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Molecular Cloning of the *nahC* Gene Encoding 1,2-Dihydroxynaphthalene Dioxygenase from *Pseudomonas fluorescens*

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Abstract The complete nucleotide sequence of the *nahC* gene from *Pseudomonas fluorescens*, the structural gene for 1,2-dihydroxynaphthalene (1,2-DHN) dioxygenase, was determined. The 1,2-DHN dioxygenase is an extradiol ring-cleavage enzyme that cleaves the first ring of 1,2-dihydroxynaphthalene. The amino acid sequence of the dioxygenase deduced from the nucleotide sequence suggested that the holoenzyme consists of eight identical subunits with a molecular weight of approximately 34,200. The amino acid sequence of 1,2-DHN dioxygenase showed more than 90% homology with those of the dioxygenases of other *Pseudomonas* strains. However, sequence similarity with those of the *Sphingomonas* species was less than 60%. The *nahC* gene of *P. fluorescens* was moderately expressed in *E. coli* NM522, as determined by enzymatic activity.

Key words: *Pseudomonas fluorescens*, 1,2-dihydroxynaphthalene dioxygenase, *nahC* gene

Pseudomonas fluorescens SM11 can assimilate naphthalene as its sole carbon source. The bacteria convert naphthalene to salicylate by a catabolic upper pathway [7]. Salicylate is further degraded by other catabolic enzymes. In the metabolic upper pathway of naphthalene, 1,2-dihydroxynaphthalene (1,2-DHN) is cleaved by a dioxygenase to an unstabe ring cleavage product, which spontaneously recyclizes to 2-hydroxychromene-2-carboxylate. This compound is subsequently converted by an isomerase to *cis-O*-hydroxybenzylidene pyruvate, which is cleaved by an aldolase, resulting in salicylaldehyde and pyruvate [8].

The pathway for the metabolism of 1,2-dihydroxynaphthalene is based on the study of Barnsley [2] and differs from the pathway proposed earlier by Davies and Evans [6]. Cleavage of the aromatic ring is one of the most important functions of dioxygenases. The extradiol enzymes cleave the aromatic ring between one hydroxylated carbon and another adjacent nonhydroxylated carbon. It appears that the extradiol enzymes contain ferrous ion as a prosthetic group. Moreover, the enzymes are usually multimers of a single type of subunit, and one atom of ferrous ion is associated with each subunit [12].

Yen and Gunsalus [21] determined genes for naphthalene metabolism located on the NAH7 plasmid. The genes were organized into two clusters and they were under the control of salicylate. The genes in two clusters encode proteins involved in the conversion of naphthalene to salicylate, and the ring fission generates pyruvate and acetaldehyde. Grund and Gunsalus [10] reported two gene operons encoding naphthalene catabolism by the plasmid pIG7. Yen and Gunsalus [22] described that Tn5 insertion mutations at the regulatory gene of the naphthalene catabolic pathway encoded by the NAH7 plasmid were mapped within a small NAH7 plasmid region. The *nahR* controls the induction of both the *nahABCDEF* and *nahGHIJK* operons [18, 22]. Harayama and Rekick [12] reported the complete nucleotide sequence of the *nahC* gene from the NAH7 plasmid of *Pseudomonas putida* and the deduced amino acid sequence of this enzyme. The deduced amino acid sequence of this enzyme was compared with those of other ring-cleavage enzymes.

Here, we describe the identification and characterization of the *nahC* gene, which is a structural gene for the extradiol enzyme, 1,2-dihydroxynaphthalene dioxygenase, from *P. fluorescens*. The complete nucleotide sequence of the *nahC* gene was determined and compared with those of the oxygenases from other bacterial strains.

