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Bacterial Chemotaxis Enhances Naphthalene Degradation in a Heterogeneous Aqueous System

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Chemotaxis has the potential to enhance the bacterial degradation of organic pollutants in systems in which the pollutants are distributed heterogeneously. However, experimental evidence to confirm this potential has not been documented. In the present study, we evaluated the role of chemotaxis in naphthalene degradation by *Pseudomonas putida* G7 (*Pp*G7) in aqueous systems that supplied naphthalene from a glass capillary tube. Wild-type *Pp*G7 degraded naphthalene more rapidly than two mutant strains, one deficient in chemotaxis to naphthalene and the other deficient in motility. This result was not due to differences in inherent naphthalene degradation kinetics, as all three strains degraded naphthalene at similar rates in a well-mixed system. In the heterogeneous system, a 90% reduction in the amount of naphthalene initially present took 6 h with the wild-type *Pp*G7 at an initial concentration of 4×10^6 cfu/mL, while a similar reduction with either mutant strain at the same concentration took approximately 30 h. Only the systems inoculated with the wild-type strain exhibited degradation rates in excess of the rate of naphthalene diffusion from the capillary. This suggests that chemotaxis to a priority pollutant can overcome the mass transfer limitations that may govern biodegradation rates in a number of situations.

Introduction

Hydrophobic pollutants are often distributed heterogeneously in contaminated environments, generally by association with nonaqueous phases (*1, 2*). In such cases, any process that decreases the average distance between degrader organisms and the dissolved pollutant molecules is expected to improve the degradation rate (*3*). One such process may be bacterial chemotaxis. Chemotactic organisms can control their spatial distribution relative to that of a chemical attractant by moving toward higher concentrations of the compound. If the organism degrades the compound and is not inhibited by it, a higher concentration of the compound results in a more rapid degradation rate. By bringing organisms closer to sources of higher pollutant concentrations, chemotaxis could also increase the concentration gradient, the corresponding rate of mass transfer, and consequently the overall rate of biodegradation (*4*).

Between 3% and 5% of the bacterial genome in chemotactic bacteria is devoted to chemotaxis and motility (*5*), despite the energy cost associated with the expression and operation of these systems. Such costs are sufficiently great that nonflagellated mutants of *Escherichia coli* grow significantly faster than wild-type strains in mixed systems (*6*). There is evidence, however, that motility can confer an advantage to organisms due to improved access to substrate. Motile (*7, 8*) and flagellated (*9*) strains have exhibited more rapid growth in unmixed systems as compared to mutants lacking the respective characteristic. In a similar comparison using a chemotactic strain and its nonchemotactic mutant in an unmixed system, the growth rates were the same, but the nonchemotactic mutant stopped growing sooner than the wild type (*10*). Genetic evidence has also suggested that chemotaxis may be closely associated with substrate biodegradation (*11, 12*). Nonetheless, experimental observation of the direct involvement of chemotaxis in substrate degradation is lacking. The magnitude of such effects, if any, and the spatial scales over which they are manifested are not yet known. In a recent study on carbon tetrachloride degradation by a denitrifying organism in a laboratory column system (*13*), it was suggested that chemotaxis of the organism to nitrate enhanced the degradation of carbon tetrachloride. However, the rate of degradation was not compared to that of a nonmotile strain, so the extent to which the loss of substrate was attributable to either chemotactic or random motility is not known.

Pseudomonas putida G7 (*Pp*G7) has been shown to be chemotactic to naphthalene (*14*−*16*), a priority pollutant (*17*) found at abandoned manufactured-gas plants (*18*) and wood-treatment sites (*19*). In this study, we evaluated the role of chemotaxis in naphthalene degradation by *Pp*G7 in an aqueous system in which naphthalene concentration gradients were imposed. Naphthalene degradation by the wild-type strain was compared to that by a nonmotile mutant and by a mutant deficient in naphthalene chemotaxis. This work is a key step toward evaluating the potential role of chemotaxis in pollutant biodegradation in more complex systems.

