A SELECTIVE PLATING METHOD TO ENUMERATE TARGET MICROORGANISMS IN AN ENVIRONMENT

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A sensitive method using selective plating media to detect a genetically engineered microorganism (GEM) released into the environment was developed. In experiments, a strain of Pseudomonas sp. capable of assimilating monofluoroacetate (FA) as a sole carbon source was chosen as the GEM model. Two sorts of Japanese paddy soil were used as a microcosm. In a trial when serial 10-fold soil dilutions were spread on minimal FA agar plates and incubated, native oligotrophic microorganisms (10^5–10^6 CFU/g of dry weight of soil) grew on the plates. So, it was difficult to pick out a few populations of target bacteria from such a high number of background microbial communities. A further examination showed that those indigenous microorganisms colonized even on plates containing only water and agar. For this reason, we tested silica-gel instead of agar as an agent to solidify the selective media. On minimal FA silica-gel plates, the number of indigenous oligotrophic colonies was greatly decreased. When a definite number of target Pseudomonas cells was mixed with the soil dilutions and cultured on minimal FA silica-gel plates, only the target colonies were appeared. We applied the most-probable-number technique to the plate-counting method to attain as low as 4 CFU/g of dry weight of soil as the statistical limit of detecting FA-assimilating bacteria. This selective plating method is suitable for environmental monitoring of GEMs which assimilate FA aerobically, as it is on highly sensitive, specific and feasible.

Much concern about intentional release of genetically engineered microorganisms (GEMs) into natural environments have been expressed in this decade, particularly about introducing a foreign species into a new environment. A major

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problem is how to trace the fate of the GEMs. Various techniques for monitoring GEMs in nature have been suggested \((1, 2, 9, 11, 12)\). When the target organisms are culturable on plating media, traditional plate-counting methods are advantageous, being easy to perform, sensitive and inexpensive.

But certain metabolic markers are essential in the organism to be detected on an appropriate plating medium. Bacterial resistance to antibiotics or heavy metal ions is usually used as a detection marker, but these drug-resistant phenotypes, especially antibiotic-resistant ones, are commonly observed in various environmental microflora. Therefore, the selective detection of the target bacterium is very hard in complex ecosystems. Moreover, it may be undesirable to release such drug-resistant organisms into the nature because of the difficulty in controlling their survival. Another plating method depends upon the microbial ability to assimilate some organic compound as a sole source of energy. Particularly, degradation of environmental pollutants or toxic chemicals is a good marker, since no hazardous accident would happen if such degrading genes were broadly dispersed in environments. Organic compounds which can be used as assimilating markers, for instance, \(n\)-alkanes, toluene, xylene, naphthalene and so on, are usually degraded by plasmid-mediated oxidation of the microorganisms \((3)\). Although the bacterial degradation organisms are well known, those properties may not always be unique markers. Besides most organic compounds biodegraded by bacteria are hydrophobic, hence it is troublesome to prepare selective plating media.

In a trial to establish a more specific and feasible selective plating method to detect a GEM from soil, we focused upon bacterial dehalogenation of haloacetate. Monofluoroacetate (FA) has a chemically stable bond between carbon and fluorine, so that it is rarely biodegraded. A strain of \(Moraxella\) sp. isolated from a fluorine factory assimilates FA as a single source of carbon \((6)\). This bacterium harbors plasmid pU01 which encodes two types of haloacetate dehalogenase (EC 3.8.1.3), H-1 and H-2 \((6)\). The H-1 enzyme acts preferably on FA; the H-2 enzyme does not \((6)\). The H-1 gene appears to be uniform in nature \((5)\). In addition, FA is soluble in water, making selective plates easy to prepare. So we chose FA-assimilating \(Pseudomonas\) sp. E harboring plasmid pU012, one of the deletion mutants of pU01, encoding only H-1 gene \((8)\), as a model of GEMs. We investigated the detection limit of the target bacteria in two kinds of paddy soil using the minimal FA plating medium.

