Identification of Critical Members in a Sulfidogenic Benzene-Degrading Consortium by DNA Stable Isotope Probing[▽]

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Stable isotope probing (SIP) was used to identify the active members in a benzene-degrading sulfidogenic consortium. SIP-terminal restriction fragment length polymorphism analysis indicated that a 270-bp peak incorporated the majority of the ¹³C label and is a sequence closely related to that of clone SB-21 (GenBank accession no. AF029045). This target may be an important biomarker for anaerobic benzene degradation in the field.

Benzene is one of the monoaromatic compounds in gasoline (32) and is a carcinogen. Although anaerobic benzene degradation has been studied extensively for the last 2 decades, there is a very limited understanding about the mechanisms of degradation and the organisms that are involved in this process (for reviews, refer to references 4 and 20). Isolates capable of mineralizing benzene under denitrifying conditions have been obtained (5, 11); however, no pure culture capable of benzene degradation under iron-reducing or sulfate-reducing conditions has been identified thus far. The current understanding of anaerobic benzene degradation is based mostly on enrichment cultures, with very little insight into the roles that different microorganism execute in the mineralization of benzene under anaerobic conditions. In this report, DNA-based stable isotope probing (SIP) and terminal restriction fragment length polymorphism (TRFLP) analysis were used to distinguish the active microorganism(s) in a benzene-degrading sulfidogenic consortium (21). Prior molecular characterization of this original enrichment suggested that the enrichment was made of diverse phylotypes distributed among the classes *Gammaproteobacteria*, *Deltaproteobacteria*, and *Epsilonproteobacteria*, the order *Cytophagales*, and low-G+C gram-positive organisms. Among the 12 bacterial phylotypes identified at that time, 4 were sulfate reducers (22). However, all attempts to obtain a pure culture of a sulfate-reducing benzene degrader have been unsuccessful to date.

To perform the SIP experiment, all cultures were grown in a modified Widdel and Pfennig marine medium (33) at 30°C, with 4.0 g/liter of Na₂SO₄. A master culture was grown on [¹²C]benzene as the sole carbon source, and then it was starved for 21 days. Fifteen 40-ml serum bottles with 24 ml of culture were prepared, of which 5 bottles were amended with [¹²C]benzene (Chromasolv high-performance liquid chromatography, ≥99%; Sigma, St. Louis, MO), and 10 were amended with uniformly labeled [¹³C]benzene (99 atom% of ¹³C; Isotec, Maimis-

burg, OH), both to a concentration of 134 μM. Samples were taken on days 0, 4, 8, 11, and 15 to determine benzene concentrations by gas chromatography-flame ionization detector analysis, using the method described for toluene (6) but with 0.1 mM fluorobenzene as the internal standard. The remaining biomass was used for genomic DNA extraction, using a modified phenol-chloroform procedure (12, 25). Sample DNA (150 ng) was combined with 10 to 20 μg of ethidium bromide, ¹²C-labeled and ¹³C-labeled archaeal carrier DNA (60 ng and 300 ng of *Halobacterium salinarum* DNA, respectively) (10), and 30 ng of *Escherichia coli* DNA as internal indicator (27). DNA was separated by CsCl density gradient centrifugation as described by Gallagher et al. (10) and Tierney (29). The separated [¹²C]DNA and [¹³C]DNA bands were dialyzed (10), and equal volumes of dialyzed samples were used for 16S rRNA gene PCR and TRFLP analysis using the *Bacteria*-specific primers 27F (AGAGTTTGATCMTGGCTCAG), with a 6-carboxyfluorescein label at the 5' end, and 1100R (GGGTT GCGCTCGTTG). TRFLP analysis was performed by digestion of equal volumes of PCR product with HaeIII restriction enzyme and precipitation as described by McGuinness et al. (15). TRFLP fingerprinting was carried out on an ABI 310 genetic analyzer (Applied Biosystems, Foster City, CA) using Genescan software. Terminal restriction fragments (TRFs) between 50 and 500 bp long with a count of more than 50 per unit area were used for analysis. 16S rRNA gene PCR was also performed using the *E. coli* species-specific primers ECA75F and ECR619R (24, 27) to test for cross-contamination of separated DNA bands.

The 16S rRNA genes from the master culture were amplified using 27F and 1525R (AAGGAGGTGWTCCARCC) and cloned into pCR4-TOPO (Invitrogen, Carlsbad, CA). Sequence-ready plasmid DNA was purified using a Flexi Prep kit (Amersham Biosciences, Piscataway, NJ). TRFs of individual inserts were verified by TRFLP analysis, and 16S rRNA genes were sequenced on an ABI 3100 genetic analyzer (Foster, CA). Unambiguously assembled 500-bp sequences with unique TRFs were aligned with those from the SILVA 95 database (<http://www.arb-silva.de>), and a phylogenetic tree was constructed by using ARB software (14). A 16S rRNA gene com-

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TABLE 1. Utilization of benzene over the course of SIP incubation^a