Methods

Media. Tryptone broth and phosphate buffer were prepared as described previously (*15*). The media used to grow bacterial cells contained mineral salts (*12*) and 5 mM sodium salicylate. Saturated solutions of naphthalene in phosphate buffer were made by autoclaving 1 g of naphthalene (Aldrich, Milwaukee, WI) in a 20-mL (23 × 46 mm) glass vial that was sealed with a butyl rubber stopper and aluminum crimp top (National Scientific, Lawrenceville, GA). After the melted substrate recrystallized at room temperature, it was rinsed several times with methanol and distilled water to remove any fine crystals. Sterile phosphate buffer (2 mL) was added, allowed to soak the crystal for 16−24 h, and subsequently supplemented with chloramphenicol (Sigma-Aldrich, St. Louis, MO) at 10 μg/mL, a concentration that prevented growth of *Pp*G7. A 2.1-cm glass fiber microfilter (Whatman, Kent, England) was placed between the naphthalene crystal and buffer to prevent naphthalene flakes from entering the buffer.

Capillary Preparation. Capillaries were prepared by removing 8 mm from one end of a 10-μL microcapillary (Drummond Scientific, Broomall, PA) and flame-sealing the remaining piece at the broken end. The capillary was then placed on a small glass Petri dish that rested on a heated stir plate, which was set at the highest heat setting. After being heated for 15 min, the capillaries were immersed, open-side down, into the saturated naphthalene solution. The amount of naphthalene contained in each individual capillary was estimated from the length of the liquid plug.

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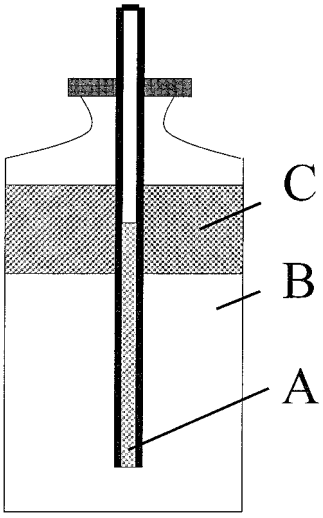

FIGURE 1. Apparatus for naphthalene degradation experiments in an unmixed, heterogeneous system. The 1-cm liquid plug (A) in the capillary initially contained a solution of naphthalene at aqueous saturation (218 μM). The capillary has a 0.56 mm diameter opening, and the liquid plug occupied a volume of approximately 2 μL. A 1-mL culture reservoir (B) initially contained all the bacterial cells, if any. A 0.5-mL layer of heptamethylnonane (C) trapped the nondegraded naphthalene in the culture reservoir and minimized losses due to volatilization.

Cultures. Wild-type *Pp*G7 and the mutant strain C1 (pHG100), which is nonchemotactic to naphthalene, were obtained from Caroline Harwood (University of Iowa). A nonmotile mutant strain, R2, was generated spontaneously in an overnight culture grown in tryptone broth. Strain R2 was isolated from a set of colonies that were tested for motility on a swarm plate (*20*) containing 5 mM salicylate. All three strains are stored cryogenically (−80 °C) in 1.5-mL aliquots of overnight cultures that were supplemented with dimethyl sulfoxide to 10% (v:v). Each strain was tested for chemotaxis and random motility in conventional capillary assays (*21*) as described previously (*15*). Initial cell concentrations in these assays were approximately 10^6 cfu/mL.

Overnight cultures were grown in tryptone broth (*15*), which was supplemented with 25 mg/mL tetracycline (Sigma-Aldrich, St. Louis, MO) for strain C1 (pHG100) only, from frozen stocks. The overnight cultures were then washed and grown in 5 mM salicylate to an optical density (590 nm) of 0.2−0.4. Cells were prepared for experiments by centrifuging and resuspending them in phosphate buffer (pH 6.8−7.0) that was supplemented with chloramphenicol (10 μg/mL).

