A DNA PROBE SUITABLE FOR MONITORING
GENETICALLY ENGINEERED MICROORGANISMS
IN THE ENVIRONMENT

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(Received April 13, 1992)

A bacterial haloacetate dehalogenase (H-1 type enzyme, EC 3.8.1.3) was
proved to be one of the useful probes for detecting a genetically en-
gineered microorganism (GEM) from environmental samples. A mono-
fluoroacetate assimilating bacterium (Pseudomonas sp.), characteristical-
ly encoding H-1 gene on its plasmid pUO12, was applied to two sorts of
intact paddy soil and was detected by colony hybridization probing 32P-
labeled H-1 DNA fragment (1.6kb). Target bacteria could be quan-
titatively counted by autoradiography with the samples of no less than
10^-3-fold soil dilution while using nutrient agar plates for colonization.
Thus, the detection limit of the target bacteria was regarded as 400 CFU
(colony-forming unit) per gram of dry weight of soil introducing the
principle of most-probable-number method. When the same experiment
was carried out using minimal agar containing monofluoroacetate as a
sole source of carbon, target colonies were clearly detected even in the
10^-1 soil dilution and thus, the detection limit was improved to as low as
4 CFU per gram of dry weight of soil. These results suggested that H-1
gene probe was very valuable for tracing a GEM in the environment
because of its unique nucleotide sequence and high sensitivity.

The outdoor use of novel recombinant organisms may bring many benefits to
industry and agriculture in the near future. However, on introducing genetically
engineered microorganisms (GEMs) into nature, it is most important to trace their
survival and distribution in complex environmental microflora for safety assurance.
Several methods for monitoring GEMs from environmental samples, i.e., selective
media, DNA probes, fluorescent antibodies are recommended, although each of

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them has both advantages and disadvantages (2, 5, 12, 13, 15). Taking it into account that GEMS keep modified gene, probably in characteristic gene, DNA probe method seems to be a most specific technique for detecting GEMs.

In addition to high specificity, the most significant element needed for monitoring technology is high sensitivity. To obtain high sensitivity for DNA probe method, it is necessary to employ a unique nucleotide sequence as a gene marker, since there must be many kinds of sequences derived from indigenous organisms in complex ecosystems like soil. For probing target microorganisms in soil or sediment, already tested markers were TOL and NAH plasmids (17), mercuric reductase gene (mer) (3), kanamycin resistance gene (nptII), a large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase gene (rbcL) (7), and a herbicide 2,4,5-T degrading gene (18). Some of them, however, are broadly distributed in nature; TOL and NAH plasmids were cross-hybridizable (17), and rbcL kept a high degree of homology to a variety of autotrophic organisms (1).

We have searched for the most unique gene suitable for a monitoring marker in nature. Consequently, bacterial haloacetate dehalogenase (H-1) gene was selected, which seemed uniform in nature (8). A haloacetate-assimilating bacterium, Pseudomonas sp. E, harboring H-1 gene on its plasmid pU012 (11), was also selected as a target microorganism. Pseudomonas sp. E/pU012 is capable of assimilating merely biodegradable haloacetate, monofluoroacetate (FA), as a sole carbon source. By utilizing this property, we developed a selective plating method for enumerating Pseudomonas sp. E/pU012 on minimal FA plates (16). Since, much higher sensitivity was required, we have endeavored to improve the method by fully utilizing previous findings.

In the present paper, the feasibility of H-1 DNA as a probe for detecting the target bacteria in soil samples was examined by plate-culturing followed by employing colony hybridization assays. Firstly, the background in the soil microbial community was measured. Secondly, detection limit was determined by a combined enumeration method of plate-counting and the most-probable-number (MPN), and finally, inoculation and recovery test was carried out to confirm the present detection strategy.

MATERIALS AND METHODS

Soil. Two sorts of Japanese paddy soil, named thereafter as Kyoto soil and Kobe soil (16) were used. Their characteristics are shown in Table 1. The preparation of model paddy ecosystems (microcosms) was made with 1 g (dry weight) of each soil as previously described (16).

