Comparative study of five polycyclic aromatic hydrocarbon degrading bacterial strains isolated from contaminated soils

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Abstract: Five polycyclic aromatic hydrocarbon (PAH) degrading bacterial strains, *Pseudomonas putida* 34, *Pseudomonas fluorescens* 62, *Pseudomonas aeruginosa* 57, *Sphingomonas* sp. strain 107, and the unidentified strain PL1, were isolated from two contaminated soils and characterized for specific features regarding PAH degradation. Degradation efficiency was determined by the rapidity to form clearing zones around colonies when sprayed with different PAH solutions and the growth in liquid medium with different PAHs as sole source of carbon and energy. The presence of plasmids, the production of biosurfactants, the effect of salicylate on PAH degradation, the transformation of indole to indigo indicating the presence of an aromatic ring dioxygenase activity, and the hybridization with the SphAb probe representing a sequence highly homologous to the naphthalene dioxygenase ferredoxin gene *nahAb* were examined. The most efficient strain in terms of substrate specificity and rapidity to degrade different PAHs was *Sphingomonas* sp. strain 107, followed by strain PL1 and *P. aeruginosa* 57. The less efficient strains were *P. putida* 34 and *P. fluorescens* 62. Each strain transformed indole to indigo, except strain PL1. Biosurfactants were produced by *P. aeruginosa* 57 and *P. putida* 34, and a bioemulsifier was produced by *Sphingomonas* sp. strain 107. The presence of salicylate in solid medium has accelerated the formation of clearing zones and the transformation of indole by *Sphingomonas* sp. strain 107 and *P. aeruginosa* 57 colonies. Plasmids were found in *Sphingomonas* sp. strain 107 and strain PL1. The SphAb probe hybridized with DNA extracted from each strain. However, hybridization signals were detected only in the plasmidic fraction of *Sphingomonas* sp. strain 107 and strain PL1. Using a polymerase chain reaction (PCR) approach, we determined that several genes encoding enzymes involved in the upper catabolic pathway of naphthalene were present in each strain. Sequencing of PCR DNA fragments revealed that, for all the five strains, these genes are highly homologous with respective genes found in the *pah*, *dox*, and *nah* operons, and are arranged in a polycistronic operon. Results suggest that these genes are ordered in the five selected strains like the *pah*, *nah*, and *dox* operons.

Key words: polycyclic aromatic hydrocarbons, biodegradation, polymerase chain reaction, naphthalene catabolic genes.

Résumé : Cinq souches bactériennes capables de dégrader les hydrocarbures aromatiques polycycliques (HAP), *Pseudomonas putida* 34, *Pseudomonas fluorescens* 62, *Pseudomonas aeruginosa* 57, *Sphingomonas* sp. souche 107 et la souche PL1 non identifiée, ont été isolées à partir de deux sols contaminés. Nous avons étudié leurs caractéristiques face à la dégradation des HAP. L'efficacité de dégradation a été déterminée en se basant sur la rapidité de formation de zones d'éclaircissement autour de colonies lors de la vaporisation avec différentes solutions de HAP, ainsi que par la croissance bactérienne en milieu liquide avec différents HAP comme seule source de carbone et énergie. Nous avons aussi examiné la présence de plasmides, la production de biosurfactants, l'effet du salicylate sur la dégradation des HAP, la transformation de l'indole en indigo indiquant la présence de l'activité d'une dioxygénase du noyau aromatique, et l'hybridation avec la sonde SphAb représentant une séquence hautement homologue au gène *nahAb* codant pour la ferrédoxine de la naphtalène dioxygénase. La souche la plus efficace pour la spécificité de substrats et la rapidité à dégrader différents HAP était *Sphingomonas* sp. souche 107, suivie de la souche PL1 et de *P. aeruginosa* 57. Les souches les moins efficaces étaient *P. putida* 34 et *P. fluorescens* 62. Chaque souche a transformé l'indole en indigo, excepté la souche PL1. *Pseudomonas putida* 34 et *P. aeruginosa* 57 ont produit des biosurfactants, tandis qu'une activité bioémulsifiante a été observée chez *Sphingomonas* sp. souche 107. La présence de salicylate en milieu solide a accéléré la formation de zones d'éclaircissement et la transformation de l'indole par les colonies de *Sphingomonas* sp. souche 107 et *P. aeruginosa* 57. Des plasmides ont été trouvés chez *Sphingomonas* sp. souche 107 et la souche PL1. La sonde SphAb a hybridé avec l'ADN extrait de chacune des souches. Cependant, des signaux d'hybridation ont été détectés uniquement dans la fraction plasmidique de *Sphingomonas* sp. souche 107 et de la souche PL1. Par la réaction

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en chaîne de la polymérase (PCR), nous avons déterminé que plusieurs gènes codant pour la voie catabolique supérieure du naphtalène étaient présents dans chaque souche. La séquence des produits de PCR a révélé que, pour toutes les souches, ces gènes sont hautement homologues aux gènes respectifs retrouvés dans les opérons *dox*, *pah* et *nah*, et qu'ils sont arrangés en un opéron polycistronique. Nos résultats suggèrent également que ces gènes soient ordonnés dans les cinq souches de façon similaire aux opérons *pah*, *dox* et *nah*.

Mots clés : hydrocarbures aromatiques polycycliques, biodégradation, réaction en chaîne de la polymérase, gènes responsables du catabolisme du naphtalène.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are widespread pollutants resulting from human activities. They are released in large quantities from fossil fuel combustion and various industrial processes. Several PAHs are known or suspected to be toxic, mutagenic, and carcinogenic. Exposure to PAHs can be hazardous to human health, and therefore, their elimination is considered a priority in many countries (White 1986).

Many bacterial strains have been isolated for their ability to degrade and use PAHs as source of carbon and energy. The catabolic pathway of naphthalene has been elucidated for *Pseudomonas putida* G7, while for other PAHs this has been partly done (see reviews in Yen and Serdar 1988; Cerniglia 1992; Pothuluri and Cerniglia 1994). Naphthalene catabolism in *P. putida* G7 involves hydroxylation of one aromatic ring to produce 1,2-dihydroxy-1,2-dihydronaphthalene by the naphthalene dioxygenase. This multimeric enzyme is composed of a reductase (encoded by *nahAa*), a ferredoxin (*nahAb*), and two subunits of iron–sulfur protein (*nahAc* and *nahAd*). After the dehydrogenation of the 1,2-dihydroxy-1,2-dihydronaphthalene to 1,2-dihydroxynaphthalene (by a dehydroxygenase encoded by *nahB*), the hydroxylated ring is cleaved by the 1,2-dihydroxynaphthalene dioxygenase (*nahC*). Further reactions involving an isomerase (*nahD*), a hydratase–aldolase (*nahE*), and a dehydrogenase (*nahF*) lead to the formation of salicylate and pyruvate (Eaton and Chapman 1992; Eaton 1994). Salicylate is further transformed to catechol, which is then degraded by *ortho* or *meta* cleavage depending on the bacterial strains; this is referred to as the lower pathway (Williams and Sayers 1994). The catabolic pathway of naphthalene to salicylate, referred to as the upper pathway, occurs also with other PAHs. For example, it is involved in the transformation of phenanthrene and anthracene to 1-hydroxy-2-naphthoic acid and 2-hydroxy-3-naphthoic acid, respectively, in *Pseudomonas fluorescens* 5R (Menn et al. 1993).

