Combined Application of PCR-Based Functional Assays for the Detection of Aromatic-Compound-Degrading Anaerobes

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Received 15 February 2011/Accepted 11 May 2011

To explore the reliability of assays that detect aromatic-compound-degrading anaerobes, a combination of three functional-gene-targeting assays was applied to microcosms from benzene-contaminated aquifers. Results of the assays were consistent and suggest that species related to the genera Azoarcus and Geobacter dominated benzene degradation at the individual sites.

Aromatic compounds comprise many highly toxic and persistent contaminants in aquatic and terrestrial environments. Key reactions in the aerobic degradation of aromatic compounds, such as ring hydroxylation and ring cleavage, are catalyzed by mono- or dioxygenases. In contrast, under anoxic conditions, for example, in groundwater or sediments, microorganisms use a completely different set of enzymatic reactions for the catabolism of aromatic growth substrates (4, 6, 8).

In aromatic-compound-degrading anaerobes (ACDA), the majority of aromatic growth substrates, such as benzene, toluene, ethylbenzene, and xylenes (BTEX), are converted to the central intermediate benzoyl coenzyme A (CoA). In the past years, the anaerobic degradation pathways of toluene, xylenes, and ethylbenzene were characterized in some detail (10). Initial evidence has been obtained in recent studies that anaerobic benzene degradation proceeds via carboxylation to benzoic acid (1, 17). Independently of the initial activation reaction, the complete anaerobic degradation of benzene is accomplished in the so-called benzoyl-CoA degradation pathway (5, 7, 16, 18, 25). The upper part of this pathway comprises four enzymatic steps (Fig. 1), including the reductive dearomatization of benzoyl-CoA to cyclohexa-1,5-diene-1-carboxyl-CoA and the cleavage of the ring structure catalyzed by 6-oxocyclohex-1-ene-1-carboxyl-CoA hydrolase. The dearomatization step is catalyzed either by ATP-dependent class I benzoyl-CoA reductases (BCRs; in facultative anaerobes, encoded by bcrA to -D) (3) or by ATP-independent class II BCRs (in obligate anaerobes, encoded by bamB to -I) (13, 15).

PCR-based assays that target key enzymes of anaerobic aromatic degradation are valuable molecular tools for the culture-independent detection of ACDA. The limitations of the assays available for detecting functional marker genes of ACDA (Fig. 1) are discussed below.

bssA assay. The bssA-targeting assays detect the gene coding for the large subunit of benzyllsucinate synthases and probably related enzymes which catalyze the addition of the methyl group attached to an aromatic ring to fumarate (2, 14, 24). However, use of the bssA assays is limited to the detection of ACDA that degrade toluene and probably some related methylated aromatic compounds. Moreover, a recent study indicated that the bssA-targeting assay appears not to be applicable for all bssA-like enzyme-containing organisms (11).

bcr/bsd assays. With bcr/bsd-targeting PCR assays, subunits of class I BCRs from facultative ACDA were detected in environmental samples (12, 19), with bcr referred to as genes coding for the Thauera type and bsd referred to as genes coding for the Azoarcus type of BCRs. However, the use of the assays is problematic, as positive results were obtained with Geobacter species or sulfate-reducing bacteria that do not contain a class I BCR (12). Furthermore, class I BCRs were detected with this assay in organisms that are not known to degrade aromatic compounds under anoxic conditions (e.g., Pseudomonas or Bradyrhizobium sp.) (19).

bamA assay. The ring-cleaving hydrolase of the benzoyl-CoA degradation pathway is encoded by the bamA gene and appears to be present in all ACDA (14). Due to the high conservation of bamA genes on the nucleotide level, a single degenerate oligonucleotide primer set was designed which amplified a 300-bp fragment in some Gram-negative pure cultures (14). Very recently, Geobacter species and Georhizcula toluolica were detected in a leachate-contaminated aquifer near a landfill using a bamA-targeting assay (20). However, the applicability of this assay to Gram-positive ACDA has not been tested yet.

bamB assay. A PCR assay has recently been developed that targets the bamB gene coding for the active-site subunit of class II BCRs from obligate anaerobes (15). That study revealed that in many ACDA, including Gram positives, several bamB gene duplications that have diverged in sequence were present. The bamB-targeting assay has not been applied to environmental samples yet.

