Review Article

Journal of Molecular Microbiology and Biotechnology

J Mol Microbiol Biotechnol 2016;26:180–194
DOI: 10.1159/000441946

Published online: March 10, 2016

Functional Gene Markers for Fumarate-Adding and Dearomatizing Key Enzymes in Anaerobic Aromatic Hydrocarbon Degradation in Terrestrial Environments

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Key Words
Catabolic marker genes · Anaerobic aromatic metabolism

Abstract
Anaerobic degradation is a key process in many environments either naturally or anthropogenically exposed to petroleum hydrocarbons. Considerable advances into the biochemistry and physiology of selected anaerobic degraders have been achieved over the last decades, especially for the degradation of aromatic hydrocarbons. 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. These approaches have mainly 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 or dearomatizing aryl-coenzyme A 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 among 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 environ-

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ment. 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 stabilized by high-resonance energy levels within the delocalized π-electron system of the aromatic ring. Notwithstanding, many bacteria are in fact able to utilize aromatic hydrocarbons as 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 capitalize on such substrates.

Catabolic Pathways

Under oxic conditions, aromatic compounds are first activated by the addition of hydroxyl groups to destabilize 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 metabolized by various peripheral pathways into a few di-hydroxylated central intermediates. The ring structure is then opened by either meta- or ortho-cleavage, and breakdown products are further degraded before entering central metabolism, mostly as succinyl- or acetyl-coenzyme A (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].

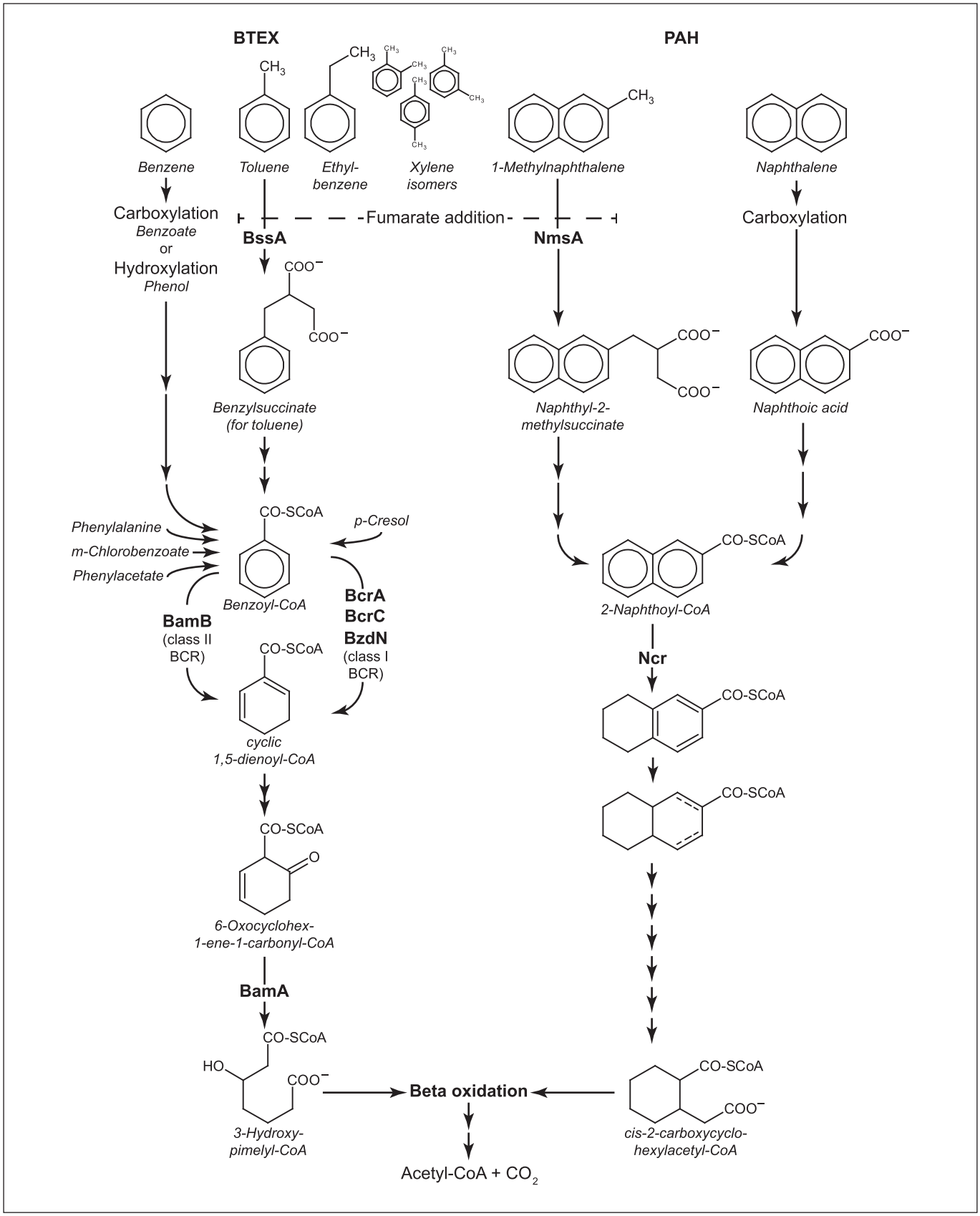
In contrast, when hydrocarbon loads exceed the availability of oxygen, anaerobic biodegradation of aromatic hydrocarbons becomes a pivotal process, particularly in contaminated subsurface environments (i.e. groundwater). However, the microbes involved in anaerobic degradation and their biochemistry eluded scientific investigators for most of the last century. Beginning from the late 1980s, the 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 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 catalyzed 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 (FAEs) 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,

