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Assessment of anaerobic benzene degradation potential using 16S rRNA gene-targeted real-time PCR

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Summary

Benzene is a common groundwater pollutant that is often recalcitrant under the anaerobic conditions that prevail at hydrocarbon-contaminated aquifers. Thus, determining the potential for anaerobic benzene degradation is important to assess the feasibility of intrinsic bioremediation. In this work we developed a 16S rRNA biomarker to estimate the concentration of putative benzene degraders in a methanogenic consortium that has been enriched on benzene for several years. Primers were designed based on phylogenetic information from this consortium. The primers and probe were obtained by sequencing the dominant denaturing gradient gel electrophoresis band of this consortium, which corresponded to *Desulfobacterium* sp. clone OR-M2. No hybridization was observed with DNA samples from negative controls (i.e. toluene-degrading and dehalorespiring methanogenic consortia that do not degrade benzene). Samples from an anaerobic aquifer column that was bioaugmented with this benzene-degrading consortium showed a strong correlation between benzene degradation activity and the concentration of the target organism. Although our data do not prove that *Desulfobacterium* sp. is a benzene degrader, its enrichment as a result of benzene consumption and its correlation to anaerobic benzene degradation activity suggest that it either initiates benzene degradation or is a critical (commensal) partner. Therefore, the utility of this primers and probe set to assess anaerobic benzene degradation potential was demonstrated. This is the first report of the use of real-time quantitative PCR for forensic analysis of anaerobic benzene degradation. Whether this biomarker will be adequately selective and broadly applicable to assess benzene degradation potential under strongly anaerobic (sulfate reducing and methanogenic) conditions will require further research.

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Introduction

Benzene is one of the most common groundwater pollutants, ranking third in priority (after vinyl chloride and polychlorinated biphenyls) in the 2005 CERCLA List of Hazardous Organic Substances found at Superfund sites (http://www.atsdr.cdc.gov/cercla/05list.html). Groundwater contamination by benzene is also prevalent at about 450 000 sites in the USA that are contaminated with gasoline leaks from underground storage tanks (UST) (USEPA, 2005). Such widespread contamination of potential drinking water sources is a major concern because benzene is a known carcinogen that can cause leukaemia in humans (USEPA, 1998a). Consequently, benzene is often the single compound that drives the need for corrective action at sites contaminated with petroleum product releases.

Monitored natural attenuation (MNA) is often the most cost-effective approach to manage groundwater contamination by benzene and other monoaromatic hydrocarbons. Monitored natural attenuation relies primarily on intrinsic anaerobic bioremediation processes that proceed without human intervention, and has been selected to remediate more than 50% of the existing gasoline-contaminated UST sites (USEPA, 2000). However, there is a tendency to over-prescribe MNA due to its relatively low cost, even though intrinsic bioremediation does not always proceed sufficiently fast to provide adequate risk protection (NAS, 2000). Indeed, MNA is not a panacea, and its regulatory and public acceptance requires thorough documentation that natural degradation processes (rather than dilution or sorption) are effective at a given site (USEPA, 1998b).

Although anaerobic benzene degradation has been proven to occur (Grbic′-Galic′ and Vogel, 1987; Edwards and Grbic′-Galic′, 1992; Lovley *et al*., 1995; Kazumi *et al*., 1997; Anderson and Lovley, 2000), the need to demonstrate such catabolic potential on a case by case basis is important because benzene is frequently recalcitrant under anaerobic conditions that prevail at hydrocarbon-contaminated sites (Alvarez and Vogel, 1995; Heider *et al*., 1998). The recalcitrance of benzene is exacerbated by the presence of ethanol, which is increasingly being

added to gasoline to replace methyl tert-butyric ether as a fuel oxygenate and meet Clean Air Act and renewable fuel requirements (Powers *et al*., 2001a,b). The high electron acceptor demand exerted by ethanol at sites contaminated with reformulated gasoline rapidly induces methanogenic conditions that decrease the thermodynamic feasibility for benzene bio-oxidation (Corseuil *et al*., 1998; Da Silva and Alvarez, 2002). This contributes to longer benzene plumes and a greater risk of exposure (Ruiz-Aguilar *et al*., 2002). Thus, evaluating the potential for *in situ* benzene degradation under anaerobic conditions is increasingly important to ensure that MNA is an appropriate strategy and to assess the performance of anaerobic biological treatment. For example, determining whether a site has the potential for rapid or slow anaerobic benzene degradation is crucial for determining the need for implementing more aggressive remediation approaches.

