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A Methyl-Accepting Protein Is Involved in Benzoate Taxis in *Pseudomonas putida*

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***Pseudomonas putida* is attracted to at least two groups of aromatic acids: a benzoate group and a benzoylformate group. Members of the benzoate group of chemoattractants stimulated the methylation of a *P. putida* polypeptide with an apparent molecular weight of 60,000 in sodium dodecyl sulfate-polyacrylamide gels. This polypeptide is presumed to be a methyl-accepting chemotaxis protein for several reasons: its molecular weight is similar to the molecular weights of *Escherichia coli* methyl-accepting chemotaxis proteins, the amount of time required to attain maximal methylation correlated with the time needed for behavioral adaptation of *P. putida* cells to benzoate, and methylation was stimulated by benzoate only in cells induced for chemotaxis to benzoate. Also, a mutant specifically defective in benzoate taxis failed to show any stimulation of methylation upon addition of benzoate. Benzoylformate did not stimulate protein methylation in cells induced for benzoylformate chemotaxis, suggesting that sensory input from this second group of aromatic-acid attractants is processed through a different kind of chemosensory pathway. The chemotactic responses of *P. putida* cells to benzoate and benzoylformate were not sensitive to external pH over a range (6.2 to 7.7) which would vary the protonated forms of these weak acids by a factor of about 30. This indicates that detection of cytoplasmic pH is not the basis for aromatic-acid taxis in *P. putida*.**

Aromatic compounds are major components of plants and constitute a substantial proportion of the toxic wastes present in the environment (8, 18). Many soil bacteria can grow on naturally occurring aromatic compounds, and the enzymology and genetic control of pathways governing the breakdown of a number of aromatic acids and aromatic hydrocarbons have been worked out (4, 9, 31, 39). On the basis of this knowledge, experimental strategies have been devised and implemented to increase the range of substrates that can be attacked by microorganisms (32, 33). Chemotaxis is another process that might be manipulated to enhance the biodegradation capabilities of selected bacteria.

Aromatic acids are chemoattractants for *Pseudomonas putida*, and the compounds detected can be placed into two groups based on patterns of induction (13). A benzoate group of attractants includes benzoate, 4-hydroxybenzoate, 3-toluate, 3-chlorobenzoate, and salicylate; a benzoylformate group of attractants includes benzoylformate, L-mandelate, and β-phenylpyruvate. β-Ketoadipate, an intermediate in the chromosomally encoded pathway of benzoate and 4-hydroxybenzoate breakdown (Fig. 1), and adipate, its nonmetabolizable analog, are inducers of benzoate taxis. These compounds also induce the synthesis of many of the enzymes required for benzoate and 4-hydroxybenzoate catabolism (25). Benzoylformate and L-mandelate are inducers of the benzoylformate tactic response (13).

The ability to sense and swim towards aromatic acids is widespread among bacteria and has also been seen in *Rhizobium*, *Bradyrhizobium*, and *Agrobacterium* spp. (1, 2, 27, 28). However, positive chemotactic responses to these compounds are not part of the behavioral repertoires of *Escherichia coli* and *Salmonella typhimurium*, the two microorganisms in which mechanisms of chemotaxis have been most thoroughly studied. In fact, benzoate is a chemorepellent for these enteric bacteria (17, 30).

A unifying theme in bacterial chemotaxis is that cells respond to chemical stimuli via a signaling process that is initiated when attractant interacts with receptor sites. The signal directs the bacterial flagella to change the direction of their rotation, and this results in a biasing of random motile behavior such that cells accumulate in the region of attractant (22, 29). Responses to chemical stimuli are often mediated by transmembrane proteins called transducers. These proteins, which have been studied intensively in *E. coli* and *S. typhimurium*, bind chemoeffectors or chemoeffector-binding protein complexes to initiate transmission of chemosensory signals. They are also involved in adaptation to environmental stimuli. The adaptation phase of chemotaxis is correlated with a change in the level of methylation of transducer proteins, and for this reason they are also termed methyl-accepting chemotaxis proteins (MCPs) (14, 22).

