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Environmental Contamination and Toxicology

Characterization of Bacteria Capable of Degrading Soil-Sorbed Biphenyl

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Bioavailability of soil-sorbed chemicals is an important determinant of the environmental fate of contaminants and the successful bioremediation of contaminated soils. Soil-sorbed organic contaminants have been considered unavailable for biodegradation until recently. Experimental evidence indicates that this generalization may be inappropriate and should be reexamined. Several reports have shown that sorbed contaminants can be degraded by microorganisms, or at least that desorption into bulk solution is not a prerequisite for biodegradation (Guerin and Boyd 1992; Calvillo and Alexander 1996; Tang et al. 1998; Feng et al. 2000; Park et al. 2001). Different bacteria degrade sorbed compounds to different extents. Specific physiological properties of the bacteria involved in the degradation may contribute to the enhanced availability of the compound. Bioavailability refers to the accessibility of a chemical for assimilation by organisms (Alexander 2000). Wick et al. (2002) suggested that high-affinity uptake systems, adhesion to solid surfaces, and biosurfactant production may be the underlying mechanisms for bioavailability enhancement. Cell attachment to a solid surface where contaminants are sorbed reduces the distance between cells and substrate and thus enhances substrate availability. Another mechanism to reduce the distance between cells and substrates may involve bacterial surface translocation and chemotaxis. These abilities may enable bacteria to actively seek new substrates once they are depleted in one area.

Biphenyl is an aromatic hydrophobic compound used as a fungicide for citrus in agriculture and as a chemical feedstock for organic syntheses in industry (U.S. EPA 1994). It has been used as a model compound in studies of the bioavailability of soil-sorbed chemicals (Calvillo and Alexander 1996; Feng et al. 2000). Feng et al. (2000) showed that two biphenyl-degrading bacteria, *Pseudomonas putida* strain P106 and *Rhodococcus erythropolis* strain NY05, were able to access soil-sorbed biphenyl even when desorption was accounted for, and that strain P106 had better accessibility to the pool of sorbed biphenyl. The objective of this study was to evaluate the bacterial characteristics that may be related to the differential bioavailability of soil-sorbed biphenyl to bacteria. Cell characteristics related to the above-mentioned mechanisms, such as motility, chemotaxis, biosurfactant production, attachment ability, and cell surface hydrophobicity, were evaluated.

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MATERIALS AND METHODS

Pseudomonas putida strain P106 and *Rhodococcus erythropolis* strain NY05 were grown in mineral salts media (Feng et al. 2000) supplemented with 0.2 g biphenyl/L and 0.48 g biphenyl/L, respectively. For experiments using radioactive labeled bacteria, L-[3,4,5-^{3}H] leucine stock solution (150 Ci/mmol, Sigma, St. Louis, MO) in 20-mM phosphate buffer (pH 7) was added to a mineral salts medium supplemented with biphenyl to obtain a final radioactivity level of 0.35 μCi/mL. Liquid cultures were incubated at room temperature (23 ± 1°C) with shaking at 200 rpm on a rotary shaker, and growth was monitored by measuring the optical density (OD) at 600 nm. Cells in the early stationary phase were harvested by centrifugation, washed with 20-mM sterile phosphate buffer, resuspended in the same buffer solutions, and adjusted to the appropriate OD prior to use. Optical densities of 0.05 and 0.2 correspond to 1.44 and 7.18 x 10^6 CFU/mL for strain P106, and 1.40 and 7.10 x 10^6 CFU/mL for strain NY05, respectively. All experiments were carried out with three replications and repeated at least three times unless stated otherwise.

