Cloning and Expression in *Escherichia coli* of the Naphthalene Degradation Genes from Plasmid NAH7

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The genes encoding the enzymes responsible for conversion of naphthalene to 2-hydroxymuconic acid (*nahA* through *nahI*) are contained on a 25-kilobase *EcoRI* fragment of an 85-kilobase NAH plasmid of *Pseudomonas putida*. These genes were cloned into the plasmid vectors pBR322 and RSF1010 to obtain the recombinant plasmids pKGX505 and pKGX511, respectively. To facilitate cloning and analysis, an NAH7 plasmid containing a Tn5 transposon in the salicylate hydroxylase gene (*nahG*) was used to derive the *EcoRI* fragment. The genes for naphthalene degradation were expressed at a low level in *Escherichia coli* strains containing the fragment on the recombinant plasmids pKGX505 or pKGX511. This was shown by the ability of whole cells to convert naphthalene to salicylic acid and by in vitro enzyme assays. The expression of at least two of these genes in *E. coli* appeared to be regulated by the presence of the inducer salicylic acid. In addition, high-level expression and induction appear to be mediated by an NAH plasmid promoter and a regulatory gene located on the fragment. A restriction endonuclease cleavage map of the cloned fragment was generated, and the map positions of several *nah* genes were determined by analysis of various subcloned DNA fragments.

Plasmid-encoded degradation of aromatic hydrocarbons (e.g., naphthalene, salicylate, toluene, xylene, benzoate) has been extensively described in the genus *Pseudomonas* (2, 3, 21). However, the molecular and genetic organization of these plasmids has remained largely unknown due to technical problems caused by their large size and low copy number and the lack of methodology for *Pseudomonas* plasmid isolation. Recently, two groups have cloned fragments of the TOL plasmid and initiated extensive physical and genetic mapping of the organization of this plasmid (5, 9). In addition, Yen and Gunsalus have used Tn5 transposon mutagenesis to map several genes, required for the degradation of naphthalene, that are present on the NAH7 plasmid of *Pseudomonas putida* (7, 22). These studies have suggested that both the NAH and TOL plasmids have a similar operon-like organization of the genes required for the degradation of aromatic compounds as well as positive regulatory genes that control their expression. Inouye et al. have reported that several of the TOL plasmid genes encoding the enzymes for toluene degradation are expressed and regulated in *Escherichia coli* when present as a TOL::RP4 hybrid (13) or on recombinant plasmids (9). More recently, several other genes of the TOL plasmid have been individually cloned into recombinant plasmids and expressed at low levels in *E. coli* (5, 9, 10).

Despite the importance of the NAH plasmid for aromatic chemical degradation, little information is available concerning the gene organization and regulatory mechanisms. To circumvent the problems associated with characterizing the large NAH plasmid from *P. putida* directly, a 30-kilobase (kb) *EcoRI* fragment of an NAH7::Tn5 plasmid was cloned into multicycplasmids and its expression was studied in *E. coli*. This made it possible to obtain large quantities of plasmid DNA for further cloning and genetic analysis.

Experiments presented in this communication show that a 30-kb *EcoRI* fragment of the NAH7 plasmid contains all the genetic information necessary to produce the enzymes for the degradation of naphthalene to 2-hydroxymuconic acid. These genes are expressed in *E. coli* at levels considerably below those in *P. putida*.

**MATERIALS AND METHODS**

**Bacterial strains.** *P. putida* AC10 (*met*; from A. Chakrabarty), *P. putida* 1064 (NAH7) (trp Nap* Sal*) from J. Johnston), *E. coli* C600 (thi trp thi), *E. coli* HB101 (pBR322) (pro leu recA Ap* Tc*), and *E. coli* J53 (RSF1010) (Sm* Su*) (from P. Guerry-Kopecko) were used in these studies. *P. putida* AC-

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10(NAH::Tn5/G67) was prepared by transformation with NAH::Tn5/G67 DNA (from I. C. Gunsalus; ref. 22) by the method of R. Farrell (Ph.D. thesis, University of Illinois, Urbana, 1980).

