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# Development of a PCR method for the detection and quantification of benzoyl-CoA reductase genes and its application to monitored natural attenuation

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**Abstract**

Benzoyl coenzyme A reductase (BCR) catalyzes dearomatization of benzoyl coenzyme A (benzoyl-CoA), which is the central step in the anaerobic degradative pathways for a variety of aromatic compounds. This study developed a PCR method for the detection and quantification of BCR genes in bacterial strains and environmental samples. PCR primers were designed by aligning known BCR genes in *Thauera*, *Azoarcus* and *Rhodopseudomonas* species, and their utility was assessed by amplifying BCR fragments from aromatic-hydrocarbon degrading anaerobes and other bacteria. BCR fragments with the expected sizes were obtained from denitrifying and phototrophic aromatics degraders. The positive signals were also obtained from *Geobacter metallireducens* and xylene-degrading sulfate-reducing bacterium (strain mXyS1) but not from other aromatics-degrading sulfate-reducing bacteria and aerobic bacteria. When the PCR was used for analyzing a natural attenuation (NA) site, the positive signal was obtained only from gasoline-contaminated groundwater; sequence analysis of these amplicons revealed that most of them exhibited substantial similarities to the known BCRs. Quantitative competitive PCR analysis estimated BCR-gene copies to account for 10–40% of bacterial 16S rRNA gene copies in the contaminated groundwater, indicating that bacteria possessing BCR genes were highly enriched in the contaminated groundwater. In microcosm bioremediation tests using the contaminated groundwater, the copy number of BCR gene was approximately 10-fold increased in the course of aromatics degradation under denitrifying conditions but not under sulfidogenic conditions. These results suggest the utility of the PCR method for assessing the potential of denitrifying bacteria for aromatic-compound degradation in groundwater.

## Introduction

Contamination of aquatic and terrestrial environments with aromatic hydrocarbons (AHs), such as benzene, toluene, xylenes (BTX) and polycyclic aromatic hydrocarbons (PAHs) occurs frequently and causes negative impacts on the ecosystem (Fent 2003) and human health (Ritchie et al. 2003). To address this problem, physical and/or biological treatments have been applied to remove these pollutants (Prince 1993; Vogel 1996; Franzmann et al. 2002). Monitored natural attenuation (MNA) is currently recognized as an attractive remediation strategy for BTX-contaminated groundwater due to its relatively low cost and little disturbance to the ecosystem (Filz et al. 2001; Margesin & Schinner 2001). In MNA, biological, chemical and physical parameters have been monitored for evaluating the process (Salanitro 1993), and these monitoring data have indicated

that anaerobic biodegradation significantly contributed to the BTX attenuation at MNA sites (Alexander 1994; Widdel & Rabus 2001; Lin et al. 2002). In addition, considering the limitation of oxygen-injection methods for enhancing biodegradation in underground aquifers (Lin et al. 2002), enhanced *in situ* bioremediation of BTX by injecting alternative electron acceptors, such as nitrate, have been employed (Hutchins et al. 1998; Cunningham et al. 2001).

Bacterial populations occurring at MNA sites have been analyzed using molecular ecological approaches targeting 16S rRNA genes (Becker et al. 2001; Rockne & Strand 2003). These approaches can phylogenetically identify bacterial populations present at these sites, although direct linkage of the phylogenetic information to *in situ* degradative capacity is generally difficult. A PCR method targeting the gene for benzylsuccinate synthase, one of enzymes involved in the anaerobic toluene degradation in several bacteria (Widdel & Rabus 2001), has been developed for detecting and quantifying anaerobic toluene-degrading bacteria in groundwater (Beller et al. 2002). In addition to these methods, it is desirable to develop alternative molecular approaches that facilitate the detection of catabolic genes involved in the anaerobic degradation of a wide range of aromatic compounds. Such a molecular approach is expected to provide valuable information concerning the contribution of anaerobic biodegradation to the *in situ* aromatic-compound attenuation at contaminated aquifers.

Anaerobic pathways for the aromatic-compound degradation (e.g., those for AHs, phenol, and benzoate) have been studied using several isolated strains of bacteria, including *Thauera aromatica* (Breese et al. 1998) and *Rhodopseudomonas palustris* (Egland et al. 1997). These studies have shown that benzoyl coenzyme A (benzoyl-CoA) is the central metabolite from a variety of aromatic compound (reviewed by Harwood et al. 1999). Benzoyl-CoA is further transformed by benzoyl-CoA reductase (BCR) that catalyzes the two-electron reduction of benzoyl-CoA to form cyclic dienoyl-CoA (Harwood et al. 1999). This reaction is driven by the hydrolysis of two molecules of ATP to ADP + Pi and requires a low-potential ferredoxin as an electron donor (Harwood et al. 1999). BCR has been found in *T. aromatica* (Breese et al. 1998), *Azoarcus evansii* (Harwood et al. 1999) and *R. palustris* (Egland et al. 1997). It is likely that BCR is involved in degradative pathways for several aromatic compounds in diverse bacteria, although energetic considerations have posed a question if sulfate-reducing and fermentative bacteria can also utilize BCR (Peters et al. 2004).

