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Review

Genetics of Polycyclic Aromatic Hydrocarbon Metabolism in Diverse Aerobic Bacteria

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Polycyclic aromatic hydrocarbons (PAHs), which consist of two or more fused aromatic rings, are widespread in the environment and persist over long periods of time. The decontamination of a PAH-polluted environment is of importance because some PAHs are toxic, mutagenic, and carcinogenic and therefore are health hazards. As part of the efforts to establish remediation processes, the use of aerobic bacteria has been extensively studied, and both enzymologic and genetic studies are underway for the purpose of effective biodegradation. In the last two decades, one highly conserved group of PAH-catabolic genes from *Pseudomonas* species, called the *nah*-like genes, has been well investigated, and much has been found, including the structure-function relationships and the evolutionary trails of the catabolic enzymes. However, recently, PAH-catabolic genes, which are evolutionarily different from the *nah*-like genes, have been characterized from both Gram-negative bacteria other than *Pseudomonas* species and Gram-positive bacteria, and the information about these genes is expanding. This review is an outline of genetic knowledge about bacterial PAH catabolism.

Key words: fluorene degradation; naphthalene degradation; phenanthrene degradation; polycyclic aromatic hydrocarbons; pyrene degradation

Polycyclic aromatic hydrocarbons (PAHs) are an ubiquitous class of hydrophobic organic compounds consisting of two or more fused benzene rings in linear, angular, or cluster arrangements. PAHs are found in the environment after the disposal of coal processing wastes, petroleum sludge, asphalt, creosote, and other wood preservative wastes, and they persist within the ecosystem for years owing to their low water solubility and their adsorption to solid particles. The decontamination of PAH-polluted sites is urgent because many PAH compounds are known or suspected to be toxic, mutagenic, or carcinogenic.[1] On the basis of their abundance and toxicity, 16 PAH compounds have been identified by the U.S. Environmental Protection Agency (EPA) as priority pollutants[2] (Fig. 1).

The biological treatment of soils contaminated with PAH should be an efficient, economic, and versatile alternative to physicochemical treatment, because it offers potential advantages such as the complete destruction of the pollutants, lower treatment cost, greater safety, and less environmental disturbance. Therefore, many researchers have been focusing on the biodegradation of PAHs. Microorganisms were found to degrade PAHs *via* either metabolism or cometabolism. Cometabolism is important for the degradation of mixtures of PAHs and high-molecular-weight PAHs. In contrast, several

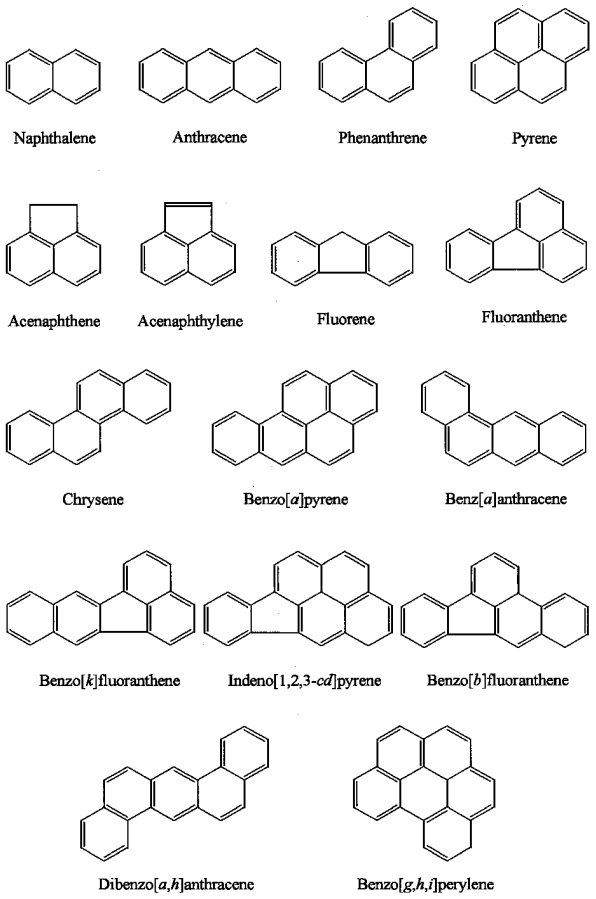

Fig. 1. Chemical Structures of 16 Priority PAH Compounds Identified by the US EPA.[2]

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Abbreviations: EPA, Environmental Protection Agency; IS, insertion sequence; ISP, iron-sulfur protein; NDO, naphthalene dioxygenase; ORF, open reading frame; PAHs, polycyclic aromatic hydrocarbons; RT, reverse transcriptase; TCA, tricarboxylic acid

PAHs, especially 2- to 4-ring PAHs, have been known for years to be growth substrates for bacteria. Although the metabolism of PAHs by a bacterial pure culture in anaerobic conditions has been reported,[3,4] most attention has been paid to metabolism of PAHs by aerobic bacteria, and various bacteria capable of utilizing PAHs have been investigated. The biochemical and biotechnological studies of this subject have been reviewed.[5–9] The common biochemical pathways for the bacterial metabolism of PAHs have been well investigated. The initial step in the aerobic metabolism of PAHs usually occurs *via* the incorporation of molecular oxygen into the aromatic nucleus by a multicomponent dioxygenase enzyme system, forming *cis*-dihydrodiol. These compounds are rearomatized through a *cis*-diol dehydrogenase to yield dihydroxylated intermediates. These dihydroxylated substrates can be cleaved by dioxygenase *via* either an *ortho*-cleavage pathway or a *meta*-cleavage pathway, finally leading to tricarboxylic acid (TCA) cycle intermediates.

Recently, several genes encoding PAH-catabolic enzymes have been characterized. Analysis of the PAH-catabolic genes is of interest in both fundamental and applied aspects. Fundamental knowledge of the PAH-catabolic genes in different species of bacteria can give useful information about the evolution of enzyme structure-function relationships and the evolution and diversity of catabolic pathway genes *via* horizontal transfer, transposition events, DNA rearrangement, gene fusion, point mutation, and so on. From such information we may suggest some molecular mechanisms by which bacteria adaptat to xenobiotic compounds. In applied studies, genetic information will be useful for the monitoring of bacterial populations that degrade PAH in contaminated soils. Most studies of bioremediation involve either stimulation of indigenous microbial populations by addition of both nutrients and electron acceptors to the environment (biostimulation) or introduction of exogenous microbial populations that degrade the pollutants (bioaugmentation). Before biostimulation of a contaminated site, the existence in the environment of bacteria that degrade PAHs should be confirmed. However, enumeration of the bacterial population in a contaminated site by traditional plate-counting methods can take an inordinate length of time, and often underestimates numbers because some bacteria can not be cultured. Therefore, a molecular approach with gene probes and the PCR is useful for the detection and characterization of degraders.[10–14] However, the development of more gene probes and primers, founded on new sequence information, is necessary. On the other hand, before bioaugmentation, the fate of augmented degraders in the new environment also should be investigated. Quantitative PCR with specific gene primers originating from different degrading bacteria[15] could help in the monitoring of degraders in a contaminated environment. It may be possible to create both robust microorganisms and effective degrading enzymes by modification of existing ones[16] until they can be used in bioremediation.

In the last two decades, genetic analyses of PAH degradation by aerobic bacteria have focused on naphthalene catabolic genes from *Pseudomonas* species. However, the analysis of a single, highly homologous group of genes may not reflect the true catabolic diversity of PAH-catabolic genes among bacteria. Knowledge about many PAH-catabolic genes from Gram-negative bacteria other than those in the genus *Pseudomonas* and also from Gram-positive bacteria is accumulating. However, until now, there have been no reviews summarizing such information on PAH-catabolic pathway genes in aerobic bacteria. Here, we outline recent genetic studies on the bacterial degradation of PAHs, including novel degradative genes of PAHs other than the well-studied compounds, naphthalene and phenanthrene.

Bacterial Metabolism of Naphthalene, Phenanthrene, and Anthracene

Naphthalene is a bicyclic aromatic hydrocarbon commonly found in the environment; it has been used often as a model compound of PAH degradation. The bacterial degradation of naphthalene is well understood. The degradation of naphthalene in soil pseudomonads was first reported by Davies and Evans.[17] Although it was suggested by Annweiler *et al.*[18] that *Bacillus thermoleovorans* strain Hamburg 2 degrades naphthalene *via* another pathway, by initial dioxygenation at the 2,3-position in addition to the known one, one primary pathway is known as the ''upper'' catabolic pathway of naphthalene (Fig. 2(A)). In the first catabolic step, an oxygen molecule is introduced at the 1,2-position of the aromatic nucleus to produce *cis*-(1*R*,2*S*)-dihydroxy-1,2-dihydroxynaphthalene (*cis*-naphthalene dihydrodiol) (Fig. 2(A), compound A-II) by naphthalene dioxygenase (NDO). This dioxygenase system consists of three components, a ferredoxin reductase, a ferredoxin, and an iron sulfur protein (ISP) composed of two nonidentical subunits, α and β. The electron transport is initiated by a single two-electron transfer from NAD(P)H to FAD in a ferredoxin reductase, which generates a fully reduced form of the FAD. The reduced FAD provides one electron each to the [2Fe-2S] cluster of a ferredoxin. These electrons are finally transferred to an ISP and used in its active site to facilitate the addition of an oxygen molecule to naphthalene (Fig. 3). The substrate specificity of NDO has been investigated in detail,[19] and the three-dimensional structure of the NDO components also was identified.[20] Parales *et al.*[21] found that Asp-205

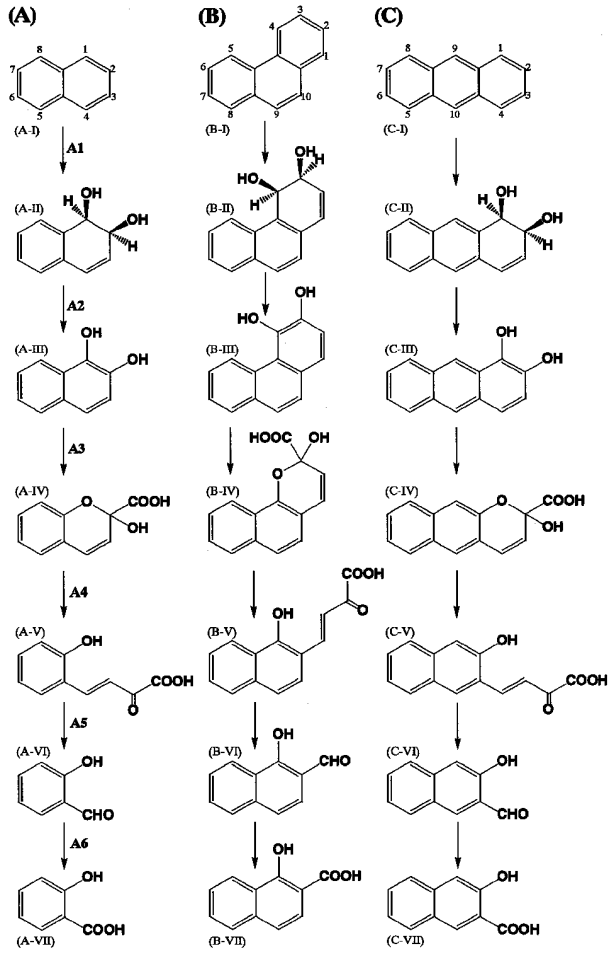

Fig. 2. Proposed Upper Catabolic Pathways of Naphthalene (A), Phenanthrene (B), and Anthracene (C) by Aerobic Bacteria.

