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Application of real-time PCR, DGGE fingerprinting, and culture-based method to evaluate the effectiveness of intrinsic bioremediation on the control of petroleum-hydrocarbon plume

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ABSTRACT

Real-time polymerase chain reaction (PCR), denaturing gradient gel electrophoresis (DGGE), and the culture-based method were applied in the intrinsic bioremediation study at a petroleum-hydrocarbon contaminated site. The genes of phenol hydroxylase (PHE), ring-hydroxylating toluene monooxygenase (RMO), naphthalene dioxygenase (NAH), toluene monooxygenase (TOL), toluene dioxygenase (TOD), and biphenyl dioxygenase (BPH4) were quantified by real-time PCR. Results show that PHE gene was detected in groundwater contaminated with benzene, toluene, ethylbenzene, xylene isomers (BTEX) and methyl tert-butyl ether (MTBE), and this indicates that intrinsic bioremediation occurred at this contaminated site. Results from DGGE analyses reveal that the petroleum-hydrocarbon plume caused the variation in microbial communities. In this study, MTBE degraders including *Pseudomonas* sp. NKNU01, *Bacillus* sp. NKNU01, *Klebsiella* sp. NKNU01, *Enterobacter* sp. NKNU01, and *Enterobacter* sp. NKNU02 were isolated from the contaminated groundwater using the cultured-based method. Results from MTBE biodegradation experiment show that the isolated bacteria were affected by propane. This indicates that propane may influence the metabolic pathway of MTBE by these bacteria. Knowledge and comprehension obtained from this study will be helpful in evaluating the occurrence and effectiveness of intrinsic bioremediation on the remediation of petroleum-hydrocarbon contaminated groundwater.

1. Introduction

Accidental releases of petroleum products from pipelines and fuel-oil storage tanks are among the most common causes of ground water contamination. Petroleum hydrocarbons contain benzene, toluene, ethylbenzene, and xylene isomers (BTEX), which are hazardous substances regulated by many nations including Taiwan [1]. Gasoline contains approximately 10–20% of BTEX, and thus, the residual amount of BTEX persists in a pure liquid phase [commonly referred as non-aqueous-phase liquids (NAPLs)] within pore spaces or fractures at many gasoline spill sites. The slow dissolution of residual BTEX results in a contaminated plume of ground water. In addition to BTEX, methyl tert-butyl ether (MTBE), naphthalene, 1,3,5-trimethylbenzene (1,3,5-TMB), and 1,2,4-trimethylbenzene (1,2,4-TMB) are also toxic to humans [2,3]. Among those gasoline constituents, MTBE is the most commonly used oxygenate now due to its low cost, convenience of transfer, and ease of blending and production [1,4]. Currently, MTBE has become a prevalent ground water contaminant because it is widely used, and it has been disposed inappropriately. MTBE is a highly water soluble compound and its biodegradation rate is low in many cases. Consequently, a MTBE plume typically results in longer remediation periods [5]. Because MTBE is a possible human carcinogen, U.S. Environmental Protection Agency (USEPA) has set its advisory level for drinking water at 20–40 μg/L [6].

In Taiwan, Taiwan Environmental Protection Administration (TEPA) has classified MTBE as the Class IV toxic chemical substances. The Class IV toxic chemical substances are defined as those chemical substances that may pollute the environment or endanger human health [7–9]. Although the ban of MTBE use is under evaluation by the TEPA, no criterion for MTBE is established up to now. Thus, MTBE is not a regulated compound in the "Soil and Ground water Remediation Act" which was established in the year of 2000 by TEPA. The unique characteristics of MTBE make its behavior in

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the subsurface different from other BTEX. MTBE is a highly water soluble and low adsorption compound in comparison with BTEX. The biodegradability of MTBE is low due to the stable ether linkage and tert-butyl structure. Thus, it usually migrates a longer distance than BTEX and results in more difficult for remediation at gasoline-contaminated sites. Given that it is often not possible to locate and remove the residual BTEX or MTBE, remediation must focus on preventing further migration of the dissolved contamination. This plume control must be maintained for a long period of time. Therefore, some more economic approaches are desirable for ground water remediation to provide for long-term control of contaminated groundwater.

Bioremediation is an attractive remediation option because of its economic benefit [10,11]. Recently, intrinsic bioremediation has been considered as one of the potential methods for the cleanup of petroleum-hydrocarbon contaminated sites. If the intrinsic bioremediation rate is limited by in situ environmental factors (e.g., oxygen, nutrients, and electron acceptors), enhanced in situ bioremediation can be applied to stimulate contaminants biodegradation [12]. BTEX are biodegradable under both aerobic and anaerobic conditions. Nevertheless, rates of BTEX biodegradation under aerobic conditions are higher than those under anaerobic conditions [13]. Organic compounds are removed more completely under aerobic biodegradation. Moreover, microbiological investigations of aquifer sediments have revealed the presence of microbial communities capable of degrading a broad range of naturally occurring and xenobiotic compounds under a broad range of environmental conditions [14].

