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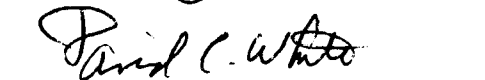
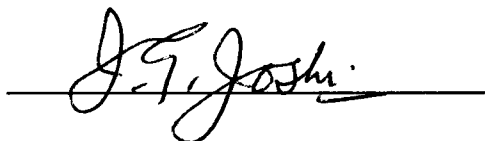
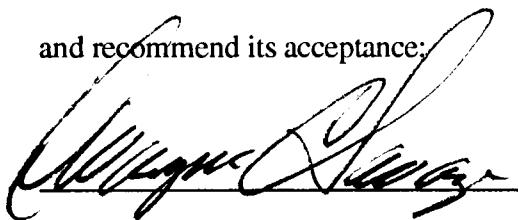
I am submitting herewith a dissertation written by Yeonghee Ahn entitled " Microbial and Molecular Basis of Polycyclic Aromatic Hydrocarbon Biodegradation in Contaminated Soils". I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment for the degree of Doctor of Philosophy, with a major in Microbiology.



Gary S. Sayler, Major Professor

We have read this dissertation

and recommend its acceptance:



Accepted for the Council:



Associate Vice Chancellor

and Dean of The Graduate School

MICROBIAL AND MOLECULAR BASIS OF POLYCYCLIC AROMATIC
HYDROCARBON BIODEGRADATION IN CONTAMINATED SOILS

A Dissertation
Presented for the
Doctor of Philosophy Degree
The University of Tennessee, Knoxville

Yeonghee Ahn
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Finally I thank sponsors of this research, the United States Air Force Office of Scientific Research and the University of Tennessee Waste Management Research and Education Institute.

ABSTRACT

Studies were undertaken to investigate the microbial and molecular basis of polycyclic aromatic hydrocarbon (PAH) biodegradation in contaminated soils. A culture collection of bacteria isolated from PAH-contaminated soils was characterized genotypically and phenotypically. The collection consisted of 141 putative PAH-degraders originally isolated using the PAH spray plate and the naphthalene vapor plate methods. The characterizations were designed to analyze genotypes and phenotypes of PAH-degraders, to screen HMW (high molecular weight) PAH degraders as sources for understanding of HMW PAH degradation, and to screen nonNAH7-hybridizing PAH-degraders as sources for the future development of new gene probes. The methods used to achieve the purpose were as follows: PAH spray plate method, ^{14}C -PAH mineralization assay, indigo test, and DNA hybridization. NAH7-derived gene probes were used in the hybridization. The results of the analyses showed that number of PAH-degraders with NAH7-like genotype was dominant in the contaminated soils and that the *nahA* gene probe could be used to determine the microbial potential to degrade not only naphthalene but also 3- or 4-ring PAHs. The results support the continued use of the NAH7-derived gene probes for contaminated soils to monitor genetic potential of *in situ* microorganisms to degrade PAHs. However, some PAH-degraders did not hybridize with NAH7-derived probes, showing the limitation of NAH7-derived gene probes. This suggested that considering the numerous unculturable microorganisms in nature, NAH7-derived gene probes might underestimate *in situ* microbial potential to catabolize PAHs. Therefore it is necessary to develop new gene probes for PAH-degraders to better understand *in situ* microbial potential to catabolize PAHs.

Strain A8AN3 was chosen for further study by virtue of its non-hybridization with NAH7-derived gene probes and ability to degrade a broad range of PAHs including

benzo[*a*]pyrene (BaP). A8AN3 was determined to be most similar to *Sphingomonas paucimobilis* based on phenotypic and chemotaxonomic traits. The 16S rDNA of A8AN3 was partially sequenced to determine the phylogenetic relationship of the strain with bacteria currently in the Ribosomal Database Project database. Comparative sequence analysis of the 16S rDNA suggested that A8AN3 was closest to *S. adhaesiva* in the database.

To investigate PAH-degradative genes in A8AN3 at the molecular level, random Tn5-mutagenesis was performed and gene libraries were constructed. When Tn5 mutagenesis was performed, a mutant strain of A8AN3 was obtained which lost the ability to convert indole to indigo (Ind⁻). Furthermore, the mutant could no longer mineralize BaP. Southern blot analyses suggested that Tn5 was inserted in the chromosome of the mutant. Based on the phenotype of the mutant and the result of Southern blot analysis, Tn5 is likely inserted in the initial dioxygenase genes or upstream of the genes in an operon. As a result of constructing the gene libraries of A8AN3 and the Ind⁻ mutant, two different genes (*cdo1* and *cdo2*) of extradiol dioxygenase and short flanking-regions of Tn5 were isolated from the libraries. The flanking regions can be used for future study to isolate genes encoding initial dioxygenase which attacks BaP. Southern blot analyses suggested that *cdo1* gene was isolated from chromosomal DNA and *cdo2* gene was from plasmid DNA in A8AN3. Nucleotide sequences of the two genes were determined. Analyses of their deduced amino acid sequences showed that the chromosome-encoded extradiol dioxygenase (CDO1) was most closely related (92.2% identity) to XylE of *S. yanoikuyae* B1. The plasmid-encoded extradiol dioxygenase (CDO2) had also most similarity (74.9% identity) to XylE of *S. yanoikuyae* B1 although the similarity was less than chromosome-encoded CDO1. A dendrogram produced by comparison of amino acid sequences showed considerable diversity of *Sphingomonas* extradiol dioxygenases from extradiol dioxygenases of the other strains.

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Part 1: General Introduction

I. INTRODUCTION AND OBJECTIVES

Bioremediation is a process for the cleanup of hazardous pollutants using the metabolic versatility of microorganisms. The increasing application of bioremediation has been fostered by the knowledge of how specific chemicals are degraded by microorganisms. The isolation of microorganisms capable of degrading the target compounds, as well as biochemical and genetic information about the degradation, may lead to the development of new bioremediation technologies for contaminated sites.

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants. Environmental concern exists about their persistence and toxicity, especially high-molecular-weight (HMW) PAHs. However, few studies on microbial degradation of HMW PAHs have been conducted. Benzo[*a*]pyrene (BaP), an HMW PAH, is one of the priority pollutants on the list compiled by the United States Environmental Protection Agency (US EPA) (Natl. Res. Council, 1991) and is one of the potential human carcinogens (IARC, 1987). In spite of the realization of the carcinogenic effects of BaP metabolites produced by mammals (Williams & Weisburger, 1986), few reports are available to understand how bacteria degrade BaP (Gibson et al., 1975; Schneider et al., 1996). Little is known about the extent of BaP mineralization by bacterial pure cultures (Sherrill, 1982; Ahn et al., 1994; Ye et al., 1996).

To understand the microbial degradation of PAHs, a literature review on PAH biodegradation is presented with emphasis on HMW PAH degradation by bacteria. The significance of PAHs as pollutants is also briefly reviewed. The overall goal of this research is to provide new information about bacterial degradation of HMW PAHs. In this regard, three specific objectives are

- 1) to isolate HMW PAH degraders as sources for new gene probe development and HMW-PAH-metabolic information,

- 2) to characterize BaP (a HMW PAH)-mineralizing strain A8AN3 isolated in this study, and
- 3) to isolate genes involved in PAH degradation in A8AN3.

To isolate HMW PAH degraders which can be sources for new gene probe development and metabolic pathway information, a bacterial culture collection was genotypically and phenotypically analyzed. Results of the analyses supported the continued use of the NAH7-derived gene probes for PAH-contaminated soils to monitor genetic potential of *in situ* microbial communities to degrade PAHs. The analyses also showed limitation of NAH7-derived gene probes, suggesting necessity to develop new gene probes for HMW PAH-degraders. Through the analyses, a BaP-mineralizing strain, A8AN3 was found. The strain did not hybridize with NAH7-derived gene probes. A8AN3 was identified as a *Sphingomonas* sp. strain. Genes encoding extradiol dioxygenases (*meta*-ring cleavage enzymes) were isolated from A8AN3. The genes were molecularly characterized. The results of this research have important implications for the potential degradation of PAHs by bacteria. The knowledge gained will aid in PAH bioremediation and environmental sustainability.

II. LITERATURE REVIEW

1. Significance of PAHs as environmental pollutants.

PAHs consist of two or more fused-benzene rings (Fig. 1). They are ubiquitous environmental pollutants mainly produced from incomplete combustion of organic materials (Benner et al., 1990; Edwards, 1983). Because of their persistence in the environment and their toxicity, remediation of PAH-contaminated environments is important.

1.1 Source and distribution

Numerous environmental samples show global distribution of PAHs (Harvey, 1991). They are found in air, water, sediment (soil), food, cigarette smoke, and fossil fuels. In nature, PAHs are present as complex mixtures that vary in concentration of individual components (Harvey, 1991). Major sources of PAHs are anthropogenic such as fossil fuel combustion, automobile exhaust, waste incineration and coal tar. Natural sources of PAHs are minor. PAHs are produced by forest fires and volcanic activities. PAHs also occur as natural constituents of fossil fuels. Increasing industrial development results in a constantly rising rate of production and accumulation of PAHs, particularly in urban and industrialized areas (Jones et al., 1989; Linz et al., 1991).

Although PAHs are ubiquitous, they are found at particularly high levels at coal gasification sites and wood treatment sites. Manufactured gas plants (MGP) sites are major sites of PAH contamination. MGPs generated gas from coal and oil from the mid-19th through the mid-20th century for public and industrial uses. It has been estimated that the United States had over 1,500 MGP sites during the time. The major waste

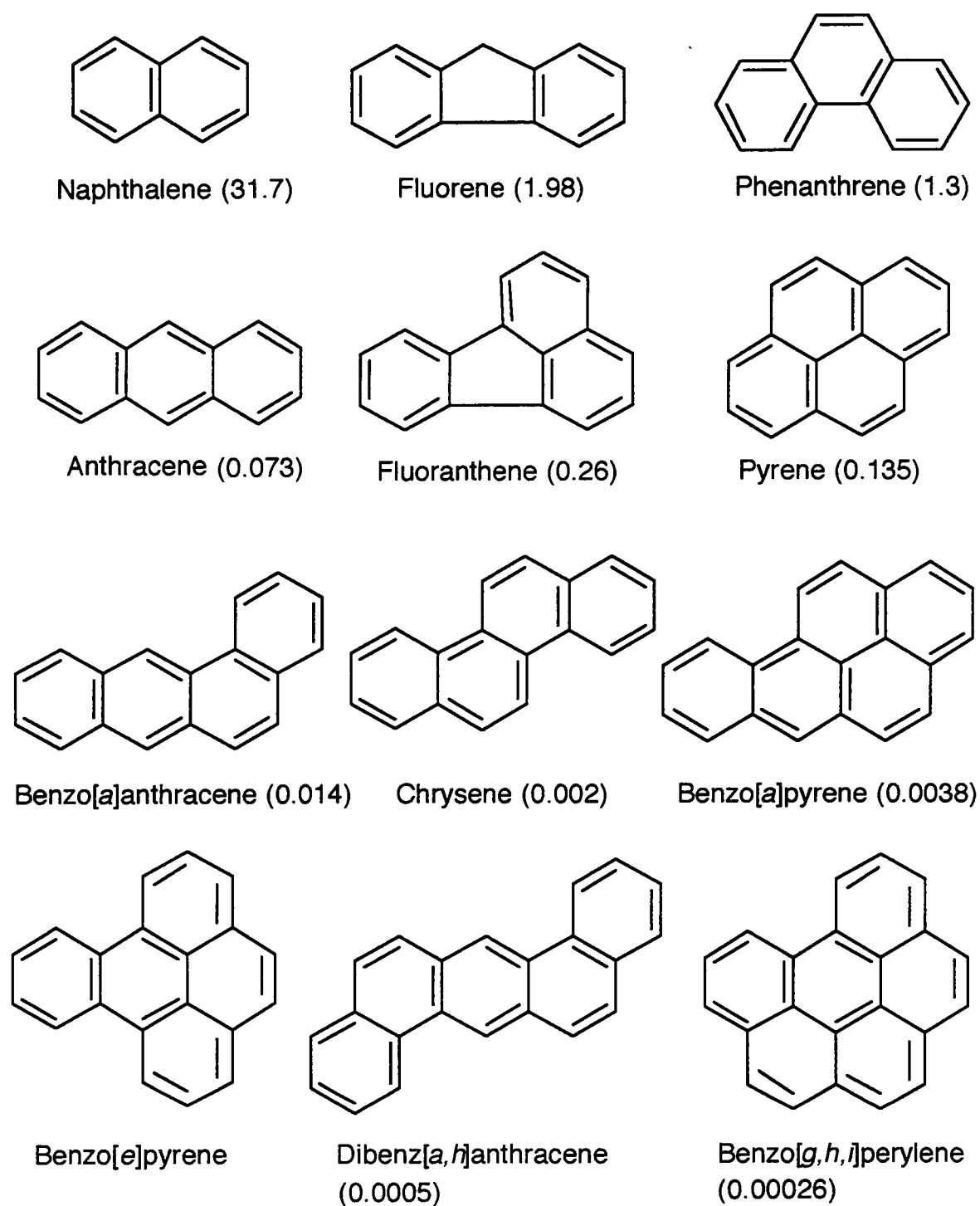


Figure 1. Chemical structures and water-solubilities (Mackay & Shiu, 1977; Sims & Overcash, 1983) of some polycyclic aromatic hydrocarbons. Water-solubilities of PAHs (mg/L) are in parentheses.

products from the gasification process were tars and sludges mainly composed of PAHs (Linz et al., 1991). Cleanup of the MGP sites has become a priority: over 400 MGP sites were listed on the Superfund National Priority List (US EPA, 1985). PAH contamination in soil and ground water was reported at wood treatment facilities where creosote was used (Goerlit et al., 1985; Mueller et al., 1989). PAHs are major constituents of creosote, a commonly used wood preservative. Creosote is a complex mixture produced by the distillation of coal tar. Creosote contains approximately 85% PAHs by weight (Nestler, 1974). Bioremediation of PAH-polluted sites has received attention as an alternative to a traditional cleanup method of incineration.

1.2 Physicochemistry and toxicology

PAHs have low solubilities in water (Fig. 1); generally, as molecular weight increases, water-solubility decreases. Especially, HMW PAHs (four- or more ring PAHs) are extremely insoluble in water and are persistent in the environment (Herbes, 1981; Heitkamp & Cerniglia, 1987; Shiaris, 1989). The water-solubilities of naphthalene (a two-ring PAH) and BaP (a five-ring PAH) are 31.7 mg/L and 3.8 μ g/L at 25°C, respectively (Mackay & Shiu, 1977). Because of their hydrophobicity, they adsorb onto soils and particulates, which limits their bioavailability and contributes to their persistence (Means et al., 1980; Weissenfels et al., 1992).

Toxicological concern exists about the persistence and toxicity of PAHs, especially HMW PAHs. Many HMW PAHs such as BaP and benz[*a*]anthracene are known as potent carcinogens (Miller & Miller, 1981; Williams & Weisburger, 1986; Pahlman & Pelkonen, 1987). They are classified as (pro)carcinogens and require metabolic activation to produce their DNA-reactive metabolites in mammals (Sims et al., 1974; Booth & Sims, 1974; Phillips, 1983; Williams & Weisburger, 1986; Jernstrom &

Graslund, 1994). Sixteen PAHs including BaP are listed on the US EPA priority pollutant list (Natl. Res. Council, 1991). BaP is listed on the IARC potent human carcinogen list (IARC, 1987).

2. Biodegradation of PAHs

The metabolism of PAHs has been studied in mammalian, fungal, algal, and bacterial systems. This literature review will focus on microbial degradation of PAHs, especially bacterial degradation of HMW PAHs. However, mammalian degradation of PAHs will be briefly mentioned for comparison. Bacteria show differences from fungal and mammalian systems in PAH degradation. It seems that generally, eukaryotic PAH degradation is detoxification and excretion of PAHs, whereas bacterial PAH degradation is ring fission and assimilation. Complete removal of PAHs to harmless compounds is important due to their persistence in nature and genotoxicity in the animals. Microbial biodegradation appears to be a major removal mechanism of PAHs in nature (Park et al., 1990; Sims et al., 1990); although PAH removal in nature can be attributed to abiotic factors such as volatilization, adsorption, chemical oxidation, and photolysis (Pitts et al., 1978; Dzombak, 1984; Valerio et al., 1984). Bioremediation can take advantage of the metabolic versatility and wide distribution of microorganisms. For bioremediation strategies, it is necessary to understand PAH degradation by microorganisms. As a model compound for PAHs, naphthalene (a low-molecular-weight PAH) degradation is the best studied of the PAHs in microbial system.

2.1 Mammalian PAH degradation

Three different mechanisms have been proposed for the metabolic activation of

PAHs (Stansbury et al., 1994; Jernstrom & Graslund, 1994; Cavalieri & Rogan, 1995). The most widely accepted activation mechanism is ring epoxidation catalyzed by cytochrome P450 (Fig. 2). The other two are relatively less studied; radical cation formation by one-electron oxidation and benzylic ester formation by substitution reactions.

In mammals, PAHs are subjected to complex steps before PAHs encounter cytochrome P450 which can initially attack PAHs enzymatically (Hankinson, 1994, 1995; Okey et al., 1994; Fujii-Kuriyama et al., 1994). Since isolations of AHR (aryl hydrocarbon receptor) and ARNT (AHR nuclear translocator) genes were achieved recently in 1991 and 1992, respectively, many questions about them have been unanswered. According to a proposed model for the AHR action mechanism (Fig. 3), a PAH binds to AHR in a multimeric protein complex consisting of one or more molecules of AHR, one or more molecules of heat shock protein, and possibly other proteins. The AHR-PAH complex is released from the multimeric complex to bind to ARNT, before entering the nucleus. AHR in the nucleus can bind the xenobiotic responsive element (XRE) of DNA. The AHR binding to XRE induces transcription of PAH-metabolic enzyme genes such as genes encoding cytochrome P450s (e.g., *CYP1A1*, *CYP1A2*, and *CYP1B1*) and genes encoding non-cytochrome P450 (e.g., *GST-Ya* encoding glutathione S-transferase Ya subunit and *Ugt1*06* encoding UDP-glucuronosyl transferase).

2.2 Microbial PAH degradation.

The biodegradability of PAHs is reported to be inversely related to the number of rings (Heitkamp & Cerniglia, 1987; Shiaris, 1989; Sanseverino et al., 1993b). PAHs with more rings were reported to have higher resonance energy and lower water-solubility (Lee et al., 1981). Since it is generally believed that microorganisms use only PAHs

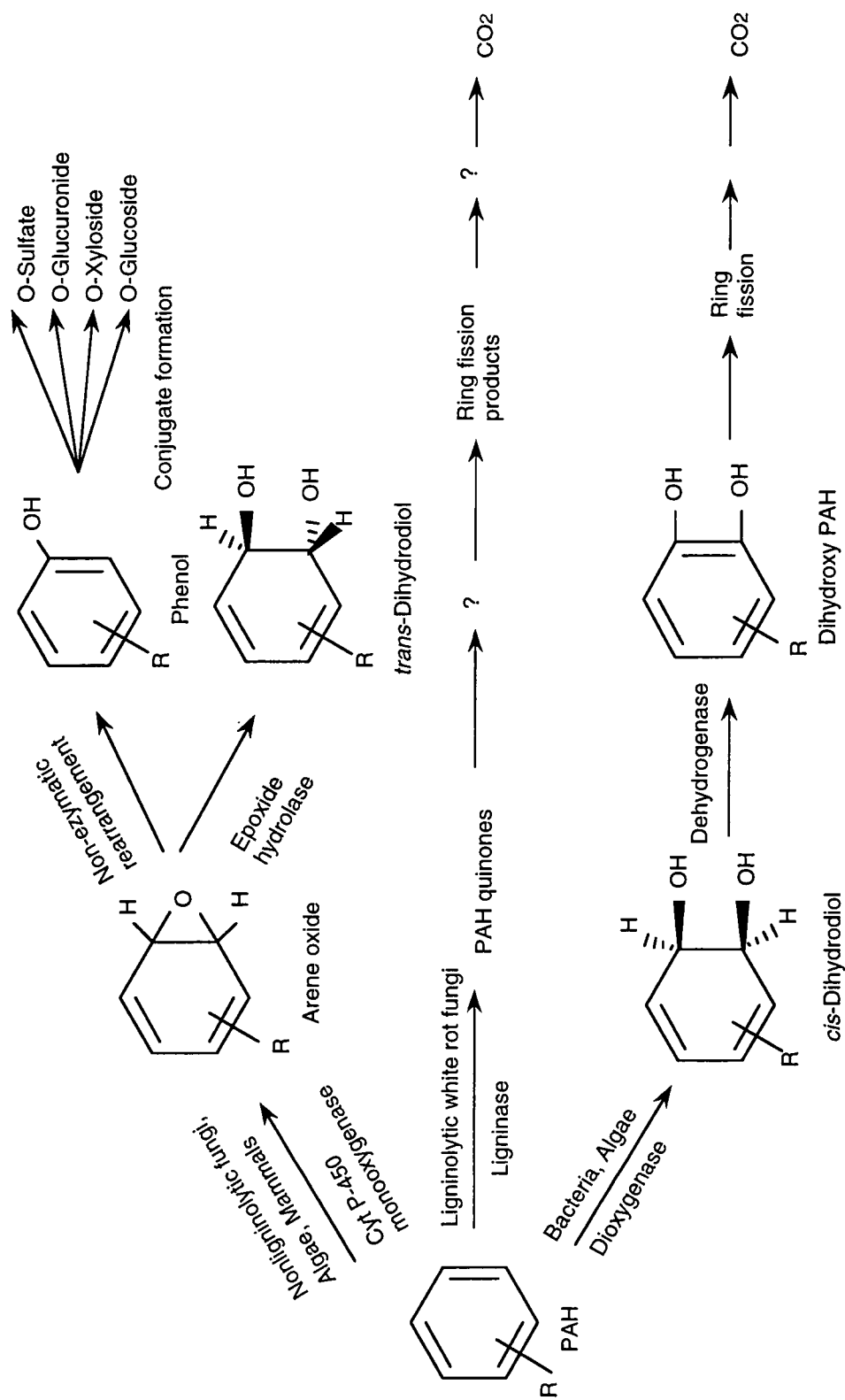


Figure 2. Diverse pathways of PAH degradation (after Cerniglia, 1993). R, aryl group. Two arrows between metabolites indicate multiple reaction steps.

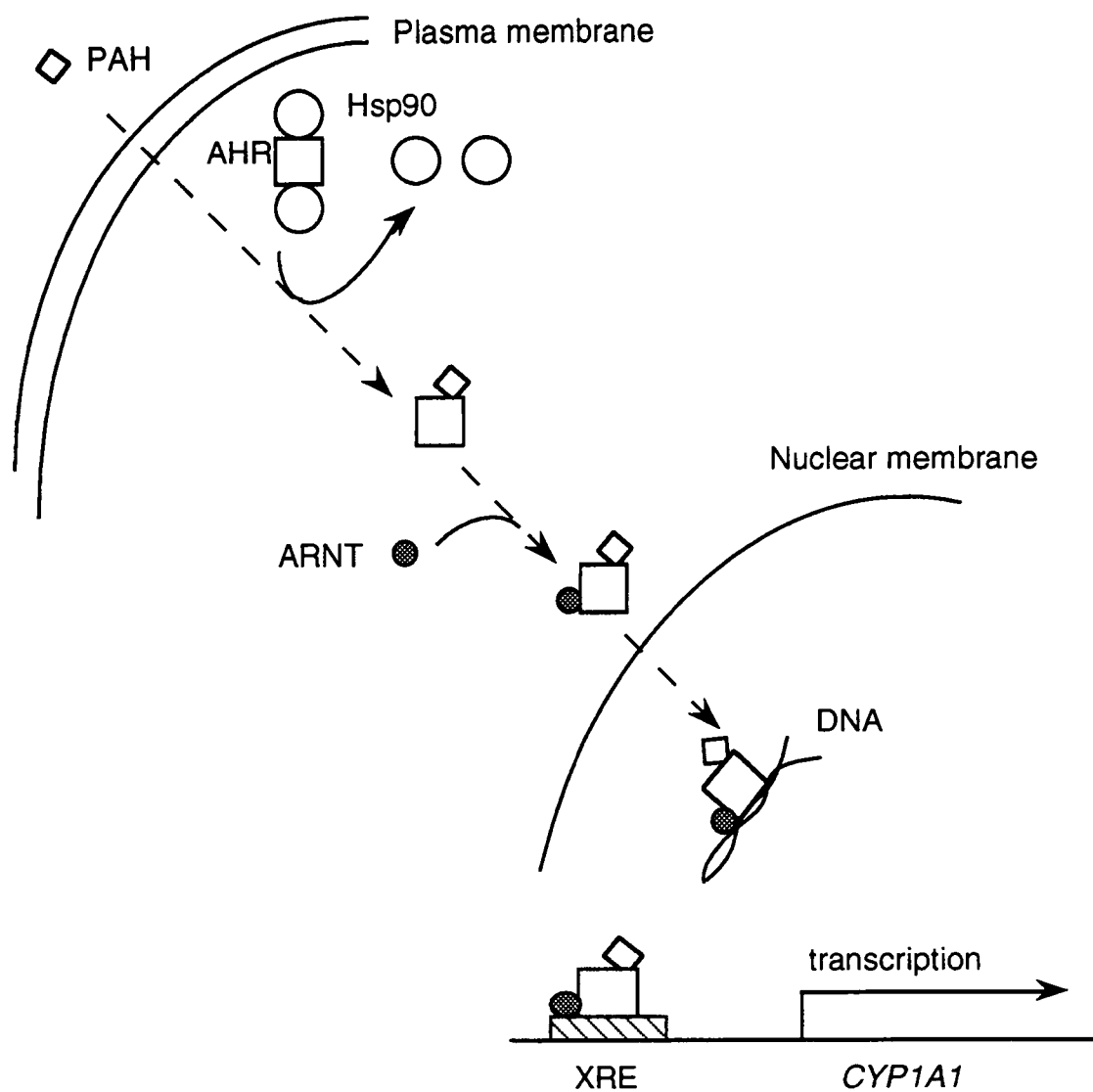


Figure 3. Model of AHR (aryl hydrocarbon receptor) action mechanism (adapted from Hankinson, 1994). PAH, polycyclic aromatic hydrocarbon; Hsp90, heat shock protein 90; ARNT, AHR nuclear translocator; XRE, xenobiotic responsive element.

dissolved in aqueous phase (Wodzinski & Bertolini, 1972; Wodzinski & Coyle, 1974), HMW PAHs may be less bioavailable and more recalcitrant because of their lower water-solubilities. Added (co-occurring) PAHs inhibited mineralization of pyrene, a HMW PAH (Molina et al., 1993; Kelley & Cerniglia, 1995). Kelley and Cerniglia (1995) reported that *Mycobacterium* sp. strain PYR-1 mineralized individual PAHs more slowly in PAH mixtures than in individual PAHs alone, suggesting possible competitive inhibition or chemical interaction of PAHs in the environment where PAHs exist as mixtures.

Bacteria appear to play a predominant role in PAH degradation in sediments, relative to eukaryotic microorganisms (Heitkamp et al., 1987; MacGillivray & Shiaris, 1994). PAH-degrading bacteria were found to be widely distributed in nature (Kiyohara et al., 1992; Kastner et al., 1994), although the numbers of PAH degraders were different from site to site. The bacteria can be isolated from the environment using the PAH spray plate method (Kiyohara et al., 1982). The PAH spray plate method consists of an agar plate sprayed with a 1% (w/v) PAH dissolved in acetone. Bacteria capable of degrading the PAH will form clear zones around the colonies on the plate covered with the PAH.

PAHs are generally believed to enter bacterial cells by simple diffusion, although not much study has been done on PAH transport into bacterial cells. Bateman et al. (1986) studied naphthalene uptake using a *Pseudomonas* (NAH7) strain and showed that ATP, membrane potential, and protein carrier were not required for the uptake. Therefore, passively diffused PAHs into cells are believed to serve as substrates of PAH degradative enzymes in the cells. Generally, bacteria initiate PAH degradation by incorporating two atoms of oxygen by a dioxygenase, forming *cis*-dihydrodiol (Jerina et al., 1971; Gibson et al., 1975; Jerina et al., 1976) (Fig. 2). The second step in bacterial PAH oxidation is to form a dihydroxylated intermediate from the *cis*-dihydrodiol via a dehydrogenase (Davies & Evans, 1964; Evans et al., 1965). The dihydroxylated PAH is subjected to ring fission

(Evans et al., 1965; Dagley & Gibson, 1965; Barnsley, 1976a, b). Further catabolism converts the ring fission product to small aliphatic compounds (e.g. pyruvate and acetaldehyde) that can enter the tricarboxylic acid (TCA) cycle.

Dioxygenases play important roles in microbial PAH degradation. Aromatic ring dioxygenase catalyzes the initial reaction and ring fission dioxygenase catalyzes ring-cleavage reaction in the PAH degradation. The aromatic ring dioxygenase catalyzing the initial reaction is a multicomponent enzyme system that consists of a flavoprotein, a ferredoxin, and an iron sulfur protein (Yeh et al., 1977; Crutcher & Geary, 1979; Ensley et al., 1982; Harayama et al., 1992). Aromatic ring dioxygenase requires a cofactor, NAD(P)H as an electron donor in the initial reaction. Many aromatic ring dioxygenases can convert indole to indigo (blue dye), an oxidized product of indole, when indole is added to the cultures of aromatic hydrocarbon degraders (Ensley et al., 1983; Eaton & Timmis, 1986; Tan & Mason, 1990; Denome et al., 1993). Aromatic ring dioxygenases oxidize indole to produce dihydroxylated indole. The dihydroxylated indole is subject to spontaneous dehydration to yield indoxyl. Air oxidation converts indoxyl to indigo (Ensley et al., 1983).

Two classes of ring fission dioxygenases exist on the basis of their action position in ring cleavage. Ring-cleavage can occur either between two dihydroxylated carbons or between a hydroxylated carbon and its adjacent nonhydroxylated carbon (Fig. 4). The former case is called *ortho*-cleavage and is catalyzed by an intradiol cleavage dioxygenase such as catechol 1,2-dioxygenase. The later is called *meta*-cleavage and is catalyzed by an extradiol cleavage dioxygenase such as catechol 2,3-dioxygenase. In contrast to initial dioxygenases, ring fission dioxygenases do not require NAD(P)H, but do require iron for their activity. *Ortho*-cleavage dioxygenases are homomultimers or heteromultimers and contain Fe^{3+} ions (Harayama & Rekik, 1989). *Meta*-cleavage dioxygenases are homomultimers and each subunit is associated with Fe^{2+} ion. Extradiol

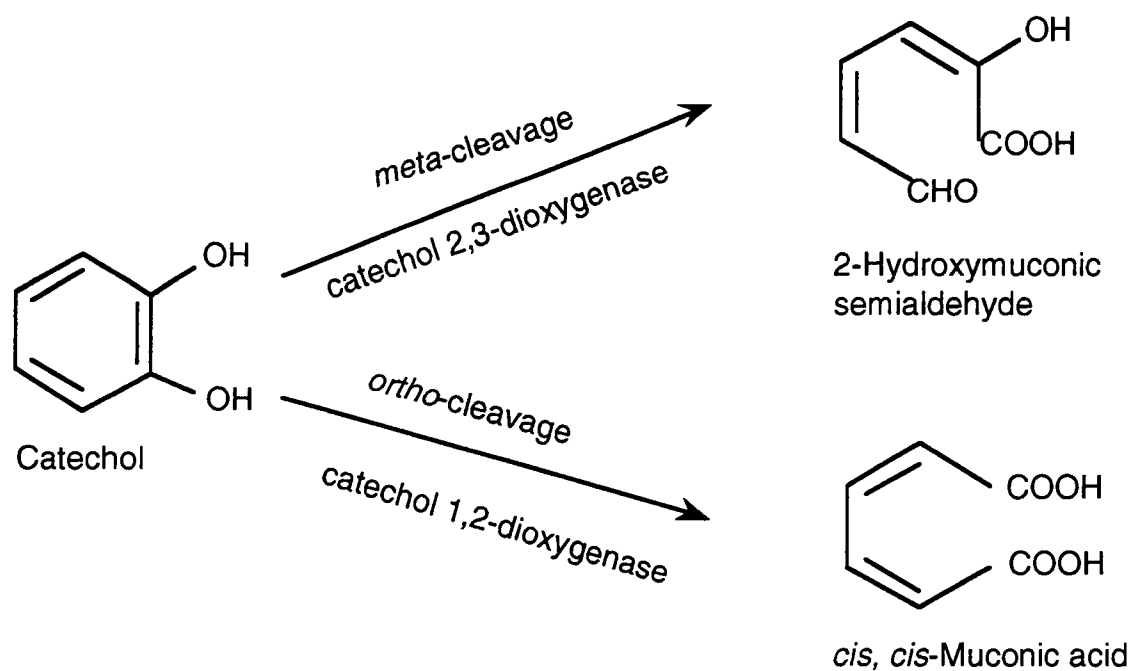


Figure 4. The *ortho*- and *meta*-pathways for aromatic ring cleavage of catechol.

dioxygenases produce distinguishable yellow ring cleavage products (e.g., 2-hydroxymuconic semialdehyde) from dihydroxylated aromatic hydrocarbons such as catechol (Dagley & Gibson, 1965; Barnsley, 1976b; Patel & Barnsley, 1980; Eaton & Chapman, 1992) (Fig. 4). Besides the importance of dioxygenases in the reactions (initial and ring fission) of PAH degradation, their abilities to produce blue or yellow color have been used as useful chromogenic tools to screen clones containing initial dioxygenase genes, *meta*-cleavage dioxygenase genes, or even whole operons (Ghosal et al., 1987; Irie et al., 1987; Denome et al., 1993; Kiyohara et al., 1994; Yang et al., 1994; Kim & Zylstra, 1995; Goyal & Zylstra, 1996).

PAH degradation pathways of fungi are less studied than those of bacteria. Fungi do not grow on PAHs as sole carbon and energy sources but cometabolize PAHs. Weber et al. (1995) reported that the fungus *Cladosporium sphaerospermum* could grow on toluene, an one-ring aromatic hydrocarbon, as a source of carbon and energy. They used CO₂ production in a minimal liquid medium to measure growth. The growth result is inconclusive since enzymes can be active and produce CO₂ although cells are not growing. A diverse group of ligninolytic and nonligninolytic fungi can degrade PAHs (Cerniglia & Gibson, 1979; Bumpus et al., 1985; Aust, 1990; Morgan et al., 1993; Lange et al., 1994; Bezalel et al., 1996). In fungal PAH degradation, two major groups of enzymes are involved in the initial attack on PAHs; cytochrome P450 monooxygenases and ligninases. Both enzymes have broad substrate specificities and oxidize a variety of PAHs.

Nonligninolytic fungi, such as *Cunninghamella elegans*, oxidize PAHs via a cytochrome P450 monooxygenase by incorporating one atom of oxygen into the PAHs (Fig. 2). The oxidation results in arene oxide formation (Cerniglia & Gibson, 1978; Cerniglia & Heitkamp, 1989). Arene oxides are further transformed either into *trans*-dihydrodiols or phenols. The *trans*-dihydrodiols can then be oxidized into *trans*-

dihydrodiol oxides via a cytochrome P450 monooxygenase. Phenolic PAHs can be conjugated with sulfate, glutathione, glucuronide, glucose, or xylose (Cerniglia & Crow, 1981; Cerniglia et al., 1982a, 1985a; Sutherland et al., 1992b; Pothuluri et al., 1995). The formation of water-soluble conjugates of phenolic PAHs suggests that conjugation reactions may be important in the fungal detoxification and excretion of PAHs (Cerniglia et al., 1982a, 1985a). These mechanisms of PAH metabolism are similar to those shown for mammals (Jerina et al., 1970; Sims et al., 1974; Booth & Sims, 1974; Williams & Weisburger, 1986). Some of the minor fungal metabolites are more mutagenic than either the parent PAHs or major metabolites. They include benz[*a*]anthracene-3,4-dihydrodiol (Cerniglia et al., 1980e), benzo[*a*]pyrene *trans*-7,8-dihydrodiol 9,10-oxide (Cerniglia & Gibson, 1980a), 7,12-dimethylbenz[*a*]anthracene *trans*-3,4-dihydrodiol (Wong et al., 1983), and 1-hydroxy-3-methylchloanthrene *trans*-9,10-dihydrodiol (Cerniglia et al., 1982b). Although the minor fungal metabolites are mutagenic and carcinogenic, most fungal PAH metabolites are less mutagenic than the parent PAHs (Cerniglia et al., 1985a, b; Pothuluri et al., 1992).

Ligninolytic white rot fungi have shown the ability to mineralize a variety of PAHs (Bumpus et al., 1985; Aust, 1990; Morgan et al., 1991; Field et al., 1992; Bezalel et al., 1996). A white rot fungus, *Phanerochaete chrysosporium* is the best studied of ligninolytic fungi. The exact mechanism of fungal PAH mineralization is not fully understood. The enzyme system involved in lignin degradation is thought to function nonspecifically on lignin that is a complex aromatic compound (Kirk & Farrell, 1987). Likewise, the enzyme system is thought to attack various PAHs nonspecifically. Extracellular ligninases initiate PAH degradation in *P. chrysosporium* (Haemmerli et al., 1986; Hammel et al., 1986; Aust, 1990). The enzymes catalyze one-electron oxidations of PAHs to form cation radicals. The radicals then undergo spontaneous non-enzymatic rearrangements to form quinones (Haemmerli et al., 1986; Hammel et al., 1986, 1992;

Field et al., 1992). Quinones produced from pyrene degradation were shown to be more toxic than the parent compound (Okamoto & Yoshida, 1981). Other ligninolytic extracellular enzymes, such as laccases and manganese peroxidases, were also implicated to be involved in PAH degradation (Kirk & Farrell, 1987; Moen & Hammel, 1994; Bogan & Larmar, 1995). Ligninolytic activities of white rot fungi were correlated with the ability to degrade PAHs (Field et al., 1992; Sack & Gunther, 1993).

However, *P. chrysosporium* may contain other enzymes involved in PAH degradation besides extracellular ligninolytic enzymes. Sutherland et al. (1991) reported that *P. chrysosporium* degraded phenanthrene to phenols and *trans*-dihydrodiols in nonligninolytic cultures, suggesting the involvement of monooxygenase and epoxide hydrolase activities. Recently Masaphy et al. (1996) actually showed that fraction of cytochrome P450 from the fungus produced 3-hydroxyBaP (a phenol) from BaP, suggesting a BaP degradation similar to BaP degradation by *Cunninghamella elegans*. White rot fungi have received attention because of their advantageous potential for the bioremediation of PAH-polluted soils (Davis et al., 1993; Morgan et al., 1993). White rot fungi exist throughout the environment and degrade various PAHs to CO₂. Besides, their extracellular ligninases may eliminate bioavailability problem resulted from adsorption of PAHs onto soil particles. Therefore, fungi may be as important as bacteria in the bioremediation of PAH-contaminated environments. However, more biochemical information is needed along with information on toxicity of metabolites before using white rot fungi for PAH bioremediation.

Algal PAH degradation is less studied than fungal PAH degradation, although algae are widely distributed in aquatic environment and they have been shown to degrade PAHs (Cerniglia et al., 1980a, b). Two different lines of data suggest that algal PAH degradation is similar to either monooxygenase-catalyzed fungal and mammalian systems or dioxygenase-catalyzed bacterial system. Cyanobacteria and eukaryotic algae degraded

naphthalene under photoautotrophic conditions and produced 1-naphthol as a major metabolite (Cerniglia et al., 1980a, c, d; Narro et al., 1992b). A cyanobacterium *Oscillatoria* sp. strain JCM produced 1-naphthol as a major metabolite of naphthalene (Narro et al., 1992b) and another cyanobacterium *Agmenellum quadruplicatum* PR-6 formed *trans*-9,10-dihydrodiol and 1-methoxyphenanthrene as major metabolites of phenanthrene (Narro et al., 1992a). These data suggest that algal PAH degradation is more similar to fungal and mammalian PAH degradation. However, *cis*-1,2-dihydrodiol was identified as a minor metabolite of naphthalene in the culture of *Oscillatoria* sp. strain JCM (Narro et al., 1992b). Green alga *Selenastrum capricornutum* degraded BaP predominantly to *cis*-dihydrodiols, suggesting the involvement of dioxygenase similar to the bacterial system.

PAH degradation by yeast is the least studied among microbial systems. PAH degradation by yeast is similar to cytochrome P450-mediated fungal PAH degradation. *Candida lipolytica* and *Saccharomyces cerevisiae* produced phenols and *trans*-dihydrodiols from the degradation of naphthalene and BaP (Wiseman & Woods, 1979; Woods & Wiseman, 1979; Cerniglia & Crow, 1981).

3. Microbial degradation of BaP

The study of PAHs began in the 1920s to determine the structures of the carcinogenic compounds in coal tar, after high incidences of skin cancer were reported among workers in the oil refining, shale oil, and coal tar industries in the 19th century (Phillips, 1983). As one of the compounds, BaP was first purified from coal tar in 1933 (Cook et al.) and shown to be carcinogenic in animal experiments. Since then BaP has become the most thoroughly studied PAH in the mammalian system. However, little is known about microbial BaP degradation. This is partly because of the fact that a few

BaP-degraders have been isolated, together with recalcitrance of HMW PAHs due to their complex structures and low water-solubilities.

Instead of carcinogenic HMW PAHs, naphthalene has been the best studied of the PAHs in the microbial system because it is the simplest and the most soluble PAH (Fig. 1). Information from the studies of bacterial degradation of naphthalene has been used for understanding and predicting the pathways in the degradation of the three- or more ring PAHs (Fig. 5). Naphthalene-degrading bacteria were first reported by Tausson (1927). Since then, the ubiquitous presence of naphthalene-degraders has been shown (Kiyohara et al., 1992; Kastner et al., 1994). The bacterial degradation of naphthalene has been well characterized for the naphthalene catabolic enzyme system encoded by the plasmid NAH7 of *Pseudomonas putida* PpG7 (Dunn & Gunsalus, 1973; Yen & Gunsalus, 1982; Yen & Serdar, 1988). The naphthalene degradation pathway has been determined (Davies & Evans, 1964; Yen & Gunsalus, 1982; Eaton & Chapman, 1992), and the genes involved have been isolated (Grund & Gunsalus, 1983; Schell, 1983). Based on biochemical and genetic information of naphthalene degradation, NAH7-derived gene probes have been used to determine the *in situ* microbial potential to degrade naphthalene (Sayler & Layton, 1990; Fleming et al., 1993; Sanseverino et al., 1993b).

Recent studies have shown that NAH system can degrade not only naphthalene but also other compounds such as phenanthrene, anthracene, dibenzothiophene, fluorene, and methylated naphthalene (Menn et al., 1993; Sanseverino et al., 1993a; Denome et al., 1993; Yang et al., 1994; Kiyohara et al., 1994; personal communication with J. Leblond). The studies suggested that NAH7-derived gene probes could be used to determine the *in situ* microbial potential to degrade not only 2-ring PAHs but also 3-ring PAHs. However, many PAH-degraders were not detected by NAH7-derived gene probes (Foght & Westlake, 1988, 1991; King et al., 1991; Fredrickson et al., 1991; Ahn et al., 1994; Goyal & Zylstra, 1996). Considering reports on numerous unculturable microorganisms in

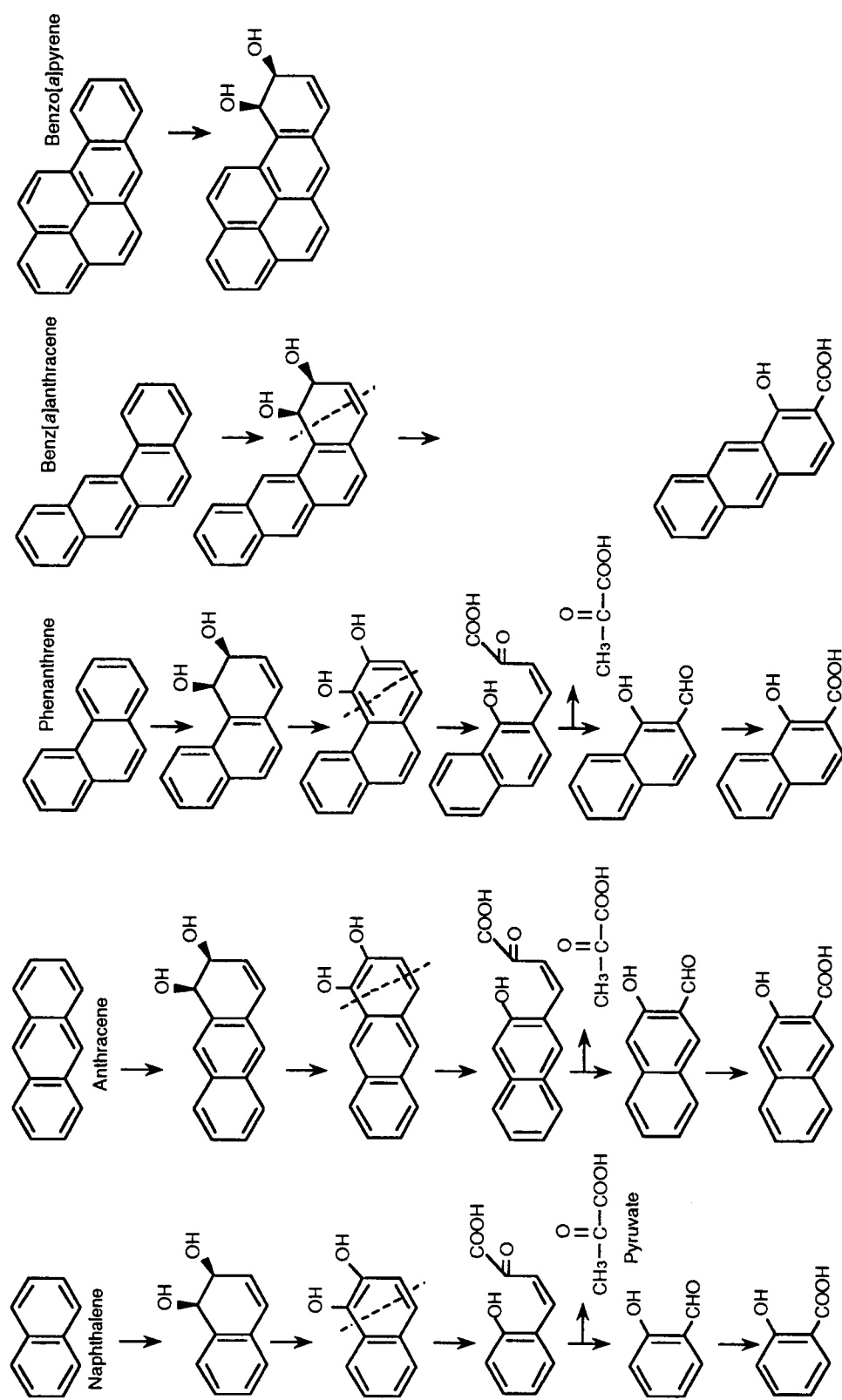


Figure 3. Structural similarities of initial reactions in the proposed bacterial degradation of PAHs (adapted from Davis & Evans, 1964; Evans et al., 1965; Mahaffey et al., 1988; Gibson et al., 1975). Major isomers of dihydrodiol (first metabolites) and planes of ring cleavage (dashed lines) are shown.

nature (Roszak et al., 1984; Ward et al., 1990), the current NAH7-derived gene probes may underestimate *in situ* microbial potential to catabolize PAHs. Moreover, NAH7-derived gene probes do not assure detection of HMW PAH degraders. Therefore, biochemical and genetic information of HMW PAH degradation is necessary for the development of new biotechnologies which can provide a better understanding of microbial activity in the environment.

3.1 BaP degradation by bacteria

Although the microbial metabolism of low-molecular-weight (LMW) PAHs (two- or three-ring PAHs) has been well studied over the last 30 years, investigations on the degradation of HMW PAHs have begun only recently. Few studies on the biodegradation of HMW PAHs have been reported. Therefore, less is known about the potential for HMW PAH degradation. Only a few pure bacterial isolates have been found that are capable of growing solely on HMW PAHs: pyrene- and fluoranthene-degrading *Mycobacterium* sp. strain BB1 (Boldrin et al., 1993); pyrene-, chrysene-, and fluoranthene-degrading *Rhodococcus* sp. strain UW1 (Walter et al., 1991); chrysene-degrading *Pseudomonas fluorescens* (Caldini et al., 1995); fluoranthene-degrading *Sphingomonas paucimobilis* EPA 505 (Mueller et al., 1990) and *Alcaligenes denitrificans* (Weissenfels et al., 1990).