(A): The compounds are naphthalene (A-I), *cis*-1,2-dihydroxy-1,2-dihydronaphthalene (*cis*-naphthalene dihydrodiol) (A-II), 1,2-dihydroxynaphthalene (A-III), 2-hydroxy-2*H*-chromene-2-carboxylic acid (A-IV), *trans-o*-hydroxybenzylidenepyruvic acid (A-V), salicylaldehyde (A-VI), and salicylic acid (A-VII). The enzymes involved in each reaction step are naphthalene dioxygenase (NahAaAbAcAd) (step A1), *cis*-naphthalene dihydrodiol dehydrogenase (NahB) (A2), 1,2-dihydroxynaphthalene dioxygenase (NahC) (A3), 2-hydroxy-2*H*-chromene-2-carboxylate isomerase (NahD) (A4), *trans-o*-hydroxybenzylidenepyruvate hydratase-aldolase (NahE) (A5), and salicylaldehyde dehydrogenase (NahF) (A6). (B): The compounds are phenanthrene (B-I), *cis*-3,4-dihydroxy-3,4-dihydrophenanthrene (B-II), 3,4-dihydroxyphenanthrene (B-III), 2-hydroxy-2*H*-benzo[*h*]chromene-2-carboxylic acid (B-IV), *trans*-4-(1-hydroxynaph-2-yl)-2-oxobut-3-enoic acid (B-V), 1-hydroxy-2-naphthoaldehyde (B-VI), and 1-hydroxy-2-naphthoic acid (B-VII). (C): The compounds are anthracene (C-I), *cis*-1,2-dihydroxy-1,2-dihydroanthracene (C-II), 1,2-dihydroxyanthracene (C-III), 2-hydroxy-2*H*-benzo[*f*]chromene-2-carboxylic acid (C-IV), *trans*-4-(2-hydroxynaph-3-yl)-2-oxobut-3-enoic acid (C-V), 2-hydroxy-3-naphthoaldehyde (C-VI), and 2-hydroxy-3-naphthoic acid (C-VII).

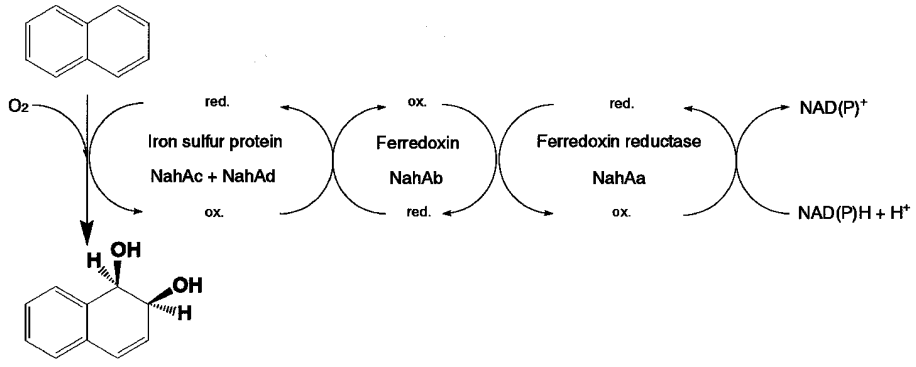

Fig. 3. Initial Oxidation of Naphthalene to *cis*-1,2-Dihydroxy-1,2-dihydronaphthalene by Naphthalene Dioxygenase.

in the catalytic domain of NDO is essential for its activity, and the crystal structure showed that an alternative NDO substrate, indole, binds near the mononuclear iron, suggesting that the residue is the oxygen-activating site.[22] When site-directed mutagenesis is done to measure the contributions of several active-site residues to catalysis, substitutions at Phe-352 result in the formation of *cis*-naphthalene dihydrodiol with altered stereochemistry and also changes the site of oxygenation of biphenyl and phenanthrene.[16] Such results provide a good structural model of how NDO binds a substrate in its oxygenase component. Recently, the chemistry of O_2 activation and substrate hydroxylation by the oxygenase component of NDO also has been reported.[23]

cis-Naphthalene dihydrodiol is then dehydrogenated to 1,2-dihydroxynaphthalene (Fig. 2(A), compound A-III) by *cis*-naphthalene dihydrodiol dehydrogenase. 1,2-Dihydroxynaphthalene is *meta*-cleaved by 1,2-dihydroxynaphthalene dioxygenase, and the resulting ring-cleavage product spontaneously recyclizes to form 2-hydroxy-2*H*-chromene-2-carboxylase (compound A-IV). Enzymatic reactions

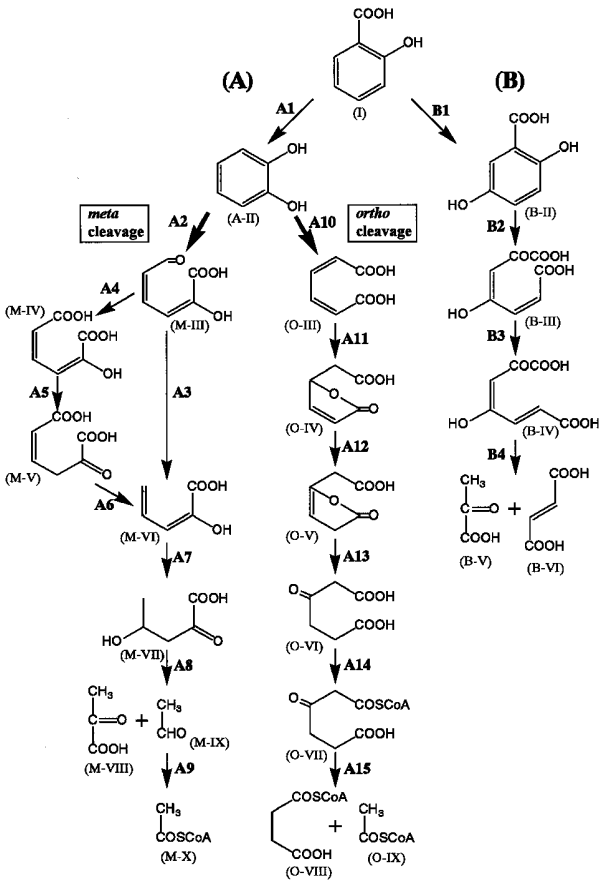

Fig. 4. Proposed Catabolic Pathways of Salicylic Acid *via* Catechol (A) and Gentisic Acid (B) as the Lower Pathways of Naphthalene Degradation.

(A): The compounds are salicylic acid (I), catechol (A-II), 2-hydroxymuconic-semialdehyde (M-III), 2-hydroxymuconic acid (M-IV), 4-oxalocrotonic acid (M-V), 2-oxo-4-pentenoic acid (M-VI), 4-hydroxy-2-oxovaleric acid (M-VII), pyruvic acid (M-VIII), acetaldehyde (M-IX), acetyl-CoA (M-X), *cis,cis*-muconic acid (O-III), muconolactone (O-IV), β-ketoadipate-enol-lactone (O-V), β-ketoadipic acid (O-VI), β-ketoadipyl-CoA (O-VII), succinyl-CoA (O-VIII), and acetyl-CoA (O-IX). The enzymes involved in each reaction step are salicylate hydroxylase (NahG) (step A1), catechol 2,3-dioxygenase (NahH) (A2), hydroxymuconic semialdehyde hydrolase (NahN) (A3), hydroxymuconic semialdehyde dehydrogenase (NahI) (A4), 4-oxalocrotonate isomerase (NahJ) (A5), 4-oxalocrotonate decarboxylase (NahK) (A6), 2-oxopent-4-enoate hydratase (NahL) (A7), 2-oxo-4-hydroxypentanoate aldolase (NahM) (A8), acetaldehyde dehydrogenase (NahO) (A9), catechol 1,2-dioxygenase (A10), *cis,cis*-muconate lactonizing enzyme (A11), muconolactone isomerase (A12), β-ketoadipate-enol-lactone hydrolase (A13), β-ketoadipate:succinyl-CoA transferase (A14), and β-ketoadipyl-CoA thiolase (A15). (B): The compounds are gentisic acid (B-II), maleylpyruvic acid (B-III), fumarylpyruvic acid (B-IV), pyruvic acid (B-V), and fumaric acid (B-VI). The enzymes involved in each reaction step are salicylate 5-hydroxylase (NagGHAaAb) (B1), gentisate 1,2-dioxygenase (NagI) (B2), maleylpyruvate isomerase (NagL) (B3), and fumarylpyruvate hydrolase (NagK) (B4).