Many physical and chemical remediation technologies such as air sparging, chemical oxidation or pump and treat have been used to treat MTBE-contaminated groundwater. However, bioremediation is a more attractive option for MTBE because of its economic benefit. Therefore, biological systems, including intrinsic and enhanced approaches, offer the possibility of a cost-effective destruction technology for groundwater remediation. MTBE is biodegradable under both aerobic and anaerobic conditions. Nevertheless, rates of MTBE biodegradation under aerobic conditions are higher than those under anaerobic conditions [13,15]. Based on the above description, in situ aerobic bioremediation is a feasible technology to clean up MTBE-contaminated sites if MTBE degrader exists at the site.

The objectives of this field-scale study were to (1) assess the potential of intrinsic bioremediation at a BTEX-contaminated aquifer by quantitative real-time polymerase chain reaction (PCR), (2) determine the dominant native microorganisms at different locations of the petroleum-hydrocarbon contaminated aquifer through microbial identification via denaturing gradient gel electrophoresis (DGGE) and culture-based methods, (3) isolate the potential MTBE-degrading bacteria from the studied site.

2. Materials and methods

2.1. Study site

A government owned tank farm facility site located in southern Taiwan was selected for this study. In early 2000, leakage from a gasoline pipeline resulted in the ground water contamination by petroleum hydrocarbons (mainly BTEX and MTBE). On-site borings encountered up to 25 m of mostly brownish to graying, fine to medium sand to silty sand. The average ground water elevation within the shallow aquifer is approximately 3–4 m below land surface. Ground water in the unconfined aquifer, according to the groundwater elevation in site monitor wells, flows to the southwest. The measured effective porosity is 0.3, and the average hydraulic conductivity for the surficial, unconfined aquifer is

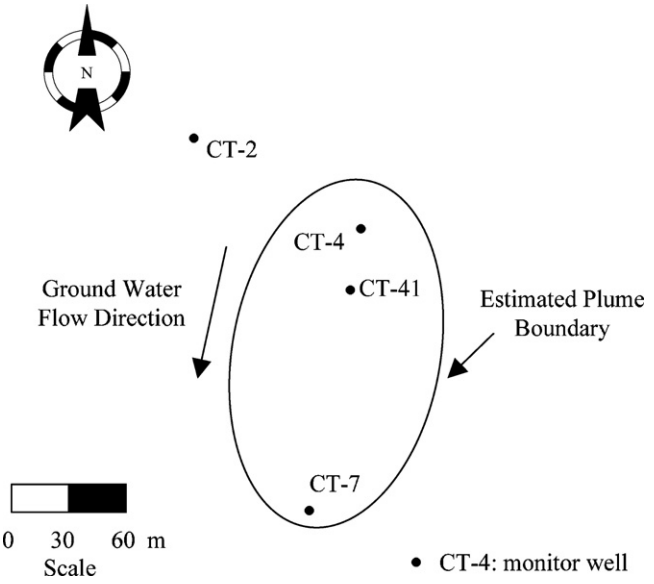

Fig. 1. Site map showing the contaminant source area, groundwater flow direction, and the sampling locations.

2.9×10^{-5} m/s. The calculated site groundwater flow velocity is 3.7×10^{-7} m/s. The measured ground water temperature in the surficial aquifer varies from 17 to 29 °C. Fig. 1 presents the site map showing the estimated plume boundary, locations of representative monitor wells, and groundwater flow direction. Groundwater samples were collected from four representative monitor wells CT-4, CT-41, CT-7, and CT-2, which were located at the source area, mid-plume area, downgradient area, and background area, respectively. All selected wells were sampled quarterly during the one-year investigation period.

2.2. DNA extraction and conventional PCR

DNA extractions were performed with 0.5 g of the groundwater solids using Power Soil™ extraction kit (MoBiol, USA). The PCR primers for aromatic oxygenase genes and PCR protocols were according to the previously published paper [16]. The individual primer sets were allowed to amplify of fragments of naphthalene dioxygenase (NAH), toluene dioxygenase (TOD), toluene monooxygenase (TOL), ring-hydroxylating toluene monooxygenase (RMO), phenol hydroxylase (PHE), and biphenyl dioxygenase (BPH4). These primer sets were listed in Table 1. Annealing temperature, the amounts of DNA, and the concentration of $MgCl_2$ and primer were optimized based on the protocols of Baldwin et al. [16].

Conventional PCR was performed with the following temperature program: (i) 10 min at 95 °C; (ii) 30 cycles, with 1 cycle consisting of 1 min at 95 °C, 1 min at the optimum annealing temperatures, and 2 min at 72 °C, and (iii) a final extension step of 10 min at 72 °C. All experiments included control reaction mixtures without added DNA. PCR products were routinely visualized by running 10 μL of PCR mixtures on 1% agarose gels in 1× Tris–acetate–EDTA (TAE) buffer stained with ethidium bromide. Reproducibility was confirmed by performing PCR with positive-control DNAs in triplicate as a minimum.

2.3. Real-time qPCR with SYBR green I

Real time PCR was performed on a Light Cycler® 1.5 Instrument (Roche, USA). The Light Cycler® Fast Start DNA Master SYBR Green I kit (Roche, Germany) was used for the real time PCR reac-

Table 1
Primers of aromatic oxygenase gene and expected product size for real-time PCR.