Bacteria motility was evaluated by the swarm plate method (Gerhardt et al. 1994), the filter paper bridge method (Atlas et al. 1995), and microscopic observation. Swarm plates contained mineral salts medium or half-strength nutrient broth and were solidified with 0.3, 0.5, or 0.7 % agar. Biphenyl was supplied on the lid of Petri plates for mineral salts media. Bacteria were inoculated by stabbing with a straight inoculating needle in the center of the agar plate. For the paper bridge method, a 1-cm wide agar strip was removed from the middle of a Petri plate containing nutrient agar medium. A strip of wet sterile filter paper (1 x 10 cm) was used to bridge the two halves of the agar media. Bacteria were then inoculated on one end of the filter paper. Motile bacteria are able to move across the filter paper strip and develop colonies on the un-inoculated side of the agar medium.

Chemotaxis assays were performed using the capillary method (Adler 1973) to determine the chemotactic response of the two bacterial strains at three biphenyl concentrations (5, 10, 15, and 20 μM) and two cell densities (OD_{600} = 0.05 and 0.2). Bacterial suspensions were placed in chambers formed by placing a U-shaped glass tube between a microscope slide and a cover slip. Triplicate 1-μl capillaries, flame-sealed at one end, were immersed, open end down, into biphenyl solutions of different concentrations for 5 min. The capillaries were then individually placed in the culture chambers. The system was kept at room temperature (23 ± 1°C) for 30 min. The capillaries were removed, rinsed with phosphate buffer, broken, and emptied into 2-mL phosphate buffer. The suspensions were diluted 10 or 100 fold, if necessary, and plated onto nutrient agar plates. The relative chemotactic response (RCR) was calculated as the ratio of the colony forming units (CFU) obtained from the biphenyl-containing capillary to CFU obtained from the capillary containing buffer only. Values of RCR higher than 2 are considered significantly chemotactic.

Cell surface hydrophobicity of the biphenyl-degrading bacteria was tested with bacterial adhesion to hydrocarbons (BATH) (van der Mei et al. 1993) and hydrophobic interaction chromatography (HIC) (Stenström 1989) assays. For the BATH assay, hexadecane (150 μL) was added to 3 mL bacterial suspensions adjusted to appropriate densities (OD_{600} ranged from 0.05 to 0.9) and vortexed for 10 sec. The mixture was left to stand for 10 min to allow the phases to separate. This procedure was repeated to a total vortexing time of to 60 sec; the OD of the aqueous phase was then measured. The percentage of bacterial cells associated with the hydrocarbon phase was used as a measure of their hydrophobicity. To perform the HIC assay, Octyl-Sepharose gel was packed into a Pasteur pipet plugged with glass wool in ethanol suspension (2 mL), washed with water, and equilibrated with 20 mM phosphate buffer. ^{3}H-labeled cell suspensions (1 mL) with OD_{600} of 0.05 or 0.2 were applied to the column and eluted with 20 mM phosphate buffer. The radioactivity in the original cell suspension and the eluate collected were measured. A high percentage of cells retained in the gel column indicated high hydrophobicity.

The soil attachment assay was performed using four agricultural soils collected from Michigan. The chemical and physical properties of these soils were reported previously (Feng et al. 2000). The ^{3}H-labeled cell suspension (1 mL) was loaded on a column packed with 10 g soil in a 20 mL syringe and the column was then eluted with 20 mM phosphate buffer. The percentage of cells retained by each soil was calculated based on the radioactivity of the initial cell suspension and that of the eluate.

The surface tension of the cell cultures was determined using a DuNoüy tensiometer at room temperature. The radioactivity was determined by liquid scintillation counting. The biphenyl concentration in the buffer solution was measured using a spectrophotometer at 248 nm.

RESULTS AND DISCUSSION

Both *P. putida* P106 and *R. erythropolis* NY05 were found to be motile by light microscopy observation and the filter paper bridge method. After a two-day incubation, both organisms showed the ability to grow by swarming on soft agar plates, with colonies having larger diameters at lower agar concentrations (Table 1). On nutrient agar, the P106 colonies were 75, 109, and 65% larger than NY05 at agar concentrations of 0.3, 0.5, and 0.7%, respectively. On mineral salts media, the differences in colony size were smaller; P106 colonies were 12, 24, and 10% larger than NY05 for the same agar concentrations. These results suggest that both organisms were motile in the liquid solution and on the agar surface, and that P106 moved faster on soft agar surfaces than NY05.