Degradation of HMW PAHs has primarily been observed in microbial cultures provided with an alternative growth-supporting compound, i.e., cometabolism: BaP-degrading A8AN3 (Ahn et al., 1994) and *S. paucimobilis* EPA 505 (Ye et al., 1996); fluoranthene- and pyrene-degrading *Mycobacterium* sp. strain PYR-1 (Heitkamp & Cerniglia, 1988); pyrene- and BaP-degrading *Mycobacterium* sp. strain RJGII-135 (Grosser et al., 1991); benz[*a*]anthracene- and BaP-degrading *Beijerinckia* sp. strain B1

(Gibson et al., 1975; Mahaffey et al., 1988). HMW PAHs have extremely low water-solubilities (Fig. 1). Therefore, in the absence of other carbon sources, their aqueous-phase concentrations may not be sufficient to support bacterial growth (Wodzinski & Coyle, 1974; Volkerling et al., 1992). Little is known about the genetic and regulatory mechanisms in bacterial degradation of HMW PAHs. Isolation of genes involved in HMW PAH degradation has not been reported.

Most pure culture studies of bacterial HMW PAH degradation have been aimed at elucidating biochemical pathways by isolating and identifying metabolites (Gibson et al., 1975; Mahaffey et al., 1988; Heitkamp et al., 1988; Kelley et al., 1993; Schneider et al., 1996). BaP was shown to be oxidized initially into *cis*-dihydrodiols by B836, a mutant of *Beijerinckia* sp. strain B1 that was recently re-identified as *Sphingomonas yanoikuyae* (Wang & Lau, 1996). The dihydrodiols were *cis*-9,10-dihydrodiol (major) and *cis*-7,8-dihydrodiol (minor) (Fig. 6) (Gibson et al., 1975). *Beijerinckia* sp. strain B1 was originally isolated as a biphenyl degrader, and shown to grow on naphthalene, phenanthrene, or anthracene as sole sources of carbon and energy (Gibson et al., 1973). The strain could cometabolize HMW PAHs such as BaP and benz[a]anthracene. Its mutant strain B836 accumulated the corresponding *cis*-dihydrodiols from naphthalene, phenanthrene, anthracene, benz[a]anthracene, and BaP (Gibson et al., 1975).

Recently, ring cleavage products of BaP were reported (Schneider et al., 1996). An initial metabolite (*cis*-7,8-BaPdihydrodiol) and three different ring cleavage products (4,5-chrysene dicarboxylic acid, hydroxypyrenyl oxobutenoic acid, and dihydropyrene carboxylic acid) were identified in the culture of *Mycobacterium* sp. strain RJGII-135 (Fig. 7) that could mineralize BaP (Grosser et al., 1991). Consistent with BaP degradation by the *Beijerinckia* sp. (Gibson et al., 1975), *cis*-7,8-BaPdihydrodiol was identified as an initial metabolite, suggesting the involvement of initial dioxygenase activity. However, the major isomer, *cis*-9,10-BaPdihydrodiol formed by the *Beijerinckia* sp., was not

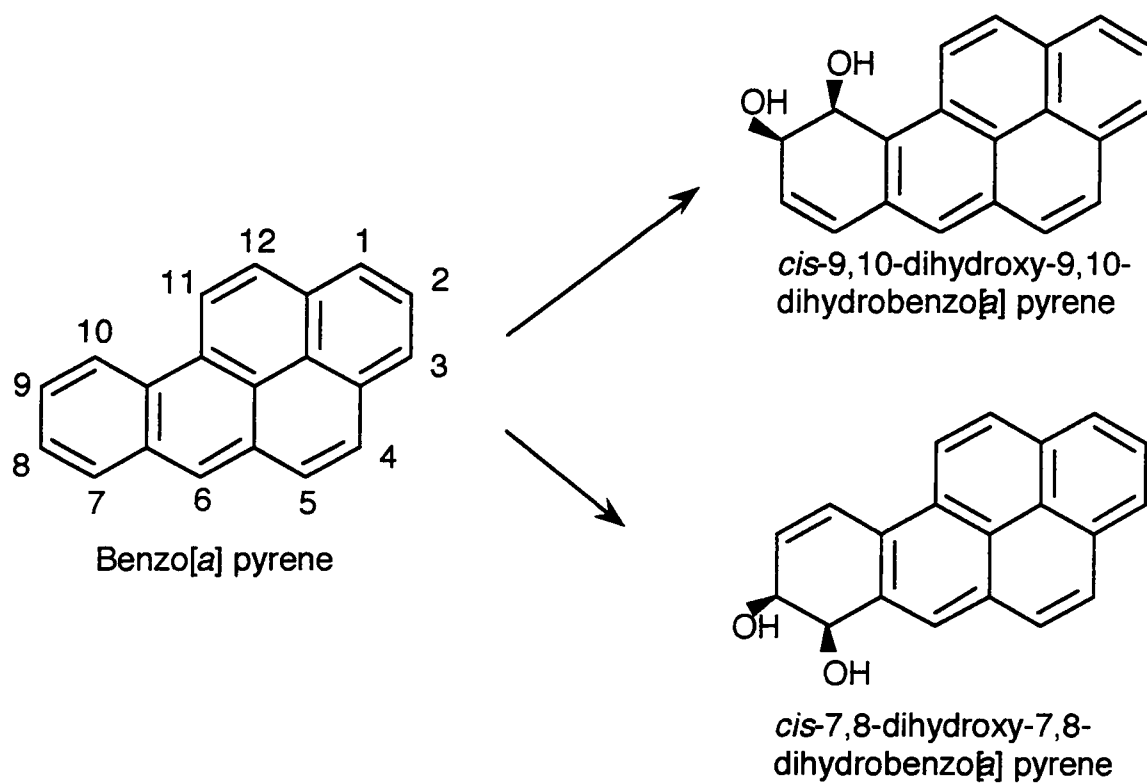


Figure 6. Bacterial oxidation of BaP (adapted from Gibson et al., 1975).

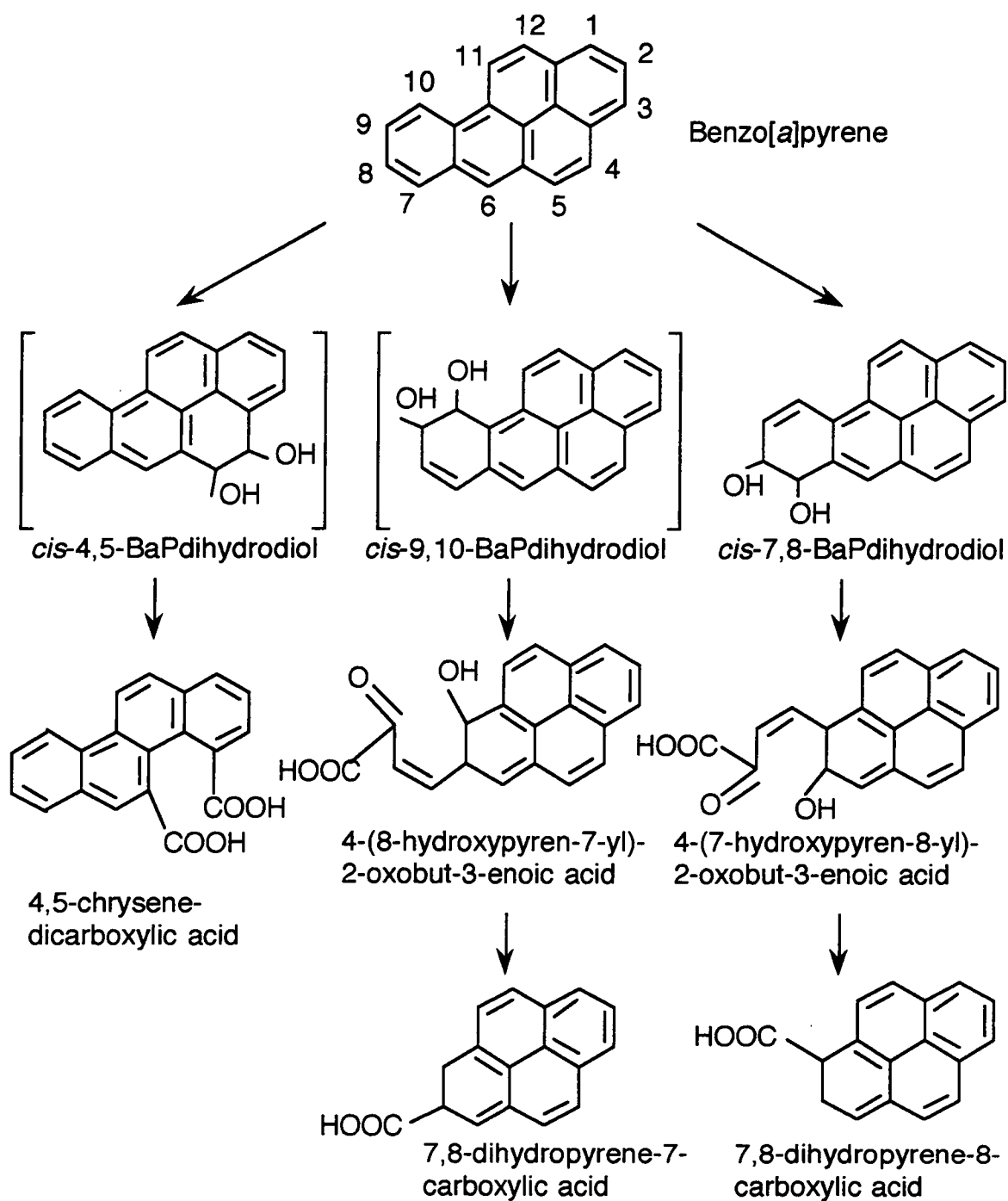


Figure 7. Proposed BaP degradation pathways by *Mycobacterium* sp. strain RJGII-135 (adapted from Schneider et al., 1996). Compounds in brackets are unidentified. No distinctive identifications were made between two hydroxypyrenyloxobutenoic acids and between two dihydropyrene carboxylic acids.

identified in the *Mycobacterium* culture. Although *cis*-4,5-BaPdihydrodiol was not identified, a ring cleavage product, 4,5-chrysene dicarboxylic acid implicated production of the metabolite by the *Mycobacterium* sp. The formation of 4,5-chrysene dicarboxylic acid suggested *ortho*-cleavage (intradiol cleavage) of the dihydroxylated BaP metabolite. The other ring fission products were from *meta*-cleavage of BaP dihydrodiol. However, no distinctive identifications were made between two hydroxypyrenyloxobutenoic acids [4-(8-hydroxypyrenyl-7-yl)-2-oxobut-3-enoic acid and 4-(7-hydroxypyrenyl-8-yl)-2-oxobut-3-enoic acid] and between two dihydropyrene carboxylic acids (7,8-dihydropyrene-7-carboxylic acid and 7,8-dihydropyrene-8-carboxylic acid). Although dioxygenase activity was suggested in the initial BaP degradation by the *Mycobacterium* sp., another *Mycobacterium* sp. strain PYR-1 produced both *trans*- and *cis*-dihydrodiols from pyrene (Heitkamp et al., 1988) and naphthalene (Kelley et al., 1990), suggesting involvement of both dioxygenase and monooxygenase in the initial attack.

Little is known about the extent of BaP mineralization by bacterial pure cultures (Grosser et al., 1991). Biological as well as physicochemical factors make the study of bacterial BaP degradation difficult. First of all, the extremely low water-solubility (3.8 µg/L at 25°C) of BaP complicates the study of BaP degradation and metabolite analysis. BaP adsorbs readily from aqueous solution onto the wall of its container. May et al. (1979) estimated that BaP solution of the concentration of a 1 µg/L decreased by 53% when it was kept in a glass vessel for an hour. Therefore, mineralization is better evidence for the degradation of BaP than decreased concentration of BaP. However, currently available ¹⁴C-BaP ([7-¹⁴C] or [7,10-¹⁴C]BaP) may yield limited information on BaP mineralization due to localized ¹⁴C label(s) at only one ring of five-ringed BaP (Fig. 6). Secondly, few BaP degraders have been isolated. To date, only four bacterial strains have been reported to be able to mineralize BaP. They are three *Sphingomonas* strains and one *Mycobacterium* strain: *Beijerinckia* sp. strain B1 (re-identified as a

Sphingomonas strain later, Wang & Lau, 1996), *Mycobacterium* sp. strain RJGII-135, A8AN3 (identified as a *Sphingomonas* strain in this study, Ahn et al., 1994), and *S. paucimobilis* EPA 505. The BaP degraders were isolated from a polluted stream (Gibson et al., 1973), a coal gasification site (Grosser et al., 1991), a MGP site (King et al., 1991), and a creosote waste site (Mueller et al., 1990), respectively. The strains were originally isolated as degraders of simpler compounds (e.g., biphenyl, anthracene, fluoranthene, and pyrene). Later their ability to mineralize BaP was demonstrated (Sherrill, 1982; Grosser et al., 1991; Ahn et al., 1994; Ye et al., 1996). A decline of BaP concentration was reported in pure culture of *Pseudomonas putida* NCIB 9816 (Barnsley, 1975). *Mycobacterium* sp. strain PYR-1 was reported to produce unidentified BaP metabolites (Heitkamp & Cerniglia, 1988). However, no mineralization of BaP has been observed with these strains.

A complete BaP degradation pathway has not been proposed to date. Attempts to identify other BaP metabolites have been unsuccessful, suggesting that BaP metabolites might be short-lived or could not be detected by the analytical methodology employed in previous studies. Bacteria capable of growing with BaP as the sole carbon and energy source or forming clear zones on BaP spray plates have not been reported. For better understanding of bacterial BaP-degradation, more BaP-degraders need to be isolated.

3.2 BaP degradation by eukaryotic microorganisms

The fungus *Cunninghamella elegans* degraded BaP to several metabolites. Major metabolites were sulfate and glucuronide conjugates and minor ones were dihydrodiols (*trans*-7,8-dihydrodiol and *trans*-9,10-dihydrodiol), quinones (1,6-quinone and 3,6-quinone), phenols (3-hydroxyBaP and 9-hydroxyBaP), and unidentified metabolites (Cerniglia & Gibson, 1979). Cytochrome P450 catalyzes the further oxidation of BaP

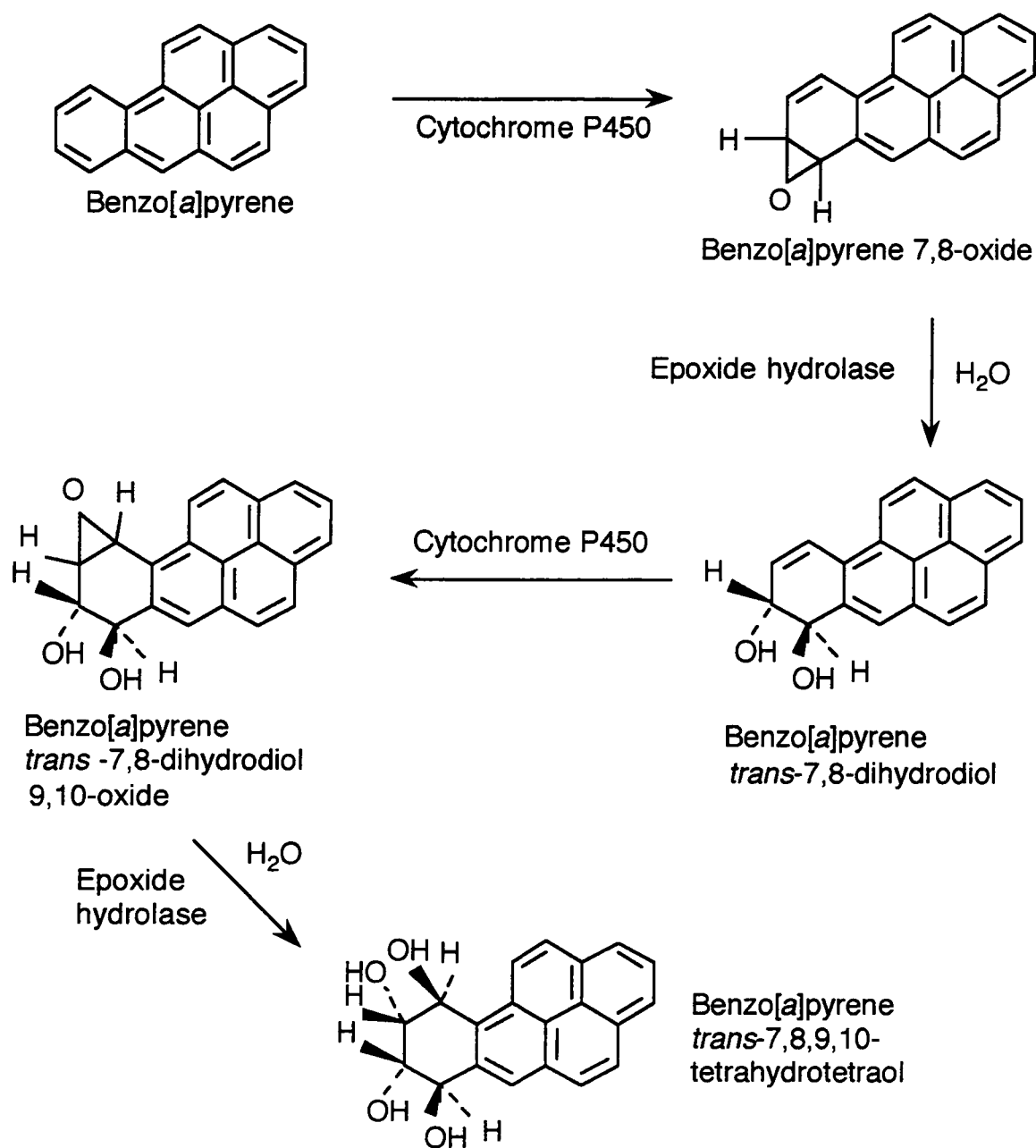


Figure 8. Formation of a carcinogenic dihydrodiol oxide, Benzo[a]pyrene *trans*-7,8-dihydrodiol 9,10-oxide, by *Cunninghamella elegans* (adapted from Cerniglia & Gibson, 1980a; Sutherland, 1992a).

trans-dihydrodiols and produces BaP *trans*-dihydrodiol oxides (Cerniglia & Gibson, 1980a) (Fig. 8). BaP *trans*-7,8-dihydrodiol 9,10-oxide has been implicated to be a carcinogenic and mutagenic metabolite of BaP in mammals (Williams & Weisburger, 1986; Sims et al., 1974). However, BaP *trans*-9,10-dihydrodiol 7,8-oxide was less mutagenic than the isomer (Cerniglia & Gibson, 1980b). Epoxide hydrolase catalyzes hydrolysis of dihydrodiol oxides to tetrahydrodiols that are less mutagenic than the dihydrodiol oxides (Cerniglia & Gibson, 1980b). Although a BaP degradation pathway by *Cunninghamella elegans* has been proposed (Fig. 8), the BaP degradation pathway by ligninolytic white rot fungi has not been established. *Phanerochaete chrysosporium* was reported to mineralize BaP (Bumpus et al., 1985; Aust, 1990). Metabolites, BaP 1,6-, 3,6- and 6,12-quinones were identified from the culture of *P. chrysosporium* (Haemmerli et al., 1986). Other white rot fungi (*Bjerkandera* sp., *Trametes versicolor*, and *Trametes* sp.) produced unknown BaP metabolites (Field et al., 1992; Morgan et al., 1993). After growth under non-ligninolytic conditions, the microsomal fraction of cytochrome P450 from *P. chrysosporium* produced a phenolic metabolite, 3-hydroxyBaP from BaP (Masaphy et al., 1996), suggesting more than ligninolytic enzyme system is involved in PAH degradation depending on environmental conditions.

Algal and yeast systems have shown their ability to degrade BaP, although BaP mineralization has not been reported. The green alga *Selenastrum capricornutum* oxidized BaP to *cis*-11,12-BaPdihydrodiol as a major metabolite (Lindquist & Warshawsky, 1985; Warshawsky et al., 1988). As minor metabolites, *cis*-9,10-, *cis*-7,8-, and *cis*-4,5-BaPdihydrodiols were also identified along with the major isomer. The formation of *cis*-dihydrodiols suggests a dioxygenase pathway similar to that found in prokaryotes rather than the monooxygenase pathway in other eukaryotes. Interestingly, the green alga produced sulfate and glucoside conjugates of the *cis*-4,5-BaPdihydrodiol (Warshawsky et al., 1990).

Although only few reports are available, BaP degradation by yeast seems to be similar to the cytochrome P450-mediated fungal BaP metabolism. *Saccharomyces cerevisiae* produced *trans*-7,8-BaPdihydrodiol from BaP as a major metabolite (Wiseman & Woods, 1979; Woods & Wiseman, 1979). *Candida lipolytica* degraded BaP to the phenolic compounds, 3-hydroxyBaP and 9-hydroxyBaP (Cerniglia & Crow, 1981).

**Part 2: Analyses of PAH-degrading Bacteria Isolated
from Contaminated Soils**

I. INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants. They are produced from incomplete combustion of organic materials (Benner et al., 1990; Edwards, 1983). Major sources of PAHs are anthropogenic such as fossil fuel combustion, automobile exhaust, waste incineration and coal tar. They are found in air, water, sediment (soil), food, cigarette smoke, and fossil fuels. (Harvey, 1991).

Although PAHs are ubiquitous, they are found at particularly high levels at coal gasification sites and wood treatment sites. Manufactured gas plants (MGP) generated gas from coal and oil from the mid-19th through the mid-20th century for public and industrial uses. It has been estimated that the United States had over 1,500 MGP sites during the time. The major waste products from the gasification process were tars and sludges mainly composed of PAHs (Linz et al., 1991). Cleanup of the MGP sites has become a priority: over 400 MGP sites were listed on the Superfund National Priority List (US EPA, 1985). PAH contamination in soil and ground water was reported at wood treatment facilities where creosote was used (Goerlit et al., 1985; Mueller et al., 1989). PAHs are major constituents of creosote, a commonly used wood preservative. Creosote contains approximately 85% PAHs by weight (Nestler, 1974). Bioremediation of PAH-polluted sites has received attention as an alternative to a traditional cleanup method of incineration.

Environmental concern exists about persistence and toxicity of PAHs, especially high-molecular-weight (HMW) PAHs. HMW PAHs (four- or more ring PAHs) are extremely insoluble in water and are persistent in the environment (Herbes, 1981; Heitkamp & Cerniglia, 1987; Shiaris, 1989). The water-solubilities of naphthalene (a two-ring PAH) and BaP (a five-ring PAH) are 31.7 mg/L and 3.8 µg/L at 25°C, respectively (Mackay & Shiu, 1977). Because of their hydrophobicity, they adsorb onto

soils and particulates, which limits their bioavailability and contributes to their persistence (Means et al., 1980; Weissenfels et al., 1992).

Many HMW PAHs such as BaP and benz[a]anthracene are known as potent carcinogens (Miller & Miller, 1981; Williams & Weisburger, 1986; Pahlman & Pelkonen, 1987). They are classified as (pro)carcinogens and require metabolic activation to produce their DNA-reactive metabolites in mammals (Sims et al., 1974; Booth & Sims, 1974; Phillips, 1983; Williams & Weisburger, 1986; Jernstrom & Graslund, 1994). Sixteen PAHs including BaP are listed on the US EPA priority pollutant list (Natl. Res. Council, 1991). BaP is listed on the IARC potent human carcinogen list (IARC, 1987). In spite of their persistence and toxicity, few studies on microbial degradation of HMW PAHs have been conducted. Together with recalcitrance of HMW PAHs due to their complex structures and low water-solubilities, this is also partly because of the fact that a few HMW PAH degraders have been isolated.

Instead of carcinogenic HMW PAHs, naphthalene has been the best studied of the PAHs in microbial system because it is the simplest and the most soluble PAH. Besides, naphthalene degraders are relatively easy to isolate. Information from the studies of bacterial degradation of naphthalene has been used for understanding and predicting the pathways in the degradation of the three- or more ring PAHs. Since naphthalene-degrading bacteria were first reported by Tausson (1927), the ubiquitous presence of naphthalene-degraders has been shown (Kiyohara et al., 1992; Kastner et al., 1994). The bacterial degradation of naphthalene has been well characterized for the naphthalene catabolic enzyme system encoded by the plasmid NAH7 of *Pseudomonas putida* PpG7 (Dunn & Gunsalus, 1973; Yen & Gunsalus, 1982; Yen & Serdar, 1988). The naphthalene degradation pathway has been determined (Davies & Evans, 1964; Yen & Gunsalus, 1982; Eaton & Chapman, 1992), and the genes involved have been isolated (Grund & Gunsalus, 1983; Schell, 1983). Based on biochemical and genetic information of

naphthalene degradation, NAH7-derived gene probes have been used to determine the *in situ* microbial potential to degrade naphthalene (Sayler & Layton, 1990; Fleming et al., 1993; Sanseverino et al., 1993b).

Recent studies has showed that NAH system can degrade not only naphthalene but also other compounds such as phenanthrene, anthracene, dibenzothiophene, fluorene, and methylated naphthalene (Menn et al., 1993; Sanseverino et al., 1993a; Denome et al., 1993; Yang et al., 1994; Kiyohara et al., 1994; personal communication with J. Leblond). The studies suggested that NAH7-derived gene probes could be used to determine the *in situ* microbial potential to degrade not only 2-ring PAHs but also 3-ring PAHs. However, many PAH-degraders were not detected by NAH7-derived gene probes (Foght & Westlake, 1988, 1991; King et al., 1991; Fredrickson et al., 1991; Ahn et al., 1994; Goyal & Zylstra, 1996). Even considering reports on numerous unculturable microorganisms in nature (Roszak et al., 1984; Ward et al., 1990), NAH7-derived gene probes may underestimate *in situ* microbial potential to catabolize PAHs. Moreover, NAH7-derived gene probes do not assure detection of HMW PAH degraders. Therefore, biochemical and genetic information of HMW PAH degradation is necessary for the development of new biotechnologies which can provide a better understanding of microbial activity in the environment.

Bioremediation is a process for the cleanup of hazardous pollutants using the metabolic versatility of microorganisms. The increasing application of bioremediation has been fostered by the knowledge of how specific chemicals are degraded by microorganisms. The isolation of microorganisms capable of degrading the target compounds, as well as biochemical and genetic information about the degradation, may lead to the development of new bioremediation technologies for contaminated sites. In this regard, a bacterial culture collection was characterized phenotypically and genotypically. The characterization was designed 1) to analyze genotypes and phenotypes

of PAH-degraders in the collection, 2) to screen HMW PAH degraders as sources for understanding of HMW PAH degradation, and 3) to screen nonNAH7-hybridizing PAH degraders as sources for the future development of new gene probes.

II. MATERIALS AND METHODS

1. Bacterial strains

Table 1 shows sources and characteristics of strains used in this study.

Pseudomonas putida strains PpG7 (NAH7) and PB2440 were employed for certain PAH-degradation tests as positive and negative controls, respectively. The strains were also used in the colony hybridization as positive and negative controls for NAH7-derived gene probes. *Sphingomonas paucimobilis* ATCC 29837 was used as a type strain for the identification of A8AN3. For motility test, strains PpG7 and *Rhodococcus* sp. Sm-1 (obtained from K. J. Malachowsky) were used as positive and negative controls, respectively.

A bacterial collection was used to analyze PAH-degrading bacteria. The collection consisted of 141 unidentified bacterial strains that were isolated as putative PAH-degraders (King et al., 1991). The bacteria in the collection were originally isolated from PAH-contaminated soils by previous workers (King et al., 1991) using the PAH spray plate method (Kiyohara et al., 1982) or naphthalene vapor plate method. PAHs used in the spray plate method were phenanthrene, anthracene, and pyrene. The naphthalene vapor plate method consisted of a minimal agar medium containing naphthalene crystals (sole carbon source) in the lid of the plate. The soil samples were from six MGP site soils and one creosote-contaminated site soil. The physical and chemical characterization of the soils was described in detail elsewhere (Cushey & Morgan, 1990). General soil characteristics are described in Table 2. Soils A - E, and N were from MGP sites. Soil G was the creosote-contaminated soil. The concentration of total organic carbon ranged from 4 ± 4 to 426 ± 789 g/kg soil. Total PAH concentration ranged from 233 ± 149 to $49,000 \pm 2,800$ mg/kg soil. BaP concentration was in the range

Table 1. Strains and plasmid used in the study.

Strain	Relevant characteristic	Reference
<i>P. putida</i>		
PB2440	<i>r⁻ m⁺ PIA⁺</i> ^a , plasmidless	Bagdasarian et al., 1981
PpG7(NAH7)	Nah ⁺ ^b	Dunn & Gunsalus, 1973
<i>S. paucimobilis</i>		
ATCC 29837	Type strain	ATCC
<i>Rhodococcus</i> sp. Sm-1		Malachowsky, 1992
Bacterial isolates	Putative PAH-degraders ^c	King et al., 1991

^a Growth on *Pseudomonas* isolation agar.

^b Ability to degrade naphthalene.

^c Isolated as putative PAH-degraders using the PAH spray plate method (Kiyohara et al., 1982) or the naphthalene vapor plate method. The naphthalene vapor plate method consisted of a minimal agar medium containing naphthalene crystals (sole carbon source) in the lid of the plate. Detailed information on each bacterial strain is in Appendix.

Table 2. Characteristics of soils used in the isolation of PAH degraders.

Soil	MGP					Creosote	
	Soil A	Soil B	Soil C	Soil D	Soil E	Soil N	Soil G
Date collected	4/20/89	3/27/89	10/12/89	Unknown	11/16/88	Unknown	10/9/89
pH	7.1	8.1	9.0	8.0	7.0		7.1
Total organic carbon (g TOC/kg soil)	426 ± 789	6 ± 1	33 ± 5	65 ± 91	4 ± 4	585	112 ± 33
Total PAHs (mg PAH/kg soil)	3,730 ± 907	233 ± 149	3,621 ± 1,866	334 ± 85	454 ± 190	19,200	49,000 ± 2,800
PAH (mg PAH/kg soil)							
Naphthalene	1,153 ± 481	11 ± 7	1,080 ± 553	28 ± 18	17 ± 24	321.3	4,100 ± 300
Phenanthrene	236 ± 209	17 ± 23	341 ± 367	26 ± 20	41 ± 61	220.2	9,200 ± 1,000
Anthracene	315 ± 141	33 ± 21	349 ± 214	29 ± 24	47 ± 60	653	8,800 ± 2,300
Pyrene	219 ± 141	22 ± 18	184 ± 103	32 ± 12	47 ± 63	87.7	3,200 ± 360
BaP	86 ± 39	3 ± 1	67 ± 30	8 ± 7	8 ± 8	64.9	330 ± 44
Silt (%)	4	2	38	17	3	8.8	20
Sand (%)	75	70	59	70	79	91.2	60
Gravel (%)	21	28	3	12	18	0	20

Source: Cushey & Morgan, 1990.

of 3 ± 1 to 330 ± 44 mg/kg soil. The bacterial collection was stored at -70°C before used for tests.

2. Chemicals and enzymes

All chemicals were purchased from Millinckrodt Chemical, Inc. (Paris, KY) unless otherwise specified. BaP (purity 99%) was obtained from Eastman Kodak Co. (Rochester, NY). The other PAHs were purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI). All solvents and chemicals were the highest purity available.

Except for ^{14}C -BaP, ^{14}C -labeled compounds were purchased from Sigma Chemical Company (St. Louis, MO). Radiochemical purities and specific activities of the compounds were as follows: $[1-^{14}\text{C}]$ naphthalene (10.1 mCi/mmol; purity, >98%), $[9-^{14}\text{C}]$ phenanthrene (10.4 mCi/mmol; purity, >99%), $[\text{U}-^{14}\text{C}]$ anthracene (15.0 mCi/mmol; purity, >98%), $[4,5,9,10-^{14}\text{C}]$ pyrene (10.4 mCi/mmol; purity, >98%), and $[\text{U}-^{14}\text{C}]$ salicylic acid (1-10 mCi/mmol; purity, >98%). $[7,10-^{14}\text{C}]$ BaP (60 mCi/mmol; purity, >98%) was obtained from Amersham (Arlington Heights, IL). $[\alpha-^{32}\text{P}]$ dCTP purchased from ICN Biomedicals Inc. (Irvine, CA) was used for radiolabeled gene probe preparations.

Restriction endonucleases were purchased from GIBCO BRL (Gaithersburg, MD). *GeneAmp*[®] PCR reagent kit with *AmpliTaq*[®] DNA polymerase was obtained from Perkin-Elmer Cetus (Norwalk, CT) was used for the polymerase chain reaction (PCR) in gene probe preparations.

3. Media and growth conditions

Minimal basal salts (MBS) medium (pH 7.2) was composed of (per liter) 4.0 g of

NaNO₃, 1.5 g of KH₂PO₄, 0.005 g of FeCl₃·6H₂O, 0.2 g of MgCl₄·7H₂O, 0.01 g of CaCl₂·2H₂O, and 0.5 g of Na₂HPO₄. MBS agar plates were prepared by adding 1.7% Noble agar (Difco Lab., Detroit, MI). Yeast extract-Polypeptone-Glucose (YEPG) medium (pH 7.0) contained (per liter) 0.2 g of yeast extract, 2.0 g of polypeptone, 1.0 g of glucose, and 0.2 g of NH₄NO₃. YEPG was used as a multipurpose growth medium. It was used at 25% strength for mineralization assays. Bacterial strains were grown in YEPG at 28°C.

To examine possible bacterial growth on PAHs, MBS medium containing a PAH (0.1%, w/v) as a sole carbon and energy source, the medium was prepared and used as follows: PAHs were dissolved in acetone (10%, w/v) to obtain PAH stock solutions. One ml of the stock solutions were added into the sterile 250 ml flasks. After total evaporation of the organic solvent, 100 ml of autoclaved MBS medium was added into the flasks. The flasks were shaken for 1 day at 28°C and 150 rpm in the dark. Cells were inoculated in the flasks and incubated at the same conditions. To prepare cells for the inoculation, cells grown in YEPG broth were harvested when the optical density of the culture was 0.8 at 600 nm. The cells were washed twice in MBS medium. The washed cells were resuspended in MBS medium and were inoculated into the flasks. The bacterial growth was monitored by measuring the OD at 600 nm with a Beckman DU-70 spectrophotometer.

4. DNA isolation and analysis

Total DNA was prepared by the procedure of Ausubel et al. (1989). Large-scale plasmid DNA was prepared by of an alkaline lysis method (Promega, 1992). The alkaline lysis method was modified for better recovery of HMW DNA: extracted DNA was directly subjected to ethanol precipitation without extraction steps with

phenol/chloroform and saturated chloroform. DNA was further purified by EtBr-CsCl gradient ultracentrifugation followed by butanol extraction and ethanol precipitation (Sambrook et al., 1989). For small-scale plasmid preparations, the boiling method (Holmes & Quigley, 1981) was employed. DNA was resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0) and stored at - 20°C until use.

For RFLP (restriction fragment length polymorphism) analysis, DNA was digested by restriction enzymes (*EcoRI*, *EcoRV*, and *BamHI*) as recommended by the manufacturer (Gibco BRL). Two µg of each sample plasmid DNA was digested by 1 µl (8-10 Unit) of each enzyme at 37°C for 2 h. Agarose gel electrophoresis was performed in the TBE buffer to separate DNA fragments as described (Sambrook et al., 1989). Gels were prepared with 0.8% agarose. Size markers were DNA fragments produced from *Lambda* DNA digested with *HindIII*.

5. Characterization of a bacterial culture collection

A bacterial culture collection was characterized phenotypically and genotypically as shown in Figure 1 (Menn et al., 1992). As phenotypic tests, PAH spray plate, ¹⁴C-PAH mineralization, and indigo tests were employed. As a genotypic test, DNA hybridization was performed with NAH7-derived gene probes.

5.1 Phenotypic tests

The PAH spray plate method (Kiyohara et al., 1982) was employed to test the PAH degradation ability of isolates in the collection. The PAHs used in the spray plate methods were phenanthrene, fluorene, anthracene, pyrene, and BaP. Bacterial colonies were pre-grown on YEPG agar plates and then sprayed with a 1% (w/v) PAH dissolved

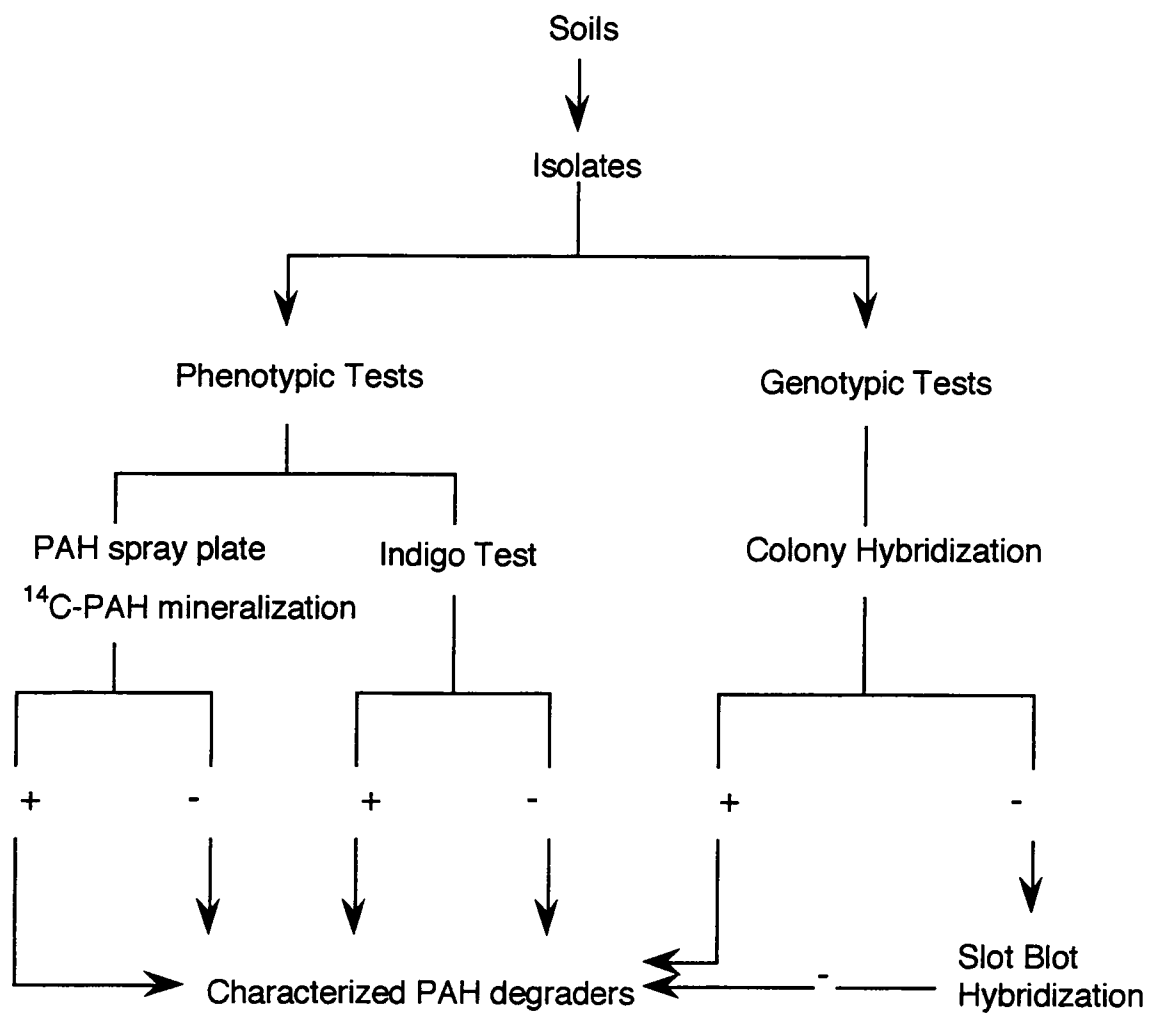


Figure 1. Flow diagram for the characterization of PAH degraders. Positive and negative results are indicated by + and -, respectively.

in acetone. The spraying resulted in an opaque layer on the surface of the plate. After evaporating the acetone by placing the plates in the hood for 1 minute, the plates were placed in plastic bags and incubated up to 3 weeks. The plates were observed daily. Colonies producing clear zones by degradation of the PAH were scored positive. As a negative control strain, *P. putida* PB2440 was used.

To test the ring cleavage of PAHs, ^{14}C -PAH mineralization (conversion of ^{14}C -PAHs to $^{14}\text{CO}_2$) assays were performed as follows. Cells were grown in 50 ml of YEPG medium to an OD_{600} of 0.8. The cells were washed once and resuspended in 25% YEPG broth to an OD_{600} of 0.4. Four ml of the suspension was added to a 25-ml EPA vial (Pierce, Rockford, IL). A CO_2 trap (8-ml vial) containing 0.5 ml of 0.5 M NaOH was placed inside the EPA vial. Before the EPA vial was sealed with a Teflon-lined silicone septum, an appropriate amount of ^{14}C -PAH was added: BaP, 65K dpm; pyrene, 14K dpm; naphthalene, 100K dpm; phenanthrene, 16K dpm; anthracene, 65K dpm. Figure 2 shows the numbering and ^{14}C -labeled positions of the test compounds. The assays were performed in triplicate. A negative control consisted of cells killed with 1 M H_2SO_4 . The vials were incubated at 28°C with shaking at 150 rpm for 7 days for BaP and pyrene and 3 days for the other PAHs. The assays were stopped by injecting 0.5 ml of 1 M H_2SO_4 through the septa.

Mass balance was performed to measure total recovery of ^{14}C at the end of the mineralization assays. This was achieved by measuring radioactivity from $^{14}\text{CO}_2$, the aqueous portion, and the ethyl acetate-extractable portion of the mineralization assay. After the assays were stopped, vials were shaken for 1 h to drive off $^{14}\text{CO}_2$ into the traps. Four ml of ethyl acetate was injected, and the vials were shaken for another 1 h. The NaOH solution in the CO_2 traps and 0.5 ml of the aqueous phase were removed and then added to scintillation vials containing 1 ml of distilled water. Beckman ReadySafe scintillation fluor (10 ml) was added to each vial. The organic (ethyl acetate) phase (0.5

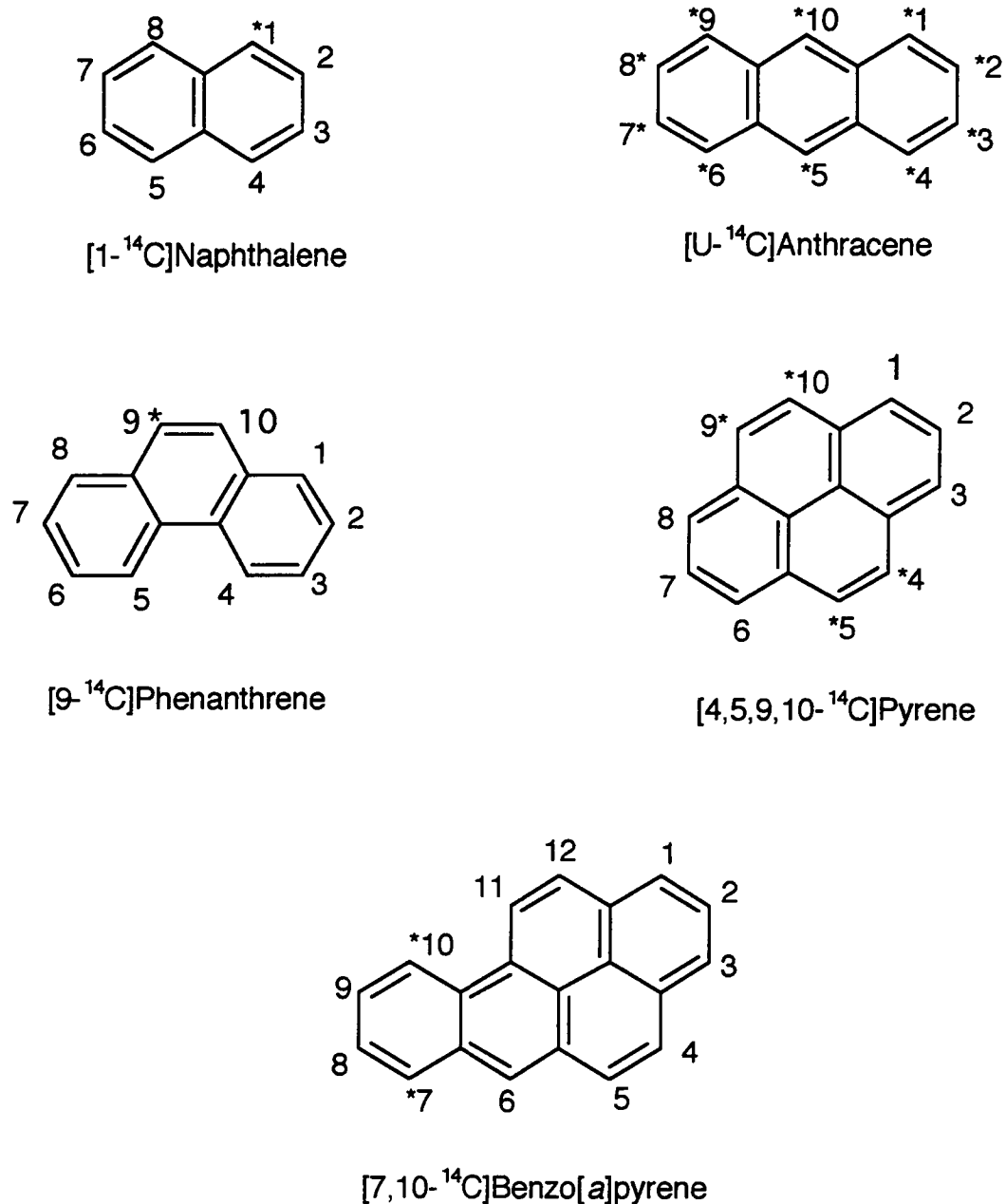


Figure 2. Positions of ¹⁴C labels in the PAHs used for mineralization assays. Numbering (Loening et al., 1990) and ¹⁴C location (*) are indicated.

ml) was added to 10 ml of Econofluor (Dupont) scintillation fluor. The scintillation vials were placed in the dark for 24 h before the measurement of their radioactivity. The ^{14}C -radioactivity was measured with a Beckman model LC5000 scintillation counter. To calculate the percentage of ^{14}C -PAHs mineralized, values from the killed control vials were subtracted as background.

The indigo test was used to examine aromatic ring dioxygenase activity because the formation of indigo from indole is presumptive for aromatic ring dioxygenase (Ensley et al., 1983). Dioxygenase activity of each isolate was examined by exposure to indole vapor. Bacterial colonies were pre-grown on YEPG agar plates, and then indole crystals were placed on the lid of the petri dish. After 1 day of incubation at room temperature, colonies that produced a blue color were scored positive. As positive and negative controls, *P. putida* strains PpG7 (NAH7) and PB2440 were used, respectively.

5.2 Genotypic tests

Gene probing was used to determine if the isolates contained NAH7-like PAH catabolic genes. All DNA hybridizations were performed as previously described (Church & Gilbert, 1984; Sanseverino et al., 1993). DNA was blotted on nylon membranes (ICN Biomedicals, Inc., Costa Mesa, CA). The membranes were then baked at 80°C for 1 h and stored at room temperature until use. One buffer (0.5 M NaH_2PO_4 , 1 mM EDTA, and 7% sodium dodecyl sulfate [SDS]; pH 7.2) was used for prehybridization and hybridization at 65°C. Washing of membranes were carried out at 65°C. For rehybridization, probes on the membranes was stripped by boiling in 0.1% SDS as manufacturer's protocol. Stripped membranes were monitored for complete removal of radiolabeled probes by extended autoradiography before rehybridization with different probes.