NAH-F NAH-R	Naphthalene dioxygenase	5′-CAAAA(A/G)CACCTGATT(C/T)ATGG 5′-A(C/T)(A/G)CG(A/G)G(C/G)GACTTCTTTCAA	377
TOD-F TOD-R	Toluene dioxygenase	5′-ACCGATGA(A/G)GA(C/T)CTGTACC 5′-CTTCGGTC(A/C)AGTAGCTGGTG	757
TOL-F TOL-R	Xylene monooxygenase	5′-TGAGGCTGAAACTTTACGTAGA 5′-CTCACCTGGAGTTGCGTAC	475
BPH2-F BPH2-R	Biphenyl dioxygenase	5′-GACGCCCGCCCCTATATGGA 5′-AGCCGACGTTGCCAGGAAAAT	724
BPH4-F BPH3-R[b]	Biphenyl dioxygenase	5′-CCGGGAGAACGGCAGGATC 5′-TGCTCCGCTGCGAACTTCC	452
RMO-F RMO-R	Toluene monooxygenase	5′-TCTC(A/C/G)AGCAT(C/T)CAGAC(A/C/G)GACG 5′-TT(G/T)TCGATGAT(C/G/T)AC(A/G)TCCCA	466
PHE-F PHE-R	Phenol monooxygenase	5′-GTGCTGAC(C/G)AA(C/T)CTG(C/T)TGTTC 5′-CGCCAGAACCA(C/T)TT(A/G)TC	206

tion. All of the procedures including annealing and polymerization temperatures, primers concentrations, and $MgCl_2$ concentration for qPCR were done following the procedures of the manufactures. The PCR regime consisted of the following steps: 10-min incubation at 95 °C followed by 40 cycles of 10 s at 95 °C, 10 s at 60 °C, and 8 s at 72 °C and, finally, the dissociation curve step, with 65 °C for 15 s. The PCR Master Mix was composed of 4 μL of the forward (0.2 μM) and reverse primers (0.2 μM) and 2 μL of 1× SYBR Green I (Roche, USA), as well as sterile nuclease-free water to a final volume of 20 μL. Finally, 3 μL of cDNA was added to this mixture. The specificity of the PCR product was verified (apart from dissociation curve analysis) periodically using electrophoresis (data not shown).

2.4. Denaturing gradient gel electrophoresis

Total bacterial DNA from 1 g of collected ground water samples were extracted with a Power Soil™ extraction kit (MoBiol, USA). Before DGGE analysis, the V3 region of 16S rDNA was amplified with the primer sets (341f, forward: 5′-CCTACGGGAGGCAGCAG-3′ containing a GC clamp of 40-nucleotide GC-rich sequence; 534r, reversed: 5′-ATTACCGCGGCTGCTGG-3′). DGGE was performed with D-Code™ universal mutation detection system (BioRad Lab., USA). PCR product was loaded on to 8% (w/v) polyacrylamide gels with a denaturing gradient of 30–60% (100% denaturant according to 7 M urea plus 40% formamide in 1× TAE buffer) and were run for 7 h at 150 V and at 60 °C in 1× TAE buffer (pH 7.4). After electrophoresis, the gels were stained with SYBE Safe™ (Invitrogen, UK), and then were put in a shaker at 150 rpm for 10 min, followed by excision of the bands from the gel with a pipette tip under UV illumination. The excised bands were directly placed in 1.5 mL microcentrifugation tubes containing 50 μL ultrapure DNase/RNase-free sterile water and incubated at 4 °C for overnight to elute DNA. Ten microliters of the supernatants was used as template for the re-amplification (as described above). The resulting amplicons were again electrophoresed on a DGGE gel to verify the position of the original band. Subsequently, the amplicons were purified using the Gel/PCR DNA fragments extraction kit (Geneaid, Taiwan). The PCR products were then cloned into pGEM®- T Vector System (Promega, USA). Clones were screened according to the manufacture's instruction. Recombinant plasmids were isolated from overnight culture by alkaline lysis and inserts were detected by PCR amplification with M13 primer set. Subsequently, 16S rDNA gene fragment were sequenced by Mission Biotech. Co. in Taiwan. All sequences were compared to those in the GenBank database (http://www.ncbi.nlm.nih.gov).

2.5. DGGE and phylogenic analysis

DGGE banding patterns were analyzed using Quantity One software (BioRad, USA). For cluster analysis, PCR-DGGE of one primer set was performed in duplicate. Calculation of the pairwise similarities was based on the Dice correlation coefficient. Dendrograms were created using the algorithm of unweighted pair-group method using arithmetic averages (UPGMA) [17]. Database searches of these16S rDNA gene sequence determined were conducted by a BLAST program using the GenBank database. The profiled alignment technique of Clustal W was used to align the sequences [17]. Phylogenetic trees were constructed by Maximum Parsimony method.

