A Real-Time Polymerase Chain Reaction Method for Monitoring Anaerobic, Hydrocarbon-Degrading Bacteria Based on a Catabolic Gene

HARRY R. BELLER,*,† STACI R. KANE,† TINA C. LEGLER,† AND PEDRO J. J. ALVAREZ‡

Lawrence Livermore National Laboratory, P.O. Box 808, L-542, Livermore, California 94551, and Department of Civil and Environmental Engineering, University of Iowa, Iowa City, Iowa 52242

We have developed a real-time polymerase chain reaction (PCR) method that can quantify hydrocarbon-degrading bacteria in sediment samples based on a catabolic gene associated with the first step of anaerobic toluene and xylene degradation. The target gene, *bssA*, codes for the α-subunit of benzylsuccinate synthase. The primer−probe set for real-time PCR was based on consensus regions of *bssA* from four denitrifying bacterial strains; *bssA* sequences for two of these strains were determined during this study. The method proved to be sensitive (detection limit ca. 5 gene copies) and had a linear range of >7 orders of magnitude. We used the method to investigate how gasohol releases from leaking underground storage tanks could affect indigenous toluene-degrading bacteria. Microcosms inoculated with aquifer sediments from four different sites were incubated anaerobically with BTEX (benzene, toluene, ethylbenzene, and xylenes) and nitrate in the presence and absence of ethanol. Overall, population trends were consistent with observed toluene degradation activity: the microcosms with the most rapid toluene degradation also had the largest numbers of *bssA* copies. In the microcosms with the most rapid toluene degradation, numbers of *bssA* copies increased 100- to 1000-fold over the first 4 days of incubation, during which time most of the toluene had been consumed. These results were supported by slot blot analyses with unamplified DNA and by cloning and sequencing of putative *bssA* amplicons, which confirmed the real-time PCR method's specificity for *bssA*. Use of a companion real-time PCR method for estimating total eubacterial populations (based on 16S rDNA) indicated that, in some cases, ethanol disproportionately supported the growth of bacteria that did not contain *bssA*. The real-time PCR method for *bssA* could be a powerful tool for monitored natural attenuation of BTEX in fuel-contaminated groundwater. To our knowledge, this is the first reported molecular method that targets anaerobic, hydrocarbon-degrading bacteria based on a catabolic gene.

* Corresponding author e-mail: beller2@llnl.gov; phone: (925)-422-0081; fax: (925)423-7998.

† Lawrence Livermore National Laboratory.

‡ University of Iowa.

Introduction

A major challenge for regulatory acceptance of in situ bioremediation is to demonstrate that decreases in the concentrations of groundwater contaminants truly represent biological metabolism of these compounds rather than abiotic processes such as sorption or dilution (*1*, *2*). Approaches proposed for monitoring bioremediation of gasoline-contaminated aquifers include measurement of distinctive metabolites in groundwater (e.g., refs *3*−*5*) or stable carbon isotope ratios of dissolved inorganic carbon or residual hydrocarbons (e.g., refs *6* and *7*). Another approach is to show that the indigenous microbial community includes bacteria known to be capable of degrading the contaminants and, ideally, that these bacteria are enriched in contaminated zones. Molecular (i.e., nucleic acid-based) techniques are preferred for such monitoring because they preclude laboratory isolation and cultivation of bacteria, which can be extremely time-consuming and often unsuccessful at including the majority of bacterial species in environmental samples. Preferably, molecular techniques would focus on catabolic genes that code for specific pollutant-degrading enzymes (e.g., refs *8* and *9*); however, to date, molecular ecology techniques have focused overwhelmingly on phylogeny and have relied on 16S rRNA (or 16S rDNA) to identify bacterial species in environmental samples (e.g., refs *10*−*12*).

In this study, we were interested in in situ hydrocarbon biodegradation associated with leaking underground fuel tank (LUFT) sites and therefore focused on detection of a gene associated with anaerobic toluene degradation, which is highly relevant to such sites (*4*). Understanding of the biochemistry and genetics of anaerobic toluene degradation has increased dramatically in the past five years. A novel enzyme that catalyzes the first step of anaerobic toluene degradation, benzylsuccinate synthase (BSS), has been reported in cultures that degrade toluene under denitrifying (*13*, *14*), sulfate-reducing (*15*, *16*), anoxygenic phototrophic (*17*), ferric iron-reducing (*18*), and methanogenic (*19*) conditions; all of these studies involved in vitro enzyme assays. BSS has been purified from two denitrifying bacteria (*20*, *21*), and the gene encoding for the large (α) subunit of BSS (*bssA* or *tutD*) has been sequenced from two strains of the denitrifying bacterium *Thauera aromatica* (*20*, *22*). Notably, BSS has recently been shown to catalyze the first step of anaerobic xylene degradation (*23*, *24*). Thus, detection of bacteria harboring genes for BSS are relevant to anaerobic xylene as well as toluene degradation. To date, no enzyme other than BSS has been identified that catalyzes the first (activation) step of anaerobic toluene degradation. Although another activation step was proposed for *Azoarcus tolulyticus* strain Tol-4 (*25*), the proposed enzyme activity has not been demonstrated with in vitro experiments, and in fact, this strain has been shown to contain BSS activity (*15*) and genes (this study).

Real-time, quantitative polymerase chain reaction (PCR) analysis (*26*−*28*), specifically the 5′-nuclease or TaqMan assay, was chosen for this study because it offers many advantages over traditional methods used to detect specific bacterial populations in environmental samples, such as DNA:DNA hybridization. A primary disadvantage of membrane-based, DNA:DNA hybridization methods, such as slot blot hybridization, is low sensitivity: detection limits are on the order of 10^5 or greater for functional genes, which typically occur at a frequency of only one copy per cell. The PCR (*29*) has been used to increase the quantity of low abundance genes for detection. Most PCR-based, quantitative methods

10.1021/es025556w CCC: $22.00
Published on Web 00/00/0000

rely on amplification of target DNA to a sufficient concentration to allow end-point detection. However, quantification based on end-point detection can be compromised by several factors, including (i) reagent depletion and (ii) PCR biases caused by the competition of amplicons with PCR primers for template annealing (e.g., refs *30* and *31*). The real-time PCR method precludes problems associated with end-point detection by quantifying amplified DNA in an early, exponential phase of the reaction. In addition to high sensitivity, real-time PCR has several favorable characteristics, including a linear range of ≥5 orders of magnitude, high specificity, relatively rapid analysis, and accuracy in a complex DNA background (*30*, *32*−*34*). Although most real-time PCR applications to date have involved the detection and quantification of pathogenic bacteria in food or animal tissue, the technique has recently been used to quantify specific bacteria in environmental samples based on their 16S rDNA (*31*, *32*, *34*) or on a species-specific nitrate reductase gene (*33*).

