Chemotaxis of *Pseudomonas* spp. to the Polyaromatic Hydrocarbon Naphthalene

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Two naphthalene-degrading bacteria, *Pseudomonas putida* G7 and *Pseudomonas* sp. strain NCIB 9816-4, were chemotactically attracted to naphthalene in drop assays and modified capillary assays. Growth on naphthalene or salicylate induced the chemotactic response. *P. putida* G7 was also chemotactic to biphenyl; other polyaromatic hydrocarbons that were tested did not appear to be chemoattractants for either *Pseudomonas* strain. Strains that were cured of the naphthalene degradation plasmid were not attracted to naphthalene.

The bacterial degradation of a number of toxic organic compounds, including toluene, naphthalene, and chlorinated biphenyls, has been extensively studied. Many of the enzymes involved in the degradation of these compounds have been purified, and the genes encoding these proteins have been cloned and sequenced (17, 22, 24). Although the actual pathways of catabolism are well-known, an aspect of degradation that has been overlooked is chemotaxis. Chemotaxis enhances the ability of motile bacteria to locate and degrade low concentrations of organic compounds, and it is reasonable to expect that it also directs the movement of motile bacteria to toxic, but metabolizable, compounds present in contaminated environments. Naphthalene is a priority pollutant commonly found in industrial effluents and is a constituent of coal tar (23). It is often used as a model compound for studies of in situ biodegradation of polyaromatic hydrocarbons (PAHs) because it is relatively easily degraded by bacteria (26). Naphthalene's relatively high solubility compared to those of other PAHs (15) and the fact that the naphthalene degradation genes are plasmid encoded (4, 5, 8, 16, 18, 21, 26–28) have contributed to a rapid pace of laboratory research on naphthalene degradation. In this study we examined the abilities of two *Pseudomonas* strains to respond chemotactically to naphthalene and other PAHs in two chemotaxis assays.

The naphthalene-degrading strains used, *Pseudomonas putida* G7 (4) and *Pseudomonas* sp. strain NCIB 9816-4 (20), as well as their naphthalene degradation plasmid-cured derivatives *P. putida* G7.C1 (18, 26) and *Pseudomonas* sp. strain 9816-4 C84 (19), were obtained from D. T. Gibson of the University of Iowa. All strains were motile by means of polar flagella. Cells were grown in a basal mineral salt medium (11) at 30°C with shaking at 250 rpm. All compounds used as growth substrates or tested as possible attractants in chemotaxis assays were obtained from Sigma Chemical Co. (St. Louis, Mo.). Salicylate and succinate were used as growth substrates at final concentrations of 5 and 10 mM, respectively. Naphthalene was provided as a carbon source by direct addition of naphthalene crystals to liquid basal medium.

Chemotaxis was tested with a drop assay (6) and a modified capillary assay. For the drop assay, 40 ml of cells in the logarithmic phase of growth were harvested and resuspended in 12 ml of chemotaxis buffer (100 mM potassium phosphate [pH 7.0], 20 μM EDTA). For strain 9816-4, 10 mM succinate was added to the chemotaxis buffer as an energy source, since this strain's motility decreased rapidly in unamended buffer. A 1% aqueous solution of hydroxypropylmethylcellulose (formulated to give a viscosity of about 4,000 cP in 2% aqueous solution) (Sigma Chemical Co.) was added to the cell suspension to give a final volume of 15 ml. The viscous cell suspension was then layered on the bottoms of 60-mm-diameter petri dishes to a depth of about 3 mm. A small amount of a test attractant was added to the center of a dish either as crystals, for the poorly soluble PAHs, or as 10-μl drops of 500 mM salicylate, a 10% Casamino Acids solution, or chemotaxis buffer. A chemotactic response of cells to the added compound resulted in the formation of a ring of turbidity near the center of the petri dish after about an hour. To photograph drop assays, petri dishes were placed on a transparent plastic sheet with supports and black paper was placed under the supports.

