

Chemotaxis in polycyclic aromatic hydrocarbon-degrading bacteria isolated from coal-tar- and oil-polluted rhizospheres

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Received 11 November 2002; received in revised form 5 March 2003; accepted 28 March 2003

First published online 16 April 2003

Abstract

The limited mass transfer in polycyclic aromatic hydrocarbon (PAH)-contaminated soils during bioremediation treatments often impedes the achievement of regulatory decontamination end-points. Little is known about bioavailability of these hydrophobic pollutants in phytoremediation systems. This work attempts to evaluate, for the first time, chemotaxis as a bioavailability-promoting trait in PAH-degrading bacteria from the rhizosphere. For this aim, 20 motile strains capable of degrading different PAHs were isolated from rhizosphere soils contaminated with coal tar and oil. Three representative *Pseudomonas* strains were selected, on the basis of their faster growth and/or range of PAHs degraded, for detailed chemotaxis studies with PAHs (naphthalene, phenanthrene, anthracene, and pyrene), bacterial lipopolysaccharide and root exudates from seven different plants. The chemotactic response was quantified with a new densitometric method. The results indicate that chemotaxis is a relevant mobilizing factor for PAH-degrading rhizosphere bacteria. © 2003 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Polycyclic aromatic hydrocarbon; Chemotaxis; Rhizosphere; Bioremediation; Bioavailability

1. Introduction

Phytoremediation, or the use of plants and/or associated rhizosphere to decontaminate polluted sites, is considered today a realistic, low-cost alternative to treating extensive areas of pollution by organic chemicals [1]. This technology is based on the catabolic potential of root-associated microorganisms, which are supported by the organic substrates in root excretions and by a favorable micro-environment in the rhizosphere. Soils polluted by polycyclic aromatic hydrocarbons (PAHs) are suitable for treatment by phytoremediation, since several scientific studies, performed with well-designed controls, have specifically shown higher rates of PAH biodegradation in whole soils planted with a variety of species. From these studies a stimulatory effect of the rhizosphere on pollutant dissipation has been inferred [2–5]. However, whereas the ecology of plant–microorganism interactions is very well known in cases such as legume–*Rhizobium* systems [6], there is very

limited knowledge regarding plants and microbes active in phytoremediation of PAH-polluted sites.

Biodegradation of PAHs in soils is often limited by the slow mass transfer of these hydrophobic compounds towards degrading microbes. This slow process may lead to bioavailability restrictions, even in the conditions of massive contamination often faced by bioremediation technologies. Little is known about bioavailability in phytoremediation systems. Specific bioavailability-promoting mechanisms, operating in soils with PAH-degrading populations, may be responsible for increased rates of pollutant transformation. These include an increased bacterial adherence to the pollutants [7,8], and production of biosurfactants by bacteria [9,10] or by plants [11]. The possible role of chemotaxis in the bioavailability of PAHs to the degrading microbial populations has so far remained relatively unexplored. It is conceivable, however, that the capability of moving along chemical gradients through soil may allow chemotactic PAH-degrading bacteria to increase net mass transfer rates.

Whereas chemotaxis is a well-known phenomenon in most bacteria with flagellar motility, the ecological significance of this process has been determined only rarely. There is plenty of evidence for chemotaxis as a virulence

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factor in the intestinal and urogenital tract pathogens *Vibrio cholerae* and *Salmonella* species [12]. Chemotaxis can also play a relevant ecological role in plant-associated bacteria, such as *Rhizobium* and *Agrobacterium* species, by increasing their efficiency for root colonization [13,14]. In addition, recent results with the biodegradation of carbon tetrachloride [15] and naphthalene [16] have shown the potential of chemotaxis to enhance biodegradation in laboratory-scale microcosms. Indeed, chemotaxis has already been studied in bacteria able to degrade a wide variety of organic pollutants, such as naphthalene [17], BTEX compounds [18] and pesticides [19]. However, no chemotactic bacteria have so far been described with more hydrophobic pollutants, such as high-molecular-mass PAHs. Therefore, our aim was to study the chemotaxis of PAH-degrading microorganisms present in polluted rhizosphere soils. Root exudates, PAHs, and bacterial lipopolysaccharide (LPS) were chosen as representative chemoeffectors, because of their possible relevance for the microbial ecology of phytoremediated, PAH-polluted sites.