In the chemotaxis assay, accumulations of P106 and NY05 cells in the capillaries containing biphenyl ranged from 149 to 1464 CFU per capillary for all the biphenyl and cell concentrations tested. These numbers are comparable to those

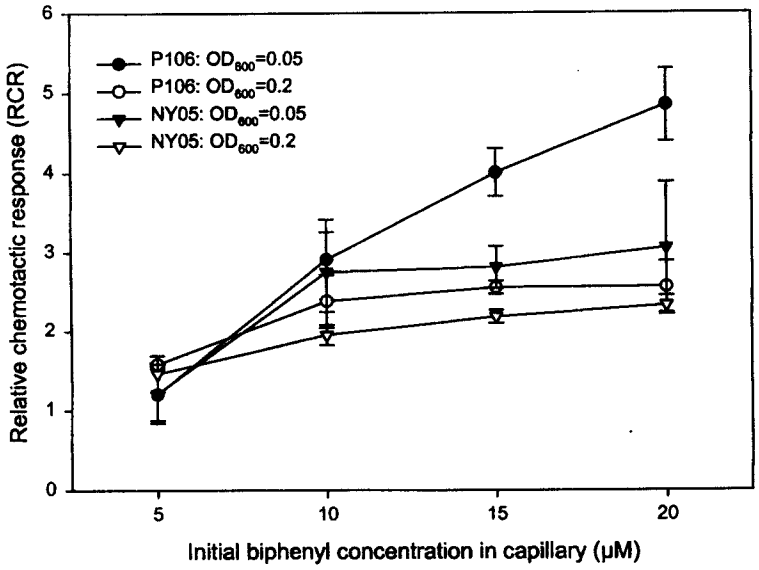

Figure 1. Chemotactic responses of *P. putida* P106 and *R. erythropolis* NY05 towards biphenyl.

Table 1. Swarming motility of *P. putida* P106 and *R. erythropolis* NY05 on soft agar plates

Organism	Agar concentration	Diameter of colonies (cm)	
		Nutrient medium	Mineral salts medium
P106	0.3 %	5.1 ± 0.2	5.5 ± 0.4
	0.5 %	4.8 ± 0.2	5.1 ± 0.3
	0.7 %	2.8 ± 0.2	3.3 ± 0.3
NY05	0.3 %	2.9 ± 0.2	4.9 ± 0.3
	0.5 %	2.3 ± 0.2	4.1 ± 0.3
	0.7 %	1.7 ± 0.2	3.0 ± 0.2

previously reported for *Pseudomonas putida* G7, which is chemotactic towards naphthalene (Marx and Aitken 1999). The chemotaxis assays showed that both P106 and NY05 had significant chemotactic responses towards biphenyl (RCR > 2) at three of the four biphenyl concentrations tested (Fig. 1). RCR values for P106 increased from 1.2 to 4.8 when the biphenyl concentration increased from 5 to 20 μM at OD_{600} = 0.05. The RCR values for NY05 at OD_{600} = 0.05 increased from 1.2 to 3.1 when the biphenyl concentration increased from 5 to 20 μM. At an OD_{600} of 0.2, the RCR values for strains P106 and NY05 changed from 1.6 to 2.7 and 1.5 to 2.3 with increasing biphenyl concentration, respectively. At both