Media and cell growth conditions for *E. coli* have been described previously (17). Antibiotic selections were performed at 100 μg/ml for kanamycin (Km), 100 μg/ml for ampicillin (Ap), 25 μg/ml for tetracycline (Tc), and 50 μg/ml for streptomycin (Sm). *P. putida* was grown at 30°C in L-broth or basal salts media (22).

Preparation of plasmid DNA. NAH plasmid DNA was isolated from *P. putida* AC10(NAH::Tn5/G67) by the method of R. Farrell (thesis). Recombinant plasmids pKGX505 and pKGX511 were isolated from *E. coli* as described by Schell and Wilson (17), as were the vectors pBR322 (1) and RSF1010 (6).

Restriction endonuclease digestion and analysis. Plasmids were digested with restriction endonucleases in the buffers supplied by the suppliers. After digestion at 37°C for 2 h with 2 U of enzyme per μg of DNA, the reaction mixtures were heated at 65°C for 10 min. Samples were then electrophoresed at 100 V for 5 h on 0.7% agarose gels in Tris-acetate buffer (40 mM Tris-acetate, 5 mM sodium acetate, pH 8.3), followed by staining with ethidium bromide and photography of the DNA bands in UV light. Molecular weights of the resultant fragments were determined by comparison with the migration of known molecular weight markers generated by digestion of a phage DNA with EcoRI or HindIII.

Transformation. *E. coli* strain HB101 or C600 was transformed with ligated plasmid DNA as described previously (17). *P. putida* AC10 was transformed by the method of R. Farrell (thesis).

Preparation of cell extracts and enzyme assays. Cells were grown at 30°C in L-broth containing the appropriate antibiotic (kanamycin or ampicillin) with or without 0.1% (wt/vol) sodium salicylate as an inducer. Late-exponential-phase cultures (absorbancy at 600 nm = 0.8) were harvested by centrifugation, washed with 50 mM sodium-potassium phosphate buffer (pH 7.0), and stored at −80°C until used. Cell pellets were suspended in 2 volumes of 50 mM sodium-potassium phosphate buffer (pH 7.0) containing 10% (wt/vol) ethanol, 10% (wt/vol) glycerol, and 1 mM glutathione and disrupted at 18,000 lb/in² with a French pressure cell. Cellular debris was removed by centrifugation at 28,000 × g for 20 min at 4°C, and the supernatant fractions, clarified by centrifugation for 2 h at 105,000 × g, were used for enzyme assays. For salicylaldehyde dehydrogenase assays, extracts were prepared in 50 mM potassium phosphate (pH 7.0).

The following enzymes were assayed spectrophotometrically as previously described: catechol 2,3-dioxygenase (nahH) (4, 8), 2-hydroxyxymuconic semialdehyde dehydrogenase (nahI) (14), salicylaldehyde dehydrogenase (nahF) (18), salicylate hydroxylase (nahG) (20), and cis-naphthalene dihydrodiol dehydrogenase (nahB) (15). Protein concentrations were determined by the method of Lowry et al. (12) or Kalb and Bernlohr (11). One unit of activity is defined as the amount of enzyme required to produce 1 nmol of product per min.

Naphthalene metabolite analysis. Salicylate production was monitored by colorimetric analysis. Culture supernatants (10 to 100 μl) were diluted to 1 ml with water. Then 0.2 ml of 1% FeNH4(SO4)2 in 5% (vol/vol) acetic acid was added; after 5 min the absorbance at 540 nm was measured.

Naphthalene metabolites were qualitatively analyzed and verified by thin-layer chromatography analysis. Samples of medium were acidified to pH 2 with 1 N HCl, extracted with 2 volumes of ether, dried, and redissolved in 95% ethanol. Samples were chromatographed on cellulose F-254 thin-layer plates in isopropanol-NH4OH-water (20:1:2), and naphthalene-derived metabolites were detected in UV light or by spraying with 0.2% 2,6-dibromo-N-chloro-p-benzoquinoneimine in methanol followed by 10% Na2CO3.