In this paper, we describe the development and use of a real-time PCR method to assess anaerobic, toluene-degrading bacteria in aquifer sediment based on the abundance of *bssA*. The molecular method was used to investigate how gasohol releases from leaking underground fuel tanks could affect indigenous toluene-degrading bacteria. Microcosms inoculated with aquifer sediments from four sites with different histories of exposure to fuel hydrocarbons were incubated anaerobically with BTEX and nitrate in the presence and absence of ethanol. Real-time PCR analysis was used to quantify bacteria containing the *bssA* gene as well as total eubacteria (represented by a universal eubacterial probe based on 16S rDNA). To our knowledge, this is the first instance of a molecular method that targets anaerobic, hydrocarbon-degrading bacteria based on a functional (catabolic) gene.

Experimental Section

Microcosm Construction, Sampling, and Chemical Analysis. Microcosms (constructed in duplicate) consisted of 40 mL of artificial groundwater and 10 g of homogenized aquifer sediment; they were contained in 125-mL amber glass bottles sealed with Teflon Mininert valves (Alltech Associates, Inc., Deerfield, IL) and were prepared and incubated under anaerobic conditions in a glovebox with an atmosphere composed of N_2 (80%), CO_2 (10%), and H_2 (10%). The artificial groundwater, which was rigorously deoxygenated before use, had a composition similar to that described by von Gunten and Zobrist (*35*), except that lactate was excluded and the bicarbonate, nitrate, ammonium, and phosphate concentrations were 7.3, 5.3, 0.3, and 0.02 mM, respectively. All microcosms were amended with BTEX at concentrations of 0.4−3 mg/L (for individual compounds); when added, ethanol was present at 50−100 mg/L. Analytical chemistry monitoring was performed on a parallel series of microcosms that were identical except for the following: the "analytical" microcosms were twice the size and were prepared in triplicate. BTEX, ethanol, and nitrate were monitored regularly; nitrate was added again if it was determined to be depleted. For analysis of BTEX and ethanol, aqueous samples (1 mL) were collected using gastight syringes and were analyzed with a Hewlett-Packard 5890 series II gas chromatograph equipped with an HP 19395A headspace autosampler and flame ionization and photoionization detectors. Nitrate was analyzed by ion chromatography (Dionex series 4500i) with anionic micromembrane suppression and conductivity detection.

Microcosms were inoculated with aquifer sediments from four sites with different histories of exposure to fuel hydrocarbons: (i) a LUFT site at Travis Air Force Base (AFB) in California (*36*); (ii) a LUFT site in Sacramento, CA (*37*); (iii) an ethanol- and fuel-contaminated terminal in the Pacific Northwest (Northwest Terminal) (*5*, *38*); and (iv) a background, uncontaminated site in Tracy, CA. For each site, microcosms were constructed with and without ethanol. In general, sacrificial sampling of microcosms for real-time PCR analysis was based on the depletion of ethanol and toluene. Sampling times conformed to the following guidelines: (i) control microcosms were sacrificed without incubation (initial conditions); (ii) when ethanol was depleted in the ethanol-amended microcosms, both ethanol-amended *and* unamended microcosms were sacrificed; and (iii) when toluene was depleted in either ethanol-amended or unamended microcosms, those microcosms were sacrificed. Sacrificed microcosms were centrifuged, and the pelleted aquifer material was frozen and stored at −40 °C until shipment to Lawrence Livermore National Laboratory (LLNL) in coolers filled with ice. After receipt at LLNL, centrifuged microcosm samples were stored at −80 °C until DNA extraction.

DNA Extraction. The DNA extraction protocol was modified from Zhou et al. (*39*). Five grams of wet sediment from each microcosm was extracted three times using sodium dodecyl sulfate and heating at 65 °C. Prior to extraction, PCR Inhibitor Removal Solution (Mo Bio Laboratories, Inc., Carlsbad, CA) was added at a 10% final concentration. In addition, 10 ng of a 500-bp PCR product (position: 7131−7630) amplified from bacteriophage λ was added as an internal standard to assess DNA recovery on a sample-specific basis (*40*). The supernatants from each of the three extractions were combined and mixed with an equal volume of chloroform:isoamyl alcohol (24:1, vol/vol). Phase-lock gel (Heavy, Eppendorf AG, Hamburg, Germany) was added prior to centrifugation (1500*g*, 3 min) to form a solid interface between the upper aqueous phase and the lower organic phase. DNA was precipitated from the aqueous phase by adding 2-propanol (0.6 vol) and linear acrylamide (25 μg, Ambion, Austin, TX) to facilitate precipitation, incubating for 1 h, and centrifuging (16000*g*, 20 min, room temperature). The DNA pellet was dried under vacuum (Speed Vac DNA120, Savant, Farmingdale, NY) and resuspended in 500 μL of TE buffer (Tris-OH, 10 mM; EDTA, 1 mM; pH 8). The crude DNA extract was further purified and concentrated 5-fold (to 100 μL final volume) using a Mo Bio Spin Filter following the manufacturer's protocols.

Real-Time PCR. The real-time PCR method uses the 5′-nuclease activity of *Taq* DNA polymerase (from *Thermus aquaticus*) to cleave an internal dual-labeled "probe" annealed to the target DNA sequence as it polymerizes through the region (*26*−*28*). The probe has two fluorescent dyes, a "reporter" at the 5′ end and a "quencher" at the 3′ end. Energy absorbed by the reporter is transferred to the quencher when the two dyes are in close proximity. However, when the probe is cleaved, the quencher is no longer associated with the reporter and fluorescence is emitted. The amount of fluorescence is proportional to the number of PCR amplicons produced. A threshold cycle (C_T) is identified as the PCR cycle at which the fluorescence emitted exceeds a threshold value (for this system, the sample-specific threshold was defined as 10 times the standard deviation of the background fluorescence). The amount of gene target originally present in the sample was determined by reference to a calibration curve of log target concentration versus C_T.

Primers and probes were designed based on examination of sequences of *bssA* in a variety of toluene-degrading, denitrifying bacteria: *Azoarcus* sp. strain T (*23*), *Thauera aromatica* strains T1 and K172 (*20*, *22*), and *Azoarcus tolulyticus* strain Tol-4 (*25*). The *bssA* sequences for strains T1 and K172 are available in the GenBank database (accession numbers AF113168 and AJ001848, respectively), whereas those for strains T and Tol-4 were determined in this study by designing PCR primers based on strains T1 and K172.

