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Molecular Cloning of the *nahG* Gene Encoding Salicylate Hydroxylase from *Pseudomonas fluorescens*

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A gene encoding the salicylate hydroxylase was cloned from the genomic DNA of *Pseudomonas fluorescens* SME11. The DNA fragment containing the nahG gene for the salicylate hydroxylase was mapped with restriction endonucleases and sequenced. The DNA fragment contained an ORF of 1,305 bp encoding a polypeptide of 434 amino acid residues. The nucleotide and amino acid sequences of the salicylate hydroxylase revealed several conserved regions with those of the enzyme encoded in *P. putida* PpG7: The homology of the nucleotide sequence is 83% and that of amino acid sequence is 72%. We found large conserved regions of the amino acid sequence at FAD and NADH binding regions. The FAD binding site is located at the amino terminal region and a lysine residue functions as a NADH-binding site.

Keywords: *nahG* Gene; *Pseudomonas fluorescens* SME11; Salicylate Hydroxylase.

Introduction

Degradation of hydrocarbons has been extensively described in the genus *Pseudomonas* (Chakrabarty, 1976; Dunn and Gunsalus, 1973; Mark, 1983). The genes for the early steps of their degradation are frequently found on metabolic plasmids (Chakrabarty, 1972; Rheinwald *et al*., 1973; Yen and Gunsalus, 1982). Naphthalene and salicylate serve as aromatic structures that support growth of microorganisms as carbon sources (Davies and Evans, 1964). Strains carrying a *nah* plasmid grow on both naphthalene and salicylate, whereas those carrying a *sal* plasmid grow on salicylate but not naphthalene. Plasmid DNA isolated from *nah* and *sal*-carrying strains are comparable in restriction sites (Heinaru *et al*., 1978; Mark, 1983) and hybridization homology (Heinaru *et al*., 1978; Mark, 1986).

Transposon mutagenesis has been used for the analysis of plasmid gene organization. Molecular manipulation has established the plasmid location of the genes encoding the first 11 steps of naphthalene oxidation and determined the gene organization and direction of the transcription (Yen and Gunsalus, 1982). The catabolic genes for the metabolism of aromatic hydrocarbons on NAH7 and TOL plasmids are organized in two operons (Franklin *et al*., 1981; Yen and Gunsalus, 1982; 1985). On NAH7, *nah* operon encodes enzymes for the metabolism of naphthalene to salcylate; whereas *sal operon* encodes enzymes for the metabolism of salicylate to intermediates of the tricarboxylic acid cycle (Kim *et al*., 1997; Lee, *et al*., 1997; Mark, 1986).

Genetic evidence suggests that the *nahR* regulatory genes of the NAH7 plasmid encodes an inducer protein, which activates transcription of both catabolic operons of *nah* and *sal* (Mark, 1986). The positive regulatory genes, *nahR*, which is divergently transcribed from the *sal* operon, are located between the *nah* and *sal* operons (Mark, 1986; Yen and Gunsalus, 1982; 1985).

The *nahG* gene of *sal* operon codes for salicylate hydroxylase. Salicylate hydroxylase, a flavoprotein, converts salicylate to catechol via decarboxylative hydroxylation. The *nahG* gene of plasmid NAH7 was cloned and mapped by restriction analysis (Yen and Gunsalus, 1982; 1985). A DNA sequence analysis of the *Hin*dIII fragment containing the *nahG* locus reveals an open reading frame for salicylate hydroxylase (You *et al*., 1991).

Recently we cloned a salicylate hydroxylase gene from

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Abbreviations: PCR, polymerase chain reaction, ORF, open reading frame.

the chromosomal DNA of *Pseudomonas putida* KF715 (Lee *et al*., 1996). In this study, the *nahG* gene encoding salicylate hydroxylase was cloned from the chromosome of the *P. fluorescens* SME11, and its nucleotide sequence was analyzed by comparison with the hydroxylase genes from other bacterial sources.

Materials and Methods

Bacterial strains and plasmids The *Pseudomonas fluorescens* SME11 that was isolated from the soil sample (Kim *et al*., 1995) was grown in a LB medium or minimal medium containing 0.1% naphthalene or salicylate as a sole carbon and energy source. The *E. coli* NM522 was used as a host harboring each of recombinant plasmids. For antibiotic selections, ampicillin with 50 μg/ml, or kanamycin with 100 μg/ml as a final concentration, was supplemented to a LB medium (Sambrook *et al*., 1989). pT7Blue (R) was used as a cloning vector in this experiment.