The second chemotaxis assay, developed as a modification of the classical capillary assay (1), allowed qualitative assessment of chemotaxis with a phase-contrast microscope. Cells grown to mid-logarithmic phase were harvested and resuspended in chemotaxis buffer (strain G7) or chemotaxis buffer with 10 mM succinate (strain 9816-4) to an A_{660} of approximately 0.1. The suspension of motile cells was placed in a small chamber formed by placing a U-shaped glass tube between a microscope slide and coverslip. A heat-sealed 1-μl capillary tube was filled with 100 mM potassium phosphate buffer (pH 7.0), and then the open end was packed with finely ground crystals of naphthalene or another insoluble aromatic compound by pressing the capillary tube into a mound of crystals. The phosphate buffer was drawn in first to avoid the formation of air pockets around the naphthalene crystals, as this could result in aerotaxis. Phosphate buffer agar capillaries were prepared by drawing 1.5% molten Noble agar (Difco, Detroit, Mich.) dissolved in 100 mM potassium phosphate into a heated capillary as described by Masduki et al. (14). A capillary was then inserted into a chemotaxis chamber that had been placed on a microscope stage. A dark background was achieved by setting the optical objective at ×10 magnification and the phase-contrast ring at the setting normally used for ×100 magnification. The tip of the capillary was brought into focus and photographed at appropriate times. A positive chemotactic response was visualized by the accumulation of a cloud of motile cells around the mouth of the capillary tube over a period of about 10 min.

P. putida G7 and *Pseudomonas* sp. strain 9816-4 cells were motile when grown with naphthalene as a sole source of carbon

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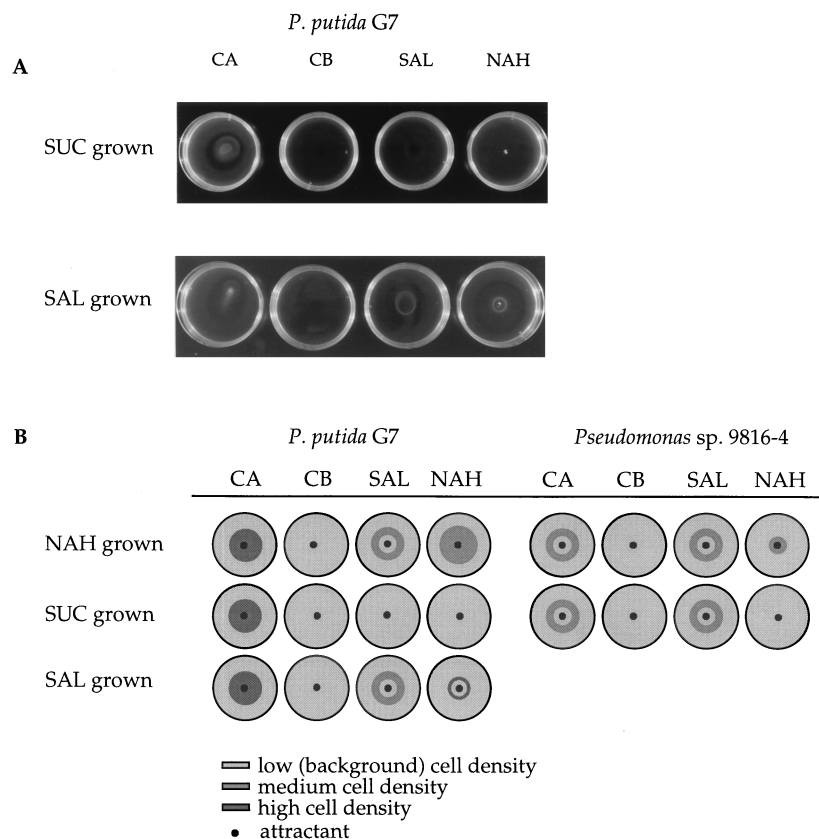
FIG. 1. Chemotactic responses of *Pseudomonas* strains to naphthalene and salicylate in drop assays. (A) Photographs of drop assay plates showing responses of *P. putida* G7 to test compounds. (B) Diagram of drop assay plates. Drawings were used because often only a small amount of contrast was visible between the accumulated cells and the background. Note that naphthalene-grown cells of strain G7 form a broader and more diffuse ring around naphthalene than do salicylate-grown cells. Abbreviations: CA, 10% Casamino Acids; CB, chemotaxis buffer; SAL, salicylate; NAH, naphthalene; SUC, succinate.