cell densities, RCR values of strain P106 were higher than those of strain NY05 for the same biphenyl concentrations (Fig. 1). Higher RCR values indicate that strain P106 moved faster towards biphenyl by chemotaxis than strain NY05. A higher chemotactic response might be attributed to higher swimming speed or higher sensitivity of cells to biphenyl. This would give P106 an advantage in accessing and utilizing biphenyl. A higher chemotactic response for strain P106 may contribute to its more complete access of soil-sorbed biphenyl than strain NY05. Although most studies on chemotaxis have focused on hydrophilic substrates that are not pollutants, bacterial chemotaxis towards environmental pollutants such as naphthalene, benzene, toluene, trichloroethylene have been observed (Marx and Aitken 1999; Parales et al. 2000). Chemotaxis has the potential to enhance the bacterial degradation of organic pollutants in systems where the pollutants are not uniformly distributed. Chemotaxis brings organisms closer to where pollutants are concentrated and enables bacteria to actively seek a fresh supply of substrate when it is depleted in a local area.

Both strains P106 and NY05 showed higher RCR values at lower cell concentrations for biphenyl concentrations of 10, 15, and 20 μM (Fig. 1). This may be due to the difference in biphenyl concentration gradients between the capillary and the assay chamber for different bacterial concentrations. A higher cell concentration resulted in greater sorption and metabolism of biphenyl and thus decreasing the biphenyl concentration in the capillary compared to that seen for a lower cell concentration. With the reduced concentration gradient, the number of bacteria going into the capillary would be fewer relative to the control. At a lower cell density, the concentration gradient is steeper and more cells enter the capillary, resulting in a higher RCR value. Alteration of the attractant gradient due to bacterial consumption has been reported by Adler (1973) and Rivero-Hudec and Lauffenburger 1986). Lower bacterial concentration is preferred when performing chemotaxis assays in cases where the test chemical is being metabolized.

BATH assays showed that NY05 was considerably more hydrophobic than P106 (Fig. 2). P106 attachment to hexadecane was the highest (26%) at the lowest cell density (OD_{600} = 0.05). Cell attachment decreased to less than 5% at cell densities greater than 0.1. Attachment of NY05 to hexadecane varied between 81 and 88 % for all cell densities. Adhesion of strains P106 and NY05 to hexadecane was cell-density independent (Fig. 2), except at a very low cell density for P106. HIC assays confirmed these results. P106 cells retained by Octyl – Sepharose gel columns were 66.3 ± 2.65% and 73.8 ± 3.69% at OD values of 0.05 and 0.2, respectively. For NY05, the percentages were 99.0 ± 2.97% and 98.3 ± 1.97% at OD_{600} of 0.05 and 0.2, respectively. The difference in cell hydrophobicity could be due to the differences in cell wall structures of Gram-positive (NY05) and Gram-negative (P106) bacteria. Although cell appendages (e.g., pili and flagella) and extracellular materials (e.g., capsules and slime layers) may also affect cell surface hydrophobicity, electromicrographs of these two organisms did not reveal the presence of either pili or flagella. No

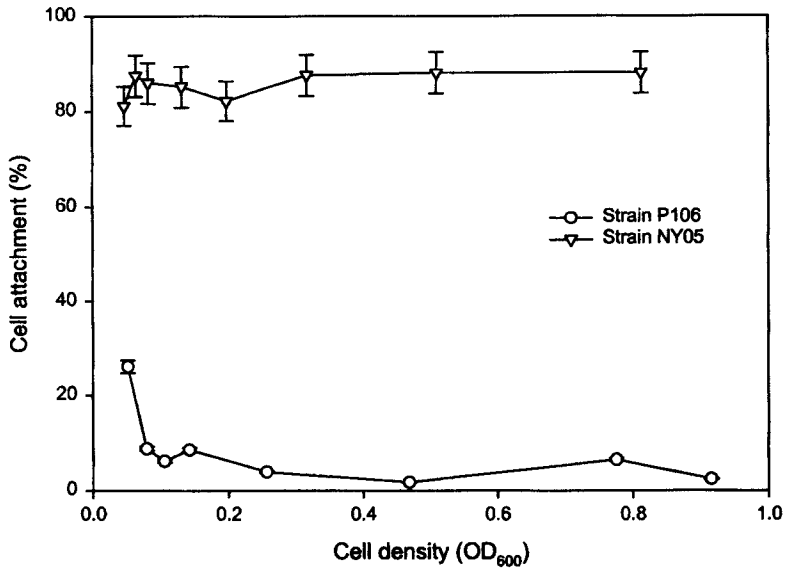

Figure 2. Percent cell attachment to hexadecane in the bacterial adhesion to hydrocarbon assays.

differences between P106 and NY05 were observed when they were stained for capsules and slime layers.

