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NahY, a Catabolic Plasmid-Encoded Receptor Required for Chemotaxis of *Pseudomonas putida* to the Aromatic Hydrocarbon Naphthalene

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***Pseudomonas putida* G7 exhibits chemotaxis to naphthalene, but the molecular basis for this was not known. A new gene, *nahY*, was found to be cotranscribed with *meta* cleavage pathway genes on the NAH7 catabolic plasmid for naphthalene degradation. The *nahY* gene encodes a 538-amino-acid protein with a membrane topology and a C-terminal region that resemble those of chemotaxis transducer proteins. A *P. putida* G7 *nahY* mutant grew on naphthalene but was not chemotactic to this aromatic hydrocarbon. The protein NahY thus appears to function as a chemoreceptor for naphthalene or a related compound. The presence of *nahY* on a catabolic plasmid implies that chemotaxis may facilitate biodegradation.**

The ability of bacteria to recognize and swim toward aromatic hydrocarbons is possibly an important prelude to their degradation, but this has not been proven. Recently we reported that naphthalene, a polyaromatic hydrocarbon and a U.S. Environmental Protection Agency priority pollutant (8), is a chemoattractant for *Pseudomonas putida* G7 (5). Naphthalene is produced in large quantities by the petrochemical industry as a bulk chemical for use in organic syntheses and has been a model compound for studies of biodegradation because it is relatively easily metabolized by soil bacteria (11, 21). The genes required for its complete degradation are typically located on large self-transmissible catabolic plasmids and, in the case of the catabolic plasmid NAH7, have been shown to reside within the boundaries of a defective transposon (17, 20). A mutant derivative of *P. putida* G7 (G7.C1) that was cured of NAH7 was not chemotactic to naphthalene. Moreover, a 25-kb subclone of the 83-kb NAH7 plasmid conferred on *P. putida* G7.C1 the ability to grow on naphthalene but did not restore naphthalene chemotaxis to the strain (5). This suggested that an additional gene or genes required for naphthalene chemotaxis might be present on NAH7. Here, we describe work on the molecular basis for chemotaxis to naphthalene and report the identification of a chemotaxis protein transducer gene on the catabolic plasmid NAH7.

Bacterial strains and experimental procedures. *P. putida* G7 and its plasmid NAH7-cured derivative, *P. putida* G7.C1, were obtained from D. T. Gibson at the University of Iowa. *P. putida* G7.C1(pHG100) has been previously described (5). Cells were grown in Luria broth (1a) or minimal mineral salt medium (7) supplemented with a carbon source. Succinate was used as a growth substrate at a final concentration of 10 mM. Naphthalene was provided as a carbon source by the direct addition of naphthalene crystals to liquid minimal medium, due to the limited solubility of this compound. Liquid cultures were grown at 30°C with shaking at 250 rpm.

The bacterial strains and plasmids used are described in Table 1. Antibiotics were added when necessary at the following concentrations: kanamycin, 100 μg/ml; gentamicin, 10 μg/ml; and tetracycline, 50 μg/ml for *P. putida* and 25 μg/ml for *Escherichia coli*. Plasmids were introduced into *P. putida* from *E. coli* S17-1 by conjugation. Mating mixtures were spread on minimal medium containing succinate and either kanamycin, gentamicin, or both. In the case of plasmid pHG97, which was used to create the *nahY* mutant *P. putida* G7 Y1, exconjugants were screened for sensitivity to gentamicin to ensure that the suicide delivery vector, pSUP102-Gm, had been lost. A gentamicin-susceptible (Gm^s) kanamycin-resistant (Km^r) colony was then passaged on a minimal medium plate containing succinate and kanamycin for about a week to allow for complete loss of any wild-type NAH7 plasmid that might still be present in the strain.

Southern analysis was carried out with the Genius hybridization kit according to the manufacturer's instructions (Roche Molecular Biochemicals, Inc., Indianapolis, Ind.). Plasmids were isolated from *E. coli* and *P. putida* with the Qiagen (Santa Clarita, Calif.) miniprep spin or midiprep system. Nucleotide sequencing was performed by the University of Iowa DNA Core Facility. Sequence data were analyzed with software from Textco, Inc. (West Lebanon, N.H.), Genetics Computer Group (Madison, Wis.), and the National Center for Biotechnology Information (Bethesda, Md.).

