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Transposon and spontaneous deletion mutants of plasmid-borne genes encoding polycyclic aromatic hydrocarbon degradation by a strain of *Pseudomonas fluorescens*

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Abstract

Pseudomonas fluorescens strain LP6a, isolated from petroleum condensate-contaminated soil, utilizes the polycyclic aromatic hydrocarbons (PAHs) naphthalene, phenanthrene, anthracene and 2-methylnaphthalene as sole carbon and energy sources. The isolate also co-metabolically transforms a suite of PAHs and heterocycles including fluorene, biphenyl, acenaphthene, 1-methylnaphthalene, indole, benzothiophene, dibenzothiophene and dibenzofuran, producing a variety of oxidized metabolites. A 63 kb plasmid (pLP6a) carries genes encoding enzymes necessary for the PAH-degrading phenotype of *P. fluorescens* LP6a. This plasmid hybridizes to the classical naphthalene degradative plasmids NAH7 and pWW60, but has different restriction endonuclease patterns. In contrast, plasmid pLP6a failed to hybridize to plasmids isolated from several phenanthrene-utilizing strains which cannot utilize naphthalene. Plasmid pLP6a exhibits reproducible spontaneous deletions of a 38 kb region containing the degradative genes. Two gene clusters corresponding to the archetypal naphthalene degradation upper and lower pathway operons, separated by a cryptic region of 18 kb, were defined by transposon mutagenesis. Gas chromatographic-mass spectrometric analysis of metabolites accumulated by selected transposon mutants indicates that the degradative enzymes encoded by genes on pLP6a have a broad substrate specificity permitting the oxidation of a suite of polycyclic aromatic and heterocyclic substrates.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are present in fossil fuels, their derivatives such as creosote, and in combustion products (Edwards 1983). Two- and three-ring PAHs (e.g. naphthalene and phenanthrene) are generally susceptible to biodegradation, and various microbes capable of growing on or co-metabolizing such compounds have been reported (Cerniglia 1992). Higher molecular weight PAHs and substituted PAHs are more resistant to biodegradation (Fedorak & Westlake 1981) and may persist in the environment (Cerniglia 1992), while certain PAHs are suspected of being carcinogens (Sutherland et al. 1995). For these reasons, the possibility of using bacteria to remove PAHs from contaminated environments is being examined, making it necessary to understand the fundamentals of bacterial metabolism of PAHs. Two approaches are being considered for the biological removal of PAH compounds (Mueller et al. 1994): the stimulation of indigenous PAH-degrading flora, and the introduction of microorganisms with enhanced PAH-degrading capabilities. The latter option can be made attractive if we understand the biochemical, physiological and molecular biological characteristics of candidate PAH-degrading organisms.

Research over the past decade has focused primarily on the molecular biology and biochemistry of naphthalene biodegradation, and genes encoding enzymes for degradation of naphthalene have been identified in different *Pseudomonas* species. In several well-studied cases these genes are organized into two operons borne on isofunctional NAH plasmids, comprising a related family of catabolic plasmids (Yen & Serdar 1988),

while in some strains the genes are chromosomal (Kiyohara et al. 1994). In contrast, the existence of genes encoding enzymes specific for non-naphthalene PAHs, such as phenanthrene, has been suggested (Kiyohara et al. 1983; Foght & Westlake 1991) and recently reported in *Comamonas testosteroni* GZ38A (Goyal & Zylstra 1996).

Naphthalene degradation proceeds via hydroxylation and ring cleavage through salicylate to central metabolic intermediates (Yen & Serdar 1988; Eaton & Chapman 1992). Some naphthalene-utilizing strains have been shown to possess broad-specificity 'upper pathway' enzymes permitting oxidation of alternate aromatic substrates such as phenanthrene and anthracene (Menn et al. 1993; Sanseverino et al. 1993; Kiyohara et al. 1994), dibenzothiophene (Denome et al. 1993) and fluorene (Yang et al. 1994) through pathways parallel to conversion of naphthalene to salicylate. Although different names have been assigned to two of these newly described gene clusters (i.e. *dox*, dibenzothiophene oxidation [Denome et al. 1993] and *pah*, PAH degradation [Kiyohara et al. 1994; Takizawa et al. 1994]), the genes are strikingly similar to previously described naphthalene degradation (*nah*) genes. They share sequence homology with *nah* genes and are organized into analogous upper and lower pathway operons. However, despite genetic similarities among these naphthalene utilizers, differences in aromatic substrate ranges have been reported (Yang et al. 1994).

The bacterium LP6a, identified as a member of the *Pseudomonas fluorescens* group, was isolated from soil contaminated with petroleum condensate (a hydrocarbon mixture that is gaseous in the reservoir but condenses into fluid when produced, with a gravity of $\geq 60^{\circ}$ API; Hunt 1979). This organism grows on naphthalene, phenanthrene and anthracene and cometabolically oxidizes a variety of PAHs and heterocycles. Because of this broad PAH oxidizing activity LP6a is an interesting strain for genetic and enzymatic study, as it could be used in the preparation of mixtures of PAH-degrading organisms for bioremediation purposes. It already has been included in a mixed culture inoculum used for testing the efficacy of commercial crude oil spill bioremediation products (Blenkinsopp et al. 1995).

The research presented here compares isolate *P. fluorescens* LP6a with two archetypal naphthalene-utilizing *Pseudomonas putida* strains and several other phenanthrene-utilizing species. Its catabolic plasmid, PAH substrate range, and the specificity of some of its PAH-oxidizing enzymes were examined using a cured strain, and transposon and spontaneous deletion mutants.

Materials and methods

Media and bacterial strains

Mineral Medium, B + NP liquid medium and BYP agar have been described (Foght & Westlake 1988). Mineral Medium was solidified with Purified or Noble Agar (Difco) at 15 g l^{-1}.

Isolate LP6a was purified (Foght & Westlake 1991) from a mixed population enrichment culture derived from petroleum condensate-contaminated soil due to its formation of colored metabolites from dibenzothiophene. The isolate now has been identified as a member of the *Pseudomonas fluorescens* group using standard taxonomic tests (Smibert & Krieg 1981).

Other bacterial strains used in this study (and their donors where applicable) include: *Escherichia coli* HB101(pGS9) (Selvaraj and Iyer, 1983; V.N. Iyer, Carleton University); *P. putida* NCIB 9816-3, carrying pWW60 and a smaller cryptic plasmid, pWW61 (Cane & Williams 1982; Yen & Serdar 1988); *P. putida* 1064(NAH7) (Yen & Gunsalus 1982) and *Pseudomonas alcaligenes* DM201(pC1) (Finnerty & Robinson 1986; W.R. Finnerty & M. Schell, University of Georgia); *Pseudomonas (Sphingomonas) paucimobilis* WW3 (Weissenfels et al. 1990; M. Beyer & J. Klein, DMT-Gesellschaft Für Forschung und Prüfung mbH, Germany); *Mycobacterium* sp. RJGII135 (Grosser et al. 1991; R.J. Grosser & J.R. Vestal, University of Cinicinnati); *Pseudomonas* spp. HL7b and D2, and unidentified strain HL4 (Foght & Westlake 1991).

