REVIEW ARTICLE

Microbial biodegradation of polyaromatic hydrocarbons

Ri-He Peng, Ai-Sheng Xiong, Yong Xue, Xiao-Yan Fu, Feng Gao, Wei Zhao, Yong-Sheng Tian & Quan-Hong Yao

Shanghai Key Laboratory of Agricultural Genetics and Breeding, Agro-Biotechnology Research Institute, Shanghai Academy of Agricultural Sciences, Shanghai, China

Correspondence: Quan-Hong Yao, Shanghai Key Laboratory of Agricultural Genetics and Breeding, Agro-Biotechnology Research Center, Shanghai Academy of Agricultural Sciences, 2901 Beidi Rd, Shanghai, China. Tel./fax: +086 021 62203180; e-mail: yaoquanhong_sh@yahoo.com.cn

Received 8 March 2008; revised 14 April 2008; accepted 10 June 2008.
First published online 25 July 2008.

DOI:10.1111/j.1574-6976.2008.00127.x

Editor: David Gutnick

Keywords
polycyclic aromatic hydrocarbons; biodegradation; microorganisms; pathway; genetic regulation.

Abstract

Polycyclic aromatic hydrocarbons (PAHs) are widespread in various ecosystems and are pollutants of great concern due to their potential toxicity, mutagenicity and carcinogenicity. Because of their hydrophobic nature, most PAHs bind to particulates in soil and sediments, rendering them less available for biological uptake. Microbial degradation represents the major mechanism responsible for the ecological recovery of PAH-contaminated sites. The goal of this review is to provide an outline of the current knowledge of microbial PAH catabolism. In the past decade, the genetic regulation of the pathway involved in naphthalene degradation by different gram-negative and gram-positive bacteria was studied in great detail. Based on both genomic and proteomic data, a deeper understanding of some high-molecular-weight PAH degradation pathways in bacteria was provided. The ability of nonligninolytic and ligninolytic fungi to transform or metabolize PAH pollutants has received considerable attention, and the biochemical principles underlying the degradation of PAHs were examined. In addition, this review summarizes the information known about the biochemical processes that determine the fate of the individual components of PAH mixtures in polluted ecosystems. A deeper understanding of the microorganism-mediated mechanisms of catalysis of PAHs will facilitate the development of new methods to enhance the bioremediation of PAH-contaminated sites.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are an all-pervading group of hydrophobic organic compounds consisting of two or more combined benzene rings in linear, angular or cluster arrangements. PAHs are the most important components of crude oil, creosote, asphalt and coal tar. They contaminate the soil via many routes, including the burning of fossil fuels, the manufacture of gas and coal tar, wood processing, escaped automobile gasoline and the incineration of waste (Harvey, 1991). There are more than 100 diverse PAH compounds, and most of them persist in the ecosystem for many years owing to their low aqueous solubility and their absorption to solid particles (Volkering *et al.*, 1992, 1993; Bosma *et al.*, 1997). The presence of PAHs in contaminated soil and sediment poses a significant risk to the soil, because many PAH compounds are known or suspected to be toxic, mutagenic and, in some cases, carcinogenic (Cerniglia & Heitkamp, 1989; Patnaik, 1992). On the basis of their abundance and toxicity, 16 PAH compounds have been included in the United States Soilal Protection Agency's list of priority pollutants (Keith & Telliard, 1979).

The elimination of PAHs from contaminated soil is essential during its remediation according to relevant clean-up standards. The biological treatment of soil contaminated with PAHs should be a more efficient, financially affordable and adaptable choice than physicochemical treatment because it presents potential advantages such as the complete degradation of the pollutants, lower treatment cost, greater safety and less soil disturbance (Habe & Omori, 2003). Many bacteria have been discovered that degrade PAHs via either metabolism or cometabolism. The ordinary biochemical pathways of the bacterial metabolism of PAHs have been scrutinized. Low-molecular-weight (MW) PAHs, such as naphthalene, phenanthrene and anthracene, are usually

readily degraded by bacteria in soil and under laboratory conditions (Cerniglia, 1984, 1992; Sutherland *et al.*, 1995). Since the initial studies by Heitkamp *et al.* (1988a, b) on the bacterial degradation of pyrene, there have been numerous reports describing the microbial oxidation of four-ring PAH. Recently, a *Sphingomonad* endowed with the remarkable ability to grow on four-ring PAH chrysene as its sole carbon and energy source was isolated (Willison, 2004). However, less is known about the bacteria capable of utilizing PAHs containing five or more rings as a carbon and energy source, such as benzo[α]pyrene and benz[α]anthracene. The mechanisms involved in the degradation of PAHs with more than five rings are unclear.

Diverse fungi capable of utilizing PAHs have been investigated as well. Some filamentous fungi, basidiomycetes, white-rot fungi and deuteromycetes have been shown to remove PAHs more competently than bacteria. PAHs susceptible to fungal biodegradation include naphthalene, phenanthrene, anthracene, pyrene, benzo[α]pyrene, fluorene, dibenzothiophene, catechol, benzo[α]anthracene, benzo[ghi]-perylene, chrysene, benzo[b]fluoranthene and benzo[k]-fluoranthene (Cerniglia, 1997; Zheng & Obbard, 2002, 2003). At least two mechanisms are involved in PAH biodegradation: one utilizes the cytochrome P-450 system (Yadav *et al.*, 2006) and the other uses the soluble extracellular enzymes of lignin catabolism, including lignin peroxidase, manganese peroxidase (Steffen *et al.*, 2003) and laccase (Gianfreda *et al.*, 1999).

In the past decade, more and more microorganisms capable of mineralizing PAH compounds have been isolated, and knowledge about many PAH-catabolic genes from bacteria and fungi is accumulating. However, until now, there have been no reviews summarizing this progress. In this review, we will summarize the recent advances in understanding the natural diversity and capabilities of bacteria and fungi for degrading PAH compounds.

Bacterial catabolism of polycyclic aromatic hydrocarbons

Numerous bacteria have been found that degrade PAHs, and some can utilize low-MW PAHs as their sole carbon source. The common biochemical pathways for the bacterial degradation of PAHs such as naphthalene (Resnick *et al.*, 1996; Annweiler *et al.*, 2000), phenanthrene (Menn *et al.*, 1993; Kiyohara *et al.*, 1994; Pinyakong *et al.*, 2003a, b), anthracene and acenaphthene (Dean-Ross *et al.*, 2001; Pinyakong *et al.*, 2004) have been well investigated. Biodegradation mechanisms require the presence of molecular oxygen to initiate the enzymatic attack of PAH rings. In the initial step, dioxygenase-catalyzed oxidation of arenes generally takes place in aerobic bacterial systems to yield vicinal *cis*-dihydrodiols as the early bioproducts by a multicomponent enzyme system. These dihydroxylated intermediates may then be cleaved by intradiol or extradiol ring-cleaving dioxygenases through either an *ortho*-cleavage pathway or a *meta*-cleavage pathway, leading to central intermediates such as protocatechuates and catechols that are further converted to tricarboxylic acid (TCA) cycle intermediates (Cerniglia, 1992; Eaton & Chapman, 1992; Gibson & Parales, 2000).

Dioxygenases responsible for the formation of *cis*-dihydrodiols from arenes of PAH substrates appear to be the most ubiquitous in bacteria. They have usually been found to contain multicomponent enzyme systems involving several proteins, nonheme iron, and require NADH (Gibson & Subramanian, 1984; Gibson *et al.*, 1990; Resnick *et al.*, 1994). To date, dioxygenase-containing organisms have been grown on a range of carbon sources, and these enzymes have been classified accordingly, e.g. toluene (TDO), naphthalene (NDO) and biphenyl (BPDO). TDO is confirmed to be an extremely wide-ranging substrate enzyme. The TDO system is most suitable for *cis*-dihydroxylation of substituted benzene substrates and bicyclic arenes. However, there is a size limitation on substrate acceptability for TDO; bulky and bendy molecules fit poorly into the active site of this enzyme. For larger PAHs, both NDO and BPDO are capable of catalyzing dihydroxylation, with only the latter enzyme capable of metabolizing tetracyclic or larger examples. By contrast, the NDO enzyme appears to be generally incapable of catalyzing the *cis*-dihydroxylation of monocyclic aromatic rings (Boyd & Sheldrake, 1998).

Low-MW PAH dioxygenase system

The bacterial naphthalene dioxygenase system is particularly useful for oxidizing bi- and tri-cyclic PAH substrates, such as naphthalene, phenanthrene and anthracene. The naphthalene dioxygenase system is a multicomponent enzyme, generally including an NADH oxidoreductase, a ferredoxin and an oxygenase component that contains the active site. This catalytic portion of the naphthalene dioxygenase system, termed NDO, is composed of large and small subunits – α and β, respectively – that are in an α3β3 configuration. NDO is a member of a large family of oxygenases whose α subunits contain a Rieske [2Fe–2S] center and mononuclear nonheme iron, and determine the substrate specificity of NDO (Butler & Mason, 1997; Parales *et al.*, 1998). Generally, two electrons from the reduced pyridine nucleotide are transferred via the reductase, the ferredoxin, and the Rieske center to the Fe (II) ion at the active site during a catalytic cycle. The reducing equivalents allow the activation of molecular oxygen, which is a prerequisite to dihydroxylation of the substrate (Carredano *et al.*, 2000; Ferraro *et al.*, 2005, 2006). Interestingly, the *AaAb* genes, which encode the ferredoxin and reductase components of the multicomponent PAH initial dioxygenase, are not present on the *phn*

locus of the *Burkholderia* sp. strain RP007 (Laurie & Lloyd-Jones, 1999). Currently, we are trying to improve the efficiency of the electron transport functions of the *nahAcAd* gene products by DNA shuffling.

