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Chemotaxis of *Pseudomonas putida* toward Chlorinated Benzoates

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The chlorinated aromatic acids 3-chlorobenzoate and 4-chlorobenzoate are chemoattractants for *Pseudomonas putida* PRS2000. These compounds are detected by a chromosomally encoded chemotactic response to benzoate which is inducible by β-ketoadipate, an intermediate of benzoate catabolism. Plasmid pAC27, encoding enzymes for 3-chlorobenzoate degradation, does not appear to carry genes for chemotaxis toward chlorinated compounds.

Aromatic compounds occur naturally, principally as the major monomeric component of plant lignin (12). They are also prevalent in the biosphere as products of human industrial activities (6). Toxic chlorinated aromatic compounds, which are used as herbicides, pesticides, and solvents, figure prominently in this second category. Bacteria from several genera have been isolated that can degrade selected chlorinated aromatics (5, 16, 17), often by utilizing genetic information borne on transmissible plasmids (4, 7). However, most chlorinated compounds are recalcitrant to biodegradation, and the construction and introduction into the environment of bacterial strains with improved degradative abilities is one strategy that has been proposed to alleviate environmental pollution (13).

The success of such an approach depends not only on the improved catabolic abilities of the constructed strains, but also on their ability to compete with indigenous microflora. One trait that may be important in this regard is chemotaxis, the ability of motile bacteria to sense and swim towards organic compounds. Behavioral sensing of aromatic compounds may give cells a competitive advantage in natural environments by enabling them to locate low concentrations of compounds that can be used as growth substrates. This could be especially important in situations in which pollutants have become dispersed throughout a wide area.

We have noted that 3-chlorobenzoate is a chemoattractant for *Pseudomonas putida* PRS2000 (9). This strain cannot, however, metabolize 3-chlorobenzoate; it does not harbor any plasmids and apparently lacks any chromosomally encoded ability to catabolize chlorinated compounds. In the present study, we have examined the physiological basis for the behavioral responses of PRS2000 to chlorinated aromatics. Also, we determined that the transmissible plasmid pAC27, encoding enzymes for the degradation of 3-chlorobenzoate, does not appear to carry genes for chemotaxis toward chlorinated compounds.

Attraction to 3-chlorobenzoate. Chemotactic responses to 3-chlorobenzoate were assayed by two methods. The quantitative capillary assay measures the accumulation of cells in a microcapillary tube in response to a spatial gradient of attractant that forms as a chemical diffuses from the capillary mouth (1, 11). Cells grown in defined mineral medium with 5 mM benzoate as the sole carbon and energy source (15) showed a concentration-dependent response to 3-chlorobenzoate. Cells grown on glucose (10 mM) did not respond to 3-chlorobenzoate (Fig. 1).

Behavioral responses of cells were also measured with a temporal chemotaxis assay. With this method, alterations in the swimming behavior of cells that occur in response to a temporal addition of chemical are analyzed by computer-assisted motion analysis (10). This system analyzes the swimming paths of individual cells, and behavior is assessed quantitatively in terms of the average number of changes of direction of swimming per second for a population of cells. A high value (above 0.4 change of direction per second, for most strains) reflects random swimming behavior, while a low value (below 0.3 change of direction per second) corresponds to chemotactic stimulation (10). *P. putida* cells that were grown with 5 mM 4-hydroxybenzoate and suspended in chemotaxis buffer (50 mM potassium phosphate [pH 7.0], 10 μm EDTA) showed a drastic modification of swimming behavior upon addition of 3-chlorobenzoate. Cells changed direction much less frequently and swam smoothly for prolonged periods. As in the capillary assay, the response to temporal additions of 3-chlorobenzoate was concentration dependent. The smooth-swimming response was maximal at 500 μM 3-chlorobenzoate and was not detectable at 3-chlorobenzoate concentrations below about 50 μM (Fig. 2).

3-Chlorobenzoate is a member of the benzoate group of chemoattractants. A benzoate chemotaxis system which recognizes benzoate, 4-hydroxybenzoate, 3- and 4-toluate, and salicylate has been defined in *P. putida* (10, 11). β-Ketoadipate, an intermediate in the chromosomally encoded pathway of benzoate and 4-hydroxybenzoate catabolism by PRS2000, and adipate, its nonmetabolizable analog, induce benzoate chemotaxis (11). Several lines of evidence indicate that 3-chlorobenzoate is also recognized by the benzoate chemotaxis system. First, cells grown under conditions (e.g., with either benzoate or 4-hydroxybenzoate) in which β-ketoadipate was generated as a metabolic intermediate responded behaviorally to 3-chlorobenzoate (Fig. 1 and 2), as did cells that were grown with glucose in the presence of adipate (Table 1). Cells grown on glucose only, on the other hand, had no measurable response to 3-chlorobenzoate (Table 1). Second, a chemotaxis mutant (PCH603) specifically defective in responses to the benzoate group of attractants (9) failed to respond to 3-chlorobenzoate when grown under conditions that induced chemotaxis in the wild-type strain (Table 1). Finally, we have shown previously that 3-chlorobenzoate, benzoate, 4-hydroxybenzoate, 4-toluate, and salicylate all stimulate methylation of a *P. putida* methyl-accepting chemotaxis protein (9). This protein does not become methylated in response to other *P. putida* chemoattractants, such as benzoylformate or succinate. It is likely that a single chemoreceptor recognizes the benzoate group