Naphthalene Degradation Kinetics in a Mixed System. A 2-mL aliquot of culture (1.2−1.3 × 10^6 cfu/mL) was mixed in a cuvette with a small volume (<20 μL) of methanol containing dissolved naphthalene. The optical absorbance at 218 nm was monitored for 60 s to determine the initial rate of naphthalene removal (*22*). The rate was normalized by the cell concentration as measured by plate counts on R2A agar (Difco, Detroit, MI). Removal rates by controls (wild-type grown on 5 mM succinate or killed by acidification with 0.2% H_3PO_4) at a naphthalene concentration of 5 μM were less than 2% of that by live, salicylate-grown cells.

Naphthalene Degradation in a Heterogeneous System. Aliquots (1 mL) of culture were placed in 2-mL amber glass vials at a cell concentration of 4 × 10^6 cfu/mL. On top of each vial was placed a septum, which had been pierced with a 22-gauge syringe needle and into which a glass microcapillary containing a saturated naphthalene solution had been inserted (see Figure 1). With the mouth of the capillary immersed in the culture fluid, 500 μL of 2,2,4,4,6,8,8-heptamethylnonane (Sigma-Aldrich, St. Louis, MO) was carefully pipetted into the vial. The vial was then crimp-sealed.

At designated time points, triplicate vials were sacrificed by removing, breaking, and expelling the contents of the capillaries into 300-μL limited volume inserts (Laboratory Supply Distributors, Mt. Carmel, NJ) that each contained 200 μL of high-pressure liquid chromatography (HPLC) grade methanol (Sigma-Aldrich, St. Louis, MO). The crimp seal was then removed from each vial, and the vial was vortexed. About 200 μL of the heptamethylnonane layer was removed with a 100-μL glass melting-point capillary tube and placed into an empty insert. The samples were sealed in 4-mL HPLC vials and stored at −10 °C until the completion of the experiment.

HPLC Analysis. HPLC analysis was performed with a Waters (Milford, MA) 600E system using a Supelcosil LC18 column (25 × 0.46 cm, 5 μm media). Injections were 25 μL, and the mobile phase was 70:30 acetonitrile:water at 1 mL/min for 10 min. Naphthalene concentrations were quantified against external standards. For concentrations lower than the quantification limit (0.08 μM), a value of one-half the quantification limit was assigned. To calculate the percentage of naphthalene remaining in each system, the masses of naphthalene recovered in the vial and the capillary were compared to the mass of naphthalene estimated to be present initially in the capillary.

Model for Diffusion of Naphthalene from a Capillary. A model was developed to predict the loss of naphthalene from the capillary tubes due to diffusion alone. Applying Fick's second law (*23*) to the liquid plug in the capillary (A in Figure 1) yields

$$\frac{\partial C}{\partial t} = D\frac{\partial^2 C}{\partial y^2} \tag{1}$$

where C is the naphthalene concentration, D is the naphthalene diffusivity, y is the vertical coordinate, and t is time. The general solution to eq 1 is of the form (*24*):

$$C(y,t) = G + Hy + (I\cos\kappa y + J\sin\kappa y)\mathrm{e}^{-\kappa^2 Dt} \tag{2}$$

where G, H, I, J, and κ are constants. To determine the values of the constants, boundary and initial conditions were applied. The following boundary conditions hold if there is assumed to be no flux at the interior boundary ($y = 0$) and the concentration at the mouth ($y = -L$) is maintained at 0:

$$\frac{\partial C}{\partial y}(0,t) = 0 \tag{3}$$

$$C(-L,t) = 0 \tag{4}$$

The latter boundary condition (eq 4) has been shown to be approximately correct (*25, 26*) and reflects the fact that the solute diffuses from the capillary into a much larger volume. As the capillary is initially filled with a homogeneous solution of aqueous naphthalene at saturation (C_{sat}), the initial condition is

$$C(y,0) = C_{sat} \tag{5}$$

The constants in eq 2 were determined by applying eqs 3−5 sequentially. The resulting expression for the concentration of naphthalene, relative to saturation, at any time and point in the capillary is (*24*)

$$\frac{C(y,t)}{C_{sat}} = \sum_{n=1,3,5,\ldots}^{\infty} \frac{4}{n\pi}(-1)^{((n+3)/2)}\cos\left(\frac{n\pi y}{2L}\right)\mathrm{e}^{-(n\pi/2L)^2 Dt} \tag{6}$$