Genetics

Plasmids were isolated as described previously (Foght & Westlake 1991) and chromosomal DNA was prepared by the method of Marmur (1961). Selective media containing antibiotics and (or) sole carbon sources were used to ensure maintenance of transposons and plasmids. Electrophoresis of DNA was carried out using standard methods (Sambrook et al., 1989) and restriction endonuclease digestions were performed according to manufacturers' instructions. A restriction map of a 38 kb region of plasmid pLP6a was constructed using *Hin*dIII, *Bam*H1, *Eco*R1 and

*Bgl*II enzymes. DNA :: DNA hybridization on Hybond N (Amersham) nylon membranes was performed as described previously (Foght & Westlake 1991) using non-radioactive labelling and detection (Boehringer, Mannheim).

Transposon *Tn5* on the suicide vector pGS9 (Selvaraj & Iyer 1983) was conjugally transferred from *E. coli* HB101 to *P. fluorescens* LP6a wild type (ampicillin resistant). Putative transconjugants were selected on BYP agar containing kanamycin (50 μg ml^{-1}) plus ampicillin (35 μg ml^{-1}) and subsequently screened for phenotypic changes. Transposon insertion points were determined by restriction endonuclease digestion of pLP6a :: *Tn5* plasmids from PAH phenotype negative mutants.

Spontaneous deletion mutants were generated by transferring isolate LP6a daily for seven days at 28 °C in liquid Mineral Medium plus 5 mM benzoic acid (Fisher). The last transfer was plated onto BYP agar and isolated colonies were screened for phenotypic changes by replica plating. A cured derivative of LP6a was isolated by incubating a wild type culture of LP6a in Tryptic Soy Broth (Difco) at 35 °C, subsequently screening for colonies on BYP which did not produce colored metabolites from dibenzothiophene.

Determination of wild type phenotypes

The ability of wild type bacterial isolates to utilize pure PAHs as sole carbon source was determined using both solid and liquid Mineral Medium. For solid medium, naphthalene (Sigma, > 99%) was provided as a vapor by supplying crystals in the lid of an inverted petri plate; salicylic acid (Aldrich, > 99%) was incorporated at 2.5 mM; phenanthrene (Aldrich, $\geq$ 98%), anthracene (Aldrich, > 99.9%), and 1- and 2- methylnaphthalene (Aldrich, > 98%) were provided as a surface layer applied either by spraying (Kiyohara et al. 1982) or spreading ethereal solutions. Petri plates were sealed with laboratory film and incubated in separate plastic bags at 30 °C up to two weeks (with substrate replenishment as needed) before scoring for growth. Inoculated substrate-free Mineral Medium plates were included in all cases as controls for comparison with test plates. Luxurious growth on a substrate was qualitatively scored as '++', growth significantly greater than that observed on the control was scored as '+', and growth equivalent to that on the control plate was scored '-'. Growth in liquid medium similarly was determined by monitoring optical density compared with a 'no substrate' control, and, in the case of alkyl-naphthalenes, was confirmed by measuring protein content in cell lysates using bicinchoninic acid (Pierce, Rockford, IL).

Co-metabolic oxidation of PAHs and heterocycles was screened by spraying colonies pre-grown on BYP agar plates with ethereal solutions of the non-growth substrates dibenzothiophene (Fluka, > 98%), fluorene (Aldrich, 98%), catechol (Aldrich, > 99%), dibenzofuran (Aldrich), biphenyl (Eastman), or indole (MCB). The plates were incubated in the dark up to 7 days and observed for production of characteristic colored metabolites and (or) clearing of the opaque substrate layer. Mass spectrometric analysis of co-metabolic products from fluorene, dibenzofuran, 1-methylnaphthalene, acenaphthene (Aldrich, 99%) and benzothiophene (Aldrich, 97%) was performed as described in the section following.

Determination of P. fluorescens *LP6a mutant phenotypes*

Growth of transposon, deletion, and cured mutants of *P. fluorescens* LP6a on aromatic substrates was determined as for wild type isolates, compared with controls lacking a carbon source.

Oxidation of aromatic substrates and production of pathway intermediates were screened initially by spraying induced mutant colonies with aromatic substrates and observing clearing of the substrate layer and (or) production of colored metabolites. In further qualitative tests to identify the metabolites accumulated by various mutants, cultures were grown overnight in Tryptic Soy Broth supplemented with 0.5 mM 2-aminobenzoate (BDH, $\geq$ 99%) plus kanamycin where appropriate, harvested, then washed and resuspended in 3 mM phosphate buffer (pH 8). Aromatic substrates were added as N,N-dimethylformamide (Sigma, > 99%) solutions to final concentrations of 5 mM (nominal) for dibenzothiophene or 0.25 mM for salicylaldehyde (Sigma) and salicylate, and incubated at 30 °C for 30 to 60 min and 24 h. Suspensions were clarified by centrifugation, then scanned on a Philips PU8740 spectrophotometer, blanked against phosphate buffer. Alternatively, transposon mutants 11 and 16 were used to produce dibenzothiophene-dihydrodiol and *trans*-4-[2-(3-hydroxy)-thianaphthenyl]-2-oxo-3-butenoic acid (*trans*-HTOB), respectively. Induced, washed cell suspensions of 'upper pathway' mutants were incubated with phosphate buffered solutions of these metabolites and their further oxidation was determined by spectrophotometric scans. Absorption max-

ima for substrates and metabolites were compared with literature values. Positive control cultures (wild type LP6a and *P. putida* strains carrying NAH7 and pWW60) and putative negative controls (a deletion mutant and cured LP6a) were included for reference. Dibenzothiophene was used as a surrogate substrate for the naphthalene catabolic genes because several of its products are colored, allowing visual assessment of substrate oxidation in addition to spectrophotometric analysis. The use of dibenzothiophene as a surrogate was justified by results indicating the broad substrate specificity of the PAH catabolic enzymes in LP6a (see Results and discussion).

