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Effect of Humic Fractions and Clay on Biodegradation of Phenanthrene by a *Pseudomonas fluorescens* Strain Isolated from Soil

JOSE-JULIO ORTEGA-CALVO* AND CESAREO SAIZ-JIMENEZ

Instituto de Recursos Naturales y Agrobiologia, C.S.I.C., Apartado 1052, 41080 Seville, Spain

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The mineralization of phenanthrene in pure cultures of a *Pseudomonas fluorescens* strain, isolated from soil, was measured in the presence of soil humic fractions and montmorillonite. Humic acid and clay, either separately or in combination, shortened the acclimation phase. A higher mineralization rate was measured in treatments with humic acid at 100 μg/ml. Humic acid at 10 μg/ml stimulated the transformation only in the presence of 10 g of clay per liter. We suggest that sorption of phenanthrene to these soil components may result in a higher concentration of substrate in the vicinity of the bacterial cells and therefore may increase its bioavailability.

Soils are complex living media and contain large amounts of microorganisms. In this medium, most bacteria are associated with particles, with more microorganisms attached to the clay size fraction than to silt or sand (15).

Adsorption of bacteria in soils is often considered in terms of soil physical properties, and the degree of adsorption between microorganisms and soil particles is broadly related to the surface area and surface charge properties of the particles (5). The major soil components that affect bacterial adsorption are the organic matter and clay fractions. Both these components possess large surface areas and consist primarily of negatively charged particles.

Microorganisms are the major agents mineralizing organic pollutants in terrestrial and aquatic environments (1), and their adsorption on soil colloids is of great importance. Previous studies have shown biodegradation of pollutants in the presence of either humic acids (2) or clays (11). However, no data exist on the effect of humic acid-clay complexes on the microbial degradation of hydrophobic pollutants. Hence, a study was conducted to compare rates of biodegradation of phenanthrene, a polycyclic aromatic hydrocarbon widely present in contaminated soils, in the presence of soil colloids and to reveal possible mechanisms by which microorganisms mineralize polycyclic aromatic hydrocarbons in soils.

The phenanthrene-degrading bacterium was isolated from a Cambisol soil (pH 6.4; 15.5% organic matter and 13.4% clay) from Guadalajara (Spain). A few grams of soil were incubated in 100 ml of an inorganic salts solution (pH 5.6) (whose composition was described previously [14]) containing 0.1 g of phenanthrene per ml. The enrichment culture was incubated for 2 weeks at 31°C on a rotary shaker operating at 120 rpm. After two serial transfers to fresh medium, the enrichment culture was streaked on plates containing 0.3% (wt/vol) Trypticase soy broth, 1.5% agar, and the inorganic salts solution. A pure culture was obtained from a single colony after incubation.

* Corresponding author. Mailing address: Instituto de Recursos Naturales y Agrobiologia, C.S.I.C., Apartado 1052, 41080 Seville, Spain. Phone: 345 462 47 11. Fax: 345 462 40 02. E-mail: jjortega@irnase.csic.es.

The bacterium was characterized by standard microbiological methods (8). The phenanthrene-utilizing isolate shows 0.9- by 1.2- to 2-μm rods, which are single or in pairs or chains. The isolate is nonmotile and showed no spores in 24 h. It is gram negative, weakly oxidase reaction positive, and catalase reaction positive. Ubiquinones Q-9, Q-8, and Q-10 in a ratio of 96:3:1 were found, which confirms that the isolate is gram negative. Ubiquinone Q-9 is a major compound in many *Pseudomonas* species, including *Pseudomonas fluorescens* (19). Biolog (Hayward, Calif.) identification indicated that the isolate was *P. fluorescens* type G, with a similarity of 0.798 after 24 h of incubation and 0.906 after 48 h of incubation. API (BioMérieux SA, Marcy l'Étoile, France) identified the isolate as *P. fluorescens* with a similarity of 0.942. These scores are very good and sufficiently high for acceptance of the identification result.

The bacterium was able to use phenanthrene as a sole carbon and energy source for growth and was maintained in phenanthrene-containing liquid medium identical to that used for enrichment culture.