Currently, the assessment of bioremediation and MNA relies on indirect evidence of biodegradation (e.g. geochemical footprints, numerical modelling and stable isotope fractionation) (Alvarez and Illman, 2005). However, such techniques are not universally applicable and could be complemented by less costly emerging molecular approaches that detect and quantify specific degraders. This paper describes the first molecular approach to detect and quantify bacteria that are closely associated with benzene degradation under strongly anaerobic (methanogenic and sulfate reducing) conditions. The objectives of this project were to: (i) develop a phylogenetic primer and probe set to detect such bacteria; (ii) test the specificity and selectivity of this set; and (iii) determine whether anaerobic benzene degradation activity and the number of gene copies that hybridize with the primers-probe set are correlated, using samples from anaerobic aquifer column experiments.

Results and discussion

Primers and probe design

In previous work, 16S rRNA gene clone library analysis of the benzene-enriched methanogenic consortium revealed the presence of an operational taxonomic unit, clone OR-M2, which was affiliated with *Desulfobacterium anilini*, *Desulfosarcina variabilis* and *Desulfobacula toluolica* (Ulrich and Edwards, 2003). These strains are capable of degrading metabolites of anaerobic benzene degradation known to be produced by this consortium, such as toluene (*Desulfobacula toluolica*), phenol (*Desulfobacterium anilini*) and benzoate (*Desulfosarcina variabilis*) (Chakraborty and Coates, 2005; Ulrich *et al*., 2005). A clone most similar to OR-M2, called SB-21, was also detected in a different sulfate-reducing consortium capable of degrading benzene (Phelps *et al*., 1998). Therefore, considering the relatively limited catabolic diversity of methanogenic Archaea, it was postulated that a sulfate-reducing bacterium capable of fermentative growth (Bryant *et al*., 1977; Jones *et al*., 1984; Archer and Powell, 1985; Guyot, 1986) was responsible for the initial attack on the benzene ring. Thus, primers were designed based on the 16S rRNA gene sequence of the sulfate-reducers that were phylogenetically associated with OR-M2 as a starting point (Table 1).

The gene sequence of this uncultured species (*Desulfobacterium* sp. clone OR-M2, GenBank AY118142) was also used to design primers and probe; this sequence was obtained from the dominant denaturing gradient gel electrophoresis (DGGE) band of the DNA extracted from the benzene-enriched methanogenic consortium (Fig. 1).

Primers designed based on the gene sequence of *Desulfobacula toluolica* did not amplify with the DNA extracted from the benzene-enriched methanogenic consortium (Table 2). The primers designed based on the gene sequence of *D. anilini* amplified only with DNA from the benzene-enriched methanogenic consortium (Table 2). However, the low copy numbers obtained ($1.4 \pm 0.6 \times 10^2$ cell ml^{-1}) represented less than 1% of the total bacteria (Table 2), suggesting that these primers were not sufficiently selective to target the benzene degraders. Primers based on the gene sequence of *Desulfosarcina variabilis* showed amplification with DNA from the benzene-enriched consortium, but also amplified with negative control samples (Table 2). Overall, the results show that these primers were not suitable to quantify the concentration of anaerobic benzene degraders in the starting culture.

Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis analysis of the 16S rRNA gene sequence from the benzene-enriched methanogenic consortium grown in batch reactor (Fig. 1) was conducted as an alternative approach to identify the gene sequence of the putative benzene degrader. Note that the DGGE primers used here are ineffective for the amplification of *Archaea* sequences (Ferris *et al*., 1996); thus the dominant bands corresponded to *Bacteria*.

The amount of methane produced (2.8 mM) during the degradation of benzene (869 μM) (Fig. 1) was close to the maximum theoretical value of 3.3 mM (ignoring anabolism and assuming a ratio of 3.75 mol of methane per mole of benzene degraded: $C_6H_6 + 4.5\ H_2O \rightarrow 2.25\ CO_2 + 3.75\ CH_4$). Bacterial concentrations increased from $4.7 \pm 0.48 \times 10^9$ (no benzene present) to $1.4 \pm 0.07 \times 10^{10}$ cells (51 days after benzene addition) as determined by real-time quantitative PCR (RTQ-PCR) (Fig. 1B). This final microbial concentration (X) is similar to the theoretical value of 5.0×10^{10} cells, which was calculated based on

Table 1. Primers and probe sequences used for RTQ-PCR.

