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

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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):

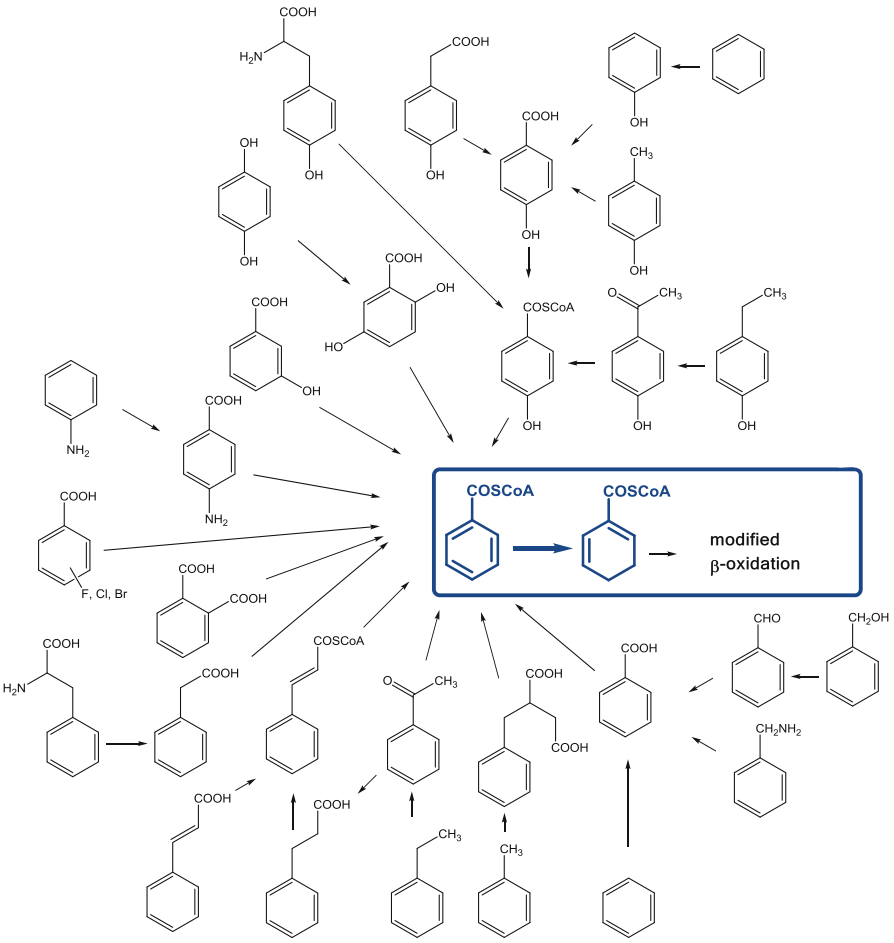

Fig. 1 Degradation of selected MAC via benzoyl-CoA. The central intermediate is dearomatized by different classes of benzoyl-CoA reductases yielding the same cyclohexa-1,5-diene-1-carboxyl-CoA (1,5-dienoyl-CoA) product

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

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

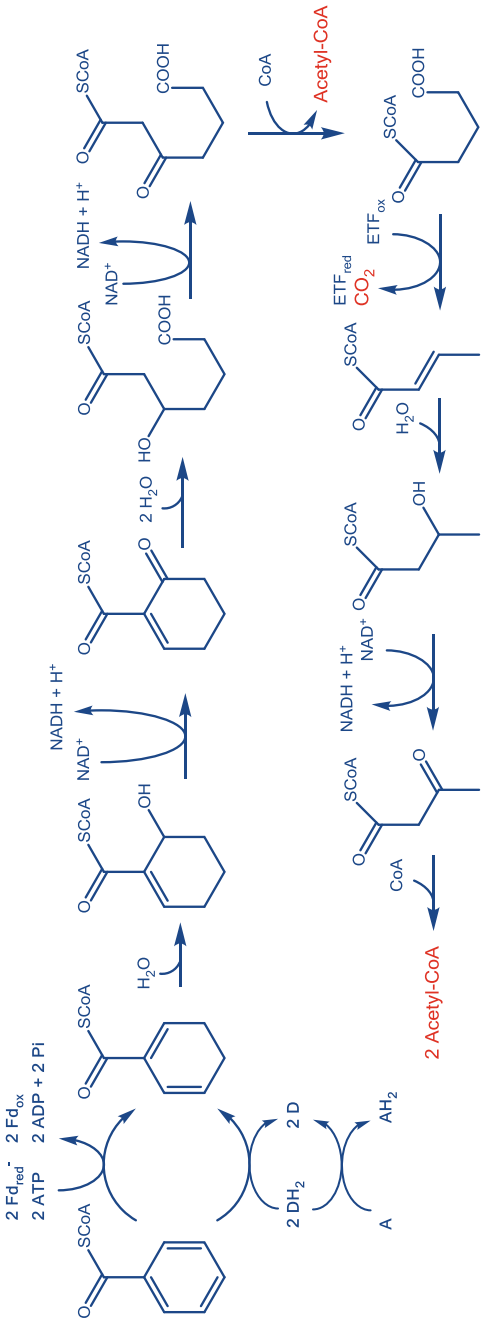

Fig. 2 Enzymatic reactions involved in the benzoyl-CoA degradation pathway. Benzoyl-CoA is reduced to 1,5-dienoyl-CoA either by ATP-dependent class I (upper reaction) or ATP-independent class II BCR (lower reaction); the latter may drive endergonic electron transfer by electron bifurcation, using an 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)