Construction of recombinant plasmids. (i) pKGX505. Plasmid pKGX505 was obtained as follows. EcoRI-digested pBR322 DNA (1.5 μg in 17 μl of EcoRI buffer: 0.1 M Tris-hydrochloride, pH 7.5; 6 mM MgCl2; 50 mM NaCl) was added to EcoRI-digested NAH7::TnS/G67 plasmid DNA (0.5 μg in 45 μl of EcoRI buffer). After heating at 65°C for 10 min and cooling on ice, the DNA mixture was adjusted to 50 mM Tris-hydrochloride (pH 7.5), 50 mM NaCl, 12 mM MgCl2, 10 mM diethiothreitol; 0.05 mM ATP, 100 μg of bovine serum albumin per ml, and 300 μl of T4 DNA ligase per ml. After ligation overnight at 12°C, *E. coli* HB101 cells were transformed with the ligated DNA (17), and transformants were selected by growth on plates of L-agar containing 0.2% glucose and 100 μg of kanamycin per ml at 37°C for 24 to 36 h.

(ii) pKGX511. To construct plasmid pKGX511, EcoRI-digested RSF1010 DNA (5 μg in 20 μl of TE: 10 mM Tris-hydrochloride, pH 7.5; 1 mM EDTA) was mixed with EcoRI-digested pKGX505 DNA (0.2 μg in 10 μl of TE). The DNA mixture was heated and then cooled on ice, and the solution was adjusted as described above. After incubation at 12°C for 2 h, the mixture was diluted 20-fold with ligation buffer (50 mM Tris-hydrochloride, pH 7.5; 50 mM NaCl; 12 mM MgCl2; 10 mM diethiothreitol; 0.05 mM ATP; 100 μg of bovine serum albumin per ml). After addition of 300 U of T4 DNA ligase per ml, the reaction was incubated overnight at 12°C. *E. coli* HB101 was transformed, and colonies were selected for kanamycin resistance as described above. This two-step dilution method of ligation was found to greatly increase the number of Km’ transformants obtained.

(ii) Deletion plasmids. Deletion plasmids (pKGX530, pKGX561, pKGX563, pKGX565, and pKGX567) were constructed as follows. pKGX505 DNA (1.0 μg in 10 μl of TE) was completely digested with the appropriate restriction enzyme. The digests were heated at 65°C for 10 min, chilled on ice, and then diluted to 0.2 ml with ligation buffer. T4 DNA ligase was added to 300 U/ml, and samples were incubated overnight at 12°C. The dilute ligation reaction was used to enhance only recircularization of the digested plasmids. Since the restriction enzymes used in this procedure have no sites in pBR322 (19), all restriction fragments not contiguous with the vector are digested out from the reaction. Transformation and selection for Ap’ clones were performed as described above. Restriction enzymes used to generate each deletion plasmid type were *XmaI* for pKGX530, *SstI* for pKGX561, *BglII* for pKGX563, *XhoI* for pKGX565, and *BamHI* for pKGX567.

Chemicals and reagents. Restriction endonucleases, agarose, CsCl, lambda DNA, and bovine serum albu-
min were obtained from Bethesda Research Laboratories (Rockville, Md.). T4 DNA ligase came from New England Biolabs (Beverly, Mass.). Naphthalene, catechol, and salicylic acid were obtained from (Mal- linekrodt Chemical Co. (Paris, Ky.). Salicylaldehyde (redistilled in vacuo and stored at −20°C) was obtained from Eastman Chemicals (Rochester, N.Y.). Ampicillin, kanamycin, tetracycline, dithiothreitol, ATP, and amino acids came from Sigma Chemical Co. (St. Louis, Mo.). 2,6-Dibromo-N-chloro- p-benzoquinoneimine was a product of Aldrich Chemical Co. (Milwaukee, Wis.). Medium materials were obtained from Difco Laboratories (Detroit, Mich.); TLC plates came from E. M. Laboratories (Elmsford, N.Y.). All other chemicals were of reagent-grade purity.