Target	Forward primer (5′–3′)	Reverse primer (5′–3′)	Probe[a] (5′–3′)
Bacteria[b]	CGGTGAATACGTTCYCGG	GGWTACCTTGTTACGACTT	FAM-CTTGTACACACCGCCCGTC-BHQ1
Archaea[c]	CGGTGAATACGTCCCTGC	AAGGAGGTGATCCTGCCGCA	FAM-5-CTTGTACACACCGCCCGTC3-BHQ-1
Desulfosarcina variabilis	TAGTCCACGCAGTAAACGGTGA	CCCGAGATGTCAAATCCAGGTA	SYBR Green
Desulfobacula toluolica	GGCGTGCTTAACACATGCAAGT	CGCATAGAAACTGCATCCGGTA	SYBR Green
Desulfobacterium anilini	CGTGGGTAACCTCCCTTTGAATCT	GCGTCGAATTAAACCACATGCTCC	SYBR Green
Desulfobacterium sp.	CCACGAAAGTCGATTATACCAGAA	AACCATACCTTAGGCGCTTATCTC	FAM-CGCTGGTTTAACCCCCGATTTATCTCG-TAMRA
Phage (λ)[d] Internal standard	ACGCCACGCGGGATG	AGAGACACGAAACGCCGTTC	TET-ACCTGTGGCATTTGTGCTGCCG-TAMRA

a. The reporter dye used were FAM (6-carboxyfluorescein) or TET (tetrachloro-6-carboxyfluorescein) and, the quencher dye were either TAMRA (6-carboxy tetramethyl rhodamine) or BlackHole Quencher-1.
b. Forward primer (BACT1369F), reverse primer (PROK1492R) and probe (TM1389F) were developed by Suzuki and colleagues (2000).
c. The forward primer ARCHMIX1369F (ARCH1-1369F and ARCH2-1369F), reverse primer PROK1541R, and probe TM1389F were developed by Suzuki and colleagues (2000).
d. The forward and reverse primers as well as the probe were designed by Beller and colleagues (2002).

the amount of benzene consumed (ΔS) of 708 μM, a measured bacterial yield coefficient (Ŷ) of 9.4 g cells mol benzene^{-1} (Ulrich and Edwards, 2003), and a dry cell weight (dcw) of 1.33×10^{-13} g cell^{-1} (Bratbak, 1985) [i.e. $X = (Y \times \Delta S/dcw)$].

DNA samples extracted from this consortium at time 0 (no benzene present) and 51 days after addition of 869 μM of benzene were used for DGGE analysis (Fig. 1). Two salient bands were observed in the acrylamide gel, both of which were enriched after benzene was consumed (Fig. 1C). Band 1 had 94% sequence identity (SI) with DNA from an uncultured bacterium (clone Eub 2) found in a toluene-enriched methanogenic consortium that could not degrade benzene (Ficker *et al.*, 1999). This sequence was also found later in anaerobic soil column samples that degraded toluene but not benzene (data not shown). Thus, its enrichment in Fig. 1C was postulated to be due to growth on benzene metabolites. Band 2 matched closest with *Desulfobacterium* sp. clone OR-M2 (SI = 100%), which had previously been circumstantially implicated as a benzene degrader by Ulrich and Edwards (2003). This band also had similarity to an uncultured bacterium clone ZZ14C7 (SI = 98%) found in benzene-contaminated groundwater (Alfreider and Vogt, 2003), and was dominant at the beginning of the experiment due to years of enrichment on benzene. Therefore primers and probe were designed based on the gene sequence of this band 2 and tested for its selectivity using DNA from the positive and negative controls.

The gene copy numbers measured with these primers were higher in the benzene-enriched methanogenic consortium; representing 88% of the total bacteria (16S rRNA). Amplification was negligible for the negative controls, representing (as a percentage of the total Bacteria gene copies) $< 0.0006\%$ for the PCE- and 0.3% for the toluene+*o*-xylene-enriched consortia respectively (Table 2). Therefore, this set of primers and probe was chosen as biomarker to quantify the concentration of organisms that are closely associated with anaerobic benzene degradation (either initiating it or participating as critical, commensal partners) and to explore the correlation between such concentrations and anaerobic benzene degradation activity.