As a step towards elucidating the mechanisms responsible for the detection of aromatic acids by *P. putida*, we have examined the possible participation of MCPs. The results reported here show that sensory information from the benzoate group of attractants is processed through a methyl-accepting chemotaxis system. Benzoylformate chemotaxis, on the other hand, does not appear to involve MCP participation. We also show that pH taxis is not responsible for the responses of *P. putida* cells to either benzoate or benzoylformate.

MATERIALS AND METHODS

Bacterial strains. *P. putida* wild-type strain PRS2000 has been characterized with respect to chemotaxis towards aromatic compounds as well as to metabolism of aromatic acids (7, 12, 13, 15, 25). Mutant strain PRS61, derived from PRS2000, lacks mandelate dehydrogenase and is therefore unable to convert L-mandelate to benzoylformate (16). Strains PCH601 and PCH603, derived from PRS2000, were isolated after mutagenesis with the transposable promoter probe Tn*5*-VB32 (5). PCH603 is kanamycin resistant when grown on glucose in the presence of adipate but kanamycin sensitive when grown on glucose alone. PCH601 is kanamycin resistant under all growth conditions tested. Strain

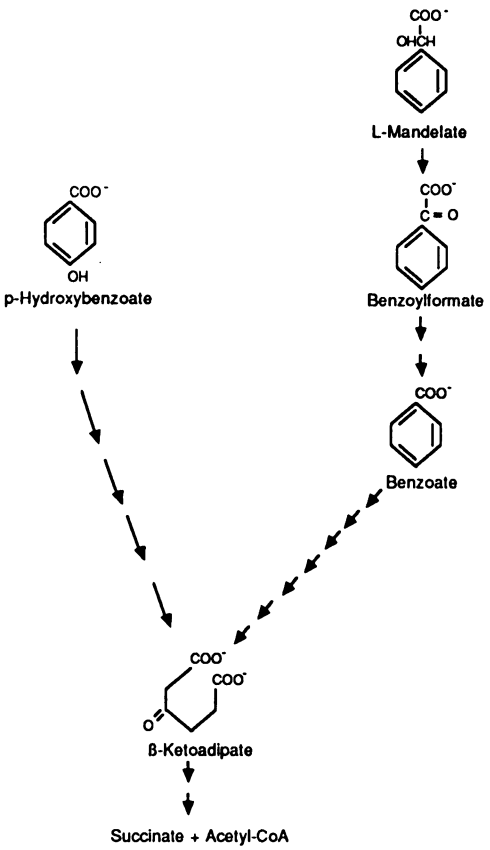

FIG. 1. Metabolism of aromatic acid chemoattractants in *P. putida* PRS2000. Each arrow represents a specific enzymatic step.

PCH603 fails to respond to benzoate, 4-hydroxybenzoate, or 3-chlorobenzoate in temporal chemotaxis assays but has wild-type responses to Casamino Acids and succinate. Strain PCH601 has chemotactic responses that are indistinguishable from those of the wild type. *E. coli* K-12 has been described elsewhere (3).

Culture conditions. *P. putida* strains were cultivated in defined mineral medium (26). Aromatic acids were added to culture media to a final concentration of 5 mM, glucose was added to a final concentration of 10 mM, and adipate was added to a final concentration of 20 mM. Kanamycin was added to media at a final concentration of 100 μg/ml. *E. coli* cells were grown in LB medium (23). Liquid cultures were grown in 10-ml volumes of medium in 50-ml Erlenmeyer flasks, with constant aeration provided by a gyratory shaker.

***methyl*-^{3}H labeling of proteins.** Protein methylation assays were based on published procedures (19, 35). Cultures of motile cells in the mid-logarithmic phase of growth were harvested, washed once, and suspended in chemotaxis buffer (50 mM potassium phosphate [pH 7.0], 10 μM disodium EDTA) to a concentration of 10^9 cells per ml. After being incubated for 15 min at 30°C in the presence of 200 μg of chloramphenicol per ml, 0.5-ml samples of cell suspensions were placed in microcentrifuge tubes, and 10 μCi of L-[*methyl*-^{3}H]methionine (specific activity, 85 Ci/mmol; Amersham Corp.) was added. After 60 min, 5-μl additions of water or chemicals (5 mM, final concentration) were made as indicated in the figure legends. After an additional incubation period, cells were harvested in a microcentrifuge, and methionine incorporation was arrested by suspending the cell pellets in 60 μl of sodium dodecyl sulfate (SDS) sample buffer (21) and boiling them for 2 min. Samples of solubilized cells were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (0.7-mm gels; 10% acrylamide–0.08% [wt/vol] bisacrylamide). Gels were soaked in Autofluor (National Diagnostics, Somerville, N.J.), dried, and exposed to X-ray film at −70°C for 4 to 7 days.

