Journal of Molecular Microbiology and Biotechnology

"Special Issue - Anaerobic Hydrocarbon Degradation"

Functional gene markers for fumarate-adding and dearomatising key enzymes in anaerobic aromatic hydrocarbon degradation in terrestrial environments

Frederick von Netzer[a,d] , Kevin Kuntze[b,e], Carsten Vogt[b], Hans H. Richnow[b], Matthias Boll[c], Tillmann Lueders[a]

[a] Helmholtz Zentrum München – German Research Center for Environmental Health, Institute for Groundwater Ecology, Neuherberg, Germany

[b] UFZ - Helmholtz Centre for Environmental Research, Department of Isotope Biogeochemistry, Leipzig, Germany

[c] Institute for Biology II - Microbiology, University of Freiburg, Freiburg, Germany.

[d] present address: University of Washington, Department of Civil and Environmental Engineering, Seattle, United States

[e] present address: Isodetect GmbH Leipzig, Leipzig, Germany.

Correspondence: Tillmann Lueders, Helmholtz Zentrum München (GmbH), Institute of Groundwater Ecology, Ingolstädter Landstr. 1, 85764 Neuherberg, Germany.

E-Mail: tillmann.lueders@helmholtz-muenchen.de

Abstract

Anaerobic degradation is a key process in many environments either naturally or anthropogenically exposed to petroleum hydrocarbons. Especially for the degradation of aromatic hydrocarbons, considerable advances into the biochemistry and physiology of selected anaerobic degraders have been achieved over the last decades. However, researchers have only recently begun to explore the ecology of complex anaerobic hydrocarbon degrader communities directly in their natural habitats, as well as in complex laboratory systems using tools of molecular biology. Mainly, these approaches have been facilitated by the establishment of a suite of targeted marker gene assays, allowing for rapid and directed insights into the diversity as well as the identity of intrinsic degrader populations and degradation potentials established at hydrocarbon-impacted sites. These are based on genes encoding either peripheral or central key enzymes in aromatic compound breakdown, such as fumarate-adding benzylsuccinate synthases, dearomatising aryl-CoA reductases, or on aromatic ring-cleaving hydrolases. Here, we review recent advances in this field, explain the different detection methodologies applied, and discuss how the detection of site-specific catabolic gene markers has improved the understanding of processes at contaminated sites. Functional marker gene-based strategies may be vital for the development of a more elaborate population-based assessment and prediction of aromatic degradation potentials in hydrocarbon-impacted environments.

Introduction – anaerobic aromatic hydrocarbon degradation by microbes

Petroleum hydrocarbons are amongst the most important and ubiquitous contaminants in aquatic and terrestrial systems. Hydrocarbons typically occur as complex mixtures of aliphatic compounds (alkanes, alkenes and cycloalkanes), monoaromatic hydrocarbons (i.e. classical BTEX compounds like benzene, toluene, ethylbenzene and xylenes) and polycyclic aromatic hydrocarbons (PAHs, e.g. naphthalene, anthracene, etc.) in the environment. Petroleum hydrocarbons are generally harmful for organisms and can be very persistent in the environment [Wilkes and Schwarzbauer, 2010], especially with increasing molecular complexity. Hydrocarbons with a high molecular weight or long chain aliphatic compounds (> C_{16}) are mostly hydrophobic, solid waxes with a low mobility and bioavailability. Aromatic hydrocarbons are generally stabilised by high resonance energy levels within the delocalised π-electron system of the aromatic ring. Notwithstanding, many bacteria are in fact able to utilise aromatic hydrocarbons as an electron donors and carbon sources under a variety of redox conditions [Fuchs et al., 2011; Schink, 2002; Widdel et al., 2010]. Since aromatic compounds lack functionalization by carboxyl-, hydroxyl- or amine groups to facilitate biochemical attack [Wilkes and Schwarzbauer, 2010], degraders have evolved a number of catabolic pathways allowing them to capitalise on such substrates.

Catabolic pathways

Under oxic conditions, aromatic compounds are first activated by the addition of hydroxyl groups to destabilise the aromaticity by various mono- or dioxygenases [Pérez-Pantoja et al., 2010]. Oxygen is strictly necessary as a co-substrate for ring activation reactions. The ring systems of the activated compounds are then metabolised by various peripheral pathways into a few di-hydroxylated central intermediates. The ring structure is then opened by either a meta- or ortho-cleavage, breakdown products are then further degraded before entering central metabolism mostly as succinyl- or acetyl-CoA. Due to the superior redox potential of the H_2O/O_2 redox couple, molecular oxygen is a thermodynamically and kinetically preferred electron acceptor for biodegradation [Fuchs et al., 2011].

In contrast, when hydrocarbon loads exceed the availability of oxygen, anaerobic biodegradation of aromatic hydrocarbons becomes a pivotal process, particularly in contaminated subsurface environments (i.e. in groundwater),. Yet the microbes involved in anaerobic degradation and their biochemistry eluded scientific investigators for most of the last century. Beginning from the late 1980s, first systematic insights became apparent [Evans and Fuchs, 1988]. Similar to aerobic pathways, anaerobic aromatic compound degradation involves a number of peripheral funnelling pathways, where the crucial initial activation occurs (Fig. 1). The activated compounds are then converted to central metabolites, which are then further degraded via conserved pathways to acetyl-CoA for assimilation, complete oxidation to CO_2 , or for release to methanogenic partners [Fuchs et al., 2011].

Currently, three alternative strategies for the anaerobic activation of aromatic hydrocarbons are known [Heider, 2007; Widdel and Rabus, 2001]: (1.) The addition of a methyl or methylene group of substituted aromatic compounds across the double bond of fumarate (so-called “fumarate-addition”); (2.) hydroxylation independent of molecular oxygen in the degradation of substituted benzenes such as ethylbenzene [Johnson et al., 2001; Kniemeyer and Heider, 2001]; and (3.) carboxylation, described for the activation of non-substituted naphthalene and benzene [Abu Laban et al., 2010; Holmes et al., 2011; Meckenstock and Mouttaki, 2011] and phenanthrene [Davidova et al., 2007]. Fumarate-addition was first described for the activation of toluene catalysed by the enzyme benzylsuccinate synthase (BSS) of *Thauera aromatica* strain K172 [Biegert et al., 1996]. Benzylsuccinate is then further degraded via CoA-thioesters to benzoyl-CoA, which is the central metabolite in anaerobic aromatic hydrocarbon degradation [Fuchs et al., 2011].

Activation by fumarate-adding enzymes is not only found for aromatic compounds such as toluene, xylenes and ethylbenzene [Heider, 2007; Widdel and Rabus, 2001]. The same mechanism is also used for the activation of 2-methylnaphthalene by naphthylmethylsuccinate synthases (NMS) [Annweiler et al., 2000; Musat et al., 2009], for the degradation of cresols [Müller et al., 1999] and also for the activation of *p*-cymene in certain denitrifiers [Strijkstra et al., 2014]. Fumarate addition is also known

to be involved in the activation of non-aromatic compounds. These include short- and long-chain alkanes [Callaghan et al., 2008; Grundmann et al., 2008; Kniemeyer et al., 2007; Kropp et al., 2000; Rabus et al., 2001], cyclic alkanes [Musat et al., 2010; Rios-Hernandez et al., 2003] and also linear alkylbenzenesulfonate detergents [Lara-Martin et al., 2010]. A dual nomenclature is in use for alkane-activating enzymes: alkylsuccinate synthases, ASS [Callaghan et al., 2008] and 1-methylalkylsuccinate synthases, MAS [Grundmann et al., 2008]. In summary, the genes of fumarate-adding enzymes (FAE) can be considered as widely applicable catabolic markers for anaerobic hydrocarbon degradation in the environment [von Netzer et al., 2013].

Though not yet directly demonstrated by *in vitro* assays, numerous studies including the identification of upregulated genes encoding carboxylases from the same enzyme family as enzymes involved in decarboxylation reactions in ubiquinone biosynthesis (UbiD-like carboxylases) [Schühle and Fuchs, 2004] support the concept of carboxylation as conserved initial reaction during anaerobic degradation of benzene and naphthalene [Abu Laban et al., 2010; Bergmann et al., 2011a; Luo et al., 2014; Meckenstock and Mouttaki, 2011; Mouttaki et al., 2012]. Upon carboxylation of benzene, benzoate can be directly funnelled into the central benzoyl-CoA degradation pathway by the action of an ATP-dependent benzoate CoA-ligase. An alternative activation mechanism, anaerobic hydroxylation of benzene to phenol has been reported for *Geobacter metallireducens* [Zhang et al., 2013]. However, the enzymes or genes involved in such a reaction are still unknown.

All known peripheral pathways for anaerobic degradation of aromatic compounds converge at the level of benzoyl-CoA or benzoyl-CoA analogues as central intermediates (Fig. 1). In the so-called benzoyl-CoA degradation pathway, the aromatic ring is first desaturated by benzoyl-CoA reductases. There are two enzyme classes known for the initial dearomatization step [Boll et al., 2014; Fuchs et al., 2011]: Either the ATP-dependent benzoyl-CoA reductase BcrCBAD/BzdNOPQ/BadDEFG (class I BCR) in facultative anaerobes like *Thauera aromatica*, *Azoarcus* and *Aromatoleum* spp. and *Rhodopseudomonas palustris*, or the ATP-independent benzoyl-CoA reductase BamBCDEFGHI (class II BCR) in strict anaerobes like *Geobacter metallireducens*. Subsequently, the ring cleaving hydrolase

(BamA/BzdY/Oah) precedes successive β-oxidation-like reactions, yielding CO_2 and three molecules of acetyl-CoA which can be funnelled into central metabolism (Fig. 1).

The degradation of PAHs substantially differs from that of monocyclic aromatic compounds and has so far only been studied for the bicyclic model compound naphthalene (Fig. 1). Here the 2-naphthoic acid formed by carboxylation is thought to be activated by an ATP-dependent, 2-naphthoyl-CoA forming ligase. The subsequent degradation pathway involves three different aryl-CoA reductases, one of which is ATP-dependent [Eberlein et al., 2013a; Eberlein et al., 2013b]. The 2-naphthoyl-CoA reductase (NCR) is a member of the old yellow enzyme (OYE) family, which are flavoproteins with a flavin mononucleotide cofactor [Stott et al., 1993]. The NCR dearomatises the non-activated ring of the bicyclic 2-naphthoyl-CoA by a two-electron reduction to 5,6-dihydronaphthoyl-CoA (DHNCoA). The latter is subsequently reduced by a second OYE to 5,6,7,8-tetrahydronaphthoyl-CoA (THNCoA) [Estelmann et al., 2015]. The reduction of the activated ring of THNCoA is then accomplished by an ATP-dependent THNCoA reductase forming a hexahydronaphthoyl-CoA product [Eberlein et al., 2013b]. The genes encoding an ATP-dependent class I benzoyl-CoA reductase of the *Azoarcus*-type are present in the genome of sulphate-reducing enrichment culture N47 [Bergmann et al., 2011b] as well as the pure culture NaphS2 [DiDonato et al., 2010]. The ATP-dependence of THNCoA reduction was additionally demonstrated *in vitro* [Eberlein et al., 2013b]. The degradation of the hexahydronaphthoyl-CoA has not been demonstrated *in vitro* yet, but is likely to proceed via cyclohexane ring-containing intermediates as evidenced by metabolite analyses [Annweiler et al., 2002].