Naphthalene-degrading bacteria are broadly distributed in nature. So far, a few naphthalene dioxygenases have been purified and extensively characterized from different strains of bacteria, and genetic control of the pathways involved in naphthalene degradation has been studied in detail. The naphthalene catabolic genes (*nah*) of NAH7 are organized into two operons: the *nal* operon encoding the upper pathway enzymes involved in conversion of naphthalene to salicylate, and the *sal* operon encoding the lower pathway enzymes involved in the conversion of salicylate to pyruvate and acetyl coenzyme A. The two operons are closely genetically linked to each other and to their common regulatory gene, *nahR*. The *nahR* gene encodes a *trans*-acting positive regulator belonging to the LysR family of transcriptional regulators that are widely distributed in bacteria. NahR is required for the induction of the *nah* genes by salicylate and the high-level expression of those genes in bacteria (Yen & Gunsalus, 1982, 1985; Grund & Gunsalus, 1983).

The nucleotide sequences of genes encoding the upper pathway enzymes from several *Pseudomonas* strains have been reported: *ndo* genes from *Pseudomonas putida* NCIB 9816 (Yang *et al*., 1994), *nah* genes from *P. putida* G7 (Menn *et al*., 1993), NCIB9816-4 (Simon *et al*., 1993), ND6 (Li *et al*., 2004), BS202 (Kosheleva *et al*., 1986; Balashova *et al*., 2001), and *P. putida* sp. (Ono *et al*., 2007), *dox* genes from *Pseudomonas* sp.C18 (Denome *et al*., 1993), *pah* genes from *P. putida* OUS82 (Kiyohara *et al*., 1994; Takizawa *et al*., 1994) and *Pseudomonas aeruginosa* Pak1 (Takizawa *et al*., 1999), and *nah* genes from *Pseudomonas stutzeri* AN10 (Bosch *et al*., 1999a, b, 2000). All the *nah* structural and regulatory genes on the former plasmid are located on a 39-kb class II transposon, Tn4655 (Tsuda & Iino, 1990; Sota *et al*., 2006). The nucleotide sequences of the upper pathway genes are more than 90% identical. All these genes are arranged in a similar order: *nahAa* (ferredoxin reductase)- *nahAb* (NDO ferredoxin)- *nahAc* (the α-subunit of NDO)- *nahAd* (the β-subunit of NDO)- *nahB* (naphthalene-*cis*-dihydrodiol dehydrogenase)- *nahF* (salicylaldehyde dehydrogenase)- *nahC* (1,2-dihydroxynaphthalene dioxygenase)- *nahQ* (unknown gene)- *nahE* (*trans*-*o*-hydrobenzylidenepyruvate hydratase aldolase)- *nahD* (2-hydroxychromene-2-carboxylate isomerase) (Habe *et al*., 2003) (Fig. 1). The genetic regulation of this pathway was also studied in detail for different bacterial strains (Fig. 2). In *Ralstonia sp*. U2, the naphthalene dioxygenase genes (*nag* gene) contained all of the genes corresponding to the classical *nah* gene of *Pseudomonas* strains in the same order, with the exception of two extra genes inserted between the ferredoxin reductase gene and ferredoxin gene. The two additional genes, named *nagG* and *nagH*, are structural subunits of salicylate-5-hydroxylase and can help the host convert naphthalene to gentisate (Fuenmayor *et al*., 1998; Zhou *et al*., 2001). In the *Comamonas testeroni* strain GZ42, the upper pathway genes are similar to those of *Ralstonia* sp. U2 (Goyal & Zylstra, 1997). However, in *C. testeroni* strain GZ39, the pathway genes are quite different from those of the typical *nah* genes. In this strain, several genes (such as extradiol dioxygenase) are not within the cluster, but a glutathione-*S*-transferase gene was inserted into the cluster (Goyal & Zylstra, 1996; Zylstra *et al*., 1997).

It was determined from the sequences of *P. putida* G7, NCIB9816-4, ND6, and *P. stutzeri* AN10 strains that the lower pathway genes consist of 11 genes organized in the order *nahGTHINLOMKJY*. *nahY* is not a catabolic gene, but a naphthalene chemotaxis gene (Grimm & Harwood, 1999; Boronin, 2001) (Fig. 2). In the strains AN10 and ND6, there is another salicylate hydroxylase gene (*nahW*) outside but close to the *sal* operon. It was suggested that isofunctional enzyme might be advantageous for the host to adjust its metabolism to extreme conditions (Bosch *et al*., 1999a, b; Li *et al*., 2004).

A number of PAH-degrading phenotypes cannot be explained genotypically by comparison with *nah*-like sequences in contaminated soils. The *nah*-like probe can only provide a monitor for 45% of strains isolated with a naphthalene-degrading phenotype, and yet the same probe was only useful for 15% of strains isolated with a phenanthrene-degrading phenotype (Lloyd-Jones *et al*., 1999, 2000). The enzymes involved in the conversion of naphthalene to salicylate can also degrade phenanthrene to naphthalene-1,2-diol. However, it was found that the sequence similarity and gene organization of the naphthalene catabolic genes were very different between the *Burkholderia* sp. RP007 strain and some classical naphthalene degradation strains. The strain RP007's naphthalene catabolic genes (*phn* gene) encode iron–sulfur protein α and β subunits of a PAH initial dioxygenase, but lack both ferredoxin and reductase components. The ferredoxin and reductase genes for electron transport functions of the *phn* gene may be located elsewhere in the RP007 genome or may be supplied by cellular housekeeping genes (Laurie & Lloyd-Jones, 1999, 2000).

Different routes of naphthalene degradation have also been illustrated. The *Alcaligenes faecalis* AFK2 strain makes use of phenanthrene as its sole carbon source through the *o*-phthalate pathway (Kiyohara *et al*., 1982). Some unique genes, such as 3,4-dihydroxyphenanthrene dioxygenase (phnC), 2-carboxybenzaldehyde dehydrogenase (phnI), and *trans*-2-carboxybenzal-pyruvate hydratase aldolase (phnH), appear in this pathway, which may make the strain prefer phenanthrene to naphthalene (Nagao *et al*., 1988;

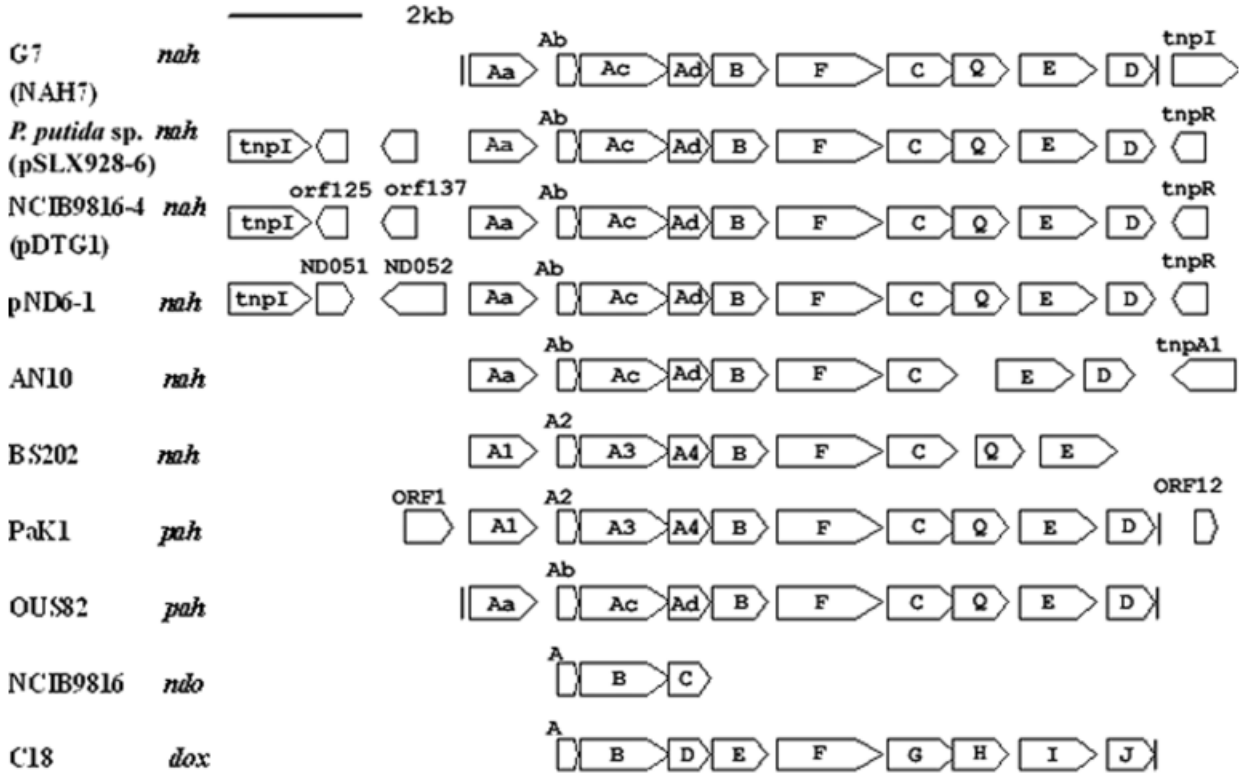

Fig. 1. Gene clusters for naphthalene/phenanthrene upper catabolic pathway in *Pseudomonas* strains. The pentagon represents the size, location, and direction of transcription of the ORF. The *tnpI* and *tnpR* genes encode the site-specific tyrosine and serine recombinases, respectively (Nash, 1996). The bold line represents the 84-bp sequence reported Eaton (1994). References for the sequences are as follows: *nah* genes from *Pseudomonas putida* G7 (NAH7) (AB237655), *P. putida* sp. (pSLX928-6) (AB255564), *P. putida* NCIB9816-4 (pDTG1) (AF491307), *Pseudomonas* sp. ND6 (pND6-1) (AY208917), *Pseudomonas stutzeri* AN10 (AF039533), and *P. putida* BS202 (AF010471); *pah* genes from *Pseudomonas aeruginosa* PaK1 (D84146) and *P. putida* OUS82 (AB004059); *ndo* genes from *P. putida* NCIB9816 (M23914); and *dox* genes from *Pseudomonas* sp. strain C18 (M60405). Enzyme designations are described in the text.

