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Detection of *Nocardia* sp. H17-1 by PCR during Bioremediation of Crude Oil-Contaminated Soil

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For the detection of the oil-degrading bacterium, *Nocardia* sp. H17-1, inoculated during the bioremediation of oil-contaminated soil, a species-specific primer was constructed based on the 16S rDNA sequence of this strain. Two forward primers and two reverse primers were designed and tested against both closely and distantly related bacterial strains. All the primers designed were specific to the *Nocardia* sp. H17-1. Particularly, primer sets NH169F-NH972R and NH575F-NH972R could be used to detect 50 fg of template DNA and 1.2×10^4 CFU/g of sandy soil. These two PCR primer sets successfully detected the H17-1 strain in the oil-contaminated soil samples containing heterogeneous DNA. We also conformed the primer specificity by restriction-enzyme cleavage of the PCR products and denaturing gradient gel electrophoresis.

Key words: 16S rDNA, denaturing gradient gel electrophoresis (DGGE), detection, *Nocardia* sp., PCR, species-specific primer

Bioaugmentation - the addition of pollutant-degrading microorganisms - has proven successfully at the remediation of petroleum hydrocarbons in soil, but there have been numerous cases where this strategy has failed [1, 3]. The main difficulty with the being effective and of surviving under harsh environmental conditions. Therefore, culture-based monitoring techniques lack the specificity and sensitivity required for the accurate tracking of inoculants during bioremediation of contaminated sites. Recently developments in molecular techniques such as DNA hybridization and PCR have made it easier to identify and assess the survival of strains introduced into the environment [4, 5, 12]. In particular, PCR based on 16s rDNA gene sequences has become an attractive tool for the detection of specific microorganisms in environmental samples.

Nocardia species are aerobic actinomycetes - members of the genus *Dietzia*, *Gordona*, *Mycobacterium*, *Rhodococcus*, and *Tsukamurella* - that are distributed in worldwide. These bacteria are effective at the high pollutant concentrations that prevail at industrial sites [2]. We previously isolated the *Nocardia* sp. H17-1 from oil-contaminated soil as a crude oil degrader [7]. While bioremediation with *Nocardia* sp. H17-1 is proceeding in oil-contaminated soil, it is difficult to distinguish *Nocardia* sp. H17-1 from some of the most closely related genera using selective plating. The objective of the present study was to detect *Nocardia* sp. H17-1 by PCR using species-specific primers based on 16S rDNA gene sequences during the course of bioremediation of crude oil-contaminated soil.

The 16S rDNA sequence of strain H17-1 (AF487704) was compared with the sequences in the Ribosomal Database Project (RDP) [10] using the SEQUENCE_MATCH service and the GenBank nucleotide database. Specific alignments of 16S rDNA sequences were retrieved by the SUB_ALIGNMENT routine from RDP, and potential PCR primer target regions were manually determined. The designed primers were tested for their species specificity against three *Nocardia* species and ten other strains obtained from KCTC (Korea Culture Type Collection, Daejeon, Korea). The bacterial strains used to design species-specific primers and to evaluate the specificity of PCR primers were as follows; *N. beijingensis* KCTC 19931, *N. flavorosea* KCTC 9371, *N. ignorata* KCTC 19935, *R. rhodochrous* KCTC

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9086, *R. erythropolis* KCTC 9077, *M. dierhoferi* KCTC 9506, *Corynebacterium* sp. KCTC 1450, *Pseudomonas putida* KCTC 1770, *Burkholderia vietnamiesnsis* KCTC 2974, *E. coli* JM109, *Alcaligenes faecalis* subsp. *faecals* KCTC 2678, *Bacillus subtilis* C9 [6] and *Acinetobacter* sp. A54 [8]. The selected primers from 16S rDNA of *Nocardia* sp. H17-1 were NH169F, NH575F, NH972R and NH1079R (Genotech, Korea) (Table 1). The length of the fragments amplified from four primer sets, NH169F-NH972R, NH575F-NH972R, NH169F-NH1079R and NH575R-NH1079R, was 803-, 397-, 910- and 504-bp, respectively. As shown in Fig. 1, NH169F-NH972R, NH575F-NH972R, and NH169F-NH1079R primer sets did not amplify any DNA from the other strains tested, whereas primer set NH575F-NH1079R

Table 1. Primer sequence used in this study.

