johnson et al. 2001; Kniemeyer and Heider 2001). Besides a subunit containing the molybdenum cofactor and a [4Fe-4S] cluster, the enzyme consists of two electron-transferring subunits containing FeS centers and heme b, respectively (Boll et al. 2014). It was demonstrated that the hydroxyl group is generated from water. Other reported substrates for this type of enzyme are steroids (Dermer and Fuchs 2012) and *p*-cymene (Strijkstra et al. 2014).

Anaerobic hydroxylation of benzene resulting in phenol was reported for *Geobacter metallireducens* under iron-reducing conditions (Zhang et al. 2013), but an enzyme catalyzing this reaction has not been characterized yet.

Recently, carbon fractionation factors for the anaerobic hydroxylation of *p*-cresol by nitrate-reducing model strains containing flavocytochrome c hydroxylases were shown to be substantial (Table 1; Wei et al. 2016) and in the same range observed for methyl group oxidation by a monooxygenase (Table 1; Mancini et al. 2006; Morasch et al. 2002; Vogt et al. 2008). Unfortunately, hydrogen isotope analysis for anaerobic *p*-cresol hydroxylation could not be determined yet due to analytical constraints (Wei et al. 2016). Similar carbon and hydrogen isotope fractionation factors were detected in microbial cultures hydroxylating the ethyl side chain of ethylbenzene by ethylbenzene dehydrogenase. Under nitrate-reducing conditions, carbon and hydrogen fractionation factors were extraordinarily high (Table 1; Dorer et al. 2014a, b), demonstrating that the cleavage of the C-H bond is the rate-limiting step of the reaction mechanism. Notably, carbon and hydrogen isotope fractionation factors were considerably lower if nitrate was replaced by manganese or iron as electron acceptor in the same microbial cultures (Dorer et al. 2016). The lambda values were however similar for all conditions, a result which can be explained by considerable masking of isotope fractionation by diffusion-controlled, rate-limiting ethylbenzene availability due to the use of solid manganese (IV) or ferric iron (III) particles as electron acceptors, leading to attachment of cells, biofilm formation, and decreased ethylbenzene degradation rates (Dorer et al. 2016).

2.2.2 Anaerobic Hydroxylation of Alkanes

Anaerobic hydroxylation of alkanes at the subterminal position was also suggested for the sulfate-reducer *Desulfococcus oleovorans* Hdx3 using a molybdenum-comprising enzyme similar to ethylbenzene dehydrogenase (Heider et al. 2016b). Isotope fractionation factors for this reaction have not been determined yet.

2.2.3 Addition of Alkyl-Aromatic Hydrocarbons to Fumarate

The addition of alkyl-substituted aromatic hydrocarbons or of alkanes to fumarate yielding alkylaryl- or alkylsuccinates is a key reaction of anaerobic hydrocarbon degraders. A high number of substrates were reported to be activated by this reaction (Heider et al. 2016a). The reaction is catalyzed by glycyl-radical enzymes of the pyruvate formate lyase family (Leuthner et al. 1998). The reaction was discovered in toluene-degrading nitrate-reducing strains (Beller and Spormann 1997; Biegert et al. 1996); the enzyme catalyzing the activation of toluene to benzylsuccinate, benzylsuccinate synthase, is composed of a large, glycyl-radical-containing subunit (bearing the active center of the enzyme) and two smaller FeS cluster-containing subunits (Boll et al. 2014). This structure is unique for all fumarate-adding enzymes detected so far (Heider et al. 2016a), although sequence variations of the large subunit indicate that several isoenzymes exist (von Netzer et al. 2016). Besides toluene, a large number of aromatic hydrocarbons with alkyl side chains were shown to be activated by addition to fumarate, e.g., xylenes, ethylbenzene, or 2-methylnaphthalene (Heider et al. 2016a).

Several carbon and hydrogen enrichment factors for fumarate addition of alkylated aromatics have been determined up to now (Table 1). For all cultures and substrates investigated, carbon and hydrogen isotope fractionation were observed, suggesting that the homolytic cleavage of the C-H bond is a rate-determining step of the reaction mechanism. Enrichment factors differed however up to an order of magnitude ($AKIE_C$ ranging from 1.004 to 1.046, $AKIE_H$ ranging from 1.2 to 4.7, Table 1). Notably, these differences cannot be solely caused by simple masking of carbon and hydrogen isotope fractionation, as lambda values differed also considerably, e.g., for toluene from 4 ± 3 to 41 ± 8 (Table 1). Thus, the considerably different lambda values were interpreted as variations of the reaction mechanisms of benzylsuccinate synthase due to isoenzymes occurring in distinct ecophysiological groups of bacteria, e.g., nitrate-reducing Betaproteobacteria and sulfate-reducing or iron-reducing Deltaproteobacteria (Dorer et al. 2016; Kümmel et al. 2013; Vogt et al. 2008). Such variability in isotope fractionation of enzymes catalyzing the same reaction may complicate the interpretation of in situ isotope effects in hydrocarbon-contaminated environments but offers also the chance to detect distinct isoenzymes (Vogt et al. 2008).

2.2.4 Addition to Fumarate of Alkanes

In addition to alkylated aromatics, several alkanes were shown to be activated by addition to fumarate; in most cases, fumarate was added in subterminal position (C2), but also additions at C1 (terminal) or C3 position were observed as side reactions (Callaghan 2013). Fractionation factors were determined for fumarate addition to propane, *n*-butane, and *n*-hexane (Table 1; Jaekel et al. 2014; Vieth and Wilkes 2006). Similar to fumarate addition to alkyl side chains of aromatics, addition of alkanes to fumarate was linked to carbon and hydrogen isotope fractionation – but the magnitude differed considerably ($AKIE_C$ ranging from 1.003 to 1.026, $AKIE_H$ ranging from 1.05 to 2.14). Since the corresponding lambda values were roughly similar (4.9 ± 1.2 to 11.9 ± 0.2), this variety in isotope fractionation was interpreted as an effect of cultivation conditions (static or mixed cultures); hence the physicochemical transfer of the short-chain alkanes from the gas phase to the liquid phase was shown to be partially rate-determining, masking carbon and hydrogen isotope fractionation considerably (Table 1; Jaekel et al. 2014).

2.2.5 Carboxylation

Carboxylation of the aromatic ring under anoxic conditions was demonstrated for phenolic compounds and naphthalene and proposed for benzene. Phenylphosphate carboxylase (Ppc) of the nitrate-reducing strain *Thauera aromatica* involved in anaerobic phenol degradation is well investigated. Firstly, phenylphosphate is formed from phenol by an ATP-dependent phenylphosphate synthase (Pps), followed by carboxylation to 4-hydroxybenzoate (Boll et al. 2014). Ppc consists of four subunits related to UbiD-like decarboxylases (Boll et al. 2014). Pps and Ppc were detected in the phenol-degrading iron-reducer *Geobacter metallireducens* GS-15, too, although the Ppc was shown to be of different architecture than the

enzyme in *Thauera aromatica* (Schleinitz et al. 2009). The same phenol degradation pathway was found to be active also in a sulfate-reducing strain (Ahn et al. 2009).

Carbon isotope fractionation of the Pps/Ppc activation system was determined for *Thauera aromatica* (Table 1; Wei et al. 2016). The intermediary dephosphorylation of phenylphosphate to a phenolate anion is proposed to be the rate-determining step of the reaction sequence from phenol to 4-hydroxybenzoate (Schmeling et al. 2004), a reaction in which no C bonds are actually cleaved, explaining the small carbon enrichment factor observed (-0.7 ± 0.1‰). Even a slight inverse carbon enrichment factor was determined during phenol degradation by the sulfate-reducer *Desulfosarcina cetonica* (Table 1; Wei et al. 2016) for which the phenol degradation pathway has not been determined yet; due to the missing primary carbon isotope effect, *D. cetonica* may also use the pathway initiated by Pps and Ppc, as shown for a strain of *Desulfobacterium anilii* (Ahn et al. 2009).

Similar to the carboxylation of phenylphosphate, a UbiD-like carboxylase was tentatively identified to catalyze the carboxylation of naphthalene by the sulfate-reducing freshwater enrichment culture N47 (Meckenstock et al. 2016; Mouttaki et al. 2012), a reaction suggested also for naphthalene-degrading, marine sulfate reducers (Musat et al. 2009). Carbon and hydrogen isotope fractionation of the freshwater enrichment culture N47 and the marine strain NaphS6 were in a similar range – very low carbon and considerable hydrogen isotope fractionation (Table 1; Bergmann et al. 2011; Kümmel et al. 2016) – indicating naphthalene carboxylation by similar enzymes. Notably, carbon isotope fractionation of strain NaphS2 was of significantly higher magnitude (whereas hydrogen isotope fractionation was in the range observed for the other two tested cultures) (Bergmann et al. 2011). This could be explained by slight variations of the reaction mechanism of the putative UbiD-like carboxylases.

Carboxylation of benzene by UbiD-like carboxylases has been also proposed for iron- and nitrate-reducing enrichment cultures, although the reaction could not be confirmed biochemically in enzyme assays (Abu Laban et al. 2010; Luo et al. 2014). These two cultures showed identical isotope enrichment factors: strong carbon and considerable hydrogen isotope fractionation, leading to highly similar lambda values of 17 ± 1 and 16 ± 2, respectively (Table 1; Bergmann et al. 2011; Mancini et al. 2008). This indicated mechanistically similar enzymes and that cleavage of benzene C-H bonds is the rate-determining step upon catalysis. Lambda values for other nitrate-reducing benzene-degrading enrichment cultures are in the same range (8 ± 2 to 19 ± 3, Mancini et al. 2008), suggesting similar carboxylating enzymes in nitrate and iron reducers. It would have been important to determine isotope fractionation of benzene degradation by the iron-reducer *Geobacter metallireducens* GS-15 for which an initial hydroxylation step was proposed (Zhang et al. 2013); possibly, benzene hydroxylation shows a different fractionation pattern. Notably, carbon and hydrogen isotope fractionation factors of sulfate-reducing and methanogenic benzene-degrading cultures (for which the activation mechanism has not been determined yet) are always higher than those of nitrate- and iron-reducing cultures (22 ± 3‰ to 39 ± 5‰, Fischer et al. 2008, 2009; Mancini et al. 2003, 2008). This leaves open the possibility of distinct activation mechanisms in facultative vs. strictly anaerobic microorganisms.

2.2.6 Alkane Activation by Methyl-Coenzyme M Reductases

Anaerobic oxidation of methane (AOM) is a major global methane sink, most frequently observed in marine sediments in the sulfate-methane transition zone (Reeburgh 2007) but also occurring with nitrate, nitrite, iron, or manganese as electron acceptors (Callaghan 2013; Ettwig et al. 2016; Welte et al. 2016). AOM with sulfate as terminal electron acceptor is performed by specific anaerobic methanotrophic archaea (ANME) (Knittel and Boetius 2009). AOM under sulfate-reducing conditions proceeds by reversed methanogenesis, i.e., the reversal of the biochemical reactions by which carbon dioxide is reduced to methane; thus, the initial step of methane oxidation is catalyzed by a nickel-containing methyl-coenzyme M reductase (MCR) (Fig. 2; Ettwig et al. 2016; Thauer and Shima 2008; Welte et al. 2016). The MCR-catalyzing methane oxidation differs from the methane-releasing enzyme by a modified F430 cofactor and a cysteine-rich region (Krüger et al. 2003; Shima et al. 2012). Sulfate-dependent AOM was shown to be associated with strong carbon and hydrogen isotope fractionation (Table 1; Holler et al. 2009) which in principle allows detecting this reaction in the environment by CSIA. However, due to similar isotope fractionation with aerobic methane oxidation by MMO, it is difficult to differentiate aerobic and anaerobic methane oxidation by CSIA alone, without analyzing additional biogeochemical parameters (Feisthauer et al. 2011).

Recently, marine thermophilic archaea were shown to activate butane to butyl-coenzyme M (Laso-Perez et al. 2016), demonstrating that the methyl-coenzyme M-catalyzed reaction is not restricted to methane as substrate. Considering the strong isotope effects linked to AOM, it can be expected that also the activation of short-chain alkanes by methyl-coenzyme M reductase is associated with strong isotope fractionation. Whether these patterns are different from those determined for the activation of short-chain alkanes by addition to fumarate (Jaekel et al. 2014) needs to be investigated.

3 Conclusions and Research Needs

In the last 15 years, the known aerobic and anaerobic enzymatic hydrocarbon activation steps have been characterized by CSIA. Especially anaerobic reactions were shown to be linked to considerable carbon and/or hydrogen isotope fractionation (Table 1). Since anaerobic biodegradation is thought to be the main driver of natural attenuation at hydrocarbon-polluted sites, e.g., contaminated aquifers (Aronson and Howard 1997), the data strongly indicate that in situ biodegradation can be principally proven by CSIA; some reactions may be even identified and/or quantified. The strength of the CSIA concept can be exemplified by benzene as model compound. Benzene is a widespread and toxic hydrocarbon; its anaerobic degradation is often slow (Vogt et al. 2011). At a benzene-contaminated site in Zeitz, Eastern Germany, biodegradation of benzene under strictly anoxic conditions has been proven and quantified by CSIA in samples containing different benzene concentrations along the groundwater flow path (Fischer et al. 2007). Benzene isotope enrichment factors determined in situ were considerably lower compared to laboratory experiments

(Fischer et al. 2009), indicating masking of isotope fractionation in the field. Such masking effects can be due to partially rate-limiting mass transfer processes at different scales (Thullner et al. 2012; Thullner et al. 2013; Vogt et al. 2016). Other constraints potentially negatively influencing the in situ application of CSIA are related to the heterogeneity of the environmental system (e.g., complex hydrogeological conditions or multiple hydrocarbon sources). As discussed above, a few hydrocarbon activation mechanisms are also characterized by rather small carbon and/or hydrogen isotope fractionation or variable lambda values, hampering the application of CSIA for its identification. Simultaneously expressed different degradation pathways may complicate the interpretation of isotope signals, too. Currently, only a few hydrocarbons contaminated sites were investigated by carbon and hydrogen isotope analysis (Vogt et al. 2016); hence more field data are needed to assess the strengths and weaknesses of CSIA from a practical point of view. Besides applying CSIA for monitoring biodegradation at spill sites, the concept may work also to monitor hydrocarbon degradation reactions upon underground gas storage, oil reservoirs, deep geological sections, or during hydraulic fracturing ("fracking") operations.

Future research may focus also on studying reaction mechanisms on a molecular scale by CSIA. The $AKIE_{C,\ H}$ determined in degradation experiments can be used for the analysis of kinetic rate limitations prior to catalysis and bond cleavage (Northrop 1981; Swiderek and Paneth 2013). The analysis of binding isotope effects (BIE) offers opportunities to study the binding of a substrate to the active center of the enzyme, and the quantification of equilibrium isotope effects (EIE) enables to study enzymatic reaction mechanisms with complex reaction coordinates (Swiderek and Paneth 2013) typical for hydrocarbon activation reactions. The use of stable isotope fractionation concepts for studying enzymatic hydrocarbon transformation reaction mechanisms on a molecular level is still in its infancy. There is potential to characterize various reaction steps of C-H bond cleavage mechanism combining ^{2}H and ^{13}C fractionation pattern. Multi-element stable isotope fractionation analysis is suitable to indicate bond cleavage mechanisms by cancelling out rate limitations prior to catalysis (Elsner 2010) if these rate limitations are not isotope sensitive. A promising concept for mechanistic analysis of enzymatic reactions might be stable isotope fractionation studies with pure enzymes. Multi-isotope fractionation pattern could be used to investigate the molecular mechanism of binding and complex transition stages by interpreting BIE, EIE, and KIE, respectively (Swiderek and Paneth 2013). Comparing isotope effects gained from experimental studies and KIEs calculated by quantum mechanical/molecular mechanical models (QM/MM) may allow precisely characterizing the mechanism of bond cleavage and transition stages of the whole reaction (Swiderek and Paneth 2013). In a further step, QM/MM modeling of enzyme reactions may be applied for elucidating the chemical nature of bond cleavage when the crystal structure of the enzyme is available.

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Compound-Specific Stable Isotope Analysis (CSIA) for Evaluating Degradation of Organic Pollutants: An Overview of Field Case Studies

15

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Contents

Abstract

Compound-specific stable isotope analysis (CSIA) is an advanced monitoring tool for evaluating natural and stimulated degradation of organic pollutants at contaminated field sites. CSIA enables the decipherment and quantification of degradation processes and the assignment of organic pollutant sources and polluters, respectively. Since the end of 1990s, around 200 field studies on CSIA as well as over 40 reviews on its basics have been published illustrating the wide range of application and reliability of this monitoring tool. Increasingly, multielement CSIA (ME-CSIA) is applied, which allows the differentiation of degradation pathways. For most organic pollutants, anaerobic biodegradation can be distinguished from aerobic biodegradation and abiotic transformation using

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ME-CSIA. CSIA is often the key monitoring tool for multiple-line-of-evidence approaches (MLEA) allowing a comprehensive evaluation of degradation processes of organic pollutants, which is required for most effective remediation strategies. Due its advantages, CSIA is recommended in numerous guidelines and directives from environmental agencies and associations. However, there is still a need for implementation of CSIA within the contaminated site management in order to increase the benefits of this method for a thorough development of conceptual site models and success control of natural and stimulated degradation of organic pollutants. This chapter provides an overview and recent developments on CSIA applied for the assessment of pollutant degradation at field sites including an extensive review of literature.

1 Introduction

In subsurface environments, biodegradation is the main process for a sustainable removal of organic pollutants. Since the supply of oxygen in soil, groundwater systems, and sediments is often limited, these compartments predominantly exhibit anoxic conditions. Thus, anaerobic biodegradation is the key removal process of organic pollutants and needs to be included in the fate and risk assessment of contaminants. In order to prove and quantify anaerobic pollutant biodegradation as well as to distinguish it from other decomposition processes (such as aerobic biodegradation, chemical transformation) or physical attenuation (e.g., sorption, dilution, dispersion, volatilization), appropriate monitoring tools are required. In combination with conventional methods (e.g., determination of pollutant concentration and redox parameters), advanced monitoring tools should be applied for the evaluation of pollutant biodegradation (Bombach et al. 2010; Fischer et al. 2016; Wittebol and Dinkla 2017; Bouchard et al. 2018a). Especially, compound-specific stable isotope analysis (CSIA) becomes increasingly popular for assessing pollutant degradation at contaminated field sites. The state of the art and future trends about CSIA of organic pollutants have been described in more than 40 review articles (Table 1) and even textbooks (Aelion et al. 2010; Jochmann and Schmidt 2012). CSIA is a well-accepted monitoring tool and recommended by several environmental authorities and associations for the implementation and success control of innovative management and remediation concepts, like *Monitored Natural Attenuation* (MNA) and *Enhanced Natural Attenuation* (ENA) (e.g., Beck and Mann 2010; EA-UK 2010; UBA 2011; NJDEP 2012; ITCR 2013; US-EPA 2013; Adamson and Newell 2014; ASTM 2015; Döberl et al. 2016). Moreover, fact sheets or guidelines on CSIA have been published by environmental agencies or associations (US-EPA 2008; ITCR 2011; Haderlein and Buchner 2015; Watzinger and Leitner 2015).