In conclusion, the assays available for functional genes from ACDA have their limitations, as they produced either false-negative or false-positive results or their application is restricted to the detection of physiological subgroups of ACDA. To provide a reliable and general PCR-based tool for detecting ACDA at environmental sites, the bcrC/bsdN- and bamA-targeting assays were modified and their reliability was verified with DNA from a number of Gram-negative and Gram-positive pure cultures. In a second step, a combination of
Improvement of bcrC/bzdN- and bamA-targeting assays. The primer pairs used throughout this study, including the modified bcrC/bzdN- and bamA-targeting assays, are listed in Table 1. The following PCR conditions for the primer sets were optimized empirically: 5 min at 95°C and 35 cycles of 30 s at 95°C; 30 s at 52°C (bamA-700), 54°C (bamA-800), or 70°C (bcrC/bzdN); 60 s at 72°C; and 10 min at 72°C.

The updated phylogenetic tree of bcrC/bzdN sequences shows the two subclasses of class I BCRs (Fig. 2). The new primer pair bzdNf/bzdNr for the γ subunit of the Azoarcus-type BCRs exclusively yielded a 700-bp amplicon using DNA from Azoarcus and Aromatoleum species as the template. In contrast, primer pair bcrCf/bcrCr, designed for detecting the γ subunit of the Thauera-type class I BCR, exclusively yielded approximately 800-bp amplicons with DNA from Thauera, Magnetospirillum, and Rhodopseudomonas species. In conclusion, both were specific for bcrC and bzdN, respectively.

In a previously designed assay, a 300-bp fragment of the bamA gene, referred to here as bamA-300, was amplified using the DNA of some facultative (Thauera aromatica, Azoarcus evansii, Aromatoleum aromaticum, and Magnetospirillum strains TS-6 and CC26) and obligate (Geobacter metallireducens, Syntrophus aciditrophicus, and Desulfococcus multivorans) ACDA as the templates (14). A slightly modified assay was used to detect Geobacter species and Georgfuchsia toluolicola in a leachate-contaminated aquifer (25). To explore the general applicability of the bamA-targeting assay, chromosomal DNA from Gram-negative (Desulfosarcina cetonica, Desulfosarcina ovala, and Desulfobacterium anulatum) and Gram-positive (Desulfotomaculum gibsoniae and Desulfotomaculum thermobenzoicum) sulfate-reducing ACDA without sequenced genomes served as the templates in PCRs (Table 1). While the DNA from all of the Gram-negative ACDA tested yielded the expected 300-bp DNA fragment, the assay failed to detect bamA from the Gram-positive sulfate-reducing strains. For this reason, two novel bamA-targeting primer sets were designed using the same forward oligonucleotide primer but different reverse oligonucleotide primers for PCRs. The two assays were highly selective for two phylogenetic subclusters of bamA and yielded ~700- and ~800-bp amplicons, respectively (Fig. 2), i.e., (i) the GMT (Geobacter, Magnetospirillum, and Thauera) cluster and (ii) the SA (Gram-negative/Gram-positive sulfate-reducing bacteria and Syntrophus, Azoarcus, and Aromatoleum) cluster. In all cases, the amplified bamA fragments obtained were verified by sequencing. No false-positive result was obtained with DNA from Geobacter sulfurreducens, Escherichia coli, or Desulfovibrio gigas.

In situ microcosms (BACTRAP) from test field sites. The combined application of the PCR-based assays was carried out using in situ microcosms (BACTRAP) incubated at two different tar oil- and BTEX-contaminated anoxic aquifers referred to as Gneisenau and BPP (benzene production plant). The microcosms were each deployed in a monitoring well of the individual contaminated aquifers for a period of 100 days at an 8-m depth. Preparation of the in situ microcosms was carried out as described previously (21). Bio-Sep beads (K. Sublette, University of Tulsa) were loaded with 13C-labeled benzene (≥99% 13C; Campro Scientific) or unlabeled benzene (100

FIG. 1. Degradation of selected aromatic compounds via the benzoyl-CoA degradation pathway and the availability of functional-gene-targeting assays. The bssA-targeting assay is applicable for detecting ACDA that degrade toluene and related alkylated aromatic compounds. The bcr and bzd assays target class I BCRs from facultatively anaerobic ACDA, and the bamB assay targets class II BCRs from obligately anaerobic ACDA. The bamA assay targets the ring-hydrolyzing enzyme present in all ACDA. Benzene is initially activated either via carboxylation to benzoate or via methylation to toluene; in each case, benzene will be degraded via enzymes of the benzoyl-CoA degradation pathway.
mg/g of beads) via gas phase under reduced pressure as explained elsewhere (9).