[Achong et al. (*24*) published the *bssA* sequence for strain T after this study was completed.] After PCR amplification, the amplicons putatively containing *bssA* from strains T and Tol-4 were cloned, sequenced, and aligned with known *bssA* sequences to determine consensus regions for real-time PCR primer and probe design. Primer and probe sequences are shown in Table 1. The 28-base fluorogenic probe was 100% homologous to *bssA* genes in all four denitrifiers, whereas the 18-base PCR primers were degenerate, containing one or two alternate bases in order to be homologous to all four *bssA* genes in the denitrifying bacteria. The estimated melting temperatures of *bssA* primers were ca. 60 and 61 °C for the forward and reverse primers, respectively, whereas that of the probe was ca. 71 °C. The specificity of the *bssA* primers and probe were confirmed with database (blastn) searches in GenBank, which showed that the combination of primer and probe sequences (including the relative spacing of primer and probe regions) only had significant similarity with *bssA* sequences from the denitrifying, toluene-degrading strains that were used for primer and probe design.

Reactions were also performed using primers and a probe developed by Suzuki et al. (*31*) for 16S rDNA of eubacteria (Table 1). A primer−probe set specific to bacteriophage λ was used to quantify the recovery of the internal standard in a separate PCR analysis (Table 1).

A 5-μL volume of extracted, undiluted DNA (*bssA*) or 10-fold diluted DNA (16S rDNA or λ) was subjected to real-time PCR using a Smart Cycler System (Cepheid, Sunnyvale, CA). The PCR mixture contained 0.625 μM of each primer, either 0.2 μM probe (*bssA* or 16S rDNA) or 0.195 μM probe (λ), 1.25 U *Taq* DNA polymerase and 1× PCR buffer A (Fisher Scientific, Pittsburgh, PA), 1 mM of each deoxynucleoside triphosphate, 1× Cepheid additive reagent [0.2 mg/mL bovine serum albumin (BSA), 150 mM trehalose, 0.2% Tween 20, and 0.2 mM Tris buffer; pH 8.0], and sterile water to give a final volume of 25 μL. For amplification of 16S rDNA, BSA and trehalose were omitted because they contributed to the fluorescence background in negative control reactions that lacked an added template. The PCR conditions were as follows: 95 °C for 10 min; 50 cycles of 95 °C for 15 s and 58 °C for 60 s. For selected samples, serial dilutions of DNA ranging over 3 orders of magnitude were analyzed to test whether the results for undiluted (*bssA*) or 10-fold diluted samples were affected by PCR inhibition.

Quantification and Underlying Assumptions. Dilutions of *T. aromatica* strain K172 genomic DNA were used to generate calibration curves for *bssA* quantification. The quantity of DNA in the standards was determined using a Hoefer DyNA Quant 200 fluorometer (Amersham Pharmacia, Piscataway, NJ) according to the manufacturer's protocols. The following assumptions were used in calculating the number of *bssA* copies in a sample: (i) the approximate size of the strain K172 genome was 4.6 Mbp [the genome size of *Escherichia coli* (*41*)]; (ii) there was one copy of *bssA* per genome; and (iii) the *bssA* calibration curve for strain K172 was representative of other bacteria containing *bssA*. Data supporting these assumptions will be addressed in the Results and Discussion section. The last assumption is necessary given the genetic diversity that will inevitably be encountered in environmental samples (e.g., the natural diversity of *bssA* sequences among toluene-degrading, anaerobic bacteria). Similar calibration curves were used for 16S rDNA; in this case, genomic DNA from *E. coli* strain DH5α was used for standard dilutions (10^3−10^7 copies of 16S rDNA), and analogous assumptions were used for quantification. Real-time PCR analysis of the internal standard λ was used to correct the *bssA* and 16S rDNA data for efficiency of DNA recovery. Specifically, when the recovery of λ was <100%, *bssA* and 16S rDNA data were normalized to correct for recovery. However, no correction was made for samples in

TABLE 1. Primer and Probe Sequences Used for Real-Time PCR

target	forward primer[b]	reverse primer[c]	probe[d]
bssA	5′ACGACGGYGGCATTTCTC3′	5′GCATGATSGGYACCGACA3′	FAM-5′ CTTCTGGTTCTTCTGCACCTTGGACACC 3′-TAMRA[e]
16S rDNA[a]	5′CGGTGAATACGTTCYCGG3′	5′GGWTACCTTGTTACGACTT3′	FAM-5′CTTGTACACACCGCCCGTC3′-BHQ-1[f]
bacteriophage λ	5′ACGCCACGCGGGATG3′	5′AGAGACACGAAACGCCGTTC3′	TET-5′ACCTGTGGCATTTGTGCTGCCG3′-TAMRA[g]

[a] The forward primer (BACT1369F), reverse primer (PROK1492R), and probe (TM1389F) were developed by Suzuki et al. (*31*). [b] Y = C or T. [c] S = C or G; Y = C or T; W = A or T. [d] The probes were synthesized by BioServe Biotechnologies, Ltd. (Laurel, MD) and Integrated DNA Technologies, Inc. (Coralville, IA). [e] The reporter dye was FAM (6-carboxyfluorescein), and the quencher dye was TAMRA (6-carboxytetramethylrhodamine). [f] The reporter dye was FAM, and the quencher dye was BlackHole Quencher-1. [g] The reporter dye was TET (tetrachloro-6-carboxyfluorescein), and the quencher dye was TAMRA.

which λ recoveries exceeded 100%. Unlike the quantification method used for *bssA* and 16S rDNA, no assumptions had to be made for λ quantification because the λ standard solutions contained DNA fragments of known length (not genomic DNA), and natural genetic diversity was not an issue (λ DNA added to samples was identical to that in the standard solutions).

Slot Blot Analyses for *bssA* in Unamplified DNA. As an independent check on real-time PCR results for *bssA* in selected microcosms, slot blot analyses were performed. The DNA extracts (50-μL portions) from Travis AFB microcosms along with genomic standards from *T. aromatica* strain K172 (5-fold dilutions from ca. 8×10^4 to 8×10^6 copies of *bssA*) and *E. coli* genomic DNA (10^8 copies) as a negative control were analyzed by slot blot hybridization as described by Kane et al. (*18*). In this study, the 1060-bp PCR product amplified from the *bssA* gene of *T. aromatica* strain K172 (GenBank accession no. AJ001848, position 8007-9066) was labeled using a Megaprime DNA labeling kit (Amersham Pharmacia) with 50 μCi (62.5 pmol) of both [α-^{32}P]-dCTP and [α-^{32}P]-dATP according to manufacturer's protocols. The hybridization signals were quantified using a Phosphorimager SI with ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Cloning and Sequence Analysis of Real-Time PCR Amplicons. To confirm that the real-time PCR primers and probe were specifically targeting *bssA*-like sequences in the microcosms (i.e., false positive detections were not occurring), amplicons from both Travis AFB and Tracy microcosm extracts were cloned into the TA cloning vector, pCRII (Invitrogen, Carlsbad, CA). Individual clones were blotted (*42*) and probed as described above except that a 28-base oligonucleotide identical to the *bssA* probe (Table 1) was labeled using a 5′ end-labeling kit (Amersham Pharmacia) according to manufacturer's protocols. Plasmid DNA was isolated from *bssA*-positive clones and sequenced as described by Kane et al. (*18*).