2. Materials and methods

2.1. Isolation and characterization of PAH-degrading bacteria from polluted rhizospheres

PAH-degrading bacteria were isolated from soil in close contact with the roots of plants growing on creosote- and oil-polluted lands. The crops and locations, sampled during the vegetation season of the year 2000, included alfalfa (*Medicago sativa* growing on coal-tar-contaminated soil in Rybinsk, Russia, and on oil-products-contaminated soil in Serpukhov, Russia), white clover (*Trifolium album*; coal tar; Kemerovo, Russia), red clover (*Trifolium pratense*; coal tar; Makeyevka, Ukraine), pea (*Pisum arvense*; oil products; Novokuybyshevsk, Russia), wheat (*Triticum aestivum*; coal tar; Volgograd region, Russia), oats (*Avena sativa*; coal tar; Donetsk, Ukraine), and meadow fescue (*Festuca pratensis*; oil products; Kapotnya, Russia). Samples were placed in sterile vessels and stored in the dark at 4–8°C for no more than 24 h until use.

The plants were removed from the PAH-polluted soil and shaken to remove soil loosely adhering to the roots. The roots, with the remaining adherent soil, were separated from the shoots and transferred to 50-ml screw-cap test tubes containing 20 ml of 0.12 M phosphate-buffered saline solutions. The tubes were shaken with a vortex mixer for 10 s to dislodge the rhizosphere soil. The resulting suspension was filtered through Whatman no. 3 filter paper, and all the roots were removed. These filtered suspensions of rhizosphere soil were used as a source of microorganisms for enrichment cultures. Twenty-milliliter tubes containing 6 ml of liquid L9 medium inoculated with the suspensions and supplemented with 0.05% (w/v) crystals of naphthalene, phenanthrene, anthracene or py-

rene were incubated on a rotary shaker operating at 180 rpm and 25°C. The composition of L9 medium was (per liter of distilled water): 8.8 g of $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 3.0 g of KH_2PO_4 , 0.5 g of NaCl, 1.0 g of NH_4Cl , 1.0 ml of 1 M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 2.5 ml of microelements solution (containing, per liter, 23 mg of $\text{MnCl}_2 \cdot 2\text{H}_2\text{O}$, 30 mg of $\text{MnCl}_4 \cdot \text{H}_2\text{O}$, 31 mg of H_3BO_3 , 36 mg of $\text{CoCl}_2 \cdot \text{H}_2\text{O}$, 10 mg of $\text{CuCl}_2 \cdot \text{H}_2\text{O}$, 20 mg of $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 30 mg of $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ and 50 mg of ZnCl_2). The medium was adjusted to pH 7.0. Incubation periods ranged from 7 days to 2 months. Pure PAH-degrading strains were obtained by plating dilutions of the enrichments on agar plates with rich medium (L9+0.5% fish fermentative hydrolysate). The capability of isolated strains for transformation of PAHs was tested with a modification of Kiyohara's method: isolated colonies were transferred to solid L9 medium whose surface was previously sprayed with a 2% PAH solution in acetone:hexane (1:1 v/v), after evaporating the organic solvent. PAH utilization was followed by the formation of dissolution halos in the PAH coating. The isolated PAH-degrading microorganisms were identified using standard microbiological methods [20]. The characteristics that were determined included Gram staining, morphology, sporulation, catalase, cytochrome oxidase, β -galactosidase, urease activities, ability to dilute gelatin, nitrification reaction (NO_3/NO_2), denitrification, capability of formation of H_2S , acetone or indole, ability for hydrolysis of esculin or arginine, assimilation of glucose, arabinose, mannose, maltose, *N*-acetyl-glucosamine, mannitol, gluconate, capronate, adipate, malate, citrate, Simmone's citrate or phenylacetate, and decomposition to acids of saccharose, glucose, arabinose, mannose, maltose, fructose, rhamnose, galactose, sorbitol, mannitol and glycerol. Stock cultures of each strain were stored at –20°C in 30% (v/v) glycerol until use.

2.2. Determination of PAH-degrading capability

The capability of a selected set of strains for PAH metabolism was studied in liquid culture. Bacterial cells were grown in liquid L9 medium with addition of those PAHs from which they were isolated. The grown cultures were filtered through cotton wool to eliminate PAH crystals and washed twice with 0.01 M MgSO_4 . Cells were resuspended with L9 medium and diluted to an optical density of 0.3 at 590 nm. Microbial cell suspension (0.5 ml) was added into glass tubes containing 2.5 ml of L9 medium and the corresponding PAH at a final concentration of 20 mg l^{-1} . The test compound had been added to the bottom of the tubes as a solution in hexane, which was left to evaporate before the addition of the medium. After a 5-day incubation on a shaker at 28°C in the dark, the remaining amount of PAHs was extracted twice with hexane and determined by gas chromatography. Culture liquors both with inactivated microbial cells and without cells were taken as controls. Chemical analysis of in-

dividual PAHs was performed on a 'Crystal 5.000' gas chromatograph, equipped with a flame ionizing detector and a Supelco capillary column (PTE-5, 30 m \times 0.25 mm i.d., 0.25 μ m film). Carrier: helium at 1.15 ml min⁻¹ (29.0 cm s⁻¹); oven: 70°C (2 min) to 300°C (8°C min⁻¹), 300°C (3 min; split ratio: 60/1; injection: 2 μ l, 250°C; detector: FID, 310°C). Quantitative data were obtained by the external standard method with standard PAH solutions (Supelco).