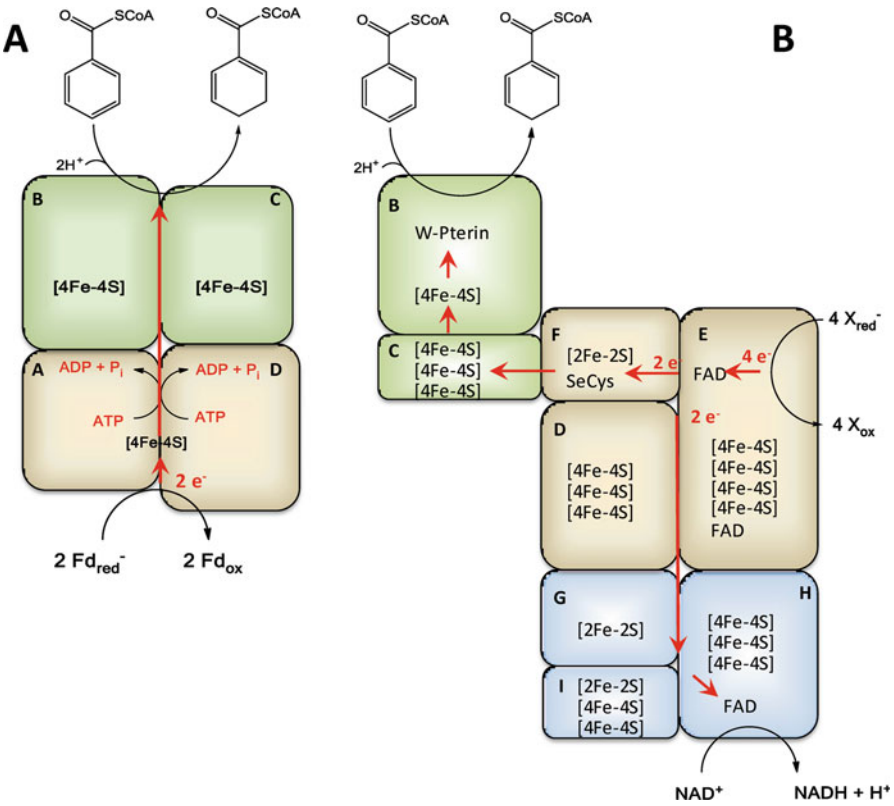

Fig. 3 Molecular architectures of class I (**a**) and class II BCRs (**b**). In class I BCR (BcrABCD subunits) endergonic electron transfer from reduced ferredoxin ($E' \approx -500$ mV) to benzoyl-CoA ($E^{\circ\prime} = -622$ mV) is driven by stoichiometric ATP hydrolysis. In class II BCR (BamBCDEFGHI), 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

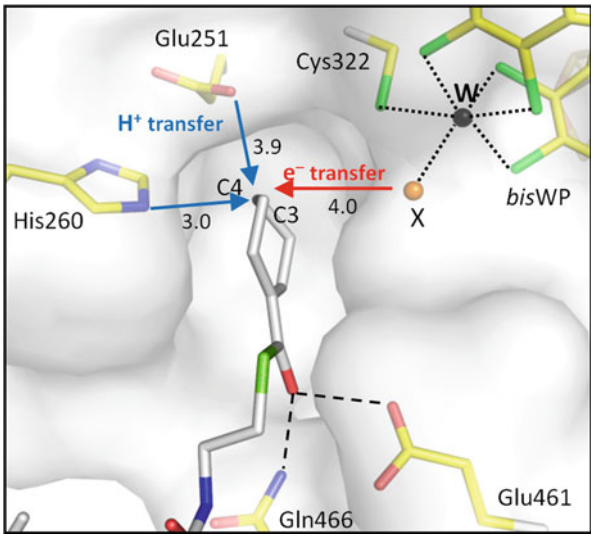

Fig. 4 Crystal structure of the active site BamB component from*G. metallireducens* class II BCR. The ring of a benzoyl-CoA analogue (a cyclic monoenoyl-CoA) is bound in an aprotic cavity. Electrons for aromatic ring reduction are transferred from the reduced W-atom, coordinated by five sulfur ligands (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).

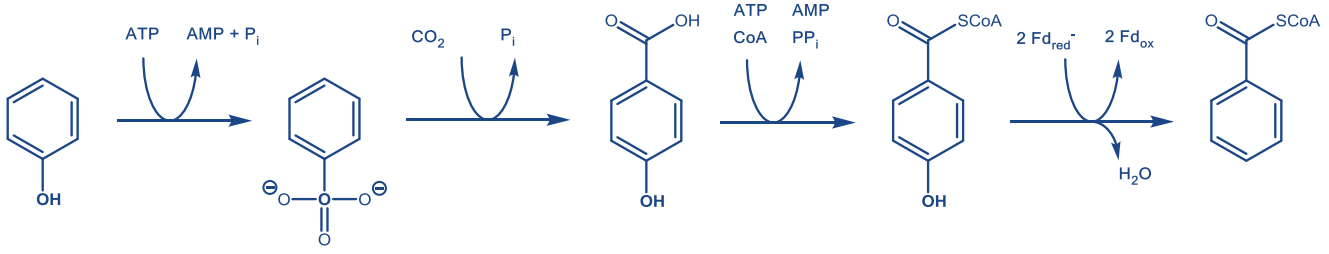

Fig. 5 Enzymatic reactions involved in the conversion of phenol to benzoyl-CoA. Phenylphosphate synthase catalyzes the first step, followed by a carboxylation reaction by phenylphosphate carboxylase. The generated product 4-hydroxybenzoate is then activated to 4-hydroxybenzoyl-CoA, which is reductively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

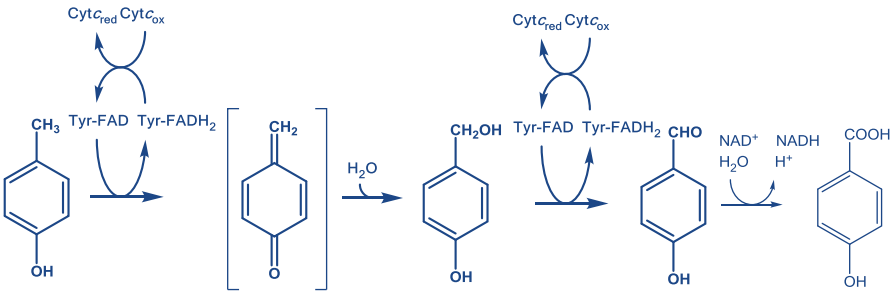

Fig. 6 Enzymatic reactions involved in the conversion of *p*-cresol to 4-hydroxybenzoate in aerobic and facultatively anaerobic bacteria. The *p*-cresol methylhydroxylase catalyzes hydride abstraction from *p*-cresol by the covalently bound FAD cofactor, yielding an enzyme-bound quinone methide; addition 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).