In the present study, a PCR method was developed for the detection and quantification of BCR genes in bacterial strains and environmental samples. The purpose was to investigate the distribution of known types of BCR genes in anaerobic aromatic compound-degrading bacteria with different types of energy metabolism. Moreover, we aimed at evaluating this PCR assay for assessing the potential and contribution of BCR-bearing bacteria to BTX attenuation in groundwater.

## Materials and methods

### *Materials*

Bacterial strains used in this study are listed in Table 1. These strains were obtained from culture collection centers; German Collection of Microorganisms and Cell Cultures (DSMZ), American Type Culture Collection (ATCC) and Marine Biotechnology Institute Culture collection (MBIC).

Groundwater samples were obtained in November 2002 and March 2003 from a gasoline-contaminated aquifer (from wells 16, 17 and 29) undergoing MNA in Kumamoto, Japan. Control groundwater samples were obtained upstream (well 7) and downstream (well 60) of the contaminated zone. The temperature, pH, dissolved oxygen (DO) and reduction/oxidation potential (Eh) of each groundwater sample were measured using a multi-monitoring system (U-22, Horiba) immediately after sampling. Groundwater samples used for other analyses were stored in screw-cap bottles at 4 °C immediately after sampling. By minimization of the headspace in these bottles, the groundwater samples could be kept anaerobic; the Eh was almost unchanged during the storage. The total direct count (TDC) of microorganisms was determined by using fluorescence microscopy after microorganisms were stained with 4′,6′-diamidino-2-phenylindole (DAPI) (Watanabe et al. 2000).

*Table 1.* Bacterial strains used in this study

| Organism[a] | Hydrocarbon utilization[b] | Type of metabolism[c] | BCRα-PCR[d] |
|---|---|---|---|
| Aromatic compound-degrading bacteria | | | |
| *Azoarcus evansii* (DSM 6898) | Bz , Ph, *p*Cr | DN | + |
| *Azoarcus toluvorans* (DSM 15124) | To, Ph | DN | + |
| *Thauera aromatica* (DSM 6984) | To, Ph, *p*Cr | DN | + |
| *Desulfobacula phenolica* (DSM 3384) | Ph, Bz, To | SR | − |
| *Desulfobacula toluolica* (DSM 7467) | To, *p*Cr | SR | − |
| Strain mXyS1 (DSM 12567) | *m*Xy, Eb, To | SR | + |
| Strain NaphS2 (DSM 14454) | Np | SR | − |
| *Desulfotignum balticum* (DSM 7044) | Bz | SR | − |
| *Rhodopseudomonas palustris* (DSM 123) | Bz | Ph | + |
| *Blastochloris sulfoviridis* (DSM 13255) | To | Ph | + |
| *Geobacter metallireducens* (DSM 7210) | To | FR | + |
| *Pseudomonas putida* (ATCC 11172) | To, *p*Cr, Ph | AE | − |
| Aromatic compound non-degrading bacteria | | | |
| *Rhodocyclus tenuis* (DSM 109) | − | DN | − |
| *Thauera mechernichensis* (DSM 12266) | − | DN | − |
| *Thauera terpenica* (DSM 12139) | − | DN | − |
| *Desulfobulbus propionicus* (DSM 2554) | − | SR | − |
| *Geobacter chapellei* (DSM 13688) | − | FR | − |
| *Zoogloea ramigera* (DSM 287) | − | AE | − |
| *Escherichia coli* (DH5α) | − | AE | − |
| *Acidaminococcus fermentans* | − | FM | − |
| *Clostridium symbiosum* | − | FM | − |

[a]Degrading bacteria, bacteria capable of aromatic-hydrocarbon degradation; non-degrading bacteria, bacteria incapable of aromatic-hydrocarbon degradation.
[b]Bz benzenoate; To = toluene; Ph = phenol; Np = naphthalene; *p*Cr = *p*-crezol; *m*Xy = *m*-xylene; Eb = ethylbenzene.
[c]DN = denitrification; FR = Fe(III) reduction; Ph = Photosynthesis; SR = sulfate reduction; AE = aerobic respiration; FM = fermentation.
[d]+ = a fragment with the expected size was obtained; − = not obtained.

BTX concentrations were determined using a purge-and-trap system (Shimadzu) and gas chromatograph (GC; GC-17A, Shimadzu) equipped with a 30-m capillary column (0.53 mm inner diameter, 3-$\mu$m film thickness, DB-624; J&W Scientific) and a flame ionization detector (FID). The concentrations of nitrate and sulfate were determined by ion chromatography with an IA-100 ion analyzer (DKK Toa).