Cell extract preparation and enzyme assay Strains were grown in a PAS minimal medium containing 2.5 mM sodium salicylate. The cells were harvested at 5°C, washed with a 0.1 M potassium phosphate buffer at pH 7.0, resuspended in the same buffer, and ruptured by sonication with sonic dismembrator (model 300, Fisher.) The cell debris was sedimented at 25,000 × *g* for 30 min and the supernatant was used for an enzyme assay.

Salicylate hydroxylase assays were according to the procedure of White-Steven and Kamin (1972). Enzyme assays were performed with a spectrophotometer (UV-1201, Shimadzu.) The reaction mixture was composed of 1 mM EDTA, 133 μM sodium salicylate, 147 μM NADH, and 0.05 μM potassium phosphate buffer, pH 7.0 in a volume of 3 ml. One unit of enzyme activity represented the oxidation of 1 μmole of NADH per min measured at 340 nm at 30°C (White-Stevens and Kamin, 1972). The protein concentration was determined according to the method of Lowry (Lowry *et al*., 1951).

DNA manipulation The plasmid was isolated by the alkali lysis method (Sambrook *et al*., 1989) or by using a kit from Qiagen. The DNA cleavage and ligation were accomplished under standard conditions recommended by the supplier (Boehringer Mannheim.) The DNA was resolved in 0.7% or 1% agarose gel by electrophoresis, and identified by staining with ethidium bromide followed by UV irradiation (Sambrook *et al*., 1989). Transformation was accomplished by the calcium chloride method (Sambrook *et al*., 1989).

Cloning of *nahG* gene Previously, the *nahG* gene from *P. putida* was cloned (Lee *et al*., 1996; Suzuki *et al*., 1996; Yen and Gunsalus, 1982; You *et al*., 1991) and the nucleotide sequence was determined. From the sequence data of *P. putida* KF715 (Lee *et al*., 1996), we designed oligonucleotide primers (forward: 5'-AAGCGGCAGATCGTACATTCTCCCC-3', reverse: 5'-GGGGCCCCAGCCCGCGAACGCATCGAGCAT-3'). The template DNA (1 μg) and primers (10 pmol of each) were incubated in a 50 μl reaction mixture containing 25 mM $MgCl_2$, 2 mM dNTP, and *Taq* polymerase (Bioline Co., Nevata, USA) in a thermal cycler at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. After thirty cycles, the PCR product was obtained from the genomic DNA of *P. fluorescens* SME11.

Southern hybridization Southern hybridization of genomic DNA that was digested with *Eco*RI and *Hin*dIII was carried out with the probe, about 800 bp DNA fragment that was generated by PCR. This fragment was obtained by amplification of a DNA segment of *P. fluorescens* SME11 with primers that were designed against start and stop sequences of *nahG*. The probe DNA was labeled with a labeling reagent by using the ECL direct nucleic acid of the labeling system (Life Science, Amersham). Digested genomic DNA was separated by electrophoresis through 0.8% agarose gel, and then transferred to a Hybond-N^+ Nylon transfer membrane (Life Science). After the membrane was baked at 80°C for 2 h, hybridization was performed at 42°C for 24 h.

Nucleotide sequencing and analysis Unidirectional deletion mutants were constructed from *Eco*RV fragments in the polycloning site of the pNYI by using an Erase-a-Base system (Promega Co., Madison, W1. USA). Both strands of the DNA segments containing the nahG gene were sequenced with an automated-sequencing apparatus (Pharmacia Biotech Inc. Piscataway, NJ. USA). The nucleotide sequences obtained were analyzed by using the BLAST program.

Results

Molecular cloning of *nah*G gene *Pseudomonas*. sp. degrades naphthalene via the upper and lower pathway to the tricarboxylic acid cycle (Mark, 1986). To understand the structure, function, and induction mechanism of the salicylate hydroxylase in *P. fluorescens* SME11 at the molecular level, we cloned about 1.6 kb DNA fragment containing the corresponding gene from the chromosomal DNA of the strain SME11.

Amplification of the genomic DNA with primers generated the DNA fragment, about 800 bp. Using the PCR product as a probe, a 1.6-kb *Eco*RV fragment was obtained from the chromosomal DNA by Southern hybridization. This fragment was inserted into the T-cloning vector, pT7Blue (R), in order to generate a recombinant plasmid, pNY1, and was transformed into *E. coli* NM522. Color selection was examined by spraying sodium salicylate to produce a dark brown color due to catechol accumulation and auto-oxidation (Cane and Peter, 1986). When 2.5 mM salicylate was incorporated into the agar plates, the presence of salicylate hydroxylase (*nah*G gene product) produced a black color on colonies (data is not shown). Moreover, the transformed *Escherichia coli* cells harboring the recombinant plasmid exhibited a low activity of salicylate hydroxylase and the addition of salicylate to the cells induced the enzyme activity (Table 1).