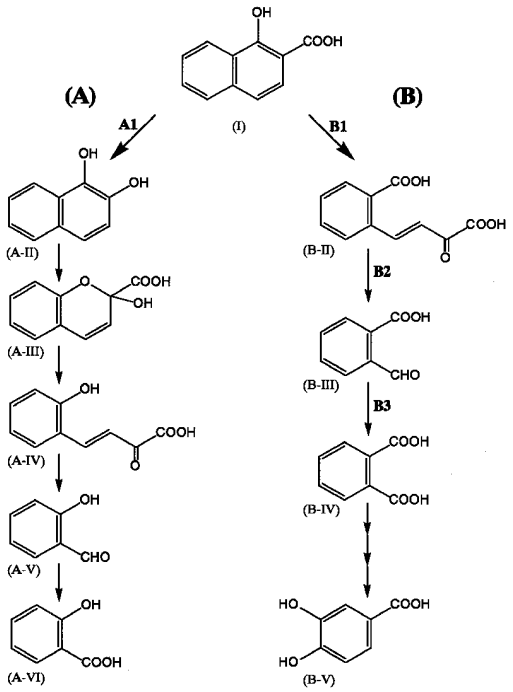

Fig. 5. Proposed Catabolic Pathways of 1-Hydroxy-2-naphthoic Acid as Degradation Pathways of Phenanthrene.

(A): The compounds are 1-hydroxy-2-naphthoic acid (I), 1,2-dihydroxynaphthalene (A-II), 2-hydroxy-2*H*-chromene-2-carboxylic acid (A-III), *trans-o*-hydroxybenzalpyruvic acid (A-IV), salicylaldehyde (A-V), and salicylic acid (A-VI). The enzyme involved in the first reaction step is 1-hydroxy-2-naphthoate hydroxylase (step A1). (B): the metabolites are *trans*-2′-carboxybenzalpyruvic acid (B-II), 2-carboxybenzaldehyde (B-III), *o*-phthalic acid (B-IV), and protocatechuic acid (B-V). The enzymes involved in each reaction step are 1-hydroxy-2-naphthoate dioxygenase (PhdI) (B1), 2′-carboxy-benzalpyruvate hydratase-aldolase (PhdJ) (B2), and 2-carboxybenzaldehyde dehydrogenase (PhdK) (B3).

by an isomerase and a hydratase-aldolase result in the production of salicylaldehyde (compound A-VI), which is then transformed to salicylate (compound A-VII) by salicylaldehyde dehydrogenase. Salicylate is further metabolized *via* catechol (Fig. 4, compound A-II) or gentisate (compound B-II) to TCA cycle intermediates.

Phenanthrene also has been used as a model compound in PAH biodegradation studies. The enzymes involved in the conversion of naphthalene to salicylate can degrade phenanthrene and anthracene to 1-hydroxy-2-naphthoate and 2-hydroxy-3-naphthoate, respectively, through similar catabolic steps[24–27] (Fig. 2(B), (C)). Although it was suggested by Pinyakong *et al.*[28] that *Sphingomonas* sp. strain P2 degrades phenanthrene *via* another pathway, by dioxygenation at the 1,2-position in addition to the known one, a pathway *via* dioxygenation at the 3,4-position is the major catabolic pathway of phenanthrene (Fig. 2(B)). In general, phenanthrene is degraded to 1-hydroxy-2-naphthoate (compound B-VII), which is further metabolized through two pathways. One catabolic pathway involves the hydroxylation of 1-hydroxy-2-naphthoate with the formation of 1,2-dihydroxynaphthalene (Fig. 5(A), compound A-II), which then enters the naphthalene degradation

Table 1. Gene Location, Growth Substrates, and Enzyme Substrate Specificity of Genetically Characterized PAH-Degrading Bacteria

Bacterial strain[a]	Gene name	Genotype	Location[b]	Growth substrates[c] Nap/Phe/Ant	Enzyme substrates[d] Nap/Phe/Ant	Reference
Pseudomonas putida G7	*nah*	*nah*(G7)	NAH7	+/n/n	+/+/+	24, 39, 44
P. putida NCIB9816-4	*nah*	*nah*(G7)	pDTG1	+/n/n	(+)/(+)/(+)	19, 39, 44
P. putida NCIB9816	*nah*	*nah*(G7)	pWW60-1	+/+/n	+/+/n	27, 39
Pseudomonas sp. C18	*dox*	*nah*(G7)	Plasmid	+/n/n	+/+/n	46
P. putida OUS82	*pah*	*nah*(G7)	Chromosome	+/+/−	+/+/+	26, 47, 48
P. aeruginosa PaK1	*pah*	*nah*(G7)	Chromosome	+/−/−	+/+/+	48
P. stutzeri AN10	*nah*	*nah*(G7)	Chromosome	+/n/n	n/n/n	49
P. putida BS202	*nah*	*nah*(G7)	NPL-1	+/n/n	n/n/n	AF010471[e]
Comamonas testosteroni GZ39	*phd*	*phd*(GZ39)	n	+/+/−	(+)/(+)/n	56, 58, 59
C. testosteroni GZ42	*nah*	*nag*(U2)	n	+/+/−	n/n/n	56, 58, 59
C. testosteroni H	*pah*	*nag*(U2)	n	+/+/n	n/(+)/n	12, 13
Ralstonia sp. U2	*nag*	*nag*(U2)	Plasmid	+/n/n	n/n/n	60, 61
Burkholderia sp. RP007	*phn*	*phn*(RP007)	n	+/+/+	(+)/(+)/n	63
Alcaligenes faecalis AFK2	*phn*	*phn*(AFK2)	Plasmid	−/+/n	n/n/n	65, AB024945
Rhodococcus sp. NCIMB12038	*nar*	*nar*(NCIMB12038)	n	+/n/n	n/n/n	83, 86, 87
Nocardioides sp. KP7	*phd*	*phd*(KP7)	Chromosome	−/+/n	(+)/(+)/(+)	93, 94, 96

[a] Genetically characterized PAH-degrading bacteria treated in this review are listed.
[b] "n" indicates "to our knowledge, it has not been reported".
[c] Nap, Phe, and Ant indicate naphthalene, phenanthrene, and anthracene, respectively; "+" and "−" indicate "growth" and "no growth", respectively.
[d] "+" indicates that the clone carrying the upper-pathway-enzyme-encoding genes can transform naphthalene to salicylic acid, phenanthrene to 1-hydroxy-2-naphthoate, or anthracene to 2-hydroxy-3-naphthoate. "(+)" indicates that at least the initial dioxygenolytic attack for each PAH compound has been observed.
[e] Accession number.

pathway. In contrast, the aromatic ring of 1-hydroxy-2-naphthoate is directly cleaved by 1-hydroxy-2-naphthoate dioxygenase in another catabolic pathway (Fig. 5(B)). Later enzymatic reactions result in the production of phthalate (compound B-IV), which is further metabolized *via* protocatechuate (compound B-V) to TCA cycle intermediates.

Through the initial dioxygenolytic attack at the 1,2-position, anthracene is converted to 1,2-dihydroxyanthracene (Fig. 2(C), compound A-III). Although it was suggested by Dean-Ross *et al.*[29] that a *Rhodococcus* strain degrades 1,2-dihydroxyanthracene *via* the *ortho*-cleavage pathway, a *meta*-cleavage pathway of 1,2-dihydroxyanthracene that later produces 2-hydroxy-3-naphthoate (compound A-VII), is known as the catabolic pathway of anthracene. 2-Hydroxy-3-naphthoate is converted to 2,3-dihydroxynaphthalene, which is further metabolized to salicylate and catechol.

Growth substrate specificities of genetically analyzed bacteria that degrade PAH and transformation capabilities of their upper-pathway enzymes towards naphthalene, phenanthrene, and anthracene are summarized in Table 1. For most bacteria listed, both naphthalene and phenanthrene can be growth substrates. However, phenanthrene-degrading bacteria using the phthalate pathway (*e.g.*, strains AFK2 and KP7) cannot grow on naphthalene as the sole carbon source.

(1) PAH-catabolic genes of Gram-negative bacteria

(i) nah-*like genes of* Pseudomonas *strains*

The metabolism of naphthalene has been well studied genetically in *Pseudomonas putida* strain G7 because a transmissible plasmid coding for naphthalene catabolism was isolated by Dunn and Gunsalus.[30] The catabolic genes are organized in three operons on the 83-kb plasmid, NAH7: one encoding the upper-pathway enzymes involved in the conversion of naphthalene to salicylate, the second encoding the lower-pathway enzymes involved in the conversion of salicylate to a TCA cycle intermediate *via meta*-ring cleavage, and the third encoding a regulatory protein (NahR).[31–33] Both upper and lower operons are regulated by a *trans*-acting positive control regulator encoded by the *nahR* gene, which is between the two operons. NahR is needed for the high-level expression of the *nah* genes and their induction by salicylate.[34–36] Moreover, a 37.5-kb region containing a set of naphthalene catabolic genes on the NAH7 plasmid is on the defective class II transposon, Tn*4655*, which lacks the gene for the *trans*-acting factor for cointegration (*tnpA*) but encodes a cointegrate resolution system with a novel site-specific recombination.[37]

The naphthalene catabolic plasmids (called NAH plasmids), such as pWW60 derivatives from *P. putida* strain NCIB 9816,[38–40] pDTG1 from *P. putida* strain NCIB 9816-4,[39,41,42] and pKA1 from *P. fluorescens* strain 5R,[24] also were analyzed, and the plasmids were found to be very similar to the NAH7 plasmid from strain G7. Later, nucleotide sequences of genes encoding the naphthalene upper-catabolic enzymes from several *Pseudomonas* strains were reported: *ndo* genes from *P. putida* strain NCIB 9816,[43] *nah* genes from *P. putida* strain G7 and