Kiyohara *et al.*, 1990). The *Nocardioides* sp. KP7 strain degrades phenanthrene via the phthalate pathway as well (Iwabuchi & Harayama, 1997, 1998a, b). The *phd* genes of this strain belong to a new class of PAH-catabolic genes due to variation in gene organization and sequence similarity. The *PhdA* and *PhdB* genes, which encode the α- and β-subunit of phenanthrene dioxygenase, are essential for enzyme activity. Although the basic sequence features of each protein family are conserved, each of the subunits of the dioxygenase shows a modest ($< 60\%$) sequence identity to all of the known dioxygenase subunits. The ferredoxin component of the dioxygenase *PhdC* belongs to the [3Fe–4S] or [4Fe–4S] type of ferredoxin, but not to the [2Fe–2S] type found in most components of PAH dioxygenase (Iwabuchi & Harayama, 1998a, b; Saito *et al.*, 1999). The dispensability of the ferredoxin and ferredoxin reductase components of *Escherichia coli* suggests the relatively low specificity of the electron transport systems toward the oxygenase components (Saito *et al.*, 2000).

It has been observed that a high proportion of the PAH-degrading isolates in soil belong to the sphingomonads. Unlike other gram-negative bacteria strains, members of the genus *Sphingomonas* are able to degrade a wide range of natural and xenobiotic compounds, such as biphenyl, (substituted) naphthalene(s), fluorene, (substituted) phenanthrene(s), pyrene, (chlorinated) diphenylether(s), (chlorinated) furan(s), (chlorinated) dibenzo-p-dioxin(s), carbazole, estradiol, polyethylenglycols, chlorinated phenols and different herbicides and pesticides (Basta *et al.*, 2005). It seems possible that PAH-degrading sphingomonads are adapted to the oligotrophic environment by having high-affinity uptake systems and an ability to simultaneously take up mixed substrates rather than being particular specialists for the degradation of hydrophobic aromatic compounds (Eguchi *et al.*, 1996; Schut *et al.*, 1997; Fredrickson *et al.*, 1999; Pinhassi & Hagstrom, 2000). Many sphingomonads degrade naphthalene, phenanthalene, and anthracene via common pathways found in other gram-negative bacteria (Pinyakong *et al.*, 2003a, b). It was demonstrated that large plasmids present in xenobiotic-degrading *Sphingomonas* strains are responsible for the degradative capabilities (Kim *et al.*, 1996; Feng *et al.*, 1997;

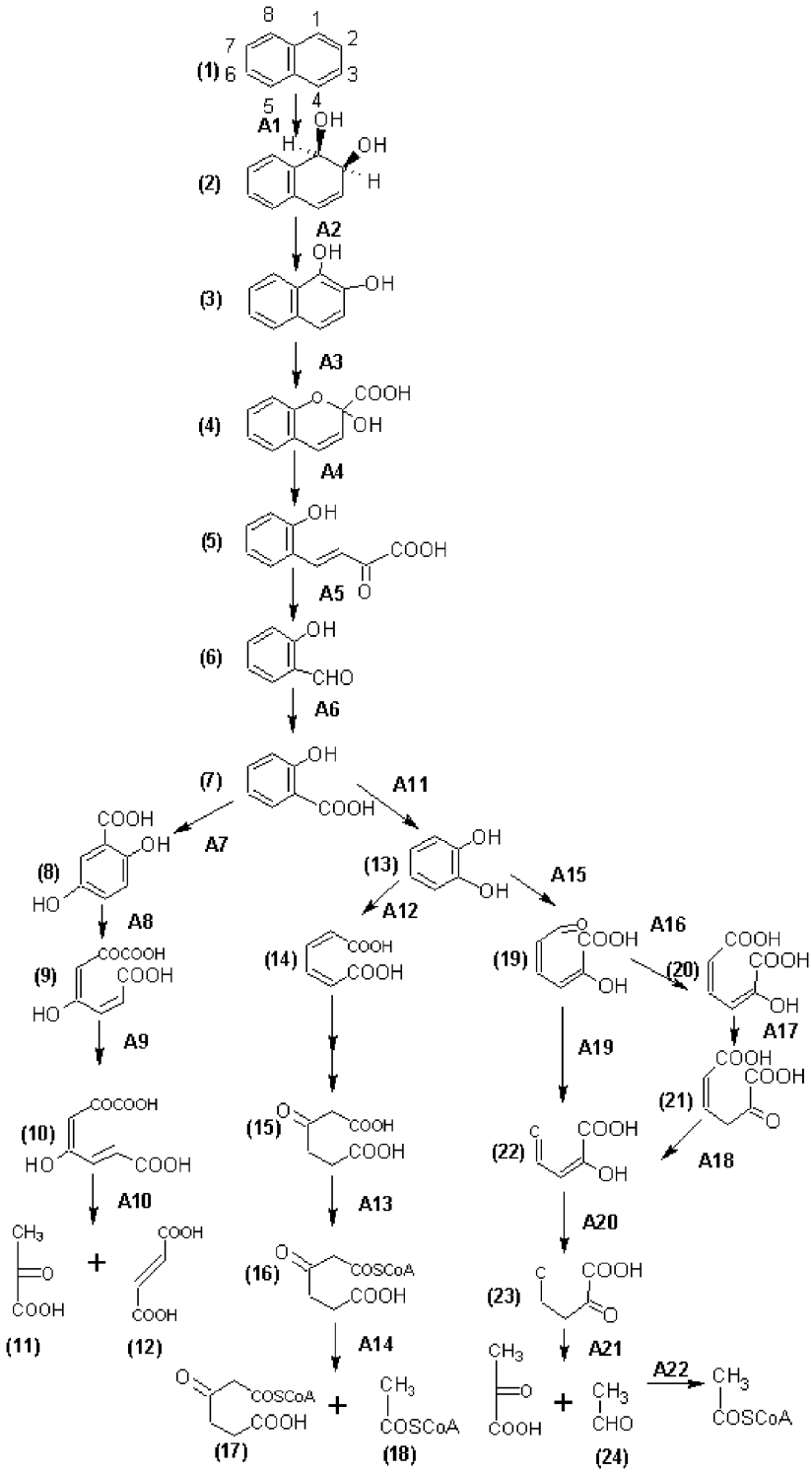

Fig. 2. Proposed catabolic pathways of naphthalene by aerobic bacteria. The compounds are naphthalene (1), *cis*-1,2-dihydroxy-1, 2-dihydronaphthalene (*cis*-naphthalene dihydrodiol) (2), 1,2-dihydroxynaphthalene (3), 2-hydroxy-2*H*-chromene-2-carboxylic acid (4), *trans*-*o*-hydroxybenzylidenepyruvic acid (5), salicylaldehyde (6), salicylic acid (7), gentisic acid (8), maleylpyruvic acid (9), fumarylpyruvic acid (10), pyruvic acid (11), fumaric acid (12), catechol (13), *cis,cis*-muconic acid (14), β-ketoadipic acid (15), β-ketoadipyl-CoA (16), succiny-CoA (17), acetyl-CoA (18), 2-hydroxymuconic-semialdehyde (19), 2-hydroxymuconic acid (20), 4-oxalocrotonic acid (21), 2-oxo-4-pentenoic acid (22), 4-hydroxy-2-oxovaleric acid (23), and acetaldehyde (24). The enzymes involved in each reaction step are naphthalene dioxygenase (NahAaAbAcAd) (step A1), *cis*-naphthalene dihydrodiol dehydrogenase (NahB) (A2), 1, 2-dihydronaphthalene dioxygenase (NahC) (A3), 2-hydroxy-2*H*-chromene-2-carboxylate isomerase (NahD) (A4), *trans*-*o*-hydroxybenzylidenepyruvic hydratase-aldolase (NahE) (A5), salicylaldehyde dehydrogenase (NahF) (A6), salicylate 5-hydroxylase (NagGHAaAb) (A7), gentisate 1, 2-dioxygenase (NagI) (A8), maleylpyruvate isomerase (NagL) (A9), fumarylpyruvate hydrolase (NagK) (A10), salicylylate hydroxylase (NahG) (A11), catechol 1,2-dioxygenase (A12), β-ketoadipate:succinyl-CoA transferase (A13), β-ketoadipyl-CoA thiolase (A14), catechol 2, 3-dioxygenase (NahH) (A15), hydroxymuconic-semialdehyde dehydrogenase (NahI) (A16), 4-oxalocrotonate isomerase (NahJ) (A17), 4-oxalocrotonate decarboxylase (NahK) (A18), hydroxymuconic-semialdehyde hydrolase (NahN) (A19), 2-oxo-4-pentenoate hydratase (NahL) (A20), 2-oxo-4-hydroxypentenoate aldolase (NahM) (A21), and acetaldehyde dehydrogenase (NahO) (A22).

Romine *et al*., 1999; Ogram *et al*., 2000; Basta *et al*., 2004). For example, a 40-kb DNA region was found to be related to aromatic catabolism in *Sphingobium yanoikuyae* strain B1, in which two dioxygenase genes are predicted to be required for conversion of PAHs and biphenyl to simple aromatic acid, and *meta*-cleavage genes are required for conversion of aromatic acids to the tricarboxylic acid cycle (TAC) intermediate (Yen & Serdar, 1988; Assinder & Williams, 1990; Zylstra & Kim, 1997; Kim & Zylstra, 1999). However, these aromatic-degradative genes for catabolic pathways are often

localized in the plasmid separately from each other, or, at least, are not organized in coordinately regulated operons. There is no evident biochemical function of the individual gene clusters. In plasmid pNL1 of strain F199, there are at least 13 gene clusters predicted to encode enzymes associated with the degradation of aromatic compounds (Romine *et al.*, 1999). Several parts of the DNA sequence in pNL1 regions encoding aromatic catabolic genes are similar to those in other *Sphingomonas* strains (Habe *et al.*, 2003). The 'flexible' gene organization (e.g. different combinations of conserved gene clusters) could be one of the mechanisms that allows sphingomonads to adapt quickly and efficiently to novel compounds in the environment (Basta *et al.*, 2005).