This result confirms that the genes responsible for salicylate hydroxylase are cloned and expressed in *E. coli* cells.

Construction of the physical map Restriction enzyme digestion and electrophoretic analysis of the plasmid pNYI were used to generate a physical map of the cloned fragment of the *nah*G. Analysis was performed with

Table 1. Induced levels of the nahG gene product in the strains.

Strain	Salicylate hydroxylase (specific activity: U/mg protein)	
	NI	I
E. coli NM522	NT	NT
E. coli NM522(pNYI)	0.3	0.5
P. fluorescens SME11	0.8	3.6

Extracts were prepared from cells grown in the presence (I) or absence (NI) of inducer salicylate. Specific activities represent enzyme unit per mg of protein.

restriction endonucleases *Eco*RV, *Stu*I, *Kpn*I, and *Pvu*II, employing double and triple digestions in conjugation with the enzymes.

The sizes of the restriction fragments of pNY1 produced by endonuclease were examined (data is not shown). This new structural information will be valuable for structural analysis of this DNA fragment, including the *nah*G gene, and for nucleotide sequencing.

Nucleotide sequence of the salicylate hydroxylase gene

The salicylate hydroxylase activity was expressed in *E. coli* cells harboring pNY1 on the plates. Accordingly, the DNA fragment was examined for its nucleotide sequence as shown in Fig. 1.

Only one ORF was found to be of sufficient length to encode the enzyme. The ORF starts with the ATG codon at position one and ends at nucleotide position 1,305 with the TAA codon. The *nah*G gene nucleotide sequence and the deduced amino acid sequence are presented in Fig. 1. The G+C content of the structural gene was 58.3%. A putative sequence of an RBS, complementry to the 3'-end of the 16S rRNA of *P. fluorescens* was identified 18 nucleotide upstream from the ATG start codon.

The *nahG* gene of *Pseudomonas fluroscences* SME11 can encode a polypeptide of about 48 kDa molecular mass containing 434 amino acid residues. The alignment among the amino acid sequences exhibited highly conserved residues, as shown in Fig. 2. The amino acid sequence of salicylate hydroxylase from *P. fluorescens* SME11 showed 80%, 72% and 70% identify with those of the