Molecular Cloning of *nahC* Gene

Pseudomonas sp. degrades naphthalene via upper and lower pathways to the tricarboxylic acid cycle [4, 6, 19]. To understand the structure, function, and induction mechanism of the 1,2-dihydroxynaphthalene (1,2-DHN) dioxygenase in *P. fluorescens* at the molecular level, we cloned a 2.9 kb DNA fragment containing the corresponding gene from

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the chromosomal DNA of the strain SME11, by color selection with 3-methylcatechol. For the construction of the genomic library, the chromosomal DNA of *P. fluorescens* SM11 [5] was purified as described previously [16]. The chromosomal DNA (50 μg) was partially digested with *Sal*I. Then, appropriate DNA fragments isolated by agarose gel electrophoresis were ligated into the *Sal*I site of a pUC19 vector [23, 24]. The ligated DNA was introduced into *E. coli* NM522 by the transformation method [16], and subjected to selection of the *nahC* gene with 3-methylcatechol.

The selected plasmid carried an insert of 2.9 kb. The resulting recombinant plasmid, pNA1 (5.7 kb), contained a 2.9 kb *Sal*I fragment carrying the *nahC* gene functionally associated with the degradation of 1,2-dihydroxynaphthalene. A 2.3 kb fragment of pNA1 digested with *Sal*I and *Sph*I was subcloned into the pUC19 vector to generate pNA2. In addition, the recombinant plasmids of pNA3 and pNA4 were constructed from pNA1 by digestion of the plasmid with various enzymes. The result of the color test for the subclones is shown in Fig. 1.

E. coli NM522 was transformed with these recombinant plasmids, and the 1,2-DHN dioxygenase activities in these transformants were detected by spraying 3-methylcatechol to the cells as follows [8]. Cells of *E. coli* NM522 transformed with this recombinant plasmid were grown on LB plates containing ampicillin (100 μg/ml), and a solution of 100 mM 3-methylcatechol was sprayed directly onto the developing colonies on the plates. Some colonies turned yellow within 60 min. The plasmids were isolated and analyzed. According to the results of color selection for the recombinant plasmids as shown in Fig. 1, the insert DNA of *Sal*I-*Sca*I of the pNA1 carried the *nahC* region. Restriction enzyme digestion and electrophoretic analysis of the plasmid pNA1 were used to generate a physical map of the cloned fragment carrying the *nahC* gene. Analysis was performed with restriction endonucleases *Sal*I, *Pst*I, *Sac*I, *Bam*HI, *Sca*I, *Hin*dIII, *Apa*I, *Bgl*I, *Ava*I, *Spl*I, *Cla*I, and *Eco*RV. Comparison of its restriction map with that of the NAH plasmid [12] demonstrated that this recombinant plasmid carried a region of DNA containing the *nahC* gene.

For sequencing, unidirectional deletions were generated from the fragment at the polycloning site of the pUC19 by using an Erase-a-Base system (Promega Co., Madison, WI, U.S.A.). Both strands of the DNA segments containing the *nahC* gene were sequenced with an automated-sequencing apparatus (Pharmacia Biotech Inc., Piscataway, NJ, U.S.A.). The nucleotide sequences obtained were analyzed by using the BLAST program. The nucleotide sequence of the *nahC* gene from *P. fluorescens* SME11 was found to be 924 bp, as shown in GenBank accession number AY048760. The *nahC* gene appeared to encode a polypeptide chain with a molecular mass of 34.2 kDa, consisting of 308 amino acid residues. A putative ribosome binding sequence, GGA, was identified 10 bp upstream from the initiation codon of the gene. The subunit of the 1,2-DHN dioxygenase from *P. fluorescens* has a molecular weight of 33,000, and the holoenzyme consists of eight identical subunits [13]. Therefore, in this study, it was assumed that the 1,2-DHN dioxygenase was composed of eight subunits with a molecular weight of about 34,200.

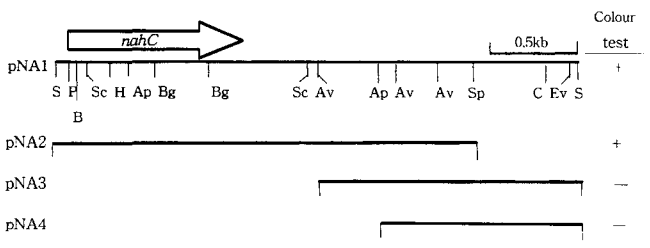

Fig. 1. Physical maps of pNA1 and its subclones.
Abbreviations: S, *Sal*I; P, *Pst*I; B, *Bam*HI; Sc, *Sca*I; H, *Hin*dIII; Ap, *Apa*I; Bg, *Bgl*I; Av, *Ava*I; Sp, *Sph*I; C, *Cla*I; Ev, *Eco*RV.

