

Monitoring of Microorganisms Added into Oil-Contaminated Microenvironments by Terminal-Restriction Fragment Length Polymorphism Analysis

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Abstract Terminal-restriction fragment length polymorphism (T-RFLP) analysis was used to monitor inoculated oil-degrading microorganisms during bioremediation treatability tests. A pair of universal primers, fluorescently labeled 521F and 1392R, was employed to amplify small subunit rDNA in order to simultaneously detect two bacterial strains, *Corynebacterium* sp. IC10 and *Sphingomonas* sp. KH3-2, and a yeast strain, *Yarrowia lipolytica* 180. Digestion of the 5'-end fluorescence-labeled PCR products with *Hha*I produced specific terminal-restriction fragments (T-RFs) of 185 and 442 bases, corresponding to *Corynebacterium* sp. IC10 and *Y. lipolytica* 180, respectively. The enzyme *Nru*I produced a specific T-RF of 338 bases for *Sphingomonas* sp. KH3-2. The detection limit for oil-degrading microorganisms that were inoculated into natural environments was determined to be 0.01% of the total microbial count, regardless of the background environment. When three oil-degrading microorganisms were released into oil-contaminated sand microenvironments, strains IC10 and 180 survived for 35 days after inoculation, whereas strain KH3-2 was detected at 8 days, but not at 35 days. This result implies that T-RFLP could be a useful tool for monitoring the survival and relative abundance of specific microbial strains inoculated into contaminated environments.

Key words: Oil-degrading microorganisms, specific detection, terminal-restriction fragment length polymorphism (T-RFLP)

Detection and monitoring of specific microorganisms in natural environments is one of the most important topics in microbial ecology, particularly bioremediation applications [20, 29, 33, 34]. Bioaugmentation, using microbial strains,

requires knowledge of how long the strains survive in the target environment. To monitor specific bacteria introduced into environments, as well as indigenous microbial groups, microbial strains must be detected specifically and sensitively. Traditionally, microorganisms have been monitored by cultivation techniques such as plate counting [7]. However, because most naturally occurring microorganisms are not cultivated by standard culture techniques, alternative methods must be used to detect specific microorganisms [13, 14]. Therefore, molecular biology techniques that do not require microorganism cultivation are valuable for detecting microorganisms in natural environments [1].

Terminal-restriction fragment length polymorphism (T-RFLP) analysis, based on 16S rDNA, was developed to identify bacterial species [2]. Subsequently, it has been used for structure analysis and differentiation of bacterial communities in soil [8, 9, 32], flooded paddy soil cores [23], marine bacterioplankton [30], and various other samples, including activated sludge, a contaminated aquifer, and termite guts [21, 22]. This technique has been applied not only to archaeal communities in the digestive tracts of marine fish [40], an anoxic rice field [6], and seawater [31] but also to eukaryal communities in activated sludge [26]. Clearly, T-RFLP analysis is an increasingly popular method for rapid comparison of microbial communities; its automated sequencer yields high resolution and throughput, and its Web-based analysis function utilizes sequence databases [24, 27]. Furthermore, an analytical procedure has been developed that reduces variation and extracts a reproducible subset of data from replicate T-RFLP profiles [10].

In this study, we used the T-RFLP method to simultaneously detect three microorganisms (two bacterial strains and a yeast strain) in the presence of natural microbial populations. We also attempted to monitor the survival of oil-degrading microorganisms introduced

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into oil-contaminated sand during a bioremediation treatability test.

MATERIALS AND METHODS

Microorganisms

We used the oil-degrading microbial strains *Corynebacterium* sp. IC10 [19], *Sphingomonas* sp. KH3-2 [37], and a yeast *Y. lipolytica* 180 [15, 16]. The strains were grown in Luria-Bertani (LB) medium [4] to early stationary phase, and then harvested by centrifugation, washed twice in phosphate-buffered saline (PBS) buffer, and resuspended in 10% glycerol for inoculation. The number of cells was determined by plate counting. The mixed microbial suspensions, containing equal numbers of cells per strain, were prepared in the same buffer and stored at -70°C .

