

Anaerobic Microbial Degradation of Hydrocarbons: From Enzymatic Reactions to the Environment

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Key Words

Aromatic compounds · *n*-Alkanes · Anaerobic bacteria · Reactions · Enzymes · Crystal structure · Stereochemistry · Phylogeny · Alkyl-/arylalkylsuccinate synthases · Stable isotope probing · Microbial diversity · Stable isotope fractionation

Abstract

Hydrocarbons are abundant in anoxic environments and pose biochemical challenges to their anaerobic degradation by microorganisms. Within the framework of the Priority Program 1319, investigations funded by the Deutsche For-

schungsgemeinschaft on the anaerobic microbial degradation of hydrocarbons ranged from isolation and enrichment of hitherto unknown hydrocarbon-degrading anaerobic microorganisms, discovery of novel reactions, detailed studies of enzyme mechanisms and structures to process-oriented in situ studies. Selected highlights from this program are collected in this synopsis, with more detailed information provided by theme-focused reviews of the special topic issue on 'Anaerobic biodegradation of hydrocarbons' [this issue, pp. 1–244]. The interdisciplinary character of the program, involving microbiologists, biochemists, organic chemists and environmental scientists, is best exemplified by the studies on alkyl-/arylalkylsuccinate synthases. Here, research topics

ranged from in-depth mechanistic studies of archetypical toluene-activating benzylsuccinate synthase, substrate-specific phylogenetic clustering of alkyl-/arylalkylsuccinate synthases (toluene plus xylenes, *p*-cymene, *p*-cresol, 2-methylnaphthalene, *n*-alkanes), stereochemical and co-metabolic insights into *n*-alkane-activating (methylalkyl)succinate synthases to the discovery of bacterial groups previously unknown to possess alkyl-/arylalkylsuccinate synthases by means of functional gene markers and in situ field studies enabled by state-of-the-art stable isotope probing and fractionation approaches. Other topics are Mo-cofactor-dependent dehydrogenases performing O₂-independent hydroxylation of hydrocarbons and alkyl side chains (ethylbenzene, *p*-cymene, cholesterol, *n*-hexadecane), degradation of *p*-alkylated benzoates and toluenes, glycol radical-bearing 4-hydroxyphenylacetate decarboxylase, novel types of carboxylation reactions (for acetophenone, acetone, and potentially also benzene and naphthalene), W-cofactor-containing enzymes for reductive dearomatization of benzoyl-CoA (class II benzoyl-CoA reductase) in obligate anaerobes and addition of water to acetylene, fermentative formation of cyclohexanecarboxylate from benzoate, and methanogenic degradation of hydrocarbons.

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Introduction

Role of Hydrocarbons in Natural Environments and the Technosphere

Aliphatic and aromatic hydrocarbons and other aromatic compounds represent the most abundant small organic molecules on earth and occur predominantly in anoxic (devoid of O₂) terrestrial soils, marine sediments or deep subsurface environments. This global abundance substantiates the importance of their biodegradation for a balanced global carbon budget. Hydrocarbons are highly valuable natural resources for energy generation and chemical industry. They represent the major constituents of natural oil, coal and gas, with about 10¹² t of carbon stored in worldwide reservoirs that have formed over geological time scales. Hydrocarbons are also recently formed either biologically (e.g. by bacteria, animals and plants) or by abiotic thermogenic processes in deep-sea sediments [Wilkes and Schwarzbauer, 2010]. Thus, such compounds are potential and ubiquitous substrates for microbial metabolism albeit often considered as recalcitrant under anoxic conditions. Anthropogenic activities (e.g. transport and storage of oil or gasoline) increasingly lead to contamination of ground water ecosystems (po-

tentially impairing drinking water supplies) and oceanic water bodies by accidental spills. As aromatic hydrocarbons exhibit a relatively higher water solubility and toxicity than alkanes, it is important to understand their biodegradation pathways and the factors controlling elimination processes in the environment. On the other hand, the biodegradation in oil reservoirs affects the quantity and quality of fossil fuels, in particular crude oil, and thus gives rise to fundamental geological and industrial interest.

Biochemical Challenge of Anaerobic Degradation of Hydrocarbons

The chemical inertness of hydrocarbons poses an energetic and mechanistic challenge for microbial metabolism. This is particularly true for the initial activation and eventual cleavage of the apolar C–H bond, where high energy barriers have to be overcome. The initial functionalization is instrumental for channeling the hydrocarbon substrates into central catabolic routes. In oxic environments, C–H bond activation is mainly accomplished by O₂-dependent oxygenase-catalyzed reactions, which are largely irrelevant under anoxic conditions. The only exceptions are the recently discovered ‘intra-aerobic’ anaerobes which apparently derive oxygen species from utilizing chlorate or nitrite to employ monooxygenases for attacking hydrocarbon substrates (e.g. during anaerobic growth of *Candidatus Methyloirabilis oxyfera* with methane [Ettwig et al., 2010], gammaproteobacterial strain HdN1 with *n*-hexadecane [Zedelius et al., 2011] and *Pseudomonas chloritidis*mutans AW-1^T with *n*-decane [Mehboob et al., 2015]). Accordingly, anaerobic degradation of hydrocarbons involves a variety of intriguing biochemically unprecedented reactions, as indicated by previous microbiological and biochemical research on some model compounds. Examples of such anaerobic reactions are the addition of toluene or *n*-alkanes to fumarate, the O₂-independent hydroxylation of ethylbenzene and the ATP-dependent and ATP-independent variants of reductive dearomatization of the central intermediate benzoyl-CoA. These novel enzymatic reactions may represent blueprints for biomimetic examples of C–H bond activation and Birch type reductions that in current organic synthesis typically requires expensive transition metal catalysts and does not exhibit enantioselectivity [Labinger and Bercaw, 2002].

Scope of this Synopsis

This synopsis aims at providing a brief overview of the results from research on anaerobic hydrocarbon degradation conducted within the framework of the Priority Pro-

Table 1. Pure and enriched cultures of bacteria used to study anaerobic degradation of hydrocarbons coupled to nitrate, sulfate or iron reduction or to fermentative or syntrophic growth

Organism	Key substrate(s)	e ⁻ acceptor	Phylogeny	Reference
<i>Aromatoleum aromaticum</i> EbN1	Toluene, ethylbenzene	NO ₃ ⁻	Betaproteobacteria	Rabus and Widdel, 1995
<i>Aromatoleum aromaticum</i> pCyN1	<i>p</i> -Cymene	NO ₃ ⁻	Betaproteobacteria	Harms et al., 1999a
<i>Aromatoleum</i> sp. strain HxN1	<i>n</i> -Hexane	NO ₃ ⁻	Betaproteobacteria	Ehrenreich et al., 2000
<i>Aromatoleum</i> sp. strain mXyN1	Toluene, <i>m</i> -xylene	NO ₃ ⁻	Betaproteobacteria	Rabus and Widdel, 1995
<i>Aromatoleum</i> sp. strain ToN1	Toluene	NO ₃ ⁻	Betaproteobacteria	Rabus and Widdel, 1995
<i>Azoarcus</i> sp. strain T	Toluene	NO ₃ ⁻	Betaproteobacteria	Dolfing et al., 1990
<i>Desulfobacula toluolica</i> Tol2	Toluene	SO ₄ ²⁻	Deltaproteobacteria	Rabus et al., 1993
<i>Desulfococcus biacutus</i>	Acetone	SO ₄ ²⁻	Deltaproteobacteria	Platen et al., 1990
<i>Desulfococcus oleovorans</i> Hxd3	<i>n</i> -Hexadecane	SO ₄ ²⁻	Deltaproteobacteria	Aeckersberg et al., 1991
Enrichment culture BF	Benzene	Fe ³⁺	<i>Peptococcaceae</i>	Kunapuli et al., 2007
Enrichment culture N47	Naphthalene, 2-methyl-naphthalene	SO ₄ ²⁻	Deltaproteobacteria	Meckenstock et al., 2000
Enrichment culture Prop12-GMe	Propane	SO ₄ ²⁻	Deltaproteobacteria	Kniemeyer et al., 2007
Enrichment culture Propane 60-GuB	Propane	SO ₄ ²⁻	Deltaproteobacteria	Kniemeyer et al., 2007
Enrichment culture But12-GuMe	<i>n</i> -Butane	SO ₄ ²⁻	Deltaproteobacteria	Kniemeyer et al., 2007
Enrichment culture But12-HyR	<i>n</i> -Butane	SO ₄ ²⁻	Deltaproteobacteria	Jaekel et al., 2013
<i>Geobacter metallireducens</i> GS-15	Benzoate	Fe ³⁺	Deltaproteobacteria	Lovley et al., 1993
<i>Magnetospirillum</i> sp. strain pMbN1	<i>p</i> -Methylbenzoate	NO ₃ ⁻	Alphaproteobacteria	Lahme et al., 2012
<i>Pelobacter acetylenicus</i>	Acetylene	Fermenting	Deltaproteobacteria	Schink, 1985
<i>Peptoclostridium difficile</i> ^a	<i>p</i> -Hydroxyphenylacetate	Fermenting	<i>Peptostreptococcaceae</i>	Yutin and Galperin, 2013
<i>Sterolibacterium denitrificans</i> Chol-1S ^T	Cholesterol	NO ₃ ⁻	Betaproteobacteria	Tarlera and Denner, 2003
Strain BuS5	<i>n</i> -Butane	SO ₄ ²⁻	Deltaproteobacteria	Kniemeyer et al., 2007
Strain mXyS1	Toluene, <i>m</i> -xylene	SO ₄ ²⁻	Deltaproteobacteria	Harms et al., 1999b
Strain NaphS2	Naphthalene, 2-methyl-naphthalene	SO ₄ ²⁻	Deltaproteobacteria	Musat et al., 2009
Strain OcN1	<i>n</i> -Octane	NO ₃ ⁻	Betaproteobacteria	Ehrenreich et al. 2000
Strain oXyS1	Toluene, <i>o</i> -xylene	SO ₄ ²⁻	Deltaproteobacteria	Harms et al., 1999b
Strain TD3	<i>n</i> -Decane	SO ₄ ²⁻	Deltaproteobacteria	Rueter et al., 1994
<i>Syntrophus aciditrophicus</i> SB	Benzoate	Syntrophic	Deltaproteobacteria	Hopkins et al., 1995
<i>Thauera aromatica</i> K172	Toluene	NO ₃ ⁻	Betaproteobacteria	Anders et al., 1995
<i>Thauera aromatica</i> T1	Toluene	NO ₃ ⁻	Betaproteobacteria	Song et al., 1998
<i>Thauera</i> pCyN2	<i>p</i> -Cymene	NO ₃ ⁻	Betaproteobacteria	Harms et al., 1999a

^a Renamed from *Clostridium difficile*.

gram 1319 'Biological transformation of hydrocarbons in the absence of oxygen: from the molecular to the global scale' funded by the Deutsche Forschungsgemeinschaft from 2009 to 2015. This priority program has early roots in ground-breaking studies from the 1990s by the groups of G. Fuchs (Freiburg) and F. Widdel (Bremen), which provided the first enzymatic evidence for fumarate-dependent activation of toluene to benzylsuccinate [Biegert et al., 1996], phenylphosphate formation preceding carboxylation during anaerobic phenol degradation [Lack and Fuchs, 1994] and reductive dearomatization of benzoyl-CoA [Boll and Fuchs, 1995], as well as the isolation of the first pure culture degrading *n*-alkanes anaerobical-

ly [Aeckersberg et al., 1991], the discovery of sulfate-reducing bacteria thriving on hydrocarbons from crude oil [Rueter et al., 1994] and the culture-based evidence of methanogenic degradation of *n*-alkanes [Zengler et al., 1999].

The program was based on an interdisciplinary collaboration of microbiologists, biochemists, organic chemists and environmental scientists, and benefited from the availability of a variety of pure cultures (partly genome sequenced) anaerobically degrading model hydrocarbons and other aromatic/aliphatic compounds of interest (table 1). While keeping a clear focus on the key enzymatic reactions rendering anaerobic degradation of hydrocar-

bons (saturated, unsaturated or aromatic) and other aromatic/aliphatic compounds possible, the research topics were embedded in a larger ecophysiological context. Program topics ranged from process-oriented in situ investigations via the isolation of novel anaerobic bacteria to detailed studies of enzyme mechanisms and structures. Theme-focused reviews are compiled in the special topic issue 'Anaerobic biodegradation of hydrocarbons' of the *Journal of Molecular Microbiology and Biotechnology* [this issue, pp. 1–244]. For reasons of succinctness, only selected results are highlighted, and the relevant primary literature is largely only provided in the respective theme-focused reviews rather than in this synopsis.

Metabolism of Aromatic Compounds

Toluene-Activating Benzylsuccinate Synthase, the Archetype of Alkyl-/Arylalkylsuccinate Synthases

An overview of structural and functional properties of toluene-activating benzylsuccinate synthase (BSS) and other related glycy radical-bearing alkyl-/arylalkylsuccinate synthases is provided by Heider et al. [2016b]. BSS and glycy radical enzymes in general carry a conserved glycine residue close to the C terminus of the catalytic subunit for generating an organic (glycyl) radical and a structurally close-by cysteine residue, assumed to function as reactive (thiyl) radical. The ($\alpha\beta\gamma$)₂ heterohexameric and O₂-sensitive BSS is best studied in the betaproteobacterial strains *Thauera aromatica* K172, *T. aromatica* T1 and *Azoarcus* sp. strain T, and was previously shown to stereospecifically add toluene to the cosubstrate fumarate forming (*R*)-benzylsuccinate.