Strains, culture, and probe preparation. Pseudomonas sp. E/pU012 as a model of GEMS was kindly provided by Dr. Tonomura. Its culture condition in minimal media containing FA as a sole source of carbon was previously described (16). Nutrient agar—0.3% Bacto-beef extract, 0.5% Bacto-peptone, 1.5% agar—was purchased from Difco Laboratories. Probe DNA fragment was prepared from a
subclone of pUO12, named as pBREF1 by Kawasaki et al. (10), in E. coli C600 strain (also a gift from Dr. Tonomura). Plasmid extraction and purification were performed according to the procedure of Birnboim and Doly (4). The subclone of approximately 1.6 kb from EcoRI to SalI site covering almost the whole coding region of H-1 dehalogenase gene was obtained by restriction enzyme treatment and electrophoretic separation.

Colony hybridization. Colonization, lysis, and DNA fixation were performed according to the methods of Hanahan and Meselson (6). Autoclaved nitrocellulose filters (Millipore, HATF) were placed on the surface of the agar media in plastic Petri dishes. Target bacterial cells and/or soil dilutions were spread on nitrocellulose filters covering the agar. After colonization, filters were stripped and treated by alkalis, and denatured DNAs were bound to the filters (6). Prior to hybridization, filters were washed in 3×SSC (1×SSC—0.15 M NaCl, 0.015 M sodium citrate) plus 0.1% SDS (sodium dodecyl sulfate) to remove lysed cell debris. Probe DNA was 32P-labeled by nick translation (14) using the commercially supplied kit (Takara Shuzo). [α-32P]Deoxycitidine triphosphate at 111 TBq (3,000 Ci) per millimole was used as a sole labeling nucleotide. The specific radioactivity of the labeled probe was 2.0×10⁸ cpm/µg. Filters were set in plastic bags, and longer than 2 h of prehybridization was carried out at 65°C in 2 ml per filter of buffer solution: 4×SSC, 10×Denhardt (1×Denhardt—0.02% Ficoll 400, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin) and 100 µg/ml of sonicated calf thymus DNA. Then, sufficient amount (over 10⁵ cpm/filter) of labeled probes was added with new hybridization buffer (1.2 ml/filter), and hybridized with target bacterial DNA by incubation at 65°C for 16 h with a gentle shaking. Unhybridized probe molecules were washed out twice by a large volume of 2×SSC plus 0.1% SDS at 65°C for 20 min with a gentle shaking. Filters were air-dried for 1 h, followed by placing between Whatman 3MM paper and Saran Wrap. Autoradiographs were developed on Kodak SB-5 X-ray films.

Background measurement. Microcosms were suspended by a reciprocating shaker at 200 cycles/min for 30 s. Serial 10-fold dilutions of the soil were prepared with sterile distilled water. In the first experiment, 0.5 ml of each dilution was spread on nitrocellulose filters covering nutrient agar. Indigenous microbial colonies were grown by incubation at 30°C for 24 h. After hybridization, background colonies which keep H-1 gene were screened. In the second experiment, 0.5 ml of soil dilutions were spread on minimal FA agar plates directly. Colonization was performed at 30°C for 48 h. Colonies were replicated on nitrocellulose filters,
and the replicates were set on new minimal FA agar plates. Further incubation was done at 30°C for several hours, and the same background measurement was carried out.

**Detection limit determination.** A definite number of viable *Pseudomonas* sp. E/pU012 cells (0.05 ml), prepared as previously described (16), was mixed with 0.45 ml of a 9-fold dilution of each soil (1 g, dry weight) to give 0.5 ml of the 10-fold diluted mixture of each soil on a plate with or without nitrocellulose filters. Seven series of the 9-fold soil dilution were prepared by 10-fold dilution to a rate of 10⁻³ with sterile distilled water. Each 0.45 ml of them was mixed with the 0.05 ml of target bacterial cell suspension and spread on plates. After plate-culture and hybridization, spots on autoradiograph of target bacterial colonies were enumerated. The detection limit was determined by combination of conventional plate-counting method with the MPN method as previously reported (16); When five replicates of successive three 10-fold bacteria-soil dilutions were prepared, the minimum number estimated from MPN score was determined as the detection limit.

**Inoculation and recovery test.** Various number of viable *Pseudomonas* sp. E/pU012 cells were inoculated in the water phase of the microcosms. The inocula were immediately mixed by shaking and diluted with sterile distilled water. Serial 10-fold dilutions (0.5 ml each) were spread on minimal FA agar plates, and colony hybridization was carried out. Recovery of target bacteria was enumerated in CFU by plate-counting method or by combined method with MPN as described above.

