Assay for Detection and Enumeration of Genetically Engineered Microorganisms Which Is Based on the Activity of a Deregulated 2,4-Dichlorophenoxyacetate Monooxygenase

R. J. KING,1* K. A. SHORT,2† AND R. J. SEIDLER2
NSI Technology Services Corp.1 and Environmental Research Laboratory,2 U.S. Environmental Protection Agency, 200 S.W. 35th Street, Corvallis, Oregon 97333

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An assay system was developed for the enumeration of genetically engineered microorganisms expressing a deregulated 2,4-dichlorophenoxyacetate (TDF) monooxygenase, which converts phenoxyacetate (PAA) to phenol. In PAA-amended cultures of Pseudomonas aeruginosa PAO1C(pRO103) and Pseudomonas putida PPO301(pRO103), strains which express a deregulated TDF monooxygenase, phenol production was proportional to cell number. Phenol was reacted, under specific conditions, with a 4-aminoantipyrine dye to form an intensely colored dye-phenol complex (AAPPC), which when measured spectrophotometrically could detect as few as 106 cells per ml. This assay was corroborated by monitoring the disappearance of PAA and the accumulation of phenol by high-performance liquid chromatography and gas chromatography. The AAPPC assay was modified for use with plate cultures and clearly distinguished colonies of PPO301(pRO103) and PAO1C(pRO103) from a strain expressing a regulated TDF monooxygenase. Colonies of P. putida PPO301(pRO101) remained cream colored, while colonies of PPO301(pRO103) and PAO1C(pRO103) turned a distinct red.

The use of genetically engineered microorganisms (GEMs) in the environment is accompanied by the need for methods to accurately and economically detect them and track their fate. Incorporation of antibiotic resistance markers may not be suitable because of their general inability to maintain marker expression (7, 15). Also of concern are the long-term fate and the potential for dissemination in ecosystems of antibiotic resistant phenotypes (9, 10).

The lacZY (3) and xylE (12, 19) systems are two gene marker systems described for the enumeration of GEMs from environmental samples. The lacZY system relies on lactose cleavage and generally incorporates an antibiotic to increase sensitivity. The xylE system plasmid constructs are often unstable or necessitate concentrating cells on filters to increase sensitivity.

The uncertainty over the ability to culture target organisms from environmental samples favors direct detection methods. The method we have developed allows for direct enumeration of GEMs. It is based on the activity of a deregulated 2,4-dichlorophenoxyacetate (TDF) monooxygenase, expressed by bacterial strains harboring plasmid pRO103 (8, 14). The deregulated TDF monooxygenase converts phenoxyacetate (PAA) to phenol in the absence of the inducers TDF and 3-chlorobenzoate (8). Phenol can be measured by direct UV spectrophotometry, high-performance liquid chromatography (HPLC), or gas chromatography (GC) analysis. The limit of detection for each of these methods was increased by reacting phenol with 4-aminoantipyrine to produce an intensely colored leuco dye (6).

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and plasmids used in this study are described in Table 1. Frozen stock cultures of PPO301(pRO103), PPO301 (pRO101), and PAO1C(pRO103) were cultured at 30°C with shaking at 200 rpm on brain heart infusion broth (BHI; Difco Laboratories, Detroit, Mich.) and supplemented with Hg (25 μg/ml) as HgCl2 until an optical density at 660 nm of approximately 1.0 was obtained.

For spread plate assays or plate counts, 100 μl of the appropriate serial dilutions was added, and the plates were incubated for 24 h at 30°C. For liquid culture assays, 500 μg of PAA per ml was added to bacterial cultures prepared in triplicate that were serially diluted with 10 mM phosphate buffer, pH 7, and the cultures were shaken at room temperature for 6 h at 200 rpm to allow time for enzymatic conversion of PAA to phenol.

Description of colorimetric assay. Condensation of 4-aminoantipyrine with a phenol in the presence of alkaline oxidizing agents produces a red antipyrine dye (AAPPC) in what is known as the Emerson reaction (4, 5). This reaction is more sensitive than other color reactions of phenols (16). Color development occurs within 2 min and in aqueous solutions is most sensitive in the range of 0.2 to 2 μg/ml. The color changes with time but not significantly within an hour (11). Factors influencing the assay are the concentration and ratio of reagents and phenol, the order in which the reagents are added, the concentration of reagents relative to the phenol concentration (6, 17), and the buffering capacity of the reaction mix, which should maintain an optimal pH range of 7.5 to 9.5 (17). It is also necessary to have at least a 10-fold excess of 4-aminoantipyrine and the oxidizing agent relative to phenol. However, too great an excess can result in a reaction of the 4-aminoantipyrine and the oxidizing reagent (17). For this reason, a blank should be run with any series of determinations by mixing 4-aminoantipyrine, buffer, and
the oxidizing agent in the absence of sample. The reagents and phenol standards were prepared just prior to their use. The reagents, in the order in which they were added to a phenol solution, were pH 10.0 buffer solution (3.09 g of boric acid-3.73 g of potassium chloride-44 ml of 1 N sodium hydroxide brought to 1 liter in distilled water), 2% 4-aminopyridine solution in water, and 8% potassium ferricyanide in water (18).