Amino Acid Sequence Homology of 1,2-Dihydroxynaphthalene Dioxygenase

As shown in Fig. 2, the amino acid sequence of the 1,2-DHN dioxygenase deduced from the nucleotide sequence was aligned with seven corresponding enzymes in other bacterial strains. The product of the *nahC* gene showed high sequence similarities to known bacterial dioxygenase enzymes. The deduced amino acid sequence of the enzyme produced by *P. fluorescens* SM11 exhibited a 98% identity with that of the enzyme by *P. putida* OUS82 [20], 97% with that of the enzyme from *P. putida* plasmid NAH7 [12], and 90% with that of the enzyme from *P. stutzeri* AN10 [3]. However, the homology of the sequences was less than 60% when compared to the corresponding enzymes of *Sphingomonas macrogoltabidus* [1] and *Sphingomonas* strain [15].

Harayama and Rekik compared the complete amino acid sequence of four extradiol enzymes [12]. They observed a striking similarity between the amino acid sequence of NahC and 2,3-DHN dioxygenase. However, the NahC sequence of *P. fluorescens* SM11 could also be aligned with those of catechol 2,3-dioxygenase with 16% homology. The enzymatic reactions involved in the oxidation of polycyclic aromatic compounds such as naphthalene and biphenyl resemble those involved in oxidation of monocyclic aromatic compounds [12]. For example, the first reactions in the degradation of naphthalene and biphenyl, which introduce two hydroxyl groups into the first ring of these compounds, are analogous to the double hydroxylation reactions that commonly occur in the initial steps of oxidative degradation of monocyclic aromatic compounds. Furthermore, enzymes catalyzing the subsequent steps in the degradation of naphthalene and biphenyl are similar to those catalyzing by the *meta*-cleavage pathway. The reactions catalyzed by 1,2-DHN dioxygenase and 2,3-dihydroxybiphenyl dioxygenase are very similar to those of catechol 2,3-

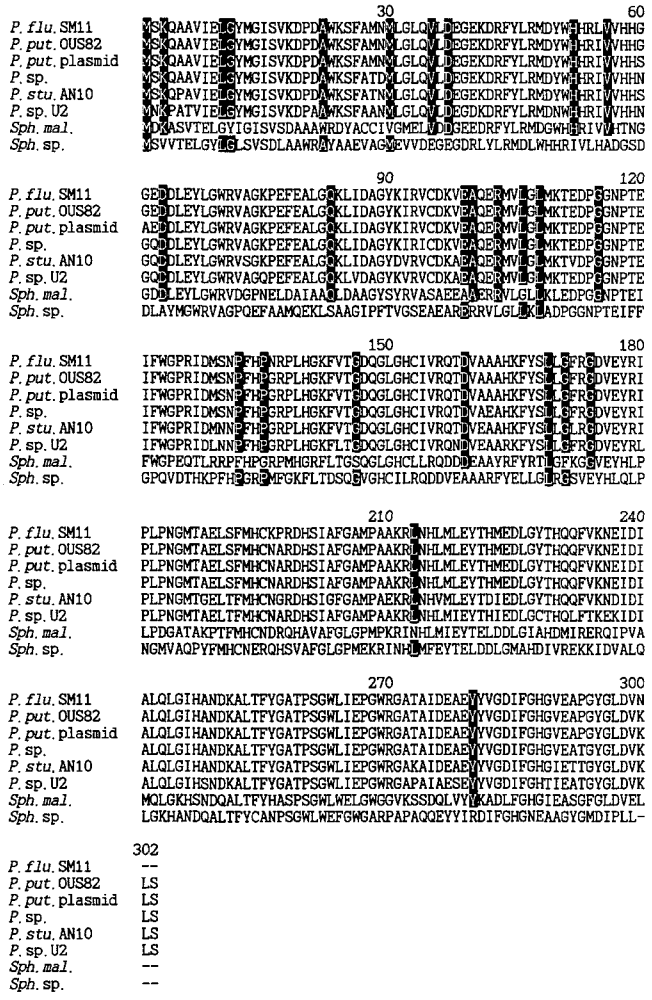

Fig. 2. Alignment of amino acid sequences of 1,2-dihydroxynaphthalene dioxygenase (*nahC*) as calculated by the Clustal program. The numbers above the sequences refer to positions in the alignment.