Correlation between benzene degradation and 16S rRNA gene copy numbers

Flow-through aquifer columns used to simulate the bioattenuation of BTEX and ethanol mixtures (Da Silva and Alvarez, 2002) were used to evaluate the potential of our primers to estimate benzene degradation activity. Figure 2A shows benzene and ethanol concentration profiles in a column that was bioaugmented 3 years ago with the benzene-enriched methanogenic consortium

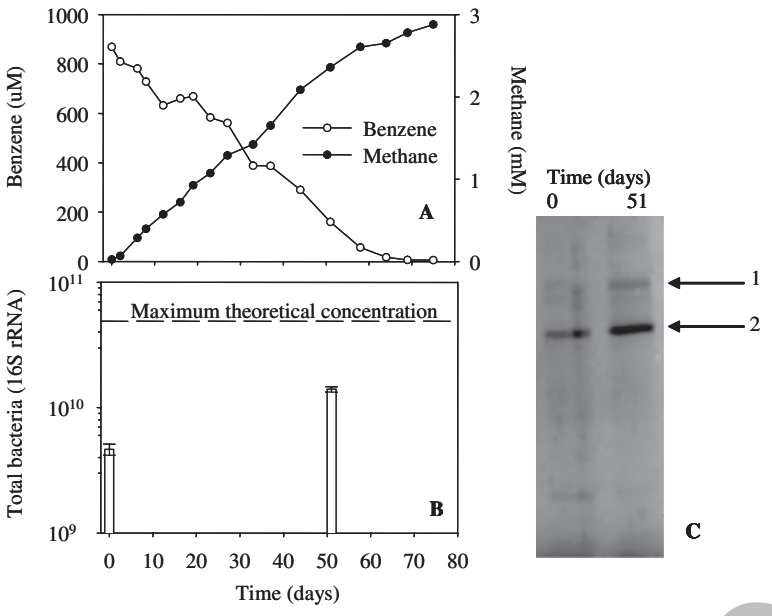

Fig. 1. DNA samples from a benzene-enriched methanogenic consortium (A) were used for quantification of total bacteria using RTQ-PCR (B) and for DGGE analysis (C). Samples were taken before (t = 0) and after (t = 51 days) the degradation of 708 mM of benzene. Gene sequencing of band 1 showed 94% SI with an uncultured bacterium (clone Eub 2) found in a toluene-enriched methanogenic consortium that could not degrade benzene. Band 2 showed 100% SI with the putative benzene degrader *Desulfobacterium* sp. clone OR-M2, and was used to design primers and probe to quantify anaerobic benzene degradation potential (Table 1). Error bars in panel B depict the range of duplicate analyses.

(Da Silva and Alvarez, 2004). Toluene was not added to this column because its presence inhibits benzene degradation by this consortium.

The highest bacterial counts (~5×10^5 cells g soil^{-1}) were measured in the column's inlet and it decreased along the column as the concentration of substrate decreased (Figs 2 and 3). The highest number of benzene degraders (~2×10^5 cells g soil^{-1}) were measured with our primer and probe set at the inoculation port, which is the location that exhibited the highest benzene degradation activity (A = 0.69 mg day^{-1} between the 14 cm and 21 cm sampling ports) (Fig. 2). This concentration of benzene degraders is close to the theoretical population (X) of 2.5×10^5 cells g soil^{-1}, estimated based on ΔS of 5.74 μmol of benzene consumed and assuming Y = 9.4 g cells mol benzene^{-1} (Ulrich and Edwards, 2003), dcw = 1.33×10^{-13} g cell^{-1} (Bratbak, 1985), and a soil bulk density (ρ_b) of 1.6 kg l^{-1} [i.e. $X = (Y \times \Delta S/\rho_b \times dcw)$].

Consistent with the notion that the target sequence corresponded to benzene degraders, little benzene degradation occurred away from the inoculation port (Fig. 2A) where copy numbers were relatively low (10–100 copies g soil^{-1}) (Fig. 2B). It is unclear why the concentration of putative benzene degraders was so low at these points where benzene was present at significant levels. Potential reasons include insufficient experimental time in relation to bacterial migration and colonization kinetics, and inhibitory conditions such as the presence of (easily degradable) ethanol upstream and bacterial washout (or stress due to oxygen intrusion) downstream of the inoculation port.

