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Bacterial degradation of polycyclic aromatic hydrocarbons on agar plates: the role of biosurfactants

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Summary

Colonies of a polycyclic aromatic hydrocarbon (PAH) degrading and biosurfactant producing strain of *Pseudomonas marginalis* PD-14B, pre-incubated on nutrient agar plates, formed zones of clearing when the agar surface was coated with phenanthrene. Application of a drop of the cell-free biosurfactant solution to the agar surface, followed by coating with phenanthrene film also produced a clear zone against the opaque background of the PAH coating. The results indicate that bacterial colonies generate transparent haloes not only as a result of PAH degradation, as is generally concluded from such tests, but also by solubilization of these hydrophobic compounds, mediated by biosurfactants released by the cells into the agar zone surrounding the colony.

Introduction

Biodegradation of polycyclic aromatic hydrocarbon (PAHs) is limited by the low water solubility and rates of dissolution of the hydrophobic substrates (Stucki and Alexander, 1987). The insolubility of PAHs has caused problems in the development of solid media for selection and enumeration of PAH degrading bacteria in which PAHs are uniformly dispersed. Consequently, PAH degraders are usually isolated by enrichment procedures. In the case of other insoluble xenobiotics, such as polychlorinated biphenyls, a rapid screening method for the direct isolation of p-chlorobiphenyl (p-CB) degrading bacteria from the natural environment has been described (Sylvestre, 1980). This method was based on the observation that, following spraying of p-CB solution over previously inoculated plates, clear zones developed in the film of deposited p-CB around some of the colonies. The same principle has been applied to isolation of phenanthrene (Kiyohara *et al.*, 1982; Shiaris and Cooney, 1983) and pyrene (Heitkamp and Cerniglia, 1988) utilizing bacteria. Zones of clearing on agar plates covered by a thin layer of PAHs have been used to detect PAH degrading strains. Some authors incorporated bacteria together with fine particles of phenanthrene into an agarose overlayer and poured the mixture over a mineral salt underlayer (Bogardt and Hemmingsen, 1992), while others sprayed a solution of PAHs in acetone on the surface of agar plates prior to inoculation (Kastner *et al.*, 1994).

It is believed that formation of clear zones is closely related to the ability of bacteria to degrade hydrocarbons. Most of the isolates producing clear zones were able to use PAHs as a single source of carbon or degrade the compounds cometabolically (Shiaris and Cooney, 1983). Another possible interpretation of the phenomenon is that clear zone generation may also result from solubilization of the hydrocarbon through production of extracellular surface-active substances by the bacterial colony. We have previously described a surface-active extracellular factor produced by a PAH degrading bacteria *P. marginalis* PD-14B (Burd and Ward, 1996). This high-molecular weight LPS-containing factor, having an approximate composition: protein, 70%; lipid, 25% and polysaccharide (5%), was capable of preventing flocculation of a water dispersion of different PAHs and emulsifying some liquid hydrocarbons. In this communication we describe experiments that demonstrate that surface-active factor produced by PAH degrading bacteria, generates clear zones on agar plates covered by a PAH layer.

Materials and Methods

P. marginalis PD-14B was maintained on nutrient agar. Isolation, identification and properties of the strain were described in Burd and Ward (1996). The solid medium used for bacterial colony growth was also nutrient agar. The PM-factor solution was prepared in 10 mM Tris-HCl buffer, pH 7.1, with $MgCl_2$ (1 mM) and dithiothreitol (0.1 mM). Concentration of the factor was 3.45 mg protein/ml.

The amount of phenanthrene on agar plates was estimated by gas chromatography. Three agar cores from the transparent areas of the phenanthrene coated plates where the factor was applied (experimental) and three control cores from coated agar, not treated with PM-factor, were sampled by means of a metallic round bore (d = 1.6 cm). The cores were cut in small pieces with a blade, extracted with 5 ml of methylene chloride by rigorous vortexing in screw-capped 10 ml glass tubes, evaporated to dryness under a nitrogen stream and dissolved in 0.2 ml of hexane. Recovery of phenanthrene was at least 90%. One microlitre samples were injected into a GC-14A gas chromatograph (Shimadzu, Kyoto, Japan), equipped with a flame ionization detector and DB5 column (J & W Scientific, CA, 30.0 m by 0.32 mm inside dia.). Helium was used as the carrier gas (10 ml/min) with nitrogen as a make-up gas. The oven temperature was programmed at 120°C for 4 min, followed by a linear increase of 20°C/min to 300°C, and held at 300°C for 5 min. Column injection and detection temperatures were 250°C and 275°C, respectively.

Results

The PD-14B strain was incubated at 30°C for 48 h on nutrient agar plates. The agar surface was then sprayed with a phenanthrene (2% in acetone). Plates were incubated at 30°C for a further 2-3 h. Transparent haloes were observed around the colonies (Figure 1).