Primer	Primer sequence (5'3')	Target site	Specificity
9F	GAGTTTGATCCTGGCTCAG	9 - 28 (*E. coli*)	Universal
NH169F	TCCTATCGCATGGTGGGT	169 - 187	Strain H17-1
341F-GC[a]	CTACGGGAGGCAGCAG	341 - 357 (*E. coli*)	Universal
NH575F	ACCAGCAGCTCAACTGCT	575 - 591	Strain H17-1
536R	GAATTACCGCGGCAGCTG	536 - 544 (*E. coli*)	Universal
907R	CCGTCAATTCATTTGAGTTT	887 - 907 (*E. coli*)	Universal
NH972R	GCCACATCTCGTCAGCTT	989 - 972	Strain H17-1
NH1079R	GCTGGCAACATAAGATAG	1096 - 1079	Strain H17-1

[a]The GC clamp sequence is CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGC.

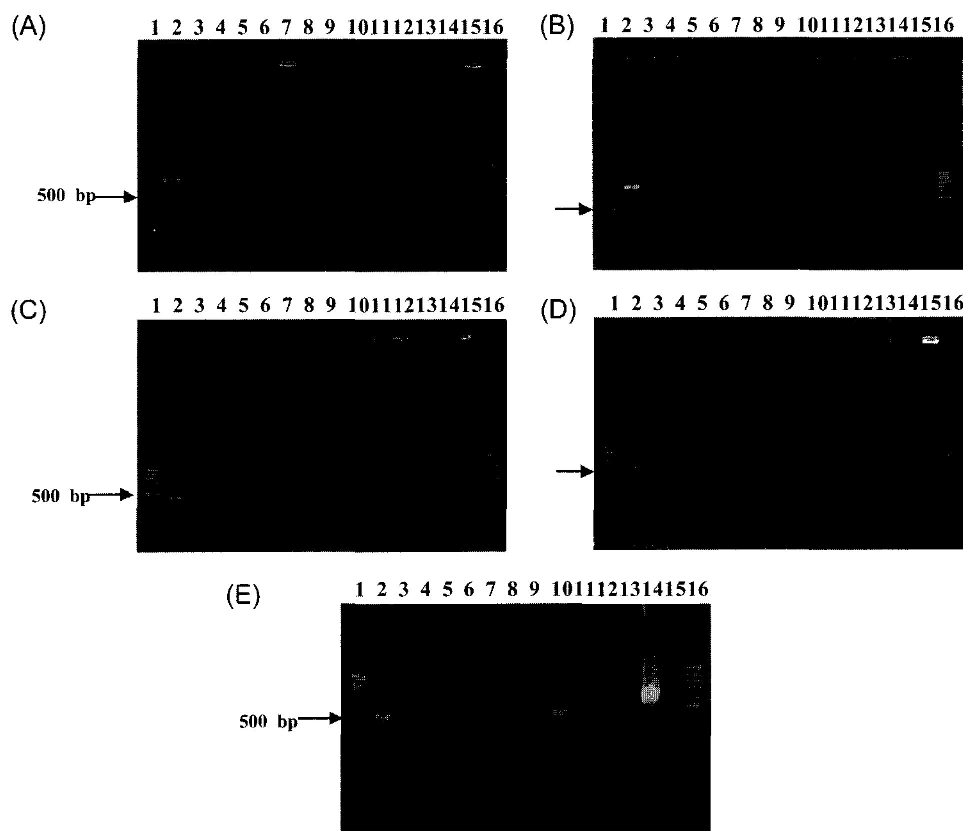

Fig. 1. Specific detection of *Nocardia* sp. H17-1 by PCR with primer set NH169F-NH972R (A), NH169F-NH1079R (B), NH575F-NH972R (C), NH575F-NH1079R (D), and *E. coli* universal primer 9F- 536R (E). Ethidium bromide stained 1% agarose gel. Lanes: 1 & 16, 100-bp marker; 2, *Nocardia* sp. H17-1; 3, *R. erythropolis* KCTC 9077; 4, *R. rhodochrous* KCTC 9086; 5, *N. beijingensis* KCTC 19931; 6, *N. flavorosea* KCTC 9371; 7, *N. ignorata* KCTC 19935; 8, *Mycobacterium dierfoeri* KCTC 9506; 9, *Corynebacterium* sp. KCTC 1450; 10, *Pseudomonas putida* KCTC 1770; 11, *Burkholderia vietnamiensis* KCTC 2974; 12, *E. coli* JM109; 13, *Alcaligenes faecalis* subsp. *faecals* KCTC 2678; 14, *Bacillus subtilis* C9; 15, *Acinetobacter* sp. A54.