2.6. Enrichment of MTBE-degrading cultures

Site groundwater in well CT-4 was used to enrich for MTBE-degrading microorganisms in sterile FTW minimal salts to which 100 mg/L MTBE (Sigma, USA) was added. The components of the FTW medium (g/L) included K_2HPO_4, 0.225 (BIO BASIC, Canada); KH_2PO_4, 0.225 (J.T. Baker, USA); $(NH_4)_2SO_4$, 0.225 (BIO BASIC, Canada); $MgSO_4{\cdot}7H_2O$, 0.05 (Genemark, Canada); $CaCO_3$, 0.005 (MERCK, Germany); $FeCl_2{\cdot}4H_2O$, 0.005 (J.T. Baker, USA) and 1 mL of trace elements solution [18]. The mixtures of trace elements solution (g/L) were $MnSO_4{\cdot}H_2O$, 169.02 (MERCK, German); $ZnSO_4{\cdot}7H_2O$, 287.56 (MERCK, German); $CuSO_4$, 159.6 (MERCK, German); $NiSO_4{\cdot}6H_2O$, 26.285 (MERCK, German); $CoSO_4{\cdot}7H_2O$, 28.11 (MERCK, German); $Na_2SO_4{\cdot}2H_2O$, 24.195 (MERCK, German). The pH of the medium was adjusted to 7.4. The aerobic culture was incubated at ambient temperature (25 °C) with orbital shaking (150 rpm) for 4 weeks. Thereafter, 1 mL of the culture was transferred onto fresh sterile FTW enrichment medium including 100 mg/L of MTBE, and further incubated for two weeks. Subsequently, this enrichment culture was used for isolation of single strains.

2.7. MTBE-degrading monocultures

To isolate single strains from the enrichment culture, 1 mL of aliquot of the enrichment culture was centrifuged (10,000 rpm, 10 min) using a micro-centrifugation tube. The supernatant was removed and the residue was re-suspended in 50 μL of sterile FTW mineral salts medium by vortexing. The resulting suspension was plated on agar plates which were made from the mixtures of FTW medium, 1.5% agar and sterile MTBE (100 mg/L). Agar plates were incubated under aerobic conditions at 25 °C and colonies appearing within 3–4 days were isolated. The screening of isolates

Table 2
Laboratory analytical results for four monitor wells.

Monitor well	CT-4	CT-41	CT-7	CT-2
Distance to CT-4 (m)	0	30	135	–[a]
Location	Source	Mid-plume	Downgradient	Background
Benzene (μg/L)	399 ± 41[b]	35 ± 12	BDL[c]	BDL
Toluene (μg/L)	22 ± 13	BDL	BDL	BDL
Ethylbenzene (μg/L)	60 ± 24	3 ± 1.4	BDL	BDL
M + p-Xylene (μg/L)	24 ± 8	BDL	BDL	BDL
o-Xylene (μg/L)	5 ± 1.3	BDL	BDL	BDL
MTBE (μg/L)	255 ± 49	25 ± 17	7 ± 2.5	BDL
DO (mg/L)	0.02 ± 0.02	0.3 ± 0.2	1.4 ± 0.4	2.2 ± 1.1
Carbon dioxide (mg/L)	421 ± 87	337 ± 57	258 ± 43	139 ± 34
Methane (mg/L)	14.2 ± 1.5	6.2 ± 4.1	0.7 ± 0.2	0.02 ± 0.01
pH	6.45 ± 0.24	6.4 ± 0.17	6.5 ± 0.15	6.6 ± 0.2
Redox potential (mV)	−174 ± 43	−20 ± 32	132 ± 38	241 ± 44
Ferrous iron (mg/L)	12 ± 4.6	6 ± 1.3	0.2 ± 0.1	BDL
Nitrate (mg/L)	0.1 ± 0.03	0.3 ± 0.15	5.6 ± 1.6	7.7 ± 2.2
Sulfate (mg/L)	3.7 ± 2.3	2.8 ± 1.1	7.5 ± 3.5	9.6 ± 3.2
Alkalinity (mg/L as $CaCO_3$)	298 ± 32	247 ± 27	187 ± 19	156 ± 13
Ammonia nitrogen (mg/L)	0.15 ± 0.03	0.17 ± 0.04	0.26 ± 0.06	0.31 ± 0.07

[a] "–": not available.
[b] Mean ± standard deviation.
[c] BDL: below detection limit.

for MTBE degradation in liquid culture was conducted according to the procedures of Okeke and Jr. Frankenberger [18] with minor modification. The isolates were then pre-grown in FTW mineral salts media to which yeast extract (300 mg/L), ethanol (300 mg/L) and MTBE (100 mg/L) were added. Cells were harvested by centrifugation (5000 rpm, 15 min) and washed in 40 mL FTW mineral salts solution. Subsequently, cells were re-suspended in the same medium to an in initial OD of approximately (2.0). This cell suspension (1 mL) was inoculated into 59 mL FTW mineral salts media in a 250 mL Erlenmeyer flask sealed with the rubber septa. Cultures were incubated (25 °C, 150 rpm) for the indicated days.