Reverse transcriptase PCR (RT-PCR) was used to determine if *nahX* and *nahY* were cotranscribed with *nahJ*, a structural gene of the lower naphthalene degradation pathway. RNA was isolated from naphthalene-grown cells by using the SV Total RNA isolation system from Promega (Madison, Wis.). Cells were sonicated after being resuspended in lysis buffer to improve RNA yield. RT-PCR was performed with the Access RT-PCR system from Promega according to the manufacturer's instructions. The primers used for RT-PCR were SH-R2 (5′-GCGCCGACTAGCATTAAAAG-3′) and Sal3R-2 (5′-CGCCCAGTTGTACATCCTC-3′) for the region from *nahJ* to *nahX*. Primers NahXN (5′-CCATGATCATCTCGAC CCTCGAAAC-3′) and NahYN2 (5′-CGTCATAGGCACGC TTTGATTCC-3′) were used for the region from *nahX* to *nahY*.

Chemotaxis to naphthalene was tested by a modified capil-

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
P. putida		
G7	Wild-type strain containing the NAH7 catabolic plasmid	3
G7.C1	NAH7-cured derivative of G7	D. T. Gibson
G7 Y1	*nahY*::Km mutant	This work
E. coli		
DH5α	F^- λ^- *recA1* Δ(*lacZYA-argF*)*U169 hsdR17 thi-1 gyrA96 supE44 endA1 relA1* ϕ80*lacZ*ΔM15	Gibco-BRL
S17-1	*thi pro hdsR hdsM*$^+$ *recA* chromosomal insertion of RP4-2(Tc::Mu Km::Tn*7*)	14
Plasmids		
pBBR1-MCS2	Km^r; multicopy broad-host-range vector	9
pBBR1-MCS5	Gm^r; multicopy broad-host-range vector	9
pLAFR1	Tc^r; low-copy-number broad-host-range cosmid	4
pSUP102::Gm	Gm^r Cm^r; suicide vector in *Pseudomonas*	13
pUC19	Ap^r; ColE1 replicon	19
pHG59	Km^r; pBBR1-MCS2 containing a 5.9-kb *Eco*RI fragment from NAH7[b]	This work
pHG60	Km^r; pBBR1-MCS2 containing a 4-kb *Sma*I-*Eco*RI fragment from pHG59 cloned into *Hin*cII-*Eco*RI sites	This work
pHG70	Ap^r; the insert of pHG60 is cloned into the *Kpn*I-*Bam*HI sites of pUC19, resulting in *Eco*RI sites on either side of the insert	This work
pHG95	Ap^r Km^r; pHG70 with the 3′ end of *nahY* removed with *Nsi*I and replaced with a GenBlock Km^r cassette from Pharmacia that had been digested with *Pst*I	This work
pHG97	Km^r Gm^r; the *Eco*RI insert of pHG95 (containing *nahY*::Km) cloned into pSUP102::Gm	This work
pHG100	Tc^r; pLAFR1 containing a 25-kb *Eco*RI fragment from NAH7[b]	5
pHG125	Gm^r; 1.7-kb *Bsi*WI fragment from pHG59 that contains *nahY* introduced into the *Acc*651 (*Kpn*I) site of pBBR1-MCS5	This work

[a] Ap^r, ampicillin resistant; Gm^r, gentamicin resistant; Cm^r, chloramphenicol resistant, Tc^r, tetracycline resistant.
[b] See Fig. 1.

lary assay (5) and also by the agarose-in-plug assay described by Yu and Alam (23). Chemotaxis to salicylate was tested only with the agarose-in-plug assay. Cells used in these assays were grown on naphthalene as a sole source of carbon and energy to mid-logarithmic phase, harvested, and resuspended in chemotaxis buffer (100 mM potassium phosphate [pH 7.0], 20 μM EDTA). Cells were motile in chemotaxis buffer for at least 1 h in the absence of an exogenous energy source. With the capillary method, the open end of a buffer-filled 1-μl capillary tube was packed with finely ground crystals of naphthalene and introduced into a chamber containing a suspension of motile cells. Chemotaxis was determined microscopically and defined as the accumulation of a cloud of cells around the tip of the capillary tube over a period of 10 to 20 min. With the agarose-in-plug method, a blob of melted agarose containing an attractant, either crystals of naphthalene or 50 mM salicylate, was placed between a microscope slide and a glass coverslip that was laid across two strips of plastic to form a bridge. After the agarose solidified to form a plug, a suspension of cells was introduced into the space between the slide and coverslip so that it flooded around the plug. Cells that responded to the naphthalene or salicylate formed a dense band, visible to the naked eye, about 0.5 mm from the plug within a period of 5 min. Cells did not respond to a plug of agarose prepared with chemotaxis buffer alone. Soft agar swarm plates were used to assess chemotaxis to succinate, 4-hydroxybenzoate, and benzoate, as described previously (2).

