

**DETERMINATION OF THE PRESENCE OF A NAPHTHALENE  
DIOXYGENASE GENE IN MICROORGANISMS ISOLATED FROM  
CONTAMINATED SOILS ON THE CENTRAL COAST OF CALIFORNIA**

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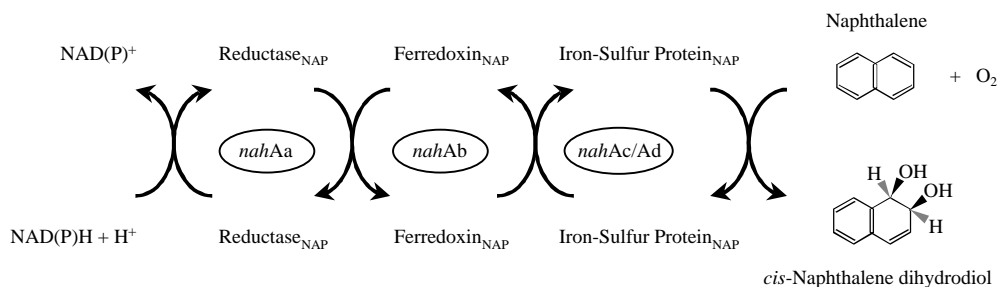
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**Abstract**

Forty-one bacterial isolates from the petroleum contaminated soils of Guadalupe, CA were tested for the presence of a naphthalene dioxygenase gene (*ndo*) by PCR amplification. *Pseudomonas putida* was used as a positive control and five isolates produced similar sized PCR products. The PCR products from these five isolates were sequenced and two were homologous to the naphthalene dioxygenase gene. Strains L2PS4A and L2PS4B produced PCR fragments whose sequences were both 99% homologous to the *Pseudomonas fluorescens ndoC2* gene, which encodes the large subunit of the iron-sulfur protein (ISP). These strains were also determined to be *Pseudomonas* sp. by both 16S rDNA and FAME analysis. According to a phylogenetic tree which was constructed, L2PS4A and L2PS4B were both grouped with other naphthalene degrading genes which appeared to be genomic rather than plasmid-borne.

## Introduction

Naphthalene is a fused ring bicyclic aromatic hydrocarbon that is commonly found in crude oil and oil products. It is part of a large class of polynuclear aromatic hydrocarbons (PAH) consisting of two or more fused benzene rings. Microbial metabolism of these products is of special interest since certain PAHs are strong human carcinogens (5).



**Figure 1.** Oxidation of naphthalene to *cis*-naphthalene by naphthalene dioxygenase multienzyme complex

Extensive research has been conducted on naphthalene oxidation in *Pseudomonas* sp. Studies have revealed that naphthalene 1,2 dioxygenase (ND) is the first enzyme in the naphthalene degradation pathway and it is a multicomponent enzyme system. As shown in Fig. 1 electrons from NADH or NADPH are transferred to an iron-sulfur flavoprotein (reductase<sub>NAP</sub>) which passes single electrons to an iron-sulfur protein designated ferredoxin<sub>NAP</sub>. Electrons from ferredoxin<sub>NAP</sub> are transferred to an iron sulfur protein (ISP<sub>NAP</sub>) which catalyzes the oxidation of naphthalene to *cis*- naphthalene dihydrodiol (6). This terminal oxygenase component, ISP<sub>NAP</sub>, consists of two large ( ) and two small ( ) subunits. Figure 1 is an example of the plasmid-encoded ND genes specific to *P. putida* G7 and *P. putida* 9816-4 . However, there are many other organisms that use dioxygenase enzyme systems to form *cis*-dihydrodiols from aromatic hydrocarbons. The nomenclature of the genes for each organism is different (Table 1), but the functions of the genes are very similar. An experiment by Takizawa et. al (7) indicated that the

*pahA* genes encode ND- type enzymes and that *pahAc* was very similar to *ndoB* (8). Simon et. al. concluded that the nucleotide sequences of *nahAc* and *ndoB* are 95% homologous (4).

**Table 1.** Identification of the genes from specific organisms and the compounds which they act upon.

Gene Name	Organism	Compound
<i>ndoC1, ndoC2</i> <i>ndoC3</i>	<i>P. fluorescens</i>	naphthalene
<i>nahAa, nahAb</i> <i>nahAc, nahAd</i>	<i>P. putida</i> G7 or <i>P. putida</i> 9816-4	naphthalene
<i>doxA, doxB,</i> <i>doxC</i>	<i>Pseudomonas</i> sp. Strain C18	naphthalene?
<i>pahAa, pahAb,</i> <i>pahAd, pahAc</i>	<i>P. putida</i> OUS82	poly aromatic hydrocarbons
<i>dntAa, dntAb,</i> <i>dntAc, dntAd</i>	<i>Pseudomonas</i> sp. or <i>Burkholderia</i> sp. Strain DNT	dinitrotoluene
<i>nagAa, nagAb,</i> <i>nagAc, nagAd</i>	<i>Pseudomonas</i> sp. Strain U2	salicylate

The primary objectives of this study were to determine the presence of a naphthalene dioxygenase gene in microorganisms isolated from petroleum contaminated sites using PCR amplification and to characterize the isolates that contained orthologs to the *ndo* gene.