2.3. Preparation of chemoeffector solutions: PAH, LPS and root exudates

Solutions of PAH were prepared by saturating chemotaxis buffer (6.1 mM K₂HPO₄, 3.9 mM KH₂PO₄ and 20 μ M EDTA; pH 7.0) with excess crystals for several days, and passing them through polycarbonate membrane filters (0.45 μ m) to remove the remaining solid PAHs. These solutions were diluted from 1:1 to 1:100 (in chemotaxis buffer) for chemotaxis determinations. The PAHs used have the following aqueous solubilities (mg l⁻¹): naphthalene, 30.00; phenanthrene, 1.29; anthracene, 0.07; pyrene, 0.14 [21].

LPS from cells of *Pseudomonas putida* 10D was prepared by a water-based extraction method used to obtain bacterial capsules [22,23]. Frozen stock cultures were transferred onto agar plates with rich medium (L9+0.5% fish fermentative hydrolysate). After incubation for 24 h at 28°C, cells were taken from the nutrient agar plate and inoculated into 300-ml Erlenmeyer flasks containing 100 ml of liquid rich medium. The flasks were incubated overnight on a rotary shaker (150 rpm, 28°C) to obtain bacterial cultures in the late exponential phase of growth. Cells were then centrifuged at 10 000 $\times g$ for 20 min at 4°C, and resuspended in 50 ml of water. The cell suspension, containing 5 \times 10¹² colony-forming units (CFU), was vigorously stirred in a boiling water bath for 30 min and then cooled in an ice bath with stirring for another 90 min, after which the cell residue was removed by centrifugation (10 000 $\times g$, for 30 min at 4°C). The supernatant was adjusted to 1% acetic acid, and crude polysaccharides were precipitated with 2.5 volumes of ethanol for 24 h at 20°C. After centrifugation (10 000 $\times g$, for 30 min at 4°C), the precipitate was dried, dissolved in 1.0 ml of nuclease buffer (0.01 M Tris pH 7.8, 10 mM MgCl₂), and incubated for 16 h at 37°C with DNase (2 μ g ml⁻¹) and RNase I (10 μ g ml⁻¹). The sample was adjusted to include 0.5% sodium dodecyl sulfate for incubation with proteinase K (50 μ g ml⁻¹) for 16 h at 42°C. A phenol–chloroform mixture (1:1 v/v) was used to remove hydrolytic enzymes, and LPS in the aqueous phase collected after centrifugation was precipitated with 2.5 volumes of ethanol for 16 h at 20°C. The precipitate was centrifuged (10 000 $\times g$, for 10 min at 4°C) and resuspended in 200 μ l of water. This solution was diluted with enough volume of chemotaxis buffer to give a final concentration of 10 μ g ml⁻¹ in the chemoeffector

solution. Approximately 1.8 mg of LPS was recovered from 2 l of culture broth.

To prepare root exudates, seeds of wheat, oats, pea, alfalfa, white clover, red clover, and meadow fescue were surface-sterilized. Seeds were incubated in 96% ethanol (1 min), washed three times in demineralized water, incubated in 3% H₂O₂ solution (30 min), and washed again in demineralized water. Surface-sterilized seeds were aseptically transferred to sterile Petri dishes containing Whatman paper soaked with demineralized water. The plates were incubated at 25°C and assessed for bacterial contaminants after 48–72 h. When the seeds had germinated and the roots reached the plant culture solution, the seedlings were transferred aseptically to sterile tubes containing 2 g of perlite and 20 ml of M9 medium. The tubes were placed under a light bank (30 W m⁻²) with a 16-h photoperiod and were incubated at 20°C and 80% relative humidity for 8–10 days. Root exudates were aseptically recovered from soaked perlite by suction under vacuum and checked for bacterial contaminants. All exudates were sterilized by membrane filtration (pore size 0.45 μ m) and stored at –20°C until use for capillary assays. Then, 1:1 dilutions were prepared with a chemotaxis buffer solution and were used as chemoeffector solutions. These dilutions were arbitrary and were not intended to reflect any natural situation.