*DNA extraction*

DNA was extracted from bacterial cultures (0.1 l) and groundwater samples (0.8 l) according to the method described elsewhere (Watanabe et al. 2000). The procedure included collection of cells by filtration, disruption of cells by hot detergent treatment, phenol/chloroform extraction, ethanol precipitation and RNase treatment. The quantity and quality of the extracted DNA were checked by measuring UV-absorption spectrum (Sambrook et al. 1989).

*PCR amplification*

DNA fragments coding for the alpha subunit of BCR were amplified by PCR using the following primers: BCR-1F (5′-GTYGGMACCGGC-TACGGCCG-3′, corresponding to *T. aromatica* alpha subunit of BCR gene positions 697–716 [Breese et al. 1998]) and BCR-2R (5′-TTCTKVGCIACICCDCCGG-3′ [I = inosine],

corresponding to positions 1160–1178 (Breese et al. 1998). Amplification was performed with a Progene thermal cycler (Techne) in a 50-$\mu$l reaction mix containing 2.5 U of Taq DNA polymerase (AmpliTaq Gold; Perkin-Elmer), 15 mM Tris–HCl (pH 8.0), 50 mM KCl, 2.5 mM $MgCl_2$, 0.02% (w/v) bovine serum albumin, each deoxynucleoside triphosphate at a concentration of 250 $\mu$M, 25 pmol of each primer, and 50 ng of DNA. The PCR conditions used were as follows: step 1, 10 min activation of the polymerase at 94 °C, step 2, three cycles consisting of 30 s at 94 °C, 30 s at 58 °C, 1 min at 72 °C, step 3, three cycles consisting of 30 s at 94 °C, 30 s at 54 °C, 1 min at 72 °C, step 4, 30 cycles consisting of 1 min at 94 °C, 1 min at 50 °C, 2 min at 72 °C, step 5, 10 min of extension at 72 °C. The PCR products were checked by electrophoresis through 2.0% (w/v) agarose gels (Seakem ME agarose; Takara) in a TBE buffer (Sambrook et al. 1989) and staining with ethidium bromide.

*Cloning and sequencing of PCR products*

The PCR products were ligated into the pGEM-T vector (Promega) as described in the manufacturer's instructions, and the ligation product was cloned into *Escherichia coli* JM109. Selection of vector-harboring clones and recovery of inserts by PCR were performed as described previously (Watanabe et al. 2000). The nucleotide sequences of PCR products were determined as described previously (Watanabe et al. 2000). Database searches for related BCR gene sequences were conducted using the BLAST program (Zhang & Madden 1997) and the GenBank database. Multiple alignment of the sequences were carried out using the ClustalW ver. 1.7 program (Thompson et al. 1994). A phylogenetic tree was constructed by the neighbor-joining method (Saitou & Nei 1987) using the njplot program in ClustalW.

*Competitive PCR (cPCR)*

The competitor fragment (538 bp) was produced by PCR amplification from a template DNA supplied in a competitive DNA construction kit (Takara) using two primers; BCR-1FC (5′-GTYGGMACCGGCTACGGCCGGTACGGTCATCATCTGACAC-3′) and BCR-2RC (5′-TTCTKVGCIACICCDCCGGAATACATCAAACGCCGCGAC-3′). The purification and quantification of the competitor fragment were conducted according to the instruction of the kit. The composition of PCR mixture was as described above, except for the competitor fragment being added at a known copy number. The PCR conditions were also as described above. The PCR products were separated by electrophoresis through 3.0% (w/v) agarose gels and stained with ethidium bromide. The band intensities of the target and competitor fragments were quantified by using the Multianalyst program supplied with Gel Doc 2000 (Bio-Rad). The copy number of the target sequence was estimated by considering band intensities, fragment lengths, relative amplification efficiency of the target to the competitor and an amount of competitor added to PCR according to the method of Lee et al. (1996). At least, five cPCR reactions with different competitor concentrations (decimal dilutions) were conducted to determine the target copy number. The copy number of bacterial 16S rRNA genes in the same samples was also estimated by cPCR as described elsewhere (Watanabe et al. 2002).

*Microcosm bioremediation test*

Groundwater obtained from well 29 in November 2002 was used. Immediately after sampling, 125-ml vials were completely filled with groundwater and each vial was supplemented with 1 mg $l^{-1}$ of ammonium chloride and 0.2 mg $l^{-1}$ of sodium phosphate. Some vials were also supplemented with 2 mM sodium nitrate or 2 mM sodium sulfate as a terminal electron acceptor. After sealing with sterilized teflon-coated butyl rubber stoppers and aluminum caps, they were incubated at 20 °C for 11 weeks. Aliquot samples were periodically taken for the TDC, cPCR, GC and ion chromatography analyses (as described above).

*Nucleotide sequence accession numbers*

The nucleotide sequences reported in this paper have been deposited in the DDBJ, EMBL and NCBI nucleotide sequence databases under accession numbers AB166660 to AB166662 (*Blastochloris sulfoviridis*, *A. toluvorans*, and sulfate-reducing bacterium strain mXyS1), and AB186473–AB186484 (environmental clones).