## Materials and Methods

### ISOLATION OF TPH DEGRADING BACTERIA

Microorganisms derived from petroleum contaminated soil and groundwater samples were isolated from Guadalupe, CA using the contaminant as the sole carbon source. Three of the samples (S1, S3, S5) were from dry soil taken at a 6 inch depth. S1 was a pristine sample and did not contain any oil. S3 contained diluent (C10-C40) but did not contain any PAHs. S5 was taken from a contaminated pile of soil that contained crude oil. A liquid sample (L2) was groundwater taken from a depth of 20 feet and did contain a small amount of PAHs. Bacteria were isolated using the spread plate method on a Minimum Diluent Agar (MDA) as the isolation medium; 50mM KH<sub>2</sub>PO<sub>4</sub>, 50mM Na<sub>2</sub>HPO<sub>4</sub>, 80mM NH<sub>4</sub>Cl, 0.036mM FeSO<sub>4</sub>, 0.40 mM MgSO<sub>4</sub>, 0.50 mM CaCl<sub>2</sub>, 0.008% yeast, and 0.500 mL filter-sterilized diluent. After twenty-one days, colonies were picked from the MDA based on differences in colony morphology. Isolates were transferred to Trypticase Soy Agar (TSA) for growth before DNA extraction using the Fast Prep method (Bio 101, Alta Vista, CA).

### PCR AMPLIFICATION

16S rDNA. PCR was performed using the primers PAF and 531R(2) and resulted in an amplified sequence of approximately 530 bp. Final concentrations of the reaction mix were 10mM Tris HCl (pH 8.0), 1 mM dNTP, 20ug/mL BSA, 2.5 mM MgCl<sub>2</sub> and 2 U Amplitaq (PE Applied Biosystems). Forty cycles were performed at the following temperatures and times: 94°C for 2 min, 94°C for 30 sec, 56°C for 30 sec, 72°C for 30 sec, 72°C for 7 min.

ndo genes. PCR was performed to determine the presence of a catabolic gene (*ndo*) which is one of the genes responsible for the degradation of the PAH naphthalene. Primers *ndo*-F (CACTCATGATAGCCTTGATTCCTGCCCGGCG) and *ndo*-R

(CCGTCCCACAACACACCCATGCCGCTGCCG) were used corresponding to positions 622 to 1663 on *P. putida* ATCC 17484 16S rRNA (9). Final concentrations of the reaction mix were 10mM Tris-HCl (pH 8.0), 800 mM dNTP, 2.5 mM MgCl<sub>2</sub> and 2.0 U Amplitaq (PE Applied Biosystems). Reaction temperatures and times were performed as follows: 96° C for 2 min, thirty cycles of 94° C for 1 min, 55°C for 1 min, 72° C for 1 min, and one cycle of 72° C for 7min.

#### NUCLEOTIDE SEQUENCING of 16S rDNA

DNA sequencing was performed using the Dye Terminator Cycle Sequencing Ready Reaction with polymerase FS (PE Applied Biosystems). The 16S rDNA sequences were used to conduct searches in non-redundant DNA databases (BLAST) and analyzed using the maximum likelihood program Phylip 3.5.

#### CLONING of PCR PRODUCTS

Amplified fragments were extracted from the agarose gel using the QIA gel extraction kit (Qiagen, Chatsworth, CA). The DNA fragments were then ligated using the Ready-To-Go kit (Amersham Pharmacia Biotech, Piscataway, NJ) and transformed using XL-1 Blue Competent Cells (Stratagene, La Jolla, CA). The cells were then spread onto x-gal plates made in duplicate with 25µl, 50µl and 100µl per plate. After 17 hours of incubation at 37° C and 2-4 hours of refrigeration, white colonies, and one blue colony to be used as a negative control, were picked and the DNA was amplified by PCR.