This chapter provides an overview and recent developments on CSIA applied for the assessment of natural and stimulated degradation of organic pollutants. The main focus is on field studies illustrating the practical application of CSIA in order to highlight options to stakeholders (e.g., practitioners, consultants, field site owners,

Table 1 Review articles on CSIA of organic pollutants and their main topics

CSIA review	Main topic
Slater 2003	Source apportionment and evaluation of volatile organic compound (VOC) degradation
Meckenstock et al. 2004	Evaluation of degradation
Schmidt et al. 2004	Analytical basics, source apportionment, and evaluation of degradation
Elsner et al. 2005	Deciphering of degradation pathways
Aranami et al. 2006	Source apportionment and evaluation of organochlorine degradation
Miljevic and Golobocanin 2007	Analytical basics, source apportionment, and evaluation of degradation
Philp 2007	Source apportionment and evaluation of degradation
Philp et al. 2007	Evaluation of degradation
Rosell et al. 2007	Evaluation of fuel oxygenate degradation
Blessing et al. 2008	Analytical basics
Hofstetter et al. 2008	Deciphering of degradation pathways
Hunkeler 2008	Evaluation of degradation with strong focus on quantification
Kuder and Philp 2008	Evaluation MTBE and TBA biodegradation
Thullner et al. 2009	Evaluation of degradation with strong focus on quantification
Elsner 2010	Deciphering of degradation pathways
Meckenstock and Richnow 2010	Evaluation of hydrocarbon degradation
Hofstetter and Berg 2011	Deciphering of degradation pathways
Braeckevelt et al. 2012	Applicability for field studies
Cincinelli et al. 2012	Analytical basics of $^{37}Cl/^{35}Cl$ and $^{81}Br/^{79}Br$ for organohalogen compounds
Elsner et al. 2012	Analytical basics and need for further developments
Négrel et al. 2012	Source apportionment
Schmidt and Jochmann 2012	Analytical basics, source apportionment, and evaluation of degradation
Thullner et al. 2012	Quantification of in situ pollutant degradation
Buczynska et al. 2013	Analytical basics of $^{13}C/^{12}C$ for polyaromatic hydrocarbons (PAHs)
Hatzinger et al. 2013	Applicability for field studies
Thullner et al. 2013	Influence of mass transfer processes
Bernstein et al. 2014	Evaluation of explosive degradation
Elsner et al. 2014	Deciphering of degradation pathways
Hofstetter et al. 2014	Deciphering of degradation pathways
Badea and Danet 2015	Basics of enantioselective stable isotope analysis (ESIA)
Elsner and Imfeld 2016	Evaluation of micropollutant degradation
Fischer et al. 2016	Applicability for field studies
Gauchotte-Lindsay and Turnbull 2016	Analytical basics on carbon position-specific stable isotope analysis
Hunkeler 2016	Source apportionment and evaluation of organochlorine degradation

(*continued*)

Table 1 (continued)

CSIA review	Main topic
Musat et al. 2016	Deciphering of degradation pathways for saturated and alkylated aromatic hydrocarbons
Nijenhuis and Richnow 2016	Deciphering of degradation pathways for halogenated pollutants
Nijenhuis et al. 2016	Analytical basics for multielement CSIA of organohalogen compounds
Renpenning and Nijenhuis 2016	Evaluation of reductive dehalogenation
Schwarzenbach et al. 2016	Evaluation of degradation
Vogt et al. 2016	Deciphering of degradation pathways for hydrocarbons
Kohli et al. 2017	Evaluation of hexachlorocyclohexane (HCH) degradation
Pati et al. 2017	Deciphering of enzymatic oxygenation reactions
Gao et al. 2018	Source apportionment of PAHs
Teixeira and de Abreu 2018	Source apportionment of VOC
Vogt et al. 2018	Evaluation of hydrocarbon degradation
Wanner and Hunkeler 2019	Isotope fractionation due to aqueous phase diffusion

authority representatives) for their usage with the implementation, conception, and success control of MNA (Thornton and Rivett 2008; Beck and Mann 2011) and remediation of contaminated field sites (EA-UK 2010; US-EPA 2013).

2 Analytical Basics of CSIA

Different isotopes of an element have the same number of protons but vary in the number of neutrons, and therefore they have different masses (Fig. 1). Organic pollutants mainly consist of carbon and hydrogen and can additionally contain chlorine, bromine, oxygen, sulfur, and nitrogen in their functional groups. All these elements exhibit at least two stable isotopes.

The quotient between the heavy and the light isotope is called isotope ratio or isotope signature (e.g., $^{13}C/^{12}C$, $^{2}H/^{1}H$), which is expressed as delta notation (δ_{sample}) relative to an international standard according to Eq. 1 (Coplen 2011).

$$\delta_{sample} = \frac{R_{sample}}{R_{standard}} - 1 \tag{1}$$

R_{sample} and $R_{standard}$ are the isotope ratios of the sample and an international standard, respectively. For example, the delta notation of the carbon isotope ratio ($^{13}C/^{12}C$) is given as $\delta^{13}C$-value with regard to the international standard V-PDB (Vienna-Pee Dee Belemnite, $^{13}C/^{12}C = 0.0111802$) (Fig. 2). Because variations in natural isotope abundance are typically small, δ-values are mostly reported in per mil (‰) or milli-Urey (mUr) (Brand and Coplen 2012).

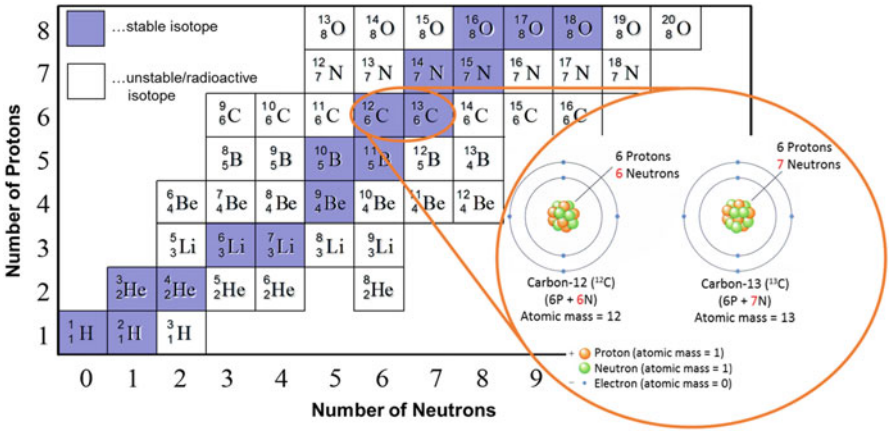

Fig. 1 Overview on isotopes of chemical elements ranging from hydrogen to oxygen. The enlarged excerpt shows a simple atomic model of the two stable carbon isotopes with the derivation of their atomic masses based on the different numbers of neutrons. P, protons; N, neutrons. (Reproduced from Fischer et al. 2019 with permission from DBFZ)

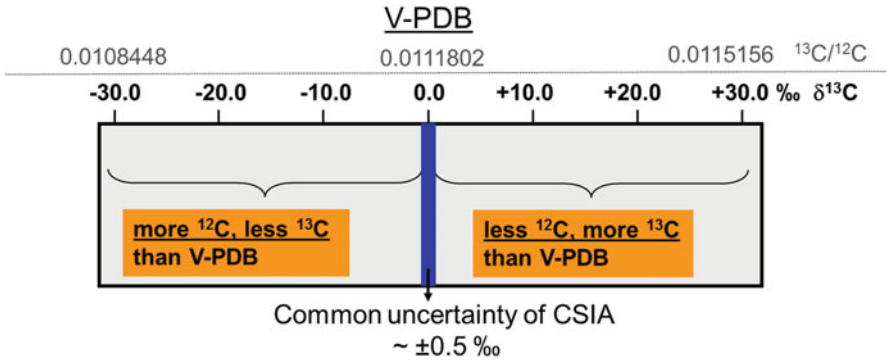

Fig. 2 Illustration of the delta scale for stable carbon isotope ratios ($^{13}C/^{12}C$) as an excerpt ranging from −30 to +30 ‰. The anchor of the $\delta^{13}C$-scale is V-PDB (Vienna-Pee Dee Belemnite, $^{13}C/^{12}C = 0.0111802$). (Reproduced from Fischer et al. 2019 with permission from DBFZ)

CSIA of organic pollutants in environmental samples (e.g., groundwater, soil) mainly relies on gas chromatography-isotope ratio mass spectrometry (GC-IRMS) (Blessing et al. 2008; Elsner et al. 2012; Schmidt and Jochmann 2012). CSIA of carbon is well-established for VOC (e.g., benzene, toluene, ethylbenzene, xylenes, BTEX; chlorinated ethenes, ethanes, and methanes) as well as fuel oxygenates (e.g., methyl *tert*-butyl ether, MTBE; ethyl *tert*-butyl ether, ETBE) using purge and trap (P&T) as extraction and pre-concentration technique (Zwank et al. 2003; Meyer et al. 2017). The P&T method is also state of the art for CSIA of hydrogen for volatile hydrocarbons (e.g., BTEX) and fuel oxygenates (e.g., MTBE) (Kujawinski et al. 2010; Bouchard et al. 2018b). Due to the limitations of

GC-IRMS for polar organic pollutants, recent developments have focused on the application of liquid chromatography-isotope ratio mass spectrometry (LC-IRMS) (Kujawinski et al. 2013; Gilevska et al. 2014; Mogusu et al. 2015; Melsbach et al. 2019) or derivatization techniques allowing CSIA of polar pollutants by GC-IRMS (Spahr et al. 2013; Maier et al. 2014; Mogusu et al. 2015; Melsbach et al. 2019). Recent optimization of CSIA for chlorine, bromine, and hydrogen of halogenated pollutants (Gelman and Halicz 2010; Kuder and Philp 2013; Renpenning et al. 2015a, b, 2017, 2018; Zakon et al. 2016; Heckel et al. 2017; Horst et al. 2017) will increase their applications for the assessment of pollutant degradation and source identification at contaminated field sites. Since the sensitivity of CSIA is often significantly lower compared to concentration analysis, novel sampling, pre-concentration, and cleanup techniques have been developed in order to reach better performance for CSIA of organic pollutants in environmental samples (e.g., Schreglmann et al. 2013; Ivdra et al. 2014; Passeport et al. 2014; Bakkour et al. 2018; Torrentó et al. 2019).

3 Isotope Fractionation and Interpretation of Isotope Data

(Bio)chemical reactions lead to a fractionation of stable isotopes, i.e., a change in δ-values. This isotope fractionation is caused by a kinetic isotope effect (KIE) (for review, see Elsner 2010). KIEs depend on rate differences for the cleavage of chemical bonds substituted by compounds only consisting of light isotopes (e.g., ^{12}C, ^{1}H) or bearing a heavy isotope (e.g., ^{13}C, ^{2}H). Due to the preferential transformation of the lighter isotope species, compounds bearing a heavy isotope especially in the reactive position accumulate in the residual fraction of the reactant (*normal isotope effect*). Thus, KIEs result in an enrichment of heavy isotopes (e.g., ^{13}C, ^{2}H) in the non-degraded residual fraction of the pollutant leading to changes in the isotope ratio toward more positive δ-values (Fig. 3). In few cases, degradation of pollutants leads to an enrichment of light isotopes (e.g., ^{12}C, ^{1}H) in the non-degraded residual fraction (*inverse isotope effect*), which has been so far mainly observed for nitrogen isotope fractionation of nitrogen-bearing pollutants (Meyer et al. 2009; Pati et al. 2012) as well as hydrogen isotope fractionation during dihydroxylation of aromatic hydrocarbons (Kümmel et al. 2016).

For conventional GC- and LC-IRMS analysis, organic compounds are converted to a measuring gas, for which the isotope ratio is determined (e.g., CO_2 for CSIA of carbon, H_2 for CSIA of hydrogen). Due to the total conversion to a measuring gas, position-specific changes in the isotope composition of a target compound cannot be ascertain. Therefore, the extent of stable isotope fractionation decreases with an increasing number of atoms of this element located in non-reactive entities of a molecule as illustrated by the carbon isotope fractionation during biodegradation of chlorinated alkanes with increasing number of carbon atoms (Abe et al. 2009a). For example, carbon isotope fractionation due to degradation is not detectable by conventional CSIA of carbon for organic pollutants with a carbon number >12. Future developments in position-specific stable isotope

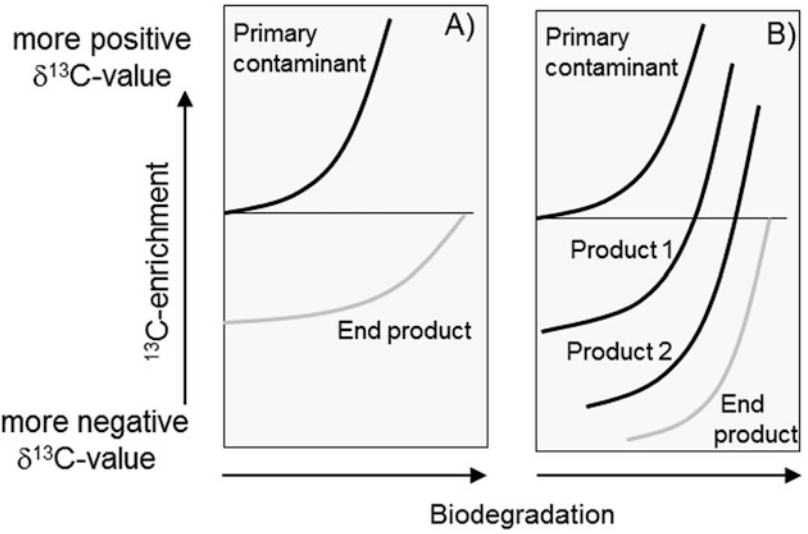

Fig. 3 Evolution of stable carbon isotope ratios during (**a**) degradation with negligible accumulation of transformation products (e.g., mineralization of toluene to CO_2) and (**b**) transformation chain: degradation with significant accumulation of transformation products (e.g., reductive dechlorination of TCE to ethene). (Reproduced from Fischer et al. 2016 with permission from Elsevier)

analysis (PSIA) may enable the evaluation of degradation for molecules with a high carbon number (Gauchotte-Lindsay and Turnbull 2016).

The interpretation of field isotope data is based on the comparison of δ-values and thus on their distinctness. For the appraisement of field isotope data, US-EPA recommended that the difference of δ-values should be more than four times larger than the analytical uncertainty in order to minimize the possibility of an erroneous interpretation (US-EPA 2008). For example, taking into account general uncertainty of ±0.5 ‰ for δ^{13}C-values of organic pollutants, the evaluation of carbon isotope data can be accomplished as follows:

- Changes in δ^{13}C-values of $> +2$ ‰ along a groundwater flow path between two sampling points provide evidence of pollutant degradation, if the influence of a secondary contaminant source with a more positive isotope signature can be excluded. Smaller changes in δ^{13}C-values ($> +1$ to $\leq +2$ ‰) can be interpreted as indication for pollutant degradation.
- Primary carbon isotope signatures of organic contaminants are mostly more negative than −22 ‰ (Thullner et al. 2012). Hence, δ^{13}C-values that are more positive than −20 ‰ can be explained by degradation.

These criteria can be adapted to the assessment of field isotope data for other elements taking into account their uncertainty of the corresponding CSIA method or the primary isotope signatures of organic contaminants.

4 Impact of Physical Processes on Evaluation of Degradation Using CSIA

In order to assess pollutant degradation at contaminated field sites, isotope fractionation due to processes other than degradation should be negligible; otherwise, changes in isotope ratios of pollutants may be misinterpreted. In most of the cases, isotope fractionation due to degradation is larger than for physical processes like sorption (Schüth et al. 2003; Kopinke et al. 2005; Höhener and Yu 2012; Imfeld et al. 2014; Liu et al. 2016; Kopinke et al. 2017; Wanner et al. 2017), water-air partitioning (Kuder et al. 2009; Jeannottat and Hunkeler 2012, Jeannottat and Hunkeler 2013; Julien et al. 2015; Horst et al. 2016; Julien et al. 2016; Horst and Lacrampe-Couloume 2018), and diffusion in water (LaBolle et al. 2008; Jin et al. 2014; Wanner and Hunkeler 2015; Xu et al. 2016; Rolle and Jin 2017; Wanner et al. 2017; Xu et al. 2017; Kopinke et al. 2018; Wanner and Hunkeler 2019) and in gas phase (Bouchard et al. 2008a; Kuder et al. 2009; Jeannottat and Hunkeler 2012; Jeannottat and Hunkeler 2013; Liang et al. 2017; Khan et al. 2018), respectively. These findings do not a priori imply that physical processes cause only negligible changes in isotope ratios at a contaminated site.

If contaminants are subjected to sorption in an aquifer, the degree of isotope fractionation due to sorption is predicted to be generally small and possibly insignificant within a stationary contaminant plume (Kopinke et al. 2005). It is expected that sorption might lead to relevant carbon isotope fractionation for BTEX at the front of an expanding contaminant plume or of a breakthrough curve of a contamination pulse (Kopinke et al. 2005; van Breukelen and Prommer 2008; Höhener and Atteia 2010), while for MTBE carbon isotope fractionation by sorption is predicted to be insignificant (Kopinke et al. 2005). The higher the retardation is, the stronger the sorption-induced isotope fractionation becomes (van Breukelen and Prommer 2008). It was shown that retardation factors of BTEX derived from field tracer tests may be generally lower and lie close to 1 (Thierrin et al. 1995, Fischer et al. 2006) as retardation factors calculated by the organic carbon-water partitioning coefficient (K_{OC}) and typical organic matter content of aquifer sediments. If so, sorption would not lead to significant isotope fractionation (Höhener and Atteia 2010). Recently, changes in $\delta^{13}C$-values of toluene were observed for a transient pulse experiment in a mesoscale tank (Qiu et al. 2013, Eckert et al. 2013). Enrichment of ^{13}C in the front of the toluene breakthrough curve was partly referred to carbon isotope fractionation by sorption. Since toluene was also influenced by biodegradation under the oxic conditions and thus subjected to carbon isotope fractionation, direct evidence for sorption-induced carbon isotope fractionation could not be derived; particularly aerobic toluene degradation might yield variable carbon isotope fractionation due to different reaction mechanisms (Vogt et al. 2008). For achieving a more clear indication of carbon isotope fractionation by sorption, it would have been better to choose a more persistent compound for the transient pulse experiment. Finally, sorption-induced isotope fractionation of organic pollutants seems to be negligible at most of the contaminated field sites.

Besides sorption, water-air partitioning may cause significant changes in concentrations of organic pollutants within contaminated aquifers. As investigated in column experiments, advective and diffusive volatilization of MTBE lead to negligible to small ^{13}C-enrichment as well as small to moderate ^{2}H-enrichment within the aqueous phase, while air sparging seems to cause higher ^{2}H-enrichment (Kuder et al. 2009). Both column experiments and aquifer investigations have revealed that water-air partitioning of chlorinated ethenes lead to negligible to small changes in carbon and chlorine isotope ratios for steady-state conditions (Hunkeler et al. 2011a; Jeannottat and Hunkeler 2012; Jeannottat and Hunkeler 2013). Hence, water-air partitioning seems to lead only to minor or negligible changes in isotope ratios of organic pollutants in the water-saturated zone.

The spreading of pollutants within aquifers exhibiting a very low groundwater flow velocity is dominated by diffusion. At slow but realistic groundwater velocities, the contribution of effective molecular diffusion to transverse dispersion cannot be neglected (Cirpka et al. 2006). Laboratory studies revealed fractionation for non- and perdeuterated isotopologues of organic pollutants. Aqueous diffusion coefficients were determined for the different deuterated isotopologues based on kinetic "square root" relation or an empirical correlation from Worch (1993), in order to describe the fractionation behavior (LaBolle et al. 2008; Rolle et al. 2010). The concepts derived for diffusion-induced fractionation of different deuterated isotopologues were transferred to isotope effects for pollutants with natural isotope abundance, and the impact of diffusion and of transverse dispersion, respectively, on stable isotope ratios of organic pollutants were modelled for various scenarios of groundwater contamination (LaBolle et al. 2008; Rolle et al. 2010; Eckert et al. 2012; Van Breukelen and Rolle 2012; Centler et al. 2013). It was hypothesized that significant changes in the isotope ratios may occur due to diffusion-induced isotope fractionation. Recent laboratory studies have shown a significant lower isotope fractionation by diffusion for organic pollutants with natural isotope abundance in comparison to theoretical concepts (Jin et al. 2014; Wanner and Hunkeler 2015; Wanner et al. 2017). Therefore, previous modelling studies based on kinetic "square root" relation or the empirical correlation have overestimated isotope effects during diffusive transport of organic compounds in water-saturated systems (Wanner and Hunkeler 2015; Wanner et al. 2017; Xu et al. 2017; Wanner and Hunkeler 2019). Moreover, diffusive fractionation of deuterated isotopologues obtained from previous lab experiments (LaBolle et al. 2008; Rolle et al. 2010) could not be confirmed by a recent study (Kopinke et al. 2018). Simulations indicated that diffusion-induced isotope fractionation in water-saturated sediment with low permeability only impairs the identification of pollutant degradation using CSIA during short diffusion periods, in which the diffusion-rated isotope shifts are largest and isotope fractionation related to degradation might still be small (Wanner and Hunkeler 2015). Anyway, diffusion-induced isotope fractionation of organic pollutants seems to be negligible at most of the contaminated field sites. Carbon and chlorine isotope fractionation of 1,2-dichloroethane (1,2-DCA) and carbon isotope fractionation of dichloromethane (DCM) due to diffusion and sorption were investigated at a field

site within water-saturated, low permeability sediments below two DNAPL sources (Wanner et al. 2017). The observed shifts of carbon isotope ratios due to the physical processes were in the range of the threshold value of +2 ‰, which is often used for identifying degradation (US-EPA 2008). Thus, minor changes in carbon isotope ratios toward more positive δ^{13}C-values of 1,2-DCA and DCM can be expected at field sites, at which the contaminant transport through the water-saturated zone is dominated by diffusion and sorption. In contrast to the δ^{13}C-values, Wanner et al. 2017 observed more negative δ^{37}Cl-values of 1,2-DCA moving away from the DNAPL. Thus, the influence of diffusion and sorption on carbon isotope ratios seems to be indicated by CSIA of chlorine.