Inoculation of Microorganisms into Natural Samples

To test detection limits of oil-degrading microorganisms in natural microbial communities, sandy and muddy marine sand samples were collected from the intertidal zone in Ohee-Do, Kyong-gi Bay. Total direct bacterial counts were determined by epifluorescence-microscopy after staining with 4',6'-diamidino-2-phenylindole (DAPI) [42]. Total microbial counts in sand samples were estimated as 2.2×10^9 and 1.2×10^{11} g/dry in sandy and muddy samples, respectively. All sand samples were aliquoted (0.5 ml) into 2-ml sterilized Eppendorf tubes. The mixed microbial suspensions were 10-fold serially diluted and inoculated into the treatment tubes; control samples were not inoculated. The cell concentration of each inoculated strain was adjusted to between 3.2×10^7 and 3.2×10^3 g/dry in treated sand samples, and between 1.7×10^8 and 1.7×10^3 g/dry in treated muddy samples.

Microenvironment Test

We also tested the survival of microbial strains added during the bioremediation process. Sea sand [gravel/sand/mud=0.14:88.97:10.89; wet weight=50 g; water content=0.88% (w/w); water holding capacity=19.1% (w/w)] was artificially contaminated with Arabian light crude oil to 6% (w/w) in 250-ml Erlenmeyer flasks. Slow-release fertilizer (SRF) was added for a C/N/P ratio of 100:10:3.

Tween 80 [$1 \times \text{CMC}$ (critical micelle concentration; 0.075% of water)] and the three microbial strains were added to 1×10^8 CFU g sand $^{-1}$. Control sea sand was not altered at all. Both sand samples were adjusted to 66% of their water-holding capacity, using seawater/distilled water mixture (1:1). Mineralization of the crude oil was measured by CO_2 production [3]. Subsamples were collected 0, 8, and 35 days after incubation at ambient temperature and were stored at -70°C until DNA could be extracted.

DNA Extraction and rDNA Amplification

Genomic DNA was prepared from pure cultures by using a Wizard Genomic DNA kit (Promega), according to the manufacturer's instructions. Total DNA was extracted mechanically from natural samples, containing microbial strains and microenvironment sea-sand samples, to avoid selective cell disruption. A bead-beating protocol [17] was adapted to omit the hot-detergent treatment step (which greatly degraded the DNA). DNA was extracted by phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1), and was then precipitated. Humic substances that inhibit PCR were removed from the crude DNA extracts, using polyvinylpyrrolidone micro spin columns [18]. The yield of extracted DNA was estimated to be about 2 μg per gram of wet sediment. We selected the forward primer 518F (5'-CCA GCA GCC GCG GTA ATW C-3') (*Escherichia coli* base numbers 518-536) and the reverse primer 1392R (5'-ACG GGC GGT GTG TRC-3') (*E. coli* base numbers 1392-1406), based on small subunit ribosomal DNA (SSU rDNA) sequences [1]. Table 1 shows the sequence alignment of the primers and target SSU rDNA sequences of three strains, *E. coli* and *Saccharomyces cerevisiae* SSU rDNAs. The fluorescent primer (518F-Hex) was prepared by 5'-end labeling with a phosphoramidite fluorochrome, 5-hexachloro fluorescein (Perkin Elmer, U.S.A.).

T-RFLP Analysis

Fifty μl of PCR mixture contained 0.2 μM each of primers, 50 mM Tris-HCl (pH 9.0), 1.5 mM MgCl_2 , 200 μM each of deoxynucleotides, 5% DMSO (dimethylsulfoxide), and 2.5 U of *Taq* DNA polymerase (Promega Corp., Madison, WI, U.S.A.). DNA was amplified by PCR as follows:

Table 1. A primer set adopted for simultaneous amplification of SSU rDNA sequences.

