APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1983, p. 706–710
0099-2240/83/020706-05$02.00/0

Vol. 45, No. 2

# Replica Plating Method for Estimating Phenanthrene-Utilizing and Phenanthrene-Cometabolizing Microorganisms†

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Received 4 June 1982/Accepted 26 October 1982

A replica plating method was developed for detecting and enumerating phenanthrene-degrading microorganisms. The method is designed to discriminate between aquatic organisms that utilize phenanthrene as the sole carbon and energy source and organisms that cometabolize phenanthrene. The method was used to demonstrate that phenanthrene utilizers and phenanthrene cometabolizers coexist in estuarine sediments.

Cometabolism, the subject of several reviews and critiques (1, 13, 15, 20), is generally regarded as a potentially important mechanism of pollutant degradation in the environment. One class of persistent pollutants consists of the polynuclear aromatic hydrocarbons, some of which are toxic and mutagenic (2, 27). Reports of incomplete oxidation of polynuclear aromatic hydrocarbons by microorganisms (3, 8, 10) suggest that these hydrocarbons may be degraded in natural ecosystems by cometabolic processes. Indeed, a number of hydrocarbons are degraded by cometabolic processes under laboratory conditions (1, 4, 7, 9, 14, 21). Only one report, to our knowledge, provides direct evidence of cometabolism occurring in the environment; Jacobson et al. (16) has found evidence that four herbicides are degraded in sewage sludge by the cometabolic action of a sewage microbial community.

To address the question of cometabolism in natural populations, it is desirable to determine whether the ability to cometabolize aromatic hydrocarbons is prevalent in a population. Zones of clearing on agar plates have been used to detect microorganisms that degrade environmental pollutants such as alkyl benzene sulfonate (19), monochlorobiphenyl (25), and phenanthrene (18). In this work, we describe an agar plate method which, when combined with replica plating, can be used to screen large numbers of microorganisms rapidly and to distinguish organisms which in the environment are potential phenanthrene utilizers and phenanthrene cometabolizers.

**Sampling and initial plating.** Triplicate Van Veen grabs of estuarine sediments were taken from three Chesapeake Bay sites: the harbor at Solomons, Md., 25 m downstream from a marina gasoline pump; the Patuxent River, at the research pier of the Chesapeake Biological Laboratory at Solomons, Md.; and Baltimore Harbor, near an oil refinery. With a sterile spatula, the surface (1 cm) of undisturbed sediment in the center of the sample (ca. 150 g [wet weight]) was collected into glass bottles with aluminum foil-lined screw caps. All plating was performed within 20 min of sampling. Samples were diluted serially in filter-sterilized estuarine water and plated (10 plates per dilution) on the surface of estuarine salt water agar (ESWA) plates (24). The medium contained 0.05 g of sodium glycerophosphate, 0.5 ml of glycerol, 0.1 g of proteose peptone, 0.1 g of yeast extract, 15 g of agar, and 1 liter of aged estuarine water. ESWA was chosen because it produced numbers of colonies comparable to those produced by other commonly employed marine media. ESWA also contains relatively low concentrations of organic carbon, thereby promoting the development of small, nonspreading colonies (<1- to 2-mm diameter) suitable for replica plating.

**Replica plating.** Plates were incubated at 25°C. The colonies on five of the plates were counted after 2 weeks to determine total plate counts. The remaining five plates were replicated onto several agar media which contained phenanthrene (PA agar). PA agar plates were prepared by spreading phenanthrene onto the surface of prepoured, air-dried plates. The agar medium consisted of an estuarine salts solution (ESS) (26), 1.8% (wt/vol) purified agar (Difco Labora-

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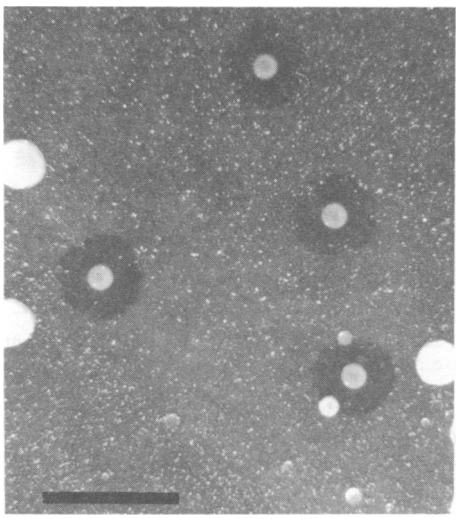