2.8. Ground water analysis

Ground water samples were collected quarterly and analyzed for organic compounds and geochemical indicators including MTBE, BTEX, methane (CH_4), CO_2, nitrate, ammonia, sulfate, Fe(II), pH, Eh (redox potential), alkalinity, and dissolved oxygen (DO). Methane was analyzed on a Shimadzu GC-9A GC using headspace techniques. Ion chromatography (Dionex) was used for anions analyses. Perkin-Elmer Plasma II Inductively Coupled Plasma-Argon Emission Spectrometer (ICP-AES) was used for Fe (II) analyses following Standard Methods [19]. DO, Eh, pH, CO_2, and temperature were measured in the field. An Accumet 1003 pH/Eh meter (Fisher Scientific) was used for pH and Eh measurements, an Orion DO meter (Model 840) was used for DO and temperature measurements, and a Hach digital titrator cartridge was used for CO_2 measurements. Ground water samples were collected in 40 mL of volatile organic analyte (VOA) bottles with Teflon® lined septa. The VOA analyses were performed within two days (48 h) of sample collection. The samples were analyzed using an Agilent 6890 gas chromatograph coupled with a 5973N mass selective detector (MSD) and ChemStation for control and data acquisition. Analyte compound separation was achieved with a 0.32 mm i.d., 30 m, fused silica capillary column (5% diphenyl–95% dimethyl polysiloxane) with a 0.25 μm film thickness (HP-5MS, J&W Scientific). The temperature program included a 2 min hold time at 26 °C, and temperature ramping at 1 °C/min to 40 °C with 1 min hold time, followed by a ramp to 100 °C at 10 °C/min with 1 min hold time, and a final ramp to 150 °C at 10 °C/min with a 1 min hold time. Helium was used as carrier gas at a flow rate of approximately 1.1 mL/min. The electron energy was set at 70 eV. The detector scanned from 35 to 300 amu. The electron multiplier voltage was 1235 eV.

2.9. Statistic analysis

Student *t*-test was used for the evaluation of differences between the amounts of residual MTBE after biodegradation with or without propane addition and their corresponding control.

3. Results and discussion

3.1. Ground water analysis

Table 2 shows the averaged results of ground water analyses in four representative wells. Results show that the BTEX and MTBE concentrations in the representative monitor wells reached a relatively stable condition, and no significant decrease or increase in BTEX and MTBE concentrations was observed during the investigation period. Compared to CT-4 and CT-41, the downgradient monitor well CT-7 and background well CT-2 contained higher O_2 concentrations (up to 2.2 mg/L). This indicates that the downgradient area of the plume was under the oxidized stage and aerobic biodegradation was the dominant biodegradation process within that area. The decline in Eh (−174 mv in CT-4) and DO (0.02 mg/L in CT-4) near the source area reflects the change from oxidizing to reducing conditions. The increased CH_4 (up to 14.2 mg/L) and CO_2 (up to 421 mg/L) concentrations, increased alkalinity (298 mg/L as $CaCO_3$) and decreased pH (pH 6.45) in CT-4 suggest that the methanogenesis was the dominant biodegradation process within the most contaminated zone. This also indicates that significant microbial activity and intrinsic bioremediation occurred in this area. The lower nitrate and sulfate concentrations within the plume reveal that both nitrate and sulfate were used as the electron acceptors after the depletion of oxygen. Higher ferrous iron concentrations were detected in CT-4 (12 mg/L) and CT-41 (6 mg/L). This indicates that ferric iron might have been also used as the electron acceptor around the source area. Moreover, relative higher concentration of methane was also detected in CT-4. This indicates that mixed anaerobic biodegradation processes occurred within the most contaminated zone. The decrease in BTEX and MTBE concentrations from CT-4 to CT-7 suggests the occurrence of natural attenuation of BTEX and MTBE.