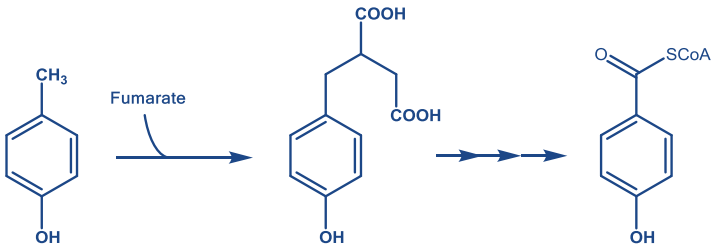

Fig. 7 Enzymatic reactions involved in the conversion of *p*-cresol to 4-hydroxybenzoyl-CoA in sulfate-reducing bacteria. The reactions leading from 4-hydroxybenzylsucinate to 4-hydroxybenzoyl-CoA are similar to those of benzylsuccinate to benzoyl-CoA involved in toluene degradation (Sect. 2.8.1)

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).

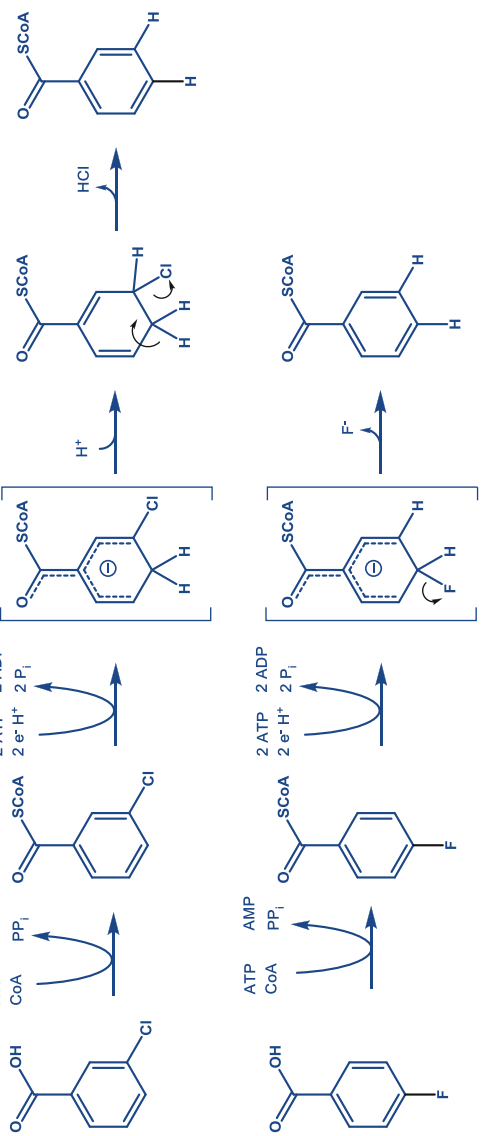

Fig. 8 Enzymatic reactions involved in the conversion of halobenzoates to benzoyl-CoA. After activation of 3-Cl- or 4-F-benzoate to the corresponding CoA-thioesters by AMP-forming CoA ligases, the 3-Cl-/4-F-benzoyl-CoA formed are converted by promiscuous class I BCR to the common, enzyme-bound anionic 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

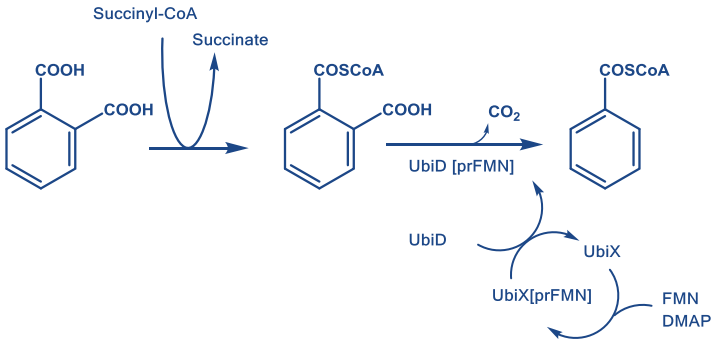

Fig. 9 Enzymatic reactions involved in the conversion of phthalate to benzoyl-CoA. The phthaloyl-CoA formed by a specific, succinyl-CoA dependent CoA transferase is decarboxylated by the oxygen-sensitive UbiD-like phthaloyl-CoA decarboxylase possibly carrying a prenylated FMN cofactor (prFMN). The prenylated 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.