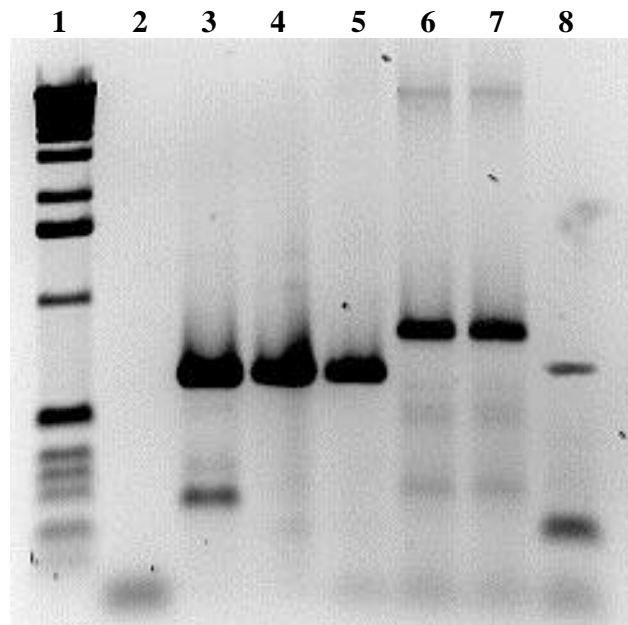
#### FAME ANALYSIS

Strains were grown on Trypticase Soy Broth plus Agar (TSBA) for 24 hours or until good but nonconfluent growth was achieved. Approximately 50 mg of wet cell weight was harvested and extracted according to standard operating procedures of MIDI, Inc., (Newark, NJ).

The Sherlock microbial identification system was used for separation, detection and identification of the fatty acids in the cell extracts. All parameters, settings and procedures were followed as recommended by MIDI, Inc. FAME profiles obtained were compared to a standard library (MIDI Inc.) using the Sherlock (MIDI, Inc.) software system. The profiles of the unknown organisms were compared to known library profiles, generating similarity indices for each unknown.

## Results

Forty-one isolates from 4 different sites were tested for the presence of *ndo*. Out of those 41 isolates 5, or 12%, produced a PCR product (fig. 2). Some isolates resulted in amplification products of about 1100 bp and 640 bp.



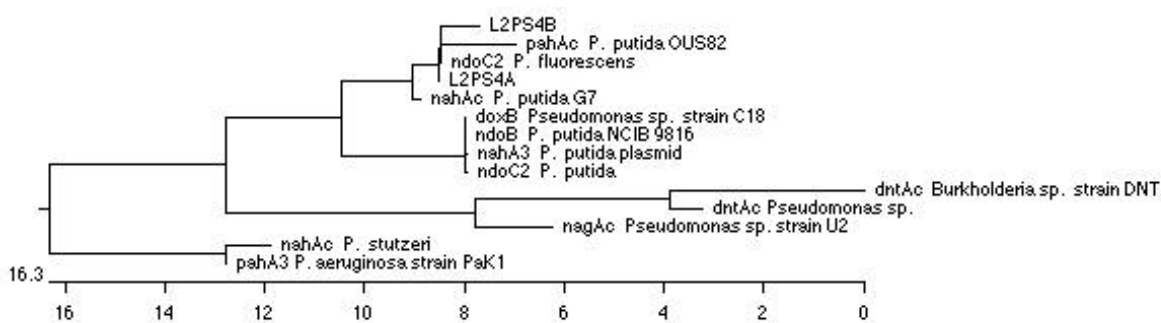
**Figure 2.** Agarose gel electrophoresis of PCR products. Lanes:1, 1kb DNA ladder; 2, negative control; 3, *P. putida*; 4, L2PS4A; 5, L2PS4B; 6, S5MM9; 7, S5MM14; 8, S1RS12

Sequencing was performed on all PCR products which had a similar fragment size to the one produced by *P. putida* (~ 640 bp). Upon sequencing the PCR products, 2 out of the 5, or approximately 5%, had homology to the *ndo* gene. The genes in L2PS4A and L2PS4B were 99% homologous to *ndoC2*, the large subunit of the ISP from *P. fluorescens*, (Table 2).

**Table 2.** Identification of apparent *ndo* positive DNA fragments

Isolate	BLAST ID	% homology
L2PS4A	<i>Pseudomonas fluorescens ndoC2</i>	99.0
L2PS4B	<i>Pseudomonas fluorescens ndoC2</i>	99.0

The *ndo* sequences from the two isolates, L2PS4A and L2PS4B, were aligned with each other and 12 other previously characterized sequences and a phylogenetic tree was constructed (Figure 3). The sequences selected were *ndoC2* from *P. fluorescens*, *ndoB* from *P. putida* NCIB 9816, *doxB* from *Pseudomonas* sp. Strain C18, *pahAc* from *P. putida* OUS82, *nahAc* from *P. putida* G7, *nahA3* from *P. putida* plasmid, *ndoC2* from *P. putida*, *dntAc* from *Pseudomonas* sp., *nahAc* from *P. stutzeri*, *pahA3* from *P. aeruginosa* strain PaK1, *nagAc* from *Pseudomonas* sp. strain U2 and *dntAc* from *Burkholderia* sp. strain DNT (10). The phylogenetic tree showed that there were two main groups of naphthalene degraders. The Group I genes appeared to be genomic while the Group II genes appeared to be plasmid-borne. The genes from L2PS4A and L2PS4B both fall into Group I and are closely related with *pahAc* from *P. putida* OUS82 and *ndoC2* from *P. fluorescens*.