Colony hybridization was performed using NAH7-derived gene probes: *nahA* (encoding naphthalene dioxygenase), *nahG* (encoding salicylate hydroxylase), *nahH* (encoding catechol-2,3-dioxygenase), and *nahR* (encoding regulator protein). Probe *nahA* was used in the initial colony hybridization assay. Non*nahA*-hybridizing strains were further analyzed with the other NAH7-derived gene probes. For strains that did not hybridize with any of NAH7-derived gene probes, slot blot hybridization was used to confirm negative results from colony hybridization. Because the degree of cell lysis is variable depending on strains, poor or no cell lysis may yield false negative results from colony hybridization assays. Total DNA isolated from the bacteria was subjected to slot blot hybridization using the same probes. Negative and positive controls were total DNA from *P. putida* strains PB2440 and NAH7 DNA from *P. putida* PpG7, respectively. For slot blot hybridization, each total DNA sample was blotted onto a nylon membrane by using the slot blotting system (BioDot SF Microfiltration Apparatus, BioRad, Richmond, CA) in accordance with manufacturer's protocol. The blotted DNA on the membranes was subject to DNA hybridization. To prove the availability of the slot blotted DNA on the membranes, the membranes were rehybridized with a ³²P-labeled 23S rRNA gene probe (Festl & Schleifer, 1986) under low-stringency conditions: prehybridization, hybridization, and washing were performed at 45°C.

PCR amplification by DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk, CT) was employed to obtain the NAH7-derived gene probes: *nahA*, *nahG*, *nahH*, and *nahR*. Gene *nahA* encodes the first enzyme (naphthalene dioxygenase) of the naphthalene degradation pathway on plasmid NAH7. Genes *nahG* and *nahH* encode the enzymes at the branch points in naphthalene degradation pathways so far reported (Fig. 3), so that probes *nahG* and *nahH* determine further degradation of naphthalene metabolites. Plasmid NAH7 was used as a template to prepare the gene probes. Table 3 shows primers and PCR conditions used for probe preparations. The amplified products were then

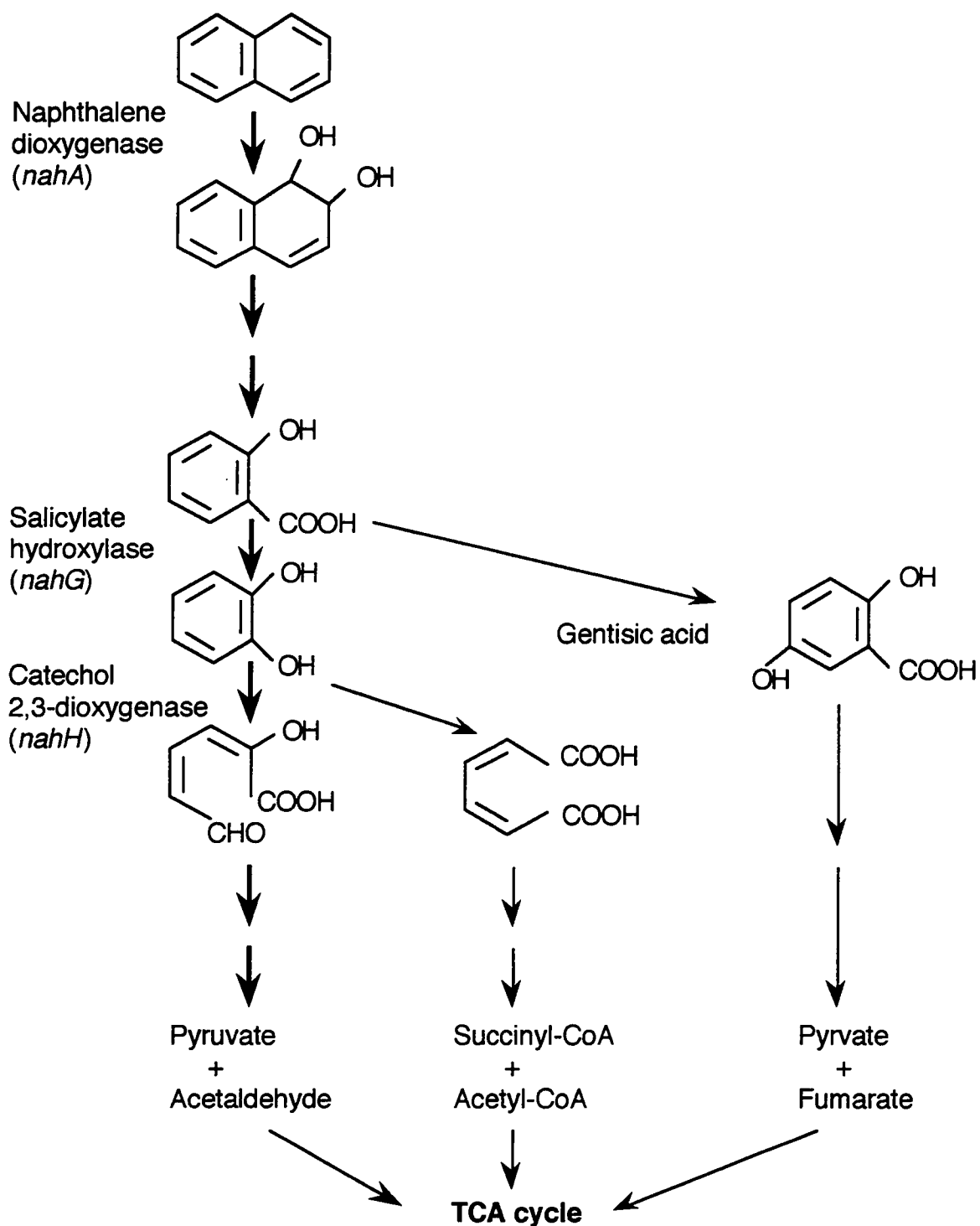


Figure 3. Naphthalene degradation pathways in bacteria (Davies & Evans, 1964; Starovoitov et al., 1975; Barnsley, 1976a ; Grund et al., 1992). Bold arrows indicate NAH7-encoded naphthalene degradation pathway.

Table 3. Preparations of gene probes used in genotypic characterization.

Probe (size)	Template	Primers (reference)	PCR condition ^a
<i>nahA</i> (1 kb)	NAH7	5'CCCTAGCGCGTAACTACCCC3' 5'GGTCCAGACCTCGGTGGTG3' (Simon et al., 1993)	94°C (1 min), 37°C (30 sec), & 72°C (1 min)
<i>nahG</i> (2.1 kb)	NAH7	5'GCGCATCGGTATCGTCGGCGGCGG3' 5'CGTGGCCGGGGCGCATTACACC3' (You et al., 1991)	94°C (1 min), 55°C (30 sec), & 72°C (3 min)
<i>nahH</i> (1 kb)	NAH7	5'GTATCGAGCGTTACTAGTAGCCG3' 5'CGTCCTAGGTCATAACGGTCATG3' (You et al., 1991; Ghosal et al., 1987)	94°C (1 min), 37°C (30 sec), & 72°C (1 min)
<i>nahR</i> (1.1 kb)	NAH7	5'ATGGAACTGCGTGACCTG3' 5'TCAATTCTCTCTATCCTGCG3' (You et al., 1988)	94°C (1 min), 37°C (30 sec), & 72°C (1 min)
23S rDNA (0.36 kb)	pHF360	5'AAGCTTGCTGGAGGTATC3' 5'TTGGAAGCATGGCATCAA3' (Festl & Schleifer, 1986)	94°C (30 sec), 42°C (30 sec), & 72°C (30 sec)

^a PCR conditions consisted of 30 cycles. Before the 30 cycles, preheating of 94°C (5 min) was employed. After the 30 cycles, final chain elongation was conducted at 72°C for 5 min.

detected by 1% agarose gel electrophoresis. The sizes of *nahA*, *nahH*, and *nahR* probes were about 1 kb each. The size of *nahG* probe was 2.1 kb. The *nahA* gene probe was derived from the gene locus encoding iron sulfur protein α (ISP α) of naphthalene dioxygenase. Multicomponent enzyme, naphthalene dioxygenase consists of flavoprotein, ferredoxin and ISP. ISP consists of ISP α and ISP β : $\alpha 2\beta 2$. ISP α contains the enzyme activity site (Ensley & Gibson, 1983; Furukawa et al., 1993). The 1 kb of *nahA* probe ranges about 30% of the whole genes (*nahAaAbAcAd*, approximately 3.5 kb in size) encoding naphthalene dioxygenase and 75% of the gene (*nahAc*) encoding ISP α . The *nahG*, *nahH*, and *nahR* probes cover almost of each gene. The *nahG* probe ranges from the start of *nahG* to the start of *nahH*.

One μ l of PCR-amplified product was used as a template for double amplification using the same PCR condition. Double amplified products were used as templates to make radiolabeled probes. Radiolabeled DNA probes were generated by asymmetric amplification using only one primer to generate single-stranded radiolabeled probes. ^{32}P -dCTP was used for the labeling. The labeled probes were purified using a NucTrap Push Column (Stratagene, La Jolla, CA). The specific activities of the probes were quantified by a Beckman model LS 5000 liquid scintillation counter. Specific activity of the labeled probes was approximately 10^8 disintegration per minutes (dpm) μg^{-1} of DNA. Plasmid pFH360 (Festl & Schleifer, 1986) was used as a template for 23S rDNA gene probe. The probe was prepared by the same procedure used to obtain NAH7-derived gene probes. The size of 23S rDNA gene probe was 360 bp.

6. Strain identification

The following methods were employed to identify strains: API Rapid NFT test, Biolog analysis (Bochner, 1989), and fatty acid analysis. As preliminary identification,

biochemical and growth characteristics were determined by API Rapid NFT kit (API analytab products, Ltd., Plainview, NY) according to the manufacturer's instruction. Gram staining and motility tests (semisolid agar and wet mount methods) were performed as described (Smibert & Krieg, 1981). Morphology of strains were examined by a light microscope (Nikon Optiphot, Nikon Inc., Tokyo, Japan). Biolog analysis was performed by Microbe Inotech Lab Inc. (St. Louis, MO) and Analytical Services Inc. (Williston, VT). Fatty acid analysis was performed by Microbe Inotech Lab Inc. (St. Louis, MO) and CEB (Center for Environmental Biotechnology) fatty acid analysis facility.

Pigments of cells were analyzed for fluorescence production and absorbance spectra. Fluorescence of pigments was tested on medium B of King et al. (1954). The absorbance spectra of pigments were examined as described by Holmes et al. (1977). Cells were grown in 10 ml of YEPG for 24 h and harvested by centrifugation. Cells were then resuspended and washed with sterile distilled water. The pigments were extracted from the cells by adding 5 ml of methanol and by heating to 50°C on a water bath for 5 min. Cell debris was removed by centrifugation. The absorbance spectra of the extracts in methanol were determined by Beckman DU-70 spectrophotometer. Yellow pigment extracted from *S. paucimobilis* ATCC 29837 was used as a positive control for nostoxanthin. In addition to all of the above assays, A8AN3 was further examined for sphingolipid, quinone, and flagellar morphology.

Sphingolipid analysis was carried out as described (Kawahara et al., 1991). Lyophilized cells (630 mg) were extracted with chloroform:methanol (2:1, v/v). The resulting residue was further extracted with methanol:chloroform (3:1, v/v) at 80°C for 1 h. The extracts were then fractionated using preparative TLC (thin layer chromatography) with stepwise elution using methanol:chloroform mixtures. The obtained fractions were screened for sphingolipids by 2-hydroxy (2-OH) fatty acid and long-chain base (dihydro-sphingosine) analyses using GC-MS (gas chromatography-mass spectrometry). Before

the analysis by GC-MS, dihydrosphingosine and 2-OH fatty acid were trimethyl-silylated with BSTFA [*N,O*-bis(trimethylsilyl)trifluoroacetamide] (Pierce Chemical Co., Rockford, IL). Samples were injected into a Hewlett-Packard HP5890 series II GC interfaced with Hewlett-Packard HP5971 mass selective detector. The GC was equipped with a 60-m Restek Rt_X-1 capillary column (inside diameter, 0.2 mm; film thickness, 0.1 µm). The column was programmed from an initial temperature of 100 to 150°C, held isothermally for 1 min, and then increased by 3°C/min to 280°C at which it was held isothermally for 3 min. The mass detector was run at 70 eV with positive ion electron impact ionization.

Isoprenoid quinone was analyzed as described (Collins et al., 1985). Lyophilized cells (50 mg) were extracted with chloroform:methanol (2:1, v/v) for 2 h. The solvent was collected by filtering and evaporated to dryness using a rotary evaporator at 40°C. The dried residue was resuspended in chloroform:methanol (2:1, v/v) and spotted onto a silica gel 60 (F254) plate (Aldrich Chemical Co., Milwaukee, WI). Chromatography was developed in hexane:diethyl ether (8:2, v/v) and observed under UV light (254 nm). A band within *R_f* 0.3 - 0.4 was recovered as ubiquinone by scraping off the band. The recovered band was eluted with 1 ml of chloroform and dried under N₂ gas. The residue was analyzed by high pressure liquid chromatography (HPLC). Spherisorb ODS (5 µm) column was employed for the separation of the residue using methanol:1-chlorobutane (10:1, v/v) as mobile phase (1.5 ml/min). Ubiquinones were monitored with UV detector at 270 nm.

The presence and arrangement of flagella were determined with a transmission electron microscope (Univ. of Tennessee Dept. of Zoology EM Facility). Cells were grown to mid-log phase in YEPG broth. Cells were prepared on copper grids coated with formvar and carbon. The cell preparation was negatively stained with 0.5% (w/v) phosphotungstic acid (pH 7.0) and observed with a Hitachi H-600 electron microscope (Hitachi Co., Tokyo, Japan).

III. RESULTS

1. Phenotypic characterization of a bacterial culture collection

Putative PAH degraders in a bacterial culture collection were phenotypically characterized by the use of PAH spray plate, mineralization, and indigo tests. The strains in the collection were previously isolated from PAH-contaminated soils (King et al., 1991) using the PAH spray plate method or the naphthalene vapor plate method. For phenotypic characterization of the strains, each of the 141 strains was subjected to the spray plate method and the indigo test. Fifty-nine randomly selected strains were subjected to the ^{14}C -naphthalene mineralization test; this study was focused on HMW PAH degraders and a convenient screening method, the spray plate method was not suitable for naphthalene degradation test because of its fast vaporization. Further mineralization assays were performed to determine ring-cleavage activities of non*nahA*-hybridizing strains or HMW PAH degraders. Phenanthrene, anthracene, pyrene, and BaP were employed for the further mineralization assays.

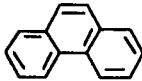
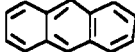
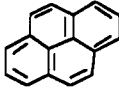
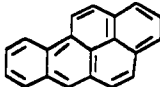
Among a total of 141 bacteria isolated previously as putative PAH-degraders (King et al., 1991), 89 strains showed PAH degradation ability as determined by PAH spray plate method and ^{14}C -naphthalene mineralization assay in this study. Not all the strains were subjected to the naphthalene degradation test in this study; therefore, the other 52 strains could be naphthalene degraders. It is also possible that the 52 strains are not PAH degraders because the strains in the collection were isolated by previous workers using the PAH spray plate method or the naphthalene vapor plate method. The methods occasionally produce false results due to overspreading of clear zones to other bacterial colonies which do not degrade PAHs in the spray plate method or cross-feeding of strains on the naphthalene vapor plates.

The results of the phenotypic tests suggested that the PAH degraders in the collection showed multiple phenotypes in terms of PAH degradation (Table 4). The majority of the naphthalene degraders from the collection were able to degrade 3-ring PAHs as determined by the spray plate method and the ^{14}C -naphthalene mineralization assays. All naphthalene degraders (27 strains) and anthracene degraders (13 strains) formed clear zones on the phenanthrene spray plates.

A few HMW PAH degraders were found: three pyrene-degraders were found; one of the pyrene degraders could mineralize BaP. The HMW PAH degraders could degrade a broad range of PAHs (i.e., naphthalene, fluorene, phenanthrene, anthracene, and pyrene) as determined by the spray plate method or ^{14}C -PAH mineralization assay (Table 4, 6, and 7). Three strains (A8AN3, B1PH6, and N1-4PH) produced clear zones on the pyrene spray plates (Fig. 4). They mineralized ^{14}C -PAHs used for the spray plate method (fluorene mineralization was not tested). However the pyrene degraders did not mineralize [4,5,9,10- ^{14}C]pyrene in a month. Strains B1PH6, and N1-4PH did not mineralize [9- ^{14}C]phenanthrene in 3 days (Table 7), although they formed clear zones on phenanthrene spray plates (Table 6), suggesting no ring-cleavage or unlabeled-ring cleavage in the incubation time. Among the pyrene degraders, A8AN3 could mineralize [7,10- ^{14}C]BaP although the strain did not produce clear zones on BaP spray plates. No strain produced clear zones on the BaP spray plates. In the spray plate assays, the time required for the formation of clear zones was as follows: 1 day for fluorene, 2-3 days for phenanthrene, 3-4 days for anthracene, and 3 weeks for pyrene. All 3 HMW PAH-degraders showed dioxygenase activity, as judged by indole conversion to indigo.

The results of the indigo test indicated that 98% (87/89) of the PAH degraders showed dioxygenase activity (Table 5). Of the 141 strains in the collection, 109 strains were positive in the indigo test. Among 109 Ind⁺ strains, 87 strains were PAH-degraders in this study. The remaining 22 strains (Ind⁺) could be naphthalene degraders because not

Table 4. Multiple phenotypes of PAH degraders.

Assay (positive strains/tested strains)	Phenotype	Number of strains	
Mineralization			
¹⁴ C-Naphthalene (27/59)	Nah ⁺ Phn ⁺	6	(22) ^a
	Nah ⁺ Phn ⁺ Flu ⁺	13	(48)
	Nah ⁺ Phn ⁺ Ant ⁺	1	(4)
	Nah ⁺ Phn ⁺ Flu ⁺ Ant ⁺	5	(19)
	Nah ⁺ Phn ⁺ Flu ⁺ Ant ⁺ Pyr ⁺	2	(7)
	Spray Plate		
Fluorene (35/141)	Flu ⁺	6	(17)
	Flu ⁺ Phn ⁺	22	(63)
	Flu ⁺ Phn ⁺ Ant ⁺	4	(11)
	Flu ⁺ Phn ⁺ Ant ⁺ Pyr ⁺	3	(9)
	Phenanthrene (83/141)		
	Phn ⁺	50	(60)
	Phn ⁺ Flu ⁺	21	(25)
	Phn ⁺ Ant ⁺	4	(5)
	Phn ⁺ Flu ⁺ Ant ⁺	5	(6)
	Phn ⁺ Flu ⁺ Ant ⁺ Pyr ⁺	3	(4)
Anthracene (13/141)			
	Ant ⁺ Phn ⁺	5	(38)
	Ant ⁺ Phn ⁺ Flu ⁺	5	(38)
	Ant ⁺ Phn ⁺ Flu ⁺ Pyr ⁺	3	(23)
Pyrene (3/141)			
	Pyr ⁺ Phn ⁺ Flu ⁺ Nah ⁺ Ant ⁺	3	(100)
	Benzo[a]pyrene (0/141)		
		0	

^a Percentage of the strains that positively scored for the indicated phenotype.

Abbreviations: Nah, naphthalene; Flu, fluorene; Phn, phenanthrene; Pyr, pyrene; Ant, anthracene.

A

B



A

C



Figure 4. Clear zone formation on pyrene spray plates. A, *Pseudomonas putida* PB2440; B, A8AN3; C, B1PH6; D, N1-4PH. Cells grown in YEPG broth were harvested and suspended in YEPG. The suspended cells were spotted on YEPG plates. After 2 day incubation of the plates at 28°C, pyrene was sprayed over the plates and the plates were incubated. The pictures were taken on the 24th day after the spraying.

A

D



Figure 4. (Continued).

Table 5. Comparative analysis of PAH-phenotypic tests and *nahA* gene probing.

Phenotypic tests	Number of strains			
	Tested	Positive	<i>nahA</i> Probe positive ^a (%)	<i>nahA</i> Probe negative (%)
Indigo test	141	109	95 (87)	14 (13)
Spray plate				
Anthracene	141	13	10 (77)	3 (23)
Phenanthrene	141	83	70 (84)	13 (16)
Fluorene	141	35	31 (89)	4 (11)
Pyrene	141	3	2 (67)	1 (33)
Benzo[<i>a</i>]pyrene	141	0	0	0
¹⁴ C-Naphthalene mineralization	59	27	21 (78)	6 (22)

^a Of 141 strains, 96 strains showed positive from colony hybridization using *nahA* probe.

Table 6. Phenotypes and genotypes of selected strains.

Strain	Indigo test	<i>nah</i> Probes ^a				¹⁴ C-PAH mineralization				Spray plate		Growth on PIA ^b
		A	G	H	R	Nah	Phn	Ant	BaP	Flu	Pyr	

Non <i>nahA</i> -hybridizing HMW PAH degrader												
A8AN3	+	-	-	-	-	+	+	+	+	+	+	-

Non <i>nahA</i> -hybridizing PAH degraders												
A5PH1	+	-	-	-	-	+	+	-	-	-	-	-
B1PH2	+	-	-	-	-	+	+	+	-	+	-	-
B10AN1	+	-	-	-	-	+	+	-	-	-	-	-

HMW PAH degraders												
B1PH6	+	+	*	*	*	+	-	+	-	+	+	*
N1-4PH	+	+	+	*	*	+	-	+	-	+	+	*

^a NAH7-derived gene probes.

^b *Pseudomonas* isolation agar. * Not determined.

Abbreviations: Nah, naphthalene; BaP, benzo[*a*]pyrene; Phn, phenanthrene; Ant, anthracene; Flu, fluorene; Pyr, pyrene.

Table 7. Mineralization of ^{14}C -PAHs by selected strains.

Strain	% $^{14}\text{CO}_2$ recovered from ^{14}C -labeled:				
	Nah	Phn	Ant	Pyr	BaP
A5PH1	60.6 ± 3.3^a (96.1 ± 2.1) ^b	11.2 ± 0.7 (98 ± 3.2)	0	0	0
A8AN3	74.0 ± 6.2 (93 ± 5.7)	53.3 ± 15 (119 ± 7.3)	17.0 ± 2.7 (102 ± 1.2)	0	45.0 ± 2 (91.0 ± 0.6)
B1PH2	61.2 ± 1.2 (97.2 ± 3.6)	57.0 ± 0.6 (104 ± 1.2)	13.1 ± 0.3 (95 ± 2.1)	0	0
B1PH6	56 ± 0.8 (83.4 ± 2.6)	0	14.8 ± 2.4 (87.7 ± 3.2)	0	0
B10AN1	46.1 ± 1.7 (87 ± 2.6)	9.0 ± 0.3 (97.1 ± 0.5)	0	0	0
N1-4PH	60 ± 2.1 (95.1 ± 2.7)	0	12.3 ± 0.9 (97.1 ± 2.3)	0	0
PB2440 ^c	0	0	0	0	0

^a Data are given as mean $\pm$ standard deviation (n=3).

^b Total ^{14}C recovery (expressed as percentages). ^c *P. putida* PB2440.

Abbreviations: Nah, naphthalene; BaP, benzo[*a*]pyrene; Phn, phenanthrene; Ant, anthracene; Flu, fluorene; Pyr, pyrene.

all 141 strains were subjected to the naphthalene degradation test in this study. Of the 22 strains could be MAH (monocyclic aromatic hydrocarbon) degraders. Aromatic ring dioxygenases are reported to convert indole to indigo (Ensley et al., 1983); besides, PAH degraders (Ensley et al., 1983; Denome et al., 1993; Kiyohara et al., 1994), MAH degraders contain dioxygenases that can convert indole to indigo (Ensley et al., 1983; Eaton & Timmis, 1986; Zylstra et al., 1989; Tan & Mason, 1990). Although indole-converting ability to indigo is known as dioxygenase activity, Yen et al. (1991) reported that toluene-4-monooxygenase could also produce indigo from indole. Of 32 Ind⁻ strains in the culture collection, 2 strains were PAH degraders as determined by the phenanthrene spray plate method, suggesting no or low (under detection limit) dioxygenase activity under the conditions used.

2. Genotypic characterization of a bacterial culture collection

Gene probing was used to determine if the isolates contained NAH7-like PAH catabolic genes. Overall, the PAH-degrading strains from the culture collection showed a positive hybridization response to the *nahA* probe. Of the 141 strains examined, 96 strains tested positive from the *nahA* gene probing (Table 5). Of the 96 *nahA*⁺ strains, 76 strains were PAH degraders in this study, indicating that 85% (76/89) of culturable PAH-degraders were detected by the *nahA* gene probe: of the 141 strains in the collection, 89 strains were PAH degraders in this study. Because the PAH degradation phenotype in this study was based mainly on PAHs with more than 2-rings, the percentage could be higher if all 141 strains were subjected to the naphthalene-degradation test; only 59 randomly selected strains were subjected to the naphthalene degradation test. The *nahA* gene probe could detect 78% of naphthalene-degraders. The probe also detected bacteria that could degrade more than 3-ring PAHs: 89% of fluorene-degraders; 84% of phenanthrene-

degraders; 77% of anthracene degraders; 67% of pyrene degraders. However, BaP degraders were not detected by the probe. Almost all of the strains (99%, 95/96) detected by the *nahA* probe showed dioxygenase activity as determined by the ability to convert indole to indigo. However, 13% (14/109) of strains showing dioxygenase activity were not detected by the gene probe, suggesting that the strains contained different dioxygenase genes.

Some PAH-degraders were not detected by *nahA* gene probing: roughly, as molecular weight (number of rings) of PAHs increases, percentage of detection decreases. The followings are percentages of PAH-degraders that were not detected by the *nahA* gene probe: 22% of naphthalene-degraders; 11% of fluorene-degraders; 16% of phenanthrene-degraders; 23% of anthracene-degraders; 33% of pyrene-degraders. Fifteen % of PAH-degraders in the culture collection did not hybridize with the *nahA* probe: 13 strains out of 89 PAH-degraders. Four PAH-degraders did not hybridize with any of the NAH7-derived gene probes (*nahA*, *nahG*, *nahH*, and *nahR*): A5PH1, A8AN3, B1PH2, and B10AN1 (Fig. 5, 6, 7, and 8) under high (65°C)-stringency conditions. The strains did not hybridize with the *nahA* probe under low (55°C)-stringency conditions, suggesting dissimilar PAH degradative genes in the strains. As a positive control for the presence of DNA, the filter was probed with a 23S rDNA probe under low stringency hybridization condition (Fig. 9).

Table 6 illustrates genotype and phenotypes of selected *nahA*⁻ PAH-degraders or HMW PAH degraders. All of the 6 strains showed aromatic ring dioxygenase activities: Ind⁺. ¹⁴C-PAH mineralization by the 6 strains is shown in Table 7. Four non*nahA*-hybridizing strains could mineralize more than 72% of ¹⁴C-salicylate added (33K dpm) in 25% YEPG broth. However, they did not grow on minimal medium containing sodium salicylate (0.1%, w/v) as a sole carbon and energy source, suggesting that there might be a problem associated with transfer of salicylate from medium into the cells. The 4 *nahA*⁻

strains did not grow on *Pseudomonas* isolation agar plates (PIA). PIA contains a broad spectrum antibiotic, Irgasan (Furia & Schenkel, 1968). The strains were also sensitive to following antibiotics: kanamycin, 50 µg/ml; ampicillin, 100 µg/ml; tetracycline, 15 µg/ml; rifampicin, 75 µg/ml. Among the 6 strains of *nahA*⁻ PAH-degraders or HMW PAH degraders, A8AN3 was a HMW-PAH-degrader that did not hybridize any of the NAH7-derived gene probes.

Restriction endonuclease analysis was conducted using plasmids isolated from the four PAH-degraders which did not hybridize with any of the NAH7-derived gene probes. Plasmids often carry the genetic information for the degradation of aromatic hydrocarbons in bacteria (Dun & Gunsalus, 1973; Eaton & Timmis, 1986; Ghosal et al., 1987; Tan & Mason, 1990; Wallace & Sayler, 1992; Denome et al., 1993). All of the 4 strains harbored plasmids. A8AN3 harbored more than 2 plasmids, while each of three strains appeared to contain a single plasmid. Plasmid DNA isolated from the strains were subjected to restriction enzyme digestion. The length of DNA fragments produced by restriction enzymes (*EcoRI*, *EcoRV*, and *BamHI*) were compared to that of DNA fragments from NAH7 digestion. The plasmids from 4 strains produced different restriction patterns from that of NAH7 (Fig. 10). Gene probing (Fig. 5 - 9) and RFLP analysis may suggest dissimilar genes or gene organization in the 4 strains.

3. Identification of selected non*nahA*-hybridizing PAH-degraders

Four non*nahA*-hybridizing strains were subjected to identification. Because they did not hybridize with any of NAH7-derived gene probes, identification of the strains was of interest. All four strains were Gram-negative rods and formed yellow colonies. Except A5PH1, three strains showed motility (Table 8). The API Rapid NFT test produced initial identification results of the 4 non*nahA*-hybridizing strains (Table 8). Except A5PH1,

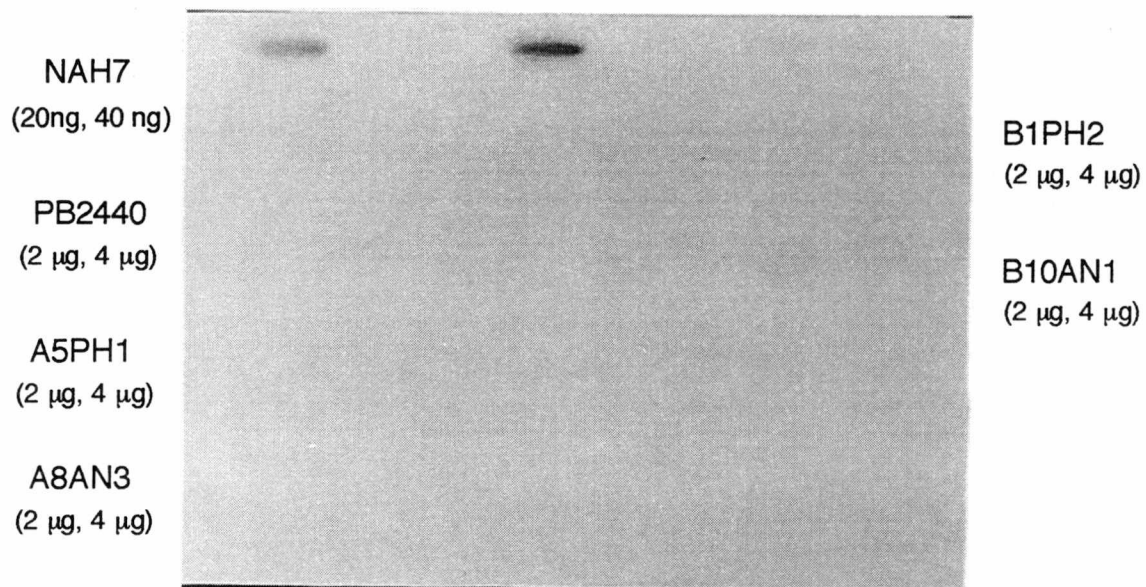


Figure 5. Slot blot hybridization probed with *nahA* probe. This was performed to confirm *nahA*⁻ genotype of four strains (A5PH1, A8AN3, B1PH2, and B10AN1) in colony hybridization. NAH7, plasmid NAH7 DNA; PB2440, *P. putida* PB2440. NAH7 DNA and *P. putida* PB2440 total DNA were used as positive and negative controls, respectively. Sample DNA was total DNA isolated from each isolate. The amount of DNA used for the hybridization was indicated in parentheses.

NAH7
(20ng, 40 ng)

PB2440
(2 µg, 4 µg)

A5PH1
(2 µg, 4 µg)

A8AN3
(2 µg, 4 µg)

B1PH2
(2 µg, 4 µg)

B10AN1
(2 µg, 4 µg)

Figure 6. Slot blot hybridization probed with *nahG* probe. This was performed to confirm *nahG*⁻ genotype of four strains (A5PH1, A8AN3, B1PH2, and B10AN1) in colony hybridization. NAH7, plasmid NAH7 DNA; PB2440, *P. putida* PB2440. NAH7 DNA and *P. putida* PB2440 total DNA were used as positive and negative controls, respectively. Sample DNA was total DNA isolated from each isolate. The amount of DNA used for the hybridization was indicated in parentheses.

NAH7
(20ng, 40 ng)

PB2440
(2 µg, 4 µg)

A5PH1
(2 µg, 4 µg)

A8AN3
(2 µg, 4 µg)

B1PH2
(2 µg, 4 µg)

B10AN1
(2 µg, 4 µg)

Figure 7. Slot blot hybridization probed with *nahH* probe. This was performed to confirm *nahH*⁻ genotype of four strains (A5PH1, A8AN3, B1PH2, and B10AN1) in colony hybridization. NAH7, plasmid NAH7 DNA; PB2440, *P. putida* PB2440. NAH7 DNA and *P. putida* PB2440 total DNA were used as positive and negative controls, respectively. Sample DNA was total DNA isolated from each isolate. The amount of DNA used for the hybridization was indicated in parentheses.

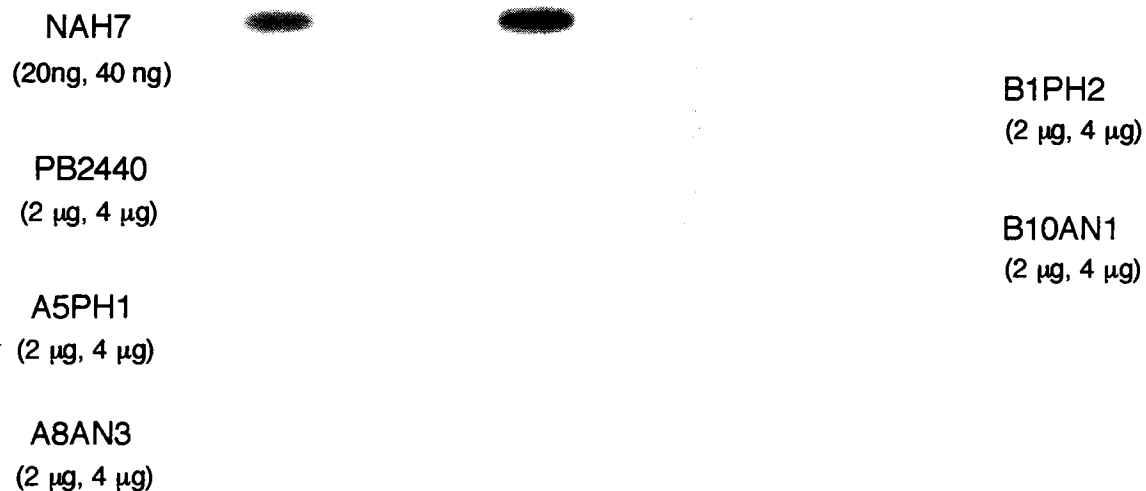


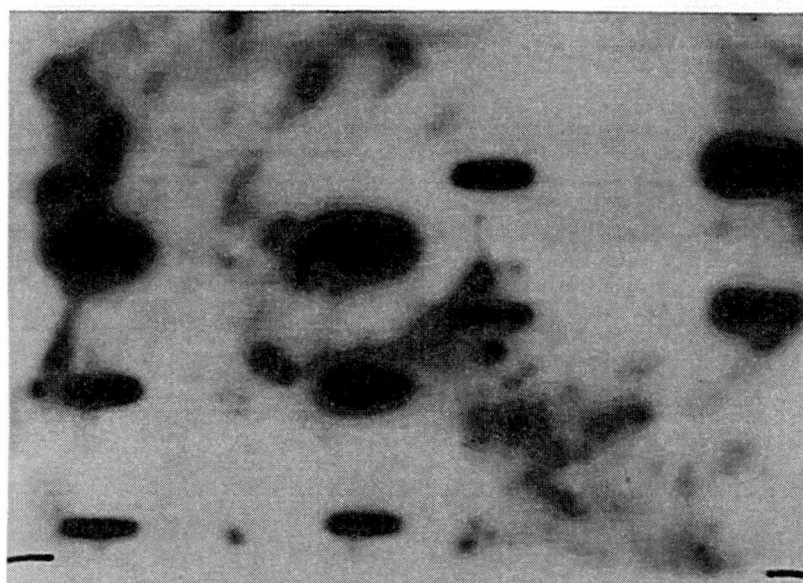
Figure 8. Slot blot hybridization probed with *nahR* probe. This was performed to confirm *nahR*⁻ genotype of four strains (A5PH1, A8AN3, B1PH2, and B10AN1) in colony hybridization. NAH7, plasmid NAH7 DNA; PB2440, *P. putida* PB2440. NAH7 DNA and *P. putida* PB2440 total DNA were used as positive and negative controls, respectively. Sample DNA was total DNA isolated from each isolate. The amount of DNA used for the hybridization was indicated in parentheses.

NAH7
(20ng, 40 ng)

PB2440
(2 μ g, 4 μ g)

A5PH1
(2 μ g, 4 μ g)

A8AN3
(2 μ g, 4 μ g)



B1PH2
(2 μ g, 4 μ g)

B10AN1
(2 μ g, 4 μ g)

Figure 9. Slot blot hybridization probed with 23S rDNA probe. It was to confirm the presence of sample DNA on the filter used in NAH7-derived gene probing. The hybridization was conducted under low stringency conditions as described in the text.

three strains yielded acceptable or good identification (id) from the test: A5PH1, *Flavobacterium indologenes* (unacceptable id); A8AN3, *Sphingomonas paucimobilis* (acceptable id); B1PH2, *S. paucimobilis* (good id); B10AN1, *Pseudomonas vesicularis* (acceptable id). Two pyrene degraders (B1PH6 and N1-4PH) were identified as *Pseudomonas fluorescens* (unacceptable id) by the API tests (data not shown).

The API kit is a commercial mutitest system containing 20 biochemical and growth tests for the identification of Gram-negative nonfermentative bacteria. The kit contains 20 cupules of dehydrated substrates for biochemical tests to determine enzyme activity and growth tests to judge growth in single carbon sources. Saline-suspended cells are added to the cupules and incubated for 24 - 48 h at 30°C. Biochemical tests yield metabolic end products (e.g., acids) which produce color change (due to pH indicator) as an indication of enzyme activity. The growth tests are based on turbidity formation due to cell growth in the presence of single sources of carbon. The quantity of each carbon source is in the range of 0.8 - 2.3 mg (API analytab products, Ltd., Plainview, NY). Since approximately 100 µl of cell suspension is added to the each dehydrated carbon source, the concentration of carbon sources are much higher than real environment. Moreover, monomeric sugars (e.g., D-glucose) and amino acids are rarely found in natural environments and their concentrations are low if found (Paul & Clark, 1989). Therefore, environmental isolates may produce variations resulting in poor matches. However, the kit has proved useful for the identification of clinical isolates (Appelbaum & Leathers, 1984; Von Graevenitz & Zollinger-Iten, 1985) for which the kit was originally designed. Although the test kit is environmentally not relevant, it was used for a preliminary identification method due to its convenient and fast procedure.

Biolog analyses of the 4 strains were performed by Microbe Inotech Lab Inc. (September, 1992). The results were shown in Table 9. A5PH1 was identified as *Brucella abortus* biovar 2. Absence of 3-OH fatty acids in A5PH1 (Table 10) rejected the result.

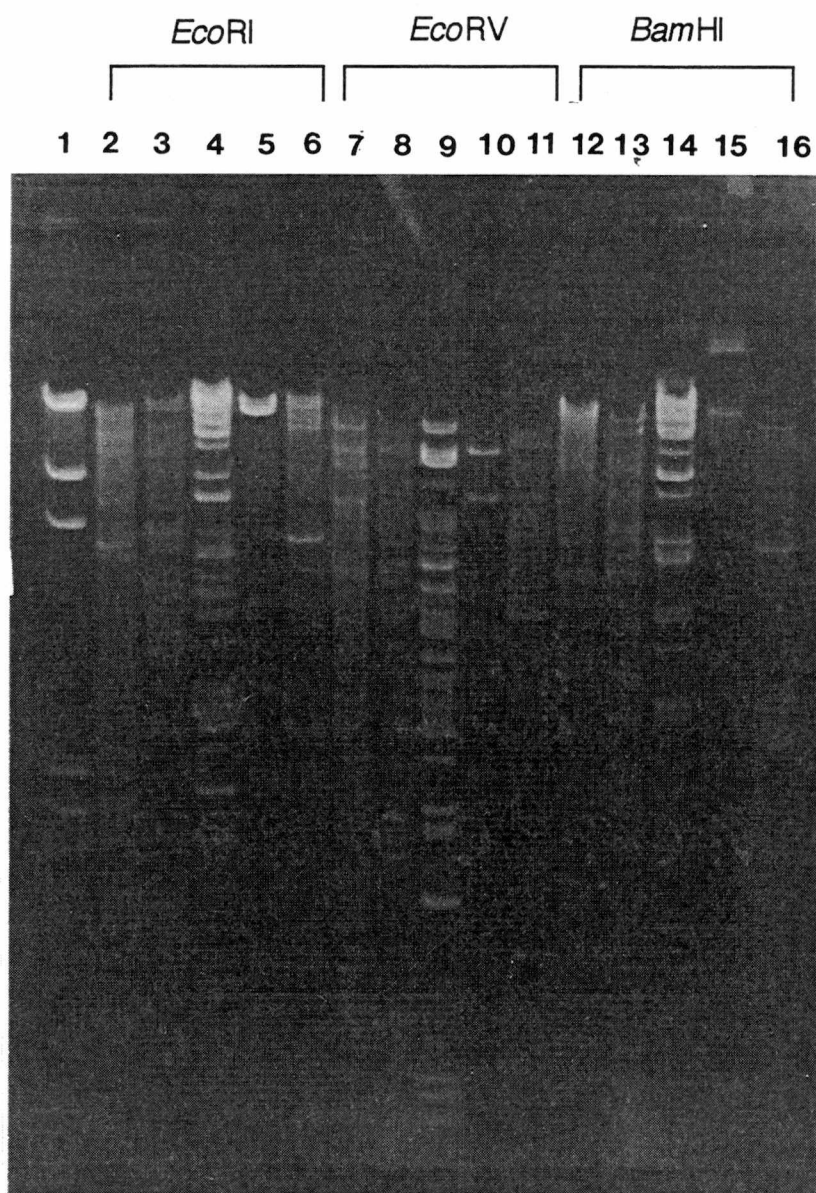


Figure 10. Restriction fragment length polymorphism of plasmid NAH7 and plasmids isolated from nonnahA-hybridizing PAH-degraders. Lanes: lane 1, *Hind*III digest of *Lambda* DNA; lanes 2, 7, and 12, NAH7; lanes 3, 8, and 13, A5PH1; lanes 4, 9, and 14, A8AN3; lanes 5, 10, and 15, B1PH2; lanes 6, 11, and 16, B10AN1; lanes 2-6, *Eco*RI digest; lanes 7-11, *Eco*RV digest; lanes 12-16, *Bam*HI digest.

Table 8. Growth, biochemical, and morphological characterization of selected *nonnahA*-hybridizing strains.

Characteristics	A5PH1	A8AN3	B1PH2	B10AN1
Bacteriological test				
Motility	-	+	+	+
Gram stain	-	-	-	-
Morphology	Rods	Rods	Rods	Rods
Color of colonies	Yellowish	Yellow ^a	Yellow ^a	Yellow ^a
Growth tests*				
D-Glucose	+	+	+	+
L-Arabinose	+	+	+	-
D-Mannose	-	-	-	-
D-Mannitol	-	-	-	-
N-Acetyl-D-glucosamine	-	-	-	-
Maltose	+	+	+	+
D-Gluconate	-	-	-	-
Caprate	-	-	-	-
Adipate	-	-	-	-
L-Malate	-	-	-	-
Citrate	-	-	-	-
Phenylacetate	-	-	-	-
Biochemical tests*				
Nitrate reduction	-	-	+	-
Tryptophanase	-	-	-	-
Glucose fermentation	-	-	-	-
Arginine dihydrolase	-	-	-	-
Urease	+	-	-	-
Esculine hydrolysis	+	+	+	+
Gelatinase	-	-	-	+
β -Galactosidase	-	+	+	+
Oxidase	+	+	+	+

Table 8. (Continued).

Characteristics	A5PH1	A8AN3	B1PH2	B10AN1
Identified as	<i>F. indologenes</i>	<i>S. paucimobilis</i>	<i>S. paucimobilis</i>	<i>P. vesicularis</i>
(using API Rapid NFT)**	(% id=63.3)	(% id=86.4)	(% id=91.9)	(% id=84.5)

^a Nostoxanthin as determined by absorbance spectrum of pigment. Pigment was extracted from cells as described (Holmes et al., 1977).

* By API Rapid NFT (a test system for the identification of Gram-negative, non fermentative bacteria).

** Identification comment on the value of the identification percentage (% id): excellent, ≥ 99.9 ; very good, ≥ 99.0 ; good, ≥ 90 ; acceptable, ≥ 80 .

A8AN3 produced a poor match to *Acinetobacter calcoaceticus* genospecies 1. Since A8AN3 possessed a polar monotrichous flagellum (Fig. 11) and showed mobility (Table 8), the Biolog result of nonmotile *Acinetobacter calcoaceticus* was rejected. Consistent with API tests, B1PH2 was identified as *S. paucimobilis* by Biolog analysis. B10AN1 produced excellent match to *Xanthomonas campestris* pv. *xanthosoma*. However, absence of 3-OH fatty acids in B10AN1 (Table 10) rejected the result of *Xanthomonas campestris* pv. *xanthosoma* that contains abundant 3-OH fatty acid (Wilkinson, 1988). Biolog analysis of A8AN3 was re-performed (August, 1993) by Analytical Services Inc. (Williston, VT). The result was 'no match'.

Like API tests, the Biolog analysis has advantage over traditional biochemical tests. Biolog analysis is convenient and fast compared to traditional biochemical tests that are labor-intensive and slow. Whereas the API kit employs 20 tests, Biolog analysis uses 95 tests so that Biolog analysis provides more information. Biolog analysis is based on utilization of chemicals by bacteria. The analysis employs a 96-well microplates with a different dehydrated chemical (carbon source) in each well along with tetrazolium redox indicator. Saline-suspended cells (150 µl) are added into the each well, and the plate is incubated for 4 h or 24 h. Reduction of the colorless tetrazolium indicator to purple formazan indicates which substrates have been oxidized. Substrate concentrations are 0.167 - 0.83% (final concentration after adding cell suspension) in Biolog plate wells (Personal communication with B. Bochner, Biolog Inc., Hayward, CA). Biolog analysis and API tests require that organisms are metabolically active with the carbon sources under conditions that are quite different from those in the environment in concentration and type of substrates. Slow-growing environmental isolates are difficult to identify at the incubation periods recommended by manufacturers. Therefore, Biolog analyses and API tests may not reflect the metabolic capabilities of the environmental isolates.

Table 9. Biolog analysis results of the selected *nonnahA*-hybridizing strains.

Strain	Identified by Biolog	Similarity coefficient*
A5PH1	<i>Brucella abortus</i> biovar 2	0.7
A8AN3	<i>Acinetobacter calcoaceticus</i> genospecies 1	0.38
B1PH2	<i>Sphingomonas paucimobilis</i>	0.6
B10AN1	<i>Xanthomonas campestris</i> pv <i>xanthosoma</i>	0.67

* Identification comment on a similarity coefficient value: ≥ 0.5 , excellent match (to Biolog database); 0.4 - 0.5, good match; 0.3 - 0.4, poor match; ≤ 0.3 , unsuccessful.

Table 10. Cellular fatty acid compositions of selected non*nahA*-hybridizing strains.

Fatty acid	A5PH1	A8AN3	B1PH2	B10AN1
12:0 2OH			0.4 ^a	
14:0	1.91	1.11		
14:0 2OH	7.89	10.38	6.78	3.48
15:0 2OH			4.56	
16:1 ω 7c	28.48	36.01	24.19	39.41
16:1 ω 5c	1.59	3.39		4.24
16:0	8.28	7.63	5.8	8.65
17:1 ω 8c			6.01	
17:1 ω 6c	6.37	1.40	23.24	
16:1 2OH		0.99		
16:0 iso 3OH		0.62		
18:1 ω 7c/ ω 9t/ ω 12t	45.47	36.75	29.42	44.22
% Peak identified	97.44	98.46	100.00	100.00

^a Peak area presented as the percent of total peak identified.

The fatty analysis was performed by Microbe Inotech Lab Inc. (St. Louis, MO).