The NAH7 plasmid contains a gene or genes necessary for chemotaxis to naphthalene. To determine whether plasmid NAH7 included genes that were required for chemotaxis to naphthalene, we started with a strain [*P. putida* G7.C1 (pHG100)] that we had previously constructed which was cured of the NAH7 plasmid but could grow on naphthalene because it carried all of the upper and most of the lower naphthalene pathway genes on pHG100, a subclone of NAH7 (5) (Fig. 1). We presume that *ortho* cleavage genes located on the chromosome allowed the strain to circumvent the need for all of the lower pathway genes. This strain did not exhibit chemotaxis to naphthalene. We then introduced additional subclones of NAH7 into strain G7.C1(pHG100) on a compatible plasmid and tested for naphthalene chemotaxis. Naphthalene chemotaxis was restored by a subclone (pHG59) that contained a 5.9-kb *Eco*RI fragment that mapped immediately adjacent to the 25-kb *Eco*RI fragment used to make pHG100 (Fig. 2). Introduction of the vector pBBR1-MCS2, used to construct pHG59, into strain G7.C1(pHG100) did not restore chemotaxis to naphthalene. Strain G7.C1 carrying only pHG59 sometimes exhibited a weak chemotactic response to naphthalene. However, a consistently strong response was seen only when pHG100 was present together with pHG59 in strain G7.C1.

Genes present on the chemotaxis-complementing clone. Nucleotide sequencing of pHG59 revealed several genes on the 5.9-kb *Eco*RI insert that had not previously been described (Fig. 3). As expected, several lower naphthalene pathway genes required for the degradation of the intermediate salicylate via a *meta* cleavage pathway were found, their locations having previously been roughly mapped to the region of the 5.9-kb *Eco*RI fragment (21). The order of the genes *nahOMKJ* was slightly different from that previously reported based on an analysis of Tn*5* mutations in this region (21). The sequences of the *nahO* and *nahM* genes, encoding acetaldehyde dehydrogenase and 4-hydroxy-2-oxovalerate aldolase, respectively, from the naphthalene catabolic plasmid pWW60-22 had already been reported (10). The deduced amino acid sequences of the *nahK* and *nahJ* genes show a high level of identity (about 90%) to those of homologous *meta* pathway genes involved in phenol and toluene degradation (6, 12). *nahJ* encodes 4-oxalocroto-