Results and Discussion

Sensitivity, Linear Range, and Specificity of the Real-Time PCR Method for *bssA*. Representative PCR amplification progress curves for *bssA* standards are presented in Figure 1A. These data include standards ranging from ca. 8 to 7.7 $\times 10^7$ template copies along with a no-template control. Figure 1B shows the inverse relationship between C_T and log *bssA* copies and demonstrates linearity ($r^2 = 0.997$) over 7 orders of magnitude. The detection limit for *bssA* was estimated as ~5 templates per 5-μL sample; therefore, the method detection limit was ~200 templates per 50-mL microcosm (accounting for dilution). The no-template control, which was analyzed along with each batch of samples, always gave a similar result to that shown in Figure 1A, demonstrating that *bssA* contamination was not introduced into real-time PCR samples.

The specificity of the *bssA* primer−probe set was tested by analyzing 2×10^4 genome copies of *E. coli* as a negative control. *E. coli* DNA was used as a negative control because it contains a gene coding for pyruvate formate-lyase (PFL), which has strong similarity to the C-terminal region of BssA (*43*) but plays no role in anaerobic toluene metabolism. As was the case for no-template controls, no increase in fluorescence was detected, demonstrating that there was no significant cross-reactivity with the *pfl* gene (or any other genes) present in *E. coli*.

Assessment of Assumptions Involved in *bssA* Quantification by Real-Time PCR. The assumptions (discussed previously) that there was one copy of *bssA* per genome in the strain K172 standards and that the genome size was 4.6 Mbp were assessed by comparing the genomic DNA standards (Figure 1) to a set of plasmid-based standards that contained 1060-bp inserts of *bssA* from strain K172 (GenBank accession no. AJ001848, position 8007-9066, which includes the PCR target region). The slopes and intercepts of calibration curves for the genomic and plasmid-based standards agreed within 0.6% (data not shown). Since the absolute concentration of *bssA* copies in the plasmid-based standards was known to a high degree of certainty, the close agreement of the genomic and plasmid-based calibration curves suggests that both assumptions are reasonable. Furthermore, the number of *bssA* copies per cell did not vary significantly as a function of growth phase ($P > 0.05$), based on quantification of *bssA* in logarithmic and stationary phase cultures of *Azoarcus* sp. strain T (data not shown).

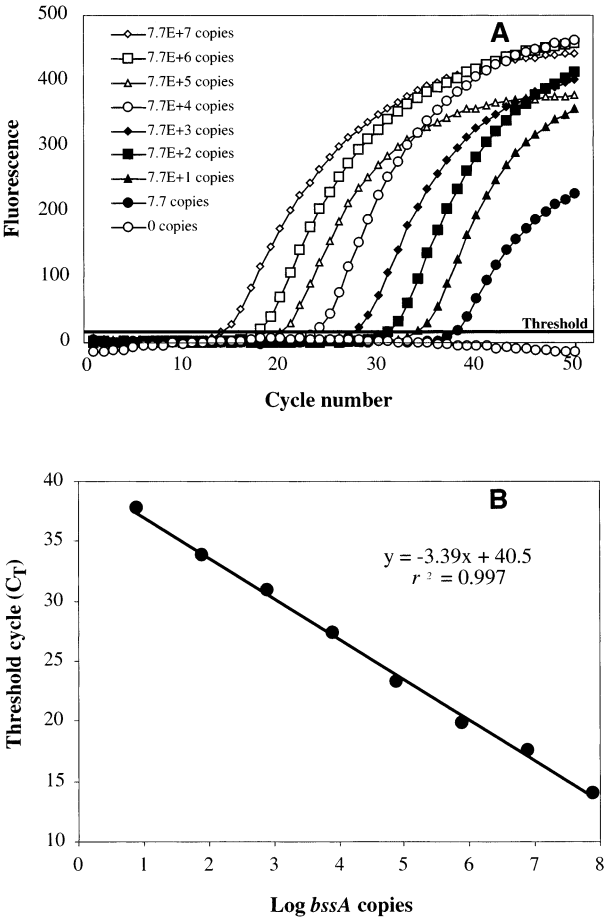

FIGURE 1. Example of calibration data for *bssA* (using genomic DNA from *Thauera aromatica* strain K172). (A) Fluorescence vs PCR cycle number for eight *bssA* standard solutions and a no-template control. The threshold level (see text) is denoted by a line; the threshold cycle (C_T) is defined as the PCR cycle at which fluorescence exceeds this threshold level. (B) Threshold cycle vs log *bssA* copies for the data presented in panel A.

To address the assumption that *bssA* standards derived from strain K172 are representative of *bssA* in other bacteria, we compared calibration curves generated with dilutions of genomic DNA from three toluene-degrading, denitrifying bacterial strains (*T. aromatica* strain K172, *Azoarcus* sp. strain T, and *A. tolulyticus* strain Tol-4). The *bssA* calibration slopes and intercepts for the three strains were similar [3.7% and 6.7% relative standard deviation (RSD), respectively] over the range of 10^2−10^6 genome copies, which indicates that the calibration based on strain K172 used in this study would accurately quantify *bssA* in at least two other toluene-degrading strains.

Performance of the Real-Time PCR Method for the 16S rDNA and λ (Internal Standard) Primer−Probe Sets. Similar calibration curves were generated for 16S rDNA and λ as

