Enhanced Gene Detection Assays for Fumarate-Adding Enzymes Allow Uncovering of Anaerobic Hydrocarbon Degraders in Terrestrial and Marine Systems

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The detection of anaerobic hydrocarbon degrader populations via catabolic gene markers is important for the understanding of processes at contaminated sites. Fumarate-adding enzymes (FAEs; i.e., benzylsuccinate and alkylsuccinate synthases) have already been established as specific functional marker genes for anaerobic hydrocarbon degraders. Several recent studies based on pure cultures and laboratory enrichments have shown the existence of new and deeply branching FAE gene lineages, such as clostridial benzylsuccinate synthases and homologues, as well as naphthylmethylsuccinate synthases. However, established FAE gene detection assays were not designed to target these novel lineages, and consequently, their detectability in different environments remains obscure. Here, we present a new suite of parallel primer sets for detecting the comprehensive range of FAE markers known to date, including clostridial benzylsuccinate, naphthylmethylsuccinate, and alkylsuccinate synthases. It was not possible to develop one single assay spanning the complete diversity of FAE genes alone. The enhanced assays were tested with a range of hydrocarbon-degrading pure cultures, enrichments, and environmental samples of marine and terrestrial origin. They revealed the presence of several, partially unexpected FAE gene lineages not detected in these environments before: distinct deltaproteobacterial and also clostridial *bssA* homologues as well as environmental *nmsA* homologues. These findings were backed up by dual-digest terminal restriction fragment length polymorphism diagnostics to identify FAE gene populations independently of sequencing. This allows rapid insights into intrinsic degrader populations and degradation potentials established in aromatic and aliphatic hydrocarbon-impacted environmental systems.

Hydrocarbon contaminants are harmful to organisms and the environment, but they are also substrates for microbial degradation under both oxic and anoxic conditions. The aerobic degradation of hydrocarbons occurs readily but is constrained by oxygen limitation, especially in subsurface habitats. Therefore, degradation at many sites either naturally or anthropogenically impacted by hydrocarbons relies on anaerobic pathways. Investigating the structure and function of the involved anaerobic hydrocarbon-degrading microbial communities is thus crucial for the understanding of environmental hydrocarbon breakdown (e.g., see reference 1).

During the last few decades, several pathways for anaerobic hydrocarbon catabolism have been unraveled (2). Consequently, researchers have begun to trace the respective microbes in environmental systems based on the detection of the involved catabolic genes. Currently, the genes of enzymes for different peripheral and central catabolic reactions have been established as so-called functional markers for anaerobic hydrocarbon degradation (summarized by Kuntze et al. [3]). Among these, glycyl radical enzymes responsible for the initial activation of hydrocarbons with methyl- and methylene groups via addition to fumarate (i.e., fumarate addition) are currently the most widely used (see references 4 to 6 and see Table S1 in the supplemental material). This approach was initially introduced to target benzylsuccinate synthase (BSS) genes involved in toluene degradation by *Betaproteobacteria* (4). The utility of targeting fumarate-adding enzymes (FAEs) is limited not only to the detection of organisms involved in the oxidation of toluene, xylenes, and ethylbenzene (2). The same substrate acti-

vation principle is also used in alkylsuccinate synthases (ASS [7]; also named methylalkylsuccinate synthases [MAS] [8]) for long-chain as well as short-chain alka(e)nes (9) and in naphthylmethylsuccinate synthases (NMS) for 2-methylnaphthalene activation (10).

A number of primers targeting the α subunit of BSS and ASS genes for the analysis of environmental degrader populations have been developed (see Table S1 in the supplemental material). The first PCR and quantitative PCR (qPCR) primers targeting *bssA* genes of nitrate-reducing *Betaproteobacteria* were introduced by Beller et al. (4). The assay of Winderl et al. (6) extended the range of detectable hydrocarbon-degrading microbes to iron- and sulfate-reducing *Deltaproteobacteria* and revealed partially novel, site-specific degrader populations at three different tar oil-impacted aquifers in Germany. Staats et al. (11) applied an altered primer targeting *bssA* of iron-reducing degraders first developed

TABLE 1 FAE-gene targeted primer sets designed and employed in this study

Primer set	Annealing temp (°C)	Primer, position[a]	Sequence (5′–3′)	Targeted FAE lineages[b]
bssA[c]	58/52	7772f	GAC ATG ACC GAC GCS ATY CT	*bssA* sensu lato
		8546r	TC GTC GTC RTT GCC CCA YTT	
FAE-B	58	7768f	C AAY GAT TTA ACC RAC GCC AT	Clostridial *bssA*, *bssA* sensu lato, *nmsA*
		8543r	TC GTC RTT GCC CCA YTT NGG	
FAE-N	58	7363f	TC GCC GAG AAT TTC GAY TTG	*nmsA* sensu stricto
		7374f	TTC GAY TTG AGC GAC AGC GT	
		8543r	TC GTC RTT GCC CCA YTT NGG	
FAE-KM	58	FAE-Kf, 7757f-1	TCG GAC GCG TGC AAC GAT CTG A	*assA*
		FAE-Kf, 7757f-2	TCG GAC GCG TGC AAC GCC CTG A	
		FAE-Mf, 7766f	TGT AAC GGC ATG ACC ATT GCG CT	
		8543r	TC GTC RTT GCC CCA YTT NGG	

[a] *Thauera aromatica* K127 *bss* operon position.
[b] Targets intended *in silico*; see lineages in Fig. 1.
[c] Primer set of Winderl et al. (6, 15).

by Botton et al. (12) at an aquifer contaminated by landfill leachate. Here, retrieved *bssA* sequences were related to the betaproteobacterial *bssA* sequence type of *Georgfuchsia toluolica* (13) rather than the *Geobacter* populations expected from 16S rRNA gene studies. Recently, Callaghan et al. (5) also introduced assays for ASS genes, evolved from existing *bssA* primers, on the basis of the small number of pure-culture *assA* sequences available. These optimized primer sets were applied to DNA extracted from propane- and paraffin-degrading enrichments as well as several aquifers and freshwater and estuarine habitats contaminated with alkanes, revealing an environmental diversity of *assA* genes similar to that known for *bssA* genes.