## RESULTS

**Background of H-1 gene in paddy soil**

*Pseudomonas* sp. E/pU012 was colonized under the culture conditions at 30°C for 24 h on nutrient agar and at 30°C for 48 h on minimal FA agar (16), respectively. The background of H-1 gene in both paddy soils was measured under these conditions. No colony hybridizable with 1.6 kb of H-1 DNA probe was detected in indigenous microorganisms on either nutrient or minimal FA agar. This meant that there was no aerobically culturable bacteria encoding H-1 gene in both soils.

**Detection limit of target colonies on nutrient agar**

On nutrient agar, 2.6×10⁷ and 2.3×10⁷ CFU/g of dry weight of soil of indigenous microorganisms from Kyoto and Kobe soil grew respectively under the settled culture conditions. Serial 10-fold dilutions of each soil (1 g, dry weight) containing a definite number of target bacteria were prepared from a rate of 10⁻¹ to 10⁻⁷. Each 0.5 ml of the above dilutions included approximately 10⁶ to 10⁹ CFU of indigenous microorganisms and the definite number of *Pseudomonas* sp. E/pU012. Colony hybridization assays clarified the number of non-target colonies to interfere the detection of target colonies. As shown in Fig. 1, target colonies were quantitatively probed from a rate of 10⁻³ of both Kyoto and Kobe soil dilutions.
Though target spots were observed on each $10^{-2}$ dilution, they were remarkably less in number compared with that of inoculated cells (Table 2). No colony was detected on the $10^{-1}$ soil dilution. These results indicated the detection limit of *Pseudomonas* sp. E/pU012 from paddy soil on nutrient agar was 400 CFU/g of dry weight of soil by the combined method of plate-counting with the MPN.

**Detection limit of target colonies on minimal FA agar**

Even on minimal FA agar, $2.9 \times 10^6$ and $2.3 \times 10^5$ CFU/g of dry weight of soil of background bacteria from Kyoto and Kobe soil grew, respectively (16). Compared with nutrient agar, however, indigenous bacteria decreased in number at about one-tenth for Kyoto soil and about one-hundredth for Kobe soil (16). The same assay as in nutrient agar was performed using minimal FA agar except for some small modifications; Serial 10-fold soil dilutions containing a definite number of target bacteria were prepared to a rate of $10^{-6}$ (for Kyoto soil) or $10^{-5}$ (for Kobe soil), and each 0.5 ml of the above dilutions was plated directly on the medium. Colonies grown on a minimal FA plate were replicated on a nitrocellulose filter and grown again on a new plate.
As shown in Fig. 2 and Table 3, target bacterial colonies were enumerated quantitatively on autoradiographs of every bacteria-soil mixed dilution. Therefore, the detection limit of *Pseudomonas* sp. E/pU012 from paddy soil on selective agar was 4 CFU/g of dry weight of soil.

**Recovery of target bacteria from microcosms**

Target bacteria inoculated into microcosms were extracted immediately. The recovery measured by colony hybridization using minimal FA agar is shown in Table 4. In the extent from $3.7 \times 10^1$ to $3.7 \times 10^5$ of inoculated *Pseudomonas* sp. E/pUO12 cells, almost all target bacterial colonies were detected correctly by conventional plate-counting method or the combined method of plate-counting with the MPN.

**DISCUSSION**

One of the most important elements for detecting specific microorganisms in soil by DNA probe technique is the probe specificity. H-1 gene selected as a probe
DNA Probe for Monitoring GEMs

was isolated from microorganisms grown in a particular environment, i.e. fluorine factory (9). Kawasaki et al. suggested the uniformity of H-1 gene through cross-hybridization assays between H-1 gene and other bacterial dehalogenase genes (8). Although they searched for a DNA sequential homology to H-1 gene in nucleic acid data-base (EMBL and LASL), no homologous sequence was found (Kawasaki, personal communication). In the present study, we investigated the possibility of existence of H-1 gene in indigenous bacteria in two sorts of paddy soil by colony hybridization, but none of them was probed by H-1 DNA fragment. When the same background measurement had been carried out as in the present study in three sorts of field soil, there were no DNAs hybridized with H-1 gene probe (Nakamura, unpublished data). Thus, H-1 gene seems to maintain high specificity to the target bacteria.