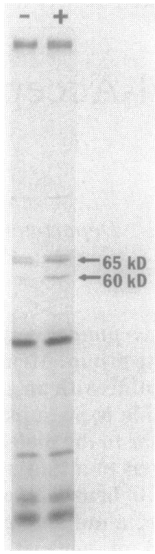

FIG. 2. Methylation of *P. putida* PRS2000 proteins. Cells were treated with chloramphenicol and incubated with L-[*methyl*-^{3}H] methionine for 60 min. Water (−) or 5 mM benzoate (+) was added 5 min before the methylation reaction was stopped. Samples were then subjected to SDS-PAGE, and an autoradiogram of the gel was prepared. PRS2000 was grown in defined mineral medium with 4-hydroxybenzoate. The arrows indicate the 60- and 65-kDa polypeptide bands that became methylated when benzoate was added.

Chemotaxis assays. Capillary assays were carried out as described previously (13).

RESULTS

Protein methylation. *P. putida* cells incubated with L-[*methyl*-^{3}H]methionine in the presence of the protein synthesis inhibitor chloramphenicol and then examined by SDS-PAGE and autoradiography had fewer radiolabeled polypeptides (Fig. 2) than did cells incubated in the absence of chloramphenicol (data not shown). Labeling of several polypeptide bands from the chloramphenicol-treated cells was stimulated by the addition of benzoate to cell suspensions. Methylation of a band with a molecular weight corresponding to 60,000 was consistently stimulated by the benzoate group of chemoattractants. In some experiments, additional bands with apparent molecular weights of about 65,000 also became more heavily labeled when benzoate was added (Fig. 2). Under our experimental conditions, a series of *E. coli* polypeptide bands became more heavily methylated when the *E. coli* chemoattractant serine was added. These bands, which have the characteristics of the *E. coli* MCP Tsr (taxis to serine and repellents) (22), had about the same molecular weights as the labeled *P. putida* polypeptides. Maximal levels of radiolabeling of *P. putida* proteins were attained within 2 min of the addition of benzoate. This is about the same amount of time required for *P. putida* to adapt behaviorally to the addition of benzoate (12). Removal of benzoate from cell suspensions resulted in a decrease in the labeling of the bands (data not shown).

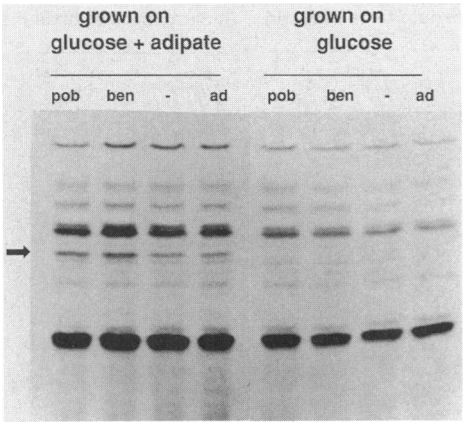

FIG. 3. Comparison of methylation in cells grown under conditions in which benzoate chemotaxis is induced (grown with glucose and adipate) or not induced (grown with glucose only). 4-Hydroxybenzoate (pob), benzoate (ben), water (−), or adipate (ad) (a nonattractant) was added 5 min before the methylation reaction was stopped. The samples were then subjected to SDS-PAGE, and an autoradiogram of the gel was prepared. The arrow indicates the position of the 60-kDa methylated polypeptide band.