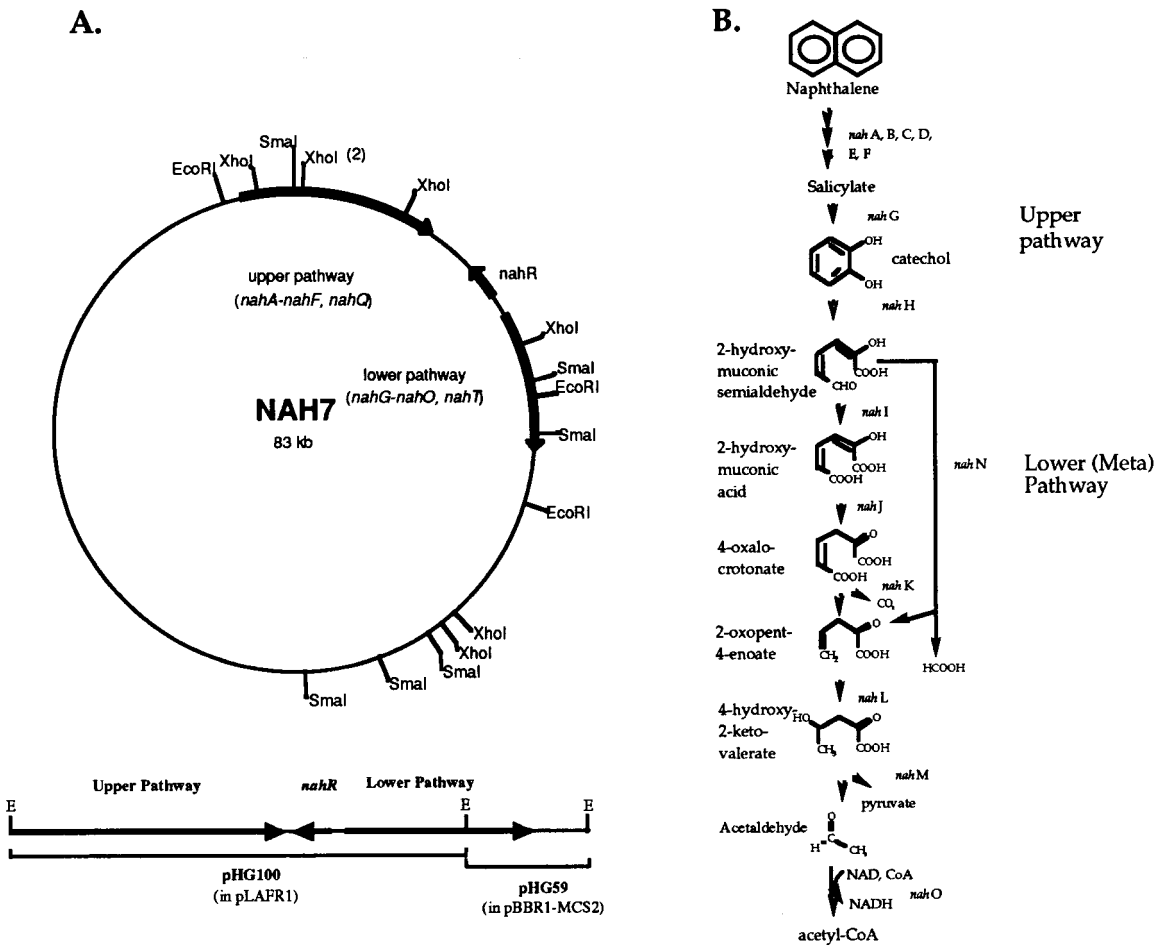

FIG. 1. The naphthalene catabolic plasmid and degradation pathway in *P. putida* G7. (A) NAH7. The region of NAH7 involved in naphthalene degradation is indicated by arrows. Two subclones made from NAH7, pHG100 and pHG59, are shown. Only *Eco*RI sites in or around the naphthalene degradation genes are shown (E, *Eco*RI). (B) Naphthalene catabolic pathway. The *meta* pathway, which converts catechol to acetyl-coenzyme A (acetyl-CoA) and pyruvate, is encoded on the NAH7 plasmid.

nate tautomerase, and *nahK* encodes 4-oxalocrotonate decarboxylase.

Downstream of *nahOMKJ* were two new genes that we have termed *nahX* and *nahY*. *nahX* is predicted to encode a 140-amino-acid protein that resembles (38% amino acid identity) a *Sphingomonas* sp. protein (CmpX) of undefined function. It may be noteworthy that CmpX is encoded by an open reading frame that is also located among plasmid-borne *meta* cleavage genes (22).

nahY shows 28% deduced amino acid identity with PctB, a

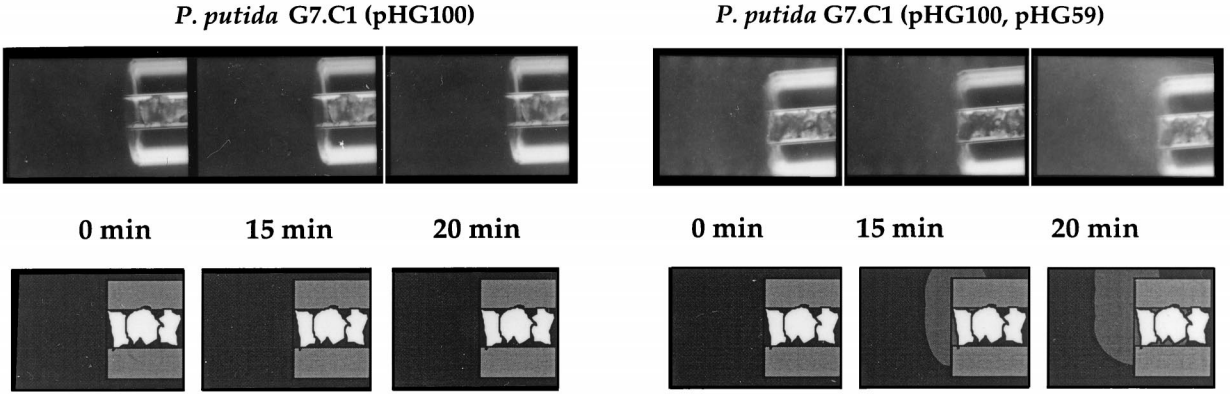

FIG. 2. Chemotactic responses of *Pseudomonas* strains to naphthalene in modified capillary assays. Naphthalene chemotaxis was restored when pHG59 was introduced into *P. putida* G7.C1(pHG100). Cells were grown on naphthalene. The results are shown in both photographic (top) and schematic (bottom) form. Naphthalene crystals are visible inside the mouths of the capillary tubes. The accumulation of a cloud of cells around the mouth of a capillary tube over time indicates a chemotactic response.