FIG. 1. Zones of clearing on PA agar replica plate inoculated from a plate containing ESWA. Bar, 5 mm.

tories, Detroit, Mich.), and potential growth substrates at 0.1 g liter$^{-1}$; to prevent precipitate formation, phosphate salts were autoclaved separately and added aseptically before pouring plates. Plates were allowed to dry overnight. Phenanthrene was distributed evenly as a thin, visible overlay by pipetting 0.2 ml of a filter-sterilized acetone solution (5 g of phenanthrene liter$^{-1}$) onto the agar surface while plates were spinning on a turntable. Plates were dried overnight at 30°C to allow the acetone to evaporate.

Each ESWA plate was replicated onto four PA agar plates. One contained no organic carbon other than phenanthrene. The other three media contained phenanthrene plus D-glucose, sodium benzoate, or nondetergent motor oil and kerosene (1:1 [vol/vol]). To achieve a homogeneous and fairly clear emulsion, sterile PA agar medium with oil and kerosene was mixed in a blender before pouring plates. By filling the blender jar to the top and eliminating headspace, air bubble formation in the medium was avoided. Plates were incubated at 25°C and examined daily for 2 weeks for zones of phenanthrene-clearing. Each phenanthrene-clearing colony was marked so the ability to degrade phenanthrene on each medium could be recorded. Organisms able to degrade phenanthrene without an additional growth source were scored as phenanthrene utilizers. Organisms which did not degrade phenanthrene unless an additional growth source was present were scored as phenanthrene cometabolizers.

Zones of phenanthrene-clearing were easily discerned on PA agar plates (Fig. 1). Phenanthrene degradation on replica plates was first observed after 2 or 3 days of incubation. The majority of phenanthrene degraders produced zones of clearing within 5 days. To ensure maximum counts, plates were monitored daily for 2 weeks, after which colonies were selected for isolation. After 2 weeks, large colony size and spreading prohibited accurate counts, and zones of clearing were no longer discernible as the phenanthrene overlay became thin by sublimation or volatilization.

The effect of the phenanthrene overlay on total plate counts was determined by comparing sediment counts on three media: ESWA, ESWA with a phenanthrene overlay, and EWSA with an acetone overlay. No significant differences in total plate counts among the media were detected after incubation periods of 1 or 2 weeks. Each PA agar plate contained approximately 1 mg of phenanthrene. Apparently, growth of estuarine microorganisms is not markedly affected by relatively high levels of phenanthrene, which tends to persist in aquatic systems (12). This is consistent with a report that 100 ppb of phenanthrene (100 ng/ml) has no effect on the heterotrophic activity of freshwater bacterial populations (22).

**Comparison with cultures in liquid medium.** Seventy-five phenanthrene degraders were streaked onto ESWA plates for isolation and maintained on slants of ESWA plus phenanthrene. To confirm that zones of clearing on replica PA agar plates indicated phenanthrene degradation, five isolates were chosen for further examination. All were gram-negative rods, but each had a different cellular morphology, was isolated from a different site, or appeared to cometabolize phenanthrene when incubated with a different growth substrate (Table 1).

Isolates were grown in estuarine salt water broth and centrifuged at 1,000 × *g*. The cell pellet was suspended in sterile ESS and washed twice in ESS. Washed cells were suspended in ESS to a final concentration of approximately $10^6$ cells ml$^{-1}$. The suspension was used to inoculate ESS broth with or without one of the growth substrates. Broth (50 ml) in flask cultures was the same as for plates, but without agar. An acetone solution of phenanthrene was added to the broth to give a final concentration of 50 mg liter$^{-1}$. Flasks were prepared in triplicate and incubated at 25°C. Some cultures contained emulsions which interfered with estimation of growth by determining optical density. Therefore, growth was estimated visually (see Table 2). After 2 weeks, the contents of each flask were extracted with 2 100-ml volumes of ethyl acetate by shaking for 1 h on a wrist-action shaker. The two extracts were combined and dried over anhydrous $Na_2SO_4$ and condensed to approximately 10 ml in a Kuderna-Danish concentrator (Kontes Co., Vineland, N.J.). Further concentration to 1.0 ml was accomplished by