Compared to the water-saturated zone, gas diffusion is expected to cause more significant changes in the isotope ratios of organic pollutants within the unsaturated zone. It seems to be more pronounced for petroleum hydrocarbons than for chlorinated ethenes (Bouchard et al. 2008b; Hunkeler et al. 2011a). This suggests that, in the water-unsaturated zone, the interpretation of degradation of petroleum hydrocarbons using isotope data should be based on a model combining gas-phase diffusion and degradation (Bouchard et al. 2011; Khan et al. 2018).

In conclusion, the impact of physical processes is minor or negligible for the assessment of pollutant degradation based on CSIA in most of the cases. For field sites that are strongly influenced by water-air partitioning, sorption, and diffusion, it is recommended to verify the findings on pollutant degradation derived by CSIA using other monitoring tools.

5 Multielement CSIA for Differentiation of Degradation Pathways

The correlation of isotope fractionation for two or more elements is a valuable tool for deciphering degradation pathways (Musat et al. 2016; Nijenhuis and Richnow 2016; Vogt et al. 2016). Therefore, multielement CSIA (ME-CSIA) has a great potential for differentiation of anaerobic biodegradation from aerobic biodegradation and abiotic transformation, respectively. CSIA for carbon and hydrogen (2D-CSIA) has been employed for characterizing predominant degradation pathways for fuel additives (MTBE, ETBE) and BTEX in contaminated aquifers or constructed wetlands (Kuder et al. 2005; Zwank et al. 2005; Fischer et al. 2007; Fischer et al. 2009; Kujawinski et al. 2010; Jechalke et al. 2011; Rakoczy et al. 2011; Fayolle-Guichard et al. 2012; Feisthauer et al. 2012; Van Keer et al. 2012; Xiong et al. 2012; Bombach et al. 2015; Wei et al. 2015; Bouchard et al. 2018b; Shayan et al. 2018). CSIA for carbon and chlorine has been used for the evaluation of predominant reaction mechanisms of degradation for chlorinated hydrocarbons (Hunkeler et al. 2011b; Lojkasek-Lima et al. 2012a; Lojkasek-Lima et al. 2012b; Wiegert et al. 2012; Palau et al. 2014; Badin et al. 2016; Kaown et al. 2016; Palau et al. 2016; Clark et al. 2017; Doğan-Subaşı et al. 2017; Rodríguez-Fernández et al. 2018; Schiefler et al. 2018; Segal et al. 2018). Abiotic and biotic degradation processes of chlorinated ethenes have been distinguished within a contaminated aquifer with a Fe(0) barrier

using CSIA for carbon, chlorine, and hydrogen (3D-CSIA) (Audí-Miró et al. 2015). 3D-CSIA was also applied for source identification and deciphering of degradation processes for chlorinated ethenes within two contaminated aquifers located at disposal sites of by-products from the chloro-chemical industry (Filippini et al. 2018). Degradation pathways of nitroaromatic explosives at a contaminated field site were assessed using 3D-CSIA for carbon, nitrogen, and hydrogen (Fig. 4) (Wijker et al. 2013). Latest attempts for evaluating degradation processes at contaminated field sites using ME-CSIA have focused on pesticides (Alvarez-Zaldívar et al. 2018; Wu et al. 2018) and flame retardants (Balaban et al. 2016). Recently, Ojeda et al. 2019 provided recommendations for standardizing, calculating, and interpreting dual-isotope data. Modelling approaches have been developed to estimate individual contributions of different pathways to the overall degradation based on ME-CSIA (van Breukelen 2007; Centler et al. 2013; Jin and Rolle 2014).

6 Quantification of Pollutant Degradation

Using the Rayleigh-equation approach, pollutant degradation (B[%]) within groundwater systems can be estimated by changes in isotope ratios (Thullner et al. 2012) according to Eq. 2:

$$B[\%] = \left(1 - \frac{C_{Bx}}{C_0}\right) \cdot 100 = \left[1 - \left(\frac{\delta_x + 1}{\delta_0 + 1}\right)^{\left(\frac{1}{\varepsilon}\right)}\right] \cdot 100 \tag{2}$$

in which δ_0 is the initial isotope ratio of the contaminant in time or space (e.g., source zone), δ_x is the isotope ratio of a contaminant at a certain time or space (groundwater well downgradient of a source zone), C_{Bx}/C_0 is the fraction of substrate remaining during degradation at a certain time or space, and ε is the isotope enrichment factor correlating changes in concentrations and isotope ratios. Moreover, distance-dependent in situ first-order degradation rate constants (λ_s) can be estimated by changes in isotope ratios over the distance between the initial and observation points (s) using the Rayleigh-equation approach according to Eq. 3 (Hunkeler 2008).

$$\lambda_s = -\frac{1}{\varepsilon \cdot s} \ln\left(\frac{\delta_x + 1}{\delta_0 + 1}\right) \tag{3}$$

Time-dependent in situ first-order degradation rate constants (λ_t) can be determined taking into account the travel time of the pollutants along the groundwater flow path according to Eq. 4:

$$\lambda_t = -\frac{1}{\varepsilon \cdot s} \ln\left(\frac{\delta_x + 1}{\delta_0 + 1}\right) \tag{4}$$

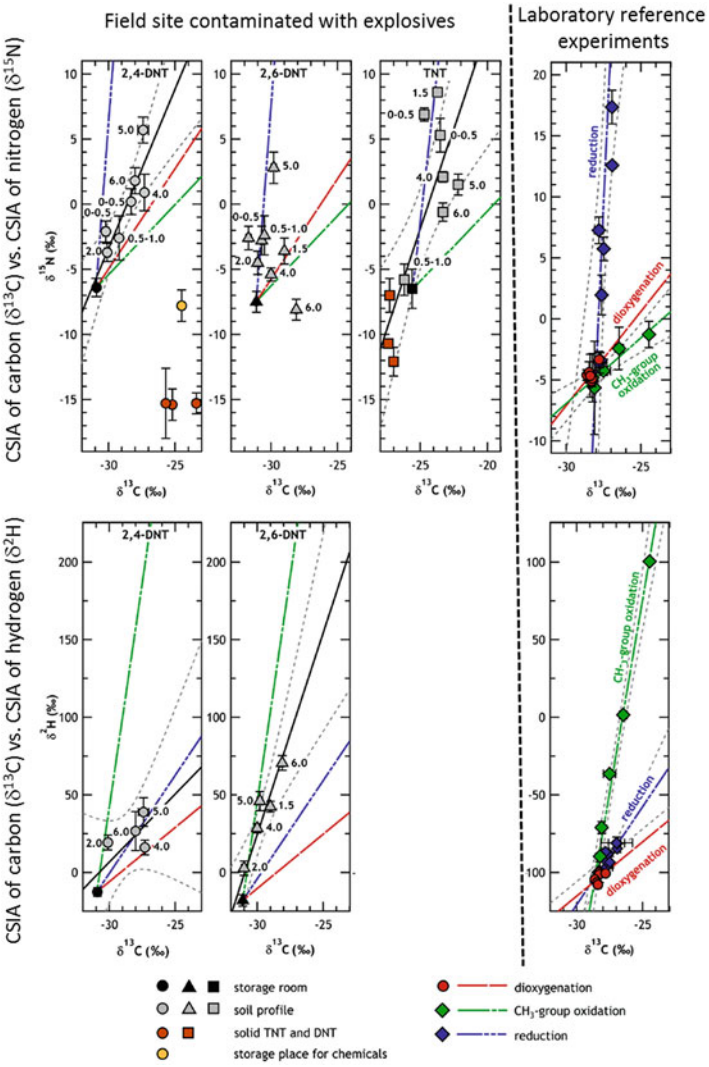

Fig. 4 $\delta^{13}C$- vs. $\delta^{15}N$-values of 2,4-DNT (circles), 2,6-DNT (triangles), and TNT (squares) as well as $\delta^{13}C$- vs. $\delta^{2}H$-values of 2,4-DNT (circles) and 2,6-DNT (triangles) from different depths of a subsurface profile (in m below surface) at a former explosives production site. Black lines indicate regression lines for field isotope data. The red, blue, and green dashed lines indicate trends of carbon vs. nitrogen as well as of carbon vs. hydrogen isotope fractionation for different biodegradation pathways of the investigated explosives obtained from laboratory reference experiments: 2,4-DNT dioxygenation (red circles/regression line), 4-NT reduction (blue diamonds/regression line), and CH_3-group oxidation of 4-NT (green diamonds/regression line). Dashed lines are 95% confidence intervals of regression lines (not shown for dioxygenation). Initial values for surface isotope signature (black markers) represent samples taken in a former storage room. Orange markers are isotope signatures of 2,4-DNT and TNT measured from solid TNT and DNT found in 1.5 m depth. The yellow marker stands for the isotope signature of 2,4-DNT from a sampling point in 100 m distance of the subsurface profile. (Reproduced from Wijker et al. 2013 with permission from American Chemical Society)

in which the travel time (t) can be approximated using the groundwater flow velocity (v) and the distance between the initial and observation points (s) according to Eq. 5:

$$t \approx \frac{s}{v} \tag{5}$$

For a proper quantification of pollutant degradation, appropriate source isotope signatures (δ_0) and isotope enrichment factors (ε) have to be selected because δ_0-values can vary due to multiple pollutant sources (Van Keer et al. 2012) and the choice of the ε-value can be limited by a wide range of values (Cichocka et al. 2008). In principle, a conservative estimation is obtained by using the isotope enrichment factor exhibiting the highest isotope fractionation and the source isotope signature with the most positive δ_0-value. Furthermore, the higher the observed changes in isotope ratios along groundwater flow paths are, the smaller are the differences for calculated degradation obtained from different constellations of isotope enrichment factors and source isotope signatures (Thullner et al. 2012). Uncertainties due to multiple source isotope signatures can be reduced by a proper source zone identification using hydrological and/or historical information, as well as by knowledge of the pollutant patterns and the distribution of the contaminant plume. Recently, a model was developed, which enables the quantification of the extent of degradation when it follows instantaneous mixing of two sources (Lutz and Van Breukelen 2014a, b). For the selection of appropriate ε-values, the following recommendations should be considered (Thullner et al. 2012):

- The dependency of isotope enrichment factors on reaction mechanisms requires knowledge of dominating degradation processes. For many pollutants, degradation pathways are linked to specific redox conditions. Therefore, the evaluation of hydrogeochemical data (e.g., concentrations of various electron acceptors and their reduced redox equivalents) can provide information for confining the range of possible ε-values for quantification using the Rayleigh-equation approach.
- The detection of metabolites or transformation products that are specific for a degradation pathway can help to determine appropriate ε-values.
- Information on pollutant degrading microorganisms might also be helpful for the proper selection of specific ε-values.
- ME-CSIA is a valuable tool for determining degradation pathways and hence could help to select appropriate ε-values.

When degradation of a pollutant is accomplished by several pathways and those exhibit different isotope fractionation, extended Rayleigh-type equations taking into account multiple competing degradation pathways with varying ε-values can be applied for a proper quantification (van Breukelen 2007; Centler et al. 2013).

The Rayleigh-equation approach has also been applied for quantifying degradation of transformation chains, for which carbon isotope ratios can be measured for a primary pollutant and its transformation products (Eq. 6), e.g., reductive dechlorination of chlorinated ethenes or benzenes (Stelzer et al. 2009; Aeppli et al. 2010).

$$\sum {}^{13}C_x[‰] = \frac{\sum \left(C_i * \delta^{13}C_i\right)}{C_x} \quad (6)$$

According to Eq. 6, the sum carbon isotope signature ($\Sigma^{13}C_x$-value) is calculated by summation of the primary pollutant and all transformation products gained from their molar concentrations (C_i) and their respective carbon isotope ratios ($\delta^{13}C_i$) and dividing it by the total molar concentration of all compounds included in the transformation chain (C_x). However, the selection of an appropriate ε-value for the transformation chain is challenging. Recently, improved constraints have been provided for the derivation of proper ε-values for the quantification of pollutant degradation of transformation chains (Höhener et al. 2015).

When mass transfer processes (e.g., transverse mixing, diffusion, dissolution, retardation, cell membrane passage) act as the rate-limiting step in the overall contaminant removal, the Rayleigh-equation approach results in an underestimation of degradation (Aeppli et al. 2009; Eckert et al. 2012; Thullner et al. 2013; Ehrl et al. 2019). Several studies provide recommendation and extensions of the conventional Rayleigh-equation approach in order to deal with aquifer heterogeneities due to mass transfer limitations, variable degradation kinetics, nonaqueous phase liquid (NAPL) presence, or dispersion (Mak et al. 2006; Abe and Hunkeler 2006; Fischer et al. 2007; van Breukelen and Prommer 2008; Höhener and Atteia 2010; Thullner et al. 2012; Hwang et al. 2013; Heße et al. 2014; Höyng et al. 2015; Zhou et al. 2017; Thouement et al. 2019).

7 CSIA Field Studies

The first field applications of CSIA for assessing degradation of organic pollutants at contaminated field sites were carried out in the end of the 1990s (Kelley et al. 1997; Sturchio et al. 1998; Hunkeler et al. 1999; Miyares et al. 1999; Richnow and Meckenstock 1999; Stehmeier et al. 1999a, b) (Table 2). Subsequently, changes in stable isotope ratios of contaminants were applied for the quantification of in situ pollutant degradation (Richnow et al. 2000; Sherwood-Lollar et al. 2001; Richnow et al. 2002) as well as ME-CSIA for deciphering degradation pathways (Kuder et al. 2005; Zwank et al. 2005; Fischer et al. 2007; Hunkeler et al. 2011b). Since then, CSIA has gained increasing interest in the evaluation of pollutant degradation at contaminated sites as indicated by around 200 research articles on CSIA field studies (Table 2). The majority of CSIA field studies focused on the most abundant groundwater pollutants: chlorinated aliphatic hydrocarbons (Hunkeler 2016; Nijenhuis and Richnow 2016; Nijenhuis et al. 2016; Renpenning and Nijenhuis 2016), BTEX (Meckenstock and Richnow 2010; Musat et al. 2016; Vogt et al. 2016; Vogt et al. 2018), and fuel oxygenates (Rosell et al. 2007; Kuder and Philp 2008). In these studies, CSIA has been established as widely used monitoring tool. CSIA has also been applied for chlorinated benzenes (Alberti et al. 2017) PAHs (Buczynska et al. 2013; Gao et al. 2018) and nitroaromatic explosives (Bernstein et al. 2014). Moreover, recent studies revealed the applicability of CSIA for assessing degradation of

Table 2 Applications of CSIA for evaluating sources and sinks of organic pollutants at contaminated field sites

Compound class	Identification/differentiation of pollutant sources	Assessment of degradation		
		Qualitative	Quantitative	ME-CSIA
Volatile chlorinated aliphatics (mainly chlorinated ethenes and ethanes)	👍👍👍👍 👎 due to degradation Beneteau et al. 1999; Hunkeler et al. 2004; Eberts et al. 2008; Blessing et al. 2009; Wang and Smith 2010; Hunkeler et al. 2011a; McHugh et al. 2011; Nijenhuis et al. 2013; Wang 2013; Kaown et al. 2014; Badin et al. 2015; Beckley et al. 2016; Filippini et al. 2018	👍👍👍👍 Sturchio et al. 1998; Hunkeler et al. 1999; Song et al. 2002; Kirtland et al. 2003; Kirchholtes et al. 2004; VanStone et al. 2005; Chapman et al. 2007; Martac et al. 2007; McKelvie et al. 2007a; Nijenhuis et al. 2007; Imfeld et al. 2008; Carreon-Diazconti et al. 2009; Kuhn et al. 2009; Morrill et al. 2009; Courbet et al. 2011; Mundle et al. 2012; Damagaard et al. 2013; McLoughlin et al. 2014; Révész et al. 2014; Velimirovic et al. 2014; Lee et al. 2015; Filippini et al. 2016; Puigserver et al. 2016; Atashgahi et al. 2017; Sonne et al. 2017; Pierce et al. 2018; Schaefer et al. 2018a; Vogel et al. 2018; Blázquez-Pallí et al. 2019; Buchner et al. 2019; Richards et al. 2019; Wilkin et al. 2019	👍👍👍👍 Sherwood-Lollar et al. 2001; Hunkeler et al. 2003; Vieth et al. 2003; Chartrand et al. 2005; Hunkeler et al. 2005; Morrill et al. 2005; Martin et al. 2006; Hirschorn et al. 2007; Abe et al. 2009b; Mäurer et al. 2009; Pooley et al. 2009; Aeppli et al. 2010; Elsner et al. 2010; Aeppli et al. 2011; Amaral et al. 2011; Hamonts et al. 2012; Wiegert et al. 2012; Patterson et al. 2013; Broholm et al. 2014; Torrentò et al. 2014; Hermon et al. 2018; Schaefer et al. 2018b; Wanner et al. 2018	👍👍👍👍 Hunkeler et al. 2011b; Lojkasek-Lima et al. 2012a; Lojkasek-Lima et al. 2012b; Wiegert et al. 2012; Petitta et al. 2013; Palau et al. 2014; Audí-Miró et al. 2015; Badin et al. 2016; Kaown et al. 2016; Palau et al. 2016; Clark et al. 2017; Doğan-Subaşı et al. 2017; Filippini et al. 2018; Rodríguez-Fernández et al. 2018; Schiefler et al. 2018; Segal et al. 2018

(*continued*)

Table 2 (continued)

Compound class	Identification/differentiation of pollutant sources	Assessment of degradation		
		Qualitative	Quantitative	ME-CSIA
BTEX	👍👍👍👍 👎 due to degradation Kelley et al. 1997; Mancini et al. 2008; Lutz and Van Breukelen 2014b; Beckley et al. 2016	👍👍👍👍 Richnow and Meckenstock 1999; Stehmeier et al. 1999a; Stehmeier et al. 1999b; Bugna et al. 2004, 2005; Micic et al. 2007; Beller et al. 2008; Gelman and Binstock 2008; Berghoff et al. 2014; Kolhatkar and Schnobrich 2017; Moshkovich et al. 2018; BenIsrael et al. 2019	👍👍👍👍 Richnow et al. 2000; Vieth et al. 2001; Mancini et al. 2002; Meckenstock et al. 2002; Richnow et al. 2002; Richnow et al. 2003a; Richnow et al. 2003b; Fischer et al. 2004; Griebler et al. 2004; Peter et al. 2004; Steinbach et al. 2004; Vieth et al. 2005; Fischer et al. 2006; Mak et al. 2006; Stelzer et al. 2006; Fischer et al. 2007; Bouchard et al. 2008b; Batlle-Aguilar et al. 2009; Blum et al. 2009; Mäurer et al. 2009; Prommer et al. 2009; De Biase et al. 2011; Morasch et al. 2011; Rakoczy et al. 2011[a]; Van Keer et al. 2012; Batlle-Aguilar et al. 2014; Lutz and Van Breukelen 2014a,b; Ponsin et al. 2015; Gilevska et al. 2019	👍👍👍👍 Mancini et al. 2002; Steinbach et al., 2004; McKelvie et al. 2005; Fischer et al. 2007; Fischer et al. 2009; Rakoczy et al. 2011[a]; Feisthauer et al. 2012; Van Keer et al. 2012; Xiong et al. 2012; Wei et al. 2015[a]; Bouchard et al. 2018b; Shayan et al. 2018; Wei et al. 2018; Wanner et al. 2019

