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EXPERIMENTAL ARTICLES

# The P-7 Incompatibility Group Plasmids Responsible for Biodegradation of Naphthalene and Salicylate in Fluorescent Pseudomonads

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**Abstract**—Analysis of seven plasmids (77 to 135 kb in size) of the P-7 incompatibility group that are responsible for the biodegradation of naphthalene and salicylate has shown that the main natural host of IncP-7 plasmids is the species *Pseudomonas fluorescens.* The IncP-7 plasmids are structurally diverse and do not form groups, as is evident from their cluster analysis. The naphthalene catabolism genes of six of the IncP-7 plasmids are conservative and homologous to the catabolic genes of NAH7 and pDTG1 plasmids. The pAK5 plasmid contains the classical *nahA* gene, which codes for naphthalene dioxygenase, and the salicylate 5-hydroxylase gene (*nagG*) sequence, which makes the conversion of salicylate to gentisate possible.

*Key words*: IncP-7 plasmids, fluorescent pseudomonads, biodegradation, naphthalene catabolism genes.

Developing industries heavily pollute the environment with waste, including polycyclic aromatic hydrocarbons (PAHs). In nature, PAHs are mainly degraded by microorganisms. The transfer and rearrangement of genetic material in biodegradative microorganisms largely determine the adaptation of microbial communities to a polluted environment. The distribution of biodegradative traits among microorganisms is often mediated by plasmids, which play an important part in the formation of new genetic combinations and acquisition of new metabolic properties. It is known that most PAH biodegradation plasmids belong to the P-2, P-7, and P-9 incompatibility groups [1] and that the conjugative plasmids of the P-9 incompatibility group responsible for naphthalene biodegradation are widely spread among fluorescent pseudomonads [2]. The organization of catabolic genes has mainly been studied with reference to IncP-9 plasmids NAH7 and pDTG1, whereas naphthalene catabolism plasmids belonging to the other incompatibility groups are as yet insufficiently studied. The incompatibility of plasmids is due to the specific organization of their basic replicons. The affiliation of a plasmid to a certain incompatibility group determines its possible bacterial hosts and the mode of its interaction with other plasmids occurring in the host cell. In turn, this determines the plasmid's fate in different environments and the possibility of combining different traits in one microbial strain. The distribution of biodegradation plasmids over different incompatibility groups remains unknown.

The aim of this work is to study the plasmids from the P-7 incompatibility group that control the biodegradation of naphthalene and salicylate.

## MATERIALS AND METHODS

The bacterial strains and plasmids used in this work are listed in Table 1.

The strains were grown at 28°C overnight in LB broth [3] or a mineral Evans medium [4] on a shaker, with 1 g/l naphthalene or salicylate used as a source of carbon. Upon cultivation on agar plates, naphthalene was placed on the inner side of the lids of inverted petri dishes.

Total bacterial DNA was isolated according to the method described in the handbook [5]. Plasmid DNA was isolated using a slightly modified alkaline lysis method [3]. The DNA was quantified by using a TKO-100 fluorimeter (Hoefer Scientific Instruments, United States) and the dye Hoechst 33258 (Bio-Rad, United States) according to the manufacturer's instructions.

The plasmids were conjugally transferred to recipient *P. putida* KT2442 cells by incubating them overnight on Millipore filters placed onto the surface of agar plates. After the incubation, the bacterial cells were washed off the filters with 1 ml of Evans medium and plated onto Evans medium containing kanamycin

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**Table 1.** The bacterial strains and plasmids used in this work

| Strain | Relevant phenotype | Plasmid | Plasmid size, kb | Source |
|---|---|---|---|---|
| *P. putida* PpG7 | $Nah^+Sal^+$ | NAH7, IncP-9β | 83 | I.C. Gunsalus, United States |
| *P. fluorescens* NK33 | $Nah^+Sal^+$ | pNK33 IncP-7 | 100 | Hizhnekamskneftekhim sludge deposit |
| *P. fluorescens* NK43 | $Nah^+Sal^+$ | pNK43 IncP-7 | 123 | |
| *P. fluorescens* OS18P$^+$ | $Nah^+Sal^+Phn^+Hna^+Tol^+$ $mXyl^+oXyl^+Ebz^+Tra^+$ | pOS18 IncP-7 | 135 | |
| *P. fluorescens* OS19P$^+$ | $Nah^+Sal^+Phn^+Hna^+$ | pOS19 IncP-7 | 122 | |
| *P. fluorescens* FME4 | $Nah^+Sal^+2MeN^+Phe^+$ | pFME4 IncP-7 | 77 | |
| *P. fluorescens* FME5 | $Nah^+Sal^+2MeN^+$ | pFME5 IncP-7 | 80 | |
| *P. putida* AK5 | $Nah^+Sal^+2MeN^+Gen^{+/-}$ | pAK5 IncP-7 | 115 | |
| *P. putida* KT2442 | $Nah^-Sal^-gfpKm^R$ | – | | K. Smalla, Germany |

