

Nitrobenzoates and Aminobenzoates Are Chemoattractants for *Pseudomonas* Strains

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Three *Pseudomonas* strains were tested for the ability to sense and respond to nitrobenzoate and aminobenzoate isomers in chemotaxis assays. *Pseudomonas putida* PRS2000, a strain that grows on benzoate and 4-hydroxybenzoate by using the β -keto adipate pathway, has a well-characterized β -keto adipate-inducible chemotactic response to aromatic acids. PRS2000 was chemotactic to 3- and 4-nitrobenzoate and all three isomers of aminobenzoate when grown under conditions that induce the benzoate chemotactic response. *P. putida* TW3 and *Pseudomonas* sp. strain 4NT grow on 4-nitrotoluene and 4-nitrobenzoate by using the *ortho* (β -keto adipate) and *meta* pathways, respectively, to complete the degradation of protocatechuate derived from 4-nitrotoluene and 4-nitrobenzoate. However, based on results of catechol 1,2-dioxygenase and catechol 2,3-dioxygenase assays, both strains were found to use the β -keto adipate pathway for the degradation of benzoate. Both strains were chemotactic to benzoate, 3- and 4-nitrobenzoate, and all three aminobenzoate isomers after growth with benzoate but not succinate. Strain TW3 was chemotactic to the same set of aromatic compounds after growth with 4-nitrotoluene or 4-nitrobenzoate. In contrast, strain 4NT did not respond to any aromatic acids when grown with 4-nitrotoluene or 4-nitrobenzoate, apparently because these substrates are not metabolized to the inducer (β -keto adipate) of the chemotaxis system. The results suggest that strains TW3 and 4NT have a β -keto adipate-inducible chemotaxis system that responds to a wide range of aromatic acids and is quite similar to that present in PRS2000. The broad specificity of this chemotaxis system works as an advantage in strains TW3 and 4NT because it functions to detect diverse carbon sources, including 4-nitrobenzoate.

With few exceptions, nitroaromatic compounds are man-made chemicals that have been used extensively only for the last century. Nitrobenzene, nitrotoluenes, nitrobiphenyls, nitrophenols, and nitrobenzoates have been used as industrial solvents and in the production of dyes, polymers, fungicides, pesticides, herbicides, and explosives (19, 39). In addition, soil and groundwater contamination by trinitrotoluene and dinitrotoluenes has resulted from their extensive production and use as explosives during World Wars I and II. Various nitroaromatic compounds have been shown to be toxic, mutagenic, and possibly carcinogenic to a range of organisms, from bacteria to humans (19, 39, 48), and as a result, seven nitroaromatic compounds are included on the U.S. Environmental Protection Agency's November 2002 list of priority pollutants (<http://www.epa.gov/region1/npdes/permits/generic/prioritypollutants.pdf>).

Since these xenobiotic compounds have been present in the environment for a relatively short period of time, microorganisms have not had long to evolve pathways for their degradation. Although nonspecific reduction of nitroaromatic compounds appears to be quite common (4, 37), until recently most nitroaromatic compounds were thought to be nonbiodegradable. It has only been within the last 15 years that bacteria capable of growth on compounds such as nitrobenzene and nitrotoluenes have been isolated, and such strains have been isolated only from locations with a history of exposure to nitroaromatic contamination (27, 45, 46).

The role of chemotaxis in the biodegradation of pollutants

and man-made chemicals has not been extensively explored. Organic chemicals such as sugars and amino acids are strong chemoattractants for the enteric bacteria (23). In contrast, *Pseudomonas* and related nonenteric bacteria have been shown to be attracted to aromatic acids, many of which are growth substrates for particular strains. *Pseudomonas putida* PRS2000 has been the model organism for studying chemotaxis to aromatic acids (12–14, 16, 17). Attractants for PRS2000 include the growth substrates benzoate, *p*-hydroxybenzoate, and benzoylformate as well as the nonmetabolizable compounds 2-hydroxybenzoate (salicylate), *m*- and *p*-toluate, 3- and 4-chlorobenzoate, and 3-fluorobenzoate (16, 17). Other aromatic attractants for various *Pseudomonas* and related *Proteobacteria* include the aromatic hydrocarbons naphthalene (9, 32, 43), toluene and benzene (32), and the herbicide 2,4-dichlorophenoxyacetic acid (18). Recent reports have demonstrated chemotaxis to nitrocatechol, nitrobenzoate, and nitrophenols by a *Ralstonia* sp. (2, 30, 42). The aim of this study was to determine whether nitrobenzoates and aminobenzoates are recognized as chemoattractants by the aromatic acid chemotaxis system in *P. putida* PRS2000 and whether these compounds serve as attractants for specific *Pseudomonas* strains capable of 4-nitrobenzoate degradation. The results reported here indicate that nitrobenzoates and aminobenzoates are detected as chemoattractants by strains that do not degrade these compounds, as well as by strains that can grow on and mineralize 4-nitrotoluene and 4-nitrobenzoate. This finding is significant because bacterial strains with the ability to detect the presence of 4-nitrobenzoate may have an increased growth and survival advantage and could contribute to the biodegradation of 4-nitrotoluene and 4-nitrobenzoate in the environment (31, 33).