2.4. Chemotaxis assays

2.4.1. Densitometry chemotaxis assay

The selected strains were cultured in 50-ml screw-cap tubes containing 20 ml of L9 medium and 0.05% (w/v) naphthalene as growth substrate, maintained at 25°C on a rotary shaker operating at 180 rpm. Microbial cultures from the middle exponential growth phase were centrifuged at 5000 $\times g$ for 10 min, washed three times with potassium phosphate buffer solution (pH 7.0), and resuspended in the same solution to give a final cell concentration of 10⁹ cells ml⁻¹ (OD₅₉₀ = 0.3). These suspensions were stored at 4°C for no more than 24 h before chemotactic examination. Each densitometric chamber (a 5-ml quartz cuvette) was then filled completely with chemotactic buffer solution (5 ml) that contained 50 μ l of the chemoeffector solution. The bacterial suspension (200 μ l) was added at the bottom of the chamber by micropipette. Chambers with only chemotactic buffer solution were used as a control. The distribution of bacterial cells in the densitometric chamber was followed at 28°C after different time intervals with a densitometer (2222-010 Uktroscan XL, LKB Producter AB, Bromma, Sweden).

On the basis of the densitograms obtained, the speed (V) of the bacterial front was calculated from the formula:

$$V = (X_n - X_1) / (t_n - t_1) \text{ [mm h}^{-1}\text{]}$$

where X_n and X_1 are the position of the bacterial front at time moments t_n and t_1 , respectively. Experiments were

repeated no fewer than three times. To facilitate comparison between experiments having differences in control cell velocities, the ratio of the speed of the bacterial front in the experimental assay (with chemoeffector) to the speed of the bacterial front of the buffer controls (chemotaxis index, CI) was also calculated:

$$CI = V_{\text{exp}}/V_{\text{control}}$$

where V_{exp} and V_{control} are, respectively, the speed in the presence of chemoeffector solution and in the control with buffer. A CI value of 1 indicates no response; a value greater than 1 indicates positive chemotaxis; and a value less than 1 indicates negative chemotaxis. Relative error in CI determinations ranged from 11 to 20%. Statistical com-

parisons with control values were performed with a *t*-test at $P = 0.95$.

2.4.2. Capillary chemotaxis assay

The density of bacterial suspensions, prepared as already stated for densitometry assays, was brought up to approximately 5×10^7 cells ml^{-1} . The assays, which followed the classical capillary method [24], were performed by placing a few drops of these suspensions in a small chamber comprising a U-shaped glass tube between a microscope and coverslip. The chemotactic response was measured by placing the open end of a 1- μl capillary tube containing the chemoeffector solution in the pool of bacterial cells present in the chamber.

Table 1
Characterization and identification of chemotactic PAH-degrading bacteria isolated from polluted rhizospheres

Characteristic	Strain		
	8A	9A	10D
Origin ^a	Oats, coal-tar, Donetsk, Ukraine	Alfalfa, coal-tar, Rybinsk, Russia	White clover, coal-tar, Kemerovo, Russia
Gram	—	—	—
Morphology	rods	rods	rods
Motility	+	+	+
Oxidase	+	+	+
Catalase	+	+	+
Fluorescent diffusible pigment	—	—	+
Diffusible non-fluorescent pigments	—	—	—
Non-diffusible non-fluorescent pigments	—	—	—
Poly- β -hydroxybutyrate accumulation	—	—	—
Extracellular poly- β -hydroxybutyrate hydrolysis	—	—	—
Autotrophic growth with H_2	—	—	—
Growth at 41°C	+	+	—
Growth at 4°C	ND ^b	ND	+
Organic growth factors required	—	—	—
Levan formation from sucrose	—	—	—
Arginine dihydrolase	+	—	+
Denitrification	—	+	—
Gelatin hydrolysis	+	—	—
Starch hydrolysis	—	+	—
Utilization of:			
Glucose	—	+	+
Trehalose	—	—	—
2-Ketogluconate	—	—	+
Meso-inositol	—	—	—
Geraniol	—	—	—
L-Valine	—	+	+
β -Alanine	+	—	+
L-Arginine	+	—	+
Nitrate (nitrogen source)	+	+	+
Naphthalene	+	+	+
Phenanthrene	+	+	+
Anthracene	—	+	—
Fluoranthene	+	—	+
Pyrene	—	—	+
Lecithinase	—	—	—
Ortho cleavage catechol	ND	+	+
Ortho cleavage protocatechuate	ND	ND	+
Meta cleavage protocatechuate	ND	ND	—
Identification	<i>P. alcaligenes</i>	<i>P. stutzeri</i>	<i>P. putida</i>

^aSamples from which the strain was isolated, indicating crop, type of pollution and location sampled.