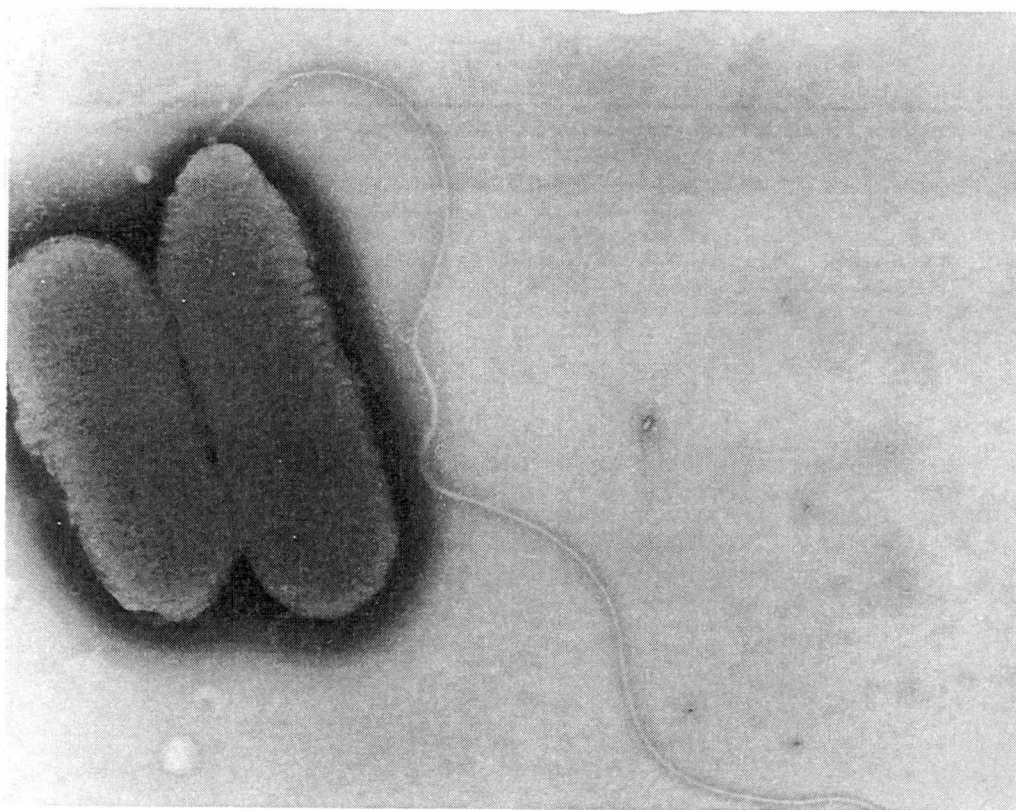


Figure 11. Transmission electron micrograph of negatively stained A8AN3 showing a polar monoflagellum.

The Microbe Inotech Lab Inc. (September, 1992) and CEB lipid analysis facility (October, 1992) performed fatty acid analyses of the 4 strains that did not hybridize with any of NAH7-derived gene probes. Two independent analyses of fatty acid produced results of 'no match' for the 4 strains. Table 10 shows fatty acid profiles of the 4 strains. All 4 strains contained 18:1 as a major cellular fatty acid and 2-hydroxymyristic acid (2-OH-14:0) as a major 2-OH fatty acid. Except for A8AN3, three strains did not contain 3-OH fatty acids that were major fatty acid components of lipopolysaccharide (LPS). Small amount of 3-OH-16:0 was detected in A8AN3. Since fatty acid analysis is based on retention time (RT) of a compound in gas chromatography analysis, the small amount of 3-OH fatty acid detected from A8AN3 could be other compounds that had the same RT: no 3-OH fatty acid was detected from A8AN3 when fatty acid analysis was performed by CEB fatty acid analysis facility.

Although the results of fatty acid analyses were no 'match' for the 4 strains, the yellow color of colonies, absence of 3-OH fatty acids, and dominance of 14:0 as a 2-OH fatty acid rendered the 4 strains suspected to be *Sphingomonas* strains. Because the genus *Sphingomonas* was proposed in 1990 (Yabuuchi et al.), the bacterial identification systems (e.g., fatty acid analysis, API tests, and Biolog analysis) used in this study might not have included sufficient database for *Sphingomonas* strains when the identification of the 4 strains were performed. The genus contains sphingolipid as a characteristic component of cellular lipids (Yabuuchi et al., 1990). Dihydrosphingosine and 2-OH-14:0 are components of sphingolipids in the genus. Yellow pigment, nostoxanthin, motility, and major ubiquinone, Q10 are also the characteristics of the genus, although *S. yanoikuyae* does not have the yellow pigment and *S. capsulata* is immotile (Yabuuchi et al., 1990).

The yellow pigment of the strains was investigated. The strains did not produce diffusable fluorescent pigment on King B medium. Except for strain A5PH1, methanol-

extracted yellow pigment of the strains showed an absorbance spectrum that matched nostoxanthin (Holmes et al., 1977), the carotenoid pigment of *S. paucimobilis* ATCC 29837 (Fig. 14): a major peak at 448 to 450 nm, a secondary peak at 475 to 478 nm, and a slight inflection at 415 to 420 nm. Along with characteristics of fatty acid profile, the pigment analysis result supported the identification of B1PH2 as *Sphingomonas paucimobilis*. Identification of nostoxanthin also agreed with identification of A8AN3 as *Sphingomonas paucimobilis*. Based on absence of 3-hydroxy fatty acids, dominance of 2-OH-14:0 as a major 4-OH fatty acid, and identification of nostoxanthin, B10AN1 was identified as a *Sphingomonas* sp. strain. However, identification of A5PH1 was uncertain.

Since A8AN3 did not hybridize with any of NAH7-derived gene probes and did not mineralize BaP, identification of the strain was of particular interest. Further analyses were performed to identify the strain. A8AN3 contained dihydrosphingosine and 2-OH-14:0 (Fig. 12 and 13). Isoprenoid quinone analysis performed by HPLC showed that ubiquinone Q10 was the major type of ubiquinone in A8AN3 (data not shown). Taken together, A8AN3 was determined to be most similar to *S. paucimobilis* based on phenotypic characteristics (Table 8) as well as chemotaxonomic traits such as sphingolipid, nostoxanthin, and major ubiquinone (Fig. 12, 13, and 14).

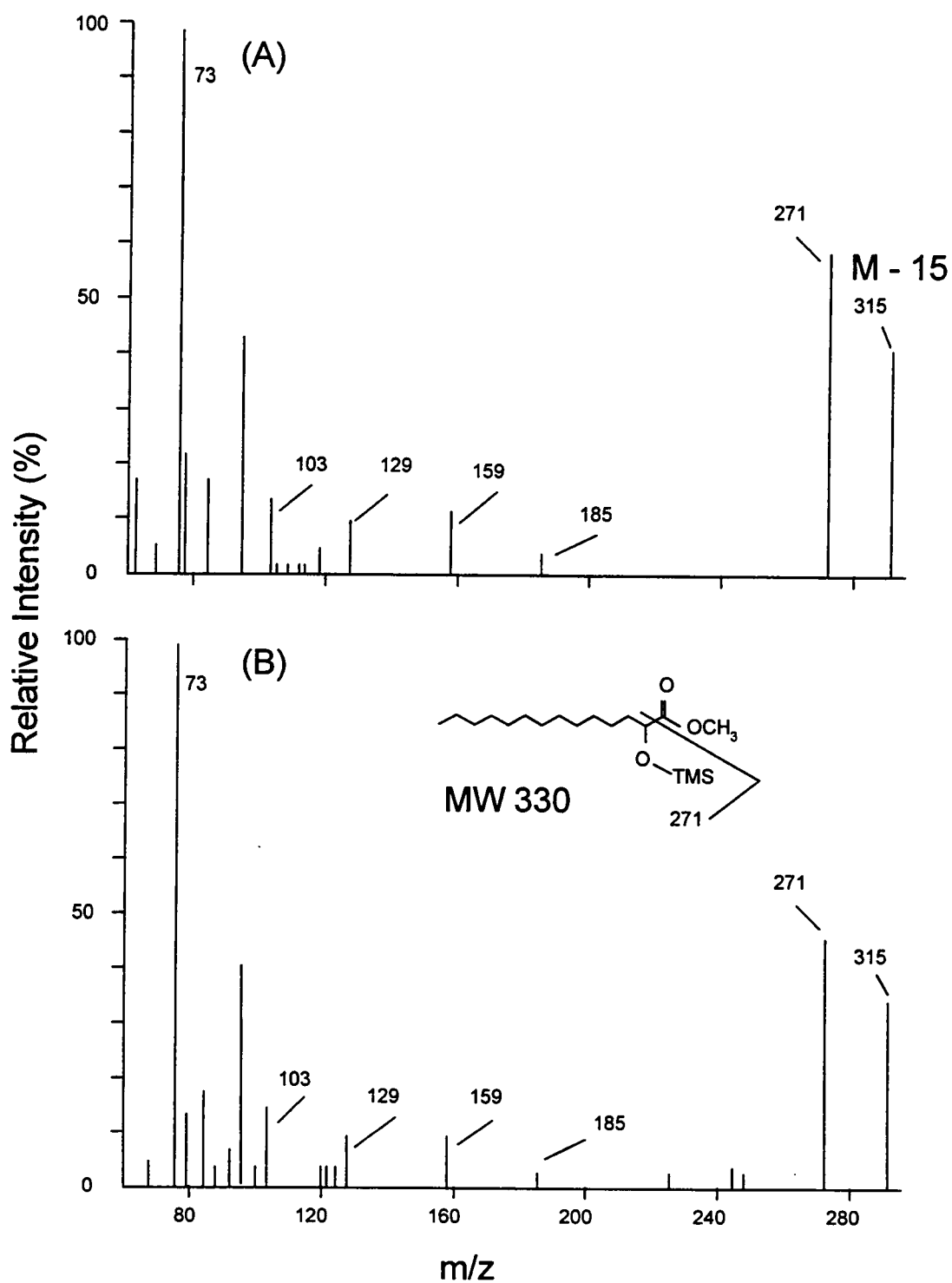


Figure 12. Mass spectrum of trimethylsilane (TMS) derivative of 2-hydroxymyristic acid methyl ester. (A), *Sphingomonas paucimobilis* ATCC 29837. (B), A8AN3.

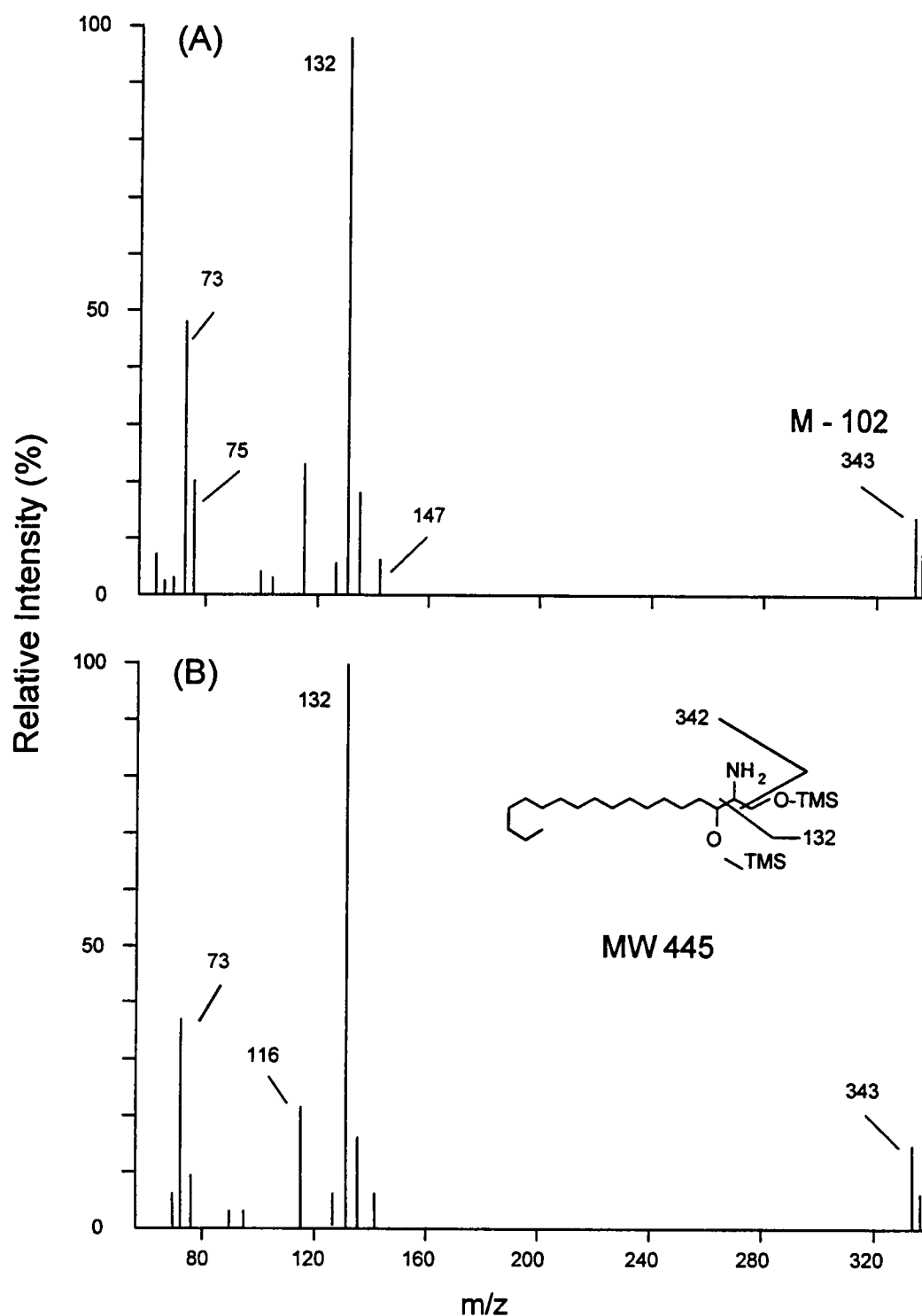


Figure 13. Mass spectrum of trimethylsilane (TMS) derivative of dihydrosphingosine. (A), *Sphingomonas paucimobilis* ATCC 29837. (B), A8AN3.

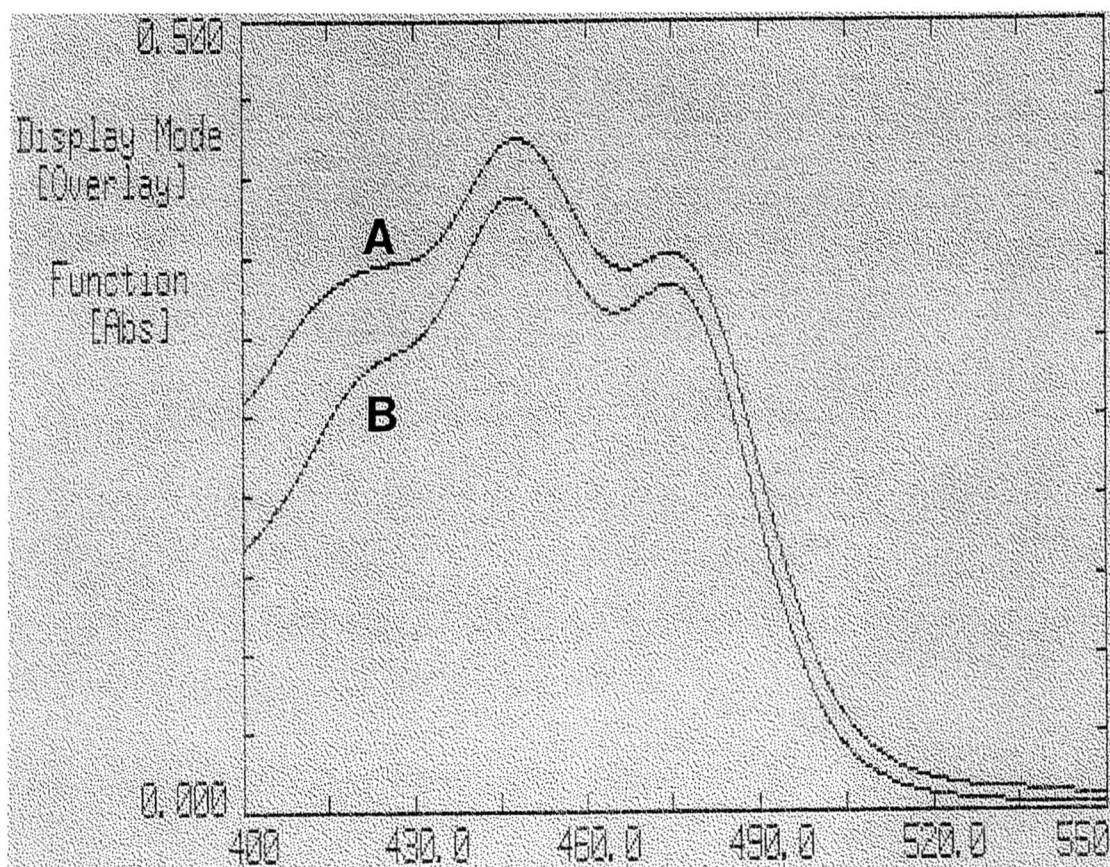


Figure 14. Absorbance spectra of pigment extracts from A8AN3 and *Sphingomonas paucimobilis* ATCC 29837. The spectra were measured in methanol. X axis is wavelength (nm). Y axis is absorbance. (A), A8AN3. (B), *Sphingomonas paucimobilis* ATCC 29837.

IV. DISCUSSION

To clean up environmental contamination, the demand for new approaches has been growing. The demand has resulted in the increased use of bioremediation technology. Because bioremediation is environmentally sound and cost-effective, bioremediation has an advantage over conventional cleanup technology such as incineration and air stripping. Bioremediation requires the knowledge of how specific chemicals are degraded by microorganisms. The isolation of microorganisms capable of degrading the target compounds, as well as biochemical and genetic information about the degradation, may lead to the development of new bioremediation technologies for contaminated sites.

PAH degraders isolated from PAH contaminated soils were analyzed with phenotypic and genotypic tests. The analyses was designed to screen HMW PAH degraders as sources for understanding of HMW PAH degradation and development of new gene probes. In this study, pyrene and BaP were used as HMW PAHs. Along with the HMW PAHs, LMW PAHs were also used in the analyses to determine the capability of current NAH7-derived gene probes to detect various PAH degraders.

The majority of PAH-degraders showed ability to degrade more than one PAH (Table 4). All naphthalene-degraders and anthracene-degraders could degrade phenanthrene. Various PAHs can be degraded by a single bacterial strain that has one enzyme system of broad substrate specificity. Although complete degradation of PAHs was not proven, the genes involved in the naphthalene degradation (*nah* genes) were reported to be involved in the degradation of 3-ring PAHs such as anthracene, phenanthrene, and fluorene (Menn et al., 1993; Sanseverino et al., 1993a; Denome et al., 1993; Yang et al., 1994; Kiyohara et al., 1994). A mutant of *Beijerinckia* sp. strain B1, accumulated the corresponding *cis*-dihydrodiols from naphthalene, phenanthrene,

anthracene, benz[*a*]anthracene, and BaP (Gibson et al., 1975). A single bacterium may degrade various PAHs by more than one enzyme systems in which each enzyme system has narrow substrate specificity.

Three HMW PAH degraders were found in this study using pyrene spray plates and ^{14}C -BaP mineralization assays. The finding of HMW PAH degraders suggested that *in situ* microorganisms have potential to degrade HMW PAHs. Three pyrene-degraders formed clear zones on the pyrene spray plates but did not evolve $^{14}\text{CO}_2$ from [4,5,9,10- ^{14}C]pyrene, perhaps indicating that the pyrene was transformed or that unlabeled ring(s) of the pyrene were cleaved within the incubation period (4 weeks). Initial ring-cleavage of pyrene can occur in either the 1,2- or 4,5-positions, based on reported ring-cleavage products (Heitkamp et al., 1988; Walter et al., 1991; Schneider et al., 1996). Heitkamp et al. (1988) reported 4-hydroxyperinaphthenone and 4-phenanthroic acid as ring-cleavage products of pyrene by *Mycobacterium* sp. strain PYR-1. Schneider et al. (1996) showed that *Mycobacterium* sp. strain RJGII-135 formed 4-phenanthrene carboxylic acid and 4,5-phenanthrene dicarboxylic acid as ring-cleavage products of pyrene. A pyrene metabolite, 4-hydroxyperinaphthenone provides evidence of ring cleavage in the 1,2-position of pyrene. Ring cleavage in the 4,5-position of pyrene is indicated by identification of 4-phenanthroic acid, 4-phenanthrenecarboxylic acid, and 4,5-phenanthrene dicarboxylic acid.

The 3 pyrene-degraders found in this study produced unknown pyrene-metabolite(s). The production was suggested when culture extracts of the strains were scanned by spectrophotometer or radiochromatogram. To identify any accumulated pyrene metabolites, large-scale pyrene biotransformation was performed using the pyrene degraders in phosphate buffer (pH 7.5) containing 25% YEPG medium (P-25% YEPG). The cultures became gray or black in color after 1 week incubation at 28°C. However abiotic (no cells) and biotic (no pyrene) controls did not produce the color change. The

compound(s) contributed to the color of the culture was suspected to be oxidized catechol-form-compound(s) resulting in quinone (dark gray color). When the culture supernatants were scanned by spectrophotometer, two major peaks were detected: absorbances of the peaks were 338 nm and 320 nm, respectively. To identify the pyrene metabolite, the culture supernatants were extracted with ethyl acetate as described (Menn et al., 1993) and separated by preparative HPLC. The separated each compound was analyzed by GC-MS. However, identification of pyrene metabolites was unsuccessful. After ^{14}C -pyrene mineralization assay, the culture supernatant of A8AN3 was extracted with ethyl acetate and dried under gentle stream of N_2 gas. The extract was dissolved in acetone and developed on a TLC plate using a solvent system of chloroform:methanol (9:1, v/v). The plate was then scanned by radiochromatogram analyzer (Bioscan Imaging System 200, Bioscan Inc., Washington, D.C.). A putative metabolite peak was shown at $R_f=0.06$ region, whereas substrate ^{14}C -pyrene was at $R_f=0.45$.

A HMW PAH degrader, A8AN3 was originally isolated from an MGP soil (soil A) by previous workers because of its ability to form clear zones on the anthracene spray plates. The strain was then kept in the bacterial collection. Its ability to mineralize [7,10- ^{14}C]BaP was shown in this study. However, it did not produce clear zones on BaP spray plates. The degradation rate of BaP by A8AN3 may not be high enough to form a clear zone due to the extremely low solubility of BaP. Considering the positions of ^{14}C labels, the mineralization result indicates that at least one ring of BaP can be cleaved by A8AN3. No information is available about cleavage of the other rings in five-ringed BaP. Attempts to identify BaP metabolites from A8AN3 were unsuccessful. Initial oxygenated-metabolites and ring cleavage products of BaP were reported from the cultures of *Beijerinckia* sp. strain B836 (Gibson et al., 1975) and *Mycobacterium* sp. strain RJGII-135 (Schneider et al., 1996). However, a complete BaP degradation pathway has not been proposed to date. Bacteria capable of growing with BaP as the sole carbon

and energy source or forming clear zones on BaP spray plates have not been reported. A8AN3 degraded a broad range of PAHs (Table 6 and 7); naphthalene, fluorene, phenanthrene, anthracene, pyrene, and BaP. However, the strain could grow only on either naphthalene or phenanthrene as sole carbon and energy sources among the PAHs (growth on fluorene was not examined). The strain showed dioxygenase activity as determined by ability to convert indole to indigo.

The indigo test indicated a strong relationship between PAH-degrading bacteria and their dioxygenase activity: 98% of PAH-degraders showed dioxygenase activity. The high percentage suggests that majority of bacteria degrade PAHs via dioxygenases. Many aromatic ring dioxygenases showed an ability to convert indole to indigo (Ensley et al., 1983; Eaton & Timmis, 1986; Tan & Mason, 1990; Denome et al., 1993; Goyal & Zylstra, 1996). However, toluene-4-monooxygenase could also produce indigo from indole (Yen et al., 1991).

The results of the genotypic tests showed that the NAH7-like degradative genes were dominant among PAH-degraders in the culture collection (Table 3). The results suggested long-term selection or enrichment of PAH-degraders containing NAH7-like PAH degradative genes in the PAH-contaminated sites. Because plasmid NAH7 is self-transmissible (Dunn & Gunsalus 1973), the horizontal transfer of NAH7 or NAH7-like plasmids might have occurred among bacterial strains in the soils, contributing to the dominance of NAH7-like genes among PAH-degraders. Most PAH-degraders in the collection contained indigenous plasmids (King et al., 1991). However, the function of the plasmids has not been investigated.

The results of the genotypic tests support the continued use of the NAH7-derived probes to monitor genetic potential of *in situ* microorganisms to degrade PAHs in contaminated soils. Using the *nahA* gene probe, this study actually proved NAH7-derived gene probes could be used to determine microbial potential to degrade not only

naphthalene but also 3- or 4-rings. The probe was especially effective for detecting phenanthrene degraders or fluorene degraders among 3 or more ring PAH-degraders. However, 15% (13/89) of the PAH degraders did not hybridize with the *nahA* gene probe. Four PAH-degraders including a BaP degrader did not hybridize with any of NAH7-derived gene probes used in this study. Therefore, considering reports on the numerous unculturable microorganisms in nature (Ward et al., 1990) and the non-NAH7 hybridizing PAH degraders (Fredrickson et al., 1991; Foght & Westlake, 1988, 1991; King et al., 1991; Goyal & Zylstra, 1996), the current NAH7-derived gene probes may underestimate *in situ* microbial potential to degrade PAHs.

Strain identification was performed to place the taxonomic positions of 4 strains (A5PH1, A8AN3, B1PH2, and B10AN1), that did not hybridize any of NAH7-derived gene probes used in this study. A8AN3, B1PH2, and B10AN1 were identified as *Sphingomonas* sp. strains: A8AN3 and B1PH2 were most similar to *S. paucimobilis*. However, A5PH1 produced taxonomic uncertainty. Difficulty in reliable identification of environmental isolates can be found in many reports. The difficulty was alleviated by comparative 16S rDNA analysis (Ward et al., 1986; Fredrickson et al., 1995; Wang & Lau, 1996). For example, a PAH-degrading *Sphingomonas capsulata* strain F199 was originally thought to be a Gram-positive bacterium (Fredrickson et al., 1991). A HMW PAH degrader *Beijerinckia* sp. strain B1 (Gibson et al., 1973) was recently re-identified as *S. yanoikuyae* (Wang & Lau, 1996). A naphthalenesulfonate-degrading *P. vesicularis* strain BN6 (Nortemann et al., 1993) was found to be a *Sphingomonas* strain (Heiss et al., 1995).

The application of bioremediation ultimately depends on understanding microbial community structure and activity. It is important to prove the presence of degradative microorganisms or genes. It is also important to determine that their degradability is active because the rate of PAH-biodegradation depends on physical and chemical

parameters of the site. This study analyzed a collection of culturable PAH-degraders isolated from PAH-contaminated soils. The analyses described in this study have the potential bias of depending on culturable PAH-degraders. Considering the fact that the majority of environmental microorganisms are unculturable, the collection of bacteria may not represent the diversity or activity of PAH-degraders in the habitats where the bacteria were isolated. Therefore, new gene-probe development is necessary for better understanding of *in situ* microbial potential to degrade PAHs, especially for HMW PAH degraders. New gene-probes along with NAH7-derived gene probes can be used in determining the genetic potential of *in situ* microbial communities to degrade PAHs, which will provide better understanding of the *in situ* microbial potential. A BaP-mineralizing strain A8AN3 was isolated from soil in which degradation of PAHs including BaP was observed. Biochemical and genetic characterization of the strain will provide insight on the efficacy of an A8AN3 population to degrade BaP.

**Part 3: Characterization of a BaP-mineralizing Strain A8AN3
and Phylogenetic Analysis of Its 16S rRNA Gene**

I. INTRODUCTION

PAHs are ubiquitous environmental pollutants mainly produced from incomplete combustion of organic materials. Sources of PAHs include crude oil, motor oil, automobile exhaust, waste incineration, coal tar, and cigarette smoke. Increasing industrial development results in a constantly rising rate of production and accumulation of PAHs, particularly in urban and industrialized areas. Bioremediation of PAH-polluted sites has received attention as an alternative to a traditional cleanup method of incineration.

Environmental concern exists about persistence and toxicity of PAHs, especially HMW PAHs (four- or more ring PAHs). The biodegradability of PAHs was reported to be inversely related to the number of rings (Heitkamp & Cerniglia, 1987; Shiaris, 1989; Sanseverino et al., 1993b). HMW PAHs are extremely insoluble in water and are persistent in the environment. The solubilities of naphthalene (a two-ring PAH) and BaP (a five-ring PAH) in water are 32 mg/L and 3.8 µg/L at 25 °C, respectively. BaP is one of the priority pollutants on the list compiled by the US-EPA and is one of the potential human carcinogens. Considering the persistence and toxicity of BaP, it is important to study BaP-degrading bacteria. However, few studies on microbial degradation of HMW PAHs have been conducted. Little is known about BaP-mineralization by bacteria. This is partly because of the fact that few BaP-degraders have been isolated, along with recalcitrance of HMW PAHs due to their complex structures and low water-solubilities.

Although the microbial metabolism of low-molecular-weight (LMW) PAHs (two- or three-ring PAHs) has been well studied over the last 30 years, investigations on the degradation of HMW PAHs have begun only recently. Few studies on the biodegradation of HMW PAHs have been reported. Therefore, less is known about the potential for HMW PAH degradation. Only a few pure bacterial isolates have been found that are

capable of growing solely on HMW PAHs: fluoranthene-degrading *Sphingomonas paucimobilis* EPA 505 (Mueller et al., 1990) and *Alcaligenes denitrificans* (Weissenfels et al., 1990); pyrene-, chrysene-, and fluoranthene-degrading *Rhodococcus* sp. strain UW1 (Walter et al., 1991); pyrene- and fluoranthene-degrading *Mycobacterium* sp. strain BB1 (Boldrin et al., 1993); chrysene-degrading *Pseudomonas fluorescens* (Caldini et al., 1995).

Degradation of HMW PAHs has primarily been observed in microbial cultures provided with an alternative growth-supporting compound, i.e., cometabolism: benz[*a*]anthracene- and BaP-degrading *Beijerinckia* sp. strain B1 (Gibson et al., 1975; Mahaffey et al., 1988); fluoranthene- and pyrene-degrading *Mycobacterium* sp. strain PYR-1 (Heitkamp & Cerniglia, 1988); pyrene- and BaP-degrading *Mycobacterium* sp. strain RJGII-135 (Grosser et al., 1991); BaP-degrading A8AN3 (Ahn et al., 1994) and *S. paucimobilis* EPA 505 (Ye et al., 1996). HMW PAHs have extremely low water-solubilities. Therefore, in the absence of other carbon sources, their aqueous-phase concentrations may not be sufficient to support bacterial growth (Wodzinski & Coyle, 1974; Volkering et al., 1992). Little is known about the genetic and regulatory mechanisms in bacterial degradation of HMW PAHs. Isolation of genes involved in HMW PAH degradation has not been reported.

Most pure culture studies of bacterial HMW PAH degradation have been aimed at elucidating biochemical pathways by isolating and identifying metabolites (Gibson et al., 1975; Mahaffey et al., 1988; Heitkamp et al., 1988; Kelley et al., 1993; Schneider et al., 1996). BaP was shown to be oxidized initially into *cis*-dihydrodiols by B836, a mutant of *Beijerinckia* sp. strain B1. The dihydrodiols were *cis*-9,10-dihydrodiol (major) and *cis*-7,8-dihydrodiol (minor) (Gibson et al., 1975). Recently, ring cleavage products of BaP were reported (Schneider et al., 1996) in the culture of *Mycobacterium* sp. strain RJGII-135 that could mineralize BaP (Grosser et al., 1991).

Little is known about the extent of BaP degradation by bacterial pure cultures (Sherrill, 1982; Grosser et al., 1991; Ahn et al., 1994; Ye et al., 1996). Bacteria capable of growing with BaP as the sole carbon and energy source or forming clear zones on BaP spray plates have not been reported. Considering the extremely low water-solubility (3.8 µg/L at 25°C) of BaP, mineralization is better evidence for BaP degradation than decreased BaP concentration. To date, only four bacterial strains have been reported to be able to mineralize BaP. They are three *Sphingomonas* strains and one *Mycobacterium* strain: *Beijerinckia* sp. strain B1 (Sherrill, 1982) (re-identified as a *Sphingomonas* strain later, Wang & Lau, 1996), *Mycobacterium* sp. strain RJGII-135 (Grosser et al., 1991), A8AN3 (identified as a *Sphingomonas* strain, Ahn et al., 1994), and *S. paucimobilis* EPA 505 (Ye et al., 1996). The phylogenetic relationship of BaP-degrading sphingomonads has not been described.

In this regard, the objectives of this study were 1) to establish phylogenetic placement of BaP-mineralizing strain A8AN3, 2) to characterize BaP-mineralization by A8AN3, and 3) to localize BaP-degradative genes in A8AN3. To achieve the objectives, the 16S rRNA gene (rDNA) of A8AN3 was partially sequenced for comparative analysis of nucleotide sequence. BaP mineralization assays were performed using two different radiochemicals, [7,10-¹⁴C]BaP and [7-¹⁴C]BaP. Random Tn5 mutagenesis was performed to localize BaP-degradative genes in A8AN3.

II. MATERIALS AND METHODS

1. Strains and plasmids

Through the analyses of a culture collection, a HMW PAH degrader, A8AN3 was previously found (Ahn et al., 1994). The strain was determined to be most similar to *S. paucimobilis* based on phenotypic characteristics as well as chemotaxonomic traits. A8AN3 was chosen for further study by virtue of its ability to degrade a broad range of PAHs including BaP and non-hybridization with NAH7-derived gene probes. For a triparental conjugation experiment, *Escherichia coli* strains C600 (pRZ102) and HB101 (pRK2013) were used as donor and helper strains, respectively. *E. coli* DH5 α was used as a host strain for gene cloning. A cloning vector pCRTM II (Invitrogen Co., San Diego, CA) was used for cloning of PCR products. Strains and plasmids used in this study are listed in Table 1.

2. Chemicals and enzymes

All chemicals were purchased from Millinckrodt Chemical, Inc. (Paris, KY) unless otherwise specified. BaP (purity, 99%) was obtained from Eastman Kodak Co. (Rochester, NY). The other PAHs were purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI). All solvents and chemicals were the highest purity available.

Except for ¹⁴C-BaP, ¹⁴C-labeled compounds were purchased from Sigma Chemical Company (St. Louis, MO). Radiochemical purities and specific activities of the compounds were as follows: [1-¹⁴C]naphthalene (10.1 mCi/mmol; purity, >98%), [9-¹⁴C]phenanthrene (10.4 mCi/mmol; purity, >99%), [U-¹⁴C]anthracene (15.0 mCi/mmol; purity, >98%), and [4,5,9,10-¹⁴C]pyrene (10.4 mCi/mmol; purity, >98%), and [U-

Table 1. Strains and plasmids used in the study.

Strain or plasmid	Relevant characteristic	Reference
A8AN3	BaP ⁺ ^a Ind ⁺ ^b Nd ^r	This study
2123	BaP ⁻ Ind ⁻	This study
<i>E. coli</i>		
DH5 α	<i>hadR recA lacZ</i> ΔM15 Nal ⁺	BRL Inc.
HB101	<i>hadS recA</i>	Sambrook et al., 1989
C600	<i>hadR</i>	Sambrook et al., 1989
plasmid		
pRK2013	Km ^r Tra ⁺ , helper plasmid	Figurski & Helinski, 1979
pRZ102	Km ^r Mob, suicide Tn5 vector	Jorgensen et al., 1979
pCR TM II	Am ^r , ColEI replicon	Invitrogen Co.

^a Ability to mineralize ¹⁴C-BaP.

^b Dioxygenase activity as determined by ability to convert indole to indigo.

Nd^r, nalidixic acid-resistant; Km^r, kanamycin-resistant; Am^r, ampicillin-resistant.

^{14}C]salicylic acid (1-10 mCi/mmol; purity, >99%). [7,10- ^{14}C]BaP (60 mCi/ mmol; purity, >98%) and [7- ^{14}C]BaP (58.6 mCi/ mmol; purity, >99%) were obtained from Amersham (Arlington Heights, IL) and Chemsyn (Lenexa, KS), respectively. [α - ^{32}P]dCTP purchased from ICN Biomedicals Inc. (Irvine, CA) was used for radiolabeled gene probe preparations.

Restriction endonucleases were purchased from GIBCO BRL (Gaithersburg, MD). *GeneAmp*[®] PCR reagent kit with *AmpliTaq*[®] DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT) was used for the polymerase chain reaction (PCR).

3. Media and growth conditions

Minimal basal salt medium (MBS, pH 7.2) and Yeast extract-Polypeptone-Glucose medium (YEPG, pH 7.0) were described in part 2. MBS agar plates were prepared by adding 1.7% Noble agar (Difco Lab., Detroit, MI). YEPG was used as a multipurpose growth medium. It was used at 25% strength for mineralization assays. A8AN3 was grown in YEPG at 28°C. Unless otherwise specified, *E. coli* strains were grown in Luria-Bertani (LB) broth (Sambrook et al., 1989) at 37 °C. Antibiotics were added at the following concentrations when needed: ampicillin (Am), 100 µg/ml; kanamycin (Km), 50 µg/ml; nalidixic acid (Nd), 30 µg/ml.

To monitor cell growth, cells were grown in 250 ml flasks containing 40 ml of YEPG or 25% YEPG broth. The flasks were shaken at 28°C and 150 rpm. One ml of culture was withdrawn from each flask every two hours and cell growth was monitored by measuring the OD at 600 nm with a Beckman DU-70 spectrophotometer. The growth measurement was performed in triplicate.

4. DNA isolation and analysis

Total DNA and plasmid DNA were prepared as described in Part 2. DNA was resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0) and stored at - 20°C until use. Agarose gel electrophoresis was performed in the TBE buffer to separate DNA fragments as described (Sambrook et al., 1989).

Southern blot hybridization was performed as described in Part 2. For re-hybridization, probes on the membranes was stripped by boiling in 0.1% SDS as described in manufacturer's protocol. Stripped membranes were monitored for complete removal of radiolabeled probes by extended autoradiography before re-hybridization with different probes.

5. PCR amplification of 16S rDNA

PCR-amplified 16S rDNA was employed for 16S rDNA sequence analysis. Approximately 1.5 kb of 16S rDNA was amplified using genomic DNA of A8AN3 or its Tn5 mutant strain, 2123 as the template. For the PCR (total vol. 100 µl), 100 ng of template and 80 ng of primers were used. The primers were 5'AGTTTGATCCTGGCTC3' (*E. coli* positions 10 to 25, Brosius et al., 1978) and 5'AAGGAGGTGATCCAGCC3' (complementary to *E. coli* positions 1541 to 1525) (Lane, 1991). Reaction conditions consisted of 30 cycles of 94°C (1 min), 58°C (2.5 min), and 72°C (2.5 min). Before the 30 cycles, preheating of 94°C (2.5 min) was employed. After the 30 cycles, final chain elongation was conducted at 72°C for 5 min. The size of the amplified product was determined by agarose (1%, w/v) gel electrophoresis. The PCR product of A8AN3 was cloned by using TA cloning kit

(Invitrogen, San Diego, CA) in accordance with the manufacturer's protocol. Insert of the clone was analyzed by electrophoresis after digestion with *EcoRI*.

6. Nucleotide sequence determination and analysis

The cloned 1.5 kb of 16S rDNA was partially sequenced by the dideoxy-chain termination method (Sanger et al., 1977) either manually or automatically. The following primers were used to determine nucleotide sequences of both strands in the positions 1099 to 1510: 5'GCAACGAGCGCAACCC3' (*E. coli* positions 1099 to 1114) and 5'GGCTACCTTGTTACGA3' (complementary to *E. coli* positions 1510 to 1495) (Lane, 1991). The primers were synthesized by the Oligo 1000 DNA Synthesizer (Beckman Instruments Inc., Fullerton, CA) at the Center for Environmental Biotechnology (CEB), University of Tennessee, Knoxville. For the manual sequencing, Sequenase 2.0 (United States Biochemical Co., Cleveland, OH) and [α^{33} -P]dATP were used. Automated sequencing was performed by an Applied Biosystems model 373A DNA sequencer (Foster City, CA) (Univ. of Tennessee Dept. of Microbiology Molecular Biology Resource Facility).

The resulting sequence was screened against the Ribosomal Database Project (RDP) database using the program Similarity_Rank (Maidak et al., 1994) to search the most similar sequences in the database. Computer analyses of 16S rDNA sequences were accomplished using the Genetics Computer Group package (Devereux et al., 1984). Sequences were aligned using the program PILEUP. Evolutionary distances were calculated using the program DISTANCES by the method of Jukes and Cantor (1969) and a phylogenetic tree was constructed using the program GROWTREE by unweighted pair-group method using arithmetic average (UPGMA) (Sneath & Sokal, 1973). The tree

was prepared based on comparison of the partial 16S rDNA sequences of strains from positions 1099 to 1510 (*E. coli* numbering).

7. BaP mineralization assay

BaP mineralization assays was performed in 25% YEPG broth for 7 days as previously described in the Part 2. Approximately 80,000 dpm (approximately 0.15 μg , 0.03 ppm) of [7,10- ^{14}C]BaP (specific activity, 60 mCi/mmol) and approximately 71,000 dpm (approximately 0.14 μg , 0.035 ppm) of [7- ^{14}C]BaP (specific activity, 58.6 mCi/mmol) were used for the assays, respectively. Since the water-solubility of BaP is 3.8 μg per liter (Mackay & Shiu, 1977), all BaP concentrations used in mineralization assays were oversaturation concentrations. Approximately 64,700 dpm of [7,10- ^{14}C]BaP was used for a time course experiment of BaP mineralization. At certain time intervals, 0.5 ml of 0.5 M NaOH solution was withdrawn from the CO_2 trap vial and fresh NaOH solution (0.5 ml) was added into the vial.

8. Tn5 mutagenesis

Using the Tn5 carrier plasmid pRZ102 (Goldberg & Ohman, 1987), Tn5 (Km^{r}) was introduced into the Nd^{r} resistant A8AN3. The narrow-host-range plasmid pRZ102 (Mob^+) was mobilized by triparental filter mating (Ditta et al., 1980) from *E. coli* to A8AN3, using the conjugative helper plasmid pRK2013 (Tra^+). The donor strain, *E. coli* C600 (pRZ102), and the helper strain, *E. coli* HB101 (pRK2013), were grown to mid-log phase in 5 ml of LB broth containing kanamycin. The recipient strain, A8AN3, was grown to mid-log phase in 5 ml of YEPG broth containing nalidixic acid. The donor, helper, and recipient strains were harvested and washed twice in YEPG broth. The strains

were suspended in 2.5 ml of YEPG broth and mixed together (donor:helper:recipient, 1:1:10) in a sterilized tube. The mixture was filtered through a sterile membrane (pore size, 0.2 μ m). The membrane was placed on a YEPG plate and incubated at 28°C for 3 days. Cells on the filter were harvested by centrifugation in YEPG broth. The harvested cells were spread on YEPG plates containing kanamycin and nalidixic acid. $Km^r Nd^r$ transconjugants were screened for the absence of the indole oxidation function (Ind^-) or *meta*-cleavage activity: transconjugants were exposed to indole vapor for 1 day by placing indole crystals in the lids of plates. The plates were also subjected to catechol spray plates to detect transconjugants that were incapable of producing the yellow ring-cleavage metabolite (2-hydroxymuconic semialdehyde) from catechol. An Ind^- mutant was subsequently analyzed for [7,10- ^{14}C]BaP mineralization. Auxotrophic mutants were eliminated from the selection by plating transconjugants on minimal-medium containing glucose and kanamycin.

To verify Tn5 transposition, Southern blot hybridization was performed using an *Hpa*I fragment of Tn5 as a probe. The loss of the pRZ102 vector DNA in the mutant was confirmed by performing hybridization with a vector-specific probe, ~ 3 kb *Eco*RI-*Pst*I fragment of pRZ102 (Jorgensen et al., 1979). The probe was labeled by the random primer labeling method (Feinberg & Vogelstein, 1983) using Prime It® (Stratagene, La Jolla, CA) according to the manufacturer's protocol.

III. RESULTS

1. Comparative 16S rDNA analysis

PCR-amplified 16S rDNA was used to determine the 16S rDNA sequence of A8AN3. The size of the PCR product was 1.5 kb. The product was cloned into a vector plasmid pCR™II. Gel electrophoresis of an *Eco*RI-digested clone showed the expected 1.5-kb insert and 3.9-kb vector on 1% agarose gel. The 16S rDNA of A8AN3 was partially sequenced to determine its phylogenetic relationship with bacteria currently in the RDP database. Positions 1099 to 1510 (*E. coli* numbering) were employed for the partial sequencing. The PCR-amplification and nucleotide sequencing primers were located at highly conserved regions of the 16S rDNA (Lane, 1991). The sites have proven useful as target sites for PCR and sequence determination (Lane, 1991; Weisburg et al., 1991; Takeuchi et al., 1993). The sequenced positions 1099 to 1510 (*E. coli* numbering) contain positions 1220 to 1376 (*E. coli* numbering) that are characterized by a high number of variable nucleotides in different species belonging to the α , β , and γ subgroups of the *Proteobacteria* (Brosius et al., 1978; Toschka et al., 1988; Yabuuchi et al., 1990; Busse et al., 1992).

Figure 1 shows 412 bases of the partial 16S rDNA sequence of A8AN3. Both strands of 16S rDNA were sequenced except the first 32 bases and last 36 bases of the 412 bases. The nucleotide sequence was deposited in GenBank under accession number U73007. Figure 2 shows the 157-nucleotide sequence (from 1220 to 1376, *E. coli* numbering) of 16S rDNA from A8AN3 and other sphingomonads: *Sphingomonas paucimobilis* ATCC 29837 is the type species of the genus *Sphingomonas* and all shown sphingomonads are type strains of their species. For comparison, the figure also includes the 16S rDNA sequence of *Pseudomonas aeruginosa* ATCC 10145, the type species of

the genus *Pseudomonas*. The 16S rDNA sequence of A8AN3 had a deletion of a nucleotide at position 1290 with respect to *P. aeruginosa*. Yabuuchi et al. (1990) reported that sphingomonads were characterized by deletions at this position.

The 16S rDNA sequence of A8AN3 was subjected to a Similarity_Rank analysis of the RDP database to determine the most similar sequence in the database (April 21, 1996: 11:14:11). The Similarity_Rank analysis showed a match of 0.829 with *Sphingomonas adhaesiva* JCM 7370 (= ATCC 51229) as a closest relative. The other very similar sequences were 16S rDNA sequences of *Sphingomonas* sp. strains SYK6 and SS86; the 16S rDNA sequence of an azo dye-degrading isolate C7 (Govindaswami et al., 1993) was also similar to the sequence of A8AN3. The strain SYK6 was reported to degrade various dimeric lignin compounds such as biphenyl, pinoresinol, and diarylpropane (Katayama et al., 1988). *Sphingomonas* sp. strain SS86 was a lindane-degrader (Senoo & Wada, 1989).

A GenBank search was also performed (April 5, 1996: 15:03:21) for comparison with recently deposited 16S rDNA sequences. Because the RDP database is updated roughly once a year (personal communication with B. L. Maidak, RDP service manager, Univ. of Illinois Department of Microbiology), recently deposited sequences of many interesting PAH-degraders were not found in the RDP database when the sequence of A8AN3 was analyzed. The most similar sequence in the GenBank database was the 16S rDNA sequence of *Sphingomonas* sp. strain BN6 (95% identity). BN6 was reported to degrade naphthalenesulfonate (Nortemann et al., 1993). The second most similar sequence was the 16S rDNA of *Sphingomonas adhaesiva* JCM 7370.