detected *Rhodococcus erythropolis* KCTC 9077 as well as H17-1. With the universal primer, 9F-536R, the amplification product was obtained from all strains used (Fig. 1E). From these results, all of designed primers were specific to the *Nocardia* sp. H17-1.

To determine the lower limit of detection for *Nocardia* sp. H17-1, serial 10-fold dilutions of genomic DNA from *Nocardia* sp. H17-1 were amplified with four primer sets, and detected on agarose gels with ethidium bromide staining. Two primer sets, NH169F-NH972R and NH575F-NH972R, were able to detect 50 fg of DNA (Fig. 2), but the detection limit of the primer set NH169F-NH1079R and NH575F-NH1079R was above 500 fg (Table 2). It is suggested that the primer NH972R is responsible for the high sensitivity to genomic DNA of H17-1.

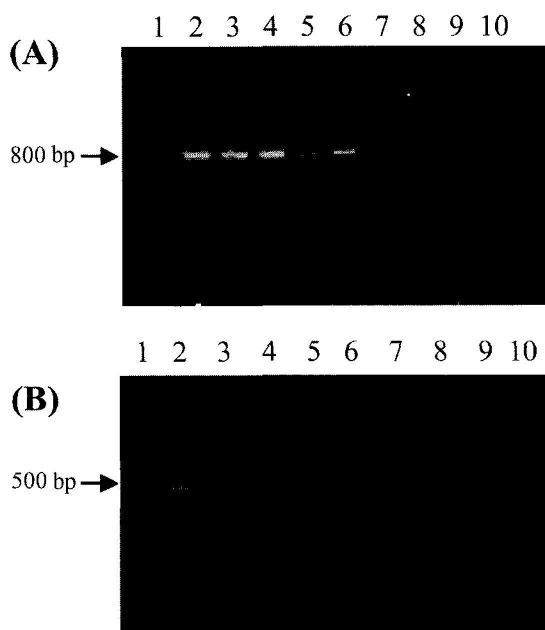

Fig. 2. Sensitivity of primer sets NH169F-NH972R (A) and NH575F-NH972R (B) on template genomic DNA isolated from a *Nocardia* sp. H17-1 pure culture. Ethidium bromide stained 1% agarose gel. Lanes: 1, PCR marker (50-2000 bp); 2, 4.5 ng; 3, 450 pg; 4, 45 pg; 5, 4.5 pg; 6, 450 fg; 7, 45 fg; 8, 4.5 fg; 9, 0.45 fg; 10, 0 (no DNA).

Table 2. Detection limit of template DNA using different primer sets.