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MATERIALS AND METHODS

Bacterial strains and growth conditions. *P. putida* PRS2000 is a wild-type strain that grows on benzoate and 4-hydroxybenzoate by using the β -ketoadipate pathway (29). *P. putida* TW3 (38) and *Pseudomonas* sp. strain 4NT (11) are wild-type isolates that each grow on 4-nitrotoluene and 4-nitrobenzoate. Prior to the initiation of experiments, highly motile populations of all three strains were enriched on diluted L-agar swarm plates (1, 17). A variation of auxanography (36) was used to test for growth of PRS2000, 4NT, and TW3 on aminobenzoates, nitrobenzoates, and 4-nitrotoluene. Overnight cultures grown in MSB minimal medium (47) with succinate as the carbon source were harvested by centrifugation, washed with MSB, resuspended in MSB to a concentration of approximately 10^{10} cells/ml and spread (50 μ l/plate) on MSB medium solidified with 1.8% Noble agar (Difco Laboratories, Detroit, Mich.). Potential growth substrates (2-, 3-, and 4-aminobenzoate; 2-, 3-, and 4-nitrobenzoate; and 4-nitrotoluene) were applied as crystals at the edge of the plate. To control plates, 4-hydroxybenzoate crystals were added as the carbon source. Plates were incubated at 30°C for up to 1 week. Growth on auxanography plates was verified in liquid MSB containing crystals of the substrate. For chemotaxis assays, *P. putida* PRS2000 was grown to mid-logarithmic phase in MSB with 5 mM benzoate, 5 mM 4-hydroxybenzoate, or 10 mM succinate. *P. putida* TW3 and *Pseudomonas* sp. strain 4NT were grown under similar conditions or with crystals of 4-nitrobenzoate or 4-nitrotoluene as the carbon source.

Chemotaxis assays. Soft agar swarm plates consisted of MSB medium containing 0.1% rather than 1% Hutner's mineral base and 0.3% Noble agar (7). Benzoate was provided as a potential attractant in swarm plates at a final concentration of 1 mM, and 4-nitrobenzoate was provided at a final concentration of 0.5 mM. Cells were inoculated at the center of the plates; plates were incubated at 30°C overnight. Modified capillary assays were carried out as previously described (9, 32). Capillaries (1 μ l) contained attractant in either 2% low-melting-temperature agarose (NuSieve GTG Agarose; FMC Bioproducts, Rockland, Maine) dissolved in a chemotaxis buffer (50 mM potassium phosphate buffer [pH 7.0], 10 μ M disodium EDTA, 0.05% glycerol) (32) or as crystals in chemotaxis buffer. Freshly grown cells were harvested in log phase (when the optical density at 660 nm [OD₆₆₀] was between 0.3 and 0.5), suspended in chemotaxis buffer to an OD₆₆₀ of approximately 0.05, and placed in a chamber formed by a microscope slide, a glass U-tube, and a coverslip. A capillary containing the attractant was inserted into the pool of cells. Control capillaries contained 2% low-melting-temperature agarose in the chemotaxis buffer. Cell behavior was observed at a magnification of $\times 40$. Chemotaxis was measured quantitatively by using a temporal assay that monitors adaptation to the chemoattractant (44). Briefly, cells were harvested by centrifugation and resuspended in the chemotaxis buffer to an OD₆₆₀ of approximately 0.05. After addition of attractant, cells were either viewed directly or videotaped, and the time required for approximately 50% of the population to return to prestimulus behavior was determined.

Preparation of cell extracts. *Pseudomonas* cultures (150 ml) grown in MSB containing the appropriate carbon source were harvested in the exponential phase of growth (OD₆₆₀ of 0.4 to 0.6). Cells were washed once with MSB, and pellets were stored at -20°C until just prior to disruption. Thawed cells were resuspended in a solution of 50 mM Tris-HCl (pH 7.5)-1 mM dithiothreitol. Chilled cell suspensions were disrupted by sonication, and cell debris was removed by centrifugation.

Enzyme assays. Catechol 1,2- and catechol 2,3-dioxygenase activities were assayed spectrophotometrically at 260 and 375 nm as previously described (24, 28). Assays were carried out in 1-ml quartz cuvettes containing 50 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, and 1 mM catechol. In each case, 1 unit is defined as the amount of enzyme that oxidizes 1.0 μ mol of catechol per min at 25°C. Protein concentrations were determined by using the method of Bradford (3) with bovine serum albumin as standard.

