APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1982, p. 454–457
0099-2240/82/020454-04$02.00/0

Vol. 43, No. 2

Rapid Screen for Bacteria Degrading Water-Insoluble, Solid Hydrocarbons on Agar Plates

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Received 22 June 1981/Accepted 14 October 1981

A rapid procedure was devised for detecting on solid media bacteria able to degrade water-insoluble, solid hydrocarbons such as the polycyclic aromatic hydrocarbons phenanthrene, anthracene, and biphenyl. After *Alcaligenes faecalis* AFK2 was inoculated on a plate containing mineral salts agar, an ethereal solution of phenanthrene (about 10%, wt/vol) was sprayed on the surface of the plate, and the plate was incubated at 30°C for 2 to 3 days. Colonies showing degradation were surrounded with clear zones on the opaque plate. A similar clear zone also was formed around colonies which had been grown on a succinate-mineral salts agar or nutrient agar, followed by spraying of the ethereal solution of phenanthrene and further incubating for 1 day. Other phenanthrene-assimilating bacteria, including *Beijerinckia* Bwt and *Pseudomonas* SPM64, also formed clear zones on phenanthrene-covered agar plates. This method was applicable to detection of bacteria able to assimilate anthracene, naphthalene, and biphenyl.

Bacterial degradation of some aromatic hydrocarbons, toluene (16, 17), xylenes (4), ethylbenzene (9), and *p*-chlorobiphenyl (8) has been biochemically and genetically investigated. Extrachromosomal gene locations for these pathways are frequently found. On the other hand, there are only a few reports on the metabolism and genetics of polyaromatic hydrocarbons (PAHs), some of which are recognized as potential carcinogens. Of these compounds, smaller PAHs such as naphthalene (1, 7), phenanthrene (3, 7, 13), and anthracene (3, 7) have been studied with regard to their degradation by bacteria, early oxidation products, and aliphatic intermediates. In particular, the dissimilation of naphthalene has been shown to be encoded by a plasmid, NAH (2). However, no genetic studies of pure culture isolations have been reported for the bacterial dissimilation of larger PAHs such as phenanthrene.

Due to the volatilization of alkylbenzenes, biphenyl, and naphthalene at 30 to 37°C, temperatures ordinarily used for bacterial cultivation, these hydrocarbons are often provided separate from the growth medium, and they are assumed to be utilized from the vapor phase. Larger PAHs are, however, nonvolatile and water insoluble at these temperatures. Naphthalene dissolves in water at a concentration of 98 μM, but phenanthrene, anthracene, and naphthacene dissolve at concentrations of only 9.0, 0.45, and 0.066 μM, respectively (14). Therefore, no basal solid media in which phenanthrene and larger PAHs are uniformly dispersed have been developed. If an emulsifier is added in a medium to disperse such a hydrocarbon, bacteria might be subject to inhibition of growth by the emulsifier or could grow at its expense without utilizing the hydrocarbon. When an agar medium containing a larger amount of a solid hydrocarbon is autoclaved, the melted hydrocarbon forms large masses on the surface of agar on cooling. The phenanthrene-assimilating (Phn^+) bacteria we used did not grow on a mineral salts agar plate saturated with phenanthrene at 9 μM.

To study bacterial degradation of PAHs, we have studied the catabolism of phenanthrene (11, 13). We have often encountered the disappearance of the Phn^+ phenotype of bacterial cultures during prolonged storage on nutrient agar slants or by successive subcultures in liquid media containing a growth substrate other than phenanthrene. However, Phn^+ and Phn^- strains in a population could not be directly detected because of the lack of a solid medium suitable for distinguishing the two types. This paper describes a rapid procedure that uses a solid medium for the detection of Phn^+ bacteria.

(This study was presented in part at the Annual Meeting of the Society of Fermentation Technology, 1979, Osaka, Japan, and at the 6th International Fermentation Symposium, 1980, London, Canada.)