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ATATATAATATAGGGATTAGTGTATTTATCAATAGTTATGGCTTCGCTAC
TGTCGAAGATATCCCAATAATAAAGCCATGACAGGTATTGCATGAACGAC
1                                  RBS                39
ATG AAC GAC ATG AAC GCT AAG AAG CCA GCC TTG CGC GTC
 M   N   D   M   N   A   K   K   P   A   L   R   V
40                                                    78
GCT ATA GTC GGC GGC GGA ATT TCA GGC CTT GCC TTG GCA
 A   I   V   G   G   G   I   S   G   L   A   L   A
79                                                   117
TTG AGC TTG TGC AAA CAC TCC CAT CTT AAT GTG CAG CTA
 L   S   L   C   K   H   S   H   L   N   V   Q   L
118                                                  156
TTC GAG GCT GCC CCG GCG TTA GGT GAA GTC GGT GCT GGT
 F   E   A   A   P   A   L   G   E   V   G   A   G
157                                                  195
GTG TCC TTC GGG CCT AAC GCA GTG CGC GCC ATT GTC GGT
 V   S   F   G   P   N   A   V   R   A   I   V   G
196                                                  234
TTG GGT TTG GGT CAA GCC TAC TTT CAG GTT GCT GAT CGG
 L   G   L   G   Q   A   Y   F   Q   V   A   D   R
235                                                  273
ACT CCG CAG CCT TGG GAG GAT ATT TGG TTT GAA TGG CGG
 T   P   Q   P   W   E   D   I   W   F   E   W   R
274                                                  312
CCG GTA CAG TCC AGG CTA TCT AGG CCC CAC CAT TGC GGC
 P   V   Q   S   R   L   S   R   P   H   H   C   G
313                                                  351
GTA GGT CAG TCC TCT GTA CAC AGG GCT GAT TTC CTT GAC
 V   G   Q   S   S   V   H   R   A   D   F   L   D
352                                                  390
GCC CTA GTA AAA CAC CTT CCA GAA GGT ATC GCC CAA TTT
 A   L   V   K   H   L   P   E   G   I   A   Q   F
391                                                  429
AGG AAG CGT GCC ACC CAA ATC GAG CAG CAG GGT GAT GAA
 R   K   R   A   T   Q   I   E   Q   Q   G   D   E
430                                                  468
CTG CAA GTG CTA TTC GCC GAC GCG ACA GAG TAC CGC TGC
 L   Q   V   L   F   A   D   A   T   E   Y   R   C
469                                                  507
GAT CTT CTA ATT GGC GCC GAC GCG ATT AAG TCA GCG CTT
 D   L   L   I   G   A   D   A   I   K   S   A   L
508                                                  546
CGT AGC TAT GTG CTG GAA GGT CAG GGG CTG GAT CAT TTA
 R   S   Y   V   L   E   G   Q   G   L   D   H   L
547                                                  585
GAA CCA CGT TTT AGC GGT ACC TGT GCA TAC CGG GGC ATG
 E   P   R   F   S   G   T   C   A   Y   R   G   M
581                                                  624
GTA GAT AGC CTG CAA CTG CGC GAA CGC TAT AGA ATA AAT
 V   D   S   L   Q   L   R   E   R   Y   R   I   N
625                                                  663
GGT ATT GAC GAG CAC TTG GTG GAT GTC CCG CAG ATG TAC
 G   I   D   E   H   L   V   D   V   P   Q   M   Y
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664                                                  702
TTA GGG CTC TAT GGC CAT ATT CTT ACC TTC CCG GTG AGA
 L   G   L   Y   G   H   I   L   T   F   P   V   R
703                                                  741
AAA GGC CGC ATT GTC AAC GTG GTA GCG TTC ACC TCC GAC
 K   G   R   I   V   N   V   V   A   F   T   S   D
742                                                  780
CGT AGC CAG CCG GAA CCG ACT TGG CCC GCG GAC GCT CCC
 R   S   Q   P   E   P   T   W   P   A   D   A   P
781                                                  819
TGG GTA CGG GAA GCG AGC CAG CCG GAG ATG CTC GAT GCG
 W   V   R   E   A   S   Q   P   E   M   L   D   A
820                                                  858
TTC GCG GGC TGG GGC GAT CGG CGC CGC TTA CTG GAG TGC
 F   A   G   W   G   D   R   R   R   L   L   E   C
859                                                  897
ATC CCG GCC GCC CAA CTC TCT GGG CAG TGC ATG ACC TGC
 I   P   A   A   Q   L   S   G   Q   C   M   T   C
898                                                  936
CAG AAC TGC CAG GCT ACG TAC ACG GTC GGG TTG CCT GAT
 N   C   Q   A   T   Y   T   V   G   L   Q   P   D
937                                                  975
CGG CGA CGC AGC GAC GCA TGC TGC CGC AAC CAA GGG GCC
 R   R   R   S   D   A   C   C   R   N   Q   G   A
976                                                 1014
GGT GCA GGG CAA GGG CTT GAG GAC GCC TAT TTC CTC GCC
 G   A   G   Q   G   L   E   D   A   Y   F   L   A
1015                                                1053
CGG CTG TTG GGA CTA GTC GGA CCG AAA CAG GCA ACC TCC
 R   L   L   G   L   V   G   P   K   Q   A   T   S
1054                                                1092
CCG AGC TGC TTG GAG CTT ACG CAC GAC CTG CGC CGC CCT
 P   S   C   L   E   L   T   H   D   L   R   R   P
1093                                                1131
CAT GCC TGT CGT GTG CAA CGA ACC ACT GTG GAA ACC GGC
 H   A   C   R   V   Q   R   T   T   V   E   T   G
1132                                                1170
GAG TTA TAC GAG TTG CGC GAC CCC ATT GTA GGT GCG GAC
 E   L   Y   E   L   R   D   P   I   V   G   A   D
1171                                                1209
GAA CAG CTG GTG GGG GAA ATA CTG GCG ACT CGT TTC GAC
 E   Q   L   V   G   E   I   L   A   T   R   F   D
1210                                                1248
TGG CTA TGG AAC CAT GAT CTC GAT GCC GAT GTG GCT GAG
 W   L   W   N   H   D   L   D   A   D   V   A   E
1249                                                1287
GCC CGA CTG CGC ATG GGT TGG GAG GCG CAT GAG CAA ATT
 A   R   L   R   M   G   W   E   A   H   E   Q   I
1288                1305
GCG CTG CGT CAA GGG TAA GAGGCCGTGTCGATAGTGGCTAAGGTT
 A   L   R   Q   G  ***
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Fig. 1. Nucleotide sequence of the salicylate hydroxylase gene from *Pseudomonas fluorescens* SEM11 and predicted amino acid sequence. RBS denotes a putative ribosomal binding site. Termination codon is indicated by asterisks (Genbank Accession No. 376974).