Cell surface hydrophobicity is presumed to play an important role in the initial adhesion of bacteria to hydrophobic surfaces. Bacteria with higher cell surface hydrophobicity may adhere more easily to soil organic matter where biphenyl is sorbed. Higher cell surface hydrophobicity also enhances the sorption of hydrophobic molecules like biphenyl to cell surfaces, and increases the available substrate for bacterial degradation. However, higher cell surface hydrophobicity may also facilitate the interactions between bacterial cells and cause cell aggregation. NY05 was observed to form cell aggregates in liquid suspension much more easily than P106. The formation of aggregates significantly reduced the total cell surface area and therefore reduced the effective cell surface area available to absorb hydrophobic molecules such as biphenyl. This may be one reason for the relatively low accessibility of sorbed biphenyl by NY05 compared to that of P106.

P106 and NY05 showed similar adhesive abilities for the soils tested. Cell attachment to four soils ranged from 96.1% to 98.4% for P106 and from 96.1 to 98.3% for NY05 (Table 2). These soils had similar soil texture and mineralogy but differed in the soil organic carbon contents, which ranged from 7.80 for Colwood A to 0.4 for Capac B. Although the two organisms differed in

hydrophobicity according to BATH and HIC assays, the difference is not reflected in their attachment to soils. The relationship between bacterial hydrophobicity and bacterial transport or adhesion to soil has been studied by several researchers, but no consensus has been reached. Stenström (1989) reported that high hydrophobicity coincides with enhanced adhesion to the soil mineral particles. Gannon et al. (1991) showed no correlation between bacterial transport in soil columns and hydrophobicity determined by BATH and HIC assays.

Table 2. Percentage of cells attached to soils for *P. putida* P106 and *R. erythropolis* NY05

Strain	Colwood A	Capac A	Schoolcraft A	Capac B
P106	96.1 ± 3.8	98.1 ± 3.7	98.4 ± 4.3	98.2 ± 4.9
NY05	97.7 ± 4.3	96.1 ± 3.8	98.3 ± 4.4	ND

ND: not determined.

Production of biosurfactant by either organism was not evident, since no decrease in surface tension of cell cultures was observed. Surface tensions for 24- and 48-hr cultures of P106 and NY05 ranged from 70.3 to 71.1 dynes/cm, while mineral salts medium and distilled water had surface tensions of 70.7 and 69.9 dynes/cm, respectively. The presence of biosurfactant has been reported to reduce the surface tension of cell cultures by more than 20 dynes/cm (Al-Tahhan et al. 2000). Thus, access to soil sorbed biphenyl by both P106 and NY05 cannot be attributed to the production of biosurfactant. Lack of biosurfactant production has also been observed for *Mycobacterium* sp. LB501T, which grew on solid anthracene (Wick et al. 2002).

Little is known about the influence of bacterial characteristics on the bioavailability of soil-sorbed chemicals. Our previous study has shown the bioavailability of soil-sorbed biphenyl to both *P. putida* P106 and *R. erythropolis* NY05 (Feng et al. 2000). In this study, both P106 and NY05 showed strong tendencies to attach to soils. P106 was more motile and had a higher chemotactic response to biphenyl than NY05. It appears that attachment to soil, motility, and chemotaxis may be important characteristics that influence the bacterial access of soil-sorbed biphenyl. Bacteria (P106 in this case) with higher chemotactic response and moderate cell surface hydrophobicity may access soil-sorbed biphenyl more efficiently.

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