521F (521-536)	5' CCA GCA GCC GCG GTA ATW C 3'	
1392R (1392-1406)		3'CRT GTG TGG CGG GCA 5'
Strain KH3-2	5' CCA GCA GCC GCG GTA ATA C 3'	5'GTA CAC ACC GCC CGT 3'
Strain IC-10	5' CCA GCA GCC GCG GTA ATA C 3'	5'GTA CAC ACC GCC CGT 3'
Strain 180	5' CCA GCA GCC GCG GTA ATT C 3'	5'GTA CAC ACC GCC CGT 3'
<i>E. coli</i>	5' CCA GCA GCC GCG GTA ATA C 3'	5'GTA CAC ACC GCC CGT 3'
<i>S. cerevisiae</i>	5' CCA GCA GCC GCG GTA ATT C 3'	5'GTA CAC ACC GCC CGT 3'

initial denaturation at 94°C for 5 min, 35 cycles (denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min), and a final extension at 72°C for 7 min. This PCR procedure generated SSU rDNA products of 891, 898, and 1,007 bp, theoretically corresponding to the strains KH3-2, IC-10, and 180, respectively. Amplified products were verified by electrophoresis on 0.8% agarose gel, using 1× TAE buffer. The PCR products were purified with a Wizard PCR purification kit (Promega). Each digest mixture contained 5 µl of cleaned PCR product, 5 U of restriction enzymes (Promega), the respective restriction buffer at 1×, and enough deionized water for a final volume of 20 µl. For complete digestion, the mixtures were incubated at 37°C for 2 h, supplemented with an additional 2 U of restriction enzyme, and further incubated for 1 h. Digested PCR products were precipitated by adding 1/10 volume of 3 M sodium acetate (pH 4.6) and 2.5 volumes of 95% ethanol. The DNA pellet was air-dried at room temperature after washing with 70% ethanol.

The precise T-RF lengths were determined by electrophoresis with an automated sequencer [model 377; Applied Biosystems Instruments (ABI), Foster City, CA, U.S.A.]. The pellet of digested DNA from each sample was suspended in 3 µl of loading mixture [10 µl of deionized formamide, 1 µl of loading buffer (ABI), and 1 µl of DNA fragment length standard (Rox 2500; ABI)]. This mixture was denatured at 95°C for 2 min and immediately chilled on ice prior to electrophoresis. Aliquots (1 µl) of the mixture were loaded onto a 36-cm 4.5% denaturing polyacrylamide gel and electrophoresed for 4 h. The length of fluorescently labeled T-RFs was determined by comparison with internal standards, using GeneScan analysis software (version 3.1).

RESULTS

T-RFLP Analysis of Oil-Degrading Microorganisms

A universal primer set (521F-Hex and 1392R, based on SSU rDNA) was adapted for simultaneous detection of two bacterial strains (*Corynebacterium* sp. IC10 and *Sphingomonas* sp. KH3-2) and a yeast strain (*Yarrowia lipolytica* 180) by T-RFLP analysis (Fig. 1). The primer set

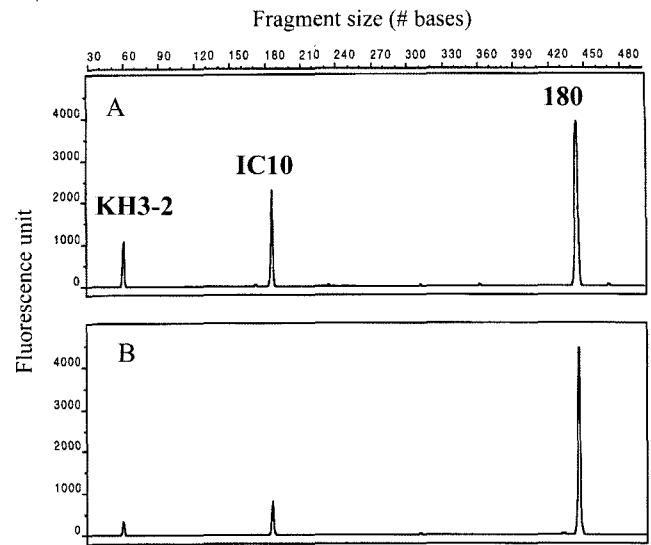


Fig. 1. Electropherograms of T-RFLP by *HhaI* digestion of SSU rDNA amplified from model communities, made by equivalent genomic DNAs (A) and cell numbers (B) of three microbial strains.