## Results and discussion

### *Design of PCR primers*

Genes coding for BCR have been cloned from two denitrifying bacteria, *T. aromatica* (Breese et al. 1998) and *A. evansii* (published in the databases, accession number AJ428529) and a phototrophic bacterium, *R. palustris* (Egland et al. 1997), and these genes have been named *bcrCBAD*, *bzdNOPQ* and *badDEFG*, respectively. BCRs are composed of four subunits, namely alpha, beta, gamma and delta subunits (Figure 1). We aligned predicted amino acid sequences of the corresponding subunits of the three enzymes and found several conserved regions. PCR primers were designed based on sequences in the conserved regions, and all combinations of these primers were examined whether or not amplicons with expected sizes were obtained from the three bacterial strains. Accordingly, we selected several candidate sets of PCR primers, which were further assessed using bacterial strains listed in Table 1. Among them, we finally chose one primer set (BCR-1F and BCR-2R) that could amplify approximately 480-bp fragment from all denitrifying aromatic-compound degrading bacteria in Table 1.

Primer set BCR-1F/BCR-2R recovers the central part of the alpha subunit of BCR (BCRα). This subunit is an ATP dehydratase-activase-like protein that catalyzes the ATP hydrolysis to generate the energy for the dearomatizing reaction (aromatic-ring reduction) (Harwood et al. 1999).

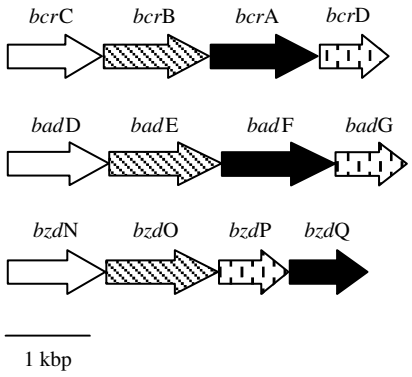


*Figure 1.* Comparison of the gene organization of operons coding for BCR. Arrows indicate the direction of transcription. *bcr*CBAD, *bad*DEFG, and *bzd*NOPQ are BCR of *T. aromatica*, *R. palustris* and *A. evansii*, respectively. Homologous subunits are marked with a same hatching pattern; ■, alpha subunit (ATP hydrolysis); ▨, beta subunit (substrate reduction); □, gamma subunit (substrate reduction); ▥, delta subunit (ATP hydrolysis).

### *BCR fragments amplified from isolated bacteria*

The utility of primers BCR-1F and BCR-2R for amplifying BCRα-gene fragments was assessed using bacterial strains listed in Table 1. Among the 19 strains, amplicons with the expected sizes were obtained from seven strains (Table 1); these included three denitrifiers, one sulfate reducer, two phototrophs and one iron reducer, all of which have been known to be capable of anaerobicaromatic-compound degradation. Sequence analysis of these amplicons showed that those amplified from *A. evansii*, *T. aromatica* and *R. palustris* were identical to *bzdQ*, *bcrA* and *badF*, respectively (Figure 2). Amplicons from the other four strains exhibited substantial sequence similarities to the known BCR alpha subunits (Figure 2), suggesting that they also possess BCRα. No amplicon was obtained from denitrifiers incapable of aromatic-compound degradation and a *Pseudomonas* strain that aerobically degrades aromatic compounds.

Among the five aromatic compound-degrading sulfate-reducing bacteria, only one (strain mXyS1) was positive in BCRα-PCR. Currently, scientists pose a question as to the utilization of BCR by aromatic compound-degrading sulfate reducers (Peters et al. 2004). The negative results of BCRα-PCR in the four aromatic compound-degrading sulfate-reducing bacteria suggest a possibility that these bacteria do not have BCR. Alternatively, the four strains may have BCR whose sequences are markedly different than those used to design our PCR method.

A putative BCRα fragment was also obtained from *Geobacter metallireducens*, an iron-reducing bacterium (Lovley et al. 1993), while the genomic sequence analysis of this bacterium have annotated the corresponding ORF as a gene for an activator of 2-hydroxyglutaryl-CoA dehydratase (accession number ZP_00080618). We therefore surveyed the flanking regions in the *Geobacter* genome and found ORFs homologous to BcrC/BadD/BzdN (approximately 50% similar in predicted amino acid sequence), suggesting that *G. metallireducens* has the BCR pathway.