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 as follows: BssA – BSS α-subunit; NmsA – NMS α-subunit; BamB – ATP-independent benzoyl-CoA reductase β-subunit; BcrA/BcrC/BzdN – ATP-dependent BCR subunits; BamA – ring-cleaving 6-oxocylcohex-1-ene-1-carbonyl-CoA hydrolase.

(For figure see next page.)



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DOI: 10.1159/000441946

the genes of FAEs 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 a 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 (BCR) BcrCBAD/BzdNOPQ/BadDEFG (class I BCR) in facultative anaerobes like *T. aromatica*, *Azoarcus* and *Aromatoleum* spp. and *Rhodopseudomonas palustris*, or the ATP-independent benzoyl-CoA reductase BamBCDEFGHI (class II BCR) in strict anaerobes like *G. 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, b]. 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 dearomatizes 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 the 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 mineralize aromatic hydrocarbons under anoxic conditions using various electron acceptors. Generally, hydrocarbons can be metabolized by single organisms or by syntrophic consortia. Organisms capable of initially attacking and metabolizing 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 recognized 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 the 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 mineralize 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 recognized as key organisms for BTEX mineralization under iron-reducing conditions [Weelink et al., 2010]. However, distinct members of the *Rhodocyclaceae* (*Georgfuchsia* sp.) within the *Betaproteobacteria* have also 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 as being capable of mineralizing 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, for example 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] or 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 ASS) have been developed (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 an amplicon of ~800 bp) 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 from Botton et al. [2007] to detect a diversity of iron- and nitrate-reducing aromatic compound degraders in a landfill leachate plume.

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

Detection Assays for Markers of Central Pathways of Monocyclic Aromatic Compound Degradation

Apart from FAE genes, functional markers targeting conserved enzymes of the benzoyl-CoA degradation pathway have also been successfully employed to detect anaerobic mono-aromatic compound degraders. These assays allow insights into the diversity and identity of degrader populations even when fumarate addition is not involved in upstream catabolism. The first primers introduced for a qualitative detection of subunits of class I BCRs *(bcr/bzd)* were published by Hosoda et al. [2005]

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-* and *Deltaproteobacteria, Clostridia*	GACATGACCGACGCSATYCT TCGTCGTCRTTGCCCCAYTT	800	tested
Beller et al., 2008	SRBf SRBr	*bssA*	sulphate-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 (G. toluolica)*	TCGAYGAYGGSTGCATGGA TTCTGGTTYTTCTGCAC	500	tested
von Netzer et al., 2013	FAE-B 7768f 8543r	*bssA* s.l.	Clostridial *bssA, bssA* s.l., *nmsA*	CAAYGATTTAACCRACGCCAT TCGTCRTTGCCCCAYTTNGG	800	
	FAE-N 7363f 7374f 8543r	*nmsA*	*nmsA* s.str.	TCGCCGAGAATTTCGAYTTG TTCGAYTTGAGCGACAGCGT TCGTCRTTGCCCCAYTTNGG	1,200	
Fowler et al., 2014	MBssA1F MBssA1R	*bssA*	*Desulfosporosinus* spp. (methanogenic enrichment)	ATGCCCTTTGTTGCCAGTAT GCTGCATTTCTTCGAAACCT	223	tested
Song and Ward, 2005	bzAQ41F bzAQ4R	*bcrA* s.l.	*bcrA* homologues of *Alpha-*, *Beta-* and *Gammaproteobacteria*	GTGGGCACCGGNTAYGGNMG GGTTCTTGGCGAYNCCNCCNGT	450	
Hosoda et al., 2005	bcr-1f bcr-2r	*bcrA*	*T. aromatica, Azoarcus evansii, R. palustris*	GTYGGMACCGGCTACGGCCG TTCTKVGCIACICCDCCGG	480	tested
Kuntze et al., 2008	bamA-SP9-f bamA-ASP1-r	*bamA*	*Alpha-, Beta-* and *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 (with bamA-SP9-f) bamA-ASP33-r	*bamA*	GMT cluster SA cluster	TTTTCCTTGTTGVSRTTCC CAKYYSGGGAASAGRTTKG	800 700	
	bzdNf bzdNr	*bzdN*	*Azoarcus*-type class I BCR	GAGCCGCACATCTTCGGCAT TRTGVRCCGGRTARTCCTTSGTCGG	700	tested
	bcrCf bcrCr	*bcrC*	*Thauera*-type class I BCR	CGHATYCCRCGSTCGACCATCG CGGATCGGCTGCATCTGGCC	600	
Morris et al., 2014	Ncr_for Ncr_rev	*Ncr*	*Deltaproteobacteria* (N47, NaphS2)	TGGACAAAYAAAMGYACVGAT GATTCCGGCTTTTTTCCAAVT	320	tested