Note: $Nah^+$, $2MeN^+$, $Sal^+$, $Phn^+$, $Tol^+$, $mXyl^+$, $oXyl^+$, and $Ebz^+$ indicate the ability to grow on naphthalene, methylnaphthalene, salicylate, phenanthrene, toluene, *m*-xylene, *o*-xylene, and ethylbenzoate respectively. $Km^R$ and $Tra^+$ stand for kanamycin resistance and capability for conjugal transfer, respectively. Gfp stands for green fluorescent protein.

**Table 2.** The PCR primers used in this work

| Gene | Primer | Nucleotide sequence | PCR product size, kb | Ref. |
|---|---|---|---|---|
| 16S rRNA | 8f<br>1492r | 5'-AGA GTT TGA TCM TGG CTC AG-3'<br>5'-TAC GGH TAC CTT GTT ACG ACT T-3' | 1484 | [6] |
| IncP-9 *repAB* | repF<br>repR | 5'-CCAGCGCGGTACWTGGG-3'<br>5'-GTCGGCAICTGCTTGAGCTT-3' | 554 | [7] |
| IncP-7 rep-region | Upper<br>Lower | 5'-CCCTATCTCACGATGCTGTA-3'<br>5'-GCACAAACGGTCGTCAG-3' | 524 | C.M. Thomas (United Kingdom) |
| *nahAc* | Ac149f<br>Ac1014r | 5'-CCC YGG CGA CTA TGT-3'<br>5'-CTC RGG CAT GTC TTT TTC-3' | 865 | [8] |
| *nahG* | shc1_up<br>shc1_lo | 5'-CGG CKT THG GTG ARG TCG GTG C-3'<br>5'-GGC GAG GAA RTA GGC GTC CTC AAG 3' | 893 | [2] |
| *nagG* | 458f<br>1224r | 5'-CCT GAC CAA GCT SAA GGT-3'<br>5'-CGT YTC GGT SAC CAT GTG-3' | 766 | This work |
| *nahR* | nahR_1f<br>nahR_585r | 5'-ATG GAA CTG CGT GAC CTG G 3'<br>5'-GCC GTA GGA ACA GAA GCG 3' | 585 | [2] |
| *nahH* | 23OF<br>23OR | 5'-ATG GAT DTD ATG GGD TTC AAG GT-3'<br>5'-ACD GTC ADG AAD CGD TCG TTG AG-3' | 721 | [9] |
| *catA* | C12O_UP<br>C12O_LOW2 | 5'-GCG HAC VAT CGA AGG NCC RYT GTA-3'<br>5'-TCR CGS GTN GCA WAN GCA AAG TC-3' | 462 | This work |

(50 μg/ml) and either naphthalene or salicylate as the sole source of carbon and energy. The selected transconjugants were tested for the presence of plasmids and the *gfp* gene.

The enzymes and buffer solutions used in this study were purchased from Amersham (United Kingdom). All the procedures were carried out according to the manufacturer's protocols.

Polymerase chain reactions (PCRs) were performed under standard conditions in a GeneAmp PCR System 2400 thermal cycler (Perkin-Elmer, United States). The reaction mixture contained 200 μM of each deoxyribonucleotide triphosphate; 1.5 mM $MgCl_2$; and, in some experiments, 5% dimethylsulfoxide (Sigma, United States). The oligonucleotide primers used are listed in Table 2.

The DNA samples were electrophoresed in 0.8% agarose gel in a 0.5× Tris–borate buffer [3]. DNA bands were visualized by staining the developed agarose gels with ethidium bromide. DNA fragments were extracted from the agarose gel with the aid of the QIAEX II Gel Extraction Kit (Qiagen, Germany) according to the recommendations of the manufacturer.

DNA sequencing was carried out using the CEQ™ 2000XL DNA Analysis System and Dye Terminator Cycle Sequencing (DTCS) Kit (Beckman, United States) according to the manufacturer's protocol.