To measure mineralization of phenanthrene in the presence of humic fractions and clay, 250 μl of dichloromethane with [9-^{14}C]phenanthrene (8.3 mCi/mmol; radiochemical purity, >98%; Sigma, Deisenhofen, Germany) and sufficient unlabeled phenanthrene to give a final concentration of 1.0 μg/ml were left to evaporate at the bottom of 250-ml Erlenmeyer flasks. The phenanthrene concentration used was below the aqueous solubility of the chemical, 1.3 μg/ml (16). Forty-five milliliters of sterile inorganic salts solution and 4 ml of a humic fraction solution and/or clay suspension were then added. The flasks were closed with Teflon-lined stoppers and equilibrated at 25°C for 2 h on a rotary shaker operating at 80 rpm. For the experiments, 1 ml of a suspension containing 3×10^6 phenanthrene-preconditioned bacteria was added and the flasks were incubated in darkness at 25°C on a rotary shaker (80 rpm). $^{14}CO_2$ production was measured as radioactivity appearing in an alkali trap (14). Radioactivity was measured with a liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.; model LS5000TD). The results are expressed as means of duplicate measurements. Maximum rates of mineralization were calculated as previously described (14). The acclimation phase was considered the length of time between the start of experiments and the onset of the phase of maximum mineral-

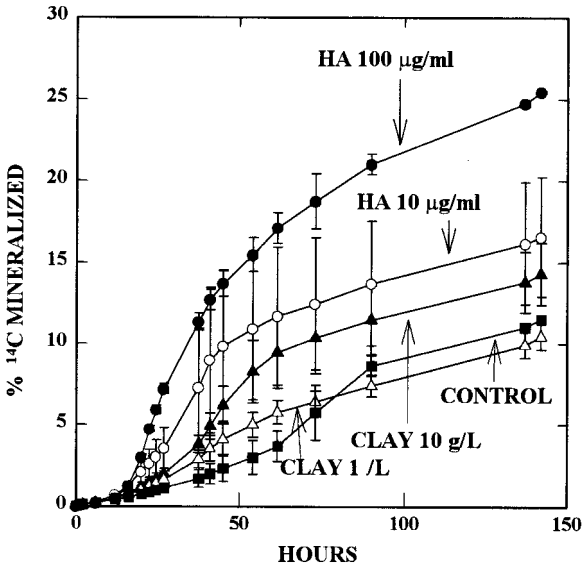

FIG. 1. Effect of different concentrations of dissolved humic acid (HA) and suspended montmorillonite (CLAY) on the mineralization of 1 μg of phenanthrene per ml by *P. fluorescens*. The error bars represent the standard deviations of duplicate experiments.

ization. Statistical analyses were performed at $P = 0.05$ with a *t* test.

Humic fractions were isolated from soils. Humic acid was extracted from a Typic Xerochrept soil, and fulvic acid was extracted from a Typic Xerorthent soil. A detailed description of methods and chemical characteristics of the samples has been published elsewhere (17). Prior to incubation, 1-mg/ml stock solutions of humic acid were prepared in 0.1 M NaOH, and the pH was adjusted to 6 with HCl. The desired concentrations were obtained by diluting the stock solution with sterile salt medium (pH 6). *P. fluorescens* was unable to use the isolated humic fractions as the sole source of carbon and energy for growth.

Sodium-montmorillonite from Crook County, Wyo. (Source Clay Minerals Repository, University of Missouri—Columbia, Columbia), was purified by gravity settling in deionized water to remove particles larger than 2 μm. The clay was saturated with 1 M $CaCl_2$, dialyzed to remove excess anions, and lyophilized. Clay suspensions were prepared aseptically by adding sterile salt medium to known amounts of dry clay.

The mineralization of phenanthrene in the presence of soil components is shown in Fig. 1. Humic acid stimulated the transformation at 100 μg/ml. The acclimation phase was shortened, and the maximum rates and extents of mineralization were statistically higher than those of the control. The effect of humic acid at 10 μg/ml was observed only in the length of the acclimation period. In this treatment, the maximum rates and extents of mineralization were not statistically different from those of the control. Mineralization in clay suspensions at both 10 and 1 g/liter was characterized by a shortening of the acclimation phase, although no statistically significant differences from the control were observed in maximum rates of mineralization.