Primer set	Pure culture (fg)	Seeded sterile-soil (cells/g soil)
NH169F-NH972R	50	5.6×10^{5}
NH575F-NH972R	50	1.2×10^{4}
NH169F-NH1079R	500	1.2×10^{7}
NH575F-NH1079R	500	N.D

N.D, not detect.

To determine the sensitivity of primer sets for detecting cells of *Nocardia* sp. H17-1 in soil, a known number of H17-1 cells (ranging from 0 to 3×10^{8} CFU/g of soil) were inoculated into sterile soil. The detection limits of primer sets NH169F-NH972R and NH575F-NH972R for cells of H17-1 were 5.6×10^{5} and 1.2×10^{4} CFU/g of soil, respectively (Table 2).

The applicability of these primer sets to nonsterile crude oil-contaminating sandy soil during 55 days was then tested. PCR amplifications were achieved with a primer set, NH575F-NH972R for the DNA samples extracted from soil treated with *Nocardia* sp. H17-1, whereas no amplification was observed for the DNA samples from untreated soil (Fig. 3A). Restriction endonuclease analysis was performed to verify the specificity of the 398-bp amplified fragment using the NH575F-NH972R primer set (Fig. 3B). *Nocardia* sp. H17-1 has an *Eco R*I recognition site on 16S rDNA sequence, resulting in the production of two DNA fragments (348 bp and 50 bp) by *Eco R*I digestion. In H17-1-treated soil, the fragment produced by *Eco R*I digestion consistent with those of that obtained from pure culture DNA, and as expected, no restriction fragments were produced in

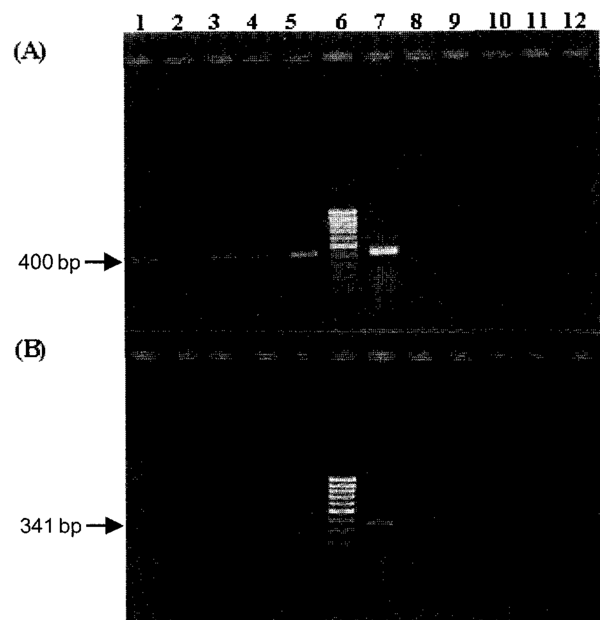

Fig. 3. Agarose-gel electrophoresis of PCR products (A) and *EcoR*I digestion (B). PCR was performed for the sandy-soil DNA with the primer set NH575-972R during the biodegradation of crude oil. Ethidium bromide stained 1.7% agarose gel. Lanes: 1-5, treatment with H17-1; 8-12, no treatment (indigenous microorganisms); 1 & 8, 1 days; 2 & 9, 14 days; 3 & 10, 28 days; 4 & 11, 42 days; 5 & 12, 55 days; 6, 100-bp marker; 7, NH575-972R PCR product of H17-1 pure culture DNA.

untreated soil.