and energy. Cells that were tested for chemotaxis with the drop assay formed a turbid ring around a crystal of naphthalene or a drop of salicylate within an hour of the addition of these compounds to the center of a petri dish. The cells also responded to 10% Casamino Acids but did not exhibit a visible response to an added drop of chemotaxis buffer (Fig. 1). In the modified capillary assay, naphthalene-grown cells of both *P. putida* G7 and strain 9816-4 accumulated around the mouths of 1-μl capillary tubes that had been filled with naphthalene crystals (Fig. 2). The motility of cells near the tips of the capillaries appeared equal to or greater than those far away from the naphthalene crystals. Cells did not accumulate around the

mouths of tubes that contained only phosphate buffer or phosphate buffer agar (data not shown).

P. putida G7 cells that were grown on salicylate, an intermediate of naphthalene degradation, formed a much denser ring of turbidity around the site of naphthalene addition than did naphthalene-grown cells in drop assays (Fig. 1). Succinate-grown cells of strain G7 did not respond to naphthalene and had only a weak response to salicylate (Fig. 1). Similar results were seen in the capillary assay (data not shown). These results indicate that salicylate, the compound that directly induces naphthalene degradation (2), is also an inducer of naphthalene chemotaxis.
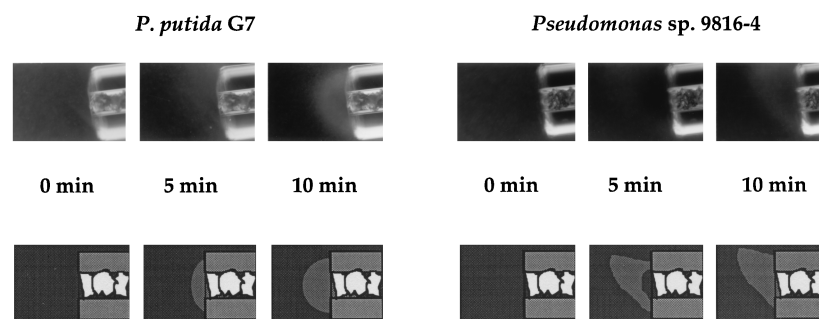

FIG. 2. Chemotactic responses of *Pseudomonas* strains to naphthalene in modified capillary assays. Cells were grown on naphthalene. The results are shown in both photographic (top) and illustrative (bottom) form. Crystals of naphthalene are visible inside the mouths of the capillary tubes.
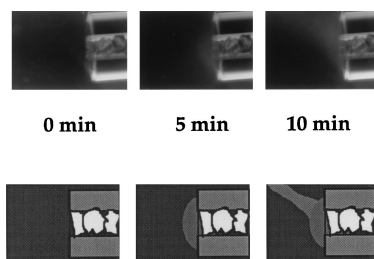
FIG. 3. Chemotactic response of *P. putida* G7 to biphenyl in a modified capillary assay. Cells were grown on naphthalene. The results are shown in both photographic (top) and illustrative (bottom) form.

Pseudomonas sp. strain 9816-4 cells grown on succinate had little to no response to naphthalene, indicating that this strain also has an inducible response to this PAH (Fig. 1). Cells grown on 5 mM salicylate alone had very poor motility, as viewed microscopically, and so were not tested in chemotaxis assays. However, 9816-4 cells grown with 5 mM benzoate and 0.5 mM salicylate were motile and attracted to both naphthalene and salicylate (data not shown). In contrast to the situation with naphthalene, succinate-grown cells had a good response to salicylate (Fig. 1). This suggests that chemotaxis to salicylate may be a constitutive property of strain 9816-4.

Anthracene, biphenyl, carbazole, dibenzothiophene, fluorene, and phenanthrene were also tested as possible chemoattractants for strains G7 and 9816-4. None of these compounds can serve as the sole carbon and energy sources for these strains. Drop assays were performed with naphthalene-grown cells, and modified capillary assays were carried out with naphthalene- and salicylate-grown cells of strain G7 and naphthalene-grown cells of 9816-4. Of the compounds tested, only biphenyl elicited a visible chemotactic response, and only from strain G7 (Fig. 3). Salicylate-grown cells responded more strongly to biphenyl in the modified capillary assay than did cells that were grown on naphthalene. The drop assay was not sufficiently sensitive to detect biphenyl chemotaxis by strain G7. Typically, we found the modified capillary assay to be more sensitive to weak responses. The lack of a detectable response to the other PAHs may be due to their low solubilities relative to that of biphenyl. The aqueous solubilities of naphthalene and biphenyl are 250 and 45 μM, respectively, whereas the solubilities of the other compounds tested were on the order of 7 μM or less (15).