Figure 3A shows benzene, toluene and ethanol concentration profiles in the control column that was not bioaugmented. Toluene was the only aromatic hydrocarbon to be degraded in this column after 2 years of acclimation. The lack of benzene degraders in this column as determined by our PCR method (detection limit 47 ± 1.8 gene copies g soil^{-1}) agreed with the absence of benzene degradation (Fig. 3). This supports the notion that our primers and probe were specific enough to detect bacteria associated with benzene degradation with no misleading amplification caused by mismatches with other monoaromatic hydrocarbon degraders.

Our data do not prove that the target organism, *Desulfobacterium* sp. is a benzene degrader. However, its enrichment as a result of benzene consumption (Fig. 1) and its correlation to anaerobic benzene degradation activity (Fig. 2) suggest that it either initiates benzene degradation or is a critical (commensal) partner (e.g. an organism that consumes by-products of benzene degradation to minimize feedback inhibition and/or enable the thermodynamic feasibility of the process). Thus, including this organism among those targeted for forensic analysis of anaerobic benzene degradation is desirable.

We recognize that the primer-probe set designed in this project will not detect all anaerobes that degrade benzene, such as *Dechloromonas* strains JJ and RCB which can degrade benzene under nitrate-reducing conditions (Coates *et al*., 2001; Chakraborty and Coates, 2005) or *Geobacter* spp. which can oxidize benzene under iron-reducing conditions (Rooney-Varga *et al*., 1999). Our primer-probe set targets bacteria associated with anaerobic benzene degradation under strongly anaerobic (sulfate-reducing and methanogenic) conditions that prevail near the source zone of BTEX-contaminated sites, especially when ethanol (which exerts a high electron acceptor demand) is present in the spilled fuel.

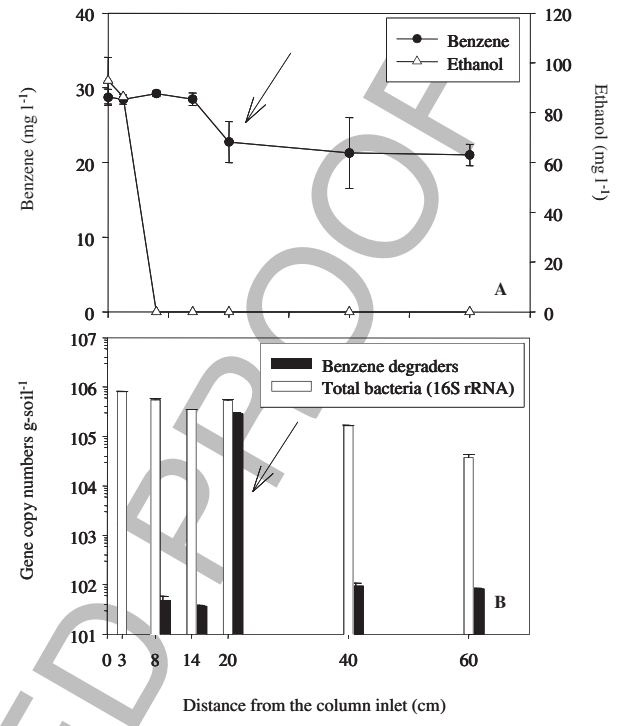

Fig. 2. Benzene and ethanol concentration profiles in a column bioaugmented with the benzene-enriched methanogenic consortium (Da Silva and Alvarez, 2004) (A) and bacterial gene distribution (B) along the length of the column. Error bars depict the range of values from duplicate analyses. Arrow shows inoculation port. Benzene losses in the sterile control column were negligible ($< 10\%$).

In summary, this is the first report of the use of RTQ-PCR for forensic analysis of anaerobic benzene degradation. Our probe targets a 16S rRNA gene sequence of *Desulfobacterium* sp. that was prevalent not only in a benzene-enriched methanogenic consortium (Ulrich and Edwards, 2003) but also in aquifer material samples from columns bioaugmented with this consortium (Fig. 2) and in contaminated sites exhibiting anaerobic benzene degradation activity (Alfreider and Vogt, 2003). This suggests the potential for broad applicability of this biomarker for forensic analysis of the natural attenuation of strongly anaerobic benzene plumes. However, further tests with contaminated site samples are needed to ascertain the specificity and selectivity of this biomarker and determine if it could be used to infer potential degradation rates in support of intrinsic bioremediation performance evaluation.