Genes encoding the lower and upper pathways in *P. putida* G7 are arranged in two respective polycistronic operons, *sal* and *nah*, located on the plasmid NAH7. Control of expression of the two operons is regulated by the *nahR* gene product. The activity of NahR depends on the intracellular concentration of salicylate (Schell 1985). PAH-degradation genes have been isolated from other pseudomonads. These genes are arranged in polycistronic operon such as (*i*) the *nah* operon on the pDTG1 plasmid in *P. putida* NCIB 9816-4 (Harayama and Rekik 1989; Simon et al. 1993; Eaton 1994); (*ii*) the *pah* operon located on the chromosome in *P. putida* OUS82 (Takizawa et al. 1994), (*iii*) the *ndo* operon located on plasmid in *P. putida* NCIB 9816 (Kurkela et al. 1988); and (*iv*) the *dox* operon located on a plasmid in *Pseudomonas* strain C18 (Denome et al. 1993). Nucleotide and the deduced amino acid sequences are highly homologous between the respective genes of the *nah*, *pah*, *ndo*, and *dox* operons. The gene order of the *nah* operon is *nahAa-nahAb-nahAc-nahAd-nahB-nahF-nahC-nahQ-nahE-nahD* (Eaton 1994). This arrangement occurs also in the *pah*, *dox*, and *ndo* operons. PAH-degradation genes are encountered in other *Pseudomonas* species. For example, strong hybridization signals were found between a *nah* probe and plasmids recovered from three *Pseudomonas* strains involved in the mineralization of naphthalene, phenanthrene, and anthracene (Sanseverino et al. 1993*a*). Similar results were observed with strains of *Pseudomonas stutzeri* involved in degradation of naphthalene (Rossello-Mora et al. 1994). The only published study of PAH catabolic genes from nonpseudomonads is on *Comamonas testosteroni* strains that have the ability to degrade naphthalene, phenanthrene, and anthracene. Although the PAH catabolic pathway is similar to pseudomonads, Goyal and Zylstra (1996) showed that no hybridization signal was detected with the *nah* probe in these bacterial strains and that the gene arrangement was different. These observations suggest that substantial differences occur at the gene level in *C. testosteroni* that may reflect a different approach of PAH degradation such as gene regulation and enzymatic specificity.

We were interested in finding highly efficient, PAH-degrading bacterial strains with a broad range of substrate specificity. We were also interested in determining whether important changes at the gene level can be found in these strains that could be related to the efficiency of PAH degradation. In this paper, we investigated five bacterial strains chosen from a pool of 65 PAH-degrading bacterial isolates derived from two contaminated soils. Comparison of these strains revealed differences in their substrate specificity and their efficiency to degrade PAHs.

Materials and methods

Isolation of PAH-degrading bacterial strains

Soils came from a sandpit heavily contaminated with oil refinery wastes and an area contaminated with aromatic hydrocarbons. Soil samples were agitated with four volumes of Bushnell-Haas minimal salt medium (BH) at 150 rpm for 10 days and 30°C in presence of 100 ppm of different PAHs such as phenanthrene, fluorene, pyrene, fluoranthene, and chrysene. Dilutions of soil slurry were inoculated on BH medium plates supplemented with 0.05% w/v yeast extract (BHY). Colonies were chosen for their capacity to form clearing zones and (or) produce a color change when sprayed with different PAH solutions (Sylvestre 1980). These solutions were prepared (2% w/v in acetone) with naphthalene, three-ring PAHs (dibenzothiophene, fluorene, phenanthrene, and anthracene), and four-ring PAHs (fluoranthene, pyrene, and benz[*a*]anthracene). PAHs were purchased from Aldrich Chemical Co. (Milwaukee, Wis.). Colonies were also examined for the expression of aromatic ring dioxygenase activity by the formation of blue indigo when they were transferred onto 1 mM indole agar plates (Ensley et al. 1983). Colonies were

checked at regular time intervals for the appearance of clearing zones and blue-indigo color.

Identification of bacterial strains

PAH-degrading bacterial strains were characterized based on colony morphology and pigmentation, Gram staining, API (Analytical Profile Index) 20E strips (bioMérieux Vitek, Inc., Hazelwood, Mo.), and several physiological and biological tests (Krieg and Holt 1984).

Effect of salicylate on PAH degradation

Bacteria were plated on BHY solid medium containing 100 μg/mL of sodium salicylate (Aldrich Chemical, Milwaukee, Wis.) and incubated at 30°C, except at 25°C for strain PL1, for 1–4 days. Colonies were sprayed with the same PAH solutions as enumerated before. They were checked at regular time intervals for the appearance of the clearing zones. Controls were BHY agar plates without salicylate inoculated with bacteria.

Growth in liquid culture with PAH as sole source of carbon

Bacterial strains were grown at 30°C (or 25°C, strain PL1) on BHY agar plates for 1–4 days. Three to five colonies were picked and added to 25 mL of BH medium containing 200 ppm of respective PAH and agitated in the dark at 200 rpm and 30°C (or 25°C, strain PL1). The PAHs used were the same as enumerated before. Cultures were regularly checked for bacterial growth (turbidity), appearance of color (reflecting appearance of intermediates), and disappearance of nonsolubilized PAH crystals. Negative controls were noninoculated media.

Biosurfactant production

Presence of surface tension lowering activity in bacterial cultures was determined with the drop-collapsing test (Jain et al. 1991) and measured with a Fisher tensiometer by the De Nouy ring method. Emulsifying activity was estimated visually as described by Déziel et al. (1996). The positive control was the biosurfactant-producing strain *Pseudomonas aeruginosa* 19SJ (Déziel et al. 1996).