RESULTS

Growth of *Pseudomonas* strains on nitrobenzoates and aminobenzoates. *P. putida* PRS2000 is known to be chemotactic to aromatic acids that serve as growth substrates as well as several others that do not (16, 17). We tested this strain for the ability to grow with nitro- and aminobenzoates and 4-nitrotoluene and found that none of these compounds were utilized as carbon sources (Table 1). The two 4-nitrotoluene-degrading strains *P. putida* TW3 and *Pseudomonas* sp. strain 4NT grew

TABLE 1. Growth of *Pseudomonas* strains on various aromatic compounds^a

Substrate	Mean doubling time (min) \pm SD of strain:		
	PRS2000	4NT	TW3
Benzoate	77 \pm 11	54 \pm 2	113 \pm 8
4-Hydroxybenzoate	123 \pm 24	118 \pm 14	92 \pm 9
4-Nitrobenzoate	—	180 \pm 36	124 \pm 13
3-Nitrobenzoate	—	—	—
2-Nitrobenzoate	—	—	—
4-Aminobenzoate	—	— ^b	— ^c
3-Aminobenzoate	—	—	—
2-Aminobenzoate	—	—	—
4-Nitrotoluene	—	179 \pm 23	296 \pm 16

^a Growth was assessed by auxanography as described in Materials and Methods. Positive growth was verified in liquid culture (MSB containing 5 mM benzoate or 4-hydroxybenzoate or saturating 4-nitrobenzoate or 4-nitrotoluene at 30°C with shaking at 250 rpm); —, no growth.

^b Reported in reference 11.

^c Reported in reference 38.

with 4-nitrobenzoate and 4-nitrotoluene as previously reported (11, 38) but not with 2- or 3-nitrobenzoate or 2-, 3-, or 4-aminobenzoate (Table 1).

Benzoate and 4-nitrobenzoate chemotaxis by strains TW3 and 4NT. Chemotaxis to benzoate and 4-nitrobenzoate was initially tested by swarm plate assays. Both strains TW3 and 4NT were chemotactic to benzoate, as seen by the swarm patterns (Fig. 1A). The larger swarm formed by strain 4NT is consistent with its faster growth rate on benzoate (Table 1). Even though both strains are able to grow with 4-nitrobenzoate, only strain TW3 showed a chemotactic response in the swarm plate assay (Fig. 1B).

Nitrobenzoates and aminobenzoates are attractants for PRS2000, TW3, and 4NT. Previous studies have shown that PRS2000 expresses an inducible chemotactic response to benzoate and other aromatic acids (12, 16, 17). After growth of PRS2000 in minimal medium with benzoate (induced) or succinate (uninduced), cells were harvested by centrifugation and resuspended in buffer and chemotaxis was assayed in two ways. The smooth swimming response assay (44) provides a quantitative temporal assay that measures the strength of the attractant response. In this assay, cells were visualized microscopically immediately after the addition of a potential attractant. Upon addition of an attractant, the cells respond by swimming smoothly, i.e., cells that are responding change direction much less frequently as they bias the rotation of the flagellar motor. The time required for approximately 50% of the cells to adapt (return to unstimulated behavior) is the smooth swimming response time. The length of time taken for the cells to adapt is a general measure of the strength of the attractant.

When grown with benzoate, PRS2000 responded to benzoate as expected, and one-half of the population adapted within approximately 1 min (Table 2). This result is consistent with the results of computer-assisted motion analysis studies, which demonstrated that PRS2000 adapted within 1 to 2 min after stimulation with benzoate (12). Benzoate-grown PRS2000 was also attracted to 3- and 4-nitrobenzoate (Table 2). Similarly, strain TW3 also responded to benzoate and to 3- and 4-nitrobenzoate when grown with benzoate but not when grown with succinate. TW3 was also attracted to the same subset of chem-