In contrast to *Pseudomonas* and other gram-negative bacteria whose structural genes required for naphthalene utilization are usually clustered, the gram-positive bacteria *Rhodococcus* strains NCIMB12038, P200 and P400 display only three structural genes required for naphthalene utilization (*narAa, narAb* and *narB*) in the region (Larkin *et al.*, 1999; Kulakov *et al.*, 2005). The *narAa* and *narAb* genes encode the α- and β-subunits of the naphthalene dioxygenase (NDO) catalytic component of iron–sulfur protein (ISPNAR). Both subunits of the NCIMB12038 NDO showed only 30% amino acid identity to the corresponding *P. putida* NDO subunits. The *narB* gene has 39% amino acid identity to *NahB* from the *P. putida* G7 strain. No genes were found in the *Rhodococcus* strains that corresponded to the genes encoding the electron transport components reductase and ferredoxin of NDO in *Pseudomonas*. It was found that the *nar* region is not organized into a single operon, but there are several transcription units that differ in the *Rhodococcus* strains. The *narA* and *narB* genes were found to be transcribed as a single unit through different start sites, and their transcription was induced by naphthalene. Putative regulatory genes (*narR1* and *narR2*) are transcribed as a single mRNA in naphthalene-induced cells (Kulakov *et al.*, 2000; Larkin *et al.*, 2005).

As described above, many bacteria containing the enzymes involved in the conversion of naphthalene to salicylate can degrade phenanthrene as well. There are three pathways that can convert phenanthrene dihydrodiols to naphthalene-1,2-diol: initial dioxygenation at the 1,2-position, 3,4-position and 9,10-position of phenanthrene (Pinyakong *et al.*, 2000; Seo *et al.*, 2006) (Fig. 3). In studies of the *Burkholderia* sp. C3 strain, the initial dioxygenation at the 3,4-C positions may be dominant in the first three days of incubation because the concentrations of *cis*-phenanthrene-3,4-dihydrodiol were higher than *cis*-phenanthrene-1,2-dihydrodiol. However, *cis*-phenanthrene-1,2-dihydrodiol was more abundant than *cis*-phenanthrene-3,4-dihydrodiol at the 7th day. The result of a constant accumulation of 1-hydroxy-2-naphthoic acid rather than 2-hydroxy-1-naphthoic acid in the phenanthrene-supplemented media suggested that degradation of 2-hydroxy-1-naphthoic acid might not be a limiting step. Slow degradation of 1-hydroxy-2-naphthoic acid probably facilitated the 1,2-dioxygenation metabolic pathway but 3,4-dioxygenation. Naphthalene-1,2-diol was further degraded through *meta*- and *ortho*-cleavages. The *meta*-cleavage of naphthalene-1,2-diol yielded 2-hydroxybenzalpyruvic acid, which was then transformed to gentisic acid through salicylic aldehyde. This reaction was presumably catalyzed by hydratase–aldolase, which works in the naphthalene degradation pathway. The *ortho*-cleavage formed 2-carboxycinnamic acid, which was then converted to phthalic acid (Seo *et al.*, 2007). The *Arthrobacter* sp. P1-1 strain can degrade phenanthrene through dioxygenation on the 1,2-, 3,4-, and 9,10-C positions. The intermediate products phenanthrene-1,2-, 3,4- and 9,10-diols mainly undergo *meta*-cleavage and convert into naphthalene-1,2-diol, except for a limited amount of *ortho*-cleavage that produces a trace amount of *o*-carboxyvinylnaphthoates and diphenic acid (Seo *et al.*, 2006). As in the *Burkholderia* sp. C3 strain, the degradation of naphthalene-1,2-diol was branched again to yield a dominant *ortho*-cleavage pathway to produce phthalate and a minor meta-cleavage to salicylate. Phenanthrene-degrading bacteria using the *ortho*-cleavage pathway cannot grow when naphthalene is the sole carbon source (Keum *et al.*, 2005; Xia *et al.*, 2005).

Anthracene is found in high amounts in PAH-contaminated environments, and a variety of bacterial species have the ability to utilize anthracene as their sole source of carbon and energy (Tongpim & Pickard, 1999; Wick *et al.*, 2002). The initial reactions in the degradation of anthracene are catalyzed by multicomponent dioxygenases to produce *cis*-1,2-dihydrodiols. The enzymatic attack at the C-1 and C-2 positions of the anthracene moiety is similar to the naphthalene dioxygenase pathways. The product 1,2-dihydroxyanthracene can be cleaved by *meta*-ring cleavage and converted to 2-hydroxy-3-naphthaldehyde and then to 2-hydroxy-3-naphthoic acid, which is further metabolized to salicylate. Identification of metabolites by comparison with authentic standards and transient accumulation of *o*-phthalic acid by the *Mycobacterium* sp. strain during growth on anthracene suggest a pathway through *o*-phthalic acid and protocatechuic acid (van Herwijnen *et al.*, 2003a, b). An alternate route of enzymatic attack by the *Mycobacterium* sp. strain PYR-1 at the C-9 and C-10 positions of anthracene was found to form the dead-end product 9, 10-anthraquinone. A small number of side product 6,7-benzocoumarin can also be detected during the *meta*-ring cleavage process. The accumulation of 1-methoxy-2-hydroxyanthracene provided further evidence for the dioxygenation of anthracene by the *Mycobacterium sp*. strain PYR-1 (Moody *et al.*, 2001). On the other hand, *ortho*-cleavage of 1,2-dihydroxyanthracene into 3-(2-carboxyvinyl) naphthalene-2-carboxylic acid has also

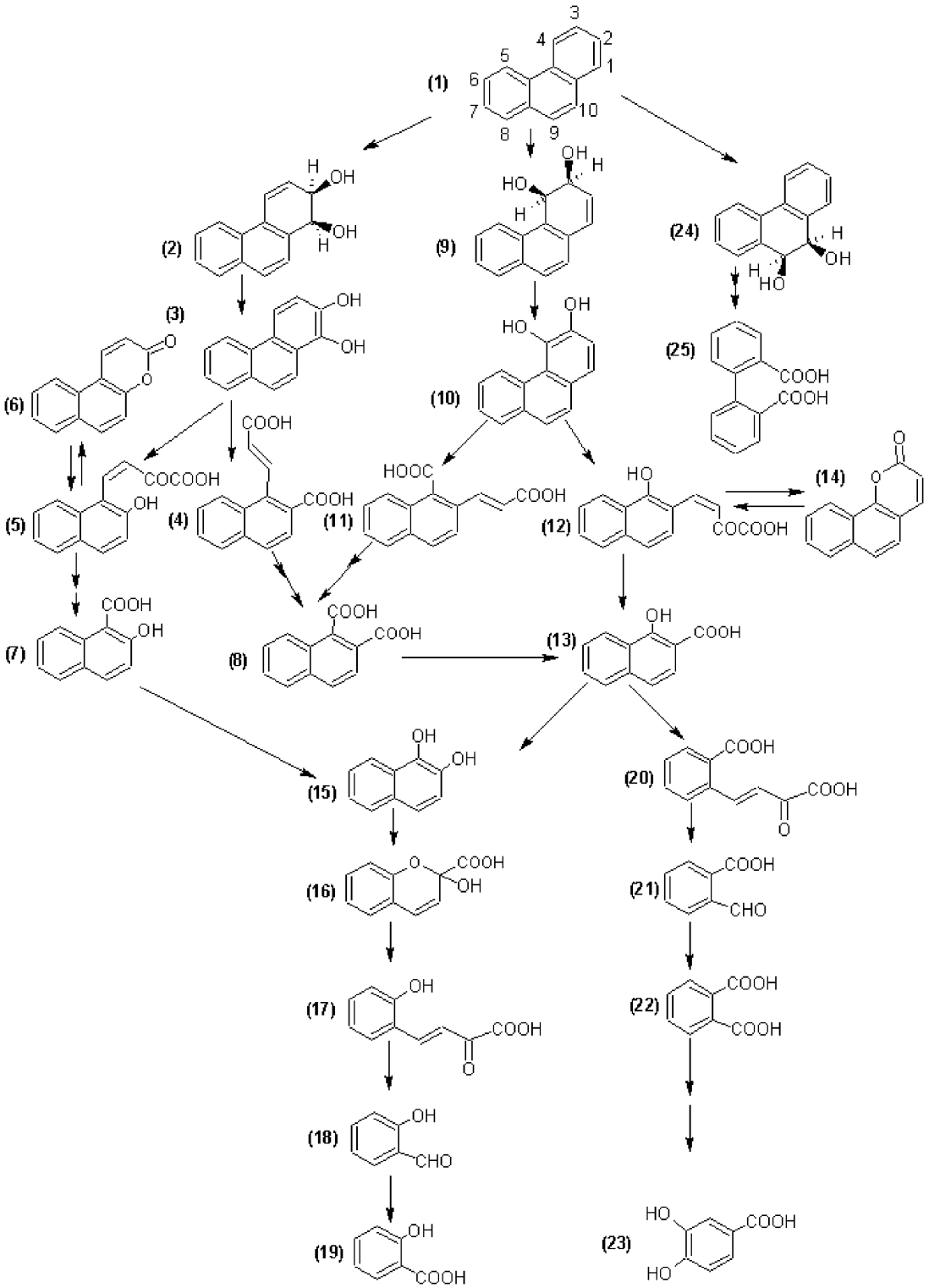

Fig. 3. Proposed catabolic pathways of phenanthrene by aerobic bacteria. The compounds are phenanthrene (1), *cis*-1,2-dihydroxy-1,2-dihydrophenanthrene (2), 1,2-dihydroxyphenanthrene (3), 2-[(*E*)-2-carboxyvinyl]-1-naphthoic acid (4), *trans*-4-(2-hydroxynaph -1-yl)-2-oxobut-3-enoic acid (5), 5,6-benzocoumarin (6), 2-hydroxy-1-naphthoic acid (7), naphthalene-1,2-dicarboxylic acid (8), *cis*-3,4-dihydroxy-3,4-dihydrophenanthrene (9), 3,4-dihydroxyphenanthrene (10), 1-[(*E*)-2-carboxyvinyl]-2-naphthoic acid (11), *trans*-4-(1-hydroxynaph-2-yl)-2-oxobut-3-enoic acid (12), 1-hydroxy-2-naphthoic acid (13), 7,8-benzocoumarin (14), 1,2-dihydroxynaphthalene (15), 2-hydroxy-2H-chromene-2-carboxylic acid (16), *trans*-*o*-hydroxybenzalpyruvic acid (17), salicylaldehyde (18), salicylic acid (19), *trans*-2-carboxybenzalpyruvic acid (20), 2-carboxybenzaldehyde (21), *o*-phthalic acid (22), protocatechuic acid (23), *cis*-9,10-dihydroxy-1,2-dihydrophenanthrene (24), and 2,2′-diphenic acid.