The study conducted by Winderl et al. (6) revealed several unassigned, deeply branching *bssA* lineages, the so-called T and F clusters. F1 *bssA* was later identified to belong to the dominating *Desulfobulbaceae* degrader population (14) on-site, while F2 was identified as a novel *bssA* sequence type of the *Peptococcaceae* (15). Other studies on hydrocarbon-contaminated aquifers by Callaghan et al. (5) and Yagi et al. (16) and on xylene-degrading enrichments by Herrmann et al. (17) also corroborated the existence of new and deeply branching FAE lineages, in addition to the described BSS, NMS, and ASS lineages. Furthermore, some new anaerobic hydrocarbon degraders belonging to the *Clostridia* were recently discovered: *Desulfitobacterium aromaticivorans* UKTL, using fumarate addition for toluene activation (18), and strain BF, possessing a *bss*-homologous gene (19), despite oxidizing benzene and not toluene. We observed that *bssA* or homologous gene amplicons are not readily obtained from these strains with the available *bssA* primers. Moreover, the NMS genes recently described in naphthalene-degrading marine strains NaphS2, NaphS3, and NaphS6 (20) and aquifer sediment enrichment strain N47 (21) are not targeted by available primers at all. Consequently, the potential of FAE genes as markers for the degradation of various hydrocarbons by putatively novel species may not yet be fully exploited by existing assays.

FAE gene lineages dominating at given sites indicate relevant degradation potentials and should not be missed, if optimum bioremediation strategies are to be designed. Thus, there is a need for the development and application of more comprehensive primers for FAE gene detection in general. Qualitative gene detection is also a prerequisite for the design of quantitative (qPCR) assays, which may be useful for the inference of *in situ* degradation rates (4, 22).

The aim of this work was to assess FAE gene pools in different terrestrial and marine environments impacted by hydrocarbons via more universal detection assays. We hypothesize that the existing FAE detection systems do not yet optimally recover the full diversity of catabolic lineages, such as clostridial *bssA* and also *nmsA* homologues. We test whether one universal primer pair can be developed or whether several primer sets targeting overlapping amplicons in the FAE gene alignment are needed to recover most lineages. Moreover, for the routine and possibly high-throughput detection of FAE genes in the field and microcosms, rapid diagnostics via fingerprinting may be helpful to trace hydrocarbon degrader communities in space and time. Thus, we propose a novel, dual-digest terminal restriction fragment length polymorphism (T-RFLP) fingerprinting method for sequencing-independent diagnostics of major FAE gene lineages.

MATERIALS AND METHODS

Primer design. Primers were designed using an updated version of the *bssA* ARB database originally set up by Winderl et al. (6). Several new *bssA*, *nmsA*, and *assA* (also known as *masD*) sequences were added (see Fig. 1). The primer sets FAE-B, FAE-N, and FAE-KM, presented in Table 1, were designed to match the same target region as the original primers of Winderl et al. (6), which were also applied in this study. The binding sites for the new primers were found by searching the FAE alignment for conserved, distinctive protein motifs common for major gene clusters and using the DNA sequences of those protein motifs for primer design.

Strains, samples, and DNA extraction. *Desulfotignum toluenicum* DSMZ 18732, *Desulfatibacillum alkenivorans* DSMZ 16219, *Desulfatibacillum aliphaticivorans* DSMZ 15576, and *Geobacter metallireducens* DSMZ 7210 were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany). The strains were grown anaerobically in 120-ml serum bottles with butyl stoppers and the media specified by DSMZ. A toluene-degrading *Desulfosporosinus* sp. enrichment (15), *Desulfitobacterium aromaticivorans* UKTL, *Azoarcus* sp. strain T, strain BF, and strain N47 originated from our institute strain collection (Institute of Groundwater Ecology). The *Desulfosporosinus* sp. enrichment and *D. aromaticivorans* UKTL were cultivated anaerobically as previously described (15, 18). DNA was extracted from collected biomass by bead beating and a phenol-chloroform extraction (23). *Azoarcus* sp. T was cultivated and extracted as described by Winderl et al. (6). Strain BF was cultivated as described by Kunapuli et al. (24), and

the DNA was extracted as previously described (19). Strain N47 was cultivated and extracted as described by Selesi et al. (21).

Flingern tar oil-contaminated aquifer sediment was sampled in June 2009 and extracted as described by Pilloni et al. (25) with a total of four replicate sediment DNA extracts per chosen depth. Three depths (6.85 m, 7.15 m, and 7.25 m below the surface) spanning the lower plume fringe were chosen, as those depths were previously identified to be benzene, toluene, ethylbenzene, and *o*-, *m*-, and *p*-xylene (BTEX) degradation hot spots (26, 27). Furthermore, DNA from different terrestrial hydrocarbon-degrading enrichments (Paclele Mici mud volcano and Gölzau refinery-contaminated aquifer) was extracted with a FastDNA Spin kit for soil (MP Biomedicals, Irvine, CA). Further information on the sampling sites and enrichments are provided by Alain et al. (28) and Feisthauer et al. (29). Gulf of Mexico hydrocarbon seep sediment was sampled from station 161 as described by Orcutt et al. (30). DNA was extracted according to the protocol of Zhou et al. (31), and cleanup was performed with a Wizard DNA cleanup system (Promega, Fitchburg, WI). Marine samples from the Nyegga area methane seeps were taken at pockmarks located on the edge of the Norwegian continental slope; the sampling is described by Van Gaever et al. (32). The DNA was extracted with a Power soil RNA extraction kit and DNA elution accessory kit (MO BIO Laboratories, Carlsbad, CA). Sediment samples from the Amon mud volcano in the eastern Nile deep-sea fan (station 929, dive 240 [33]) and Guaymas Basin hydrocarbon seeps in the Gulf of California (dive 5473 [34]) were collected from below a microbial mat. The DNA was extracted as described by Kleindienst et al. (S. Kleindienst, F.-A. Herbst, F. von Netzer, R. Amann, J. Peplies, M. von Bergen, T. Lueders, J. Seifert, F. Musat, and K. Knittel, unpublished data) from a 2- to 20-cm sediment depth (Amon mud volcano) and from a 0- to 10-cm sediment depth (Guaymas, fine-grained sediments) according to the protocol described by Zhou et al. (31) and based on mechanical, chemical, and enzymatic lyses.

PCR amplification. FAE gene fragments were amplified via PCR using available and newly developed primer sets (Table 1) in a Mastercycler ep gradient (Eppendorf, Hamburg, Germany) with the following cycling conditions: a 3-min initial degradation, 30 to 40 cycles of amplification (30 s at 94°C, 30 s at 52°C or 58°C, 60 s at 72°C), and 5 min at 72°C of terminal extension. The annealing temperature was 58°C for all primers, as initially published (6), but also lowered comparatively to 52°C (15) for the *bssA* primer set. The 50-µl PCR mixture contained nuclease-free water (Promega, Fitchburg, WI), 1× PCR buffer, 1.5 mM MgCl$_2$ (Fermentas, Thermo Fisher Scientific, Waltham, MA), 10 µg bovine serum albumin (Roche, Basel, Switzerland) 0.1 mM deoxynucleoside triphosphates, 0.3 µM primer (biomers.net, Ulm, Germany), 1.25 U *Taq* polymerase, and DNA in the range of ~0.2 to 20 ng. Amplicon quality was checked with gel electrophoresis in a 1.5% agarose gel. Cleanup of the amplicons was done with a PCRextract kit (5Prime, Hamburg, Germany) according to the manufacturer's protocol before further processing.