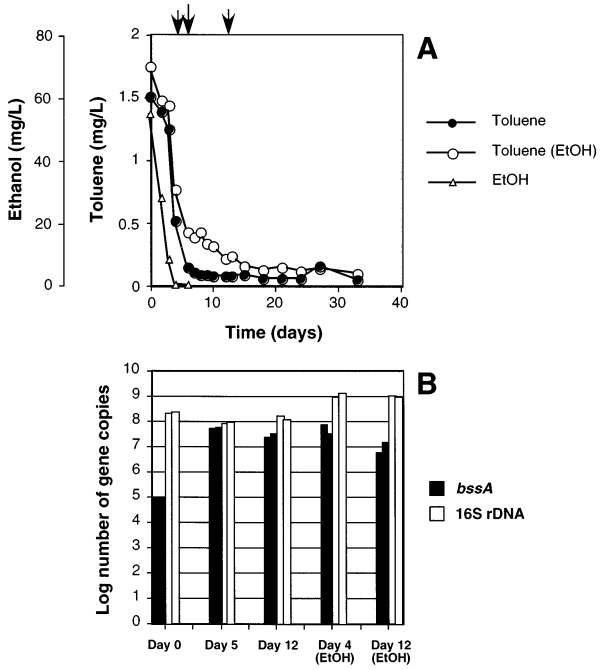

FIGURE 2. Results for microcosms inoculated with aquifer sediment from Travis AFB and incubated under denitrifying conditions. (A) Concentrations of toluene (in the presence and absence of ethanol) and ethanol are shown. Data points represent the averages of results of triplicate measurements. The median RSD for all toluene data was 17%. Nitrate (not shown) was depleted in the ethanol-amended microcosms and was added again on day 10. Arrows designate sampling times for real-time PCR analysis. (B) Numbers of copies (per 50-mL microcosm, expressed as a base 10 logarithm) of *bssA* (a gene associated with anaerobic toluene degradation) or eubacterial 16S rDNA, based on real-time PCR analysis. All individual replicates are shown. Recoveries of the internal standard for DNA (λ) ranged from 49 to 101% for all replicates shown.

those described for *bssA* quantification, with standards ranging from 10^3 to 10^7 genome copies of *E. coli* or from 10^4 to 10^8 copies of the 500-bp λ fragment, respectively. Calibration curves for 16S rDNA and λ were linear ($r^2 \geq 0.992$). The no-template controls for 16S rDNA analysis were always nondetect (no fluorescence increase), whereas those for λ analysis occasionally had C_T values ≥ 40, which was negligible (λ copies present in microcosm samples were >7 orders of magnitude higher than those calculated for the no-template control).

Results for Travis AFB Microcosms. Ethanol and toluene were largely depleted within the first 5 days of incubation (Figure 2A); the two compounds were degraded concurrently, and the toluene degradation rate was similar in the presence and absence of ethanol. A very steep increase in the number of *bssA* copies occurred between day 0 (i.e., the controls) and days 4−5 (Figure 2B): copies of *bssA* increased from ca. 10^5 initially to between 10^7 and 10^8. Analyses of two additional replicates confirmed the control *bssA* data (<3% RSD for all four values of log *bssA* at day 0). The presence of ethanol had no clear effect on numbers of *bssA* copies, which is consistent with its lack of effect on toluene degradation activity.

The steep increases in *bssA* copies indicated by real-time PCR (Figure 2B) are supported by slot blot analyses conducted with unamplified DNA. For the four Travis AFB samples collected on days 4 and 5, quantification of *bssA* copies by real-time PCR ($5.2 \times 10^7 \pm 1.7 \times 10^7$; mean ± SD) and slot blot analysis ($3.1 \times 10^7 \pm 2.5 \times 10^7$) agreed within a factor of 2. Furthermore, cloning and sequencing of putative *bssA* amplicons from these microcosms demonstrated that *bssA* copies were indeed being quantified. Of 25 clones analyzed, 15 had *bssA* sequences identical to that of *Azoarcus* sp. strain T (GenBank accession no. AY032676). Overall, for the 25 amplicons (132 bp), there were 1.4 ± 2.5 mismatches (mean ± SD) relative to the sequence for strain T.

The rapid increases in *bssA* copies (Figure 2B) are also consistent with reported yields and generation times for toluene-degrading, denitrifying bacteria. For example, an increase on the order of 10^7−10^8 cells is plausible, given the reported range of cell yields for toluene-degrading, denitrifying strains (ca. 50−100 g cells/mol toluene; *44*), the mass of toluene consumed in these microcosms (Figure 2A), and the approximate mass of a bacterium (*45*). Assuming an average of one copy of *bssA* per cell, the observed 100- to 1000-fold increase in 4 days is reasonable; reported doubling times of denitrifying bacteria growing on toluene (*44*) suggest that a 1000-fold increase (i.e., 10 doublings) would occur in ≤2.5 days.

In contrast to its apparent lack of effect on toluene degradation, ethanol had an effect on total eubacterial populations (represented by 16S rDNA). In the absence of ethanol, the numbers of copies of 16S rDNA did not change notably from the initial value of ca. 2×10^8 (Figure 2B). However, for ethanol-amended microcosms, there was a 4- to 5-fold increase relative to the initial conditions. The apparent number of additional eubacterial cells resulting from growth on ethanol (Figure 2B) is generally consistent with the estimate of 6×10^9 cells based on a thermodynamic calculation method described by McCarty (*46*), the known mass of ethanol consumed under denitrifying conditions in this experiment (Figure 2A), and the assumption that the number of 16S rDNA copies/genome was the same in the samples and the real-time PCR standards.

Results for Sacramento Microcosms. The toluene degradation rates for microcosms inoculated with aquifer sediment from a LUFT site in Sacramento were considerably slower than those observed for Travis AFB microcosms (Figure 3A). The rates for ethanol-amended and unamended microcosms were similar until day 30, at which time the rate appeared to increase somewhat in ethanol-amended microcosms (Figure 3A).

Overall, there was not a wide range of *bssA* values among all the samples and controls (Figure 3B). Ethanol degradation may have slightly increased the number of *bssA* copies; at day 3 (after ethanol was degraded), the numbers of *bssA* copies in ethanol-amended microcosms were approximately 170% those in unamended microcosms. As was observed for Travis AFB microcosms, growth on ethanol appreciably increased the populations of total eubacteria. Numbers of 16S rDNA copies in ethanol-amended microcosms were, on average, approximately 50 times those of the controls (Figure 3B). Smaller increases in total eubacteria were also observed for unamended microcosms (Figure 3B), presumably as a result of growth on indigenous organic matter in the aquifer sediment.

Results for Tracy Microcosms. The Tracy site was chosen to be representative of aquifer materials that have not been exposed to LUFT contamination. Relatively slow toluene degradation was observed under denitrifying conditions, and ethanol had no apparent effect on the rate (Figure 4A).