Anaerobic degraders

A wide diversity of bacterial cultures and enrichments is known to mineralise aromatic hydrocarbons under anoxic conditions using various electron acceptors. Generally, hydrocarbons can be metabolised by single organisms or by syntrophic consortia. Organisms capable of initially attacking and metabolising aromatic hydrocarbons are typically to be found within the *Rhodocyclaceae* (*Betaproteobacteria*), *Geobacteraceae*, *Desulfobacteraceae, Syntrophobacteraceae*

(*Deltaproteobacteria*) and *Peptococcaceae* (*Clostridia*) [Heider and Schühle, 2013; Weelink et al., 2010; Widdel et al., 2010]. *Betaproteobacteria* (especially *Thauera* and *Azoarcus* strains) are recognised as key organisms for BTEX degradation under nitrate-reducing conditions [Weelink et al., 2010]. The strains described so far activate toluene or xylene isomers by fumarate addition, and ethylbenzene by anaerobic hydroxylation of the side chain [Weelink et al., 2010]. Notably, *Betaproteobacteria* have also been reported as secondary degraders in two benzene-degrading enrichment cultures, apparently drawing on metabolites produced by initial degradation of benzene by bacteria within the *Peptococcaceae* [Luo et al., 2014; van der Zaan et al., 2012] (see below).

Several sulphate-reducing *Deltaproteobacteria* enriched from the terrestrial subsurface have been described to mineralise BTEX compounds [Abu Laban et al., 2015; Beller et al., 1996; Bombach et al., 2010; Bozinovski et al., 2012; Sun and Cupples, 2012; Weelink et al., 2010] or PAHs [Meckenstock and Mouttaki, 2011]. Members of the *Geobacteriaceae* (*Geobacter* spp.), also belonging to the *Deltaproteobacteria*, are generally recognised as key organisms for BTEX mineralization under iron-reducing conditions [Weelink et al., 2010]. However, also distinct members of the *Rhodocyclaceae* (*Georgfuchsia* sp.) within the *Betaproteobacteria* have recently been substantiated as respective iron-reducing degraders [Pilloni et al., 2011; Weelink et al., 2009].

Gram-positive *Peptococcaceae* also represent a major group involved in anaerobic aromatic compound degradation under various electron-acceptor conditions. Sulphate-reducing or iron-reducing *Peptococcaceae* have been described being capable of mineralising and assimilating carbon from toluene, benzene, xylenes and cresols [Abu Laban et al., 2009; Kunapuli et al., 2010; Pilloni et al., 2011; Sun et al., 2014b; Weelink et al., 2010; Winderl et al., 2010].

Additionally, several *Peptococcaceae* have been identified as primary degraders of BTEX compounds in syntrophic consortia under different electron-acceptor conditions, e.g. for benzene degradation coupled to nitrate reduction [Luo et al., 2014; van der Zaan et al., 2012], sulphate reduction [Herrmann et al., 2010; Taubert et al., 2012], iron reduction [Kunapuli et al., 2007] or for toluene

degradation under methanogenic conditions [Abu Laban et al., 2015; Fowler et al., 2014; Sun et al., 2014b].

Gene markers for anaerobic aromatic hydrocarbon degradation

The discovery and functional identification of a number of key-enzymes involved in the catabolic pathways detailed above has led to the successful establishment of several specific functional marker gene assays now widely used to detect natural populations of anaerobic aromatic compound degraders in the environment. In the following, these are reviewed for both peripheral and central reactions of anaerobic aromatic compound catabolism.

Detection assays for markers of peripheral pathways

The detection of FAE genes is arguably the most commonly used strategy for detecting anaerobic aromatic compound degraders, because of their well-defined functional affiliation and widespread occurrence [Callaghan et al., 2010; von Netzer et al., 2013; Winderl et al., 2007]. Their unique reaction mechanism – the addition of a hydrocarbon substrate radical to fumarate – is linked to conserved protein motifs, facilitating the development of specific functional marker gene PCR assays. Indeed, several primer sets targeting genes of the Bss α-subunit (partially detecting also alkylsuccinate synthases, Ass) have been developed (see Table 1). The first primers for a qualitative (and quantitative) detection of a relatively short (~130 bp) fragment of the *bssA* gene were published for denitrifying *Betaproteobacteria* by Beller et al. [2002]. Later, this assay was extended also towards recovering *bssA* of sulphate-reducing aromatic compound degraders [Beller et al., 2008]. While the primers of Washer and Edwards [2007] were specifically designed for fermenting toluene degraders within a methanogenic enrichment culture, the PCR assay of Winderl et al. [2007] (generating a ~800 bp amplicon) was the first to recover a wide diversity of catabolic gene lineages affiliated to iron- and sulphate-reducing *Deltaproteobacteria* from several contaminated aquifers. Staats et al. [2011] targeted a distinct ~500 bp fragment of the *bssA* gene using primers modified

195 from Botton et al. [2007], to detect a diversity of iron- and nitrate-reducing aromatic compound
196 degraders in a landfill-leachate plume.

197 The utility of the *bssA* gene as a functional marker for anaerobic aromatic compound degraders has
198 also been demonstrated for a wider diversity of contaminated field samples and enrichment cultures
199 from terrestrial and marine systems, along with the introduction of a number of new primers capable
200 of detecting alkylsuccinate synthase (*assA*) [Callaghan et al., 2010] and also naphthylmethylsuccinate
201 synthase (*nmsA*) genes [von Netzer et al., 2013]. Yet today, the optimization of existing FAE detection
202 assays for a more comprehensive recovery of catabolic gene diversities at contaminated sites is still
203 ongoing [von Netzer et al., 2013].

204 ### Detection assays for markers of central pathways of monocyclic aromatic compound degradation

205 Apart from FAE genes, functional markers targeting conserved enzymes of the benzoyl-CoA
206 degradation pathway have also been successfully employed to detect anaerobic mono-aromatic
207 compound degraders. These assays allow insights into the diversity and identity of degrader
208 populations even when fumarate-addition is not involved in upstream catabolism. The first primers
209 introduced for a qualitative detection of subunits of class I BCRs (*bcr/bzd*) were published by Hosoda
210 et al. [2005] and Song and Ward [2005]. However, the use of these assays was not straightforward,
211 as non-BRC gene fragments were also recovered at high frequency. To provide a more reliable PCR-
212 based approach targeting class I BCRs in environmental DNA, these assays were later modified by
213 Kuntze et al. [2011] with the design of two independent primer pairs targeting *Azoarcus*-type BCRs
214 (*Azoarcus* spp. and "*Aromatoleum aromaticum* EbN1") as well as *Thauera*-type class I BCRs (*Thauera*,
215 *Magnetospirillum* and *Rhodopseudomonas* spp.). Specific primers were also developed by Löffler et
216 al., targeting the *bamB* gene coding for the active site subunit of class II BCRs from obligate
217 anaerobes [Löffler et al., 2011].

218 The design of degenerate primer sets to amplify conserved gene fragment of the ring-cleaving
219 hydrolases of Gram-negative monoaromatic compound degraders has also been possible [Kuntze et

al., 2008; Staats et al., 2011], due to the conservation of respective *bamA/bzdY/oah* genes. Two additional assays selective for two phylogenetic subclusters of *bamA*, the "*GMT*-cluster" (targeting the genera *Geobacter, Magnetospirillum, Thauera, Rhodomicrobium*) and the "*SA*-cluster" (Gram-negative/Gram-positive sulphate-reducing degraders as well as *Synthrophus*, *Azoarcus* and *Aromatoleum* spp.) were designed later to recover a wider diversity of degraders, including also Gram-positives [Kuntze et al., 2011]. Thus in the meantime, the *bamA* gene is also established as a widely applied biomarker for anaerobic degradation of monocyclic aromatic compounds [Andrade et al., 2012; Li et al., 2012; Porter and Young, 2013, 2014; Sun et al., 2014a].

Assays for functional marker of anaerobic PAH degradation

In comparison to the well-studied anaerobic degradation pathways of monocyclic aromatic hydrocarbons, knowledge with respect to the genes/enzymes involved in anaerobic PAH degradation is still rather limited. Until very recently, the lack of any experimentally verified enzyme involved in anaerobic PAH catabolism largely hampered the development of respective functional marker gene assays. The recent discovery of the dearomatising 2-naphthoyl-CoA reductase (NCR) and its gene, *ncr*, has now opened a door for designing targeted assays for detecting anaerobic naphthalene degraders [Eberlein et al., 2013b]. NCRs are highly conserved among the established naphthalene degrading, sulphate-reducing cultures N47 and NaphS2 (amino acid sequence identities >65%, highest amino acid sequence identities to other enzymes <40%). They can therefore be readily distinguished from other related enzymes of the OYE family, which have different functions [Stott et al., 1993]. Based on these findings, a primary PCR-based assay has been developed for the targeted detection of *ncr* genes in environmental samples, which reliably detected *ncr* genes in the known sulphate-reducing, naphthalene-degrading pure cultures and enrichments: NaphS2, S3 and S6 and N47 [Morris et al., 2014]. False-positive results were not obtained with DNA from organisms harbouring most closely related genes (e.g. *Thauera aromatica* encoding a cyclohexa-1,5-dienoyl-CoA oxidase) [Thiele et al., 2008]. The new assay has been applied to degrader enrichments from

different contaminated groundwater systems, successfully identifying *ncr* genes closely related to that of the naphthalene-degrading culture N47 [Morris et al., 2014].