A phylogenetic tree was constructed (Fig. 3) based on a comparison of the partial 16S rDNA sequences of closest relatives of A8AN3 and PAH-degrading sphingomonads. The tree was obtained from the analysis of the 16S rDNA sequences on positions 1099 to 1510 (*E. coli* numbering). The 16S rDNA sequences of high similarity values in RDP and

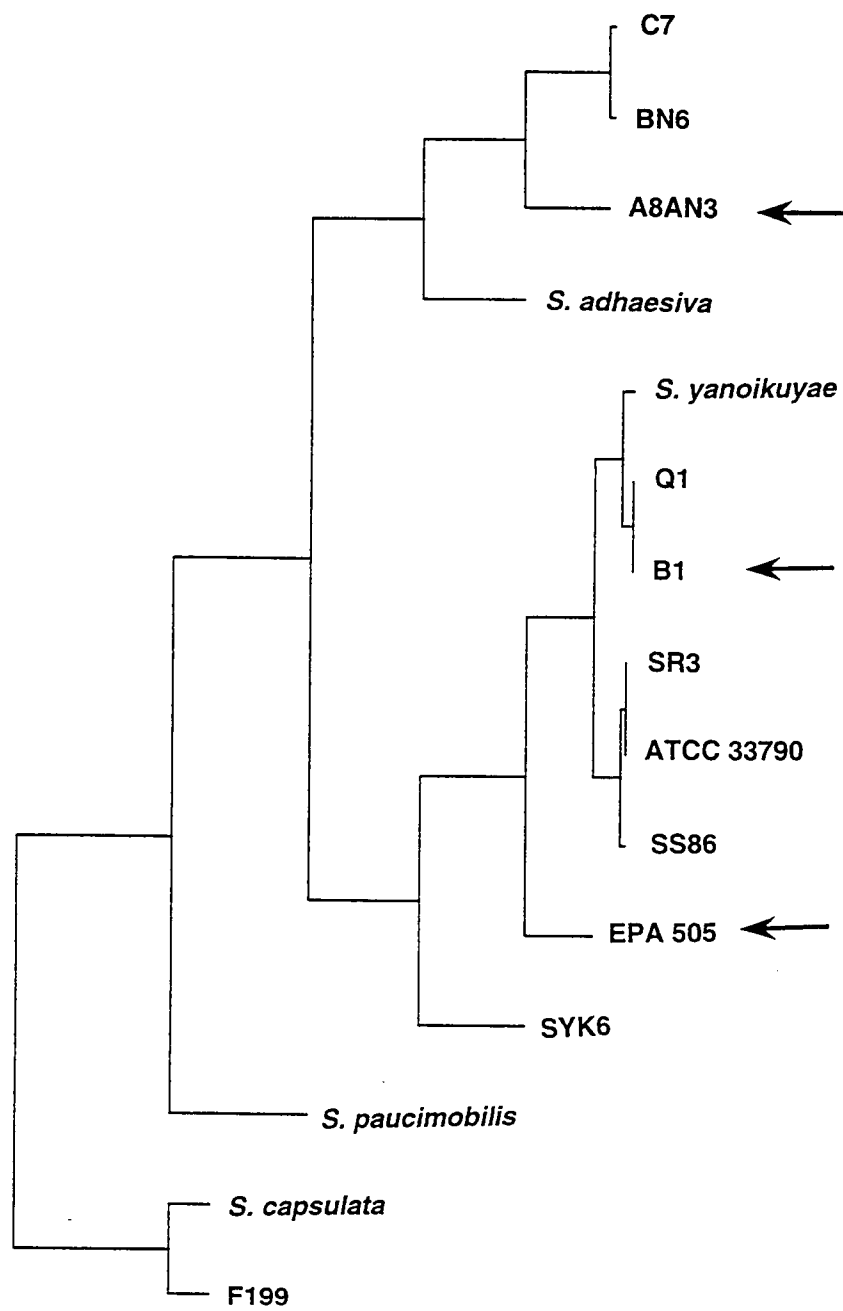
1 GCAACGAGCG	11 CAACCCTCGC	21 CTTTAGTTGC	31 CAGCATTGG	41 TTGGGTACTC
51 TAAAGGAACC	61 GCCGGTGATA	71 AGCCGGAGGA	81 AGGTGGGGAT	91 GACGTCAAGT
101 CCTCATGGCC	111 CTTACGCGCT	121 GGGCTACACA	131 CGTGCTACAA	141 TGGCGACTAC
151 AGTGGGCAGC	161 AACTCCGCGA	171 GGGGAAGCTA	181 ATCTCCAAAA	191 GTCGTCTCAG
201 TTCGGATTGT	211 TCTCTGCAAC	221 TCGAGAGCAT	231 GAAGGCGGAA	241 TCGCTAGTAA
251 TCGCGGATCA	261 GCATGCCGCG	271 GTGAATACGT	281 TCCCAGGCCT	291 TGTACACACC
301 GCCCCTCACA	311 CCATGGGAGT	321 TGGATTCACC	331 CGAAGGCGCT	341 GCGCTAACTC
351 GCAAGAGAGG	361 CAGGCGACCA	371 CGGTGGGTTT	381 AGCGACTGGG	391 GTTGAATCGT
401 AACAAGGTAG	411 CC			

Figure 1. Partial 16S rDNA sequence of A8AN3. Primers used to determine the nucleotide sequence were described in the text. The nucleotide sequence was deposited in GenBank under accession number U73007.

	1220	1230	1240	1250	1260	1270
A8AN3	GGGCUACACA	CGUGCUACAA	UGGCGACUAC	AGUGGGCAGC	AACUCCGCCG	GGGGAAGCUA
S. adh	GGGCUACACA	CGUGCUACAA	UGGCGACUAC	AGUGGGCUGC	AAUCCAGCGA	GGGUGAGCUA
S. pau	GGGCUACACA	CGUGCUACAA	UGGCAACUAC	AGUGGGCAGC	GACCCUGCGA	GGGCGAGCUA
S. yan	GGGCUACACA	CGUGCUACAA	UGGCGACUAC	AGUGGGCAGC	CACCUCCGCA	GAGGGAGCUA
P. aer	GGGCUACACA	CGUGCUACAA	UGGUCGGUAC	AAAGGUUGC	CAAGCCGCCG	GUGGGAGCUA
	1280	1290	1300	1310	1320	1330
A8AN3	AUCUCCAAAA	. GUCGUCUCA	GUUCGGAUUG	UUCUCUGCAA	CUCGAGAGCA	UGAAGGCGGA
S. adh	AUCUCCAAAA	. GUCGUCUCA	GUUCGGAUUG	CUCUCUGCAA	CUCGAGAGCA	UGAAGGCGGA
S. pau	AUCCCCAAAA	. GUUGUCUCA	GUUCGGAUUG	UUCUCUGCAA	CUCGAGAGCA	UGAAGGCGGA
S. yan	AUCUCCAAAA	. GUCGUCUCA	GUUCGGAUCG	UUCUCUGCAA	CUCGAGAGCG	UGAAGGCGGA
P. aer	AUCCCAUAAA	ACCGAUCGUA	GUCCGGAUCG	CAGUCUGCAA	CUCGACUGCG	UGAAGUCGGA
	1340	1350	1360	1370		
A8AN3	AUCGCUAGUA	AUCGCGGAUC	AGCAUGCCGC	GGUGAAU		
S. adh	AUCGCUAGUA	AUCGCGGAUC	AGCAUGCCGC	GGUGAAU		
S. pau	AUCGCUAGUA	AUCGCGGAUC	AGCAUGCCGC	GGUGAAU		
S. yan	AUCGCUAGUA	AUCGCGGAUC	AGCAUGCCGC	GGUGAAU		
P. aer	AUCGCUAGUA	AUCGUGAAUC	AGAAUGUCAC	GGUGAAU		

Figure 2. Nucleotide sequences of 16S rRNA at positions 1220 to 1376 (*E. coli* numbering). S. adh, *S. adhaesiva* JCM 7370; S. pau, *S. paucimobilis* ATCC 29837; S. yan, *S. yanoikuyae* JCM 7371 (= ATCC 51230); P. aer, *Pseudomonas aeruginosa* ATCC 10145. The sequence of A8AN3 was determined in this study. The other sequences were available from the RDP or GenBank databases. GenBank accession number of each strain is as follows: P. aer, X06684; S. adh, D16146; S. pau, D16144; S. yan, D16145.

Figure 3. Phylogenetic tree showing the relationship of A8AN3 with other sphingomonads. The tree was prepared according to UPGMA based on a comparison of 16S rDNA sequence at positions 1099 to 1510 (*E. coli* numbering). The strains used for the tree construction were as follows: C7, strain C7; BN6, *Sphingomonas* sp. BN6; A8AN3, *Sphingomonas* sp. A8AN3; *S. adhaesiva* JCM 7370; *S. yanoikuyae* JCM 7371; Q1, *S. paucimobilis* Q1; B1, *S. yanoikuyae* B1 (formerly *Beijerinckia* sp. B1); SR3, *Pseudomonas* sp. SR3; ATCC 33790, *Arthrobacter* sp. ATCC 33790; SS86, *S. paucimobilis* SS86; EPA 505, *S. paucimobilis* EPA 505; SYK6, *S. paucimobilis* SYK6; *S. paucimobilis* ATCC 29837; *S. capsulata* ATCC 14666; F199, *Sphingomonas* sp. F199. See the text for re-identified strain names and references. Arrows indicate BaP-degraders.



GenBank searches were retrieved from the databases. The 16S rDNA sequences of PAH-degraders retrieved from GenBank are as follows (their sequences were not found in the RDP databases at the time): *Beijerinckia* sp. strain B1 (re-classified as *S. yanoikuyae*, Wang & Lau, 1996); *S. paucimobilis* Q1 (re-classified as *S. yanoikuyae*, Wang et al., 1996); *S. paucimobilis* EPA 505, *Sphingomonas* sp. strain F199; *Sphingomonas* sp. strain BN6; *Pseudomonas* sp. strain SR3 (re-identified as *S. chlorophenolica*, Karlson et al., 1996); *Arthrobacter* sp. ATCC 33790 (re-identified as *S. chlorophenolica*, Karlson et al., 1996). A dibenzo-*p*-dioxin degrader, *Sphingomonas* sp. strain RW1 (Moore et al., 1993) was excluded due to regions with many unidentified nucleotides. The tree showed three BaP-degrading sphingomonads (B1, A8AN3, and EPA 505) emerging as two clusters: EPA 505 and B1 were in the same cluster with *S. yanoikuyae*, whereas A8AN3 was in the cluster with *S. adhaesiva*.

2. Growth of A8AN3

Growth of A8AN3 in full-strength YEPG or 25% YEPG broth was monitored by observing the increase of OD at 600 nm. YEPG medium was routinely used to grow A8AN3. For mineralization assay, 25% YEPG medium was employed. Growth curves of A8AN3 in 25% YEPG and full-strength YEPG are shown in Figures 4 and 5, respectively. A8AN3 showed relatively slow growth; doubling times were approximately 4 h and 30 min in 25% YEPG and approximately 4 h in YEPG, respectively. Cell growth in both media reached stationary phase within 20 hours after incubation.

3. BaP mineralization by A8AN3

The ¹⁴C-BaP mineralization assay is the method of choice to test the ability of

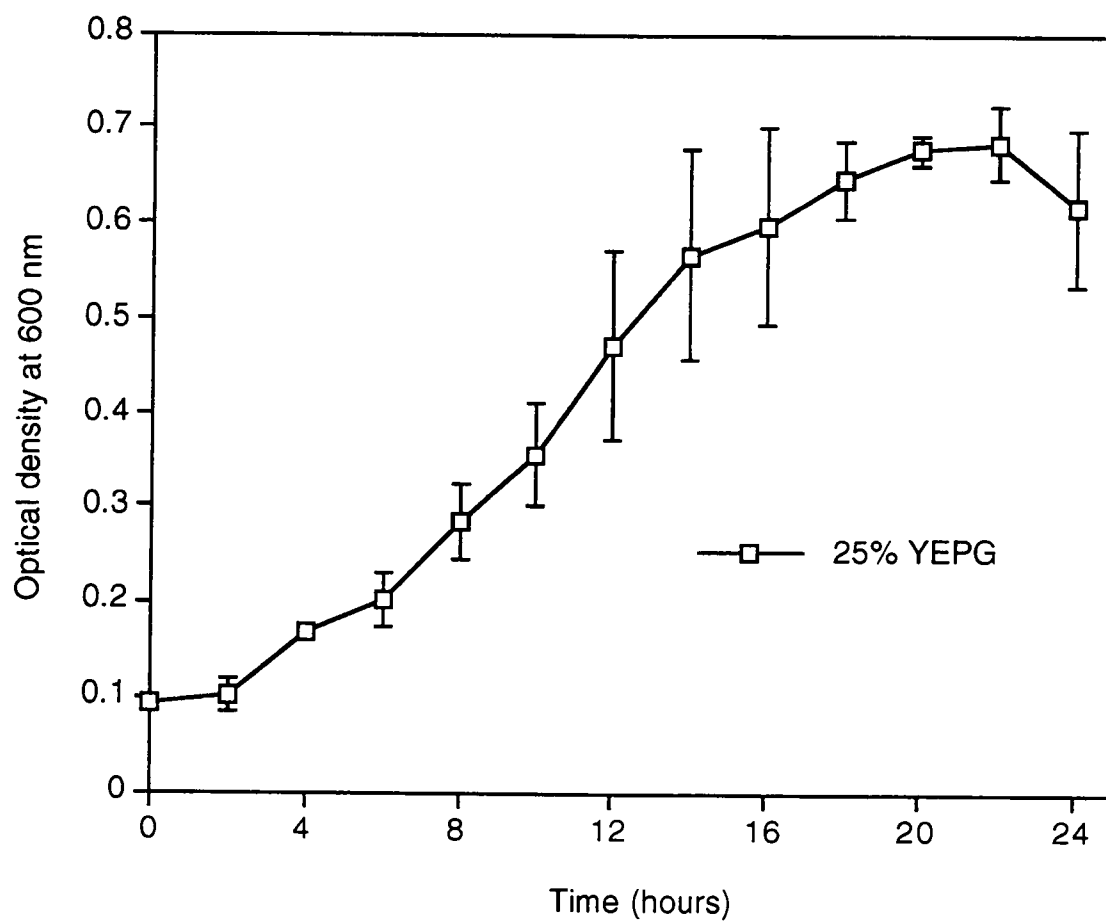


Figure 4. Growth curve of A8AN3 in 25% YEPG broth. Data are given as mean $\pm$ standard deviation (n=3).

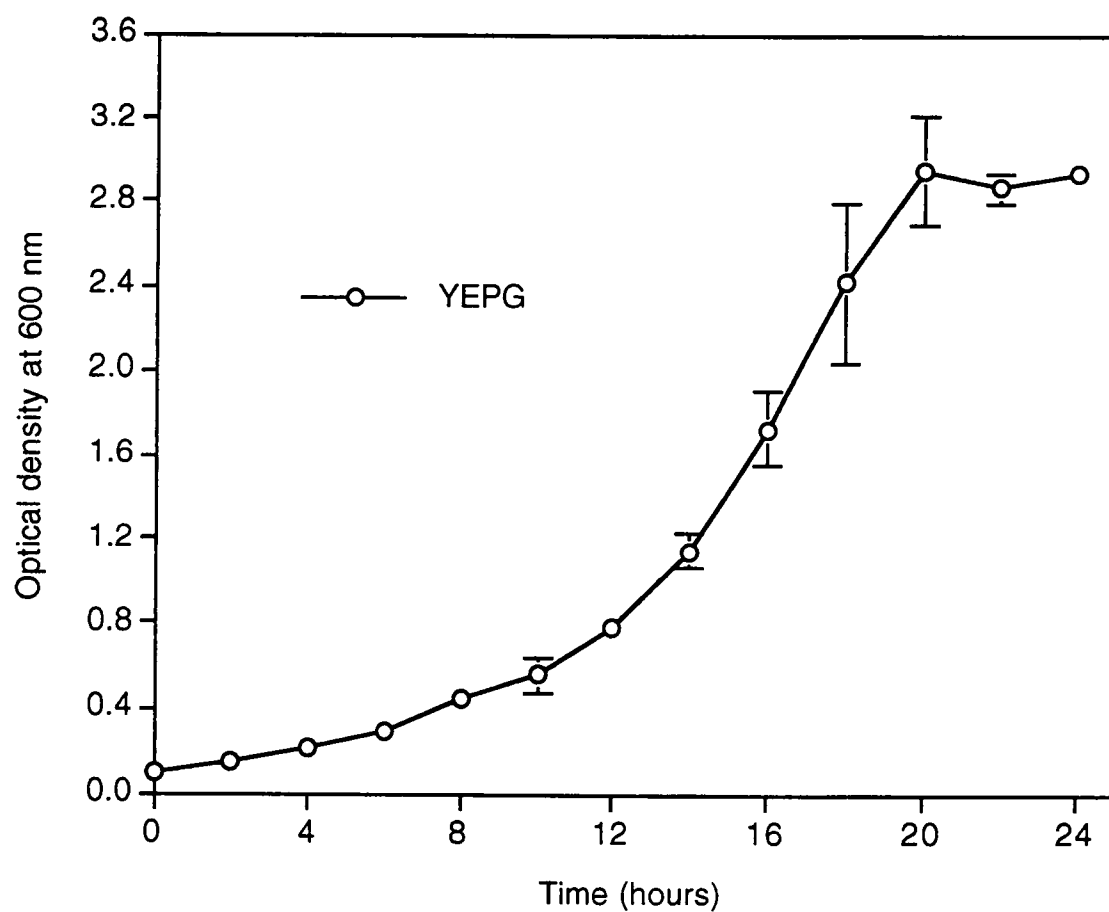


Figure 5. Growth curve of A8AN3 in YEPG broth. Data are given as mean $\pm$ standard deviation ($n=3$).

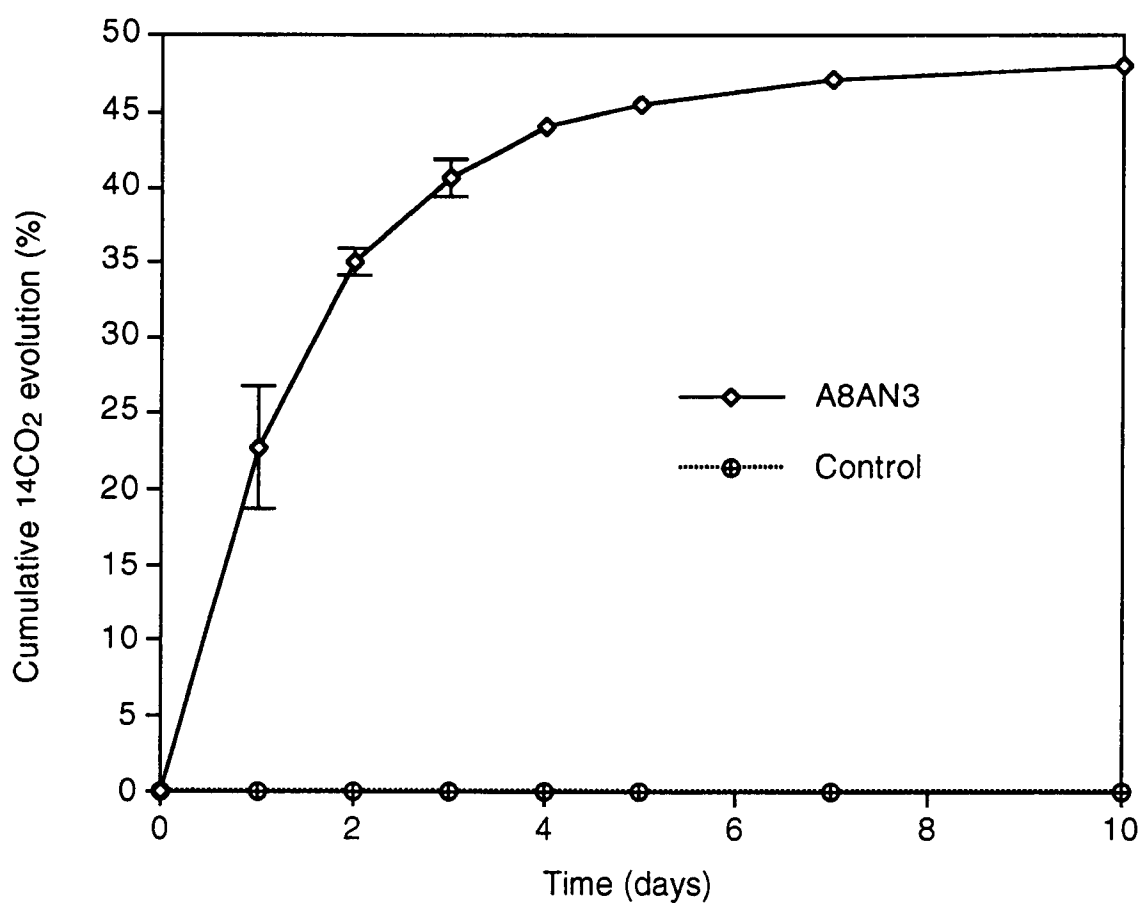


Figure 6. Time course of [7,10- ^{14}C]BaP mineralization by A8AN3 in 25% YEPG broth. Data are given as mean $\pm$ standard deviation ($n=3$). To calculate the percentage of ^{14}C -BaP mineralized, values from the killed control vials were subtracted as background. Control plot was produced by subtracting 0 time values of the control vials as background.

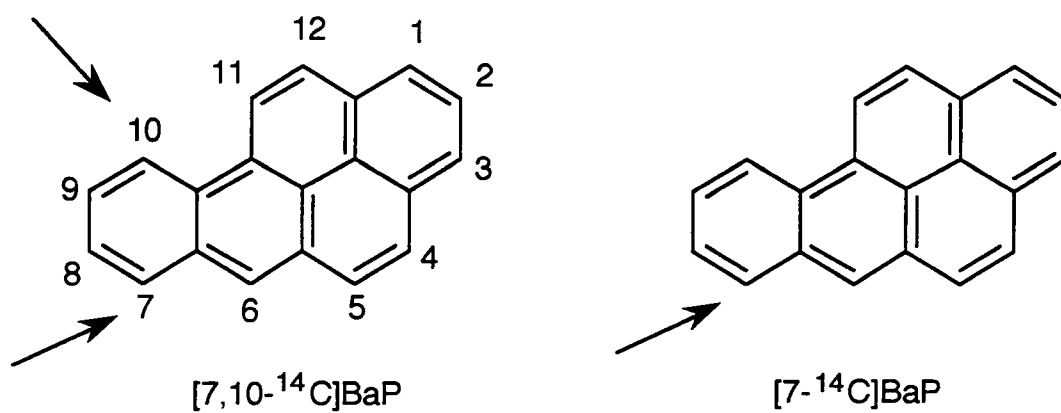


Figure 7. ^{14}C -Benzo[*a*]pyrene used in mineralization studies. Arrows indicate ^{14}C -labeled positions.

A8AN3 to degrade BaP, because the strain does not grow on BaP as a sole carbon and energy source and does not form clear zones on BaP spray plates. [7,10- ^{14}C]BaP mineralization was performed in 25% YEPG broth to determine the time course of BaP-mineralization. BaP mineralization started rapidly within 1 day after incubation (Fig. 6). Evolution of $^{14}\text{CO}_2$ was slightly decreased thereafter, eventually reaching a plateau on the 4th day of incubation. Approximately 45% of the added ^{14}C radioactivity was evolved as $^{14}\text{CO}_2$ after 5 days of incubation. A small amount of $^{14}\text{CO}_2$ evolution was observed after the evolution reached plateau.

The result of $^{14}\text{CO}_2$ evolution from [7,10- ^{14}C]BaP suggested that A8AN3 could cleave at least one ring of the 5-ring BaP. However, the source of the $^{14}\text{CO}_2$ was not determined: either the C-7 or C-10 position or only one of the two. If either C-7 or C-10 is the origin of $^{14}\text{CO}_2$, it remains to be determined which position is preferred for ring cleavage. To determine the origin of $^{14}\text{CO}_2$, BaP mineralization was performed using [7,10- ^{14}C]BaP and [7- ^{14}C]BaP (Fig. 7). Because they were the only commercially available ^{14}C -BaP, [10- ^{14}C]BaP could not be employed in the assay. The result of BaP mineralization is shown in Table 2. A8AN3 produced very similar values of total evolved $^{14}\text{CO}_2$ from [7,10- ^{14}C]BaP and [7- ^{14}C]BaP; $41.3 \pm 2.8 \%$ ($\sim 0.25 \mu\text{mol}$ of $^{14}\text{CO}_2$) of added [7,10- ^{14}C]BaP and $42.9 \pm 1.6 \%$ ($\sim 0.24 \mu\text{mol}$ of $^{14}\text{CO}_2$) of added [7- ^{14}C]BaP. However, the Ind⁻ Tn5-mutant (2123) of A8AN3 did not mineralize any of the radiolabeled BaP used in this study.

Because of the specific activity of radiochemical [7,10- ^{14}C]BaP used in this study, interpretation of the results is difficult. Radiochemical [7,10- ^{14}C]BaP used in the study contained not only [7,10- ^{14}C]BaP but also [7- ^{14}C]BaP and [10- ^{14}C]BaP (communication with technical service department in Amersham). It is assumed that the radiochemical [7,10- ^{14}C]BaP used in the study contained [7,10- ^{14}C]BaP as a major component and [7- ^{14}C]BaP, and [10- ^{14}C]BaP as minor components. Since it is

Table 2. BaP mineralization by A8AN3.

	[7, 10- ¹⁴ C]BaP	[7- ¹⁴ C]BaP
Purchased from	Amersham	Chemsym
Radiochemical purity (%)	98.5	99.9
Spe. activity (mCi/mmol)	60	58.6
Total % mineralized (in 7 days)		
by A8AN3	41.3 ± 2.8	42.9 ± 1.6
by 2123*	0	0

*Incapable of converting indole to indigo.

technically difficult to separate each radiolabeled BaP from the mixture of [7,10- ^{14}C]BaP, [7- ^{14}C]BaP, and [10- ^{14}C]BaP, the ratio of each radiolabeled-BaP in commercial radiochemical [7,10- ^{14}C]BaP is unknown. The mineralization results suggested that either C-7 or C-10 can be converted to CO_2 by A8AN3, based on the results that wild type A8AN3 could evolve $^{14}\text{CO}_2$ from both [7,10- ^{14}C]BaP and [7- ^{14}C]BaP and the mineralization rate of [7,10- ^{14}C]BaP was more than half of the mineralization rate of [7- ^{14}C]BaP. However, it is difficult to determine the preferred position for ring cleavage based on the mineralization result because of the specific activity of [7,10- ^{14}C]BaP. Since the mutant strain of A8AN3 could not convert [7,10- ^{14}C]BaP and [7- ^{14}C]BaP to $^{14}\text{CO}_2$, a single dioxygenase with broad position-specificity might be involved in the oxidation of C-7 and C-10 in the BaP molecule.

3. Random Tn5-mutagenesis of A8AN3

Random Tn5 mutagenesis was conducted to determine if dioxygenase activity, as judged by the formation of indigo from indole (Ensley et al., 1983), was involved in BaP mineralization in A8AN3. If so, it may be possible to use the phenotype as a screening method in the isolation of the genes involved in BaP degradation. The mutagenesis experiment was also designed to determine if common enzymatic step(s) existed in the degradation of different PAHs by A8AN3. In addition to the indigo test, the catechol spray plate method was employed to screen mutants with blocked *meta*-cleavage activity.

When Tn5 was introduced into A8AN3, one Ind⁻ mutant (2123) was found among Km^r Nd^r transconjugants (Fig. 8); however, no transconjugants showed blocked *meta*-cleavage activity as determined by the catechol spray plate method. The Ind⁻ mutant was subsequently analyzed for [7,10- ^{14}C]BaP mineralization. The Ind⁻ mutant did not mineralize the ^{14}C -BaP and the other PAHs (Table 3); no clear zones were observed on

PAH spray plates (Fig. 9). The mutant was unable to grow on naphthalene vapor plates. The mutant could grow on minimal medium containing glucose, eliminating the possibility of auxotroph. Although the mutant could not degrade PAHs that were degraded by wild type A8AN3, its activity with salicylate and catechol was the same as wild type. When [U- ^{14}C]salicylate mineralization assay was performed as described in the Materials and Methods, except the assay was stopped in 1 day, the mutant 2123 mineralized ^{14}C -salicylate similarly to the wild type strain: total evolved $^{14}\text{CO}_2$ by the wild type and mutant strains were 50.1 ± 2.1 and 49.7 ± 0.8 , respectively. The mutant also produced yellow colonies (due to *meta*-cleavage product) in the catechol spray plate method. The results shown in Table 3 suggest that there may be a common enzymatic step for the degradation of all PAHs tested in A8AN3.

Together with colony morphology and color, RFLP analysis of PCR-amplified 16S rDNA (1.5 kb) indicated that the Tn5 mutant 2123 was derived from A8AN3. When PCR products of A8AN3 and 2123 were digested with restriction enzymes (*Eco*RI, *Hind*III, *Bam*HI, or *Sal*I), the two PCR-products produced the same restriction patterns (data not shown), suggesting that A8AN3 and 2123 have the same 16S rDNA.

To verify Tn5 transposition, Southern blot hybridization was performed using an *Hpa*I fragment of Tn5 as a probe. One band (approximately 18 kb DNA fragment) was detected from *Eco*RI-digested total DNA of the mutant (there is no *Eco*RI site in Tn5) (Fig. 10 and 11). However, no band was detected from the plasmid DNA of the mutant, suggesting Tn5 was inserted into chromosomal DNA. The location of Tn5 on chromosomal DNA is inconclusive because Tn5 might be located on unrecovered HMW plasmid DNA. The loss of the pRZ102 vector DNA in the mutant was confirmed by performing hybridization with a vector-specific probe (Fig. 11B). The mutant study suggests that it would be possible to use the indole oxidation function and Tn5-flanking regions to screen clones able to degrade BaP in gene banks of A8AN3 or 2123 DNA.

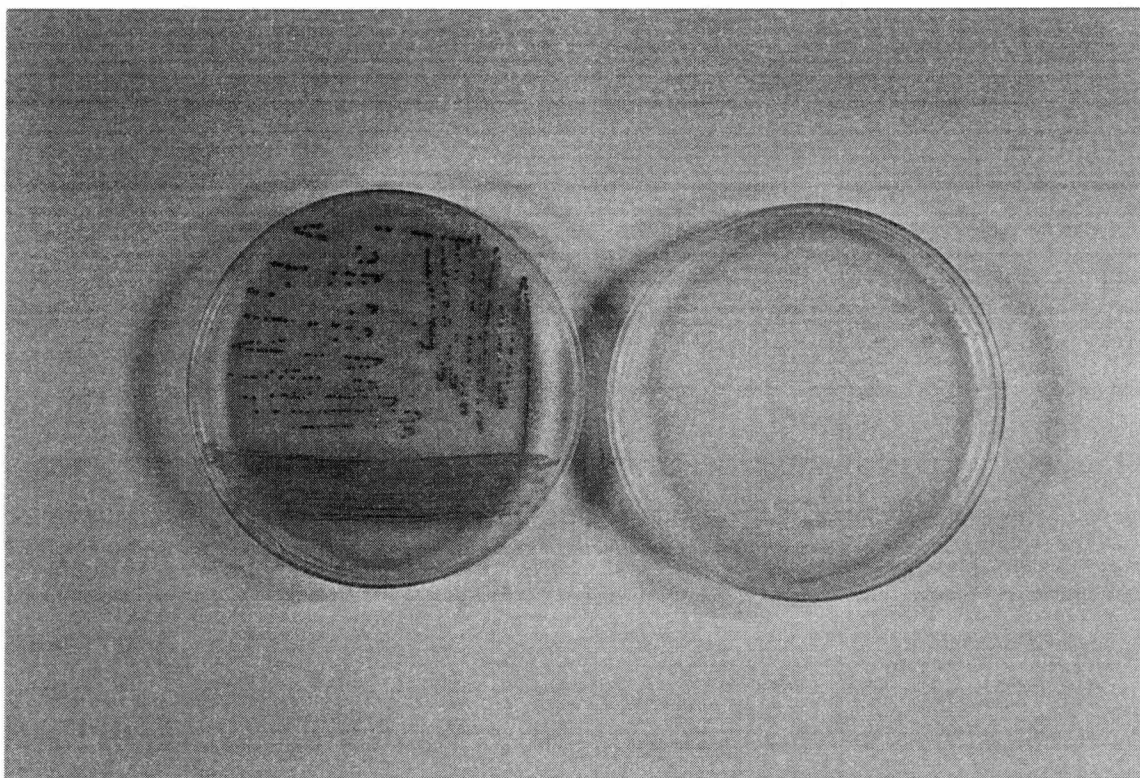
A**B**

Figure 8. Indigo formation from indole by A8AN3. A, A8AN3; B, 2123 (Tn5 mutant of A8AN3, Ind⁻). Both plates were exposed to indole vapor by placing indole crystals in the lids of the plates.

Table 3. Degradative phenotypes of A8AN3 and its Tn5 mutant (Ind⁻).

Strain	¹⁴ CO ₂ evolution from radiochemicals						Spray plate ^a				Growth medium		
	BaP	Pyr	Ant	Phn	Nah	Sal	BaP	Pyr	Ant	Phn	Cat	Naphthalene vapor plate	Glucose MM
A8AN3 (Wild type)	+	-	+	+	+	+	-	+	+	+	+	+	+
2123 (Ind ⁻ mutant of A8AN3)	-	NA	-	-	-	+	NA	-	-	-	+	-	+

Abbreviations: NA, not applicable; Glucose MM, minimal medium containing glucose (1%, w/v); Pyr, pyrene; Ant, anthracene; Phn, phenanthrene; Nah, naphthalene; Sal, salicylic acid; Cat, catechol.

^a Positive in the spray plate indicates clear zone formation on the PAH spray plates or yellow ring-cleavage product formation upon catechol spray. The naphthalene vapor plate method consists of a minimal agar medium containing naphthalene crystals (sole carbon source) in the lid of the plate.

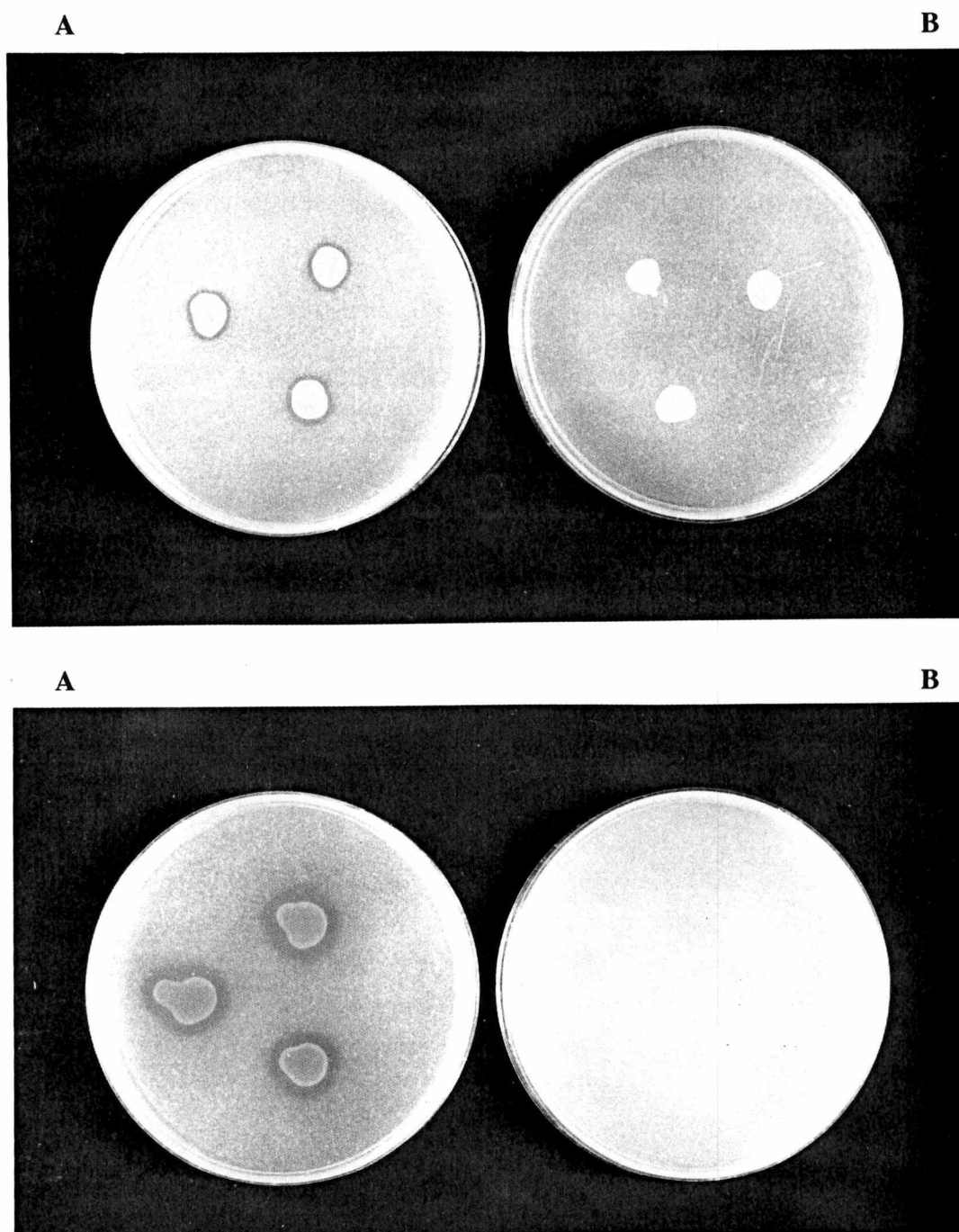


Figure 9. Clear zone formation on phenanthrene and fluorene spray plates. Top: phenanthrene spray plates of A8AN3 (A) and 2123 (Tn5 mutant of A8AN3, Ind⁻) (B). Bottom: fluorene spray plates of A8AN3 (A) and 2123 (B). Cells were spotted on YEPG plates and incubated at 28°C for 2 days. The PAHs were sprayed over the plates and the plates were re-incubated. The pictures were taken on the next day after the spraying.



Figure 10. DNA analysis of an Ind⁻ mutant of A8AN3. DNA from the mutant (2123) and A8AN3 was separated on a 0.7% agarose gel and stained with ethidium bromide. Lanes: lane 1, *Hind*III digest of *Lambda* DNA; lanes 2-5, *Eco*RI digest; lane 6, *Hind*III digest of pRZ102; lanes 7-10, uncut; lanes 2 and 7, total DNA of 2123; lanes 3 and 8, total DNA of A8AN3; lanes 4 and 9, plasmid DNA of 2123; lanes 5 and 10, plasmid DNA of A8AN3.

A

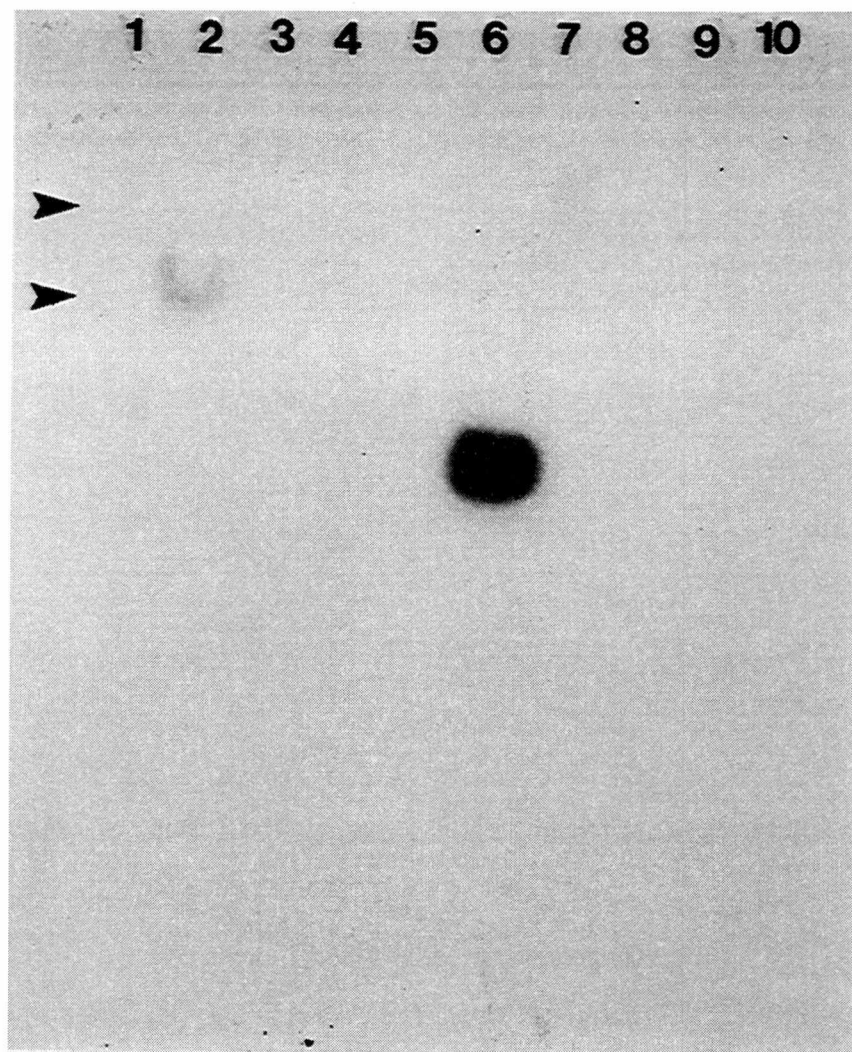


Figure 11. Southern blot analysis of an *Ind*⁻ mutant of A8AN3. This is an autoradiograph of Southern blot hybridization of the gel in Fig. 10. DNA from the mutant (2123) and A8AN3 was probed with a Tn5-specific probe (A) and vector-specific probe (B) of Tn5-carrier plasmid pRZ102. Lanes: lane 1, *Hind*III digest of *Lambda* DNA; lanes 2-5, *Eco*RI digest; lane 6, *Hind*III digest of pRZ102; lanes 7-10, uncut; lanes 2 and 7, total DNA of 2123; lanes 3 and 8, total DNA of A8AN3; lanes 4 and 9, plasmid DNA of 2123; lanes 5 and 10, plasmid DNA of A8AN3. Arrows indicate Tn5-containing *Eco*RI fragment of 2123 total DNA (lane 2) and Tn5-containing uncut total DNA of 2123 (lane 7).

B

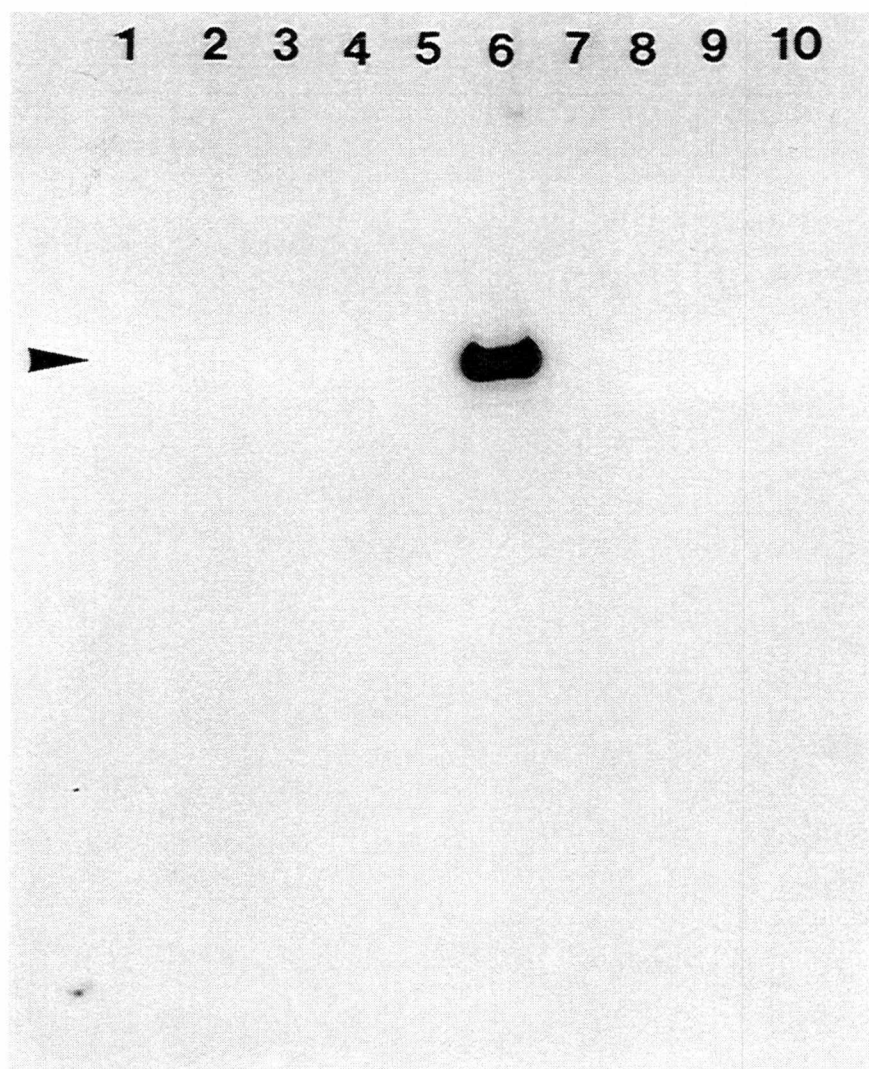


Figure 11. (Continued). Arrow indicates vector region DNA of Tn5 carrier plasmid pRZ102.

IV. DISCUSSION

Small subunit rRNA, 16S rRNA serves as a useful tool to establish phylogenetic relationships among microorganisms, because it occurs universally with constant function and it contains regions of invariable and variable sequences, in which sequences change at different rate (Woese, 1987). Previously A8AN3 was determined to be most similar to *Sphingomonas paucimobilis* based on phenotypic characteristics and chemotaxonomic traits (Ahn et al., 1994). The placement of A8AN3 in the genus *Sphingomonas* was further indicated by comparative 16S rDNA sequence analysis. Partial 16S rDNA nucleotide sequence of A8AN3 was determined and its phylogenetic placement was established. Comparative sequence analysis of the 16S rDNA suggested that the sequence of A8AN3 is closest to *S. adhaesiva* in the RDP database.

A phylogenetic tree was constructed based on comparative 16S rDNA analyses. The tree was obtained according to UPGMA of 15 taxa. Placement in the tree further suggested that A8AN3 belong to the genus *Sphingomonas*. Three BaP-degrading sphingomonads emerged as two clusters. A8AN3 emerged separately for the other BaP degraders, strains EPA 505 and B1. The BaP-degrading sphingomonads were isolated from different geographical locations. They were isolated from a polluted stream (Gibson et al., 1973), a creosote waste site (Mueller et al., 1990), a coal gasification site (Grosser et al., 1991), and a MGP site (King et al., 1991), respectively. It is interesting that among known 4 BaP-degraders, all three Gram-negative strains belong to the genus *Sphingomonas*; a Gram-positive BaP-degrader is *Mycobacterium* sp. strain RJGII-135 (Grosser et al., 1991).

Yabuuchi et al. (1990) proposed the new genus *Sphingomonas* due to its different taxonomic characteristics such as 16S rRNA sequence and sphingolipid. Based on 16S rRNA information, the genus belongs to the α -subgroup that is distant from γ -subgroup

of the *Proteobacteria*. The most remarkable characteristics of the genus is the presence of sphingolipid and the absence of lipopolysaccharide (LPS). Glycosphingolipids are found as membrane components of eukaryotic cells (Hakomori, 1983). They are located in the outer leaflet of the membrane and function in the immune response, cellular interactions, and differentiation. Sphingomonads have LPS-lacking cell envelope structures, although they are Gram-negative. Characteristic components of LPS are not found in the strains; 3-OH fatty acid (major fatty acid component of lipid A), 2-keto-3-deoxyoctonate (KDO, linkage between lipid A and polysaccharide), and L-glycero-D-manno-heptose (L,D-Hep) (Kawahara et al., 1991). It was assumed that instead of LPS, sphingomonads contained sphingolipid. Using immunoelectron microscopic technique, Kawasaki et al. (1994) actually demonstrated the presence of sphingolipid in the outer leaflet of the outer membrane of *S. paucimobilis*.

Current trend of paying attention to *Sphingomonas* strains is because not only they have unique sphingolipids but also they show wide presence in the environment and metabolic potential for bioremediation and environmental sustainability. Sphingomonads were found in (surface and subsurface) soils (Fredrickson et al., 1991, 1995; King et al., 1991), water (Gibson et al., 1973), and clinical specimens (Yabuuchi et al., 1990). They have been shown to degrade a variety of aromatic hydrocarbons (Gibson et al., 1975; Mueller et al., 1990; Fredrickson et al., 1991; Ahn et al., 1994). Studies on metabolic versatility and phylogenetic relationships of *Sphingomonas* strains yield the significance of the α -subgroup of *Proteobacteria* in the environment as well as previously better studied γ -subgroup bacteria.

