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Enumeration of Phenanthrene-Degrading Bacteria by an Overlayer Technique and Its Use in Evaluation of Petroleum-Contaminated Sites

ANNE H. BOGARDT AND BARBARA B. HEMMINGSEN*

Department of Biology and The Applied Microbiology Laboratory, San Diego State University, San Diego, California 92182-0057

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Bacteria that are capable of degrading polycyclic aromatic hydrocarbons were enumerated by incorporating soil and water dilutions together with fine particles of phenanthrene, a polycyclic aromatic hydrocarbon, into an agarose overlayer and pouring the mixture over a mineral salts underlayer. The phenanthrene-degrading bacteria embedded in the overlayer were recognized by a halo of clearing in the opaque phenanthrene layer. Diesel fuel- or creosote-contaminated soil and water that were undergoing bioremediation contained 6×10^6 to 100×10^6 phenanthrene-degrading bacteria per g and ca. 5×10^5 phenanthrene-degrading bacteria per ml, respectively, whereas samples from untreated polluted sites contained substantially lower numbers. Unpolluted soil and water contained no detectable phenanthrene degraders (desert soil) or only very modest numbers of these organisms (garden soil, municipal reservoir water).

The pollution of soil and water with crude oil and petroleum products is a problem of increasing magnitude (10). In situ cleanup may include bioremediation (6), which can be defined as the intentional stimulation of resident hydrocarbon-degrading bacteria by oxygen, water, and nutrient additions. Usually, the addition of laboratory-grown bacteria that have appropriate degradative abilities is not useful (12), although such additions are beneficial in certain circumstances (8).

The evaluation of a polluted site before bioremediation often involves the detection and enumeration of the hydrocarbon-degrading bacteria. A pilot study may be devised to ascertain the appropriate levels of oxygen or nutrients to be added. That the bacteria are actually removing the contaminant during treatment requires showing an increase in microbial populations (6, 7), especially the hydrocarbon degraders, together with a decrease in pollutant concentration. Such studies require an enumeration technique that produces accurate results as rapidly, safely, and inexpensively as possible.

Molecular biology techniques (11) may allow the detection of specific genes, but the presence of these genes does not guarantee that the bacteria possessing them are viable or that the genes are being expressed in situ. Cultural methods for detecting bacteria with appropriate genes can supply viability data and are, in general, less expensive and within the reach of more laboratories, but the cultural methods that are currently in use are flawed. Incubation of inoculated mineral agar plates in hydrocarbon vapors (17) or inoculation of oil-containing mineral agar (18) yields numerous colonies which are often assumed to consist solely of hydrocarbon-degrading bacteria. While some colonies do contain such bacteria, others may be growing on organic compounds that contaminate the agar or the laboratory atmosphere or on excreted metabolic products of other bacteria present on the plates. Short of examining every colony on the plates, there is no way to tell which colonies contain true hydrocarbon-degrading bacteria. Therefore, these methods probably overestimate the true number of hydrocarbon-degrading bacteria (9, 13). The most-probable-number technique, in which liquid mineral medium to which a hydrocarbon is added (9) is used, overcomes the problem of trace amounts of contaminating organic compounds, but is extremely labor and glassware intensive, and incubation times of 1 to 2 months may be required for satisfactory results.

Easier, more accurate methods of enumerating hydrocarbon-degrading bacteria often use nonvolatile hydrocarbons (e.g., phenanthrene) (2). Previous workers have sprayed or spread acetone or ethanolic solutions of phenanthrene onto minimal agar plates either before or after inoculation; the fine opaque precipitate disappeared from the vicinity of the colonies that contained bacteria capable of phenanthrene degradation after 1 to 3 weeks of incubation (14). However, it is difficult to inoculate plates that are prespread with phenanthrene without disturbing the layer, and the spraying of already inoculated plates either before (5) or after (2) colony appearance exposes the bacteria to high, albeit transient, levels of the organic solvent. Also, spraying contaminates the atmosphere and fume hood with solvent and phenanthrene. In addition, as the bacteria are physically unrestrained on the surfaces of the plates, spreaders and swarmers can be a problem (14).

In this paper we describe a modification of the phenanthrene technique that solves some of the problems discussed above. In our technique bacteria are added to molten, cooled agarose which contains fine particles of phenanthrene; this is then poured onto the surface of an already solidified agar underlayer. Thus, the concentration of the solvent that is used to dissolve the phenanthrene is low, the colonies that develop are constrained from spreading, and halos of phenanthrene disappearance are easy to recognize against a background of evenly dispersed phenanthrene particles.

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* Corresponding author.