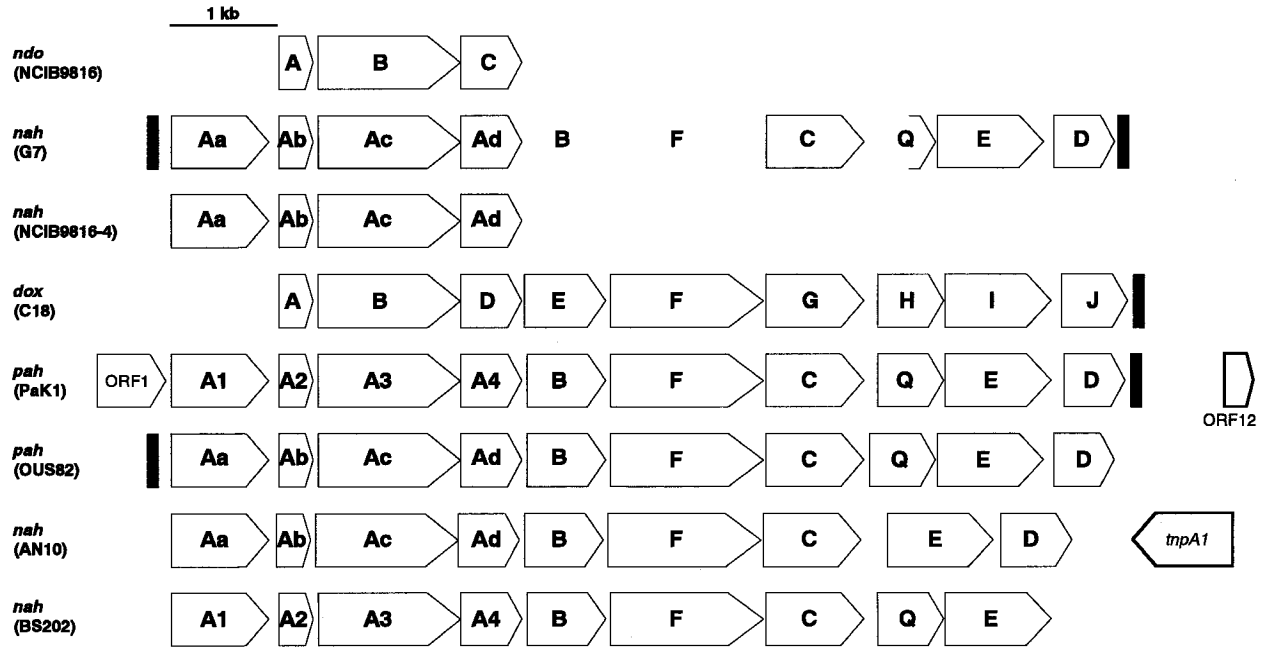

Fig. 6. Naphthalene Upper Catabolic Pathway Genes of *Pseudomonas* Strains.

The sequences from the different strains are more than 90% identical. The pentagons indicate the size, location, and direction of transcription of ORFs. The bold pentagons indicate the ORFs similar to genes encoding transposase-like enzymes. The bold lines indicate the 84-bp direct repeated sequences reported by Eaton.[45] References for the sequences are as follows: *ndo* genes from *P. putida* strain NCIB9816[43] (M23914), *nah* genes from *P. putida* strain G7[44,45] (M83949, U09057), *nah* genes from *P. putida* strain NCIB9816-4[44] (M83950, U49496), *dox* genes from *Pseudomonas* sp. strain C18[46] (M60405), *pah* genes from *P. aeruginosa* strain PaK1[48] (D84146), *pah* genes from *P. putida* strain OUS82[47,48] (AB004059), *nah* genes from *P. stutzeri* strain AN10[49] (AF039533), and *nah* genes from *P. putida* strain BS202 (AF010471). Enzyme designations are described in the text. [Note: *nahE* and *nahD* genes from strain BS202 were assigned to the *trans-o*-hydroxybenzylidenepyruvate hydratase-aldolase and 2-hydroxy-2*H*-chromene-2-carboxylate isomerase genes, respectively (AF010471). However, comparison of their size and sequences with those of other *nah*-like genes suggested that these are functionally unknown *nahQ*-like genes and *trans-o*-hydroxybenzylidenepyruvate hydratase-aldolase genes, respectively. Hence, in this figure, *nahE* and *nahD* have been changed to *nahQ* and *nahE*, respectively.]

NCIB 9816-4,[44,45] *dox* genes from *Pseudomonas* sp. strain C18,[46] *pah* genes from *P. putida* strain OUS82 and *P. aeruginosa* strain PaK1,[26,47,48] *nah* genes from *P. putida* strain BS202 [accession number AF010471], and *nah* genes from *P. stutzeri* strain AN10.[49] Each gene name was chosen according to the strain's feature of substrate use: *nah* for naphthalene degradation, *ndo* for naphthalene dioxygenation (equivalent to *nah*), *dox* for dibenzothiophene oxidation, and *pah* for polycyclic aromatic hydrocarbon (phenanthrene) degradation (later, all of these strains were found to grow on naphthalene). Among these genes, the upper-pathway gene sequences were completely designated for strains OUS82, PaK1, and AN10, but only partial sequences were analyzed in other strains. The gene organization (Fig. 6) and sequence similarity (about 90%) among the upper catabolic pathway genes of these strains were similar to those of the *nah* genes from the NAH7 plasmid of strain G7. These genes are usually called "classical *nah*-like genes". As shown in Fig. 6, in the upper catabolic pathway, the genes coding for NDO ferredoxin reductase (*nahAa*), NDO ferredoxin (*nahAb*), the α subunit of NDO (*nahAc*), the β subunit of NDO (*nahAd*), naphthalene *cis*-dihydrodiol dehydrogenase (*nahB*), salicylaldehyde dehydrogenase (*nahF*), 1,2-dihydroxynaphthalene dioxygenase (*nahC*), an unknown ORF (*nahQ*), *trans-o*-hydroxybenzylidenepyruvate hydratase-aldolase (*nahE*), and 2-hydroxychromene-2-carboxylate isomerase (*nahD*), are arranged in this order (*nahAaAbAcAdBFCQED*), with the exception of a *nahQ*-like gene deletion in strain AN10.

For the lower pathway genes, the complete sequence was determined in only strain AN10,[50,51] and partial sequences were analyzed in strains G7[52–55] and NCIB9816.[40] In the lower-catabolic pathway of strain AN10, the genes encoding salicylate hydroxylase (*nahG*), chloroplast ferredoxin-like protein (*nahT*), catechol 2,3-dioxygenase (*nahH*), hydroxymuconic semialdehyde dehydrogenase (*nahI*), hydroxymuconic semialdehyde hydrolase (*nahN*), 2-oxopent-4-enoate hydratase (*nahL*), acetaldehyde dehydrogenase (*nahO*), 2-oxo-4-hydroxypentanoate aldolase (*nahM*), 4-oxalocrotonate decarboxylase (*nahK*), and 4-oxalocrotonate isomerase (*nahJ*) are arranged in this order (*nahGTHINLOMKJ*).[51] Besides *nahG*, another salicylate hydroxylase gene (*nahW*), which is outside the lower-pathway operon but close to the operon, was identified in strain

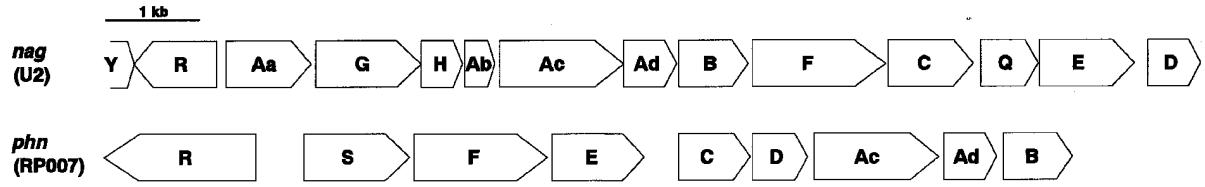

Fig. 7. Gene Organization of the Upper Pathways for Naphthalene Degradation in *Ralstonia* sp. Strain U2 and for Phenanthrene Degradation in *Burkholderia* sp. Strain RP007.

The pentagons indicate the size, location, and direction of transcription of ORFs. References for the sequences are as follows: *nag* genes from *Ralstonia* sp. strain U2[60] (AF036940), and *phn* genes from *Burkholderia* sp. strain RP007[63] (AF061751). Enzyme designations are described in the text.

AN10.[50] Downstream of the *nahJ* of strain G7, two additional genes coding for an unknown function (*nahX*) and chemotaxis transducer protein (*nahY*) were found.[55]

(ii) phd *genes of* Comamonas teststeroni *strain GZ39*

Comamonas teststeroni strains GZ38A, GZ39, and GZ42, all capable of utilizing phenanthrene as its sole carbon source, were isolated; these strains did not contain any genes very simmilar to the classical *nah*-like genes from *P. putida* strain NCIB 9816-4.[56] Genes responsible for the initial conversion of naphtharene and phenanthrene from strain GZ39 (*phd* genes) were cloned on the basis of the formation of both indigo on L agar plates[57] and clear zones on phenanthrene-sprayed plates, and hybridization analyses were done with genomic DNAs from strains GZ38A and GZ42. The results indicated that the genes for phenanthrene degradation in strain GZ38A are similar (but not identical) to those from strain GZ39, but that strain GZ42 did not have any genes similar to the *phd* genes from strain GZ39 (see next section). The three *C. testosteroni* strains thus are in at least two new classes of genes involved in PAH degradation.[56,58,59] In strain GZ39, the genes coding for ferredoxin (*phdAb*), ferredoxin reductase (*phdAa*), *cis*-dihydrodiol dehydrogenase (*phdB*), the α subunit of ISP (*phdAc*), the β subunit of ISP (*phdAd*), isomerase (*phdD*), an unknown ORF, glutathione-*S*-transferase, and hydratase-aldolase (*phdE*) were arranged in this order. This gene order was quite different from that of the *nah*-like genes, and several genes such as extradiol dioxygenase (*nahC* analogue) were not within the cluster.[58,59] Comparison of the *phd* genes with known genes indicated that the PhdAc sequence falls into the family of naphthalene dioxygenases (although very distantly related) but that PhdAd and PhdAb sequences have little similarity to isofunctional proteins of other aromatic-ring dioxygenases.[58] However, a more detailed comparison is not possible (*e.g.*, elucidation of similarity with other genes) because the sequence of the *phd* gene from strain GZ39 is not available on any databases.