The anoxic aquifer at the Gneisenau site is located near a former coking plant in the vicinity of a coal mine in the Ruhr area (Germany). The hydrological environment was previously described (21). In situ microcosms were incubated in well P36F, located at the fringe of a BTEX plume in the quaternary anoxic aquifer. In the investigated monitoring well, the concentrations of organic contaminants were as follows: benzene, 1 µg liter⁻¹; toluene, <0.1 µg liter⁻¹; polycyclic aromatic hydrocarbons (PAH), <0.1 µg liter⁻¹. The concentrations of potential electron acceptors were as follows: sulfate, 497 mg liter⁻¹; nitrate, <0.5 mg liter⁻¹; total dissolved iron, 5 mg liter⁻¹. Elevated concentrations of the latter are indicative of bacterial reduction of Fe(III) to Fe(II). The potential for benzene and toluene degradation in the center and at the fringes of the contamination plume was demonstrated in previous experiments using BACTRAP microcosms (21). The second anaerobic aquifer was located close to a former BPP in the vicinity of a coal mine in the Ruhr area. In the monitoring well, the concentrations of organic contaminants were as follows: benzene, 1,040 µg liter⁻¹; toluene, <0.1 µg liter⁻¹; PAH, <0.1 µg liter⁻¹. The concentrations of potential electron acceptors were as follows: sulfate, 6 mg liter⁻¹; dissolved iron, 9 mg liter⁻¹; nitrate, <0.5 µg liter⁻¹.

**Functional marker genes/16S rRNA gene analyses in microcosms from benzene-contaminated aquifers.** The carbon isotope signatures of fatty acids (δ¹³C FA) extracted from the BACTRAPS amended with [¹³C]benzene were significantly enriched in δ¹³C: up to +35% at the Gneisenau site and up to +699% at the BPP site, demonstrating the assimilation of benzene by the microbial consortia established.

DNA was extracted from these microcosms according to a procedure described previously (23). PCR products obtained with the DNA from microcosms where cloned into E. coli JM109 (Promega) cells. For each sample, 25 to 45 clones were randomly selected. To compare the ACDA detected by the bamN, bamA, and bamB assays with the entire bacterial community in each microcosm, a 16S rRNA gene-targeting PCR assay was applied using the primer set UniBac27/UniV1492r (23).

**Gneisenau site.** The Gneisenau site was chosen as a test case for natural community analysis at low benzene concentrations. Using the bamB-targeting assay, nine different clones were obtained. Most of them were closely related to bamB homologs from Geobacter species (Fig. 2). The amplified DNA fragment from clone Gneisenau bamB 1 was related to a bamB gene from G. metallireducens (15), while the fragments of the bamB2-bamB9 clones were most similar to bamB genes from Geobacter daltonii and D. cetonica. These results indicate that various bamB genes from obligately anaerobic bacteria were present at the Gneisenau site.

Using the assays for genes encoding class I BCRs only with the bcrC assay but not with the bamN assay, a single fragment from a Thauera-type bcrC gene was obtained (Gneisenau bcrC 1; the highest identity to known bcrC sequences was 70%; Fig. 2). This result implies that Thauera-like facultative anaerobes containing a class I BCR are present at the Gneisenau site, albeit at much lower diversity than the Geobacter-like obligate anaerores responsible for aromatic-compound metabolism.

For the detection of bamA genes, the newly developed GMT and SA bamA cluster-targeting assays were applied. In accordance with the results obtained with the SA cluster-targeting assays (Fig. 2), the clones analyzed contained a DNA fragment assigned to the GMT cluster-targeting assays. A number of clones containing a bamA fragment of the GMT cluster were obtained with the highest similarities to the G. metallireducens species. The bamA fragment from the Gneisenau bamA 1 clone showed the highest sequence identity to that from G. metallireducens (88%), whereas the bamA fragments from the five additional clones were highly similar to each other, with the highest similarities to G. metallireducens (85 to 88%). Notably, no appropriate fragment was amplified with the SA cluster-targeting assay, although the SA cluster-targeting assay yielded clones with bamB amplicons most closely related to bamB from D. cetonica. The failure to identify bamA genes of the SA cluster demonstrates again that the bamB assay alone is not suitable for detailed phylogenetic assignments.