The alpha subunit is known to be homologous to a subunit (HgdC) of hydroxyglutaryl-CoA

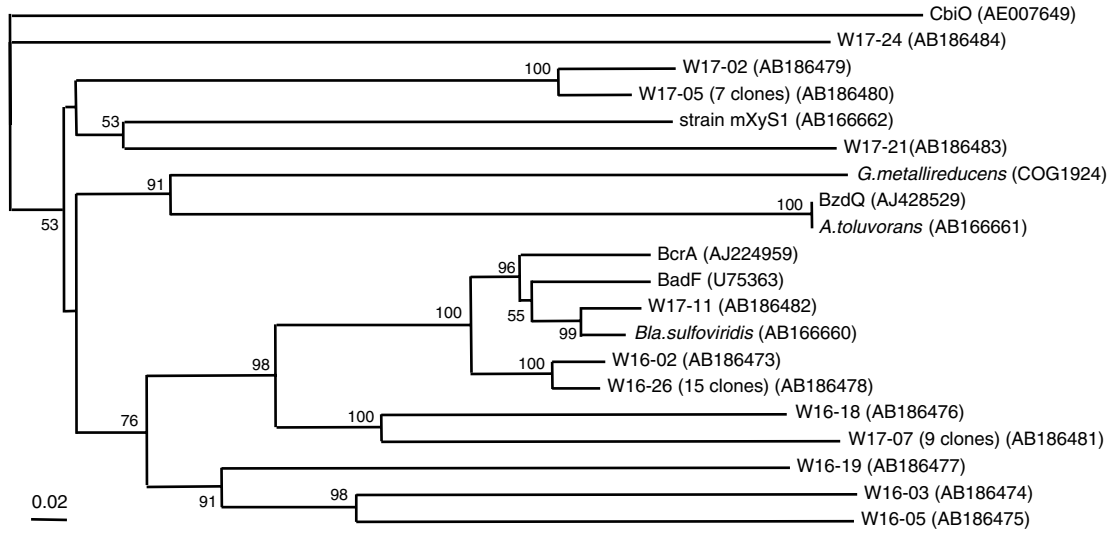


*Figure 2.* A dendrogram based on predicted amino-acid sequences showing phylogengetic relationships among BCR$\alpha$ and groundwater clones. Groundwater clones are designated with four numbers, e.g., W17–24. Abbreviations are as follows: BadF, BCR$\alpha$ of *R. palustris* (U75363); BcrA, BCR$\alpha$ of *T. aromatica* (AJ224959); BzdQ, BCR$\alpha$ of *A. evensii* (AJ428529); CbiO, an arsenic-reductase subunit of *C. acetobutylicum* (AE007649). CbiO was used as the outgroup. The number at the branch nodes are bootstrap values (per 100 trials); only values greater than 50 are shown. The scale bar indicates 0.02 substitutions per site.

dehydratase involved in the glutamate fermentation in several bacteria, e.g., *Acidaminococcus fermentans* (Bendrat et al. 1993) and *Clostridium symbiosum* (Hans et al. 1999). Sequences of *hgdC* genes, including putative ones, were compared with the sequences of the BCR primers, showing that all of these have several mismatches. In addition, no expected fragment was amplified from DNAs extracted from *A. fermentans* and *C. symbiosum* (Table 1).

### *Analysis of BCR fragments at the MNA site*

Groundwater samples obtained from the BTX-contaminated aquifer undergoing MNA were used to examine the utility of the BCR$\alpha$-PCR for assessing the contribution of BCR to MNA. Characteristics of the groundwater samples were summarized in Table 2. As shown in this table, groundwater samples obtained from wells 16, 17 and 29 (these wells are situated in a BTX-contaminated zone) were contaminated with BTX, and the BTX concentrations were stable in recent years (data not shown). The electron acceptors were almost completely depleted in groundwater in the contaminated zone. Control groundwater (well 7) was obtained upstream of the contaminated zone and therefore not affected by the contamination. In contrast, well 60 was situated at immediate downstream of the BTX-contaminated zone. Despite the fact that BTX were not detected in groundwater obtained from well 60, it may have been affected by the BTX contamination, because some parameters (DO and TDC) of well 60 were different from those of well 7. These chemical and microbiological data indicated that BTX-degrading populations have been developed in the contaminated zone.

Figure 3 shows electrophoretic patterns of PCR products amplified from the groundwater samples, showing that amplicons with the expected size were obtained from the groundwater in the BTX-contaminated zone (wells 16, 17 and 29) but not from the control groundwater (well 7) and groundwater obtained from well 60 in November 2002. A weak band was amplified from groundwater obtained from well 60 in March 2003.

Copy numbers of BCR$\alpha$ genes in the groundwater samples were estimated by cPCR, and the relative abundance to bacterial 16S rRNA gene copies (also determined by cPCR) was estimated (Figure 4). This index is considered useful to show to what extent BCR genes are enriched in a bacterial community. It is shown in Figure 4 that the relative abundance of BCR$\alpha$ genes to bacterial 16S rRNA gene in BTX-contaminated groundwater

