J. Microbiol. Biotechnol. (2009), **19**(4), 339–345
doi: 10.4014/jmb.0807.423
First published online 13 January 2009

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Monitoring Bacterial Population Dynamics Using Real-Time PCR During the Bioremediation of Crude-Oil-Contaminated Soil

Baek, Kyung-Hwa, Byung-Dae Yoon, Dae-Hyun Cho, Byung-Hyuk Kim, Hee-Mock Oh, and Hee-Sik Kim*

Environmental Biotechnology Research Center, KRIBB, Daejoen 305-806, Korea

Received: July 8, 2008 / Revised: July 31, 2008 / Accepted: August 15, 2008

We evaluated the activity and abundance of the crude-oil-degrading bacterium *Nocardia* sp. H17-1 during bioremediation of oil-contaminated soil, using real-time PCR. The total petroleum hydrocarbon (TPH) degradation rate constants (k) of the soils treated with and without H17-1 were 0.103 d^{-1} and 0.028 d^{-1}, respectively. The degradation rate constant was 3.6 times higher in the soil with H17-1 than in the soil without H17-1. In order to detect and quantify the *Nocardia* sp. H17-1 in soil samples, we quantified the genes encoding 16S ribosomal RNA (16S rRNA), alkane monooxygenase (*alkB4*), and catechol 2,3-dioxygenase (23CAT) with real-time PCR using SYBR green. The amounts of H17-1 16S rRNA and *alkB4* detected increased rapidly up to 1,000-folds for the first 10 days, and then continued to increase only slightly or leveled off. However, the abundance of the 23CAT gene detected in H17-1-treated soil, where H17-1 had neither the 23CAT gene for the degradation of aromatic hydrocarbons nor the catechol 2,3-dioxygenase activity, did not differ significantly from that of the untreated soil (α=0.05, p>0.22). These results indicated that H17-1 is a potential candidate for the bioaugmentation of alkane-contaminated soil. Overall, we evaluated the abundance and metabolic activity of the bioremediation strain H17-1 using real-time PCR, independent of cultivation.

Keywords: Bioaugmentation, crude oil, *Nocardia* sp., real-time PCR, total petroleum hydrocarbon

Bioaugmentation (the addition of pollutant-degrading microorganisms) is an effective method for remediating petroleum hydrocarbon pollutants in soil; however, this strategy also presents numerous challenges [1, 10, 11]. The main difficulty associated with bioaugmentation is ensuring that the introduced microbes are effective and can survive the harsh environmental conditions. Currently used culture-based monitoring techniques lack the specificity and sensitivity required to track the inoculants accurately. Previously, we demonstrated that *Nocardia* sp. H17-1 isolated from crude-oil-contaminated sites can degrade *n*-alkanes and alkylated aromatics [3, 5]. We used the traditional plate count method to detect H17-1; however, it was difficult to distinguish *Nocardia* sp. H17-1 from related species. Moreover, this method is laborious, time consuming, and expensive, which severely limits its application.

Advances in molecular biology and gene technology have created exciting possibilities for rapidly detecting and identifying specific microorganisms. Recent studies have demonstrated the possibility of detecting very low concentrations of microorganisms in contaminated environments using quantitative PCR. Real-time PCR allows continuous monitoring of the sample during PCR using hybridization probes [9, 12, 15] or DNA-binding dyes, such as SYBR Green I [6, 16]. In particular, the SYBR green assay is a simple, fast, and sensitive method because it relies on the fluorescence signal produced as the dye binds to double-stranded DNA during the extension step [21]. Thus, it can be used with any specific PCR primers with only minor modifications of the described protocols.

Until recently, most studies about bioaugmentation of petroleum-contaminated sites have quantified the 16S rRNA genes of total and specific populations and catabolic genes involved in the degradation of aromatic compounds such as BTEX (benzene, toluene, ethylbenzene, and xylenes), naphthalene, and phenanthrene [6, 7, 14, 23]. In this study, we evaluated the degradation activity and quantified the density of *Nocardia* sp. H17-1 during bioremediation of crude-oil-contaminated soil using gene- and species-specific primers. In our previous report [5], we showed that *Nocardia* sp. H17-1 possessed the *alkB* gene responsible for degrading *n*-alkanes, but not the catechol-2,3-dioxygenase (23CAT gene, *xylE*) or *cis*-naphthalene dihydrodiol dioxygenase (*nar*) genes [2], involved in degrading aromatic hydrocarbons such as benzene, toluene, xylene, and naphthalene. Therefore, we monitored the *alkB4* and 16S rRNA gene content to evaluate the effectiveness of *Nocardia* sp. H17-1 in bioremediating

*Corresponding author
Phone: +82-42-860-4326; Fax: +82-42-860-4594;
E-mail: hkim@kribb.re.kr

crude oil-contaminated soil. We also wanted to quantify the catabolic genes involved in degrading aromatic compounds because oil-contaminated sites contain high concentrations of aromatics. Therefore, we used the primers designed by Mesarch *et al.* [17] to detect the 23CAT gene in soil with and without *Nocardia* sp. H17-1. We compared the levels of *Nocardia* sp. H17-1 and indigenous microorganisms by monitoring the 16S rRNA gene, *alkB4*, and 23CAT gene content.