produced PCR products of 891, 898, and 1,007 bp for the strains KH3-2, IC-10, and 180, respectively; the sequence length was verified via agarose gel electrophoresis. The expected size of terminal-restriction fragments (T-RFs) cut with restriction enzymes (*AluI*, *HaeIII*, *HhaI*, *MspI*, *RsaI*, and *NruI*) for each strain was based on SSU rDNA sequence data and compared with observed T-RF data from cut genomic DNA (Table 2). The experimentally observed T-RF lengths agreed well (± 4 bases) with the expected values. Two enzymes, *MspI* and *AluI*, were found to be inappropriate for T-RFLP analysis, possibly because either the PCR product did not contain the restriction site or the T-RF generated by the enzyme tested was too short. T-RFLP analysis was conducted, using a chosen *HhaI* from two model microbial communities (Fig. 1): The one was a mixture of equal amounts of genomic DNAs and the other was made by equal numbers of cells of the three strains. The peaks specific for the three oil-degrading strains were found in these samples (Figs. 1A and 1B). Strain KH3-2 had a peak smaller than the other

Table 2. Predicted and observed lengths (numbers of bases) of 5'-T-RFs for three oil-degrading microorganism strains, cut by various restriction enzymes.

Strain	PCR-product (bp)	Predicted (and observed) 5'-TRFs					
		<i>HhaI</i> (GCG▼C)	<i>MspI</i> (C▼CGG)	<i>AluI</i> (AG▼CT)	<i>HaeIII</i> (GG▼CC)	<i>RsaI</i> (GT▼AC)	<i>NruI</i> (TCG▼CGA)
KH3-2	891	60(60)	95(95)	312(313)	412(414)	374(377)	488(488)
IC-10	898	184(185)	38(38)	60(60)	387(383)	383(379)	641(637)
180	1,007	441(442)	NC*	22	95(95)	420(423)	NC*

*NC=not cut; PCR product contains no restriction site.

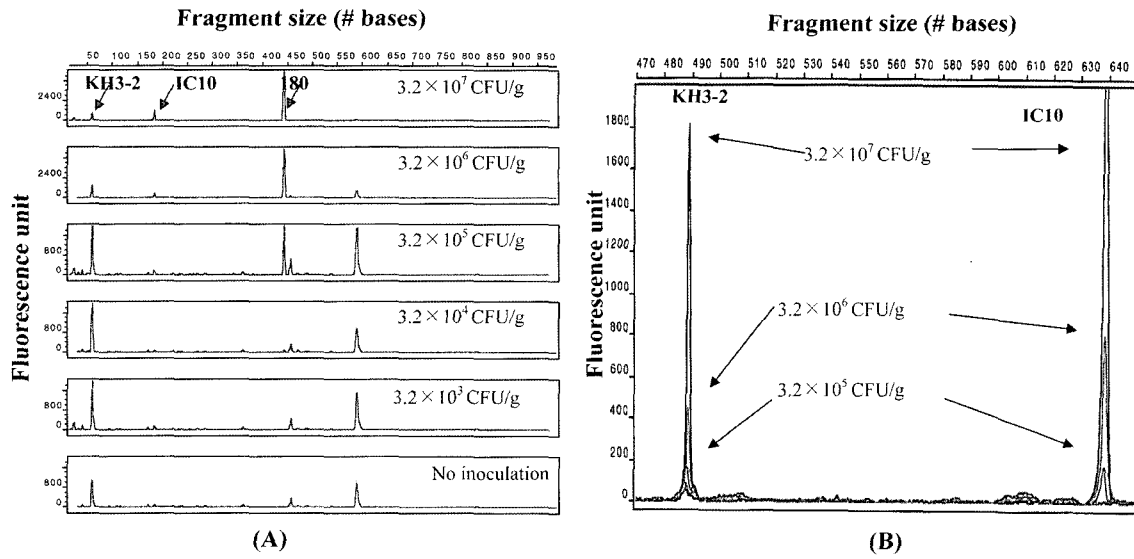


Fig. 2. Electropherogram of T-RFs produced by *HhaI* (A) and *NruI* (B) for the strains KH3-2, IC-10, and 180 inoculated into natural sand (3.2×10^3 to 3.2×10^7 CFU/g; total cell count 2.2×10^9 /g).

strains, and the peak for strain 180 was the highest. The ratio of relative peak area between strains 180 and KH3-2 was 16.8, despite the same number of cells per strain tested.