The amino acids in positions with more than seven identical sequences are considered identical (highlighted). Accession numbers and references in order of the aligned sequences are as follows: *P. flu.*, *Pseudomonas fluorescens* SM11 (AY048760); *P. put.* OUS, *P. putida* OUS82 (AB004059, 19); *P. put.* plasmid, *P. putida* plasmid NAH7 (J04994, 12); *P.* sp., *P.* strain (M60405, 7); *P. stu.* AN10, *P. stutzeri* AN10 (AF039533, 3); *P.* sp. U2, *P.* sp. strain U2 (AF036940, 9); *S. mal.*, *Sphingomonas macrogoltabidus* (AF157565, 1); *S.* sp., *Sphingomonas* strain (AB035677, 14).

dioxygenase. Therefore, the hypothesis that catabolic enzymes for oxidation of the polycyclic aromatic ring evolved from those for oxidation of monocyclic aromatic compounds was proposed [10].

Expression of 1,2-Dihydroxynaphthalene Dioxygense Gene in *E. coli*

In cells of *P. fluorescens* SM11, naphthalene is degraded via 1,2-dihydroxynaphthalene to generate yellow intermediates. Thus, *E. coli* NM522 cells carrying the recombinant plasmid pNA1 were tested for their ability to convert 1,2-dihydroxynaphthalene to 2-hydroxychromene-2-carboxylate. *E. coli* cells containing pUC19-recombinant plasmids carrying the *nahC* gene were grown in LB-broth containing 100 μg/ml ampicillin at 30°C to the late exponential growth phase and were induced with 0.1 mM IPTG for 24 h for the expression of the *nahC* gene, which was under the control of the *lac* promoter of the pUC vector. Cell-free extracts were prepared by sonication (sonic dismembrator, model 300, Fisher, N.Y., U.S.A.) and the pellet was suspended in 100 mM potassium phosphate (pH 7.0) containing 10% acetone. The cell debris was sedimented at 25,000 ×g for 30 min and the supernatant was used for an enzyme assay. 1,2-Dihydroxynaphthalene dioxygenase was determined spectrophotometrically as follows. To a cuvette containing 100 mM potassium phosphate buffer (pH 7.0), cell-free extract (usually 50 μl) was added to give a volume of 2.85 ml. 1,2-Dihydroxynaphthalene (15 μl, 40 mM) was added and the initial rate of decrease of the absorbance at 285 nm was measured [19]. One unit of the enzyme activity represented conversion of 1 μmol of 1,2-dihydroxynaphthalene per min at 30°C. The protein concentration was determined according to the method of Lowry *et al.* [14].

Table 1. Induced expression of the *nahC* gene in *E. coli* NM522[a].

Strain	1,2-Dihydroxynaphthalene dioxygenase (units/protein)	
	NI	I
E. coli NM522	NT	NT
E. coli NM522 (pNA1)	0.3	2.3
P. fluorescens SM11	2.8	2.9

[a]Cell-free extracts were prepared from cells grown in the presence (I) or absence (NI) of IPTG. Specific activities represent enzyme unit per mg of protein. NT: not detected.

The results in Table 2 showed that the gene for 1,2-dihydroxynaphthalene dioxygenase from *P. fluorescens* SM11 was expressed at a low level in the cells of *E. coli* NM522 in the presence of IPTG induction, whereas the parental strain, NM522, lacked the enzyme activity. Therefore, this result demontrates that *E. coli* has the ability to express the *nahC* gene from *P. fluorescens*, but only at a low level.

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