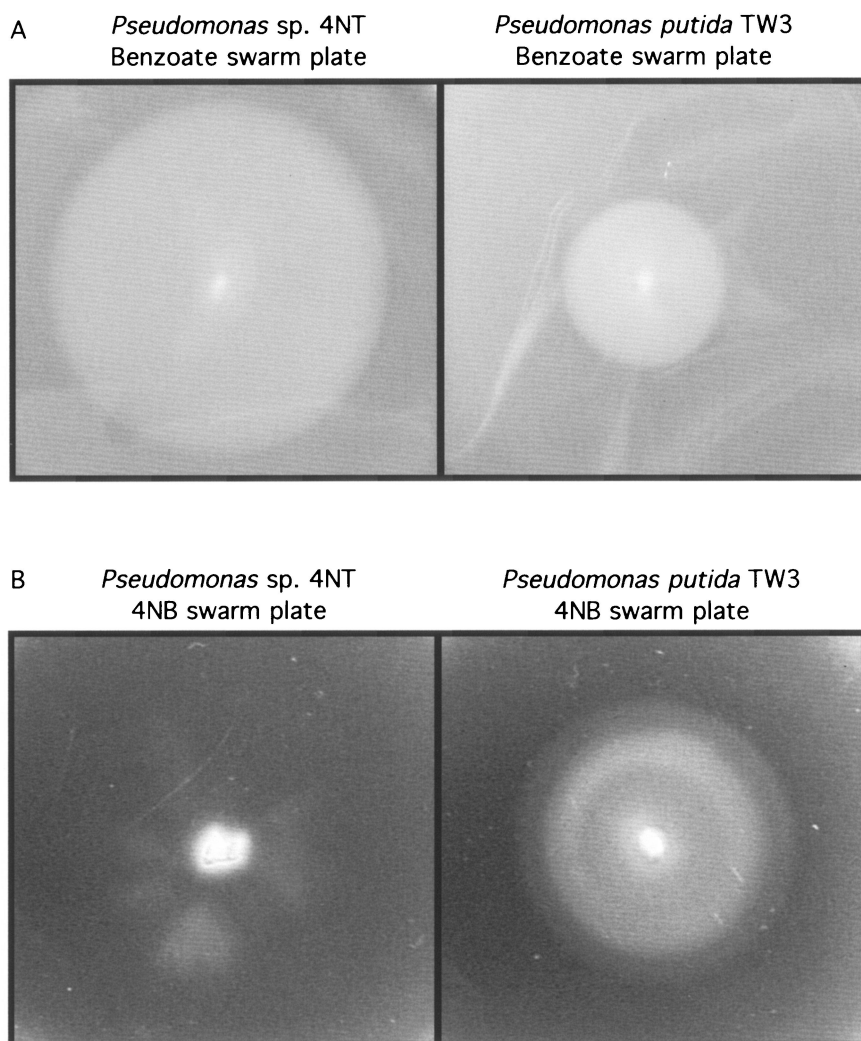


FIG. 1. Chemotactic responses of *Pseudomonas* sp. strain 4NT and *P. putida* TW3 to 1 mM benzoate (A) and 0.5 mM 4-nitrobenzoate (B) in swarm plates.

icals after growth with 4-nitrobenzoate or 4-nitrotoluene (Fig. 2A). In contrast, strain 4NT was chemotactic to benzoate and 3- and 4-nitrobenzoate only after growth with benzoate (Fig. 2B). After growth with succinate, all three strains responded only to the addition of Casamino Acids (Fig. 2; Table 2), indicating that the chemotactic response to aromatic compounds was inducible in all of the *Pseudomonas* strains. We tested the ability of the three strains to detect the structurally related aminobenzoate isomers and found that 2-, 3-, and 4-aminobenzoate were attractants for all three strains when grown under the conditions that elicited benzoate and nitrobenzoate chemotaxis (Fig. 2; Table 2).

Chemotactic responses were verified with the modified capillary assay, which allows a direct visualization of the accumulation of cells in response to an attractant present in a 1- μ l capillary. Benzoate- and 4-hydroxybenzoate-grown PRS2000 cells accumulated at the mouths of capillaries containing either 5 mM benzoate or 4-nitrobenzoate (Fig. 3A; Table 3). No response to either benzoate or 4-nitrobenzoate was detected with succinate-grown cells (Table 3). Similar results were seen

with strain TW3 (Fig. 3B; Table 3). 4-Nitrobenzoate- and 4-nitrotoluene-grown TW3 cells were also clearly attracted to benzoate and 4-nitrobenzoate (Fig. 3C and D; Table 3). In contrast, strain 4NT responded to benzoate and 4-nitrobenzoate after growth with benzoate or 4-hydroxybenzoate but not 4-nitrobenzoate or 4-nitrotoluene (Fig. 3E and F; Table 3).

No response by any of the strains was detected to 2-nitrobenzoate in temporal assays or modified capillary assays (Table 2; data not shown), and no response to any of the aromatic compounds was detected with succinate-grown cells with either assay method (Fig. 2; Table 3).

Benzoate degradation pathway in strains TW3 and 4NT. Although strains TW3 and 4NT were previously shown to grow with benzoate (11, 38), the benzoate degradation pathways present in these strains have not been described in detail. Strain TW3 was reported to have elevated catechol 1,2-dioxygenase activity after growth with benzoate compared to succinate-grown cells (22). Catechol 1,2- and catechol 2,3-dioxygenase assays were carried out with both strains after growth with either succinate or benzoate. In both strains, catechol 1,2-

TABLE 2. Temporal response of PRS2000 to nitro- and aminobenzoates^a

Attractant	Mean smooth swimming response time (s) ± SD (no. of independent assays) after growth with:	
	Succinate	Benzoate
None (buffer control)	– (3)	– (3)
Casamino Acids (0.2%)	185 ± 30 (11)	142 ± 19 (11)
Benzoate (1 mM)	– (2)	66 ± 21 (9)
4-Nitrobenzoate (1 mM)	– (3)	100 ± 11 (8)
3-Nitrobenzoate (1 mM)	– (3)	57 ± 12 (9)
2-Nitrobenzoate (1 mM)	– (5)	– (2)
4-Aminobenzoate (1 mM)	– (5)	64 ± 12 (6)
3-Aminobenzoate (1 mM)	– (5)	40 ± 7 (6)
2-Aminobenzoate (1 mM)	– (4)	41 ± 6 (6)

^a Amount of time for approximately 50% of the cells to adapt to the added attractant; –, no response detected.

dioxygenase activity was induced during growth with benzoate (Table 4). Extracts of succinate-grown strain TW3 had a low but detectable level of the enzyme, while the activity was undetectable in succinate-grown strain 4NT. Catechol 2,3-dioxygenase activity was not detectable in strain 4NT grown under either condition. Very low levels of catechol 2,3-dioxygenase were detected in strain TW3 grown under both conditions (Table 4). These results indicate that strains TW3 and 4NT utilize the β -keto adipate pathway for the degradation of benzoate.