Materials and Methods

Soil and Inoculum Preparation

We used sandy loam soil (3.8% organic matter, pH 7.1) that had been passed through a 2-mm sieve and air-dried. A 10-kg aliquot was inoculated with 3% (w/w) Arabian light oil (SK Chem., Korea) and homogenized thoroughly by hand mixing. Soil samples (1 kg contaminated soil) were kept in 2.25-l polyethylene containers with lid. To determine the effect of the indigenous microorganisms, three containers received no treatment (no inoculums). Three containers were inoculated with *Nocardia* sp. H17-1. Three containers containing autoclaved soil (121°C, 45 min) were prepared as abiotic control. In addition three autoclaved soils were inoculated with H17-1. The water content of all containers was adjusted to 50% of the maximum water holding capacity using sterile water. All the soil samples were kept at 23±2°C during the experiments. Containers were regularly aerated with a sterilized spatula to provide sufficient air and oxygen, and were moistened by addition of sterile water until the end of the experiment.

To prepare the inoculum, *Nocardia* sp. H17-1 was grown to the late exponential phase in Luria-Bertani liquid medium. The cells were collected by centrifugation at 10,000 ×*g* for 20 min, and washed twice in 20 ml of sterile water. The bacterial suspension was applied in droplets to the soil surface at approximately 1×10^6 cells per gram of soil, and then the soil sample was thoroughly mixed manually.

For taking soil samples, each soil was homogenized with a hand mix at clean bench to inhibit contamination. Total heterotrophic bacteria were estimated based on periodic colony formation on tryptic soy agar after 72 h incubation at 30°C.

Analysis of Total Petroleum Hydrocarbons

At various intervals, 5-g (dry wt) soil samples were transferred to 25-ml vials and mixed with anhydrous sodium sulfate. Then, 20 ml of dichloromethane was added to the vials; the vials were tightly capped and thoroughly mixed for 10 min using a vortex mixer, followed by sonication for 1 h in a water bath. The supernatant was passed through a 0.45-μm Teflon filter, and 1 μl of extract was analyzed using a gas chromatograph (Varian 3400CX; Varian, U.S.A.) equipped with a flame ionization detector and a DB-1 column (30 m×0.32 mm and 0.25 μm film thickness; J&W Scientific, U.S.A.). During the analysis, the injector and detector temperatures were maintained at 250°C and 300°C, respectively. The column temperature was programmed to increase from 40°C to 170°C in 6°C/min increments, stabilize at 170°C for 3 min, increase from 170°C to 300°C in 8°C/min increments, and finally hold at 300°C for 10 min.

Quantification of Bacterial 16S rRNA and Functional Genes by Real-Time PCR

Total DNA was extracted using a FastSPIN DNA kit for soil (Q-Biogene, U.S.A.) following the manufacturer's instructions. The quantity and purity of DNA were determined by measuring absorbance at 260 and 280 nm (NanoDrop ND-1000 spectrophotometer, U.S.A.). Real-time PCR was performed using the DNA Engine Opticon Continuous Florescence Detection System [Bio-Rad (formerly MJ research), U.S.A.]. The 20-μl reaction mixtures contained 0.2 μM of each primer (see Table 1), 1 μl of DNA (all 1 ng/μl), and 10 μl DyNAmo HS SYBR Green qPCR Kit (Finzymes, U.S.A.). The real-time PCR conditions for detecting the *Nocardia* sp. H17-1-specific 16S rRNA gene and *alkB4* were 5 min at 95°C, 40 cycles at 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min. For the total bacterial 16S rRNA gene assay, the PCR program was 5 min at 95°C, 40 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min. For 23CAT gene detection, the PCR program was 5 min at 95°C, 40 cycles at 95°C for 1 min, 50°C for 1 min, and 72°C for 1 min. All samples were measured three times during each assay, and negative controls without template were included in each PCR run. Because the detection by SYBR green is not fragment specific, we verified the length and quality of the amplicons by electrophoresis and melt-curve analysis.

The standard curve for total bacterial 16S rRNA genes was made using genomic DNA extracted from *E. coli*. The standard curves for the H17-1-specific 16S rRNA gene and *alkB4* were made using genomic DNA extracted from *Nocardia* sp. H17-1. The standard curve for the 23CAT gene was made using genomic DNA extracted from *P. putida* mt-2 (ATCC33015). DNA standards for each gene were prepared from serial dilution of pGEM T-easy vector (Promega Co., Madison, WI, U.S.A.) carrying each target gene product. The

Table 1. Primers used in this study.

Primer	Target	Sequence (5'→3')	Annealing temp. (°C)	Expected product size (bp)	References
1055f	Eubacteria	ATGGCTGTCGTCAGCT	55	352	[9]
1392r		ACGGGCGGTGTGTAC			
NH575f	*Nocardia* sp. H17-1	ACCAGCAGCTCAACTGCT	60	397	[3]
NH972r		GCCACATCTCTGCAGCTT			
alkB4f	Alkane monooxygenase	TACGGTCACTTCTACATCGAG	60	440	[5]
alkB4r		ATTCGCGTGGTGGTCGGAGT			
23cat-f	Catechol 2,3-dioxygenase	CGACCTGATCTCCATGACCGA	52	238	[6]
23cat-r		TCAGGTCAGCACGGTCA			

number of copies of each target gene per PCR reaction was calculated by comparison of threshold cycles obtained in each PCR reaction from standard DNA. The PCR efficiency (E) was determined from the slope of the external calibration curve according to $E=10^{(1/\text{-slope})}-1$. In addition the real-time PCR detection limit for each target gene in soil was determined using dilutions of sample DNA.