Based on biochemical, structural and modeling data, the following mechanistic concept for the catalytic cycle of BSS has been conceived (fig. 1a, upper panel). Upon substrate binding (first fumarate, then toluene in a long active site cavity of BSS from *T. aromatica* T1 [Funk et al., 2015]), a reactive thiyl radical (Cys⁴⁹³) is generated at the expense of the spatially close-by resting-state glycy radical

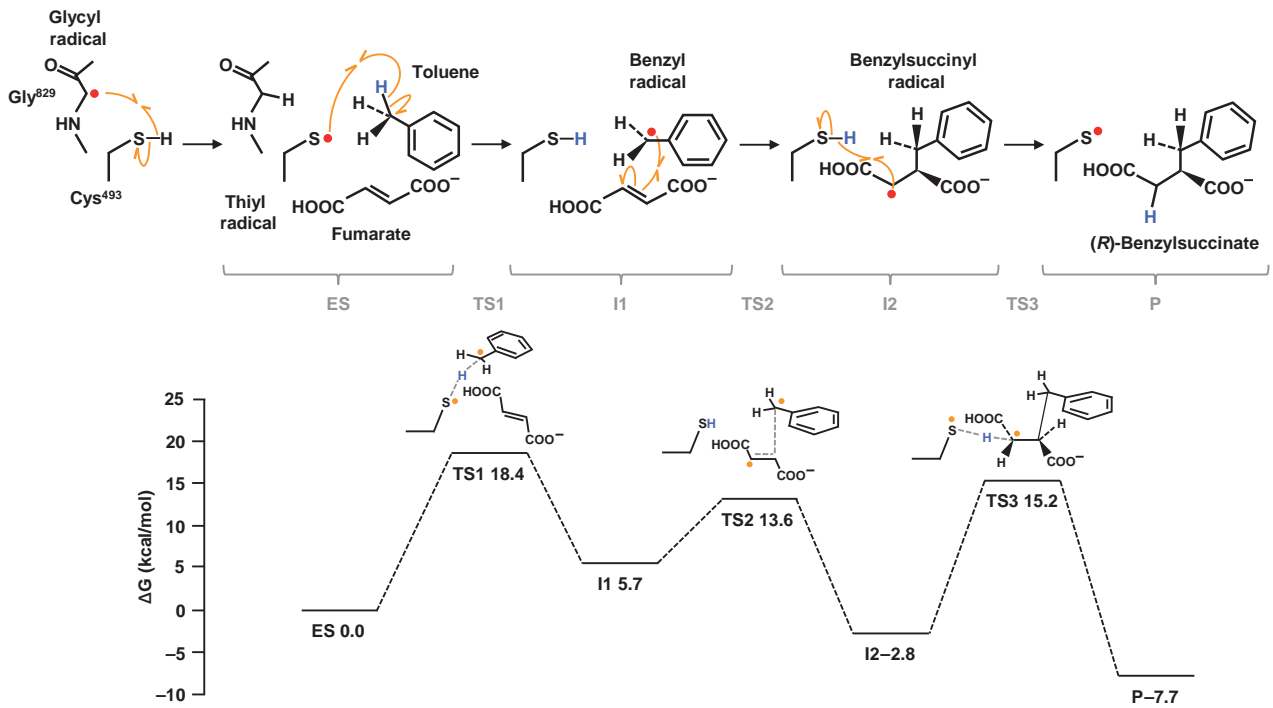
character (Gly⁸²⁹). In the initial step controlling the reaction rate, the thiyl radical abstracts a hydrogen atom from the methyl group, yielding a first transition state (fig. 1a, lower panel) with the highest energy level and subsequently an enzyme-bound benzyl radical intermediate. This transient intermediate subsequently attacks the double bond of fumarate, which is oriented in the pro-*R* position in the active site. This attack generates a new C–C bond to yield a benzylsuccinyl radical, with inverted stereochemistry at the former methyl group. Finally, back transfer of the initially abstracted hydrogen atom by *syn*-addition to the succinate moiety leads to the formation of the final product, (*R*)-benzylsuccinate, and reestablishment of the thiyl radical at the conserved Cys. The catalytically relevant Gly⁸²⁹ and Cys⁴⁹³ residues of the BSS α -subunit reside on the tips of two finger loops extending into a 10-stranded β/α -barrel, as represented in the core fold of all known glycy radical enzymes. The enzyme-bound fumarate provides a charged COO[−] group interacting with the positively charged neighboring Arg⁵⁰⁸, a universally conserved residue of alkyl-/arylalkylsuccinate synthases.

The reaction principle of BSS is widespread from a threefold perspective. (i) All anaerobic degraders of toluene and methyl-substituted monoaromatic hydrocarbons investigated to date employ the fumarate-dependent activation (except for *p*-cymene-degrading *Aromatoleum aromaticum* strain pCyN1, see below). Phylogenetic analysis (fig. 1b) revealed BSS orthologs to form a monophyletic clade with the branching order of the proteins reflecting the taxonomic affiliation of the source bacteria. (ii) Proteogenomic analysis of novel isolates of nitrate- or sulfate-reducing bacteria has revealed further clades (fig. 1b) of alkyl-/arylalkylsuccinate synthases specific for *p*-cymene ([4-isopropylbenzyl]succinate synthase, Ibs), *p*-cresol ([4-hydroxybenzyl]succinate synthase, Hbs), 2-methylnaphthalene ([2-naphthylmethyl]succinate synthase, Nms) and *n*-alkanes ([1-methylalkyl]succinate synthase, Mas). (iii) Alkylsuccinate synthases of *n*-alkane degraders have an unprecedented wide range of hydrocarbons that can be co-metabolically acti-

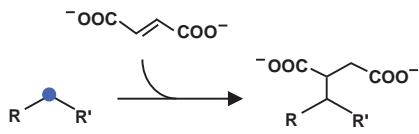
Fig. 1. Alkyl- and arylalkylsuccinate synthases adding hydrocarbons to fumarate. **a** Overall reaction mechanism for archetypical BSS from *T. aromatica* K172 (upper panel) and calculated energy values of the proposed transition states and intermediates (lower panel). ES = Enzyme substrate complex; TS1 = first transition state; I1 = first intermediate; TS2 = second transition state; I2 = second intermediate; TS3 = third transition state; P = product. Orange fishhook arrows (homolytic bond cleavage/formation) indicate reallocation of bonds; dots indicate full (red) and partial (orange)

radical character. Gray dashed lines indicate transitional bonding. Modified from Heider et al. [2016b]. **b** Phylogenetic relationship of currently known alkyl-/arylalkylsuccinate synthases based on the respective catalytically active α -subunits. Compound names: 1 = toluene, *m*-xylene or *p*-xylene; 2 = *p*-cymene; 3 = *p*-cresol; 4 = 2-methylnaphthalene; 5 = *n*-alkanes with C₅ to C₁₆ chain length. Blue dots indicate the reactive carbon atom at the hydrocarbon substrate. Enzyme names of alkyl-/arylalkylsuccinate synthases are provided in the text. Modified from Rabus et al. [2016].

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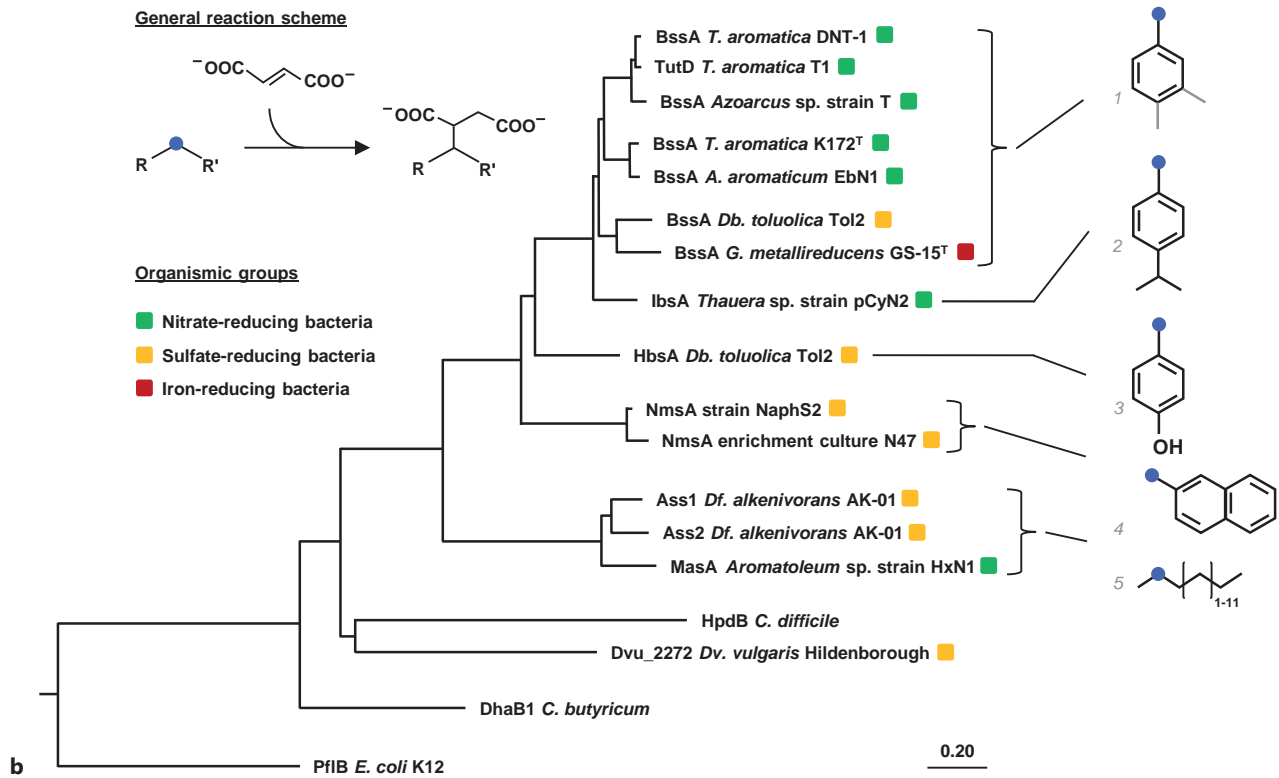


General reaction scheme



Organismic groups

- Nitrate-reducing bacteria
- Sulfate-reducing bacteria
- Iron-reducing bacteria



vated to the respective succinate derivatives (see below section ‘Stereochemistry of anaerobic activation of *n*-alkanes and co-metabolic capacities of anaerobic *n*-alkane degraders’). Recent homology modeling on the basis of the BSS X-ray crystal structure [Funk et al., 2015] revealed active site substitutions (2, 4 and 6, respectively) in Ibs, Hbs and Nms that could specifically tailor the binding sites for the corresponding hydrocarbon substrate. However, for Mas a completely different shape of the active site appears to be required for accommodating the alkyl chain and for binding fumarate.

Ethylbenzene Dehydrogenase, Archetype for O₂-Independent Hydroxylation

Mechanistic and structural insights into ethylbenzene dehydrogenase (EBDH) from denitrifying *A. aromaticum* EbN1 and the relevance of EBDH for the O₂-independent hydroxylation of other hydrocarbons are summarized by Heider et al. [2016c]. The soluble heterotrimeric EBDH contains a Mo-*bis*-MGD cofactor (MoCo) in the α -subunit and is closely related to membrane-anchored nitrate reductase from *Escherichia coli* (NarGHI) of the DMSO reductase subfamily II. In EBDH, liganding of Mo involves the dithiolenes of the two pterins as well as conserved Asp²²³. EBDH catalyzes the O₂-independent hydroxylation of its natural substrate ethylbenzene to enantiomerically pure (*S*)-1-phenylethanol. Notably, the enzyme converts >30 further ring-substituted mono- and bicyclic aromatic compounds enantioselectively to the respective alcohols with (*S*)-configuration, which are catalytic capacities with strong biotechnological potential.

According to structural and modeling studies, the catalytic mechanism of EBDH based on homolytic C–H bond cleavage is currently perceived as follows (fig. 2a). Initially, the C–H bond of the methylene group is activated via a first transition state characterized by transfer of a hydrogen atom from the methylene group of ethylbenzene to the oxidized MoCo (Mo^{VI}=O state) yielding a