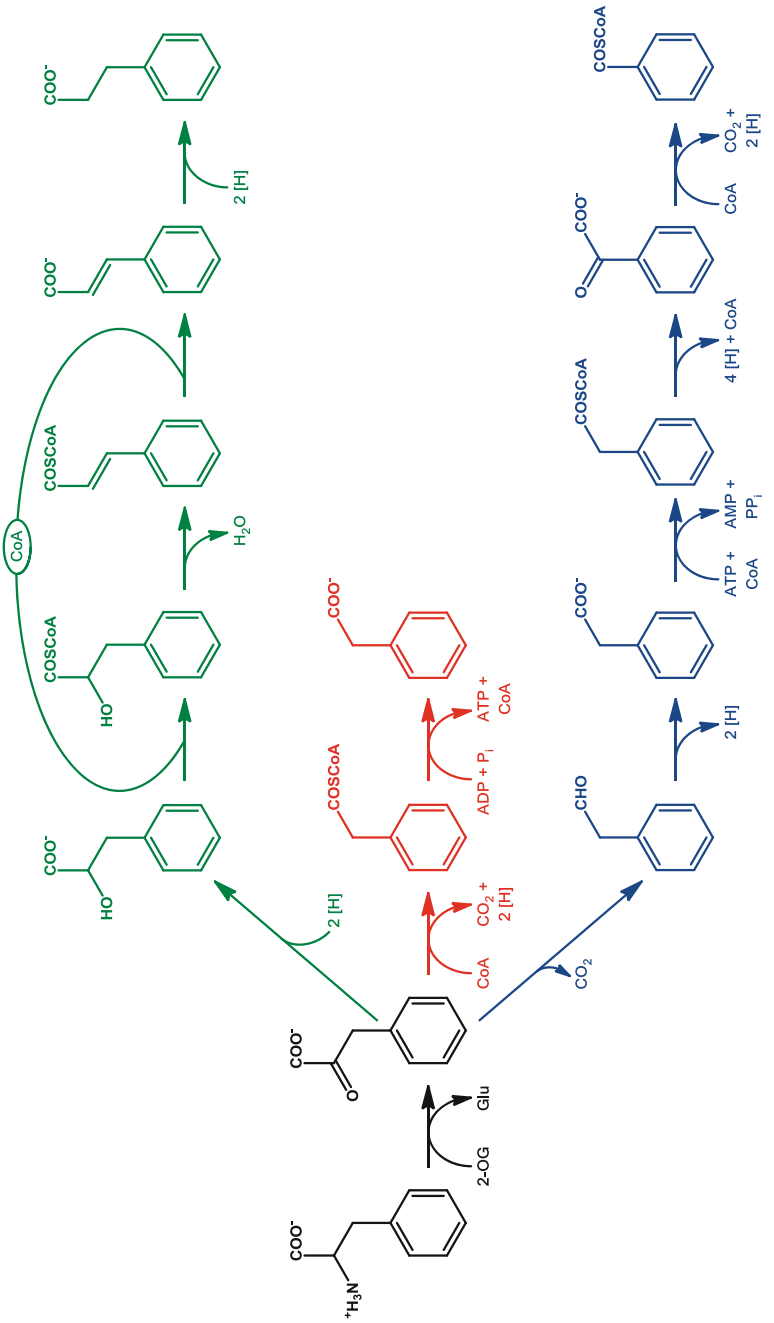

Fig. 10 Anaerobic degradation of aromatic amino acids. Reactions in green: reductive branch during fermentative transformations via Stickland fermentation; reactions in red: oxidative branch during fermentative Stickland reactions or during oxidative transformations in hyperthermophilic archaea; reactions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

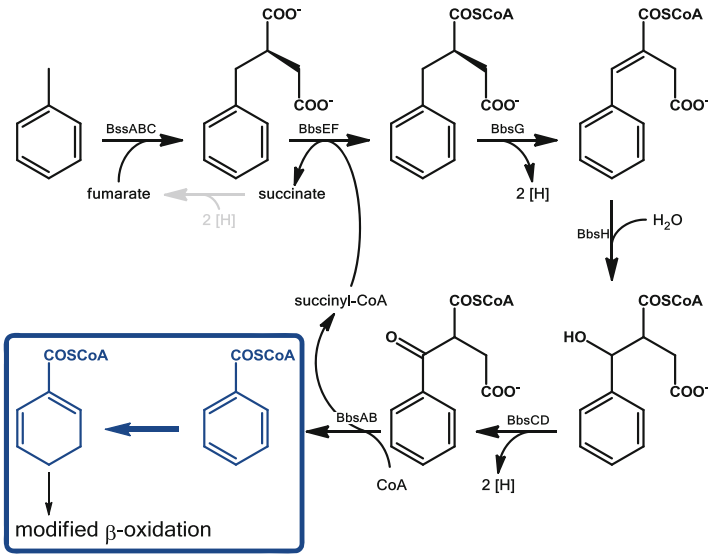

Fig. 11 Anaerobic degradation of toluene. Reactions are shown that are catalyzed by enzymes encoded by genes of the *bss* and *bbs* operons

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

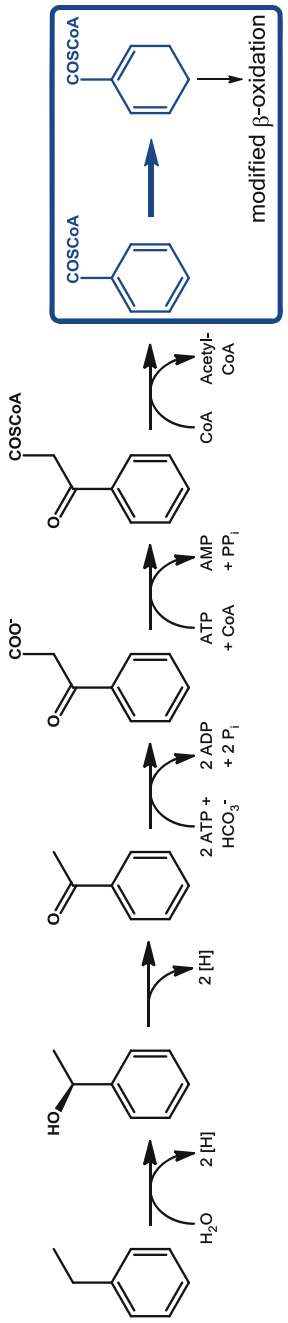

Fig. 12 Anaerobic degradation of ethylbenzene. The degradation of p-ethylphenol (4-hydroxyethylbenzene) proceeds in analogous reactions in an induced pathway to 4-hydroxybenzoyl-CoA