Day	Mean concn (μ M) of [$^{12}\text{C}_6$]benzene	SD	Amt of day 0 benzene degraded (%)	Samples used for analysis	Mean concn (μ M) of [$^{13}\text{C}_6$]benzene	SD	Amt of day 0 benzene degraded (%)	Samples used for analysis
0	134.8	21.2		5	121.5	16.1		10
4	71.7	11.0	47	4	52.2	20.8	57	8
8	16.9	2.6	87	3	6.2	13.8	95	6
11	ND	0	100	2	15.7	—	87	1
15	—	—		—	ND	—	100	1

^a Means and standard deviations (SD) of benzene concentrations shown in samples taken over a 15-day period from [$^{12}\text{C}_6$]benzene and [$^{13}\text{C}_6$]benzene cultures. No samples were taken for analysis after the benzene concentrations in the previous samples were below the detection limit. ND, not detected; —, not determined.

munity fingerprint was also prepared with the genomic DNA from the subcultures.

Gas chromatography-flame ionization detector analysis of the SIP samples showed that nearly half (47 and 57% of [^{12}C]- and [^{13}C]benzene, respectively) was utilized by day 4 and almost all substrate (87 and 95% of [^{12}C]- and [^{13}C]benzene, respectively) was utilized within 8 days (Table 1). These data confirm that benzene was degraded by the cultures in the time frame chosen for the SIP experiment.

After DNA was centrifuged for 20 to 24 h, two distinct DNA bands were observed for each gradient under UV transillumination. The ^{12}C -labeled archaeal carrier DNA, the *E. coli* [^{12}C]DNA, and the bacterial [^{12}C]DNA formed a separate [^{12}C]DNA band, while the [^{13}C]DNA band contained the ^{13}C -labeled archaeal carrier DNA and any ^{13}C -labeled bacterial DNA from the consortium. To test for contamination of the [^{13}C]DNA band by any [^{12}C]DNA, *E. coli* species-specific 16S rRNA gene PCR was performed with equal volumes of the separated DNA bands after dialysis. No detectable PCR product was obtained from the [^{13}C]DNA bands. *E. coli* PCR product was observed only in the [^{12}C]DNA bands of the gradients. This is an important control, demonstrating that [^{13}C]DNA bands were satisfactorily separated in the CsCl gradients, with ^{12}C cross-contamination below the PCR detection limit (10), and that differences seen in the TRFLP profiles of separated DNA bands are a consequence of the anaerobic degradation of benzene. Figure 1 shows the results obtained for the 16S rRNA gene PCR on day 11 with *Bacteria*-specific primers. In this gel, lanes 1 and 3 demonstrate positive PCR products obtained from [^{12}C]DNA bands with [^{12}C]- and [^{13}C]benzene amendments, respectively. No amplicons were detected in lane 2 from PCR amplification of the [^{13}C]DNA band, when cultures were amended with [^{12}C]benzene. This was an additional control, which indicates that any cross-contamination was below the detection limit. Lane 4 demonstrates that PCR product from the [^{13}C]DNA band is obtained only when cultures are amended with [^{13}C]benzene, indicating that newly synthesized ^{13}C [DNA] is detected.

After being amplified, all 16S rRNA gene PCR products were subjected to TRFLP analysis as shown in Fig. 2. The ^{12}C -labeled control is the TRFLP of the [^{13}C]DNA band with [^{12}C]benzene amendment. It has no detectable TRFs and acts as an important control in this analysis, as described earlier. TRFLP was also performed using amplicons from [^{13}C]DNA bands from day 4 to day 15 cultures with [^{13}C]benzene amendment, and results show changes in the active members of this consortium (Fig. 2). No TRFs were obtained from the day 4 sample, which indicates that the level of ^{13}C -labeled DNA in

the day 4 sample is below our PCR detection limits. On days 8, 11, and 15, analysis detected 8, 10, and 7 different TRFs, respectively, in the samples. TRFs detected in more than one sample include TRFs of 108, 110, 131, 237, 270, 272, and 421 bp. Of these, the 270-bp TRF dominates the TRFLP profiles at all time points. It accounts for 46 to 59% of the total area of the TRFLP profile, and its appearance and decline are coincident with the decrease in benzene concentrations in the samples taken during the course of the experiment (Table 1). Community fingerprinting (Fig. 2, Master culture panel) and phylogenetic analysis of the 16S rRNA gene (Fig. 3) were used to identify three of these SSU genes (clones SB-9, SB-21, and SB-29) that were present in the original consortium (22). The 16S rRNA gene sequence, represented by a TRF of 270 bp, was found to be 99% similar to that of clone SB-21 (GenBank accession no. AF029045, from the family *Desulfobacteraceae*) (3). Our SIP experiment indicates that this bacterium was the first to derive the bulk of ^{13}C -labeled benzene for DNA synthesis and likely plays a critical role in anaerobic benzene degradation (17).