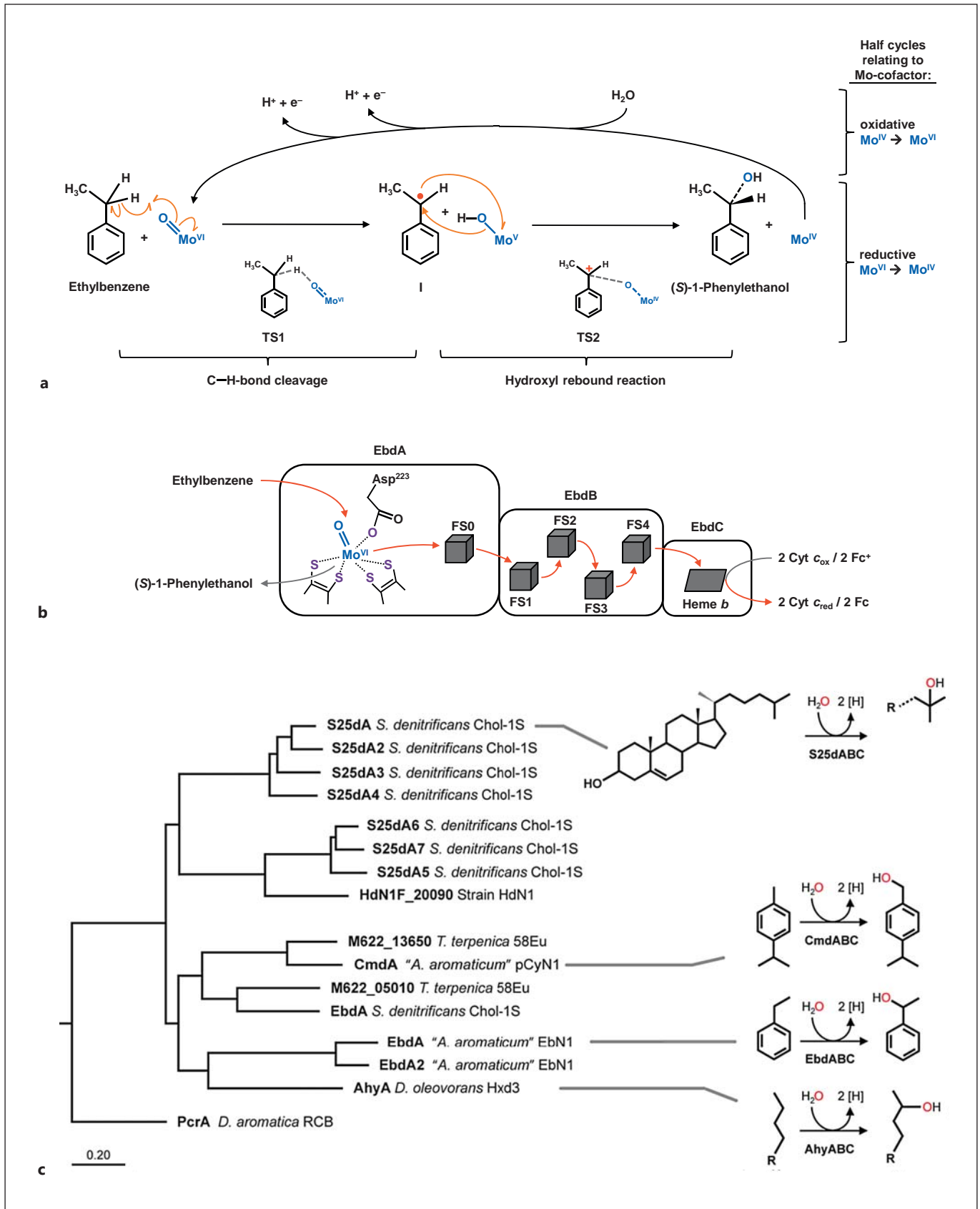
radical-state hydrocarbon intermediate and semireduced MoCo (Mo^V-OH state). A subsequent hydroxyl rebound reaction from the MoCo to the radical intermediate proceeds via a carbocation state intermediate complexed with the reduced MoCo (Mo^{IV}-OH; second transition state) to finally yield (*S*)-1-phenylethanol and Mo^{IV}. From the perspective of the Mo cofactor, the C–H bond cleavage described and the hydroxyl rebound reaction together represent the reductive half-cycle of the reaction catalyzed by EBDH. The oxidative half-cycle is responsible for coordination of H₂O to the Mo cofactor and its oxidation (from Mo^{IV} to Mo^{VI}) by two 1-electron transfers to the FS0-[Fe₄S₄] cluster additionally present in the α -subunit (EbdA). Further electron transfer to external cytochrome *c* (fig. 2b) then proceeds via the 4 Fe-S clusters in the β -subunit (EbdB) and the heme *b* group in the γ -subunit (EbdC). QM:MM modeling indicated the initial C–H bond cleavage as decisive for enantioselectivity, because removal of the pro-*R* hydrogen atom of the methylene group of ethylbenzene is considerably slower than that of the pro-*S* hydrogen atom.

Recently, several further hydrocarbon-hydroxylating, Mo-cofactor-containing enzymes (fig. 2c) related to archetypical EBDH have been reported. (i) The steroid C25 hydroxylase (S25d) from betaproteobacterial *Sterolibacterium denitrificans* anaerobically hydroxylates the tertiary carbon atom 25 in the alkyl side chain of cholesterol subsequent to its initial oxidation/isomerization to cholest-4-en-3-one. Notably, further degradation of the alkyl side chain is assumed to involve an unprecedented O₂-independent shift of the hydroxyl group from C25 to C26. Potential application of the S25d enzyme arises from synthesis of 25-hydroxyvitamin D₃, the circulating and active form of vitamin D₃. (ii) *p*-Cymene dehydrogenase (CmdABC) was recently discovered in *A. aromaticum* pCyN1 by combined proteogenomic and metabolite analysis to anaerobically hydroxylate the benzylic methyl groups of *p*-cymene (*p*-isopropyltoluene) and *p*-ethyltol-

Fig. 2. Anaerobic hydroxylation of hydrocarbons by ethylbenzene dehydrogenase (EBDH) and other Mo cofactor-containing dehydrogenases. **a** Overall mechanism of EBDH. TS1 = First transition state; I = intermediate; TS2 = second transition state. Fishhook (homolytic bond cleavage/formation) and normal (heterolytic bond cleavage/formation) arrows (orange) indicate reallocation of bonds. Red dots indicate full radical character. Dashed lines (gray or blue) indicate transitional bonding. Modified from Heider et al. [2016c]. **b** Electron flow (red) from ethylbenzene through the three subunits of EBDH via FeS clusters (FS) and heme to the natural (Cyt *c*) or artificial (ferricenium, Fc) electron acceptors. The Mo

cofactor (blue) in the α -subunit (EbdA) is ligated (purple) by the dithiols of 2 molybdopterin moieties and the carboxyl group of Asp²²³. **c** Phylogenetic relationship of currently known Mo cofactor-containing dehydrogenases, based on the respective catalytically active α -subunits. Compound names: 1 = cholesterol; 2 = *p*-cymene; 3 = 4-isopropylbenzyl alcohol; 4 = ethylbenzene; 5 = (*S*)-1-phenylethanol; 6 = *n*-alkane; 7 = alkan-2-ol. Enzyme names: S25dABC = steroid C25 hydroxylase; CmdABC = *p*-cymene dehydrogenase; EbdABC = EBDH; AhyABC = presumptive alkane hydroxylase. Blue dots indicate the reactive carbon atom at the hydrocarbon substrate. Modified from Rabus et al. [2016].

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uene. Considering the mechanism of EBDH described above, the hyperconjugative effect of the *p*-alkyl groups is assumed to stabilize the resonance structures of the intermediate carbenium ion. (iii) Sulfate-reducing *Desulfococcus oleovorans* Hxd3 is the sole currently known anaerobic bacterium that degrades *n*-alkanes independently of addition to fumarate or anaerobic generation of oxygen species. Recent proteogenomic analysis confirmed the absence of an alkylsuccinate synthase and rather indicated the involvement of an EBDH ortholog. A putative alkane C2-methylene hydroxylase (AhyABC) is proposed to activate the *n*-alkane at the subterminal carbon atom to the respective alkan-2-ol.

Anaerobic Degradation of 4-Alkylbenzoates and 4-Alkyltoluenes

Organisms, elucidation of pathways and regulatory studies on the anaerobic degradation of *p*-alkylated monoaromatic compounds are compiled by Rabus et al. [2016]. Previous research on alkylbenzenes had revealed *p*-xylene as particularly recalcitrant substrate, but also showed the feasibility of an initial fumarate-dependent activation to (4-methylbenzyl)succinate and its subsequent conversion to 4-methylbenzoate. Therefore, the anaerobic degradation of the latter was regarded as the major challenge of the degradation pathway. In accord with this assumption, class I benzoyl-CoA reductase (BCR) from *T. aromatica* K172 is apparently incapable of acting on 4-methylbenzoyl-CoA. The *p*-alkyl group of 4-methylbenzoate may not be reconcilable with the properties of the substrate binding site and the proposed reaction mechanism of BCR due to its electron-releasing and space-filling properties (see below section ‘The W-containing BCRs and acetylene hydratase’). Thus, anaerobic degradation of 4-methylbenzoate may involve a specific variant of BCR.

Denitrifying *Magnetospirillum* sp. strain pMbN1 (Alphaproteobacteria) oxidizes 4-methylbenzoate completely to CO₂. Applying a combination of proteogenomics, targeted metabolite analyses and enzyme activity measurements enabled the discovery of a specific 4-methylbenzoyl-CoA pathway in addition to the classical central benzoyl-CoA pathway in strain pMbN1. The coding genes for the two pathways are organized in distinct genomic clusters. Remarkably, the *p*-methyl group of 4-methylbenzoate is retained beyond dearomatization, ring cleavage and β-oxidation to the level of 3-methylglutaryl-CoA (fig. 3a), which is assumed to be further metabolized via the leucine/isovalerate pathway. Phylogenetic analysis revealed that an apparent 4-methylbenzoyl-CoA reductase (MbrBCAD) is formed in the respective

cells and branches distinctly off the archetypical class I BCR (BcrBCAD) in sequence comparison. The central metabolic role of benzoate/benzoyl-CoA in the anaerobic degradation of aromatic compounds may account for the ‘regulatory’ observation that the presence of benzoate represses utilization of 4-methylbenzoate (and also simultaneously of succinate from a ternary substrate mixture) in strain pMbN1. Repression is apparently executed at multiple levels, i.e. by inhibition of 4-methylbenzoate and succinate uptake as well as succinate conversion to acetyl-CoA (via pyruvate).

While the betaproteobacteria *A. aromaticum* pCyN1 and *Thauera* sp. strain pCyN2 are both known to anaerobically degrade the plant-derived hydrocarbon *p*-cymene (4-isopropyltoluene), they employ two different strategies for its conversion to 4-isopropylbenzoyl-CoA. In *A. aromaticum* pCyN1, the benzylic methyl group is hydroxylated by a putative *p*-cymene dehydrogenase (CmdABC), while in *Thauera* sp. strain pCyN2 it is added to fumarate by (4-isopropylbenzyl)succinate synthase (IbsABCDEF; fig. 3b). Both enzymes are new representatives of EBDH-like and BSS-like hydrocarbon-activating enzymes (fig. 1b, 2c). Further transformation of the initial intermediates (4-isopropyl)benzyl alcohol and (4-isopropylbenzyl)succinate involves dehydrogenations and β-oxidation-like reactions, respectively, leading to (4-isopropyl)benzoyl-CoA in both cases.

Decarboxylation of 4-Hydroxyphenylacetate

Structural and mechanistic insights into the glycyl radical enzyme 4-hydroxyphenylacetate decarboxylase (4Hpad) and its respective activase (4Hpad-AE) are summarized by Selvaraj et al. [2016]. Concurrently with activases of other glycyl radical enzymes (e.g. pyruvate formate-lyase and BSS), 4Hpad-AE possesses a conserved N-terminal [4Fe-4S]^{2+/1+} RS-cluster for reductive ([4Fe-4S]²⁺ → [4Fe-4S]¹⁺) cleavage of *S*-adenosylmethionine into methionine and a transient 5'-deoxyadenosyl radical. Possibly, the reactivity of 4Hpad-AE is controlled by a (downregulating) additional ferredoxin-like domain to assure full activation only after complex formation of 4Hpad-AE with 4Hpad. The 5'-deoxyadenosyl radical generated by 4Hpad-AE abstracts a hydrogen atom from conserved Gly⁸⁷³ of 4Hpad, leading to formation of the storage radical Gly^{873•}. While residing in two distinct β-hairpin loops, Gly⁸⁷³ and Cys⁵⁰³ are spatially arranged in close proximity to establish the glycyl/thiyl radical dyad prosthetic group. Hydrogen atom transfer from Cys⁵⁰³-SH to the Gly^{873•} radical generates the reactive Cys⁵⁰³-S[•] radical.