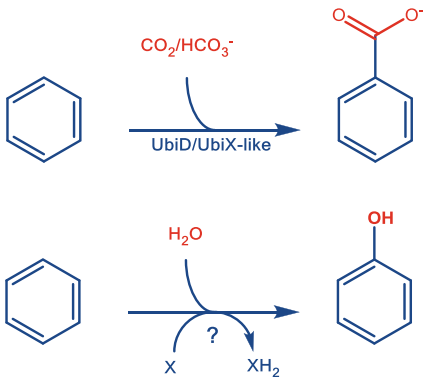

Fig. 13 Scenarios for the initial conversion of benzene without oxygen. The two alternative reactions in benzene degrading Fe(III)-reducing/methanogenic enrichment cultures (carboxylation) or *G. metallireducens* (hydroxylation) are shown. In the case of benzene carboxylation, the induction of *ubiD*-like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

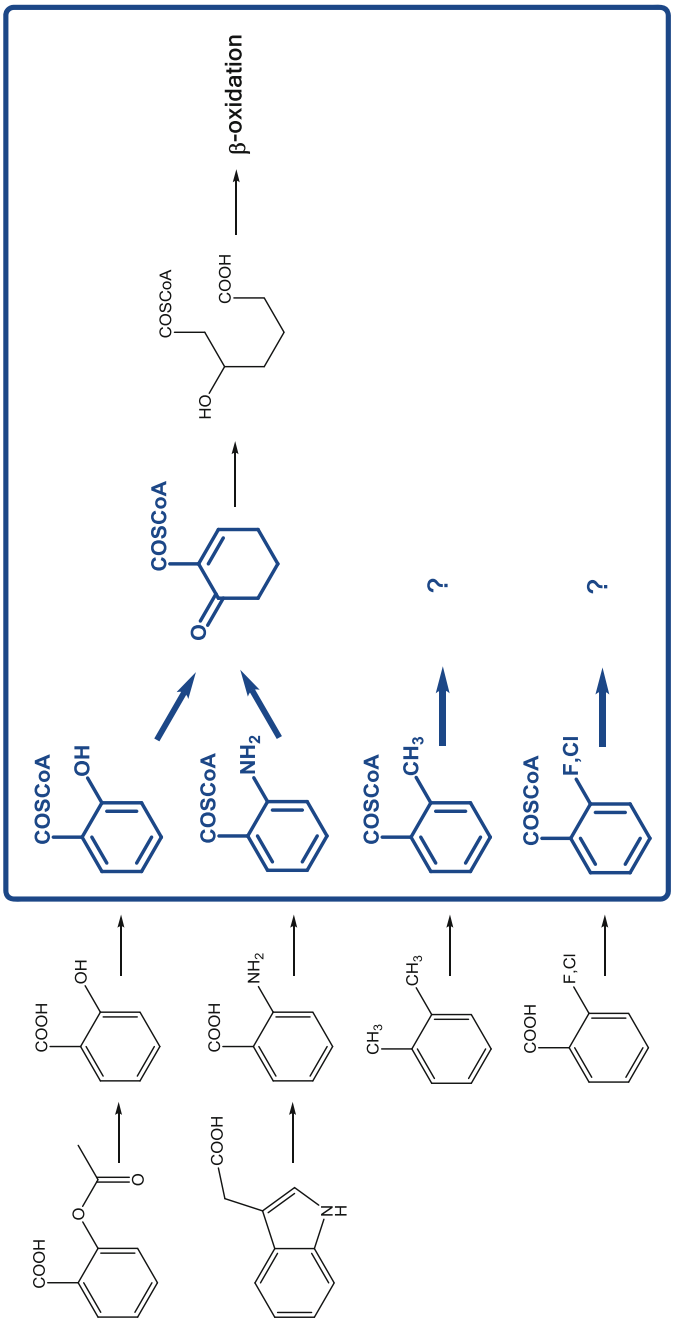

Fig. 14 Degradation of MAC via *ortho*-substituted benzoyl-CoA intermediates

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

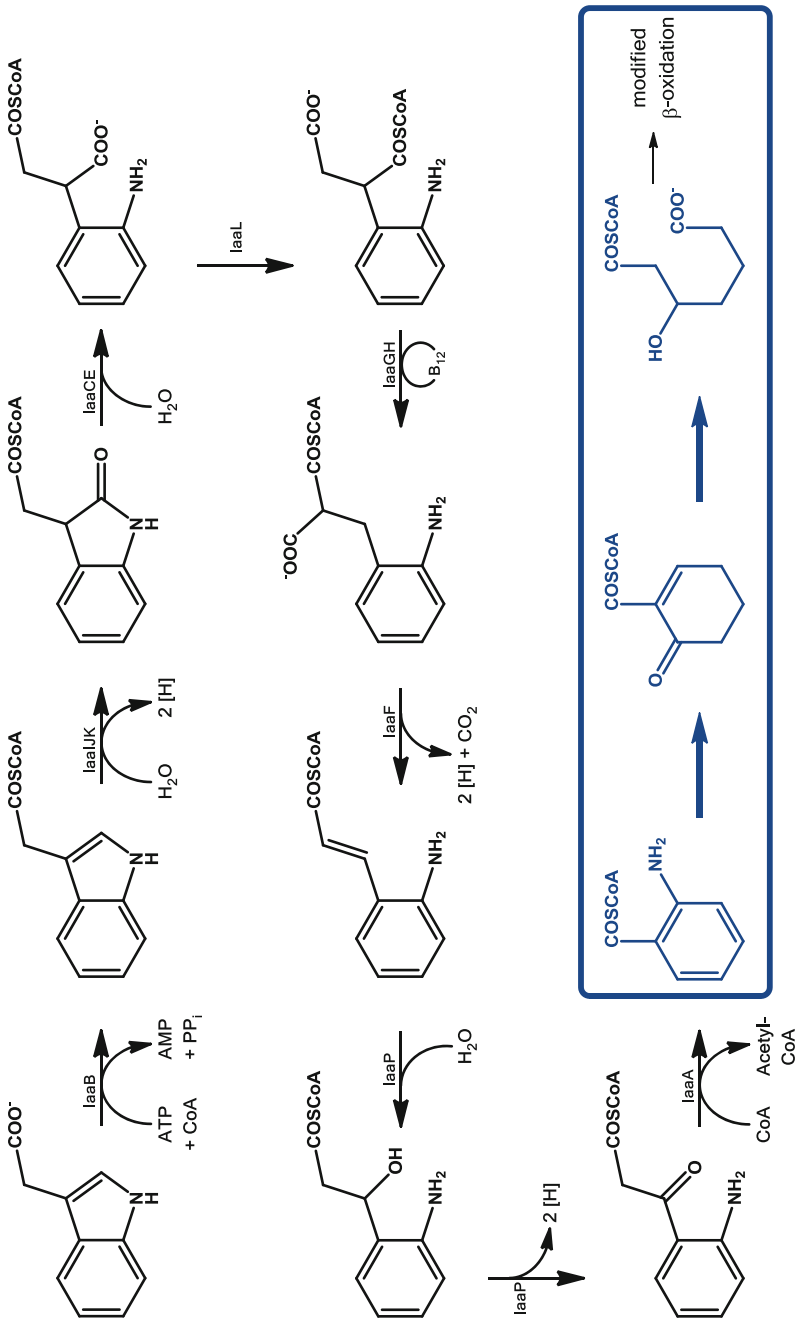

Fig. 15 Conversion of indoleacetate to 2-aminobenzoyl-CoA. The reactions catalyzed by the Iaa-gene products are indicated