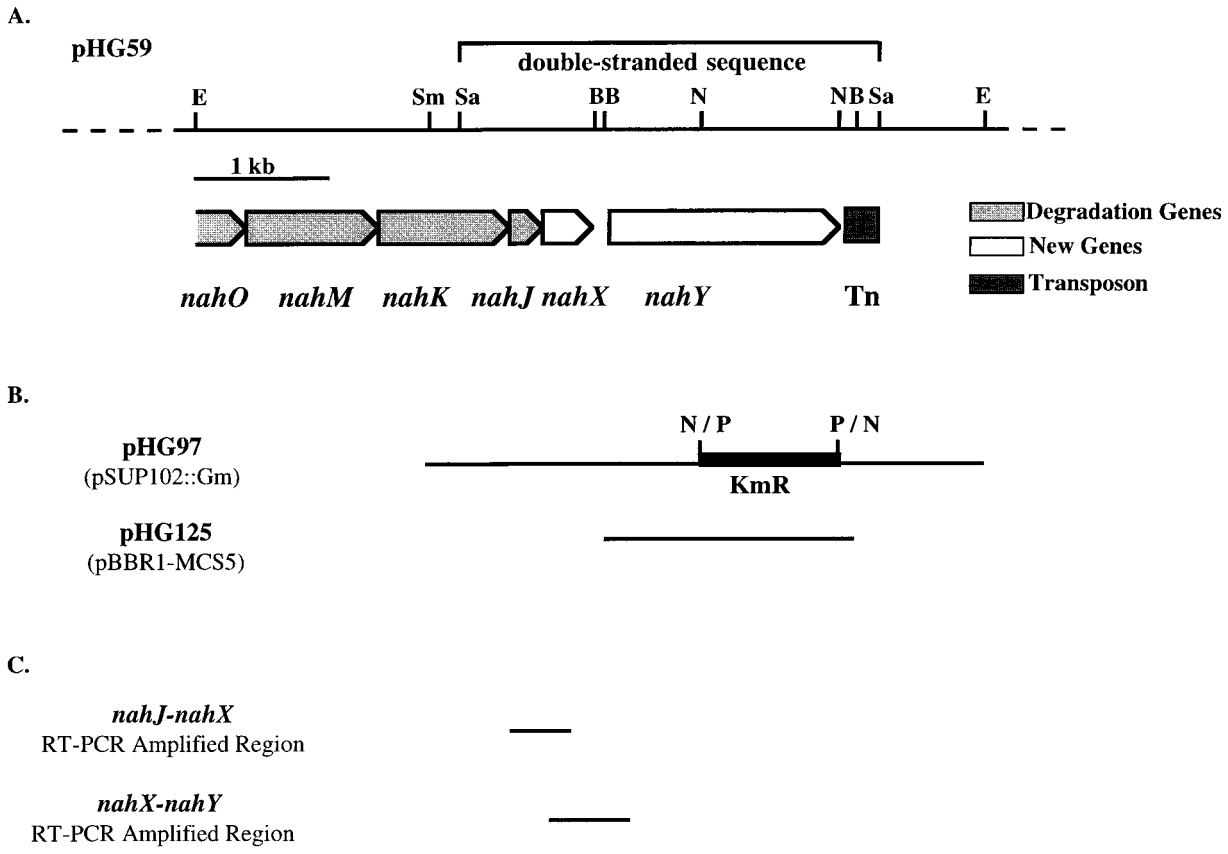

FIG. 3. Plasmid pHG59. (A) Restriction and gene map of pHG59. Sufficient sequencing was done to determine the locations of the starts and stops of all the genes illustrated. B, *Bsi*WI; E, *Eco*RI; N, *Nsi*I; P, *Pst*I; Sa, *Sal*I; Sm, *Sma*I. Not all *Sal*I or *Nsi*I sites are shown. (B) Subclones of pHG59. Plasmid pHG97 (see Table 1 for construction details) was derived from a 4-kb *Sma*I-*Eco*RI fragment of pHG59. A kanamycin resistance cassette was introduced into the *Nsi*I sites of this 4-kb fragment. The second clone, pHG125, contains *nahY* and was used for complementation analysis. (C) Transcript analysis of *nahJ*, *nahX*, and *nahY*. The intergenic regions between *nahJ* and *nahX* and between *nahX* and *nahY* that were amplified by RT-PCR are shown.