Experimental procedures

Primers and probe design

To date, no pure culture of benzene-degrading sulfatereducing -bacteria has been isolated. Thus, primers and probe were obtained by analysing 16S rRNA nucleotide sequences

Table 2. 16S rRNA gene copy numbers (±range of duplicate samples) per ml estimated by RTQ-PCR.

Methanogenic consortia enriched with	*Desulfosarcina variabilis*	*Desulfobacula toluolica*	*Desulfobacterium anilini*	*Desulfobacterium* sp.	Total bacteria	Archaea (methanogens)
Benzene (Ulrich and Edwards, 2003)	$1.9 \pm 0.6 \times 10^4$	ND	$1.4 \pm 0.6 \times 10^2$	$1.8 \pm 0.3 \times 10^6$	$1.5 \pm 0.2 \times 10^6$	$6.7 \pm 3.5 \times 10^5$
Toluene + *o*-xylene (Edwards and Grbić-Galić, 1994)	$7.0 \pm 0.9 \times 10^2$	$3.8 \pm 0.4 \times 10^4$	ND	$3.7 \pm 0.4 \times 10^4$	$1.2 \pm 0.5 \times 10^7$	$2.4 \pm 6.4 \times 10^6$
PCE (Adamson and Parkin, 1999)	$4.8 \pm 1.2 \times 10^4$	$1.8 \pm 2.3 \times 10^3$	ND	$1.9 \pm 0.2 \times 10^3$	$3.4 \pm 0.4 \times 10^8$	$3.2 \pm 0.2 \times 10^{10}$
No DNA	< 10	< 10	< 1	< 1	$5.4 \pm 5.0 \times 10^2$	< 10

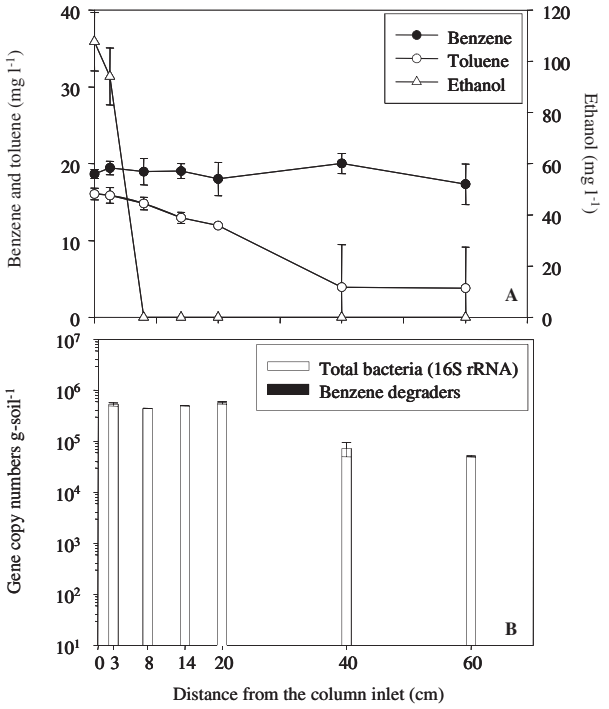

Fig. 3. Benzene, toluene and ethanol concentration profiles in the control column (not bioaugmented) (A) and bacterial gene distribution (B) along the length of the column. Error bars in panel B depict the range of values from duplicate analyses.

from several bacteria suspected of having the capacity for benzene degradation under sulfate-reducing and methanogenic conditions (Phelps *et al*., 1998; Ulrich and Edwards, 2003) (Table 1). A benzene-enriched methanogenic consortium (donated by Elizabeth Edwards, University of Toronto), which was enriched from sulfate-reducing microcosms, was used as a starting point. The sediment-free culture was kept inside a Coy anaerobic chamber (80% N_2, 10% H_2 and 10% CO_2) in 120 ml serum bottles capped with 20 mm Teflon-coated septa and aluminium crimps. The culture medium was prepared as described previously (Edwards *et al*., 1992) except that no sulfate was added. Benzene (~800 μM every 2 months) was fed as sole carbon and energy source.

Molecular analysis of this consortium showed the presence of four Archaeal species (that grouped with acetoclastic and hydrogenotrophic methanogens) and two predominant bacterial species associated with sulfate reduction (Ulrich and Edwards, 2003). One of these bacteria grouped with *Desulfosporosinus* sp. (which typically utilizes lactate, pyruvate, ethanol, or certain fatty acids as electron donors), and the second bacterium grouped with *Desulfobacterium anilini* (which can utilize aromatic compounds such as aniline and phenol as substrates).