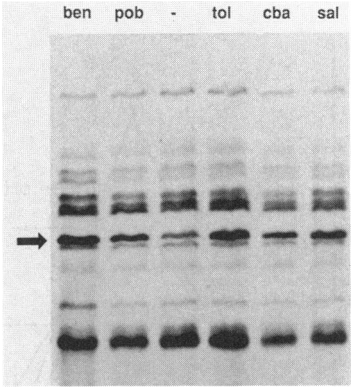

FIG. 4. Effects of various aromatic chemoattractants on protein methylation. Benzoate (ben), 4-hydroxybenzoate (pob), water (−), 3-toluate (tol), 3-chlorobenzoate (cba), and salicylate (sal) were added 5 min before the methylation reaction was stopped. The samples were then subjected to SDS-PAGE, and an autoradiogram of the gel was prepared. Cells were grown with benzoate as the carbon source. The arrow indicates the position of the 60-kDa methylated polypeptide band.

It has been known for some time that amino acid and sugar chemoattractants stimulate *S*-adenosylmethionine-mediated methylation of chemotaxis proteins in *E. coli* and *S. typhimurium* and that this process is essential for normal behavioral responses to occur (37). By analogy, it seemed likely that the *P. putida* 60-kilodalton (kDa) polypeptide plays a role in chemotaxis to benzoate. This inference was tested by comparing methylation levels in cells that were induced and not induced for benzoate chemotaxis, by looking at the specificity of protein methylation, and by examining protein methylation in a benzoate taxis mutant. Protein methylation was stimulated by benzoate in cells grown on glucose in the presence of adipate (an inducer of benzoate taxis) but not in cells grown with glucose alone (Fig. 3). In fact, the 60-kDa polypeptide appeared not to be present at all in glucose-grown cells. This could be because a much lower basal level of protein methylation existed in uninduced cells or because the 60-kDa protein was not synthesized by these cells. Salicylate, 4-hydroxybenzoate, and 3-toluate—aromatic acids that had previously been classified as being recognized by a single benzoate chemoresponse (13)—stimulated methylation of polypeptides in the 60- to 65-kDa region of gels (Fig. 4). 3-Chlorobenzoate, which we recently found to elicit a positive response in a temporal assay for chemotaxis (data not shown), also stimulated methylation (Fig. 4). None of these compounds is degraded by benzoate-grown PRS2000 cells, so the stimulation of methylation cannot be attributed to some nonspecific effect of metabolism. A benzoate chemotaxis mutant (PCH603) had an altered pattern of labeled proteins in the 60-kDa region of gels and failed to show any stimulation of methylation upon addition of attractant (Fig. 5), indicating that protein methylation is directly involved in chemotaxis.

Effect of pH on aromatic acid chemotaxis. A possible basis for the stimulation of protein methylation in *P. putida* is suggested by work on the repellent responses of enteric bacteria to benzoate. It has been shown that *E. coli* and *S. typhimurium* respond to benzoate in its capacity as a membrane-permeant weak acid (17, 30). The lowered cytoplasmic pH that ensues when benzoic acid diffuses into cells triggers a repellent response. An MCP has been identified as the chemoreceptor for cytoplasmic pH in *E. coli* (35). Because *P. putida* cells can respond differentially to benzoate and benzoylformate (13), two compounds with very similar weak-acid characteristics, it seemed unlikely that pH taxis would be involved in aromatic-acid chemoattraction. This was confirmed by assaying chemotaxis to benzoate and benzoylformate at three external pHs (6.2, 7.0, and 7.7), a range over which the protonated (membrane-permeant) forms of these weak acids would vary by a factor of about 30. These pH differences had essentially no effect on the magnitude of the chemotactic responses (Fig. 6).