Fuel oxygenates (mainly MTBE)	👍👍👍 👎 due to degradation Oudijk 2008	👍👍👍👍 Day et al. 2002; Kolhatkar et al. 2002; Day and Gulliver 2003; Spence et al. 2005; McKelvie et al. 2007a; Gelman and Binstock 2008; De Biase et al., 2011[a]; Thornton et al. 2011; Lu et al. 2016; Moshkovich et al. 2018; Van der Waals et al. 2018	👍👍👍👍 Kuder et al. 2005; Wilson et al. 2005; Zwank et al. 2005; McKelvie et al. 2007b; Gafni et al. 2016	👍👍👍👍 Berg et al. 2005; Kuder et al. 2005; Zwank et al. 2005; Lesser et al. 2008; Kujawinski et al. 2010; Jechalke et al. 2011[a]; Fayolle-Guichard et al. 2012; Bombach et al. 2015
PAHs	👍👍👍👍 O'Malley et al. 1996; Hammer et al. 1998; McRae et al. 2000; Fabbri et al. 2003; Walker et al. 2005; Saber et al. 2006; Bosch et al. 2015	👍👍 👎 for PAHs with more than 12 C-atoms not suitable Richnow et al. 2003a; Richnow et al. 2003b; Micic et al. 2007; Morasch et al. 2011; Berghoff et al. 2014	👍👍 Griebler et al. 2004; Blum et al. 2009	👍👍 Steinbach et al. 2004
Heterocyclic compounds	👍 👎 due to degradation	👍👍 Griebler et al. 2004; Steinbach et al. 2004; Gedalanga et al. 2016	👍 	👍👍 Wang 2016
Chlorinated benzenes	👍👍👍 👎 due to degradation Mancini et al. 2008; Alberti et al. 2017	👍👍👍 Kaschl et al. 2005; Braeckevelt et al. 2007a[a]; Stelzer et al. 2009; Passeport et al. 2016; Alberti et al. 2017	👍👍👍 Braeckevelt et al. 2007b[a]; Gilevska et al. 2019	👍
Explosives	👍👍 👎 due to degradation Coffin et al. 2001	👍👍👍 Amaral et al. 2009	👍👍👍 Bernstein et al. 2010; Sagi-Ben Moshe et al. 2010; Wijker et al. 2013; Hatzinger et al. 2019	👍👍👍 Miyares et al. 1999; Pennington et al. 2001; Wijker et al. 2013; Hatzinger et al. 2018

(*continued*)

Table 2 (continued)

Compound class	Identification/differentiation of pollutant sources	Assessment of degradation		
		Qualitative	Quantitative	ME-CSIA
Pesticides	👍👍 👎 due to degradation HCHs: Badea et al. 2011; Bashir et al. 2015; Chartrand et al., 2015; Liu et al. 2017 Diazinon: Kawashima and Katayama 2010	👍👍 HCHs: Chartrand et al., 2015; Liu et al. 2017; Wu et al. 2019 Dichlorprop: Milosevic et al. 2013 Metolachlor/acetochlor/alachlor: Elsayed et al. 2014[a] DDT: Niu et al. 2016 Chlorpyrifos: Tang et al. 2017[a]	👍👍 HCHs: Bashir et al. 2015 (*S*)-Metolachlor: Alvarez-Zaldívar et al. 2018 Parathion: Wu et al. 2018	👍👍 (*S*)-Metolachlor: Alvarez-Zaldívar et al. 2018 Atrazine: Schreglmann et al. 2013 Bentazone/2,6-dichlorobenzamide (BAM): Schürner et al. 2016[a] Parathion: Wu et al. 2018
Polychlorinated biphenyls	👍👍 (👎 due to degradation) Zeng et al. 2013	👍👍 Zeng et al. 2013	👍	👍
Flame retardants	👍👍 👎 due to degradation Zeng et al. 2013	👍👍 Zeng et al. 2013; Huang et al. 2019	👍	👍👍 Balaban et al. 2016
Pharmaceuticals	👍	👍👍	👍	👍👍 Diclofenac/ibuprofen: Schürner et al. 2016[a]

👍👍👍👍 – Well-established
👍👍👍 – Approved
👍👍 – Preliminary studies available
👍 – Analytical methods and/or laboratory studies available, but field study missing
👎 – Limitations possible or not applicable
[a] – (model) wetland or mesocosm study

micropollutants (such as pesticides, pharmaceuticals) at contaminated field sites (Elsner and Imfeld 2016; Kohli et al. 2017). A multitude of man-made chemicals are chiral and exhibit enantioselectivity due to biodegradation. Thus, enzymatic processes often prefer one enantiomer against the other. Badea et al. 2011 firstly introduced the concept of enantiomer-specific stable carbon isotope analysis (ESIA) for evaluating the biodegradation of chiral pollutants. Recently, the applicability of ESIA has been illustrated in field studies (Milosevic et al. 2013; Liu et al. 2017).

8 Conclusions

The reliability of CSIA for evaluating degradation as well as identifying sources of organic pollutants at contaminated sites is highlighted in around 200 field studies (Table 2). Besides the assessment of natural degradation, CSIA is increasingly applied in order to ascertain pollutant removal during in situ biological or chemical remediation measures. There are also increasing applications for the usage of ME-CSIA for deciphering degradation pathways. In most of the studies, anaerobic biodegradation could be distinguished from aerobic biodegradation or abiotic transformation. CSIA is one of the few methods allowing quantification of in situ pollutant degradation, which is essential for reliable risk assessment and site-specific MNA as well as success control of remediation measures. CSIA has been applied within multiple-line-of-evidence approaches (MLEA), mostly even as the key monitoring tool. Therefore, it has been recommended as a promising technique to quantify degradation at the field scale (Fischer et al. 2016; Eisenmann and Fischer 2018a, b; Ottosen et al. 2019). Moreover, it can be applied for forensic investigations to distinguish contamination events and identify liable polluters (Philp 2007; Négrel et al. 2012; Gao et al. 2018; Teixeira and de Abreu 2018; McLoughlin 2019).

Due to the advantages of CSIA, it has already been recommended by environmental authorities and associations as an appropriate monitoring tool for the implementation and success control of innovative management and remediation concepts of contaminated field sites. However, CSIA is still not routinely applied in the context of contaminated site management; it is an "add-on tool" in most of the cases. The main reason seems to be the limited knowledge on the advantages and the extent of applications of CSIA for a large part of authorities, consultancies, and field site owners. In order to overcome this obstacle, CSIA needs to be further explained and discussed in concrete instructions for handling by environmental authorities. Moreover, the CSIA guideline by the US-EPA from 2008 should be updated, and other countries ought to establish CSIA guidelines. A European guideline or directive on CSIA is long overdue.

9 Research Needs

Especially for micropollutants (e.g., pesticides, pharmaceuticals, healthcare products, perfluorinated tensides), appropriate methods for ME-CSIA need to be established and isotope enrichment factors be determined for quantification

of degradation and differentiation of reaction mechanisms (Elsner and Imfeld 2016). In order to improve the accuracy and interlaboratory conformity of CSIA for organic pollutants, future efforts should focus on the establishment of certified, compound-, or at least compound-class-specific standards with known isotope ratios and of internationally standardized guidelines for calibration of GC- and LC-IRMS, respectively (Elsner et al. 2012). Moreover, future developments should rely on the establishment of pollutant-class- and subsurface-compartment-specific MLEAs including CSIA that are optimized regarding the compatibility and synergistic effects of single monitoring methods. This would support cost-efficient and reliable assessment of pollutant degradation and, thus, increase the attention of stakeholders dealing with the management and redevelopment of contaminated field sites.

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Signature Metabolite Analysis to Determine In Situ Anaerobic Hydrocarbon Biodegradation

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Lisa M. Gieg and Courtney R. A. Toth

Contents

Abstract

Anaerobic hydrocarbon-biodegrading microorganisms can contribute substantially to the remediation of hydrocarbon-contaminated sites such as groundwater aquifers. However, documenting that such in situ degradation actually occurs can be challenging. Identifying metabolites diagnostic for the anaerobic biodegradation of specific hydrocarbons within contaminant plumes is an approach that can be used to provide strong evidence for in situ anaerobic hydrocarbon bioremediation. Here, we overview the key mechanisms of hydrocarbon metabolism under anoxic conditions, describe how metabolites identified in these pathways can be used to determine

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whether in situ biodegradation is occurring within hydrocarbon-containing environments, and summarize the reports documenting the successful use of diagnostic hydrocarbon metabolites to garner evidence in support of in situ bioremediation.

1 Introduction

The widespread use of petroleum to fulfill our energy and feedstock needs within the last century has led to contamination of hundreds of thousands of environmental sites across the globe with various fuel components. These include crude oil itself, and a variety of refined mixtures (such as gasoline, diesel, condensate, naphtha, creosote, tar oil, etc.). Fuel mixtures are mainly comprised of hydrocarbons, which in the strictest sense are defined as molecules that contain only C and H atoms, and can be classified as aromatic (monoaromatic and polycyclic aromatic), aliphatic (linear or branched alkanes), or alicyclic (cyclic alkanes). Many fuel mixtures also contain molecules harboring N, S, and/or O atoms (e.g., heterocyclic compounds, naphthenic acids, resins/asphaltenes). The majority of components within fuel mixtures are associated with some degree of toxicity, mutagenicity, and/or carcinogenicity to human and other life forms (Webb et al. 2014; Achten and Andersson 2015); thus, there is a great need to remediate fuel-contaminated environments (US EPA 2016; EEA 2016).

Bioremediation is one approach that can be used to treat fuel-contaminated environments, relying on the abilities of naturally occurring microorganisms to metabolize fuel components (Meckenstock et al. 2015). Decades of research have clearly demonstrated that hydrocarbons are readily biodegraded aerobically (wherein O_2 serves as both a strong oxidant and a terminal electron acceptor) (e.g., Gibson and Parales 2000). However, fuel-contaminated sites such as groundwater aquifers typically become depleted of dissolved O_2 due to the response of aerobic hydrocarbon-degrading microorganisms. Perhaps partly driven by this observation, hundreds of investigations in the last 30 years have now shown that hydrocarbons can be biodegraded in the absence of O_2 (for reviews see Foght 2008; Widdel and Musat 2010; Widdel et al. 2010; Meckenstock and Mouttaki 2011, Heider and Schühle 2013; Callaghan 2013a; Musat 2015; Rabus et al. 2016a). Thus, hydrocarbon-degrading anaerobic microbial communities indigenous to the subsurface can also contribute to fuel-contaminated site remediation (Meckenstock et al. 2015).

This chapter focuses on the use of signature metabolites of anaerobic hydrocarbon degradation pathways to determine whether in situ biodegradation is occurring at a contaminated site and builds on other recent reviews on this topic (Gieg and Suflita 2005; Martus and Schaal 2010; Bombach et al. 2010; Callaghan 2013b; Agrawal and Gieg 2013). As knowledge in this field has progressed, other hydrocarbon-containing environments such as crude oil reservoirs, coal seams, and contaminated marine sediments have also been interrogated for evidence of in situ biodegradation using a signature metabolite approach; thus, these environments are

considered here as well. A brief overview of anaerobic hydrocarbon metabolic pathways is presented as an introduction to the types of metabolites that can be sought in hydrocarbon environments. This is followed by a description of the rationale for utilizing signature metabolites, details about how metabolites can be detected, and presents a summary of the studies that have to date reported on the identification of signature metabolites in hydrocarbon-containing environments.

2 Pathways of Anaerobic Hydrocarbon Biodegradation

While scientists in the 1940s and 1950s demonstrated that hydrocarbons could serve as carbon sources under various anoxic conditions (e.g., Zobell 1946; Muller 1957), it was not until the late 1980s/early 1990s that research investigating the anaerobic biodegradation of hydrocarbons came into the forefront as more reports on this kind of metabolism emerged (e.g., Grbić-Galić and Vogel 1987; Dolfing et al. 1990; Evans et al. 1991; Beller et al. 1992; Edwards et al. 1992). Since then, it has been demonstrated that various classes of hydrocarbons (aromatic and saturated hydrocarbons) can be metabolized via the reduction of nitrate, Fe(III), sulfate, and/or via methane production. Studies using pure cultures or highly enriched consortia not only revealed that substrate loss could be coupled with these electron-accepting processes, but led to the discovery of novel biochemical mechanisms and metabolic pathways. Within the last decade, several detailed reviews have described pathways for the metabolism of different classes of hydrocarbons (e.g., Foght 2008; Widdel and Musat 2010; Widdel et al. 2010; Meckenstock and Mouttaki 2011, Heider and Schühle 2013; Callaghan 2013a; Musat 2015; Rabus et al. 2016a and references therein). Thus, this section only briefly overviews the key mechanisms and metabolic pathways currently known or reported for anoxic hydrocarbon biodegradation. The biodegradation pathways for the fuel components most well studied to date are shown for monoaromatic hydrocarbons, polycyclic aromatic hydrocarbons, and saturates in Figs. 1–3, respectively. It should be noted that a detailed understanding of the enzymes and genes associated with many of the biotransformation steps are known and a diagnostic biodegradative gene approach is also used to interrogate hydrocarbon-containing environments. As here we focus on a chemical approach for metabolite detection, the reader is referred to recent papers or reviews describing the use of diagnostic biodegradative genes to assess environmental samples for biodegradation potential (Callaghan et al. 2010; Callaghan 2013b; von Netzer et al. 2013; Johnson et al. 2015; Stagars et al. 2016; von Netzer et al. 2016).

3 Initial Activation Mechanisms

Under anoxic conditions, it has now been shown that (1) fumarate addition, (2) carboxylation, (3) hydroxylation, and (4) methylation are different mechanisms by which microorganisms may initially activate hydrocarbons in the absence of O_2. Of these, fumarate addition (also referred to as “addition to fumarate”) is the most

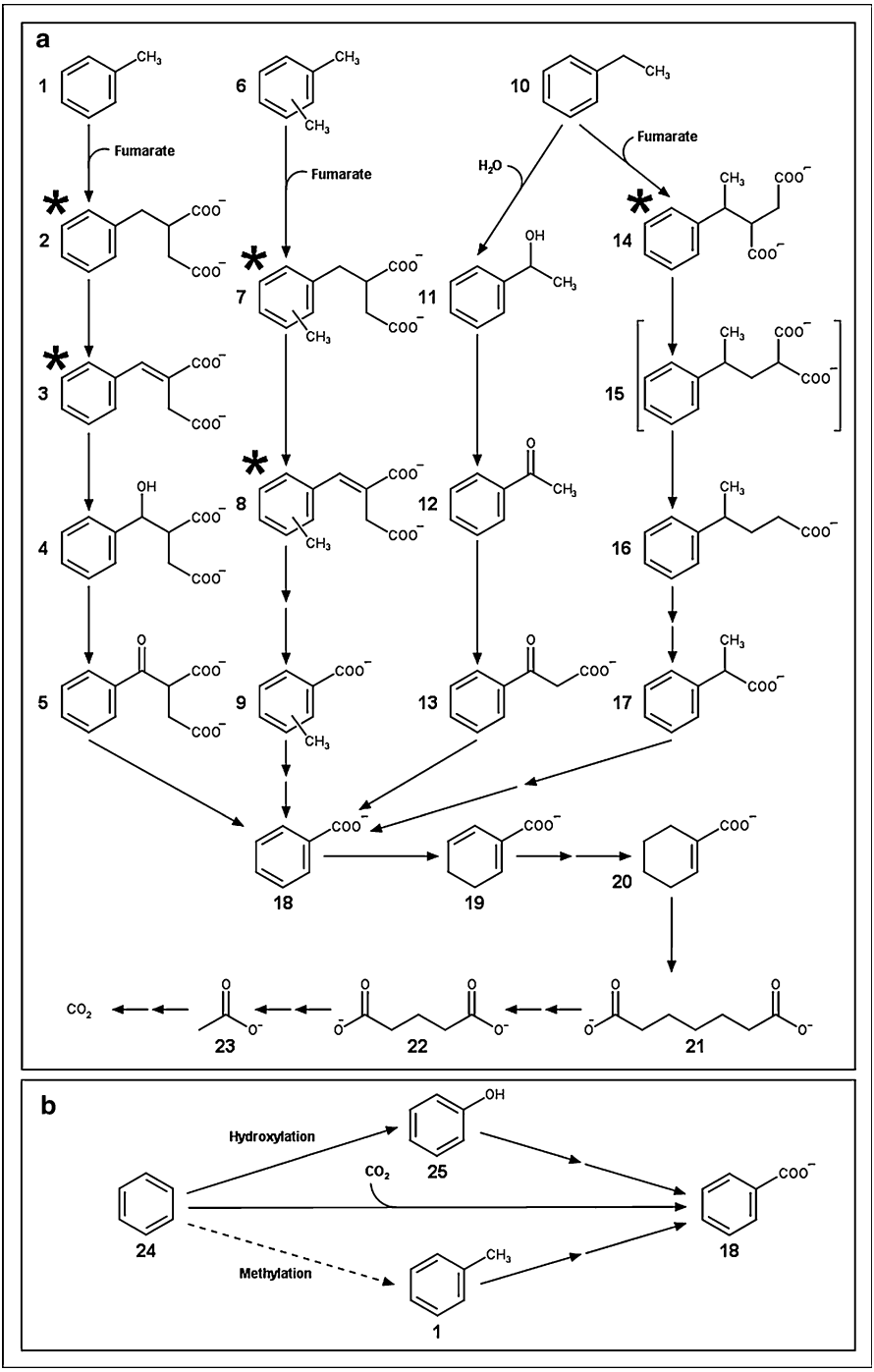

Fig. 1 (continued)

widely reported mechanism used by diverse anaerobic taxa to activate alkyl-branched aromatic compounds (alkylbenzenes, methylnaphthalenes, etc.) or alkanes (linear and cyclic) (Heider and Schühle 2013). For a methyl-substituted aromatic hydrocarbon such as toluene or 2-methylnaphthalene, the methyl group is added via a glycyl-radical-driven C–C addition reaction to the double bond of fumarate, forming benzylsuccinic acid or naphthyl-2-methylsuccinic acid (e.g., Biegert et al. 1996; Beller and Spormann 1997; Achong et al. 2001; Kane et al. 2002; Annweiler et al. 2000; Beller and Edwards 2000; Figs. 1a and 2a). For xylenes, the corresponding methylbenzylsuccinic acids are formed (Krieger et al. 1999; Achong et al. 2001), while for ethylbenzene (under sulfate-reducing conditions), the methylene group reacts with fumarate to form (1-phenylethyl)succinic acid (Elshahed et al. 2001a; Kniemeyer et al. 2003) (Fig. 1a). In the case of *n*-alkanes, fumarate addition typically occurs at the subterminal C_2 position yielding the corresponding (1-methylalkyl)succinate (we use the more simple term "alkylsuccinate" here; Kropp et al. 2000; Rabus et al. 2001; Cravo-Laureau et al. 2005; Callaghan et al. 2006) (Fig. 3a). Fumarate addition may also occur to a lesser extent at C_3 (Rabus et al. 2001), and for the gaseous alkane *n*-propane, fumarate addition occurs at the C_2 or terminal position (Fig. 3c; Kniemeyer et al. 2007; Savage et al. 2010; Musat 2015). *Iso*-alkanes have also been reported to undergo fumarate addition reactions (Abu Laban et al. 2015). During the anaerobic metabolism of the cyclic alkanes studied to date (cyclopentane, cyclohexane, methylcyclopentane, methylcyclohexane, ethycyclopentane), fumarate addition products have also been reported (Rios-Hernandez et al. 2003; Wilkes et al. 2003; Gieg et al. 2009; Musat et al. 2010; Jaekel et al. 2015; Jarling et al. 2015; Tan et al. 2015) (Fig. 3b, c).

Hydroxylation as an activation mechanism has been most well characterized for ethylbenzene metabolism under nitrate-reducing conditions (Ball et al. 1996; Heider et al. 2016). Here, the O atom arises from water, and the metabolism proceeds through a variety of reactions yielding benzoate (benzoyl-CoA) (Fig. 1). Hydroxylation has also been suggested for the activation of benzene to phenol (Caldwell and Suflita 2000) (Fig. 1b). While this metabolism was recently confirmed for benzene degradation by the Fe(III)-reducer *Geobacter metallireducens* using isotopically heavy water and genetic analysis (Zhang et al. 2013), other reports attributed phenol formation to abiotic reactions (Kunapuli et al. 2008; Abu Laban et al. 2010).