T-RFLP. The reverse primers 8546r and 8543r were labeled for T-RFLP with 5′-6-carboxyfluorescein (FAM). After amplicon purification, these were digested in two separate preparations with TaqI (Fermentas, Thermo Fisher Scientific, Waltham, MA) and HaeIII (New England BioLabs, Ipswich, MA) for 2 h in a thermocycler at 65 and 37°C, respectively. The digests were purified with DyeEx columns (Qiagen, Hilden, Germany). The T-RFLP run was conducted on an ABI 3730 DNA analyzer (Applied Biosystems, Foster City, CA) as described by Pilloni et al. (14). The electropherograms were processed with the PeakScanner (version 1.0) and GeneMapper (version 4.0) programs (Applied Biosystems, Foster City, CA). Further analysis was performed with the T-REX online software (35) using the default settings. Noise filtering was done on the basis of the peak height with the standard deviation multiplier set to 1. Terminal restriction fragments (T-RFs) were defined by aligning peaks with a clustering threshold of 1 bp. T-RF affiliation in dual fingerprints was assigned via searching TaqI digests first for database entries with a corresponding *in silico* T-RF. If multiple entries with potentially matching T-RFs were present, the corresponding data from the HaeIII digests were used for unambiguous T-RF affiliation to a defined FAE gene lineage.
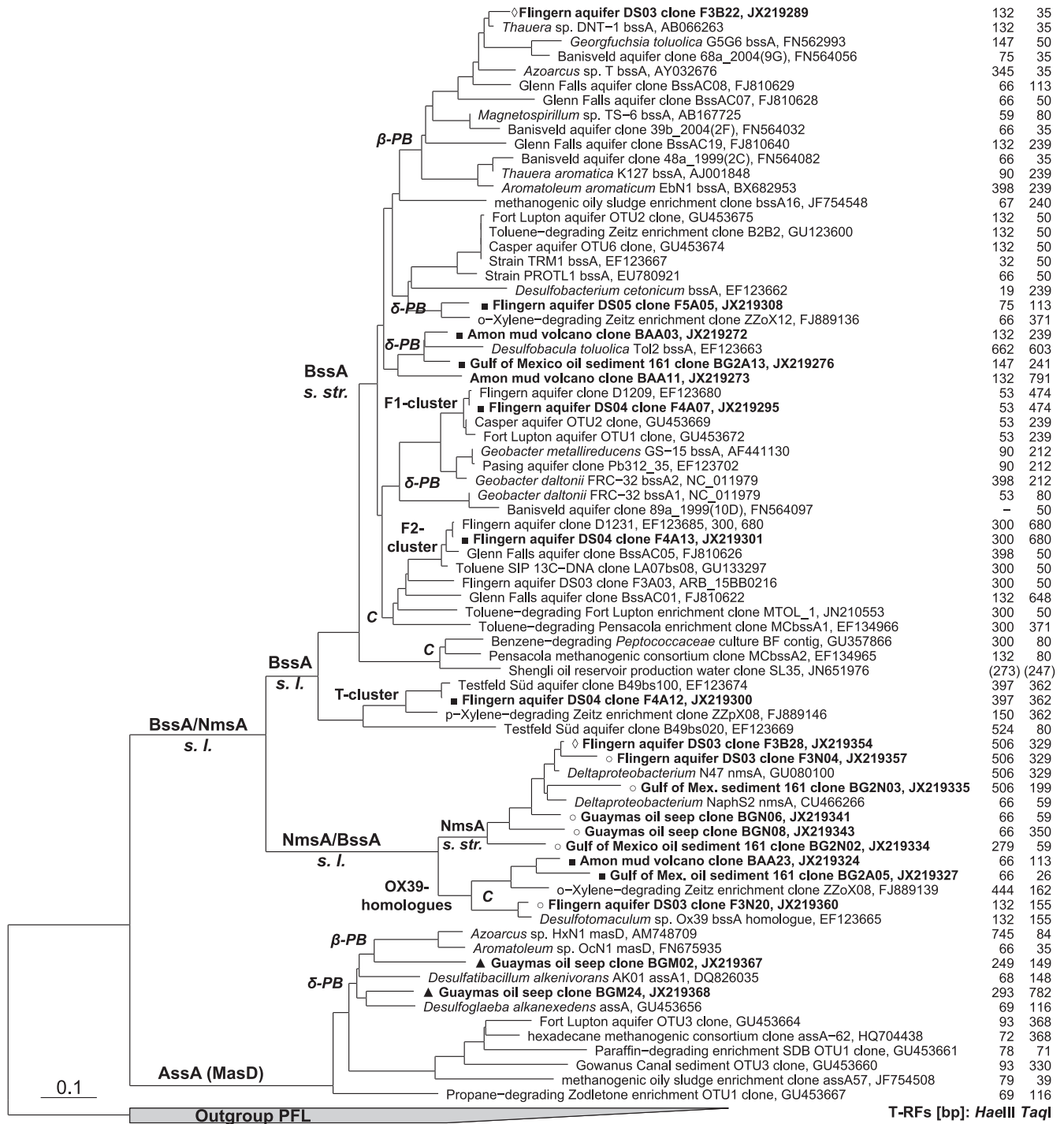
Cloning and sequencing. Selected purified, nonlabeled FAE gene amplicons were cloned with a TOPO-XL kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The sequencing of the Flingern samples (primer *bssA*, 52°C, all three depths; primer sets FAE-B and FAE-N, only the 6.85-m depth; 30 clones were picked per five samples) was performed on an ABI 3730 DNA analyzer (Applied Biosystems, Foster City, CA) as described by Winderl et al. (6). Samples of Amon mud volcano (*bssA* primers, 58°C), Gulf of Mexico hydrocarbon seeps (*bssA* and FAE-N primers, 58°C), and Guaymas hydrocarbon seeps (with primer sets FAE-N and FAE-KM) were sequenced by GATC (Konstanz, Germany). FAE gene tree reconstruction was done in ARB on the basis of an amino acid alignment via a Phylip distance matrix with the Fitch algorithm as described by Winderl et al. (6).

Nucleotide sequence accession numbers. The sequences were deposited with GenBank under accession numbers JX219271 to JX219368.