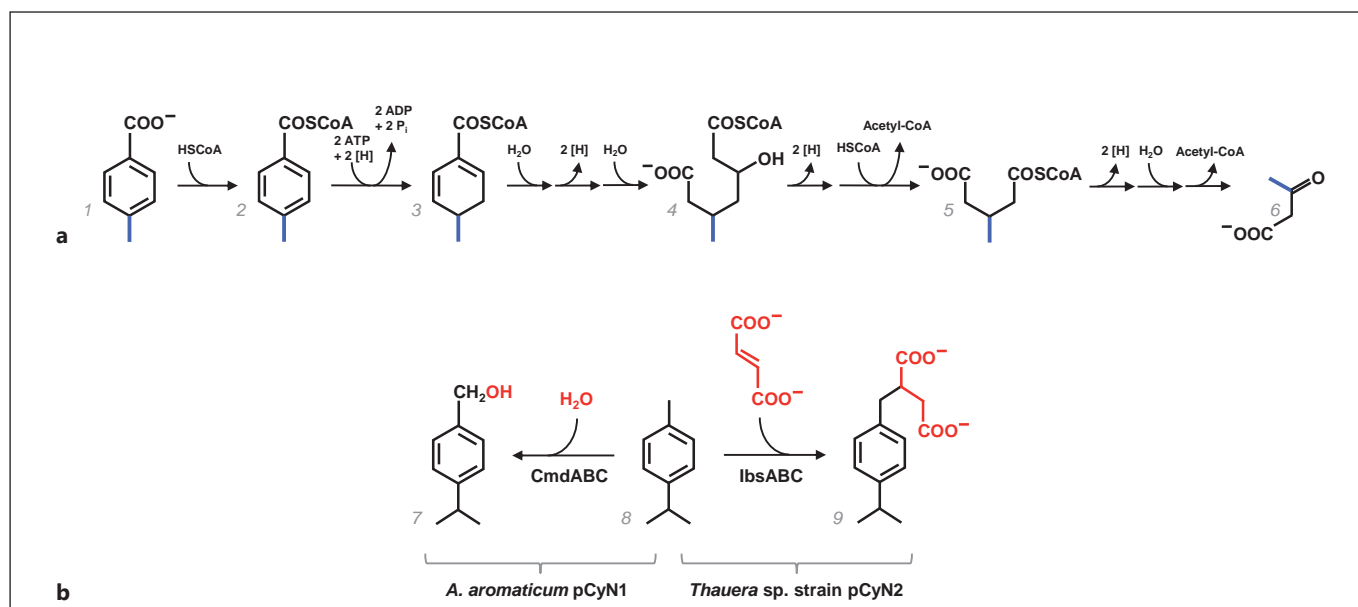


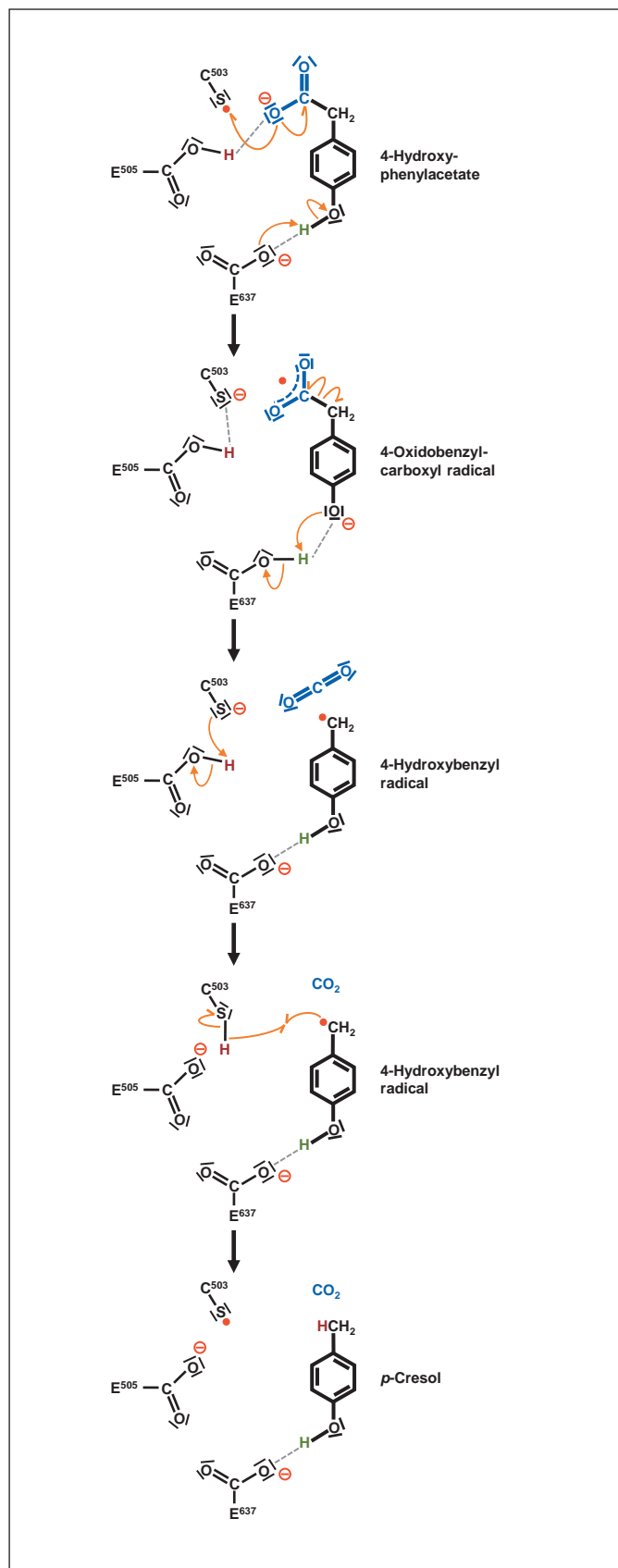
Fig. 3. Anaerobic degradation of 4-methylbenzoate and *p*-cymene in denitrifying bacteria. **a** Analogous pathways for 4-methylbenzoate (*p*-methyl group highlighted in blue) and benzoate degradation in *Magnetospirillum* sp. strain pMbN1. Note that reactions from compounds 5 to 6 differ for glutaryl-CoA and 3-methylglutaryl-CoA, respectively. Compound names: 1 = benzoate or 4-methylbenzoate; 2 = benzoyl-CoA or 4-methylbenzoyl-CoA; 3 = cyclohexa-1,5-diene-1-carboxyl-CoA or 4-methylcyclohexa-1,5-diene-1-carboxyl-CoA; 4 = 3-hydroxypimelyl-CoA or 3-hydroxy-

5-methylpimelyl-CoA; 5 = glutaryl-CoA or 3-methylglutaryl-CoA; 6 = acetoacetate (preceding elimination of acetyl-CoA in case of 4-methylbenzoate). Modified from Rabus et al. [2016]. **b** Initial reactions in anaerobic degradation of *p*-cymene. CmdABC = *p*-cymene dehydrogenase; IbsABC = (4-isopropylbenzyl)succinate synthase. Compound names: 7 = 4-isopropylbenzyl alcohol; 8 = *p*-cymene; 9 = (4-isopropylbenzyl)succinate. Modified from Rabus et al. [2016].

4Hpad is a ($\beta\gamma$)₄ tetramer of heterodimers that catalyzes the conversion of 4-hydroxyphenylacetate to *p*-cresol. Crystal structure analysis revealed close proximity of the reactive Cys⁵⁰³-S[•] radical to the carboxyl group of 4-hydroxyphenylacetate. The current mechanistic reaction model derived from structural analysis and quantum chemical calculations is depicted in figure 4. In analogy to a Kolbe-type decarboxylation, the Cys⁵⁰³-S[•] radical oxidizes the carboxylate to a carboxyl radical, while the thiolate (Cys⁵⁰³-S⁻) is protonated to Cys⁵⁰³-SH by neighboring Glu⁵⁰⁵. The carboxyl radical breaks apart into CO₂ and a 4-hydroxybenzyl radical. The substrate-binding mode also indicated that the *p*-hydroxyl group of 4-hydroxyphenylacetate is anchored via hydrogen bonding to Glu⁶³⁷. This is relevant for the proposed reaction mechanism, as de- and backprotonation between the phenolic group of the substrate and the carboxyl group of Glu⁶³⁷ facilitates intermediate formation of the 4-hydroxybenzyl radical. Finally, abstraction of a hydrogen atom from Cys⁵⁰³-SH yields the product *p*-cresol and regenerates the reactive Cys⁵⁰³-S[•] radical for the next round of catalysis.

Anaerobic Degradation of Benzene and Naphthalene

An overview on organisms, reactions, genes and enzymes involved in anaerobic degradation of benzene and naphthalene is provided by Meckenstock et al. [2016]. The initial reaction of anaerobic degradation of benzene and naphthalene has been studied mainly with sulfate- or nitrate-reducing prokaryotes during the past decade. Among several proposed possibilities, carboxylation of the aromatic rings to benzoate and 2-naphthoate, respectively, emerged as most likely initial reaction, based on proteogenomic studies and activity assays (fig. 5). The putative carboxylase-like protein is orthologous to the catalytic α -subunit of phenylphosphate carboxylase from *T. aromatica* K172 and related to the so-called UbiD/UbiX proteins, recently demonstrated to catalyze aryl (e.g. styrene) (de)carboxylation via 1,3-dipolar cycloaddition [Payne et al., 2015]. In case of 2-methylnaphthalene, fumarate-dependent activation leads to (2-naphthylmethyl)succinyl-CoA, which is converted via a β -oxidation-like reaction sequence to naphthoyl-CoA and succinate, as known



from anaerobic toluene degradation [Heider et al., 2016c] (fig. 5).

The further degradation of the benzene-derived benzoyl-CoA can proceed via the known central benzoyl-CoA pathway, whereas the naphthalene-derived intermediate 2-naphthoyl-CoA requires 3 newly discovered and distinct reductases for 2 successive reduction steps at the nonsubstituted ring followed by 1 reduction step at the substituted ring (fig. 5). The ATP-independent naphthoyl-CoA (NCR) and 5,6-dihydronaphthoyl-CoA (DHNCr) reductases each catalyze a 2-electron reduction step: NCR reduces naphthoyl-CoA to 5,6-dihydronaphthoyl-CoA, while DHNCr further reduces the latter to 5,6,7,8-tetrahydronaphthoyl-CoA. NCR and DHNCr belong to the old yellow enzyme family and contain FAD, FMN and a [4Fe-4S] cluster as cofactors. Finally, an ATP-dependent 5,6,7,8-tetrahydronaphthoyl-CoA reductase (THNCr) similar to class I BCR forms hexahydro-naphthoyl-CoA, the isomeric structure of which remains to be resolved. Further degradation is proposed to involve reaction sequences for the successive cleavage of the 2 rings mediated by enzymes encoded in the *thn* operon of sulfate-reducing enrichment culture N47.

The W-Containing BCRs and Acetylene Hydratase

Structural and mechanistic insights into class II BCR and acetylene hydratase (ACH) are summarized by Boll et al. [2016a]. Benzoyl-CoA represents the central intermediate of most known peripheral routes for the anaerobic degradation of aromatic compounds. BCR de-aromatizes benzoyl-CoA via a Birch-like reaction to cyclohexa-1,5-diene-1-carboxyl-CoA (dienoyl-CoA). The reaction is considered to proceed by sequential transfer of single electrons and protons via radical/anionic intermediates (fig. 6a). The benzoyl-CoA/dienoyl-CoA couple exhibits a very low reduction potential ($E^{\circ} = -622$ mV). For this reason, electron transfer from any physiological reductant has to be coupled to an exergonic reaction. The well-studied class I BCR from *T. aromatica* K172 operates irreversibly with a low-potential electron transfer from reduced ferredoxin ($E^{\circ} = -500$ mV) being achieved by stoichiometric hydrolysis of 1 molecule ATP to ADP per

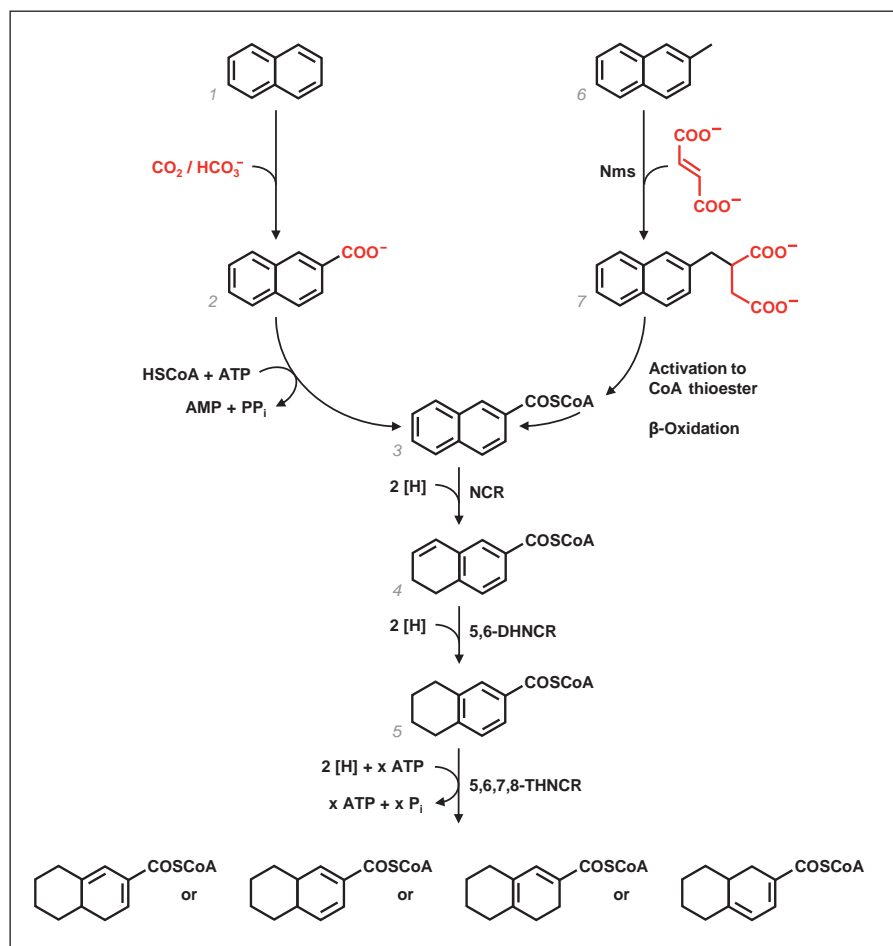
Fig. 4. Proposed catalytic mechanism of the glyceryl-radical enzyme 4-hydroxyphenylacetate decarboxylase. Fishhook (homolytic bond cleavage/formation) and normal (heterolytic bond cleavage/formation) arrows (orange) indicate reallocation of bonds. Red dots indicate full radical character. Gray dashed lines indicate transitional bonding. Modified from Selvaraj et al. [2016].