Measurement of PAHs degradation in liquid culture

The 25-mL cultures were acidified with 10 N HCl and extracted with three volumes of 10 mL diethyl ether. The final volume of the extraction was adjusted to 25 mL and dehydrated with anhydrous sodium sulfate. Residual PAHs were determined by gas chromatography (GC) (Hewlett Packard, model 5890A) with a flame ionization detector. Finally, the residual concentration of PAHs was calculated in comparison to an internal standard (9-methyl anthracene).

Plasmid extraction

Plasmids from *Sphingomonas* sp. strain 107, strain PL1, and *Escherichia coli* were extracted by alkaline lysis protocol and purified either by polyethylene glycol precipitation or a CsCl gradient as described by Sambrook et al. (1989).

Total DNA extraction

Bacterial cells were centrifuged at 8000 × *g* for 10 min, resuspended in 50 mM Tris–HCl (pH 8.0) – 20 mM EDTA – 150 mM NaCl with 5 mg lysozyme/mL, and incubated 10 min at 37°C. Sodium dodecyl sulfate (SDS), β-mercaptoethanol, and proteinase K were added to a final concentration of 2%, 1%, and 50–100 μg/mL respectively, and incubated 2–3 h at 45°C. The solution was then extracted twice with phenol (equilibrated with Tris–HCl pH 8.0), twice with phenol – chloroform – isoamyl alcohol (50:49:1), and once with chloroform – isoamyl alcohol (24:1). After RNAse treatment (5 μg/mL for 15 min at 37°C), DNA was precipitated with two volumes of ethanol 95% and resuspended in water.

Polymerase chain reaction (PCR)

Consensus sequences used to generate oligonucleotides specific for PAH-degradation genes were deduced from the alignment between sequences available from *pah*, *nah*, and *dox* operons using the PILEUP program of GCG (Genetics Computer Group, University of Wisconsin). Universal eubacterial primers for 16S rRNA gene are the following: pA, 5′AGAGTTTGATCCTGGCTCAG3′ (location 8–27 based on the *E. coli* 16S rRNA sequence); pH, 5′AAGGAGGTGATCCAGCCGCA3′ (location 1541–1522) (Bruce et al. 1992). Oligonucleotides were synthesized using a Gene Assembler (Pharmacia, Baie-d'Urfé, Que.).

Amplification was accomplished in a 50-μL reaction volume with 10 mM Tris–HCl (pH 9.0), 1.5 mM $MgCl_2$, 50 mM KCl, 200 μM dNTP, 10 pmol of each primer, and 2.5 U of *Taq* DNA polymerase (Pharmacia). Amplifications were done in a DNA thermal cycler 480 (Perkin-Elmer) at 80°C for 3 min (in which 50–100 ng of template DNA was added), 94°C for 5 min, and 55°C for 5 min; then 30–35 cycles at 72°C for 2 min, 94°C for 40 s, and 55°C for 1 min; and finally, an extension time of 10 min at 72°C. Amplification products were examined by 1.0–1.8% agarose gel electrophoresis.

PCR for amplification of large DNA fragments (more than 2 kb) was accomplished in a 50-μL volume as recommended by the manufacturer (Expand PCR, Boehringer Mannheim, Laval, Que.) with 50 mM Tris–HCl (pH 9.2), 1.75 mM $MgCl_2$, 16 mM $(NH_4)SO_4$, 350 μM dNTP, 15 pmol of each primer, 100–200 ng of template DNA, and 2.5 U of thermostable DNA polymerases. Amplifications were done in a DNA thermal cycler 480 (Perkin-Elmer) at 94°C for 2 min; 10 cycles at 94°C for 30 s, 55°C for 30 s, and 68°C for 4 min; then 25 cycles at 94°C for 30 s, 55°C for 30 s, and 68°C for 4 min with 20-s extension time/cycle; and finally an extension time of 7 min at 68°C.

Cloning of PCR products

Amplified DNA were recovered from agarose gel electrophoresis with a NaI solution and a silica matrix column as described by the manufacturer (Geneclean II, BIO 101 inc. LaJolla, Calif.). They were ligated to the TA vector (pCRII) and transformed in the *E. coli* INV αF′ host (Invitrogen, San Diego, Calif.) as recommended by the manufacturer, or ligated into the *Sma*I-digested pTZ18U (Bio-Rad) vector and transformed in *E. coli* XL1 blue as described by Sambrook et al. (1989).

DNA manipulations

Restriction endonuclease digestions and DNA separation on agarose gel electrophoresis were carried out as described by Sambrook et al. (1989). DNA was transferred from agarose gels on nylon charge membrane with ammonium acetate solution as described by Rigaud et al. (1987). The SphAb probe was prepared by PCR amplification of a 291-bp DNA fragment with the ferredoxin primers (FerD–FerG, Fig. 3) using the plasmid of *Sphingomonas* sp. strain 107. This 291-bp fragment was labelled by random primer extension with digoxigenin-11-dUTP (DIG) according to the manufacturer specifications (Boehringer Mannheim, Laval, Que.). Hybridization and detection conditions were also done according to the manufacturer specifications (Boehringer Mannheim) with the hybridization temperature carried out at 63°C with 5 × SSPE (20 × SSPE stock solution is 200 mM phosphate buffer (pH 7.0) – 3 M NaCl – 20 mM EDTA) in the hybridization buffer. The membrane was washed twice for 30 min at 63°C in 0.5 × SSPE – 0.1% SDS. Sequencing were performed on double-stranded DNA by the dideoxynucleotide method (Sanger et al. 1977).

Results

Identification of PAH-degrading bacterial strains selected

Sixty-five bacterial strains were isolated for their ability to form clearing zones around colonies when sprayed with one or

Fig. 1. 16S rRNA gene sequence analysis of *Sphingomonas* sp. strain 107. The 16S rRNA gene sequences of strain 107 and six *Sphingomonas* were aligned. The most divergent region of these 16S rRNA gene sequences is illustrated. Only the nucleotide changes are shown compared with *S. yanoikuyae* 16S rRNA gene sequence. Dots represent identity. Bars represent a gap. YANOI, *S. yanoikuyae* (GenBank accession No. D13728); 107, *Sphingomonas* sp. strain 107; S. sp., *Sphingomonas* sp. (GenBank accession No. D16148); TERRA, *S. terrae* (GenBank accession No. D13727); PARAP, *S. parapaucimobilis* (GenBank accession No. D13724); ADHAE, *S. adhaesiva* (GenBank accession No. D16146); PAUCI, *S. paucimobilis* (GenBank accession No. D16144).