RESULTS

Establishment of optimized primer sets. No universally conserved protein motif that would allow the development of a single, universal primer pair was found across the different FAE gene lineages (shown in Fig. 1). Therefore, several primer sets called FAE-B, FAE-N, and FAE-KM, shown in Table 1, were designed *in silico* to optimally target an overlapping FAE gene region for robust phylogenetic comparison. All newly developed forward primers target sequence motifs not targeted by available assays prior to this study. The new FAE-B forward primer was predicted to target betaproteobacterial *bssA*, the recently discovered clostridial *bssA* homologues (F2 cluster, strain BF), as well as *nmsA* homologues (strain N47, NaphS strains). The two new FAE-N forward primers specifically target a sequence motif found only in *nmsA* of strain N47 and NaphS strains 400 bp upstream of the other forward primer sites. This primer set was aimed to distinguish *nmsA* sensu stricto amplicons from the homologous *bssA*-*nmsA* sensu lato amplicons retrieved by set FAE-B. The new FAE-KM set contained two different forward primer combinations: forward primer combination FAE-K to target sequences related to the *assA* genes of *Desulfatibacillum alkenivorans* and forward primer FAE-M to target relatives of strain HxN1 and OcN1 *masD*. FAE-K forward primers consisted of two different primers with two positions, instead of one with two ambiguity codes, to avoid four possible homopolymers and a subsequent loss of specificity. The primer sets FAE-B, FAE-N, and FAE-KM shared the same modified reverse primer 8543r, as the targeted sequence motif is conserved in all known FAE genes. This is documented for other, similar primers targeting this region (5, 6, 36) (see Table S1 in the supplemental material).

Primer performance in pure cultures and environmental samples. The lineage-specific FAE gene performance of the different primer sets predicted *in silico* was verified, wherever possible, using pure-culture DNA, as shown in Table 2. Our previous *bssA* primer set (6) did not amplify FAE genes of *Desulfitobacterium aromaticivorans* UKTL, clostridial strain BF, strain N47, *Desulfitobacterium alkenivorans* PF2803, or *D. aliphaticivorans* CV2803. Apart from the *bssA* gene of *Azoarcus* sp. T, the new primer set FAE-B did indeed amplify the FAE genes of these selected clostridial strains and also *nmsA* of strain N47. As intended, set FAE-N amplified only *nmsA* of strain N47. Primer set FAE-KM amplified only *assA* of *D. alkenivorans* PF2803 and *D. aliphaticivorans*

	T-RFs [bp]:	HaeIII	TaqI
◇**Flingern aquifer DS03 clone F3B22, JX219289**		132	35
Thauera sp. DNT−1 bssA, AB066263		132	35
Georgfuchsia toluolica G5G6 bssA, FN562993		147	50
Banisveld aquifer clone 68a_2004(9G), FN564056		75	35
Azoarcus sp. T bssA, AY032676		345	35
Glenn Falls aquifer clone BssAC08, FJ810629		66	113
Glenn Falls aquifer clone BssAC07, FJ810628		66	50
Magnetospirillum sp. TS−6 bssA, AB167725		59	80
Banisveld aquifer clone 39b_2004(2F), FN564032		66	35
Glenn Falls aquifer clone BssAC19, FJ810640		132	239
Banisveld aquifer clone 48a_1999(2C), FN564082		66	35
Thauera aromatica K127 bssA, AJ001848		90	239
Aromatoleum aromaticum EbN1 bssA, BX682953		398	239
methanogenic oily sludge enrichment clone bssA16, JF754548		67	240
Fort Lupton aquifer OTU2 clone, GU453675		132	50
Toluene−degrading Zeitz enrichment clone B2B2, GU123600		132	50
Casper aquifer OTU6 clone, GU453674		132	50
Strain TRM1 bssA, EF123667		32	50
Strain PROTL1 bssA, EU780921		66	50
Desulfobacterium cetonicum bssA, EF123662		19	239
■ **Flingern aquifer DS05 clone F5A05, JX219308**		75	113
o−Xylene−degrading Zeitz enrichment clone ZZoX12, FJ889136		66	371
■ **Amon mud volcano clone BAA03, JX219272**		132	239
Desulfobacula toluolica Tol2 bssA, EF123663		662	603
■ **Gulf of Mexico oil sediment 161 clone BG2A13, JX219276**		147	241
■ **Amon mud volcano clone BAA11, JX219273**		132	791
Flingern aquifer clone D1209, EF123680		53	474
■ **Flingern aquifer DS04 clone F4A07, JX219295**		53	474
Casper aquifer OTU2 clone, GU453669		53	239
Fort Lupton aquifer OTU1 clone, GU453672		53	239
Geobacter metallireducens GS−15 bssA, AF441130		90	212
Pasing aquifer clone Pb312_35, EF123702		90	212
Geobacter daltonii FRC−32 bssA2, NC_011979		398	212
Geobacter daltonii FRC−32 bssA1, NC_011979		53	80
Banisveld aquifer clone 89a_1999(10D), FN564097		−	50
Flingern aquifer clone D1231, EF123685, 300, 680		300	680
■ **Flingern aquifer DS04 clone F4A13, JX219301**		300	680
Glenn Falls aquifer clone BssAC05, FJ810626		398	50
Toluene SIP 13C−DNA clone LA07bs08, GU133297		300	50
Flingern aquifer DS03 clone F3A03, ARB_15BB0216		300	50
Glenn Falls aquifer clone BssAC01, FJ810622		132	648
Toluene−degrading Fort Lupton enrichment clone MTOL_1, JN210553		300	50
Toluene−degrading Pensacola enrichment clone MCbssA1, EF134966		300	371
Benzene−degrading *Peptococcaceae* culture BF contig, GU357866		300	80
Pensacola methanogenic consortium clone MCbssA2, EF134965		132	80
Shengli oil reservoir production water clone SL35, JN651976		(273)	(247)
Testfeld Süd aquifer clone B49bs100, EF123674		397	362
■ **Flingern aquifer DS04 clone F4A12, JX219300**		397	362
p−Xylene−degrading Zeitz enrichment clone ZZpX08, FJ889146		150	362
Testfeld Süd aquifer clone B49bs020, EF123669		524	80
◇ **Flingern aquifer DS03 clone F3B28, JX219354**		506	329
○ **Flingern aquifer DS03 clone F3N04, JX219357**		506	329
Deltaproteobacterium N47 nmsA, GU080100		506	329
○ **Gulf of Mex. sediment 161 clone BG2N03, JX219335**		506	199
Deltaproteobacterium NaphS2 nmsA, CU466266		66	59
○ **Guaymas oil seep clone BGN06, JX219341**		66	59
○ **Guaymas oil seep clone BGN08, JX219343**		66	350
○ **Gulf of Mexico oil sediment 161 clone BG2N02, JX219334**		279	59
■ **Amon mud volcano clone BAA23, JX219324**		66	113
■ **Gulf of Mex. oil sediment 161 clone BG2A05, JX219327**		66	26
o−Xylene−degrading Zeitz enrichment clone ZZoX08, FJ889139		444	162
○ **Flingern aquifer DS03 clone F3N20, JX219360**		132	155
Desulfotomaculum sp. Ox39 bssA homologue, EF123665		132	155
Azoarcus sp. HxN1 masD, AM748709		745	84
Aromatoleum sp. OcN1 masD, FN675935		66	35
▲ **Guaymas oil seep clone BGM02, JX219367**		249	149
Desulfatibacillum alkenivorans AK01 assA1, DQ826035		68	148
▲ **Guaymas oil seep clone BGM24, JX219368**		293	782
Desulfoglaeba alkanexedens assA, GU453656		69	116
Fort Lupton aquifer OTU3 clone, GU453664		93	368
hexadecane methanogenic consortium clone assA−62, HQ704438		72	368
Paraffin−degrading enrichment SDB OTU1 clone, GU453661		78	71
Gowanus Canal sediment OTU3 clone, GU453660		93	330
methanogenic oily sludge enrichment clone assA57, JF754508		79	39
Propane−degrading Zodletone enrichment OTU1 clone, GU453667		69	116