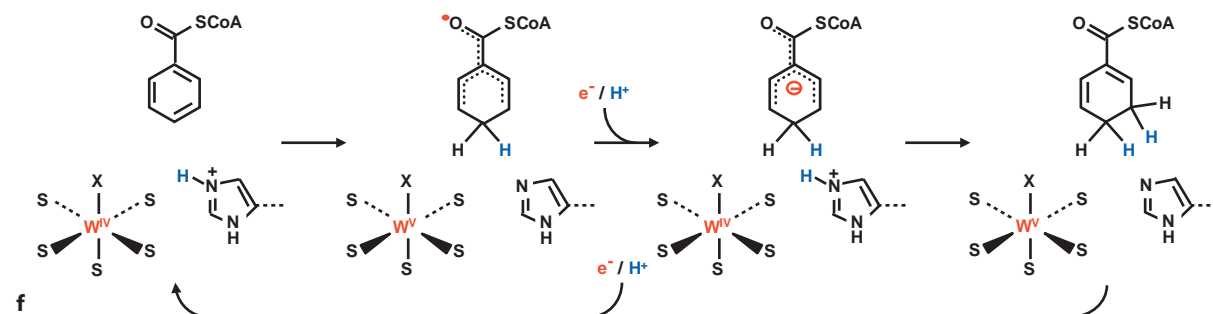
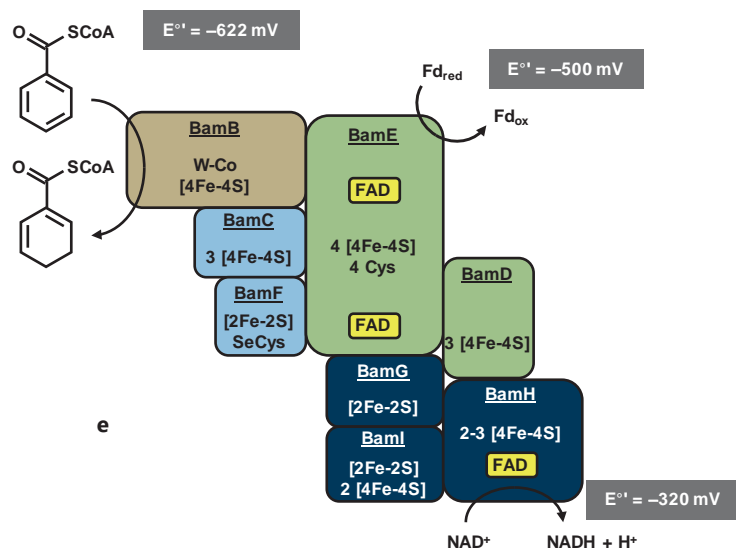
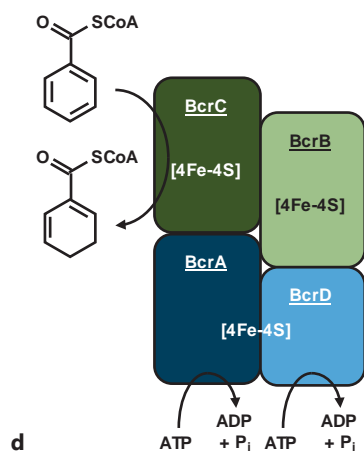
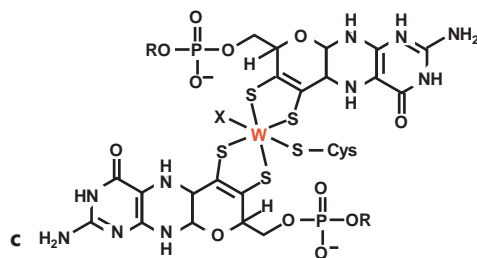
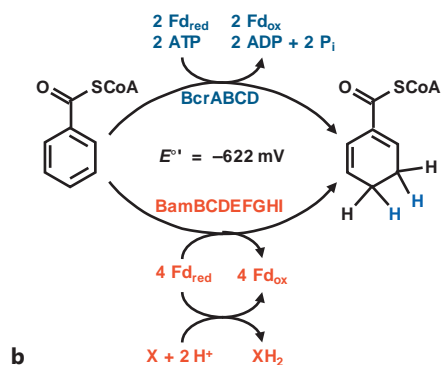
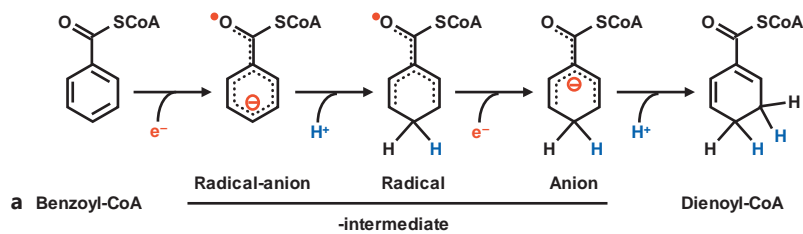
electron transferred. Class I BCR has a heteromeric $\alpha\beta\gamma\delta$ structure (fig. 6d) and is widespread among facultative anaerobes. In contrast, energy-limited obligate anaerobes employ a completely different type of BCR named class II BCR. This enzyme has a considerably more complex subunit structure, is ATP-independent and operates reversibly (fig. 6b, e). The O_2 sensitivity of both types of BCRs explains their absence in aerobic organisms. A recently discovered third class of dearomatizing arylcarboxyl-CoA reductases comprises flavin-dependent enzymes (NCR and 5,6-DHNCR) that are involved in reductive dearomatization of naphthalene (see above section ‘Anaerobic degradation of benzene and naphthalene’).

Class II BCR was mainly studied in Fe^{III} -reducing *Geobacter metallireducens* GS-15 and predicted from proteogenomic studies to be constituted by the large BamBCDEFGHI (benzoic acid metabolism) complex, with the active BamB belonging to the aldehyde:ferredoxin oxidoreductase family of W/Mo-cofactor-containing enzymes (fig. 6e). While the complete complex so far defied puri-

fication, the BamBC complex could be purified. It catalyzed the reductive dearomatization/oxidation of benzoyl-CoA and the 1,5-dienoyl-CoA, respectively, as demonstrated by isotope exchange experiments. The recently determined crystal structure of the Bam(BC)₂ heterotetramer revealed the presence of 1 *bis*-WPT cofactor (WCo; fig. 6c), 1 Zn^{2+} ion and 1 [4Fe-4S] cluster in the BamB subunit as well as 3 [4Fe-4S] clusters bound in a ferredoxin-like fold in the electron-transferring BamC subunit. Localization of the W center of WCo in an aprotic and locked cavity ensures that the low-potential electron donor in the W^{IV} state is not dissipated by protons derived from the reducing solvent. Within the WCo, W is octahedrally coordinated by 5 sulfur atoms (4 dithiolene sulfurs from 2 molybdopterin and 1 from Cys³²²) and 1 additional ligand (fig. 6c). The Zn^{2+} ion does not directly interact with the WCo but is assumed to encapsulate the active site in a protective role. Upon CoA-thioester binding, the Zn^{2+} -binding site disintegrates and the Zn^{2+} ion is released from the protein. Tight binding of benzoyl-

Fig. 5. Anaerobic degradation of naphthalene and 2-methylnaphthalene in the sulfate-reducing enrichment culture N47. Nms = (2-Naphthylmethyl)succinate synthase; NCR = 2-naphthoyl-CoA reductase; 5,6-DHNCR = 5,6-dihydro-2-naphthoyl-CoA reductase; 5,6,7,8-THNCR = 5,6,7,8-tetrahydro-2-naphthoyl-CoA reductase. Compound names: 1 = naphthalene; 2 = 2-naphthoate; 3 = 2-naphthoyl-CoA; 4 = 5,6-dihydro-2-naphthoyl-CoA; 5 = 5,6,7,8-tetrahydro-2-naphthoyl-CoA; 6 = 2-methylnaphthalene; 7 = (2-naphthylmethyl)succinate. Modified from Meckenstock et al. [2016].





6

(For legend see next page.)

CoA in the hydrophobic cavity shields C2 and C6 of the aromatic ring from a proton donor. Therefore, the 1,5-dienoyl-CoA is formed and not the 1,4-isomer as known for chemical Birch reduction of benzoate. From a mechanistic perspective, the recent structural insights on class II BCR are essentially in accord with a Birch-like reduction of benzoyl-CoA, as (i) regions potentially involved in electron and proton transfer processes are separated in the active site, and (ii) the substrate ring is spatially oriented between the WCo and conserved His²⁶⁰ as required for proton-coupled electron transfer steps possibly ensuring that a highly reactive radical-anion intermediate is avoided (fig. 6f; the found W^{IV/V} redox chemistry is compatible with single electron transfer steps). Homology between the BamDEFGHI components and the heterodisulfide-reductase/hydrogenase complex from methanogens gave rise to the hypothesis that a flavin-based electron bifurcation [Buckel and Thauer, 2013] could enable class II BCR to drive the ‘uphill’ electron transfer from reduced ferredoxin to the active site by coupling it to the ‘downhill’ electron transfer to NAD⁺.

ACH catalyzes the addition of a H₂O molecule to the triple-bond (C≡C) of acetylene (C₂H₂) yielding acetaldehyde. ACH of *Pelobacter acetylenicus*, like BamB described above, contains a bis-WPT-guanine-dinucleotide cofactor (WCo) and a [4Fe-4S] cluster. While the structure of ACH overall resembles that of other enzymes of the dimethyl sulfoxide reductase family, the access funnel traversing from the surface of the enzyme to the active site is unique. In the WCo, the tungsten atom is ligated by the 4 sulfur atoms of the dithiolene moieties and 1 Cys residue, similar as in BamB (fig. 6c). The sixth ligand in ACH is a tightly coordinated oxygen atom (from H₂O or OH⁻), while its nature in BamB is currently unresolved (possibly a cyanide; W–C≡N). Site-directed mutagenesis revealed that Asp¹³ (activates water to add to the C≡C bond) and Ile¹⁴² (substrate-binding cavity) are decisive for the reaction mechanism. Proximity to a protonated Asp¹³ would transform H₂O into an electrophile, which may have a role in the attack on the C≡C bond. Computational studies on the other hand suggest a direct η² complex of acet-

ylene with the tungsten ion and the displaced H₂O to attack the η²-acetylene complex forming an intermediate hydroxyethenyl species. Experimental verification of the mechanism is still missing as is the determination of the Asp¹³ protonation state.

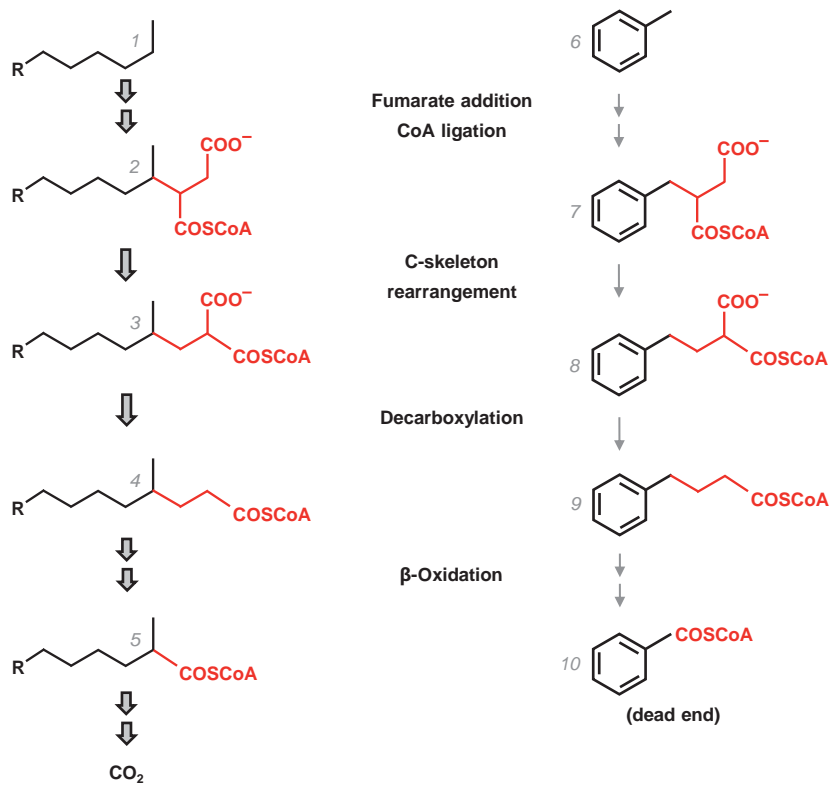
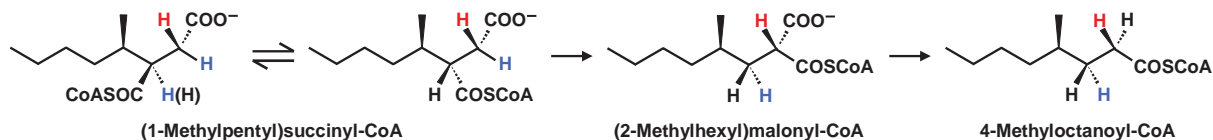
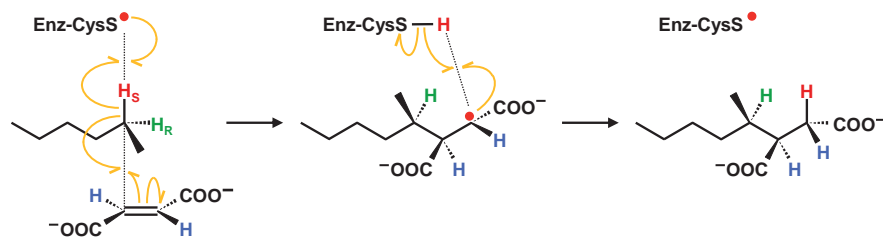
Metabolism of Aliphatic Compounds

Stereochemistry of the Anaerobic Activation of n-Alkanes and Co-Metabolic Capacities of Anaerobic n-Alkane Degradors

Recent mechanistic insights into the stereochemistry of the anaerobic activation of *n*-alkanes and the capacities of anaerobic *n*-alkanes degraders to co-metabolically functionalize a broad range of hydrocarbons other than the growth-supporting ones are summarized by Wilkes et al. [2016]. Previous studies with the denitrifying betaproteobacterium *Aromatoleum* sp. strain HxN1 had suggested that anaerobic degradation of *n*-hexane proceeds via addition of a hex-2-yl radical to fumarate leading to (1-methylpentyl)succinate (MPS). In contrast to the related transformation of toluene to (*R*)-benzylsuccinate by BSS, the MPS synthase forms 2 stereocenters at adjacent carbon atoms. Comparison to synthetic standards demonstrated formation of (2*R*,1'*R*)- and (2*S*,1'*R*)-MPS. To elucidate the stereochemical course of involved hydrogen atom abstraction, incubation experiments of *Aromatoleum* sp. strain HxN1 with chemically synthesized (*S,S*)-, (*R,R*)- and *meso-n*-(2,5-²H₂)hexane isomers were conducted. Thus, MPS synthase is proposed to homolytically cleave the pro-*S* hydrogen atom at C2 of *n*-hexane and subsequently transfer this hydrogen atom back to C3 of MPS accompanied by inversion of configuration at C2 of *n*-hexane. Based on these results, a concerted reaction mechanism was suggested where the pro-*S* hydrogen atom and fumarate localize to opposite sides of the alkyl chain (fig. 7a). The labeling pattern of *n*-hexane-derived metabolites in *Aromatoleum* sp. strain HxN1 also agreed with the previously suggested pathway downstream of MPS. Epimerization of CoA-activated MPS is suggested

Fig. 6. Mechanisms and structures of BCRs. **a** Reduction of benzoyl-CoA to cyclohexa-1,5-diene-1-carboxyl-CoA (dienoyl-CoA) via a Birch-like mechanism. **b** ATP-dependent class I BCR (BcrA–D) from *T. aromatica* K172 versus ATP-independent class II BCR (BamB–I) from *Geobacter metallireducens* GS-15. **c** Structure of the bispyranopterin (*bis*-WPT) cofactor of class II BCR and ACH. **d** Subunit and cofactor composition of class I BCR. **e** Subunit and

cofactor composition of class II BCR, with the *E*^o values indicative of a possible involvement of electron bifurcation. **f** Catalytic mechanism of class II BCR involving a proposed proton-coupled electron transfer to avoid formation of the reactive radical-anion intermediate as occurring in the chemical Birch reduction. Modified from Boll et al. [2016].



d

	Compounds transformed (not vs. growth-supporting)		
	Strain HxN1	Strain OcN1	Strain TD3
Aliphatic hydrocarbons	13 / 3	17 / 5	22 / 11
Aromatic hydrocarbons	11 / 0	32 / 0	34 / 0

to generate the proper diastereoisomer for a mutase forming (2*R*,2*R'*)-(2-methylhexyl)malonyl-CoA. The latter has been suggested to be decarboxylated to 4-methyloctanoyl-CoA (fig. 7b), which is then converted by classical β -oxidation to 3 acetyl-CoA and 1 propionyl-CoA. Channeling the propionyl-CoA through the methylmalonyl-CoA pathway and succinate dehydrogenase regenerates the fumarate cosubstrate for the initial MPS-forming reaction.