^bND, not determined.

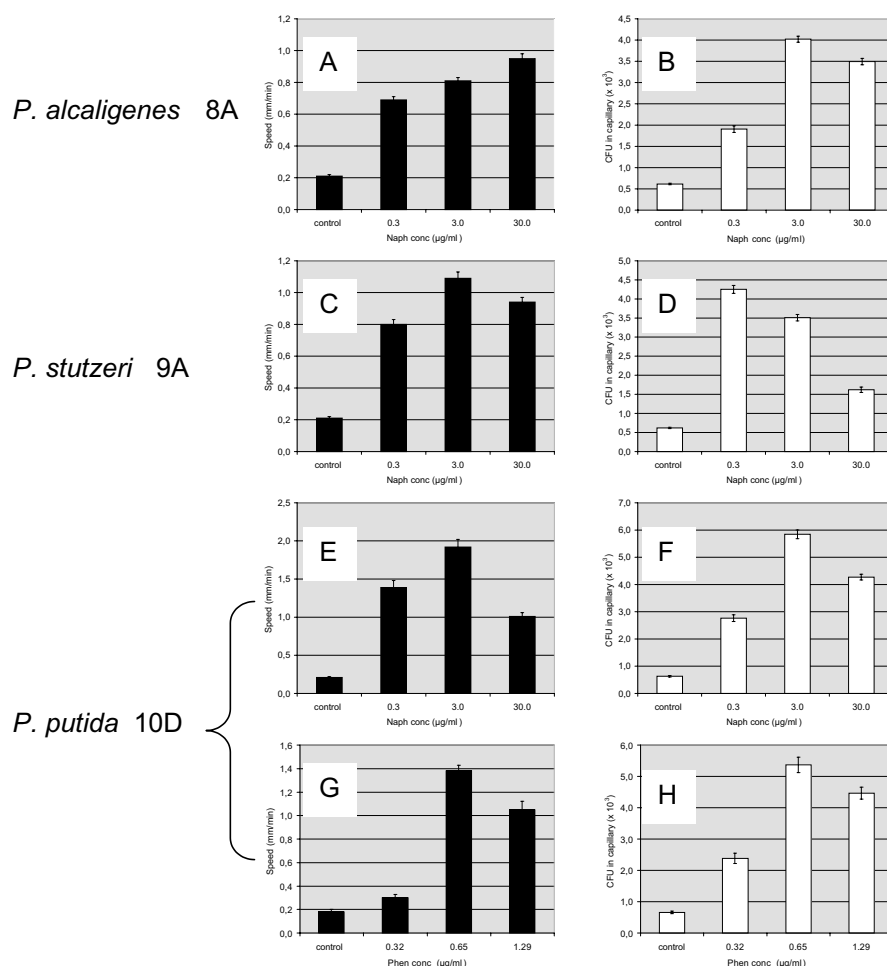


Fig. 1. Chemotaxis towards PAHs of the rhizosphere, PAH-degrading strains *P. alcaligenes* 8A (towards naphthalene – Naph), *P. stutzeri* 9A (naphthalene), and *P. putida* 10D (naphthalene and phenanthrene – Phen). A, C, E and G correspond to results from densitometric assays, and B, D, F and H to capillary assays. Error bars represent S.E.M.

After incubation for 30 min at 30°C, the contents of the capillaries were transferred to tubes of L9 mineral medium. Appropriate dilutions were prepared, and then 0.1-ml samples were spread on plates of *Pseudomonas* agar. Colonies were counted after the plates had been incubated at 30°C for 16–24 h. Results are based on averages of triplicate plate counts in each of two separate capillary assays, and are expressed as the number of cells per capillary. In all experiments, a blank (no chemoeffector present) was included. The relative error in the determinations by capillary assay was less than 20%.