To confirm the specificity of the amplicons, each purified target gene product was ligated into the pGEM T-easy vector and transformed into competent *E. coli* DH5α cells as instructed by the manufacturer. The clones containing inserts of the correct size were sequenced using an ABI Prism 377 automatic sequencer (Applied Biosystems, CA, U.S.A.). The sequences were compared with the GenBank database using the BLASTN function (http://ncbi.nlm.nih.gov).

Statistical Analysis

All statistical analyses were performed using SPSS version 11.0 (SPSS Inc. U.S.A.) and Excel (Microsoft, U.S.A.). Data followed a normal distribution, and a one-way analysis of variance (ANOVA) was performed after $\log_{10}$ transformation of the data to meet the assumption of equal variance between groups. For ANOVA testing, we set the level of significance at $p \leq 0.05$.

RESULTS

Degradation of Total Petroleum Hydrocarbons

Fig 1A shows the concentration of TPH in each treatment group at different times during the bioremediation study. At the beginning of the study, the concentration of TPH in the H17-1-treated soil was 11.26 g/kg. After 30 days, it decreased to 4.10 g/kg of soil, indicating removal of 63.5% of TPH. In the soil without added H17-1 (containing indigenous microorganisms), the TPH concentration was 10.02 g/kg at day-0 and 4.88 g/kg at day-30, showing removal of 51.6% of TPH. Although the amount of TPH remaining after 30 days was similar for both treatment groups (with and without *Nocardia* sp. H17-1), the TPH degradation rate constants (k) calculated by a previous report [4] were 0.102 d^{-1} (r^2=0.99) and 0.028 d^{-1} (r^2=0.97), respectively. k was 3.6 times higher with H17-1 treatment than without H17-1 treatment. In addition, k in the autoclaved soils treated solely with H17-1 was 0.044 d^{-1} (r^2=0.97), which was also higher than that of no-treatment soil (data not shown). We could not measure k in the autoclaved soils used as negative control owing to contamination by microorganisms after eight days.

Bacterial Growth

The oil-contaminated soil with and without H17-1 initially contained $4.5\pm3.0\pm10^6$ and $2.3\pm1.5\times10^6$ CFU/g-soil, respectively, as counted on agar plates (Fig. 1B). During the 12 days of incubation, the number of heterotrophic bacteria in both groups increased rapidly reaching over 10^9 CFU/g-soil, and then decreased slightly or stabilized over the rest of the incubation period. There was no significant difference between

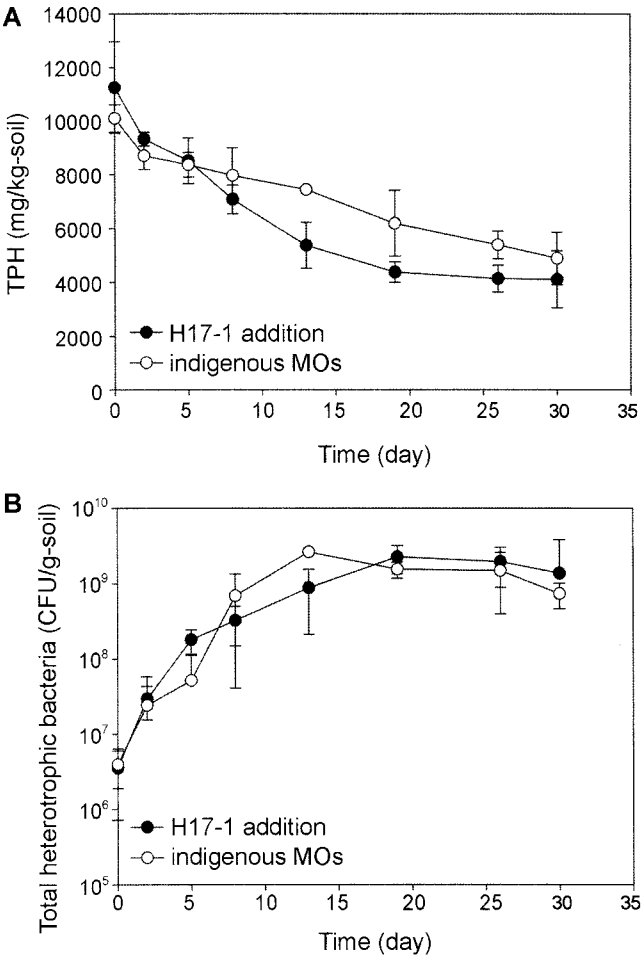

Fig. 1. Degradation of TPH **(A)** and total heterotrophic bacteria **(B)** during bioremediation of crude-oil-contaminated soil (n=3).

H17-1-treated and the nontreated groups (α=0.05, p>0.96). On the other hand, H17-1 added in the autoclaved soils at $5.8\pm0.5\times10^6$ CFU/g increased only 10-fold during the whole experiment. The autoclaved negative control soils had no colonies at the beginning of the experiment; however, colonies were detected after eight days owing to contamination (data not shown).