Though this *ncr*-targeting assay can be regarded as a pioneering tool for the monitoring of anaerobic PAH degraders, several limitations have to be taken into account. First, this *ncr*-assay was developed based on the very small number of reference sequences of *ncr* genes available, i.e. from naphthalene-degrading, sulphate-reducing *Deltaproteobacteria*. Therefore, its utility for the detection of *ncr* genes from other phylogenetic lineages stills needs to be demonstrated. Second, with the DHNCoA reductase encoded by the *dhncr* genes of the naphthalene-degraders N47 and NaphS2, a second OYE-like enzyme involved in anaerobic naphthalene degradation has recently been identified, which showed only 33-34% amino acid sequence similarity to NCR [Estelmann et al., 2015]. Future studies should test whether *dhncr* genes can also be detected by *ncr* assays. Moreover, a preliminary metagenome analysis of phenanthrene-degrading enrichment cultures has suggested the presence of multiple sequences of OYE encoding genes (M. Boll et al., unpublished data). Whether they are detected by the *ncr*-targeting assay is also still unknown.

Primer selection

The primary amino acid sequence of FAEs is more conserved towards the C-terminus [Lehtiö and Goldman, 2004]. Therefore, conserved primer motifs are more readily found towards the 3'-end of FAE genes. We currently recommend the reverse primer 8543r (Table 1), developed for more optimal performance [von Netzer et al., 2013] from previous permutations [Callaghan et al., 2010; Washer and Edwards, 2007; Winderl et al., 2007], as most suited candidate for covering a wide diversity of FAE gene lineages (Fig. 2). The selectivity of the PCR assay should thus be guided by the forward primer, and also by the annealing temperature [von Netzer et al., 2013]. For detecting a wide range of *bssA* genes *sensu stricto* (s.str., Fig. 2), the forward primer 7772f (Table 1) has been successfully used in numerous studies [Acosta-González et al., 2013; Herrmann et al., 2009; Sun et al., 2014b; Winderl et al., 2010; Winderl et al., 2007; Yagi et al., 2010]. Other f-primers are recommended to

recover the more deeply-branching *bssA* genes *sensu lato* (s.l.), i.e. the FAE-B f-primer for clostridial *bssA* and homologues, or the FAE-N f-primers for *nmsA* genes (Table 1).

The primer pair bzdNf/bzdNr, designed for amplifying a 700 bp fragment of the γ-subunit of the *Azoarcus*-type class I BCR has been successfully applied to DNA extracted from pure cultures as well contaminated sites, as well as the bcrCf/bcrCr primer pair, designed for amplifying a 800 bp fragment from the γ-subunit of the *Thauera*-type class I BCR [Fahrenfeld et al., 2014; Kuntze et al., 2011]. For class II BCRs, the developed bamBf/bamBr primers only yield a ~300 bp gene fragment of the active subunit, and several *bamB* homologs have been recovered [Löffler et al., 2011], suggesting its applicability rather on a more general level of class II BCR detection, rather than as a detailed phylogenetic marker.

For a wide range of *bamA/bzdY/oah* genes, the forward primer SP9F in combination with the reverse primer ASP1R [Kuntze et al., 2008] as well as the slightly modified combination oah_f/oan_r [Staats et al., 2011] has been successfully used in numerous studies, thus demonstrating its applicability for detecting a wide range of anaerobic monoaromatic compound degraders [Andrade et al., 2012; Higashioka et al., 2011; Li et al., 2012; Porter and Young, 2013; Sun et al., 2014a]. Two additional primer sets were designed using the same forward primer SP9F but different reverse primers ASP23R and ASP33R targeting ring opening hydrolase subclusters including Gram-positive anaerobic monoaromatic compound degraders as described above [Kuntze et al., 2011].

Screening methods

A qualitative check for the presence of potential anaerobic aromatic compound degraders within a DNA sample should always start with a simple PCR for respective catabolic genes. However, more elaborate downstream analyses of the amplicons are necessary for further details. For a phylogenetic placement of degrader lineages and a dissection of degrader diversity, cloning and sequencing of peripheral and central gene markers is well established [Callaghan et al., 2010; Kuntze et al., 2008; Kuntze et al., 2011; Porter and Young, 2013; Staats et al., 2011; Winderl et al., 2007]. However it

should be noted that amplicons generated with the often highly degenerate catabolic gene primers can be problematic in cloning [von Netzer et al., 2013; Winderl et al., 2007], and that sequence yield will be directly dependent on the specificity of the chosen primer pair for the degraders present in a given sample, and on the abundance of possibly co-amplified PCR artefacts such as primer dimers or unspecific amplicons.

Moreover, fingerprinting based on terminal restriction fragment length polymorphism (T-RFLP) analysis can be used for the rapid screening of larger numbers of FAE gene amplicons in a sequencing-independent manner [Pilloni et al., 2011]. Although T-RFLP analysis is not a strong diagnostic tool, the identity of distinct T-RFs can cautiously be elucidated via cross-referencing of fragment lengths to reference sequences digested *in silico*, possibly even based on dual-digests to increase discriminative confidence [von Netzer et al., 2013]. Similarly, denaturing gradient gel electrophoresis (DGGE) fingerprinting of *bamA* gene pools has also been applied to analyse the community structure of anaerobic degraders [Andrade et al., 2012; Li et al., 2012].

qPCR with FAE-, *bzdN*- and *bamA*- gene primers has been repeatedly employed for the quantification of anaerobic aromatic hydrocarbon degraders in environmental samples [Beller et al., 2002; Beller et al., 2008; Fahrenfeld et al., 2014; Staats et al., 2011; Sun et al., 2014a; Winderl et al., 2008]. However, compared to cloning and sequencing, qPCR reactions need to fulfil even more rigid stringency criteria, while many of the primer sets used to date are highly degenerate (Table 1). This is why quantification is often done for specific FAE sub-lineages, where less degenerate primers can be employed. qPCR detection chemistries have relied either on SYBR-Green [Beller et al., 2008; Staats et al., 2011; Sun et al., 2014a] or on lineage-specific qPCR probes [Beller et al., 2002; Winderl et al., 2008].

As a more recent development, next-generation sequencing is also increasingly applied to characterise amplicon pools of environmental functional markers. In contrast to next-generation sequencing of 16S rRNA gene amplicons, which is already well-established and many standardised

data handling pipelines are available [Caporaso et al., 2010; Cole et al., 2009; DeSantis et al., 2006; Schloss et al., 2009], next-generation sequencing of functional markers is currently still developing. While first reports of the application of next-generation sequencing strategies to aerobic hydrocarbon degradation genes [Penton et al., 2013; Wallisch et al., 2014] and also to reductive dehalogenase genes [Hug and Edwards, 2013] are published, a dedicated next-generation sequencing pipeline for peripheral or central genes in anaerobic aromatic compound degradation is currently still unpublished.

Functional marker gene-based advances in the understanding of biodegradation processes

Obviously, the notable suite of marker gene assays described above has been instrumental for the investigation of anaerobic aromatic hydrocarbon degraders in diverse laboratory cultures and directly in environmental samples. Especially in combination with isotopic labelling strategies, the first has proven of value to identify novel degraders, affiliate novel catabolic genes and to elucidate microbial interactions (e.g. syntrophy) in defined degrader assemblages. In the field, relevant insights into degrader diversity, spatial distribution of degraders and the controls of their activity in natural settings have been provided.

Catabolic marker gene-based insights into degrader cultures and enrichments

In the lab, the detectability of *Azoarcus*-related *bssA* sequence types was first demonstrated for denitrifying, toluene-degrading lab microcosms with aquifer sediment by Beller et al. [2002]. *BssA* of iron-reducing, toluene-degrading enrichments obtained from the landfill-contaminated Banisveld aquifer was affiliated to an as-of-then unidentified betaproteobacterial lineage. This was particularly interesting, as *Deltaproteobacteria* related to *Geobacter* had been previously assumed to dominate BTEX degradation at the site [Botton and Parsons, 2007]. In fact, the detected *bssA* phylotype was

only later identified to represent the novel iron-reducer *G. toluolica* [Weelink et al., 2009], now evident as key toluene degrader *in situ*.

Under sulphate reduction, *bssA* genes affiliated to members of the *Desulfobulbaceae* were detected in both toluene and xylene-degrading laboratory enrichments from the BTEX-contaminated Zeitz aquifer [Herrmann et al., 2009; Jehmlich et al., 2010]. Distinct and deeply-branching sequence types were found in xylene-degrading microcosms, related to known naphthylmethylsuccinate synthase (*nmsA*) genes and the "T-cluster" *bssA* homologues (Fig. 2) first discovered in the field [Winderl et al., 2007]. This demonstrates how the detection of catabolic gene marker in laboratory enrichments may help to affiliate previously unidentified sequence types found directly in the field to putative functions, albeit such interpretation must of course be with caution.

A novel deltaproteobacterial *bssA* sequence type, the so-called "F1-cluster" was affiliated to key toluene degraders within the *Desulfobulbaceae* by stable isotope probing (SIP) of DNA with aquifer sediments obtained from the tar oil-contaminated Flingern aquifer [Pilloni et al., 2011], thus excluding a role of presumed *Geobacteraceae* in toluene degradation *in situ*. Dominating desulfobulbal *bssA* sequence types were also identified in toluene-degrading sulphate-reducing enrichments obtained from less contaminated zones of the BTEX-contaminated Zeitz aquifer, while distinct clostridial *bssA* sequences where detected in parallel enrichments from highly contaminated zones [Kuppardt et al., 2014]. This has allowed for first insights into possible degrader niche partitioning depending on contamination levels *in situ*.

Washer and Edwards [2007] were the first to demonstrate the expression of deeply-branching *bssA* sequence types within a methanogenic, toluene-degrading enrichment culture from a contaminated Pensacola aquifer. An affiliation of this *bssA* lineage to degraders within the *Peptococcaceae* (*Clostridia*) was suggested by Winderl et al. [2010], who retrieved related "F2-cluster" *bssA* phylotypes form labelled DNA of sulfidogenic SIP incubations of tar-oil contaminated Testfeld Süd aquifer sediments dominated by *Desulosporosinus* spp.. Later, Fowler et al. [2012] substantiated the

placement of this *bssA* lineage upon catabolic analysis of a methanogenic, toluene-degrading enrichment culture from the Fort Lupton aquifer. Via RT-qPCR, Fowler et al. [2014] could even show the active expression of clostridial *bssA* mRNA in the same microcosms. Similar clostridial bssA sequences affiliated to *Desulfosporosinus* spp. were recently also detected in methanogenic toluene-degrading microcosms enriched from contaminated sludge and soil [Sun et al., 2014b], as well as in methanogenic and sulphate-reducing toluene-degrading enrichments prepared from oil sands tailing ponds [Abu Laban et al., 2015]. All of these studies highlight the paramount and previously underestimated importance of clostridial anaerobic aromatic compound degraders in contaminated terrestrial systems.