membrane-bound transducer protein from *Pseudomonas aeruginosa* that functions in chemotaxis to amino acids (16). The 538-amino-acid predicted NahY protein has several features that are conserved among chemotaxis transducer proteins, also known as methyl-accepting chemotaxis proteins (MCPs). These include two predicted membrane-spanning regions, one near amino acid 10 and the other near amino acid 200.We assume, based on homology with other MCPs, that the intervening sequence is located in the cellular periplasm. Residues 375 to 425 of NahY are about 70% identical to the chemotaxis-signaling domain of transducer proteins from *E. coli* and *Salmonella typhimurium* (15). This region, predicted to be in the cytoplasm, is highly conserved among chemotaxis transducer proteins from diverse bacteria. The C-terminal cytoplasmic regions of MCPs have two domains on either side of the signaling domain that contain glutamate or glutamine residues which are either methylated, demethylated (glutamate), or deamidated (glutamine) as part of the adaptation phase of chemotaxis (15). In the region corresponding to the methylation region designated K1 in the enteric transducers, NahY has two glutamine residues, one at position 291 and the other at position 296, that align to within one amino acid residue of the first two methylation sites of the *E. coli* transducers Tsr, Tar, and Trg. NahY has a glutamate at position 304 that aligns exactly with a glutamine that has been shown to serve as a site of methylation in each of the *E. coli* MCPs. There appears to be only one potential methylation site in the portion of NahY that corresponds to the R1 methylation region. This is a glutamate at position 506 that aligns with glutamates that are methylated in the *E. coli* MCPs. The last five amino acids (NWETF) of the major *E. coli* and *S. typhimurium* chemotaxis transducers serve as a methyltransferase binding sequence (18). NahY lacks this sequence.

Immediately downstream of *nahY* was a region of DNA that was almost identical to the left inverted repeat of Tn*4655*, a transposon previously reported to be part of the NAH7 plasmid (17).

RT-PCR showed that *nahJ*, *nahX*, and *nahY* were cotranscribed in naphthalene-grown cells. This suggests that *nahX* and *nahY* may be part of the lower naphthalene pathway operon of NAH7.

Construction of a *nahY* mutant. The deduced structural characteristics of NahY suggested that it might serve as a catabolic plasmid-encoded chemoreceptor for naphthalene. To test this, we constructed a mutant strain (G7 Y1) in which the 3′ end of *nahY*, including the domain that is highly conserved in transducer proteins, was replaced with a kanamycin resistance cassette (Table 1). Southern hybridization analysis was used to confirm the strain construction and to show that *P. putida* G7 Y1 contained no detectable wild-type copies of *nahY*. The *nahY* mutant strain grew on naphthalene at the same rate as its wild-type parent, but naphthalene-grown cells

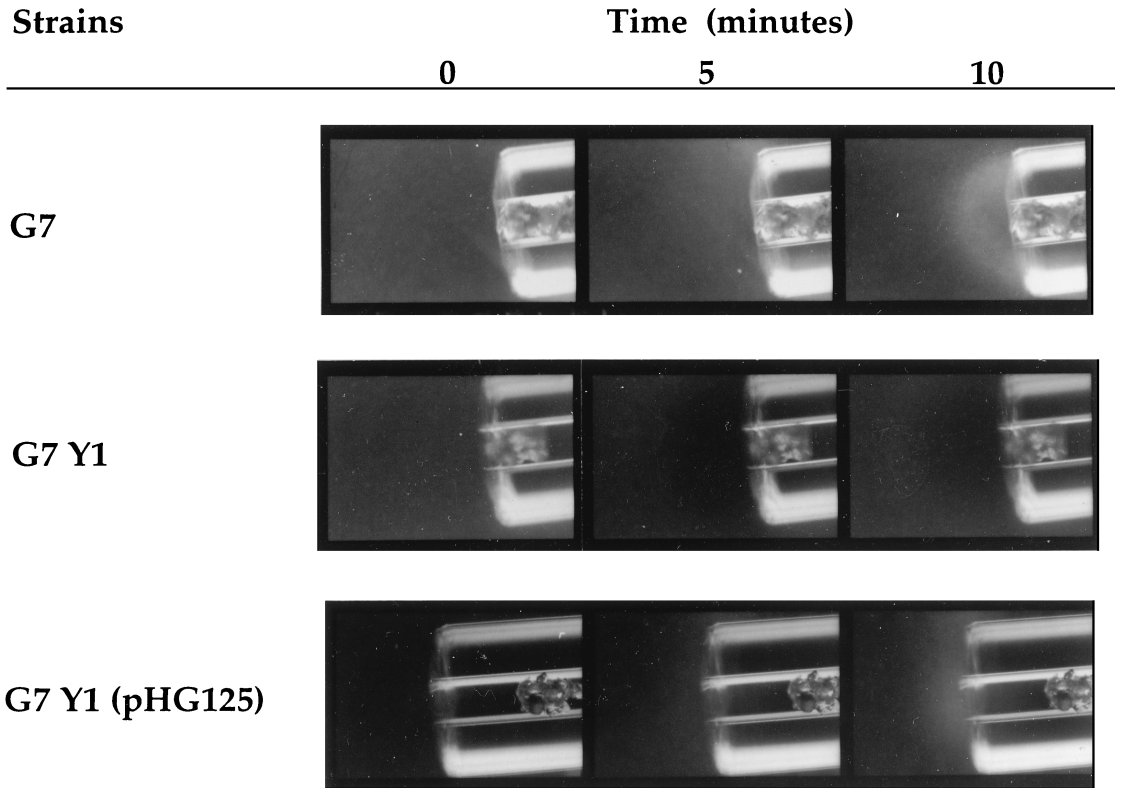

FIG. 4. NahY is necessary for naphthalene chemotaxis, as determined by capillary assay. The mutant *P. putida* G7 Y1 is not chemotactic to naphthalene, but the mutation is complemented by the introduction of pHG125, a clone containing *nahY*. The wild-type strain also exhibited a positive response to naphthalene by the agarose-in-plug method, whereas the *nahY* mutant strain G7 Y1 did not (data not shown). Cells were grown on naphthalene.