Experiments with defined substrate mixtures demonstrated that anaerobic *n*-alkane degraders can activate toluene (not growth-supporting) to benzylsuccinate and convert it to benzoyl-CoA as dead end product. For that purpose, they channel toluene through the 'alkane' route including the proposed mutase and decarboxylase reactions (fig. 7c). Probably MPS synthase-like enzymes, which cleave the stronger C–H bond (–400 kJ/mol) of an aliphatic methylene group as compared to that of a benzylic methyl group (–368 kJ/mol), are able to arbitrarily activate the not growth-supporting toluene, while BSS-like enzymes are restricted to methylated aromatic compounds. Correspondingly, anaerobic alkylbenzene degraders are apparently incapable of cotransforming an *n*-alkane. One may speculate that the more specialized BSS-type enzymes evolved from MPS synthases having a less restricted substrate range. Growth experiments with (2,3-²H₂)fumarate as cosubstrate yielded predominantly monodeuterated alkylsuccinates versus dideuterated benzylsuccinates, i.e. the hydrogen atom at C2 of the succinate adduct was completely exchanged with external hydrogen in case of *n*-alkanes, while it was retained in benzylsuccinate. This indicates that the hydrogen atom exchange is more determined by the type of substrate than the type of enzyme. Motivated by these observations on co-metabolism, comprehensive growth and metabolite studies were conducted with 11 bacterial strains provided with crude oil or binary mixtures of >40 different hydrocarbons. Alkane utilizers were found to form a much broader range

of alkyl- and arylalkylsuccinates than alkylbenzene utilizers. For example, the thermophilic sulfate-reducing strain TD3, which was previously isolated from hydrocarbon-rich deep-sea sediment, utilizes 11 *n*-alkanes (C₆–C₁₆) as growth substrates, while it is capable of co-metabolically transforming 22 aliphatic and 34 aromatic hydrocarbons that are not growth-supporting as single compounds (fig. 7d). This hitherto unknown broad range of co-metabolic transformation of hydrocarbons may benefit bacteria by relieving solvent stress (e.g. at the oil-water interface in reservoirs or hydrocarbon-bearing/-contaminated sites) or other bacteria by crossfeeding events. Moreover, this will have to be considered in future studies investigating microbial activity in hydrocarbon-rich anoxic habitats. In accord, very recent habitat studies employing an alkane-activating (methylalkyl)succinate synthase gene marker (*masD*, coding for the catalytic subunit) demonstrated ubiquitous presence and diversity of anaerobic alkane degraders in (cold) marine sediments [Gittel et al., 2015; Johnson et al., 2015] and marine seeps [Stagars et al., 2016].

Activation of Acetone and Other Simple Ketones

The current knowledge on the activation of acetone and acetophenone by different types of carboxylases from sulfate- and nitrate-reducing bacteria is summarized by Heider et al. [2016a]. Acetone carboxylase (Acx) from denitrifying *A. aromaticum* EbN1 and related enzymes from other organisms are heterohexameric ($\alpha\beta\gamma$)₂ enzyme complexes which do not contain biotin, but 2–3 metal ions per ($\alpha\beta\gamma$)₂ complex. Acx uses bicarbonate as cosubstrate to convert acetone to acetoacetate according to the following proposed mechanism. One or 2 ATP (hydrolyzed to AMP and 2 P_i) are required to activate acetone to its phosphoenol derivative and bicarbonate to carboxyphosphate. These 2 highly reactive intermediates would then combine to form acetoacetate and 2 P_i (fig. 8a). Acx orthologs appear to fall into 2 enzyme types which

Fig. 7. Mechanism, stereochemistry and co-metabolism of anaerobic degradation of *n*-alkanes. **a** Mechanistic and stereochemical model for the anaerobic transformation of *n*-hexane to MPS in denitrifying *Aromatoleum* sp. strain HxN1. Orange fishhook arrows (homolytic bond cleavage/formation) indicate reallocation of bonds; red dots indicate full radical character. H_S = Pro-S hydrogen atom; H_R = pro-R hydrogen atom. Modified from Wilkes et al. [2016]. **b** Transformation of MPS-derived (1-methylpentyl)succinyl-CoA by proposed epimerase, mutase and decarboxylase reactions to 4-methyloctanoyl-CoA. Modified from Jarling et al. [2012]. **c** Co-metabolic transformation of toluene to benzoyl-CoA

via the 'alkane pathway' by *n*-hexane-utilizing cultures of *Aromatoleum* sp. strain HxN1. Compound names: 1 = *n*-hexane; 2 = (1-methylpentyl)succinyl-CoA; 3 = (2-methylhexyl)malonyl-CoA; 4 = 4-methyloctanoyl-CoA; 5 = 2-methylhexanoyl-CoA; 6 = toluene; 7 = benzylsuccinyl-CoA; 8 = (2-phenylethyl)malonyl-CoA; 9 = 4-phenylbutanoyl-CoA; 10 = benzoyl-CoA. Modified from Rabus et al. [2011]. **d** Range of co-metabolic activation of not growth-supporting hydrocarbons by various bacteria degrading *n*-alkanes anaerobically (more details on the strains are provided in table 1). Data taken from table 1 in Wilkes et al. [2016].

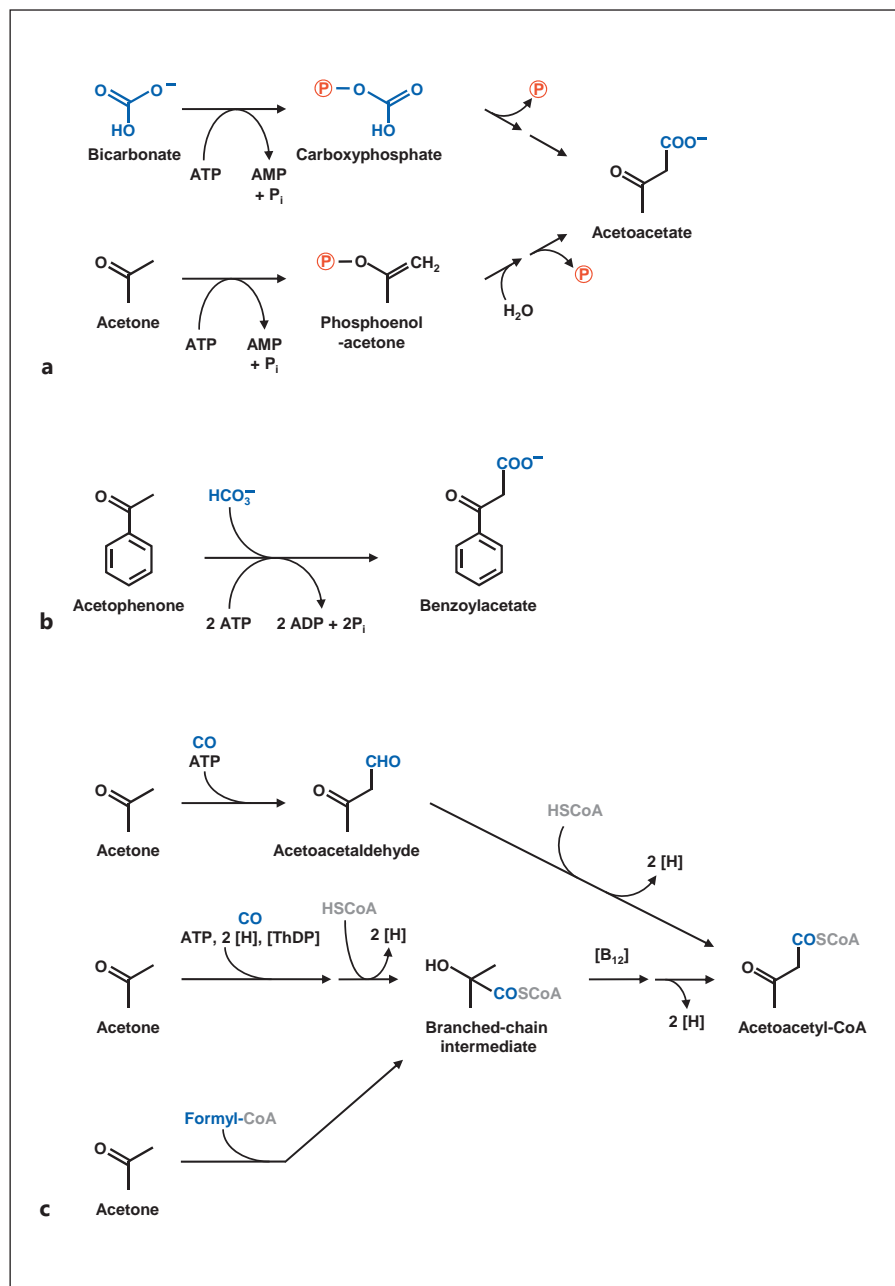


Fig. 8. Carboxylases acting on acetone and acetophenone. **a** Proposed mechanism of biotin-independent Acx (AcxABC) from denitrifying *A. aromaticum* EbN1. **b** Proposed mechanism of acetophenone carboxylase from *A. aromaticum* EbN1. In analogy to Acx, the acetophenone carboxylase-catalyzed reaction is proposed to involve intermediary formation of carboxyphosphate and phosphoenol acetophenone. **c** Proposed carbonylation or formylation of acetone to acetoacetyl-CoA in sulfate-reducing *Desulfococcus biacutus*. Modified from Heider et al. [2016a].

differ in their metal content (Zn and Fe vs. Zn, Fe and Mn) and ATP stoichiometry (2 ATP vs. 1 ATP hydrolyzed per acetone carboxylated).

Acetophenone carboxylase (Apc) from *A. aromaticum* EbN1 converts acetophenone with bicarbonate as cosubstrate to benzoylacetate (fig. 8b) as part of the anaerobic ethylbenzene degradation pathway. In contrast to Acx described above, Apc is composed of 5 subunits and hydrolyzes 2 ATP to ADP per acetophenone carbox-

ylated. Its ATPase activity is uncoupled if 1 of the 2 substrates is absent, and the reaction rate is limited by proton abstraction from the ketone. Moreover, Apc is also capable of carboxylating the methylene group of propiophenone. Notably, *A. aromaticum* EbN1 possesses a third type of a putative ketone carboxylase, the predicted biotin-dependent 4-hydroxyacetophenone carboxylase (XccABC) as part of the anaerobic *p*-ethylphenol degradation pathway.

Acetone catabolism in the sulfate-reducing *Desulfo-coccus biacutus* differs completely from that described for *A. aromaticum* EbN1 or other facultative anaerobes, as its genome does not possess any *acx*-like genes. Rather, proteogenomic, metabolite and enzymatic evidences suggest the possibilities for either carbonylation (addition of CO) at one of the methyl groups or the carbonyl group of acetone, or a formylation (addition of a formyl-CoA) of the carbonyl group of acetone (fig. 8c). The energy-limited *D. biacutus* would benefit from any of the 3 proposed reactions, as these afford substantially less energy expenditure than the *Acx*-type of carboxylation reaction.

Fermentative Formation of Cyclohexanecarboxylate

The enzymology involved in fermentative formation of cyclohexanecarboxylate via cyclohexa-1,5-diene-1-carboxyl-CoA (Ch1,5CoA) from benzoate by the obligately anaerobic *Syntrophus aciditrophicus* is summarized by Boll et al. [2016b]. During growth with benzoate, *S. aciditrophicus* employs the class II BCR for reductive dearomatization of benzoyl-CoA to Ch1,5CoA and its subsequent conversion by modified β -oxidation reactions to 3-hydroxypimelyl-CoA. Further decomposition of the latter to 1 CO₂ and 3 acetyl-CoA involves biotin-dependent glutaconyl-CoA decarboxylase that couples the transformation of glutaryl-CoA (C₅) to crotonyl-CoA (C₄) to the export of H⁺/Na⁺ ions. Fermentative reduction of Ch1,5CoA to cyclohexanecarboxylate involves 2 heterotetrameric, FAD-containing dehydrogenases: the Ch1,5CoA dehydrogenase specifically reduces Ch1,5CoA to cyclohex-1-ene-1-carboxyl-CoA (Ch1CoA), while Ch1CoA dehydrogenase converts Ch1CoA to cyclohexanecarboxylate (fig. 9a). A special property of Ch1CoA dehydrogenase is the 1,4-addition (not the common 1,2) to the diene. The recently determined crystal structure of this enzyme revealed a tight association of the substrate to the FAD cofactor and identified Asp⁹¹ as the most likely proton acceptor/donor from/to C3 of the Ch1CoA/Ch1,5CoA ring. Electron transfer from NADH ($E^{\circ} = -320$ mV) to Ch1,5CoA ($E^{\circ} = -10$ mV) by Ch1CoA dehydrogenase is an exergonic process, which could hypothetically be coupled to energy conservation via electron transferring protein (ETF)-mediated flavin-based electron bifurcation [Buckel and Thauer, 2013] (fig. 9b).