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          930.        .        .        .        .      980.
YANOI  GACATCCCTATCGCGGATCGTGGAGACACTTTCCTTCAGTTCGGCTGGATAGGTGACAGG
107    ............................................................
S. sp. ..............t.tcca....tgga.a............................
TERRA  .......tg.g.....t.acca....tgg.......t.....................
PARAP  .....-gtccgga..at.tcca....tgga.ct....ct.cg..--.ac.g.aac.....
ADHEA  .....-gtccgga..at.tcca....tgga.ct..nnnn.cg..--.ac.g.aac.....
PAUCI  .....-gg..gga..ac.tcca....tgga..t....ttcgg..---acctacac.....
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several PAH solutions. Identification of these strains revealed that most of them were pseudomonads, mainly *P. aeruginosa*, *P. fluorescens*, and *P. putida*. Some *Sphingomonas* sp. strains were identified. With one strain, named PL1, the identification was uncertain (see below). Relative to each species, we have chosen for the comparative study the most efficient bacterial strain based on two characteristics. The first one was the spectrum of substrate specificity tested by the formation of clearing zones around colonies, indicating PAH degradation. The second characteristic was the relative speed of appearance of these clearing zones. These strains are 34, 57, 62, 107, and PL1.

Strain 34 is Gram negative and produces pyoverdin pigment but no pyocyanin. It grows at 5°C but not at 42°C, and uses acetamide as source of carbon but not sorbitol, trehalose, or geraniol. Together with API, it was identified as a *P. putida* species. Strain 57 is Gram negative and produces pyocyanin and pyoverdin pigments. It does not grow at 5°C but grows at 42°C. It uses acetamide and geraniol as source of carbon but not trehalose or sorbitol. With API, it was identified as a *P. aeruginosa* species. Strain 62 is Gram negative, produces pyoverdin but not pyocyanin, grows at 5°C but not at 42°C, and uses trehalose and sorbitol as sources of carbon but not geraniol or acetamide. It was identified as a *P. fluorescens* species. Strain 107 is a Gram-negative rod bacterium, forming yellow-pigmented colonies that become sticky and adhesive after 3–4 days of incubation at 29°C on tryptic soy agar plates. Excellent similarity with *Pseudomonas paucimobilis*, now *Sphingomonas paucimobilis*, was obtained with the API strips. Its 16S rRNA gene was amplified by PCR and cloned, and part of the gene was sequenced. The resulting sequence was highly homologous to the 16S rRNA gene sequence of *Sphingomonas* species. The most divergent region in the 16S rRNA gene sequence between *Sphingomonas* species (Fig. 1) is identical between strain 107 and *Sphingomonas yanoikuyae*, suggesting that strain 107 is a *S. yanoikuyae* species.

Strain PL1 is a nonpigmented, Gram-negative, motile, coccobacillus (0.4–0.5 to 0.7–1.1 μm) bacterium. Electron micrographs showed one flagellum per cell (data not shown). Identification tests with API strips showed that strain PL1 is oxidase positive. It was negative for β-galactosidase, arginine dihydrolase, ornithine decarboxylase, urease, tryptophan deaminase, gelatinase, and amylase; it was negative for production of H_2S and indole and for nitrate reduction. It uses citrate as a carbon source but none of the tested carbohydrates. Further testing with the Biolog identification system (Microlog GN; Harward, Calif.) also showed that strain PL1 did not use any carbohydrates. It uses methyl pyruvate, acetate, *cis*-aconitate, citrate, formate, α-hydroxybutyrate, β-hydroxybutyrate, α-ketobutyrate, α-ketoglutarate, D- and L-lactate, malonate, propionate, succinate, bromo succinate, succinamate, alaninamide, and D and L-alanine. It does not use any other amino acid (results were borderline for some of them). Tests with the API strips showed that strain PL1 belonged to the *Alcaligenes* genus. The Biolog system identified strain PL1 as *Oligella ureolityca/urethralis* (formerly *Moraxella urethralis* [*Neisseriaceae* family, *Moraxella* genus]; Krieg and Holt 1984; Rossau et al. 1987), with a similarity index of 0.738 that is below the index (0.75) to be considered as acceptable for species identification. Indeed, *O. ureolityca/urethralis* has no flagella and does not use malonate and citrate. Furthermore, it was found primarily in human samples (Hansen et al. 1975). Further tests are needed such as G+C content, fatty acid composition, and rRNA gene sequence analysis to identify strain PL1.

Characteristics of the five PAH-degrading bacterial strains selected

We compared these five strains on the following characteristics: (*i*) presence of plasmids on which catabolic genes might be located; (*ii*) presence of an aromatic ring dioxygenase activity such as the naphthalene dioxygenase by the appearance of blue indigo color in presence of indole (the relative speed of appearance of the color was also measured), (*iii*) the effect of salicylate added in the BHY solid medium on the time required for the appearance of clearing zones and the transformation of indole; (*iv*) production of biosurfactants that might increase the availability of PAHs; and (*v*) the time required to observe bacterial growth in BH liquid medium with phenanthrene as a sole source of carbon and energy. Table 1 summarizes the characteristics of the five selected bacterial strains.

The less efficient bacterial strains *P. fluorescens* 62 and *P. putida* 34 had a narrower spectrum of substrate specificity than the other strains. Formation of clearing zones when sprayed with different PAHs and transformation of indole to indigo took more than a day. Addition of salicylate in the solid medium neither decreased the time required for the formation of clearing zones and the conversion of indole, nor stimulated the appearance of clearing zones on new PAHs.

Table 1. Characteristics of the five selected PAH-degrading bacterial strains.

	P. putida 34	*P. fluorescens* 62	*P. aeruginosa* 57	Strain PL1	*Sphingomonas* sp. strain 107
Plasmid	No	No	No	Yes	Yes
Biosurfactant	Yes	No	Yes	No	Yes[a]
Indole[b]	+[c]	+	+++	−	+++
Specificity[d]					
Naphthalene	+[c]	+	++	++	++
Dibenzothiophene	−	+/o	++/o	++/o	+++/o
Fluorene	+/y	+/y	++/y	++/y	+++/y
Phenanthrene	+	+	++	++	+++
Anthracene	+	+	+	+	++
Fluoranthene	−	−	−	+/o	+/o
Pyrene	−	−	−	−/br	−/br
Benz[*a*]anthracene	−	−	−	+	+
Salicylate[e]	No	No	Yes	No	Yes

[a]Bioemulsifying activity.

[b]Appearance of blue indigo color of colonies transferred on 1 mM indole plate.

[c]Relative speed of appearance of blue indigo or relative speed of appearance of clearing zones and color: +, more than 1 day; ++, less than 1 day; +++, less than 1 h; −, absence of blue indigo or clearing zones after 14 days of incubation; o, orange; y, yellow; br, brown.

[d]Appearance of clearing zones and color of colonies sprayed with different PAHs.