(iii) nag *genes of* Ralstonia *sp. strain U2*

The genes for naphthalene and phenanthrene degradation from *C. testosteroni* strain GZ42 (see above) (*nah* gene), which were not the same but were similar to the classical *nah*-like gene of *Pseudomonas* strains, were cloned and sequenced by Zylstra *et al.*[58,59] The *nah* operon of strain GZ42 contained all of the genes corresponding genes to the classical *nah* operon of strain NCIB9816 in the same order, but two more genes (*nahAc2* and *nahAd2*) were found between the genes encoding ferredoxin reductase (*nahAa*) and ferredoxin (*nahAb*) of strain GZ42 operon. Sequences of *nahAc2* and *nahAd2* genes were similar to those of the α and β subunits, respectively, of ISP of other aromatic-ring dioxygenases. But these genes have been thought to be nonfunctional in strain GZ42.[58,59] Later, a naphthalene catabolic operon with the same gene organization and very similar to the *nah* operon of strain GZ42 was found in *Ralstonia* (formerly *Pseudomonas*) sp. strain U2, and it has been analyzed in detail.[60,61]

The naphthalene-utilizing bacterium *Ralstonia* sp. strain U2 was isolated from oil-contaminated soil in Venezuela.[60] By screening for *E. coli* transformants that formed indigo,[57] the naphthalene dioxygenase genes (*nag* gene) were cloned and characterized. The genes encoding ferredoxin reductase (*nagAa*), ferredoxin (*nagAb*), the α subunit of ISP (*nagAc*), the β subunit of ISP (*nagAd*), *cis*-dihydrodiol dehydrogenase (*nagB*), and aldehyde dehydrogenase (*nagF*) were arranged in this order, and two ORFs (designated *nagG* and *nagH*) that were very similar to *nahAc2* and *nahAd2* of strain GZ42 were inserted between *nagAa* and *nagAb* (Fig. 7). The *nagG* product was identical to the α subunit of other aromatic-ring dioxygenases, but the *nagH* product had limited similarity to the β subunit of other aromatic-ring dioxygenases, and recombinant *E. coli* cells carrying *nagGHAb* genes were able to convert salicylate to gentisate with the complementation of ferredoxin reductase from host cells.[60] NagG and NagH were structural subunits of salicylate 5-hydroxylase linked to electron transport proteins consisting of NagAb and NagAa.[62] Recently, Zhou *et al.*[61] reported the whole gene organization of the

nag operon. The genes for conversion of naphthalene to gentisate (*nagAaGHAbAcAdBFCQED*) in strain U2 were similar to and in the same order as the genes in the classical *nah*-like operon of *Pseudomonas* strains, with the exception of the *nagGH* insertion (Fig. 7). A further difference between *nag* and *nah* (NAH7 plasmid) operons is the location of the regulatory gene (*nagR*) and the putative chemotaxis gene (*nagY*). In strain U2, both *nagY* and *nagR* genes were upstream from *nagAa*, but in the *nah* operon, *nahR* and *nahY* genes were downstream from the upper-catabolic pathway.[55]

Downstream from the *nagD* gene encoding isomerase, a gene cluster (*nagJIKLMN*) was found; the genes were probably cotranscribed with *nagAaGHAbAcAdBFCQED* as a single operon. The *nagI*, *nagK*, and *nagL* genes encoded the enzymes involved in the further catabolism of gentisate to fumarate-pyruvate by biochemical assays (gentisate 1,2-dioxygenase, fumarylpyruvate hydrolase, and maleylpyruvate isomerase, respectively)[61] (Fig. 4(B)). The naphthalene and phenanthrene-degrader *C. testosteroni* strain H had the partial catabolic genes (*pahHAbAcAdBF*) highly similar (more than 95%) to *nagHAbAcAdBF*.[13]

(iv) phn *genes of* Burkholderia *sp. strain RP007*

Characterization of the novel *phn* operon of *Burkholderia* sp. strain RP007 expanded our knowledge of the genetics of bacterial PAH catabolism.[63,64] Strain RP007 was isolated from a PAH-contaminated site in New Zealand on the basis of its ability to degrade phenanthrene as a sole carbon and energy source. This strain also utilizes low-molecular-weight PAHs like naphthalene and anthracene as sole carbon sources. The naphthalene and phenanthrene are degraded through a common upper pathway *via* salicylate and 1-hydroxy-2-naphthoic acid, respectively. The *phn* locus on an 11.5-kb *Hin*dIII fragment, which contained nine ORFs, was cloned on the basis of the ability to form indigo from indole,[57] and it was found that the *phn* genes [encoding regulatory protein (*phnR*), regulatory protein (*phnS*), aldehyde dehydrogenase (*phnF*), hydratase-aldolase (*phnE*), extradiol dioxygenase (*phnC*), isomerase (*phnD*), ISP α subunit of initial dioxygenase (*phnAc*), ISP β subunit of initial dioxygenase (*phnAd*), dihydrodiol dehydrogenase (*phnB*), respectively, in this order] were different in sequence similarity and gene organization from previously characterized PAH-catabolic genes (Fig. 7). For example, the locus contains ISP α and β subunits of PAH-initial dioxygenase (*phnAcAd*) but lacks both the ferredoxin and reductase components. In addition, the *phnB* gene encoding *cis*-diol dehydrogenase is more closely related to the corresponding genes from biphenyl catabolic pathways than to those found in the classical *nahB*-like genes. Furthermore, *phnC*, encoding the PAH extradiol dioxygenase, had a phylogeny not seen before among extradiol dioxygenases from any PAH or biphenyl catabolic pathways. Besides this novel extradiol dioxygenase, two catechol 2,3-dioxygenase genes, which are predicted to be involved in lower pathways for aromatic degradation, also have been cloned and characterized.[64] The involvement of the *phn* genes in PAH degradation of strain RP007 has been demonstrated by either the transcriptional analysis of the genes with reverse transcriptase-PCR (RT-PCR) when the strain was grown on both naphthalene and phenanthrene, or the biotransformation experiment of both naphthalene and phenanthrene with recombinant *E. coli* cells carrying the *phn* genes.

Upstream of the *phn* catabolic genes, there are two putative regulatory genes, *phnR* and *phnS*. Sequence similarity suggested that PhnS is a LysR-type transcriptional activator and that PhnR is a member of the σ^{54}-dependent family of positive transcriptional regulators.[63] Although the *phn* gene cluster was suggested by RT-PCR experiments to be under regulatory control, which may involve PhnR and PhnS, the detailed regulatory mechanism of the *phn* genes has not been described.

(v) phn *genes of* Alcaligenes faecalis *strain AFK2*

Strain AFK2 can utilize phenanthrene as the sole carbon source through the *o*-phthalate pathway, but not naphthalene.[65] Information on the *phn* genes from strain AFK2 is available only on databases (No. AB024945). The *phn* genes have a novel gene structure, and the genes coding for ferredoxin (*phnAb*), ferredoxin reductase (*phnAa*), *cis*-dihydrodiol dehydrogenase (*phnB*), the α subunit of NDO (*phnAc*), the β subunit of NDO (*phnAd*), putative 2-hydroxychromene-2-carboxylate isomerase (*phnD*), glutathione-*S*-transferase (*gst*), *trans*-2-carboxybenzalpyruvate hydratase-aldolase (*phnH*), 1-hydroxy-2-naphthoate dioxygenase (*phnG*), 2-carboxybenzaldehyde dehydrogenase (*phnI*), 3,4-dihydroxyphenanthrene dioxygenase (*phnC*) (2.2 kb apart from the *phnI* gene), 1-hydroxy-2-naphthoaldehyde dehydrogenase (*phnF*), and putative *trans*-*o*-hydroxybenzylidenepyruvate hydratase-aldolase (*phnE*) (6.7 kb apart from the *phnF* gene) are arranged in this order. However, details have not been reported.

(vi) PAH-catabolic genes of Sphingomonas *and its related species*

Unlike other Gram-negative bacterial strains, the members of the genus *Sphingomonas* and related species can utilize a wide variety of aromatic compounds, including PAHs, as the carbon and energy sources. For example, *Novosphingobium aromaticivorans* (formerly *Sphingomonas aromaticivorans*) strain F199 can grow on toluene, all

isomers of xylene, *p*-cresol, biphenyl, naphthalene, dibenzothiophene, fluorene, salicylate, and benzoate,[66,67] and similarly, *S. yanoikuyae* strain B1 can grow on 1,2,4-trimethylbenzene, toluene, *p*-ethyltoluene, *m*- and *p*-xylene, biphenyl, naphthalene, phenanthrene, and anthracene.[68,69] *S. paucimobilis* strain EPA505 utilizes fluoranthene, naphthalene, and phenanthrene as the sole carbon sources for energy and growth.[70] Elucidation of the genetics of PAH-degrading sphingomonads is under way to learn how these bacteria can utilize such a variety of aromatic compounds.

Recently, the complete sequence of a 184-kb catabolic plasmid, pNL1, from strain F199 was identified.[71] At least 13 gene clusters were predicted to encode enzymes associated with degradation of aromatic compounds in strain F199 and were complexly arranged in pNL1. Seven sets of oxygenase components seemed to interact with the only set of ferredoxin and reductase components in pNL1. Interestingly, several parts of the DNA sequence in pNL1 regions encoding aromatic catabolic genes were similar to those in *S. yanoikuyae* strain B1,[69,72,73] *S. paucimobilis* strain EPA505,[74] *S. paucimobilis* strain Q1,[75] *Sphingomonas* sp. strain HV3,[76] *Sphingomonas* sp. strain DJ77,[77–79] *S. paucimobilis* strain TNE12,[80] *Sphingobium* sp. strain P2 (Pinyakong, O., Habe, H., Yoshida, T., Nojiri, H., Omori, T., in preparation). These results suggest that the unusual arrangement of various genes from different catabolic pathways may be typical of *Sphingomonas* species. Although several functional analyses of aromatic degradation gene expression in sphingomonads have been reported,[73,81] the function of each initial dioxygenase remains unclear. Although its enzymatic activity has not been confirmed, BphA1f has been predicted to be the α subunit of naphthalene initial dioxygenase in strain F199.[81]

(2) PAH-catabolic genes of Gram-positive bacteria

(i) nar *genes of* Rhodococcus *sp. strain NCIMB12038*

The genus *Rhodococcus* is a diverse Group of Gram-positive soil bacteria that degrade many xenobiotic compounds. Although *Rhodococcus* species utilize naphthalene as their sole carbon and energy source,[82–85] to the best of our knowledge, the PAH catabolic genes from these have not been characterized until recently. Larkin *et al.*[86] reported the purification and characteristics of a novel ISP of naphthalene dioxygenase from *Rhodococcus* sp. strain NCIMB12038 and the nucleotide sequences of the *narAa* and *narAb* genes encoding the α and β subunits of ISP, respectively. Similarity analysis showed that the products of the *narAa* and *narAb* genes are 31–39% identical to the α and β subunits of a number of aromatic-ring dioxygenases. Despite the low overall similarity of α subunits between NarAa and NahAc from *P. putida* strain 9816-4, they have conserved key catalytic residues such as a Rieske [2Fe-2S] center.[86] The β subunits of these naphthalene dioxygenases had slightly less sequence similarity than the corresponding α subunits. Kulakov *et al.*[87] reported the cloning and sequencing of the gene, *narB*, encoding *cis*-naphthalene dihydrodiol dehydrogenase; the gene was found 290 bp downstream of the *narAb*. The *narB* gene has 39% amino acid identity with NahB from *P. putida* strain G7. The order of naphthalene catabolic genes in strain NCIMB12038, *narAaAbB*, was the same as the *nah* genes of *Pseudomonas* species.