Attempts to measure naphthalene chemotaxis with a quantitative capillary assay similar to that described previously for use with *P. putida* (10) were not successful. Cells did not consistently accumulate to significant numbers above background in capillaries filled with a solution of naphthalene in chemotaxis buffer up to 250 μM, the maximum solubility of naphthalene. Although a higher concentration of dissolved naphtha-
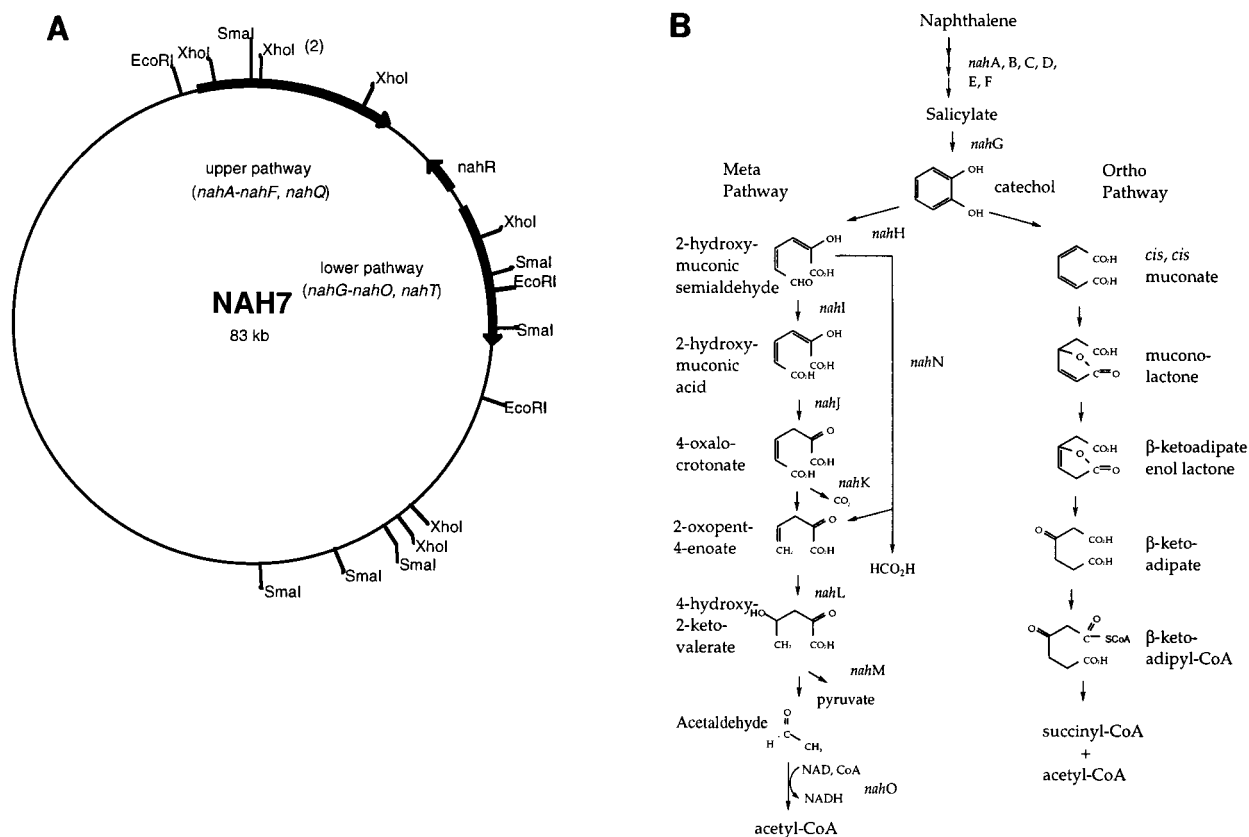