[e]Effect of salicylate on the speed of forming clearing zones and producing indigo color.

In BH medium containing phenanthrene, bacterial growth was apparent after more than a week. The culture had an orange coloration characteristic of degradation intermediates and nonsolubilized phenanthrene crystals had disappeared. Biosurfactants were produced by *P. putida* 34 and no plasmid was observed in both strains.

Pseudomonas aeruginosa 57 and strain PL1 colonies formed clearing zones faster than the preceding strains. It took less than a day to see clearing zones with naphthalene, dibenzothiophene, phenanthrene, and fluorene, and more than a day with anthracene. No clearing zones were formed with any four-ring PAHs tested with *P. aeruginosa* 57. Strain PL1 colonies formed clearing zones with biphenyl, acenaphtylene, and dibenzofuran, and with the four-ring PAHs fluoranthene and benz[*a*]anthracene. A brown coloration with pyrene was apparent, indicating formation of degradation intermediates. However, in liquid medium with pyrene, fluoranthene, or benz[*a*]anthracene as sole source of carbon, no growth of strain PL1 was observed after 3 weeks of incubation. Both strains grew in phenanthrene−BH liquid medium after 5−6 days, concomitant with the presence of orange coloration and the disappearance of nonsolubilized phenanthrene crystals. Analysis by gas chromatography showed that 100% of phenanthrene and 50% of fluorene were degraded after 8 days in strain PL1 liquid cultures containing these respective PAHs as substrate.

Pseudomonas aeruginosa 57 transformed indole to indigo in less than a day. We observed that clearing zones and formation of indigo appeared more rapidly when this strain was plated on BHY solid medium containing salicylate. However, strain 57 colonies did not form clearing zones with new PAHs in the presence of salicylate. Finally, strain 57 produced biosurfactants and no plasmid was observed. Strain PL1 did not transform indole to indigo. Salicylate had no effect on the formation of clearing zones. Extrachromosomal DNAs were extracted from strain PL1. However, this DNA never produced the usual two plasmidic bands but rather a broad band of 5−10 mm on CsCl gradient. Figure 2C (lane 11) shows that this DNA preparation was composed of 5- to 10-kbp DNA fragments and a DNA smear of 2−4 kbp long. The nature of such DNA remains to be clarified.

For *Sphingomonas* sp. strain 107, the time required for the formation of clearing zones and for the transformation of indole was in general faster than the other strains. Formation of clearing zones was observed in less than a day with naphthalene and anthracene, and in less than an hour with dibenzothiophene, fluorene, and phenanthrene. Transformation of indole was observed in less than an hour, usually 10 min after transferring colonies on indole plates. Clearing zones were seen with fluoranthene and benz[*a*]anthracene. A brown color was observed on colonies sprayed with pyrene. *Sphingomonas* sp. strain 107 colonies also formed clearing zones with biphenyl, acenaphthene, acenaphthylene, and dibenzofuran. No clearing zone was observed when sprayed with the five-ring PAH benzo[*a*]pyrene. Bacterial growth in phenanthrene−BH liquid medium was apparent after 4 days with the usual orange coloration and disappearance of phenanthrene crystals. In contrast to strain PL1, *Sphingomonas* sp. strain 107 was able to grow in BH liquid medium with fluoranthene and pyrene as substrate. Addition of salicylate in BHY solid medium accelerated the formation of clearing zones and transformation of indole. A plasmid was found in this strain (Fig. 2A). Digestion with different restriction enzymes revealed that the plasmid is approximately 55 kbp long.

Sphingomonas sp. strain 107 cultures were grown in BH liquid medium with 200 ppm of pyrene. After 7 days of incubation, 20 ppm of pyrene was degraded. As pyrene is poorly soluble in water, Tween 80 (200 μg/mL) was added to increase the availability of pyrene for microbial degradation. The same was done with 200 ppm of fluoranthene. After 7 days of incubation, 40 ppm of pyrene and 25 ppm of fluoranthene

were degraded. With the three-ring PAHs fluorene and phenanthrene, no trace of these PAHs were present after 10 days of incubation in BH with Tween 80. Finally, addition of salicylate (100 μg/mL) in the BH with Tween 80 showed an increase in pyrene degradation to 95 ppm after 7 days of incubation. Analysis of pyrene degradation intermediates by GC – mass spectrometry showed several peaks. However, none of these intermediates have been identified yet.

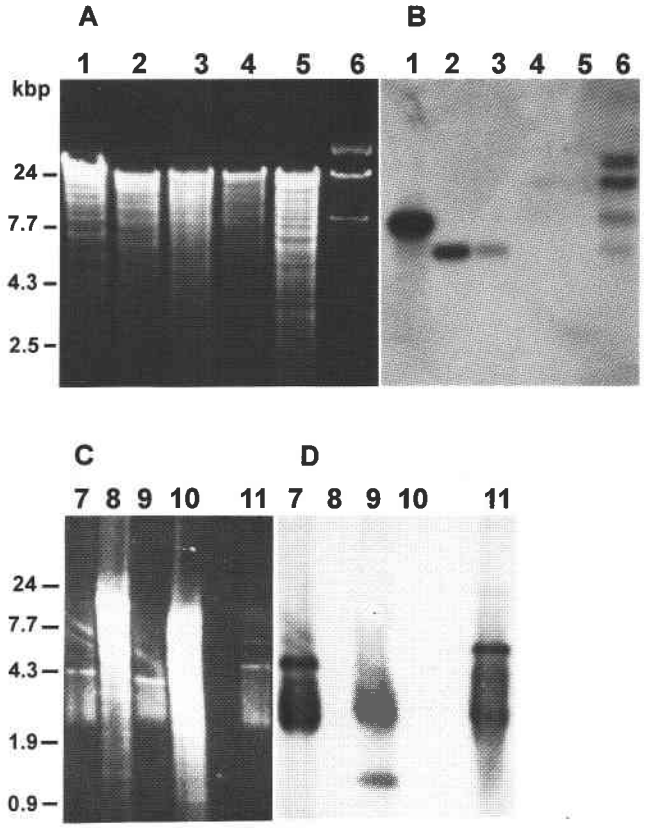
Hybridization with the *nahAb*-related probe

PCR on the *Sphingomonas* plasmid with the FerD and FerG primers specific to the naphthalene dioxygenase ferredoxin gene such as *nahAb*, *pahAb*, and *doxA* produced a DNA fragment of approximately 300 bp. Cloning and sequencing of this DNA fragment revealed a homology of 92% with the *nahAb* nucleotide sequence. This fragment was used as a probe, named SphAb, to detect the presence of ferredoxin gene in the other four strains. Total and plasmid DNAs were extracted from each strain, digested with *Hin*dIII, and hybridized with the SphAb probe. Hybridization signals were observed with DNA extracted from each strain (Fig. 2). The SphAb probe hybridized exclusively to the plasmid DNA of strain PL1 and *Sphingomonas* sp. strain 107. However, hybridization signals were observed with total DNA of these two strains when a longer exposure of the hybridized membrane was done (data not shown). These signals corresponded to the plasmid DNA that coextracted with the chromosomal DNA. Several hybridization DNA fragments were observed with the *Sphingomonas* plasmid DNA that correspond to the digested and different forms of the undigested circular plasmid. A weaker hybridization signal was observed with *P. putida* DNA that might be the result of divergence between the probe and the target sequence. Interestingly, the hybridization signals on the plasmid fraction of strain PL1 did not correspond to one of the major DNA fragments but rather to a DNA fragment not visible by ethidium bromide staining. It also produced a strong hybridization signal on the DNA smear.