Numbers of *bssA* copies in all incubated samples were roughly equal to numbers in the control (initial) samples (Figure 4B). The variability apparent in *bssA* values among samples may be due to the fact that they are approaching detection limits in this sample matrix (e.g., *bssA* was not detectable in one replicate collected at day 51 despite the favorable 85% λ recovery in this sample). The detection of *bssA* in aquifer material that has not been exposed to LUFT contamination is not particularly surprising, as toluene-degrading, denitrifying bacteria have been found in a range

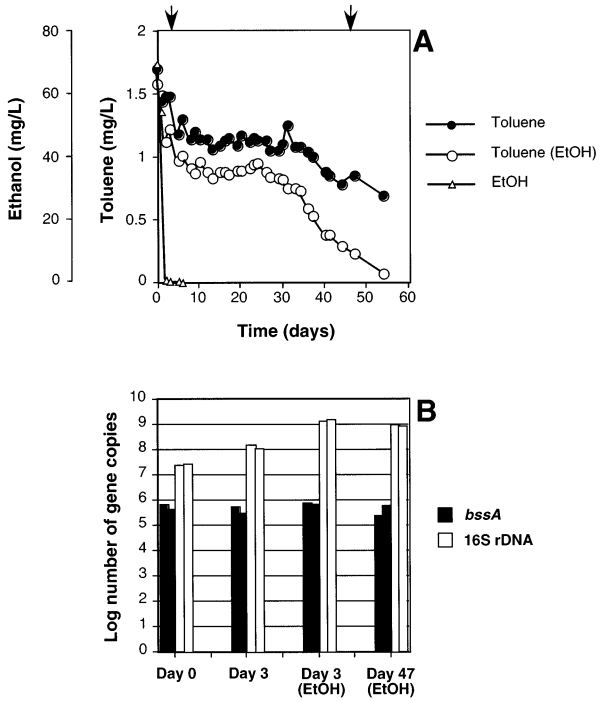

FIGURE 3. Results for microcosms inoculated with aquifer sediment from the Sacramento site and incubated under denitrifying conditions. (A) Concentrations of toluene (in the presence and absence of ethanol) and ethanol are shown. Data points represent the averages of results of triplicate measurements. The median RSD for all toluene data was 28%. Nitrate (not shown) was depleted in the ethanol-amended microcosms and was added again on day 5. Arrows designate sampling times for real-time PCR analysis. (B) Numbers of copies (per 50-mL microcosm) of *bssA* or eubacterial 16S rDNA, based on real-time PCR analysis. All individual replicates are shown. Recoveries of the internal standard for DNA (λ) ranged from 25 to 41% for all replicates shown.

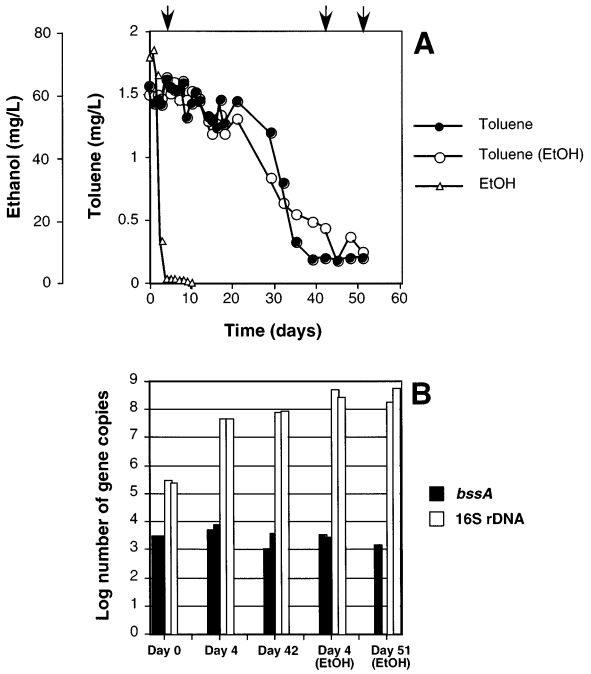

FIGURE 4. Results for microcosms inoculated with aquifer sediment from the Tracy site and incubated under denitrifying conditions. (A) Concentrations of toluene (in the presence and absence of ethanol) and ethanol are shown. Data points represent the averages of results of triplicate measurements. The median RSD for all toluene data was 5.2%. Nitrate (not shown) was largely (>90%) depleted in the ethanol-amended microcosms and was added again on day 8. Arrows designate sampling times for real-time PCR analysis. (B) Numbers of copies (per 50-mL microcosm) of *bssA* or eubacterial 16S rDNA, based on real-time PCR analysis. All individual replicates are shown. Recoveries of the internal standard for DNA (λ) ranged from 21 to 85% for all replicates shown.

of pristine environments (*47*). Furthermore, cloning and sequencing of putative *bssA* amplicons (132 bp) from these microcosms indicated that false positive detections were not of concern. For 23 clones analyzed, two had *bssA* sequences identical to that of *Azoarcus* sp. strain T, whereas the most divergent clone had 15 mismatches relative to strain T.

It appears that ethanol enhanced the numbers of eubacterial 16S rDNA copies, as the average abundance in ethanol-amended microcosms was ca. 5.5 times higher than the average in the unamended microcosms. However, total eubacteria also increased in unamended microcosms (Figure 4B).

Results for Northwest Terminal Microcosms. In contrast to results for microcosms from all other sites, ethanol had a dramatic and positive effect on anaerobic toluene degradation in Northwest Terminal microcosms (Figure 5A). In the presence of ethanol, toluene was mostly degraded by day 15, whereas in the absence of ethanol, there was no clear degradation in over 50 days (Figure 5A). The differences in toluene concentrations between ethanol-amended and unamended conditions cannot be explained by analytical error, as the median RSD for all toluene data was 2.5%.

If ethanol promoted toluene degradation by fortuitously supporting the growth of anaerobic, toluene-degrading bacteria, this is not reflected in the data for *bssA*. Indeed, the numbers of *bssA* copies at day 5 (when ethanol was depleted) were 2−3 times lower in ethanol-amended microcosms than in unamended ones (Figure 5B). Unfortunately, control (i.e., initial) data for *bssA* could not be generated, presumably because some compound(s) in the extracts of these samples inhibited PCR amplification. As an indication of inhibition in the control extracts, 16S rDNA was not detectable in 10^{-1} and 10^{-2} dilutions but was detectable in 10^{-3} and 10^{-4} dilutions; the abundance of *bssA* was apparently not sufficiently high to be detectable in the 10^{-3} dilution. Notably, such inhibition was not observed for samples from other sites. For example, serial dilution of genomic DNA from Travis AFB extracts (dilutions of 10^{0}, 10^{-1}, 10^{-2}, and 10^{-3}) resulted in comparable real-time PCR results for *bssA* and λ over this entire range (r^2 values for linear regressions of gene copy abundance versus dilution level were ≥0.9999).

There are several possible explanations for the lack of an apparent increase in *bssA* copies in ethanol-amended microcosms despite their enhanced toluene degradation activity: (i) a minor increase actually occurred but was within the range of experimental error for these samples, (ii) ethanol stimulated toluene degradation by some means unrelated to growth (e.g., by induction of genes associated with toluene degradation, although this seems unlikely based on current knowledge of the anaerobic toluene degradation pathway; *43*, *44*), (iii) the primer−probe set used for real-time PCR did not efficiently match the actual *bssA* sequences of the toluene-degrading bacteria in these particular microcosms (although the primer−probe set was based on a consensus sequence for four different denitrifying strains), or (iv) anaerobic toluene degradation proceeded by a metabolic pathway that did not involve BSS (although there is currently no in vitro or genetic evidence for such a pathway).