BaP-degraders were originally isolated as degraders of simpler compounds (e.g., biphenyl, anthracene, fluoranthene, and pyrene) (Gibson et al., 1973; Mueller et al., 1990; Grosser et al., 1991; King et al., 1991). Later their ability to mineralize BaP was demonstrated (Sherrill, 1982; Grosser et al., 1991; Ahn et al., 1994; Ye et al., 1996). A

decline of BaP concentration was reported in pure culture of *Pseudomonas putida* NCIB 9816 (Barnsley, 1975). *Mycobacterium* sp. strain PYR-1 was reported to produce unidentified BaP metabolites (Heitkamp & Cerniglia, 1988). However, no mineralization of BaP has been observed with PYR-1; no mineralization of BaP has been demonstrated with NCIB 9816.

Considering BaP toxicity and persistence in the environment, the extent of BaP mineralization by A8AN3 was of interest. A8AN3 mineralized BaP although it did not form clear zones on the BaP spray plates. The maximum $^{14}\text{CO}_2$ recovery from [7,10- ^{14}C]BaP mineralization was 47% in A8AN3 culture (Fig. 6). Figure 6 shows the time course of [7,10- ^{14}C]BaP mineralization by the strain. A8AN3 produced $^{14}\text{CO}_2$ rapidly during exponential growth. When cell growth reached stationary phase after 20h of incubation $^{14}\text{CO}_2$ evolution was continuously increasing, although the evolution rate was decreased. Considering the radiolabeled positions, the result indicated that A8AN3 mineralized at least one ring of 5-ringed BaP molecule. Mineralization was performed using two radiochemicals ([7,10- ^{14}C]BaP and [7- ^{14}C]BaP) and compared evolved the amounts of $^{14}\text{CO}_2$. A8AN3 similarly mineralized [7,10- ^{14}C]BaP and [7- ^{14}C]-BaP (Table 2). It suggested that considering the specific activity of radiochemical [7,10- ^{14}C]BaP, either C-7 or C-10 could be converted to CO_2 by A8AN3.

A complete BaP degradation pathway has not been proposed to date. However, according to a previous report (Gibson et al., 1975), bacterial oxidation of BaP has been shown to produce an isomeric mixture of *cis*-dihydrodiols. The dihydrodiols were 9,10-dihydrodiol (major) and 7,8-dihydrodiol (minor). Based on ring cleavage products produced by a *Mycobacterium* sp. strain RJGII-135 (Schneider et al., 1996), it is possible that either C-7 or C-10 could be converted to CO_2 . Since the mutant strain of A8AN3 could convert none of [7,10- ^{14}C]BaP and [7- ^{14}C]-BaP to $^{14}\text{CO}_2$, a single dioxygenase

with broad position-specificity might be involved in the oxidation of C-7 and C-10 in a BaP molecule.

The potential for the accumulation of metabolites, their toxicity and subsequent biodegradation require assessment. Attempts to identify BaP metabolites were unsuccessful, evidently because of the low water-solubility (3.8 $\mu\text{g/L}$) of BaP. Although BaP-mineralizing bacteria have been reported, it is unknown if they can completely mineralize BaP. Currently available ^{14}C -BaP ([7- ^{14}C] or [7,10- ^{14}C]BaP) may yield limited information on BaP mineralization due to localized ^{14}C label(s) at only one ring of the five-ringed BaP. ^{14}C -BaP labeled at different positions (e.g., [10- ^{14}C]BaP, [3- ^{14}C]BaP, [5- ^{14}C]BaP, [6- ^{14}C]BaP, [12- ^{14}C]BaP, etc.) will give us better information about BaP mineralization by microorganisms.

Tn5 mutagenesis was performed to localize BaP-degradative genes. The mutagenesis was done to facilitate the isolation of genes involved in BaP degradation. The isolated genes could be used to study BaP metabolites and to develop new gene-probes. Previous studies showed that A8AN3 did not hybridize with NAH7-derived gene probes (Ahn et al., 1994). The mutagenesis experiment was also designed to investigate if common enzymatic step(s) existed in the degradation of different PAHs by A8AN3. Through Tn5 mutagenesis, a mutant strain of A8AN3 was obtained which lost the ability to oxidize indole and to mineralize BaP. The Ind⁻ mutant did not degrade other PAHs degraded by wild type A8AN3, suggesting a common enzymatic step for the degradation of all PAHs tested in A8AN3. When Southern blot analysis was performed using a Tn5-specific probe, total DNA from the mutant showed positive hybridization but plasmid DNA did not. The results suggested that Tn5 was inserted into the chromosomal DNA of the Ind⁻ mutant. However, the Southern blot analysis results did not exclude the possibility of Tn5-insertion into unrecovered HMW plasmid DNA.

When the salicylate mineralization assay and catechol spray plate method were performed, the Ind⁻ mutant showed activity with salicylate and catechol that are known to be the most common metabolites of PAHs. Its ability to degrade salicylate and produce *meta*-cleavage product of catechol suggested that at least two operons might be present in PAH-degradative genes of A8AN3. No transconjugant showed blocked extradiol dioxygenase activity as screened by the catechol spray method. This suggested that A8AN3 might contain more than one extradiol dioxygenase gene in different operons. NAH7 from *P. putida* PpG7 contains two extradiol dioxygenase genes; *nahC* and *nahH* (Yen & Gunsalus, 1982). *S. yanoikuyae* B1 possesses two extradiol dioxygenase genes; *bphC* and *xylE* (Kim & Zylstra, 1995). Both extradiol dioxygenase genes in each strain show activity with both catechol and dihydroxylated 2-ring compounds (e.g., dihydroxylated naphthalene or dihydroxylated biphenyl), although relative activity was different (Ghosal et al., 1987; Kuhm et al., 1991; Kim & Zylstra, 1995).

Tn5 is likely inserted in the initial dioxygenase genes or upstream of the genes in an operon where the initial dioxygenase genes are present. Tn5 is unlikely inserted in a regulatory gene in A8AN3 because 2123 does not show the basal level of initial dioxygenase activity that is observed in wild type A8AN3. A8AN3 is routinely grown in YEPG which is a complex medium. When indole crystals are placed in the lids of YEPG plates, A8AN3 produces indigo, suggesting a basal level of initial dioxygenase gene expression without induction. Although the inducer for PAH-degradative genes in A8AN3 was not determined, growth in YEPG medium is assumed to be an uninduced condition because generally inducers for degradative-genes are metabolites or substrates themselves.

In this study, phylogenetic placement of previously isolated BaP-mineralizing *Sphingomonas* strain A8AN3 was established and the BaP mineralization ability of the strain was tested. Besides, BaP-degradative genes were localized by random Tn5

mutagenesis of A8AN3. Since strain A8AN3 was isolated from soil in which degradation of PAHs including BaP was actually observed, the results of this study will provide insight into the efficacy of an A8AN3 population to degrade PAHs including BaP. A naphthalene-degradative plasmid NAH7 is from a γ -subgroup fluorescent pseudomonad and is well studied in genetics and biochemistry. NAH7-derived gene probes have been used to determine *in situ* microbial potential to degrade PAHs. The microbial potential to degrade PAHs has been underestimated by current NAH7-derived gene probes because some PAH-degrading microbial populations do not hybridize with the probes. The A8AN3 population is one of those populations and is not considered in determination of *in situ* microbial potential to degrade PAHs when NAH7-derived gene probes are used. Further molecular characterization of PAH-degradative genes in A8AN3 is warranted for development of new biotechnology which can provide better understanding of *in situ* microbial potential and activity of PAH degradation.

**Part 4: Molecular Characterization of Extradiol Dioxygenase Genes
Isolated from A8AN3 and Its Mutant Strain**

I. INTRODUCTION

PAHs are ubiquitous environmental pollutants mainly produced from incomplete combustion of organic materials. Major sources of PAHs are anthropogenic such as fossil fuel combustion, automobile exhaust, waste incineration and coal tar. Increasing industrial development results in a rising rate of production and accumulation of PAHs. Bioremediation of PAH-polluted sites has received attention as an alternative to a traditional cleanup method of incineration. Many bacteria show metabolic versatility to degrade various aromatic hydrocarbons. Bioremediation can take advantage of metabolic versatility and wide occurrence of microorganisms to clean up PAH-contaminated sites. Better understanding of microbial degradative pathways and genes involved in PAH-degradation can provide better bioremediation strategies.

Bacterial degradation of PAHs has been well characterized for the naphthalene catabolic enzyme system encoded by the plasmid NAH7 of *Pseudomonas putida* PpG7. Based on biochemical and genetic information of the NAH7-encoded enzyme system, NAH7-derived gene probes can be used to determine the *in situ* microbial potential to degrade naphthalene as well as other PAHs such as methylated naphthalene, phenanthrene, anthracene (Sayler & Layton, 1990; Fleming et al., 1993; Sanseverino et al., 1993b; personal communication with J. Leblond). NAH7-derived gene probes were used to characterize PAH-contaminated MGP sites. Studies showed that PAH-degraders with NAH7-like genotype were present in the sites (King et al., 1990; Sanseverino et al., 1993a) and dominant among culturable PAH-degraders (King et al., 1991; Ahn et al., 1994). The *nah* gene frequency was positively correlated with PAH-degradation activity (Fleming et al., 1993; Sanseverino et al., 1993b). However, there is a potential that PAH-degraders with dissimilar PAH-catabolic genes or pathways also exist and contribute to *in situ* PAH-degradation. Indeed, some PAH-degraders were shown not to hybridize with

NAH7-derived gene probes (Foght & Westlake, 1991, 1988; King et al., 1991; Fredrickson et al., 1991; Ahn et al., 1994; Goyal & Zylstra, 1996) and such PAH-degraders were isolated from MGP sites (King et al., 1991; Ahn et al., 1994). Therefore, biochemical and genetic information on those PAH-degraders is necessary for the development of new biotechnology which will provide a better understanding of microbial activity in the environment.

Biodegradation of aromatic hydrocarbons is known to converge to a few intermediates such as catechol or substituted catechols (Evans et al., 1965; Dagley & Gibson, 1965; Barnsley, 1976; Sutherland et al., 1995). These intermediates serve as substrates of ring-cleavage dioxygenases. Further catabolism converts ring-fission products to small aliphatic compounds (e.g., pyruvate and acetaldehyde) that can enter the tricarboxylic acid (TCA) cycle. Many aromatic hydrocarbons such as naphthalene, biphenyl, benzene, and toluene can be channeled into ring cleavage via conversion to catechol by degradative enzymes (Catelani et al., 1971; Dagley, 1971; Williams & Murray 1974; Barnsley, 1976).

Two classes of ring-fission dioxygenases exist on the basis of their action position in ring cleavage. Ring-cleavage can occur either between two dihydroxylated carbons or between a hydroxylated carbon and its adjacent nonhydroxylated carbon. The former case is called *ortho*-cleavage and is catalyzed by an intradiol dioxygenase such as catechol 1,2-dioxygenase. The later is called *meta*-cleavage and is catalyzed by an extradiol dioxygenase such as catechol 2,3-dioxygenase. Catechol degradation pathway through *meta*-cleavage is called the *meta*-pathway. Ring fission dioxygenases require iron for their activity except for three manganese-dependent dioxygenases (Que et al., 1981; Qi, 1991; Harayama et al., 1992; Boldt et al., 1995).

Ring-cleavage dioxygenases have been studied and found in both Gram-positive and -negative strains (Nakai et al., 1983; Harayama & Rekik, 1989; Candidus et al., 1994;

Boldt et al., 1995). Most studies of ring-cleavage dioxygenases have been achieved with those isolated from Gram-negative bacteria, especially γ -subgroup fluorescent pseudomonads (e.g., *P. putida* and *P. aeruginosa*) in *Proteobacteria* (Nakai et al., 1983; Furukawa & Arimura, 1987; Harayama & Rekik, 1989; Zylstra & Gibson, 1989). Well-characterized catechol 2,3-dioxygenases are XylE encoded by the toluene-degradative plasmid pWWO of *P. putida* mt-2 (Nakai et al., 1983; Harayama & Rekik, 1989) and NahH encoded by the naphthalene-degradative plasmid NAH7 of *P. putida* PpG7 (Ghosal et al., 1987; Harayama et al., 1987a). XylE and NahH are closely related at both the levels of nucleotide and amino acid sequences (Ghosal et al., 1987; Harayama et al., 1987a).

Since Yabuuchi et al. proposed the new genus *Sphingomonas* in 1990, *Sphingomonas* strains have been receiving increasing attention not only because of their unique sphingolipids but also because of their wide presence in the environment and metabolic potential for bioremediation. Sphingomonads, an α -subgroup of *Proteobacteria*, have been found in (surface and subsurface) soils (Fredrickson et al., 1991, 1995; King et al., 1991; Kastner et al., 1994), rhizosphere (Takeuchi et al., 1995), water (Gibson et al., 1973, Holmes et al., 1977), and clinical specimens (Holmes et al., 1977; Yabuuchi et al., 1990). They have been shown to degrade a variety of aromatic hydrocarbons (Gibson et al., 1973; Mueller et al., 1990; Fredrickson et al., 1991; Ahn et al., 1994). Studies on the metabolic versatility of *Sphingomonas* strains show the significance of the α -subgroup of *Proteobacteria* in the environment as well as the better studied γ -subgroup fluorescent pseudomonads. In contrast to γ -subgroup fluorescent pseudomonads, not much information is available about the sphingomonad genes involved in degradation of aromatic hydrocarbons.

An MGP-site soil isolate *Sphingomonas* sp. strain A8AN3 showed ability to degrade a broad range of PAHs including BaP (Ahn et al., 1994). Presence of dissimilar

PAH-degradative genes in A8AN3 was suggested by previous hybridization studies with plasmid NAH7-derived gene probes. To investigate the genes at molecular level, genomic libraries of A8AN3 and its Tn5-mutant were constructed. In this study, genes encoding extradiol dioxygenases were isolated from gene libraries of A8AN3 and 2123 (Tn5-mutant of A8AN3) and molecularly characterized. Nucleotide sequences of the genes were determined and deduced amino acid sequences were compared to the other extradiol dioxygenases. Enzyme assays were performed to analyze substrate specificity of extradiol dioxygenases encoded by the genes.

II. MATERIALS AND METHODS

1. Strains and plasmids

A HMW PAH degrader, *Sphingomonas* sp. strain A8AN3 was previously found through the analyses of a culture collection (Ahn et al., 1994). The strain showed ability to degrade a broad range of PAHs including BaP. Its Ind⁻ mutant strain 2123 (Ind⁻ BaP⁻ Km^r) was obtained by random Tn5-mutagenesis of A8AN3 as described in Part 3. Together with colony morphology, color, and indigenous plasmid profile, RFLP analysis of PCR-amplified 16S rDNA (1.5 kb) verified that the Tn5-mutant 2123 was derived from A8AN3. As determined by PAH-mineralization assay and PAH spray plate method, the mutant could no longer degrade PAHs that wild type strain A8AN3 could degrade. However, the mutant produced yellow *meta*-cleavage product (2-hydroxymuconic semialdehyde) as shown by the catechol spray plate method. Table 1 shows sources and characteristics of strains and plasmids used in this study. *E. coli* DH5 α was used as a host strain for all gene cloning experiments. Vector plasmid pBluescript II SK(+) was used for gene library construction. For subcloning experiments, vectors pBluescript II SK(+) or pUC19 were used. 5-Bromo-4-chloro-3-indolyl- β -D-thiogalactopyranoside (X-gal, 40 μ g/ml) was added into LB plates containing ampicillin to isolate subclones.

2. Chemicals and enzymes

All chemicals were purchased from Millinckrodt Chemical, Inc. (Paris, KY) unless otherwise specified. 1,2-Dihydroxynaphthalene (purity, 99.3%) and 3-methylcatechol (purity, 99%) were purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI). Catechol (purity, 99%) and 4-methylcatechol (purity, 98.1%) were

Table 1. Strains and plasmids used in the study.

Strain or plasmid	Relevant characteristic	Reference
A8AN3	BaP ⁺ a Ind ⁺ b C23O ⁺ c	This study
2123	BaP ⁻ Ind ⁻ C23O ⁺ Tn5 mutant of A8AN3	This study
<i>E. coli</i>		
DH5 α	<i>hadR recA lacZ</i> AM15 Nal ⁺	BRL Inc.
HB101	<i>hadS recA</i>	Sambrook et al., 1989
C600	<i>hadR</i>	Sambrook et al., 1989
plasmid		
pBluescript II SK(+)	Am ^r , ColE1 replicon	Stratagene
pUC19	Am ^r , ColE1 replicon	BRL Inc.
pRZ102	Tn5::ColEI	Jorgensen et al., 1979
pUTK14	4.42-kb <i>Sau</i> 3AI fragment from A8AN3 plasmid DNA inserted into <i>Bam</i> HI site of pBluescript. Hybridize with pS3 or pUTK15. C23O ⁺	This study
pUTK15	12.42-kb <i>Sst</i> I fragment from 2123 plasmid DNA inserted into <i>Sac</i> I site of pBluescript. C23O ⁺	This study
pUTK141	2.3-kb <i>Sma</i> I- <i>Sst</i> I fragment of pUTK14 inserted into <i>Sma</i> I- <i>Sst</i> I sites of pBluescript. C23O ⁺	This study
pUTK142	2.3-kb <i>Sma</i> I- <i>Sst</i> I fragment of pUTK14 inserted into <i>Sma</i> I- <i>Sst</i> I sites of pUC19. C23O ⁻	This study
pY143	1.1-kb <i>Sst</i> I- <i>Bam</i> HI fragment of pUTK141 inserted into <i>Sac</i> I- <i>Bam</i> HI sites of pBluescript. C23O ⁻	This study

Table 1. (Continued).

Strain or plasmid	Relevant characteristic	Reference
pY144	1.3-kb <i>EcoRI-SmaI</i> fragment of pUTK141 inserted into <i>EcoRI-SmaI</i> sites of pBluescript. C23O ⁻	This study
pS3	13.85-kb <i>SstI</i> fragment from 2123 plasmid DNA inserted into <i>SacI</i> site of pBluescript. Hybridize with pUTK14. C23O ⁻	This study
p5-1	6.6-kb <i>SstI</i> fragment from 2123 genomic DNA inserted into <i>SacI</i> site of pBluescript. Hybridize with Tn5. Km ^r C23O ⁻	This study
pUTK16	13.45-kb <i>SstI</i> fragment from 2123 genomic DNA inserted into <i>SacI</i> site of pBluescript. C23O ⁺	This study
pUTK161	7.45-kb <i>SmaI-SstI</i> fragment of pUTK16 inserted into <i>SmaI-SstI</i> sites of pBluescript. C23O ⁺	This study
pUTK162	2.8-kb <i>NotI</i> fragment of pUTK161 inserted into <i>NotI</i> site of pBluescript. Opposite direction to pUTK163. C23O ⁺	This study
pUTK163	2.8-kb <i>NotI</i> fragment of pUTK161 inserted into <i>NotI</i> site of pBluescript. Opposite direction to pUTK162. C23O ⁺	This study
pY164	1.1-kb <i>NotI-SalI</i> fragment of pUTK162 inserted into <i>NotI-SalI</i> sites of pBluescript. C23O ⁻	This study
pUTK169	2.2-kb <i>NotI-SalI</i> fragment of pUTK162 inserted into <i>NotI-SalI</i> sites of pBluescript. C23O ⁺	This study

Table 1. (Continued).

Strain or plasmid	Relevant characteristic	Reference
pY170	1.9-kb <i>Bam</i> HI- <i>Sma</i> I fragment of pUTK162 inserted into <i>Bam</i> HI- <i>Sma</i> I sites of pBluescript. C23O ⁻	This study

^a Ability to mineralize ¹⁴C-BaP.

^b Dioxxygenase activity as determined by ability to convert indole to indigo.

^c Ability to produce catechol 2,3-dioxygenase activity as determined by catechol spray plate method (Zukowski et al., 1983).

obtained from Sigma Chemical Company (St. Louis, MO). 2,3-Dihydroxybiphenyl (purity, 98%) was purchased from Wako Pure Chemical Industry Ltd. (Osaka, Japan). All solvents and chemicals were the highest purity available. 2,3-Dihydroxynaphthalene (95%, ICN Biomedicals Inc., Irvine, CA) was purified by Dr. F. -M. Menn using the sublimation method.

[α -³²P]dCTP purchased from ICN Biomedicals Inc. was used for radiolabeled gene probe preparations. Restriction endonucleases, T4 DNA ligase, and calf intestinal alkaline phosphatase (CIAP) were purchased from GIBCO BRL (Gaithersburg, MD) and used as manufacturer suggested. *GeneAmp*® PCR reagent kit with *AmpliTaq*® DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT) was used for the PCR in gene probe preparations as described in Part 2. Low-melting-temperature (LMT) agarose, Sea Plaque GTG was obtained from FMC BioProducts (Rockland, ME).

3. Media and growth conditions

A8AN3 was grown in YEPG medium at 28°C as described in Part 2. YEPG containing kanamycin was employed to grow its Tn5-mutant strain 2123. *E. coli* strains were grown in Luria-Bertani (LB) broth (Sambrook et al., 1989) at 37 °C. Antibiotics were added into media at the following concentrations when needed: ampicillin (Am), 100 µg/ml; kanamycin (Km), 50 µg/ml.

4. DNA isolation and analysis

Total DNA and plasmid DNA were prepared as described in Part 2. DNA hybridization (colony or Southern blot hybridization) was also performed under high stringency conditions as described in Part 2. Gene probes were prepared by the random

primer labeling method (Feinberg & Vogelstein, 1983) unless otherwise specified. DNA digested with appropriate restriction enzyme(s) was subject to LMT agarose (1%, w/v) gel electrophoresis to separate DNA fragments. The fragment of interest was recovered from the agarose gel and labeled using Prime It® (Stratagene, La Jolla, CA) according to manufacturer's protocol. The labeled probes were purified using a NucTrap Push Column (Stratagene, La Jolla, CA).

PCR was also employed to obtain specific probes for extradiol dioxygenase genes on plasmids pUTK169 and pUTK141. Probe preparation by PCR was performed as described in Part 2 except as follows. For the PCR (total vol. 100 µl), 100 ng of template DNA (pUTK169 or pUTK141) and 60 ng of each primer were used. Table 2 shows the primer sets used to amplify extradiol dioxygenase genes on plasmids pUTK141 and pUTK169. Reaction conditions consisted of 25 cycles of 96°C (10 sec), 50°C (5 sec), and 60°C (4 min). Before the 25 cycles, preheating of 94°C (5 min) was employed. After the 25 cycles, final chain elongation was conducted at 72°C for 5 min. The sizes of the amplified products were determined by agarose (1%, w/v) gel electrophoresis. The primers used in PCR were synthesized by the Oligo 1000 DNA Synthesizer (Beckman Instruments Inc., Fullerton, CA) at the Center for Environmental Biotechnology (CEB), University of Tennessee, Knoxville. The Beckman Cleavage and Deprotection Kit was used to cleave synthesized primers from solid support and to remove protecting groups as described in manufacturer's protocol.

5. Gene library construction and DNA subcloning

Total genomic DNA from A8AN3 and its Ind⁻ mutant was partially digested with *Sau3AI* and *SstI*, respectively. The digested DNA was fractionated by sucrose gradient centrifugation as described (So et al., 1987). Approximately 4-10 kb *Sau3AI* fraction and

Table 2. Primer sets used to prepare gene probes.

Template	Primers	Size of PCR product
pUTK141	5'ATGGCTGTCACGGGCGTATTGC3' 5'CGACCGCTTCTTCGGTGACG3'	0.55 kb
pUTK169	5'GGTCTCAATCTCGTCAGCGTCG3' 5'CTGCATGAAAGGAAGATACCGAGA3'	0.5 kb

6-15 kb *Sst*I fraction were recovered and ligated to pBluescript II SK(+) digested with *Bam*HI and *Sac*I, respectively; *Sac*I and *Sst*I are isoschizomers. Vector DNA was treated with CIAP before ligation. Ligated DNA was transformed into library efficiency competent *E. coli* DH5 α cells (Gibco BRL).

Transformants were screened for aromatic ring dioxygenase activity on LB plates containing ampicillin and 1 mM indole. Transformants were also subjected to the catechol spray plate method (Zukowski et al., 1983) to screen clones containing extradiol dioxygenase genes. Km^r was used for clones containing Tn5-flanking regions to screen clones containing Tn5 and its flanking region DNA from 2123.

6. Nucleotide sequence determination and analysis

Nucleotide sequences of both strands were determined by the dideoxy-chain termination method (Sanger et al., 1977). Automated sequencing was performed by an Applied Biosystems model 373A DNA sequencer (Foster City, CA) (The Univ. of Tennessee Dept. of Microbiology Molecular Biology Resource Facility). M13 forward or reverse primers on pBluescript II SK(+) vector were employed for the initial sequencings. The primers were purchased from New England Biolabs Inc. (Beverly, MA). For the continual sequencings, primers were synthesized by the Oligo 1000 DNA Synthesizer at CEB as described above. Computer analyses of sequences were performed with the Genetics Computer Group (GCG) package, Version 8 (Devereux et al., 1984): the programs used were MAP and MAPSORT for restriction site identification, TRANSLATE for deduced amino acid sequence determination, FRAME for open reading frame (ORF) identification, PUBLISH for sequence presentation, GAP or BESTFIT for sequence alignment, and PILEUP for multiple sequence alignment or dendrogram construction. The BLAST program (Altschul et al., 1990) was used to search the

GenBank database (Benson et al., 1993) for similar nucleotide sequences and amino acid sequences. The program was also used to search specific nucleotide or amino acid sequences in the GenBank database. The nucleotide sequences of *cdo1* and *cdo2* (Fig. 5 and 6) were deposited in GenBank under accession numbers U73127 and U73128, respectively.

7. Enzyme assays

Crude enzyme assays were performed to quantitate activity and to determine relative activity of extradiol dioxygenases. Crude enzyme was obtained by the procedure described previously (Furukawa & Arimura, 1987). *E. coli* strains harboring recombinant DNA (pUTK141 or pUTK163) were grown in 100 ml of LB broth supplemented with ampicillin (LB_{am}). Cells were harvested when the optical density of the culture reached 1.0 at 600 nm. Harvested cells were washed in 50 ml of 50 mM phosphate buffer (pH 7.5) and suspended in 2 ml of the same buffer containing 10% acetone (v/v). The suspended cells were disrupted by sonication (Sonifier[®] cell disrupter, Model W140, Heat Systems-Ultrasonics, Inc., Plainview, L.I., NY). Twenty pulses of 30-second duration were applied; pulses were separated by 1 minute intervals. Cell suspensions were kept on ice throughout the disruption procedure. Cell debris was removed by centrifugation at 100,000 x g for 30 min at 4 °C. The supernatant was stored at -70°C until use as a crude enzyme for the assays. As a negative control, *E. coli* DH5 α [pBluescript II SK (+)] grown in LB_{am} was subjected to the same procedure.

Extradiol dioxygenase activity was determined spectrophotometrically by measuring the increase in the absorbance at the wavelength (λ_{max}) of *meta*-cleavage products formed. Reaction rates were calculated by using extinction coefficients (ϵ). Substrates used were the following: 1,2-dihydroxynaphthalene, λ_{max} = 331 nm and ϵ =

2,600 cm⁻¹ M⁻¹ (Kuhm et al., 1991); 2,3-dihydroxybiphenyl, λ_{max} = 434 nm and ϵ = 22,000 cm⁻¹ M⁻¹ (Furukawa et al., 1983); catechol, λ_{max} = 375 nm and ϵ = 33,400 cm⁻¹ M⁻¹; 3-methylcatechol, λ_{max} = 388 nm and ϵ = 13,800 cm⁻¹ M⁻¹; 4-methylcatechol, λ_{max} = 382 nm and ϵ = 28,100 cm⁻¹ M⁻¹ (Bayly et al., 1966). Substrates were freshly prepared for each assay. Substrates 1,2-dihydroxynaphthalene, 2,3-dihydroxynaphthalene, and 2,3-dihydroxybiphenyl were dissolved in dimethylformamide; the other substrates were dissolved in distilled water.

The enzyme reaction was performed in 1.0-ml quartz cuvettes with a 1-cm light path. The enzyme reaction mixture (final vol., 1 ml) contained 50 mM phosphate buffer (pH 7.5), an appropriate volume of crude enzyme and 10 μ l of 100 mM substrate. The reaction was initiated by the addition of a substrate. Reaction mixtures prepared without substrate were used as blanks. Enzyme activities were determined for 1 min. The amount of protein was measured with the BCA protein assay reagent (Pierce, Rockford, IL) according to the manufacturer's protocol. Bovine gamma globulin was used as the standard in the protein assay. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1.0 μ mol of *meta*-cleavage product per minute. Specific enzyme activity was defined as the number of enzyme units per milligram of total protein.

III. RESULTS

1. Isolation of extradiol dioxygenase genes

Genomic libraries of strains A8AN3 and its Ind⁻ mutant were constructed as described in Materials and Methods. Three randomly selected clones from each library were subjected to mini plasmid-isolation to analyze insert size in the clones. Enzyme (*Sst*I) digestion of the isolated plasmids showed that insert sizes were in the range of size fractions used for the gene library construction. To represent two genome equivalent, a total of 5300 and 3500 clones were collected from A8AN3 and 2123 gene libraries, respectively. Because the genome size of the genus *Sphingomonas* is unknown, the size was assumed to be similar to *E. coli* genome size, 4,000 kb (Sambrook et al., 1989). The following equation (Clarke & Carbon, 1976) was used to calculate the number of clones required to represent the genome size.

$$N = \frac{\ln (1-P)}{\ln (1-f)}$$

N: number of clones needed to represent one genome equivalent

f: fraction of genome in each clone (= insert size/genome size)

P: probability

Am^r transformants were screened for either initial dioxygenase activity or *meta*-cleavage activity by the indigo test and catechol spray plate method, respectively. Km^r was used to screen Tn5-flanking regions in the genomic library constructed with 2123. As results of screening gene libraries of A8AN3 or 2123, clones showing *meta*-cleavage

enzyme activity or kanamycin resistance were isolated. However, clones expressing initial dioxygenase activity were not found as determined by indigo test.

Two clones isolated from the 2123 genomic library showed resistance to kanamycin and ampicillin. DNA analyses using restriction endonucleases indicated that they were the same clones. Therefore, one of the two Km^r clones, p5-1 was chosen for further study. Clone p5-1 contained short Tn5-flanking regions and Tn5 as determined by colony hybridization and restriction analyses. The clone hybridized strongly with a Tn5-specific probe (5.75-kb *Hpa*I fragment of pRZ102) in colony hybridization (data not shown). Digestion of p5-1 with *Sst*I produced a 6.6-kb insert. Considering Tn5 does not contain an *Sst*I site and the size of Tn5 is 5.8 kb, short Tn5-flanking regions were a total of 0.8 kb. When the clone was digested with *Hpa*I or *Hind*III, the expected 5.75- and 3.3-kb DNA fragments were found. *Hpa*I sites are present at both terminal-regions of Tn5, and *Hind*III sites are present within the inverted repeats (Jorgensen et al., 1979).

Sixteen clones showed *meta*-cleavage activity with the catechol spray plate method. Some of them appeared to be the same clones as determined by DNA analyses with restriction endonucleases. Others appeared to contain overlapping common DNA fragments. Therefore, among the clones showing *meta*-cleavage activity two unique clones, pUTK15 (12.42-kb insert) and pUTK16 (13.45-kb insert) were selected for further study. The clones were determined to be different from each other as judged by restriction analyses (Fig. 1) and colony hybridization. When an 1.12-kb *Eco*RI fragment of pUTK169 was used as a gene probe, the clone pUTK16, wild type A8AN3, and the mutant strain 2123 showed positive but *E. coli* DH5 α harboring pUTK15 or pBluescript II SK (+) did not (data not shown), suggesting that the insert of pUTK16 was different from pUTK15, and that A8AN3 or 2123 contained the DNA fragment used as a gene probe. A clone pUTK14 isolated from genomic library of A8AN3 also showed *meta*-cleavage activity.

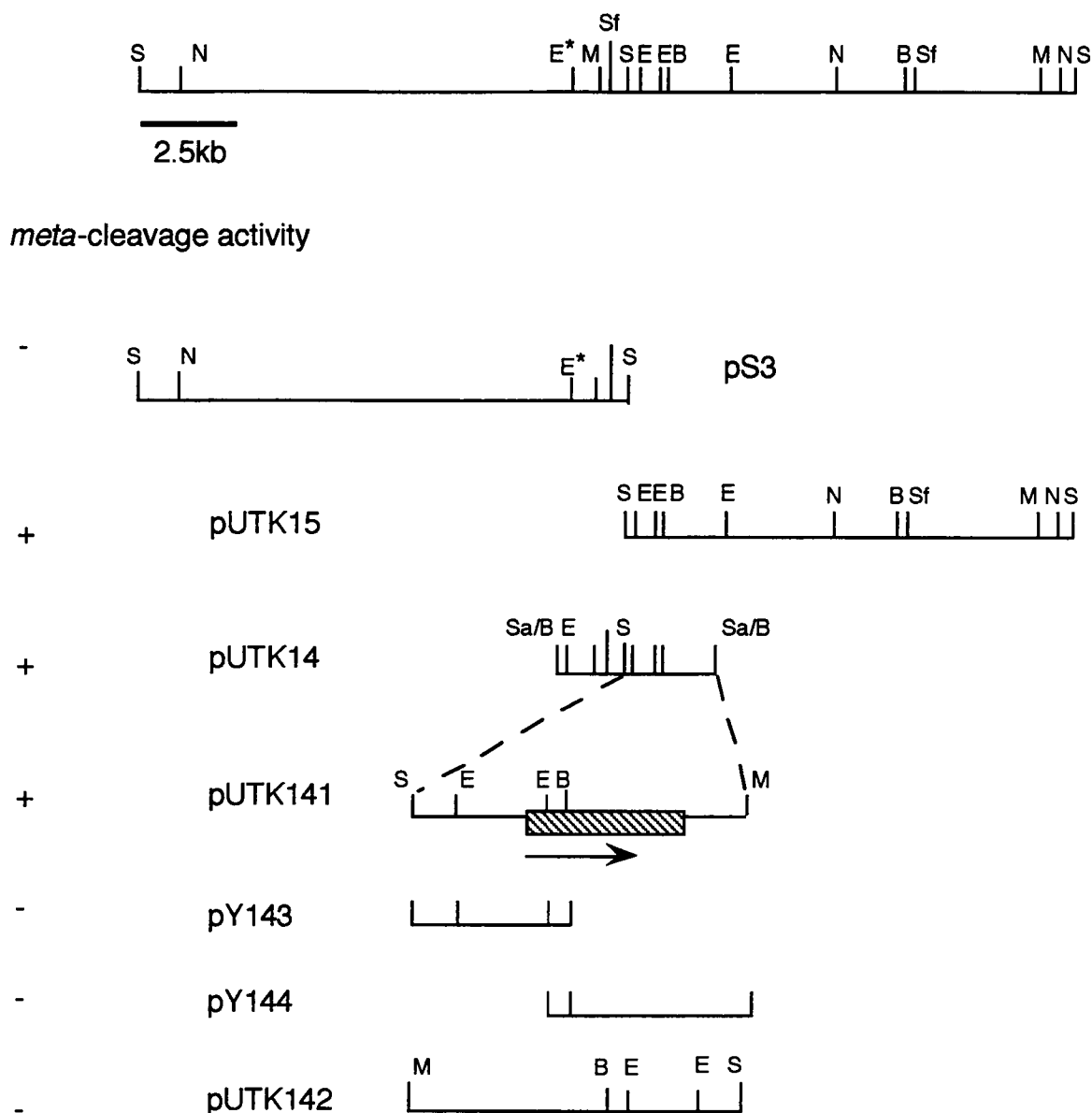


Figure 1. Location of extradiol dioxygenase genes and Tn5-flanking regions from A8AN3 and its Tn5-mutant 2123. Abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hind*III; Hp, *Hpa*I; M, *Sma*I; N, *Not*I, S, *Sst*I, Sf, *Sfi*I; Sa, *Sau*3AI. Arrows indicate transcriptional direction of the genes. The *lac* promoter on the cloning vector is located on the left of each map; transcription precedes toward right. Hatched boxes are loci of the extradiol dioxygenase genes. Asterisks denote non-unique restriction sites.

meta-cleavage activity

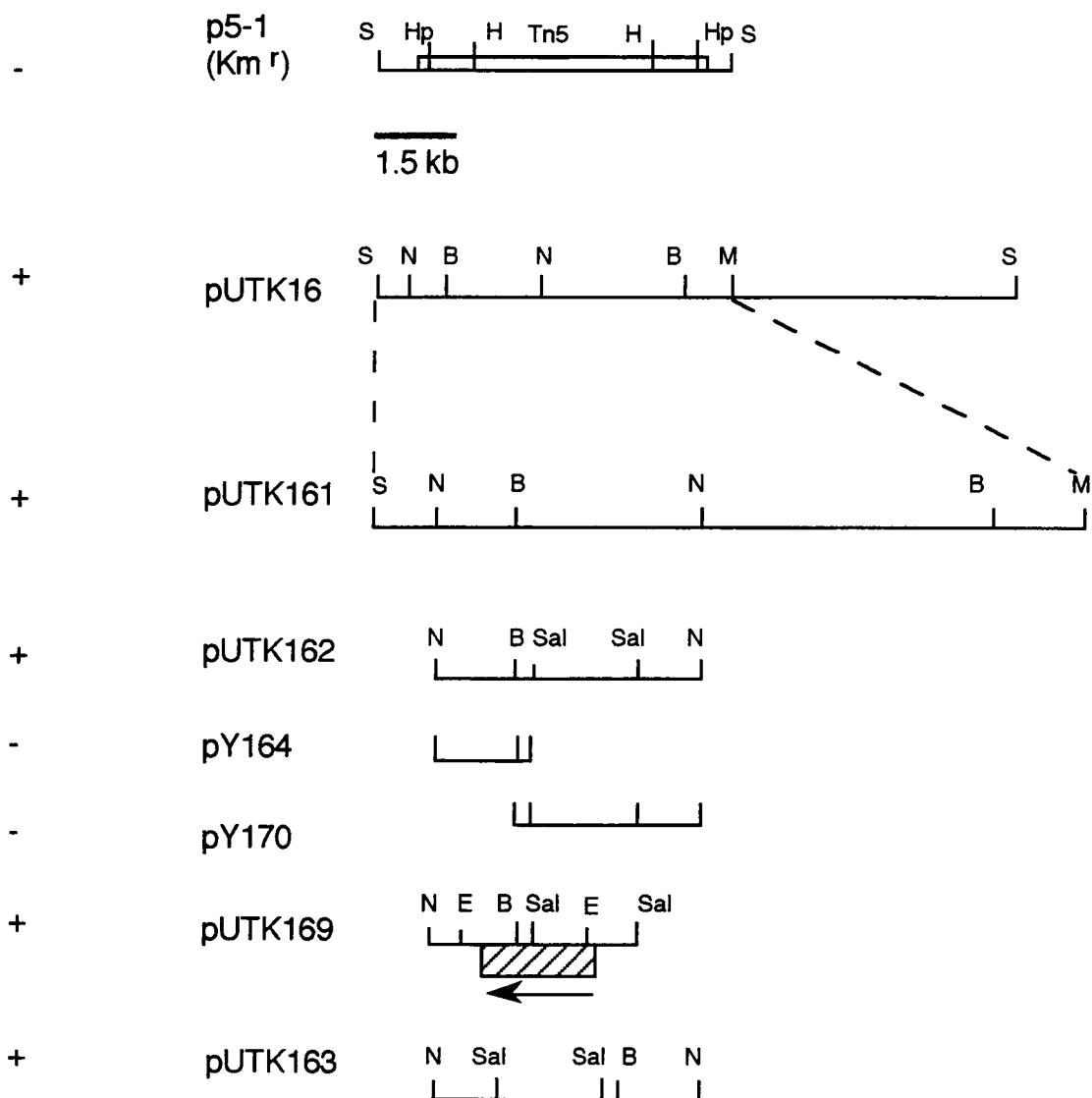


Figure 1. (Continued).

Restriction analyses revealed that the clones pUTK14 and pUTK15 contained a common DNA region (Fig. 1). The result of restriction analyses was confirmed by colony hybridization. When an 1.25-kb *Sst*I-*Bam*HI fragment of pUTK15 was used as a gene probe, *E. coli* DH5 α containing pUTK15 or pUTK14, A8AN3, and 2123 showed positive but *E. coli* DH5 α harboring pBluescript II SK (+) or pUTK16 did not (data not shown). This also suggested that clones pUTK14 and pUTK15 contained a common DNA region, and that A8AN3 or 2123 contained the DNA used as a gene probe. Using an 1.1-kb *Eco*RI-*Sfi*I fragment of pUTK14 as a probe, a clone pS3 (13.85-kb insert) was isolated from gene library of 2123. Based on common restriction sites (Fig. 1) and positive colony hybridization (data not shown), pS3 and pUTK15 were determined to be adjacent. The clone pS3 did not show *meta*-cleavage activity.

To define a locus of the extradiol dioxygenase gene in pUTK14 or pUTK15, subclones were constructed. An *Sst*I-*Sma*I fragment of pUTK14 was inserted into the vectors pBluescript II SK(+) or pUC19 that was digested with *Sst*I and *Sma*I, producing pUTK141 and pUTK142, respectively. The *Sst*I site is closer to the *lac* promoter than *Sma*I on the plasmid pBluescript whereas the *Sma*I site is closer to the promoter on pUC19. Inserting the *Sst*I-*Sma*I fragment into the two vectors allowed the fragment to be subcloned in opposite directions. The subclone pUTK141 showed *meta*-cleavage activity. However, the activity was not observed with the subclone pUTK142, suggesting an extradiol dioxygenase gene in the *Sst*I-*Sma*I fragment was expressed from the *lac* promoter on the vector plasmid pBluescript II SK (+). Subclones (pY143 and pY144) interrupted at the *Bam*HI site or *Eco*RI site near the *Bam*HI site did not show *meta*-cleavage activity. Taken together an extradiol dioxygenase gene on pUTK141 is transcribed from left to right (Fig. 1) and the gene is located in the middle region of the *Sst*I-*Sma*I fragment.

To define an extradiol dioxygenase gene locus in pUTK16, a 13.45-kb *Sst*I fragment of pUTK16 was narrowed down, producing subclones. The *Sst*I-*Sma*I fragment of pUTK16 was ligated into pBluescript II SK (+) digested with *Sst*I and *Sma*I, producing a subclone pUTK161. The subclone pUTK161 showed *meta*-cleavage activity. Since the average size of reported extradiol dioxygenase genes is approximately 0.95 kb (Harayama et al., 1987a; Boldt et al., 1995), the clone pUTK161 was continuously subjected to subcloning. *Not*I fragment (2.8 kb) of pUTK161 was ligated into *Not*I site of the vector pBluescript II SK(+) in both orientations, constructing two subclones pUTK162 and pUTK163. Both of subclones pUTK162 and pUTK163 showed *meta*-cleavage activity, suggesting that an extradiol dioxygenase gene on the *Not*I fragment was expressed from its own promoter from which the gene was expressed. A subclone pUTK169 was constructed by deleting 0.8-kb *Sal*I-*Not*I fragment of pUTK162. Subclones (pY 164 and pY 170) interrupted at the *Bam*HI site did not show *meta*-cleavage activity, suggesting an extradiol dioxygenase gene is located in the middle region of the insert of pUTK169. Subclone pY 170 was produced by ligating an 1.95-kb *Bam*HI-*Sma*I fragment of pUTK162 in to pBluescript digested with *Bam*HI and *Sma*I.

2. Southern blot analysis of the isolated extradiol dioxygenase genes

Southern blot hybridization was performed with subclones of extradiol dioxygenase genes to verify that both strains A8AN3 (wild type) and 2123 (Tn5-mutant of A8AN3) contained the two extradiol dioxygenase genes isolated in this study and that the two extradiol dioxygenase genes differed from each other. The Southern blot hybridization was also designed to determine the physical location of the 2 extradiol dioxygenase genes in the strains. The Southern blot membrane used in Part 3 to prove Tn5 insertion in 2123 was re-used in this hybridization analyses. When an 1.52-kb

EcoRI-SmaI fragment of pUTK141 was used as a gene probe, 2.02-kb *EcoRI* fragments were detected in *EcoRI*-digested total DNA and plasmid DNA of A8AN3 and 2123 (data not shown). The uncut total DNA and plasmid DNA of the 2 strains also hybridized with the probe. This suggested that strains A8AN3 and 2123 contained the DNA fragment used as the gene probe and that the DNA fragment was from plasmid DNA in the 2 strains.

When an 1.12-kb *EcoRI* fragment of pUTK169 was used as a gene probe, 1.12-kb *EcoRI* fragments were shown in *EcoRI*-digested total DNA of A8AN3 and 2123 (data not shown). The uncut total DNA of the two strains were also hybridized with the probe. However, (*EcoRI*-digested or uncut) plasmid DNA of the 2 strains did not hybridize with the probe. This result suggested that strains A8AN3 and 2123 contained the DNA fragment used as a gene probe and the DNA fragment was from chromosomal DNA in the 2 strains.

Because the probes used the above Southern blot hybridization contained DNA adjacent to the extradiol dioxygenase genes in addition to the extradiol dioxygenase genes, extradiol dioxygenase gene-specific probes were prepared by PCR after nucleotide sequences of the genes were determined. The extradiol dioxygenase gene-specific probes were amplified using the primers shown in Table 2. The primers were targeted to approximately the 5'-end region and the middle region of each extradiol dioxygenase gene. When Southern blot hybridization was re-performed using the probes, the results agreed to those from the probing with 1.52-kb *EcoRI-SmaI* fragment of pUTK141 and 1.12-kb *EcoRI* fragments from pUTK169 (Figure 2 and 3).

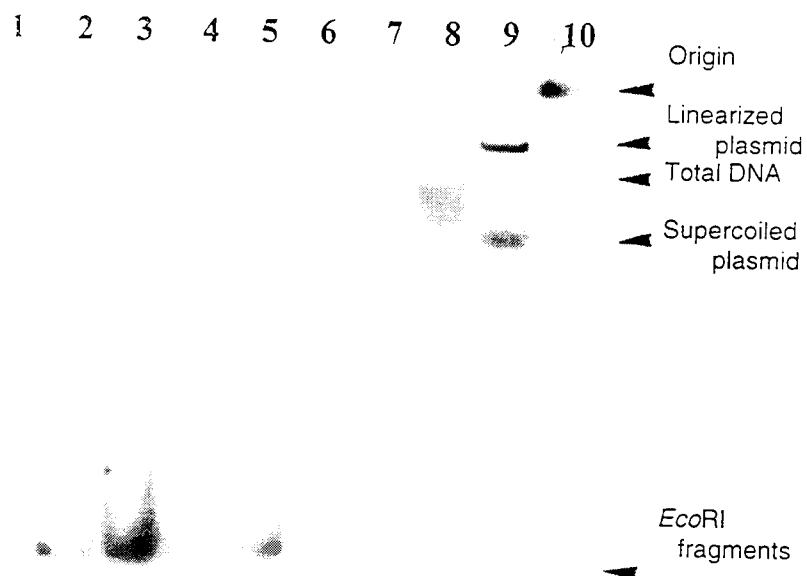


Figure 2. Southern blot analysis of an extradiol dioxygenase gene on plasmid pUTK141. DNA from Ind⁻ mutant (2123) and A8AN3 was probed with a 0.55-kb PCR product from the gene locus in pUTK141. Lanes: lane 1, *Hind*III digest of *Lambda* DNA; lanes 2-5, *Eco*RI digest; lane 6, *Hind*III digest of pRZ102; lanes 7-10, uncut; lanes 2 and 7, total DNA of 2123; lanes 3 and 8, total DNA of A8AN3; lanes 4 and 9, plasmid DNA of 2123; lanes 5 and 10, plasmid DNA of A8AN3. The picture of agarose gel used for this blot is shown in Fig. 10 of Part 3.