s.l. = Sensu lato; s.str. = sensu stricto.

and Song and Ward [2005]. However, the use of these assays was not straightforward, as non-BRC gene fragments were also recovered at high frequency. To provide a more reliable PCR-based approach targeting class I BCRs in environmental DNA, these assays were later modified by Kuntze et al. [2011] with the design of two independent primer pairs targeting *Azoarcus*-type BCRs (*Azoarcus* spp. and '*Aromatoleum aromaticum* EbN1'), as well as *Thauera*-type class I BCRs (*Thauera*, *Magnetospirillum* and *Rhodopseudomonas* spp.). Specific primers were also developed by Löffler et al. [2011], targeting the *bamB* gene coding for the active site subunit of class II BCRs from obligate anaerobes.

The design of degenerate primer sets to amplify conserved gene fragments of the ring-cleaving 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* and *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 mean-

time, the *bamA* gene was 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 Markers 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 dearomatizing 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. *T. 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 those 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 (from naphthalene-degrading, sulphate-reducing *Deltaproteobacteria*). Therefore, its utility for the detection of *ncr* genes from other phylogenetic lineages still 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 [Boll et al., unpubl. 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 the 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., 2007, 2010; Yagi et al., 2010]. Other f-primers are recommended to recover the more deeply branching *bssA* genes sensu lato (s.l.), that is, 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 and also 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* homologues have been recovered [Löffler et al., 2011], suggesting its applicability 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 prim-

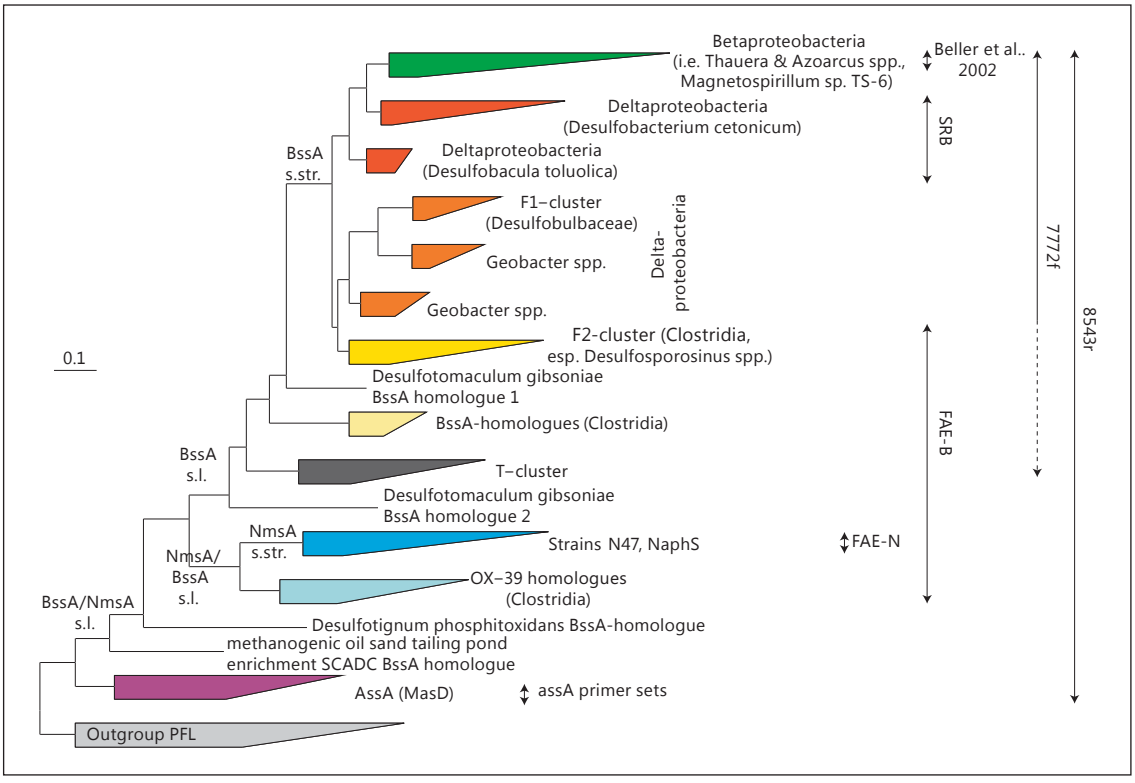

Fig. 2. Overview of the phylogeny of known pure culture and environmental FAE gene sequences. Several lineages are collapsed with only a few representatives named. Additionally, the demonstrated range of coverage for selected primer pairs is indicated. Outgroup: related pyruvate formate lyase genes.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