of the mutant were not attracted to naphthalene (Fig. 4). The chemotaxis defect appears to be specific to the attractant naphthalene, as the *nahY* mutant had a wild-type chemotactic response to succinate, benzoate, salicylate, and 4-hydroxybenzoate (data not shown). The unstimulated-swimming patterns of the *nahY* mutant were the same as those of wild-type cells, as judged by microscopic observation. Neither the wild type nor the mutant increased its swimming speed in response to the addition of any of the attractants tested. Wild-type *nahY*, supplied on a broad-host-range plasmid, complemented the naphthalene chemotaxis phenotype of *P. putida* G7 Y1 in *trans* (Fig. 4).

NahY is a catabolic plasmid-encoded chemoreceptor. The phenotype of the *nahY* mutant and the deduced amino acid sequence of NahY suggest that this protein is likely to function by binding naphthalene or a related compound on its periplasmic face to initiate chemosensory signaling in a manner analogous to that of other bacterial transducer proteins. Members of this family of proteins have been best studied in *E. coli* and *S. typhimurium*, where they bind amino acids or sugars and initiate sensory signal transduction by altering the activity of CheA, an associated histidine kinase. CheA-P phosphorylates a response regulator protein, CheY, that interacts with rotational "switch" proteins in the flagellar motor. This causes a change in swimming behavior such that cells migrate towards chemoattractants (15). A cluster of genes whose products are homologous to five of the six soluble proteins required for chemotaxis in *E. coli* have recently been identified in *P. putida* (2), suggesting that the two species process sensory information to effect chemotaxis in similar ways.

Although NahY has a signaling domain, potential methylation sites, and a membrane topology typical of chemotaxis transducer proteins, it does not show a high degree of overall amino acid identity with other MCPs. This is particularly evident if one compares the predicted N-terminal periplasmic domains of transducers and transducer-like proteins (Fig. 5). The presumed periplasmic sensing domain of NahY is clearly an outlier, rather distantly related to the N-terminal sensing domains of other bacterial and archaeal transducer proteins. This raises the possibility that NahY may have distinct chemosensing characteristics. In fact, several features of NahY chemoreceptor function remain to be explored. We still do not know, for example, exactly what NahY senses and whether it functions alone or in concert with NahX. We have been unable to consistently demonstrate naphthalene chemotaxis in a *P. putida* strain that carries the *nahY* gene in *trans* without naphthalene degradation genes also being present. This may suggest that a metabolite of naphthalene, rather than naphthalene itself, is the chemoattractant. If this is the case, then such a metabolite is likely to be an intermediate of the upper naphthalene degradation pathway, since the *nahY* mutant is not defective in chemotaxis to salicylate, the starting compound of the lower pathway.

That *nahY* is located on the NAH7 catabolic plasmid and cotranscribed with genes for naphthalene degradation suggests that chemotaxis may be an important adjunct to biodegradation. The availability of *nahY* mutants should facilitate work to critically test the possibility that chemotaxis to aromatic hydrocarbons may enhance their biodegradation in natural environments.

Nucleotide sequence accession number. The *nahKJXY* sequences have been assigned GenBank accession no. AF100302.