0.1

Outgroup PFL

FIG 1 Current overview of FAE gene phylogeny and affiliation of clones detected in this study. Sequences retrieved in this study are in bold and marked with different symbols according to the primer sets used: ◇, set FAE-B; ■, *bssA* at 52°C; ○, set FAE-N; ▲, set FAE-KM. Abbreviations of FAE lineages (as identified via enrichments or pure cultures): C, *Clostridia*; β-P, *Betaproteobacteria*; δ-P *Deltaproteobacteria*. The numbers to the right give the T-RFs predicted *in silico* for amplicons generated with the 8543r primer and a digest with HaeIII or TaqI. The T-RF sizes in parentheses are tentatively extrapolated, as some sequence entries end before the primer site. s. str. sensu stricto; s. l., sensu lato; PFL pyruvate formate lyase.

CV2803. A combination of all possible forward primers with one reverse primer in a single PCR failed to produce amplicons.

Subsequently, the performance of the primer sets was tested with samples originating from different hydrocarbon-degrading enrichments or impacted environmental systems. The samples in

Table 3 were chosen as they were exposed to and actively degrading different aliphatic or aromatic hydrocarbons: single compounds (enrichments), confined mixtures (e.g., natural gas at marine seeps), or complex mixtures of hydrocarbons (oil, asphalt). According to the known hydrocarbon substrates of those samples

TABLE 2 Experimentally tested performance of different FAE gene-targeted primers in selected strains and enrichments degrading hydrocarbons via fumarate addition

Gene	FAE lineage	Culture	Substrate	Reference	Amplicon production with primer set[a]:			
					bssA	FAE-B	FAE-N	FAE-KM
bssA sensu lato	*Proteobacteria*	*Azoarcus* sp. T	Toluene	37	+	+	−	−
		Geobacter metallireducens GS-15	Toluene	38	+	−	−	−
		Desulfotignum toluenicum H3	Toluene	39	+	−	−	−
	Clostridia	*Desulfosporosinus* sp. enrichment	Toluene	15	+	+	−	−
		Desulfitobacterium aromaticivorans UKTL	Toluene	18	−	+	−	−
		Strain BF	Benzene	19	−	+	−	−
nmsA	*Proteobacteria*	Strain N47	2-Methyl-naphthalene	21	−	+	+	−
assA		*Desulfatibacillum alkenivorans* PF2803	Alkanes	40	−	−	−	+
		Desulfatibacillum aliphaticivorans CV2803	Alkanes	41	−	−	−	+

[a] +, a clear amplicon with the correct size was observed; −, no amplicon was obtained.

and the potential FAEs supposedly involved in their degradation, we predicted expected FAE gene lineages to be recovered by our primer sets: (i) *bssA-nmsA* sensu lato amplified by primers *bssA* and FAE-B, (ii) *nmsA* sensu stricto amplified by primer set FAE-N, and (iii) *assA* amplified by primer set FAE-KM (Fig. 1). The results are presented in Table 3.

For samples exposed to confined mixtures of hydrocarbons, like those from the mud volcanoes and the Nyegga methane seeps,

amplicons were obtained only for single FAE gene lineages. In contrast, several major FAE gene lineages were obtained for samples exposed to a broader mixture of hydrocarbons. For example, all expected FAE lineages were detected for the Gölzau enrichments and respective substrates, but also additional catabolic marker lineages were always present. Multiple FAE gene lineages were also detected at the Flingern aquifer and the Gulf of Mexico hydrocarbon seep, while only both *nmsA* sensu stricto and *assA*

TABLE 3 Verification of FAE assay performance with DNA from selected hydrocarbon-impacted enrichments and environmental samples

Sample	Region	Reference(s)	Environment, sample type	Substrate(s)	Expected FAE lineage	Results[a]			
						bssA-nmsA sensu lato		*nmsA* sensu stricto with FAE-N	*assA* with FAE-KM
						bssA	FAE-B		
Paclele Mici mud volcano	Romania	28	Terrestrial, enrichment	Hexadecane	*assA*	−	−	−	+
Amon mud volcano	Mediterranean Sea, Nile deep-sea fan	42	Marine, sediment	Methane, ethane, propane, butane	*assA*-like?	+[b]	−	−	−
Nyegga methane seeps	Norwegian continental ridge	32	Marine, sediment	Methane, natural gas	*assA*-like?	−	−	−	+
Chapopote asphalt volcano station GeoB106-17-6	Gulf of Mexico	42, 43	Marine, sediment	Asphalt, alkanes, aromatics, methane	FAE	+	+	−	+
Hydrocarbon seep station 161	Gulf of Mexico	30, 42	Marine, sediment	Oil, asphalt, alkanes, aromatics	FAE	+[b]	+[b]	+[b]	+
Guaymas Basin hydrocarbon seep	Gulf of California	42	Marine, sediment	Methane, alkanes, aromatics	FAE, especially *assA*	−	−	+[b]	+[b]
Gölzau aquifer, former refinery	Germany	29	Terrestrial, enrichment	Toluene	*bssA*	+	−	+	−
				Butane	*assA*	+	+	+	+
				Methylnaphthalene	*nmsA*	+	−	+	−
Flingern aquifer, former gas work site	Germany	25	Terrestrial, sediment	BTEX, PAH[c]	*bssA-nmsA* sensu lato	+[b]	+[b]	+[b]	+[d]