Naphthalene-1,2-dioxygenase (NahA) activity was assessed by spraying colonies pre-grown on PCA plus 0.5 mM 2-aminobenzoate with indole and observing its oxidation to indigo (Ensley et al., 1983); lack of NahA activity resulted in colonies without a blue color after 24 to 48 hours incubation. NahA activity was also assessed by mass spectroscopic analysis (see below) of extracted metabolites from selected mutants. 1,2-Dihydroxy-1,2-dihydronaphthalene dehydrogenase (NahB) activity in mutants lacking 1,2-dihydroxynaphthalene dioxygenase (NahC) activity was assessed in medium containing naphthalene by observing the accumulation of naphthoquinone, a dark brown auto-oxidation product rapidly formed from 1,2-dihydroxynaphthalene (Takizawa et al. 1994). Loss of NahB activity was assessed in washed, induced cell suspensions by incubating mutants either with the alternate substrate dibenzothiophene, detecting accumulation of the dihydrodiol with absorption maxima at 315 nm and 305 nm (Laborde & Gibson 1977), or by supplying the pathway intermediate dibenzothiophene-dihydrodiol and determining its persistence or disappearance with incubation. Sequential ring cleavage activities (NahC, NahD, NahE) in induced washed cell suspensions were inferred by spectrophotometric detection of the dibenzothiophene ring cleavage products *trans*-HTOB at 475 nm, and the final product 3-hydroxy-2-formylbenzothiophene (HFBT) at 390 nm (Laborde & Gibson 1977), from either dibenzothiophene or its dihydrodiol. NahE activity was assayed by supplying the analogous dibenzothiophene *trans*-ring cleavage product (475 nm) and screening for its transformation to the final product (390 nm). Salicylaldehyde dehydrogenase (NahF) and salicylate hydroxylase (NahG) activities were determined spectrophotometrically in induced washed cell suspensions by disappearance of substrates (salicylaldehyde at 256/326 nm or salicylate at 296 nm) and transient appearance of pathway metabolites (salicylate or 2-hydroxymuconic semialdehyde at 375 nm). Catechol-2,3-oxygenase (NahH) activity was apparent in the immediate production of the bright yellow product 2-hydroxymuconic semialdehyde when pre-grown colonies were sprayed with catechol. Further metabolism of the hydroxymuconic semialdehyde was inferred when the yellow color faded with overnight incubation, whereas mutants lacking further metabolism retained the bright yellow color against a dark background of auto-oxidized catechol.

The substrate specificity of selected aromatic-oxidizing enzymes encoded on plasmid pLP6a was examined by identifying PAH metabolites using gas chromatography – mass spectrometry (GC-MS). pLP6a :: *Tn5* mutants pre-grown overnight at 30 °C in 200 ml of TSB with kanamycin were induced with 0.5 mM salicylate (200 μl of an ethanolic solution) for 3 hours. The cells were harvested aseptically and resuspended in 200 ml of 3 mM phosphate buffer (pH 8). Substrates were provided by adding 100 μL of a solution or suspension in N,N-dimethylformamide to a nominal final concentration of 5 mM. Substrate-free controls received only N,N-dimethylformamide. After 24 h incubation to allow accumulation of pathway intermediates, the suspension was clarified by filtration through glass wool and centrifugation. Pellets were retained in certain cases for analysis of cell lysates. The supernatants were extracted either at neutral pH with anhydrous diethyl ether for isolation of putative dihydrodiols, or were acidified to pH $\leq$ 2 with 4 N H_2SO_4 and extracted with pesticide-grade dichloromethane for isolation of acidic metabolites. Extracts were dried with anhydrous sodium sulfate, concentrated under nitrogen to approximately 200 μl and stored in solvent-washed glass vials with Teflon-lined caps. To chemically dehydrate putative PAH-dihydrodiol metabolites, subsamples of neutral pH extracts were evaporated to dryness under nitrogen and re-dissolved in 1.5 ml 5 N HCl (Jerina et al. 1976). This solution was heated in a sandbath at 100 °C for 8 minutes under a stream of nitrogen, then cooled to room temperature and re-extracted with ether. The extract was concentrated and stored as above.

Cell lysates were prepared from pellets by suspension in 3 mM phosphate buffer (pH 8) and physical breakage with zirconium beads in a Mini BeadBeater (Biospec Products, Bartlesville, OK). The resultant neutral lysates were immediately extracted with anhydrous ether, dried over Na_2SO_4 and stored as above.

Table 1. Growth of bacterial isolates on sole carbon sources; compilation of data from previous and current studies.

Bacterial Strain	Growth[1]			
	Naphthalene	Salicylic acid	Phenanthrene	Anthracene
Pseudomonas fluorescens LP6a (pLP6a)	++[2]	++	++[2]	++[2]
Pseudomonas putida 1064 (NAH7)	++[2]	++	-[2]	-
Pseudomonas putida NCIB 9816-3 (pWW60, pWW61)	++[2]	++	-[2]	-
Pseudomonas alcaligenes (pC1)	+	++	-	-
Pseudomonas sp. HL7b	-[3]	-[3]	+[2]	+
unidentified sp. HL4	-[2]	-	++[2]	++[2]
Pseudomonas sp. D2	-[2]	-	+[2]	+
Pseudomonas paucimobilis WW3	-[2]	-	++[2]	+[2]
Mycobacterium sp. RJGII135	-[2]	-	++[2]	++

[1] Growth determined on Mineral Medium agar as described in text: (++) good growth; (+) poor or slow growth; (-) no growth, compared with inoculated agar without added carbon source.
[2] Foght & Westlake 1991.
[3] Foght & Westlake 1990.

Ether and dichloromethane extracts were prepared for capillary gas chromatography with a flame ionization detector (GC-FID) by derivatization with N,O-*bis*(trimethylsilyl)-acetamide according to the manufacturer's instructions (Pierce, Rockford, IL). GC-FID was performed on a Model 5890 gas chromatograph (Hewlett-Packard) using a 30 m × 0.25 mm DB-5 column (J + W Scientific), with a temperature program of 90 °C for 4 min, increasing at 4 °C min^{-1} to 250 °C, then held 16 min. Electron impact GC-MS was performed as previously described (Fedorak & Westlake 1986).

Results and discussion

Comparison of wild type P. fluorescens *LP6a PAH phenotype with other aromatic-degrading isolates*

P. fluorescens LP6a was known to utilize both naphthalene and phenanthrene as sole carbon and energy source and to co-metabolize certain aromatic substrates (Foght & Westlake 1991). Bacteria with similar PAH phenotypes were selected for comparison with LP6a. Table 1 summarizes the ability of these strains to grow on naphthalene, its metabolite salicylate, and the tricyclic PAH analogues phenanthrene and anthracene. Isolate LP6a is capable of growing on all four substrates whereas the *P. putida* strains containing the catabolic plasmids NAH7 and pWW60 are limited to utilization of naphthalene and salicylate. Strain LP6a also grew on 2-methylnaphthalene (data not shown). This composite PAH-degradative phenotype of *P. fluorescens* LP6a is unusual in our collection of more than 70 aromatic hydrocarbon-degrading cultures since most of our other strains utilize either naphthalene or phenanthrene as a growth substrate but not both (Foght & Westlake 1991, and unpublished observations).

Strain LP6a possessed the ability to co-metabolize other PAHs and heterocycles when pre-grown with an alternate carbon source. Co-metabolic substrates included dibenzothiophene, biphenyl and indole (Foght & Westlake 1991) as well as fluorene, dibenzofuran, 1-methylnaphthalene, acenaphthene and benzothiophene, as demonstrated by production of characteristic colored metabolites and (or) by GC-MS analysis of culture supernatants (data not shown). The two *P. putida* strains listed in Table 1 were also capable of co-metabolizing dibenzothiophene, biphenyl and indole (Foght & Westlake 1991) as well as fluorene and dibenzofuran (data not shown). The remaining naphthalene non-utilizing strains listed in Table 1 were deficient in oxidizing one or more of the above-named co-metabolic substrates, although all but *P. alcaligenes* (pC1) still exhibited a relatively broad co-metabolic substrate range. Thus, *P. fluorescens* LP6a demonstrated growth and co-metabolic phenotypes more extensive than either the naphthalene-utilizing or the phenanthrene-utilizing reference strains shown in Table 1.