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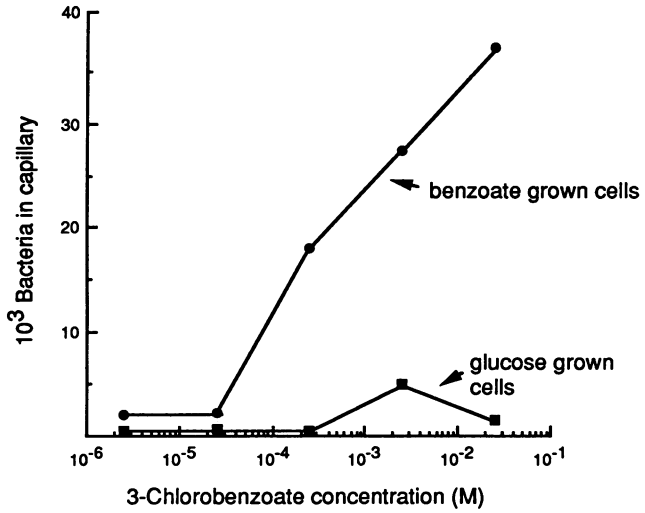

FIG. 1. Chemotactic responses of *P. putida* PRS2000 to 3-chlorobenzoate. Chemotaxis was measured by quantitative capillary assay (11). Cells were grown in defined mineral medium with benzoate (●) or glucose (■) as the carbon source. The background accumulation of cells in the absence of attractant was 2,000 for benzoate-grown cells and 1,000 for glucose-grown cells. When 5 mM benzoate was tested as an attractant for cells grown with benzoate, 62,000 cells accumulated.

of chemoattractants, including 3-chlorobenzoate, and that sensory information from this group of attractants is processed through a methyl-accepting chemotaxis system analogous to those described for *Escherichia coli* and *Salmonella typhimurium* (14).

Specificity of the response to chlorinated aromatic compounds. PRS2000 cells grown with glucose and adipate responded to 450 μM 3-fluorobenzoate (0.18 change of direction per second) and 250 μM 4-chlorobenzoate (0.15 change of direction per second) in temporal chemotaxis assays. Cells grown with glucose alone were not chemotactic toward these compounds. Cells grown with glucose and adipate did not respond behaviorally to temporal additions of 2-chlorobenzoate (final concentrations of 250 and 2,500 μM were tested), 2,4-dichlorobenzoate (final concentration, 500 μM), or 2,4-dichlorophenoxyacetate (final concentration, 500 μM). Cells remained fully motile in the presence of all compounds tested.

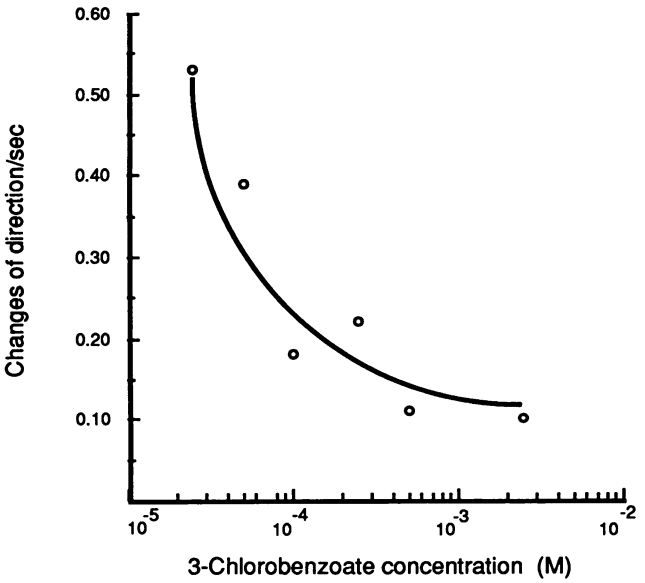

FIG. 2. Responses of *P. putida* PRS2000 cells to temporal additions of various concentrations of 3-chlorobenzoate. The temporal assay was carried out as described in the text and in reference 10. Cells were grown with 4-hydroxybenzoate as the carbon source. At least 30 s of behavior was analyzed for each point. The average number of changes of direction for cells suspended in chemotaxis buffer only was 0.50/s.