DISCUSSION

PRS2000 degrades benzoate and 4-hydroxybenzoate by using the β -keto adipate pathway (15), and neither nitrobenzoates nor aminobenzoates are growth substrates for PRS2000 (Table 1). However, metabolism of nitrobenzoates and aminobenzoates is apparently not necessary for the behavioral response to these compounds (Table 2). Previous studies have also shown that PRS2000 is attracted to several other compounds that it is unable to metabolize, including toluates, chlorobenzoates, and salicylate (14, 16, 17), and mutants of PRS2000 that are unable to grow with benzoate and 4-hydroxybenzoate were chemotactic to benzoate, provided that β -keto adipate was available to induce the chemotactic response (17). The chemotactic response to benzoate, 4-hydroxybenzoate, salicylate, toluates, and chlorobenzoates is induced when PRS2000 is grown with benzoate or 4-hydroxybenzoate (14, 16, 17). It is quite possible that the same chemotaxis system can detect the presence of the structurally related nitrobenzoates and aminobenzoates. Consistent with this possibility, only benzoate- or 4-hydroxybenzoate-grown cells responded to nitro- and aminobenzoates (Tables 2 and 3). The chemotactic response to 4-hydroxybenzoate is mediated by the PcaK protein in PRS2000 (13). PcaK is a member of the major facilitator superfamily (41) of transport proteins, and it also functions to transport 4-hydroxybenzoate and protocatechuate (25). In PRS2000, the *pcaK* gene is located nearby and coordinately regulated by β -keto adipate with several benzoate and/or 4-hydroxybenzoate degradation genes. In this strain, β -keto adipate, an intermediate in benzoate and 4-hydroxybenzoate degradation, induces the chemotactic response to substituted

benzoates as well as several of the structural genes for the degradation of these two compounds in the presence of the PcaR activator protein (10, 16, 17, 26, 34, 40).

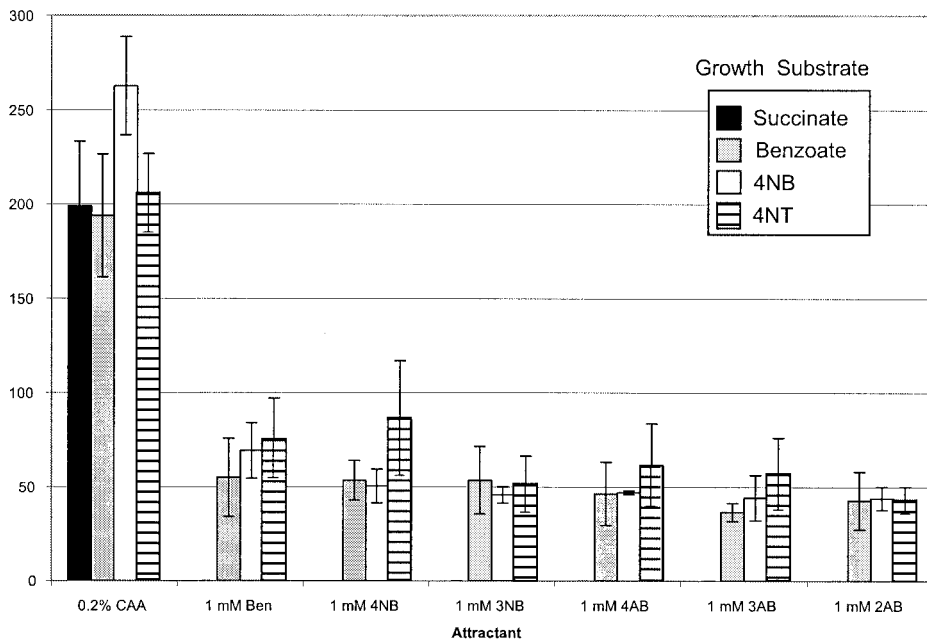
P. putida TW3 and *Pseudomonas* sp. strain 4NT degrade 4-nitrotoluene through 4-nitrobenzoate to protocatechuate, but after this step the pathways in the two strains differ (Fig. 4). In strain 4NT, protocatechuate is degraded via a *meta* ring fission pathway (11), while in TW3, protocatechuate is degraded by using the β -keto adipate pathway (38). Results of catechol dioxygenase assays indicate that strains 4NT and TW3 both degrade benzoate by using the β -keto adipate pathway (Table 4). The results of chemotaxis assays suggest that, similar to PRS2000, strains TW3 and 4NT may have a β -keto adipate-inducible chemotaxis system responding to benzoate and structurally related chemicals. Thus, strain TW3 responds to benzoate, nitrobenzoates, and aminobenzoates when grown with benzoate, 4-nitrobenzoate, and 4-nitrotoluene, all of which are degraded with the formation of β -keto adipate. In contrast, β -keto adipate is not an intermediate in the degradation of 4-nitrotoluene and 4-nitrobenzoate in strain 4NT, and this strain responds to benzoate, nitrobenzoates, and aminobenzoates only after growth with benzoate. A similar phenomenon was described for *Pseudomonas* strains carrying the TOL plasmid (14). These strains, although chemotactic to benzoate when lacking the TOL plasmid, are not chemotactic to benzoate when harboring the plasmid. This is because benzoate is preferentially degraded through a *meta* cleavage pathway in the TOL⁺ strain, and consequently, β -keto adipate is not produced as an intermediate. Therefore, under these conditions the chemotactic response is not induced. These strains exhibited normal chemotaxis to aromatic acids when grown with 4-hydroxybenzoate, which is degraded via the β -keto adipate pathway in both TOL⁺ and TOL⁻ strains.