TABLE 1. Characterization and identification of five isolates from Chesapeake Bay sediments

| Isolate | Origin of sediment | Date sampled (mo/yr) | Description | Identification |
|---|---|---|---|---|
| SPE18 | Patuxent River | 12/79 | Cocco-bacillary shaped, nonmotile, oxidase negative, catalase positive, ferments glucose, facultative anaerobe | Unidentified |
| B1 | Baltimore Harbor | 4/80 | Rods, 2 to 4 μm long, motile with polar flagella, oxidase positive, catalase positive, uses glucose oxidatively | *Pseudomonas* sp. |
| B3 | Baltimore Harbor | 4/80 | Rods, 1 μm long, nonmotile, yellow pigment, oxidase negative, catalase positive, ferments glucose | *Flavobacterium* sp. |
| G20 | Patuxent River | 3/80 | Rods, 1 to 2 μm long, nonmotile, yellow pigment, oxidase negative, catalase negative, ferments glucose | *Flavobacterium* sp. |
| I1 | Solomons Harbor | 4/80 | Rods, 2 to 4 μm long, motile with polar flagella, oxidase positive, catalase positive, uses glucose oxidatively | *Pseudomonas* sp. |

evaporating the solvent under a stream of $N_2$ gas.

Analysis of residual phenanthrene in extracts was performed on a model 5840A gas chromatograph (Hewlett-Packard, Palo Alto, Calif.) equipped with a 12-m SP2100 (Carbowax-deactivated) fused-silica capillary column and a flame ionization detector. The injection temperature was 275°C. The initial column temperature was 30°C and was programmed at 30°C $min^{-1}$ and then at 5°C $min^{-1}$ to a final column temperature of 250°C. The temperature of the flame ionization detector was 300°C. Helium was used as the carrier gas at a flow rate of 1.5 ml $min^{-1}$. Quantitative estimates of phenanthrene use were made by comparing the peak area obtained from extracts of cultures with the peak area obtained from extracts of sterile controls which had been incubated with the cultures.

To detect potential metabolites produced from phenanthrene, ethyl acetate extracts were examined by thin-layer chromatography (TLC). TLC was performed on silica gel GF plates (25 by 25 cm; Kontes Co.) which were developed with benzene–90% methanol–acetate acid (90:90:1 [vol/vol/vol]). Examination of phenanthrene standards and potential metabolites was performed under UV light.

Table 2 presents quantitative data on phenanthrene biodegradation by the five isolates in liquid media. Sterile controls were incubated and yielded an average recovery of 95.2% of the residual phenanthrene, with a 5.0% coefficient of variance. Student's *t* test was employed to test significance of phenanthrene biodegradation at the 95% confidence level. Each of the five isolates was capable of significant phenanthrene biodegradation under appropriate nutrient conditions. The extent of biodegradation was independent of the amount of growth but was dependent on the alternate growth substrate.

The presence of potential phenanthrene metabolites on TLC plates prepared from the solvent extract of the spent medium confirmed the metabolism of phenanthrene by the five isolates. Original replica plates indicated that all five isolates were phenanthrene cometabolizers. TLC of extracts from sterile media and from media lacking phenanthrene did not yield these spots.

Although the amount of phenanthrene degraded by isolate G20 was not statistically significant, the organism grew on phenanthrene as the sole carbon source and TLC plates showed one spot for a potential metabolite (Table 2); therefore, isolate G20 was scored as a false-positive phenanthrene cometabolizer. The results for four of the five isolates tested suggest that the replica plate method can be used to estimate the number of phenanthrene cometabolizers in estuarine sediments.

**Reproducibility.** To assess the reliability of the replica plating procedure, an appropriate dilution of sediment was plated on nine ESWA plates. After incubation, the plates were divided into three groups. Each plate in the first group was replicated onto six PA agar plates with no alternate growth substrate, each plate in the second group was replicated onto six PA agar plates with glucose as the potential growth substrate, and each plate in the third group was replicated onto six PA agar plates with sodium benzoate. The number of phenanthrene-degrading microorganisms was consistent in up to six replications of the same medium. Colonies of phenanthrene degraders on PA agar with glucose demonstrated identical patterns of phenanthrene degradation on all plates. A slight variability (≤8%) was observed on PA agar with benzoate and PA agar without an alternate