The ring-cleaving *bamA* genes were first shown to be detectable in a number of sulphate- [Kuntze et al., 2008] or nitrate-reducing aromatic compound-degrading enrichment cultures [Li et al., 2012]. They have also been found in a number of anaerobic monoaromatic hydrocarbon-degrading pure cultures which do not have a published genome yet, e.g. *Georgfuchsia toluolica* [Staats et al., 2011], *Desulfosarcina spp., Desulfobacterium anilini* and *strains of* the Gram-positive *Desulfotomaculum gibsoniae* and *D. thermobenzoicum* [Kuntze et al., 2011], in *p*-xylene-degrading enrichment cultures dominated by *Desulfosarcina ovata* [Higashioka et al., 2011], as well as in toluene-degrading denitrifying enrichments hosting *bamA* genes related to *T. chlorobenzoica* [Li et al., 2012]. In contrast, a diversity of different *bamA* genes has recently been reported for a number of sulphate-reducing, denitrifying, and methanogenic toluene-degrading enrichments obtained from various soil and sludge samples [Sun et al., 2014a]. As mentioned above, also the applicability of a new detection assay for N47-related *ncr* genes has been demonstrated for distinct degrader enrichments from contaminated groundwater [Morris et al., 2014]. In summary, functional marker gene analysis is a highly useful tool for the rapid and targeted screening of novel degrader isolates and enrichments for their specific catabolic potentials.

Catabolic marker gene-based insights into environmental systems

Directed catabolic gene approaches for anaerobic toluene degraders directly in the field were first applied for a number of tar-oil contaminated aquifers in Germany, revealing several hitherto unidentified and site-specific populations of intrinsic degraders especially at sites dominated by sulphate reduction [Winderl et al., 2007]. In a follow-up depth-resolved study conducted at the Flingern aquifer, Winderl et al. [2008] showed that degraders carrying the "F1-cluster" *bssA* genes were quantitatively enriched at the sulfidogenic lower fringe of the hydrocarbon plume, consistent with the hypothesis that degraders are limited by dispersive mixing of electron donors and acceptors *in situ,* and demonstrating the potential of quantitative catabolic marker gene assays to identify hot-spots of aromatic compound degradation in the field.

At the Vandenberg Air Force base (CA), Beller et al. [2008] used a qPCR assay designed to specifically detect *bssA* genes of sulphate-reducing and syntrophic BTEX degraders. They monitored comparative degrader abundance and dynamics in two field lanes of artificial BTX injection, one of them with additional ethanol amendment. While initial stimulation of degraders by the treatment was more pronounced for non-ethanol amended wells, higher absolute abundance of *bssA* genes was reached under simultaneous ethanol injection. This indicates that at least some anaerobic aromatic compound degraders may have been thriving also on the amended ethanol.

Callaghan et al. [2010] have investigated intrinsic *bssA* diversity in sediments from hydrocarbon contaminated Fort Lupton (CO) and Casper (WY) aquifers, revealing a limited diversity of *bssA* sequence types affiliated to the sulphate-reducing *Desulfobulbaceae* at both sites. In contrast, a surprising diversity of intrinsic *bssA* genes was demonstrated for the coal-tar contaminated South Glens Falls aquifer (NY). Besides unknown betaproteobacterial sequence types and such related to *Georgfuchsia toluolica*, clostridial *bssA* genes were also detected directly in the field [Yagi et al., 2010].

Investigating the landfill-leachate contaminated Banisveld aquifer, Staats et al. [2011] also revealed a low diversity of *bssA* sequence types, most of them related to *bssA* genes from *G. toluolica* and other *Betaproteobacteria*, to be found at elevated abundance within the plume. In fact, the iron-reducing *G. toluolica* was originally isolated from the same site [Weelink et al., 2009], thus demonstrating how catabolic gene detection assays can be used to query the relevance of specific degraders *in situ*. Similarly, Oka et al. [2011] used comparative qPCR with assays specific for *bssA* of betaproteobacterial (denitrifying) and deltaproteobacterial (sulphate-reducing or syntrophic) anaerobic hydrocarbon degraders. In different monitoring wells at a former coal gasification plant in Glassboro (NJ), hydrocarbon degraders were shown to be enriched by up to 2 orders of magnitude in contaminated wells. Recently, a novel *bssA* lineage distinct from previously known proteobacterial sequence types was revealed in sediments of a Swedish lake not polluted by aromatic compounds [Osman et al., 2014], thus emphasising that untapped catabolic potentials and degrader lineages may remain to be uncovered also in pristine habitats.

Besides earlier work on intrinsic *bssA* lineages, the first direct evidence for environmental *nmsA* gene pools has recently also been reported for the Flingern site [von Netzer et al., 2013]. Apart from hosting a toluene dominated plume, this site is also known to contain PAHs. In fact, a previous study on key metabolites has indicated the presence of naphthyl-2-methyl-succinic acid and methylnaphthyl-2-methylsuccinic acid, which would be expected upon the activation of 2- and 1-methylnaphtalene after addition to fumarate [Jobelius et al., 2010]. Thus the deeply-branching *nmsA* phylotypes may indeed represent the intrinsic methylnaphthalene degraders at the site.

Genes for central aromatic compound catabolism have also been utilised as functional markers for degraders in the field. Introduced by Song and Ward [2005] for contaminated estuarine sediments, the concept was demonstrated for a gasoline-contaminated aquifer in Jumamoto (Japan), by Hosoda et al. [2005], thus demonstrating that a substantial diversity of class I *bcrA* genes were detectable at this site. BcrA was significantly enriched in wells of high comparative contamination, evident also as distinct communities found between differentially impacted wells.

443 At the Banisveld site, the detection *bssA* genes mentioned above was for the first time combined
444 with a screening for intrinsic, ring-cleaving *bamA* genes [Staats et al., 2011]. Not unexpectedly, the
445 diversity of *bamA* genes was much larger than that of intrinsic *bssA* genes. Interestingly, the
446 abundance of markers in distinct plume compartments shown by qPCR was inverted: *bamA* was
447 relatively more abundant outside plume. An even more comprehensive assessment of BTEX-catabolic
448 genes (*bssA*, *bcrC*, *bamB* and *bamA*) was performed for DNA extracted from benzene-loaded *in situ*
449 microcosms incubated in two aquifers with high (Ruhr area) or low (Gneisenau) benzene
450 contamination in Germany dominated by either iron- or sulphate-reducing conditions, respectively
451 [Kuntze et al., 2011]. The results revealed a general consistency of key populations affiliated to
452 known degraders within the genera *Azoarcus* and *Geobacter* detected via the different assays for the
453 sites. Quantitative site monitoring of class I BCR (*bzdN*) genes of facultative anaerobes throughout
454 the plume of the Bemidji crude oil spill has also recently been reported [Fahrenfeld et al., 2014],
455 suggesting significantly increased gene abundances close to the oil spill. It must be noted, however,
456 that central catabolic genes such as *bamA* may be generally much more readily detectable in anoxic
457 habitats than specific peripheral markers of aromatic hydrocarbon degradation, simply because
458 benzoyl-CoA is a central intermediate not only in the anaerobic degradation of petrochemicals, but
459 also of humic acids, aromatic amino acids and of lignin monomers [Andrade et al., 2012; Porter and
460 Young, 2013].

461

462 ## Conclusions and outlook

463 Anaerobic aromatic compound degradation can be regarded as the key process reducing
464 hydrocarbon contamination in natural environments. For a better understanding of the microbes
465 conveying these important ecosystem services, comprehensive functional marker assays for their
466 detection, identification and quantification are a vital tool. The distinct marker strategies spanning
467 key mechanisms in anaerobic activation and degradation of aromatic compounds reviewed here,
468 allow for a straightforward and targeted tracing of degrader populations in space and time ("4D").

As detailed above, several key features of anaerobic aromatic compound degradation have emerged from the application of these detection strategies in lab and field. First, the importance of several novel or previously unrecognised degrader lineages especially within the *Rhodocyclaceae*, *Desulfobulbaceae* and *Peptococcaceae* has been substantiated for numerous terrestrial systems, often hosting novel catabolic gene phylotypes. Second, clear patterns of the site-specific degrader populations have become apparent, driven by a selection of both electron acceptors as well as the nature of the contamination. Third, the role of syntrophy is now recognised as a key trait in many degrader enrichments, not only under methanogenic conditions, where primary aromatic compound degradation by fermenters would be expected, but also under other anaerobic respiration modes. Potentially, this represents an ecological stabilisation of degrader assemblages under fluctuating availability of electron donors or acceptors.

It is now relevant to consider how these advances can become apparent on the level of contaminated site monitoring or bioremediation strategies. Here, functional marker gene-based approaches are still awaiting a more routine implementation. Nevertheless, a number of recent respective advances should be mentioned. Already Kazy et al. [2010] indicated a significant correlation between anaerobic toluene degradation rates and qPCR *bssA* counts in anaerobic microcosms prepared with material from a hydrocarbon contaminated aquifer. More recently, toluene degradation activity stimulated by the addition of nitrate was quantitatively monitored via *bssA* transcript-to-gene ratios across a contaminant plume directly in the field [Brow et al., 2013]. Both represent relevant advances towards a future implementation of marker gene-or transcript-based prediction of biodegradation rates in complex natural systems. Here, the observed substrate-specificity of especially the peripheral markers may be vital to functionally and quantitatively interpret field data [Acosta-González et al., 2013; Wöhlbrand et al., 2013].

To develop concepts of population-based management, a combination with other complementary strategies to assess biodegradation may become necessary, such as metabolite detection [Callaghan, 2013], the application of isotopic pathway identification [Kümmel et al., 2013], and of course high-

495 throughput metagenomics and transcriptomics. Although the application of the latter has not been
496 reported to date specifically for contaminated aquifers, respective reports for other hydrocarbon-
497 impacted systems are available [An et al., 2013; Kimes et al., 2013; Luo et al., 2014; Tan et al., 2015].
498 Such non-target, non-PCR amplification based sequencing approaches have the potential to further
499 enhance our perspective of anaerobic hydrocarbon degrader microbiomes. Only with a clear
500 understanding of the diversity, abundance and distribution of intrinsic degraders, it will become
501 possible to better understand their ecology and to possibly manipulate their activity in complex
502 contaminated systems.

503

504 Acknolwedgements

505 The authors wish to thank the Deutsche Forschungsgemeinschaft (DFG) for generous support of their
506 work within the Priority Programme “Biological transformations of hydrocarbons in the absence of
507 oxygen” (SPP-1319, grants LU 1188/4-1 & -2, BO 1565/9-1 & -2, RI 903/4-1 & 2). Furthermore, we
508 acknowledge the Helmholtz Gemeinschaft for general support.