Based on the phylogenetic association of *Desulfobacterium anilini* with an unknown strain (clone SB-21) found in a sulfate-reducing culture capable of degrading benzene (Phelps *et al*., 1998), Ulrich and Edwards (2003) postulated the bacterium that initiates benzene degradation in this consortium is a sulfate reducer capable of fermentative growth (Bryant *et al*., 1977; Guyot, 1986). Therefore, we used gene sequences from three sulfate reducers phylogenetically grouped with clone SB-21 [i.e. *Desulfobacterium anilini*, SI of 91%, *Desulfosarcina variabilis* (88.5%) and *Desulfobacula toluolica* (85%)] to design various primers. Additional primers and probes were also designed based on the 16S rRNA sequence of the dominant bacterium in this consortium (i.e. *Desulfobacterium* sp. clone OR-M2; GenBank AY118142) as determined by DGGE analysis (described below).

The software Primer Express (Applied Biosystems) was used for primers and probe design. Gene sequences from the targeted bacteria were obtained from the connected NCBI GenBank database (http://ncbi.nlm.nih.gov/Blast) and the Sequence Match facility of the Ribosomal Database Project (http://www.cme.msu.edu/RDP/). All primers and probes were obtained from Integrated DNA Technologies (Coralville, IA, USA) (Table 1).

DNA from two methanogenic consortia that do not degrade benzene and are rich in sulfate reducers [a methanogenic consortium enriched on toluene plus *o*-xylene (donated by Elizabeth Edwards, University of Toronto) (Ficker *et al*., 1999), and a tetrachloroethene-enriched methanogenic consortium (Adamson and Parkin, 1999)] were used as negative controls to test the specificity of the primers.

For Taqman PCR reactions, the mixture contained 1× Taqman PCR Master Mix® (Applied Biosystems, Foster City, CA, USA); 500 ηM forward and reverse primers, 250 ηM probe, 2 μl of sample DNA and sterile DNAase-free water to make up a final volume of 25 μl. For SYBR green PCR reactions the mixture contained 1× SYBR Green® (Applied Biosystem), 0.5 μM of each forward and reverse primer, 2 μl of sample DNA and sterile DNAase-free water to make up a final volume of 25 μl. PCR reactions were performed using a Sequence Detector (Model ABI 7500, Applied Biosystems) with the following temperature conditions: 50°C for 2 min, followed by 95°C for 10 min and 40 cycles at 95°C for 15 s, and 60°C for 1 min. The bacterial concentration in each sample was estimated based on the following equation:

$$\text{Gene copy numbers } \mu l^{-1}(\text{or g soil})^{-1} = (\mu g \text{ DNA } \mu l^{-1}/ 5.5\times10^{6} \text{ bp genome}^{-1})\times (9.1257\times10^{14} \text{ bp } \mu g \text{ DNA}^{-1}\times 2.5 \text{ genes genome}^{-1}).$$

This equation assumes that the size of the bacterial genome used as the standard in the calibration curves was 5.5×10^6 base pairs (bp) (and there are approximately 9.12576×10^{14} bp per μg of DNA), which is equivalent to the size of the *Desulfobacterium autotrophicum* genome (http://www.genomesonline.org), and that there are 2.5 gene copies per genome [Proteobacteria, Delta subdivision (http://rrndb.cme.msu.edu)]. Calibration curves using genomic DNA of *Desulfobacterium autotrophicum* (ATCC#43914D, Manassas, VA, USA) and (*Methanococcus maripaludis* ATCC#43000D) were used to estimate *Bacterial* and Archaea 16S rRNA gene copy numbers respectively. These above assumptions were also used to estimate the number of benzene degraders. Calibration curves (10^1–10^8 gene copies μl^{-1}) were prepared for all genes under consideration, yielding r^2 values ≥ 0.99.

Bacteriophage λ (500 bp) was used as an internal standard. The λ was added in the samples prior to DNA

extraction for the determination of DNA efficiency recovery. Recoveries lower than 100% were normalized to the fraction recovered. DNA recoveries ranged from 23% to 87%. Such variations in DNA recoveries are commonly reported in soil (Zhou *et al*., 1996) and are probably due to the binding of sample impurities (e.g. humic acids) that interfere with the activity of *Taq* polymerase during PCR analysis (Porteous *et al*., 1997).