RESULTS

Cloning of the naphthalene degradation genes. It has been shown that several of the naphthalene degradation genes of the NAH7 plasmid are located on a 25-kb EcoRI fragment (7, 22). To provide a selection for this EcoRI fragment in E. coli, a NAH7::Tn5 derivative (NAH::Tn5/G67) with a Tn5 (Km') transposon inserted into the salicylate hydroxylase gene (nahG) was used (22). After digestion of the NAH::Tn5/G67 plasmid DNA with EcoRI, ligation with EcoRI-digested pBR322 DNA, and transformation into E. coli HB101, recombinant clones were selected and isolated by their kanamycin resistance (Km') phenotype (Fig. 1). The selection for Km' cells eliminates the need for purification of the large fragment before ligation and the process of tedious screening for the rare plasmid containing the large 30-kb insert. Although the control transformation efficiency was normal (10^9 Ap' Te' transformants per μg of DNA) only six Km' clones per μg of DNA were obtained, four of which were also Te' Ap'. Plasmid DNA purified from the four Km' Ap' Te' clones was analyzed by restriction endonuclease digestion with EcoRI and Smal. All transformants with the Km' Ap' Te' phenotype contained both the large 30-kb EcoRI DNA fragment derived from the NAH::Tn5/G67 plasmid and the pBR322 vector in either of the two possible orientations.

One of the new plasmids was designated pKGX505 (Fig. 1). The construction of pKGX505 made it possible to purify larger quantities of the NAH7 fragment for obtaining deletions and further plasmid construction. This allowed the construction of a new plasmid in which the EcoRI fragment was inserted into the multicopy plasmid vector RSF1010 (6). The derivative, pKGX511, is particularly useful because RSF1010 has a broad host range and can be transferred to new strains by conjugative mobilization, thus extending its utility for future experiments. E. coli cells carrying either pKGX505 or pKGX511 were then analyzed for expression of the cloned genes.

Analysis of expression of naphthalene degradation in E. coli. In P. putida strains containing the NAH7 plasmid, naphthalene is degraded via salicylic acid to pyruvate and acetaldehyde by a series of 13 enzymatic reactions (shown in Fig. 2) (7, 22). P. putida strains harboring the NAH::Tn5/G67 plasmid are defective in the conversion of salicylic acid to catechol and thus accumulate salicylic acid in the medium when grown on naphthalene as the sole carbon and energy source. Therefore, strains of E. coli harboring the recombinant plasmids pKGX505 or pKGX511 were tested for their ability to convert naphthalene to salicylic acid (i.e., for expression of the cloned NAH7 plasmid genes naHA through nahF). The results (Table 1) showed that E. coli strains containing the recom-
binant plasmids pKGX505 or pKGX511 had the ability to metabolize naphthalene to salicylic acid, whereas the parental HB101 or C600 strains lacked this ability. The amount of salicylic acid produced by E. coli strains containing the recombinant plasmids was only 3% of the levels produced by P. putida containing the entire NAH::Tn5/G67 plasmid. Nonetheless, these results demonstrate that E. coli has the ability to functionally express all of the NAH7 plasmid genes required for the conversion of naphthalene to salicylic acid (nahA through nahF; Fig. 2). The identification of the material reacting with FeNH₄(SO₄)₂ as salicylic acid was confirmed by thin-layer chromatography of the naphthalene metabolites extracted from the medium.

To analyze more quantitatively the nature and magnitude of the expression and regulation of the cloned nah genes in E. coli, measurements of the specific activities of several of the naphthalene degradation enzymes were made in E. coli strains harboring the pKGX511 or pKGX505 plasmids (Table 2). The results extended the previous observations that E. coli strains harboring the recombinant plasmids pKGX511 or pKGX505 express the nahB, nahF, nahH, and nahI genes. Enzyme activities for nahB and nahF were about 10% of that observed in P. putida (NAH7). The lower relative activities for the nahH and nahI gene products, as well as their non-inducibility, may be caused by a polar effect of the Tn5 insertion in nahG. As expected, salicylate hydroxylase (nahG) activity was absent in all recombinant strains due to the Tn5 insertion.

Salicylate has been reported as the inducer for production of the naphthalene degradative enzymes of the NAH plasmid (Table 2, AC10[NAH7]; ref. 18). Attempts were made to demonstrate similar regulation of expression in E. coli cells containing the nah genes on pKGX511. E. coli cells grown in presence of salicylate consistently had two- to threefold higher activity of the nahB and nahF gene products than cells grown in the absence of salicylate. However, P. putida cells grown under similar conditions showed at least a 20-fold increase in enzyme activity when grown in the presence of salicylate.