**MATERIALS AND METHODS**

**Bacterial strain and media.** A monofluoroacetate (FA)-assimilating bacterial strain, \(Pseudomonas\) sp. E/pU012, was kindly supplied by Dr. Tonomura, University of Osaka Prefecture. The standard medium for culturing this bacterium, made according to the procedure of Kawasaki et al. \((6)\), containing 0.3% \(K_2HPO_4\), 0.1% \(KH_2PO_4\), 0.1% \((NH_4)_2SO_4\), 0.01% \(MgSO_4\cdot7H_2O\), and 0.2% FA as sodium monofluoroacetate (named minimal FA medium), purchased from Wako Pure
Chemical. Plating minimal FA medium was solidified with 1.5% purified agar (Difco Laboratories) or 90% silica-gel. The silica-gel plates were prepared by the methods of Kanagawa et al. (4). Nine hundred ml of colloidal silica (Snowtex 20, Nissan Chemical) was autoclaved at 120°C for 10 min and chilled to room temperature. The pH of the sterile silica was adjusted to 7.3 with 1.7N HCl. Stock solutions of FA and other inorganic trace elements, sterilized by filtration, were poured into the silica. Then autoclaved distilled water was added to a final volume of 1000 ml. The colloidal solution was dispensed into 25 ml per plastic Petri dishes.

**Soil.** Paddy soil delivered from the Experimental Farm Stations of Kyoto University and Kobe University, named Kyoto soil and Kobe soil respectively, was used. Their physical and chemical characteristics will be described elsewhere (Itoh et al., manuscript in preparation). A model paddy ecosystem, a microcosm, was made with 1 g dry weight of each soil and 1.5 ml of sterile distilled water in a centrifugal tube. It was preincubated at 25°C for a week in the dark.

**Background measurement.** Tubes with soil and water were shaken on a reciprocating shaker at 200 cycles/min for 30 s. Serial 10-fold dilutions of the soil suspension were prepared with sterile distilled water. One-tenth ml of each dilution was spread on minimal FA plates, then the plates were incubated at 30°C for 48 h. The background microorganisms of each soil were calculated by counting colonies.

**Detection limit determination.** *Pseudomonas* sp. E/pUO12 was liquid-cultured in 5 ml of minimal FA medium with shaking at 30°C for 48 h (until the final period of the logarithmic phase). Cells were harvested by centrifugation and washed three times with sterile distilled water. Serial 10-fold dilutions of the cell suspension were also made with sterile distilled water. A portion (0.05 ml) of a dilution containing a definite number of viable *Pseudomonas* sp. E/pUO12 cells was mixed with 0.45 ml of a 9-fold dilution of each soil (1 g, dry weight) to obtain 0.5 ml of a 10-fold dilution of each soil suspension. The mixtures were spread on minimal FA silica-gel plates and incubated under the same conditions described above. Target bacterial colonies were observed, and photographs were taken.

**Inoculation and recovery test.** Various numbers of viable *Pseudomonas* sp. E/pUO12 cells were inoculated in the water phase of each microcosm. The inocula were immediately shaken and diluted with sterile distilled water. Serial 10-fold dilutions (0.5 ml each) were spread on minimal FA silica-gel plates. Target colonies were enumerated by the normal plate-counting method or by a combination of plate-counting method with the most-probable-number (MPN) method after incubation as described above. Recovery of each inoculum was calculated.

**RESULTS AND DISCUSSION**

Almost all *Pseudomonas* sp. E/pUO12 were colonized on a minimal FA plate after incubation at 30°C for 48 h (Table 1). Thus, we decided to enumerate the target colonies under that plating-culture condition. At the beginning of the study, as a selective medium we used a minimal agar plate containing 0.2% FA as the
carbon source. Under the settled culture condition, however, many background microorganisms of both paddy soils were colonized; $2.9 \times 10^6$ CFU/g of dry weight of Kyoto soil and $2.2 \times 10^5$ CFU/g of dry weight of Kobe soil were detected (refer to Fig. 2). Therefore, it was difficult to distinguish a small population of target bacteria from such high population of background microorganisms.

The indigenous microorganisms which can grow on such a poor medium seem to be oligotrophs. They can develop at very low concentration of organic matters (10). Thus, we had to examine other medium conditions to decrease these oligotrophs.

Since FA is an inhibitor of the TCA (tricarboxylic acid) cycle, a major metabolic pathway of carbon in every organism, a high concentration of FA in the minimal agar may be critical to the indigenous oligotrophs. Thus, the FA concentration in the minimal agar was increased from 0% to 1.5% to examine the growth of target and non-target bacteria. As Fig. 1 shows, FA-assimilating \textit{Pseudomonas} sp. E/pUO12 colonized so long as the concentration of FA in the medium did not exceed 1.0%.
The colony forming unit of the bacteria on 1.0% FA medium was one-tenth of that on standard 0.2% FA medium, and no *Pseudomonas* sp. E/pO12 grew at 1.5% FA. Figure 2 shows the indigenous bacterial growth in each soil on minimal agar plates containing 0-1.0% FA. A substantial number of the background oligotrophs appeared even at the highest concentration of FA. This means that a high-concentration of FA does not effectively reduce the background microorganisms without interfering with the target bacterial growth. Also as shown in Fig. 2, the indigenous microorganisms grew well on 0% FA medium containing only minerals, water and agar. In conclusion, it appears to be impossible to enumerate correctly the target bacteria at concentrations less than $10^4$ CFU/g-soil so long as agar plates are used.