**Colorimetric assay spray technique.** Colonies of PPO301(pRO101), PPO301(pRO103), and PAO1C(pRO103) were wetted with a 1% solution of PAA in distilled water with a thin-layer chromatography sprayer. The colonies were incubated for 1 h to allow for conversion of PAA to phenol and for evaporation of excess surface fluids. Subsequently, the colonies were sprayed with an 8% potassium ferricyanide solution, again until the surface medium was just wet. The plate was allowed to air dry prior to being sprayed with a solution of pH 10.0 buffer solution, 2% 4-aminopyridine, and 8% potassium ferricyanide solution, in water (National Institute of Standards and Technology certified) to 900 µl in distilled water. The plate was maintained at 210°C, and the A442 was determined in triplicate against a blank for each replicate with a DU-8A spectrophotometer (Beckman Instruments Inc., Fullerton, Calif.).

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**UV spectral assay of phenol.** Samples from liquid cultures were prepared as described above, and the A_{308} was determined in triplicate against a blank for each replicate by using a Beckman DU-8A spectrophotometer.

**HPLC analysis of PAA and phenol.** Samples were analyzed with an HP-1090 HPLC (Hewlett-Packard Co., Avondale, Pa.) coupled to a diode-array UV-Vis detector and monitored for 268-nm (λ_{max} for PAA), 272-nm (λ_{max} for phenol), and 254-nm peaks. Samples (3 μl) analyzed in triplicate for each replicate were separated by an isocratic mobile phase (35% H₂O with 0.1% CH₃COOH-65% CH₃OH) on a C₁₈ (octadecylsilane) reverse-phase 5-μm microbore column (100 by 2.1 mm) with a C₆ (methyl octyl silane) guard column at a flow rate of 0.40 ml min⁻¹. External PAA and phenol reference standards (National Institute of Standards and Technology certified) were incorporated to bracket sample concentrations and injected in duplicate for recalibration of the standard curve every 10th sample injection. The limits of detection were 130 ng for PAA and 100 ng for monohydric phenol.

**GC analysis of phenol.** Samples were analyzed on a Perkin-Elmer Sigma 2000 GC with autosampler and flame ionization detector (Perkin-Elmer Co., Norwalk, Conn.). The column was a DB-1701 (60 m by 0.25 mm [inside diameter]) (J & W Scientific, Folsom, Calif.). The helium carrier gas column head pressure was set at 18.3 lb/in². Flows were 20 ml min⁻¹ for hydrogen and 225 ml min⁻¹ for air. The oven temperature program was initially set at 100°C, raised 10°C min⁻¹ to 210°C, and maintained for 4 min. The temperature was set at 250°C for the injector and at 260°C for the detector. A splitless open-liner configuration was used with a 3-μl injection volume. Each of three sample replicates was analyzed in triplicate. Sample quantitation was based on a method of multilevel external standards. Phenol reference standards (National Institute of Standards and Technology certified) were incorporated to bracket sample concentrations and injected in duplicate for recalibration of the standard curve every 10th sample injection. The limit of detection for phenol was 60 pg.

## RESULTS AND DISCUSSION

Bacterial colonies of cells expressing the deregulated TFD monooxygenase could be differentiated visually from colonies of cells expressing a regulated TFD monooxygenase by using the colorimetric assay spray technique. Colonies of PAO1C(pRO103) and PPO301(pRO103), strains which express the deregulated enzyme, turned red after being subjected to the AAPPC assay. Colonies of PPO301(pRO101), a strain expressing the regulated TFD monooxygenase, remained cream colored after identical treatment. A modification of the assay, for use with liquid samples, was used to quantitate cells in liquid suspension. The results of applying this assay to serial dilutions of PAO1C(pRO103) and PPO301(pRO103) showed that the assay is sensitive enough to quantify as few as 10² PAO1C(pRO103) cells per ml (Table 2). PPO301(pRO103) could not be quantified below 10⁷ cells per ml (Table 2), presumably because this bacterium can metabolize phenol and phenol transiently accumulated, min-
TABLE 2. Results of spectrophotometry, HPLC, GC, and AAPPC assays

<table>
<thead>
<tr>
<th>Assay</th>
<th>Strain</th>
<th>Sensitivity (range) (cells/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV spectrophotometry</td>
<td>PA01C(pRO103)</td>
<td>10^6 (10^5–10^6)</td>
</tr>
<tr>
<td>HPLC*</td>
<td>PA01C(pRO103)</td>
<td>10^6 (10^5–10^6)</td>
</tr>
<tr>
<td>GC*</td>
<td>PA01C(pRO103)</td>
<td>10^6 (10^5–10^6)</td>
</tr>
<tr>
<td>AAPPC</td>
<td>PA01C(pRO103)</td>
<td>10^3 (10^2–10^3)</td>
</tr>
<tr>
<td></td>
<td>PPO301(pRO103)</td>
<td>10^3 (10^2–10^3)</td>
</tr>
</tbody>
</table>

* Limit of detection for phenol was 100 ng.
* Limit of detection for phenol was 60 pg.

imizing the sensitivity of this GEM. PA01C(pRO103), however, cannot metabolize phenol, and >300 μg of phenol per ml accumulated in the medium which had been initially supplemented with 500 μg of PAA per ml (data not shown). Thus, in order to achieve maximum sensitivity, the GEM should be deficient in metabolizing phenol. The disappear-
ance of PAA was stoichiometric with the accumulation of phenol for PA01C(pRO103). Therefore, the ability to inde-
pendently measure PAA and phenol by GC and/or HPLC allows for corroboration of the AAPPC assay and is another strength of this gene tagger system. PAA is also recalcitrant to microbial degradation (13), so the specificity of this assay for the use of environmental samples should be better than the specificity of the lacZY and xylE gene tagger assay systems. The application of the tfdA monooxygenase gene tagger AAPPC system to environmental samples will be the subject of future research efforts.

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