(ii) phd *genes of* Nocardioides *sp. strain KP7*

The *phd* genes *of Nocardioides* sp. strain KP7 are the most studied PAH-catabolic genes in Gram-positive bacteria, and belong to a new class of PAH-catabolic genes because of differences in gene organization and sequence similarity. Strain KP7 was isolated on the basis of its ability to grow on phenanthrene at 40°C from marine samples, and it degrades phenanthrene *via* the phthalate pathway as shown in Fig. 5(B).[88] Biochemical and molecular analysis of phenanthrene degradation *via* the phthalate pathway made possible characterization of three purified enzymes catalyzing the three conversion steps of 1-hydroxy-2-naphthoate to phthalate; the nucleotides of the enzymes, 1-hydroxy-2-naphthoate dioxygenase (*phdI*), *trans*-2′-carboxybenzalpyruvate hydratase-aldolase (*phdJ*), and 2-carboxybenzaldehyde dehydrogenase (*phdK*) have been sequenced (Fig. 5(B)).[89–92] There have been no other reports describing the catabolic genes for the transformation of 1-hydroxy-2-naphthoate to phthalate other than *phdIJK* gene cluster of this strain. Saito *et al.*[93,94] reported the nucleotide sequences of the gene cluster *phdEFABGHCD* encoding enzymes responsible for the transformation of phenanthrene to 1-hydroxy-2-naphthoate; the cluster was 6.1 kb downstream of the *phdIJK* gene cluster (Fig. 8). The *phdA* and *phdB* genes, which encode the α and β subunits of ISP of phenanthrene dioxygenase, had less than 60% sequence identity to the α and β subunits of other aromatic-ring dioxygenases. The ferredoxin and ferredoxin reductase components of NarA from *Rhodococcus* sp. strain NCIMB12038[86] and NidA from *Mycobacterium* sp. strain PYR-1[95] (see next) have not been characterized, but the genes encoding those components in strain KP7 (*phdC* and *phdD*) were found 2.9 kb downstream of the *phdB* gene. Interestingly, PhdC had much similarity to the [3Fe-4S] or [4Fe-4S] type of ferredoxin, but not to the [2Fe-2S] type of ferredoxin found in most ferredoxin components of PAH-dioxygenases. In contrast, PhdD has moderate sequence identity (less than 40%) to the ferredoxin reductase of other isofunctional enzymes.

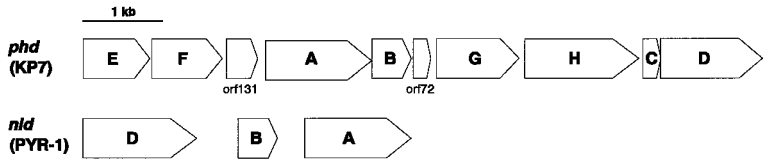

Fig. 8. Gene Organization of the Upper Pathways for Phenanthrene Degradation in *Nocardioides* sp. Strain KP7 and for Pyrene Degradation in *Mycobacterium* sp. Strain PYR-1.

The pentagons indicate the size, location, and direction of transcription of ORFs. References for the sequences are as follows: *phd* genes from *Nocardioides* sp. strain KP7[93,94] (AB017794, AB017795, and AB031319), and *nid* genes from *Mycobacterium* sp. strain PYR-1[95] (AF249300, AF249301, and AF249302). Enzyme designations are described in the text.

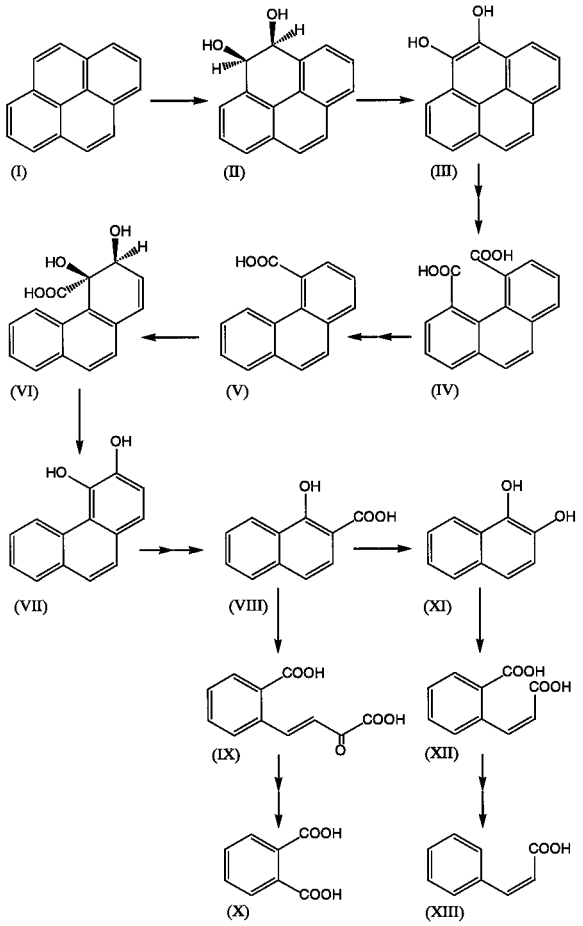

Fig. 9. Proposed Catabolic Pathway of Pyrene by Aerobic Bacteria.

The compounds are pyrene (I), *cis*-4,5-dihydroxy-4,5-dihydropyrene (II), 4,5-dihydroxypyrene (III), 4,5-phenanthrenedioic acid (IV), 4-phenanthroic acid (V), *cis*-3,4-phenanthrenedihydrodiol-4-carboxylic acid (VI), 3,4-dihydroxyphenanthrene (VII), 1-hydroxy-2-naphthoic acid (VIII), *trans*-2′-carboxy-benzalpyruvic acid (IX), *o*-phthalic acid (X), 1,2-dihydroxynaphtharene (XI), 2-carboxycinnamic acid (XII), and cinnamic acid (XIII).

All three components (PhdABCD) are necessary for the efficient dioxygenase activity that converts phenanthrene to its *cis*-diol compound.[94] The substrate specificity of PhdABCD was investigated with *E. coli* harboring these genes and with recombinant *Streptomyces lividans* expressing the *phdABCD* genes.[96,97]

PhdE, PhdF, PhdG, and PhdH had much similarity to dihydrodiol dehydrogenase, extradiol dioxygenase, hydratase-aldolase, and aldehyde dehydrogenase, respectively. It is worth noticing that the *phd* gene cluster did not contain the ORF encoding isomerase, found in all PAH-catabolic genes from Gram-negative bacteria described above.[93] Specific activities of these enzymes for phenanthrene catabolism have not been investigated.

Bacterial Metabolism of Pyrene

Bacterial degradation of PAHs consisting of more than three rings also is important. Pyrene has often been used as a model compound of high-molecular-weight PAH degradation. Heitkamp *et al.*[98] described for the first time a bacterial isolate that mineralized pyrene, and now, many pyrene-degrading bacteria have been reported. Although both *Mycobacterium* sp. strain PYR-1[99] and *Rhodococcus* sp. strain UW1[100] can degrade pyrene *via* initial dioxygenation at the 1,2-position, one primary pathway, shown in Fig. 9, is the major catabolic pathway of pyrene. Initial oxidative attack by strain PYR-1 of pyrene produces both *cis*- and *trans*-4,5-pyrenedihydrodiols.[99] Rearomatization of the dihydrodiols and subsequent *ortho*-cleavage leads to the formation of 4,5-phenanthrene dicarboxylic acid (Fig. 9, compound IV), which is further metabolized to 4-phenanthroic acid (compound V). The subsequent intermediate, *cis*-3,4-phenanthrenedihydrodiol-4-carboxylic acid, (compound VI), is formed by a second dioxygenase reaction. Rearomatization of the metabolite yields 3,4-dihydroxyphenanthrene, which is also an intermediate in bacterial phenanthrene degradation (compound VII), and further metabolism proceeds *via* catabolic pathways similar to those of phenanthrene.

nid *genes of* Mycobacterium *sp. strain PYR-1*

Recently, the genes encoding a novel polycyclic aromatic-ring dioxygenase were cloned and sequenced from *Mycobacterium* sp. strain PYR-1.[95] Strain PYR-1 can mineralize pyrene, 1-nitropyrene, fluoranthene, phenanthrene, anthrathene, and

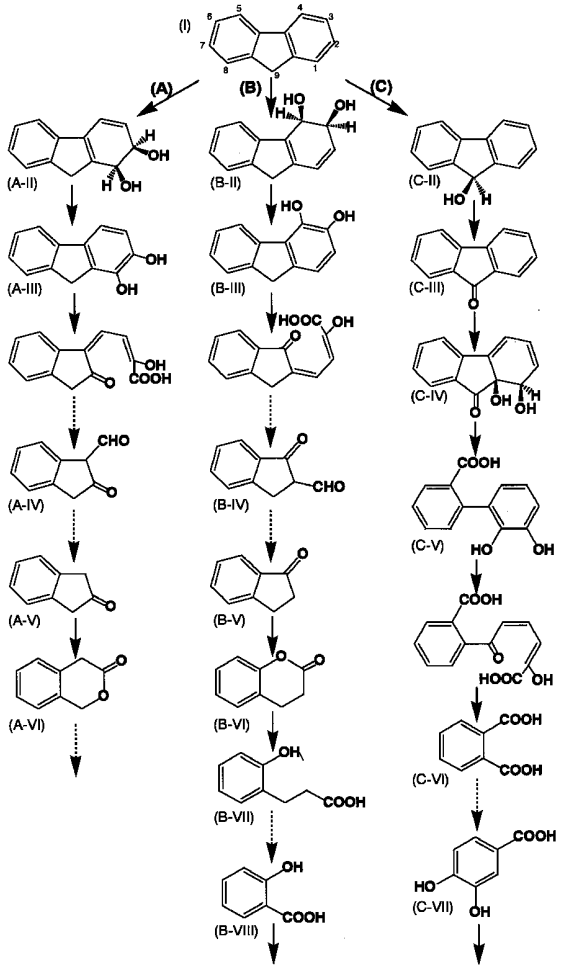

Fig. 10. Proposed Catabolic Pathways of Fluorene by Aerobic Bacteria.