Real-Time PCR Efficiency

For real-time PCR assay, equations of the regression for total bacterial 16S rRNA, H17-1-specific 16S rRNA, *alkB4*, and 23CAT gene were respectively as follows: y=46.94–3.43x (r^2=0.99, p=0.0002), y=61.71–3.83x (r^2=0.98, p=0.0019), y=50.13–3.51x (r^2=0.99, p<0.0001), and y=42.86–3.65x (r^2=0.99, p<0.0001) (Fig. 2). Optimum template concentrations and amplification efficiencies for target genes were tested using serial dilutions of genomic DNA extracted from crude-oil-contaminated soil. The detection limit for 16S rRNA genes was approximately $1\pm0.2\times10^3$ copies/g-soil, which is equivalent to approximately 1 ng per

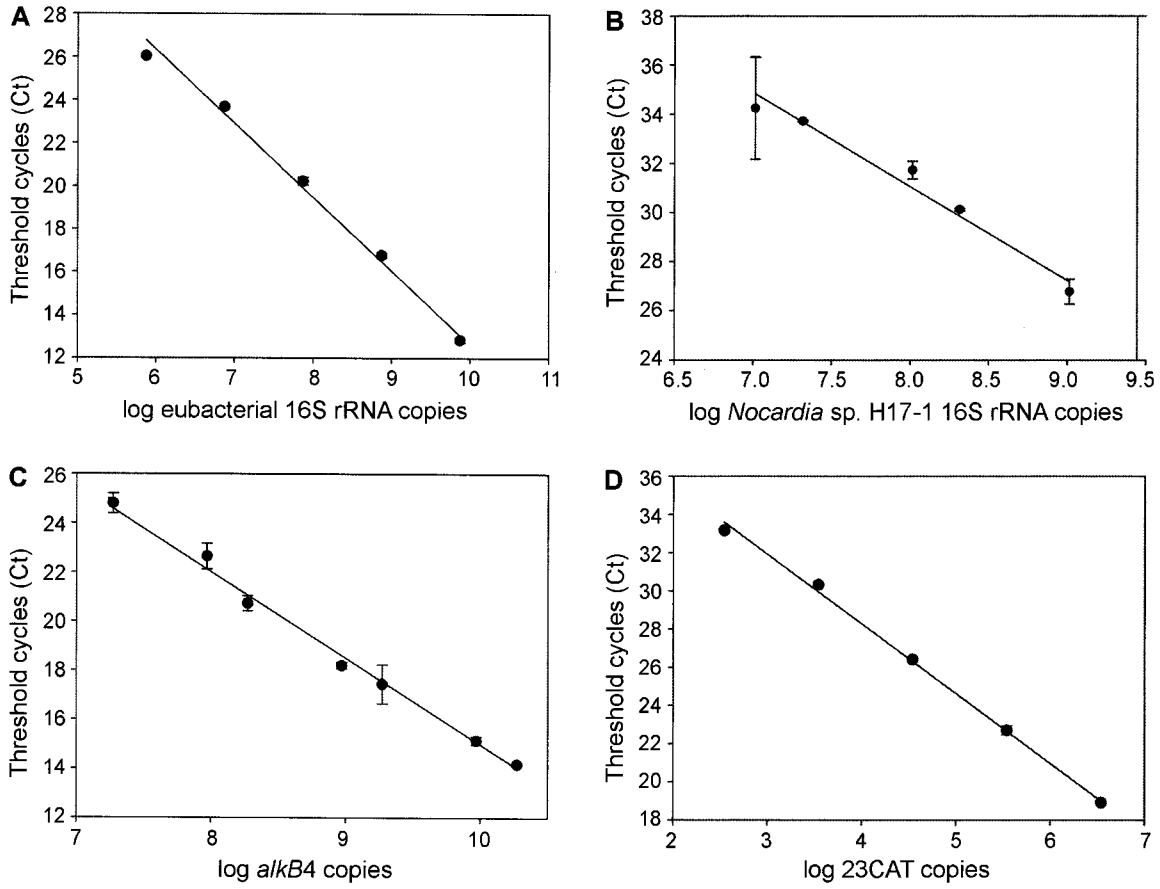

Fig. 2. Standard curves for real-time PCR assay.
A. Eubacterial 16S rRNA gene. **B.** *Nocardia* sp. H17-1 16S rRNA gene **C.** *alkB4* gene. **D.** 23CAT gene. The standard curve was generated by plotting the log amount of each gene copies against the threshold values (Ct) obtained from the analysis of three independent dilution series of standard DNA.

PCR. The *alkb4* gene was $4\pm0.6\times10^{3}$ copies/g-soil, similar to 1.2 ng per PCR. On the other hand, the detection limit for the 23CAT gene was $8\pm0.4\times10^{3}$ copies/g-soil, equal to 2 ng per PCR.

Enumeration of *Nocardia* sp. H17-1 by Real-Time PCR

The amount of total bacteria in soil containing *Nocardia* sp. H17-1, according to real-time PCR using universal bacterial 16S rRNA primers, ranged from $2.03\pm5.8\times10^{10}$ to $1.6\pm0.3\times10^{14}$ copies/g-soil during the entire experiment (Fig. 3A). On the other hand, total bacteria in the nontreated soil ranged from $1.3\pm0.4\times10^{10}$ to $6.7\pm0.7\times10^{13}$ copies/g-soil. After adding H17-1, the total bacterial gene copy number in the H17-1-treated soil was 2-fold higher than in the nontreated soils, and we detected a 2.4-fold difference between the soils at the end of the experiment.