PCR amplification of PAH-degradation genes

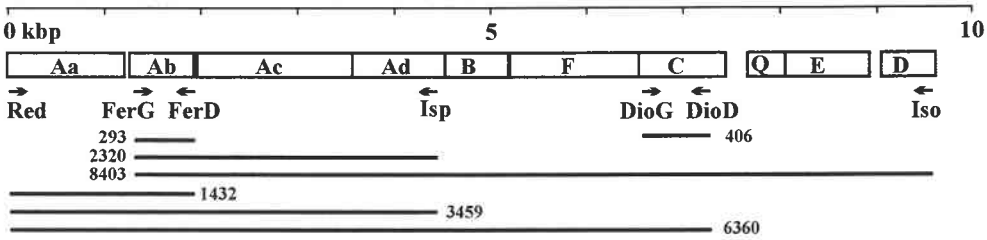
Seven oligonucleotides were synthesized based on consensus sequences found between respective genes in the *pah*, *dox*, and *nah* operons. These oligonucleotides were used as primers to do PCR on total and plasmidic DNA extracted from each strain. If PAH-degradation genes are present in these DNA and if they are arranged in polycistronic operon and ordered like the *pah*, *dox*, and *nah* operons, the length of the PCR products can be predicted based on available nucleotide sequences of these three operons (Fig. 3). Oligonucleotides (Fig. 3) are FerG and FerD, specific for the naphthalene dioxygenase ferredoxin gene; Red and Isp, specific for the gene encoding the reductase and the iron-sulfur small subunit, respectively, of naphthalene dioxygenase; DioxG and DioxD, specific for the 1,2-dihydroxynaphthalene dioxygenase gene; and Iso, specific for the *doxJ* gene and homologous to *nahD* (encoding the 2-hydroxychromene-2-carboxylate isomerase). To confirm that PCR amplified PAH-degradation genes, several PCR products with the predicted length were cloned, and one or both extremities of these products were sequenced. In all cases, nucleic acid sequence analysis confirmed the presence of the expected PAH-degradation gene. Results of PCR experiments are summarized in Table 2.

Fig. 2. Hybridization of the total and plasmid DNA with the SphAb probe. Total and plasmid DNA were run on a 0.7% agarose electrophoresis gel and stained with ethidium bromide (A, C). DNAs were transferred on a nylon membrane and hybridized with the DIG-labelled SphAb probe (B, D). *Hin*dIII-digested total DNA of *P. aeruginosa* 57 (lane 1), *P. fluorescens* (lane 2), *P. putida* 34 (lane 3), *Sphingomonas* sp. strain 107 (lane 4), and strain PL1 (lane 5). Plasmid DNA from *Sphingomonas* sp. strain 107 (lane 6). Plasmid DNA from strain PL1 digested with *Hin*dIII (lane 7), *Eco*RI (lane 9), or undigested (lane 11). Total DNA from strain PL1 digested with *Hin*dIII (lane 8) or *Eco*RI (lane 10).



PCR with the FerG–FerD and DioG–DioD primers showed that the naphthalene dioxygenase ferredoxin and 1,2-dihydroxynaphthalene dioxygenase genes are present in all strains. Amplification using the FerG–Isp primers produced in all strains a 2.2-kbp DNA fragment as predicted by the *nah*, *dox*, and *pah* operons. This suggests that the gene order of that fragment would be (using nomenclature for the *nah* operon) *nahAb-nahAc-nahAd*. Amplification using the FerG–Iso primers gave an unexpected result. A 4.2-kbp DNA fragment was obtained in all strains instead of the predicted 8.4-kbp DNA fragment. The latter fragment was observed once with *P. putida* 34 in addition to the 4.2-kbp DNA fragment. Sequence of the extremities of the 4.2-kbp fragment revealed that the Iso primers annealed in all strains to the same location in the *nahF* gene (aldehyde dehydrogenase). During the amplification of a large DNA fragment, this annealing site was preferentially amplified. Therefore, failure to obtain the 8.4-kbp DNA fragment might be due to PCR

Fig. 3. Strategy to determine the gene arrangement of PAH-degradation genes by PCR. Gene arrangement of the PAH-degradation genes is based on the *nah*, *dox*, and *pah* operons as described by Eaton (1994). Letters in the boxes correspond to the nomenclature of the *nah* operon from the NAH7 plasmid. Localization and orientation of primers are represented by arrows. Lines under the operon are the predicted DNA fragments and their length (numbers beside in bp) that should be generated by PCR with respective combination of primers. Primer sequences are shown with their location on the *nah* operon (Red primer; Simon et al. 1993) or the *dox* operon (all the other primers, Denome et al. 1993). [1]DioG and [1]DioD are consensus sequences between *nahC* and *nahH* (catechol 2,3-dioxygenase; Ghosal et al. 1987) sequences. [2]The Iso sequence was derived from *doxJ* only.




```
Red:   ATG(a/g)AACTTCTCATACAGCC (853-872)
FerG:  AAATGGATTGAAGCAGTCGC (121-140)
FerD:  ATCATTACGCGCAGGTTCTC (413-394)
Isp:   GAAAGACCATCAGATTGTG (2440-2422)
[1]DioG: GA(g/c)(a/c)A(g/a)G(a/g)CCGT(t/g)TCTATCTG (4936-4956)
[1]DioD: CC(a/g)(c/g)(a/g)(c/t)(a/g)(a/g)AAGCC(c/g)AGCA (5341-5324)
[2]Iso:  CTATTCTCCATTTACAGGCG (8523-8504)
```

Table 2. DNA fragments obtained after PCR with primers specific to PAH-degradation genes on total and plasmidic DNA isolated from the selected bacterial strains.