FIG. 4. Naphthalene catabolic plasmid and degradation pathway. (A) NAH7 catabolic plasmid (5, 16, 26). The region of the plasmid that encodes naphthalene degradation genes is shown in bold. The *Eco*RI sites in or around the naphthalene degradation pathway are shown. The segment of DNA from the *Eco*RI site just outside the upper pathway genes to the *Eco*RI site inside the lower pathway genes was subcloned to form plasmid pHG100. Arrows indicate the direction of transcription. (B) Naphthalene degradation pathway (16, 26). The *nah* genes that specify the degradation of naphthalene to pyruvate and acetyl-coenzyme A (CoA) are encoded on the NAH7 plasmid. Chromosomally encoded genes for the degradation of catechol by an *ortho* cleavage pathway are also present in all *P. putida* strains (12).

lene can be achieved in solvents, some of those tried were difficult to work with because of their volatility (acetone and dimethylformamide), and others either were strong attractants and growth substrates themselves (ethanol) or were repellents for *P. putida* G7 (isopropanol). The repellent and attractant effects of the solvents could have masked a measurable chemotactic response to naphthalene.

Naphthalene degradation is encoded by the catabolic plasmids NAH7, in *P. putida* G7 (25), and pDTG1, in *Pseudomonas* sp. strain 9816-4 (20). To determine whether naphthalene chemotaxis was plasmid associated, we tested the plasmid-cured derivatives *P. putida* G7.C1 and *Pseudomonas* sp. strain 9816-4 C84 with the drop assay. The wild-type and cured versions of each strain were grown on 5 mM benzoate and 0.5 mM salicylate (all strains were motile under these conditions). Whereas both wild-type strains formed a ring of turbidity around added naphthalene crystals in the drop assay, neither of the plasmid-cured strains responded, indicating that naphthalene chemotaxis is a plasmid-encoded trait. It is possible that chemotaxis is an intrinsic property of naphthalene degradation. For example, a flux in energy generation that accompanies naphthalene metabolism could signal a chemotactic response. Alternatively, additional genes may be needed for cells to be specifically chemotactic to naphthalene. To distinguish between these possibilities, we subcloned a 25-kb *Eco*RI fragment from the 83-kb NAH7 plasmid (Fig. 4) into the broad-host-range vector pLAFR1 (7) to generate pHG100. The cloned *Eco*RI fragment appears to include all of the naphthalene pathway genes except *nahK*, *nahM*, and *nahO* (9, 26). The enzymes encoded by the cloned genes can convert naphthalene or salicylate to a late intermediate of salicylate degradation, presumably 4-oxalocrotonate (Fig. 4). Conjugative transfer of pHG100 to *P. putida* G7.C1 resulted in the generation of a strain that grew with naphthalene as a sole source of carbon and energy, probably by using chromosomally encoded *ortho* pathway genes to degrade catechol, generated as an intermediate of naphthalene degradation. Naphthalene has been shown enter cells by diffusion (3), so a gene for naphthalene transport is probably not necessary. This strain, however, was not attracted to naphthalene in either drop assays or capillary assays (data not shown), indicating that naphthalene degradation alone is not sufficient for attraction to naphthalene. Genes in addition to those on pHG100 must be required for naphthalene chemotaxis, and such genes are presumably encoded by the NAH7 plasmid. The plasmid may encode a chemoreceptor protein that recognizes naphthalene or a metabolite of naphthalene, or it might encode a regulatory protein that is specifically required for expression of plasmid- or chromosome-encoded chemotaxis genes. It is also possible that enzymes of the lower portion of the *meta* pathway are somehow required for naphthalene chemotaxis.

This description of naphthalene chemotaxis expands the repertoire of known bacterial chemoattractants to include a U.S. Environmental Protection Agency priority pollutant (13). This report also sets the stage for further work aimed at elucidating the molecular basis for naphthalene chemotaxis. A membrane protein from *P. putida* PRS2000 that appears to function as a chemoreceptor for the aromatic acid 4-hydroxybenzoate has been described (11). It will be interesting to see if a structurally related protein is responsible for naphthalene chemotaxis. Bioremediation is a promising approach to treating environments contaminated with toxic organic compounds such as naphthalene. An eventual goal will be to determine whether bacterial chemotaxis plays a role in accelerating the rate of biodegradation of toxic wastes at contaminated sites.

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