**Subcloning of naphthalene degradation genes.** To map the nah genes on the cloned EcoRI fragment and to demonstrate the existence of a regulatory gene required for efficient expression, it was necessary to subclone various segments of the DNA. Cells containing the smaller plasmids could then be analyzed for the presence of various nah gene products. Furthermore, some instability of the large 34-kb recombinant plasmid pKGX505 made subcloning of various smaller NAH DNA fragments desirable before further study of regulation and expression. A simple method for constructing deletions in vitro was used. It involved digestion of

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**TABLE 1. Production of salicylate by various constructed strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Salicylate conc(a) (mg/ml)</th>
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<tbody>
<tr>
<td>E. coli C600</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>E. coli C600(pKGX511)</td>
<td>0.15</td>
</tr>
<tr>
<td>E. coli HB101</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>E. coli HB101(pKGX505)</td>
<td>0.11</td>
</tr>
<tr>
<td>P. putida AC10</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>P. putida AC10(NAH7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>P. putida AC10(NAH::Tn5/G67)</td>
<td>5.00</td>
</tr>
</tbody>
</table>

\(a\) Cells were grown at 30°C in L-broth containing 100 µg of kanamycin per ml to an absorbancy at 600 nm of 0.5. Naphthalene crystals were added to a final concentration of 2.0%, and the cultures were shaken vigorously for 18 h. Culture supernatants were assayed for salicylate by the FeNH₄(SO₄)₂ colorimetric method.
For the various nah gene products were performed on E. coli strains containing each deletion plasmid (Table 3). E. coli strains containing pKGX561 or pKGX563 showed significant activity of the nahB gene product, the dihydrodiol dehydrogenase enzyme, whereas strains harboring pKGX530 or pKGX565 showed no nahB gene product activity. From these data and a comparison with the physical maps of these plasmids (Fig. 3a) it can be concluded that the nahB gene is located somewhere in the region 4 to 7 kb from the left-hand EcoRI site of the cloned NAH EcoRI fragment. The induced levels of the nahB gene product in E. coli cells containing pKGX561 or pKGX563 were over 15-fold less than in cells containing plasmid

![Image of the table](http://jb.asm.org/)

**Table 2. Levels of the nah gene products in recombinant strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Enzyme sp act* (U/mg of protein)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>nahB</td>
</tr>
<tr>
<td></td>
<td>NI I</td>
</tr>
<tr>
<td><strong>E. coli C600 or HB101</strong></td>
<td>&lt;0.1 &lt;0.1</td>
</tr>
<tr>
<td><strong>E. coli C600(pKKGX511)</strong></td>
<td>10 30</td>
</tr>
<tr>
<td><strong>E. coli HB101(pKKGX505)</strong></td>
<td>0.6 24</td>
</tr>
</tbody>
</table>

| P. putida AC10 | <0.1 <0.1 | <0.1 <0.1 | <0.1 <0.1 | <0.1 <0.1 | <0.1 <0.1 |
| P. putida AC10(NAH7) | 4.0 390 | 30 700 | 10 541 | 2.2 70 | 5.0 130 |
| P. putida AC10(NAH::Tn5/G67) | 3.0 350 | 25 750 | 11 25 | 0.4 0.4 | 4.0 <0.1 | <0.1 |

*Extracts were prepared from cells grown in the presence (I) or absence (NI) of the inducer salicylate and assayed as described in the text. The following enzyme/gene product designations were used: nahB, naphthalene dihydrodiol dehydrogenase; nahF, salicylaldehyde dehydrogenase; nahG, salicylate hydroxylase; nahH, catechol 2,3-dioxygenase; nahL, 2-hydroxymuconate semialdehyde dehydrogenase.

pKGX505 with restriction enzymes that do not have sites in the pBR322 vector, ligation at very dilute DNA concentrations, and selection for Ap<sup>+</sup> transformants (for details see Materials and Methods). This procedure was carried out with the enzymes *XmaI*, *XhoI*, *SstI*, *BamHI*, and *BglII*. Plasmid DNA isolated from each of the respective transformants was analyzed by restriction enzyme digestion; a schematic summary of the resultant plasmids is shown in Fig. 3a. The map positions of the subclones relative to the 30-kb parent fragment were determined by digestion with *EcoRI*, *Smal*, and the respective enzyme used to generate deletion.