Although the majority of our studies utilized benzoate-, 4-nitrobenzoate- and 4-nitrotoluene-grown cells, strains TW3 and 4NT were also found to be chemotactic to the same set of substituted benzoates after growth with 4-hydroxybenzoate (Table 3). Most *Pseudomonas* carry both the catechol and protocatechuate branches of the β -keto adipate pathway (15), and the chemotactic responses of 4-hydroxybenzoate-grown TW3 and 4NT are most likely due to the formation of β -keto adipate from 4-hydroxybenzoate.

The genes from strain TW3 that encode the conversion of 4-nitrotoluene to 4-nitrobenzoate and from 4-nitrobenzoate to protocatechuate have been cloned and sequenced (20–22), and there are no obvious chemotaxis genes located within either cluster. Apparently, the corresponding genes from strain 4NT have been identified, but results have not yet been published (21). In strains TW3 and 4NT, the chemotactic response to substituted benzoates may be mediated by a PcaK-like protein as in PRS2000 (13), but such a protein has not yet been identified in these strains.

Ralstonia sp. strain SJ98 has been shown to be chemotactic to the nitroaromatic growth substrates 4-nitrocatechol, 2-nitrobenzoate, 4-nitrobenzoate, 4-nitrophenol, and 3-methyl-4-nitrophenol (2, 42). Although the pathways for the degradation of these compounds have not been characterized, protocatechuate was identified as an intermediate in 4-nitrobenzoate degradation and 2-nitrobenzoate was converted to 2-amino benzoate (42). The mode of protocatechuate cleavage has not

A. Strain TW3 Smooth Swimming Response



B. Strain 4NT Smooth Swimming Response

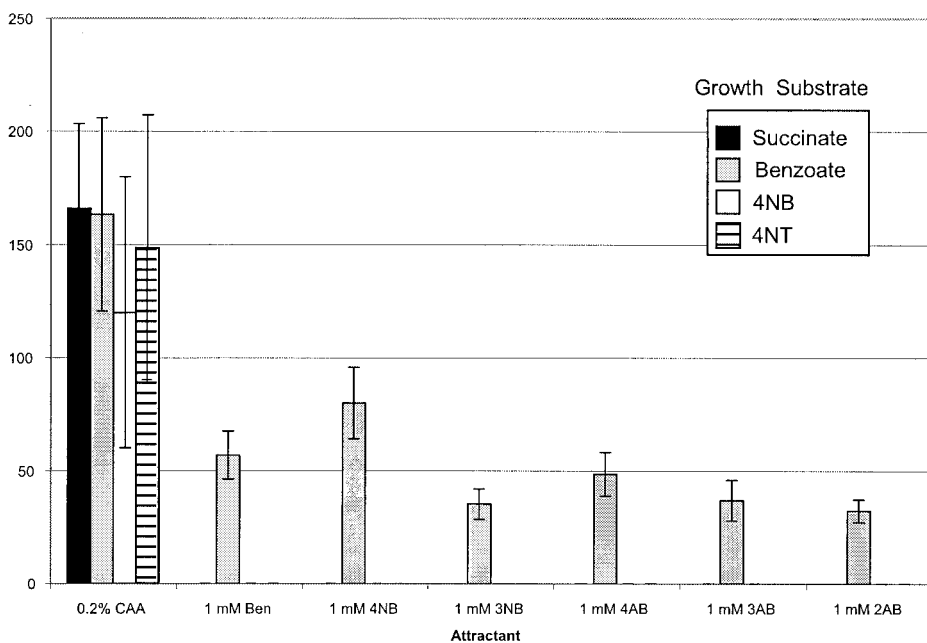
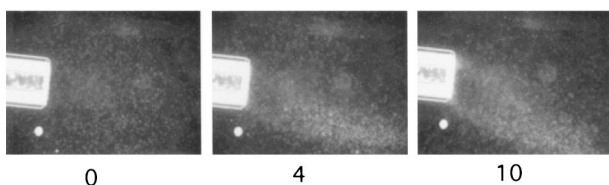
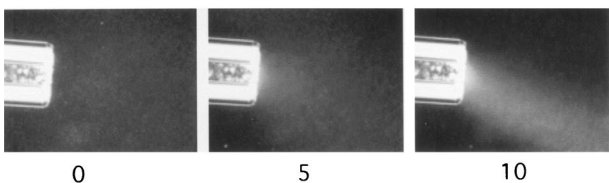