Environmental Applicability of Real-Time PCR Analysis for *bssA*. Ideally, a molecular method for in situ monitoring would target a functional gene associated with pollutant degradation, such that a strong relationship between the number of target gene copies and pollutant-degrading activity

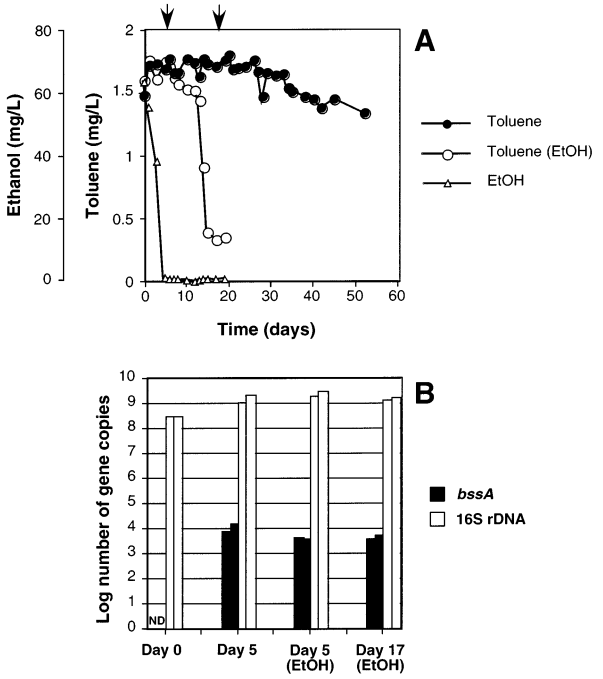

FIGURE 5. Results for microcosms inoculated with aquifer sediment from the Northwest Terminal and incubated under denitrifying conditions. (A) Concentrations of toluene (in the presence and absence of ethanol) and ethanol are shown. Data points represent the averages of results of triplicate measurements. The median RSD for all toluene data was 2.5%. Nitrate (not shown) was depleted in the ethanol-amended and unamended microcosms and was added again on day 12 (ethanol-amended) or day 6 (unamended). Arrows designate sampling times for real-time PCR analysis. (B) Numbers of copies (per 50-mL microcosm) of *bssA* or eubacterial 16S rDNA, based on real-time PCR analysis. All individual replicates are shown (ND, not detected). Recoveries of the internal standard for DNA (λ) ranged from 48 to 127% for all replicates shown.

could be confidently assumed. Our real-time PCR method for *bssA* follows this principle by targeting a key gene associated with anaerobic toluene and xylene degradation. The application of real-time PCR in this study is atypical in the sense that the primer−probe set was designed to be specific to *bssA* yet still allow for the natural genetic diversity of *bssA* among denitrifying bacteria that might be encountered in the environment. The *bssA* primer−probe set was shown to be specific enough not to detect 10^4 copies of *E. coli* genomic DNA, which contains one of the most similar genes to *bssA* that is known (i.e., *pfl*), and was also shown to perform consistently for three different toluene-degrading, denitrifying strains. Furthermore, the *bssA* primer−probe set appeared to function well when applied to microcosm samples containing indigenous (and uncharacterized) aquifer bacteria: the 100- to 1000-fold increases in *bssA* copies in Travis AFB microcosms corresponded with their rapid toluene-degrading activity and are reasonable in terms of known yields and generation times for toluene-degrading, denitrifying bacteria. Notably, the Travis AFB findings were also supported by slot blot results with unamplified DNA. Furthermore, sequence analysis of real-time PCR amplicons from Travis AFB and Tracy microcosms demonstrated sequences identical or nearly (>85%) identical to those of *bssA* genes from the reference denitrifying, toluene-degrading strains. As *bssA* sequences from a wider range of bacteria become available and the diversity of this gene is better understood, it will become possible to assess whether the primer−probe set described here will need to be altered to encompass natural diversity or whether multiple sets will be required. Regardless, real-time PCR targeted at *bssA* appears to be a promising monitoring tool for assessing natural attenuation of BTEX in fuel-contaminated groundwater.

Acknowledgments

We gratefully acknowledge T. Buscheck (ChevronTexaco Energy Research and Technology Company), Chevron Products Company, W. Day (Travis AFB), G. Mongano (U.S. Bureau of Reclamation), and M. Peterson (ETIC Engineering) for donating aquifer materials for microcosm construction; T. Alfaro, L. Balser, G. Ruiz-Aguilar, J. Fernandez-Sanchez, and M. Busi da Silva for their excellent technical assistance; R. Modali (BioServe Biotechnologies, Ltd.) for assistance with probe design; N. Quigley (University of Tennessee) for discussions about fluorometry-based DNA quantification; and J. Santo Domingo (U.S. EPA) for discussions about real-time PCR analysis based on 16S rDNA. This work was performed under the auspices of the U.S. Department of Energy by the University of California, Lawrence Livermore National Laboratory under Contract W-7405-Eng-48. Funding was provided by the California State Water Resources Control Board.