Detection of Seeded Microorganisms in Environmental Samples

To determine the detection limit of the T-RFLP method and whether it can be used to detect microorganisms added to natural environments, different numbers of cells of the three oil-degrading microorganism strains were inoculated into two types of natural sand. T-RFLP was first conducted,

using the tetrameric restriction enzyme *HhaI*. As shown in Fig. 2A, *HhaI* cut two specific T-RFs, 185 and 442 bases long, corresponding to strain IC10 and strain 180, respectively, in samples in which those microorganisms were added (3.2×10^3 to 3.2×10^7 CFU/g). The T-RF peak for strain KH3-2 could not be specified, due to a non-specific peak present even in the non-inoculated sample (Fig. 2A, bottom panel). However, *NruI*, a restriction enzyme that recognizes six bases, successfully separated the T-RFs corresponding to strains KH3-2 and IC10, from the nonspecific peaks (Fig. 2B). The detection limit of T-RFLP analysis was 1.7×10^7 CFU/g for muddy sand

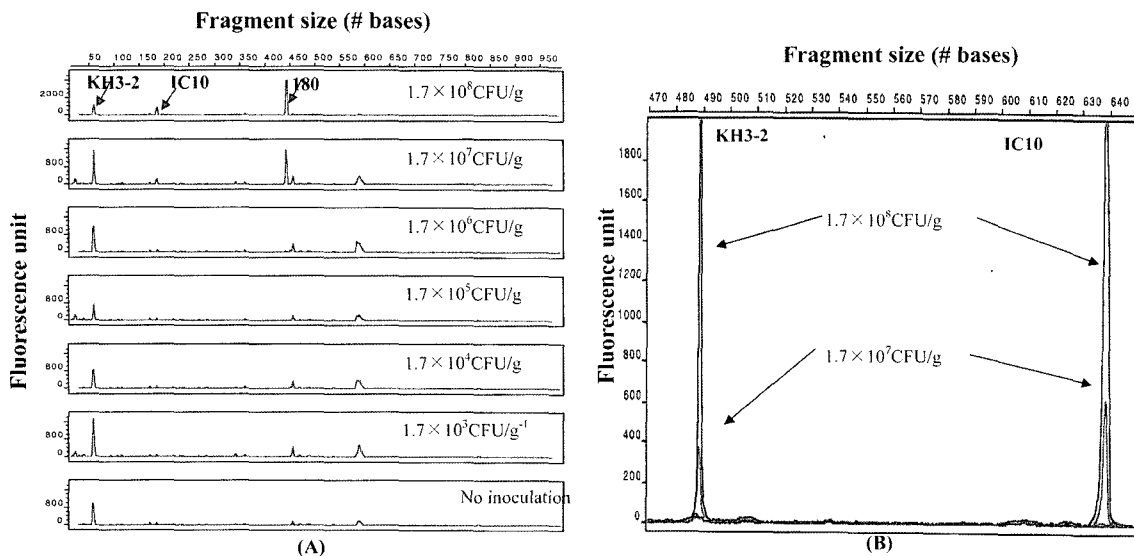


Fig. 3. Electropherogram of T-RFs produced by *HhaI* (A) and *NruI* (B) for the strains KH3-2, IC-10, and 180 inoculated into natural mud (1.7×10^3 to 1.7×10^8 CFU/g; total cell count 1.2×10^{11} /g).

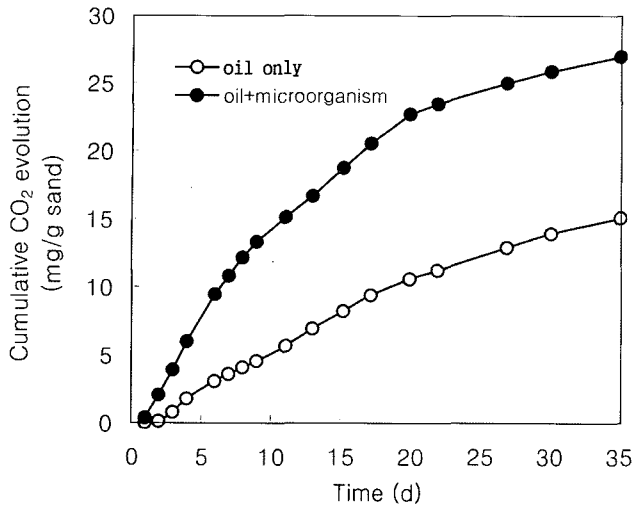


Fig. 4. Production of CO₂ from treated sand (oil plus microbes) or control sand (oil only).