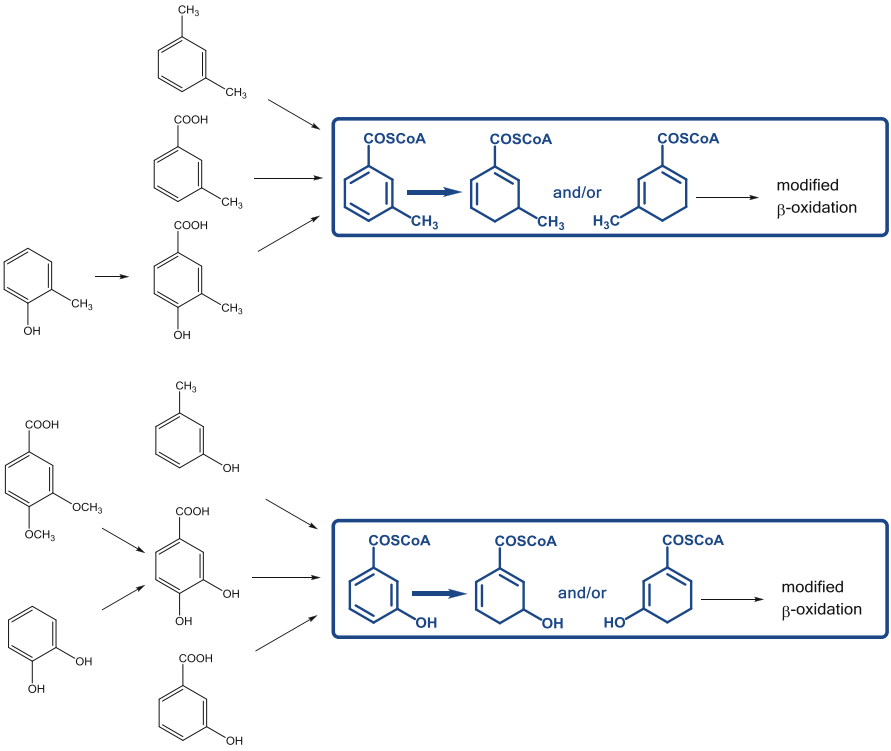

Fig. 16 Degradation of MAC via *meta*-substituted benzoyl-CoA intermediates

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

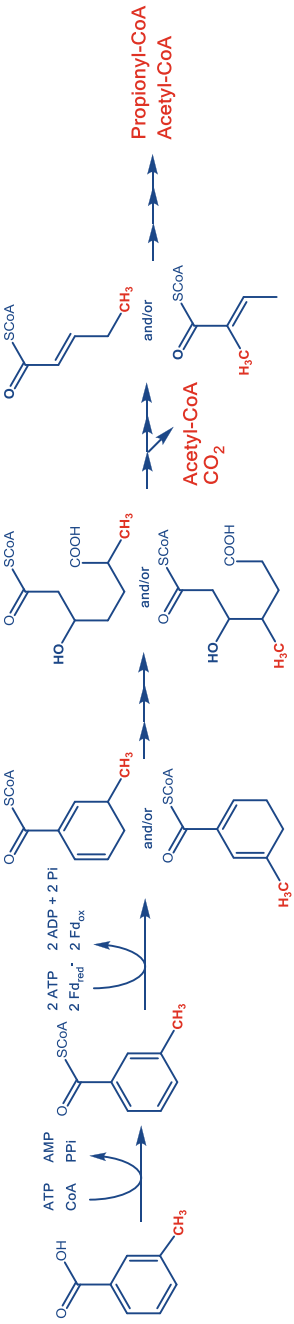

Fig. 17 Enzymatic reactions involved in the degradation of 3-methylbenzoyl-CoA to acetyl-CoA, propionyl-CoA, and CO_2. The degradation has been studied in *Azoarcus* sp. CIB

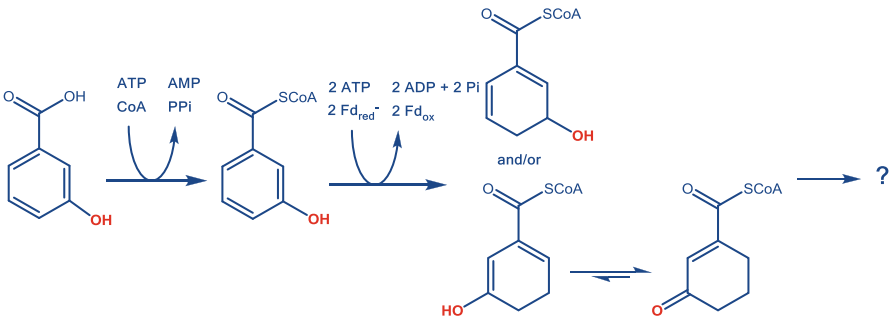

Fig. 18 Initial enzymatic reaction involved in the degradation of 3-hydroxybenzoate via 3-hydroxybenzoyl-CoA dearomatization. The degradation has been studied in *T. aromatica*; the steps involved in modified β-oxidation after ring reduction are still unknown