Fig. 1 Anaerobic biodegradation pathways of (**a**) model alkylbenzenes (**b**) proposed activation and subsequent degradation pathways for benzene. Metabolites are shown as free acids. Signature metabolites believed to be exclusively anaerobic are marked with an asterisk; theoretical metabolites are marked in brackets. Multiple arrows represent more than one enzymatic step; *dashed arrows* represent an unknown reaction. *Structure nomenclature*: **1**, toluene; **2**, benzylsuccinate; **3**, *E*-phenylitaconate; **4**, 2-[hydroxy(phenyl)methyl]succinate; **5**, benzoylsuccinate; **6**, xylene; **7**, methylbenzylsuccinate; **8**, *E*-methylphenylitaconate; **9**, toluate; **10**, ethylbenzene; **11**; 1-phenylethanol; **12**, acetophenone; **13**, benzoylacetate; **14**, (1-phenylethyl) succinate; **15**, (2-phenylpropyl)malonate; **16**, 4-phenylpentanoate; **17**, 2-phenylpropanoate; **18**, benzoate; **19**; cyclohex-1, 5-diene-1-carboxylate; **20**, cyclohex-1-ene-1-carboxylate; **21**, pimelate; **22**, glutarate; **23**, acetate; **24**; benzene; and **25**, phenol

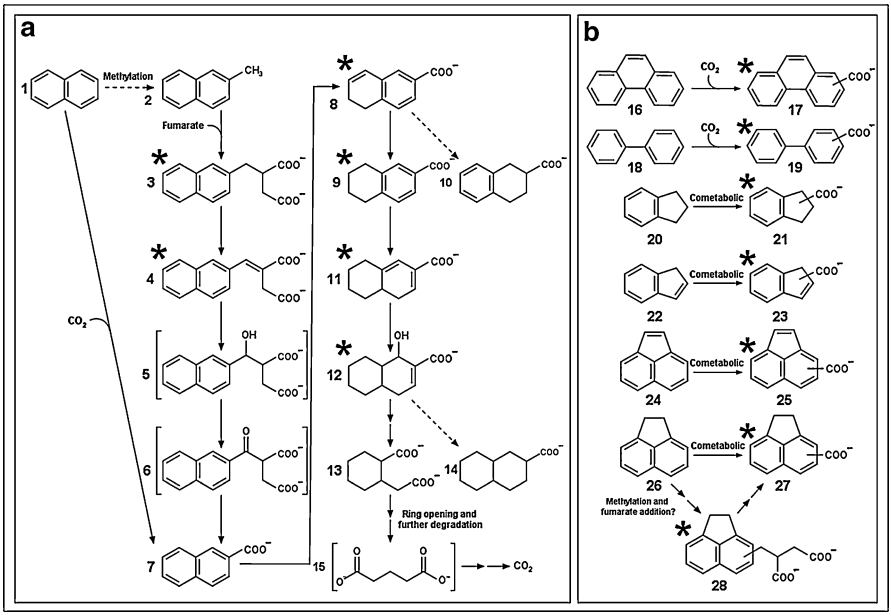

Fig. 2 Proposed anaerobic biodegradation pathways of (**a**) naphthalene/2-methylnaphthalene and (**b**) other PAHs. Metabolites are shown as free acids. Signature metabolites believed to be exclusively anaerobic are marked with an asterisk; theoretical metabolites are marked in brackets. Multiple arrows represent more than one enzymatic step. "Cometabolic" refers to metabolites observed under cometabolic conditions with naphthalene and/or 2-methylnaphthalene serving as main substrates (Safinowski et al. 2006). *Structure nomenclature*: **1**, naphthalene; **2**, 2-methylnaphthalene; **3**, naphthyl-2-methylsuccinate; **4**, naphthyl-2-methylenesuccinate; **5**, naphthyl-2-hydroxymethylsuccinate; **6**, naphthyl-2-oxomethylsuccinate; **7**, 2-naphthoate; **8**, 5,6-dihydro-2-naphthoate; **9**, 5,6,7,8-tetrahydro-2-naphthoate; **10**, 1,2,3,4-tetrahydro-2-naphthoate; **11**; hexahydro-2-naphthoate (4 isomers possible); **12**, 1-hydroxy-octahydro-2-naphthoate; **13**, *cis*-2-carboxylcyclohexylacetate; **14**, decahydro-2-naphthoate; **15**, glutarate; **16**, phenanthrene; **17**, phenanthrene carboxylate; **18**, biphenyl; **19**, biphenylcarboxylate; **20**, indane; **21**, indanoate; **22**, indene; **23**, indenoate; **24**, acenaphthylene; **25**, acenaphthylenoate; **26**, acenaphthene; **27**, acenaphthenoate; and **28**, acenaphthylmethylsuccinate

For other benzene-degrading cultures (Phelps et al. 2001; Musat and Widdel 2008; Abu Laban et al. 2010; Holmes et al. 2011) as well as for nonsubstituted polycyclic aromatics (Mouttaki et al. 2012; Davidova et al. 2007; Zhang and Young 1997), carboxylation has been reported to be the primary mechanism of activation, giving rise to benzoate from benzene (Fig. 1b), 2-naphthoic acid from naphthalene (Fig. 2a), or phenanthrene carboxylic acid from phenanthrene (Fig. 2b). A study examining the cometabolism of other PAHs by a highly enriched 2-methylnaphthalene-degrading sulfate-reducing culture (N47) also revealed a variety of carboxylic acid metabolites from indane, indene, acenaphthene, and acenaphthylene, which may have arisen from carboxylation (Safinowski et al. 2006) (Fig. 2b). It was also reported that a hexadecane-degrading sulfate reducer (*Desulfococcus*

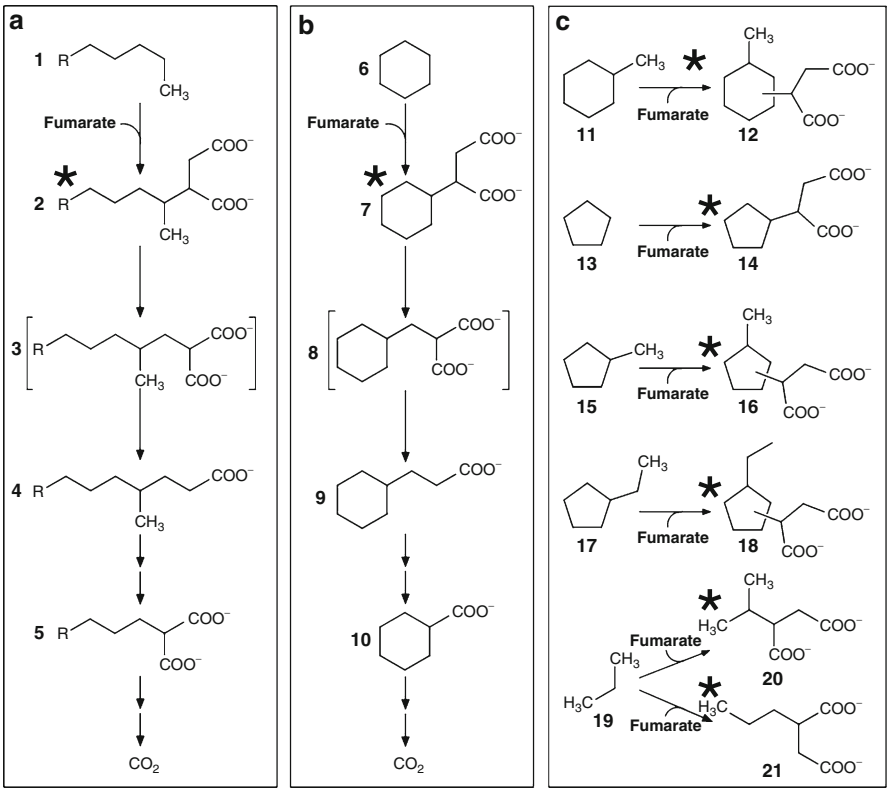

Fig. 3 Proposed anaerobic biodegradation pathways of (**a**) *n*-alkanes and (**b**) cyclohexane, and (**c**) fumarate addition of other cycloalkanes and propane. Metabolites are shown as free acids. Signature metabolites believed to be exclusively anaerobic are marked with an asterisk; theoretical metabolites are marked in brackets. Multiple arrows represent more than one enzymatic step. *Structure nomenclature*: **1**, *n*-alkane; **2**, (2-methyl)alkylsuccinate; **3**, (2-methylalkyl)malonate; **4**, 4-methylalkanoate; **5**, 2-alkylmalonate; **6**, cyclohexane; **7**, cyclohexylsuccinate; **8**, (cyclohexylmethyl)malonate; **9**, cyclohexylpropanoate; **10**, cyclohexanecarboxylate; **11**; methylcyclohexane; **12**, methylcyclohexylsuccinate; **13**, cyclopentane; **14**, cyclopentylsuccinate; **15**, methylcyclopentane; **16**, methylcyclopentylsuccinate; **17**, ethylcyclopentane; **18**, ethylcyclopentylsuccinate; **19**; propane; **20**, isopropylsuccinate; and **21**, *n*-propylsuccinate

oleovorans Hxd3) carboxylates the alkane molecule at the C_3 position, followed by a two-carbon removal ("de-ethylation") rather than degrading this alkane by fumarate addition (So et al. 2003); studies with a C_{16}-degrading nitrate-reducing culture also suggested this mechanism (Callaghan et al. 2009). Details behind this mechanism remain outstanding and may instead involve an alternate reaction (Callaghan 2013a).

A final mechanism (the least reported or understood) is that of methylation, which has been suggested to occur for the activation of benzene (wherein labeled toluene was detected from studies with labeled benzene; Ulrich et al. 2005, Fig. 1b), and naphthalene (wherein naphthyl-2-methyl-succinate was detected, presumably

following methylation to 2-methylnaphthalene; Safinowski and Meckenstock 2006, Fig. 2a). In cometabolic studies using the N47 culture, fumarate addition metabolites of the heterocycles benzothiophene, benzofuran, and indole were detected, also suggesting their formation following a methylation reaction (Safinowski et al. 2006). To date, though, a gene responsible for the expression of a methylating enzyme has not been described. Further, subsequent studies with the N47 culture and naphthalene demonstrated that carboxylation, rather than methylation, was the mechanism of naphthalene activation by this culture (Mouttaki et al. 2012).

4 Pathways Following Initial Activation

Following the addition of fumarate to toluene, benzylsuccinate is activated to benzylsuccinyl-CoA that is then degraded via modified β-oxidation reactions leading to the formation of benzoyl-CoA (Fig. 1a; e.g., Leutwein and Heider 1999, 2001, 2002), a key central metabolite in anaerobic aromatic hydrocarbon metabolism. Benzoyl-CoA subsequently undergoes a series of ring reduction reactions, starting with the conversion to cyclic 1,5-dienoyl-CoA by an ATP-dependent or independent reaction (Fuchs et al. 2011; Boll et al. 2014). Here, the pathway can diverge depending on the microorganism, ultimately leading to ring opening (Harwood et al. 1999; Fuchs et al. 2011). For the xylene isomers, analogous pathways from the methylbenzylsuccinates presumably occur, leading to the formation of toluates (Krieger et al. 1999; Elshahed et al. 2001a; Morasch et al. 2004). Detailed studies with *p*-toluic acid (4-methylbenzoate) have revealed that ring reduction and cleavage occurs in a manner that parallels that of benzoyl-CoA, with some small differences (Lahme et al. 2012; Rabus et al. 2016b). For 2-methylnaphthalene degradation, naphthyl-2-methylsuccinic acid is also activated to its CoA derivative and undergoes similar reactions to that of the toluene pathway leading to 2-naphthoyl-CoA; naphthalene biodegradation by carboxylation converges at 2-naphthoyl-CoA as well (Annweiler et al. 2000, 2002; Meckenstock et al. 2016). One ring of 2-naphthoyl-CoA then undergoes a step-wise series of recently characterized ring reduction reactions (Eberlein et al. 2013; Estelmann et al. 2015), which are presumably followed by as-of-yet uncharacterized ring opening reactions (Annweiler et al. 2002) (Fig. 2a).

For subsequent alkane degradation reactions, the formed alkylsuccinates are also presumably activated to their CoA esters (Wilkes et al. 2002). It has been postulated that this molecule then undergoes a carbon skeleton rearrangement, followed by a decarboxylation step, yielding 4-methyl-branched fatty acids that have been detected as metabolites in C_3–C_{16} alkane-amended cultures (Wilkes et al. 2002; Wilkes et al. 2003; Kniemeyer et al. 2003; Cravo-Laureau et al. 2005; Callaghan et al. 2006). These fatty acids then undergo β-oxidation to central metabolic intermediates. Interestingly, following the formation of (1-methylphenyl)succinate, ethylbenzene metabolism under sulfate-reducing conditions was hypothesized to occur in the same manner as for *n*-alkanes (rather than via the known toluene pathway) as the identified intermediate 4-phenylpentanoate (−CoA) was positively

identified (Kniemeyer et al. 2003). It is unclear whether benzoate (benzoyl-CoA) is subsequently formed from ethylbenzene as a central metabolic intermediate under sulfate-reducing conditions, although Elshahed et al. (2001a) detected benzoate in ethylbenzene-amended sulfate-reducing aquifer sediment enrichments. Following the hydroxylation of ethylbenzene to 1-phenylethanol under nitrate-reducing conditions, further metabolism leads to the generation of acetophenone, benzoylacetate, and the central metabolic intermediate benzoate (Ball et al. 1996; Heider et al. 2016).

5 Assessing In Situ Hydrocarbon Biodegradation Using Signature Metabolites

Relying on the biodegradative abilities of naturally occurring microorganisms within hydrocarbon-contaminated environments is the cornerstone of most bioremediation technologies (with the exception of bioaugmentation, wherein microbes with known biodegradative abilities are added to enhance biodegradation). When fuel mixtures are released into the subsurface (e.g., via a leaking underground storage tank), dissolved oxygen is usually rapidly consumed, resulting in anoxic environments. As discussed above, while it is now known that microorganisms can biodegrade many different hydrocarbon classes under diverse anaerobic electron-accepting processes, demonstrating or "proving" that biodegradation processes are actually occurring within a given contaminated site is crucial if a bioremediation approach is to be accepted by regulators as a feasible site clean-up strategy (NRC 1993; US EPA 2016). Bombach et al. (2010) reviewed the various approaches that have been developed in recent years to assess in situ anaerobic hydrocarbon biodegradation. These approaches have included monitoring for components indicative of electron-accepting processes (geochemical monitoring), compound-specific isotope analysis, microbial analysis, biodegradative gene analysis, the use of in situ microcosms, and via the use of signature hydrocarbon metabolites. The latter will be the focus here. When assessing a site for evidence of in situ biodegradation, it is critical that several different approaches are used to provide multiple lines of evidence, as each approach has inherent limitations that can be overcome by using different approaches (NRC 1993; Gieg et al. 1999; Weiss and Cozzarelli 2008; Beller 2000; Bombach et al. 2010; US EPA 2016). Further, measurements must be made with respect to corresponding uncontaminated areas so that any observed changes can be clearly attributed to microbial activities in response to the contamination.

6 Detection of Fumarate Addition Metabolites In Situ

A handful of landmark studies in the 1980s and early 1990s initially reported on the detection of potential hydrocarbon biodegradation products in the anoxic zones of hydrocarbon-impacted groundwaters (Reinhard et al. 1984; Cozzarelli et al. 1990; Wilson et al. 1990; Schmitt et al. 1996). These products, including aromatic acids (such as benzoate, toluate, tolylacetate, phenylacetate), along with alicyclic and

aliphatic acids, were not present in uncontaminated areas. With the discovery of fumarate addition as a novel mechanism of anaerobic activation for toluene and xylenes and the detection of these metabolites in a field site, Beller and colleagues proposed that these new metabolites (designated here as "benzylsuccinates") could serve as unique or signature metabolites indicative of in situ anaerobic hydrocarbon biodegradation (Beller et al. 1992; Beller et al. 1995). These metabolites were deemed ideal because they (1) have an unequivocal relationship to their parent hydrocarbon, (2) are not known to be present in hydrocarbon mixtures themselves/have no industrial sources, (3) are reasonably stable so they can be detected in a groundwater sample, and yet (4) are transient metabolites so they would not be expected to accumulate in the environment (NRC 1993; Beller 2000). A later independent study examining the prospects for intrinsic bioremedation (natural attenuation) in a gas condensate-contaminated aquifer substantiated the proposal put forth by Beller et al. (1995), as fumarate addition metabolites from *m*-xylene, *p*-xylene, and ethylbenzene could also be detected in contaminated groundwater samples (Elshahed et al. 2001a). By coupling the metabolite results with several other monitoring approaches, these findings indeed provided strong evidence that the site was undergoing in situ alkylbenzene biodegradation (Gieg et al. 1999; Elshahed et al. 2001a). Subsequently, many field studies have shown that such benzylsuccinates can be detected in anoxic hydrocarbon-contaminated groundwater (Table 1). It should be noted that in addition to detecting the diagnostic benzylsuccinates from toluene, xylene, and ethylbenzene, some studies have also reported fumarate addition products arising from trimethylbenzene and even more highly alkylated benzenes in contaminated groundwaters (Martus and Püttmann 2003; Gieg et al. 2009; Parisi et al. 2009).

Following the discovery that *n*-alkanes could also be activated via fumarate addition reactions (Kropp et al. 2000; Rabus et al. 2001), alkylsuccinates (including those arising from cyclic alkanes) were also sought and were detected at the same gas condensate-contaminated aquifer and at other hydrocarbon-impacted sites (Gieg and Suflita 2002; Parisi et al. 2009) (Table 2). Similarly, once it was discovered that polycyclic aromatic hydrocarbons (PAHs) such as 2-methylnaphthalene were also biodegraded via fumarate addition (Annweiler et al. 2000), the corresponding naphthylmethylsuccinates were sought and detected at contaminated sites (Griebler et al. 2004; Safinowski et al. 2006; Morasch et al. 2011; Jobelius et al. 2011). Putative fumarate addition metabolites of other PAH (from acenaphthene, dimethylnaphthalene) have also been detected in fuel-contaminated sites (Morasch et al. 2011; Jobelius et al. 2011) (Table 3). Collectively, these studies have expanded the detection of diagnostic "succinates" in groundwater environments to other classes of hydrocarbons. Notably, benzylsuccinates and/or alkylsuccinates have also been detected in produced fluids from crude oil reservoirs (Duncan et al. 2009; Gieg et al. 2010; Agrawal and Gieg 2013; Bian et al. 2015) and coal seams (Wawrik et al. 2012), as well as in oil-contaminated marine sediments (Kimes et al. 2013), extending their usefulness to pinpoint anaerobic biodegradation across a variety of hydrocarbon-laden anoxic ecosystems. Tables 1–3 list the various field studies wherein metabolites from all of these classes of hydrocarbons have been detected.