samples, containing 1.7×10^3 to 1.7×10^8 CFU/g each of the three strains (Figs. 3A, 3B). Although the detection limits varied, depending on the cell concentration, they corresponded to about 0.01% of the total microbial count of each microorganism inoculated. Differences in peak sizes among strains also did not affect the sensitivity. These results suggest that the three different microorganisms simultaneously were detected by T-RFLP analysis using two restriction enzymes. Furthermore, the sensitivity of the method depended on the concentration of added microorganisms, regardless of the background concentrations of native microbial cells.

Detection in Oil-Contaminated Sand Microenvironments

We conducted a treatability test to monitor the oil mineralization and survival of the three oil-degrading microorganism strains (IC-10, KH3-2, and 180) in sand microenvironments, artificially contaminated with oil. Significantly more cumulative CO₂ was produced in contaminated sand (oil plus microbe strains) incubated for 35 days than in control sand (oil only) (Fig. 5). T-RFLP, using *HhaI* and *NruI*, detected peaks specific for strains IC10 and 180 even after 35-days of incubation (Fig. 5). Furthermore, the IC10 peak area increased, whereas the 180 peak area decreased, during the incubation period. Strain KH3-2 was detected at 0 and 8 days, but not at 35 days (Fig. 5B).

DISCUSSION

The use of SSU rRNA is common, since the sequence of SSU rDNA can be used to distinguish genera and well-resolved species [1, 28]. The bacterial and eukaryal communities of activated sludge have been profiled by T-RFLP analysis of SSU rDNA by using domain-specific primers [21, 26]. In this study, T-RFLP analysis of SSU rDNA sequences was used to monitor oil-degrading microorganisms that were introduced into natural sand and into sand contaminated with crude oil. A universal primer set, 521F and 1392R, based on SSU rDNA sequences was used to simultaneously detect *in situ* three oil-degrading microbial strains (*Corynebacterium* sp. IC10, *Sphingomonas* sp. KH3-2, and *Yarrowia lipolytica* 180). The SSU rDNAs

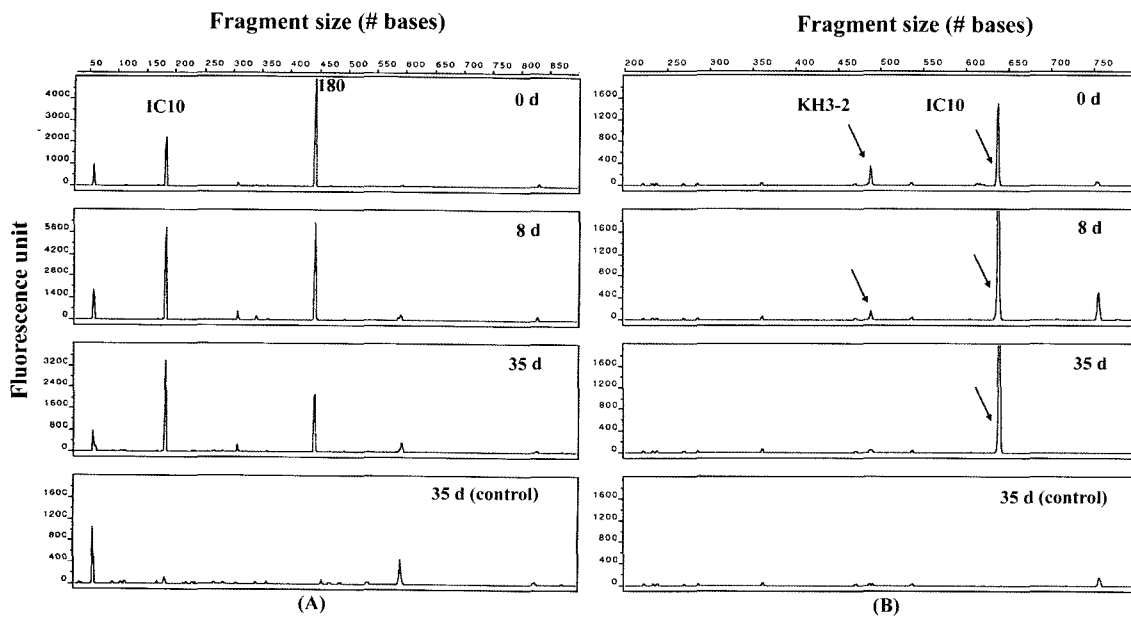


Fig. 5. Electropherogram of T-RFs produced by *HhaI* (A) and *NruI* (B) in treated sand 0, 8, and 35 days after inoculation of oil-degrading microorganisms, and in control sand, which was not inoculated, at 35 days.