The H17-1-specific 16S rRNA gene copy numbers in soil containing *Nocardia* sp. H17-1 ranged from $1.0\pm0.4\times10^{10}$ to $8.8\pm0.4\times10^{12}$ copies/g-soil (Fig. 3B). We detected fluorescence of SYBR green in nontreated soils at $1.6\pm0.3\times10^{7}$ to $9.4\pm0.6\times10^{8}$ copies/g-soil, due to the increasing amount of biomass in the samples. This phenomenon was explained using melt-curve analysis. The melting temperature (T_m) of PCR products amplified from soils with H17-1 using *Nocardia* sp. H17-1-specific primers was 84°C, but PCR products in soils without H17-1 melted at 78–84°C, indicating that the fluorescence measured from the soils without H17-1 was a false positive.

In both groups, total bacterial 16S rRNA genes increased more than 2,000 times during the experiment. The total bacterial 16S rRNA gene copies in H17-1-treated soils increased rapidly without a lag phase, whereas in nontreated soils the copy number increased after two days of incubation. In addition, the correlation between total bacterial 16S rRNA gene copy number and H17-1 16S rRNA gene copy number in H17-1-treated soil was statistically significant ($\alpha=0.05$, $p<0.033$).

After real-time PCR assay, we confirmed the specificity of target gene amplification by sequencing clones from target-gene libraries. Sixteen gene libraries were constructed (four target genes per treatment group, each at day-0 and day-30). We chose seven clones from each library for sequencing, and detected a shift in the microbial community during the bioremediation of crude oil. When we sequenced the clones

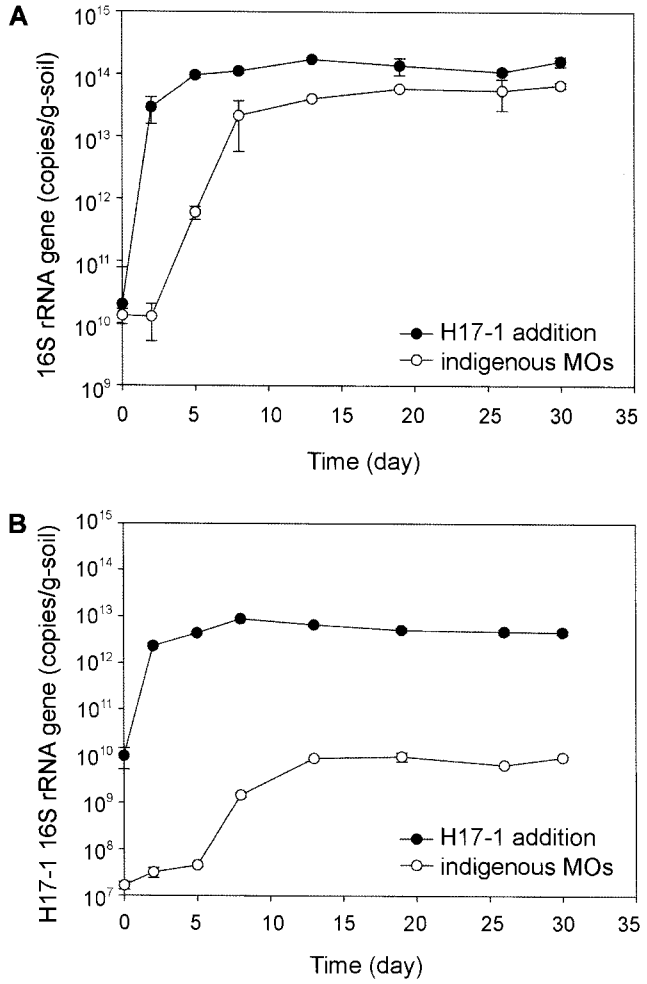

Fig. 3. Quantification of total bacterial 16S rRNA genes **(A)** and *Nocardia* sp. H17-1-specific 16S rRNA gene **(B)** using real-time PCR assay during bioremediation of crude-oil-contaminated soil (n=3).

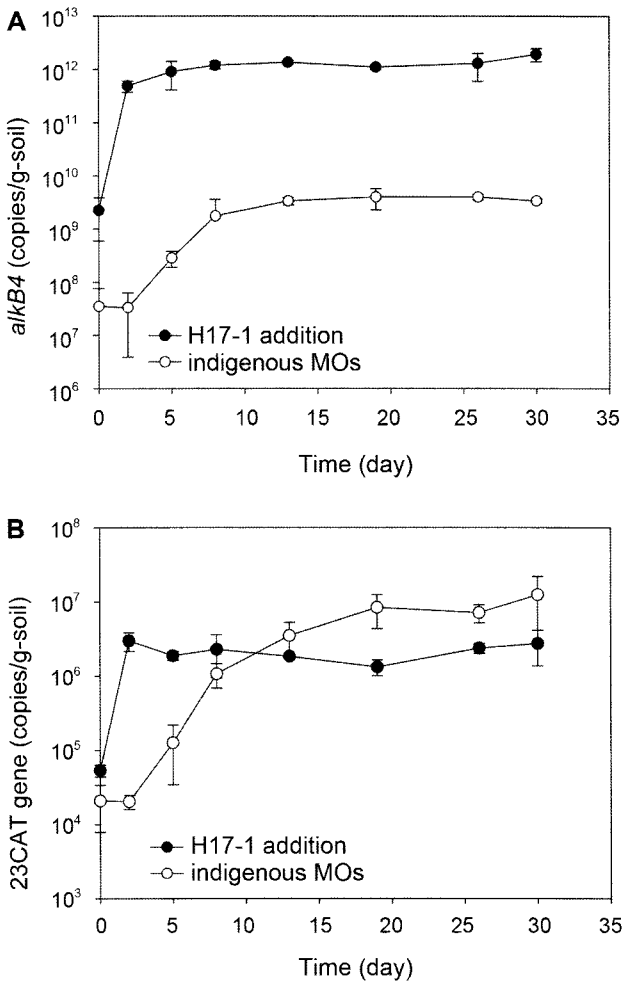

Fig. 4. Quantification of the *alkB4* gene **(A)** and 23CAT gene **(B)** using real-time PCR assay during bioremediation of crude-oil-contaminated soil (n=3).