Humic fractions (humic and fulvic acids) and clays often coexist in soil not as separate components but in a close relationship within the soil matrix. Therefore, the influence of the combination of different concentrations of humic fractions and clay on the biodegradation of phenanthrene was investigated. Stock solutions of fulvic acid were prepared by dissolving it with sterile mineral medium. Five-milliliter suspensions containing known amounts of humic fractions and montmorillonite were introduced into 10-ml screw-cap tubes and equilibrated overnight at 21°C on a rotary shaker operating at 150 rpm. These suspensions were then used for mineralization in the same way as clay suspensions in the procedure described above.

Figure 2 shows the mineralization of phenanthrene in the presence of different combinations of humic acid and clay concentrations. The maximum mineralization rates and extents were statistically higher than those of the control in the presence of 100 μg of humic acid per ml, irrespective of whether it was added together with 1 or 10 g of clay per liter. These treatments also shortened the acclimation phase in comparison with that of the control. The presence of 10 μg of humic acid per ml, with either 1 or 10 g of montmorillonite per liter, also caused an anticipation of the phase of maximum mineralization rate. However, during this phase, only with 10 g of clay per liter were the rates of transformation of phenanthrene to CO_2 statistically higher than those of the control. This was remarkable, because none of these components caused a significant stimulation at these concentrations when added separately (Fig. 1).

Figure 3 compares the mineralization of phenanthrene in the presence of dissolved humic fractions (100 μg/ml) with that in the presence of humic fractions sorbed to clay. The presence of 10 g of clay per liter induced a higher rate and extent of mineralization with both humic (Fig. 3A) and fulvic (Fig. 3B) acids. However, only with humic acid were the differences statistically significant.

Phenanthrene is a hydrophobic chemical, and this causes its strong tendency to sorb to soil surfaces when it is initially dissolved in the aqueous phase. A reduction in the water concentration due to sorption can modify the bioavailability of organic chemicals for microbial degradation (1). To assess whether sorption was occurring in our system, the aqueous concentration of phenanthrene in the aqueous phase at equi-

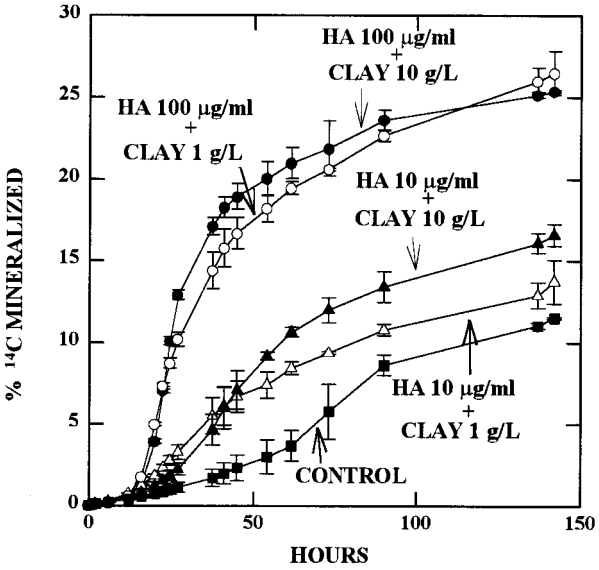

FIG. 2. Effect of combined concentrations of humic acid (HA) and montmorillonite (CLAY) on the mineralization of 1 μg of phenanthrene per ml by *P. fluorescens*. The error bars represent the standard deviations of duplicate experiments.