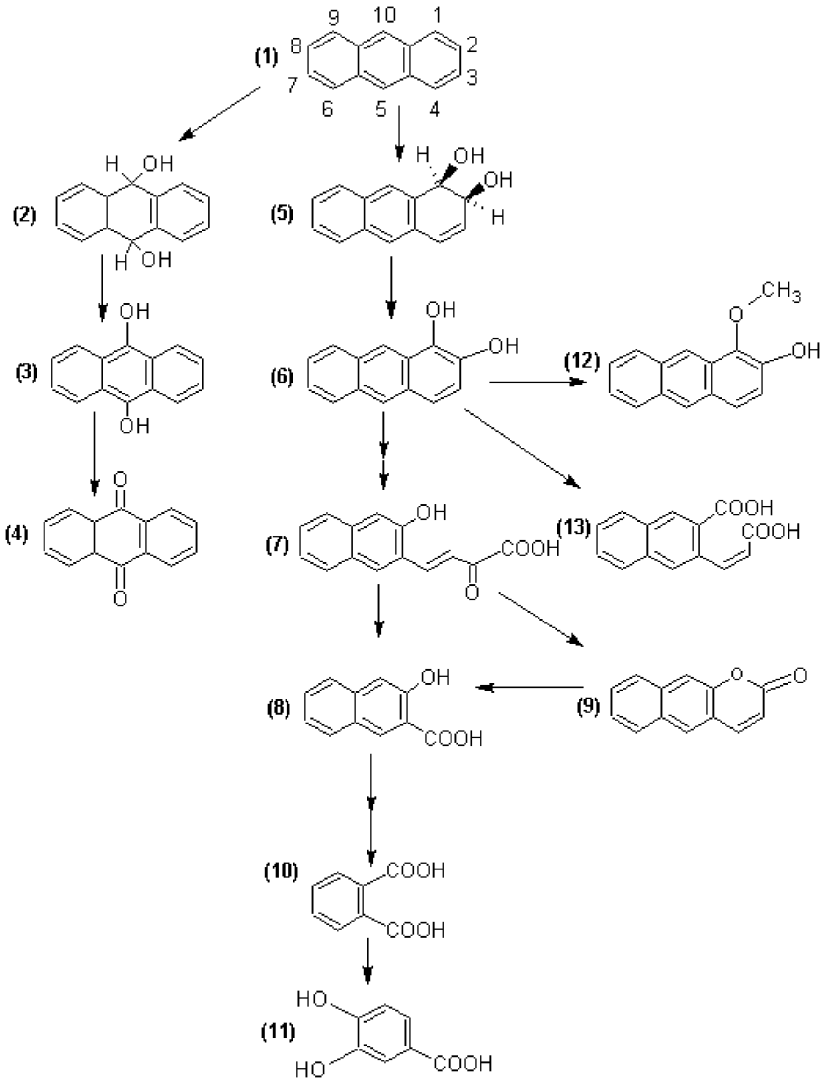

Fig. 4. Proposed catabolic pathways of anthracene by aerobic bacteria. The compounds are anthracene (1), anthracene-9,10-dihydrodiol (2), 9,10-dihydroxyanthracene (3), 9, 10-anthraquinone (4), *cis*-1,2-dihydroxy-1, 2-dihydroanthracene (5), 1,2-dihydroxy anthracene (6), cis-4-(2-hydroxynaphth-3-yl)-2-oxobut-3-enoic acid (7), 2-hydroxy-3-naphthoic acid (8), 6,7-benzocoumarin (9), *o*-phthalic acid (10), protocatechuic acid (11), 1-methoxy-2-hydroxyanthracene (12), and 3-(-2-carboxyvinyl)-naphthalene-2-carboxylic acid (13).

been reported for *Mycobacterium* sp. PYR-1 and a *Rhodococcus* species (Tongpim & Pickard, 1996; Dean-Ross *et al.*, 2001; Moody *et al.*, 2001) (Fig. 4).

Fluorene is a major component of fossil fuels and can be commonly identified in the atmosphere, fresh water, and both riverine and marine sediments (Grifoll *et al.*, 1992, 1994, 1995). Three major degradative pathways for fluorine have been proposed (Casellas *et al.*, 1997, 1998; Wattiau *et al.*, 2001; Habe *et al.*, 2004). Two of these pathways are initiated by a dioxygenation at the 1,2 or the 3,4 position. 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. Enzymatic hydrolysis of the latter results in the production of 3-(2-hydroxyphenyl) propionic acid, which is further metabolized via catechol, to connect this pathway with the central metabolism. The third route is initiated by monooxygenation at the C-9 position to produce 9-fluorenol, which is then dehydrogenated to 9-fluorenone (Fig. 5). Angular dioxygenation of 9-fluorenone leads to 1,1a-dihydroxy-1-hydro-9-fluorenone, which is dehydrogenated to 2′-carboxy-2,3-dihydroxybiphenyl and then spontaneously transformed to 8-hydroxy-3,4-benzocoumarin. Afterwards, the metabolite is catalyzed by a pathway analogous to biphenyl degradation, leading to the formation of phthalate (Engesser *et al.*, 1989; Selifonov *et al.*, 1993; Wattiau *et al.*, 2001). The genes involved in the degradation of fluorene to phthalate were characterized in the fluorine-degrader *Terrabacter* sp. strain DBF63. A 14.7-kb region containing the genes flnRB-dbfA1A2-flnED1-ORF16 that carry out fluorene degradation is found in the DBF63 strain. The initial attack on both fluorene and 9-fluorenone is