derived from each strain were successfully amplified in samples from both a model community (natural sand) and from the same sand inoculated with the strains. This result indicates that the universal primers can be used to analyze bacteria and yeast microbial community structure.

We confirmed that the expected size of T-RFs for the three oil-degrading microbe strains could be realized in experiments, using various restriction enzymes (Table 2). Experimentally observed T-RFs, measured by an automatic sequencer, were within 1 base of the expected length in restriction fragments 37 to 300 bases long, and within 4 bases in fragments 300 to 700 bases long. This variation by fragment size was probably due to differences among electrophoretic runs and heterogeneity in the standard, Rox-2500 [5]. Treatment with *HhaI* yielded co-amplified T-RFs that were 60, 185, and 442 bases long, corresponding to the strains KH3-2, IC-10, and 180, respectively (Table 2 and Fig. 1). The predicted T-RF for strain KH3-2 was the same as that derived from DNA extracted from natural sand. However, T-RFLP analysis, using *NruI*, did isolate a KH3-2 peak; it produced T-RFs of 488 and 637 bases from KH3-2 and IC10 PCR products, respectively (Figs. 2 and 3). Whereas tetrameric restriction enzymes generated T-RFs of many different sizes, and therefore, help to estimate microbial diversity, enzymes specific to six bases may be useful if specific microorganisms do not share conserved restriction sites with other strains in the community [25].

T-RFLP analysis of equivalent amounts of genomic DNA and cell numbers from the three oil-degrading microorganisms in a model community yielded different peak areas (Fig. 1). The peak-area ratios (KH3-2/IC-10/180) were 1.0:2.8:11.6 for genomic DNA and 1.0:2.5:16.8 for cell number. These differences may be due to varied genome size and numbers of rRNA operon copies among the strains [11, 12] or preferential cell lysis or PCR among species [36, 38]. Although the genome sizes and rRNA operon copy numbers are not available for these strains, we assumed that the peak area of strain 180 increased in the model community, because the yeast genome is larger than the bacterial genome.

The detection limit of T-RFLP analysis was up to 0.01% of the total microbial counts for KH3-2, IC-10, and 180 in natural sand, regardless of the concentration of indigenous microbes (Figs. 2 and 3). The actual detection limits were 10^5 to 10^7 CFU/g, which is less sensitive than other methods [39, 43]. However, T-RFLP is useful for comparative analyses of microbial communities, because it provides the percentages of the total microbial community that is occupied by specific microbial groups [8, 21]. Our results indicate that T-RFLP analysis, by using appropriate primer sets and restriction enzymes, would be useful in assessing relative abundance of a specific microbial strain and its changes over time. In the case of unspecified T-RFs, it would be possible to obtain taxonomical information by

concomitant cloning and sequencing of the 16S rDNA clones.

We tested the survival of the three oil-degrading microorganism strains, which were released into an oil-contaminated sand microenvironment (Fig. 5). Because of preferential cell lysis, DNA extraction, and PCR amplification, the proportion of oil-degrading microorganisms released in such circumstances cannot be quantified [35, 36, 38]. However, the relative peak area of *Corynebacterium* sp. IC-10 increased during incubation, whereas that of *Yarrowia lipolytica* 180 decreased. Although the survival of the strains might have been affected by environmental parameters such as temperature, soil water content, and nutrient availability [41], both strain IC10 and 180 survived well, whereas *Sphingomonas* sp. KH3-2 could not colonize the microenvironment.

These results imply that the T-RFLP method is a promising tool for monitoring microorganisms added to or indigenous to natural microbial communities. Furthermore, this method can be used to optimize the microbial inoculation step of the bioaugmentation process and to monitor changes of community structure due to environmental stresses like oil spills.

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