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

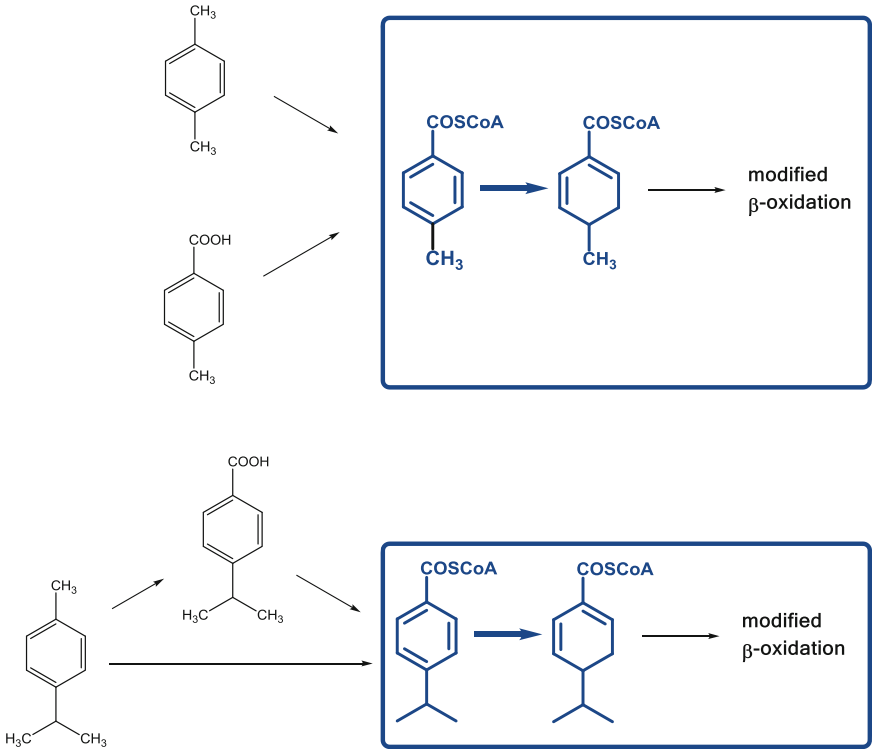

Fig. 19 Degradation of MAC via *para*-substituted benzoyl-CoA intermediates

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).

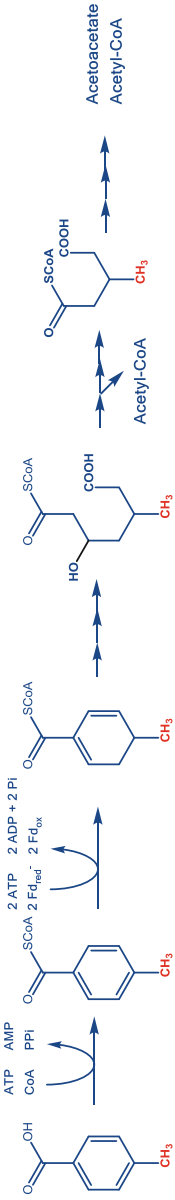

Fig. 20 Enzymatic reactions involved in the anaerobic degradation of 4-methylbenzoate. The degradation has been studied in *Magnetospirillum* pMbN1

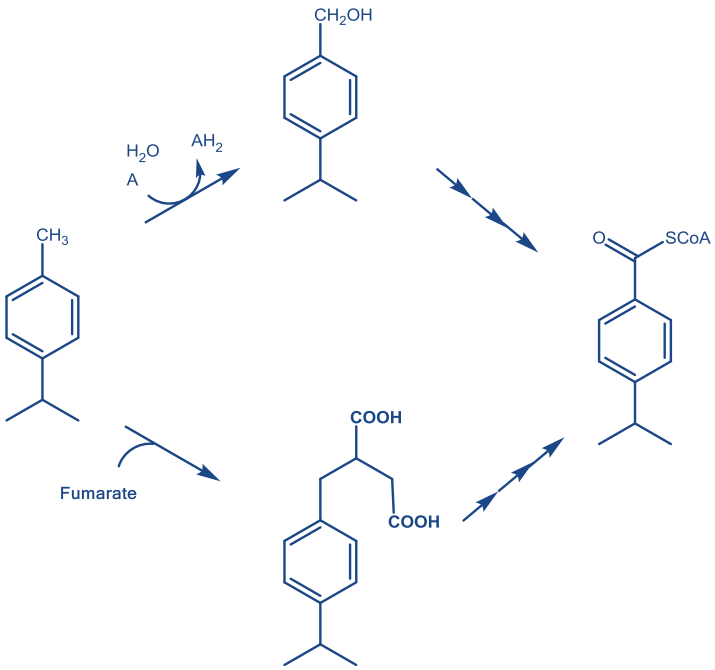

Fig. 21 Enzymatic reactions involved in the conversion of *p*-cymene to 4-isopropylbenzoyl-CoA. The upper pathway was identified in the *p*-cymene-degrading *A. aromaticum* strain pCyN1 and proceeds via anoxic hydroxylation to 4-isopropylbenzyl alcohol by a Mo-enzyme related to ethylbenzene dehydrogenase. 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).