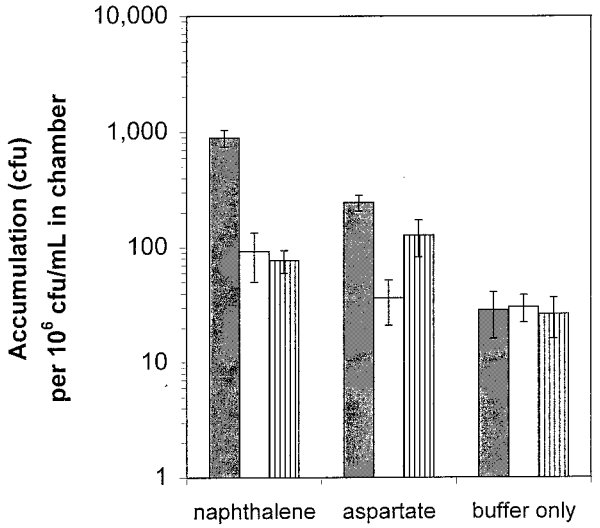

FIGURE 2. Capillary assay results for three strains of *Pseudomonas putida* G7. Assays were conducted to evaluate chemotaxis to naphthalene (present initially at its saturation concentration) and aspartate (1 mM) in comparison to accumulations of cells in capillaries containing buffer only. Accumulations in a 1-μL capillary are shown for 1-h experiments with the wild-type (solid bars), nonmotile (hollow bars), and nonchemotactic to naphthalene (striped bars) strains. Error bars represent the standard deviations of triplicate measurements.

For the experimental system shown as Figure 1, $L = 1$ cm and $D = 8.5 \times 10^{-6}$ cm^2/s (*15*). To predict the relative mass of naphthalene at any time point ($M_{cap}(t)$), the left side of eq 6 was integrated numerically over the length of the liquid plug by discretizing the length in 0.01-cm increments:

$$\frac{M_{cap}(t)}{M_{cap}(0)} = \frac{C_{cap}(t)}{C_{sat}} = \frac{1}{L}\sum_{i=1}^{100}\frac{C(y_i,t)}{C_{sat}}\Delta y \qquad (7)$$

Results and Discussion

Phenotype Characterization. To accurately determine the role of chemotaxis and motility in naphthalene biodegradation, it was necessary to confirm that each of the three strains of *Pp*G7 had the expected chemotaxis and motility phenotype. Each strain was tested with the standard capillary assay (*21*) for chemotaxis to naphthalene and aspartate (Figure 2). The wild-type strain accumulated in the naphthalene and aspartate capillaries at levels at least 10 times greater than comparable accumulations in the buffer-only capillaries, indicating that it responds chemotactically to both substrates. As expected, the accumulations of the wild-type in capillaries containing naphthalene were also an order of magnitude higher than those from either of the mutant strains. Strain C1 (pHG100), a mutant deficient in chemotaxis to naphthalene, responded chemotactically to aspartate nearly as well as the wild-type, but its response to naphthalene resembled that of the nonmotile mutant, R2. Motility phenotypes were confirmed by examination with a phase-contrast microscope.

Naphthalene Degradation Kinetics in a Mixed System. The three strains exhibited similar rates of naphthalene removal over a broad range of naphthalene concentrations under well-mixed conditions (Figure 3). This implies that the enzyme systems responsible for naphthalene removal in the mutant strains were unaffected by the mutations responsible for the respective motility phenotype. Therefore, any apparent differences in naphthalene degradation in an unmixed system would not be attributable to inherent differences in naphthalene degradation kinetics.