Hybridization of plasmid pLP6a

P. fluorescens LP6a harbors a single plasmid, pLP6a, estimated at 63 kb, as determined by restriction endonuclease digestion. This plasmid was found to

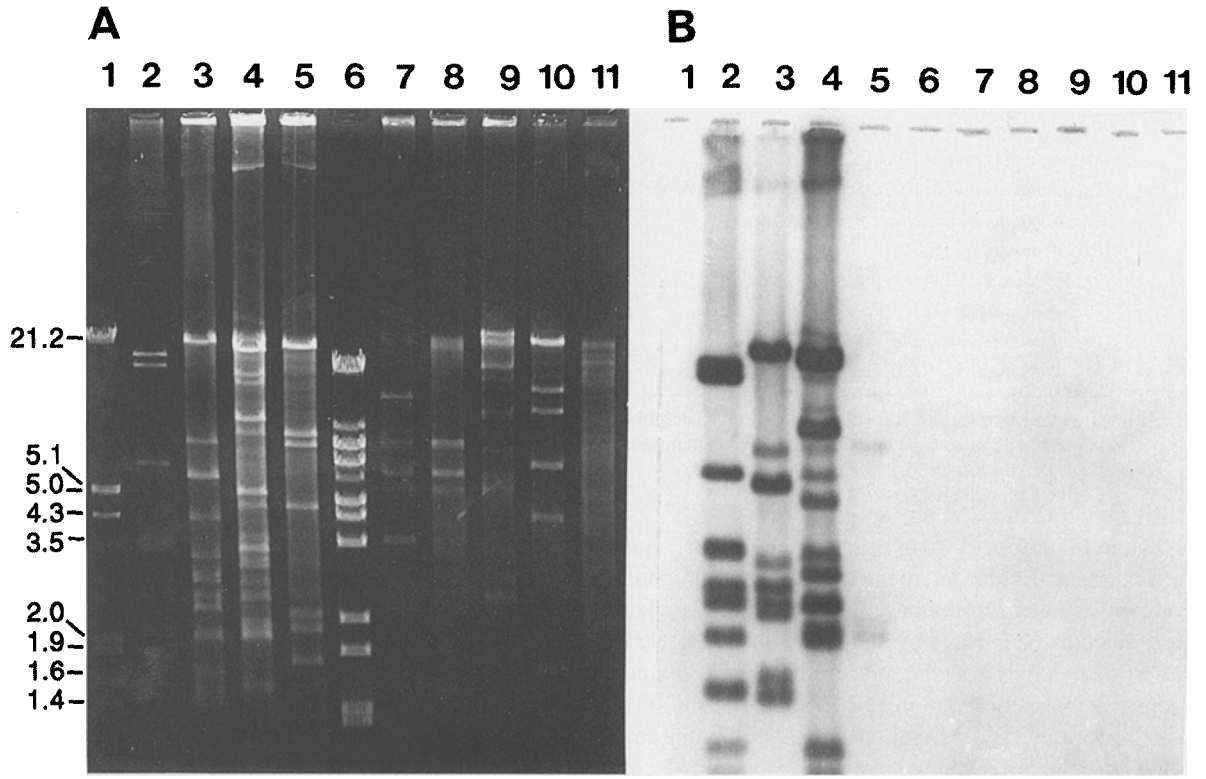

Figure 1. (*A*). Photograph of agarose gel showing *Eco*R1 restriction enzyme digests of various plasmid preparations. Lane 1, lambda *Hin*dIII/*Eco*R1 double digest (size of fragments in kb shown at left); lane 2, wild type pLP6a; lane 3, NAH7; lane 4, pWW60 (plus cryptic pWW61; Cane and Williams, 1982); lane 5, cosmid pC1; lane 6, lambda *Bst*EII digest; lane 7, *Pseudomonas* sp. HL7b; lane 8, unidentified species HL4; lane 9, *Pseudomonas* sp. D2; lane 10, *Pseudomonas* (*Sphingomonas*) *paucimobilis* WW3; lane 11, *Mycobacterium* sp. RJGII135. (*B*). Southern blot of gel shown in (A), after hybridization with purified pLP6a as probe.

encode genes for PAH degradation by strain LP6a. Figure 1A shows *Eco*R1 digestions of plasmid preparations for the PAH-degrading bacteria listed in Table 1. Plasmid pLP6a (lane 2) has an *Eco*R1 restriction pattern significantly different from the plasmids of the other aromatic-degrading bacteria, including the archetypal naphthalene-degradative plasmid NAH7 (lane 3). Digestion with *Bgl*II also revealed significant differences between pLP6a and NAH7.

Hybridization of CsCl-purified pLP6a to these fragments (Figure 1B) revealed extensive homology between pLP6a and the naphthalene-degradative plasmids NAH7 (lane 3) and pWW60 (lane 4; present with non-hybridizing cryptic plasmid pWW61). A reciprocal hybridization using purified NAH7 to probe *Eco*R1- and *Bgl*II-digested pLP6a corroborated this result. Whole pLP6a was used as a probe, rather than the isolated degradative genes, in order to maximize detection of any homology between the catabolic plasmids; in fact, a parallel digest probed with a spontaneous deletion plasmid lacking PAH catabolic genes (see deletion mutants, below) revealed that the non-catabolic region of pLP6a hybridized with many of the NAH2 and pWW60 fragments (data not shown). That is, hybridization between pLP6a and the archetypal NAH plasmids was not limited to their degradative regions.

In contrast, little hybridization was observed between pLP6a and cosmid pC1 (Figure 1B, lane 5; Finnerty & Robinson 1986) which harbors genes encoding broad-specificity naphthalene-degradative enzymes having little or no homology to pWW60 (Foght & Westlake 1990). No hybridization was observed between pLP6a and plasmid preparations from the other aromatic-degrading bacteria tested (Figure 1B, lanes 7 to 11) which grow on phenanthrene but not on naphthalene (Table 1).

The strains shown in Figure 1 and additional isolates previously had been grouped using genomic DNA hybridization under low stringency conditions: those dot-blots showed that LP6a hybridized only to strains in the NAH family (Foght & Westlake 1991). The current study confirms these observations and, further, demonstrates the extent of hybridization between the respective catabolic plasmids. Nevertheless, pLP6a is not identical to either NAH plasmid since its restriction profile is notably different.

It is significant that pLP6a did not hybridize with DNA from the five other PAH-degrading isolates, despite the fact that the strains co-metabolize similar compounds (e.g. dibenzothiophene, fluorene, biphenyl) and utilize phenanthrene. It is possible that the PAH-degradative genes are chromosomal in these latter five isolates, and therefore the plasmid digests shown in Figure 1 did not hybridize with pLP6a. However, since previous dot blots of total genomic DNA from these and other phenanthrene-degraders showed no hybridization to pLP6a or NAH plasmids (Foght & Westlake 1991), it seems more likely that the napththalene catabolic genes (including those borne on pLP6a) are significantly different from the degradative genes present in those phenanthrene degraders unable to utilize naphthalene. Goyal & Zylstra (1996) recently have demonstrated in *C. testosteroni* the presence of genes for the degradation of phenanthrene which do not hybridize to NAH7.