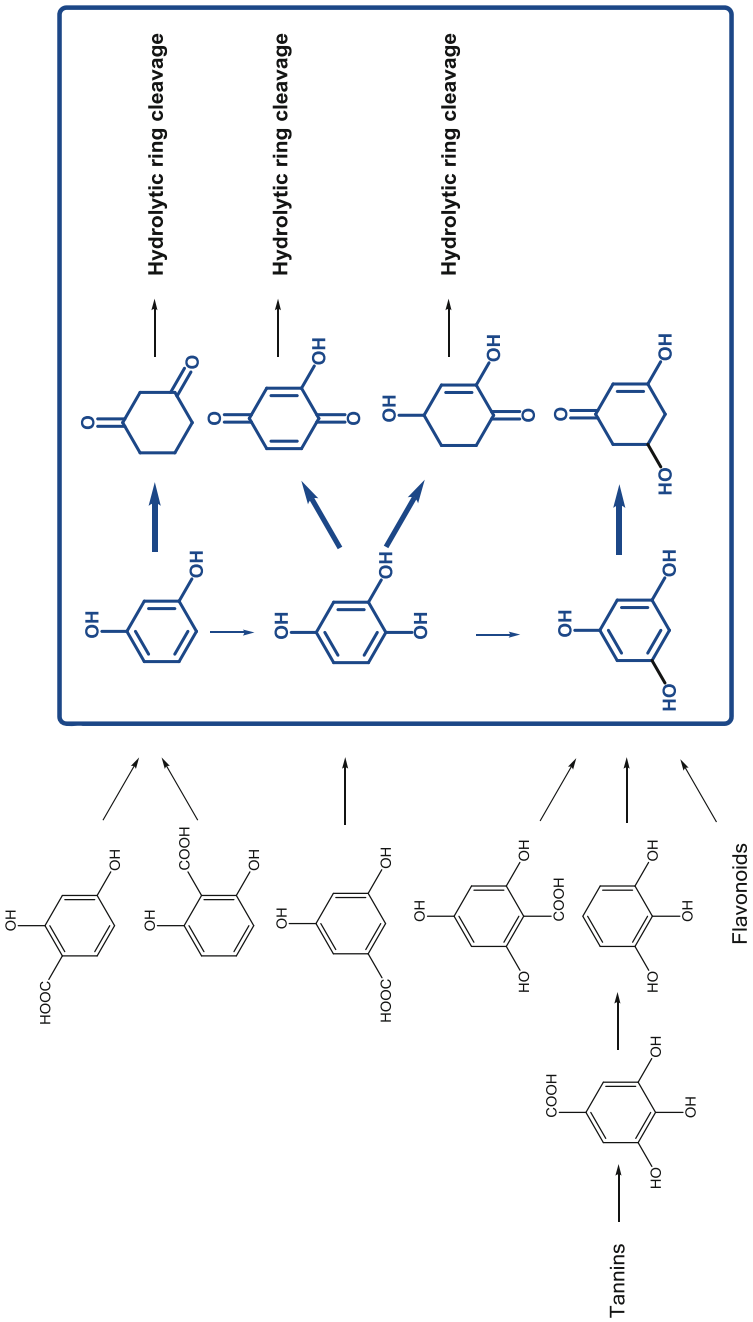

Fig. 22 Degradation of MAC via resorcinol, hydroxyhydroquinone, and phloroglucinol

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,

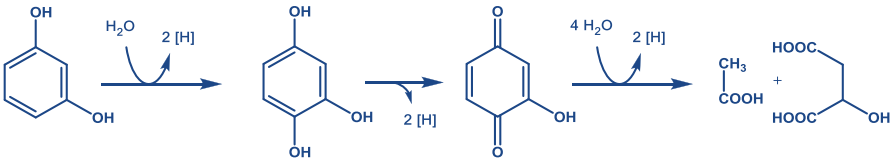

Fig. 23 Catabolism of resorcinol via the oxidative hydroxyhydroquinone degradation pathway

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.

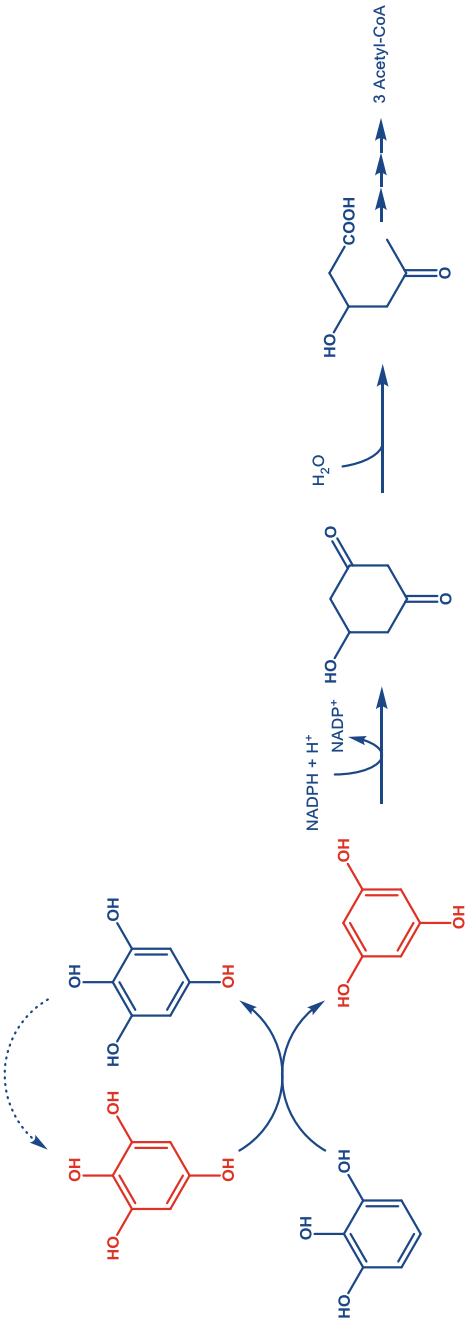

Fig. 24 Degradation of pyrogallol via the phloroglucinol degradation pathway. Pyrogallol degradation is initiated by intermolecular hydroxyl transfer catalyzed by the Mo-enzyme transhydroxylase. An enzyme-bound 1,3,4,5-tetrahydroxybenzene (in red) serves as cofactor, which after hydroxyl transfer to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