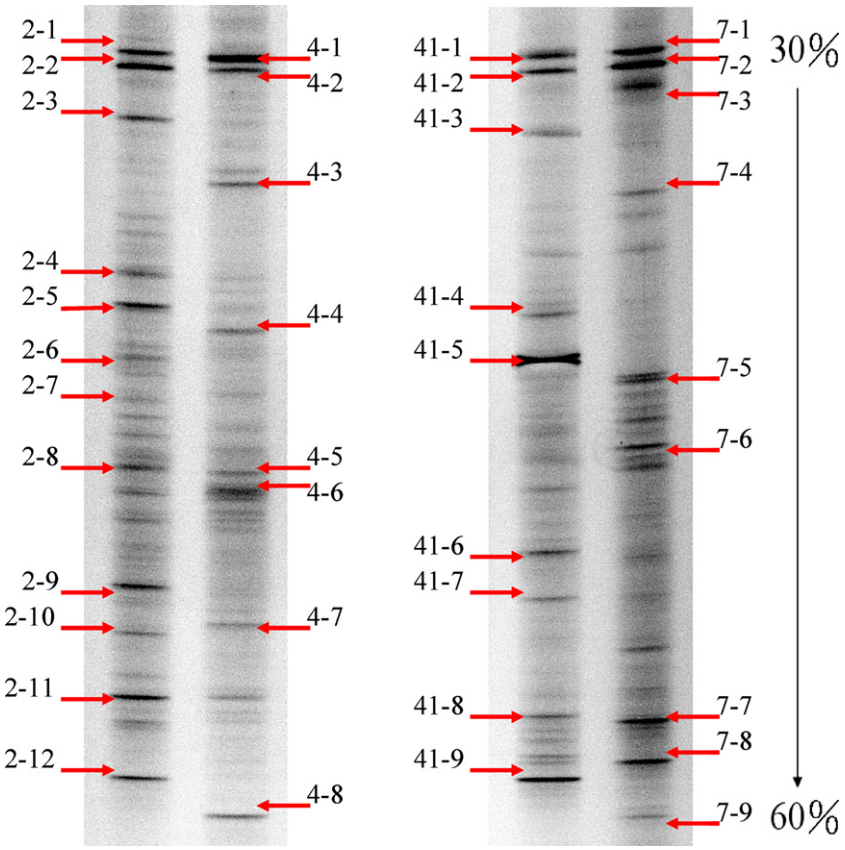

Fig. 2. DGGE profiles of the PCR-amplified for V3 region of 16S rDNA in these four sampling wells.

3.2. Evaluation of intrinsic bioremediation by real-time PCR

Kao et al. [20] reported that intrinsic bioremediation would be the major cause of the decrease in BTEX concentrations in gasoline-contaminated groundwater among the natural attenuation mechanisms. To confirm the possibility that intrinsic bioremediation occurred at the studied site, the enumeration of aromatic oxygenase genes by real-time PCR has been used for the monitoring of national attenuation at gasoline-contaminated sites [12]. As shown in (see Table 1, Supporting Information), aromatic oxygenase genes were detected in monitoring wells (CT-4 and CT-41) contaminated with BTEX, indicating that biodegradation played an important role in natural attenuation in source and mid-plume areas, although only the low copy numbers of PHE were detected. PHE, which catalyzes the further oxidation of hydroxylated intermediates of monoaromatic hydrocarbon catabolism, was also detected during the treatment of pulsed multi-phase extraction at a gasoline-contaminated site [21]. The detected genotypes of these aromatic oxygenase genes at different petroleum-hydrocarbon contaminated sites could be correlated with the selection of gasoline contamination, which warranted further investigation [16]. On the other hand, PHE was also detected in monitor well CT-7 containing undetectable BTEX compounds. As in the similar study reported by Baldwin et al. [16], they indicated that the detection of aromatic oxygenase genes in areas without BTEX contamination could be due to continual aromatic hydrocarbons flux and active biodegradation enabling low or no BTEX levels to be detected. In addition, no oxygenase genes were detected in the monitor well CT-2 (in background area) without BTEX contamination. The detection of these oxygenase genes may be the most beneficial for assessing BTEX biodegradation at petroleum-hydrocarbon impacted sites [16].

Overall, quantification of aromatic oxygenase genes under intrinsic bioremediation can demonstrate that aerobic BTEX utilizing bacteria were present, suggesting that low BTEX degradation rates may have resulted from low oxygen availability [22]. This also provides a baseline to evaluate the impact of some remedial strategies regarding enhanced bioremediation technologies including the injection of oxygen-releasing materials (ORMs) [22], multi-phase extraction [21], or in situ biosparging [20].

3.3. Bacterial communities at the studied site

To determine if bacterial community patterns in subsurface environment were changed due to the petroleum-hydrocarbon pollution, the PCR-DGGE technique was performed to investigate these patterns in four different wells (CT-4, CT-41, CT-7, and CT-2) at this studied site (Fig. 1).

Although a high number of bands appeared when performing PCR-DGGE analysis (Fig. 2), it was only possible to identify few bands by excising them from the DGGE. Weak and close bands were difficult to cut off the gel and could not be re-amplified or only produced multiple DNA sequences [23]. Thus, only the prominent bands in four sampling wells indicated in Fig. 1 were excised for DNA sequencing in this study. The V3 regions of 16S rDNA in these sequences were compared to the database of GenBank, whose results were listed (see Table 2, Supporting Information). An analyzed sequence whose similarity was less than 95% as compared to those in the known microorganisms could suggest a potentially novel genus. The sequences of these novel bacteria were related to bacterium WCHB1-69 in CT-2 and CT-4, and *Novosphingobium capsulatum* strain 213 in CT-7. A phylogenetic tree of these sequences was constructed from the analysis of DGGE banding patterns in the four sampling wells in Fig. 2 to describe the