509

510 References

511 Abu Laban N, Dao A, Foght J: DNA stable-isotope probing of oil sands tailings pond enrichment
512 cultures reveals different key players for toluene degradation under methanogenic and
513 sulfidogenic conditions. FEMS Microbiol Ecol 2015;91:doi: 10.1093/femsec/fiv1039.

514 Abu Laban N, Selesi D, Jobelius C, Meckenstock RU: Anaerobic benzene degradation by Gram-positive
515 sulfate-reducing bacteria. FEMS Microbiol Ecol 2009;68:300-311.

516 Abu Laban N, Selesi D, Rattei T, Tischler P, Meckenstock RU: Identification of enzymes involved in
517 anaerobic benzene degradation by a strictly anaerobic iron-reducing enrichment culture.
518 Environ Microbiol 2010;12:2783-2796.

519 Acosta-González A, Rosselló-Móra R, Marqués S: Diversity of benzylsuccinate synthase-like (*bssA*)
520 genes in hydrocarbon-polluted marine sediments suggests substrate-dependent clustering.
521 Appl Environ Microbiol 2013;79:3667-3676.

522 An D, Caffrey SM, Soh J, Agrawal A, Brown D, Budwill K, Dong X, Dunfield PF, Foght J, Gieg LM, Hallam
523 SJ, Hanson NW, He Z, Jack TR, Klassen J, Konwar KM, Kuatsjah E, Li C, Larter S, Leopatra V,

524 Nesbø CL, Oldenburg T, Pagé AP, Ramos-Padron E, Rochman FF, Saidi-Mehrabad A, Sensen CW,
525 Sipahimalani P, Song YC, Wilson S, Wolbring G, Wong M-L, Voordouw G: Metagenomics of
526 hydrocarbon resource environments indicates aerobic taxa and genes to be unexpectedly
527 common. Environ Sci Technol 2013;47:10708-10717.

528 Andrade L, Leite D, Ferreira E, Ferreira L, Paula G, Maguire M, Hubert C, Peixoto R, Domingues R,
529 Rosado A: Microbial diversity and anaerobic hydrocarbon degradation potential in an oil-
530 contaminated mangrove sediment. BMC Microbiol 2012;12:186.

531 Annweiler E, Materna A, Safinowski M, Kappler A, Richnow HH, Michaelis W, Meckenstock RU:
532 Anaerobic degradation of 2-methylnaphthalene by a sulfate-reducing enrichment culture. Appl
533 Environ Microbiol 2000;66:5329-5333.

534 Annweiler E, Michaelis W, Meckenstock RU: Identical ring cleavage products during anaerobic
535 degradation of naphthalene, 2-methylnaphthalene, and tetralin indicate a new metabolic
536 pathway. Appl Environ Microbiol 2002;68:852-858.

537 Beller HR, Kane SR, Legler TC, Alvarez PJ: A real-time polymerase chain reaction method for
538 monitoring anaerobic, hydrocarbon-degrading bacteria based on a catabolic gene. Environ Sci
539 Technol 2002;36:3977-3984.

540 Beller HR, Kane SR, Legler TC, McKelvie JR, Sherwood Lollar B, Pearson F, Balser L, Mackay DM:
541 Comparative assessments of benzene, toluene, and xylene natural attenuation by quantitative
542 polymerase chain reaction analysis of a catabolic gene, signature metabolites, and compound-
543 specific isotope analysis. Environ Sci Technol 2008;42:6065-6072.

544 Beller HR, Spormann AM, Sharma PK, Cole JR, Reinhard M: Isolation and characterization of a novel
545 toluene-degrading, sulfate-reducing bacterium. Appl Environ Microbiol 1996;62:1188-1196.

546 Bergmann F, Selesi D, Meckenstock R: Identification of new enzymes potentially involved in
547 anaerobic naphthalene degradation by the sulfate-reducing enrichment culture N47. Arch
548 Microbiol 2011a;193:241-250.

549 Bergmann F, Selesi D, Weinmaier T, Tischler P, Rattei T, Meckenstock RU: Genomic insights into the
550 metabolic potential of the polycyclic aromatic hydrocarbon degrading sulfate-reducing
551 Deltaproteobacterium N47. Environ Microbiol 2011b;13:1125-1137.

552 Biegert T, Fuchs G, Heider J: Evidence that anaerobic oxidation of toluene in the denitrifying
553 bacterium *Thauera aromatica* is initiated by formation of benzylsuccinate from toluene and
554 fumarate. Eur J Biochem 1996;238:661-668.

555 Boll M, Löffler C, Morris BEL, Kung JW: Anaerobic degradation of homocyclic aromatic compounds via
556 arylcarboxyl-coenzyme A esters: organisms, strategies and key enzymes. Environ Microbiol
557 2014;16:612-627.

558 Bombach P, Richnow H, Kästner M, Fischer A: Current approaches for the assessment of in situ
559 biodegradation. Appl Microbiol Biotechnol 2010;86:839-852.

560 Botton S, Parsons J: Degradation of BTX by dissimilatory iron-reducing cultures. Biodegradation
561 2007;18:371-381.

562 Botton S, van Harmelen M, Braster M, Parsons JR, Röling WFM: Dominance of *Geobacteraceae* in
563 BTX-degrading enrichments from an iron-reducing aquifer. FEMS Microbiol Ecol 2007;62:118-
564 130.

565 Bozinovski D, Herrmann S, Richnow H-H, von Bergen M, Seifert J, Vogt C: Functional analysis of an
566 anaerobic m-xylene-degrading enrichment culture using protein-based stable isotope probing.
567 FEMS Microbiol Ecol 2012;81:134-144.

568 Brow CN, O'Brien Johnson R, Johnson RL, Simon HM: Assessment of anaerobic toluene
569 biodegradation activity by *bssA* transcript/gene ratios. Appl Environ Microbiol 2013;79:5338-
570 5344.

571 Callaghan AV: Enzymes involved in the anaerobic oxidation of n-alkanes: from methane to long-chain
572 paraffins. Front Microbiol 2013;4:doi: 10.3389/fmicb.2013.00089.

573 Callaghan AV, Davidova IA, Savage-Ashlock K, Parisi VA, Gieg LM, Suflita JM, Kukor JJ, Wawrik B:
574 Diversity of benzyl- and alkylsuccinate synthase genes in hydrocarbon-impacted environments
575 and enrichment cultures. Environ Sci Technol 2010;44:7287-7294.

576 Callaghan AV, Wawrik B, Ní Chadhain SM, Young LY, Zylstra GJ: Anaerobic alkane-degrading strain AK-
577 01 contains two alkylsuccinate synthase genes. Biochem Biophys Res Commun 2008;366:142-
578 148.

579 Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena AG,
580 Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA,
581 McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA,
582 Widmann J, Yatsunenko T, Zaneveld J, Knight R: QIIME allows analysis of high-throughput
583 community sequencing data. Nat Meth 2010;7:335-336.

584 Cole JR, Wang Q, Cardenas E, Fish J, Chai B, Farris RJ, Kulam-Syed-Mohideen AS, McGarrell DM,
585 Marsh T, Garrity GM, Tiedje JM: The Ribosomal Database Project: improved alignments and
586 new tools for rRNA analysis. Nucleic Acids Res 2009;37:D141-D145.

587 Davidova IA, Gieg LM, Duncan KE, Suflita JM: Anaerobic phenanthrene mineralization by a
588 carboxylating sulfate-reducing bacterial enrichment. ISME J 2007;1:436-442.

589 DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D, Hu P, Andersen
590 GL: Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with
591 ARB. Appl Environ Microbiol 2006;72:5069-5072.

592 DiDonato RJ, Young ND, Butler JE, Chin KJ, Hixson KK, Mouser P, Lipton MS, DeBoy R, Methe BA:
593 Genome sequence of the deltaproteobacterial strain NaphS2 and analysis of differential gene
594 expression during anaerobic growth on naphthalene. PloS One 2010;5.

595 Eberlein C, Estelmann S, Seifert J, von Bergen M, Müller M, Meckenstock RU, Boll M: Identification
596 and characterization of 2-naphthoyl-coenzyme A reductase, the prototype of a novel class of
597 dearomatizing reductases. Mol Microbiol 2013a;88:1032-1039.

598 Eberlein C, Johannes J, Mouttaki H, Sadeghi M, Golding BT, Boll M, Meckenstock RU: ATP-
599 dependent/-independent enzymatic ring reductions involved in the anaerobic catabolism of
600 naphthalene. Environ Microbiol 2013b;15:1832-1841.

601 Estelmann S, Blank I, Feldmann A, Boll M: Two distinct old yellow enzymes are involved in naphthyl
602 ring reduction during anaerobic naphthalene degradation. Mol Microbiol 2015;95:162-172.

603 Evans WC, Fuchs G: Anaerobic degradation of aromatic compounds. Annu Rev Microbiol
604 1988;42:289-317.

605 Fahrenfeld N, Cozzarelli I, Bailey Z, Pruden A: Insights into biodegradation through depth-resolved
606 microbial community functional and structural profiling of a crude-oil contaminant plume.
607 Microbial Ecol 2014;68:453-462.

608 Fowler SJ, Dong X, Sensen CW, Suflita JM, Gieg LM: Methanogenic toluene metabolism: community
609 structure and intermediates. Environ Microbiol 2012;14:754-764.

610 Fowler SJ, Gutierrez-Zamora M-L, Manefield M, Gieg LM: Identification of toluene degraders in a
611 methanogenic enrichment culture. FEMS Microbiol Ecol 2014;89:625-636.

612 Fuchs G, Boll M, Heider J: Microbial degradation of aromatic compounds - from one strategy to four.
613 Nat Rev Micro 2011;9:803-816.

614 Grundmann O, Behrends A, Rabus R, Amann J, Halder T, Heider J, Widdel F: Genes encoding the
615 candidate enzyme for anaerobic activation of n-alkanes in the denitrifying bacterium, strain
616 HxN1. Environ Microbiol 2008;10:376-385.

617 Heider J: Adding handles to unhandy substrates: anaerobic hydrocarbon activation mechanisms. Curr
618 Opin Chem Biol 2007;11:188-194.

619 Heider J, Schühle K: Anaerobic biodegradation of hydrocarbons including methane; in Rosenberg E,
620 DeLong E, Lory S, Stackebrandt E, Thompson F (eds): The Prokaryotes. Springer Berlin
621 Heidelberg, 2013, pp 605-634.