TABLE 1. Effect of adipate on expression of chemotaxis toward aromatic acids by wild-type and mutant cells

Chemical stimulus[b]	Chemotaxis[a]		
	PRS2000		PCH603[c] (Glucose + adipate grown)
	Glucose grown[d]	Glucose + adipate grown	
Buffer	0.62	0.85	0.77
Benzoate	0.70	0.21	0.82
4-Hydroxybenzoate	0.67	0.19	0.65
3-Chlorobenzoate	0.74	0.14	0.74
Casamino Acids	0.13	0.18	0.12

[a] The temporal chemotaxis assay was used, as described in the text (10). Behavioral responses are expressed as the number of changes of direction of swimming per second. At least two separate cultures were analyzed for each value, and at least 25 s of behavior was included in each analysis. Each second of behavior was equally weighted.

[b] Aromatic acids were tested at a final concentration of 500 μM; Casamino Acids were tested at a concentration of 0.01%.

[c] PRS2000 is wild-type for aromatic acid chemotaxis. PCH603, a mutant defective in chemotaxis toward the benzoate group of attractants, has a Tn*5*-VB32 chromosomal insertion (2, 9). This insertion confers resistance to tetracycline (40 μg/ml) and kanamycin (100 μg/ml).

[d] Cells were grown in defined mineral medium (15) supplemented with 10 mM glucose and, where indicated, 20 mM adipate.

The chlorobenzoate plasmid and chemotaxis. The benzoate chemotaxis mutant, PCH603 (Table 1), enabled us to test whether plasmid pAC27, encoding enzymes for 3-chlorobenzoate degradation, might also carry genetic information for chemotaxis toward chlorinated aromatic compounds. It was necessary to use such a mutant, because cells growing on 3-chlorobenzoate via the plasmid-encoded pathway produce β-ketoadipate as an intermediate. In wild-type pAC27-bearing cells this would induce the chromosomally encoded chemotactic response to 3-chlorobenzoate and possibly mask detection of a plasmid-encoded response. Plasmid pAC27 was introduced into a rifampin-resistant strain of PCH603 by conjugation with *P. putida* PRS2015(pAC27) (3, 7). Exconjugants were plated on minimal medium containing 2.5 mM 3-chlorobenzoate (to select for pAC27) and 40 μg of tetracycline per ml (to select for PCH603). These cells were then checked for rifampin (50 μg/ml) resistance to ensure that a tetracycline-resistant mutant of PRS2015(pAC27) had not been selected.

PCH603(pAC27) cells were poorly motile when grown with 3-chlorobenzoate (2.5 mM) as the sole carbon source. When the growth medium also included a low concentration of Casamino Acids (0.01%, wt/vol), a greater percentage of the cells in the culture were motile, although the number of changes of swimming direction per second for cells suspended in chemotaxis buffer was somewhat low (0.30). Nevertheless, cells suspended in chemotaxis buffer responded strongly to temporal additions of Casamino Acids (0.05 change of direction per second) but did not alter their

behavior when exposed to 500 μM 3-chlorobenzoate (0.36 change of direction per second). PRS2000 cells grown with 4-hydroxybenzoate and 0.01% Casamino Acids did respond to 3-chlorobenzoate (data not shown). Plasmid pAC27 does not appear, therefore, to complement the chemotaxis defect of PCH603.

A few simple chlorinated aromatic acids are chemoattractants for *P. putida* PRS2000. Evidence presented here indicates that 3- and 4-chlorobenzoate are detected by a benzoate chemotactic response which recognizes a group of six different monosubstituted benzoates. β-Ketoadipate, a key intermediate in the chromosomally encoded β-ketoadipate pathway of aromatic acid metabolism (15) as well as the plasmid-encoded pathway of 3-chlorobenzoate metabolism (3), elicits the expression of the benzoate chemotactic response, probably by inducing the synthesis of a chemoreceptor protein.

The lowest concentration of 3-chlorobenzoate that cells were able to detect in the temporal assay (50 μM) is high in comparison with levels of chlorinated compounds that are often encountered in the environment. However, it is important to note that our experiments were performed with cells grown in batch culture in the presence of an excess of carbon and energy source. Growth substrate limitation can have a marked effect on the physiological capabilities of bacteria and can result in the expression of uptake systems and catabolic enzymes having higher substrate affinities (8). With regard to chemotaxis, the aquatic bacterium *Spirochaeta aurantia*, when grown under carbon-limited conditions, has been reported to have enhanced chemotactic responses to low concentrations of glucose and xylose (18). It would be interesting to see whether this observation can be generalized to other bacteria; perhaps *P. putida* cells grown under conditions that more closely reflect those of a soil or aquatic environment would also have enhanced chemotactic responses and be able to detect considerably lower concentrations of chlorobenzoates.

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