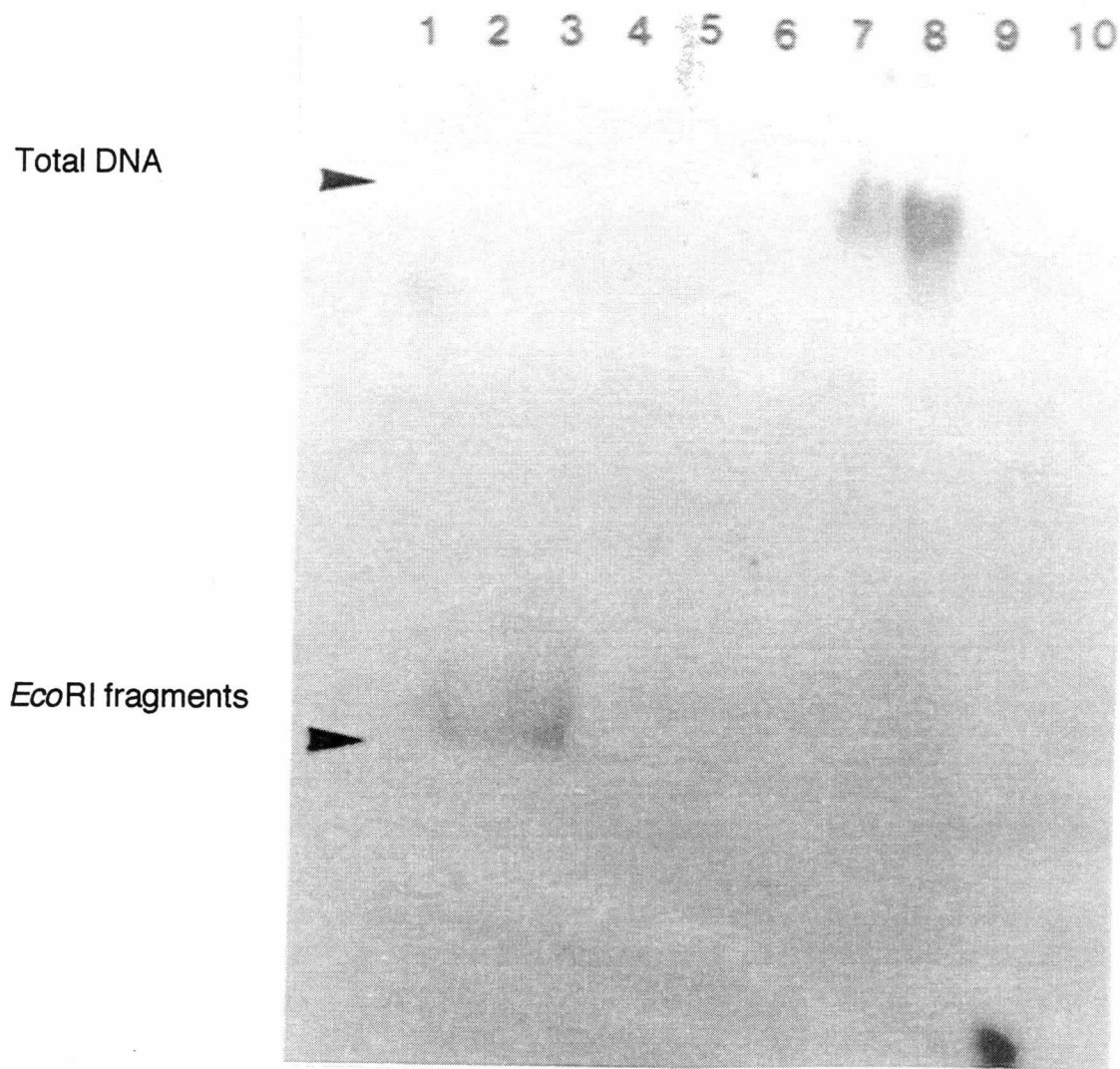


Figure 3. Southern blot analysis of an extradiol dioxygenase gene on plasmid pUTK169. DNA from Ind⁻ mutant (2123) and A8AN3 was probed with a 0.5-kb PCR product from the gene locus in pUTK169. Lanes: lane 1, *Hind*III digest of *Lambda* DNA; lanes 2-5, *Eco*RI digest; lane 6, *Hind*III digest of pRZ102; lanes 7-10, uncut; lanes 2 and 7, total DNA of 2123; lanes 3 and 8, total DNA of A8AN3; lanes 4 and 9, plasmid DNA of 2123; lanes 5 and 10, plasmid DNA of A8AN3. Top arrow indicates total DNA. Bottom arrow indicates 1.12-kb *Eco*RI fragment. The picture of agarose gel used for this blot is shown in Fig. 10 of Part 3.

3. Enzyme assays of two extradiol dioxygenases

Crude enzyme assays revealed quantitative activity and relative activity of extradiol dioxygenases encoded by two subclones (pUTK141 and pUTK163) expressing *meta*-cleavage activities. Genes encoding extradiol dioxygenases in the subclones were determined to be different as judged by restriction analysis and DNA hybridization. Results of Southern blot hybridization suggested that extradiol dioxygenase gene on pUTK141 was present on plasmid DNA, whereas the extradiol dioxygenase gene on pUTK163 was present on chromosomal DNA in A8AN3.

Six different dihydroxylated aromatic substrates were used in the enzyme assays. Conversion of the substrates to typical yellow *meta*-cleavage products was monitored by changes in absorption spectra. The products from catechol, 3-methylcatechol, 4-methylcatechol, and 2,3-dihydroxybiphenyl showed maximum absorption at 375, 388, 382, and 434 nm, respectively. These wavelengths of maximum absorbance are in good agreement with the spectral characteristics of *meta*-cleavage products formed from these substrates by other extradiol dioxygenases (Bayly et al., 1966; Sala-Trepat & Evans, 1971; Furukawa et al., 1983). The two extradiol dioxygenases showed different activity with substrates tested in this study. However, both of them primarily catalyzed *meta*-cleavage of dihydroxylated monocyclic substrates (Table 3). The plasmid-encoded extradiol dioxygenase (*E. coli* [pUTK141]) showed highest activity with 3-methylcatechol among the substrates tested, whereas the chromosome-encoded one (*E. coli* [pUTK169]) showed the highest activity with catechol. The extradiol dioxygenase encoded by pUTK141 had activity with catechol at approximately half the rate observed with 3-methylcatechol. In contrast, the enzyme encoded by pUTK169 showed activity with 3-methylcatechol at approximately half the rate observed with catechol. The extradiol dioxygenase encoded by pUTK141 had significant activity (34% of the activity

Table 3. Extradiol dioxygenase activities of crude enzymes extracted from *E. coli* DH5 α containing recombinant DNA. The activities were measured at pH 7.5.

Substrate	Specific activity ^a		
	DH5 α (pUTK141)	DH5 α (pUTK163)	DH5 α [pBluescript II SK (+)]
Catechol	2918.6 $\pm$ 79 (55.2)**	3207.6 $\pm$ 89.1 (100)	0
3-Methylcatechol	5282.7 $\pm$ 228.9 (100)	1466.5 $\pm$ 75.6 (45.7)	0
4-Methylcatechol	1798.1 $\pm$ 44.7 (34.0)	99.1 $\pm$ 32.6 (3.1)	0
2,3-Dihydroxybiphenyl	27.7 $\pm$ 4.2 (0.5)	34.2 $\pm$ 8.9 (1.1)	0
1,2-Dihydroxynaphthalene	0	0	0
2,3-Dihydroxynaphthalene	0	0	0

^a Units/mg of protein: one unit is defined as the amount of enzyme the catalyzes the formation of 1.0 μ mol of product per minute. Enzyme activities were the average from three independent experiments.

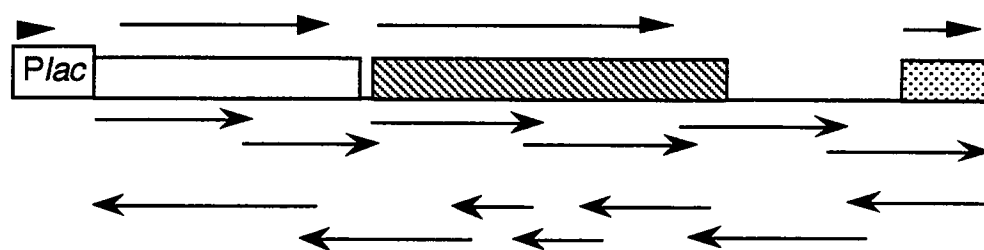
^b Relative activity reported as percentage.

with 3-methylcatechol) with 4-methylcatechol, whereas the enzyme encoded by pUTK169 showed very little activity (3.1% of the activity with catechol) with 4-methylcatechol. However, two extradiol dioxygenases showed very low or no activity with dihydroxylated 2-ring substrates. The enzymes showed very low activity with 2,3-dihydroxybiphenyl. No *meta*-cleavage activity was detected with 1,2-dihydroxynaphthalene and 2,3-dihydroxynaphthalene. Taken together, these results suggest that the two *meta*-cleavage enzyme genes encode extradiol dioxygenases that primarily catalyze ring-cleavage of monocyclic substrates. However, the two *meta*-cleavage dioxygenases have different substrate-specificity among monocyclic substrates.

4. Nucleotide sequence analyses of extradiol dioxygenase genes.

Sphingomonas sp. strain A8AN3 showed ability to degrade a broad range of PAHs including BaP (Ahn et al., 1994). Aromatic hydrocarbons are known to be biodegraded to a few intermediates that are subjected to ring cleavage (Sutherland et al., 1995). Catechol is one of the intermediates that serves as a substrate for ring-cleavage dioxygenase in degradative pathways of aromatic hydrocarbons. Bacterial ring-cleavage dioxygenases play a key role in degrading aromatic hydrocarbons for entry into the TCA cycle. In this regard, the genes encoding the extradiol ring-cleavage dioxygenase were chosen for further molecular analysis. Nucleotide sequencing was performed after subcloning each clone that showed *meta*-cleavage activity as determined by the catechol spray plate method. The subclones used for the sequencing were pUTK169 and pUTK141. The clones were in the vector plasmid pBluescript II SK (+). Figure 4 shows the nucleotide sequencing strategies used in this study. The nucleotide sequences of 2.331-kb *Sst*I-*Sma*I fragment of pUTK141 and 2.184-kb *Not*I-*Sal*I fragment of pUTK169

pUTK141



500 bp

pUTK169

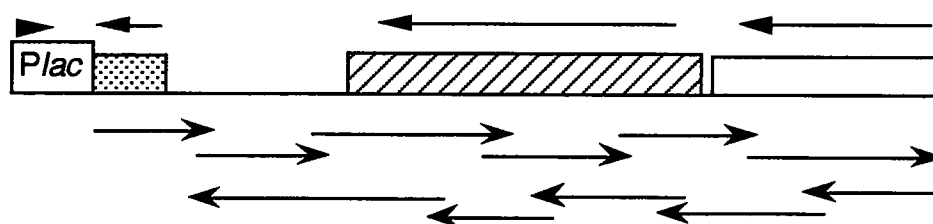


Figure 4. Nucleotide sequencing strategies for pUTK141 and pUTK169. Arrows (→) indicate direction of and length of sequence. Arrow heads indicate transcriptional direction from *lac* promoter (*Plac*) on the vector plasmid. The loci of genes in the plasmid are shown. Hatched boxes are loci of the extradiol dioxygenase genes. The other boxes (□ and ▤) are partial genes of 2-hydroxymuconic semialdehyde hydrolase and 2-hydroxymuconic semialdehyde dehydrogenase, respectively. Transcriptional direction of each gene is indicated by arrows (→). Insert sizes of pUTK141 and pUTK169 are 2.331 kb and 2.184 kb, respectively.

1 CGAGCTCGCCAAATCTTCCACGTAATTGCGCCGACATGATCGGCTTCGGCTATACCGAATCGCCAAACCGACGGCATCCGTGACAAGCCGGTCTGGGTGATCACCTGC
E L A K S F H V L A P D M I G F G Y T E S P T D G I R D K R V W V D H L L
111 TCGTTTCTCGATCGGATGCAAAATCGAAAGGCTTCGTTGGTGGCAACTCGTTTCGGCGGGGGTGAACCTCGCCTTCATGATCGCGACCCCGACCGGTGATCGC
G F L D A M Q I E K A S L V G N S F G G G L T L A F M I A H P D R V D R
221 GCGGTATTGATGGGCGCAGCCGGGCTCGAATTCGGAATACCGAAGCGCTCGACTCCGCTCGGGGCTATGAGCCGTCGTGGAAATGATGCGCCAGGCGGTGCGGTGCT
A V L M G P A G L E F P I T E A L D S V W G Y E P S L E M M R Q A I A L L
331 CGGACCGATCCGGCGCGGACCGAGGAATGGTGGCTCGGCTACGAGGCGAGCATCAGGCCGGCGGCATGAACCTATGCGGCGACGTTTCGGGCGCGCAGCCAC
A T D P A R R T E E L V R S R Y E A S I R P G A H E P Y A A T F G P Q P R
441 GCCAGAACCATATCAAAATGTTGGCGAGCCCGAGGCGGATATCGCCGCTGCCGACGAGGTGTTGATCCTGATGCAAACTCGATCGCGGTCAATCCCGCTCGATGTC
Q N H I K M L A S P E A D I A A L P H E V L I L H G K L D P V I P L D V
551 TCGGTGCGGCTCTCCAACCTGATCCCGCGCTGACCTGCACGTGTTTCGCCAGTCCGGCCATGGGTGCAGATCGAGCGCGCTGCAAGCTTCAATCGGTTAGTCACGCA
S V R L S N L I P R A D L H V F A Q C G H W V Q I E R A A S F N R L V T Q
661 GTTTTCCAAAGAGCCTGCAAGGCTGAGTGGGCTAGGAGAGTATAATGCTGTGTCAGGCGGTATTCGCGCGGGTTTCGTGCAAAATGCGCGCTGCTCGATCTCGAAGAGT
F F Q E G L Q G * *cdo2* M A V T G V L R P G F V Q M R V L D L E E S
771 CGATCCAGCATTCGCGACCGCATCGGCTTGATATGTCGGTGAGCCGCGGACGGCGCGGTTTCTTCGCGCGCTTCGATGAATTCGATCGCCACAGCGTCATCCTG
I Q H Y R D R I G L D M V G E P A D G R V F F R A F D E F D R H S V I L
881 CGCGAGGCGACACGCGGGCTCGACGATCGGCTTCAAAGTCGCCAGTGAAGCCGATCTCGATGCGTTCAAGCCCGGCTGGTCGCGATGGCGGTGCCGTGAGGGA
R E A D T P G L D Q I G F K V A S E A D L D A F K A R L V A M G V P V R D
991 TATTCCATCCGGCGAGGATCCCGGCGTCGCGCGCGCTTCAGCACGCCGACGACACATGCTTCGATCTCTACGACACAGATGACATCTCCGAATTTGCGCCAA
I P S G E D P G V G R R V R F S T P T R H V F D L Y A Q M D I S E F A P M
1101 TGACGAAAACCGGACGCTTGGCATCGCGAGCCGCGGCGATGCGTGGACCCGGTTTCGATCATTCGCGGCTCTCAACGGGATTGATATCGCGGGCTCGGCCAAGATCTTT
T K N P D V W H R E P R G M R A T R F D H C A L N G I D I A G S A K I F

Figure 5. Nucleotide sequences of 2.331-kb *Sst*I-*Sma*I fragment of pUTK141. One complete ORF (*cdo2*) and two partial ORFs were shown. The gene *cdo2* encodes catechol 2,3-dioxygenase. The nucleotide sequence of *cdo2* gene was deposited in the GenBank under the accession no. U73128. Underlined sequences are putative ribosome-binding sites. Asterisks indicate stop codons.

1211 GTCGAAGCGCTTGATTTCTCCGTACCGAAGAAGCGGTGCGACGAGTCGAGGGGCGAGCGGCTCGGTATCTTCCCTCAGTTGAGCAATAAGGCGCACGATATCGCCTTCAT
V E A L D F S V T E E A V D E S T G Q R L A I F L S C S N K A H D I A F I
1321
TGGCTACAACGAGGATGCCAAGCTTACACACCTCGTTCCGTCTGGATTCTGTGAACGATGTGGCCACGCGCGGATGTCATCACGCGTTACGACATTTCCGATCGATA
G Y N E D A K L H H T S F R L D S W N D V G H A A D V I T R Y D I S I D I
1431
TTGGCCCAACGCGCCACGGCATCACCGGTGGTCAGACAATTTACTTCTTCGATCCTTCGGGAATCGCAACGAGACCTTCAGCGGCGGCTATGATTATTATCCGGATAAT
G P T R H G I T R G Q T I Y F F D P S G N R N E T F S G G Y D Y Y P D N
1541
CCACGCGCGGTGGAGCGCAGACGAGGCGCGCAAGCGGATCTTCTACTATCAGCGTGTCTCAACGAGCGCTTCATGACCCGTGATGACCTGAACGGTGATGCAAGCGGAC
P R R V W S A D Q A G K A I F Y Y Q R V L N E R F M T V M T *
1651
ACGAACCTTGGCACCGCTAAGCGCGGAAGCTGCCCAGGCGCGGTGCGCGGTCGATGCGGGCGCGGCTGGCTGCCCGCGGTGCTGGCCCGGTGGTTCGATGCCA
1761
GCGGCGAACTGGTCGCATTCTGCGCGTGACCGGCGCGCCTTTTCATTTCGAGCGAGATCGCCCGCGACAAGGCGTTCAACGCGCAAGCTTCAAGATGCCGAGCGCGGAC
1871
GTCCACGGCCTGGTGTGGGTAGCGACGCCCCCTGCCGAAGGGATCACACGACCGCACCGCATGGTGATGTTCCGGCGGCGGCTGCCGATCGTCGTCGAGGCGAAACGGT
1981
CGGCGGTATCGGTGTCGCGGTAGTGAGGCGATGGACACGTCGTGCGCCAATGCCGCTCTCGCGGCTATCGGCGGAAGTCCTTCTGATTGGATAGGAAAGAATTGA
2091
ATGGCGGAGGTGACACCGAACTGCCCTCAGTACGGGCGAGGTGATGAATTCATCGACGGCGAGTTCGCCCGAGGATCGGAGGGCGGAGTTTCGCCAATGTTCGATCC
M A R G D T E L P S V T G E V M N F I D G E F R R G S E G R S F A N V D P
2201
CGTTCCTCGGCGGAGATCGGCGCGGTGTTTCGAAGCGAGCGGCGCGACGTGATGCGGCGGTGCGAGGCGCTCGCGGGCGCTCGAAGGACCTTGGGGCCGGATGACGA
V S G G E I G R V F E A S E A D V D A A V Q A A R R A L E G P W G R M T T
2311
CGCCCGAGCGGTCAGATCC
P E R V K I

Figure 5. (Continued).

1 GTGACCAAGATTGCGCGCTTGTACGGGCTTGGCATCGACAAGGTGTCAATGGTGGGCAATTGGTTCGGTGGCGGCAATTGGCTGGCTTTTACGATCGCGCATCCTGA
V D Q I A A L L D G L G I D K V S M V G N S F G G I A L A F T I A H P D
111 TCGAGTCGACGGCGGTCTTATGGGACCGGCGGTCTTGAGTTCCCGATCACGCCAGCGCTCGATGAGGTCTGGGGCTATGTTCCCTCGGTCGAGGCCATGCGCTCGT
R V E R A V L M G P A G L E F P I T P A L D E V W G Y V P S V E A M R S S
221 CGCTCGAATATCTTGCTGGATCACAGCGCTGACCGAGCATCTGATCAAGTCGCGATACGAAGCAAGTATGCTGCTGGTGCACAGAAACCGTATCATGCGACCTTT
L E Y L A W D H S R L T D D L I K S R Y E A S M R P G A Q E P Y H A T F
331 GCGGCGCGGACCGTCAAGCCAATATCGGATGCTGGCAAGCGCGAAGAGACATTCGGCGCTGCAACAGAAATGCTGGTGTGTCATGGCCGGTTCGATGAGGTTAT
G G A D R Q A N I A M L A S R E E D I A A L Q H E M L V L H G R F D E V I
441 TCCACTGGAGTCGAGCGTGGCAACGCTGCTGCCTCGTCTGACTTGAACGTGTCGGCGAATGCGGGCACCTGGTCCAGATTGAGCGCATGGTCAAGCTTTAACC
P L E S S V R L A T L L P R A D L N V F G E C G H W V Q I E R M V S F N R
551 GAGTTGTCTGAATTTTTCAGCAACGGCTCAAGGGCTGACGCATGCAAGGAGAACTGAAATGGCTTTGACCGGTGTAATCCGCCCTGGATATGTGCAACTGCGGGTCC
V V S E F F S N G L K G * *cdol* M A L T G V I R P G Y V Q L R V L
661 TCGATCTCGATGAGGCTATTGTTCAATTCGTGACCGGATTGGTCTCAATCTCGTCAGCGTCGAGGATGGCGTGCCTTTTCCAGGCGTTCGACGAATTCGACCGCAC
D L D E A I V H Y R D R I G L N L V S V E D G R A F F Q A F D E F D R H
771 AGCATTATCTGCGCAAGCCGATACCGTGGGCTTGACCGCATGGCTCAAGGTTCGAAAGACTCCGATCTTGATCATTTTCCGAAACGCCTGCTCGATGCGGGAGT
S I I L R E A D T A G L D R M A F K V A K D S D L D H F A E R L L D A G V
881 GAACGTGATGTTCATCCCGCTGGCGAAGATCCGGGTGTCGACGCAAAATCCGCTTCAACGCGCCGACCGCGCATGTGTTGACCTATACGCAGAAATGGAGCTGTCCG
N V D V I P A G E D P G V G R K I R F N A P T A H V F D L Y A E M E L S E
991 AAACCGGCGCGGTCCGCAATCCTGATGCTGGATAGCGCAACCGCTGGCATGCGGGCAACCCGTTTGATCACTGCGCGCTCAACGCGTGGACATCTCGGCGAGC
T G P A V R N P D V W I A E P R G M R A T R F D H C A L N G V D I S A S
1101 GCCAAGATCTTCGTGGAAGTTCTCGACTTCTCAGTAACCGAGGAACTGGTCGACGAAAGTACCGGGGACGTCCTCGGTATCTTCCCTTCATGCAGCAACAAAGGCCCATGA
A K I F V E V L D F S V T E E L V D E S T G A R L G I F L S C S N K A H D

Figure 6. Nucleotide sequences of 2.184-kb *NotI-SalI* fragment of pUTK169. One complete ORF (*cdol*) and two partial ORFs were shown. The gene *cdol* encodes catechol 2,3-dioxygenase. The nucleotide sequence of *cdol* gene was deposited in the GenBank under the accession no. U73127. Underlined sequences are putative ribosome-binding sites. Asterisks indicate stop codons. Bold nucleotides are sequences similar to σ 70-dependent promoter.

1211 TGTCGCTTTTCTGGGTTATCCGGAAGATGGCCGGATCCACCACACACTTCGTTCAACCTCGAATCCTCGAACGATGTTGGGCACGTCGGCTGACATTATCAGTCGCTAGCATA
. V A F L G Y P E D G R I H H T S F N L E S W N D V G H V A D I I S R Y D I
1321 TTTCCGCTCGACATCGGCCCGACGGGACACGGGATCACTCGCGGCAGACGATCTATTCTTCGATCCGTCGGGCAACCGCAACGAGACGTTTCAGCGCGGCTATATCTAT
. S L D I G P T R H G I T R G Q T I Y F F D P S G N R N E T F S G G Y I Y
1431 TACCCCGACAACCCGCGGTATGTGGCAGGCGAGAAAACGCGGGTAAGCGGATCTTCTATTACGAGAAGCGCTGAACGAACGCTTCATGACGGTTAAACACCTGATCATG
. Y P D N P R R M W Q A E N A G K A I F Y Y E K A L N E R F M T V N T *
1541 GACGGTTTGGTGACAATCCGGACAATCGGACACGGCTTGGCCCGACAAGCGGTTTCGGCCCGCTCGCCCAAGGGCGAGGAAGCCGGTTACCCGGTCGTTGCCGCCATCAT
. 1651 CGGTGGAGGAGGCGAACTGGCGGCTCTGCTGCGCGGAGCGGTACACCCCTTTCCCTCGTCACAAATTGCCCAAGGCAAGGCCTATACCGCCGCCAGCTTTCGGGTTGCGA
. 1761 CGCCTGACGTGTACAAGATGTTTCGGGCAATCCGGCGCTGAGCGACGGGATAACCGTTTCAGCCGGGGATCGTGATGTTTGGCGGGGACCTTCCCATCGTTATCGATGGC
. 1871 GAATTCTGTCGGTGCAATCGGCGTCTCTGGTGGATCTGAAGAACTCGACATTGCCCTGGGCCAACCGCTGGCCCTCTCCGCAATCGGCGCAGAGCAGTGAGCGGCCAGTGC
. 1981 AATAATACATTGGGACGATAGAAATCAAATGACTACCAATTCGCCCTCGACCTGTAGCCGACCAACATACTCAATTTTCATTGGGGGTTCTCTATCGCAAGGGCAGCCAGGGAA
. 2091 AGACCTTTGACAACGTCAATCCGGCAACGGGACAAACAATCGGCACGGTTTATGAGGCAAGCAAGCCGATGTCCCGCATGCAATGGCGGCGCGC
. T F D N V N P A T G Q T I G T V Y E A S E A D V A D A V A A
. M T T N S P R P V A D H I L N F I G S Y R K G S Q G K

Figure 6. (Continued).

were determined (Fig. 5 and 6). Both strands of the DNA were sequenced except the 3'-terminal (downstream of an extradiol dioxygenase gene) 204-bases of pUTK169 (Fig. 6). Nucleotide sequence analysis of the *Sst*I-*Sma*I fragment of pUTK141 revealed a complete ORF and two partial ORFs. The *Not*I-*Sal*I fragment of pUTK169 also contained a complete ORF and two partial ORFs (Fig. 4). A putative ribosome-binding site (RBS) preceded each ORF.

The *Sst*I-*Sma*I fragment of pUTK141 contained a complete ORF for an extradiol dioxygenase. The ORF was designated *cdo2*. The gene *cdo2* is most similar to *xylE* (encoding catechol 2,3-dioxygenase) from *S. yanoikuyae* B1 at both nucleotide (72.3% identity) and amino acid (74.9% identity) levels (Table 4). The *cdo2* gene was also closely related (71.0% identity for nucleotides and 74.3% identity for amino acids) to the *cmpE* gene (encoding catechol 2,3-dioxygenase) from *Pseudomonas* sp. strain HV3 (Yrjala et al., 1994). The *cdo2* gene ORF was 924-bp long. The ORF had a 60% G+C content. Nineteen-nucleotides upstream of the *cdo2* start codon was the stop codon of a partial ORF that was most similar (64.3% identity for nucleotides and 57.3% identity for amino acids) to *dmpD* (encoding 2-hydroxymuconic semialdehyde hydrolase) from *P. putida* CF600 (Shingler et al., 1992). Another partial ORF existed 458-nucleotides downstream from the *cdo2* stop codon. The partial ORF was most closely related (37% identity for amino acids) to *xylG* and *bphG* (encoding 2-hydroxymuconic semialdehyde dehydrogenase) from pWWO and pWW110, respectively (Horn et al., 1991; Carrington et al., 1994). The intergenic region of 457-nucleotides between the gene *cdo2* and its downstream partial ORF was most similar (63.9% identity for nucleotides) to the downstream region of *cmpE*. The function of the downstream region of *cmpE* is not known.

The results of nucleotide sequence analyses showed that 2.184-kb *Not*I-*Sal*I fragment of the subclone pUTK169 contained a complete ORF of an extradiol

Table 4. Comparison of extradiol dioxygenase genes and proteins.

Gene	Gene product	Nucleotides	Amino acids	% Identity with other extradiol dioxygenases ^a	
				Nucleotides	Amino acids
<i>cdo1</i>	Catechol 2,3-dioxygenase (CDO1)	924	307	<i>xylE</i> ^b , 80.5	XylE, 92.2
				<i>cmpE</i> , 78.4	CmpE, 88.6
				<i>cdo2</i> , 71.5	CDO2, 75.6
				<i>nahH</i> , 59.0	NahH, 49.8
<i>cdo2</i>	Catechol 2,3-dioxygenase (CDO2)	927	308	<i>xylE</i> , 72.3	XylE, 74.9
				<i>cmpE</i> , 71.0	CmpE, 74.3
				<i>nahH</i> , 56.6	NahH, 51.0

^a % of nucleotides or amino acids that are identical when sequences are aligned with sequences retrieved from GenBank database. Sequence alignment was performed by the GAP program of the GCG package using the algorithm of Needleman and Wunsch (1970).

^b The gene *xylE* and its gene product XylE are from *Sphingomonas yanokuyae* B1 (Kim & Zylstra, 1995). See the text for the references of the other genes and their products.

dioxygenase gene, a partial ORF of 2-hydroxymuconic semialdehyde hydrolase gene, and a partial ORF of 2-hydroxymuconic semialdehyde dehydrogenase gene (Fig. 4). The ORF of the extradiol dioxygenase gene in pUTK169 was designated *cdo1*. The ORF of *cdo1* gene was 921-bp long. The G+C content of the ORF was 57%. The gene *cdo1* had the highest similarity to *xylE* from *S. yanoikuyae* B1 at both nucleotide (80.5% identity) and amino acid (92.2% identity) levels (Table 4). The *cdo1* gene was also closely related (78.4% identity for nucleotides and 88.6% identity for amino acids) to *cmpE*. Twenty one-nucleotides upstream of the *cdo1* start codon was the stop codon of a partial ORF that was most similar (58.7% identity for nucleotides and 50.8% identity for amino acids) to *dmpD*. A partial ORF located 474-nucleotide downstream of the *cdo1* stop codon was most closely related (51% identity for amino acids) to *xylG* on pWWO (Horn et al., 1991). The region of 473 nucleotides was most closely related (70.1% identity at nucleotide level) to the downstream region of *cmpE*. Two extradiol dioxygenase CDO1 and CDO2 showed relatedness to each other (71.5% identity for nucleotides and 75.6% identity for amino acids) but they did not show significant similarity with NahH encoded by the plasmid NAH7 at either the nucleotide or amino acid levels although C-end regions showed some similarity.

The extradiol dioxygenase family contains conserved sequences at C-end regions. The consensus sequence at C-end region was proposed as signature sequence for extradiol dioxygenases (Harayama & Rekik, 1989, 1990; Boldt et al., 1995): (G, T, N, R)X(H, A)XXXXXXXX(L, I, V, M, F)YXX(D, E, T, N, A)PX(G, P)X{2, 3}E (numbers in brackets indicate a gap of 2 or 3 residues at the position in the sequence). Deduced amino acid sequences of extradiol dioxygenase genes isolated from A8AN3 showed conserved amino acid residues among all of the extradiol dioxygenases (Fig. 7).

The deduced amino acid sequences of genes *cdo1* and *cdo2* were compared with those of 22 other extradiol dioxygenases. A dendrogram showing the relationship

Figure 7. A) Amino acid sequence alignment of ten extradiol dioxygenases. NahH, *P. putida* PpG7 catechol 2,3-dioxygenase; DmpB, *P. putida* CF600 catechol 2,3-dioxygenase; XylE (mt-2), *P. putida* mt-2 catechol 2,3-dioxygenase; CDO1, A8AN3 catechol 2,3-dioxygenase in pUTK169; XylE (B1), *S. yanoikuyae* B1 catechol 2,3-dioxygenase; CmpE, *Sphingomonas* sp. strain HV3 catechol 2,3-dioxygenase; catechol 2,3-dioxygenase; CDO2, A8AN3 catechol 2,3-dioxygenase in pUTK141; BphC, *S. yanoikuyae* Q1 2,3-dihydroxybiphenyl 1,2-dioxygenase; NahC, *P. putida* PpG7 1,2-dihydroxynaphthalene dioxygenase; DbfB, *Sphingomonas* sp. strain RW1 2,2'3-trihydroxybiphenyl dioxygenase. Symbols * and † indicate amino acids conserved among all extradiol dioxygenases. Symbol † indicates potential amino acids involved in coordination of a Fe^{2+} ion in the active site. The box indicates positions that produce a C-end region consensus sequence in B). B) Consensus sequence for extradiol dioxygenase family (Harayama & Rekik, 1990; Boldt et al., 1995). The sources of the sequences are given in the Fig. 8 legend.

	1	70
NahH	. .MNKGVMRP	GHVQLRVLD. .MGKALEHYV ELLGLIEMDR DDQGRVYLKA WTEVDKFSV LREADEPGMD
DmpB	. .MKKGVMRP	GHVQLRVLN. .LESALAHYR DLLGLIEMDR DEQGRVYLKA WTEVDKFSV LREADQPGMD
Xyle (mt-2)	. .MNKGVMRP	GHVQLRVLD. .MSKALEHYV ELLGLIEMDR DDQGRVYLKA WTEVDKFSV LREADEPGMD
CD01	.MALTGVIRP	GYVQLRVLD. .LDEAIVHYR DRIGLNLVS. VEDGRAFFQA FDEFDRHSII LREADTAGLD
Xyle (B1)	.MALTGVLRP	GYVQLRVLD. .LDEAIQHYR DRIGLNLVS. VEGGRAFFQA FDEFDRHSII LREADSAGLD
CmpE	.MALTGVIRP	GYVQLRVLD. .LDEAIIHYR DRIGLNFVN. REGDRAFFQA FDEFDRHSII LREADQAGMD
CD02	.MAVTGVL RP	GFVQMRVLD. .LEESI QHYR DRIGLDMVGE PADGRVFFRA FDEFDRHSVI LREADTPGLD
BphC	. .MVAVTEL	GYLGLTVTN. .LDAWRSYAA EVAGMEIVDE GEGDRLYL RM . .DQWHHRIV LHASDDDLA
NahC	MSKQAAVTEL	GYMGISVKD. .PDPAKSFAM NMLGLQVLDE GEKDRFYLRM . .DYWHHRIV VHSASADDLE
DhbF	. .MSVKOL	GYLFECDRAD VLEOMVVVYO DIIG. AVVER DEGRALVRL . .DGRPFRI R LDGPGANRLA

[illegible]

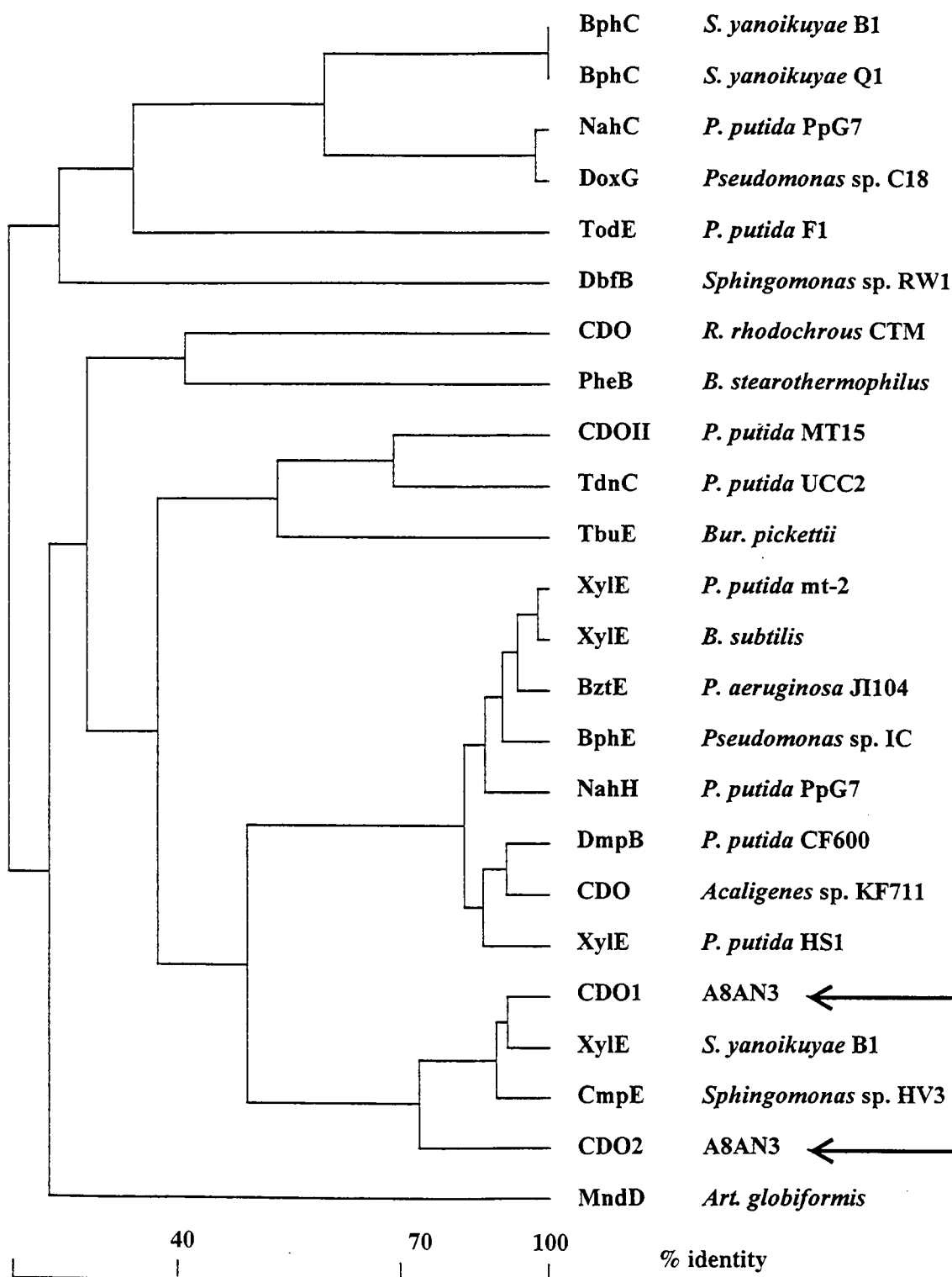
	141	210
NahH	EVNPEAWPRD LKGMAAVRFD HCLLYGDELQ ATYELFTEVL GFYLAEQV.. .VDA.DGIR LAQFLSLSTK	
DmpB	EINPEAWPRN LKGMRAVRFD HCLLYGDELQ ATYALFTEVL GFYLAEQV.. .IDD.DGTR VAQFLSLSTK	
Xyle (mt-2)	DVNPEAWPRD LKGMAAVRFD HALMYGDELQ ATYDLFTKVL GFYLAEQV.. .LDE.NGTR VAQFLSLSTK	
CD01	VRNPDVWIAE PRGMRATFRD HCALNGVDIS ASAKIFVEVL DFSVTEEL.. .VDESTGAR LGIFLSCSNK	
Xyle (B1)	VRNPDVWIAE PRGMRATFRD HCALNGVDIS ASAKIFVEAL DFSVAEEL.. .VDESTGVR MGIFLSCSNK	
CmpE	VKNPDVWVVE PRGMRATFRD HCALNGVDIA SSAKIFVDAL DFSVAEEL.. .VDESTGAR LGIFLSCSNK	
CD02	TKNPDVWHRE PRGMRATFRD HCALNGIDIA GSAKIFVEAL DFSVTEEA.. .VDESTGQR LAIFLSCSNK	
BphC	RPMYGKFVTG SE.....GLG HCILRQDDVP AAAAFYG... LLGLRGDSVEY HLQPLNGMVA QPSFMHCNER	
NahC	RPLHGKFVTG DE.....GLG HCIVRQTDVA AAHKFYS... LLGFRGDEVY RIPLPNGMTA ELYFMHCNAR	
DbfB	PVLESLFVCG EEANGIFGLG HLVVIVADRA KTOSFFTDVL GFGLSDRVTC LKPTSSSFTA TSVITPLHFR	

	211							280
NahH	AHDVAFIHHA	EKGKPHHASF	FLDTWEDVLR	AADLISMTDT	SIDIGPTRHG	LTQGKTIYFF	DPSG NRCEVF	
DmpB	AHDVAFIHCP	EKGKFHHVSF	FLETWEDVLR	AADLISMTDT	SIDIGPTRHG	LTHGKTIYFF	DPSG NRNEVF	
Xyle (mt-2)	AHDVAFIHHP	EKGRLHHVSF	HLETWEDLLR	AADLISMTDT	SIDIGPTRHG	LTHGKTIYFF	DPSG NRNEVF	
CDO 1	AHDVAFLGYP	EDGRIHTTSF	NLESWHDVGH	VADIISRYDI	SLDIGPTRHG	ITRGQTIYFF	DPSG NRNETF	
Xyle (B1)	AHDVAFLGYP	EDGRIHTTSF	FLDSWHDVGH	AADIISRYDI	SLDIGPTRHG	ITRGQTIYFF	DPSG NRNETF	
CmpE	AHDVAFLGYP	EDGKIHTTSF	NLESWHDVGH	AADIISRYDI	SLDIGPTRHG	ITRGQTIYFF	DPSG NRNETF	
CDO2	AHDIAFIGYN	EDAKLHHTSF	RLDSWHDVGH	AADVITRYDI	SIDIGPTRHG	ITRGQTIYFF	DPSG NRNETF	
BphC	QHSVAFGLGP	MEKRIINHLM	EYTDLDLGL	AHDIVRARKI	DVALQLGKHA	NDQALTFYCA	NPSGWLWEFG	
NahC	DHSIAFGAMP	AAKRLNHLM	EYTHMEDLGY	THQFVKNEI	DIALQLGKHA	NDKALTFYCA	TPSGWLIEPG	
bdfB	HRSVLSOV.	..WFNHLML	EAKSKEOVD	RFAAVKRLGY	DVIMTTTGH	NDKVYSFYMM	APAGFAVETG	

	281		321
NahH	CGGNYNYPDH	KPVTWLAKDV	GKAIFYHDRV LNERFMTVMT .
DmpB	CGGDYNYQDH	KPVTWLAKDL	GKAIFYHDRV LNERFLTULT .
Xyle(mt-2)	CGGDYNYPDH	KPVTWTDDQL	GKAIFYHDRV LNERFMTVLT .
CD01	SGGYIYYPDN	PRRMWQAENA	GKAIFYYEKA LNERFMTVNT .
Xyle(B1)	SGGYTYYPDN	PRRMWQAENA	GKAIFYYEKA LNDRFMTVNT .
CmpE	SGGYIYYPDN	PQRLWQAENA	GKAIFYYEKA LNDRFMTVNT .
CD02	SGGYDYYPDN	PRRVWSADQA	GKAIFYYQRV LNERFMTVMT .
BphC	WGARK..APS	QAEYYTR...	..DIFGHGNE AAGYGMDIPL G
NahC	WRGAT..AID	EAEYHVG...	..DIFGHGVE APGYGLDVKL S
DbfB	FGGOV..IGD	LESWWYGFDF	APSIYGHGEL IPAH

(G, T, N, R)X(H, A)XXXXXXXX(L, I, V, M, F)YXX(D, E, T, N, A)PX(G, P)X{2, 3}E

Figure 8. Dendrogram resulting from comparison of amino acid sequences of extradiol dioxygenases. The dendrogram was prepared by the Pileup program of the GCG package. Sources of the sequences are as follows: BphC, *S. yanoikuyae* B1 2,3-dihydroxybiphenyl 1,2-dioxygenase (Kim & Zylstra, 1995); *S. yanoikuyae* Q1 2,3-dihydroxybiphenyl 1,2-dioxygenase (Taira et al., 1988); NahC, *P. putida* PpG7 1,2-dihydroxynaphthalene dioxygenase (Harayama & Rekik, 1989); DoxG, *Pseudomonas* sp. strain C18 1,2-dihydroxynaphthalene dioxygenase (Denome et al., 1993); TodE, *P. putida* F1 3-methylcatechol 2,3-dioxygenase (Zylstra & Gibson, 1989); DbfB, *Sphingomonas* sp. strain RW1 2,2'-trihydroxybiphenyl dioxygenase (Happe et al., 1993); CDO, *Rhodococcus rhodochrous* CTM catechol 2,3-dioxygenase (Candidus et al., 1994); PheB, *Bacillus stearothermophilus* catechol 2,3-dioxygenase (unpublished, GenBank accession no. X67860); C23OII, *P. putida* MT15 catechol 2,3-dioxygenase (unpublished, GenBank accession no. U01826); TdnC, *P. putida* UCC2 3-methylcatechol 2,3-dioxygenase (unpublished, GenBank accession no. X59790); TbuE, *Burkholderia pickettii* catechol 2,3-dioxygenase (Kukor & Olsen, 1996); XylE, *P. putida* mt-2 catechol 2,3-dioxygenase (Nakai et al., 1983); XylE, *B. subtilis* (unpublished, patent EP0086139-A1); BztE, *P. aeruginosa* JI104 catechol 2,3-dioxygenase (unpublished, GenBank accession no. X60740); BphE, *Pseudomonas* sp. strain IC catechol 2,3-dioxygenase (Carrington et al., 1994); NahH, *P. putida* PpG7 catechol 2,3-dioxygenase (Ghosal et al., 1987); DmpB, *P. putida* CF600 catechol 2,3-dioxygenase (Bartilson & Shingler, 1989); CDO, *Alcaligenes* sp. strain KF711 catechol 2,3-dioxygenase (Moon et al., 1995); XylE, *P. putida* HS1 catechol 2,3-dioxygenase (Benjamin et al., 1991); CDO1, A8AN3 catechol 2,3-dioxygenase in pUTK169; XylE, *S. yanoikuyae* B1 catechol 2,3-dioxygenase (Kim & Zylstra, 1995); CmpE, *Sphingomonas* sp. strain HV3 catechol 2,3-dioxygenase (Yrjala et al., 1994); CDO2, A8AN3 catechol 2,3-dioxygenase in pUTK141; MndD, *Arthrobacter globiformis* CM-2 3,4-dihydroxy-phenylacetate 2,3-dioxygenase (Boldt et al., 1995). The sequences were retrieved from the GenBank database.



between the amino acid sequences of extradiol dioxygenases is shown in Fig. 8. The dendrogram clearly shows a separate cluster of the dioxygenases isolated from sphingomonads within a subfamily of extradiol dioxygenases that preferentially cleave monocyclic substrates. The dendrogram contains 2 subfamilies of extradiol dioxygenases. The subfamilies are based on substrate structures preferentially acted on by the enzymes. A subfamily (upper part of the dendrogram) contains a group of enzymes that primarily catalyze cleavage of bicyclic substrates with an exception of TodE (3-methylcatechol 2,3-dioxygenase); BphC of *S. yanoikuyae* B1, BphC of *S. yanoikuyae* Q1, NahC of *P. putida* PpG7, DoxG of *Pseudomonas* sp. C18, TodE of *P. putida* F1, and DbfB of *Sphingomonas* sp. strain RW1. TodE of *P. putida* F1 preferentially cleaves a monocyclic substrate (Zylstra & Gibson, 1989).

Within the subfamily of enzymes that preferentially catalyze cleavage of monocyclic substrates (bottom part of the dendrogram), basically 4 clusters are recognizable. The first cluster includes extradiol dioxygenases isolated from Gram-positive bacteria; CDO of *Rhodococcus rhodochrous* CTM (Candidus et al., 1994) and PheB of *Bacillus stearothermophilus* (unpublished, GenBank accession no. X67860). The second and third clusters contains extradiol dioxygenases isolated from γ - and β -subgroups of *Proteobacteria* with an exception of XylE from *B. subtilis*. The majority of extradiol dioxygenases in the clusters are from γ -subgroup. The second cluster comprises C23OII of *Pseudomonas putida* MT15 (unpublished, GenBank accession no. U01826), TdnC of *P. putida* UCC2 (unpublished, GenBank accession no. X59790), and TbuE of *Burkholderia pickettii* (Kukor & Olsen, 1996). The third and largest cluster contains XylE of *P. putida* mt-2 (Nakai et al., 1983), XylE of *B. subtilis* (unpublished, patent EP0086139-A1), BztE of *P. aeruginosa* JI104 (unpublished, GenBank accession no. X60740), BphE of *Pseudomonas* sp. strain IC (Carrington et al., 1994), NahH of *P. putida* PpG7 (Ghosal et al., 1987), DmpB of *P. putida* CF600 (Bartilson & Shingler,

1989), CDO of *Alcaligenes* sp. strain KF711 (Moon et al., 1995), and XylE of *P. putida* HS1 (Benjamin et al., 1991). The fourth cluster contains extradiol dioxygenases from sphingomonads: CDO1 of A8AN3, XylE of *S. yanoikuyae* B1, CmpE of *Pseudomonas* sp. strain HV3 (recently re-classified as *Sphingomonas* sp. strain HV3, personal communication with Dr. K. Yrjala), and CDO2 of *Sphingomonas* sp. strain A8AN3. The MndD (3,4-dihydroxyphenylacetate 2,3-dioxygenase) encoded by *mndD* from *Arthrobacter globiformis* CM-2 is distantly related to this subfamily, although it functionally prefers cleavage of monocyclic substrates (Boldt et al., 1995). The activity of MndD is Mn^{2+} -dependent, whereas all other bacterial ring-cleavage dioxygenases show iron-dependent activities.