622 Herrmann S, Kleinsteuber S, Chatzinotas A, Kuppardt S, Lueders T, Richnow H-H, Vogt C: Functional
623 characterization of an anaerobic benzene-degrading enrichment culture by DNA stable isotope
624 probing. Environ Microbiol 2010;12:401-411.

625 Herrmann S, Vogt C, Fischer A, Kuppardt A, Richnow H-H: Characterization of anaerobic xylene
626 biodegradation by two-dimensional isotope fractionation analysis. Environ Microbiol Rep
627 2009;1:535-544.

628 Higashioka Y, Kojima H, Fukui M: Temperature-dependent differences in community structure of
629 bacteria involved in degradation of petroleum hydrocarbons under sulfate-reducing
630 conditions. J Appl Microbiol 2011;110:314-322.

631 Holmes DE, Risso C, Smith JA, Lovley DR: Anaerobic oxidation of benzene by the hyperthermophilic
632 archaeon *Ferroglobus placidus*. Appl Environ Microbiol 2011;77:5926-5933.

633 Hosoda A, Kasai Y, Hamamura N, Takahata Y, Watanabe K: Development of a PCR method for the
634 detection and quantification of benzoyl-CoA reductase genes and its application to monitored
635 natural attenuation. Biodegradation 2005;16:591-601.

636 Hug LA, Edwards EA: Diversity of reductive dehalogenase genes from environmental samples and
637 enrichment cultures identified with degenerate primer PCR screens. Front Microbiol
638 2013;4:doi: 10.3389/fmicb.2013.00341.

639 Jehmlich N, Kleinsteuber S, Vogt C, Benndorf D, Harms H, Schmidt F, Von Bergen M, Seifert J:
640 Phylogenetic and proteomic analysis of an anaerobic toluene-degrading community. J Appl
641 Microbiol 2010;109:1937-1945.

642 Jobelius C, Ruth B, Griebler C, Meckenstock RU, Hollender J, Reineke A, Frimmel FH, Zwiener C:
643 Metabolites indicate hot spots of biodegradation and biogeochemical gradients in a high-
644 resolution monitoring well. Environ Sci Technol 2010;45:474-481.

645 Johnson HA, Pelletier DA, Spormann AM: Isolation and characterization of anaerobic ethylbenzene
646 dehydrogenase, a novel Mo-Fe-S enzyme. J Bacteriol 2001;183:4536-4542.

647 Kazy S, Monier A, Alvarez P: Assessing the correlation between anaerobic toluene degradation
648 activity and bssA concentrations in hydrocarbon-contaminated aquifer material.
649 Biodegradation 2010;21:793-800.

650 Kimes NE, Callaghan AV, Aktas DF, Smith WL, Sunner J, Golding BT, Drozdowska M, Hazen TC, Suflita
651 JM, Morris PJ: Metagenomic analysis and metabolite profiling of deep-sea sediments from the
652 Gulf of Mexico following the Deepwater Horizon oil spill. Front Microbiol 2013;4:50.

653 Kniemeyer O, Heider J: Ethylbenzene dehydrogenase, a novel hydrocarbon-oxidizing
654 molybdenum/iron-sulfur/heme enzyme. J Biol Chem 2001;276:21381-21386.

655 Kniemeyer O, Musat F, Sievert SM, Knittel K, Wilkes H, Blumenberg M, Michaelis W, Classen A, Bolm
656 C, Joye SB, Widdel F: Anaerobic oxidation of short-chain hydrocarbons by marine sulphate-
657 reducing bacteria. Nature 2007;449:898-901.

658 Kropp KG, Davidova IA, Suflita JM: Anaerobic oxidation of n-dodecane by an addition reaction in a
659 sulfate-reducing bacterial enrichment culture. Appl Environ Microbiol 2000;66:5393-5398.

660 Kümmel S, Kuntze K, Vogt C, Boll M, Heider J, Richnow HH: Evidence for benzylsuccinate synthase
661 subtypes obtained by using stable isotope tools. J Bacteriol 2013;195:4660-4667.

662 Kunapuli U, Jahn MK, Lueders T, Geyer R, Heipieper HJ, Meckenstock RU: *Desulfitobacterium*
663 *aromaticivorans* sp. nov. and *Geobacter toluenoxydans* sp. nov., iron-reducing bacteria capable
664 of anaerobic degradation of monoaromatic hydrocarbons. Int J Syst Evol Microbiol
665 2010;60:686-695.

666 Kunapuli U, Lueders T, Meckenstock RU: The use of stable isotope probing to identify key iron-
667 reducing microorganisms involved in anaerobic benzene degradation. ISME J 2007;1:643-653.

668 Kuntze K, Shinoda Y, Moutakki H, McInerney MJ, Vogt C, Richnow H-H, Boll M: 6-Oxocyclohex-1-ene-
669 1-carbonyl-coenzyme A hydrolases from obligately anaerobic bacteria: characterization and
670 identification of its gene as a functional marker for aromatic compounds degrading anaerobes.
671 Environ Microbiol 2008;10:1547-1556.

672 Kuntze K, Vogt C, Richnow H-H, Boll M: Combined application of PCR-based functional assays for the
673 detection of aromatic-compound-degrading anaerobes. Appl Environ Microbiol 2011;77:5056-
674 5061.

675 Kuppardt A, Kleinsteuber S, Vogt C, Lueders T, Harms H, Chatzinotas A: Phylogenetic and functional
676 diversity within toluene-degrading, sulphate-reducing consortia enriched from a contaminated
677 aquifer. Microbial Ecol 2014;68:222-234.

678 Lara-Martin PA, Gomez-Parra A, Sanz JL, Gonzalez-Mazo E: Anaerobic degradation pathway of linear
679 alkylbenzene sulfonates (LAS) in sulfate-reducing marine sediments. Environ Sci Technol
680 2010;44:1670-1676.

681 Lehtiö L, Goldman A: The pyruvate formate lyase family: sequences, structures and activation.
682 Protein Eng Des Sel 2004;17:545-552.

683 Li YN, Porter AW, Mumford A, Zhao XH, Young LY: Bacterial community structure and *bamA* gene
684 diversity in anaerobic degradation of toluene and benzoate under denitrifying conditions. J
685 Appl Microbiol 2012;112:269-279.

686 Löffler C, Kuntze K, Vazquez JR, Rugor A, Kung JW, Böttcher A, Boll M: Occurrence, genes and
687 expression of the W/Se-containing class II benzoyl-coenzyme A reductases in anaerobic
688 bacteria. Environ Microbiol 2011;13:696-709.

689 Luo F, Gitiafroz R, Devine CE, Gong Y, Hug LA, Raskin L, Edwards EA: Metatranscriptome of an
690 anaerobic benzene-degrading, nitrate-reducing enrichment culture reveals involvement of
691 carboxylation in benzene ring activation. Appl Environ Microbiol 2014;80:4095-4107.

692 Meckenstock RU, Mouttaki H: Anaerobic degradation of non-substituted aromatic hydrocarbons.
693 Curr Opin Biotechnol 2011;22:406-414.

694 Morris BEL, Gissibl A, Kümmel S, Richnow H-H, Boll M: A PCR-based assay for the detection of
695 anaerobic naphthalene degradation. Fems Microbiol Lett 2014;354:55-59.

696 Mouttaki H, Johannes J, Meckenstock RU: Identification of naphthalene carboxylase as a prototype
697 for the anaerobic activation of non-substituted aromatic hydrocarbons. Environ Microbiol
698 2012;14:2770-2774.

699 Müller JA, Galushko AS, Kappler A, Schink B: Anaerobic degradation of m-cresol by *Desulfobacterium*
700 *cetonicum* is initiated by formation of 3-hydroxybenzylsuccinate. Arch Microbiol 1999;172:287-
701 294.

702 Musat F, Galushko A, Jacob J, Widdel F, Kube M, Reinhardt R, Wilkes H, Schink B, Rabus R: Anaerobic
703 degradation of naphthalene and 2-methylnaphthalene by strains of marine sulfate-reducing
704 bacteria. Environ Microbiol 2009;11:209-219.

705 Musat F, Wilkes H, Behrends A, Woebken D, Widdel F: Microbial nitrate-dependent cyclohexane
706 degradation coupled with anaerobic ammonium oxidation. ISME J 2010;4:1290-1301.

707 Oka AR, Phelps CD, Zhu X, Saber DL, Young LY: Dual biomarkers of anaerobic hydrocarbon
708 degradation in historically contaminated groundwater. Environ Sci Technol 2011;45:3407-
709 3414.

710 Osman OA, Gudasz C, Bertilsson S: Diversity and abundance of aromatic catabolic genes in lake
711 sediments in response to temperature change. FEMS Microbiol Ecol 2014;88:468-481.

712 Penton CR, Johnson TA, Quensen JF, Iwai S, Cole JR, Tiedje JM: Functional genes to assess nitrogen
713 cycling and aromatic hydrocarbon degradation: primers and processing matter. Front
714 Microbiol 2013;4:doi: 10.3389/fmicb.2013.00279.

715 Pérez-Pantoja D, Donoso R, Junca H, González B, Pieper DH: Phylogenomics of aerobic bacterial
716 degradation of aromatics; in Timmis K (ed): Handbook of Hydrocarbon and Lipid Microbiology.
717 Springer Berlin Heidelberg, 2010, pp 1355-1397.

718 Pilloni G, von Netzer F, Engel M, Lueders T: Electron acceptor-dependent identification of key
719 anaerobic toluene degraders at a tar-oil-contaminated aquifer by Pyro-SIP. FEMS Microbiol
720 Ecol 2011;78:165-175.

721 Porter AW, Young LY: The *bamA* gene for anaerobic ring fission is widely distributed in the
722 environment. Front Microbiol 2013;4:doi:10.3389/fmicb.2013.00302.

723 Porter AW, Young LY: Benzoyl-CoA, a universal biomarker for anaerobic degradation of aromatic
724 compounds; in Sima S, Geoffrey Michael G (eds): Advances in Applied Microbiology. Academic
725 Press, 2014, vol 88, pp 167-203.

726 Rabus R, Wilkes H, Behrends A, Armstroff A, Fischer T, Pierik AJ, Widdel F: Anaerobic initial reaction
727 of n-alkanes in a denitrifying bacterium: Evidence for (1-methylpentyl)succinate as initial
728 product and for involvement of an organic radical in n-hexane metabolism. J Bacteriol
729 2001;183:1707-1715.

730 Rios-Hernandez LA, Gieg LM, Suflita JM: Biodegradation of an alicyclic hydrocarbon by a sulfate-
731 reducing enrichment from a gas condensate-contaminated aquifer. Appl Environ Microbiol
732 2003;69:434-443.