[a] +, a clear amplicon with the expected size was observed; −, no amplicon.
[b] Amplicons were actually cloned and sequenced as displayed in Fig. 1.
[c] PAH, polycyclic aromatic hydrocarbon.
[d] Forty PCR cycles were necessary to obtain an amplicon.
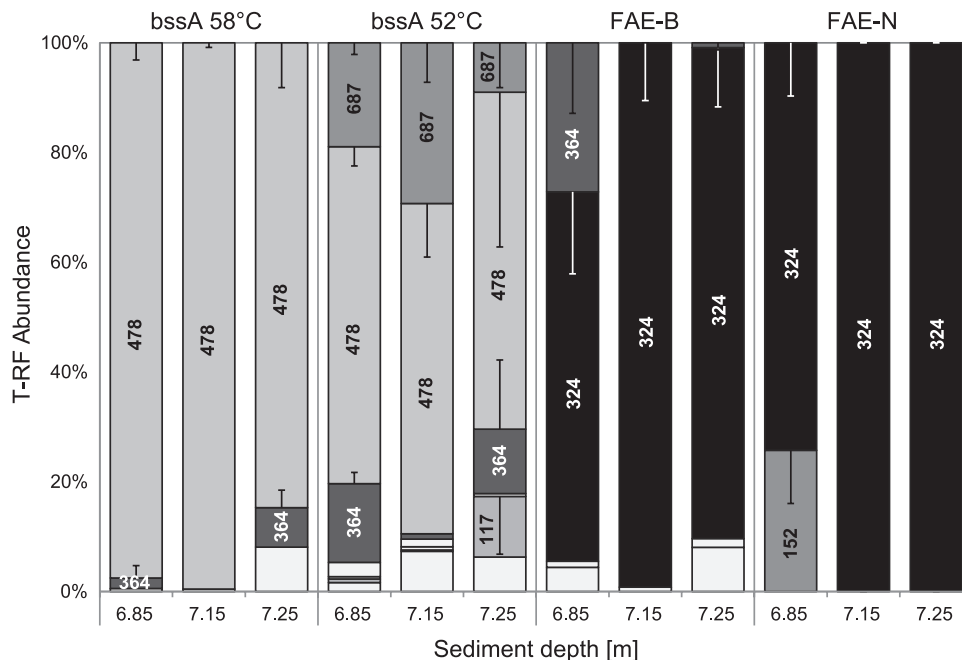
FIG 2 Comparative FAE gene T-RF retrieval from distinct depths of the tar oil-contaminated Flingern aquifer with different assays. Distinct T-RF sizes (bp) are indicated by gray scales and labeling within bars and correspond to the FAE gene lineages identified in Table 4. T-RF abundance is averaged over results from four independent DNA extractions; error bars represent standard deviations and are partially shown only as negative or only as positive standard deviations, when the graphical layout allows.

could be detected in the Guaymas Basin hydrocarbon seep sediments.

In order to substantiate the amplicon-based results, selected samples were chosen for sequencing in small clone libraries: Amon mud volcano (6 FAE sequences out of 25 clones with *bssA* primers, 58°C), the Gulf of Mexico hydrocarbon seep (9 FAE sequences out of 25 clones with *bssA* primers, 58°C; 1 FAE sequence out of 25 clones with FAE-B primers; and 6 FAE sequences out of 25 clones with FAE-N primers), and the Guaymas Basin hydrocarbon seep (8 FAE sequences out of 25 clones with FAE-N primers and 2 FAE sequences out of 25 clones with FAE-KM primers). The clones (Fig. 1) were clearly affiliated with the expected FAE lineages; the rest of the clone libraries were sequences that resulted from unspecific amplification. Amon mud volcano clones were mostly related to *Desulfobacula toluolica bssA*, with one assigned to more deeply branching *nmsA* homologues. Guaymas Basin hydrocarbon seep clones generated with set FAE-KM were related to betaproteobacterial *masD* and *Desulfoglaeba alkanexedens assA* sequences. From all three sites, clones related to *nmsA* sensu stricto of NaphS strains (all from set FAE-N) or *bssA* homologues were retrieved with primer sets FAE-N, FAE-B, and *bssA* at 52°C.

Hidden FAE gene lineages revealed by dual-digest T-RFLP analysis in the tar oil-contaminated Flingern aquifer. A more extensive assay performance test and sequencing-independent T-RFLP-based FAE gene lineage identification were conducted with sediment from the well-investigated tar oil-contaminated aquifer in Flingern (6, 44). This aquifer is known to host two specific *bssA* populations at the lower plume fringe, the hot spot of sulfidogenic BTEX degradation (26, 27): dominating *Desulfobulbaceae* F1-cluster *bssA* (6, 14) and the less abundant clostridial F2-cluster *bssA* (6, 15). As the sediment studied here is from a later sampling period (2009), we could not be sure whether to expect the same

bssA populations. We have previously introduced the use of a *bssA*-based T-RFLP fingerprinting system allowing sequencing-independent lineage identification at reasonable confidence from samples whose sequences are known from prior clone library analysis (15). However, several different *bssA* lineages were observed to display identical T-RFs with TaqI restriction (Fig. 1; e.g., a 50-bp T-RF for certain *Clostridia*, *Betaproteobacteria*, and *Deltaproteobacteria*). For better diagnostic confidence, we now introduce a second, parallel digest with HaeIII, in addition to TaqI, to unambiguously identify T-RF.

The application of the different primer sets and TaqI-digested T-RFLP for the three chosen depths from the Flingern aquifer revealed the presence of a number of tentative *bssA* T-RFs, as shown in Fig. 2. The identity of those *bssA* lineages was assigned via the T-RF sizes predicted for sequences in our ARB database and further confirmed by parallel HaeIII digest as well as, for proof of principle, by cloning and sequencing (Table 4 and Fig. 1; HaeIII digest raw data not shown). With the standard *bssA* primers and settings, the *Desulfobulbaceae* F1-cluster *bssA* T-RF (478 bp, TaqI) clearly dominated over the three chosen depths, as was expected from previous work (6, 15). However, already at a lowered annealing temperature for reduced PCR selectivity (15), a T-RF representing the clostridial F2-cluster *bssA* (687 bp, TaqI) and the unidentified Testfeld Süd T1-cluster (6) *bssA* homologues (364 bp, TaqI) became detectable. By applying the new FAE-B primer set, strain N47 *nmsA*-like sequences (324 bp, TaqI) became detectable at all three investigated depths, while the F1 and F2 clusters disappeared. With the FAE-N primers, it was, furthermore, possible to detect a T-RF (152 bp, TaqI) at the 6.85-m depth tentatively assigned to the *nmsA*-associated *bssA* homologue previously detected for *Desulfotomaculum* sp. strain OX39.