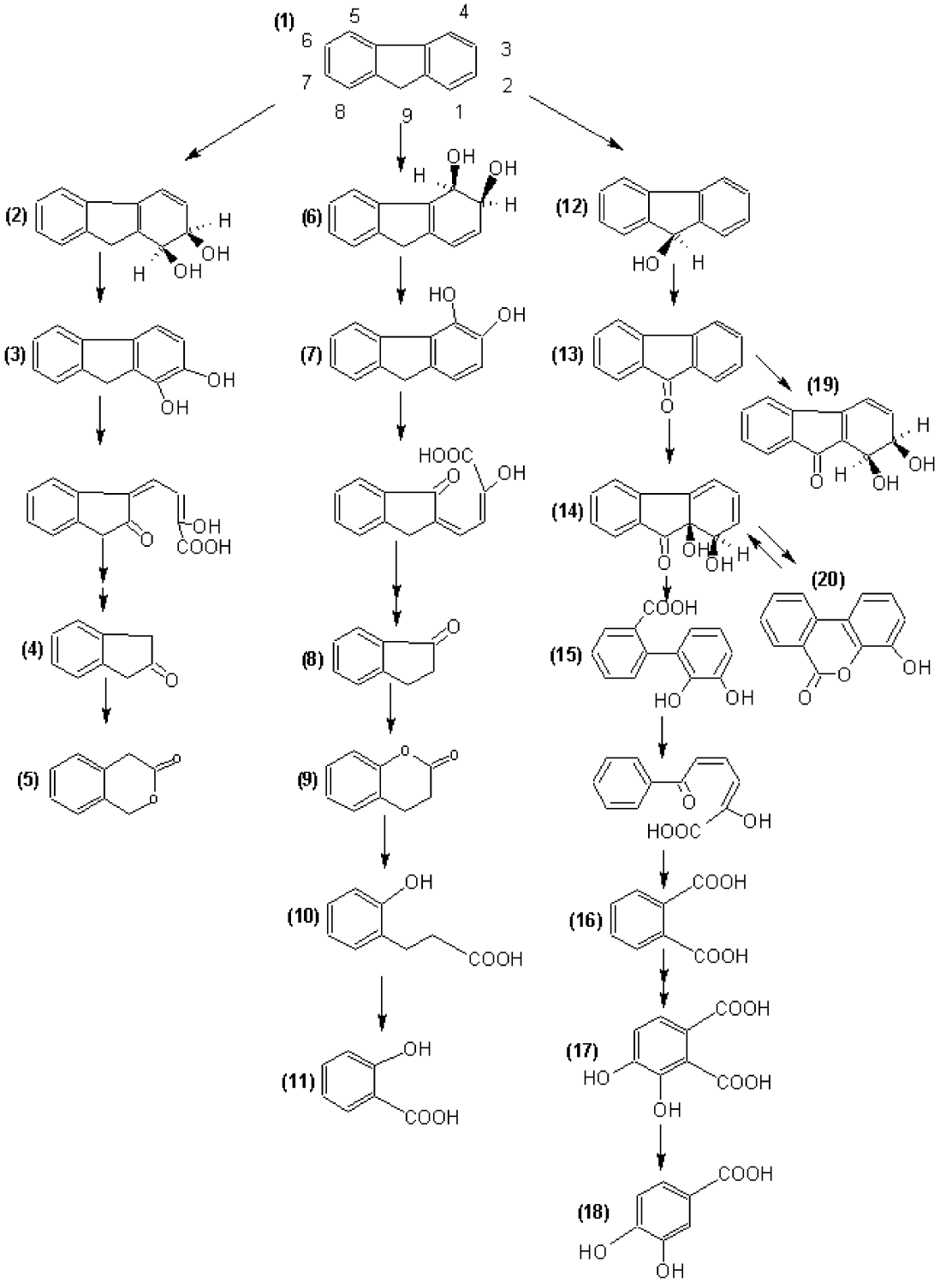

Fig. 5. Proposed catabolic pathways of fluorene by aerobic bacteria. The compounds are fluorene (1), *cis*-1,2-dihydroxy-1,2-dihydrofluorene (2), 1, 2-dihydroxy fluorene (3), 2-indanone (4), 3-isochromanone (5), *cis*-3,4-dihydroxy-3,4-dihydrofluorene (6), 3,4-dihydroxy fluorene (7), 1-indanone (8), 3,4-dihydrocoumarin (9), 3-(2-hydroxyphenyl) propionic acid (10), salicylic acid (11), 9-fluorenol (12), 9-fluorenone (13), 1,1a-dihydroxy-1-hydro-9-fluorenone (14), 2′-carboxy-2,3-dihydroxybiphenyl (15), phthalic acid (16), 4,5-dihydroxyphthalate (17), protocatechuic acid (18) 1,2-dihydro-1, 2-dihydroxy-9-fluorenone (19), and 8-hydroxy-3,4-benzocoumarin (20).

catalyzed by the angular dioxygenase (DbfA1 and DbfA2) to yield 9-fluorenol and 1,1a-dihydroxy-1-hydro-9-fluorenone, respectively. The FlnB protein exhibited activities against both 9-fluorenol and 1,1a-dihydroxy-1-hydro-9-fluorenone to produce 9-fluorenone and 2′-carboxy-2,3-dihydroxybiphenyl, respectively. FlnD1 is a heteromeric protein encoded by flnD1 and ORF16, and is a member of the class III two-subunit extradiol dioxygenases. FlnE was identified as a serine hydrolase for the metacleavage products that yield phthalate (Kasuga *et al.*, 2001; Habe *et al.*, 2003, 2004).

High-MW PAH dioxygenase system

The presence of high-MW PAHs in contaminated soils continues to pose significant problems because the persistence and genotoxicity of PAHs increase with increasing molecule size (Cerniglia, 1992). The relationship between PAH environmental persistence and increasing numbers of benzene rings is in agreement with the knowledge of environmental biodegradation rates and molecule size (Bossert & Bartha, 1986; Heitkamp & Cerniglia, 1987). For example, half-lives of the three-ring molecule phenanthrene in soil and sediment may range from 16 to 126 days, while half-lives of the five-ring molecule benzo[α]pyrene (BαP) may range from 229 to 1400 days (Shuttleworth & Cerniglia, 1995). Electrochemical stability and hydrophobicity are two crucial factors for the accretion of high-MW PAHs in the environment (Volkering *et al.*, 1992, 1993; Bosma *et al.*, 1997; Harms & Bosma, 1997).

The first report of the biodegradation of high-MW PAHs was presented in 1975. Gibson *et al.* identified two products of BαP metabolism – *cis*-9,10-dihydroxy-9,10-dihydro-benzo[α]pyrene and *cis*-7,8-dihydroxy-7,8-dihydrobenzo[α]-pyrene – from the culture solution of a mutant *Beijerinckia* sp. strain when it grew with succinate and biphenyl (Gibson *et al.*, 1975). However, in 1988 the bacterium that could degrade PAHs with four aromatic rings was isolated from sediment below an oil field by Heitkamp and Cerniglia for the first time (Heitkamp & Cerniglia, 1988). Also in 1988, Mahaffey *et al.* (1988) investigated the further catabolism of benz[α]anthracene by the *Beijerinckia* sp. strain B1 (reclassified as *S. yanoikuyae*) after induction with biphenyl, *m*-xylene, or salicylate. Nuclear magnetic resonance (NMR) and mass spectral analyses led to the identification of one major metabolite – 1-hydroxy-2-anthranoic acid – and two minor metabolites – 2-hydroxy-3-phenanthroic acid and 3-hydroxy-2-phenanthroic acid. Later, Mueller *et al.* (1989, 1990) showed that a bacterial community isolated from a creosote waste site was capable of utilizing fluoranthene as its sole source of carbon and energy for bacterial growth.

Pyrene has often been used as a model compound for high-MW PAH biodegradation because it is structurally similar to several carcinogenic PAHs. Although a number of bacterial isolates have been reported to grow on or mineralize pyrene, the majority of these isolates are nocardioform actinomycetes, such as members of the genus *Mycobacterium* and *Rhodococcus* (Heitkamp & Cerniglia, 1988; Walter *et al.*, 1991; Rehmann *et al.*, 1998).

Multiple pathways of pyrene degradation have been offered for the *Mycobacterium vanbaalenii* PYR-1 strain, including typical dioxygenation and monooxygenation (Heitkamp & Cerniglia, 1988; Brezna *et al.*, 2005). Although *Mycobacteria* strains can oxidize pyrene via initial dioxygenation at the 1,2-positions, the metabolite will form *o*-methylated derivatives of pyrene-1,2-diol or the dead end product 4-hydroxy-perinaphthenone. The chief pathway of pyrene degradation is dioxygenation at the 4,5-positions to produce both *cis*- and *trans*-4,5-pyrenedihydrodiol by dioxygenase and monooxygense, respectively. Rearomatization of the dihydrodiol and subsequent *ortho*-cleavage lead to the formation of 4,5-dicarboxyphenanthrene, which is further decarboxylated to 4-phenanthroate. Following another dioxygenation reaction, 4-phenanthroate forms *cis*-3,4-dihydroxyphenanthrene-4-carboxylate. Rearomatization of the metabolite yields 3,4-dihydroxyphenanthrene, which is further metabolized to 1-hydroxy-2-naphthoate. The subsequent enzymatic reactions, including intradiol ring cleavage dioxygenation, result in the production of *o*-phthalate. Then phthalate is further transformed to TCA cycle via the β-ketoadipate pathway (Wang *et al.*, 2000; Kim *et al.*, 2003) (Fig. 6).

Based on both genomic and proteomic data, Kim *et al.* have identified 27 enzymes necessary for constructing a complete pathway for pyrene degradation. Proteomic analysis also reveals that 18 enzymes in the pathway are upregulated more than twofold, as indicated by peptide counting when the organism was grown with pyrene. There are four ring-hydroxylating steps catalyzed by the corresponding dioxygenase NidAB2, MvanDraft_0817/0818, PhtAaAb, and NidA3B3. The electron transfer components for these four steps have been found: ferredoxin (PhtAc) and ferredoxin reductase (PhtAd). This gene arrangement of the β- and α-subunits of dioxygenase NidAB2 has rarely been found in other bacteria. NidA and NidB proteins are 40-56% similar to the corresponding isofunctional enzymes in the *Nocardioides* sp. strain KP7 and *Pseudomonas* sp. strain NCIMB12038. Three copies of the terminal subunits of ring-hydroxylating oxygenase (NidAB2, MvanDraft_0817/0818, and PhtAaAb), dihydrodiol dehydrogenase (MvanDraft_0815), and ring cleavage dioxygenase (MvanDraft_3242) were induced in pyrene-grown cells. However, no ORFs for enzymes such as 1-hydroxy-2-naphthoate hydroxylase, which is necessary for 1-hydroxy-2-naphthoate degradation through salicylate, were detected. ORFs for phthalate 4,5-dioxygenase and protocatechuate-4,5-