To confirm the primer specificity during bioremediation of oil-contaminated soils, DGGE analysis of untreated soil and soil treated with *Nocardia* sp. H17-1 was performed by using a D-code 16/16-cm gel system (Bio-Rad, USA) as described by Muyzer *et al.* [11], with following exceptions. DNA extracted directly from soil samples using FastDNA SPIN kit (Bio101, USA) were amplified using primer 341-GC (Genotech, Korea), which targets a universally conserved region (*E. coli* positions 341-358) and contains a 40-base GC clamp and primer 907r (Genotech, Korea), which target bacteria (*E. coli* positions 890-907). Gels contained 6% acrylamide and were poured with a urea-formamide gradient from 40% to 60% run at a constant voltage 60 mV for 18 h. We sequenced the 16S rDNA fragment from the expected H17-1 band, and compared this with the DNA band obtained from a pure culture of *Nocardia* sp. H17-1 on DGGE. The band expected to correspond to H17-1 was 100% homologous with the 16S rDNA sequence of *Nocardia* sp. H17-1. In the treated soil, the intensity of the H17-1 band increased with the incubation time (Fig. 4). The contrary, the H17-1 was not observed in the untreated soil as expected.

In this study, the DNA primer sequences selected from the 16S rDNA gene region appear to be highly specific for *Nocardia* sp. H17-1, enabling *Nocardia* sp. H17-1 to be distinguished from all the other organisms tested. With these selected PCR primers, 50 fg of template DNA from a pure culture can be reliably detected, and quantified at 10^4–10^5 CFU/g of sandy soil. The sensitivity of PCR amplification with these primers was higher than or similar to that achieved in other studies based on 16S rDNA genes [9, 13, 14]. In the further studies, we will evaluate the survival and degradation activity of introduced microbial species or indigenous microorganisms in bioremediation sites and quantify the individual species in oil spills and other hydrocarbon-contaminated episodes using the species-specific primers.

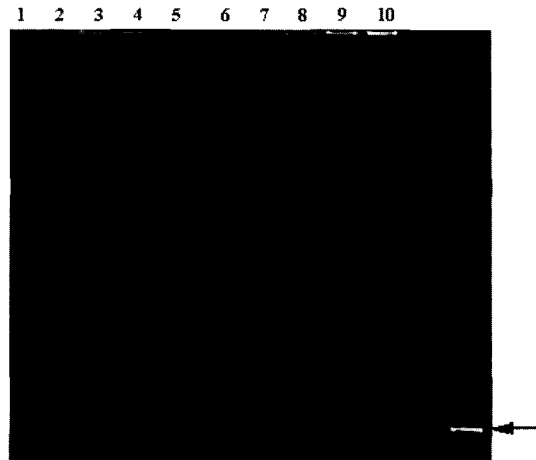

Fig. 4. DGGE profile (40% to 60% denaturant gradient) of PCR-amplified 16S rDNA fragments from H17-1-treated and untreated soil. Lanes: 1-5, no treatment (indigenous microorganisms); 6-10, treatment with H17-1 1 & 6, 1 days 2 & 7, 14 days; 3 & 8, 28 days; 4 & 9, 42 days; 5 & 10, 55 days. Arrow means the H17-1 genomic DNA from the pure culture.

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국문초록

원유 오염토양의 Bioremediation 과정 동안 PCR을 이용한 *Nocardia* sp. H17-1의 검출

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원유로 오염된 토양의 생물학적 복원과정 동안 접종된 *Nocardia* sp. H17-1 균주를 확인하기 위해 16S rDNA sequence에 기초하여 균주에 특이적인 primer를 제작하였다. 14 균주의 16S rDNA sequence 비교를 통해 제작된 4개의 primer set는 H17-1 균주를 특이적으로 검출할 수 있었다. 특히 NH169F-NH972R과 NH575F-NH972R의 primer set는 50 fg의 DNA와 1.2×10^4 cfu/g-soil의 균체농도까지 민감하게 검출할 수 있었다. 이 두 primer set는 원유로 오염된 토양의 bioremediation 과정 동안 접종된 H17-1 균주의 특이적 검출을 가능케 하였으며, 이는 사용된 primer set에 의해 증폭된 PCR 산물을 제한효소(*Eco*RI)로 절단한 결과와 DGGE를 통한 H17-1 균주의 확인을 통해 본 연구에서 제작된 primer set의 특이성을 검증하였다.

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