*Table 2.* Microbial and chemical characteristics of groundwater used in this study[a]

| Parameter | Well no. 7 | Well no. 60 | Well no. 16 | Well no. 17 | Well no. 29 |
|---|---|---|---|---|---|
| Temperature | 18.8 ± 2.26 | 19.2 ± 0.70 | 18.8 ± 1.15 | 19.3 ± 0.66 | 18.9 ± 3.09 |
| pH | 5.85 ± 0.02 | 5.73 ± 0.35 | 6.54 ± 0.21 | 6.33 ± 0.44 | 6.44 ± 0.10 |
| Eh (mV) | 166 ± 27 | 176 ± 31 | −99 ± 55 | −125 ± 30 | −84 ± 36 |
| Total direct count ($10^5$ cells $ml^{-1}$) | 0.63 ± 0.45 | 7.11 ± 2.16 | 37.5 ± 11.6 | 44.6 ± 10.3 | 20.4 ± 12.0 |
| Concentrations (mg $l^{-1}$) | | | | | |
| Benzene | 0 | 0 | 0.19 ± 0.14 | 0.06 ± 0.06 | 0.24 ± 0.08 |
| Toluene | 0 | 0 | 0 | 0.02 ± 0.02 | 0.44 ± 0.14 |
| Total xylenes | 0 | 0 | 3.43 ± 1.63 | 3.07 ± 1.33 | 11.9 ± 1.91 |
| $No_{3-}$ | 2.90 ± 0.35 | 5.73 ± 0.47 | 0.33 ± 0.58 | 0.02 ± 0.03 | 0 |
| $SO_4^{2-}$ | 13.7 ± 1.53 | 12.0 ± 1.00 | 0 | 0.67 ± 0.31 | 0 |
| DO | 7.4 ± 0.6 | 5.3 ± 0.3 | 0.3 ± 0.4 | 0.2 ± 0.2 | 0.6 ± 0.7 |

[a]Data are means ± standard deviations ($n = 3$).

ranged from approximately 10% to 40%. In well 60, the relative abundance was estimated to be approximately 3% for groundwater obtained in March 2003, while no PCR product was obtained from groundwater obtained in November 2002. Since groundwater in well 60 was aerobic and nitrate was not consumed, we assume that BCRα genes detected in groundwater from well 60 were possessed by bacteria that had migrated from the contaminated zone. Considering that bacteria generally have multiple rRNA operons per genome (from several to more than 10 (Klappenbach et al. 2001)) and the TDC values largely increased in the contaminated zone (10- to 70-fold), the data indicate that bacteria possessing BCR were greatly enriched in the contaminated zone. It is therefore conceivable that bacteria harboring BCR genes largely contributed to BTX attenuation at the MNA site.

In order to confirm that DNA fragments of BCRα genes were actually retrieved from the BTX-contaminated groundwater, amplicons were cloned into *E. coli*, and nucleotide sequences of some clones were determined (20 clones each for wells 16 and 17). Sequence analysis found that the 40 clones were divided into 13 sequence types (classified as a unique clone or group of clones with the nucleotide sequence identity of 0.95 or more); among them, sequence type W16–26 contained the largest number of clones (15 clones).

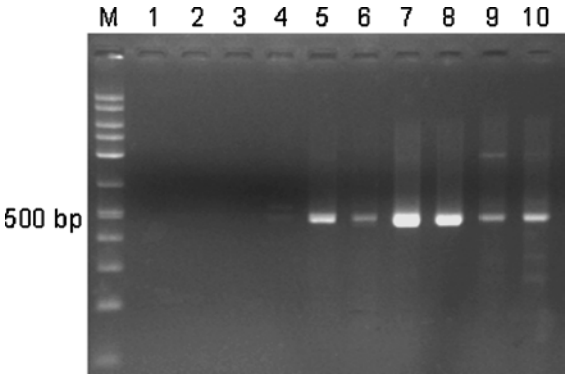


*Figure 3.* PCR amplification of BCR genes from groundwater samples. Lane M, DNA size markers (50–2 500 bp DNA Marker from 50 to 2500 bp: Takara); lane 1, well 7 (Nov. 2002); lane 2, well 7 (Mar. 2003); lane 3, well 60 (Nov 2002); lane 4, well 60 (Mar, 2003); lane 5, well 16 (Nov, 2002); lane 6, well 16 (Mar, 2003); lane 7, well 17 (Nov, 2002); lane 8, well 17 (Mar, 2003); lane 9, well 29 (Nov, 2002); lane 10, well 29 (Mar, 2003).

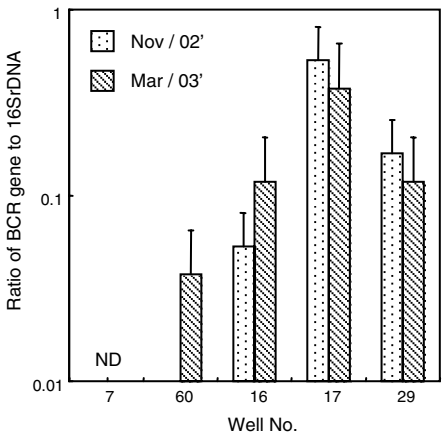


*Figure 4.* The relative abundance of BCRα genes to bacterial 16S rRNA genes as estimated by cPCR.