Using 16S rRNA gene-targeting PCR assays, almost 75% of the clones analyzed contained a DNA fragment assigned to the sequences of 16S rRNA genes 1 to 4, with the highest similarities (95% to 98% identity) to the 16S rRNA genes from Geobacter sp. strain Ply1 (gi146738018) and Geobacter psychrophilus strain P35 (gi50592582). However, these strains are not known to degrade aromatic compounds. The highest similarity of 16S rRNA genes from known ACDA to the corresponding gene from G. metallireducens was only 93%. Thus, the sequences identified may belong to a novel aromatic-compound-degrading species of the genus Geobacter. A minor part of the 16S rRNA gene clone library showed only poor similarity to known ACDA, with a maximal sequence identity to Azotobacter and Thauera species of 90.5% (16S rRNA gene clones 5 and 6). This finding is in agreement with the identification of the Gneisenau bcrC 1 DNA fragment, which was found to be

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**Table 1. Oligonucleotide primers used for PCR assays**

| Primer   | Gene target | Direction | Sequence (5'→3')                  | PCR assay | Reference |
|----------|-------------|-----------|-----------------------------------|-----------|-----------|
| bzdN     | bzdN        | Forward   | GAGCCGCGACATCTCCGGCAT             | bzdN      | This study |
| bzdNr    | bzdN        | Reverse   | TRTGYRCCGGRTARTCTTTGTCGG          | bzdN      |           |
| bcrCf    | bcrC        | Forward   | CGHATYCCRCGCTCGACCCTGCG          | bcrC      | This study |
| bcrCr    | bcrC        | Reverse   | CGGATCCGGCTGACCTGCGCC            |           |           |
| bamBf    | bamB        | Forward   | ATGMGGTAYSGASAGARACHGG           | bamB      | 1         |
| bamB     | bamB        | Reverse   | CSGCRWRYTTTCADYTCGG              |           |           |
| bamA-SP9 | bamA        | Forward   | CAGTACAAYTCTCATACAVACBG           | bamA-300  | 1         |
| bamA-ASP1| bamA        | Reverse   | CMATGCGCAGTYTCGTCGRC             | bamA-800  | (GMT cluster) |
| bamA-ASP23| bamA       | Reverse   | TTTCCTTTGTTGVSRTTCC             | bamA-700  | (SA cluster) |

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distantly related to bcrC genes from Thauera species. In summary, the results obtained indicate that the results from the combined usage of the bcrC/bzdN-, bamA-, bamB-, and 16S rRNA gene-targeting assays were mainly consistent.

**BPP site.** In contrast to Gneisenau, the BPP site contained a high concentration of benzene but lower sulfate concentrations. However, the total dissolved Fe concentrations suggested intense Fe(III) reduction. Therefore, a different microbial community composition was expected. Using the bzdN and bamB assays, fragments of the expected size were generated, whereas no amplicon was obtained with the primer set of the bcrC assays. Five different bzdN sequences were identified, with the highest similarities to the Azoarcus type of class I BCR (81 to 83%) (Fig. 2). No sequence of the Thauera type was

![Phylogenetic tree of bamB, bzdN/bcrC, bamA, and 16S rRNA gene fragments obtained with group-specific gene probes. The clones obtained from the BPP and Gneisenau sites are shown in bold. The scale bars represent 10 nt, 5 nt, and 2 nt substitutions per 100 nt. Individual groups of bamA genes of ACDA were specifically detected with bamA-targeting assays using either bamA-800 (GMT cluster) or bamA-700 (SA cluster) oligonucleotide probes.](image-url)
identified, demonstrating the specificity of the bzdN and bcrC assays. The DNA fragments from the BPP bamB clones were closely related to each other and to the DNA fragment of the Gneisenau bamB 1 clone; similarities to bamB sequences from known ACDA were only moderate, with a maximal 88% identity to bamB from *G. metallireducens*.