Possible involvement of methylation in other *P. putida* chemosensory responses. Benzoylformate and mandelate, compounds detected by a benzoylformate-inducible chemotactic response, did not stimulate protein methylation in cells

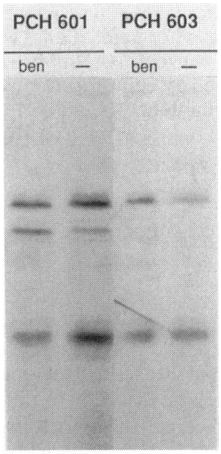

FIG. 5. Effects of aromatic acids on protein methylation in two *P. putida* strains containing Tn*5*-VB32 insertions. PCH601 has wild-type chemotactic responses. PCH603 is a mutant specifically defective in chemotaxis to benzoate and 4-hydroxybenzoate. Benzoate (ben) and water (−) were added 5 min before the methylation reaction was stopped. The samples were then subjected to SDS-PAGE, and an autoradiogram of the gel was prepared. Cells were grown in defined mineral medium with benzoate.

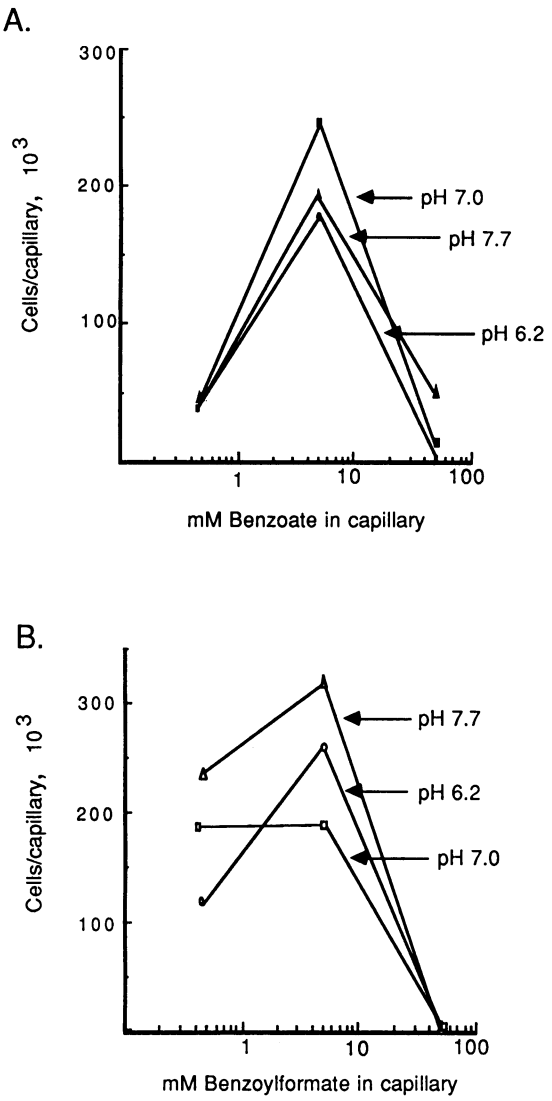

FIG. 6. Effect of external pH on responses of *P. putida* PRS2000 to benzoate and benzoylformate. Cells were grown in defined mineral medium with benzoate (A) or benzoylformate (B) as the carbon source, harvested by centrifugation, washed, and suspended in 10 mM triethanolamine-KPO_4 buffer (pH 6.2, 7.0, or 7.7) supplemented with 10 μM EDTA. Chemotaxis was measured by quantitative capillary assay.

induced for chemotaxis to either benzoate or benzoylformate (Fig. 7).

DISCUSSION

This report demonstrates the presence of a protein in *P. putida* with characteristics similar to those of the MCPs of *E. coli*. The methylation of this protein was stimulated by metabolizable and nonmetabolizable chemoattractants. The time required to attain maximal levels of methylation in *P. putida* (ca. 2 min) was about the same as the time required for MCP methylation to reach a plateau in *E. coli*, and, as in *E. coli* (11), the level of methylation in *P. putida* decreased in response to the removal of attractant. Furthermore, the apparent molecular weight of the methylated polypeptide was similar to the molecular weights of *E. coli* MCPs (34).