A 20 μl solution of the crude PM-factor was spotted on the surface of nutrient agar and allowed to absorb into the agar. The agar surface was then uniformly sprayed with a phenanthrene solution (2% in acetone). After an incubation period of 2-3 h at 30°C, a clear spot appeared against an opaque background (Figure 2). When the factor was treated with 10 mM mercuric chloride, which reduced its emulsifying activity by 80%, only faint clearing zones

were observed. The effect of concentration of the active factor (20 μl) on phenanthrene zone clearing was also investigated (Figure 3). The degree of transparency of the spot and the radius decreased with decrease in concentration of the PM-factor.

In order to prove that the clear spot generated by the PM-factor was not the result of phenanthrene degradation, PAH concentrations were determined in cores taken from PM-factor treated and untreated locations from the agar plate. The results obtained showed that the average contents of the PAHs in the control and experimental cores were 352 $\pm$ 126 and 338 $\pm$ 118 μg per circle, respectively.

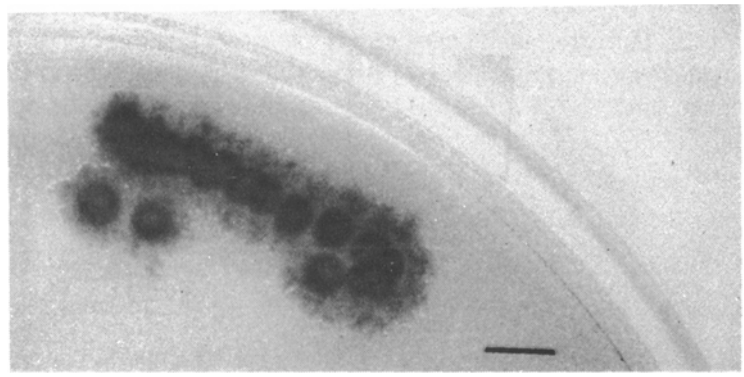

Figure 1 Photograph of PD-14B strain colonies surrounded by clear zones. Marker bar denotes 5 mm.

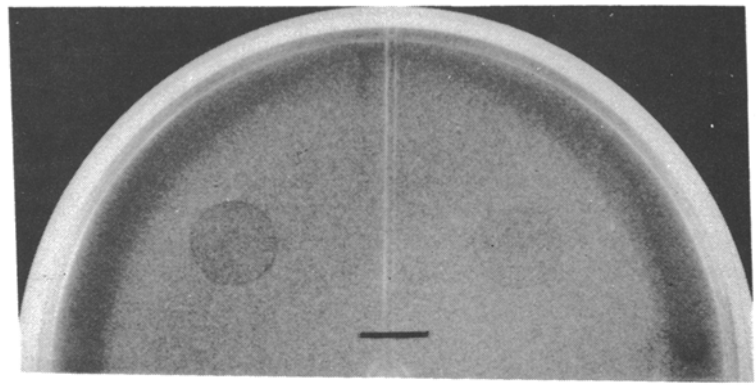

Figure 2 Generation of spots by intact (left) and $HgCl_2$ treated (right) biosurfactants. Marker bar denotes 10 mm.

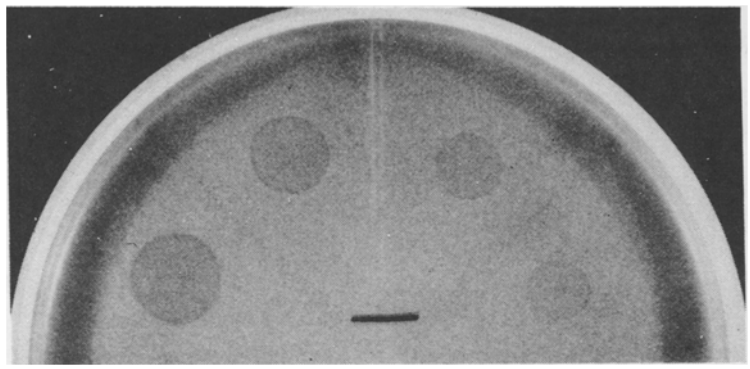

Figure 3 Effect of biosurfactant concentration on zone clearing. 20 μl volumes of surfactant solution were applied. PM-concentrations as mg protein/ml were (left to right): 3.45; 1.73; 0.86; and 0.43. Marker bar denotes 10 mm.

Discussion

The data obtained demonstrate that zones of clearing generated around colonies of *P. marginalis* PD-14B grown on phenanthrene coated agar can result from the interaction between the PAH and biosurfactant produced by the bacteria. PM-factor is a high molecular weight compound consisting mainly of proteins and lipids and appearing as a ball-shaped structure with an average diameter of 0.78 μm (Burd and Ward, 1996) and hydrophobic compounds are able to partition in membrane lipid (van der Kooi and Callis, 1974) and phospholipid vesicles (Dulfer and Govers, 1996). It is reasonable to assume that the phenanthrene, coated on the agar, dissolved directly in the lipophilic interior of PM-factor vesicles, thereby generating transparent spots on the agar surface.

Acknowledgement

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