TABLE 1. Colony formation on low-nutrient-concentration agar plates and in agarose overlayers on mineral agar

Site	Total no. of bacteria per ml or g[a]	CFU/ml or CFU/g on 1:10 TSBA[b]	CFU/ml or CFU/g in agarose overlayers on mineral agar containing[c]:		
			No addition	Ethanol[d]	Ethanol and phenanthrene[e]
Creek through urban park	3.8×10^5	7.9×10^4	4.1×10^4	2.4×10^4 (58)	1.6×10^4 (39)
Municipal reservoir	8.2×10^6	1.9×10^4	8.1×10^3	7.2×10^3 (89)	3.3×10^3 (41)
Desert spring	2.9×10^5	2.4×10^4	4.9×10^4	4.3×10^4 (88)	6.9×10^3 (14)
Gasoline- and diesel fuel-polluted water	4.4×10^5	3.2×10^5	2.8×10^5	2.9×10^5 (104)	2.1×10^5 (75)
Gasoline- and diesel fuel-polluted water (same site), 17 months after start of bioremediation	8.9×10^6	5.0×10^5	1.1×10^5	9.8×10^4 (89)	1.1×10^5 (100)
Solids from bioreactor treating a creosote-polluted aquifer	3.1×10^9	5.8×10^8	3.1×10^8	2.9×10^8 (94)	2.7×10^8 (87)
Gasoline-polluted water from a pump and treat site	2.4×10^6	ND[f]	9.4×10^4	5.8×10^4 (62)	8.2×10^4 (88)
Urban park soil	7.1×10^8	2.9×10^7	1.3×10^7	1.4×10^7 (108)	1.2×10^7 (92)
Residential garden soil	2.1×10^8	2.8×10^7	3.4×10^7	ND	2.8×10^7 (82)
Inactive land farm for grease- and oil-contaminated soil	2.7×10^8	4.5×10^7	3.3×10^7	ND	3.5×10^7 (106)
Diesel fuel-contaminated soil from a stock pile	2.0×10^8	1.9×10^7	1.9×10^7	1.7×10^7 (84)	1.5×10^7 (75)
Active land farm for creosote-polluted soil					
Sample areas 1 and 2	1.4×10^9	3.8×10^8	1.9×10^8	ND	2.3×10^8 (121)
Sample areas 3 and 4	1.3×10^9	4.5×10^8	2.8×10^8	ND	1.9×10^8 (68)

[a] As determined by the acridine orange direct-count method.
[b] 1:10 TSBA, one-tenth-strength Trypticase soy broth (BBL) solidified with 2% (wt/vol) agar.
[c] A single sample from each site was analyzed.
[d] The numbers in parentheses are percentages, which were calculated as follows: [(CFU/ml or CFU/g in overlayers containing ethanol)/(CFU/ml or CFU/g in overlayers containing no addition)] × 100.
[e] The numbers in parentheses are percentages, which were calculated as follows: [(CFU/ml or CFU/g in overlayers containing ethanol and phenanthrene)/(CFU/ml or CFU/g in overlayers containing no addition)] × 100.
[f] ND, not determined.

MATERIALS AND METHODS

Water samples were collected in sterile 200-ml bottles and were kept refrigerated until they were analyzed (usually within 3 days). Soil samples were collected in clean jars and were refrigerated until they were analyzed (within 4 days). Dry weights of soils were determined by drying known amounts in duplicate at 80°C to a constant weight.

A 1-g portion of soil in 24 ml of filtered (pore size, 0.22 μm) reverse osmosis water was shaken by hand for 1 min; this preparation was allowed to settle for 2 min in a small bottle, the upper 10 ml was removed, and decimal dilutions were prepared. Water samples were shaken by hand for 1 min and diluted as described above. Aliquots (0.1 and 0.2 ml) of the dilutions were added to 3.5 ml of 30°C 1% agarose (molecular biology grade; low gelling temperature [ca. 26°C]; obtained from, e.g., International Biotechnologies, Inc., New Haven, Conn.) in Winogradsky mineral medium (16) enriched with 0.1 g of NH_4Cl per liter and 5 ml of a trace metal solution per liter immediately after 0.2 ml of an ethanolic solution of phenanthrene (8.5 mg/ml) had been added with vortexing. The trace metal solution contained (per liter of distilled water) 200 mg of $FeSO_4 \cdot 7H_2O$, 10 mg of $ZnSO_4 \cdot 7H_2O$, 3 mg of $MnCl_2 \cdot 4H_2O$, 20 mg of $CoCl_2 \cdot 6H_2O$, 1 mg of $CuCl_2 \cdot 2H_2O$, 2 mg of $NiCl_2 \cdot 6H_2O$, 500 mg of $Na_2MoO_4 \cdot 2H_2O$, and 30 mg of H_3BO_3. After the tube contents were mixed, they were evenly distributed onto an underlayer of mineral medium that was solidified with 1.5% Noble agar (Difco) and enriched with 0.1 ml of a stock solution of vitamins per liter; the vitamin stock solution contained (per 100 ml of distilled water) 2 mg of biotin, 2 mg of folic acid, 5 mg of thiamine HCl, 5 mg of D-calcium pantothenate, 5 mg of vitamin B_{12}, 5 mg of riboflavin, 20 mg of niacin, 3 mg of pyridoxal HCl, and 2 mg of *para*-aminobenzoic acid. After the medium was autoclaved and cooled to 60°C, 0.1 g of cycloheximide (Acti-Dione; dissolved in a minimum amount of acetone) per liter was added to discourage fungal growth. After incubation at 25°C for 26 days, all of the colonies were counted by using a Quebec counter; colonies surrounded by a zone of clearing in the phenanthrene haze were counted by using a dissecting microscope (magnification, × 10).