Table 1 Mass spectral features (GC-MS, as methyl- or trimethylsilyl esters) of key metabolites associated with the anaerobic biodegradation of monoaromatic hydrocarbons, and instances where they have been detected in hydrocarbon-impacted sites

Parent hydrocarbon	Signature metabolite	Diagnostic ions (m/z)		Detected in situ
		(Di)methyl ester (MS reference)[a]	(Di)trimethylsilyl ester (MS reference)[a]	
Initial activation/early reaction, uniquely anaerobic				
Toluene	Benzylsuccinate	236 (M), 205, 176, 163, 145, 131, 117, 91 (Beller et al. 1992)	352 (M), 337 (M-15), 235, 221, 205, 190, 147, 145, 132, 73 (Gieg et al. 2009)	Beller et al. 1995, 2008; Beller 2000, 2002; Reusser et al. 2002; Baun et al. 2003; Alumbaugh et al. 2004; Ledin et al. 2005; Parisi et al. 2009; Gieg et al. 2009; Jobelius et al. 2011; Morasch et al. 2011; Wawrik et al. 2012; Jobelius et al. 2014; Bian et al. 2015
	E-phenylitaconate	234 (M), 202, 174, 115 (Beller et al. 1995)	350 (M), 335 (M-15), 305, 291, 229, 217, 147, 73	Beller et al. 1995
Xylene	Methylbenzyl-succinate	250 (M), 219, 190, 177, 159, 145, 131, 105, 91 (Krieger et al. 1999)	366 (M), 351 (M-15), 248, 235, 204, 159, 145, 105, 73	Beller et al. 1995, 2008; Gieg et al. 1999; Beller 2000, 2002; Elshahed et al. 2001a; Gieg and Suflita 2002; Gieg et al. 2009; Reusser et al. 2002; Martus and Püttmann 2003; Alumbaugh et al. 2004; Ledin et al. 2005; Young and Phelps 2005; Jobelius et al. 2011; Wawrik et al. 2012
	E-methyl-phenylitaconate	248 (M), 216, 188, 129 (Krieger et al. 1999)	*No MS data*	Beller et al. 1995
Ethylbenzene[b]	(1-phenylethyl)-succinate	250 (M), 231 (M-31), 190, 177 (M-73), 145, 131, 105 (Kniemeyer et al. 2003)	366 (M), 351 (M-15), 248, 235, 204, 145, 105, 73 (Elshahed et al. 2001a)	Elshahed et al. 2001a; Gieg and Suflita 2002; Gieg et al. 2009; Alumbaugh et al. 2004; Parisi et al. 2009; Wawrik et al. 2012

(*continued*)

Table 1 (continued)

Parent hydrocarbon	Signature metabolite	Diagnostic ions (m/z)		Detected in situ
		(Di)methyl ester (MS reference)[a]	(Di)trimethylsilyl ester (MS reference)[a]	
Trimethyl-benzene[c]	Dimethyl-benzylsuccinate	264 (M), 233 (M-31), 204, 191 (M-73), 159, 145, 119 (Martus and Püttmann 2003)	380 (M), 365 (M-15), 262, 249, 233, 218, 173, 159, 73 (Gieg et al. 2009)	Martus and Püttmann 2003; Parisi et al. 2009; Gieg et al. 2009; Kimes et al. 2013
Downstream products and/or not uniquely anaerobic				
Xylene	Methylbenzoate (Toluate)	150 (M), 119, 91, 65	208 (M), 193 (M-15), 149, 119, 91	Reinhard et al. 1984; Wilson et al. 1990; Cozzarelli et al. 1990, 1994; Beller et al. 1995; Levine et al. 1997; Gieg et al. 1999, 2009; Beller 2000; Annweiler et al. 2001; Elshahed et al. 2001a; Martus and Püttmann 2003; Griebler et al. 2004; Alumbaugh et al. 2004; Parisi et al. 2009; Wawrik et al. 2012
Benzene	Phenol	108 (M), 93, 78, 65	166 (M), 151 (M-15), 91, 77 (Caldwell and Suflita 2000)	Reinhard et al. 1984; Wilson et al. 1990; Cozzarelli et al. 1990, 1994; Baun et al. 2003; Gieg et al. 2010; Wawrik et al. 2012
Toluene, benzene, xylene, ethylbenzene	Benzoate	136 (M), 105 (M-31), 77, 51 (Beller 2000)	194 (M), 179 (M-15), 135, 105, 73	Reinhard et al. 1984; Cozzarelli et al. 1990, 1994; Wilson et al. 1990; Schmitt et al. 1996; Gieg et al. 1999, 2009, 2010; Beller 2000; Annweiler et al. 2001; Griebler et al. 2004; Alumbaugh et al. 2004; Ledin et al. 2005; Wawrik et al. 2012; Kimes et al. 2013

(*continued*)

Table 1 (continued)

Parent hydrocarbon	Signature metabolite	Diagnostic ions (m/z)		Detected in situ
		(Di)methyl ester (MS reference)[a]	(Di)trimethylsilyl ester (MS reference)[a]	
	Cyclohex-1-ene-1-carboxylate	*No MS data*	198 (M), 183 (M-15), 156, 139, 108, 75, 73 (Elshahed et al. 2001b)	Wawrik et al. 2012
	Pimelate	188 (M), 157, 128, 125, 115, 83, 74	304 (M), 289 (M-15), 245, 217, 186, 173, 155, 125, 117, 97, 73 (Elshahed et al. 2001b)	Gieg et al. 2010, Wawrik et al. 2012
	Glutarate	160 (M) 129, 100, 87, 59	276 (M), 261 (M-15), 233, 204, 186, 158, 147, 129, 97, 73 (Elshahed et al. 2001b)	Gieg et al. 2010, Wawrik et al. 2012

[a]Citation for mass spectrum figure is provided (if published).
[b]Ethylbenzene metabolism under sulfate-reducing conditions; products from nitrate reduction not detected in any field studies (known to date).
[c]Martus and Püttmann (2003) and Kimes et al. (2013) also detected more alkylated benzylsuccinates.

7 Detection of Other Metabolites In Situ

Metabolites resulting from fumarate addition reactions can be considered as the best diagnostic indicators of anaerobic in situ hydrocarbon metabolism because they are only produced in the absence of O_2 and in general have a clear relationship to their parent hydrocarbon (with a couple of exceptions for alkylsuccinates wherein cometabolism may confound their link with the parent alkane; Jarling et al. 2015). However, as described above, this reaction is not known to occur for unsubstituted aromatic hydrocarbons like benzene, naphthalene, or other unsubstituted PAHs and heterocycles whose in situ biodegradation is also of concern. Unfortunately, some products from these activation reactions (such as phenol or benzoate from benzene, naphthoic acid from naphthalene), and many intermediates resulting from reactions downstream of fumarate addition (such as fatty acids) are not uniquely anaerobic, nor exclusively related to their parent hydrocarbon. Diagnosing in situ anaerobic benzene biodegradation using metabolites is the most difficult task of all the hydrocarbons, since both known anaerobic activation metabolites (phenol or benzoate) are also known aerobic metabolites (Assinder and Williams 1990), and benzoate is a common central metabolic intermediate resulting from the

Table 2 Mass spectral features (GC-MS, as methyl- or trimethylsilyl esters) of key metabolites associated with the anaerobic biodegradation of linear or cyclic alkanes, and instances where they have been detected in hydrocarbon-impacted sites

Parent *n*-alkane	Diagnostic fragment ions (m/z)		Detected in situ
	(Di)methyl ester (MS reference)[a]	(Di)trimethylsilyl ester (MS reference)[a]	
Methane[b] (C_1)	160 (M), 129 (M-31), 101, 87 (M-73), 69, 59	276 (M), 261 (M-15), 217, 147, 73 (Duncan et al. 2009)	Duncan et al. 2009; Gieg et al. 2010; Wawrik et al. 2012; Bian et al. 2015[c]
Ethane (C_2)	174 (M), 143 (M-31), 101 (M-73), 146, 114	290 (M), 275 (M-15), 262, 217, 172, 147, 73 (Duncan et al. 2009)	Duncan et al. 2009; Gieg et al. 2010; Wawrik et al. 2012; Bian et al. 2015
Propane (C_3)	188 (M), 157 (M-31), 115 (M-73), 146, 114 (Kniemeyer et al. 2007)	304 (M), 289 (M-15), 262, 217, 172, 147, 73 (Duncan et al. 2009)	Gieg and Suflita 2002; Duncan et al. 2009; Gieg et al. 2010; Wawrik et al. 2012; Bian et al. 2015
Butane (C_4)	202 (M), 171 (M-31), 129 (M-73), 146, 114 (Wilkes et al. 2003)	318 (M), 303 (M-15), 262, 217, 172, 147, 73 (Duncan et al. 2009)	Gieg and Suflita 2002; Duncan et al. 2009; Bian et al. 2015
Pentane (C_5)	216 (M), 185 (M-31), 143 (M-73), 146, 114 (Wilkes et al. 2003)	332 (M), 317 (M-15), 262, 217, 172, 147, 73 (Berdugo-Clavijo and Gieg 2014)	Gieg and Suflita 2002; Parisi et al. 2009; Bian et al. 2015
Hexane (C_6)	230 (M), 199 (M-31), 157 (M-73), 146, 114 (Wilkes et al. 2003)	346 (M), 331 (M-15), 262, 217, 172, 147, 73 (Gieg and Suflita 2002)	Gieg and Suflita 2002; Parisi et al. 2009; Gieg et al. 2009; Bian et al. 2015
Heptane (C_7)	244 (M), 213 (M-31), 171 (M-73), 146, 114 (Wilkes et al. 2003)	360 (M), 345 (M-15), 262, 217, 172, 147, 73	Gieg and Suflita 2002; Parisi et al. 2009; Wawrik et al. 2012; Bian et al. 2015
Octane (C_8)	258 (M), 227 (M-31), 185 (M-73), 146, 114 (Wilkes et al. 2003)	374 (M), 359 (M-15), 262, 217, 172, 147, 73 (Gieg and Suflita 2002)	Gieg and Suflita 2002; Parisi et al. 2009; Wawrik et al. 2012; Bian et al. 2015
Nonane (C_9)	272 (M), 241 (M-31), 199 (M-73), 146, 114	388 (M), 373 (M-15), 262, 217, 172, 147, 73	Gieg and Suflita 2002
Decane (C_{10})	286 (M), 255 (M-31), 213 (M-73), 146, 114	402 (M), 387 (M-15), 262, 217, 172, 147, 73 (Gieg and Suflita 2002)	Gieg and Suflita 2002

(*continued*)

Table 2 (continued)

Parent *n*-alkane	Diagnostic fragment ions (m/z)		Detected in situ
	(Di)methyl ester (MS reference)[a]	(Di)trimethylsilyl ester (MS reference)[a]	
Undecane (C_{11})	300 (M), 269 (M-31), 227 (M-73), 146, 114	416 (M), 401 (M-15), 262, 217, 172, 147, 73	Gieg and Suflita 2002
Parent cyclic alkane			
Cyclopentane	214 (M), 183 (M-31), 141 (M-73), 146, 114 (Wilkes et al. 2003)	330 (M), 315 (M-15), 262, 217, 172, 147, 73	Parisi et al. 2009
Cyclohexane (or other cyclic C_6)	228 (M), 197 (M-31), 155 (M-73), 146, 114 (Musat et al. 2010)	344 (M), 329 (M-15), 262, 217, 172, 147, 73 (Gieg and Suflita 2002)	Gieg and Suflita 2002; Parisi et al. 2009
Ethyl-cyclopentane (or other cyclic C_7)	242 (M), 211 (M-31), 169 (M-73), 146, 114	358 (M), 343 (M-15), 262, 217, 172, 147, 73 (Rios-Hernandez et al. 2003)	Gieg and Suflita 2002; Rios-Hernandez et al. 2003; Parisi et al. 2009; Gieg et al. 2009; Wawrik et al. 2012
Cyclic C_8	256 (M), 225 (M-31), 183 (M-73), 146, 114	372 (M), 357 (M-15), 262, 217, 172, 147, 73 (Gieg and Suflita 2002)	Gieg and Suflita 2002; Parisi et al. 2009; Wawrik et al. 2012
Cyclic C_9	270 (M), 239 (M-31), 183 (M-73), 146, 114	386 (M), 371 (M-15), 262, 217, 172, 147, 73	Gieg and Suflita 2002; Parisi et al. 2009

[a]Citation for mass spectrum figure is provided (if published).
[b]Methylsuccinate may or may not be a fumarate addition metabolite of CH_4 (Duncan et al. 2009; Jarling et al. 2015).
[c]Bian et al. (2015) showed metabolites detected as *ethyl* esters in oilfield produced waters, but shown here since detected in field samples.

anaerobic decay of numerous aromatic substrates (Fuchs et al. 2011). Toluic acids (downstream products following fumarate addition to xylenes) while related to their parent hydrocarbon (*o*-, *m*-, or *p*-xylene) can also be produced via aerobic pathways (Assinder and Williams 1990). Similarly, 2-naphthoic acid (resulting from naphthalene carboxylation or as a downstream metabolite following fumarate addition from 2-methylnaphthalene; Fig. 3a) can form aerobically (Mahajan et al. 1994). Fatty acids resulting from subsequent biodegradation of alkylsuccinates can also be difficult to definitively attribute to in situ anaerobic hydrocarbon metabolism because these kinds of compounds are commonly found in sediment organic matter and are constituents of cell biomass. Despite these limitations, seeking these other metabolites can still be useful in documenting in situ hydrocarbon metabolism,

Table 3 Mass spectral features (GC-MS, as methyl- or trimethylsilyl esters) of key metabolites associated with the anaerobic biodegradation of PAHs and heterocycles, and instances where they have been detected in hydrocarbon-impacted sites

Parent hydrocarbon	Signature metabolite	Diagnostic fragment ions (m/z)		Detected in situ
		(Di)methyl ester (MS reference)[a]	(Di) trimethylsilyl ester (MS reference)[a]	
Naphthalene or 2-methyl-naphthalene	Naphthyl-2-methyl-succinate	286 (M), 226, 195, 167, 141, 115 (Annweiler et al. 2000)	*No MS data*	Ohlenbusch et al. 2002; Griebler et al. 2004; Jobelius et al. 2011; Morasch et al. 2011; Jobelius et al. 2014
	Naphthyl-2-methylene-succinate	284 (M), 252, 224, 165 (Annweiler et al. 2000)	*No MS data*	Jobelius et al. 2011
	Naphthoate[b]	186 (M), 155, 127 (Meckenstock et al. 2000)	244 (M), 229 (M-15), 201, 185, 155, 127 (Gieg and Suflita 2002)	Annweiler et al. 2001, Phelps et al. 2002; Ohlenbusch et al. 2002; Gieg and Suflita 2002; Griebler et al. 2004; Young and Phelps 2005; Parisi et al. 2009; Gieg et al. 2010; Jobelius et al. 2011; Morasch et al. 2011; Wawrik et al. 2012; Jobelius et al. 2014; Bian et al. 2015

(*continued*)

Table 3 (continued)

Parent hydrocarbon	Signature metabolite	Diagnostic fragment ions (m/z)		Detected in situ
		(Di)methyl ester (MS reference)[a]	(Di) trimethylsilyl ester (MS reference)[a]	
	5, 6, 7, 8-Tetrahydro-2-naphthoate	190 (M), 159, 131 (Meckenstock et al. 2000)	248 (M), 233, 189, 159, 131, 115 (Phelps et al. 2002)	Bian et al. 2015, Gieg and Suflita 2002, Gieg et al. 2010, Griebler et al. 2004, Parisi et al. 2009, Phelps et al. 2002, Wawrik et al. 2012
	1, 2, 3, 4-Tetrahydro-naphthoate	190 (M), 158, 130 (Meckenstock et al. 2000)	248 (M), 233 (M-15), 158, 130	Gieg and Suflita 2002; Griebler et al. 2004; Wawrik et al. 2012
	Hexahydro-2-naphthoate	*No MS data*	250 (M), 235 (M-15), 191, 161, 115 (Phelps et al. 2002)	Phelps et al. 2002
Dimethyl-naphthalene	Methylnaphthyl-2-methyl-succinate	300 (M), 240, 226, 21, 195, 181, 167, 153, 141 (Safinowski et al. 2006)	*No MS data*	Morasch et al. 2011
	Methylnaphthoate	200 (M), 69, 141, 115 (Safinowski et al. 2006)	258 (M), 243 (M-15), 199, 169, 141, 115 (Phelps et al. 2002)	Gieg and Suflita 2002, 2010; Phelps et al. 2002; Safinowski et al. 2006; Parisi et al. 2009; Jobelius et al. 2011; Morasch et al. 2011; Wawrik et al. 2012

(*continued*)

Table 3 (continued)

Parent hydrocarbon	Signature metabolite	Diagnostic fragment ions (m/z)		Detected in situ
		(Di)methyl ester (MS reference)[a]	(Di) trimethylsilyl ester (MS reference)[a]	
Acenaphthene	Acenaphthyl methylsuccinate	312 (M), 253, 239, 221, 207, 193, 178, 165, 152, 133, 109 (Morasch et al. 2011)	*No MS data*	Jobelius et al. 2011; Morasch et al. 2011
	Acenaphthenoate	212 (M), 181, 153 (Safinowski et al. 2006)	*No MS data*	Safinowski et al. 2006; Jobelius et al. 2011
Acenaphthylene	Acenaphthylenoate	210 (M), 179, 151 (Safinowski et al. 2006)	*No MS data*	Safinowski et al. 2006
Biphenyl	Biphenylcarboxylate	212 (M), 153, 152, 181 (Morasch et al. 2011)	*No MS data*	Morasch et al. 2011
Indane	Indanoate	176 (M), 145, 117 (Safinowski et al. 2006)	*No MS data*	Annweiler et al. 2001; Safinowski et al. 2006; Morasch et al. 2011; Jobelius et al. 2014
Indene	Indenoate	174 (M) 143, 129, 115 (Safinowski et al. 2006)	*No MS data*	Safinowski et al. 2006; Morasch et al. 2011
Benzothiophene	Carboxybenzothiophene	192 (M), 161, 133, 89 (Safinowski et al. 2006)	*No MS data*	Annweiler et al. 2001; Safinowski et al. 2006
Benzofuran	Benzofuranoate	176 (M), 145, 118, 89 (Safinowski et al. 2006)	*No MS data*	Safinowski et al. 2006; Morasch et al. 2011

[a]Citation for mass spectrum figure is provided (if published).
[b]May be formed aerobically, therefore not uniquely anaerobic.

especially when other field evidence clearly indicates that a site is anoxic due to microbial activity, and when these metabolites are only found in contaminated areas. As such, less diagnostic metabolites such as benzoate, toluates, naphthoates, benzoate degradation products (Tables 1 and 3), and fatty acids (Duncan et al. 2009; Bian et al. 2015) have been detected in many anoxic hydrocarbon-contaminated groundwaters or fossil energy reservoir fluids, suggesting in situ anaerobic BTEX (mixture of benzene, toluene, ethylbenzene, and xylenes), PAH, and/or alkane biodegradation. Some downstream metabolites of naphthalene or 2-methylnaphthalene such as partly reduced 2-naphthoic acids (e.g., 5, 6, 7, 8-tetrahydro-2-naphthoic acid) and carboxylated heterocycles or PAHs (from benzothiophene, benzofuran, acenaphthene, acenaphthylene, indane, indene) are only known to be produced anaerobically, thus can serve as signature anaerobic metabolites for these compounds (Table 3).

8 Methods of Hydrocarbon Metabolite Analysis

The detection of anaerobic hydrocarbon metabolites typically requires the use of analytical instrumentation such as a gas chromatograph (GC) or a liquid chromatograph (LC) coupled with a mass spectrometer (MS; low or high resolution). To date, low-resolution GC-MS has been most widely used to detect metabolites diagnostic of anaerobic hydrocarbon biodegradation. Here, samples (usually water samples) from the field (groundwater or produced water from oilfields, minimum of 1-L recommended) are acidified to at least pH 2 (to preserve the water samples and to protonate carboxylic acid and alcohol groups), extracted with an organic solvent (such as ether, ethylacetate, or dichloromethane), concentrated to a small volume, derivatized (using methyl esterification or silylation), and analyzed on a low-resolution GC-MS, usually using a capillary column designed for nonpolar analytes (e.g., Beller et al. 1995; Reinhard et al. 1997; Elshahed et al. 2001a; Gieg and Suflita 2002; Griebler et al. 2004). In lieu of organic solvent extraction, others have processed samples using solid-phase extraction prior to derivatization and low-resolution GC-MS analysis (Reusser et al. 2002; Ledin et al. 2005). Using low-resolution GC-MS analysis, diagnostic fragment ions specific to metabolites analyzed as their trimethylsilyl or methyl esters have been reported in numerous studies, and these can be used to identify the metabolites within environmental samples. The major diagnostic GC-MS-derived fragment ions reported for metabolites of anaerobic saturate and aromatic hydrocarbon biodegradation that can be used as a reference to interrogate field samples for their presence are shown in Tables 1–3. Recently, a high-resolution GCxGC approach has been used to examine samples for signature metabolites and/or other components that may be important in contaminated groundwater plumes (e.g., dissolved organic matter components; Mohler et al. 2013; O'Reilly et al. 2015) or biodegraded crude oil samples (West et al. 2014). This technique uses GC columns linked in tandem for improved resolution of complex mixtures, followed by detection using a high-resolution time-of-flight mass spectrometer (TOF-MS) that allows for exact mass determination (West et al. 2014).