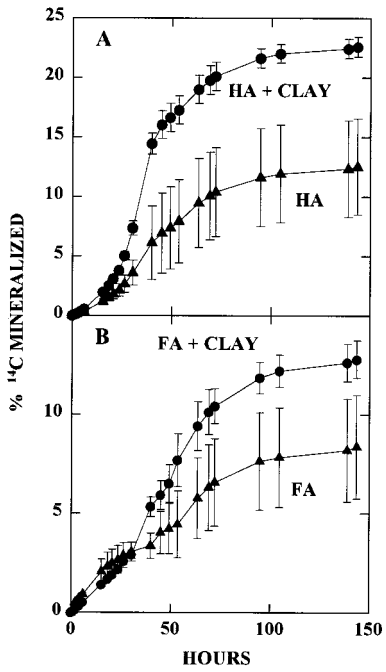

FIG. 3. Effect of humic fraction-clay complexes compared with that of dissolved humic fractions on the mineralization of 1 μg of phenanthrene per ml by *P. fluorescens*. HA, humic acid; FA, fulvic acid. Soil components were added to final concentrations of 100 μg/ml (humic fractions) and 10 g/liter (clay). The error bars represent the standard deviations of duplicate experiments.

librium was measured in humic acid solutions, clay suspensions, and controls identical to those used in mineralization experiments, but without bacteria. Aqueous phenanthrene concentration was measured in humic acid solutions by a reverse-phase separation technique (12). Six milliliters of the equilibrated solutions was passed through C_{18} Sep-Pak cartridges (Waters Associates), and the nonsorbed phenanthrene, which was retained in the cartridge, was subsequently eluted with dichloromethane and quantified by liquid scintillation counting. Aqueous concentrations in clay suspensions were measured by centrifugation and liquid scintillation counting of the supernatant. Equilibrated suspensions containing both humic acid and clay showed upon centrifugation a marked decrease in coloration of the supernatant, suggesting that most of the humic acid was sorbed onto clay. This fraction was quantified by the adsorbance at 285 nm of the supernatants after centrifugation. Controls without particles showed that humic acid remained in solution after centrifugation. This method was also used to measure the amount of humic acid adsorbed onto bacterial cells in suspensions with dissolved humic acid at 100 μg/ml. This fraction accounted for 20% of the humic acid initially in solution. The concentration of phenanthrene in the aqueous phase of suspensions of humic acid and clay was measured after centrifugation and passage of the supernatant through a Sep-Pak cartridge to discard the phenanthrene associated with humic acid in solution, i.e., not sorbed to the clay.

The results of sorption experiments are shown in Table 1. Humic acid in solution at 100 μg/ml sorbed a significant fraction of the phenanthrene initially dissolved in the aqueous phase. Sorption by humic acid was less significant at 10 μg/ml. The partition coefficient (K_{oc}) obtained was $10.5 \times 10^3 \pm 2.1 \times 10^3$ ml/g of C, slightly higher than that reported with Aldrich humic acid, 8.3×10^3 ml/g of C (12). Sorption to clay particles did not substantially cause a decrease in aqueous concentration of phenanthrene at 1 g/liter; however, at 10 g/liter the amount sorbed was half the amount initially in solution. In all cases, the combination of humic acid and clay caused a significant decrease in the aqueous concentration of phenanthrene compared to that of the control. Measurement of humic acid in solution after centrifugation revealed that 70% or more of the humic acid was sorbed to clay particles. Furthermore, these humic acid-coated clay particles contained the majority of phenanthrene sorbed in the system. Clay particles with sorbed humic acid caused higher sorption than that in suspensions containing only clay at 1 g/liter, whereas this effect was not evident at 10 g of clay per liter. The combination of 10 μg of humic acid per ml with 1 g of clay per liter caused a significant decrease in phenanthrene concentration in the water, whereas it did not occur when these components were added separately.

A plot of the data of maximum rates of mineralization versus equilibrium aqueous concentrations showed that the higher rates of mineralization observed with humic acid at 100 μg/ml (either alone or in combination with clay particles) in comparison to those of the control corresponded with lower aqueous-phase concentrations of phenanthrene (Fig. 4). However, humic acid at 10 μg/ml associated with clay caused comparable aqueous concentrations of substrate, but the rates of transformation were much lower. All these points were located well above the theoretical line which connects the control point and the origin, indicating that bacteria were consuming the sorbed compound (3). These observations suggest that the bioavailability of the compound sorbed to the humic acid-clay complexes was higher when more humic acid was sorbed to clay particles.

It has been reported that, in soils, primary particles combine into aggregates of varying size, and these aggregates are important factors in retarding soil organic matter decomposition. Also, soil humic fractions are mainly stabilized not as a result of their complex and recalcitrant structure but probably by association with metal ions and clays and aggregation (7). Evidence shown in this paper indicates that phenanthrene mineralization is enhanced in the presence of dissolved humic acids and humic acid-clay complex.