FIG. 2. Chemotactic responses of *P. putida* TW3 (A) and *Pseudomonas* sp. strain 4NT (B) in smooth swimming response assays after growth with 10 mM succinate, 5 mM benzoate, or saturating amounts of 4-nitrobenzoate (4NB) or 4-nitrotoluene (4NT). The response in this assay is the amount of time in seconds needed for approximately 50% of the cells to adapt to the added attractant. The abbreviations for the attractants in the chemotaxis buffer are as follows: 0.2% CAA, 0.2% Casamino Acids; Ben, benzoate; 4NB, 4-nitrobenzoate; 3NB, 3-nitrobenzoate; 4AB, 4-aminobenzoate; 3AB, 3-aminobenzoate; 2AB, 2-aminobenzoate. No response was seen with chemotaxis buffer alone or with 1 mM 2-nitrobenzoate (data not shown).

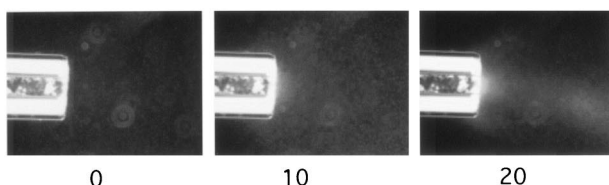
A. PRS2000 response to 4NB (Ben-grown)



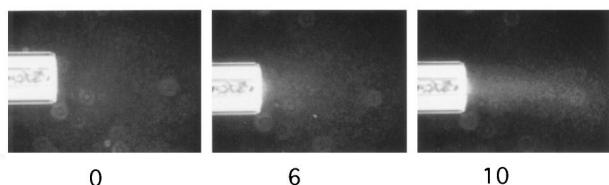
B. TW3 response to 4NB (Ben-grown)



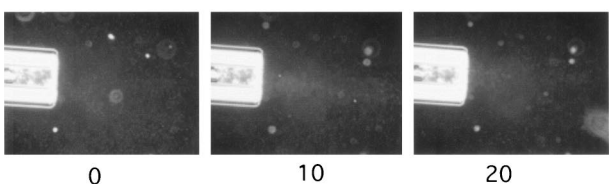
C. TW3 response to 4NB (4NB-grown)



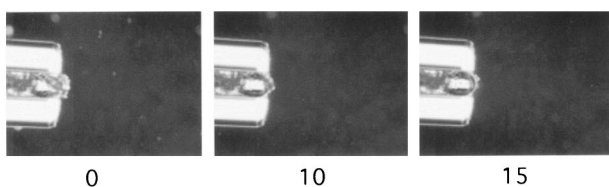
D. TW3 response to 4NB (4NT-grown)



E. Strain 4NT response to 4NB (4HB-grown)



F. Strain 4NT response to 4NB (4NB-grown)



Time (min)

FIG. 3. Time courses of the chemotactic responses of the three *Pseudomonas* strains to 4-nitrobenzoate in modified capillary assays (magnification, $\times 40$). Capillaries ($1 \mu\text{l}$) contained crystals of 4-nitrobenzoate in chemotaxis buffer. (A) Benzoate-grown PRS2000; (B) benzoate-grown TW3; (C) 4-nitrobenzoate-grown TW3; (D) 4-nitrotoluene-grown TW3; (E) 4-hydroxybenzoate-grown strain 4NT; (F) 4-nitrobenzoate-grown strain 4NT. No responses were seen when capillaries contained only agarose and chemotaxis buffer or silica in chemotaxis buffer (data not shown).

TABLE 3. Summary of modified capillary assay results

Attractant ^c	Chemotactic response ^a of <i>Pseudomonas</i> strain:												
	PRS2000 grown on ^b :			TW3 grown on ^b :					4NT grown on ^b :				
	Suc	Ben	4HB	Suc	Ben	4HB	4NB	4NT	Suc	Ben	4HB	4NB	4NT
None	-	-	-	-	-	-	-	-	-	-	-	-	-
CAA	+	+	+	+	+	+	+	+	+	+	+	+	+
Ben	-	+	+	-	+	+	+	+	-	+	+	-	-
4NB	-	+	+	-	+	+	+	+	-	+	+	-	-

^a Measured in modified capillary assays as described in Materials and Methods and shown in Fig. 3. +, positive response observed; -, no response observed.

^b Growth substrates: Suc, 10 mM succinate; Ben, 5 mM benzoate; 4HB, 5 mM 4-hydroxybenzoate; 4NB, 4-nitrobenzoate crystals; 4NT, 4-nitrotoluene crystals.

^c Attractants present in capillary: None, chemotaxis buffer in 2% agarose; CAA, 2% Casamino Acids in chemotaxis buffer and 2% agarose; Ben, 5 mM benzoate in chemotaxis buffer and 2% agarose; 4NB, 4-nitrobenzoate crystals in chemotaxis buffer and 2% agarose.

been reported, and the inducibility of the chemotactic response was not investigated. It is therefore difficult to conclude whether the chemotaxis system for the detection of nitroaromatic compounds in *Ralstonia* sp. strain SJ98 is similar to that in the three *Pseudomonas* described here.