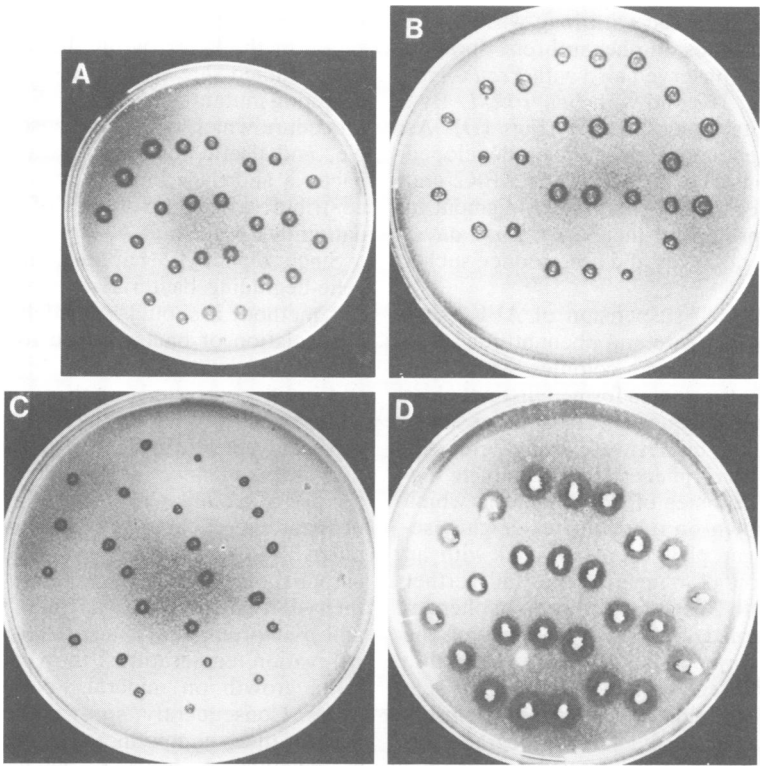

FIG. 1. Utilization of phenanthrene by bacteria on mineral salts agar plates. Immediately after cultures of the Phn$^+$ bacteria *A. faecalis* AFK2 (I), *Pseudomonas* SPM64 (II), *Beijerinckia* Bwt (III), and *Beijerinckia* B/836 (IV) were transferred onto MM2 agar plates, the plates were sprayed with an ethereal solution of phenanthrene and then incubated for 2 days.

MATERIALS AND METHODS

Organisms. The Phn$^+$ bacteria *Alcaligenes faecalis* AFK2 (12) and *Pseudomonas* SPM64, which are unable to utilize naphthalene (Nah$^-$), were isolated and identified by our laboratory. The wild strain (Bwt) and the mutant (B/836) of a *Beijerinckia* species were gifts of D. T. Gibson, of Texas University at Austin. *Pseudomonas* sp. S7K5 (Nah$^+$ Phn$^-$) has been described previously (10, 11). Strain OUS3 was an unidentified Nah$^+$ bacterium. *Pseudomonas putida* PpG1064 carrying the NAH plasmid was provided by I. C. Gunsalus, University of Illinois, Urbana.

Media and cultivation. LB medium contained 1% tryptone (Difco Laboratories), 0.5% yeast extract (Difco), and 0.5% NaCl, pH 7.2. The mineral salts solution (MM2) contained: $(NH_4)_2SO_4$, 18 mM; $FeSO_4 \cdot 7H_2O$, 1 μM; $CaCl_2 \cdot 2H_2O$; 100 μM; $MgSO_4 \cdot 7H_2O$, 1 mM; and NaCl, 8.5 mM; in 10 mM Na_2HPO_4-KH_2PO_4 buffer, pH 7.0. When agar plates were prepared, 1.5% agar was melted in MM2 by autoclaving and solidified. The MM2 agar plates contained no added substrates, and succinate- and glucose-MM2 agar plates contained 10 mM disodium succinate and glucose, respectively. The solid phenanthrene used was previously sterilized by recrystallization from ethanol-water, followed by aseptic collection by filtration and drying in vacuo in a desiccator. Incubation was always carried out at 30°C.

For growth of a Phn$^+$ bacterium on solid medium, the following procedures were used. About 10^3 Phn$^+$ cells from a culture that had been grown overnight on an LB platt and stored at 4°C were transferred with sterile toothpicks onto an MM2 agar plate. Immediately thereafter an ethereal solution of phenanthrene (about 10%, wt/vol) was uniformly sprayed over the surface of the agar plate. The ether immediately vaporized from the surface at ambient temperature, and a white, thin layer of phenanthrene remained on the entire surface. The plate was then incubated. Similar procedures were used for growth of bacteria utilizing other hydrocarbons.

Chemicals. All chemicals were obtained commercially.