er 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, the cloning and sequencing of peripheral and central gene markers is well established [Callaghan et al., 2010; Kuntze et al., 2008, 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 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 this analysis is not a strong diagnostic tool, the identity of distinct terminal restriction

fragments can cautiously be elucidated via the 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 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, 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 characterize amplicon pools of environmental functional markers. In contrast to next-generation sequencing of 16S rRNA gene amplicons, which is already well established with many standardized data-handling pipelines 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 emerging. 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] have been 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

The notable suite of marker gene assays described above has been instrumental in 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 elucidate microbial interactions (e.g. syntrophy) in defined degrader assemblages. In the field, relevant insights into degrader diversity, the spatial distribution of degraders and the control of their activity in natural settings have been provided.

Catabolic Marker Gene-Based Insights into Degrader Cultures and Enrichments

In the laboratory, the detectability of *Azoarcus*-related *bssA* sequence types was first demonstrated for denitrifying toluene-degrading laboratory 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 *Georgfuchsia toluolica* [Weelink et al., 2009], now evident as a 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, which were related to known *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 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 were detect-

ed 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 stable isotope-probing incubations of tar oil-contaminated Testfeld Süd aquifer sediments dominated by *Desulfosporosinus* 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 also recently 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-reducing [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, for example *G. toluolica* [Staats et al., 2011], *Desulfosarcina* spp., *Desulfobacterium anilini* and strains of the Gram-positive *Desulfotomaculum gibsoniae* and *Desulfotomaculum 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 *Thauera 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, the applicability of a new detection assay for N47-related *ncr* genes has also 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 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 the 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 (Calif., USA), 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, a higher absolute abundance of *bssA* genes was reached under simultaneous ethanol injection. This indicates that at least some anaerobic aromatic compound degraders may also have been thriving on the amended ethanol.

Callaghan et al. [2010] have investigated intrinsic *bssA* diversity in sediments from hydrocarbon contaminated Fort Lupton (Colo., USA) and Casper (Wyo., USA) 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 (N.Y., USA). Besides unknown betaproteobacterial sequence types and such related to *G. 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] 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. 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 (N.J., USA), hydrocarbon degraders were shown to be enriched by up to two 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 emphasizing that untapped catabolic potentials and degrader lineages may remain to be uncovered 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-methylnaphthalene after fumarate addition [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 utilized 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 was detectable at this site. BcrA was significantly enriched in wells of high comparative contamination, evident also as distinct communities found between differentially impacted wells.

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

Conclusions and Outlook

Anaerobic aromatic compound degradation can be regarded as a key process in reducing hydrocarbon contamination in natural environments. For a better understanding of the microbes conveying these important ecosystem services, comprehensive functional marker assays for their detection, identification and quantification are a vital tool. The distinct marker strategies reviewed here, spanning key mechanisms in anaerobic activation and degradation of aromatic compounds, 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 the laboratory and field. First, the importance of several novel or previously unrecognized degrader lineages, especially within the *Rhodocyclaceae*, *Desulfobulbaceae* and *Peptococcaceae*, has been substantiated for numerous terrestrial systems, often hosting novel catabolic gene phylotypes. Sec-

ond, 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 recognized 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 stabilization of degrader assemblages under the fluctuating availability of electron donors or acceptors.

It is now relevant to consider how these advances can become apparent at 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] have 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 the future implementation of a marker gene- or transcript-based prediction of biodegradation rates in complex natural systems. Here, the observed substrate specificity of the peripheral markers in particular 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 site 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-throughput metagenomics and transcriptomics. Although the application of the latter has not been reported to date for an anoxic, aromatic compound-contaminated aquifer, respective reports for other hydrocarbon-impacted systems are available [An et al., 2013; Kimes et al., 2013; Luo et al., 2014; Tan et al., 2015]. Such non-target, non-PCR amplification-based sequencing approaches have the potential to further enhance our perspective of anaerobic hydrocarbon degrader microbiomes. Only with a clear understanding of the diversity, abundance and distribution of intrinsic degraders will it become possible to better understand their ecology and to possibly manipulate their activity in complex contaminated systems.

Acknowledgements

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

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