The evolution of catabolic pathways is thought to proceed in a variety of ways. For example, genes can be recruited from existing pathways and selection of modified enzymes with extended substrate ranges can increase the number of substrates degraded. Also, modular genetic elements can combine to form complete pathways (49). Later, regulatory elements can be recruited for optimization of gene expression, and this may reduce the chance of a particular strain being outcompeted at times when no selective compounds are present (5, 6, 8, 49). In the case of the 4-nitrotoluene degradation pathway in strain TW3, the enzymes used for the conversion of 4-nitrotoluene to 4-nitrobenzoate appear to have been acquired from a TOL-plasmid upper pathway (22) with the exception of the NAD(P)⁺-independent alcohol dehydrogenase, which has no homologue in the TOL pathway (21). The genes required for the conversion of 4-nitrobenzoate to protocatechuate are apparently unlinked to those for the formation of 4-nitrobenzoate from 4-nitrotoluene in strain TW3, and they have no close homologues in the GenBank database other than those encoding isofunctional nitrobenzoate degradation proteins (20).

Chemotaxis, the ability of motile bacteria to detect and respond to specific chemicals in the environment, can increase an organism's chances of locating useful sources of carbon and energy. Thus, chemotaxis can provide an additional growth and

TABLE 4. Catechol dioxygenase activities in extracts of strains 4NT and TW3

Growth substrate (concn)	Sp act ($\mu\text{mol min}^{-1} \text{mg}^{-1}$ of protein) of indicated strain for:			
	Catechol 1,2-dioxygenase		Catechol 2,3-dioxygenase	
	4NT	TW3	4NT	TW3
Succinate (10 mM)	- ^a	0.039	-	0.005
Benzoate (5 mM)	1.34	0.330	-	0.015

^a -, no activity detected.

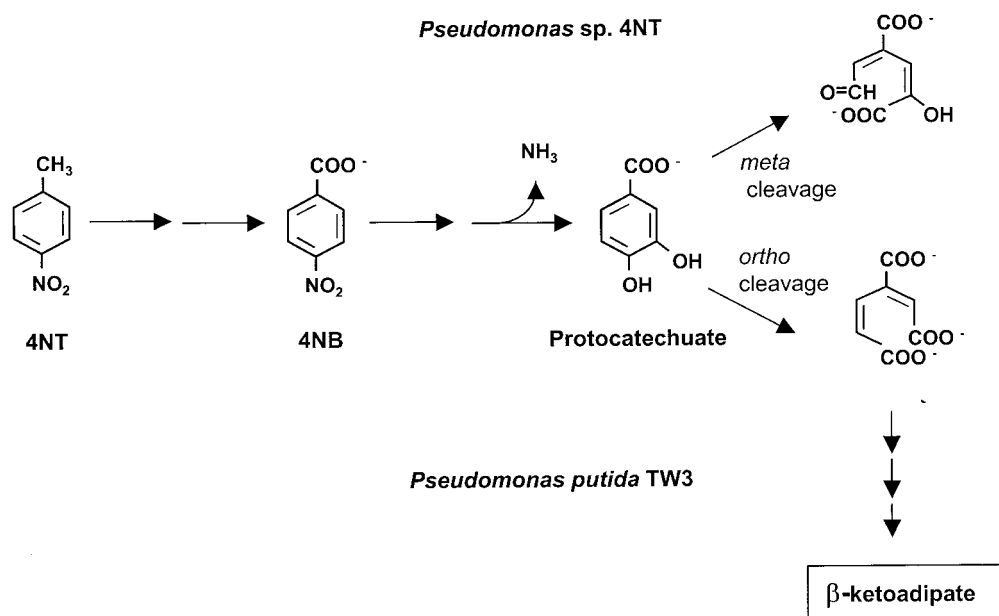


FIG. 4. Pathways for 4-nitrotoluene and 4-nitrobenzoate degradation in *P. putida* TW3 and *Pseudomonas* sp. strain 4NT. Note that *P. putida* TW3 utilizes *ortho* cleavage and *Pseudomonas* sp. strain 4NT carries out *meta* cleavage of protocatechuate.

survival advantage to bacteria and may even contribute to the dissemination of catabolic pathways, many of which are encoded by transmissible plasmids (14, 33). During the evolution of a new biodegradation pathway, it is not clear how and when specific genes for auxiliary aspects of catabolism, such as chemotaxis, are introduced. Since most catabolic genes are recruited from preexisting pathways, it seems likely that an organism would also acquire chemoreceptor functions from existing systems. It appears that an aromatic acid chemotaxis system like that in *P. putida* PRS2000 is also present in strains TW3 and 4NT. As pointed out earlier (14), the β -ketoadipate-inducible aromatic acid chemotaxis system in *Pseudomonads* is likely to be induced most of the time during the life cycle of the bacterial cells due to the abundance of plant-derived phenylpropanoids and hydroaromatic compounds in soils that are degraded through β -ketoadipate (35). The broad specificity of the system works as an advantage in the last two strains, since an existing chemotaxis system, apparently used for benzoate and 4-hydroxybenzoate chemotaxis in PRS2000, functions to detect the useful carbon source 4-nitrobenzoate.

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