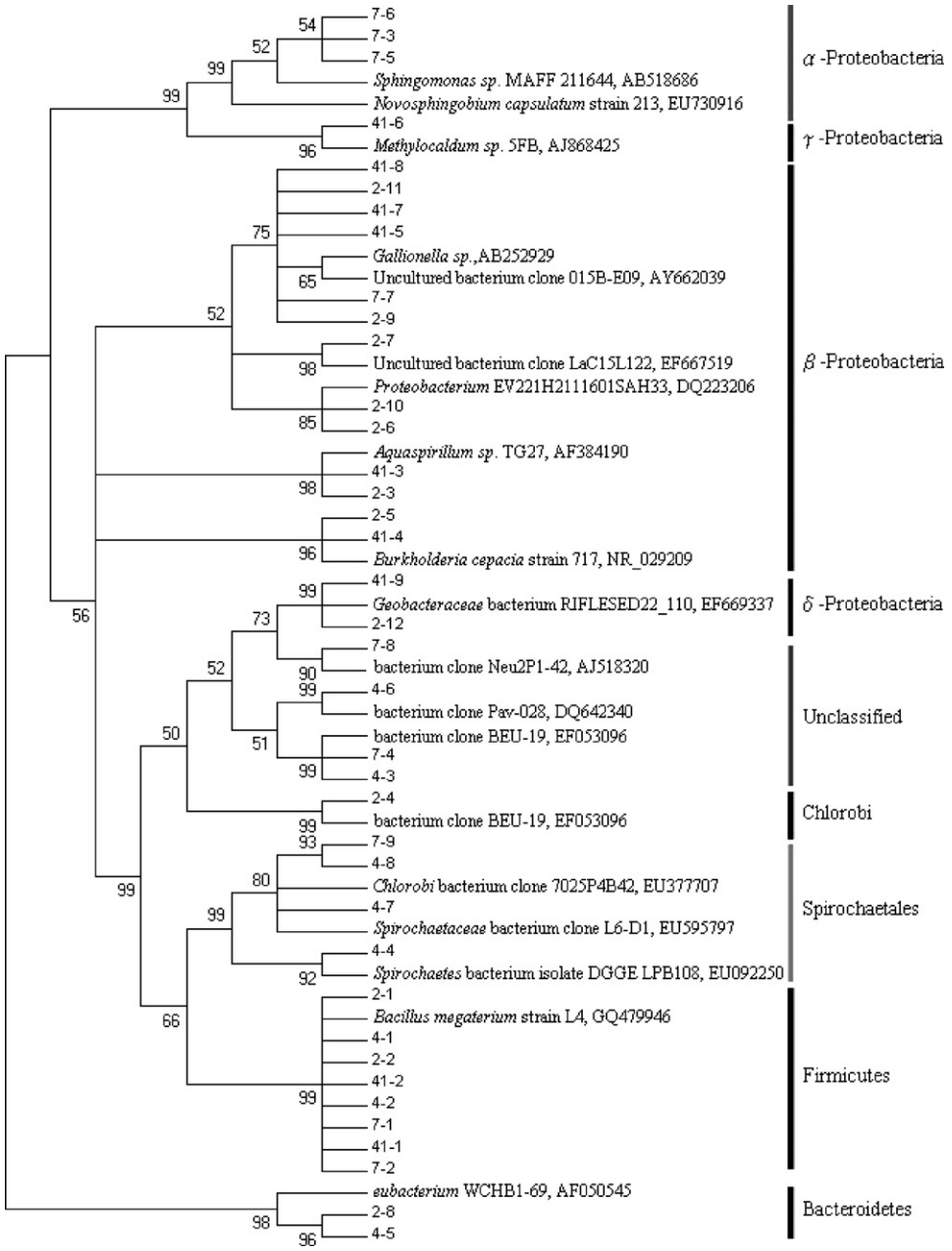

Fig. 3. The condensed phylogenetic tree was established on the basis of 16S rRNA sequences using maximum parsimony. The numbers at the nodes were bootstrap values which were expressed as percentage of 1000 replication, and bootstrap values below 50% were not shown. The codes after the bacterial species represent the GenBank accession numbers.

relationships between all the sequences (Fig. 3). Results reveal that there were two major separate phylogenetic clusters. Among them, *Geobacteraceae* has been reported to be able to biodegrade petroleum hydrocarbons with reduction of Fe (III) [24,25]. The dominant microorganisms involving petroleum-hydrocarbon degradation could be exploited and isolated for their application on the bioremediation of petroleum-hydrocarbon contaminated sites.

Fig. 4 presents the UPGMA dendrogram for illustrating theses bacterial communities in groundwater. Results show that these bacterial communities could be grouped into three major phylogenetic clusters. Monitor wells CT-4 and CT-41, which were located at source and down-gradient areas, revealed similarly bacterial communities, whereas CT-4 and CT-41 displayed the divergent communities from CT-7 and CT-2. Moreover, CT-7 and CT-2 were further grouped into two clusters, indicating that different levels

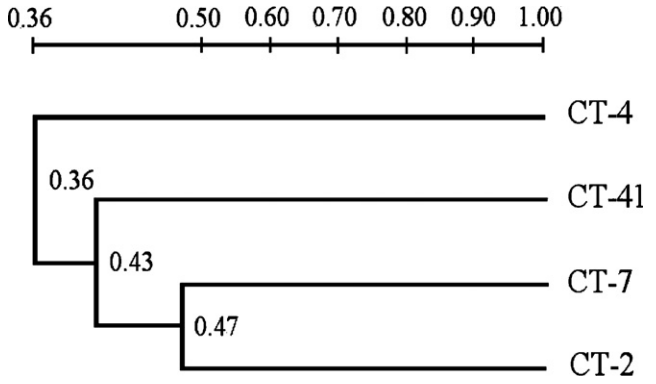

Fig. 4. The UPGMA dendrogram for illustrating relationships among microorganisms collected from four different sampling sites.