733 Schink B: Anaerobic digestion: concepts, limits and perspectives. Water Sci Technol 2002;45:1-8.

734 Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks
735 DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ, Weber CF: Introducing mothur:
736 open-source, platform-independent, community-supported software for describing and
737 comparing microbial communities. Appl Environ Microbiol 2009;75:7537-7541.

738 Schühle K, Fuchs G: Phenylphosphate carboxylase: a new C-C lyase involved in anaerobic phenol
739 metabolism in *Thauera aromatica*. J Bacteriol 2004;186:4556-4567.

740 Song B, Ward BB: Genetic diversity of benzoyl coenzyme-A reductase genes detected in denitrifying
741 isolates and estuarine sediment communities. Appl Environ Microbiol 2005;71:2036-2045.

742 Staats M, Braster M, Roling WFM: Molecular diversity and distribution of aromatic hydrocarbon-
743 degrading anaerobes across a landfill leachate plume. Environ Microbiol 2011;13:1216-1227.

744 Stott K, Saito K, Thiele DJ, Massey V: Old Yellow Enzyme. The discovery of multiple isozymes and a
745 family of related proteins. J Biol Chem 1993;268:6097-6106.

746 Strijkstra A, Trautwein K, Jarling R, Wöhlbrand L, Dörries M, Reinhardt R, Drozdowska M, Golding BT,
747 Wilkes H, Rabus R: Anaerobic activation of p-cymene in denitrifying *Betaproteobacteria*:
748 methyl group hydroxylation versus addition to fumarate. Appl Environ Microbiol 2014.

749 Sun W, Cupples AM: Diversity of five anaerobic toluene-degrading microbial communities
750 investigated using stable isotope probing. Appl Environ Microbiol 2012;78:972-980.

751 Sun W, Sun X, Cupples A: Presence, diversity and enumeration of functional genes (bssA and bamA)
752 relating to toluene degradation across a range of redox conditions and inoculum sources.
753 Biodegradation 2014a;25:189-203.

754 Sun W, Sun X, Cupples AM: Identification of *Desulfosporosinus* as toluene-assimilating
755 microorganisms from a methanogenic consortium. Int Biodeter Biodegr 2014b;88:13-19.

756 Tan B, Jane Fowler S, Laban NA, Dong X, Sensen CW, Foght J, Gieg LM: Comparative analysis of
757 metagenomes from three methanogenic hydrocarbon-degrading enrichment cultures with 41
758 environmental samples. ISME J 2015:doi:10.1038/ismej.2015.1022.

759 Taubert M, Vogt C, Wubet T, Kleinsteuber S, Tarkka MT, Harms H, Buscot F, Richnow H-H, von Bergen
760 M, Seifert J: Protein-SIP enables time-resolved analysis of the carbon flux in a sulfate-reducing,
761 benzene-degrading microbial consortium. ISME J 2012;6:2291–2301.

762 Thiele B, Rieder O, Jehmlich N, von Bergen M, Müller M, Boll M: Aromatizing cyclohexa-1,5-diene-1-
763 carbonyl-coenzyme A oxidase: characterization and its role in anaerobic aromatic metabolism.
764 J Biol Chem 2008;283:20713-20721.

765 van der Zaan BM, Saia FT, Stams AJM, Plugge CM, de Vos WM, Smidt H, Langenhoff AAM, Gerritse J:
766 Anaerobic benzene degradation under denitrifying conditions: *Peptococcaceae* as dominant
767 benzene degraders and evidence for a syntrophic process. Environ Microbiol 2012;14:1171-
768 1181.

769 von Netzer F, Pilloni G, Kleindienst S, Krüger M, Knittel K, Gründger F, Lueders T: Enhanced gene
770 detection assays for fumarate-adding enzymes allow uncovering anaerobic hydrocarbon
771 degraders in terrestrial and marine systems. Appl Environ Microbiol 2013;79:543-552.

772 Wallisch S, Gril T, Dong X, Welzl G, Bruns C, Heath E, Engel M, Suhadolc M, Schloter M: Influence of
773 compost amendments on the diversity of alkane degrading bacteria in hydrocarbon
774 contaminated soils. Frontiers in Microbiology 2014;5:doi:10.3389/fmicb.2014.00096.

775 Washer CE, Edwards EA: Identification and expression of benzylsuccinate synthase genes in a
776 toluene-degrading methanogenic consortium. Appl Environ Microbiol 2007;73:1367-1369.

777 Weelink SAB, Doesburg Wv, Saia FT, Rijpstra WIC, Röling WFM, Smidt H, Stams AJM: A strictly
778 anaerobic betaproteobacterium *Georgfuchsia toluolica* gen. nov., sp. nov. degrades aromatic
779 compounds with Fe(III), Mn(IV) or nitrate as an electron acceptor. FEMS Microbiol Ecol
780 2009;70:575-585.

781 Weelink SAB, van Eekert MHA, Stams AJM: Degradation of BTEX by anaerobic bacteria: physiology
782 and application. Rev Environ Sci Bio-Technol 2010;9:359-385.

783 Widdel F, Knittel K, Galushko A: Anaerobic hydrocarbon-degrading microorganisms: an overview; in
784 Timmis KN (ed): Handbook of Hydrocarbon and Lipid Microbiology. Springer Berlin Heidelberg,
785 2010, pp 1997-2021.

786 Widdel F, Rabus R: Anaerobic biodegradation of saturated and aromatic hydrocarbons. Curr Opin
787 Biotechnol 2001;12:259-276.

788 Wilkes H, Schwarzbauer J: Hydrocarbons: an introduction to structure, physico-chemical properties
789 and natural occurrence; in Timmis K (ed): Handbook of Hydrocarbon and Lipid Microbiology.
790 Springer Berlin Heidelberg, 2010, pp 1-48.

791 Winderl C, Anneser B, Griebler C, Meckenstock RU, Lueders T: Depth-resolved quantification of
792 anaerobic toluene degraders and aquifer microbial community patterns in distinct redox zones
793 of a tar oil contaminant plume. Appl Environ Microbiol 2008;74:792-801.

794 Winderl C, Penning H, von Netzer F, Meckenstock RU, Lueders T: DNA-SIP identifies sulfate-reducing
795 *Clostridia* as important toluene degraders in tar-oil-contaminated aquifer sediment. ISME J
796 2010;4:1314-1325.

797 Winderl C, Schaefer S, Lueders T: Detection of anaerobic toluene and hydrocarbon degraders in
798 contaminated aquifers using benzylsuccinate synthase (*bssA*) genes as a functional marker.
799 Environ Microbiol 2007;9:1035-1046.

800 Wöhlbrand L, Jacob JH, Kube M, Mussmann M, Jarling R, Beck A, Amann R, Wilkes H, Reinhardt R,
801 Rabus R: Complete genome, catabolic sub-proteomes and key-metabolites of *Desulfobacula*

802 *toluolica* Tol2, a marine, aromatic compound-degrading, sulfate-reducing bacterium. Environ
803 Microbiol 2013;15:1334-1355.

804 Yagi JM, Suflita JM, Gieg LM, DeRito CM, Jeon C-O, Madsen EL: Subsurface cycling of nitrogen and
805 anaerobic aromatic hydrocarbon biodegradation revealed by nucleic acid and metabolic
806 biomarkers. Appl Environ Microbiol 2010;76:3124-3134.

807 Zhang T, Tremblay P-L, Chaurasia AK, Smith JA, Bain TS, Lovley DR: Anaerobic benzene oxidation via
808 phenol in *Geobacter metallireducens*. Appl Environ Microbiol 2013;79:7800-7806.

809

810

811 **Table 1.** Overview of marker genes assays and primers currently used for the targeted detection of anaerobic aromatic hydrocarbon degraders.