Figure 2 presents a dendrogram based on the predicted amino acid sequences showing phylogenetic relationships among the sequence types obtained from the contaminated groundwater and BCR fragments in the bacterial strains listed in Table 1. It is shown that, except for one sequence type (W17–24 containing one clone), the sequence types obtained from the BTX-contaminated groundwater exhibited substantial sequence similarities to either BcrA/BadF or BzdQ. In particular, the most abundant sequence type in the clone library (W16–26) is quite similar to BcrA/BadF (approximately 85% similar in predicted amino acid sequence). These results confirm that the BCR$\alpha$-PCR can amplify BCR$\alpha$-gene homologues from bacteria in groundwater samples.

*Monitoring of BCR genes during BTX degradation in anaerobic microcosms*

BTX-contaminated groundwater was used for the microcosm experiments, in which BCR genes were monitored by cPCR in the course of BTX degradation. Three types of microcosms were prepared; (i) a microcosm supplemented with ammonia and phosphate (NP), (ii) that supplemented with ammonia/phosphate plus nitrate (NP + $NO_3$) and (iii) that supplemented with ammonia/phosphate plus sulfate (NP + $SO_4$). Changes in BTX, nitrate and sulfate concentrations and TDC during incubation are presented in Figure 5, in which two xylene isomers (*m*-xylene and *p*-xylene) were indistinguishable in the gas-chromatography analysis. This figure shows that benzene and *o*-xylene were not significantly degraded in any incubation (assessed by the *t*-test at $p < 0.05$). On the other hand, significant degradation of toluene and *m*, *p*-xylenes was observed in microcosm NP + $NO_3$, while only *m*, *p*-xylenes were degraded in microcosm NP + $SO_4$. The preferential degradation of xylenes (compared to toluene) in microcosm NP + $SO_4$ would be interesting, because they are generally more recalcitrant than toluene. It is likely that sulfate-reducing strains utilizing xylenes, such as strain mXyS1 (Harms et al. 1999), were enriched in groundwater microcosms. The increases in TDC values and decreases in the nitrate or sulfate concentration were observed in association with the degradation of toluene and *m*, *p*-xylenes. In microcosm NP, BTX degradation was not apparent. Benzene was not degraded in any microcosm; we assume that this was because benzene is energetically stable under anaerobic conditions compared to toluene and *m, p*- xylene (Spormann & Widdel 2000).

Figure 6 presents changes in BCR-gene copy numbers in the microcosm incubations. During the incubation, the BCR gene was gradually decreased in microcosm NP. In microcosm NP + $NO_3$, the BCR gene was approximately 10-fold increased in the course of toluene degradation, suggesting that bacteria possessing BCR genes largely contributed to the toluene degradation under denitrifying conditions. Molecular ecological analysis using 16S rRNA genes have shown that *Azoarcus* spp. were the major bacterial populations in the BTX-contaminated zone (unpublished data), implying that they may have possessed the BCR genes. *m*, *p*-xylenes were also degraded in this microcosm; however, since there is no published information concerning BCR acting on methylbenzoates, it was not evident whether or not the increased BCR genes were involved in the degradation of *m*, *p*-xylenes.

In microcosm NP + $SO_4$, the BCR gene was increased in the initial 2 weeks, while it was decreased from weeks 2 to 6 when the *m, p*-xylene degradation and sulfate consumption were observed. This result indicates that bacteria possessing PCR-detectable BCR genes were not involved in the biodegradation of xylenes under the sulfidogenic condition.

*Conclusions*

The present study has developed the BCR$\alpha$-PCR method for the detection, quantification and phylogenetic analyses of BCR genes in bacterial strains and environmental samples. The results demonstrate that this PCR method is applicable to examine whether or not a bacterial strain bears the known type of BCR genes. Recently, Peters et al. have suggested the presence of an alternative pathway for benzoyl-CoA transformation in *Desulfococcus multivorans* (Peters et al. 2004); it is therefore interesting to examine what types of bacteria bear and utilize BCR. Since measurement of the BCR enzymatic activity is generally difficult (Boll & Fuchs 1995), the PCR method developed in the present study should be useful for rapid detection the BCR genes in anaerobic aromatic compound-degrading bacteria.