of the 16S rRNA gene from the nontreated soil samples, we detected *Comamonadaceae* (AJ505858, 99% homology), uncultured *Chloroflexi* (DQ811886, 93% homology), uncultured CFB group bacteria (DQ330336, 95% homology), and *Myxobacterium* AT3-03 (AB246770, 97% homology) at day-0. We detected *Flavobacterium* sp. (DQ356494, 98% homology), uncultured *Methylophiliceace* sp. (98% homology), uncultured *Verrucomicrobium* sp. (AJ704724, 97% homology), uncultured *Chloroflexi* (DQ811886, 93% homology), and uncultured deltaproteobacteria (AY157088, 98% homology) at day-30. In the H17-1-treated soil at day-zero, we detected uncultured Bacteriodetes (DQ330336, 96% homology) and *Nocardia* sp. H17-1 (AF487704, 100% homology); at day-30, all sequenced clones were homologous to *Nocardia* sp. H17-1 (AF487704).

Using H17-1-specific primers, the PCR products that we obtained from the H17-1-treated soils at days-0 and -30 showed 100% homology to *Nocardia* sp. H17-1 (AF487704). On the other hand, no PCR products were amplified from nontreated soils using H17-1-specific primers.

Enumerating Catabolic Genes Using Real-Time PCR

We evaluated changes in the microbial community structure in response to hydrocarbon degradation using primers that target specific genes encoding enzymes commonly associated with the biodegradation of petroleum hydrocarbons (Table 1). The *alkB4* gene content in H17-1-treated soil ranged from $2.2\pm1.6\times10^{9}$ to $1.9\pm0.5\times10^{12}$ copies/g-soil and from $3.5\pm4.0\times10^{7}$ to $3.3\pm2.7\times10^{9}$ copies/g-soil in nontreated soil. There were more *alkB4* gene copies in H17-1-treated soils than in nontreated soils (Fig. 4A).

On the other hand, the 23CAT gene content in the H17-1 soil ranged from $6.1\pm0.0\times10^{4}$ to $2.7\pm1.1\times10^{6}$ copies/g-soil during the experiment (Fig. 4B), whereas the number of 23CAT genes in nontreated soil ranged from $5.3\pm1.0\times10^{4}$ to $2.7\pm1.3\times10^{7}$ copies/g-soil. There were more 23CAT genes at day-30 in nontreated soils than in H17-1-treated

soils; however, the difference was not significant (α=0.05, p>0.22).

We did not detect the *alkB4* gene in nontreated soil at day-0. However, the clones we analyzed at day-30 showed 90% homology to *Rhodococcus* sp. Q15 *alkB4* (AF388180). In H17-1-treated soils, clones from days-0 and -30 showed 100% homology to *Nocardia* sp. H17-1 *alkB4* (AY625606). We used primers designed by Mesarch *et al.* [17] to detect the 23CAT gene. When we sequenced the clones of these amplicons, we found that all sequences from both treatment groups were from *Pseudomonas* sp., as expected.

Discussion

The major concerns in this study were the survival of the added microorganisms and the maintenance of their degradation activity during the bioremediation process. It is necessary to monitor the target bacteria or genes related to the degradation of contaminants directly during the bioremediation process [24, 25]. The numbers of genes encoding the catabolic enzymes alkane monooxygenase (*alkB*), naphthalene dioxygenase (*nahH*), and catechol 2,3-dioxygenase (23CAT; *xylE*) have been used to assess the prevalence of bacteria involved in petroleum hydrocarbon degradation [13, 23]. To target specific catabolic function, we monitored the gene encoding an alkane monooxygenase, which plays an important role in the bacterial alkane degradation pathway, and the gene encoding a catechol 2,3-dioxygenase, which performs the key step in aerobic aromatic hydrocarbon degradation, as indicators of petroleum hydrocarbon catabolic potential.

We report the development of a real-time PCR assay using SYBR green to monitor the dynamics of aliphatic and aromatic hydrocarbon-degrading bacteria *via* their functional genes. SYBR green I real-time PCR analysis has many advantages and is relatively inexpensive, but can be subject to artifacts from nonspecific amplification and the formation of primer dimers [26]. Thus, it was essential that we verify the specificity of the assay by cloning and sequencing the PCR products.