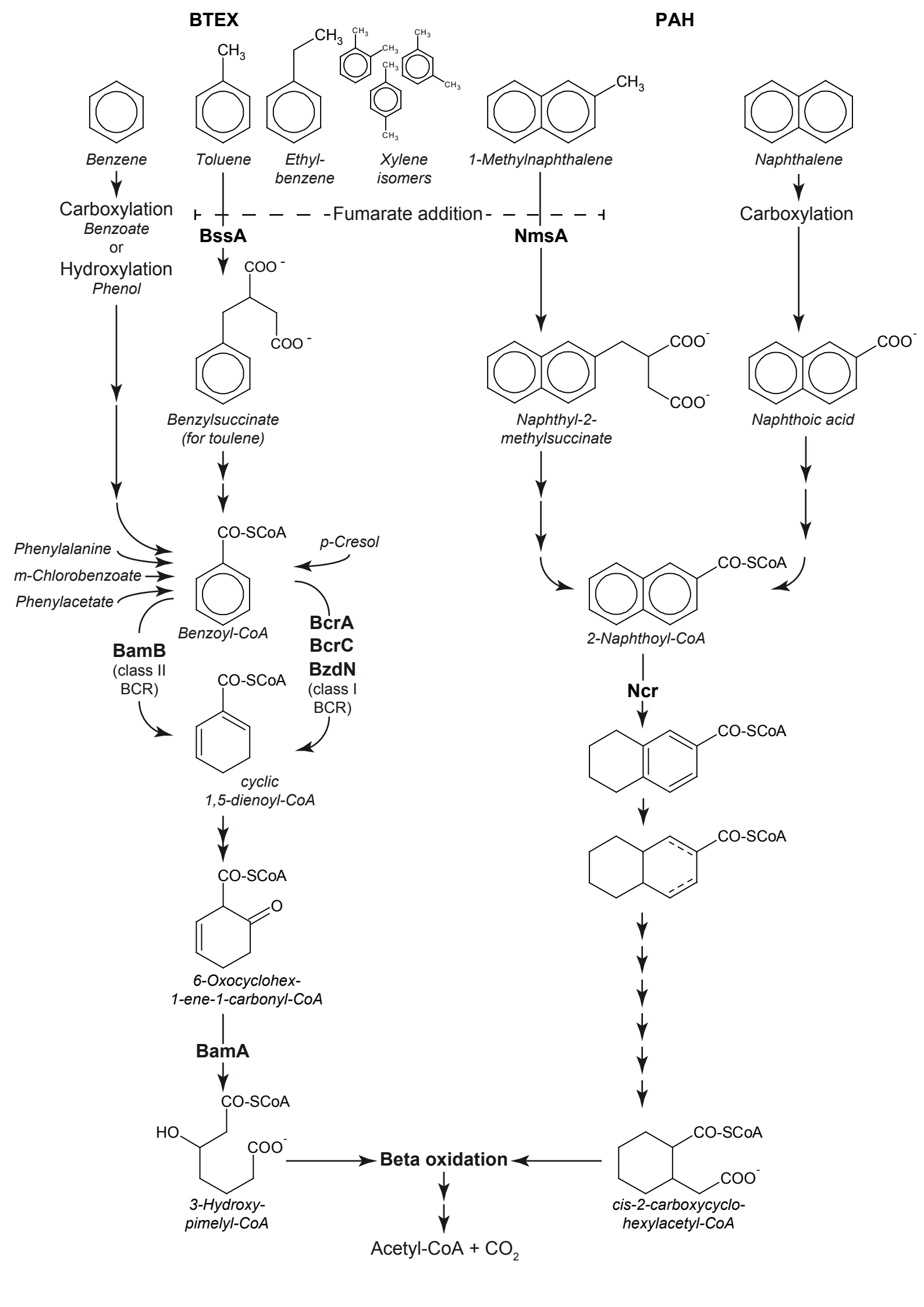
Reference	Primer name		Target gene	Target lineage	Primer sequence (5'-3')	Amplicon (bp)	qPCR applicable
Beller et al. 2002			*bssA*	denitrifying *Betaproteobacteria*	ACGACGGYGGCATTTCTC GCATGATSGGYACCGACA	130	tested
Winderl et al. 2007	7772f 8546r		*bssA*	*Beta-* & *Deltaproteobacteria, Clostridia*	GACATGACCGACGCSATYCT TCGTCGTCRTTGCCCCAYTT	800	tested
Beller et al. 2008	SRBf SRBr		*bssA*	sulfate-reducing *Deltaproteobacteria*	GTSCCCATGATGCGCAGC CGACATTGAACTGCACGTGRTCG	100	
Callaghan et al. 2010	Primer Set 1		*assA*	targets also *bssA*	TTTGAGTGCATCCGCCAYGGICT TCGTCRTTGCCCCATTTIGGIGC	700	
Staats et al. 2011	bssA3f bssAr		*bssA*	denitrifying and iron-reducing *Betaproteobacteria* (*Georgfuchsia toluolica*)	TCGAYGAYGGSTGCATGGA TTCTGGTTYTTCTGCAC	500	tested
von Netzer et al. 2013	FAE-B	7768f 8543r	*bssA* s.l.	Clostridial *bssA, bssA* sensu lato, *nmsA*	CAAYGATTTAACCRACGCCAT TCGTCRTTGCCCCAYTTNGG	800	
	FAE-N	7363f 7374f 8543r	*nmsA*	*nmsA* sensu stricto	TCGCCGAGAATTTCGAYTTG TTCGAYTTGAGCGACAGCGT TCGTCRTTGCCCCAYTTNGG	1200	
Fowler et al. 2014	MBssA1F MBssA1R		*bssA*	*Desulfosporosinus* spp. (methanogenic enrichment)	ATGCCCTTTGTTGCCAGTAT GCTGCATTTCTTCGAAACCT	223	tested
Song & Ward 2005	bzAQ41F bzAQ4R		*bcrA* s.l.	*bcrA* homologues of *Alpha-*, *Beta-* & *Gammaproteobacteria*	GTGGGCACCGGNTAYGGNMG GGTTCTTGGCGAYNCCNCCNGT	450	
Hosoda et al. 2005	bcr-1f bcr-2r		*bcrA*	*Thauera aromatica, Azoarcus evansii, Rhodopseudomonas palustris*	GTYGGMACCGGCTACGGCCG TTCTKVGCIACICCDCCGG	480	tested
Kuntze et al. 2008	bamA-SP9-f bamA-ASP1-r		*bamA*	*Alpha-, Beta-, Deltaproteobaceria*	CAGTACAAYTCCTACACVACBG CMATGCCGATYTCCTGRC	300	tested
Löffler et al. 2011	bamBf bamBr		*bamB*	*Deltaproteobacteria, Clostridia*	ATGMGGTAYGSAGARACHGG CCSGCRWRYTTCADYTCCG	320	
Staats et al. 2011	oah_f oah_r		*bamA*	iron reducers (*Rhodocyclaceae, Geobacteraceae*)	GCAGTACAAYTCCTACACSACYGABATGGT CCRTGCTTSGGRCCVGCCTGVCCGAA	350	tested
Kuntze et al. 2011	bamA-ASP23-r bamA-ASP33-r	(with bamA-SP9-f)	*bamA*	GMT cluster SA cluster	TTTTCCTTGTTGVSRTTCC CAKYYSGGGAASAGRTTKG	800 700	
	bzdNf bzdNr		*bzdN*	*Azoarcus*-type class I benzoyl-CoA reductases	GAGCCGCACATCTTCGGCAT TRTGVRCCGGRTARTCCTTSGTCGG	700	tested
	bcrCf bcrCr		*bcrC*	*Thauera*-type class I benzoyl-CoA reductases	CGHATYCCRCGSTCGACCATCG CGGATCGGCTGCATCTGGCC	600	
Morris et al. 2014	Ncr_for Ncr_rev		*Ncr*	*Deltaproteobacteria* (N47, NaphS2)	TGGACAAAYAAAMGYACVGAT GATTCCGGCTTTTTTCCAAVT	320	tested

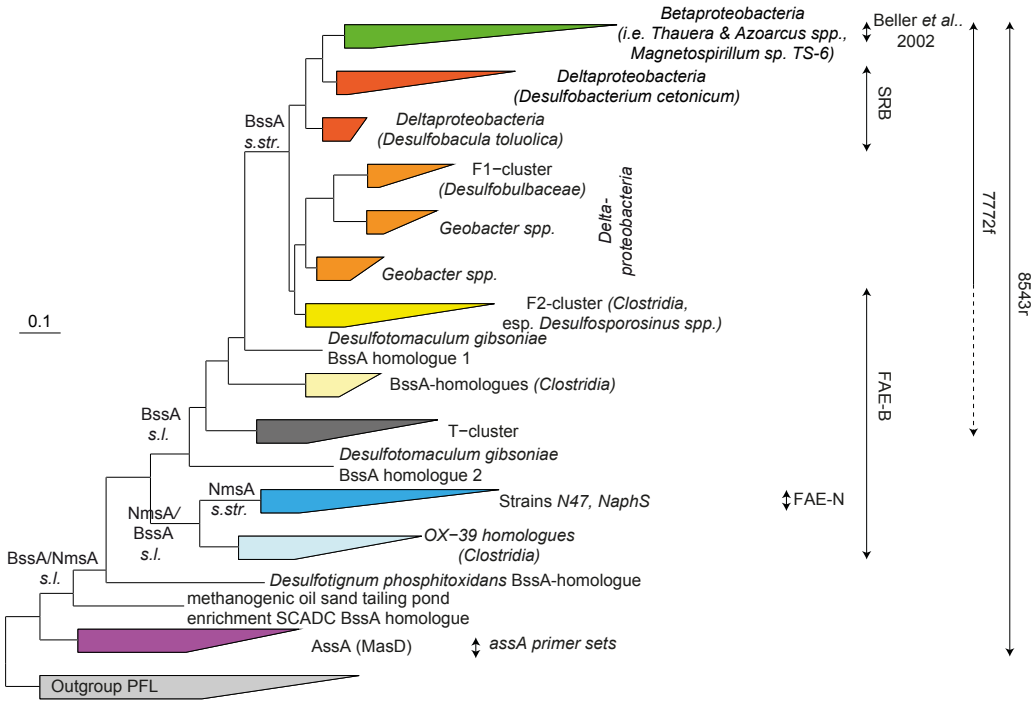
812

Figure legends

Fig. 1. Overview of important peripheral and central pathways in the anaerobic degradation of aromatic hydrocarbons. Genes of key enzyme in use as marker genes for degraders in the environment are: BssA – benzylsuccinate synthase alpha-subunit; NmsA – naphthylmethylsuccinate synthase alpha-subunit; BamB – ATP-independent benzoyl-CoA reductase beta-subunit; BcrA/BcrC/BzdN – ATP-dependent benzoyl-CoA reductase subunits; Ncr – 2-naphthoyl-CoA reductase; BamA – ring cleaving 6-oxocylcohex-1-ene-1-carbonyl-CoA hydrolase.

Fig. 2. Overview of the phylogeny of known pure culture and environmental FAE gene sequences. Several lineages are collapsed with only a few representatives named. Additionally, the demonstrated range of coverage for selected primer pairs is indicated. S.str. = *sensu stricto*, s.l. = *sensu lato*. Outgroup: related pyruvate formiate lyase genes (PFL). The scale bar represents 10% amino acid sequence divergence. The dendrogram was developed as in von Netzer et al. [2013]


BTEX
PAH
Benzene
Toluene
Ethyl-benzene
Xylene isomers
1-Methylnaphthalene
Naphthalene
Carboxylation
Benzoate
or
Hydroxylation
Phenol
Fumarate addition
BssA
NmsA
Carboxylation
Benzylsuccinate (for toulene)
Naphthyl-2-methylsuccinate
Naphthoic acid
CO-SCoA
Phenylalanine
m-Chlorobenzoate
Phenylacetate
p-Cresol
Benzoyl-CoA
BamB
(class II BCR)
BcrA
BcrC
BzdN
(class I BCR)
2-Naphthoyl-CoA
Ncr
cyclic 1,5-dienoyl-CoA
6-Oxocyclohex-1-ene-1-carbonyl-CoA
BamA
3-Hydroxy-pimelyl-CoA
Beta oxidation
cis-2-carboxycyclo-hexylacetyl-CoA
Acetyl-CoA + CO2


Betaproteobacteria
(i.e. Thauera & Azoarcus spp.,
Magnetospirillum sp. TS-6)
Beller et al..
2002
Deltaproteobacteria
(Desulfobacterium cetonicum)
SRB
BssA
s.str.
Deltaproteobacteria
(Desulfobacula toluolica)
F1−cluster
(Desulfobulbaceae)
Delta-
proteobacteria
Geobacter spp.
Geobacter spp.
7772f
8543r
0.1
F2-cluster (Clostridia,
esp. Desulfosporosinus spp.)
Desulfotomaculum gibsoniae
BssA homologue 1
BssA-homologues (Clostridia)
FAE-B
BssA
s.l.
T−cluster
Desulfotomaculum gibsoniae
BssA homologue 2
NmsA
s.str.
Strains N47, NaphS
FAE-N
NmsA/
BssA
s.l.
OX−39 homologues
(Clostridia)
BssA/NmsA
s.l.
Desulfotignum phosphitoxidans BssA-homologue
methanogenic oil sand tailing pond
enrichment SCADC BssA homologue
AssA (MasD)
assA primer sets
Outgroup PFL