(A): The compounds are fluorene (I), *cis*-1,2-dihydroxy-1,2-dihydrofluorene (A-II), 1,2-dihydroxyfluorene (A-III), 1-formyl-2-indanone (A-IV), 2-indanone (A-V), and 3-isochromanone (A-VI). (B): The compounds are *cis*-3,4-dihydroxy-3,4-dihydrofluorene (B-II), 3,4-dihydroxyfluorene (B-III), 2-formyl-1-indanone (B-IV), 1-indanone (B-V), 3,4-dihydrocoumarin (B-VI), 3-(2-hydroxyphenyl) propionic acid (B-VII), and salicylic acid (B-VIII). (C): The compounds are 9-fluorenol (C-II), 9-fluorenone (C-III), 1,1a-dihydroxy-1-hydro-9-fluorenone (C-IV), 2′-carboxy-2,3-dihydroxybiphenyl (C-V), phthalic acid (C-VI), and protocatechuic acid (C-VII).

benzo(*a*)pyrene[98,99,101–104] and has either a mono- or dioxygenase system to catalyze the initial attack on PAHs. The proteins involved in PAH degradation have been examined by the proteomic approach. At least six major proteins were induced when strain PYR-1 was grown in the presence of pyrene, phenanthrene, or dibenzothiophene.[95] In addition to the expression of an 81 kDa polypeptide encoding catalase-peroxidase (KatG) in pyrene-induced PYR-1 cell cultures,[105] a 50 kDa polypeptide the N-terminal sequence of which was similar to those of other dioxygenases was expressed. As a result of cloning and sequencing of a dioxygenase-positive clone with the probe designed from this protein sequence, the genes encoding dehydrogenase (*nidD*), the β subunit of dioxygenase (*nidB*), and the α subunit of dioxygenase (*nidA*), from the 5′- to the 3′-direction, were obtained (Fig. 8). This gene arrangement of the β and α subunits of PAH-dioxygenase (*nidBA*) has rarely been found in other bacterial PAH-dioxygenase systems with the exception of *bphA2cA1c* from *Novosphingobium aromaticivorans* strain F199.[71] NidA and NidB proteins were 40–56% similar to the isofunctional enzymes, PhdA and PhdB of strain KP7 and NarAa and NarAb of NCIMB12038; it had less identity (less than 40%) with other known large subunits. In contrast, NidD was 36–37% similar to putative aldehyde dehydrogenases of *Streptomyces* species. Functional analysis of this novel dioxygenase with clones containing the *nidDBA* genes showed that NidBA catalyzed pyrene biotransformation to pyrene *cis*-4,5-dihydrodiol.[95] However, the substrate specificity of the dioxygenase for other PAHs remains to be clarified.

Bacterial Metabolism of Fluorene

Fluorene is one of the 16 priority pollutants identified by the U.S. EPA.[2] Fluorene has been known for many years to be a growth substrate for several bacterial strains,[106–114] and three major degradative pathways have been proposed (Fig. 10). Two of these pathways are started by dioxygenation at the 1,2-(Fig. 10(A)) or 3,4-position (Fig. 10(B)), respectively. The corresponding *cis*-dihydrodiols undergo dehydrogenation and then *meta*-cleavage. After the aldolase reaction and decarboxylation of the ring-fission product, the resulting indanones are substrates for a biological Baeyer-Villiger reaction, yielding the aromatic lactones 3-isochromanone and 3,4-dihydrocoumarin (compounds A-VI and B-VI), respectively. Enzymatic hydrolysis of 3,4-dihydrocoumarin resulted in the production of 3-(2-hydroxyphenyl)propionic acid (compound B-VII), which is further metabolized to salicylic acid.

The third route (Fig. 10(C)) is initiated by monooxygenation at the C-9 position to give 9-fluorenol, which is then dehydrogenated to 9-fluorenone. Then angular dioxygenation of 9-fluorenone leads to 1,1a-dihydroxy-1-hydro-9-fluorenone (compound C-IV). Cleavage of the five-membered ring produces a 2′-carboxy derivative of 2,3-dihydroxybiphenyl, which is catabolized by reactions analogous to those of biphenyl degradation, leading to the formation of phthalate. Phthalate is then metabolized *via* protocatechuate.

dbf/fln *genes of* Terrabacter *sp. strain DBF63*

Although many bacteria utilizing fluorene have been isolated and characterized, little is known about the specific enzymes involved in the catabolism of fluorene and, especially, the genes encoding these en-

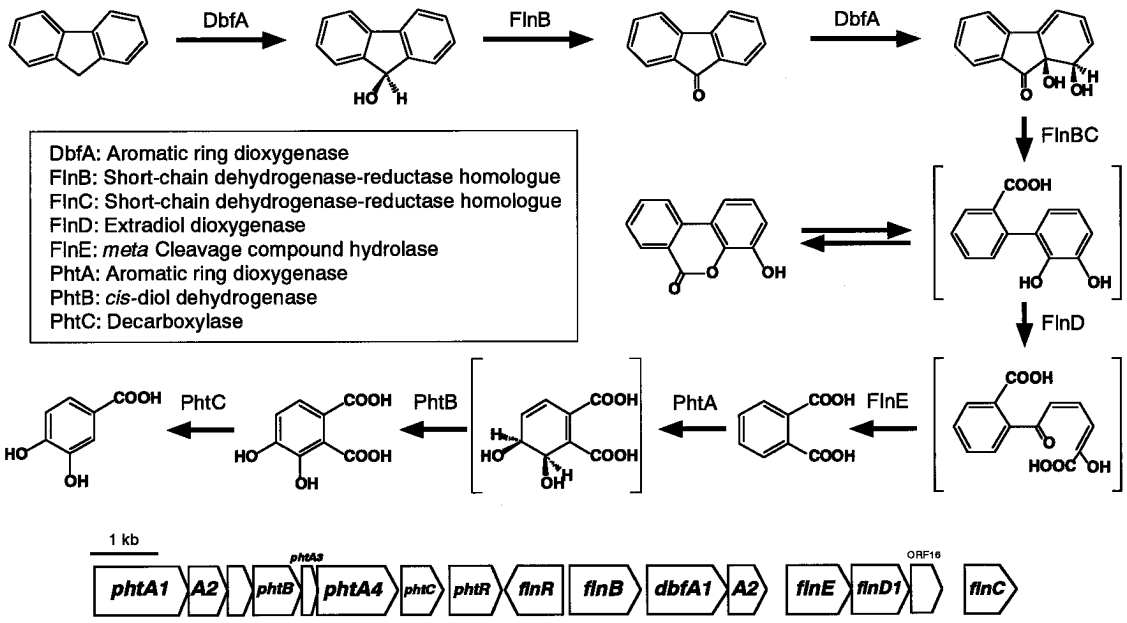

Fig. 11. Proposed Degradation Pathway of Fluorene and Gene Organization of Both *dbf*/*fln* Genes (Habe, H., Kato, H., Chung, J.-S., Kasuga, K., Yoshida, T., Nojiri, H., Omori, T., in preparation) and *pht* Genes[116] in *Terrabacter* sp. Strain DBF63.
[Note: A gene encoding the ferredoxin reductase component of DbfA (*dbfA4*) has not been identified in strain DBF63.]

zymes. Recently, a 16.5-kb DNA fragment containing genes needed for the lower pathway of fluorene degradation was isolated from *Sphingomonas* sp. strain LB126, and sequence analysis showed that protocatechuate catabolic pathway genes were in this region.[114] No information is available on the genes involved in the upper-pathway of fluorene degradation.

Terrabacter sp. (formerly *Staphylococcus auriculans*) strain DBF63 was originally isolated on the basis of its ability to utilize dibenzofuran and fluorene as the sole source of carbon and energy.[110] Recently, novel terminal oxygenase genes of angular dioxygenase, *dbfA1* and *dbfA2*, the products of which can catalyze the angular dioxygenation of dibenzofuran with the complementation of nonspecific ferredoxin and reductase components of *E. coli*, were isolated from strain DBF63 by a PCR-based strategy.[115] In further sequence analysis of the upstream region of the *dbfA1* and *dbfA2* genes, we found a gene cluster encoding enzymes for the degradation of phthalate to protocatechuate (*phtA1A2BA3A4CR*).[116] Very recently, we found a novel fluorene degradation gene cluster by the cloning, sequencing, and functional expression in *E. coli* of genes in the flanking region of the *dbfA1A2* genes. A ten-step catabolic pathway *via* angular dioxygenation and the genes encoding enzymes of each step, composed of *dbfA*, *flnB*, *flnC*, *flnD*, and *flnE*, have been proposed (Fig. 11) (Habe, H., Kato, H., Chung, J.-S., Kasuga, K., Yoshida, T., Nojiri, H., Omori, T., in preparation).

To our knowledge, this is the first genetic analysis of the upper catabolic pathway genes of bacterial fluorene degradation. For better understanding of the pathway and mechanism of fluorene catabolism by strain DBF63, biochemical studies of the purified enzymes in each catabolic step are necessary.

Evolutionary Implications Among PAH-catabolic Genes

(1) Evidence for horizontal transfer of the classical nah*-like genes*

Classical *nah*-like genes have almost identical organization or nucleotide sequence (Fig. 6). Considering that these genes are often on plasmids, called NAH plasmids, which are in incompatibility group P7 or P9, are quite large, and are self-transmissible,[39] NAH plasmids must contribute to the wide distribution of this catabolic gene cluster among naphthalene-degraders belonging to the genus *Pseudomonas*.[117] However, the size of the NAH plasmid differed with the strain,[39] and transfer of a plasmid from its bacterial host can sometimes result in its structural change.[117] These mobile plasmids may have undergone some insertions or deletions after transfer among many bacterial host strains.