**Figure 3.** Phylogenetic positioning of PAH degrading genes

Isolates producing a PCR product were also analyzed by sequencing the first 500 bp of their 16S rDNA. According to the BLAST program (1), L2PS4A and L2PS4B were 100% homologous to a *Pseudomonas* species, S5MM9 was 96% homologous to an uncultured eubacterium, S5MM14 was 97% homologous to *Sphingomonas macrogotabidus* and there was no match for S1RS12 (Table 3). These five isolates were also identified by FAME analysis. The strain L2PS4A was identified as *P. chloraphis* with an 0.825 confidence value, L2PS4B was identified as *P. fluorescens* with an 0.828 confidence value, S5MM9 was identified as *Methylobacterium maseophilicum* with an 0.035 confidence value, S5MM14 did not result in a match and S1RS12 was identified as *Nocardia* sp. with a confidence value of 0.103 (Table 3).

**Table 3.** 16S rDNA and FAME results of the isolates positive for PCR amplification

Isolate	16S rDNA BLAST ID	% homology	FAME ID	similarity index
L2PS4A	<i>Pseudomonas</i> sp.	100%	<i>Pseudomonas chloraphis</i>	0.825
L2PS4B	<i>Pseudomonas</i> sp.	100%	<i>Pseudomonas fluorescens</i>	0.828
S5MM9	uncultured eubacterium	96%	<i>Methylobacterium maseophilicum</i>	0.035
S5MM14	<i>Sphingomonas macrogotabidus</i>	97%	No Match	-
S1RS12	No Match	-	<i>Nocardia</i> sp.	0.103

## Discussion

It is not surprising that there were only two isolates positive for the *ndo* gene out of the 41 tested. The sites from which the isolates were retrieved contained little if any naphthalene. Furthermore, the only two isolates producing a PCR product of the correct sequence were derived from the liquid sample which did contain PAHs. Research suggests that the gene is mostly restricted to *Pseudomonas* sp. Pritchard et al. listed 28 organisms that degrade aromatic hydrocarbons, 9 of which were *Pseudomonas* species (5). This correlates with the results of the 16SrDNA and FAME analysis. L2PS4A and L2PS4B were both *Pseudomonas* sp., while S5MM9, S5MM14, and S1RS12 were not.

Differences in the size of the PCR products played a key role in identifying the *ndo* gene. PCR amplification resulted in multiple fragments of various sizes. The fragments of ~640 bp, similar to the size of the *ndo* positive control, were selected for extraction and sequencing. Although the sizes of the five fragments that were selected for extraction were very similar to the positive control, only those of L2PS4A and L2PS4B were identical to it (Figure 2). The DNA fragments of L2PS4A and L2PS4B, were the only ones that were actually positive for the *ndo* gene.

The sequences used to create the phylogenetic tree were grouped in a manner that resembled the phylogenetic tree used by Wilson et al. (10). The genes in Group II appear to be almost identical indicating that they may be plasmid-borne. These organisms may be obtaining the same gene through horizontal gene transfer, which is a mechanism for microorganisms to acquire new metabolic traits and may play a key role in the acclimation of microbial communities to environmental pollutants (3). Herrick et al. has reported in-situ horizontal transfer of a naphthalene-catabolic gene between bacteria isolated from a coal tar waste-

contaminated site (3). The genes in Group I still appear to be homologous, but not as homologous as those in Group II, indicating that they may be located on the genome rather than on a plasmid. L2PS4A and L2PS4B were found to be most closely related to *pahAc*, which is the large subunit of ISP<sub>NAP</sub> from *P. putida* OUS82. The sequences of L2PS4A and L2PS4B were also related to *ndoC2*, which is the large subunit of ISP<sub>NAP</sub> from *P. fluorescens*.

It has been illustrated that there are several different dioxygenases that have been characterized from *Pseudomonas* strains. They resemble each other functionally in that they all oxidize aromatic rings and contain a flavoprotein that transfers electrons. It is possible that the *ndo* genes analyzed in this experiment are unique to the isolates L2PS4A and L2PS4B.

Studies have revealed that naphthalene 1,2 dioxygenase (ND) is coded for by genes located on the NAH7 plasmid in *P. putida* (2). To further this investigation, plasmid extractions could be performed with L2PS4A and L2PS4B and PCR could be performed on the plasmid DNA to determine if the plasmid or the genome contains the *ndo* gene. If the gene is not found on the plasmid, this may indicate that the genes in L2PS4A and L2PS4B are *ndoC2* from *P. fluorescens* rather than *pahAc* from *P. putida*.

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