3. Results

3.1. Isolation and characterization of motile PAH-degrader strains

Seventy-one bacteria that were able to grow in media with PAHs as the sole source of carbon and energy were isolated from rhizosphere soil samples originating from

PAH-polluted soils. A collection was made up of 20 axenic cultures, which were selected on the basis of their active motility, as revealed by phase contrast microscopy. They were assigned to the following genera: *Pseudomonas* (nine strains), *Alcaligenes* (five), *Azospirillum* (one), *Xanthomonas* (four), and *Arthobacter* (one). Assessment of the PAH-catabolic range of these strains through the modified Kiyohara method revealed that most of them could use naphthalene and phenanthrene as the sole source of carbon and energy, whereas the less water-soluble compounds anthracene, pyrene, and fluoranthene were used as growth substrates by a much smaller number of strains. In fact, only three anthracene, five pyrene and three fluoranthene degraders were isolated. The capability of growth with these three PAHs did not coexist in any single strain. Three different *Pseudomonas* strains were selected, on the basis of their faster growth and/or range of PAH degraded, for a more detailed analysis. They were identified as *Pseudomonas alcaligenes* 8A, *Pseudomonas stutzeri* 9A, and *Pseudomonas putida* 10D. A description of each isolate is given in Table 1.

3.2. Chemotaxis towards PAHs, LPS and root exudates

The three selected strains were capable of responding chemotactically to naphthalene, being attracted by this PAH at the three concentrations tested, in both capillary and densitometric assays (Fig. 1A–F). Quantitative results obtained with the two methods were, with few exceptions, comparable. Chemotaxis, as detected in capillary assays, resulted in higher CI values, when compared with the control, than in densitometric assays. The new densitometric technique, in addition, provided quantitative data for motion rate on the chemical gradients tested. For example, the maximum speed shown in the presence of naphthalene ranged from 0.95 mm min^{-1} for *P. alcaligenes* 8A to 1.8 mm min^{-1} for *P. putida* 10D. The maximum naphthalene concentration tested ($30 \mu\text{g ml}^{-1}$) was inhibitory for *P. stutzeri* 9A and *P. putida* 10D, according to both methods, and for *P. alcaligenes* 8A in capillary tests. The same pattern of chemotaxis inhibition was seen with *P. putida* 10D against phenanthrene, which was also a growth substrate for this strain (Fig. 1G,H). In this case, however, the maximum concentration of phenanthrene tested was lower than that of naphthalene (1.29 instead of $30 \mu\text{g ml}^{-1}$), due to its lower solubility in water. In fact, a very similar maximum migration speed was detected with both PAHs, in spite of the differences in PAH concentration (1.8 mm min^{-1} for $3.0 \mu\text{g ml}^{-1}$ naphthalene and 1.35 mm min^{-1} for $0.65 \mu\text{g ml}^{-1}$ phenanthrene). No significant chemotactic attraction was observed when these three strains were tested for chemotaxis towards the other PAHs supporting their respective growth.

Chemotaxis was further studied in capillary assays involving other possible chemoeffectors that did not support growth. These included bacterial LPS (Fig. 2A) and anthracene (Fig. 2B) with *P. putida* 10D, and pyrene with *P. alcaligenes* 8A (Fig. 2C). The strain *P. putida* 10D was clearly attracted towards LPS isolated from the same strain, and this bacterial component still promoted chemotaxis in the presence of $30 \mu\text{g ml}^{-1}$ naphthalene. Anthracene, however, induced a negative chemotactic response in this strain, in spite of the low aqueous concentrations of the chemoeffector (at the ng ml^{-1} level). A negative chemotactic response was also observed with *P. alcaligenes* 8A towards pyrene, and this response was also inhibited, as was positive chemotaxis towards naphthalene and phenanthrene, at the maximum concentration tested ($0.14 \mu\text{g ml}^{-1}$). Similar results were obtained with densitometric assays (data not shown).

The three strains were also tested for chemotaxis towards root exudates of different Gramineae (wheat, oat and meadow fescue) and legumes (alfalfa, pea, white clover and red clover) in densitometric and capillary assays (Fig. 3). Again, both methods gave, in general, comparable results. The magnitude of the chemotactic response (i.e., the number of cells accumulated in capillaries and the rate of cell migration) was similar to that observed