TABLE 2. Phenanthrene degradation by bacterial isolates cultured in liquid medium[a]

| Isolate | Alternate carbon source | Growth (change in turbidity)[b] | Residual phenanthrene (mg)[c] | No. of potential metabolites on TLC plates |
|---|---|---|---|---|
| Sterile control | None | 0 | 4.76 ± 0.24 | 0 |
| SPE18 | None | 0 | 4.25 ± 0.66 | 0 |
| | Glucose | 0 | 4.52 ± 0.38 | 0 |
| | Benzoate | + | 3.71 ± 0.50[d] | 4 |
| | Oil plus kerosene | 0 | 5.33 ± 0.18 | —[e] |
| B1 | None | 0 | 3.84 ± 0.67 | 0 |
| | Glucose | ++ | 3.26 ± 0.28[d] | 4 |
| | Benzoate | 0 | 3.21 ± 0.52[d] | 4 |
| | Oil plus kerosene | + | 3.63 ± 0.96[d] | — |
| B3 | None | 0 | 5.11 ± 0.03 | 0 |
| | Glucose | +++ | 1.10 ± 0.29[d] | 9 |
| | Benzoate | + | 2.82 ± 0.68[d] | 3 |
| | Oil plus kerosene | + | 3.27 ± 0.38[d] | — |
| G20 | None | ++ | 3.02 ± 1.00[d] | 1 |
| | Glucose | +++ | 2.07 ± 0.36[d] | 8 |
| | Benzoate | + | 3.47 ± 0.56[d] | 5 |
| | Oil plus kerosene | + | 3.33 ± 0.54[d] | — |
| I1 | None | 0 | 4.29 ± 1.21 | 0 |
| | Glucose | +++ | 2.02 ± 0.91[d] | 6 |
| | Benzoate | 0 | 3.75 ± 0.80 | 0 |
| | Oil plus kerosene | 0 | 4.49 ± 0.39 | — |

[a] Duplicate Erlenmeyer flasks, each containing 50 ml of medium plus 5 mg of phenanthrene, were incubated for 2 weeks at 25°C without shaking. Alternate carbon sources were added at 100 mg liter$^{-1}$.

[b] 0, No turbidity; +, slight turbidity; ++, moderate turbidity; +++, heavy turbidity.

[c] 5.00 mg of phenanthrene was placed initially in each flask.

[d] Significantly different from sterile control ($\alpha < 0.05$).

[e] —, Potential phenanthrene metabolites on TLC plates from media containing oil plus kerosene were obscured by comigrating spots from the metabolism of these compounds.

growth source, but it was not due to loss of inoculum on successive replica plates. Thus, the initial replica plates were highly reproducible, and the occurrence of apparent false-positive phenanthrene degraders was due to other factors. Loss of plasmids encoding degradation ability (6, 17) is a possibility.

**Advantages and limitations.** The replica plating method has several advantages over enrichment techniques which have been employed commonly for examining cometabolizers (9, 11, 21). First, direct plating enables screening of a large number of colonies. Thus, the relative numbers of phenanthrene utilizers and cometabolizers in the total plate count can be estimated. Second, the test for phenanthrene degradation is performed on microorganisms only once removed from the natural environment. Third, replica plating is faster and less tedious than methods which require individual screening of large numbers of pure cultures.

The method has limitations. First, plate count procedures underestimate the number of viable organisms—in some cases, by as much as two to three orders of magnitude (5). It is assumed that enumerations of phenanthrene utilizers and cometabolizers are underestimated to the same degree as are the total plate counts. Second, the growth substrates used may not include all compounds capable of supporting cometabolism. However, use of complex growth substrates such as kerosene, which contains 5,000 to 10,000 compounds (23), should decrease such underestimates. Third, the fact that bacteria will cometabolize or utilize phenanthrene on the plates does not necessarily mean that this occurs in situ.

In spite of the limitations, the replica plating method appears useful as a tool to screen large numbers of microorganisms and to estimate the relative numbers of potential phenanthrene utilizers and cometabolizers in natural populations.

This work was partially supported through postdoctoral research award NA80AA-D-0011, *Co-oxidation of Aromatic Hydrocarbons by Estuarine Microorganisms*, awarded to M.P.S. by the National Oceanic and Atmospheric Administration, U.S. Department of Commerce; and through grant NA79AA-D-00058, *Co-oxidation of Petroleum Hydrocarbons*

*by Estuarine Microorganisms*, awarded to J.J.C. by the Maryland Sea Grant Program.

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