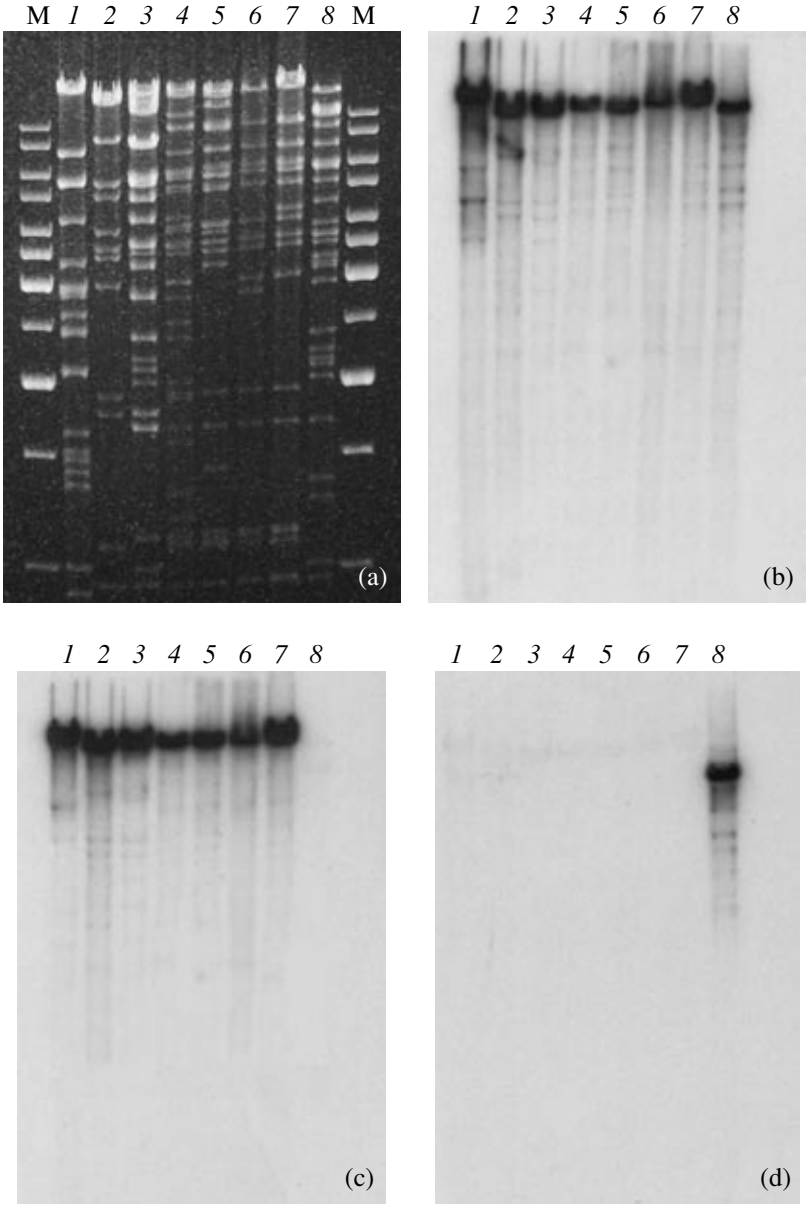


**Fig. 1.** (a) Electrophoresis of plasmid DNA digested by the *Eco*RI restriction endonuclease and hybridization with the $^{32}$P-labeled amplicons of (b) the *nahAc* gene and (c) the *nahG* gene of plasmid NAH7 and (d) the *nagG* gene of strain AK5. Lanes: (M) 1-kb DNA ladder (Invitrogen), (*1*) NAH7, (*2*) pNK33, (*3*) pNK43, (*4*) pOS18, (*5*) pOS19, (*6*) pFME4, (*7*) pFME5, and (*8*) pAK5.

The amount of DNA in the samples was 60–100 ng. The DNA was then labeled using the DECAprime II Random Primed DNA Labeling Kit (Ambion, United States) according to the manufacturer's protocol. Finally, it was hybridized on Hybond $N^+$ nylon filters (Amersham, United Kingdom). Prehybridization and hybridization procedures were carried out at 65°C, as recommended by the manufacturer.

## RESULTS

**Analysis of the naphthalene-degrading strains for the presence of IncP-7 plasmids.** The naphthalene-degrading strains used in this study were obtained from the culture collection of the Laboratory of Plasmid Biology at the Skryabin Institute of Biochemistry and Physiology of Microorganisms. The strains were isolated from oil-contaminated soils via enrichment cultures [10].