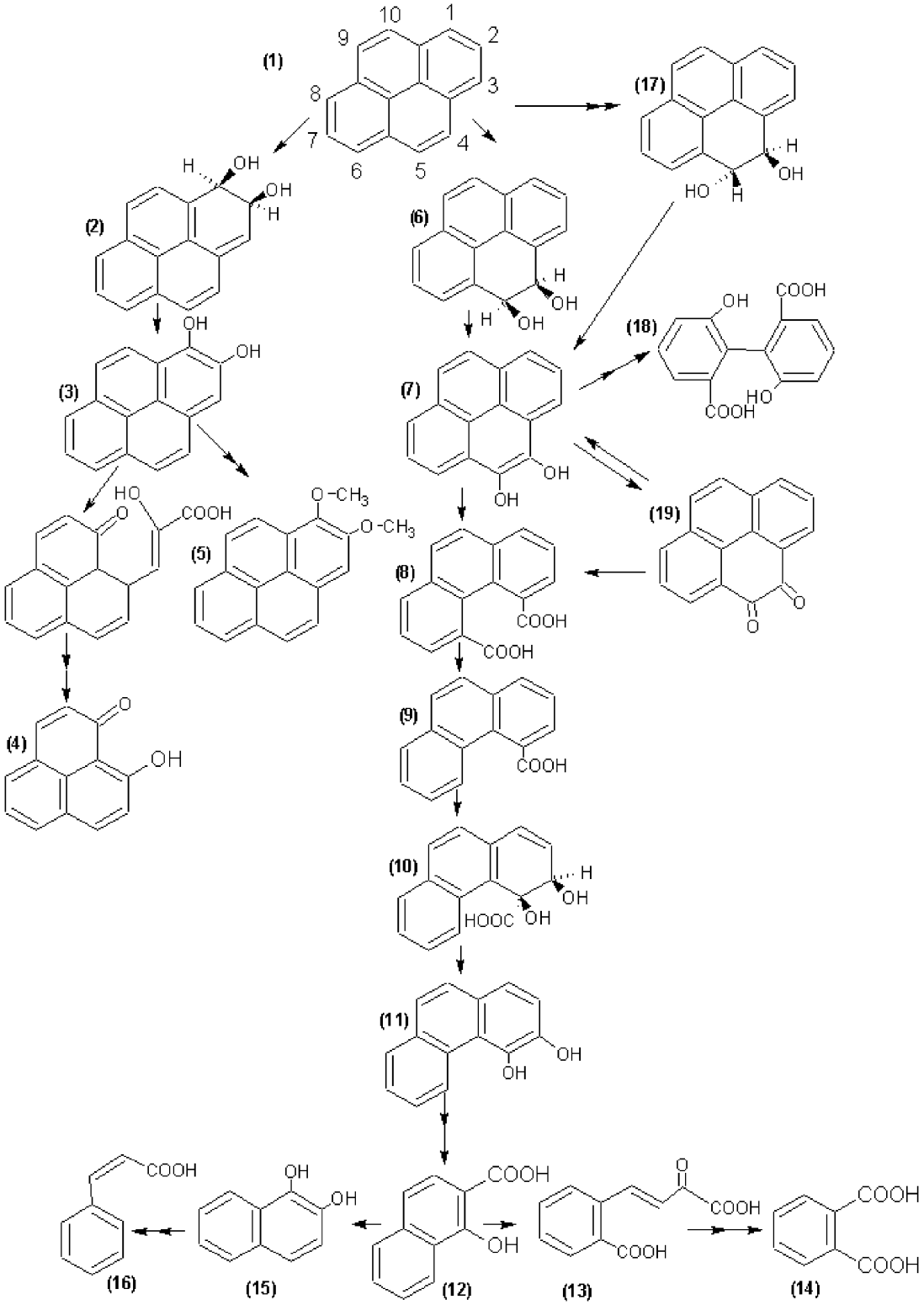

Fig. 6. Proposed catabolic pathways of pyrene by aerobic bacteria. The compounds are pyrene (1), *cis*-1,2-dihydroxy-1,2-dihydropyrene (2), 1,2-dihydroxy pyrene (3) 4-hydroxyperinaphthenone (4), 1,2-dimethoxypyrene (5), *cis*-4,5-dihydroxy-4,5-dihydropyrene (6), P2, 4,5-dihydroxypyrene (7), phenanthrene-4,5-dicarboxylate (8), phenanthrene-4-carboxylate (9), *cis*-3,4-dihydroxyphenanthrene-4-carboxylate (10), 3,4-dihydroxyphenanthrene (11), 1-hydroxy-2-naphthoate (12), *trans*-2′-carboxybenzalpyruvate (13), phthalate (14), 1,2-dihydroxynaphtharene (15), cinnamic acid (16) *trans*-4,5-dihydroxy-4,5-dihydropyrene (17), 6,6′-dihydroxy-2,2′-biphenyl dicarboxylic acid (18), and pyrene-4,5-dione (19).

dioxygenase, which are found in the optional routes for oxidation of phthalate and the meta cleavage of protocatechuate, respectively, were also not detected in the genome sequence (Kim *et al.*, 2004a, 2007).

Besides the *M. vanbaalenii* PYR-1 strain, many other *Mycobacterium* sp. strains were identified and found to mineralize pyrene. Most of the PAH-degrading mycobacteria described are fast-growing species within the genus. The mycolic acid-rich cell walls of these soil bacteria may be an important factor in their utilization of hydrophobic substrates such as PAHs.

The *Mycobacterium* sp. strain RJGII-135 is capable of degrading a wide range of PAHs. Mineralization studies using [^{14}C]pyrene added to contaminated soils, with and without the addition of this strain, indicated that this strain was able to grow on pyrene as its sole source of carbon and energy. During the mineralization of pyrene, three steady intermediates were formed within 4–8 h, which included two identified metabolites in strain PYR-1 – 4-phenanthrenecarboxylic acid and 4,5-pyrenedihydrodiol – and one special product only in the strain RJGII-135 – 4,5-phenanthrenedicarboxylic acid (Schneider *et al.*, 1996).

The *Mycobacterium* sp. strain KMS was isolated from vadosezone soil at the Champion International Superfund site (Libby, MT), and is able to degrade pyrene and other PAHs. It was determined from 2-dimensional gel electrophoresis that the first aromatic ring hydroxylating dioxygenase that oxidizes pyrene to *cis*-4,5-pyrene-dihydrodiol includes the α and β subunits, 4Fe–4S ferredoxin, and the Rieske (2Fe–2S) region, which were all induced with the addition of pyrene to the cultures. Other proteins that further pyrene degradation, such as dihydrodiol dehydrogenase, oxidoreductase, and epoxide hydrolase, were also found to be notably induced with the addition of pyrene. There were five different β-subunits and two different Rieske (2Fe–2S) regions of the α-subunit of aromatic ring hydroxylating dioxygenase, indicating that there are multiple copies of dioxygenase expressed during pyrene degradation. Pyrene-4,5-dione was identified to be a pyrene degradation metabolite of the *Mycobacterium* sp. strain KMS. Pyrene-4,5-dione can then be degraded into two major intermediates – phenanthrene-4,5-dicarboxylic acid and 4-phenanthroic acid – by a flavoprotein-like, FAD-dependent oxidoreductase. Pyrene-4,5-dione can also be reduced back to 4,5-dihydroxypyrene by quinone reductase, as reported for *M. vanbaalenii* PYR-1 (Liang *et al.*, 2006).

The *Mycobacterium* sp. strain AP1 was isolated from a pyrene enrichment culture and can grow with pyrene as its sole source of carbon and energy. However, the strain cannot remove the substrate completely, and its growth yield suggests an assimilation of about 10% of the pyrene carbon. A novel metabolite identified as 6,6-dihydroxy-2,2-biphenyl dicarboxylic acid reveals a new branch in the pathway that involves dioxygenation on 4,5 positions and 9,10 positions and cleavage of both central rings of the pyrene (Vila *et al.*, 2001).

The *Mycobacterium* sp. strain KR2 was isolated from PAH-contaminated soil originating from the area of a former gaswork plant. This isolate metabolized up to 60% of the pyrene added (0.5 mg mL^{-1}) within 8 days at 20 °C. Similar to pyrene degradation by strain PYR-1, pyrene metabolism in the KR2 strain seems to be controlled by metabolite induction. With the exception of 2-carboxybenzaldehyde and *cis*-3,4-phenanthrene dihydrodiol-4-carboxylic acid, all pyrene metabolites produced by strain KR2 have been detected in cultures of other pyrene-degrading bacteria. The formation of 4-phenanthrol from *cis*-3,4-phenanthrene dihydrodiol-4-carboxylic acid should be sterically favored under the assumption that the bulky carboxy group is extruded from the bay region, thus occupying a pseudoaxial position necessary for elimination (Rehmann *et al.*, 1998).

The *Mycobacterium* sp. strain BB1 was isolated from a past coal gasification site. Exponential growth on pyrene was observed over a 35-fold increase of CO_2 evolution in the fermentor. In addition, some nonsubstrate and harmless nonionic surfactants were found to enhance the degradation of pyrene (Boldrin *et al.*, 1993; Fritzsche, 1994; Tiehm, 1994).

The *Mycobacterium* sp. strain CH1 was isolated from PAH-contaminated freshwater sediments and could utilize pyrene or a wide range of branched alkanes and *n*-alkanes as its sole carbon and energy sources. A lag phase of at least 3 days was detected when strain CH1 was grown in pyrene; however, this lag phase was decreased to < 1 day when phenanthrene or fluoranthene was supplied in the culture media. Because there was no detectable hybridization with the *nahAc* gene, the enzyme system involved in pyrene degradation is unrelated to the naphthalene dioxygenase pathway. Furthermore, weak hybridization of the *P. oleovorans alkB* gene probe to strain CH1 DNA suggests slight homology between the genes involved in alkane oxidation. These observations suggest that the occurrence of both aromatic and aliphatic hydrocarbon-degradative capacities within a single strain may be more universal (Churchill *et al.*, 1999).

The pathway describing the biodegradation of the four-ringed PAH fluoranthene by *M. vanbaalenii* PYR-1 has been discovered recently (Kweon *et al.*, 2007) (Fig. 7). Thirty-seven fluoranthene metabolites including potential isomers were isolated from culture medium and analyzed using HPLC, GC-MS, and UV-visible absorption. Fifty-three enzymes were determined to likely be involved in fluoranthene degradation. Four proposed pathways have been proposed for the degradation of fluoranthene initiated by mono- and dioxygenation reactions. The dioxygenase attack occurs at