The same dilutions of the samples were also used to determine the total numbers of bacteria in the samples with the acridine orange direct-count method (3).

RESULTS AND DISCUSSION

Numerous colonies of both phenanthrene-degrading bacteria and bacteria that do not degrade phenanthrene formed on the plates during the 26 days of incubation (Table 1). Thus, the physical insults inherent in the plating procedure did not drastically affect the component of the bacterial population that was capable of forming colonies, a component which was considerably smaller than the total population of bacteria, as determined by the acridine orange direct-count method. This was expected as the concentration of ethanol in the 3.5 ml of agarose was 5%, well below the level required for damage to bacteria (4). The warmth (30°C) and immediate spreading of the agarose onto underlayer plates probably allowed rapid dissipation of this volatile solvent. Also, the heat shock, which was very mild for

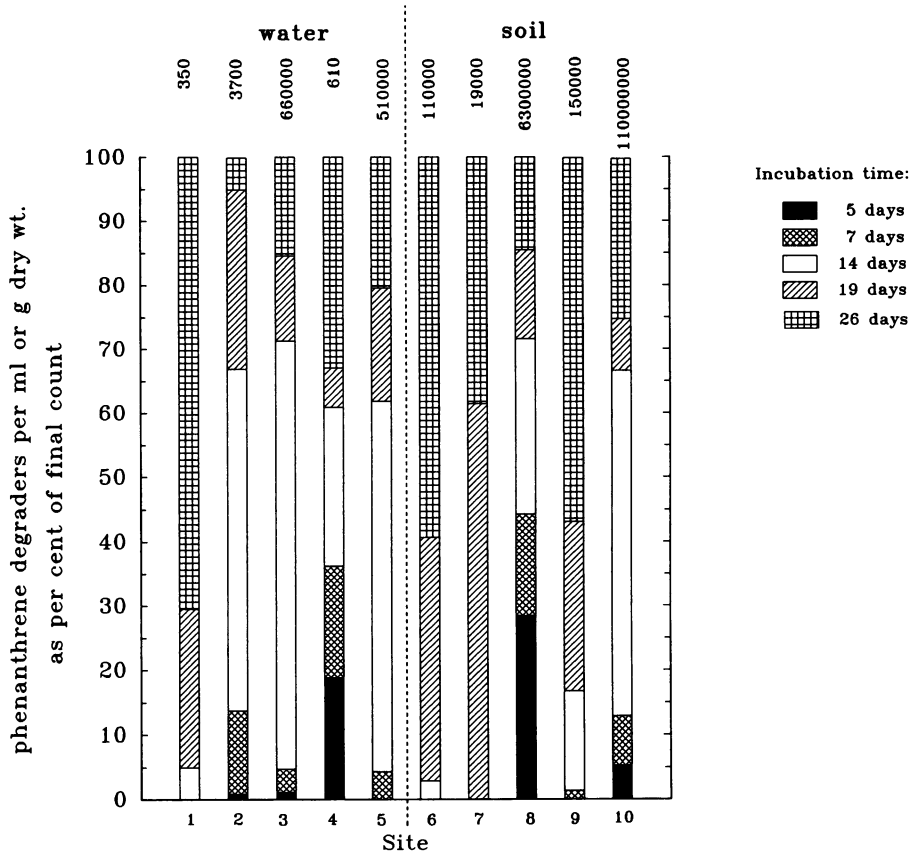

FIG. 1. Time of appearance in phenanthrene-containing agarose overlayers of phenanthrene-degrading bacteria obtained from samples of unpolluted and polluted soil and water. Site 1, seasonal creek through an urban park; site 2, gasoline- and diesel fuel-contaminated aquifer water; site 3, same as site 2, 17 months after infiltration of nutrients through contaminated soil; site 4, gasoline-contaminated water from an aquifer undergoing a pump and treatment process; site 5, diesel fuel- and gasoline-contaminated aquifer solids; site 6, urban park soil; site 7, residential garden soil; site 8, diesel fuel-contaminated soil after treatment with emulsifier and nutrients; site 9, inactive land farm that treated motor grease- and oil-contaminated soil; site 10, active land farm treating creosote-contaminated soil (four samples). Except where indicated otherwise, all of the data were obtained by using a single sample from each site. The numbers of phenanthrene-degrading colonies per milliliter of water or per gram of dry soil at the final count (26 days) are indicated at the top.