RESULTS

Growth of Phn$^+$ bacteria on phenanthrene-mineral salts agar plates. After *A. faecalis* AFK2 was incubated for 1 to 2 days on phenanthrene-sprayed MM2 agar plate, it grew at the expense of the solid phenanthrene to form colonies which were surrounded with transparent areas (clear zones) (Fig. 1A). Solid phenanthrene had disappeared from cleared areas, since they did not show the blue fluorescence characteristic of

phenanthrene illuminated with long-wavelength UV light. Clear zones on phenanthrene-sprayed MM2 plates were observed also with *Pseudomonas* SPM64 (Fig. 1B) and with *Beijerinckia* Bwt (Fig. 1C) and its mutant, B/836 (Fig. 1D). As growth increased, more clear zones developed in all four strains. The Phn^+ strains AFK2 and SPM64 produced a diffusible orange pigment in the agar after prolonged incubation for 7 days. Bwt and B/836, however, did not produce such a pigment.

After a dilute cell suspension of AFK2 was spread on an MM2 plate and phenanthrene was applied, the cells formed colonies with clear zones. Even small colonies formed distinct clear zones like phage plaques on a bacterial lawn, and therefore Phn^+ clones were easily detected.

Detection of Phn^+ phenotype on complete media. When the colonies of Phn^+ bacteria which had already grown on a succinate- or glucose-MM2 or LB agar plate were sprayed with an ethereal solution of phenanthrene and further incubated for 1 to 2 days, all utilized the phenanthrene and formed clear zones. None of the Phn^- bacteria formed such zones, and the thin layer of phenanthrene remained white. This suggests that the formation of clear zones is closely related to the ability of bacteria to degrade this hydrocarbon and allows detection of the Phn phenotype without replication of the colonies onto other mineral plates.

Detection of bacteria that degrade other solid hydrocarbons by the ethereal solution-spraying method. On an MM2 agar plate sprayed with an ethereal solution of biphenyl, the Bwt strain grew forming a distinct, clear zone as it did on a phenanthrene-sprayed plate, and it produced a diffusible, intensely yellow pigment. The mutant B/836 did not grow biphenyl because it lacks an enzyme responsible for biphenyl degradation (6).

Nah^+ bacteria could be detected on solid media sprayed with naphthalene. Nah^+ strains PpG1064, S7K5, and OUS3 grew forming clear zones. The thin layer of this hydrocarbon on the plate, however, tended to vaporize after long incubations.

A Phn^+ Nah^- strain, AFK2, was found to degrade anthracene by forming clear zones on succinate-MM2 agar sprayed with an ethereal solution of anthracene.

DISCUSSION

PAHs are among the compounds recalcitrant to biological degradation, possibly due to their insolubility in aqueous cultural media. No bacteria able to utilize as a growth substrate a PAH with more than three rings have been isolated. Although the metabolic pathways for naphthalene, phenanthrene, and anthracene have been well investigated, the gene location and organization of the latter two hydrocarbons have not been studied due to the lack of a method for detecting mutants on solid media. The detection procedure which we have devised is rapid, simple, and useful for the isolation of degrading bacteria and their Phn^- mutants, and it should contribute to genetic studies of bacterial degradation of PAHs.

Since biphenyl-, naphthalene-, and anthracene-degrading bacteria were also detected by this method, it should be widely applicable for the isolation of bacteria able to degrade other nonvolatile, water-insoluble, biologically inert, and solid compounds such as solid paraffins, wax, and larger PAHs such as benz[*a*]anthracene and benzo[*a*]pyrene. During the preparation of this manuscript Sylvestre (15) described the use of a similar procedure for the detection of bacteria capable for utilizing *p*-chlorobiphenyl. However, because smaller solid hydrocarbons, such as naphthalene and biphenyl, and their derivatives, such as methylnaphthalenes and *p*-chlorobiphenyl, are relatively volatile at cultivation temperatures, they can support bacterial growth on mineral salts agar plates in vapor. Consequently, spraying of ethereal solutions of these compounds is not required for the assimilative phenotypes. When such ethereal solutions were sprayed onto plates, the thin layer of hydrocarbons on the plate surface tended to be mottled during incubation due to the hydrocarbons' volatilities. However, this spraying method should be useful for the detection of auxotrophic mutants of such bacteria, since there is no requirement for replica plating.

ACKNOWLEDGMENTS

We express appreciation to R. Nomi, Hiroshima University, for useful suggestions. We are grateful to D. T. Gibson, Texas University at Austin, for kind gifts of the wild (Bwt) and mutant (B/836) strains of *Beijerinckia* sp. and to I. C. Gunsalus, University of Illinois, for his kind gift of *P. putida* PpG1064.

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