In contrast, several *nah*-like genes are on chromosomes; several possible mechanisms might explain the occurrence of such a phenomenon. First, the *nah* genes of NAH7 plasmid from *P. putida* strain G7 have been found on a defective class II transposon, Tn*4655*,[37] and therefore, intracellular rearrangements of catabolic genes (not only from plasmid to chromosome, but from chromosome to plasmid, or

from plasmid to plasmid) may have occurred. Second, Ravatn *et al.*[118] reported that the C-terminal 220 amino acid residues of the integrase (Int-B13) were similar to those of the ORF1 in the flanking region of the *pah* gene cluster of strain PaK1 (Fig. 6),[48] and suggested that the ORF1 is a part of a mobile element. NAH plasmids may integrate themselves into the chromosomes of *Pseudomonas* strains as observed with other degradative plasmids.[119]

Considering that *Comamonas*, *Ralstonia*, and *Sphingomonas* species also have genes similar to the classical *nah*-like genes (although these species were formerly classified as *Pseudomonas*),[13,58,120] such conjugative transfer or transposition may have occurred among these groups of bacteria. It was recently reported that *Marinobacter* sp. strain NCE312 has a *Pseudomonas*-like naphthalene dioxygenase gene.[121] Considering that *Pseudomonas* nautical was recently reclassified into the genus *Marinobacter*, the genus *Pseudomonas* and the genus *Marinobacter* may have some close relationship, and therefore, the occurrence of gene transfer between these genera may be possible.

(2) Evidence for structural changes of the classical nah*-like operons*

As described above, the mobile NAH plasmids may have undergone some insertions or deletions while being transferred among many host strains. In addition, the similarity among the classical *nah*-like operons despite the complete dissimilarity of its flanking regions suggested that the upper pathway gene cluster might have moved as a unit. Detailed sequencing analysis of the flanking regions of the *nah*-like genes found some genetic elements that might cause such structural changes in the gene organization.

Eaton[45] reported that 84-bp directly repeated sequences were located about 10 kb apart in the NAH7 plasmid from strain G7 and that they encompassed the entire upper-pathway operon. Repeated elements were found also in the downstream region of *doxJ* from strain C18, in both the upstream and downstream regions of the *pah* operon from strain OUS82, and the downstream region of *pahD* from strain PaK1 (Fig. 6).[45,48] Later, these repeated DNA segments were proposed to be the remnants of an insertion sequence (IS) element belonging to the IS*5* family.[122] In fact, some ORFs similar to transposase (*tnpA*)-like gene of the IS*5* family were found in these regions (Fig. 6). For example, in the downstream region of *pah* genes from strain PaK1, ORF12 and its upstream region were similar to the DNA region found in the IS*1472 tnpA* gene and its upstream region.[48] The 3′-terminal region of *tnpA* was truncated in ORF12, so ORF12 might be inactive and a remnant of *tnpA*. Also, *tnpA1*, *tnpA2*, and *tnpA3* are present in close proximity to the *nah* gene cluster of strain AN10, and indeed, a composite transposon composed of *tnpA3-nahW-tnpA2* was found to be mobile.[49–51] Therefore, genetic rearrangements through *tnpA* homologous recombination or *tnpA* gene deletions (or both) may have occurred to form the present operon structures.

Within the *nah* catabolic upper operon, several trails of gene rearrangement also were found. Comparison of the sequences among strains AN10, C18, and PaK1 showed that the upper-pathway operon of strain PaK1 could be the result of a recombination event between the upper pathways of the two other strains.[49] Recently, Ferreo *et al.*[123] reported that closely related naphthalene-degrading *Pseudomonas* strains isolated from the western Mediterranean might independently harbor two distinct *nahAc*-type genes, a strain-AN10-type and a strain-C18-type, and that these *nahAc* genes and other upper pathway genes may sometimes coexist in the same host strain. These results support the idea that such natural recombination occurs between catabolic pathways with enzymes having similar sequences.

There is similarity in sequence and gene order not only among the classical *nah*-like genes, but also between the *nag* genes of strain U2 and the classical *nah*-like genes. However, the similarity is observed only in genes involved in the conversion of naphthalene to salicylate (*nagAaAbAcAdBFCQE* and *nahAaAbAcAdBFCQE*) (Figs. 6 and 7). In strain U2, there are two additional genes (*nagGH*) between *nagAa* and *nagAb*, and it was found that *nagGH* encodes salicylate 5-hydroxylase, converting salicylate to gentisate.[60–62] Fuenmayor *et al.*[60] suggested that a recombination event(s) led to the acquisition of a gentisate pathway with an insertion between *nagAa* and *nagAb* of *nagG* and *nagH*, enabling their gene products to share the electron transport chain of the NDO.

(3) Phylogenetic relationships among PAH-initial dioxygenase component

A phylogenetic tree constructed from the alignment of α subunits of terminal oxygenase components is shown in Fig. 12. The alignment separates PAH-initial dioxygenase α subunits into two major groups. The first group encompasses dioxygenase components derived from the Gram-negative PAH-degrading bacteria. This group also includes two α subunit components involved in dinitrotoluene degradation (DntAc from strain DNT and NtdAc from strain JS42). Among the Gram-negative PAH-degrading bacteria, the classical NahAc-like and NagAc amino acid sequences are clustered tightly together. Although apparently different from the classical NahAc-like oxygenase branch, two PhnAc oxygenases (strains RP007 and AFK2) and BphA1f (strain F199) are near the branch of these oxygenases.

The second group is the oxygenase components

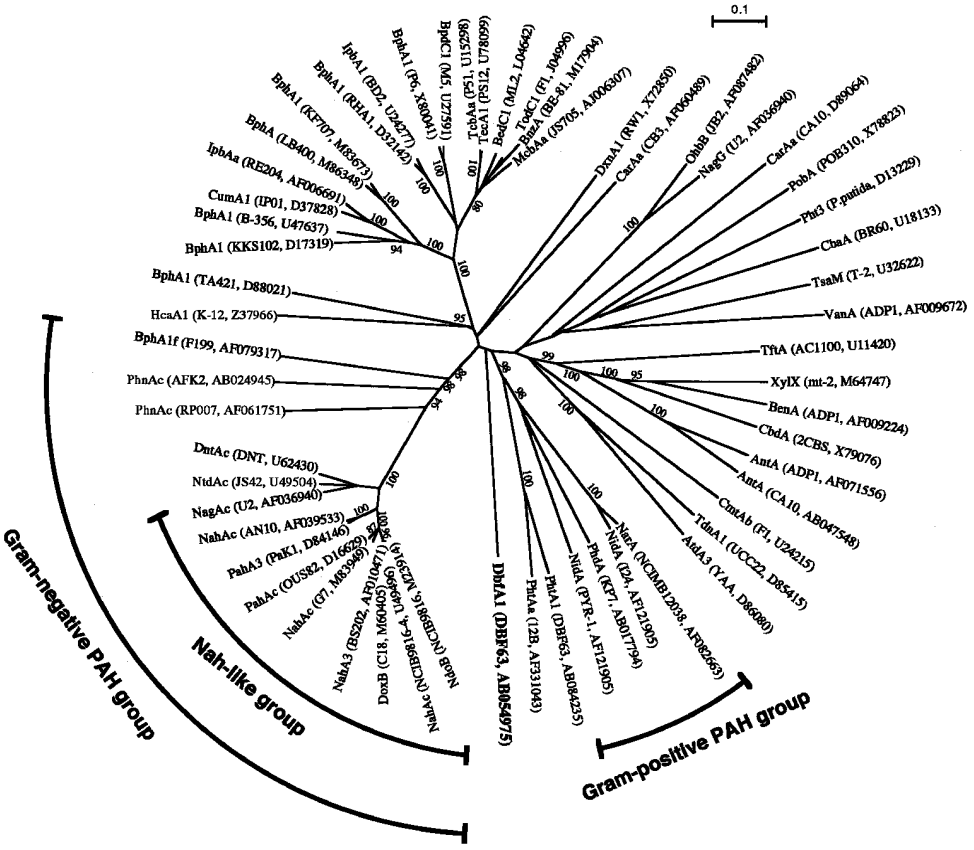

Fig. 12. Phylogenetic Tree Constructed on the Basis of the Alignment of α Subunits of Terminal Oxygenase Components of Rieske Non-heme Iron Ooxygenases.

The amino acid sequences were aligned with the CLUSTAL W package (version 1.6).[126] The tree was drawn with the program "unrooted" (http://pbil.univ-lyon1.fr/software/unrooted.html) and the layout modified with a standard graphics program. The numbers on several branches indicate the percent confidence estimated by bootstrap analysis with 100 replications. The scale bar indicates the percent divergence.

derived from Gram-positive PAH-degrading bacteria. NarAa (strain NCIMB12038), PhdA (strain KP7), and NidA (strain PYR-1) form a branch. In contrast, in the novel angular dioxygenase component involved in fluorene degradation, DbfA1 (strain DBF63), the branching point at the root of the tree is distant from other Gram-positive PAH-oxygenases.

Conclusions

For successful bioremediation, a deeper understanding is needed at the molecular level of how bacterial biodegradation proceeds in PAHs. As a result of extensive studies, the number of known PAH-catabolic genes for degrading PAHs composed of two or three rings has been increasing. It is important not only to understand the function of these genes but also to construct gene probes for monitoring of the degraders in a contaminated environment. However, knowledge about the genes for degrading PAH composed of four or more rings is still limited, although high-molecular-weight PAH-compounds are more recalcitrant and potentially carcinogenic. Moreover, within low-molecular-weight PAHs, little is known about the catabolic genes involved in the fluorene and acenaphthene-acenaphthylene degradation. Considering that PAHs exist in the environment as complex mixtures, further genetic studies on degradation of PAHs other than naphthalene and phenanthrene seem to be needed.

Recently, with PCR-based methods, many new "partial" catabolic genes involved in PAH degradation from various bacteria have been reported.[14,124,125] These results suggest the diversity of PAH-degrading bacteria in the environment, and there may still be many unidentified PAH-degrading bacteria including unculturable bacteria. Accumulation of such information is necessary for monitoring of the prevalence of different PAH-catabolic genotypes in PAH-contaminated soils, and should contribute to the effective bioremediation of PAHs.

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