S. aciditrophicus performs the described cyclohexane fermentation also during axenic growth with crotonate, resulting in an increased value of standard Gibbs-free energy change (from -24.8 to -157 kJ/reaction). When *S. aciditrophicus* grows with cyclohexanecarboxylate syn-

trophically with a methanogen, Ch1,5CoA and Ch1CoA dehydrogenases are also involved. *S. aciditrophicus* employs *re*-citrate synthase in an unusual pathway to generate glutamate for anabolic purposes.

Metabolism-Oriented Habitat Studies

Functional Gene Markers

The state-of-the-art of exploring natural communities of hydrocarbon-degrading bacteria by means of functional gene markers is summarized by von Netzer et al. [2016]. As outlined in the above sections, culture-based physiological, proteogenomic and biochemical studies have revealed a host of novel reactions – with partly in-depth mechanistic insights – involved in anaerobic degradation of hydrocarbons. To make this knowledge useful for the functional analysis of hydrocarbon-bearing or -contaminated environments, several key genes were selected as functional markers based on the hydrocarbon specificity/range or central catabolic position of their products. These functional marker genes are: *bssA*, coding for the α -subunit of toluene-activating BSS; *nmsA*, coding for the α -subunit of naphthalene-activating (2-naphthylmethyl)succinate synthase (*bssA* and *nmsA* belong to the group of genes coding for alkyl-/arylalkylsuccinate synthases); *bcrA/bcrC/bzdN*, coding for subunits of ATP-dependent class I BCRs; *bamB*, coding for the β -subunit of ATP-independent class II BCR; *ncr*, coding for 2-NCR; *bamA*, coding for ring-cleaving 6-oxocyclohex-1-ene-1-carboxyl-CoA hydrolase.

Most widely used in microbial molecular ecology are the gene markers for alkyl-/arylalkylsuccinate synthases, since a variety of aromatic and aliphatic hydrocarbons are activated by this group of enzymes (fig. 1b) and their conserved motifs facilitate primer design. Continued optimization has provided a suite of primers covering either alkyl-/arylalkylsuccinate synthases in general (reverse primer 8543r) or being more specific, e.g. *bssA* sensu strictu (forward primer 7772f; fig. 10). Screening methods for key genes range from simple PCR, terminal restriction fragment length polymorphism and qPCR to next-generation sequencing of the amplicons. Applying these assays to samples from various aromatic hydrocarbon-degrading enrichment cultures and contaminated natural systems allowed for a highly resolved phylogenetic differentiation of organisms bearing alkyl-/arylalkylsuccinate synthases (fig. 10). This included the detection of several novel lineages within

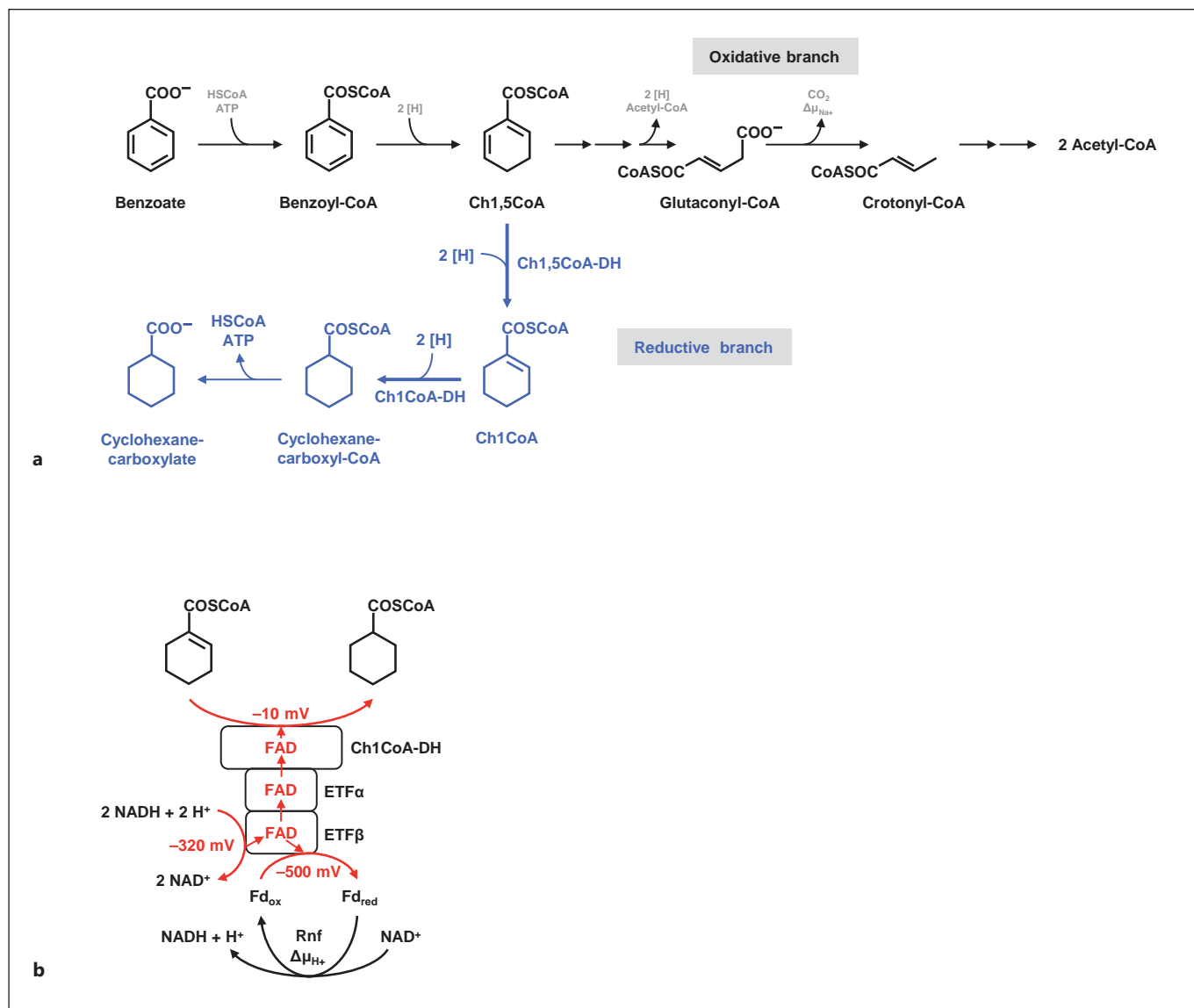


Fig. 9. Fermentative utilization of benzoate by *S. aciditrophicus*. **a** Coupling of anaerobic benzoate oxidation with the reduction of the dienoyl-CoA intermediate to cyclohexanecarboxylate as fermentation product. **b** Hypothetical (experimentally not yet prov-

en) energy conservation by coupling exergonic Ch1CoA reduction to an electron transferring protein (ETF)-mediated flavin-based electron bifurcation process. Modified from Boll et al. [2016b].

the *Rhodocyclaceae*, *Desulfobulbaceae* and *Peptococcaeae* that had hitherto not been associated with the anaerobic degradation of aromatic hydrocarbons (orange branches in fig. 10). Also for central key enzymes in the breakdown of aromatic compounds, such as dearomatizing aryl-coA reductases or aromatic ring-cleaving hydrolases, targeted detection assays are now at hand. Thus, evidence for the existence of site-specific populations of aromatic hydrocarbon-degrading bacteria has

emerged, which appear to be primarily selected by the nature of the contamination as well as the availability of electron acceptors. This has implications for the monitoring of contaminated sites and also for the design of advanced bioremediation strategies.

Stable Isotope Probing

Stable isotope probing (SIP) has matured into a powerful and widely used approach to assess metabolic activities

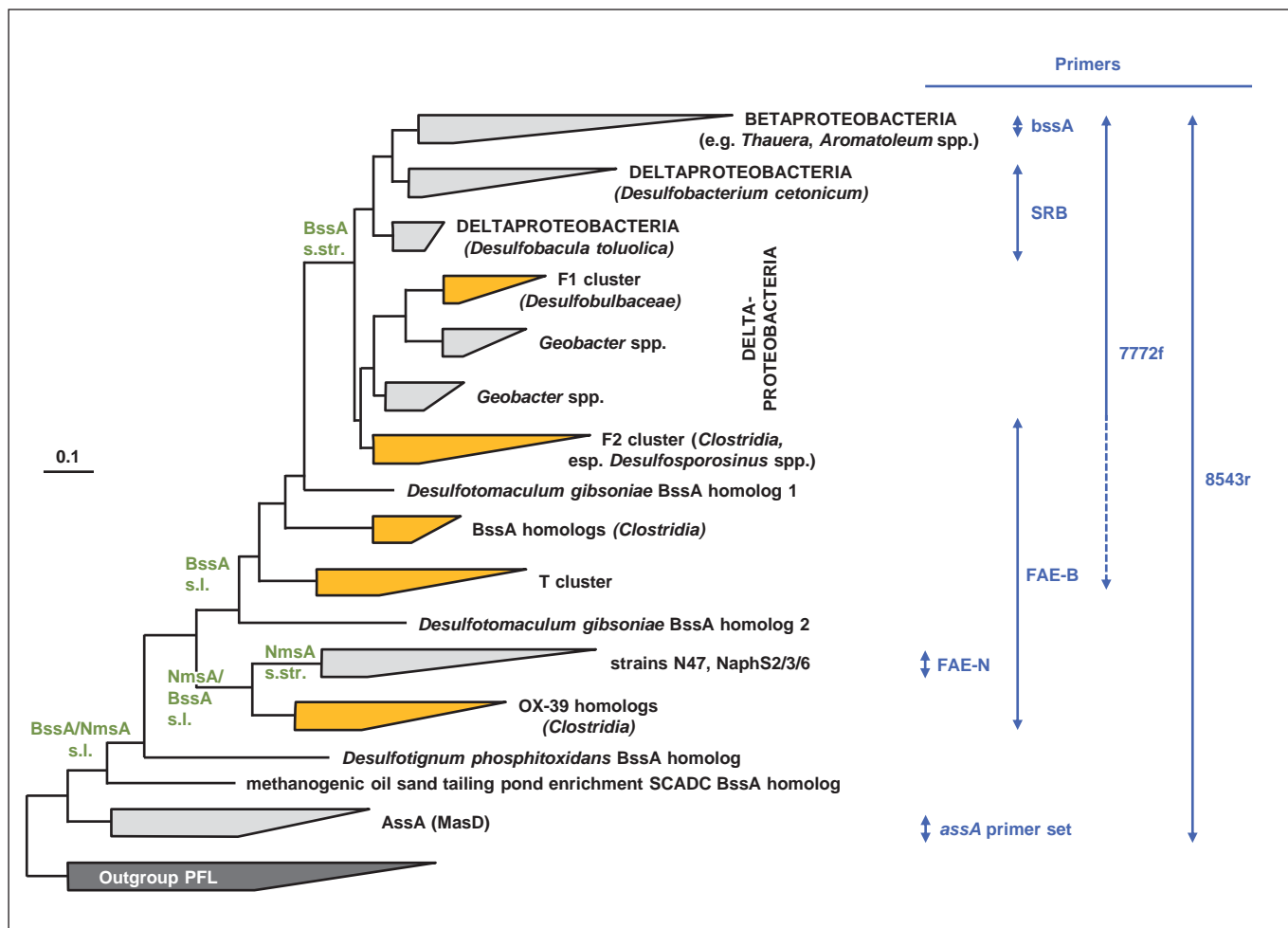


Fig. 10. Phylogeny of genes encoding alkyl-/arylalkylsuccinate synthases based on sequences retrieved from pure cultures and environmental samples. Differential gene hunting required continued optimization of primers (blue) to discriminate substrate specificity of alkyl-/arylalkylsuccinate synthases (green). Newly discovered clades of bacteria bearing alkyl-/arylalkylsuccinate syn-

thases are highlighted in orange. AssA/MasD = (1-Methylalkyl) succinate synthase; BssA = benzylsuccinate synthase; NmsA = (2-naphthylmethyl)succinate synthase; PFL = pyruvate formate-lyase; s.str. = sensu stricto; s.l. = sensu lato. Redrawn from von Netzer et al. [2016].

in conjunction with its microbial players in complex environmental samples. The latest developments and applications of SIP on the level of DNA/RNA, phospholipid fatty acids, proteins and single cells (nanoSIMS) in the field of anaerobic degradation of hydrocarbons are compiled by Vogt et al. [2016]. Complementary to the studies with functional gene markers described above, SIP approaches have contributed significantly to unraveling the diversity of anaerobic hydrocarbon degraders beyond the limits of pure cultures, in particular also in case of syntrophic processes. Key to the SIP approaches is the addition of stable isotope-labeled substrates (e.g. ^{13}C or ^{15}N label) to cultures

or in situ samples, and to trace the assimilation of the heavy label in the total biomass or specific marker biomolecules. This allows not only to identify active (with respect to the studied substrate degradation) community members, but also to decipher metabolic fluxes. The various SIP approaches differ with respect to phylogenetic coverage and sensitivity, leading to disparate application potential under different experimental settings, e.g. enriched culture versus complex in situ sample (fig. 11).

DNA/RNA SIP investigations on slowly growing enrichment cultures with different electron acceptors and under syntrophic conditions indicated a broad diversity

of bacteria to be potentially involved in anaerobic degradation of benzene, toluene and isomers of xylene, i.e. all subclasses of the Proteobacteria as well as *Peptococcaceae*. In case of a *m*-xylene-degrading sulfate-reducing enrichment culture, a combination of DNA and protein SIP allowed not only assignment of the dominant ^{13}C -label-incorporating phylotype to the *Desulfobacteraceae*, but also reconstruction of the catabolic pathway on the protein level. The latter included the alkyl-/arylalkylsuccinate synthases, the β -oxidation-like reaction sequence to 3-methylbenzoyl-CoA (upper pathway), as well as enzymes involved in the lower pathway, i.e. ring reduction and cleavage. Application of SIP to anaerobic degradation of *n*-hexadecane under methanogenic conditions identified dominant phylotypes belonging to the *Syntrophaceae* and *Methanoculleus*. In case of marine *n*-alkane-degrading sulfate-reducing sediment incubations, comprehensive SIP studies revealed the involvement of members of the habitat relevant *Desulfosarcina/Desulfococcus* clade and indicated involvement of the pathway previously proposed for anaerobic degradation of *n*-hexane in *Aromatoleum* sp. strain HxN1. Sulfate-reducing enrichment cultures anaerobically degrading polycyclic aromatic hydrocarbons are apparently dominated by phylotypes related to the enriched naphthalene-degrading strain N47 (*Desulfobacteraceae*).