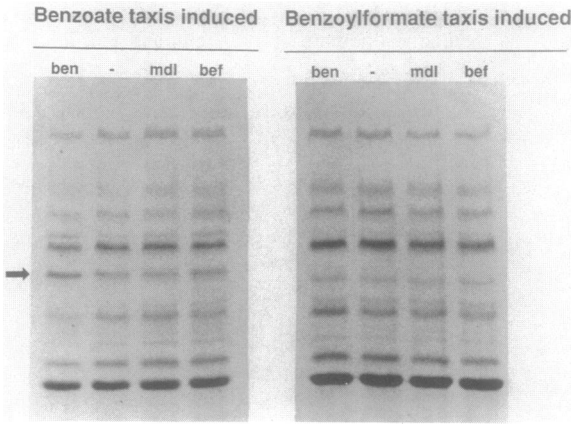

FIG. 7. Protein methylation of *P. putida* PRS61, a mutant blocked in conversion of L-mandelate to benzoylformate. Cells used in the left panel were grown with benzoate to induce benzoate chemotaxis, and cells used in the right panel were grown with L-mandelate and glucose to induce chemotaxis to benzoylformate but not benzoate. Benzoate (ben), water (−), DL-mandelate (mdl), and benzoylformate (bef) were added 5 min before the protein methylation was stopped. Cell samples were then subjected to SDS-PAGE, and an autoradiogram of the gel was prepared. The arrow indicates the position of the 60-kDa methylated polypeptide band.

Several lines of evidence indicate that an MCP participates in benzoate chemotaxis by processing sensory information from the benzoate group of chemoattractants. These attractants stimulated methylation of a 60,000-molecular-weight polypeptide, and this stimulation was seen only in cells in which the benzoate chemotactic response had been induced. Benzoate also failed to stimulate protein methylation in a *P. putida* mutant strain (PCH603) that is specifically defective in benzoate taxis.

Data presented here indicate that MCP-mediated pH taxis is not responsible for chemoattraction to benzoate. In *E. coli*, the concentration of benzoate required to elicit demethylation and a repellent response depends on external pH because benzoate (pK_a, 4.2) in the membrane-permeant weak-acid form increases as the external pH decreases. No similar dependence on external pH was observed for *P. putida*. Consistent with this is the observation that *P. putida* responds strongly to 100 μM benzoate at pH 7.0 (12), whereas considerably higher benzoate concentrations (10 mM) are required to elicit a repellent response in *E. coli* at neutral pH (17, 30). *P. putida* benzoylformate taxis was also insensitive to external pH.

In *E. coli* and *S. typhimurium*, MCPs act as primary chemoreceptors and bind amino acids directly, and they also serve as secondary chemoreceptors by interacting with periplasmic binding proteins complexed with sugars or dipeptides to initiate chemosensory signaling (22). We do not yet know whether an MCP functions in one of these ways in benzoate chemoreception or whether sensory input is processed through an MCP by some other means. We have been unable to obtain any data to suggest that a binding protein is involved in benzoate taxis, although the existence of such a protein would be of some interest because binding proteins for aromatic compounds have not been described. We also have no direct evidence to implicate an MCP as the primary chemoreceptor for benzoate. The benzoate taxis mutant we examined had an altered pattern of methylated proteins (Fig. 5), but we cannot exclude the possibilities that this may be a

secondary effect of a mutation in some other protein or that the mutation is polar and affects more than one gene.

Protein methylation was stimulated only in cells induced for benzoate taxis, either by growth in the presence of adipate or by growth under conditions which generated β-ketoadipate as a metabolic intermediate. In addition, the 60-kDa MCP band appeared not to be present at all in cells grown on glucose (Fig. 3), raising the possibility that synthesis of the methylated protein, as well as benzoate-stimulated methylation, is regulated by adipate or β-ketoadipate. However, band intensity in autoradiograms does not necessarily reflect protein concentration, and more work will be required before we can say conclusively that *P. putida* MCP synthesis is subject to regulatory control by adipate. Should this be the case, it would be a first example of an inducible MCP and would raise the evolutionary question of how a member of this group of conserved proteins, which function generally in chemotaxis in diverse bacteria (6, 10, 20, 24, 36, 38), was brought under the control of a system that also regulates the synthesis of aromatic acid catabolic genes.

We did not see any stimulation of protein methylation by benzoylformate. The most direct explanation for this is that sensory information from this second major class of aromatic-acid attractants is processed differently and that two distinct chemosensory pathways are utilized for benzoate and benzoylformate chemoreception.

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