Primers	Predicted DNA[a] fragment length (bp)	*P. putida* 34	*P. fluorescens* 62	*P. aeruginosa* 57	Strain PL1[b]	*Sphingomonas* sp. strain 107[b]
FerG–FerD	293	+[c]	+	+	+	+
DioG–DioD	406	+	+	+	+	+
FerG–Isp	2320	+	+	+	+	+
FerG–Iso	8403	+	–	–	–	–
		4188[d]	4188	4188	4188	4188
Red–FerD	1432	–	–	+	+	+
Red–Isp	3459	–	–	+	+	+
Red–DioG	6360	–	–	+	+	+

[a]Based on the *nah* and *dox* operons (Simon et al. 1993; Denome et al. 1993).
[b]PCR products generated only with the plasmidic DNA preparation.
[c]+, positive amplification of the predicted DNA fragment; –, no amplification.
[d]Unexpected 4188-bp DNA fragment generated with FerG–Iso primers (see text).

conditions used to amplify large DNA fragment. The length of a PCR product, predicted by the *nah* operon and flanked by the FerG sequence and the *nahF* sequence recognized by the Iso primer, is 4188 nucleotides. This suggests that the gene order in this 4.2-kbp fragment is *nahAb-nahAc-nahAd-nahB-nahF*. Combinations of the Red–FerD, Red–Isp, and Red–DioD primers generated the predicted 1.4-, 3.6-, and 6.4-kbp DNA fragments, respectively, with *Sphingomonas* sp. strain 107, strain PL1, and *P. aeruginosa* 57. No amplification was observed with these primers on *P. putida* 34 and *P. fluorescens* 62. This suggests that in the former three strains the gene order would be *nahAa-nahAb-nahAc-nahAd-nahB-nahF-nahG*. In *P. putida* and *P. fluorescens* the reductase gene might be missing in the operon or the reductase sequence might be different. All these results suggest that several PAH-degradation genes are present in all the studied strains and are arranged in a polycistronic operon. Our results also predict that the gene order in the five strains should be similar to the *pah*, *dox*, and *nah* operons.

Sequence homology

As mentioned before, several PCR products were cloned and their extremities were sequenced. Compared with the corresponding genes in the *pah*, *nah*, and *dox* operons, nucleotide sequences were 90–95% identical, and the deduced amino acid sequences were 93–100% identical (Fig. 4). Most of the differences were punctual and scattered through the sequences.

Discussion

Efficiency of PAH degradation of five bacterial strains isolated from contaminated soil were compared with each other. The most efficient strain in terms of (*i*) substrate specificity, (*ii*) rapidity of forming clearing zones around colonies when sprayed by PAH solutions, and (*iii*) bacterial growth in liquid medium with different PAHs as sole source of carbon and energy was *Sphingomonas* sp. strain 107, followed by the strain PL1 and *P. aeruginosa* 57. The least efficient strains were the *P. putida* 34 and *P. fluorescens* 62.