Using the PCR approach, 53 strains of fluorescent pseudomonads were tested for the presence of plasmids belonging to the P-7 incompatibility group. Primers specific to the P-7 replicon were derived based on a sequence comparison of the *ori-rep* region of plasmid Rms148 and the *repA* gene of plasmid pCAR1 [11]. Seven of the 53 strains showed the presence of IncP-7

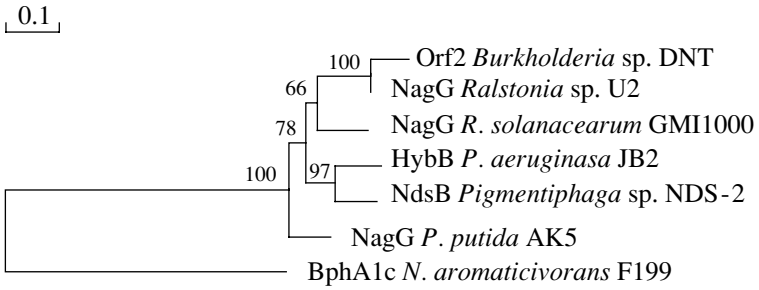


**Fig. 2.** A tree illustrating the evolutionary relationship between the amino acid sequences of the large subunit of the salicylate 5-hydroxylases of various bacterial strains. The relative distances are determined and the tree is constructed with the aid of the TREECON program [14]. The nucleotide sequences used in this work have the following GenBank accession numbers: *Burkholderia* sp. DNT (AAB09764), *Ralstonia* sp. U2 (AF036940), *R. solanacearum* GMI1000 (NP_519211), *P. aeruginosa* JB2 (AF087482), *Pigmentiphaga* sp. NDS-2, and *Novosphingobium aromaticivorans* F119 (AAD04009).

plasmids and were further tested for the presence of the P-9 replicon with the respective primers, since some plasmids (for instance, pBS2) are known to possess a fused P-7/P-9 replicon [2]. This testing, however, failed to reveal a signal specific to the P-9 replicon. The P-7 incompatibility group plasmids isolated from the seven host strains varied in size from 77 to 135 kb.

**Taxonomic identification of the host strains bearing the IncP-7 plasmids.** The seven host strains were ascribed to the genus *Pseudomonas* based on their morphological and physiological properties [12]. The species affiliation of these strains of fluorescent pseudomonads was determined by an amplified ribosomal DNA restriction analysis (ARDRA) of 16S rRNA gene amplification products with *Rsa*I, *Msp*I, and *Hae*III restriction endonucleases. The restriction fragments of 16S rDNA were electrophoresed in 2% agarose gel, using *P. putida* BS203, *P. fluorescens* 2.79, *P. chlororaphis* 17411, *P. aeruginosa* PAK NP1, and *P. aureofaciens* 30.84 as the reference strains. Strains NK33, NK43, OS18, and OS19 were identified as belonging to the species *P. fluorescens* [10]. Strains FME4 and FME5 were preliminarily ascribed to this species as well. Strain AK5 presumably belongs to the species *P. putida.*

**Location of naphthalene biodegradation plasmids.** The experiments involving the conjugal transfer of the IncP-7 plasmids to the recipient strain, *P. putida* KT2442, showed that at least one plasmid, pOS18, with a size of 135 kb, contains all the genes necessary for the conversion of naphthalene and salicylate. The frequency of the conjugal transfer of plasmid pOS18 was $10^{-4}$. The conjugation of the other six strains with KT2442 under the given experimental conditions did not give rise to transconjugants.

In order to provide evidence of the plasmid location of the naphthalene biodegradation genes, the IncP-7 plasmids were subjected to a hybridization analysis. The plasmid DNA was digested by the *Eco*RI restriction endonuclease (Fig. 1a). In these experiments, the PCR products of the *nahA* and *nahG* genes of plasmid NAH7, which were, respectively, 865 and 893 bp in size, were used as $^{32}$P-labeled probes (Table 2). This analysis showed that the naphthalene dioxygenase gene was located in the uppermost *Eco*RI fragments (from 16 to 22 kb in size) of all the IncP-7 plasmids studied (Fig. 1b). Except for pAK5, the salicylate hydroxylase gene was found in the same fragment of almost all of the IncP-7 plasmids (Fig. 1c).