Literature Cited

(1) National Research Council. *In Situ Bioremediation: When Does It Work?*; National Academy Press: Washington, DC, 1993.
(2) Madsen, E. L. *Environ. Sci. Technol.* **1991**, *25*, 1662−1673.
(3) Beller, H. R.; Ding, W.-H.; Reinhard, M. *Environ. Sci. Technol.* **1995**, *29*, 2864−2870.
(4) Beller, H. R. *Biodegradation* **2000**, *11*, 125−139.
(5) Beller, H. R. *Environ. Sci. Technol.* **2002**, *36*, 2724−2728.
(6) Aggarwal, P. K.; Hinchee, R. E. *Environ. Sci. Technol.* **1991**, *25*, 1178−1180.
(7) Meckenstock, R. U.; Annweiler, E.; Warthmann, R.; Schink, B.; Michaelis, W.; Richnow, H. H. In *Novel Approaches for Bioremediation of Organic Pollution*; Fass, R., Flashner, Y., Reuveny, S., Eds.; Kluwer Academic Publishers: New York, 1999; pp 219−226.
(8) Wilson, M. S.; Bakermans, C.; Madsen, E. L. *Appl. Environ. Microbiol.* **1999**, *65*, 80−87.
(9) Stapleton, R. D.; Sayler, G. S.; Boggs, J. M.; Libelo, E. L.; Stauffer, T.; MacIntyre, W. G. *Environ. Sci. Technol.* **2000**, *34*, 1991−1999.
(10) Olsen, G. J.; Lane, D. J.; Giovannoni, S. J.; Pace, N. R.; Stahl, D. A. *Annu. Rev. Microbiol.* **1986**, *40*, 337−365.
(11) Amann, R. I.; Ludwig, W.; Schleifer, K.-H. *Microbiol. Rev.* **1995**, *59*, 143−169.
(12) Muyzer, G.; de Waal, E. C.; Uitterlinden, A. G. *Appl. Environ. Microbiol.* **1993**, *59*, 695−700.
(13) Beller, H. R.; Spormann, A. M. *J. Bacteriol.* **1997**, *179*, 670−676.
(14) Biegert, T.; Fuchs, G.; Heider, J. *Eur. J. Biochem.* **1996**, *238*, 661−668.
(15) Beller, H. R.; Spormann, A. M. *Appl. Environ. Microbiol.* **1997**, *63*, 3729−3731.
(16) Rabus, R.; Heider, J. *Arch. Microbiol.* **1998**, *170*, 377−384.
(17) Zengler, K.; Heider, J.; Rossello-Mora, R.; Widdell, F. *Arch. Microbiol.* **1999**, *172*, 204−212.
(18) Kane, S. R.; Beller, H. R.; Legler, T. C.; Anderson, R. T. *Biodegradation* (in press).
(19) Beller, H. R.; Edwards, E. A. *Appl. Environ. Microbiol.* **2000**, *66*, 5503−5505.
(20) Leuthner, B.; Leutwein, C.; Schulz, H.; Hörth, P.; Haehnel, W.; Schiltz, E.; Schägger, H.; Heider, J. *Mol. Microbiol.* **1998**, *28*, 615−628.
(21) Beller, H. R.; Spormann, A. M. *FEMS Microbiol. Lett.* **1999**, *178*, 147−153.
(22) Coschigano, P. W.; Wehrman, T. S.; Young, L. Y. *Appl. Environ. Microbiol.* **1998**, *64*, 1650−1656.
(23) Krieger, C. J.; Beller, H. R.; Reinhard, M.; Spormann, A. M. *J. Bacteriol.* **1999**, *181*, 6403−6410.
(24) Achong, G. R.; Rodriguez, A. M.; Spormann, A. M. *J. Bacteriol.* **2001**, *183*, 6763−6770.
(25) Chee-Sanford, J. C.; Frost, J. W.; Fries, M. R.; Zhou, J.; Tiedje, J. M. *Appl. Environ. Microbiol.* **1996**, *62*, 964−973.
(26) Holland, P. M.; Abramson, R. D.; Watson, R.; Gelfand, D. H. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 7276−7280.
(27) Heid, C. A.; Stevens, J.; Livak, K. J.; Williams, P. M. *Genome Res.* **1996**, *6*, 986−994.

(28) Gibson, U. E.; Heid, C. A.; Williams, P. M. *Genome Res.* **1996**, *6*, 995−1001.
(29) Saiki, R. K.; Gelfand, D. H.; Stoffel, S.; Scharf, S. J.; Higuchi, R.; Horn, G. T.; Mullis, K. B.; Erlich, H. A. *Science* **1988**, *239*, 487−491.
(30) Becker, S.; Böger, P.; Oehlmann, R.; Ernst, A. *Appl. Environ. Microbiol.* **2000**, *66*, 4945−4953.
(31) Suzuki, M. T.; Taylor, L. T.; DeLong, E. F. *Appl. Environ. Microbiol.* **2000**, *66*, 4605−4614.
(32) Takai, K.; Horikoshi, K. *Appl. Environ. Microbiol.* **2000**, *66*, 5066−5072.
(33) Grüntzig, V.; Nold, S. C.; Zhou, J.; Tiedje, J. M. *Appl. Environ. Microbiol.* **2001**, *67*, 760−768.
(34) Hristova, K. R.; Lutenegger, C. M.; Scow, K. M. *Appl. Environ. Microbiol.* **2001**, *67*, 5154−5160.
(35) von Gunten, U.; Zobrist, J. *Geochim. Cosmochim. Acta* **1993**, *57*, 3895−3906.
(36) Parsons Engineering Science. Treatability Study To Evaluate Intrinsic Remediation at the North and South Gas Stations, Travis Air Force Base California. Prepared for the Air Force Center for Environmental Excellence, 1996.
(37) Kane, S. R.; Beller, H. R.; Legler, T. C.; Koester, C. J.; Pinkart, H. C.; Halden, R. U.; Happel, A. M. *Appl. Environ. Microbiol.* **2001**, *67*, 5824−5829.
(38) Buscheck, T. E.; O'Reilly, K.; Koschal, G.; O'Regan, G. *Proceedings of the National Ground Water Association/American Petroleum Institute Conference on Petroleum Hydrocarbons and Organic Chemicals in Ground Water*, Houston, TX, November 2001.
(39) Zhou, J.; Bruns, M. A.; Tiedje, J. M. *Appl. Environ. Microbiol.* **1996**, *62*, 316−322.
(40) Johnston, W. H.; Stapleton, R. D.; Sayler, G. S. In *Molecular Microbial Ecology Manual*; Akkermans, A. D. L., van Elsas, J. D., de Bruijn, F. J., Eds.; Kluwer: Dordrecht, The Netherlands, 1996; pp (1.3.2) 1−9.
(41) Blattner, F. R.; Plunkett, G., III; Bloch, C. A.; Perna, N. T.; Burland, V.; Riley, M.; Collado-Vides, J.; Glasner, J. D.; Rode, C. K.; Mayhew, G. F.; Gregor, J.; Davis, N. W.; Kirkpatrick, H. A.; Goeden, M. A.; Rose, D. J.; Mau, B.; Shao, Y. *Science* **1997**, *277*, 1453−1474.
(42) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning: A Laboratory Manual*, 2nd ed.; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, 1989.
(43) Heider, J.; Spormann, A. M.; Beller, H. R.; Widdel, F. *FEMS Microbiol. Rev.* **1998**, *22*, 459−473.
(44) Spormann, A. M.; Widdel, F. *Biodegradation* **2000**, *11*, 85−105.
(45) Neidhardt, F. C. In *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology, Vol. 1*; Neidhardt, F. C., Ed.; American Society for Microbiology: Washington, DC, 1987; pp 3−6.
(46) McCarty, P. L. *Prog. Water Technol.* **1975**, *7*, 157−172.
(47) Fries, M. R.; Zhou, J.; Chee-Sanford, J.; Tiedje, J. M. *Appl. Environ. Microbiol.* **1994**, *60*, 2802−2810.

Received for review January 31, 2002. Revised manuscript received July 1, 2002. Accepted July 19, 2002.

ES025556W