Since agar is a polysaccharide, it may be the carbon source for some of microorganisms. Moreover, trace amounts of organic substances present in agar as an impurity might support the growth of some bacteria. Thus, in place of agar, we tried silica-gel to solidify minimal FA medium. Silica-gel contains no organic materials, so it must not be an energy source for bacterial development, except for FA.

As shown in Table 2, *Pseudomonas* sp. E/pUO12 colonized on a minimal 0.2% FA silica-gel plate as well as it did on an agar plate. But Table 2 also shows that the background colony numbers of indigenous oligotrophs in each paddy soil were dramatically decreased on the silica-gel plates (below 20 colonies per 0.1 ml of 10-fold soil dilution). Therefore, even a small population of FA-assimilating bacteria was countable in indigenous microbial community when the minimal FA silica-gel medium was used. To prove this, a small number of *Pseudomonas* sp. E/pUO12

![Graphic](image.png)
were cultured on the silica-gel plate with 0.5 ml of a 10-fold soil dilution (1 g, dry weight). As shown in Fig. 3, the target colonies were distinguished from other microorganisms and soil particles.

The detection limit of FA-assimilating bacteria on the silica-gel FA can be improved by using the MPN technique. This is usually used to enumerate a small number of bacteria in sequential liquid culture. The target microbial population is estimated by the pattern of positive/negative bacterial growth. We applied the principle of the MPN enumeration method to plating cultures. When five replicates of three successive 10-fold dilutions (10, 10^2, 10^3) of 1 g (dry weight) of soil were made and 0.5 ml each of the dilutions was plating-cultured on the selective medium, the statistical minimal number according to MPN score would be 4 cells.

To confirm our detection procedure, inoculation and recovery tests were performed. In the range from 4.9 x 10 to 4.5 x 10^6 inoculation of Pseudomonas sp. E/pUO12 cells, almost 100% of the target colonies were enumerated by the conventional plate-counting method or the MPN method combined with plate-counting (Table 3).

In this way, minimal FA selective medium has shown high sensitivity and specificity to FA-assimilating bacteria from soil samples under the optimized culture conditions. As Dr. Tonomura and his co-workers reported (6–8), the H-1 type haloacetate dehalogenase gene was well expressed in Moraxella sp., Escherichia coli and some strains of Pseudomonas. Therefore, the H-1 gene seems to be applicable to some typical GEMs for environmental use as a detection marker. For example, in the deliberate release of engineered Pseudomonas spp. for crop protection or bioremediation, if the GEM keeps the glycolate-assimilating capability, the target bacteria will be easily detectable from environmental samples by introducing the H-1 gene into the microorganism in advance. The selective plating method, the classical method for counting bacteria, has several limitations, e.g., the target microorganisms must be culturable, only phenotypic markers are utilized, and so on. However, as suggested in this report, this technique will become a practical tool for detecting a small population of GEMs in the environment, if a unique
Fig. 3. Colonies of *Pseudomonas* sp. E/pUO12 on minimal 0.2% FA silica-gel plates.
a: pure culture,  b: pure culture with 0.5 ml of a 10-fold dilution of Kyoto soil, c: pure culture with 0.5 ml of a 10-fold dilution of Kobe soil. Arrows show target colonies.

Table 3. 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>4.9 × 10^1</td>
<td>4.6 × 10^1 (94)</td>
<td>4.6 × 10^1 (94)</td>
</tr>
<tr>
<td>4.9 × 10^2</td>
<td>3.8 × 10^2 (78)</td>
<td>3.9 × 10^2 (80)</td>
</tr>
<tr>
<td>9.0 × 10^4</td>
<td>9.0 × 10^4 (100)</td>
<td>9.7 × 10^4 (108)</td>
</tr>
<tr>
<td>4.5 × 10^6</td>
<td>3.0 × 10^6 (67)</td>
<td>3.0 × 10^6 (96)</td>
</tr>
</tbody>
</table>

^a^ “MPN-PC” means the combined method of the most-probable-number with plate-counting: statistical number from comparison MPN score with results of bacterial colonization or not on minimal FA silica-gel plates.

^b^ “PC” means conventional plate-counting method: average number of 5-replicate plates.
marker like FA-assimilation and an appropriate selective medium are used.

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REFERENCES