On the basis of previous FAE gene sequence data from the site

TABLE 4 Sequencing-independent identification of T-RF lineages from Flingern sediment via dual-digest T-RFLP[a]

Observed fragment size (bp)		In silico fragment size[b] (bp)		Candidate lineage	No. of FAE clones	Primer sets
TaqI	HaeIII	TaqI	HaeIII			
364	398	362	397	Unidentified *bssA* homologues, unaffiliated (Testfeld Süd T1)	8	*bssA*, 52°C; *bssA*, 58°C; FAE-B
478	51	474	52	*Deltaproteobacteria bssA*, *Desulfobulbaceae* (F1 cluster)	14	*bssA*, 52°C; *bssA*, 58°C
678	304	670	300	Clostridial *bssA Desulfosporosinus* sp. related (F2 cluster)	22	*bssA*, 52°C
324	505	329	506	*Deltaproteobacteria nmsA*, *Desulfobacteraceae* (N47 related)	18	FAE-B, FAE-N
152	130	155	132	Clostridial *bssA* homologues, *Desulfotomaculum* related	1	FAE-N
	132	35	132	*Betaproteobacteria bssA*, *Thauera* sp. strain DNT-1	2	FAE-B
117	76	113	75	*Deltaproteobacteria bssA*, *Desulfobacterium cetonicum bssA*	1	*bssA*, 52°C

[a] Observed T-RFs from the TaqI digest are also shown in Fig. 2; data for the HaeIII digest are not shown.
[b] In silico T-RFs are given for the labeled 8543r primer; hence, a difference of 3 bp must be considered for T-RFs delivered by the classical 8546r-labeled primer pair, as shown in Fig. 2.

(6), one minor T-RF (117/76 bp, TaqI/HaeIII), found with FAE-B primers at 7.25 m, was not assignable *in silico* and was thus identified by cloning and sequencing to represent a new relative of *Desulfobacterium cetonicum bssA* (67% amino acid sequence identity, clone F5A05; Fig. 1). Another FAE population became detectable at 6.85 m with primer set FAE-B (35-bp T-RF), which was revealed by HaeIII digestion and cloning to represent betaproteobacterial *bssA* (Table 4; clone F3B22 in Fig. 1). This population was missed in standard TaqI-digested fingerprints, as such short fragments (<50 bp) are usually not considered since they cannot be effectively differentiated from primer-dimers and other short artifacts usually observed in T-RFLP raw data. All expected HaeIII T-RFs (summarized in Table 4), corresponding to the primary TaqI T-RFs in Fig. 2 and as predicted via clones from the same samples (Fig. 1), were readily detectable (HaeIII data not shown). However, recovery of comparative T-RF abundance in both restriction assays was not semiquantitative, as illustrated for one Flingern sample in Fig. 3.

DISCUSSION

The present study focuses on functional gene analyses for degrader detection at contaminated sites. Our new primer sets are
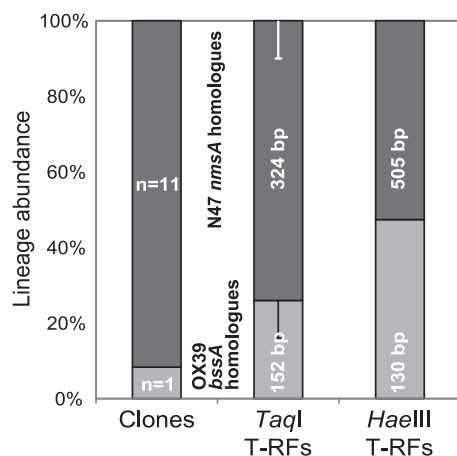
FIG 3 Exemplary comparison of semiquantitative FAE gene lineage recovery in Flingern sediments (depth, 6.85 m) retrieved with the FAE-N primer set via different methods: cloning and sequencing (a total of 12 clones) as well as T-RFLP analyses with the different restriction enzymes. Dark gray bars, abundance of FAE gene sequence types affiliated with N47 *nmsA*; light gray bars, the abundance of those affiliated with OX39 *bssA* homologues.

able to survey directly and rapidly (in fingerprinting) the structure of natural FAE gene pools in hydrocarbon-degrading systems. Among all described anaerobic hydrocarbon degradation pathways, activation by fumarate addition is presently one of the most intensively investigated. Other pathways, such as oxygen-independent hydroxylation or carboxylation, either are known for only a small selection of substances (2) or are just about to be understood biochemically (45, 46). Thus, at present, FAE genes can be regarded as the most comprehensive genetic marker for investigating anaerobic hydrocarbon degraders and degradation in the environment.

Assay performance in pure cultures and environmental samples. Our tests for FAE lineage detectability at the amplicon level (Tables 2 and 3) clearly show that major FAE gene populations are not detectable with the established *bssA* primer set alone. Thus, not all catabolic potentials for fumarate addition can be detected with this assay, as we demonstrate for terrestrial and marine samples. The clones retrieved in this study prove that the simultaneous application of our new suite of primers is able to recover a far wider range of proteobacterial and clostridial FAE gene lineages than previously described. Furthermore, our primer suite now allows a robust phylogenetic comparison of all generated amplicons. This was our main motivation to introduce the new FAE-KM primer set for *assA* detection, since the primary *assA* primers published by Callaghan et al. (5) generate FAE amplicons only partially overlapping with our other *bssA* and homologous genes.

In part, our novel primer sets are based on a combination of different forward primers, which may potentially complicate the interpretation of amplification results. Yet, mixing of different primers is commonly used in multiplex PCR (47). The application of such methods is not only common to the analysis of nutritional or clinical samples but also established for the analysis of complex environmental samples (e.g., see references 48 and 49). For example, Knaebel and Crawford (50) employed multiplex primers for the detection of aerobic functional genes. There, amplification was shown to be very sensitive but not linear for all template ratios. Still, the influence of sample origin, the targeted gene, and inhibitors from the sampled material was shown to introduce considerably greater bias to PCR results (e.g., on template ratios and detectability) than the influence of the use of multiple primers. Because of this, we consider the introduction of multiple forward primers in some sets to be connected to an acceptable bias, espe-

cially since catabolic gene analyses and fingerprinting are already known to be much less robust in a semiquantitative manner than analyses with ribosomal markers (51).

Another parameter of concern was the low frequency of actual FAE gene clones observed, especially for the libraries from the marine sites. Nonintended inserts were mostly shorter primer concatemers or other PCR artifacts. With Flingern aquifer DNA, the yield of good clones was always substantially higher (between 40 and 60%). This is why we confined the verification of our T-RFLP diagnostics to the Flingern samples, as PCR artifacts certainly complicate the interpretation of T-RFLP fingerprints. However, other studies have also reported low FAE sequence yields and cloning efficiencies for certain sample types (5, 6), which may indicate general problems in applying these degenerate primers to PCRs with complex environmental DNA. Here, a possible remedy would be the introduction of cloning-independent, high-throughput next-generation pyrotag sequencing of FAE amplicons, which would still produce sufficient amounts of good reads, even if short reads from biases in pyrotag amplification were abundant. Nevertheless, it was already shown by Winderl et al. (6) that the use of degenerate primers is important for finding previously undetected novel FAE gene lineages. This is a prerequisite for any subsequent design of lineage-specific qPCR assays (44), potentially even allowing a more quantitative inference of *in situ* biodegradation rates based on the degraders themselves (e.g., see reference 22).