Additional evidence supports the notion that phylotype SB-21

FIG. 1. Analysis using 1% agarose gel with *Bacteria*-specific 16S rRNA gene PCR product obtained from day 11 CsCl-separated DNA samples. Lanes represent different benzene amendments, as follows: 1, [^{12}C]DNA band amended with [^{12}C]benzene; 2, [^{13}C]DNA band amended with [^{12}C]benzene; 3, [^{12}C]DNA band amended with [^{13}C]benzene; 4, [^{13}C]DNA band amended with [^{13}C]benzene; 5, λ HindIII ladder; 6, positive PCR control; 7, negative PCR control.

FIG. 2. 16S rRNA gene TRFLP analysis with HaeIII restriction digests. Master culture, TRFLP profile of the culture used for setting up the experiment; ^{12}C control, TRFLP profile of ^{13}C DNA with ^{12}C benzene amendment; ^{13}C day 4 to ^{13}C day 15, TRFLP profile of ^{13}C DNA with ^{13}C benzene amendment. x axis is TRF size (values are bp), and y axis is relative peak intensity. Dominant TRFs have been identified with their sizes (bp).

(with a TRF of 270 bp) is key in the sulfidogenic metabolism of benzene. Primarily, SB-21 has been maintained in this benzene-degrading consortium over more than 10 years of subculturing (22 and this study). Second, increases in the relative peak intensity of the 270-bp TRF corresponds with an almost complete loss of benzene from the active cultures (Table 1). Finally, the 270-bp

TRF represents the most prominent peak in the ^{13}C DNA TRFLP profiles (Fig. 2), indicating that it has incorporated most of the ^{13}C from ^{13}C benzene into the DNA.

As such, all microorganisms present in this benzene-degrading enrichment could be classified using the following conceptual models: (i) organisms are strictly dependent (i.e., exhibit-

FIG. 3. Phylogenetic relationship between the clones identified in this study from the benzene-degrading sulfate-reducing enrichment (bold-face) and the members of *Deltaproteobacteria*. The 16S rRNA gene tree was constructed using maximum-likelihood analysis and 1,000 bootstraps iterations. Numbers indicate percentages of bootstrap values. Sequences represented by SB-9, SB-21, and SB-29 and SB-30 were identified in this culture's earlier characterization (22). Sequences from the study of other anaerobic benzene-degrading enrichments have been identified by an asterisk (*). GenBank accession numbers are shown in parentheses.

ing syntrophy) (1, 9, 13, 34), showing sequential degradation of metabolites (mutualism) (26), or are fastidious organisms, interdependent for growth factors or nutrients; (ii) organisms have no strict dependence but exhibit coexistence (synergy) (8, 28), possibly feeding off extracellular metabolites of degradation (19); and (iii) all microorganisms degrade benzene but with different efficiencies. Considering that not all the TRFs identified in the master culture were identified in the [^{13}C]DNA (Fig. 2, day 8 to day 15), model iii (all organism are benzene degraders) can be eliminated. Although we could narrow the possible functional models that this consortium is based on, a definitive identification of the relationship between different players needs more extensive investigation. However, we have also tested benzoate, phenol, and toluene (metabolites of benzene degradation [2, 23, 31]) as the sole carbon sources for degradation in this consortium (18). Although benzoate and phenol could be degraded, the rate of degradation was considerably lower and there was a large lag in the onset of degradation, compared to that of benzene-amended cultures. Toluene was not utilized. These results suggest that the labeling of DNA in this SIP experiment, within an 8-day period, is not due to feeding off these metabolites during benzene degradation (mutualism). Even if model i or ii is applied to this consortium, it can be definitively concluded that the phylotype represented by the TRF of 270 bp is crucial to the process of benzene degradation in this consortium, since it has incorporated the bulk of the carbon from labeled benzene into its DNA, and the change in its relative peak intensity corresponds with the loss of benzene from the culture.

Our findings that a member of the family *Desulfobacteraceae* plays a key role in benzene degradation is also supported by a recent study (16) in which a dominant phylotype (clone BznS295) in a benzene-degrading marine sulfate-reducing enrichment culture was closely related to SB-21 and SB-30 (Fig. 3). Similarly, research in a column bioaugmented with a methanogenic enrichment (7) showed a correlation between benzene degradation activity and a *Desulfobacterium*-like clone (OR-M2) (Fig. 3).

In conclusion, these collective results are evidence that SB-21-like organisms are actively involved in benzene degradation in diverse sulfate-reducing and possibly methanogenic environments. Bacteria similar to SB-21 have been identified as one of the dominant microbes in benzene-degrading enrichments established from widely dispersed environments such as a Mediterranean lagoon in France (16) and an oil refinery in Oklahoma (30). Furthermore, the abundance of these 16S rRNA gene sequences could also be linked to benzene degradation (16, 7). Thus, SB-21 potentially could serve as a biomarker for in situ biodegradation of benzene in the environment under sulfidogenic and methanogenic conditions.

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