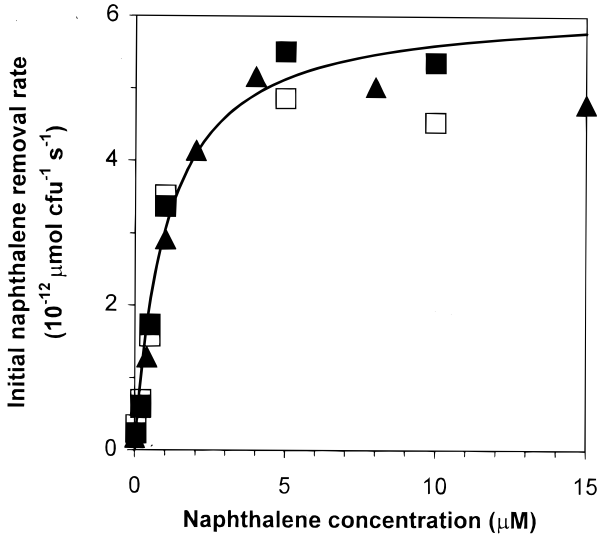

FIGURE 3. Initial specific rates of naphthalene removal as a function of naphthalene concentration. Average rates are shown for the wild-type *Pp*G7 (■), the nonmotile mutant (□), and the mutant that is nonchemotactic to naphthalene (▲). The curve represents the best fit of the Michaelis−Menten equation to data for the wild-type strain ($q_{max} = 6.2 \times 10^{-12}$ μmol cfu^{-1} s^{-1}, $K_S = 1.0$ μM).

Naphthalene Degradation in a Heterogeneous System. Naphthalene biodegradation in the heterogeneous system was measured by the loss of naphthalene over time, as shown in Figure 4. The loss of naphthalene from the capillary (Figure 4a) was essentially the same in systems containing the mutant strains as it was in the uninoculated control and matched the loss predicted by diffusion of naphthalene from the capillary. In contrast, at the 6-, 20-, and 30-h sample points, the losses from the capillaries in the systems inoculated with the chemotactic wild type were in excess of those seen for the mutant strains or the uninoculated systems as well as those predicted by diffusion alone.

In the uninoculated systems, most of the naphthalene from the capillary entered the culture reservoir over a period of approximately 20 h and was recovered in the heptamethylnonane layer (Figure 4b). The total amount of naphthalene detected in the uninoculated control was near 100% of that initially contained in the capillary over the duration of the 48-h experiment. Therefore, losses observed in the inoculated systems could not be attributed to abiotic processes. Naphthalene did not accumulate outside the capillary in any of the inoculated systems (Figure 4b). This suggests that any naphthalene entering the culture reservoir was immediately metabolized in such systems, so that an influence of chemotaxis was not manifested in this domain.

Systems inoculated with either mutant strain showed a rate of loss of naphthalene equal to the rate at which naphthalene diffused from the capillary. Hence, naphthalene biodegradation was limited by mass transfer in those systems. In contrast, the rate of naphthalene loss from the systems that were inoculated with the wild-type strain exceeded the mass transfer rate. The total naphthalene recovered in the wild-type system (not shown) was significantly lower than in the other two inoculated systems at 6 h (one tailed *t*-test assuming equal variance, $p \leq 0.001$), 20 h ($p \leq 0.01$), and 30 h ($p \leq 0.1$). Furthermore, a loss of 90% of the naphthalene originally present required only 6 h with the wild-type strain, while a similar loss in systems inoculated with the mutant strains required 30 h. These data provide direct evidence that chemotaxis can enhance the rate of degradation of an organic pollutant when it is distributed heterogeneously in an aqueous system.