The difference of detection limits of target bacteria between nutrient and minimal FA agar seems to arise from different growth condition to non-target bacteria in paddy soil. Since bacterial energy source is rich in nutrient agar, non-specific colonies grow well. High density of non-target bacteria seems to prevent target bacteria from growing sufficiently enough to be detected by labeled DNA probes. The lowest soil dilution rate for enumerating target colonies quantitatively was $10^{-3}$ of 1 g (dry weight) soil. Approximately $10^4$ CFU/plate of indigenous bacteria were developed at that dilution rate. Therefore, the detection limit of target bacteria on nutrient agar is considered 1 per $10^4$ non-target microorganisms. Sayler et al. (17) reported that the detection limit of target colony (*Pseudomonas putida*/TOL) by colony hybridization was 1 per $10^6$ nonhomologous background colonies per plate. However, they employed only a strain of *Escherichia coli* as the background. In the present study, we used a soil suspension for a purpose of investigating detection limits in environmental conditions. Therefore, a variety of indigenous bacterial colonies and soil particles was considered to reduce efficiency for picking up the target colonies.

Table 4. Inoculation and recovery test of *Pseudomonas* sp. E/pUO12 in paddy soil microcosms.

<table>
<thead>
<tr>
<th>Inoculated cell number (g dry wt soil)</th>
<th>Recovered cell number (recovery: %)</th>
<th>Enumeration method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kyoto soil</td>
<td>Kobe soil</td>
</tr>
<tr>
<td>$3.7 \times 10^4$</td>
<td>$4.4 \times 10^4$ (118)</td>
<td>$3.4 \times 10^3$ (92)</td>
</tr>
<tr>
<td>$3.7 \times 10^3$</td>
<td>$3.3 \times 10^3$ (89)</td>
<td>$3.0 \times 10^3$ (81)</td>
</tr>
<tr>
<td>$3.7 \times 10^2$</td>
<td>$3.4 \times 10^2$ (92)</td>
<td>$3.2 \times 10^2$ (86)</td>
</tr>
</tbody>
</table>

\(^a\) "MPN-PC" means combined of most-probable-number with plate-counting: statistical number from comparison MPN score with results of detection of colonies or not.

\(^b\) "PC" means conventional plate-counting method: average number of 5-replicates.

was isolated from microorganisms grown in a particular environment, i.e. fluorine factory (9). Kawasaki et al. suggested the uniformity of H-1 gene through cross-hybridization assays between H-1 gene and other bacterial dehalogenase genes (8). Although they searched for a DNA sequential homology to H-1 gene in nucleic acid data-base (EMBL and LASL), no homologous sequence was found (Kawasaki, personal communication). In the present study, we investigated the possibility of existence of H-1 gene in indigenous bacteria in two sorts of paddy soil by colony hybridization, but none of them was probed by H-1 DNA fragment. When the same background measurement had been carried out as in the present study in three sorts of field soil, there were no DNAs hybridized with H-1 gene probe (Nakamura, unpublished data). Thus, H-1 gene seems to maintain high specificity to the target bacteria.

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However, such a low detection limit as 4 CFU/g of dry weight of soil was attained in the case of minimal FA agar (selective media). On minimal FA agar, non-target colonies were decreased approximately one-tenth to one-hundredth in number, since indigenous bacteria could not utilize FA as an energy source while
Pseudomonas sp. E/pUO12 could. As a result, target bacteria grew more efficiently than nonhomologous microorganisms. Thus, it became possible to detect the target bacterial colonies by DNA-DNA hybridization at such a high sensitivity. Normal plate-counting method is usually applied to a plate where between 30 and 300 colonies appear. Reliable data will not be obtained by counting colonies beyond this extent. We employed a sensitive enumeration method, i.e. a combination method of plate-counting and MPN. The results of inoculation and recovery tests proved the usefulness of our procedures.

The strategy employed here, however, will be limitedly used for tracking certain specific bacteria which are culturable on plates and also a unique DNA probe like H-1 gene will be necessary to attain such a high specificity and high sensitivity for detecting GEMs from environmental samples.

This study was performed as a special project of the Ministry of International Trade and Industry, “Study and research on safety measures of bioindustry (Examinations on safety securing measures for the utilization of recombinant under natural environment)”. We thank Dr. Kenzo Tonomura, University of Osaka Prefecture, for kindly supplying bacterial copies.

REFERENCES

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