To establish which nah genes are located on the subcloned DNA fragments, enzyme assays

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**Fig. 3.** Physical and genetic maps of pKGX505 and various deletion subclones. (a) Each subcloned DNA fragment is shown in relationship to the 30-kb EcoRI fragment of the plasmid NAH::Tn5/G67. (b) The letter designations represent the approximate locations of various nah genes as designated in the legend of Fig. 2. R = nahR; parentheses denote approximate position. The genetic map of nah genes is taken from Yen and Gunsalus (7, 22) (Ο—Ο) and this work (Ο—Ο). (c) Restriction enzyme sites are designated as follows: H, HindIII; E, EcoRI; X, XhoI; S, Smal; B, BamHI; L, BglII; T, SstI; C, CiaI; P, Hpal.
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CLONING OF THE nah GENES

TABLE 3. Levels of nah gene products in E. coli containing recombinant plasmids

<table>
<thead>
<tr>
<th>Strain</th>
<th>Enzyme sp act* (U/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nahB</td>
</tr>
<tr>
<td></td>
<td>NI</td>
</tr>
<tr>
<td>HB101</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>HB101(pKGX530)</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>HB101(pKGX561)</td>
<td>1.2</td>
</tr>
<tr>
<td>HB101(pKGX563)</td>
<td>1.3</td>
</tr>
<tr>
<td>HB101(pKGX565)</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>HB101(pKGX567)</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

* Assays on each strain were performed as described for Table 2.

pKGX511 or pKGX505. In addition, growth in the presence of salicylate had little effect on the levels of nahB in cells containing pKGX561 or pKGX563. These results suggest the loss of some genetic information required for induction of NAH enzyme production by salicylate and high-level expression of nahB. The lost information may be the nahR gene hypothesized by Yen and Gunsalus (22). It is located in the 8- to 18-kb region of the NAH EcoRI fragment that was deleted from the pKGX561 and pKGX563 plasmids and seems to be required for full expression.

E. coli cells containing pKGX561 and pKGX563 were grown in the presence of naphthalene for 18 h, and the resultant metabolites were extracted and analyzed by thin-layer chromatography. A compound with migration characteristics identical to authentic 1,2-dihydroxy-naphthalene was detected. In addition, 1,2-naphthoquinone, an air oxidation product of 1,2-dihydroxy-naphthalene, was also detected. Cells containing pKGX530, however, did not produce any detectable naphthalene metabolites. The nahA gene(s) is therefore probably located in the 0- to 6-kb region near the left-hand EcoRI site, since pKGX561 and pKGX563 contain this region and confer upon E. coli the ability to convert naphthalene to 1,2-dihydroxy-naphthalene. Since no 2-hydroxycarboxylic acid was detected, the nahC gene probably extends beyond the 6.5-kb point into the 8- to 18-kb region.

Plasmid pKGX563 also confers on E. coli cells the ability to produce the nahH and nahI gene products, catechol 2,3-dioxygenase and 2-hydroxyacetone semialdehyde dehydrogenase, respectively. Plasmid pKGX567 also promotes synthesis of these two nah gene products. The results suggested that the nahH and nahI genes are located in the 24- to 28-kb region near the right-hand EcoRI site of the NAH EcoRI fragment (Fig. 3a). Expression of nahH or nahI genes is somewhat less in the subclones containing pKGX563 and pKGX567 than in E. coli strains containing the plasmid pKGX511 or pKGX505. These genes may be expressed by read-through from some other promoters. None of the E. coli strains harboring the deletion plasmids contained the nahF gene product, salicylaldehyde dehydrogenase, whereas cells containing the plasmid pKGX511 or pKGX505 did. This result suggests that the nahF gene probably lies in the 8- to 18-kb region deleted from the center of the NAH EcoRI fragment.

Construction of the physical map. Restriction enzyme digestion and electrophoretic analysis of the plasmid pKGX505 were used to generate a physical map of the cloned fragment of the NAH7 plasmid DNA. Analysis was performed as described in Materials and Methods with the restriction endonucleases SstI, BglII, XhoI, BamHI, Clal, HpaI, and HindIII, employing double and triple digestions in conjunction with EcoRI and Smal. A summary of the results is shown in Fig. 3c. Sites in pBR322 vector (19) and Tn5 (16) were utilized to aid in mapping and orientation of the fragments. The figure shows the restriction endonuclease cleavage map of the cloned EcoRI NAH7 DNA fragment relative to a genetic map of the nahA through nahI genes (Fig. 3b). This genetic map was derived from the transposon mutagenesis data of Yen and Gunsalus (7, 22), heteroduplex analysis (M. Sullivan, K.-M. Yen, M. A. Schell, and I. C. Gunsalus, unpublished data), and the enzyme assay data on subclones presented in the previous section. It represents a schematic summary of the present information about gene organization and location in the NAH7 naphthalene degradation genes. This new structural information will be valuable for future structural analysis of this DNA and its genes.