Fig. 4. Amino acid sequence analysis. Several PCR products were cloned and one or both extremities were sequenced. Amino acid sequence was deduced and compared with the *pah*, *dox*, and *nah* operons. Only the amino acid changes are shown compared with the *dox* or *nah* operon. The numbers refer to the amino acid sequence location in the *nah* or the *pah* operons (Simon et al. 1993; Denome et al. 1993). Dots represent identity. Empty spaces indicate that the sequence was not done. Underlined sequences in the *nahAb* gene represent the location of the iron–sulfur centres. NAH, *nah* operon of *P. putida* G7 (GenBank accession No. M83949). PAH, *pah* operon of *P. putida* OUS82 (GenBank accession No. D16629); DOX, *dox* operon of *Pseudomonas* sp. strain C18 (GenBank accession No. M60405). SPH, *Sphingomonas* sp. strain 107; AER, *P. aeruginosa* 57; PL1, strain PLi; FLU, *P. fluorescens* 62; PUT, *P. putida* 34.

```
nahAa 1
  NAH MELLIQPNNR LISFSPGANL LEVLRENGVA ISYSCMSGRC GTCRCRVTDG SVIDSGAGSG
  PAH .K........ .......... .......... .......... .......... ......T...
  SPH .......... .......... .......... .......... .......... ......T...
  AER .......... .......... .......... .......... .......... ......T...
  PL1 .......... .......... .......... .......... .......... ......T...
                                                          106
  NAH LPNLVDEHYV LACQSVLTHN CAIEIPETDE IVTHPARIIK GTVVAV
  PAH ..H....... ...R....N. .......A.. .......... .....I
  SPH ..H....... ...R....N. .......A.. .......... .....I
  AER ..H....... .P.R....N. .......A.. .......... .....T
  PL1 ..H....... ...R....N. .......A.. .......... .....I

nahAb 3
  DOX VKWIEAVALS DILEGDVLGV TVEGKELALY EVEGEIYATD NLCTHGSARM
  NAH E......... ..P....... .......... .......... ......A...
  PAH E.........C ..P....... .....D.... .......... D.....A...
  SPH F.........Y E.P....... ....R..... .......... ......A...
  AER F.........C G.P....... .....D.... .......... D.....A...
  PL1 E.........C .NP.....A. .....D.... .......... D.....A...
  FLU F.........Y E.P....... .......... .......... ......A...
  PUT     ....... .......... .......... .......... ..........
                                                        98
  DOX SDGYLEGREI ECPLHQGRFD VCTGKPL1AP VTQNIKTYPV KIENLRVM
  NAH .......... .......... ....R..... ..E.....A. ........
  PAH .......... .......... ...SR..... ..E.....A. ........
  SPH .H........ G......... ....R..... .......... .VDGH...
  AER .......... .......... ....R..... ..E.....A. ........
  PL1 .......... .......... ....R..... ..E.....A. .....L..
  FLU ...F...... .......... ....R..... .......... ...GQ...
  PUT .......... .......... .......... .......... .....

nahAd 57
  DOX VGSEVQYQVI SRELRAASER RYKLNEAMNV YNENFQQLKV RVEHQLDPQN WGNSPKLRFT RFITNVQAAM
  NAH .......... .......... .......... .......... .I........ .S........ .........R
  PAH .......... .......... .......... CD........ .......S.. .S........ .........I
  SPH .......... ....G..... .......... .......... .......S.. .S........ ..........
  AER                                                                   .. ..........
                                                                              193
  DOX DVNDKELLHI RSNVILHRAR RGNQVDVFYAAREDKWKRGE GGVRKLVQRF VDYPERILQT HNLMVFL
  NAH ..D.E..... .......... ..................... .......... .......... .....
  PAH EL..ED...V ....V..... ..................... .......... .......... .....
  SPH ....ED...V ....V..... ..................... .......... .......... .....
  AER ....ED...V ....V..... ..................... .......... .......F.. ..

nahF  198
  DOX LNYLNSSPDR SPEIADALIS AKEIRRINFT GSTRVGSIIA QKAAQHLKRC LLELGGKSPL
  PAH .......... .......... .......... .......... .......... ..........
  SPH                      .......... .......... .......... ..........
  PL1 .......... .......... .......... .......... .......... ..........
  FLU                                              . .......... ..........
                                                                          323
  DOX IVLDDADIDA AVKAAVFGSF LFQGQICMST ERLIVDEKIA DEFVAKFVEK TKRLSAGDPC VTGDCI
  PAH ......N... .......... ......
  SPH .......... .......... .......... ...V...... .......A.. .......... .....F
  PL1 .......... .......... .......... ...V...... .......A.. .......... .....F
  FLU .......... .......... .......... ...V...... .......A.. .......... .....F

nahC  40
  DOX EKDRFYLRMD YWHHRIVVHH NGQDDLEYLG WRVAGKPEFE ALGQKLIDAG YKIRICDKVE
  NAH .......... .......... SAE....... .......... .......... ....V.....
  SPH .......... .......... G.E....... .......... .......... ....V.....
                                                                    161
  DOX AQERMVLGLM KTEDPGGNPT EIFWGPRIDM SNPFHPGRPL HGKFVTGDQG LGHCIVRQTD VA
  NAH .......... .......... .......... .......... .......... .......... ..
  SPH .......... .......... .......... .......... .......... .......... ..
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Addition of salicylate in solid medium has accelerated the formation of clearing zones by *Sphingomonas* sp. strain 107 and *P. aeruginosa* 57. It has also accelerated the transformation of indole to indigo. No such effects were observed with the three other strains. However, salicylate did not broaden the range of PAH specificity for *Sphingomonas* sp. strain 107 and *P. aeruginosa* 57. This is the first report that shows the effect of salicylate on PAHs other than naphthalene. Salicylate is known to be the inducer of the expression of genes encoding the upper and lower pathway of the naphthalene catabolism in the NAH7 plasmid (Schell 1985). Our results suggest that gene regulation via salicylate might occur in *Sphingomonas* sp. strain 107 and *P. aeruginosa* 57. Ogunseitan and Olson (1993) have shown that addition of 50 μg salicylate/g of soil contaminated with naphthalene increased the naphthalene mineralization. Sanseverino et al. (1993*b*) have shown that the addition of salicylate in four contaminated soils during a slurry treatment increased the *nahA* mRNA levels in only one soil. However, they suggest that this transcript was already induced in the other soils because of high concentration of naphthalene.

The addition of synthetic or biologically produced surfactants has been shown to enhance PAH biodegradation (Tiehm 1994; Hunt et al. 1994). *Sphingomonas* sp. strain 107 secretes a bioemulsifier, whereas *P. aeruginosa* 57 and *P. putida* 34 produce extracellular surface-active agents. The ability to produce biosurfactants is often linked to the utilization of hydrophobic substrates (Hommel 1990). The capacity of forming clearing zones around the PAH-degrading bacterial colonies sprayed with PAHs may be caused by the production of biosurfactants (Burd and Ward 1996). In our study, clearing zones were generated whether the bacterial strains were producing surfactants or not. Moreover, we observed a correlation between the appearance of clearing zones with a specific PAH and the bacterial growth on liquid medium with this PAH as sole source of carbon and energy. The only exception was strain PL1 with pyrene, benz[*a*]anthracene, and fluoranthene. Finally, there is apparently no correlation between production of biosurfactants and the efficiency of PAH degradation by a bacterial strain as shown with *P. putida* 34.

A fivefold increase of pyrene degradation by *Sphingomonas* sp. strain 107 cultures occurred with the addition of a synthetic surfactant (Tween 80) and salicylate in the BH liquid medium. One explanation of this increase is that the addition of a surfactant would have solubilized more pyrene in solution making it available more rapidly for microbial degradation, and the addition of salicylate would have stimulated the expression of PAH-degradation genes. However, salicylate and Tween 80 could have been used as carbon sources, resulting in an higher cell concentration for the degradation of pyrene.

Hybridization with a *nahAb*-related probe and PCR with primers specific for PAH-degradation genes revealed that each strain contained several of these genes. Cloning and sequencing of PCR products showed high homology between PAH-degradation gene sequences found in the five selected strains and the respective gene sequences in the *nah*, *pah*, and *dox* operons. It is interesting to notice that although strain PL1 did not transform indole to indigo, genes encoding the naphthalene dioxygenase were present and parts of these genes that we sequenced are highly conserved. Indole might not be a substrate for the naphthalene dioxygenase in strain PL1 or might not enter into the cell. Results obtained by PCR suggest that PAH-degradation genes are arranged in a polycistronic operon in each strain. These same results predict that these genes are ordered like the *pah*, *nah*, and *dox* operons.

PAH-degradation genes were found on a plasmid in *Sphingomonas* sp. strain 107 and on extrachromosomal DNAs in strain PL1. No plasmids were found in *P. aeruginosa* 57, *P. putida* 34, and *P. fluorescens* 62. This study is the second one to show a PAH catabolic function on a plasmid in the *Sphingomonas* genus. Stillwell et al. (1995) have shown that *Sphingomonas* sp. strain F199 has a 180-kbp plasmid, pNL1, implicated in the degradation of PAHs. Also, no previous study showed gene arrangement and sequences of PAH-degradation genes from *P. aeruginosa*. This is the second study on PAH-degradation genes in nonpseudomonads. Although identification tests were inconclusive in determining the species with certainty, strain PL1 did not show any characteristics of pseudomonads. The only study on PAH-degradation genes in nonpseudomonads was with three *Comamonas testosteroni* strains capable to degrade phenanthrene, naphthalene, and anthracene. Using the whole *nah* operon as a probe, Goyal and Zylstra (1996) did not observe any hybridization signal with total DNA from these three strains. They also cloned the chromosomal region responsible for naphthalene and phenanthrene degradation from one of these strains and showed that the gene order was different than the *nah* operon. Moreover, this region was present in one of the other strains but absent in the third one, suggesting that two different sets of genes for the degradation of phenanthrene exist among the three *C. testosteroni* strains.

In our study, we showed that pseudomonad PAH-degradation genes can be found in other bacterial genera, and are highly conserved in their arrangement and their sequences. However, as these genes are located on a plasmid, *Sphingomonas* sp. strain 107 and strain PL1 could have obtained them by conjugation with pseudomonads.

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