Table 3
Identification of MTBE-degrading bacteria grown in the defined medium (without carbon source) by 16S rRNA gene fragments via BLAST N research in GeneBank database.

Species	Phylogenetic group	Related organism (accession no.)	Identity (%)
Pseudomonas sp. NKNU01	*γ-proteobacteria*	*Pseudomonas* sp. AHL 2 (AY379974)	100
Bacillus sp. NKNU01	*Firmicutes*	*Bacillus megaterium* (DQ789400)	99
Klebsiella sp. NKNU01	*γ-proteobacteria*	*Klebsiella pneumoniae* subsp. *pneumoniae* MGH 78578 (CP000647)	99
Enterobacter sp. NKNU01	*γ-proteobacteria*	*Enterobacter* sp. VET-7 (EU781735)	99
Enterobacter sp. NKNU02	*γ-proteobacteria*	*Enterobacter* sp. Px6-4 (EF175731)	99

Table 4
MTBE degradation by five bacteria in the presence or absence of pentane at incubation day 10 and 15.

Species	MTBE degradation (%)[a] at day 10		MTBE degradation (%) at day 15	
	− pentane	+ pentane (14.3 mg/L)	− pentane	+ pentane (14.3 mg/L)
Pseudomonas sp. NKNU01	1.67	13.40*,[b]	8.20	10.67*
Bacillus sp. NKNU01	−0.52	21.07*	3.76	17.90*
Klebsiella sp. NKNU01	2.89	14.57*	4.88	15.48*
Enterobacter sp. NKNU01	2.61	13.87*	10.20	10.71*
Enterobacter sp. NKNU02	26.02	19.42*	29.06	18.04*
mix cultures[c]	25.70	64.48*	29.32	64.24*

[a] Mean percentage (%) of degradation was calculated from the ratio (the amounts of residual MTBE with bacterial inoculum divided by its amounts without bacterial inoculum) multiplied by 100, and was derived from the average of the results in three-independent experiment, with the values of variation coefficient less than 10%.
[b] * means $p < 0.05$.
[c] The mixture of these five species of bacteria at the equivalent number.

of petroleum-hydrocarbon pollution caused the variations in bacterial communities.

3.4. Isolation of MTBE-degrading bacteria from the studied site

In this study, MTBE-degrading bacteria from the petroleum-hydrocarbon contaminated site were isolated by the culture-based method, and were identified by 16S rRNA gene sequencing (Table 3). The sequences of 16S rRNA genes of our isolates have been submitted to GeneBank, and accession numbers have been assigned to *Pseudomonas* sp. NKNU01 (FJ158035), *Bacillus* sp. NKNU01 (FJ158036), *Klebsiella* sp. NKNU01 (FJ158037), *Enterobacter* sp. NKNU01 (FJ158038), *Enterobacter* sp. NKNU02 (FJ158039). These bacteria can grow on MTBE as the sole carbon source under aerobic conditions. This indicates that those bacteria have a potential to exist in the petroleum-hydrocarbon contaminated site and degrade MTBE under aerobic conditions. However, these bacteria were not detected in the dominant bands of DGGE. This could be related to the recalcitrance of MTBE to both aerobic and anaerobic biodegradation [26,27] owing to the stability of the ether bond and the hindrance of the branched *tert*-butyl group [28].

Results from Table 4 show that pure culture was less active for MTBE degradation when compared with mixed cultures. Pruden et al. [29] also indicated that mixed cultures were usually more effective than pure culture on contaminant degradation. For this aspect, it could be the existence of many reasons. One possible scenario is that one strain may be able to degrade the compound to a certain product at which point another strain may take over with no toxicity or inhibition results [30]. MTBE can be used by bacteria either as a primary source of carbon in direct metabolism conditions, or co-metabolically when bacterial growth requires other substrates, usually an alkane [28]. Also, it was reported that MTBE in organically poor soil can be inhibited by the addition of easily degradable organic compounds [31]. In our experiment, the addition of pentane caused the decrease in MTBE degradation efficiency by *Enterobacter* spp. However, this degradation was enhanced by *Pseudomonas* sp. NKNU01, *Bacillus* sp. NKNU01, and *Klebsiella* sp. NKNU01 significantly ($p < 0.05$), as compared to the control group (without pentane addition) (Table 3). Thus, pentane may influence the metabolic pathway of MTBE by these bacteria, and *Pseudomonas* sp. NKNU01, *Bacillus* sp. NKNU01, and *Klebsiella* sp. NKNU01 can degrade MTBE more efficiently by co-metabolic process than that by direct metabolism. Co-metabolism has the advantages of reducing adaption and propagation periods for bio-treatment systems [28].

In this study, DNA from petroleum-hydrocarbon contaminated groundwater was extracted, and bacterial communities were evaluated. Knowledge and comprehension obtained from this study will be helpful in evaluating the intrinsic bioremediation system for the remediation of petroleum-hydrocarbon (e.g., MTBE, BTEX) contaminated site. With the increased knowledge on intrinsic bioremediation mechanism, less conservative regulatory decisions may be possible, and thus, costs for site remediation can be minimized.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhazmat.2010.01.096.

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