DISCUSSION

A 30-kb fragment of DNA from a 85-kb NAH7::Tn5 plasmid of P. putida was cloned in E. coli. This EcoRI fragment contained all of the genetic information required for the metabolism of naphthalene via salicylic acid to 2-hydroxy-muconic acid. Seven of these genes were shown to be expressed in E. coli at a low level when
contained in two different plasmid vectors. The nature and origin of the expression of several genes have been examined and appear to derive from an NAH plasmid promoter. This conclusion is based on two observations as follows. (i) The orientation of the cloned EcoRI fragment did not affect the level of expression of the cloned genes in E. coli. In all cloning experiments, greater than 80% of the Km' clones were initially capable of converting naphthalene to salicylate. Therefore, expression derived solely from plasmid vector promoters seems an unlikely explanation. (ii) There appeared to be an effect on levels of nahB and nahF expression in E. coli by the inducer salicylate. Although an induction of two- to three-fold was shown for the production of nahB and nahF gene products, this is substantially less induction than that observed in P. putida AC10(NAH7). One possible explanation is the inhibitory effect of the inducer salicylate on the growth of E. coli (unpublished data). Alternatively, the Pseudomonas promoters may not function as well in E. coli and may be maximally expressed in the uninduced state. The induction effect of salicylate and high-level expression were abolished by subcloning the nahB gene (nahB activity: Table 2 versus Table 3). This suggests an effect on expression in E. coli by the putative regulatory locus nahR (22), which is located on the EcoRI fragment. The efficiency of the expression of these foreign genes in E. coli as compared to P. putida is low. Although enzyme levels in E. coli were ~10% of those found in P. putida, it should be noted that the copy number of the genes in E. coli is probably at least 10-fold higher than in a P. putida strain containing NAH7. The regulated expression of these genes is consistent with the previous report of controlled expression of foreign aromatic hydrocarbon degradation genes in E. coli; cloned genes from the TOL plasmid are similarly expressed and controlled in E. coli (9, 10, 13) by a regulatory gene.

Expression of these two sets of genes in E. coli suggests a similar organization and broad host range where aromatic degradation plasmids could potentially function to allow utilization of aromatic substrates. This is supported by the fact that E. coli cells containing pKGX511 had the ability to “grow” on naphthalene; cells plated on minimal agar containing 0.1% Casamino Acids were able to form small colonies after 14 days of incubation at 25°C in the presence of naphthalene vapor, whereas control cells did not. This suggests that cells containing this DNA do not need any additional unusual metabolic systems to utilize naphthalene to generate cell mass. Transport systems for naphthalene either are not required or are encoded on the NAH7 fragment. Presumably the limiting factor for growth is the efficiency of the expression of these genes and the toxicity of accumulated salicylate.

The ability of E. coli HB101 or C600 containing the pKGX511 or pKGX505 plasmids to convert naphthalene to salicylate (full expression of nahA through nahF) was unstable. After several serial transfers on medium containing kanamycin, only a small fraction of the Km' cells retained the ability to metabolize naphthalene to salicylate. Structural changes (i.e., deletion of internal segments of DNA from the cloned EcoRI fragment), but not total loss of the entire plasmid, were detected. These changes correlated with loss of salicylate production ability.

A large segment of NAH7 DNA (>25 kb) has been cloned by utilizing a Tn5 transposon marker. The Tn5 transposon marker allowed the use of the broad host range vector RSF1010 as a cloning replicon. Direct selection for recombinants containing the desired insert was employed without construction of new vectors or screening by insertional inactivation. This broad host range recombinant plasmid may now be studied in various other organisms for expression and ability to confer naphthalene utilization.

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LITERATURE CITED