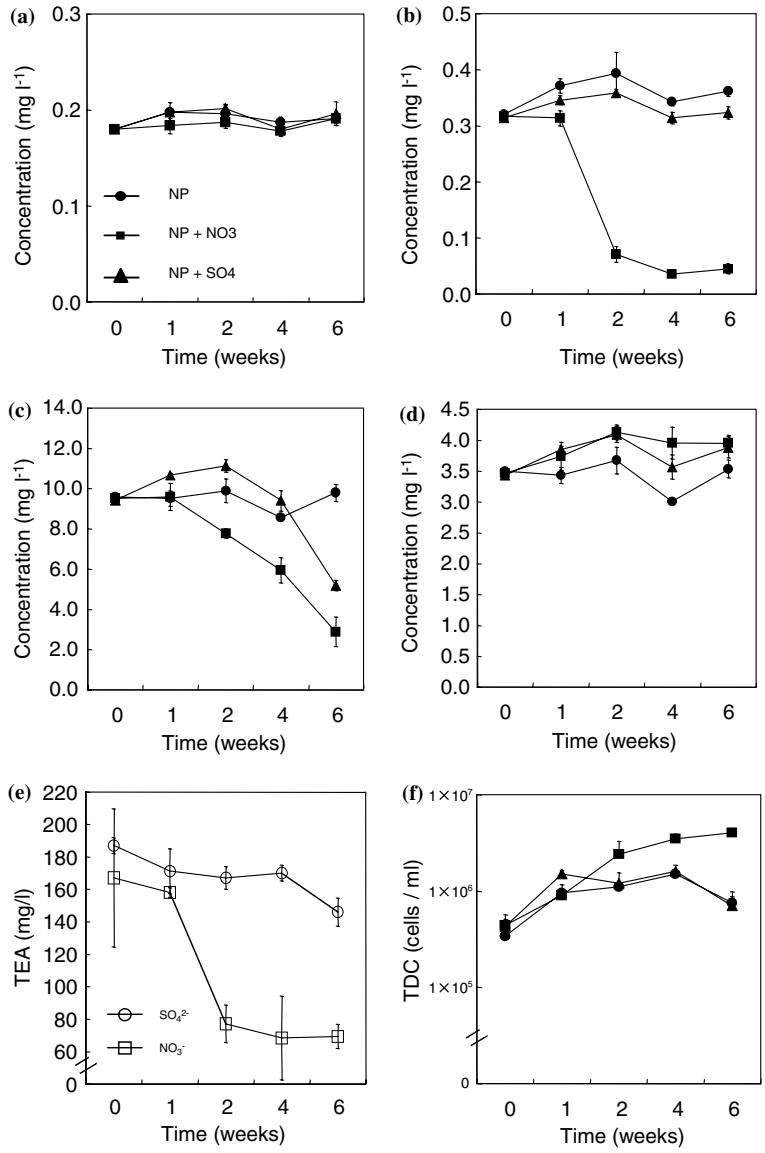


*Figure 5*. Changes in BTX, nitrate and sulfate concentrations and TDC values in microcosm incubations of BTX-contaminated groundwater. Symbols: solid circle, microcosm NP; solid square, microcosm NP + $NO_3$; solid triangle, microcosm NP + $SO_4$. Panels are changes in concentrations of benzene (a), toluene (b), *m, p*-xylene (c), *o*-xylene (d), electron acceptors (e) and TDC value (f). Data are means of triplicate incubations and error bars indicate standard errors.

The results of the MNA-site analysis and microcosm tests also suggest that BCR$\alpha$-PCR can be used for assessing the contribution of the BCR to BTX attenuation in groundwater. However, results of BCR$\alpha$-PCR should carefully be interpreted, since BCR does not catalyze the initial step of the BTX degradation and benzoyl-CoA is also produced from non-AH, such as aromatic amino acids (Harwood et al. 1999). In addition, the available data only provide strong evidence suggesting a link of BCR to toluene degradation under denitrifying conditions (Figures 5 and 6). We therefore recommend the importance of examining linkages between BTX attenuation and increases in number of the BCR genes, along with independent lines of evidence, such as compound-specific isotope analysis or signature metabolites in groundwater.

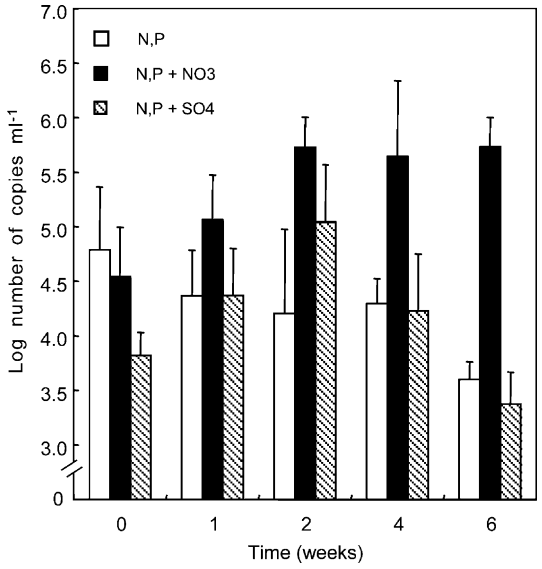


*Figure 6*. Changes in number of BCR$\alpha$-gene copies in the microcosm incubations as estimated by cPCR. The microcosms were identical to those appearing in Figure 5. Data are means of triplicate incubations and error bars indicate standard errors.

According to the results of the present study, we consider that BCR$\alpha$-PCR would be applicable to assessing MNA at nitrate-rich aquifers (Watanabe et al. 2000) and enhanced *in situ* bioremediation by injecting nitrate as the electron acceptor (Hutchins et al. 1998; Jeffrey et al. 2001). Since there have been a number of such cases, we expect that the BCR$\alpha$-PCR method will become one of monitoring tools widely used in MNA of BTX-contaminated groundwater.

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