**Amplification and RFLP analysis of the naphthalene degradation genes.** For the analysis of the key genes responsible for naphthalene degradation in the seven strains under study, we chose five genes, *nahAc, nahR, nahG, nahH*, and *catA*, that code for the large subunit of naphthalene 1,2-dioxygenase, the regulatory protein, salicylate 1-hydroxylase, catechol-2,3-dioxygenase, and catechol-1,2-dioxygenase, respectively. Amplification of the *nacAc* and *nahR* genes was observed in all the DNA samples (Table 3). The PCR product of the *nahAc* gene, which was approximately 865 bp in size, was digested by the *Hae*III restriction endonuclease [8]. This restriction analysis showed that the *nahAc* gene is conservative in all seven strains and is homologous to the *doxB* gene of *Pseudomonas* sp. C18 (GenBank accession no. M60405) and the *nahAc* gene of *P. putida* G7 (M83949) (type C18). The amplification of the *nahG* and *nahH* genes was only observed for the *P. fluorescens* strains, but not for *P. putida* AK5. Hydrolysis of the 893-bp amplicons of the *nahG* gene with the *Rsa*I and *Msp*I restriction endonucleases showed that, in all the *P. fluorescens* strains studied, the salicylate hydroxylase gene is similar to the *nahG* gene of plasmid pDTG1 (type pDTG1). The specific amplification of the *catA* gene was observed for the NK33, NK43, OS18, OS19, and AK5 strains.

**Degradation of naphthalene by *P. putida* AK5.** This strain is able to utilize naphthalene; salicylate; and, to a lesser degree, gentisate as the sole sources of carbon and energy for growth. Since strain AK5 does not contain the salicylate hydroxylase and catechol-2,3-dioxygenase genes, it has been suggested that AK5 utilizes naphthalene through salicylate and gentisate. The conversion of salicylate to gentisate is catalyzed by salicylate 5-hydroxylase. Based on the sequences of the *nagG* gene, which codes for the large subunit of the salicylate 5-hydroxylase of *Ralstonia* sp. U2 (AF036940), and the *hybB* gene of *P. aeruginosa* JB2 (AF087482),

**Table 3.** Amplification of the key genes responsible for PAH biodegradation

| Strain | *nahAc* | *nahG* | *nagG* | *nahR* | *nahH* | *catA* |
|---|---|---|---|---|---|---|
| PpG7 | ++/C18* | ++/NAH7 | – | ++ | ++ | ++ |
| NK33 | ++/C18 | ++/pDTG1 | – | ++ | ++ | ++ |
| NK43 | ++/C18 | ++/pDTG1 | – | ++ | ++ | ++ |
| OS18P$^+$ | ++/C18 | ++/pDTG1 | – | ++ | ++ | ++ |
| OS19P$^+$ | ++/C18 | ++/pDTG1 | – | ++ | ++ | ++ |
| FME4 | ++/C18 | ++/pDTG1 | – | ++ | ++ | – |
| FME5 | ++/C18 | ++/pDTG1 | – | ++ | ++ | – |
| NKAK5 | ++/C18 | – | ++ | ++ | – | + |

Note: "++" and "+" indicate very good and good amplification, respectively. C18 is the type.

* The types of *nahAc* and *nahG* genes are indicated.

we derived degenerated primers 458f and 1224r and found that the amplified fragment of the large subunit of salicylate 5-hydroxylase has 766 bp. The specific amplification of the *nagG* gene was only observed for AK5 and not for the other strains studied. The nucleotide sequence of the *nagG* amplicon of strain AK5 was 80% homologous to that of the *nagG* amplicon of *Ralstonia* sp. U2 and 72% homologous to that of the *hybB* amplicon of *P. aeruginosa* JB2. The amino acid sequence of the product of the *nagG* amplicon of strain AK5 was compared with those available in the databases with the aid of the BLAST and CLUSTAL programs [13]. A cluster analysis with the aid of the TREECON program [14] showed that the NagG product of strain AK5 forms a homology group (76 to 83% similarity) with the salicylate 5-hydroxylases of *Ralstonia* sp. U2, *P. aeruginosa* JB2, *Pigmentiphaga* sp. NDS-2, and the plant pathogen *R. solanacearum* GM1000, as well as with the ORF2 of *Burkholderia* sp. DNT (Fig. 2).