While the use of GC-MS-based approaches can readily identify the presence of signature hydrocarbon metabolites, sample preparation is laborious and time consuming. An alternate method that requires comparatively little sample preparation is through the use of an LC coupled with a tandem MS operated in the electrospray ion mode (LC-ESI-MS-MS) or with a quadrupole time-of-flight mass spectrometer (LC-ESI-Q-TOF-MS). Among these, LC-MS-MS approaches were initially used for anaerobic metabolite analysis wherein samples were injected directly (Beller 2002; Ohlenbusch et al. 2002), or were analyzed following a preconcentration step using solid-phase extraction (SPE) (Alumbaugh et al. 2004). Direct injection methods can achieve detection limits as low as 0.2 μg/L, while pretreatment of the sample with SPE allowed for the detection of metabolite concentrations approximately one order of magnitude less (e.g., 0.01 μg/L). More recently, Jobelius et al. (2011) used both LC-MS-MS and LC-ESI-Q-TOF to analyze for a variety of signature metabolites in a tar oil-contaminated aquifer. While LC-MS-MS can readily identify signature metabolites based on a parent-to-daughter ion transition (Beller 2000; Alumbaugh et al. 2004), the use of a Q-TOF-MS allows for exact mass determination (Jobelius et al. 2011). Table 4 shows the mass

Table 4 Mass spectral monitoring ions for LC-MS-MS analysis of aromatic hydrocarbon metabolites in field samples

Metabolite	Parent ion (m/z)	Daughter ion (m/z)	References
Monoaromatic hydrocarbons			
Benzylsuccinate	207	163 or 189	Beller 2000; Alumbaugh et al. 2004; Jobelius et al. 2011
Methylbenzylsuccinate, (1-Phenylethyl)succinic acid	221	177 or 203	Beller 2000; Alumbaugh et al. 2004; Jobelius et al. 2011
Toluate	135	91	Alumbaugh et al. 2004
Benzoate	121	77	Alumbaugh et al. 2004
Polycyclic aromatic hydrocarbons			
Naphthyl-2-methylsuccinate	257	169 or 213	Ohlenbusch et al. 2002; Jobelius et al. 2011
Naphthyl-2-methylene-succinate	255	211	Jobelius et al. 2011
Methylnaphthyl-2-methylsuccinate	271	227	Jobelius et al. 2011
2-Naphthoic acid	171	127	Ohlenbusch et al. 2002; Jobelius et al. 2011
Methylnaphthoic acid	185	141	Jobelius et al. 2011
Acenaphthene-5-carboxylic acid	197	153	Jobelius et al. 2011
Heterocyclic compounds			
Benzothiophenemethylsuccinate	263	219	Jobelius et al. 2011
2-Carboxybenzothiophene	177	133	Jobelius et al. 2011
Methyl-2-carboxybenzothiophene	191	147	Jobelius et al. 2011
Benzofuranmethylsuccinate	247	203	Jobelius et al. 2011
2-Carboxybenzofuran	161	117	Jobelius et al. 2011

transitions that can be used to identify various hydrocarbon metabolites by LC-MS-MS. For these various approaches, metabolite identification is generally made by comparison with authentic standards, or by comparison with mass spectral databases.

In addition to the use of GC or LC coupled with MS, variations on these approaches or different approaches altogether have been used to examine contaminant plumes for a broader range of potential metabolites (targeted analysis) and/or to identify components of dissolved organic matter (DOM) associated with contaminant plumes (e.g., nontargeted analysis; see below). For example, Jobelius et al. (2014) used a neutral loss scanning (NLS) approach in their LC-ESI-QTOF-MS analysis to specifically screen for acidic metabolites (based on the detection of mass signals associated with carboxylic and succinic acid moieties) of aromatic and heterocyclic compounds in a tar oil-contaminated aquifer. Dvorski et al. (2016) used high-resolution FT-ICR-MS (Fourier transform ion cyclotron resonance mass spectrometry, operated in both ESI and APPI [atmospheric pressure photoionization] modes), NMR (nuclear magnetic resonance) spectroscopy, and EEM (excitation emission matrix) fluorescence spectroscopy to examine the relationships between natural DOM and biodegradation processes in a tar oil-contaminated aquifer. Aside from helping to characterize DOM components at the site, these combined approaches revealed that natural DOM within the contaminant plume contained a strong sulfur signature, suggesting that the sulfate reduction reactions driving hydrocarbon biodegradation at the site were influencing the composition of organic matter (Dvorski et al. 2016).

9 In Situ Concentrations of Hydrocarbon Metabolites

Many of the various hydrocarbon metabolites detected in samples retrieved from different contaminated sites (Tables 1–3) have been quantified. Diagnostic fumarate addition metabolites of monoaromatic hydrocarbons (benzylsuccinates) have been detected in concentrations ranging from 0.1 to 220 μg/L (Beller 2000; Elshahed et al. 2001b; Gieg and Suflita 2002; Martus and Püttmann 2003; Alumbaugh et al. 2004; Ledin et al. 2005; Jobelius et al. 2011), from saturates (alkylsuccinates) ranging from 2 to 40 μg/L (Gieg and Suflita 2002) and from PAHs (e.g., naphthyl-2-methylsuccinic acid) ranging from 13 to 206 μg/L (Ohlenbusch et al. 2002; Griebler et al. 2004; Jobelius et al. 2011). Downstream metabolites such as benzoate and toluates can be detected at comparatively higher and wide-ranging concentrations. For example, in situ benzoate concentrations have been detected from as low as 3 μg/L (Annweiler et al. 2001) to as high as 6.5 mg/L (Cozzarelli et al. 1994). Toluate concentrations have been detected ranging from <1 μg/L to over 1 mg/L (Cozzarelli et al. 1994; Beller 2000; Elshahed et al. 2001a; Annweiler et al. 2001; Gieg and Suflita 2002; Griebler et al. 2004; Alumbaugh et al. 2004). Naphthoic acids (from naphthalene or methylnaphthalene metabolism) have been detected at concentrations ranging from ~0.4 to 150 μg/L (Annweiler et al. 2001; Ohlenbusch et al. 2002; Gieg and Suflita 2002; Young and Phelps 2005; Jobelius et al. 2011) while further degradation products such as tetrahydronaphthoic acids were comparatively lower (1–2 μg/L; Gieg and Suflita 2002). Carboxylic acid metabolites from other hydrocarbons and heterocycles were

detected at a former gasworks site at concentrations up to ~700 µg/L (Safinowski et al. 2006; Jobelius et al. 2011). Some studies reported that the concentrations of anaerobic hydrocarbon metabolites detected in field studies were 2–4 orders of magnitude lower than the concentrations of hydrocarbons themselves (Elshahed et al. 2001a; Martus and Püttmann 2003; Griebler et al. 2004). However, Jobelius et al. (2011) performed a ratio comparison of the molar concentrations of several parent hydrocarbons and their diagnostic metabolites in a tar oil-contaminated site and found the results to be so highly variable that no quantitative trends among the parent hydrocarbons and their daughter metabolites could be made. Thus, while in situ concentrations of metabolites can be determined, these cannot be used to directly calculate the concentrations of parent hydrocarbons consumed.

10 Limitations of the Metabolite Approach

As described herein, the detection of known metabolites of different classes of hydrocarbons can be a very useful tool for documenting in situ anaerobic biodegradation, especially those compounds that are only known to be formed anaerobically and have a distinct relationship to a particular parent hydrocarbon (fumarate addition metabolites, reduced ring metabolites for (methyl)naphthalene metabolism). However, there are some limitations in the use of this approach. One limitation is that one needs to know *a priori* which metabolites should be sought in field samples; that is, hydrocarbon metabolic pathways must first be elucidated, which under anoxic conditions is a relatively lengthy process. Another limitation is that the positive identification of the various metabolites relies on the use of comparisons with authentic standards (e.g., metabolites detected using any technique described above can only unequivocally be verified via matching chromatographic retention times and mass spectral profiles with authentic standards). Unfortunately, only some authentic standards are commercially available (e.g., benzylsuccinic acid can be purchased, but not the methylated analogs). Many researchers have chemically synthesized some metabolites of interest (e.g., methylbenzylsuccinates – Elshahed et al. 2001a; alkylsuccinates – Rabus et al. 2001; Wilkes et al. 2003; Gieg et al. 2009; Bian et al. 2014; Jarling et al. 2015; naphthyl-2-methyl/methylenesuccinates – Annweiler et al. 2000; carboxylated metabolites – Annweiler et al. 2001; Davidova et al. 2007) but many of these syntheses can be challenging (especially in the absence of proper chemistry lab facilities). An additional caveat in the use of metabolites is that the lack of their detection in situ does not necessarily indicate a lack of in situ hydrocarbon biodegradation, especially when other indicators (e.g., garnered using other approaches such as compound-specific isotope analysis) suggest that biodegradation is occurring; it is always possible that a lack of metabolite detection is due to analytical limitations. As was mentioned above, it is important that metabolite analyses are conducted along with other methods (such as assessing site biogeochemistry, performing compound-specific isotope analysis, biodegradative gene analysis, among others) (Weiss and Cozzarelli 2008; Bombach et al. 2010; Morasch et al. 2011). As all field measurement approaches will similarly

have limitations, collecting multiple lines of evidence that includes metabolite analysis will provide the strongest evidence supporting anaerobic in situ hydrocarbon biodegradation.

11 Research Needs

Compared to earlier reviews on use of signature metabolite analysis to demonstrate in situ hydrocarbon bioremediation (Beller 2000; Gieg and Suflita 2005), the list of metabolites that can be sought at field sites has expanded, largely in parallel with the increasing knowledge of anaerobic hydrocarbon metabolism. However, while many hydrocarbon metabolic pathways for some alkylbenzenes, *n*-alkanes (by fumarate addition), and (methyl)naphthalene have at least partly been elucidated, much remains to be learned about the pathways for cyclic alkanes, *iso*-alkanes, other PAHs, and for heterocyclic compounds that are also important compounds within many fuel mixtures. Thus, there is still a great research need for understanding metabolic pathways by which diverse hydrocarbons can be degraded under all anoxic electron-accepting conditions. Further, while some studies have quantified the in situ rates of metabolite formation as a way to determine rates of in situ hydrocarbon biodegradation (e.g., by using push–pull tests; Reusser et al. 2002; Alumbaugh et al. 2004; Gieg et al. 2009), more studies of this nature are needed to get a better understanding of how fast contaminants within plumes can be biologically remediated under anoxic conditions; this information could help support the use of natural attenuation as a clean-up strategy to regulators. Lastly, going beyond just looking for metabolites specific to particular hydrocarbons as evidence for in situ bioremediation is another consideration for future research. As such, understanding and/or quantifying the broader composition of "dissolved organic carbon (DOC)/DOM" has recently been put forth as an important consideration for site remediation as there is concern about the lingering toxicity of (currently undefined) polar petroleum metabolites in general (Mohler et al. 2013; Jobelius et al. 2014; O'Reilly et al. 2015; Dvorski et al. 2016; Bekins et al. 2016; Zemo et al. 2016; California Water Boards 2016). Long-term studies on contaminant plumes, for example, have found that the concentrations of polar or nonvolatile DOC exceed the concentrations of hydrocarbon contaminants by 2–10 times (Bekins et al. 2016), but the impact of this DOC on environmental receptors is unclear. Going forward, targeted and nontargeted analyses of signature metabolites and DOC, respectively, will allow for a deeper understanding of the roles that microorganisms play in restoring contaminated sites.

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Anaerobic Methane Oxidation in Freshwater Environments

17

Jörg Stefan Deutzmann

Contents

Abstract

Anaerobic methane oxidation was long thought to be limited to marine environments. Meanwhile, anaerobic methane oxidation coupled to denitrification, carried out by *Candidatus* "Methylomirabilis oxyfera"-like bacteria and *Candidatus* "Methanoperedens nitroreducens"-like archaea, has been discovered in various freshwater environments. Furthermore, this process even has been identified as the major methane sink in some environments such as lakes and peatlands.

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Anaerobic methane oxidation with sulfate or with oxidized iron and manganese species might also take place in freshwater environments, but the organisms mediating these reactions are unknown, and data on these processes at low salinities are scarce. In addition, a clear distinction between sulfate- and metal-dependent anaerobic methane oxidation has not been possible in most environments. In general, there is not much data available on the importance of anaerobic methane oxidation in freshwater habitats, but the available studies – in concert with molecular detection and quantification of anaerobic methane oxidizing organisms in a variety of freshwater habitats – indicate that anaerobic methane oxidation in freshwater environments could be a globally important methane sink.

1 Introduction

To understand the global methane cycle and the factors enabling or impeding oxidation of this effective greenhouse gas, it is essential to obtain accurate estimates of the individual sources of methane and to factor in the interplay of methane production and methane oxidation in different environments. The contribution of wetlands to global methane emissions and the share of different types of freshwater habitats in the global methane flux is a matter of ongoing investigation but has been summarized in detail by the Intergovernmental Panel on Climate Change (IPCC) (Ciais et al. 2013) and by the Global Carbon Project (Saunois et al. 2016). Overall, freshwater environments are a major source of atmospheric methane and, thus, contribute significantly to the global warming potential of methane (Saunois et al. 2016). Currently, methane emitted from natural wetlands is the largest methane source on a global scale with estimates between 100 and 260 Tg CH_4 $year^{-1}$ (Ciais et al. 2013). Global methane emission from rice fields, natural freshwater aquatic systems, and other natural wetlands was shown to be about 50, 100, and 160 Tg CH_4 $year^{-1}$, respectively (Bridgham et al. 2013). In contrast, methane emissions from oceans covering more than 70% of earth's surface is estimated to range between 4 and 15 Tg CH_4 $year^{-1}$ (Ciais et al. 2013). These studies showed that some environments, such as wetlands and rice fields, emit methane at orders of magnitude higher areal rates than oceans. The amount of methane released from an environment depends on the balance of methane formation and methane oxidation. Most methane is formed from organic substrates in anoxic environments, when external electron acceptors are missing (Thauer 2010). In stably structured environments where diffusive transport processes predominate, such as sediments and waterlogged soils, methane is then diffusing through an electron acceptor-rich zone before escaping into the atmosphere. Whenever methane and suitable electron acceptors are present, methane oxidation can take place and mitigate methane emissions. While aerobic methane oxidation was long thought to be the only methane sink in freshwater habitats, anaerobic oxidation of methane with a variety of electron acceptors has only been described recently. In this chapter, the impact of anaerobic methane oxidation (AMO) on methane cycling and global methane emissions will be discussed.

2 Electron Acceptors for AMO in Freshwater Habitats

Methane oxidation was long thought to be a purely aerobic process. However, based on thermodynamic considerations, methane oxidation is feasible with several electron acceptors including nitrate, nitrite, some oxidized metals (including Fe(III) and Mn(IV) minerals), and sulfate (Table 1). Indeed, anaerobic methane oxidation (AMO) has been postulated to occur with sulfate, nitrate, nitrite, iron, manganese, and humic substances as terminal electron acceptor to date. Which electron acceptor is used for AMO in each freshwater environment strongly depends on its availability, and concentrations of different electron acceptors vary strongly among different environments.

Nitrate concentrations, for example, vary greatly between different freshwater habitats. In pristine lake ecosystems or in rivers, nitrate concentrations are usually low with less than 30 μM (EPA 2009; Turner et al. 2003). However, nitrate concentrations of several mM can be present in areas affected by agricultural runoff or untreated sewage. In Europe, more than 50% of all groundwater sources contain more than 400 μM nitrate and more than 35% of all surface water bodies contain more than 160 μM nitrate (European Commission Report 2013).

Nitrite is likely available in environments where nitrate is available as it can be regenerated at high rates by microbial nitrate reduction and ammonium oxidation. Indeed, concentrations of nitrite are usually very low in freshwater habitats (<5 μM) but can reach hundreds of μM in nitrate-rich habitats (Georgieva et al. 2013). However, AMO with nitrite as electron acceptor is a highly exergonic process and is therefore favorable even at low nitrite concentrations.

In contrast to marine settings, where sulfate-dependent methane oxidation is widespread (Cui et al. 2015) and sulfate concentrations are usually 28 mM, sulfate concentrations in freshwater habitats are usually between 10 and 500 μM (Holmer and Storkholm 2001). Thus, given its low Gibbs free energy, AMO with sulfate

Table 1 Equations of described AMO reactions and associated Gibbs free energies

Educts		Products	$\Delta G^{\circ\prime}$ [kJ mol^{-1} CH_4]
$CH_4 + 4NO_3^-$	→	$CO_2 + 4NO_2^- + 2H_2O$	−503[a]
$3CH_4 + 8NO_2^- + 8H^+$	→	$3\ CO_2 + 4\ N_2 + 10\ H_2O$	−928[a]
$CH_4 + 8\ Fe(OH)_3 + 15\ H^+$	→	$HCO_3^- + Fe^{2+} + 21H_2O$	−572[b,c]
$CH_4 + 4\ MnO_2 + 7\ H^+$	→	$HCO_3^- + 4Mn^{2+} + 5H_2O$	−789[b,c]
$CH_4 + SO_4^{2-}$	→	$HCO_3^- + HS^- + H_2O$	−16.6[d]

[a]From Raghoebarsing et al. (2006)
[b]From Crowe et al. (2011)
[c]In situ values for M-DAMO can be much more positive because of the insolubility of most oxidized metal minerals; e.g., M-DAMO with birnessite (simplified to MnO_2) yields $\Delta G = -556$ kJ mol^{-1} and M-DAMO with ferrihydrite (simplified as $Fe(OH)_3$) $\Delta G = -270.3$ kJ mol^{-1} (Beal et al. 2009)
[d]From Cui et al. (2015)

approaches the thermodynamic minimum for biological energy conservation, especially at low substrate concentrations (Schink 2006).

Oxidized metals, in particular Fe(III) and Mn(III) and Mn(IV), are ubiquitous in freshwater environments (Thamdrup 2000). However, the oxidized forms of these metals are virtually insoluble in water and mainly present as particulates. Thus, microorganisms using these oxidized metals as electron acceptor for AMO have to access these insoluble substrates. The thermodynamics of metal-dependent methane oxidation are highly favorable (Table 1), but due to the low solubility of some oxidized metal species, such as Fe(III) minerals, the in situ energetics of Fe(III) reduction coupled to methane oxidation are far less favorable.

3 Organisms Involved in AMO in Freshwater

3.1 Organisms Coupling AMO to Denitrification (N-DAMO)

Anaerobic oxidation of methane coupled to denitrification reactions (called N-DAMO for nitrate-/nitrite-dependent anaerobic methane oxidation, Jetten 2008) has been found in many freshwater environments. N-DAMO can be carried out by two different groups of organisms with different niches: *Candidatus* "Methanoperedens nitroreducens"-like archaea (ANME 2d) (Haroon et al. 2013) and *Candidatus* "Methylomirabilis oxyfera"-like bacteria (Ettwig et al. 2008) (Table 2). Both were first enriched in a mixed culture coupling AMO to denitrification (Raghoebarsing et al. 2006). The archaeal population was lost after prolonged incubation when nitrite as electron acceptor was preferred over nitrate and AMO was attributed solely to the so called "phylum NC10 bacterium" *Candidatus* "Methylomirabilis oxyfera" (Ettwig et al. 2008, 2009). *M. oxyfera* reduces nitrite to nitric oxide which is disproportionated to molecular nitrogen and oxygen (Ettwig et al. 2010). The intracellularly produced oxygen is subsequently used for methane activation and for respiration (Ettwig et al. 2010). Thus, the gene for the β-subunit of the particulate methane monooxygenase, *pmoA*, can be used as a functional marker for *M. oxyfera*-like bacteria. Sequences of *M. oxyfera*-like *pmoA* genes can be assigned to a phylogenetic cluster distinct from the sequences of classical aerobic methanogens that also use particulate methane monooxygenase to activate methane with molecular oxygen (Luesken et al. 2011).

Candidatus "Methanoperedens nitroreducens" belongs to the ANME 2d archaea. It is related to classical ANME archaea known to oxidize methane in a syntrophic relationship with sulfate-reducing bacteria. This organism couples AMO to nitrate reduction and probably relies on nitrite-reducing organisms to remove this toxic intermediate (Haroon et al. 2013). *M. nitroreducens* has been enriched together with *M. oxyfera*-like NC10 bacteria and ANAMMOX bacteria indicating that these organisms might act as syntrophic partners (Ding et al. 2014; Haroon et al. 2013). *M. nitroreducens* uses a reverse methanogenesis pathway, and the gene for the key enzyme of methanogenesis, methyl-coenzyme M reductase *mcrA*, is used as a functional marker gene (Haroon et al. 2013).