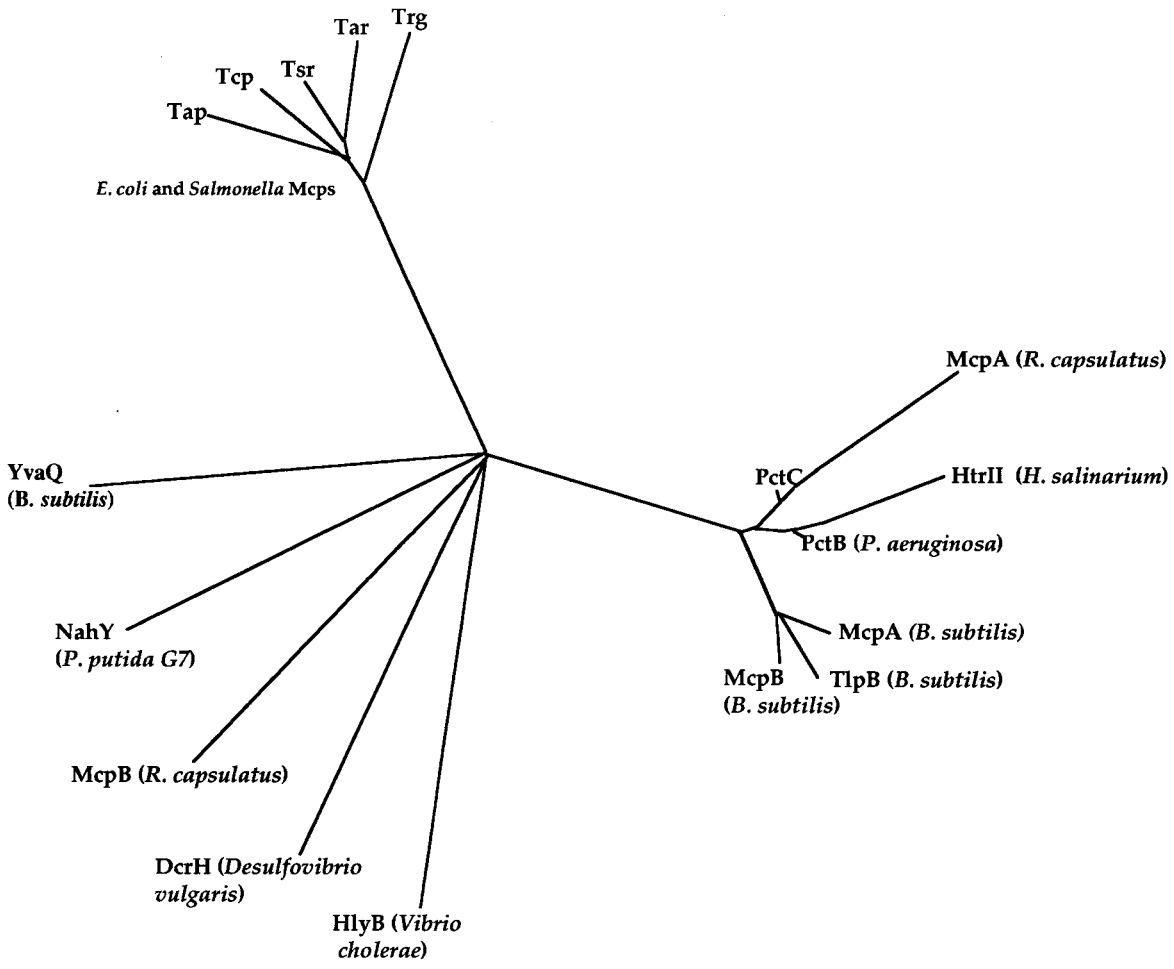

FIG. 5. Phylogenetic tree of the predicted N-terminal periplasmic domains of selected bacterial transducer proteins constructed by the AllAll program of the Computational Biochemistry Research Group (1). The proteins included, sources, and National Center for Biotechnology Information Entrez protein accession numbers are as follows: Tap, MCP IV (dipeptide chemoreceptor) from *E. coli*, 2506839; Tcp, methyl-accepting chemotaxis citrate transducer from *S. typhimurium*, 400235; Tsr, MCP I (serine chemoreceptor) from *E. coli*, 400233; Tar, MCP II (aspartate chemoreceptor protein) from *E. coli*, 2506837; Trg, MCP III (ribose and galactose chemoreceptor protein) from *E. coli*, 2506838; PctC, chemotaxis transducer protein (amino acid chemoreceptor) from *P. aeruginosa*, 2626833; PctB, chemotaxis transducer protein (amino acid chemoreceptor) from *P. aeruginosa*, 2626836; McpA, methyl-accepting chemoreceptor (presumed) from *Rhodobacter capsulatus*, 2126470; HtrII, sensory rhodopsin II transducer (methyl-accepting phototaxis protein II) from *Halobacterium salinarium*, 3023997; McpA, MCP (amino acid chemoreceptor) from *Bacillus subtilis*, 730002; McpB, MCP (amino acid chemoreceptor) from *B. subtilis*, 730003; TlpB, MCP (amino acid chemoreceptor) from *B. subtilis*, 730959; YvaQ, methyl-accepting chemotaxis-like protein from *B. subtilis*, 2635882; McpB, methyl-accepting chemoreceptor (presumed) from *R. capsulatus*, 2126471; DcrH, methyl-accepting chemoreceptor (presumed) from *Desulfovibrio vulgaris*, 887858; HlyB, hemolysin secretion protein precursor from *Vibrio cholerae*, 123206; NahY, chemotaxis transducer protein for naphthalene from *P. putida*, AF100302.

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