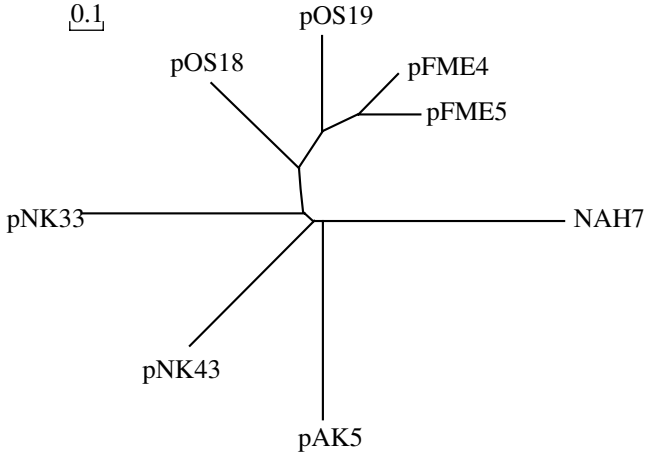


**Fig. 3.** Cluster analysis of the IncP-7 plasmids of naphthalene catabolism based on the RFLP data. The relative distances are calculated and the graph is constructed with the aid of the TREECON program [14].

In order to determine the location of the salicylate 5-hydroxylase gene, the plasmid DNA samples were hybridized with the 766-bp $^{32}$P-labeled amplicon of the *nagG* gene. The PCR product of the *nagG* gene was found to be able to hybridize only with the 10-kb *Eco*PI fragment of plasmid pAK5 (Fig. 1d).

**RFLP analysis of the IncP-7 plasmids.** A comparison between the IncP-7 plasmids was carried out via a restriction fragment length polymorphism (RFLP) analysis with the *Eco*RI restriction endonuclease (Fig. 1a). A cluster analysis with the aid of the TREECON program [14] (Fig. 3) showed that these plasmids do not form similarity groups.

## DISCUSSION

The genes responsible for the utilization of naphthalene and its derivatives in fluorescent pseudomonads are typically located on plasmids [15]. Most of the known catabolic plasmids belong to the P-9 incompatibility group, whereas little is known about the naphthalene catabolism plasmids of other incompatibility groups.

The seven IncP-7 plasmids utilizing naphthalene and salicylate studied in this work differ in their size and structural organization. The species affiliation of the host strains confirmed the recent suggestion that the main natural host of the IncP-7 plasmids is *P. fluorescens* [10]. Unlike the IncP-9 plasmids, which form three structural subgroups and show a structural correlation between the basic replicon (the IncP-9β and IncP-9δ subgroups) and the whole plasmid [2], the IncP-7 plasmids show great structural diversity and do not form similarity groups.

The IncP-7 plasmids contain an *nahAc* gene sequence similar to that of the *doxB* gene of *Pseudomonas* sp. C18 and the *nahAc* gene of *P. putida* G7. Six of the IncP-7 plasmids (pNK33, pNK43, pOS18, pOS19, pFME4, and pFME5) also contain a salicylate hydroxylase gene similar to the *nahG* gene of plasmid pDTG1, as well as the gene coding for catechol-2,3-dioxygenase (the key enzyme of the *meta*-cleavage pathway of

catechol). Thus, most of the IncP-7 plasmids show a conservative organization of their catabolic genes. At the same time, plasmid pAK5 controls the utilization pathway for converting naphthalene into gentisate and contains the salicylate 5-hydroxylase gene sequence. Most of the known salicylate hydroxylases are monomeric monooxygenases, which are flavoproteins catalyzing the conversion of salicylate to catechol. Of the two hitherto known organisms that contain the nucleotide sequence coding for salicylate 5-hydroxylase (the *nagGH* gene of *Ralstonia* sp. U2 [16] and the *hybBC* gene of *P. aeruginosa* JB2 [17]), only *Ralstonia* sp. U2 contains the entire *nag* operon, which controls the conversion of naphthalene to gentisate. The salicylate 5-hydroxylase of this organism is a component of its electron transport chain. The conversion of salicylate also requires active ferredoxin reductase (*nagAa*) and ferredoxin (*nagAb*), which are components of naphthalene dioxygenase [18]. The *nagGH* gene of this organism localizes within a cluster coding for naphthalene dioxygenase. The *nag* genes differ from the classical *nah* genes, although Zhou *et al.* [18] demonstrated that NagAa, NagAb and NahAa, NahAb are interchangeable. *P. putida* AK5 is the first natural strain containing both *nagG* and the classical *nahA* gene of naphthalene dioxygenase. Study of this phenomenon is currently in progress in our laboratory. Zhou *et al.* suggested that the *nag* and *nah* genes originated from a common *nag*-like precursor sequence [18]. A comprehensive study of the naphthalene biodegradation genes in *P. putida* AK5 may provide insight into the evolution of modern catabolic pathways.

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