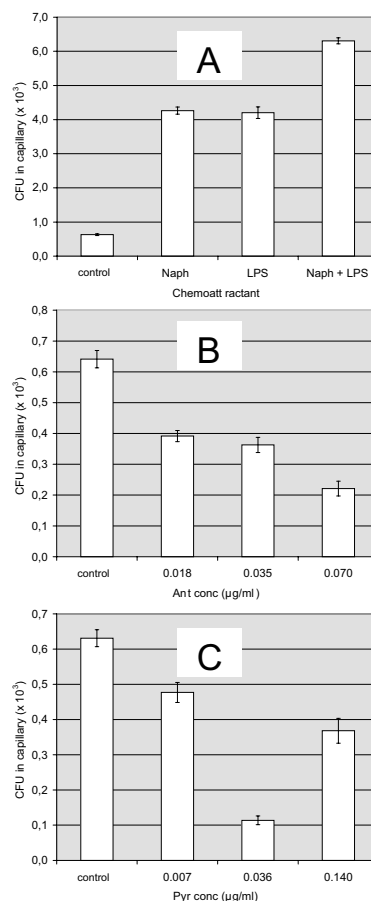


Fig. 2. Chemotaxis (capillary assays) towards PAHs of the rhizosphere, PAH-degrading strains *P. putida* 10D and *P. alcaligenes* 8A. A: Chemotaxis towards bacterial LPS (at $10 \mu\text{g ml}^{-1}$) and naphthalene (Naph – $30 \mu\text{g ml}^{-1}$) in strain 10D. B: Negative chemotaxis of strain 10D towards anthracene (Ant). C: Negative chemotaxis of strain 8A towards pyrene (Pyr). Error bars represent S.E.M.

in experiments with PAHs (Figs. 1 and 2). It is difficult to explain the causes for the few discrepancies observed here between the two methods. Thus *P. alcaligenes* 8A (towards meadow fescue) and *P. putida* 10D (towards oats and meadow fescue) were attracted in capillary assays, but were repelled in densitometric determinations. These dissimilar responses may be related to the way in which the chemical gradient was created, or to its rate, and reflect the sensitivity of the process to small changes introduced by experimental procedures, as already stated by other authors [25].

In spite of these difficulties, the new densitometric method was a useful tool to estimate quantitatively the mobilization potential of chemotaxis for rhizosphere bacteria. Therefore, the study with root exudates by densitometry was extended to the rest of the collection of motile bacteria. As a result, a significant fraction of the 20 strains showed positive chemotaxis to extracts from wheat (15 strains), oats (12), alfalfa (12), white clover (12), and red clover (11). Bacteria attracted to root exudates moved at a rate ranging from 0.38 to 0.68 mm min^{-1} , while the pos-

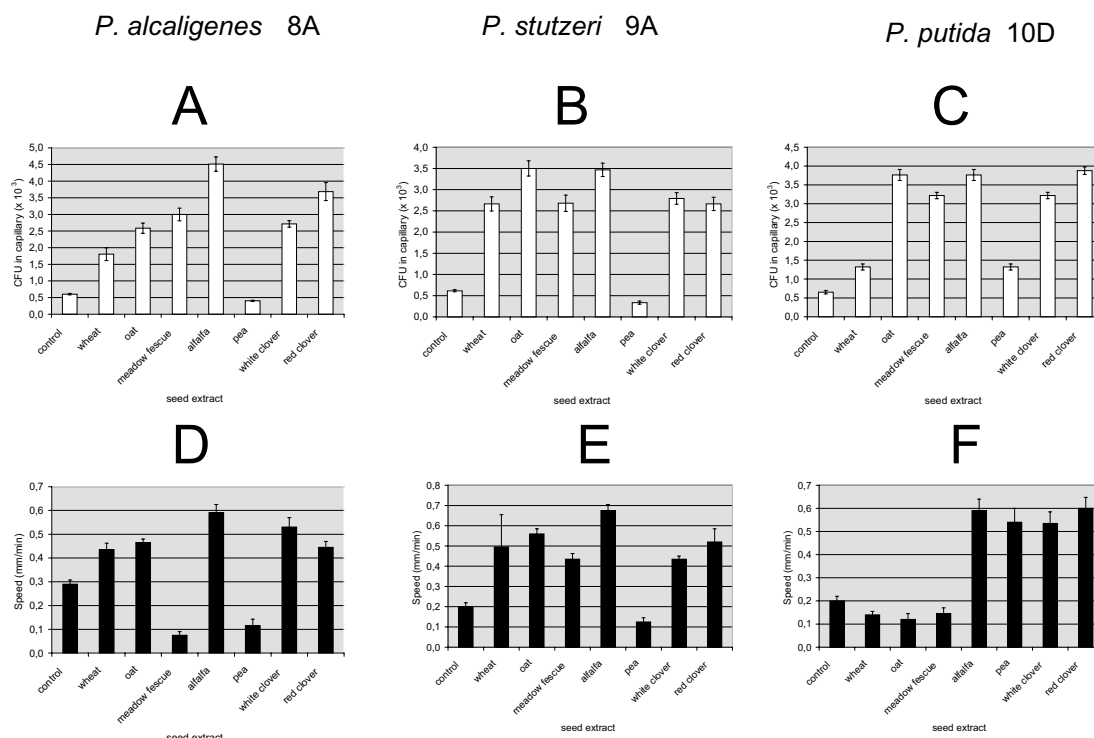


Fig. 3. Chemotaxis towards root exudates of the rhizosphere, PAH-degrading strains *P. alcaligenes* 8A, *P. stutzeri* 9A, and *P. putida* 10D. A–C correspond to results from capillary assays, and D–F to densitometric assays. Error bars represent S.E.M.