The mechanisms of the effects of these soil components on the transformation can be understood by postulating direct access by attachment of bacteria to the pool of sorbed compound. Soil microorganisms produce extracellular products which have been associated with the attachment of cells to surfaces (10). Therefore, it is suggested that direct bacterial contact with humic acid and montmorillonite particles facili-

TABLE 1. Distribution of soil humic acid and phenanthrene in suspensions and solutions with different concentrations of humic acid and clay[a]

HA concn (μg/ml)	Clay concn (g/liter)	HA sorbed to clay (μg/ml of suspension)	Phenanthrene concn	
			Sorbed to HA-clay particles (% of total sorbed)	In water (μg/ml)
0	0			1.00 ± 0.00
10	0			0.95 ± 0.01
100	0			0.64 ± 0.05
0	1			0.91 ± 0.01
0	10			0.50 ± 0.02
10	1	6.97 ± 0.00	97.8 ± 0.4	0.55 ± 0.08
10	10	7.72 ± 0.09	98.1 ± 0.1	0.43 ± 0.06
100	1	69.00 ± 1.30	86.7 ± 2.5	0.48 ± 0.03
100	10	87.20 ± 0.20	93.5 ± 1.7	0.46 ± 0.04

[a] Values are presented as means ± standard deviations. HA, humic acid.

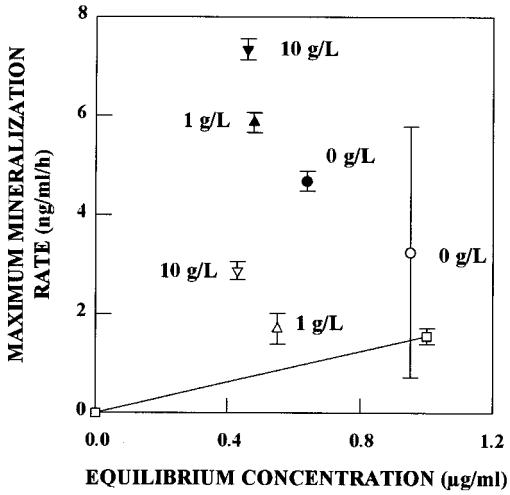

FIG. 4. Maximum rates of phenanthrene mineralization by *P. fluorescens* as a function of the concentration of phenanthrene measured in the aqueous phase at equilibrium. Symbols associated with numbers represent experiments with humic acid only (circles) or humic acid together with clay (triangles and inverted triangles). The numbers indicate the concentrations of clay added together with 10 (white symbols) and 100 (black symbols) μg of humic acid per ml. The diagonal line connects the point of the control (□) with the origin.

tated the biodegradation of the sorbed compound. The stimulation observed may be the result of an increased concentration of substrate in the vicinity of the bacterial cells, caused by the direct contact with humic acid and humic acid-clay complexes. Humic acids, which harbor both hydrophilic and hydrophobic moieties, play a key role in facilitating better access of microbes to phenanthrene sorbed to humic acid-clay complexes. Alternately, humic acid could have induced the production of enzymes involved in phenanthrene mineralization, a possibility that was not excluded by our results.

Increased pollutant transformation due to the presence of interfaces has been observed in the degradation of polycyclic aromatic hydrocarbons (9), phenol (4), and benzylamine (2, 18). An increased chemical potential of substrate for adhered bacteria has been suggested by Ortega-Calvo and Alexander (13), who studied mineralization of naphthalene, dissolved in non-aqueous-phase liquids, by bacteria present at the non-aqueous-phase liquid–water interface. The bacteria mineralized the substrate at a higher rate than that predicted by spontaneous partitioning, thus acting in a microenvironment different from that of the bulk aqueous phase. Similar observations were reported for a *Pseudomonas putida* strain able to degrade naphthalene sorbed to soil particles (6) and a surfactant-modified clay (3). Our results extend those findings and indicate that interactions between organic and inorganic soil constituents can influence the microbial degradation of hydrophobic pollutants.

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