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 the aromatic hydrocarbon 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 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<sup>+</sup>) kanamycin-resistant (Km<sup>+</sup>) colony was then passed 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 nahD, 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

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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 EcoRI fragment that mapped immediately adjacent to the 25-kb EcoRI 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 EcoRI 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 EcoRI fragment (21). The order of the genes nahOMKJ was slightly different from that previously reported based on an analysis of Tn5 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-oxalocrot-
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. 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 EcoRI sites in or around the naphthalene degradation genes are shown (E, EcoRI). (B) Naphthalene catabolic pathway. The meta pathway, which converts catechol to acetyl-coenzyme A (acetyl-CoA) and pyruvate, is encoded on the NAH7 plasmid.

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.
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 Tn4655, 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...
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 chemotactants (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 methyl-ation 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 chemotactant. 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.

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**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.
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REFERENCES