mesophilic bacteria, was brief as the agarose gelled within 2 to 3 min after its deposition on the underlayer plates. As Table 1 shows, similar numbers of colony-forming units were obtained on underlayer plates that were inoculated with dilutions suspended in agarose with and without ethanol or with and without phenanthrene. Only three unpolluted water samples had populations of bacteria that appeared to be harmed by the addition of ethanol and phenanthrene to the agarose overlayer.

Phenanthrene solubilization around colonies could show up as early as 5 days after inoculation, but usually the majority of the positive colonies appeared 7 or 14 days later, especially when we used samples obtained from polluted sites. For example, more than 60% of the phenanthrene-degrading colonies appeared by day 14 in samples obtained from sites 2 through 5, 8, and 10 (Fig. 1). In unpolluted samples, the numbers of phenanthrene degraders were often very low (Table 2), and these colonies took longer to appear (Fig. 1); less than 15% of the phenanthrene-degrading colonies appeared in samples obtained from sites 1, 6, 7, and 9 by day 14. The levels of phenanthrene degraders were below the limit of detection in unpolluted desert soil, but these organisms were more numerous in soil obtained from an urban park (site 6), in water obtained from a seasonal urban stream (site 1), in a sample obtained from a land farm for motor oil and grease just before it was closed (site 9), and in residential garden soil (site 7) (Fig. 1). In active land farm

TABLE 2. Total numbers of bacteria and numbers of phenanthrene-degrading bacteria in unpolluted sites

Site	Total no. of bacteria per ml or g[a]	No. of phenanthrene-degrading bacteria per ml of water or g of dry soil[b]
Creek through urban park	3.8×10^5	3.5×10^2
Desert spring	2.9×10^5	7.3×10^1
Municipal reservoir	8.2×10^6	1.5×10^1
Urban park soil	7.1×10^8	1.1×10^5
Residential garden soil	2.1×10^8	1.9×10^4
Desert soil from under mesquite	4.4×10^7	$<1.3 \times 10^2$

[a] As determined by the acridine orange direct-count method.
[b] The limits of detection were about 5 phenanthrene degraders per ml of water and about 130 phenanthrene degraders per g of dry soil.

soils (sites 8 and 10), phenanthrene degraders typically were very numerous, and the majority appeared within the first 14 days of incubation (Fig. 1). The phenanthrene-containing overlayer method appeared to be particularly useful for samples obtained from sites that were contaminated with creosote, diesel fuel, or other petroleum products which contain phenanthrene (1). Further study will be needed to determine whether this method yields useful information about gasoline- and jet fuel-contaminated sites, as these materials do not contain much phenanthrene.

The effects of simple organic compounds on the number of phenanthrene-degrading bacteria were tested by using an underlayer containing low concentrations of sodium glycerol phosphate, glycerol, yeast extract, and proteose peptone (14). A total of 40 different samples of polluted soil and water (i.e., gasoline- and diesel fuel-contaminated ground water, crude oil-contaminated water, soil, and tundra, and creosote-contaminated land farm soil) were plated in phenanthrene-containing overlayers on both mineral and organic plates. Usually, more colonies formed on the organic plates, but the percentages of the colonies on these plates that could solubilize phenanthrene were not significantly different ($P = 0.97$) from the percentages of phenanthrene-degrading colonies that formed on the mineral plates, as determined by the Wilcoxon signed-rank test (15) (data not shown). Thus, bacteria that only cometabolized phenanthrene did not seem to be particularly numerous in these polluted sites. We usually used just mineral underlayers for simplicity and to reduce the amount of glycocalyx formed by some bacteria, as well as to minimize the risk of fungal overgrowth. However, it is conceivable that populations of stressed bacteria (where pollutant levels are very high or some toxic material other than hydrocarbons is present) may make it difficult for the phenanthrene degraders to form adequately sized colonies on mineral plates. In such cases, parallel use of both mineral and organic underlayers may be advisable.

Large numbers of colonies (both phenanthrene degraders and bacteria that do not degrade phenanthrene) appeared on the mineral plates probably because of the nutrient content of the Noble agar by itself and the solubilization of volatile organic compounds in the agar over time. The advantage of the phenanthrene overlayer technique is that it is a safe and easy method to obtain visual evidence that a colony is able to solubilize phenanthrene, and thus enumeration of this group of bacteria can be carried out with confidence.

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