Spontaneous deletion mutants of pLP6a and cured LP6a

Plasmid pLP6a is implicated in PAH degradation by virtue of its extensive hybridization with the naphthalene catabolic plasmids (Figure 1B). Therefore, LP6a was mutagenized by generating spontaneous deletions in pLP6a and by curing the wild type of its plasmid. Serial transfer of *P. fluorescens* LP6a with benzoic acid as sole carbon source, analogous to curing of the TOL and NAH plasmids (Williams et al. 1988; Clarke & Laverack 1984), produced 12 PAH phenotype negative variants from several hundred colonies screened. None of these mutants was completely cured; instead, all had suffered identical spontaneous deletions within pLP6a (Figure 2), subsequently shown by restriction mapping to comprise a contiguous 38 kb region of the plasmid (Figure 3). This implied that the plasmid-borne degradative genes were located within a discrete 38 kb region which could reproducibly excise from pLP6a. Ten of the deletion mutants were pleiotrophically and profoundly PAH phenotype negative, having lost the ability to grow on PAHs or to oxidize indole and dibenzothiophene. Two deletion mutants demonstrated a weak PAH phenotype, growing very slowly on naphthalene or phenanthrene, and producing faint color from dibenzothiophene after prolonged incubation. These two mutants are the subjects of studies into the possible transposition of the degradative region into the LP6a chromosome, similar to the phenomenon described by Carney & Leary (1989).

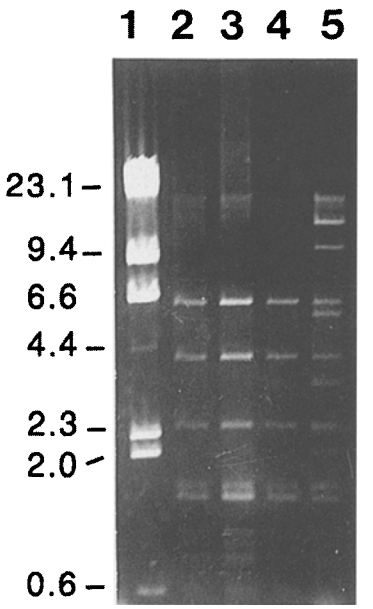

Figure 2. Photograph of agarose gel showing *Bgl*II restriction enzyme digest of plasmid preparations from wild type LP6a and three deletion mutants. Lane 1, lambda *Hin*dIII digest (size of fragments in kb shown at left); lanes 2–4, spontaneous pLP6a deletion mutants (lanes 2 and 3 are profoundly PAH phenotype negative mutants and lane 4 is a weak PAH phenotype positive mutant; see text); lane 5, wild type pLP6a. The uppermost band in the wild type lane is linearized but undigested plasmid DNA.

The degradative genes of the TOL plasmid pWW0 are contained within functional nested transposons (Tsuda & Iino 1988), whereas the catabolic genes of plasmid NAH7 are on a defective transposon (Tsuda & Iino 1990). Both these families of plasmids are known to spontaneously dissociate, producing phenotype negative deletion mutants, especially when grown on benzoic acid (Clarke & Laverack 1984; Williams et al. 1988). Similar observations of reproducible deletions in pLP6a also suggest the existence either of a functional catabolic transposon encompassing the 38 kb degradative region, or of regions containing significant homology (such as insertion sequences) which could

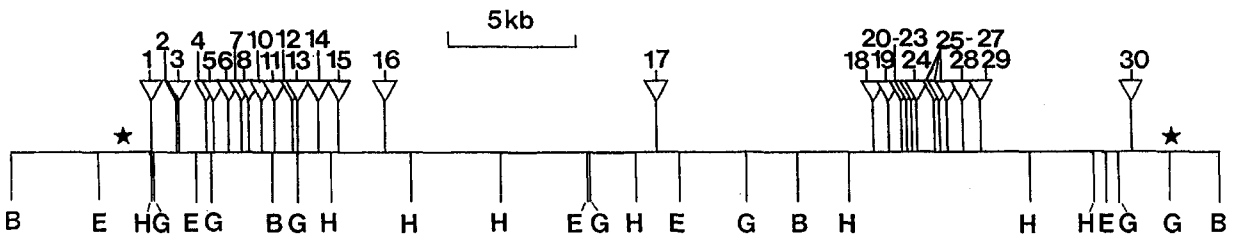

Figure 3. Restriction endonuclease digestion map of plasmid pLP6a degradative region and *Tn5* insertion points. The extent of the 38 kb catabolic region, as defined by deletion mutants, is indicated by asterisks (*). The 'upper' and 'lower pathway' clusters were defined by numbered *Tn5* insertion points, indicated by (▽). B, *Bam*H1; E, *Eco*R1; H, *Hin*dIII, G, *Bgl*II.

mediate homologous recombination events (Wyndham et al. 1994).

The stability and transferability of genes encoding PAH degradation is an important factor for microorganisms being considered as bioremediation agents. The observed instability of the degradative region of pLP6a suggests that genetic manipulation may be necessary to ensure maintenance of the degradative genes under non-selective conditions should the strain be considered for use as a bioremediation inoculant. We do not yet know whether either the plasmid pLP6a or its putative degradative cassette are conjugative.

Several unsuccessful attempts were made to completely cure isolate LP6a of its plasmid by repeated transfer in non-selective medium and by growth with sub-lethal concentrations of mitomycin-C. Two cured clones of *P. fluorescens* LP6a were eventually isolated by repeated transfer in rich medium at the sub-lethal temperature of 35 °C. These mutants were pleiotrophically PAH phenotype negative. The difficulty of completely curing LP6a of its plasmid suggests either that pLP6a has a very efficient partitioning system, or that there is an essential gene present in the non-degradative, cryptic region of the plasmid. This stable maintenance of the plasmid contrasts with the plastic nature of the degradative region, discussed above.

Transposon mutants

Fifty-nine *Tn5* insertion mutants were confirmed among 2,450 transconjugants screened for changes in PAH growth or oxidation phenotypes. In addition to these phenotypic mutants, four control mutants retaining the wild type PAH phenotype were also selected, having transposon insertions outside the catabolic genes. Plasmids isolated from phenotypic mutants were digested and probed with pGS9 to map *Tn5* insertion points. Approximately half of the *Tn5* insertions mapped at sites within the 38 kb degradative region of pLP6a. Twenty-nine of these sites are shown in Figure 3. The remaining transposon mutants were shown to have chromosomal *Tn5* insertions. These mutants were unable to grow on naphthalene and phenanthrene yet retained the ability to co-metabolically oxidize the non-growth substrate dibenzothiophene. They are being examined for chromosomally encoded functions associated *in trans* with PAH oxidation.