With the SA cluster-targeting bamA assay, eight different bamA-homologous clones (BPP bamA 1 to 8) were identified among the 40 clones analyzed. In good agreement with the results of the bzdN-targeting assay, they showed the highest similarities to *bamA* genes from species of the genera *Azoarcus* and *Aromatoleum* (75% to 78% identity, clones 3 to 8; Fig. 2). With the GMT cluster-targeting assay, two additional *bamA* fragment-containing clones were detected. Unexpectedly, they were more similar to *bamA* sequences from species of the genera *Magnetospirillum* and *Thaueria* (80% identity) than to *Geobacter* species (71% identity).

The majority of the clones obtained with the 16S rRNA gene-targeting assay contained a fragment most similar to those from uncultured bacteria classified as *Rhodocyclaceae* strains (maximum 98% identity to gi197304162 and gi239579564) and from the estradiol degrader *Denitratisoma estradiolicum* AcBE2-1 (gi58533147, 94% identity; BPP 16S rRNA gene clones 3 to 6). The highest similarity to 16S rRNA genes from known ACDA was only 90.5%, with those from *Azoarcus* species (Fig. 2). In accordance with the results obtained with the *bamB* assay, a 16S rRNA fragment with the highest similarity to the corresponding gene from *G. metallireducens* (93%, BPP 16S rRNA gene clone 1) was present. The 16S rRNA gene fragment of BPP 16S rRNA gene clone 2 showed no significant sequence identities to known ACDA (less than 78% identity to the *Betaproteobacteria* and 83.3% maximal identity to bacteria of the *Geobacter* branch). A BLAST search revealed the highest similarities to an uncultured Gram-positive bacterium (99% sequence identity, gi1362585), which most probably was not involved in anoxic benzene degradation.

With regard to the gene sequences obtained from *Azoarcus*-like species, results of the bzdN/bcrC, *bamA*, and 16S rRNA gene-targeting assays were consistent. The rather low similarities of the 16S rRNA sequences to other known ACDA suggest that they derive from a previously undescribed genus. The second group of ACDA present at the BPP site appears to be related to members of the *Geobacteraceae*, as indicated by the sequences obtained with the *bamB* and 16S rRNA gene-targeting assays. The *bamA* assay did not completely confirm this phylogenetic assignment; as expected, a positive result was obtained only with the GMT cluster-targeting assay.

**Conclusions.** A main outcome of this study is that the combined application of the improved *bamA*-, *bcrC/bzdN*, and *bamB*-targeting assays served as a general and reliable tool for detecting ACDA at the two environmental sites tested. Although the improved *bamA*-targeting assay may be applicable as a single assay for detecting ACDA, control experiments with the assays specific for obligately (*bamB*) and facultatively (*bamA, bcrC/bzdN*) anaerobic bacteria are recommended to minimize false-positive and false-negative results. As so far no assay exists for the gene involved in the initial activation of benzene in ACDA, the application of assays that target enzymes of the benzoyl-CoA degradation pathway appears to be the method of choice for detecting ACDA involved in benzene degradation. It cannot be completely ruled out that a few of the ACDA detected in the benzene-loaded microcosms used benzocate, which might be released by benzene degraders at very low concentrations. However, it is unlikely that putative benzocate degraders were selectively enriched in the microcosms at a higher abundance than benzene degraders.

Surprisingly, neither functional nor 16S rRNA genes from sulfate-reducing ACDA were detected at the Gneisenau site, although sulfate was present at high concentrations. However, the elevated concentrations of dissolved Fe determined at Gneisenau are indicative of Fe(III) reduction and fit with the detection of functional and 16S rRNA genes from *Geobacteraceae*. *Geobacter* species have been identified in earlier studies of benzene-degrading communities (18a). At the BPP site, Fe(III)-reducing *Betaproteobacteria* related to *Azoarcus* species, such as *Georgfuchisia toluolica* (22), may be involved in anaerobic benzene degradation. In a very recent study, Fe(III)-reducing *Beta*- and *Deltaproteobacteria* were shown to dominate at an anoxic BTX-contaminated site, although the concentration of Fe(II) was even lower than at the Gneisenau or BPP site (20).

**Nucleotide sequence accession numbers.** Newly obtained sequences from this study were deposited at GenBank under accession numbers HQ640662 to HQ640676 and GU057387 to GU057405.

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