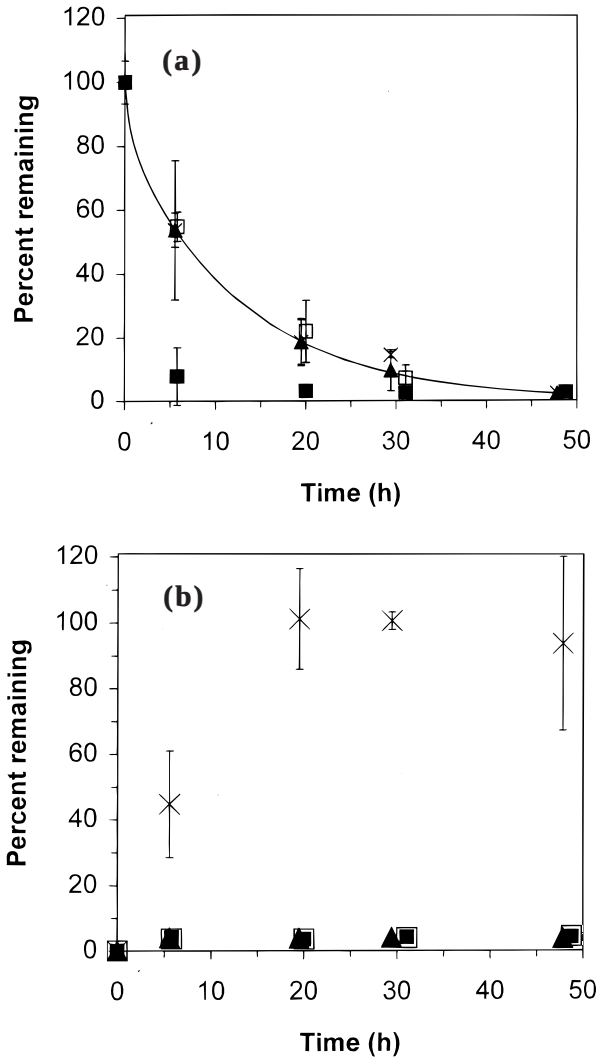

FIGURE 4. Naphthalene removal in an unmixed, heterogeneous system. The amount of naphthalene recovered relative to the amount initially present in the capillary (approximately 4.6 × 10^{-4} μmol) is shown as a function of time in the capillary (a) and the culture reservoir (b) in systems containing wild-type *Pp*G7 (■), the nonmotile mutant (□), and the mutant deficient in naphthalene chemotaxis (▲). All cell concentrations in the reservoir were 4 × 10^6 cfu/mL. Data from uninoculated controls (×) are also shown. The curve in panel a represents the predicted concentration of naphthalene in the capillary due to diffusion only, according to eq 7.

While the results shown in Figure 2 indicate that chemotaxis can lead to a dramatic increase in the number of cells that enter a capillary tube containing naphthalene, those results do not imply that cells traveled any significant distance within the tube. Using a mathematical model of the traditional capillary assay system, we recently illustrated that chemotactic cells should form a band of high cell concentration that moves further into the tube with time (*16*). Such a phenomenon probably explains the enhanced removal of naphthalene from the capillaries by the wild-type strain.

It was not expected that the rate of naphthalene loss would be equal at all times in systems inoculated with the motile but nonchemotactic strain as compared to those inoculated with the nonmotile strain. Experimental evidence has demonstrated that motile bacterial cells can diffuse more rapidly than nonmotile cells (*27*), although two different species were compared. A strain that diffuses more rapidly into the capillary would have better access to the capillary contents and therefore would be expected to degrade the substrate more rapidly for a given initial cell concentration and substrate removal kinetics. In this study, however, where strains of the same species were compared, the bacterial diffusion rate (as indicated by the accumulation of cells in the capillaries containing buffer only, Figure 2), was equal for all the strains tested. Combined with the results of the naphthalene biodegradation experiment, it appears that random motility did not enhance the net diffusion of bacteria to a significant extent over the time scales, spatial scales, and cell concentrations evaluated in this study.

Our experimental data on naphthalene degradation in the heterogeneous system illustrate the importance of the design of the experimental system in evaluating a potential role of chemotaxis in such systems. If naphthalene entered the culture reservoir more rapidly than cells approached and entered the capillary tube, chemotaxis would not have enhanced the rate of naphthalene biodegradation. Therefore, the effect of chemotaxis on substrate biodegradation depends on relative rates of substrate mass transfer and bacterial motion. The fact that the movement of naphthalene was slow relative to that of the chemotactic cells is attributed to the observation that diffusion appeared to control the movement of naphthalene in the capillary system. More rapid processes, such as convection or partial mixing, could dominate the mass transfer of naphthalene in some systems of interest. However, we have demonstrated that bacterial chemotaxis can influence biodegradation in systems characterized by small-scale heterogeneity in contaminant distribution and by a localized lack of mixing. Such conditions may be relevant to contaminated subsurface environments, although the influence of chemotaxis in porous media remains to be examined.

Acknowledgments

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