A restriction map of pLP6a :: *Tn5* insertions (Figure 3) shows that the transposon mutations map within the same 38 kb degradative region as that defined by the spontaneous deletion mutants. The 25 kb portion of pLP6a lying outside the degradative region remains cryptic.

Phenotypes of the *Tn5* mutants were determined to infer the gross PAH catabolic gene arrangement on the plasmid. Mutations in naphthalene 'upper pathway' functions, such as inability to grow on naphthalene or loss of naphthalene dioxygenase activity, clustered in one region comprising mutants 1 to 16, leaving salicylate utilization and catechol-2,3-oxygenase activity intact. Insertions causing loss of ability to utilize salicylate clustered in a second 'lower pathway' region comprising mutations 18 to 29, separated from the other cluster by a cryptic region of approximately 18 kb. This gross arrangement is similar to that reported for the 27 kb degradative region of NAH7 (Yen & Serdar 1988), although the region separating the operons is only approximately 5 kb in NAH7. In contrast, Yang et al. (1994) reported that the lower pathway genes on the plasmid from *P. putida* NCIB9816 are more than 38 kb distant from the upper pathway genes.

The general order of 'upper pathway' genes on pLP6a can be inferred from the sequential production or accumulation of pathway intermediates by

polar transposon mutations. For example, pLP6a :: *Tn5* mutants 1 through 8 were unable to effect initial oxidation of PAH substrates and did not produce indigo from indole; they likely have insertions in one of the naphthalene-1,2-dioxygenase genes (i.e. *nahAa, Ab, Ac* or *Ad* by analogy to the NAH7 upper pathway operon; Simon et al. 1993; Eaton & Chapman 1992). Mutants 10, 11 and 12 have naphthalene dioxygenase (NahA) activity but lack the subsequent 1,2-dihydroxy-1,2-dihydronaphthalene dehydrogenase (NahB) since they accumulate the dihydrodiols of naphthalene, dibenzothiophene and other substrates (see also GC-MS results, below). Mutants 13, 14 and 15 appear to have mutations affecting NahC because they accumulate naphthoquinones (spontaneous oxidation products of 1,2-dihydroxynaphthalene) when incubated with naphthalene on solid medium, and by inference then must possess NahA and NahB activity. Mutant 16 must possess NahA, NahB, NahC and NahD activity because it produces the ring cleavage intermediate *trans*-HTOB from dibenzothiophene, but apparently lacks the pyruvate-cleaving function (*cis-o*-hydroxybenzylidenepyruvate hydratase-aldolase, NahE), since it does not accumulate the final product HFBT from dibenzothiophene. No mutations in 2-hydroxychromene-2-carboxylate isomerase (NahD) or salicylaldehyde dehydrogenase (NahF) were identified using spectrophotometric and growth assays, nor did we isolate any putative promoter mutations, since all observed enzyme activities were inducible by 2-aminobenzoate.

Although *Tn5* insertion commonly results in polar mutations within operons, *Tn5* can also stimulate transcription at sites distal to the insertion due to outward-reading promoters near its termini, or may permit transcription of operon genes having internal promoters (Berg & Berg 1987). The activity of pathway enzymes not interrupted by the transposon can be inferred, with some caution, by incubating the 'upper pathway' mutants with analogues of naphthalene pathway intermediates (dibenzothiophene-dihydrodiol and *trans*-HTOB, produced by mutants 11 and 16, respectively). Putative *nahA* mutants 1 through 8 were able to transform dibenzothiophene-dihydrodiol to HFBT, indicating that they still possessed NahB, C, D, and E activity. Mutants 10, 11 and 12 were unable to further transform the dibenzothiophene-dihydrodiol, confirming their mutation in *nahB*, but yielded HFBT when incubated with *trans*-HTOB, demonstrating NahE activity. NahC and NahD activity could not be inferred for these mutants; they were not incubated with 1,2-dihydroxynaphthalene because of that intermediate's rapid auto-oxidation to naphthoquinone in aqueous solution, and we could not produce dibenzothiophene-diol which presumably undergoes similar oxidation. Mutants 13, 14 and 15 were found to possess NahB activity, since they were able to further oxidize dibenzothiophene-dihydrodiol. Unexpectedly, low levels of HFBT were observed after extended incubation of these mutants with dibenzothiophene-dihydrodiol; the expected result was accumulation of quinones as observed with naphthalene, due to *nahC* mutation. Therefore, NahC activity has been expressed as '±' in Table 2 for these mutants, which may be 'leaky', and require further study. Mutant 16 transformed the dibenzothiophene-dihydrodiol to *trans*-HTOB but not to HFBT, and was unable to further transform *trans*-HTOB when incubated with that metabolite for up to 24 hours, indicating that it lacked NahE activity. These inferred enzyme activity results are summarized in Table 2. As expected, control Mutant 17 and wild type LP6a possessed all enzyme activities tested, while the deletion mutant and cured strain of LP6a lacked all enzyme activities tested.

The gene order of the upper pathway encoded by NAH7 has been shown to be: promoter, *nahA, nahB, nahF, nahC, nahE, nahD* (Eaton & Chapman 1992). Similarly, for pLP6a the gene order is postulated to be *nahA, nahB, nahC* and *nahE*, based on inferred enzyme activities in Table 2. However, since no mutations in *nahD* or *nahF* were included in our suite of *Tn5* mutants, we have not positioned these genes in the catabolic gene cluster, and resolution of the gene order awaits sequence analysis.

All 'lower pathway' mutants were isolated on the basis of their inability to grow on salicylate. However, only two mutants (19 and 22) showed decreased salicylate hydroxylase (NahG) activity, and none were completely lacking salicylate hydroxylase. All lower pathway mutants possessed catechol-2,3-oxygenase (NahH) activity. In fact, we were unable to detect any colonies lacking this activity despite screening more than 2,000 *Tn5* mutants, and only spontaneous deletion and cured mutants failed to oxidize catechol to 2-hydroxymuconic semialdehyde. It is possible that multiple copies of a catechol-2,3-oxygenase gene or that genes for isofunctional catechol *meta*-cleavage enzymes exist within the pLP6a catabolic region. Mutants 27, 28 and 29 were defective in 2-hydroxymuconic semialdehyde metabolism and accumulated that intermediate from salicylate and from catechol. No evidence was seen of gentisate accumulation

Table 2. Phenotypes of pLP6a :: *Tn5* upper pathway mutants, wild type, profoundly PAH phenotype negative spontaneous deletion mutant of pLP6a, and completely cured variant of *P. fluorescens* LP6a.

	pLP6a :: *Tn5* mutants[1]																pLP6a	pLP6a	cured
	1	2	3	4	5	6	7	8	10	11	12	13	14	15	16	17	wildtype	deletion	LP6a
GROWTH PHENOTYPES[2]																			
Naphthalene	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
Salicylate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
INFERRED ENZYME ACTIVITIES[3]																			
naphthalene-1,2-dioxygenase (NahA)	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	-	-
1,2-dihydroxy-1,2-dihydronaphthalene dehydrogenase (NahB)	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	-	-
1,2-dihydroxynaphthalene dioxygenase (NahC)	+	+	+	+	+	+	+	+	nd	nd	nd	±	±	±	+	+	+	-	-
2-hydroxychromene-2-carboxylate isomerase (NahD)	+	+	+	+	+	+	+	+	nd	nd	nd	+	+	+	+	+	+	-	-
trans-o-hydroxybenzylidenepyruvate hydratase-aldolase (Nah E)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-

[1] see Figure 3 for positions.
[2] +, growth; -, no growth with substrate provided as sole carbon and energy source.
[3] see text; +, activity; -, no activity, ±, weak or slow activity observed with prolonged incubation (24 hours); nd, not determined.

from any mutant, nor was wild type LP6a able to grow on gentisate as sole carbon source, indicating that an alternative pathway from salicylate through gentisate (Yen & Serdar 1988) does not exist in *P. fluorescens* LP6a.