DNA extraction

DNA was extracted using the MoBio Power Soil™ kit (Carlsbad, CA, USA) according to the manufacturer's protocol. A bead-beating device (Mini Beadbeater-8, Biospec) was utilized for cell lysis. A 100 μl of DNA sample was then collected in a 1.5 ml Eppendorf vial and stored in a freezer (Isotemp® Basic) at –75°C. The concentration and purity of the DNA was measured based on the wavelength absorbance ratio (absorbance of 260 nm for DNA and 280 nm for protein) A_{260}/A_{280} using a spectrophotometer Amersham Biosciences (Model Ultraspec 2100 Pro, Piscataway, NJ, USA).

Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis analyses were conducted to determine the dominant bacteria present in the benzene-enriched methanogenic consortium. DNA was extracted from the consortium at time 0 and 51 days after feeding it with benzene (869 μM) (Fig. 1).

PCR-based DGGE was conducted using a Biometra® thermocycler (Model T-gradient, Goettingen, Germany). The PCR reaction mix consisted of 1× Qiagen® Taq PCR buffer, the bacterial forward (5′-ATGGCTGTCGTCAGCT-3′) and reverse (5′-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCCACGGGCGGTGTGTAC-3′) primers (0.5 μM each) (Ferris *et al*., 1996), 8 μl of the sample DNA, and DNAase free water to make up a final volume of 100 μl. The pair of primers used amplified a 323 bp section of the 16S rRNA genes of the members of the domain *Bacteria*. The PCR temperature conditions for this set of primers was: 94°C 5 min, 94°C 1 min, 53–43°C (–1°C $cycle^{-1}$) (total of 10 cycles), 72°C 3 min, 94°C 1 min, 43°C 1 min (total of 20 cycles), 72°C 3 min and a final extension of 72°C for 10 min. Once the PCR reaction was finished, the presence of PCR products were verified on an agarose gel prior to DGGE analysis to ensure sufficient amplified DNA. The PCR gel consisted of 1% agarose containing 1× TAE running buffer and the dye ethidium bromide (0.5 μg ml^{-1}). The presence of PCR products were visualized under UV light (EpiChemi UVP BioImaging System, Upland, CA, USA).

Denaturing gradient gel electrophoresis was performed using a BIO-RAD DCODE™ Universal mutation detection system. The acrylamide gel consisted of a high denaturant (80%) and low denaturant (30%) phase. Denaturing gradient gel electrophoresis was run for 14 h at 46 V. Fragments of DNA (bands) in the polyacrylamide gel were excised and sequenced (Lone Star Laboratories, Houston, TX, USA). Phylogenetic affiliations were determined by comparing the DNA sequences retrieved to known bacterial sequences in the Ribosomal Database Project II (http://rdp.cme.msu.edu/index.jsp) and the BLAST facility of the National Center for Biotechnology Information (http://ncbi.nlm.nih.gov/Blast). [9] [10]

Correlation between benzene degradation and 16S rRNA gene copy numbers

Benzene concentration was measured at different locations in 120-cm-long aquifer columns (7.3 days' hydraulic retention time) exposed to benzene, toluene, ethylbenzene, *o*-, *m*- and *p*-xylenes (BTEX) and ethanol (Da Silva and Alvarez, 2002; 2004). The average benzene degradation activity (A) between any two points along the length of the column was estimated as the product of the flow rate ($Q = 0.12$ l day^{-1}) and the observed decrease in benzene concentrations ($C_2 - C_1$) [i.e. $A = Q \times (C_2 - C_1)$].

One of these columns was bioaugmented 3 years ago with the same benzene-enriched methanogenic consortium used in this work to develop the primers and probe (Da Silva and Alvarez, 2004). A second column, which was not bioaugmented, showed toluene (but not benzene) degradation activity after 2 years of acclimation (Da Silva and Alvarez, 2004) and served as a negative control.

Benzene, ethanol and methane concentration profiles along the length of the columns were determined using gas chromatography (GC). Aqueous samples for benzene, toluene, ethanol and methane analyses were collected with a 1 ml gas-tight syringe directly from the columns' port vials and injected into 5 ml GC vials previously capped with 20 mm Teflon-coated septa and aluminium crimps. A 0.1 ml of headspace sample was then injected directly into a Hewlett-Packard model 5890 series II GC equipped with a Supelco 60/80 Carbopack column (6 feet and 1/8 inch diameter) and a flame ionization detector.

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