Fig. 2. Comparison of the deduced amino acid sequences of salicylate hydroxylase for *P. putida* KF715 (Lee *et al.*, 1996), *P. putida* PpG7 (You *et al*., 1991), and *P. putida* S-1 (Suzuki *et al.*, 1996). Residues that are identical in three or four represent + or *, respectively. Shade boxes indicate the peptide containing the lysine residue essential for binding of NADH.

corresponding enzyme from the *P. putida* KF715 (Lee *et al*., 1996), *P. putida* PpG7 (You *et al*., 1990), and *P. putida* S1 (Suzuki *et al.*, 1996.), respectively.

Salicylate hydroxylase, a flavoprotein, converts salicylate to catechol via decarboxylative hydroxylation. This enzyme was purified from the *P. putida* PpG7 (White-Stevens and Kamin, 1972), and later from *P. cepacia* (Tu *et al*., 1981). Mechanistic studies with the salicylate hydroxylase (isolated from *P. cepacia*) suggest that in the first step salicylate and NADH bind to salicylate hydroxylase randomly, resulting in a reduced enzyme-substrate complex. This in turn binds to oxygen leading to the production of the hydroxylation substrate, CO_2, and water (White-Stevens and Kamin, 1972).

There were at least two distinct salicylate hydroxylase enzymes reported in the papers (White-Stevens and Kamin, 1972; You *et al*., 1990). One is the *P. putida* salicylate hydroxylase, which is a flavoprotein containing one molecule of FAD. It is a monomer with an approximate molecular mass of 45 kDa (You *et al*., 1990). The other is the *P. cepacia* salicylate hydroxylase, which contains two molecules of FADs and two identical subunits in a total molecular mass of 91 kDa (Tu *et al*., 1981).

In this study, it was discovered that the *P. fluorescens* salicylate hydroxylase is the former type of salicylate hydroxylase with an approximate molecular mass of 48 kDa.

A long range homology was found among the amino acid sequences of *P. fluorescens* SEM11, *P. putida* KF715 (Lee *et al*., 1996) *P. putida* S-1 (Suzuki *et al*., 1996) and *P. putida* PpG7 (You *et al*., 1990).

The regions were also highly homologous with the region of residues 310-335 of the sequence of salicylate hydroxylase in the *P. fluorescens* SEM11, in which the region constructs the part of the substrate binding pocket (Suzuki *et al*., 1995) The region of residues 12-29 of the *P. fluorescens* SEM11 enzyme, RVAIVGGGISGLALALSL, contains the consensus sequence of the ADP binding site of FAD and is homologous to those of the salicylate hydroxylase of *P. putida* PpG7 (You *et al*., 1990) and *P. putida* S-1 (Suzuki *et al*., 1996).

The consensus sequence of the second FAD binding region of flavoprotein is found in the sequence of residues 304-314 of the *P. fluorescens* SME11, which is highly conserved in that of the *P. putida* PpG7, residues 304-314, and is similar to the sequence of the *P. putida* S-1 salicylate hydroxylase, residues 302-312 (Fig. 2).

The chemical modification of the salicylate hydroxylase in the *P. putida* PpG7 revealed the presence of a lysine residue at the 165 position in the binding site of NADH and determined the amino acid sequence around the residue (Suzuki *et al*., 1996). The sequence was found on the predicted amino acid sequence of the protein, in which the lysine residue is located at residue 166 (Suzuki *et al*., 1996) as the salicylate hydroxylase in this study. The sequence region of 155 to 169 containing the lysine residue is also conserved in the enzyme from the *P. fluorescens* SME11 in our study, *P. putida* S-1 (Suzuki *et al*., 1996), and *P. putida* PpG7 (You *et al*., 1990).

Analysis of expression of salicylate hydroxylase gene in *E. coli* In the *P. fluorescens and P. putida* strains, naphthalene is degraded via salicylate to pyruvate and acetaldehyde by a serise of enzymatic reaction (Gunsalus and Yen, 1981; Yen and Gunsalus, 1982). The *P. fluorescens* SME11 strain is able to convert salicylic acid to catechol and thus accumulate catechol in the medium when grown on salicylate as the sole carbon and energy source. Thus, *E. coli* NM522 that harbored the recombinant plasmid pNY1 was tested for their ability to convert salicylate to catechol. The results showed that the gene for salicylate hydroxylase was expressed at a low level

in the *E. coli* NM522 strain containing the recombination plasmid pNY1, whereas the parental NM522 strain lacked this ability in Table 1. Therefore, this result demonstrates that *E. coli* has the ability to express at a low level of the *nahG*.

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