In real-time PCR, gene copy numbers can be influenced by the DNA extraction method, template concentration, and the presence of PCR inhibitors [20, 22]. In this study, PCR amplification efficiencies obtained for eubacterial 16S rRNA, H17-1-specific 16S rRNA, *alkB4,* and 23CAT genes with pure culture were 0.96 (p<0.002), 0.82 (p<0.002), 0.93 (p<0.001), and 0.90 (p<0.001), respectively. The PCR amplification efficiencies of eubacterial 16S rRNA and *alkB4* were very high, indicating that this assay is a reliable measure of total bacterial biomass. The calculated amount of total bacteria using real-time PCR in both treatment groups (with and without *Nocardia* sp. H17-1) increased more than 2,000-fold over the course of the experiment. We found no significant difference in CFUs between H17-1 treatment and no treatment during the experiment (α=0.05, p>0.96); however, there was a significant difference in the total bacterial gene copy number between the treatment groups according to the real-time PCR results (α=0.05, p<0.009). We confirmed that the number of H17-1 increased up to 800-fold by quantifying the H17-1 16S rRNA gene using real-time PCR. Unexpectedly, we detected a small amount of H17-1-specific 16S rRNA genes in the soils that were not treated with H17-1. The results of melt-curve analysis and agarose gel electrophoresis showed an increase in the amount of total DNA extracted from the soils. The amounts of target genes (*alkB*, 23CAT, and 16S rRNA genes) per microgram of DNA detected by real-time PCR assay increased up to 100 times until day-8 and then decreased slightly by the end of the study (data not shown). However, the copies of target genes per gram of soil increased, because the total biomass increased continuously overtime, similarly to the 16S rRNA gene copies.

Ideally, a molecular method for *in situ* monitoring would target a functional gene associated with pollutant degradation so that a strong relationship between the number of target gene copies and pollutant-degrading activity could be assumed. Previous reports [8, 9, 18] have shown a relationship between the abundance of naphthalene dioxygenase genes and naphthalene degradation potential and active contaminant degradation. In this study, we did not fully prove the relationship between TPH degradation and functional gene abundance during the remediation process, because crude oil is a complex mixture of thousands of hydrocarbon (straight-chain alkanes, branched alkanes, cyclo-alkanes, and aromatic hydrocarbons) and non-hydrocarbon (nitrogen-, oxygen-, sulfur-, and metal-containing compounds) compounds. However, we did establish a correlation between TPH concentration and the amount of the catabolic genes measured in soil samples. As the concentration of THP in the H17-1-treated soil decreased, we detected a greater number of H17-1-specific genes. In particular, the correlation between TPH concentration and the *alkB4* copy number in H17-1-treated soil was highly statistically significant (r^2=0.94, p<0.0001), more so than the 23CAT (r^2=0.77, p=0.0039) or 16S rRNA (r^2=0.86, p=0.0009) gene, suggesting that H17-1 contributed to the degradation of aliphatic hydrocarbons. These results are similar to those from previous studies in which the amount of alkane monooxygenase genes present correlated with a reduction in the contaminant content in the polluted environment [20].

Overall, our results demonstrate a relationship between the quantitative monitoring of bacterial genes using real-time PCR and contaminant degradation. Monitoring the bacterial populations and the quantities of functional genes responsible for contaminant degradation could help to increase the efficiency of the bioremediation process and to increase our understanding of the microbial community dynamics in bioremediation sites. Successful implementation of bioremediation processes requires an understanding of the behavior of the microbial

populations responsible for target pollutant degradation. Furthermore, it is important to understand the ecology of the microbial community as a whole, because microbes not involved in degradation could influence the behavior of the degrading bacteria. As we learn more about the contaminant degradation potential of other organisms, the approach we describe will facilitate the investigation and monitoring of their behavior and activity, further enhancing bioremediation efforts.

Acknowledgments

This research was supported by the SMEs Technology Innovation Program and a grant from the KRIBB Research Initiative Program.