Identification of pathway intermediates and speculation on enzyme specificity

In theory, the observed co-metabolic substrate range of *P. fluorescens* LP6a could result either from the presence of a few oxygenases of broad specificity, such as have been reported for other pseudomonads, or a number of oxygenases with narrow specificities. Two pLP6a :: *Tn5* mutants were selected for preliminary GC-MS analysis to distinguish between these possibilities. Mutant 11 could not grow on naphthalene or phenanthrene yet possessed naphthalene dioxygenase activity (i.e., produced indigo from indole; Table 2) suggesting blockage at an early step in the degradative pathway. Mutant 17, with *Tn5* inserted in the cryptic region between the catabolic gene clusters of pLP6a, is phenotypically equivalent to the wild type and was used as a positive control.

Cell suspensions of the two mutants accumulated colorless metabolites in the culture medium when incubated with naphthalene and phenanthrene. The metabolites were extracted, derivatized by trimethylsilylation, and subjected to GC-FID and GC-MS analysis. Unidentified common minor contaminant peaks were noted in chromatograms of all derivatized ether extracts, including abiotic reagent controls. GC-FID analysis (Figure 4) revealed that control Mutant 17 did not accumulate substantial amounts of products from either naphthalene or phenanthrene, presumably because of complete metabolism of these substrates. In phenanthrene cultures, Mutant 17 accumulated very low levels of a metabolite identified by GC-MS as hydroxynaphthoic acid (Peak 4) by comparison with derivatized authentic standard. In contrast, Mutant 11 culture supernatants accumulated a single predominant metabolite from naphthalene (Peak 1) and two metabolites (Peaks 2 and 3) from phenanthrene. The naphthalene metabolite was tentatively identified, in the absence of an authentic standard, as 1,2-dihydroxy-1,2-dihydronaphthalene based on the following evidence: (i) appropriate molecular ion and mass spectrum from GC-MS analysis of trimethylsilyl (TMS)-derivatized extract (retention time 22:28 with major ions at *m/z* 306 [M], 291 [M-CH_3], 275 [M-2(CH_3)], 217 [M-TMS-O] and 203 [M-TMS-2(CH_3)]; (ii) disappearance of the single GC-FID peak in a chemically dehydrated neutral ether extract (followed by TMS derivatization); with (iii) subsequent appearance of a disproportionate profile of two GC-FID peaks which co-chromatographed with authentic 1-hydroxy- and 2-hydroxynaphthalene. These observations are consistent with the presence of a naphthalene dihydrodiol

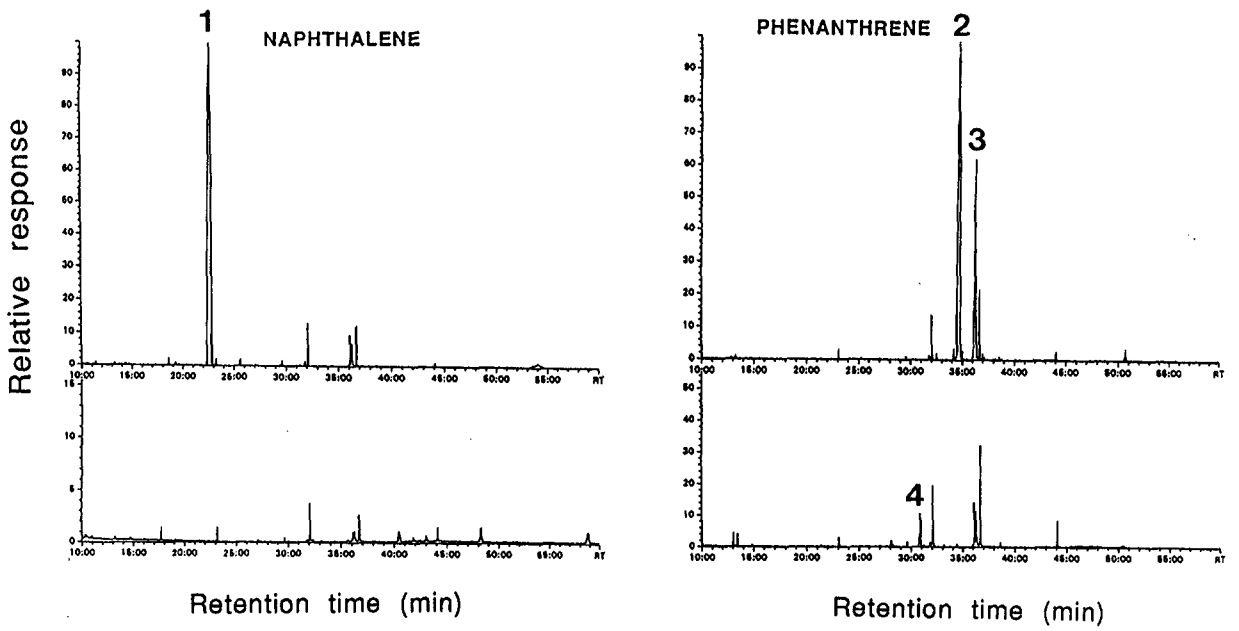

Figure 4. Gas chromatograms (GC-FID) of neutral ether extracts of culture supernatants from transposon mutants incubated with naphthalene or phenanthrene, TMS-derivatized. Upper panels, Mutant 11; Lower panels, control Mutant 17. Peaks tentatively identified by GC-MS as: 1, 1,2-dihydroxy-1,2-dihydronaphthalene; 2 and 3, phenanthrene dihydrodiol isomers; 4, hydroxynaphthoic acid.

which dehydrates to preferentially form specific monohydroxy isomers (Jerina et al. 1976).

By analogy, two phenanthrene metabolites were identified as phenanthrene dihydrodiols by GC-MS analysis (Peak 2 retention time 34:45 with major ions at *m/z* 356 [M], 341 [M-CH_3], 266 [M-TMS-O-H], 253 [M-TMS-2(CH_3)] and 178 [M-2(TMS)]; Peak 3 retention time 36:18 with the same major ions as Peak 2) and subsequent chemical dehydration to putative phenanthrols, but the isomeric positions could not be determined because neither the authentic dihydrodiols nor the corresponding phenanthrols were commercially available. Presumably the major phenanthrene metabolite is 3,4-dihydroxy-3,4-dihydrophenanthrene (Evans et al. 1965) while the lesser peak is another isomer, possibly the 1,2-dihydrodiol observed by Jerina et al. (1976). Extracts of cell lysates contained the same metabolites as were observed in the cell-free culture supernatant.