The 2.8-kb *NotI* fragment from pUTK161 showed *meta*-cleavage activity regardless of its insertional direction from the *lac* promoter on the cloning vector pBluescript II SK (+), as observed by subclones pUTK162 and pUTK163 (Fig. 1). This suggested that the *cdo1* gene was expressed from its own promoter. Subclone pUTK169 constructed by deletion of 0.8-kb *SalI-NotI* fragment from pUTK162 still showed *meta*-cleavage activity. Nucleotide sequence analyses of 2.184-kb *NotI-SalI* fragment of pUTK169 using the programs FRAME and BLAST suggested that a complete ORF of *cdo1* gene was transcribed from right to left on the map in Fig 1. Nucleotide sequences showing similarity to the consensus sequence of σ^{70} -dependent promoter in *E. coli* are underlined (Fig. 6). The consensus sequence of such a promoter is TTGACA-17 $\pm$ 1-TATAAT and is located at -10 and -35 regions (Heimann & Chamberlin, 1988). The functional role of upstream sequences of the *cdo1* gene remains to be investigated.

IV. DISCUSSION

An MGP (manufactured gas plant)-soil isolate, *Sphingomonas* sp. strain A8AN3 was shown to degrade a broad range of PAHs including BaP (Ahn et al., 1994). Its Ind⁻ mutant strain 2123 was obtained by random Tn5-mutagenesis of A8AN3 in previous study. Genomic DNA libraries of A8AN3 and 2123 were constructed using a plasmid vector, pBluescript II SK(+). The resulting libraries were screened for clones showing aromatic ring dioxygenase activity, *meta*-cleavage activity, or kanamycin resistance. As the result of screening, clones containing short Tn5-flanking regions or showing *meta*-cleavage activity were obtained. The Tn5-flanking regions can be used for future study to locate Tn5-insertion site. In this study, molecular characterization of isolated extradiol dioxygenase genes was focused.

A8AN3 shows aromatic-ring dioxygenase activity based on indigo production after exposure to indole (Ensley et al., 1983). Aromatic-ring dioxygenases are involved in initial attack of aromatic hydrocarbons, producing dihydroxylated aromatic hydrocarbons by incorporating two atoms of oxygen (Harayama et al., 1992). *E. coli* clones showing aromatic-ring dioxygenase activity were not found from gene libraries of A8AN3 in this study as determined by the indigo test, presumably because of poor or no expression of the clones in *E. coli*. No information is available about aromatic-ring dioxygenase genes isolated from sphingomonads, although an aromatic-ring dioxygenase, dibenzofuran 4,4a-dioxygenase was purified from *Sphingomonas* sp. strain RW1 (Bunz & Cook, 1993). *E. coli* expressing aromatic-ring dioxygenase genes from sphingomonads has not been reported. Inefficient expression of the genes in *E. coli* might have resulted from poor promoter recognition by *E. coli* RNA polymerase. The absence of ancillary factors such as sigma factors could cause poor promoter recognition, as could differences in promoter

sequences. Difference in RBS sequence or codon usage could also reduce gene expression in *E. coli*.

Although *E. coli* expressing aromatic-ring dioxygenase genes from sphingomonads has not been reported, a few reports on other genes including extradiol dioxygenase genes expressed in *E. coli* are available (Taira et al., 1988; Noda et al., 1990; Yrjala et al., 1994; Kim & Zylstra, 1995; Stillwell, 1995; Heiss et al., 1995). Most extradiol dioxygenase genes from sphingomonads do not appear to have expression problem in *E. coli*. However, Heiss et al. (1995) reported weak expression of *bphC* gene isolated from naphthalenesulfonate-degrading *Sphingomonas* sp. strain BN6. Kim & Zylstra (1995) reported that no activities were observed from products of *bphC* and *xylE* genes from *S. yanoikuyae* B1 when the genes were cloned in cosmid vector pH79. However, the enzyme activities were observed after subcloning into a high copy number vector plasmid pGEM.

The presence of two different extradiol dioxygenase genes (*cdo1* and *cdo2*) in A8AN3 was shown in this study. Gene *cdo2* was isolated from gene libraries of A8AN3 and its Tn5-mutant strain 2123, whereas gene *cdo1* was from a gene library of 2123. Southern blot hybridization using a *cdo1*-specific probe suggested the presence of *cdo1* gene in A8AN3. Differences in the *cdo1* and *cdo2* genes were determined on the basis of restriction sites, DNA hybridization, physical location, and nucleotide (or deduced amino acid) sequence. Their gene products, CDO1 and CDO2 showed primary activity with dihydroxylated monocyclic substrates. However, they showed different substrate specificities among the dihydroxylated monocyclic substrates tested in this study, supporting the finding that the genes *cdo1* and *cdo2* are different each other. Analysis of Southern hybridization showed that the *cdo1* gene was on chromosomal DNA, whereas the *cdo2* was on plasmid DNA of A8AN3. This is the first example that both plasmid and

chromosomal DNA are involved in aromatic hydrocarbon degradation in a *Sphingomonas* strain.

Plasmids often carry catabolic genes for various compounds (Dunn & Gunsalus, 1973; Williams & Murray, 1974; Wallace & Sayler, 1992). Little information is available for sphingomonads compared to information on degradative genes isolated from γ -subgroup fluorescent pseudomonads. However, interestingly many degradative genes in sphingomonads are chromosomally-encoded (Taira et al., 1988; Noda et al., 1990; Imai et al., 1991; Orser et al., 1993; Heiss et al., 1995; Kim et al., 1996), although two reports showed that catabolic functions were linked to plasmid DNA (Yrjala et al., 1994; Stillwell et al., 1995), based on isolation of genes encoding catechol 2,3-dioxygenase activity from plasmid DNA. Compared with plasmid-encoded genes, chromosome-encoded catabolic genes can implicate more stable maintenance of the genes and possibly more stable exhibition of degradative phenotypes. This makes sphingomonads important in bioremediation in addition to their metabolic versatility and wide occurrence.

The nucleotide sequences of the *cdo1* and *cdo2* genes were determined and amino acid sequences of the gene products were deduced. A dendrogram was constructed by comparison of amino acid sequences of extradiol dioxygenases. The dendrogram agreed with the result of enzyme assays showing that CDO1 and CDO2 were most active with monocyclic substrates. As shown in Fig. 8, the two gene products, CDO1 and CDO2 belong to a subfamily of extradiol dioxygenases that primarily catalyze ring cleavage of monocyclic substrates. CDO1 and CDO2 were most closely related to XylE (encoding catechol 2,3-dioxygenase) of *S. yanoikuyae* B1 (Kim & Zylstra, 1995) at both the nucleotide and amino acid levels. However, chromosome-encoded gene products, CDO1 and XylE (Kim et al., 1996) showed more relatedness, suggesting more diversity of plasmid-encoded extradiol dioxygenase. CDO1 and CDO2 also showed similarity to plasmid-encoded CmpE (encoding catechol 2,3-dioxygenase) from *Sphingomonas* sp.

strain HV3. The chromosome-encoded CDO1 was most active with catechol, whereas plasmid-encoded CDO2 was most active with 3-methylcatechol. Yrjala et al. (1994) reported that plasmid-encoded CmpE showed slightly more activity with methylated catechols than unsubstituted catechol.

The extradiol dioxygenases of sphingomonads, CDO1, CDO2, CmpE, and XyE, showed low similarity to other extradiol dioxygenases that primarily catalyze ring cleavage of monocyclic substrates (Fig. 8). The sphingomonad dioxygenases produced a separate cluster within a subfamily of extradiol dioxygenases that primarily catalyze ring cleavage of monocyclic substrates (Fig. 8). Compared to the extradiol dioxygenases (NahC and NahH) encoded by the NAH7 plasmid, the distance between the sphingomonad extradiol dioxygenases for monocyclic substrates and NahH is greater than the distance between the sphingomonad extradiol dioxygenases for dicyclic substrates and NahC. Although the extradiol dioxygenases of sphingomonads are diversified from the other extradiol dioxygenases, the sphingomonad dioxygenases showed evolutionary relatedness with the other extradiol dioxygenases based on conserved amino acids. The sequence alignment (Fig. 7) revealed potential amino acid residues for Fe^{2+} binding and C-end region signature sequence for extradiol dioxygenase (Harayama & Rekik, 1990). Studies on the crystal structure of BphC from *P. cepacia* LB400 (Han et al., 1995) showed three amino acid residues (H₁₄₆, H₂₁₀, and E₂₆₀) that are ligands for the ferrous iron required for enzyme activity. The amino acids of the metal ligands corresponded to (aligned with) amino acid residues, His₁₆₁, His₂₂₇, and Glu₂₇₈ shown in Figure 7 (BphC from *P. cepacia* LB400 not shown). Chemical modification of histidyl residues in catechol 2,3-dioxygenase of *R. rhodochrous* CTM also suggested the importance of the residues in ferrous iron coordination (Candidus et al., 1994).

Since strain A8AN3 can degrade a variety of PAHs including five-ringed BaP and two-ringed naphthalene, it is expected that at least another extradiol dioxygenase

responsible for cleavage of 2 or more ring substrates exists in the strain. *Sphingomonas* strains HV3, Q1, and B1 are able to degrade naphthalene, although they were originally isolated on different compounds (Gibson et al., 1973; Furukawa et al., 1983; Yrjala et al., 1994). Both genes (*xylE* and *bphC*) encoding extradiol dioxygenases responsible for monocyclic and dicyclic substrates were isolated from *S. yanoikuyae* B1 (Kim & Zylstra, 1995). However, only genes (*cmpE*, *cdo1*, and *cdo2*) encoding extradiol dioxygenases for monocyclic substrates have been isolated from HV3 (Yrjala et al., 1994) and A8AN3. Sphingomonad extradiol dioxygenases, CDO1, CDO2, CmpE, and XyE, were closely related, whereas they showed diversity from the other extradiol dioxygenases that primarily catalyze ring cleavage of monocyclic substrates. From *S. yanoikuyae* Q1, only a gene (*bphC*) encoding extradiol dioxygenase for bicyclic substrates has been isolated (Taira et al., 1988). Since *S. yanoikuyae* strains B1 and Q1 are phylogenetically very close (Wang & Lau, 1996) and their *bphC* genes are chromosomally encoded (Taira et al., 1988; Kim et al., 1996), it is not too surprising that *S. yanoikuyae* strains B1 and Q1 contained identical *bphC* genes (Kim & Zylstra, 1995). Although the chromosomal location of the genes could have resulted from transposition or recombination of plasmids, the chromosomal location could contribute to less diversity of genes than location on promiscuous plasmids. Strains Q1 and B1 contained only 2 different bases over 1410-nucleotide sequences of 16S rRNA. The previous study in Part 3 showed phylogenetic difference between *Sphingomonas* strains A8AN3 and B1 (or Q1). Therefore, it would be interesting to see their relationship in upper pathway genes such as extradiol dioxygenase genes for bicyclic substrates and aromatic-ring dioxygenase genes for the initial degradation step. Stillwell et al. (1995) reported that catechol 2,3-dioxygenases activities were found at two regions of a DNA fragment isolated from a plasmid in the naphthalene-degrading *Sphingomonas* sp. strain F199. Since only restriction maps of the fragment were presented in the paper, it is not clear whether the

extradiol dioxygenase genes are for monocyclic or dicyclic substrates. Although the relative activity was different, extradiol dioxygenases responsible for bicyclic substrates showed ring-cleavage activity with monocyclic substrates (Ghosal et al., 1987; Taira et al., 1988). Likewise extradiol dioxygenases responsible for monocyclic substrates showed ring-cleavage activity with bicyclic substrates (Kuhm et al., 1991; Carrington et al., 1994). A naphthalenesulfonate-degrading *Sphingomonas* sp. strain BN6 was suggested to contain two different extradiol dioxygenases for bicyclic substrates based on purification of 1,2-dihydroxynaphthalene dioxygenase (Kuhm et al., 1991) and isolation of a 2,3-dihydroxybiphenyl 1,2-dioxygenase (DHBD) gene (Heiss et al., 1995) from the strain. Although the 1,2-dihydroxynaphthalene dioxygenase gene has not isolated, the enzyme showed different substrate specificity from the 2,3-DHBD gene product whose amino acid sequence was different from the 1,2-dihydroxynaphthalene dioxygenase.

As yet no data are available to define the organization of genes involved in aromatic hydrocarbon degradation in A8AN3. However, some speculations are as follows. (1) Based on the phenotype of strain 2123, Southern blot analysis of 2123 DNA, and isolation of *cdo1* gene, chromosomal DNA of A8AN3 contains aromatic hydrocarbon degradative genes that encode enzymes for reactions from initial attack of PAHs to further degradation of catechol. (2) Based on isolation of the *cdo2* gene, a large plasmid (approximately 80 kb) in A8AN3 is also involved in aromatic hydrocarbon degradation. Based on the phenotype of strain 2123 and Southern blot analysis of 2123 DNA, however, the plasmid does not appear to contain genes encoding aromatic-ring dioxygenase for initial attack of PAHs. This kind of plasmid could be exemplified by SAL plasmids which encode enzymes for further degradation of salicylate, a metabolite of naphthalene degradation. Presence of 2 nonhomologous catechol 2,3-dioxygenases might provide strain A8AN3 metabolic advantage to dissimilate both substituted and unsubstituted catechols. (3) Based on high % identity of nucleotide and amino acid

sequences between chromosome-encoded genes *cdo1* and *xylE* (Kim & Zylstra, 1995), B1 and A8AN3 might have similarity in gene organization. If so, the right end region of clone pUTK16 (Fig. 1) isolated in this study might contain a gene encoding extradiol dioxygenase responsible for dicyclic substrates. Strain B1 contains two extradiol dioxygenase genes, *xylE* and *bphC* that are approximately 5.5 kb away from each other and transcribed in opposite directions.

Nucleotide sequence analysis of subclones pUTK141 and pUTK169 showed that they contain 2 partial ORFs in addition to a complete ORF of catechol 2,3-dioxygenase. Upstream of each extradiol dioxygenase gene was a partial gene of 2-hydroxymuconic semialdehyde (2-HMS) hydrolase, whereas downstream of the each extradiol dioxygenase gene was a partial gene of 2-HMS dehydrogenase. Both 2-HMS hydrolase and 2-HMS dehydrogenase are involved in further degradation of *meta*-ring cleavage product. The *meta*-cleavage product of catechol, 2-HMS can be degraded to 2-hydroxypent-2,4-dienoate by 2-HMS hydrolase or by 2-HMS dehydrogenase and two sequential enzymes (Bayly & Dagley, 1969; Sala-Trepat & Evans, 1971; Murray et al., 1972). 2-Hydroxypent-2,4-dienoate can be further converted to TCA cycle intermediates, pyruvate and acetaldehyde. Studies (Bayly & Wigmore, 1973; Wigmore et al., 1974; Bayly & DiBerardino, 1978; Harayama et al., 1987b) showed that substrate specificities of 2-HMS hydrolase and 2-HMS dehydrogenase determine further degradation of *meta*-cleavage products. The ring cleavage product of 3-methylcatechol is degraded predominantly via a hydrolase reaction, whereas ring-cleavage products of catechol and 4-methylcatechol are metabolized primarily by a dehydrogenase reaction.

Both subclones pUTK141 and pUTK169 contained a complete ORF of catechol 2,3-dioxygenase between 2 partial ORFs of enzymes involved in degradation of ring-cleavage products (Fig. 4 and 9). The order of the 2-HMS hydrolase, catechol 2,3-dioxygenase, and 2-HMS dehydrogenase genes found in pUTK161 and pUTK141 is

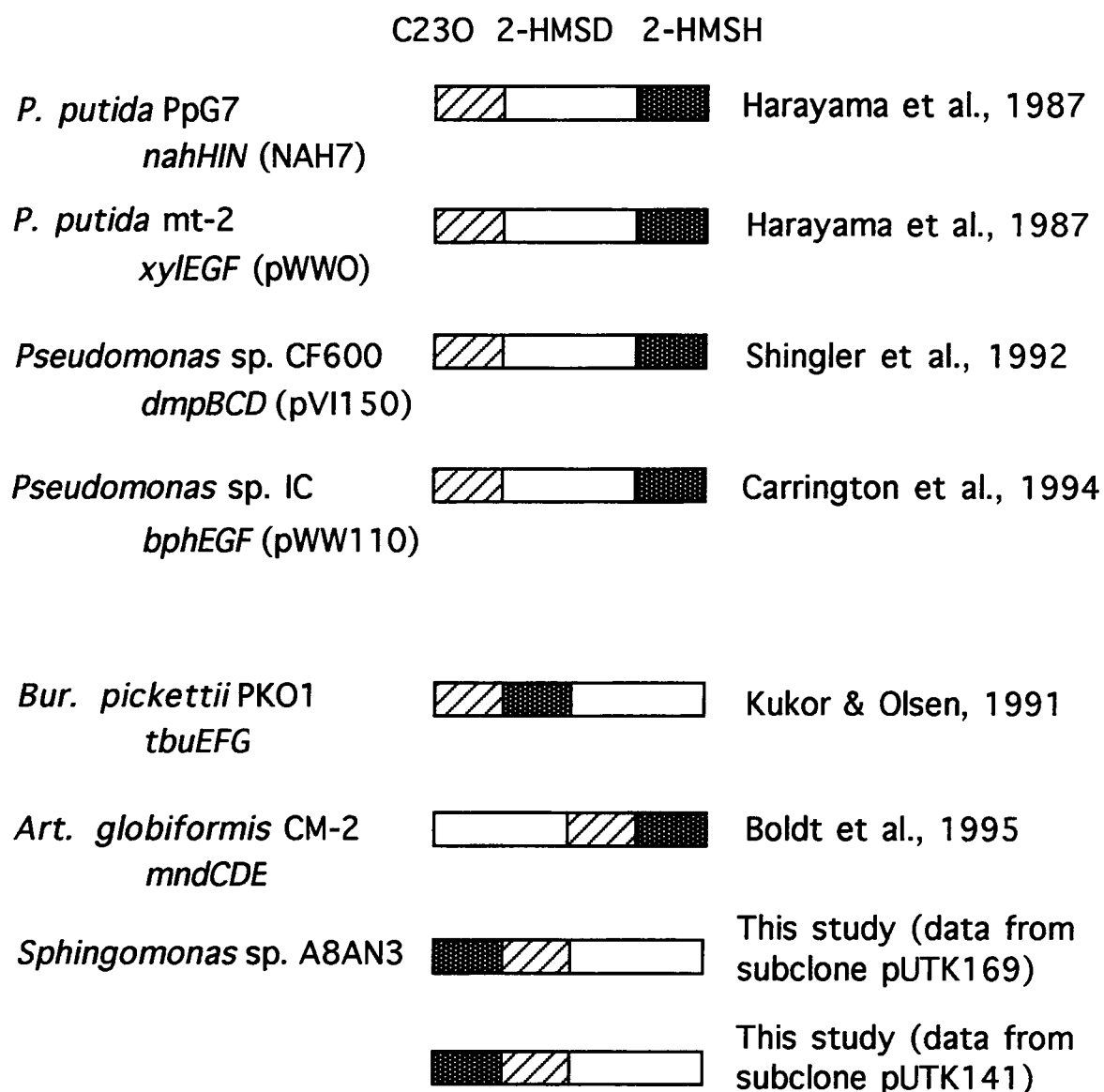


Figure 9. *Meta*-pathway gene order found in various strains. Intergenic space is not shown in the maps. Transcriptional direction is from left to right in the maps. Parentheses contain plasmids where the genes are located. C23O, catechol 2,3-dioxygenase; 2-HMSD, 2-hydroxymuconic semialdehyde (2-HMS) dehydrogenase; 2-HMSH, 2-HMS hydrolase.

different from that of the isofunctional enzyme genes found in other catabolic gene systems for aromatic hydrocarbons (Harayama et al., 1987a; Assinder & Williams, 1990; Shingler et al., 1992; Carrington et al., 1994). Many *meta*-pathways have the order of catechol 2,3-dioxygenase, 2-HMS dehydrogenase, and 2-HMS hydrolase genes: such as *nahHIN* on the naphthalene-degradative plasmid NAH7 (Harayama et al., 1987a); *xylEGF* on the toluene-degradative plasmid pWWO (Assinder & Williams, 1990); *dmpBCD* on the phenol-degradative plasmid pVI150 (Shingler et al., 1992); and *bphEGF* on the biphenyl-degradative plasmid on pWW110 (Carrington et al., 1994). The *meta*-pathway genes of the four pathways (*nah*, *xyl*, *bph*, and *dmp*) were found in γ -subgroup fluorescent pseudomonads in *Proteobacteria*. These *meta*-pathway genes are not only conserved in gene order but also are very similar in nucleotide and amino acid sequences, suggesting the genes are tightly-arranged evolutionary modules in the subgroup (Williams & Sayers, 1994). However, *meta*-pathway genes in *Sphingomonas* sp. strain A8AN3 show very distant relationship to the γ -subgroup fluorescent pseudomonads based on different gene order and less-related nucleotide sequences of catechol 2,3-dioxygenase genes. Two other strains were also reported to have different gene order in *meta*-pathway genes. A member of β -subgroup in *Proteobacteria*, *Burkholderia pickettii* PKO1 contains *tbu* genes for degradation of toluene, benzene, and phenol; *tbuEFG* genes encode catechol 2,3-dioxygenase, 2-HMS hydrolase, and 2-HMS dehydrogenase, respectively (Kukor & Olsen, 1991). Gram-positive *Arthrobacter globiformis* CM-2 contains *mndCDE* encoding 2-HMS dehydrogenase, catechol 2,3-dioxygenase, and 2-HMS hydrolase, respectively (Boldt et al., 1995). The genes *mndCDE* are involved in *meta*-pathway of 3,4-dihydroxyphenylacetate degradation.

Little information is available about promoters for catabolic genes isolated from sphingomonads. Sequences showing similarity to $\sigma 70$ -dependent promoter sequences were observed in γ -hexachlorocyclohexane-degradative genes from *S. paucimobilis* UT26

(Nagata et al., 1994). However, the functions of the putative promoters in UT26 have not been proved experimentally. A putative promoter of *cdo 1* gene is found within the coding region of the upstream gene encoding 2-HMSH in clone pUTK169 (Fig. 4 and 6). The putative promoter contains sequences similar to $\sigma 70$ -dependent promoter. The two genes are transcribed in the same direction. Such a promoter region located within an upstream gene is quite unusual. However, a few examples have been shown in *E. coli* fumarate reductase complex operon and β -lactamase gene (*frdABCD-ampC*), *E. coli* phosphoenolpyruvate-dependent phosphotransferase system genes (*ptsHI-crr*), *Rhodobacter capsulatus* photosynthesis genes (*crtEF-bchCA-puf*), and *Streptomyces clavuligerus* cephamycin biosynthetic genes (*pcbAB-pcbC*) (Grundstrom & Jaurin, 1982; De Reuse & Dunchin, 1988; Wellington & Beatty, 1991; Petrich et al., 1994). A downstream gene can be transcribed from either its own promoter present in an upstream operon or a promoter for an upstream operon; overlapping transcriptional arrangement (De Reuse & Dunchin, 1988; Wellington et al., 1991; Petrich et al., 1994). Such an overlapping transcriptional arrangement was shown to be important in regulation of gene expression (Jaurin et al., 1981; De Reuse & Dunchin, 1988; Wellington et al., 1991). The putative promoter region found in pUTK169 requires further characterization such as primer extension analysis to identify the transcriptional start site.

Aromatic ring dioxygenase catalyzes the initial reaction in the PAH degradation. Many aromatic ring dioxygenases can convert indole to indigo (Ensley et al., 1983; Eaton & Timmis, 1986; Tan & Mason, 1990; Denome et al., 1993). The initial dioxygenase is a multicomponent enzyme system that consists of a flavoprotein, a ferredoxin, and an iron sulfur protein (ISP) (Yeh et al., 1977; Crutcher & Geary, 1979; Ensley et al., 1982; Harayama et al., 1992). For example, naphthalene dioxygenase encoded by *nahAaAbAcAd* on the plasmid NAH7 consists of a flavoprotein, a ferredoxin, and an ISP. The ISP consists of ISP α and ISP β : $\alpha 2\beta 2$. Conserved amino acid sequences of each

component are known for benzene, toluene, and naphthalene dioxygenases (Irie et al., 1987; Zylstra & Gibson, 1989; Mason & Cammack, 1992; Simon et al., 1993). The enzyme activity sites of dioxygenases are present in ISP α proteins which provide substrate-specificities (Ensley & Gibson, 1983; Furukawa et al., 1993). Therefore, an ISP α -encoding gene in the initial dioxygenase genes is the source of choice to develop a gene probe for PAH degradation. The initial dioxygenase genes in A8AN3 (Ind⁺ BaP⁺) are of interest; the genes can be used to develop a gene probe to detect genetic potential to degrade BaP since the *nahA* gene probe does not detect the strain. Ind⁺ clones (supposedly containing active aromatic ring dioxygenases) have not been screened from a gene library of A8AN3 and aromatic-ring dioxygenase genes from sphingomonads seem to have expression problem in *E. coli*. Strain 2123 (Ind⁻ BaP⁻) can be used to facilitate isolation of the initial dioxygenase genes. Tn5 is likely inserted in the initial dioxygenase genes or upstream of the genes in strain 2123 (see Part 3 for more discussion). Unfortunately, clone p5-1 (Km^r) obtained in this study contains very short Tn5-flanking regions. It is necessary to get longer Tn5-flanking regions 1) to locate (or isolate) the initial dioxygenase genes, 2) to define Tn5 insertion site, and 3) to relate clone pUTK16 to clone p5-1, revealing gene organization in A8AN3. Based on superoperonic clustering of functionally related genes (Holloway & Morgan, 1986; Young et al., 1989; Averhoff et al., 1992; Eaton, 1996), clones p5-1 and pUTK16 are thought to be clustered on chromosomal DNA of strain 2123; although middle DNA fragment between the two clones has not been isolated yet. Tn5-flanking regions of clone p5-1 can be used as probes against a gene library of A8AN3 or 2123 to isolate clones containing longer Tn5-flanking region. Clone p5-1 can be also used to obtain the initial dioxygenase genes using shotgun cloning or PCR. Genomic DNA of strain 2123 is digested with *HpaI* (Tn5 has two *HpaI* sites located 185 bp away from its both ends) and another enzyme, X which cuts Tn5-flanking DNA. The resulting fragments are ligated into a vector digested with

*Hpa*I and X. Ligated DNA is transformed into competent *E. coli*. Transformants are screened by hybridization with a probe containing the terminal 185 bp of Tn5 to isolate Tn5-flanking DNA. Using genomic DNA of strain 2123 as a template, PCR can be used to amplify Tn5-flanking DNA containing a part of aromatic ring dioxygenase genes. Since nucleotide sequence of Tn5 is known (Ahmed & Podemski, 1995), a primer consisting of the terminal 15 - 20 nucleotides of Tn5 can be used in PCR together with a degenerate primer derived from conserved sequences of initial dioxygenase components. It is important to consider all possible orders of dioxygenase component genes when design degenerate primers in PCR. An arbitrary primer can be used instead of a degenerate primer if aromatic ring dioxygenase genes in A8AN3 do not contain conserved sequences (it is unlikely to happen) or if degenerate primers do not work. When an arbitrary primer is employed, chances to amplify Tn5-flanking regions are expected to be low because an arbitrary primer can bind to Tn5. The PCR products can be cloned using a TA cloning vector. After obtaining the clones from gene probing, shotgun cloning, or PCR, it is necessary to perform nucleotide sequencing or gene transfer to other host strains (e.g., an Ind⁻ *Sphingomonas* strain or strain 2123) for sequence similarity or gene expression (or complementation), which will verify the clones.

Several lines of evidence suggest that microorganisms have a high degree of diversity (Torsvik et al., 1990; Boronin, 1992; Weibner et al., 1996). Degradative genes from sphingomonads have been often reported not to hybridize with isofunctional enzyme genes from other strains (Taira et al., 1988; Noda et al., 1990; Imai et al., 1991), suggesting diversity of degradative genes in *Sphingomonas* strains. This study on extradiol dioxygenase genes of *Sphingomonas* sp. strain A8AN3 supports considerable diversity of sphingomonads. Further characterization of degradative genes in A8AN3 will provide better understanding of the molecular mechanisms for the metabolic versatility of the strain.

Part 5: Conclusions

CONCLUSIONS

Taken the results of Parts 2, 3, and 4 together, conclusions of this research are follows.

1. Among a total of 141 bacteria isolated previously as putative PAH-degraders, 89 strains showed PAH degradation ability as determined by the PAH spray plate method and the ^{14}C -naphthalene mineralization assay. The difference in the number of PAH-degraders could have resulted from false results due to overspreading of clear zones to other bacterial colonies which did not degrade PAHs in the spray plate method or cross-feeding of strains on the naphthalene vapor plates when they were originally isolated.

2. The majority of the PAH-degraders showed ability to degrade more than a single PAH as determined by the PAH spray plate method. All naphthalene degraders (27 strains) and anthracene degraders (13 strains) degraded phenanthrene.

3. A few HMW PAH degraders were found: three pyrene-degraders (A8AN3, B1PH6, and N1-4PH) were found, while A8AN3 could mineralize BaP. Finding of HMW PAH degraders suggested that *in situ* microorganisms have the potential to degrade HMW PAHs in contaminated sites. The HMW PAH degraders degraded a broad range of PAHs (i.e., naphthalene, fluorene, phenanthrene, anthracene, and pyrene) as determined by the spray plate method or the ^{14}C -PAH mineralization assay.

4. The results of indigo tests suggested strong relationship between PAH-degrading bacteria and their aromatic ring dioxygenase activity: 98% (87/89) of PAH-degraders showed aromatic ring dioxygenase activity. The high percentage suggests that the majority of bacteria degrade PAHs via dioxygenases.

5. The result of NAH7-derived gene probing suggested that PAH-degraders with NAH7-like genotype were dominant among culturable PAH-degraders: Of the 89 PAH-degraders, 76 strains were detected by the *nahA* gene probe.

6. The phenotypic and genotype analyses actually proved that the *nahA* gene probe could be used to determine the microbial potential to degrade not only naphthalene but also 3- or 4-ring PAHs. The probe was especially effective for detecting phenanthrene degraders or fluorene degraders among 3 or more ring PAH-degraders. This result supports the continued use of the NAH7-derived probes for PAH-contaminated soils to monitor genetic potential of *in situ* microorganisms to degrade PAHs.

7. Fifteen percent (13/89) of the PAH degraders were not detected with the *nahA* gene probe; roughly, as molecular weight (number of rings) of PAHs increases, the percentage of detection decreases. Four PAH-degraders (A5PH1, A8AN3, B1PH2, and B10AN1) did not hybridize with any of NAH7-derived gene probes used in this study; *nahA*, *nahG*, *nahH*, and *nahR*. Therefore, considering reports on the numerous unculturable microorganisms in nature and the non-NAH7 hybridizing PAH degraders, the current NAH7-derived gene probes may underestimate *in situ* microbial potential to degrade PAHs.

8. Strains A8AN3, B1PH2, and B10AN1 were identified as *Sphingomonas* sp. strains: A8AN3 and B1PH2 were most similar to *S. paucimobilis* based on chemotaxonomic traits and phenotypic characteristics, whereas B10AN1 was identified as a *Sphingomonas* sp. strain based on chemotaxonomic traits. However, A5PH1 produced taxonomic uncertainty.

9. Dissimilar genes or gene organization in the 4 strains (A5PH1, A8AN3, B1PH2, and B10AN1) was suggested by NAH7-derived gene probing of total DNA and RFLP analysis of plasmid DNA from the strains.

10. A8AN3 did not hybridize with any of the NAH7-derived gene probes but did degrade a broad range of PAHs; e.g., BaP, pyrene, phenanthrene, anthracene, fluorene, and naphthalene.

11. Comparative 16S rDNA sequence analysis suggested that A8AN3 was closest to *Sphingomonas adhaesiva* in the RDP database.

12. A phylogenetic tree based on partial 16S rDNA sequences of sphingomonads showed that A8AN3 has phylogenetic difference from the other two BaP-mineralizing sphingomonads.

13. A8AN3 showed slow growth; its doubling times were approximately 4 h and 30 min in 25% YEPG and approximately 4 h in YEPG, respectively.

14. A8AN3 mineralized approximately 45% of added [7-¹⁴C]BaP or [7,10-¹⁴C]BaP in 25% YEPG. The result suggested that A8AN3 could cleave at least one ring of a five-ringed BaP molecule.

15. An Ind⁻ mutant (strain 2123) of A8AN3 was obtained by Tn5 mutagenesis. Together with colony morphology, color, and indigenous plasmid profile, RFLP analysis of PCR-amplified 16S rDNA verified that the Tn5-mutant 2123 was derived from A8AN3.

16. The mutant could not degrade PAHs degraded by wild type A8AN3. However, it showed extradiol dioxygenase activity (*meta*-ring cleavage activity) as determined by the catechol spray method.

17. Southern blot analyses of the mutant DNA suggested single insertion of Tn5 into genomic DNA; Tn5 was likely inserted into the chromosomal DNA of A8AN3. Considering the phenotype of the mutant and the result of Southern blot hybridization, Tn5 was probably inserted in aromatic ring dioxygenase genes or upstream of the genes in an operon where the initial dioxygenase genes were present.

18. As the result of screening gene libraries of A8AN3 and 2123, clones showing extradiol dioxygenase activity or containing short Tn5-flanking regions were obtained. The short Tn5-flanking regions isolated in this study can be used for future study to locate the exact Tn5-insertion site.

19. Subclone pUTK141 containing an extradiol dioxygenase gene *cdo1* was isolated from gene libraries of A8AN3 and its Tn5-mutant strain 2123, whereas subclone pUTK169 containing gene *cdo1* was isolated from a gene library of strain 2123. However, presence of both *cdo1* and *cdo2* genes in A8AN3 was shown by Southern blot analyses.

20. The two extradiol dioxygenase genes (*cdo1* and *cdo2*) were determined to be nonhomologous based on analyses of restriction sites, DNA hybridization, physical location, substrate specificity, and nucleotide (or deduced amino acid) sequence.

21. Products of *cdo1* and *cdo2* genes, CDO1 and CDO2 were most active with monocyclic substrates, as determined by enzyme assays with crude enzymes extracted from *E. coli* DH5 α harboring pUTK141 or pUTK163. However, their substrate specificity was different from each other: CDO1 showed highest activity with catechol, whereas CDO2 was most active with 3-methylcatechol.

22. Southern blot analyses suggested that the *cdo1* and *cdo2* genes have different physical locations: the *cdo1* gene is likely on chromosomal DNA, whereas the *cdo2* gene is on plasmid DNA.

23. The nucleotide sequences of *cdo1* and *cdo2* genes were determined. Analyses of the deduced amino acid sequences showed that CDO1 (92.2% identity) and CDO2 were most similar to XylE from *S. yanoikuyae* B1, although the similarity of CDO2 was less than CDO1. However both CDO1 and CDO2 showed low similarity (49.8 -51.0%) to NahH encoded by the plasmid NAH7.

24. CDO1 and CDO2 contained conserved amino acid residues present in extradiol dioxygenase. These were C-end region signature sequence for extradiol dioxygenase and the potential amino acid residues for Fe²⁺ binding.

25. A dendrogram was constructed by comparison of amino acid sequences of extradiol dioxygenases. Both CDO1 and CDO2 belonged to a subfamily cluster of

extradiol dioxygenases that preferentially cleave monocyclic substrates. The dendrogram clearly showed a separate cluster of the dioxygenases isolated from sphingomonads within a subfamily of extradiol dioxygenases that preferentially cleave monocyclic substrates. This finding suggested evolutionary distance between sphingomonad extradiol dioxygenases and extradiol dioxygenases from other strains.

26. Nucleotide sequencing of subclones pUTK141 and pUTK169 revealed that each of the *cdo1* and *cdo2* genes was flanked by enzyme genes (a 2-HMS hydrolase gene and a 2-HMS dehydrogenase gene) for further degradation of ring-cleavage product. The order of the 2-HMS hydrolase, catechol 2,3-dioxygenase, and 2-HMS dehydrogenase genes found in the subclones differs from the gene order found in other *meta*-pathways, suggesting very dissimilar gene arrangement.

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APPENDIX

Summary of bacterial culture collection characterization

Source	Strain	Basis of isolation ^a	Indigo test ^b	<i>nah</i> Probes ^c			Nah miln ^d	PAH degradation ^e				
				A	G	H		Flu	Phe	Pyr	BaP	Ant
Soil A	A2	Phn	+	-		+	+	+	+	-	-	+
	A3	Phn	+	+	+			-	-	-	-	-
	A3PH5	Phn	+	+	+		+	+	+	-	-	-
	A4	Phn	+	+				-	-	-	-	-
	A5	Phn	+	+	+		+	+	+	-	-	-
	A5PH1	Phn	+	-	-	-	+	-	+	-	-	-
	A6	Phn	+	+				-	-	-	-	-
	A7	Phn	+	+				+	+	-	-	-
	A7AN6	Ant	+	+				+	+	-	-	-
	A8	Phn	-	-	-		-	-	-	-	-	-
	A8AN2	Ant	-	-			-	-	-	-	-	-
	A8AN3	Ant	+	-	-	-	+	+	+	+	-	+
	A13PY5	Pyr	+	+	+		+	+	+	-	-	-
	A17PY4	Pyr	-	-	-		-	-	+	-	-	-
Soil B	B1PH2	Phn	+	-	-	-	+	+	+	-	-	+
	B1PH3	Phn	+	+	+		+	+	+	-	-	-
	B1PH5	Phn	+	+				+	+	-	-	-
	B1PH6	Phn	+	+			+	+	+	+	-	+
	B2	Phn	+	+				-	+	-	-	+
	B2PH7	Phn	+	+	+		+	+	+	-	-	-
	B2PH8	Phn	+	+				+	+	-	-	+
	B3	Phn	-	-	-		-	-	-	-	-	-
	B3PH10	Phn	+	+			+	-	+	-	-	+
	B4	Phn	+	+	+		+	+	+	-	-	-
	B7AN4	Ant	-	-	-		-	-	-	-	-	-
	B10AN1	Ant	+	-	-	-	+	-	+	-	-	-
	B13-1	Nah	+	+				-	+	-	-	-
Soil C	C7	Nah	+	+				-	+	-	-	-
	C9-1	Nah	+	+				-	+	-	-	-
	C9-2	Nah	+	+				-	+	-	-	-
	C10	Nah	+	+				-	+	-	-	-
	C20	Nah	+	+				-	+	-	-	-
	C33-1	Nah	+	+				+	-	-	-	-
	C33-2	Nah	+	-			+	-	+	-	-	-
Soil D	D1-11	Nah	-	-	-		-	-	-	-	-	-
	D1-12	Nah	-	-	-		-	-	-	-	-	-
	D1-13	Nah	-	-	-		-	-	-	-	-	-
	D2-1	Nah	+	+	+		+	+	+	-	-	+
	D3-1	Nah	+	+				+	+	-	-	-
	D3-2	Nah	+	+				+	+	-	-	-
	D3-3	Nah	+	+	+			-	-	-	-	-
	D3-4	Nah	+	+				-	+	-	-	-
	D3-5	Nah	+	+	+			-	-	-	-	-
	D3-8	Nah	-	-	-		-	-	-	-	-	-
	D3-9s*	Nah	-	-	-		-	-	-	-	-	-
	D3-9b*	Nah	-	-	+		-	-	-	-	-	-
	D5-4	Nah	+	+				-	-	-	-	-

(Continued).

Source	Strain	Basis of isolation ^a	Indigo test ^b	<i>nah</i> Probes ^c			Nah miln ^d	PAH degradation ^e				
				A	G	H		Flu	Phe	Pyr	BaP	Ant
	D5-5	Nah	-	-	-		-	-	-	-	-	-
	D5-6	Nah	-	-	-		-	-	-	-	-	-
	D5-7	Nah	-	-	-		-	-	-	-	-	-
	D7-1	Nah	+	+				-	-	-	-	-
	D8-14	Nah	-	-	-		-	-	-	-	-	-
	D9AN1	Ant	+	+				-	+	-	-	-
	D12-1	Nah	-	-			-	-	-	-	-	-
	D13-1	Nah	+	+				-	+	-	-	+
	D13-2	Nah	+	+			+	+	+	-	-	-
	D13-3	Nah	+	+				-	+	-	-	-
	D13-4w*	Nah	-	-	-		-	-	-	-	-	-
	D13-4y*	Nah	-	-	-		-	-	-	-	-	-
	D14-1	Nah	+	+	+			-	-	-	-	-
	D14-2bk*	Nah	+	+				-	+	-	-	-
	D14-2b*	Nah	+	+				-	+	-	-	-
	D14-3	Nah	+	+	+		+	+	+	-	-	-
	D23PY4	Pyr	+	+				-	-	-	-	-
	D23PY5	Pyr	-	-	-		-	-	-	-	-	-
	D23PY6	Pyr	+	+				-	+	-	-	-
	D23PY7	Pyr	+	+	+		+	+	+	-	-	-
	D24PY1	Pyr	+	+				-	+	-	-	-
	D24-1	Nah	+	+				-	+	-	-	-
	D26-2w*	Nah	-	-	-		-	-	-	-	-	-
	D26-2y*	Nah	-	-	-		-	-	-	-	-	-
	D26-3	Nah	-	-	-		-	-	-	-	-	-
	D27-1	Nah	+	+				+	-	-	-	-
	D27-2	Nah	-	-	-		-	-	-	-	-	-
	D39-2	Nah	-	-	-		-	-	+	-	-	-
	D39-3	Nah	-	-	-		-	-	-	-	-	-
	D121	Phn	-	+	+			-	-	-	-	-
	D122	Phn	+	+				-	-	-	-	-
	D123	Phn	+	+				-	-	-	-	-
	D171	Phn	+	+	+			-	-	-	-	-
	D172	Phn	+	+	+			-	+	-	-	-
	D173	Phn	+	-	+			-	+	-	-	-
	D181	Phn	+	+	+			-	-	-	-	-
	D182	Phn	+	+				-	+	-	-	+
	D183	Phn	+	+				-	+	-	-	-
	D184	Phn	+	+				-	-	-	-	-
Soil E	E3-1	Nah	-	-	+		-	-	-	-	-	-
	E3-2	Ant	-	-	-		-	-	-	-	-	-
	E3-3	Nah	+	+				-	+	-	-	-
	E7-1	Nah	-	-	+			-	-	-	-	-
	E13-1	Ant	+	+				-	-	-	-	-
	E13-2	Ant	+	+				-	-	-	-	-
	E16-1	Nah	+	-				-	-	-	-	-
	E17	Phn	+	+				-	+	-	-	-
	E17-1	Ant	-	-	-		-	-	-	-	-	-

(Continued).

Source	Strain	Basis of isolation ^a	Indigo test ^b	<i>nah</i> Probes ^c			Nah miln ^d	PAH degradation ^e				
				A	G	H		Flu	Phe	Pyr	BaP	Ant
Soil G	E17-2	Ant	+	+	+		+	-	+	-	-	-
	E18-1	Nah	+	+				-	+	-	-	-
	E18-2	Nah	+	+				-	+	-	-	-
	E18-3	Nah	+	+				-	+	-	-	-
	E19-1	Pyr	+	+				-	+	-	-	-
	E19-2	Pyr	+	+	+		+	-	+	-	-	-
	E19-3	Pyr	+	+				-	+	-	-	-
	E21	Phn	+	+	+			-	+	-	-	-
	E22-1	Nah	+	+	+		+	+	+	-	-	-
	E22-2	Nah	+	+				-	+	-	-	-
	E28	Phn	+	+				-	+	-	-	-
	E29-1	Nah	+	+				-	+	-	-	-
	E29-2	Nah	+	+	+		+	+	+	-	-	-
	G1-1	Phn	+	-				-	+	-	-	-
	G1-2	Phn	+	+	+		+	+	+	-	-	+
	G1-3	Phn	+	+				+	+	-	-	-
	G1-4	Phn	+	-	+		-	+	+	-	-	-
	G2-1	Phn	+	+				-	+	-	-	-
	G2-2	Phn	+	+				+	+	-	-	-
	G3-1PH	Phn	+	+				+	-	-	-	-
	G4-1	Phn	+	-	+			-	+	-	-	-
	G5-1	Phn	+	+				-	-	-	-	-
	G8-1AN	Ant	+	+				+	-	-	-	-
	G8-2AN	Ant	+	-				-	-	-	-	-
	G8-3AN	Ant	+	-	+		-	-	-	-	-	-
	G9-1	Phn	+	-				-	+	-	-	-
	G11-1	Pyr	+	+				+	-	-	-	-
	G11-2	Pyr	+	+				-	-	-	-	-
	G11-3Pyr	Pyr	+	+	+		+	-	+	-	-	-
	G11-4	Pyr	+	+				-	-	-	-	-
	G16-1	Nah	+	+				+	-	-	-	-
	G16-3	Nah	+	+				-	+	-	-	-
	G19-1	Nah	-	-	-		-	-	-	-	-	-
Soil N	N1-1	Phn	+	+				-	+	-	-	-
	N1-2	Phn	+	+				-	+	-	-	-
	N1-3	Phn	+	+				-	+	-	-	-
	N1-4PH	Phn	+	+	+		+	+	+	+	-	+
	N1-5	Phn	+	+				-	+	-	-	+
	N2-1PH	Phn	+	+	+		+	+	+	-	-	-
	N2-2	Phn	+	+				-	+	-	-	-
	N2-3	Phn	+	+				-	+	-	-	-
	N2-4	Phn	+	+	+			-	+	-	-	-
	N13-1	Phn	-	-	-		-	-	-	-	-	-
	N13-2PH	Phn	+	+	+		+	+	+	-	-	-
	N13-3	Phn	+	+				-	+	-	-	-
	N13-4	Phn	+	+				-	+	-	-	-

(Continued).

Abbreviations: Nah, naphthalene; Flu, fluorene; Phe, phenanthrene; Ant, anthracene; Pyr, pyrene; BaP, benzo[a]pyrene.

^a Method used to isolate the strain from soil (King et al., 1991): Nah used in the naphthalene vapor plate method; Phn, Ant, and Pyr used in the PAH spray plate method.

^b Dioxygenase activity (Ensley et al., 1983).

^c Plasmid NAH 7-derived gene probes used for colony hybridization. *P. putida* PpG7 (NAH7, Nah + Sal +) and *P. putida* PB2440 (Nah - Sal -) were used as positive and negative controls, respectively.

^d Naphthalene mineralization.

^e Determined by the PAH spray plate method.

* An isolate designated D3-9 consisted of two different strains as determined by different colony morphology and color. They were renamed D3-9s and D3-9b in this study and treated as different strains. Similar cases were found for following isolates: D13-4, D13-4w and D13-4y; D14-2, D14-2bk and D14-2b; D26-2, D26-2w and D26-2y.

VITA

Yeonghee Ahn was born in Pusan, Korea on March 12, 1964. She graduated from Pusan Girls' High School in Pusan in February, 1983. She enrolled in the Pusan National University (PNU) in March, 1983 and received a Bachelor of Science degree in Microbiology in February, 1987. After undergraduate program, she started graduate study in Microbiology Department in PNU on March, 1987. In February 1989, she received a Master of Science degree in Microbiology. Working on environmental microbiology in graduate school in PNU led her decision to learn more about the area by working with world-leading scientists. She entered in the University of Tennessee, Knoxville (UTK) in the Fall of 1990. She did graduate research under guidance of Dr. Gary Saylor at Center for Environmental Biotechnology. She worked (Sep. 1993-Aug. 1994) as vice president for Korean Student Association in UTK. She received her Ph.D. in Microbiology in December 1996. She was a member of the American Society for Microbiology and the Association of Korean Scientists and Engineers.