3.2 Organisms Coupling AMO to Sulfate Reduction (S-DAMO)

Sulfate-dependent methane oxidation (also abbreviated as S-DAMO, Cui et al. 2015) is carried out by different classes of ANME archaea (ANME 1a and 1b, ANME 2a-2d, and ANME 3) (Table 2), often in syntrophic association with sulfate-reducing bacteria, and has been reviewed extensively (Cui et al. 2015; Knittel and Boetius 2009). Based on the energy yield of sulfate-dependent AMO ($\Delta G^{\circ\prime} = -16.6$ kJ mol^{-1} CH_4), there is still some debate if this process can be carried out by an obligate syntrophic consortium in typical freshwater environments. Because of the low concentrations of methane and sulfate in freshwater settings, S-DAMO will yield less than -16.6 kJ mol^{-1} CH_4, which is not sufficient to sustain two organisms with a minimum energy requirement of -10 kJ mol^{-1} for biological energy conservation (Schink 2006). However, ANME 2 have been reported to use a direct electron transfer mechanism to transfer the electrons derived from methane oxidation to the syntrophic partner organism (McGlynn et al. 2015). It has been shown that ANME archaea can be decoupled from their syntrophic partner when soluble electron shuttles such as AQDS, Fe(III)-citrate, or humic acids were added to the culture (Scheller et al. 2016). ANME have also been described to produce S(0) as an intermediate in AMO, which might enable AMO without a syntrophic partner organism (Milucka et al. 2012). These findings indicate that S-DAMO might be carried out by single ANME archaea in freshwater environments.

3.3 Organisms Coupling Anaerobic Methane Oxidation to Metal Reduction (M-DAMO)

To date, no organism has been identified to directly couple Fe or Mn reduction to AMO (Table 2), but indirect reduction of metals via a cryptic sulfur cycle has been

Table 2 Overview of the different AMO processes

Process	Electron acceptor	Organism	Phylogenetic group	Marker gene (representative accession no.)
N-DAMO	Nitrite	*Methylomirabilis oxyfera*-like bacteria	NC10 phylum	*pmoA*, 16S rRNA (Genome: FP565575.1)
N-DAMO	Nitrate	*Methanoperedens nitroreducens*-like archaea	ANME 2d, AAA	*mcrA*, 16S rRNA (Genome: NZ_JMIY00000000)
			GoM-Arc1 group	
			Methanosarcinales	
S-DAMO	Sulfate	ANME 1, 2, 3, AOM-associated archaea (AAA)?	ANME 1	*mcrA*, 16S rRNA (ANME 2a genome project: PRJNA219628)
			Methanosarcinales	
M-DAMO	Fe(III), Mn (III + IV)	Unknown	Unknown	Unknown

proposed (Beal et al. 2009; Norði et al. 2013). In addition, mediators such as humic substances could indirectly couple methane oxidation to metal reduction (Kappler et al. 2004; Smemo and Yavitt 2011). However, ANME archaea have been shown to reduce chelated Fe(III) recently (Scheller et al. 2016). Thus, metal-dependent methane oxidation (also abbreviated as M-DAMO, Cui et al. 2015) could be carried out directly by ANME archaea, if the proposed direct electron transport can be coupled directly to the reduction of insoluble metal species or mediated by soluble electron shuttles such as humic substances (Scheller et al. 2016). In addition, there is the possibility that a syntrophic partnership exists of ANME archaea and metal-reducing organisms performing direct electron transfer, such as *Geobacter* or *Shewanella.* In fact, a co-culture of N-DAMO organisms and *Shewanella oneidensis* MR-1 has been shown to couple methane oxidation to iron reduction (Fu et al. 2016).

4 AMO Rates in Freshwater Habitats

Direct measurement of AMO rates in freshwater habitats is a challenge in its own. In many freshwater habitats, different potential electron acceptors are present in close proximity due to the steep redox gradients in typical freshwater environments (Deutzmann et al. 2014). Consequently, standard sampling might encompass a wide range of redox species, redox processes, and the associated microbial communities. Therefore, high-resolution measurements and sampling strategies are needed. In addition, concentrations of possible electron acceptors are low in situ (<1 mM), which requires replenishment or fast recycling of the electron acceptor to sustain high microbial activities. This cannot be easily mimicked in standard batch incubations in the laboratory, and dynamics between the mixed microbial communities likely differ drastically from in situ conditions. A convenient and elegant way to measure AMO in these systems is tracer studies with labeled methane. However, the low cellular turnover rates during AMO require tracer studies to be conducted carefully over an extended period of time (days to weeks or even months), and appropriate controls are needed to correct for the label exchange during methanogenesis, called trace methane oxidation. To facilitate the identification of the main process and the organisms involved, many studies on freshwater AMO were conducted in settings where one possible electron acceptor is abundant while others are not (Schubert et al. 2011; Hu et al. 2014; Timmers et al. 2016).

4.1 Rates of Nitrate-/Nitrite-Dependent Methane Oxidation

N-DAMO has been reported in sewage treatment reactors and has also been proposed to occur in ground water years before the discovery of the responsible microorganisms (Smith et al. 1991). In Lake Constance, a deep nitrate-rich (~80 μM) lake, high potential N-DAMO rates were measured via radiotracer incubations only with nitrate as electron acceptor and only in profundal (80 m water depth) sediment slurries (Deutzmann and Schink 2011). In contrast, littoral samples

showed no substantial N-DAMO activity. In a second study, intact profundal sediment cores were investigated and high-resolution profiles of oxygen, nitrate, nitrite, and methane were recorded under in situ temperature. N-DAMO rates of 31–437 μmol m^{-2} d^{-1} were inferred from high-resolution methane profiles and the abundance of *M. oxyfera*-like bacteria peaked at 1–10% of the total microbial community in the 1–2 cm wide zone where AMO took place. AMO rates were sufficient to oxidize all the methane flux from deeper sediment layers rendering N-DAMO the major methane sink in profundal Lake Constance sediments (Deutzmann et al. 2014). However, Lake Constance also harbors a diverse community of aerobic methanotrophs in profundal sediments which is more than able to completely oxidize the entire methane formed deeper in the sediments. The interplay of aerobic and anaerobic methane oxidation requires further research to explain the high abundance of aerobic methanotrophs despite the fact that AMO has been shown to oxidize up to 100% of the methane flux before it reached the oxic zone. In agreement with these findings, it has also been shown in a freshwater pond that N-DAMO consumed a substantial part of the methane in intact sediment cores after enrichment with 1–2 mM nitrate over 16 months (Norði and Thamdrup 2014).

N-DAMO was also shown to be the dominant methane sink in a minerotrophic peatland (Zhu et al. 2012). This peatland is fed by nitrate-rich groundwater (0.4–0.6 mM nitrate) which is overflowing the sampling site. In this environment, gradients of nitrate and methane stretched across more than 40 cm soil depth and clearly indicated N-DAMO and complete oxidation of methane in the anoxic zone. In addition, the abundance of *M. oxyfera*-like bacteria peaked where high N-DAMO rates were recorded.

In three different Chinese wetlands, N-DAMO activity was measured with stable isotope tracer experiments and nitrite as electron acceptor. Rates were in the range of 0.31–5.43 nmol of CO_2 $g_{dw}{}^{-1}$ soil d^{-1} and highest rates have been found in deeper soil layers between 50 and 100 cm depth (Hu et al. 2014). Consistently, highest AMO rates were measured in soil depths below 50 cm in Xiazhuhu wetland (Shen et al. 2015a). AMO rates measured in ^{13}C tracer experiments in nitrite-amended soil slurries were translated to N-DAMO rates of 51–137 μmol m^{-2} d^{-1} and amounted to the oxidation of 2.7–4.3% of the methane flux in this wetland (Shen et al. 2015a).

Very recently, N-DAMO carried out by *M. nitroreducens*-like archaea has been reported for the first time in an environmental sample. In slurry incubations of soil from a paddy field, 80 nmol $g_{dw}{}^{-1}$ d^{-1} were consumed by N-DAMO (Vaksmaa et al. 2016).

This indicates that N-DAMO plays an important role in mitigating methane emissions, especially in nitrate-rich environments.

4.2 Rates of Sulfate- and Metal-Dependent Methane Oxidation

Both, S-DAMO and M-DAMO have been reported for freshwater habitats, but a cryptic sulfur cycle cannot be excluded in M-DAMO (Norði et al. 2013). Thus, both processes will be discussed together in this paragraph. Very recently, it has been shown for the first time that sulfate-dependent AMO mediated by ANME archaea

takes place in a low salinity freshwater environment (Timmers et al. 2016). In this study, long-term ^{13}C tracer incubations (>168 days) of water and sediment from a freshwater gas source clearly showed a sulfate dependency of net AMO. Trace methane oxidation as result of net methanogenesis has been found without sulfate addition. However, no in situ rates have been determined in this study. Reports on AMO are also available for surface water environments. S-DAMO has been reported in sulfate-rich lakes, e.g., Lago di Cadagno (Schubert et al. 2011) and Lake Plußsee (Eller et al. 2005), but no in situ rates have been reported in these studies. In wetlands, S-DAMO has been found to greatly reduce methane emissions with an absolute amount of 200 Tg y^{-1} or 50% of their potential methane emissions (Segarra et al. 2015). However, not only sulfate but potentially also oxidized metals were available in the investigated soils and could contribute to AMO in these environments. Iron-dependent methane oxidation has also been reported to take place deep in sediment cores, below the zone of methanogenesis, in Lake Kinneret (Sivan et al. 2011). In Lake Ørn, 90% of the diffusive methane flux was oxidized in the absence of oxygen or nitrate in a sulfate- and iron-rich zone in the sediment (Norði et al. 2013), while in Lake Matano, methane is presumably oxidized at the pycnocline with Fe(III) as electron acceptor at rates of 0.5 μmol L^{-1} d^{-1} (Crowe et al. 2011).

5 Estimation of AMO Potential from Molecular Abundance Data

Another approach to investigate AMO in various freshwater habitats is molecular detection of the microorganisms that perform AMO. However, it is important to keep in mind that the detection of an organism's DNA does not reflect activity of this organism at the time of sampling. Although marker-based estimations of abundance and distribution of key organisms cannot be used to infer in situ AMO rates, these data can be used to estimate potential AMO rates and, to a certain degree, to generalize findings inferred from studies that accurately measured rates and the abundance of the respective organisms in similar habitats. The most common markers used in AMO research are generally the 16S rRNA gene as universal marker and *pmoA* as functional marker for *M. oxyfera*-like bacteria or *mcrA* as functional marker for ANME archaea.

5.1 Estimation of N-DAMO Potential Based on Abundance of *M. oxyfera*-Like Bacteria

Most molecular studies on N-DAMO targeted the NC10 bacterial group related to *M. oxyfera*, mainly due to early availability of suitable sequence information enabling detection of this group's 16S rRNA and *pmoA* gene levels (Deutzmann and Schink 2011; Ettwig et al. 2009; Luesken et al. 2011). In the meantime, numerous studies have detected these organisms in various freshwater environments

(Shen et al. 2015b; Zhu et al. 2015). However, only a small subset quantified the abundance of organisms catalyzing AMO which can be used to estimate potential AMO rates. To allow comparisons of potential N-DAMO rates across studies, 16S rRNA gene copy numbers or *pmoA* gene copy numbers are multiplied here by the most conservative estimate of cellular N-DAMO rates of 0.09 fmol CH_4 day^{-1} $cell^{-1}$ (Ettwig et al. 2009).

In a natural environment, *M. oxyfera*-like bacteria were first detected in Lake Constance by molecular methods where *M. oxyfera*-like bacteria were quantified based on *pmoA* as well as 16S rRNA gene abundances (Deutzmann et al. 2014). Based on gene copy numbers, potential N-DAMO rates of 660–4890 μmol m^{-2} d^{-1} were estimated to be roughly one order of magnitude higher than rates obtained by gradient measurements (Deutzmann et al. 2014). Interestingly, qPCR also revealed that in littoral, shallow water sites, abundances were low (ca. 10^5–10^6 cells ml^{-1}). Only at a water depth of more than 20 m N-DAMO bacteria constituted a significant fraction of the total bacterial community and could efficiently mitigate methane emissions. A similar pattern has been observed in Lake Biwa: *M. oxyfera*-like bacteria have been detected only at profundal sites, although at abundances of 10^6 cells per ml sediment, resulting in a potential methane oxidation capacity of 5 μmol m^{-2} d^{-1} (Kojima et al. 2012). In the water level fluctuation zone of the Three Gorges Dam, N-DAMO abundances increased from about 1×10^3 to up to 5×10^4 copies per g sediment after 6 months of continuous flooding, translating to a potential methane oxidation capacity of up to 0.5 μmol m^{-2} d^{-1} (Wang et al. 2016). Together, these results indicate that in lakes stable environmental conditions benefit the N-DAMO process.

M. oxyfera-like bacteria have also been detected in a variety of other freshwater habitats (Cui et al. 2015; Shen et al. 2015b). One of the most extensive studies on the distribution of *M. oxyfera*-like bacteria encompassed 25 freshwater environments in China including rivers, lakes, littoral and riparian zones, groundwater, estuaries, reservoirs, swamps, and wetlands (Zhu et al. 2015). This study found widely varying abundances of NC10 bacteria in the different samples with 10^3–10^7 copies g_{dw}^{-1}. In lakes, a canal, groundwater, and constructed wetland *M. oxyfera*-like bacteria constituted up to 0.4% of the total bacterial community, indicating a more important role of N-DAMO in these habitats than in others. However, no statistically significant differences have been found between different types of ecosystems, because variability within each ecosystem type was large. In Xiazhuhu wetland, the vertical distribution showed highest abundances of *M. oxyfera*-like bacteria (up to 5×10^7 g_{dw}^{-1}) corresponding to a potential methane oxidation rate of up to 170 μmol m^{-2} d^{-1} in a soil layer of 10 cm. Considering the distribution of *M. oxyfera*-like bacteria over several 10 cm soil depth in this study (quantitative data are missing for some soil horizons), the potential rates derived from qPCR data are several times higher than rates measured in slurry incubations (Shen et al. 2015a). High abundances of *M. oxyfera*-like bacteria have also been found in rice fields with up to 10^8 16S copies g_{dw}^{-1}, corresponding to up to 4% of the total bacterial community (Zhou et al. 2014). Taken together, these data indicate a great potential role for N-DAMO in mitigating methane emissions from a variety of freshwater habitats.

5.2 Estimation of S-DAMO and M-DAMO Potential in Freshwater Environments

S-DAMO was thought to be a purely marine process, and unequivocal evidence for the involvement of ANME archaea in freshwater AMO was lacking until very recently (Timmers et al. 2016). Consequently, only few studies quantified ANME in freshwater habitats (Schubert et al. 2011; Eller et al. 2005 #44). In addition, with the current lack of knowledge about the physiology of sulfate- or metal-reducing freshwater ANME, it is impossible to derive potential methane oxidation rates from DNA copy numbers or cell counts. It is not even possible to reliably determine the potential electron acceptor for AMO from phylogenetic information. For example, the ANME 2d/AOM-associated archaea have been shown to mediate N-DAMO in enrichment cultures (Haroon et al. 2013) and in paddy soil (Vaksmaa et al. 2016), but they have also been implicated also in S-DAMO in Lago di Cadagno (Schubert et al. 2011) and in a freshwater gas source (Timmers et al. 2016). Furthermore, it is unclear which ANME are responsible for M-DAMO (Beal et al. 2009). Thus, more data on the abundance of the different groups of ANME and on their metabolism are required to estimate their impact on methane emissions from freshwater habitats.

6 Environmental Factors Influencing AMO

All microbial processes are strongly influenced by environmental parameters, because the environmental parameters create the niches the organisms live in. To understand the adaptations of the different anaerobic methanotrophs to the niches they occupy, careful measurements of the environmental parameters are needed. This has to be combined with laboratory experiments determining the range of environmental parameters strains can thrive in. To date, environmental parameters limiting N-DAMO activity in the environment are still largely unknown. In addition, virtually nothing is known about S-DAMO or M-DAMO in freshwater environments due to the uncertainty as to which organisms perform this activity in freshwater environments. The following factors are known to influence N-DAMO: Oxygen should be absent or present in only very low concentrations (Luesken et al. 2012), temperatures between 30 and 4 °C are suitable for active N-DAMO (Deutzmann et al. 2014; Ettwig et al. 2008), freshwater adapted strains do not tolerate high salinity, and abundance of *M. oxyfera*-like bacteria correlates with the concentration of their electron acceptor in the environment (Shen et al. 2015b). In addition, stable environments seem to benefit N-DAMO, likely because the generation times of *M. oxyfera*-like bacteria are in the range of weeks to months and populations would need a long time to recover from events that kill off a large number of cells (e.g., sediment resuspension, bioturbation, drying out). Environmental factors enabling or limiting S-DAMO or M-DAMO are basically unknown and require extensive research.

7 Impact of AMO on Methane Emissions from Freshwater Environments

Anaerobic methane oxidation is the dominant methane sink in some freshwater environments (Deutzmann et al. 2014; Segarra et al. 2015; Zhu et al. 2012), but only marginally reduces emissions of this greenhouse gas in others (Shen et al. 2015a). In some wetlands, N-DAMO is the dominant methane sink (Zhu et al. 2012), in others S-DAMO (Segarra et al. 2015). These two examples already show clearly that the current understanding of AMO in freshwater habitats is not sufficient to allow conclusions on the impact of AMO on methane emission on a global scale. However, it becomes increasingly evident that AMO is indeed a major methane sink in some environments. In addition, DNA-based studies find sequences related to *M. oxyfera*, *M nitroreducens*, and other AMO-related organisms in different environments and often at considerable abundances. Thus, AMO could play a role in mitigating methane emissions on a larger scale, but additional research is needed to obtain a clearer picture of AMO and its impact on methane emissions from freshwater environments.

8 Research Needs

Due to the large quantity of recent findings, AMO in freshwater environments certainly is a highly interesting and dynamic field of microbial ecology that needs extensive research. While the number of studies on AMO in freshwater habitats steadily increases and the potential impact of AMO in freshwater environments is being established, several key questions remain unanswered.

1. The physiology of S-DAMO and M-DAMO organisms is unclear, and even their phylogenetic identity is largely unknown. Recent data suggests that direct electron transfer might play a role in AMO by ANME archaea (McGlynn et al. 2015; Scheller et al. 2016). If direct electron transfer turns out to be the active mechanism, AMO by one organism might not be tightly coupled to one specific electron acceptor: Anaerobic methanotrophs could donate "free" electrons to soluble electron shuttles or diverse partner organisms that, in turn, reduce the terminal electron acceptor. Studies that unequivocally link specific strains or phylogenetic groups to S-DAMO and to M-DAMO are required before molecular studies can be employed to detect these organisms in the environment. In addition, comprehensive data on their physiology is needed to predict the niches of these organisms in their natural habitats and to predict or model their response to environmental parameters.
2. To unravel the importance of each process in mitigating methane emissions from freshwater habitats, studies combining the commonly used molecular tools for community analysis (PCR, qPCR, NGS) with accurate measurements of AMO activities are needed. In addition, fine-scale resolution of the respective organisms

and of all redox species involved is required in some environments, preferably in situ. Only these activity and distribution measurements in concert with the growing number of marker gene sequences deposited in the databases (with accompanying metadata) will allow a more accurate assessment of the importance of this process. Furthermore, these studies could be complemented by tracer experiments not only measuring AMO rates but also incorporation of the tracer into biomass to identify the key players in methane turnover. In addition, most studies focused on the NC10 bacterial "*M. oxyfera*-like" bacteria, and more studies on the environmental distribution and activity of *M. nitroreducens*-like archaea are needed.

3. To better understand methane dynamics in the diverse habitats, it might prove necessary to study not AMO as a single process but in concert with methanogenesis and aerobic methane oxidation. Lake Constance, for example, harbors a diverse community of aerobic methanotrophs in profundal sediments. This community is more than able to completely oxidize the entire methane formed deeper in the sediment despite the fact that AMO has been shown to oxidize up to 100% of the methane flux before it reaches the oxic zone. Investigation of the entire methane cycle, including methane formation rates, methane fluxes and distribution, and methane oxidation rates with different electron acceptors, would be needed to understand the dynamics of this ecosystem.

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