REFERENCES

1. Aamand, J. G., G. Bruntse, M. Jepsen, C. Jorgensen, and B. K. Jensen. 1995. Degradation of PAHs in soil by indigenous and inoculated bacteria, pp. 121–127. *In* R. E. Hinchee, J. Fredrickson, and B. C. Alleman (eds). *Bioaugmentation for Site Remediation* Battelle Press, Columbus, OH.
2. Andreoni, V., S. Bernasconi, M. Colombo, J. B. van Beilen, and L. Cavalca. 2000. Detection of genes for alkane and naphthalene catabolism in *Rhodococcus* sp. strain 1BN. *Environ. Microbiol.* **2:** 572–577.
3. Baek, K. H., Y. K. Lee, I. S. Lee, H. M. Oh, B. D. Yoon, and H. S. Kim. 2004. Detection of *Nocardia* sp. H17-1 by PCR during bioremediation of crude oil-contaminated soil. *Kor. J. Microbiol. Biotechnol.* **32:** 91–95.
4. Baek, K.-H., H.-S. Kim, S.-H. Moon, I.-S. Lee, H.-M. Oh, and B.-D. Yoon. 2004. Effects of soil types on the biodegradation of crude oil by *Nocardia* sp. H17-1. *J. Microbiol. Biotechnol.* **14:** 901–905.
5. Baek, K. H., B. D. Yoon, I. S. Lee, H. M. Oh, and H. S. Kim. 2006. Biodegradation of aliphatic and aromatic hydrocarbons by *Nocardia* sp. H17-1. *Geomicrobiol. J.* **23:** 253–259.
6. Baldwin, B., C. H. Nakatsu, and L. Nies. 2003. Detection and enumeration of aromatic oxygenase genes by multiplex and real-time PCR. *Appl. Environ. Microbiol.* **69:** 3350–3358.
7. Beller, H. R., S. T. Kane, T. C. Legler, and P. J. Alvarez. 2002. A real-time polymerase chain reaction method for monitoring anaerobic hydrocarbon-degrading bacteria based on a catabolic gene. *Environ. Sci. Technol.* **36:** 3977–3984.
8. Debruyn, J. M., C. S. Chewning, and G. S. Sayler. 2007. Comparative quantitiative prevalence of Mycobacteria and functionally abundant *nidA*, *nahAc*, and *nagAc* dioxygenase genes in coal tar contaminated sediments. *Environ. Sci. Technol.* **41:** 5426–5432.
9. Dionisi, H. M., C. S. Chewning, K. H. Morgan, F. Menn, J. P. Easter, and G. S. Sayler. 2004. Abundance of dioxygenase genes similar to *Ralstonia* sp. strain U2 *nagAc* is correlated with naphthalene concentrations in coal tar-contaminated freshwater sediments. *Appl. Environ. Microbiol.* **70:** 3988–3995.
10. Fantrossi, S. E. and S. Agathos. 2005. Is bioaugmentation a feasible strategy for pollutant removal and site remediation? *Curr. Opin. Microbiol.* **8:** 268–275.
11. Goldstein, R. M., L. M. Mallory, and M. Alexander. 1985. Reasons for possible failure of inoculation to enhance biodegradation. *Appl. Environ. Microbiol.* **50:** 977–983.
12. Harms, G., A. C. Layton, H. M. Dionisi, I. R. Gregory, V. M. Garrett, S. A. Hawkins, K. G. Robinson, and G. S. Sayler. 2003. Real-time PCR quantification of nitrifying bacteria in a municipal wastewater treatment plant. *Environ. Sci. Technol.* **37:** 343–351.
13. Heiss-Blanquet, S., Y. Benoit, C. Marechaux, and F. Monot. 2005. Assessing the role of alkane hydroxylase genotypes in environmental samples by competitive PCR. *J. Appl. Microbiol.* **99:** 1392–1403.
14. Laurie, A. D. and G. Lloyd-Jones. 2000. Quantification of *phnAc* and *nahAc* in contaminated New Zealand soils by competitive PCR. *Appl. Environ. Microbiol.* **66:** 1814–1817.
15. Layton, A. C., H. M. Dionisi, H. W. Kuo, K. G. Robinson, V. M. Garrett, A. Meyers, and G. S. Salyer. 2005. Emergence of competitive dominant ammonia-oxidizing bacterial populations in a full-scale industrial wastewater treatment plant. *Appl. Environ. Microbiol.* **71:** 1105–1108.
16. Lopez-Gutierrez, J. C., S. Henry, S. Hallet, F. Martin-Laurent, G. Catroux, and L. Philippot. 2004. Quantification of a novel group of nitrate-reducing bacteria in environment by real-time PCR. *J. Microbiol. Methods* **57:** 399–407.
17. Mesarch, M. B., C. H. Nakatsu, L. Nies. 2000. Development of catechol 2,3-dioxygenase-specific primers for monitoring bioremediation by competitive quantitative PCR. *Appl. Environ. Microbiol.* **66:** 678–683.
18. Nyssönen, M., R. Piskonen, and M. Itävaara. 2006. A targeted real-time PCR assay for studying naphthalene degradation in the environment. *Microb. Ecol.* **52:** 533–543.
19. Nerella, S., A. L. Wright, and R. W. Weaver. 1995. Microbial inoculants and fertilization for bioremediation of oil in wetlands, pp. 31–38. *In* R. E. Hinchee, J. Fredrickson, and B. C. Alleman, (eds). *Bioaugmentation for Site Remediation* Battelle Press, Columbus, OH.
20. Powell, S. M., S. H. Ferguson, J. P. Bowman, and I. Snape. 2006. Using real-time PCR to assess changes in the hydrocarbon-degrading microbial community in the Antarctic soil during bioremediation. *Microb. Ecol.* **52:** 523–532.
21. Sharkey, F. H., I. M. Banat, and R. Marchant. 2004. Detection and quantification of gene expression in environmental bacteriology. *Appl. Environ. Microbiol.* **70:** 3795–3806.
22. Smith, C. J., D. B. Nedwell, L. F. Dong, and A. M. Osborn. 1999. Molecular screening for alkane hydroxylase genes in Gram-negative and Gram-positive strains. *Environ. Microbiol.* **1:** 307–317.
23. Stapleton, R. D., G. S. Sayler, J. K. Boggs, E. L. Libelo, T. Stauffer, and W. G. Macintyre. 2000. Changes in subsurface catabolic gene frequencies during natural attenuation of petroleum hydrocarbons. *Environ. Sci. Technol.* **34:** 1991-1999.
24. Watanabe, K. and N. Hamamura. 2003. Molecular and physiological approaches to understand of the ecology of pollutant degradation. *Curr. Opin. Biotechnol.* **14:** 289–295.
25. Widada, J., H. Nojiri, and T. Omori. 2002. Recent developments in molecular techniques for identification and monitoring of xenobiotic-degrading bacteria and their catabolic genes in bioremediation. *Appl. Microbiol. Environ.* **60:** 45–59.
26. Zhang, T. and H. H. P. Fang. 2006. Amplification of real-time polymerase chain reaction for quantification of microorganisms in environmental samples. *Appl. Microbiol. Biotechnol.* **70:** 281–289.