Stable Isotope Fractionation

Carbon and hydrogen stable isotope fractionation resulting from C–H bond cleavage during microbial degradation of hydrocarbons and its prospects for monitoring respective degradation processes in situ are summarized by Musat et al. [2016]. An intrinsic property of enzymes is the faster turnover of the lighter isotopologue of the substrate, resulting in an enrichment of the heavier isotopologue in the residual substrate pool. In case of hydrocarbons as substrates, this isotopic fractionation is moderate for carbon and significant for hydrogen. Correlation of carbon and hydrogen stable isotope fractionation by the so-called lambda value (Λ) even renders differentiation between aerobic and anaerobic degradation reactions possible (fig. 12).

The first reaction step of anaerobic ethylbenzene degradation in denitrifying *A. aromaticum* EbN1 proceeds via initial homolytic C–H bond cleavage at the methylene group prior to O_2 -independent hydroxylation yielding (*S*)-1-phenylethanol (see section ‘Ethylbenzene dehydrogenase, archetype for O_2 -independent hydroxylation’). This reaction translates in a Λ_{bulk} value of 60 ± 5 for cultures of *A. aromaticum* EbN1 anaerobically growing with

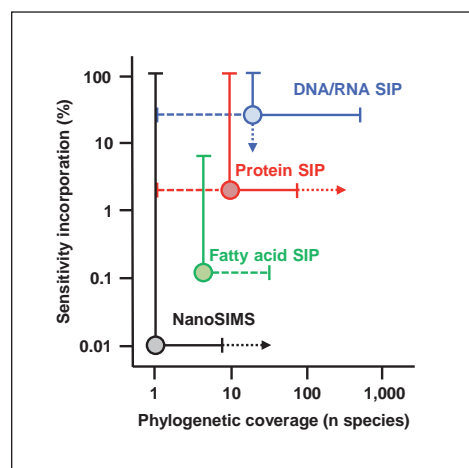


Fig. 11. SIP techniques for targeted detection and identification of metabolic activity in the environment differ with respect to sensitivity and phylogenetic coverage. Redrawn from Vogt et al. [2016].

ethylbenzene. Similar Λ_{bulk} values (40 ± 3 and 46 ± 5 , respectively) for *Georgfuchsia toluolica* G5G6 and an enrichment culture dominated by *Azoarcus* spp. implied that the same reaction principle is used for anaerobic ethylbenzene degradation in these nitrate-reducing bacteria. Notably, the lower Λ_{bulk} value of 35 ± 9 for *Pseudomonas putida* NCIB 9816 aerobically growing with ethylbenzene suggested that stable isotope fractionation could be used to distinguish between O_2 -independent and O_2 -dependent hydroxylation of ethylbenzene, i.e. between EBDH and ethylbenzene monooxygenase.

Anaerobic degradation of the gaseous hydrocarbons propane and *n*-butane has to date only been demonstrated for sulfate-reducing bacteria. Relatives of the presently sole pure culture *Desulfosarcina* sp. BuS5 (*n*-butane-degrading) were shown to occur in various marine sediments. Metabolite analysis with strain BuS5 revealed fumarate-dependent transformation of propane and *n*-butane at the subterminal carbon atom to isopropylsuccinate and (1-methylpropyl)succinate, respectively; surprisingly, activation at the terminal carbon atom to *n*-propylsuccinate was observed for *n*-propane. Λ_{bulk} values for the anaerobic degradation of propane and *n*-butane by strain BuS5, enrichment cultures and with sediment incubations ranged between approximately 5 and 10, which is markedly lower than the values given above for anaerobic hydroxylation of ethylbenzene, but still higher than for aerobic degradation of propane (3.1).

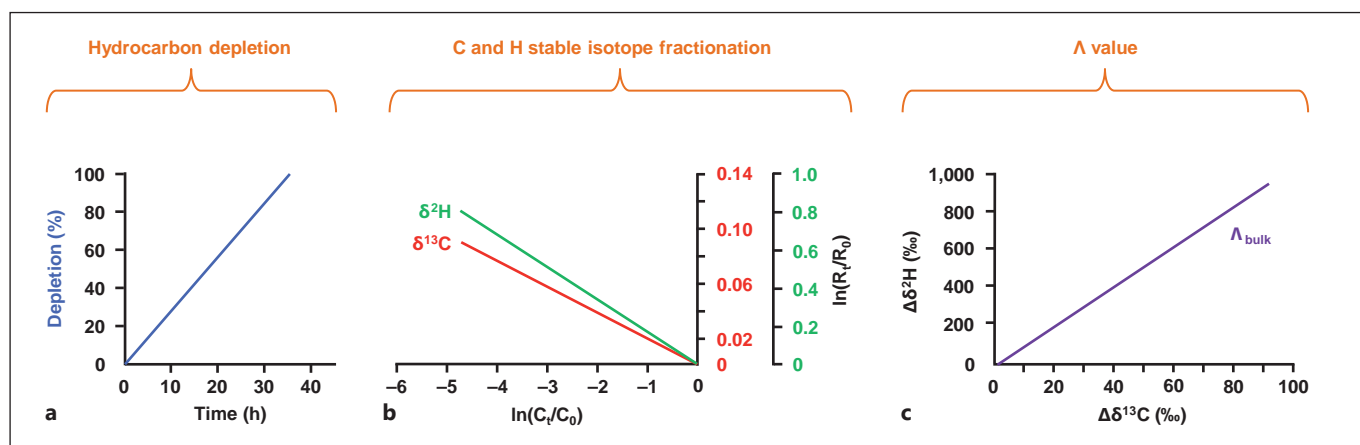


Fig. 12. Stable isotope fractionation to monitor anaerobic degradation of hydrocarbons in situ. **a** Depletion (blue) of hydrocarbon substrate during incubation of cultures or environmental sample. **b** Carbon ($\delta^{13}\text{C}$; red) and hydrogen ($\delta^2\text{H}$; green) stable isotope fractionation. **c** Correlation of the changes of carbon and hydrogen

stable isotope fractionation by the lambda value (Λ_{bulk} ; purple). $\Delta\delta^{13}\text{C}$, $\Delta\delta^2\text{H}$ and Λ_{bulk} can be used to study C–H bond cleavage during anaerobic degradation of hydrocarbons. Modified from Musat et al. [2016].

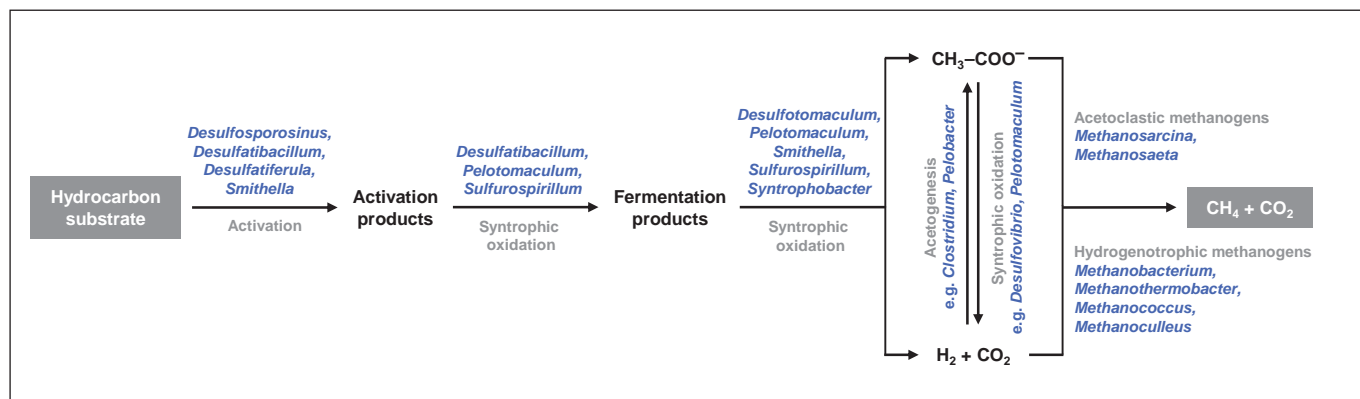


Fig. 13. Scheme of anaerobic degradation of hydrocarbons under syntrophic (methanogenic) conditions emphasizing the complexity of the involved microbial community. Modified from Jiménez et al. [2016].

Methanogenic Degradation of Hydrocarbons

Current concepts and insights into anaerobic degradation of hydrocarbons coupled to methanogenesis are summarized by Jiménez et al. [2016]. The process of methanogenic degradation of hydrocarbons prevails at sites that are depleted of electron acceptors (e.g. NO_3^- , SO_4^{2-} , Fe^{3+}), a condition typically encountered in oil reservoirs, coal deposits, groundwater aquifers and deep sediments. Due to the slowness and syntrophic nature of this process, progress in the understanding of methanogenic degradation of hydrocarbons greatly depended on integrating microbial community analysis by molecular tools with SIP and fractionation approaches.

The CH_4 production rates were found to depend on the type of hydrocarbon source provided. Essentially, the rates were highest with light oils harboring the most easily degradable hydrocarbons. Cultures enriched from Chinese oil reservoirs degraded C_{10} to C_{36} *n*-alkanes coupled to a CH_4 production rate of $76 \pm 6 \mu\text{mol/day/g oil}$. Next to *n*-alkanes, also BTEX and 2-methylnaphthalene can support methanogenesis in culture-based experiments. Carbon and hydrogen stable isotope fractionation analysis of methanogenic hydrocarbon-degrading enrichment cultures suggested co-occurrence of acetoclastic and CO_2 -reducing methanogenesis, as also evidenced from investigation of in situ samples.

Microbial diversity analysis indicated that similar types of prokaryotes are involved in methanogenic hydrocarbon-degrading communities, including Firmicutes, Proteobacteria, Bacteroidetes and Spirochaetes. Their particular role in the syntrophic process of methanogenic degradation of hydrocarbons is shown in figure 13. Essentially, members of the Clostridiales and Syntrophobacterales are assigned to the initial activation of the hydrocarbons and their conversion to the fermentation products H₂ and CO₂ on the one side and acetate on the other side. Acetate conversion to CH₄ and CO₂ involves acetoclastic methanogens (*Methanosarcina*, *Methanosae-ta*), while that of H₂/CO₂ is performed by hydrogenotrophic methanogens (e.g. *Methanobacterium*). The inter-conversion of H₂/CO₂ and acetate via acetogenesis (e.g. *Pelobacter*) and syntrophic oxidation (e.g. *Desulfovibrio*) should be considered in the overall picture.

Future Perspectives

Based on the current insights into anaerobic degradation of hydrocarbons achieved within the framework of the Priority Program 1319, a wealth of new research questions has emerged, which is detailed in the theme-focused reviews of this special topic issue [pp. 1–244]. Selected head points are listed in the following.

- The structure of BSS including bound toluene and fumarate and its substrate specificity and modeled reaction mechanism will have to be tested by analyzing mutant variants. The structural determinants for substrate recognition of the other alkyl-/arylalkylsuccinate synthase clades (Ibs, Hbs, Nms and Mas) need to be elucidated.
- The substrate-specific structural determinants and mechanisms of the EBDH-like anaerobic hydroxylases require continued research, including the analysis of mutant variants.
- The *p*-methyl group in 4-methylbenzoate facilitates stereochemical studies with synthetic isotope-labeled substrates on the dearomatization reactions; the further degradation pathway of 4-isopropylbenzoyl-CoA needs to be elucidated, and a detailed proteogenomic comparison of the *A. aromaticum* strains EbN1 and pCyN1 should be performed.
- The mechanism of generating a persistent radical in 4Hpad and other glycol radical enzymes by the respective activating enzymes and its stabilization is currently unknown and should be pursued in various model enzymes.
- The carboxylation of naphthalene needs to be studied at the enzyme level; the hypothesized carboxylation of benzene needs verification at the in vitro level. In particular the question of the ATP dependency of the reactions needs to be resolved.
- The reaction mechanism of ACH awaits definite elucidation. The nature of the sixth ligand of the tungsten atom in class II BCR and the mechanism for transferring 2 electrons from the Mo/W cofactors to the substrate require future research. Furthermore, the role of BamBCDEF components in class II BCRs and the possible involvement of electron bifurcation need to be resolved.
- The proposed anaerobic *n*-alkane degradation pathway by addition to fumarate needs to be demonstrated also on the proteogenomic and enzymatic level, amongst others to substantiate the assumed epimerization of the (1-methylalkyl)succinate intermediate. Moreover, the presence of a potential alternative pathway involving O₂-independent alkane hydroxylation needs to be confirmed.
- The differences between acetophenone, 4-hydroxyacetophenone and acetone carboxylation (also carbonylation of the latter) require further studies at the level of enzyme structures and mechanisms.
- A still unresolved question in fermentative formation of cyclohexanecarboxylate is whether and how electron bifurcation contributes to electron transfer from NADH to the enoyl-CoA.
- The successful strategy of applying functional gene markers should be complemented with nontarget, non-PCR amplification-based sequencing approaches to achieve a holistic perspective on functional capacities, diversity, abundance and distribution of intrinsic hydrocarbon degraders in the natural environment.
- SIP will continuously improve as coupled mass spectrometric devices are further advanced. Combination of the different SIP approaches according to the specific features of individual samples has to be further optimized.
- Studies on how differential (growth phase-/condition-dependent) formation of isoenzymes affects stable isotope fractionation will help interpretation of environmental data.
- Characterizing the dissemination of methanogenic degradation of hydrocarbons requires further field studies to estimate its role in the global carbon cycle. The metabolic interactions and interdependencies of the syntrophic partners need to be dissected at the systems biology level.

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