itive CI values ranged from 1.27 to 3.39. The extracts from meadow fescue and pea predominantly repelled bacterial cells (i.e., their CI was significantly lower than 1), with only six and seven strains, respectively, showing positive chemotaxis. The results showed that the chemoeffector properties of the exudates were, to a great degree, specific for each strain. No clear relationship could be observed between these strain-specific spectra and the plant dominating the sample from which they were isolated.

4. Discussion

During phytoremediation of PAH-polluted soil, positive chemotaxis towards PAHs, bacterial components such as LPS and root exudates may play a stimulatory role in biodegradation due to the existence of a favorable niche for microorganisms around the plant root. Indeed, plants can induce the mobilization of PAHs in soil, leading to their accumulation (as a more bioavailable form) in zones close to the roots, possibly by the combined effect of water flow towards the roots and the pseudosolubilization of PAHs by plant-derived organic compounds [11]. Other studies have determined an enhanced PAH biodegradation rate and/or an enrichment of PAH-degrading populations in the rhizosphere. For example, enhanced rates of phenanthrene biodegradation and increased numbers of phenanthrene degraders were observed in soil zones close to the roots of slender oats (*Avena barbata*), as compared with unplanted bulk soil controls [26]. A similar result

was obtained in another study with rye grass (*Lolium perenne*), which found higher numbers of PAH degraders (phenanthrene, anthracene, fluorene, and fluoranthene) and higher PAH biodegradation rates in the rhizospheric than in non-rhizospheric soil after a period of pollutant aging [27].

The evidence provided in our study for chemotactic motion rates of these soil bacteria towards PAHs in the order of 1 mm min^{-1} is remarkable, considering the average size of a microbial cell (approximately $1 \mu\text{m}$ in length), and the typical average distance between individual microbial colonies in soil ($100 \mu\text{m}$, for a population density of 10^7 – $10^8 \text{ cells g}^{-1}$ [28]). For a PAH-polluted soil undergoing bioremediation, this suggests that chemotactic PAH-degrading bacteria may be able to access in a few seconds a significant fraction of the aqueous volume surrounding bacterial colonies, and eventually detect distant PAH concentration gradients created by desorption from soil particles. This capability may mean an advantage in terms of biodegradation, considering that the aqueous concentrations of these hydrophobic pollutants available to microorganisms are usually low, even in heavily polluted soils, explaining the common occurrence in soil of first-order kinetics of biodegradation [29]. The net result of chemotactic attraction towards PAHs would therefore be an increase in their bioavailability and, as a consequence, biodegradation rates, as has already been shown for naphthalene biodegradation in a study with a diffusion-limited aqueous system [16].

Bacteria can be attracted to a metabolizable substrate

not only by direct chemotactic sensing but also as a response to higher cell energy levels, the so-called energy taxis [30]. Moreover, a repellence reaction can also appear as a result of slower movement due to chemical inhibition. It is difficult, on the basis of these results, to determine the exact contribution of these unspecific influences of the compounds on bacterial motility to the detected chemotactic response towards PAHs. However, the occurrence of chemotaxis in our system is supported by the marked differences with appropriate controls observed using the two independent methods to measure the chemotactic reaction, which usually suggests direct chemotactic sensing. These findings also agree with those from previous studies (on chemotaxis towards aromatic organic pollutants, such as naphthalene, toluene and 2,4-dichlorophenoxyacetate) which establish a natural link between chemotaxis and biodegradation by showing the inducible nature of the chemotactic response and the occurrence of a common regulation of chemoreceptor and biodegradation genes [25].

Chemotaxis seems, as revealed by its widespread occurrence in the isolated strains, an important mobilizing factor for microbes in PAH-polluted rhizospheres. Active chemotactic transport induced by root exudates, LPS, or PAHs themselves may increase the rates of pollutant dissipation by soil microbial populations. It is conceivable, therefore, that a possible way to optimize PAH phytoremediation is the use of selected plants and microbial inoculants with specific chemotactic affinities.

Acknowledgements

Support for this research was provided by the International Science and Technology Center (ISTC Grant 1429), the European Union (Contract QLRT-1999-00326) and Spanish CICYT (BIO2000-1857-CE).

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