In addition to transformation of naphthalene and phenanthrene, Mutant 11 also accumulated metabolites from anthracene, acenaphthene, biphenyl, benzothiophene and dibenzothiophene, which were tentatively identified as the respective dihydrodiol isomers on the basis of GC-MS analysis (data not shown). Interruption of the degradation of these structurally related aromatic substrates by a single transposon mutation implies that the naphthalene dioxygenase of LP6a has broad substrate specificy. This is similar to reports of naphthalene-degradative enzymes in several *Pseudomonas* species which transform alternate substrates such as dibenzothiophene (Denome et al. 1993), phenanthrene (Sanseverino et al. 1993), and fluorene (Yang et al. 1994), and suggests that the phenomenon of broad-specificity PAH-degradative enzymes is common in that genus.

GC-MS analysis of metabolites accumulated by Mutant 22 incubated with 2-methylnaphthalene suggests that not only the first enzymes of the pathway have broad substrate specificity. After dichloromethane extraction of acidified, clarified culture supernatant and TMS-derivatization, three major peaks were observed by gas chromatography (Figure 5), plus small common contaminant peaks. The major peaks were identified by their mass spectra as: Peak 1, residual substrate 2-methylnaphthalene, with retention time 9:19 and *m/z* 142 (M); Peak 2, TMS-derivative of residual inducer salicylate (which is not metabolized because of the 'lower pathway' mutation), with retention time 16:19 and major ions at *m/z* 267 (M-CH_3), 209 (M-

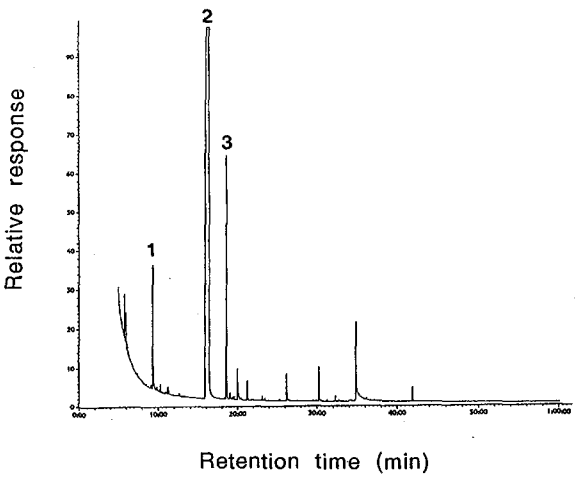

Figure 5. Gas chromatogram (GC-FID) of acidic dichloromethane extract of culture supernatant from Mutant 22 incubated with 2-methylnaphthalene, TMS-derivatized. Peaks identified by GC-MS as: 1, residual substrate 2-methylnaphthalene; 2, residual inducer salicylate; 3, product 4-methylsalicylate.

TMS), and 194 (M-TMS-O); and Peak 3, the putative TMS derivative of 4-methylsalicylate with retention time 18:33 and major ions at *m/z* 281 (M-CH_3), 223 (M-TMS), 207 (M-TMS-O) and 149 (M-TMS-O-TMS), which would result from cleavage of the unsubstituted benzene ring. The latter peak was tentatively indentified by comparison of its retention time and mass spectrum with authentic derivatized 3-methyl and 4-methylsalicylate and by analogy to the 2-methylnaphthalene degradative pathway (Cane & Williams 1982). Production of the corresponding methylsalicylate from 2-methylnaphthalene suggests that all the upper pathway enzymes recognize that particular alkylaromatic and its metabolites. It is likely that the lower pathway enzymes also recognize certain methyl-substituted metabolites, since 2-methylnaphthalene serves as a carbon source for the wild type strain. However, 1-methylnaphthalene is not utilized by wild type LP6a, indicating the specificity of the degradative enzymes for alkyl-substituent positions. As well, the number of substrates which are oxidized but do not serve as sole carbon sources (e.g. fluorene, acenaphthene) suggests that enzyme specificity must be more stringent beyond the first oxidative reactions.

Mutant 11 oxidized naphthalene (presumably the preferred growth substrate) to yield a single dihydrodiol, whereas phenanthrene (a poorer growth substrate) and benzothiophene (which is not a growth substrate) each yielded two putative dihydrodiols (Figure 4 and unpublished observations). It is interesting to speculate that differing substrate specificity of degradative pathway enzymes towards alternate or non-growth substrates may lead to the formation of a mixture of oxidized products, thus leading to the phenomenon of co-metabolism. The ability of natural microbial flora to oxidatively attack a family of related compounds (e.g. PAHs) through broad-specificity enzymes is likely advantageous to the organism *in situ*. This characteristic would allow growth on alternate carbon souces, perhaps sequentially as preferred substrates are exhausted, or in the face of competitive pressure from other microorganisms. The substrate range of degradative organisms is an important consideration for bioremediation of environments contaminated with a mixture of chemicals, such as in petroleum spills or creosote treatment sites where a single organism such as LP6a can utilize or oxidize a broad suite of aromatic contaminants. However, coincident with the ability to co-metabolically oxidize various substrates there is the potential for accumulation of metabolic isomers of unknown toxicity and increased water solubility, which may not be detected by GC-FID or GC-MS without derivatization, and which may or may not be further metabolized *in situ* by other members of the natural flora.

It is important to note that the presence of broad-specificity naphthalene-degradative enzymes in *P. fluorescens* LP6a does not imply a similar strategy in other PAH-degrading bacteria. Isolates such as *Pseudomonas* sp. HL7b or *P. paucimobilis* WW3 cannot grow on naphthalene or salicylate as sole carbon source (Table 1) and do not produce indigo from indole. Therefore phenanthrene metabolism in these strains likely does not proceed via broad-specificity naphthalene-degradative enzymes. However, many of these strains also have broad substrate range PAH-oxidizing phenotypes, and whether or not their phenanthrene-degradative genes are part of another, naphthalene-independent broad-specificity enzyme suite remains to be investigated.

Conclusions

P. fluorescens LP6a has the ability to utilize and co-metabolically oxidize a wide range of aromatic hydrocarbons, similar to several described naphthalene-utilizing *Pseudomonas* sp. isolates, by virtue of broad-specificity aromatic-degrading enzymes. The gross

arrangement of its naphthalene catabolic genes into two widely separated clusters is analogous to classical NAH plasmids. However, *P. fluorescens* LP6a is distinct from archetypal NAH-containing strains in several ways: it is capable of growing on both naphthalene and phenanthrene; its plasmid pLP6a has a unique restriction digestion pattern despite extensive homology to two NAH plasmids; and a 38 kb catabolic region of the plasmid undergoes large spontaneous, reproducible deletions. Further studies with strain LP6a could increase its potential for use in bioremediation purposes or as a source of enzymes for commercial applications.

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