Degrader diversity and on-site hydrocarbon degradation processes. As the present study was entirely based on gene detection, assumptions on actually ongoing hydrocarbon-degrading processes in the examined samples are not straightforward. Functional implications may have been stronger for transcript detection, but respective attempts were unsuccessful and indicate general problems with extraction of mRNA from these anaerobic systems.

Several distinct FAE gene lineages were detected in the Gölzau enrichments, even though they had been cultivated on monosubstrates for over a year. This either suggests a surprising diversity of catabolic potentials involved in substrate degradation in these enrichments or indicates a memory effect from initially diverse degrader populations *in situ*. At present, a more detailed investigation of the catabolic genes and potentials in the Gölzau samples is still under way.

Assumptions on ongoing hydrocarbon-degrading processes are also not facile for the gas seepage sites. The occurrence of hydrocarbons ($>C_4$) other than natural gas is known for the Paclele Mici mud volcano (28), but for the Amon mud volcano and Nyegga methane seeps, there have been no assessments for the presence of hydrocarbons higher than C_4 to date (32, 33, 52). While the detection of putative *assA* homologues is expected for natural gas seepages due to the degradation of short-chain alkanes via fumarate addition (5, 53), the discovery of only *bssA* genes related to *Desulfobacula toluolica* in Amon mud volcano sediments was unexpected. This could imply two different scenarios: (i) the microbes carrying this *bssA* sequence type may actually be involved in the degradation of short-chain alkanes, despite the phylogenetic placement of their marker genes with toluene-degrading *bssA* sensu stricto, or (ii) the detection of these FAE gene types could be attributed to the actual presence of aromatic hydrocarbons via carryover from nearby oil deposits, i.e., by gas ebullition, as is known for the Amon mud volcano's neighbor

mud volcano Isis (54). The latter would imply that the true alkane-catabolizing gene markers at the site remained undetected, despite our comprehensive parallel PCR screening. Although FAE substrate specificity for methylated aromatics and nongaseous alkanes does not usually overlap among degrader isolates (55), this may still be possible for short-chain alkanes like butane or propane, which would favor the first scenario.

Finally, although our FAE-B primer set does detect a selection of several clostridial *bssA* genes and homologues *in silico* as well in pure-culture screenings, we were able to find the clostridial F2-cluster *bssA* sequences only in Flingern sediments using our classical *bssA* primer pair at a lowered annealing temperature. It seems that the FAE-B primer set has, despite its proven ability to amplify clostridial *bssA* in pure-culture DNA, a bias for *nmsA* in environmental samples, as observed with T-RFLP data retrieved from Flingern (Fig. 2). A lowered annealing temperature, as also successfully employed for extending the coverage of the *bssA* primer set, may help to overcome this limitation and is currently being tested.

Novel *nmsA* sequences found in different environments. With our new primer sets FAE-B and FAE-N, we provide the first evidence that *nmsA* and homologous sequences can be detected in the environment, regardless of sample origin. This raises a question about the relevance of the respective catabolic pathway: addition of fumarate to methylnaphthalene. Recent work by Oka et al. (56) showed metabolites of 2-methylnaphthalene carboxylation to be present at a contaminated aquifer. Additionally, methylation and subsequent fumarate addition were first presumed to be the degradation pathway for naphthalene (57). However, recent results suggest that carboxylation (46) is the principal degradation mechanism in *nmsA*-containing strain N47 (21), abating the potential for *nmsA* to be a marker for naphthalene degradation. DiDonato et al. (58) found that fumarate addition to 2-methylnaphthalene and toluene is possible with NMS alone for strain NaphS2. Therefore, the *nmsA* sequences retrieved in this study could potentially play a role not only in 2-methylnaphthalene catabolism but also in toluene degradation. This concept may also help to better explain the phylogenetic placement of the *bssA* homologue previously retrieved from the toluene-degrading clostridial strain OX39 (6), which does not degrade 2-methylnaphthalene (59). Yet, its *bssA*-homologous sequence is untypically placed with respect to the now widely documented typical clostridial *bssA* sequence type (F2 cluster; Fig. 1). Thus, the new sequences related to OX39 that were found (Amon mud volcano clone BAA23, Gulf of Mexico sediment clone BG2A05, and Flingern aquifer clone F3N20) may also be also monoaromatics-utilizing degraders of clostridial affiliation.

Hidden lineages revealed by sequencing-independent T-RFLP screening in Flingern sediment. Our results for the Flingern samples demonstrate, in contrast to previous findings (6), that there are indeed different intrinsic FAE gene populations to be found in this contaminated aquifer. The apparent dominance of the *Desulfobulbaceae* F1-cluster *bssA* reported previously may thus be an effect of PCR selection. We show that the clostridial F2-cluster *bssA* sequences are not consistently detected over the three selected depths by our standard *bssA* primer set (58°C), while putative toluene degraders related to *Desulfosporosinus* spp. have been identified in sediments from the same site sampled in 2006 (14) and are abundant in 16S rRNA pyrotag libraries of sediments taken in 2009 (25).

The identification of degrader lineages on the basis of T-RFLP results was straightforward at the Flingern site, as there are already ample *bssA* sequence data available from that site (6, 44). We cannot claim that our new approach has an absolute diagnostic capability; sequencing will still be necessary to identify FAE lineages for sites with no *a priori* sequence information available. Nevertheless, the double digest can add diagnostic security in case of overlapping T-RF sizes for unrelated FAE lineages (e.g., an HaeIII T-RF of 132 bp or a TaqI T-RF of 50 bp could both represent distinct beta- and deltaproteobacterial as well as clostridial *bssA* lineages, as illustrated in Fig. 1), where dual-digest results can mutually exclude options. Thus, the double-digest T-RFLP described here can be used as a tool for monitoring FAE gene populations at sites with minimized sequencing effort.

Conclusions. In essence, we demonstrate that the simultaneous use of parallel FAE primer sets is necessary to recover a wide diversity of FAE gene lineages in hydrocarbon-impacted environmental systems. Via amplicon screening as well as sequencing-independent T-RFLP diagnostics, we reveal that distinct FAE gene lineages may be missed with available *bssA* primer sets, especially clostridial *bssA* and proteobacterial *nmsA* homologues. The detection of these additional degrader populations in distinct hydrocarbon-impacted systems allows extending our grasp of the biodiversity and ecology of bacteria involved in anaerobic hydrocarbon degradation in the environment, which is relevant for a better understanding of natural attenuation processes.

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