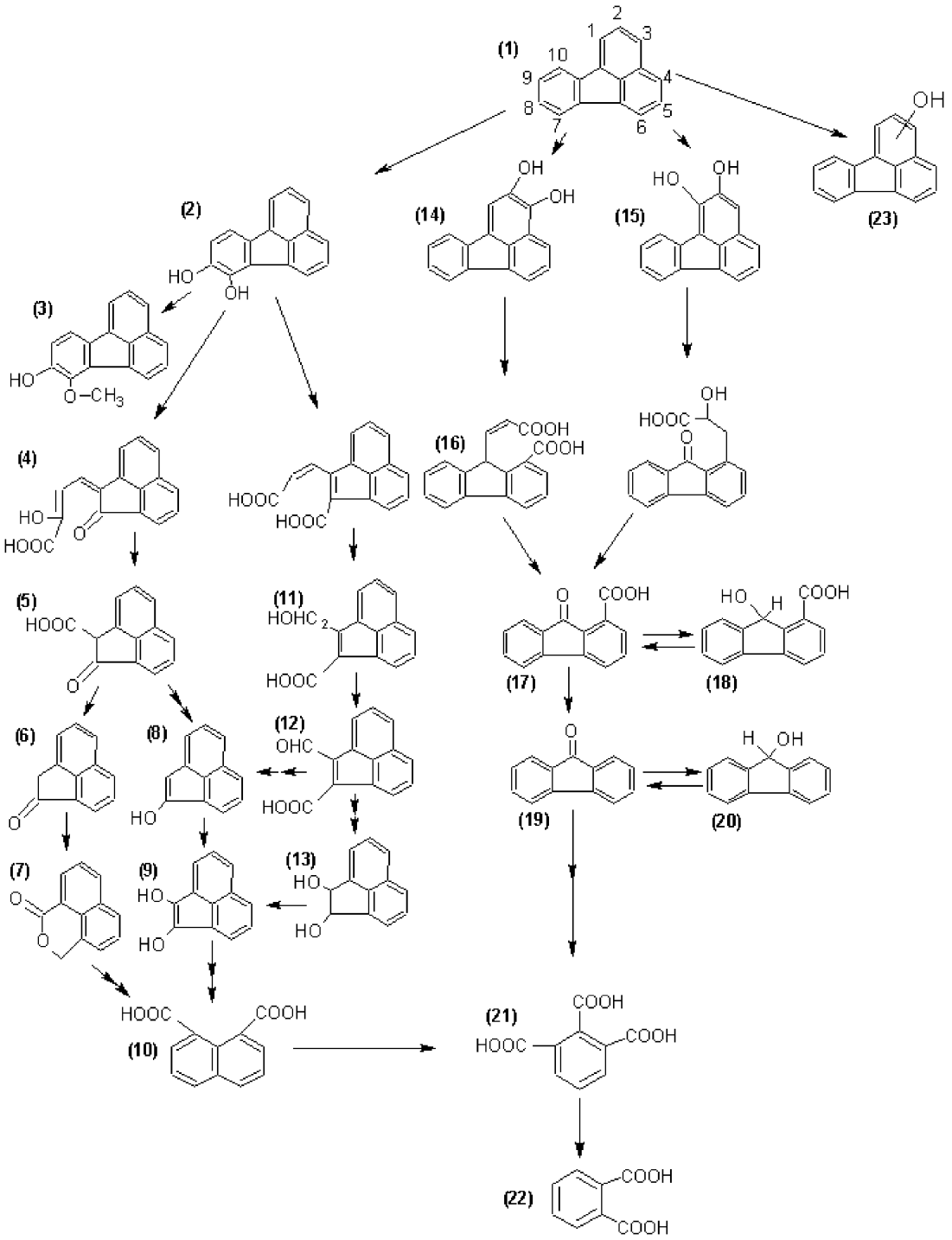

Fig. 7. Proposed catabolic pathways of fluoranthene by aerobic bacteria. The compounds are fluoranthene (1), 7,8-dihydroxy fluoranthene (2), 7-methoxy-8-hydroxy -fluoranthene (3), (2Z, 4Z)-2-hydroxy-4-(2-oxoacenaphthylen-1(2*H*)-ylidene) but-2-enoic acid (4), 1-acenaphthenone-2-carboxylic acid (5), acenaphthylene-1(2*H*)-one (6),1*H*,3*H*-benzo[de] isochromen-1-one (7), acenaphthylen-1-ol (8), acenaphthylen-1,2-diol (9), naphthalene-1,8-dicarboxylic acid (10), 2-(hydroxymethy)-acenaphthylene-1-carboxylic acid (11), 2-formylacenaphthylene-1-carboxylic acid (12), 1,2-dihydroacenaphthylene-1,2-diol (13), 2,3-dihydroxy fluoranthene (14), 1,2-dihydroxy fluoranthene (15), (9E)-9-(carboxymethylene)-9*H*-fluorene-1-carboxylic acid (16), 9-fluorenone-1-carboxylic acid (17), 9-hydroxy-9*H*-fluorene-1-carboxylic acid (18), 9-fluorenone (19), 9-hydroxyfluorene (20), 1,2,3-benzenetricarboxylic acid (21), phthalic acid (24), and monohydroxyfluoranthene (22).

the C-1,2, C-2,3, and C-7,8 positions of the fluoranthene. The C-1,2 and C-2,3 dioxygenation routes degrade fluoranthene via fluorene-type metabolites, whereas the C-7,8 route oxidizes fluoranthene via acenaphthylene-type metabolites by extra- and intradiol ring cleavages. The major site of dioxygenation is the C-2,3 dioxygenation route, which consists of 18 enzymatic steps via 9-fluorenone-1-carboxylic acid and phthalate with the initial ring-hydroxylating oxygenase, NidA3B3, oxidizing fluoranthene to fluoranthene *cis*-2,3-dihydrodiol. Six cytochrome P450 genes, including CYP51, are responsible for monooxygenation of fluoranthene. Then the monooxygenation product is transformed to monohydroxyfluoranthenes in the catalysis of ring-hydroxylating oxygenases and chemical dehydration of *cis*- or *trans*-dihydrodiols (Kweon *et al.*, 2007).

There are only limited studies that document extensive mineralization of PAHs that have more than four rings (Kanaly & Harayama, 2000). Most research is focused on the five-ring benzo[α]pyrene (BαP) because it is one of the most important environmental contaminants, and because its mutagenesis and carcinogenesis that cause ubiquitous xenobiotics are well recognized (Kalf & Crommentuijn, 1997; NTP, 2002). All reported BαP oxidization by bacteria has occurred when the bacteria were grown on other substrates, which could induce microorganisms to produce some enzymes that can degrade BαP. An early observation of BαP biodegradation was its oxidation to dihydrodiols by *S. yanoikuyae* B8/36 (formerly *Beijerinckia* sp. strain B8/36), a strain that can be induced with biphenyl, *m*-xylene, or salicylate. However, no ring cleavage products were detected (Gibson *et al.*, 1975; Mahaffey *et al.*, 1988; Gibson, 1999). Schneider *et al.* (1996) identified *cis*-7,8-dihydrodiol from growing cultures of the *Mycobacterium* sp. RJGII-135 strain where pyrene was used to maintain PAH degradation. It was suggested that the *Mycobacterium* sp. RJGII-135 strain was capable of transforming BαP to initial ring oxidation and ring cleavage products by enzymatically attacking at the C-4,5, C-7,8, and/or C-9,10 position (Schneider *et al.*, 1996). When grown in a mixture of yeast extract, peptone, and soluble starch, *M. vanbaalenii* PYR-1 can biotransform 0.5 mg BαP L^{-1} to 24.7% aqueous and organic-extractable metabolites. This strain was also shown to slightly degrade BαP in a six-component PAH mixture. Recently, Moody used resting *M. vanbaalenii* PYR-1 cultures induced with phenanthrene to produce several dihydrodiols and one ring-cleavage product, 10-oxabenzo[def]chrysene-9-one, from benzo[α] pyrene (Moody *et al.*, 2003, 2004).

The *M. vanbaalenii* strain PYR-1 initially oxidized BαP with dioxygenases and monooxygenases at C-4,5, C-9,10, and C-11,12 (Fig. 8). It was determined from the metabolites separated by reversed-phase HPLC and characterized by UV-visible, mass, NMR, and circular dichroism spectral analyses that the major intermediates of benzo[α]pyrene metabolism were benzo[α]pyrene *cis*-4,5-dihydrodiol, benzo[α]pyrene *cis*-11,12-dihydrodiol, benzo[α]pyrene *trans*-11,12-dihydrodiol, 10-oxabenzo-[*def*] chrysen-9-one, and hydroxymethoxy and dimethoxy derivatives of BαP. The reaction of dioxygenation in the K region (4,5 positions) to form the *cis*-dihydrodiol and *ortho*-cleavage to form chrysene 4,5-dicarboxylic acid are consistent with phenanthrene and pyrene degradation. The *ortho*-ring fission products 4-formylchrysene-5-carboxylic acid and 4,5-chrysene-dicarboxylic acid and a monocarboxylated chrysene product were formed when replacement culture experiments were conducted with benzo[α]pyrene *cis*-4,5-dihydrodiol. Chiral stationary-phase HPLC analysis of the dihydrodiols indicated that benzo[α]pyrene *cis*-4,5-dihydrodiol had 30% 4S,5R and 70% 4R,5S absolute stereochemistry. The initial dioxygenation of BαP at the C-9 and C-10 positions yields benzo[α]-pyrene *cis*-9,10-dihydrodiol. Dehydration of the dihydrodiol to the dihydroxy intermediate with subsequent *meta*-cleavage and aromatic-ring closure may lead to the formation of 10-oxabenzo-[*def*] chrysen-9-one. The PYR-1 strain also oxidizes BαP to *cis*- and *trans*-11,12-dihydro-11,12-dihydroxybenzo[α]pyrene, which then form *cis*- and *trans*-dihydrodiols. Benzo[α]pyrene *cis*-11,12-dihydrodiol adopted an 11S,12R conformation with 100% optical purity. The enantiomeric composition of benzo[α]pyrene *trans*-11,12-dihydrodiol was an equal mixture of 11S,12S and 11R,12R molecules. The identification of the *cis*- and *trans*-dihydrodiols is an indication that dioxygenation and monooxygenation reactions occurred in naphthalene, anthracene, phenanthrene, and pyrene degradation. The formation of the benzo[α]pyrene *trans*-11,12-dihydrodiol was probably due to the oxidation by cytochrome P450 to form benzo[α]pyrene-11,12-epoxide and the successive hydrolysis of the latter by epoxide hydrolase (Moody *et al.*, 2004).

Additional experiments with [^{14}C]7-benzo[α]pyrene demonstrated that 3.8% mineralization of BαP will take place by the *S. yanoikuyae* JAR02 strain grown in the presence of both succinate (2.0 mM) and salicylate (2.5 mM) over 7 days. Benzo[α]pyrene-*cis*-4,5-dihydrodiol and benzo[α]pyrene-*cis*-7,8-dihydrodiol were observed using HPLC/MS. Further characterization of the radio-labeled metabolite using HPLC/MS and HPLC/MS/MS identified radio-labeled pyrene-8-hydroxy-7-carboxylic acid and unlabeled pyrene-7-hydroxy-8-carboxylic acid as novel ring-cleavage metabolites. The similar chirality of these metabolites suggested that the enzymatic attack position will be at C-7,8 and C-9,10 (Rentz *et al.*, 2008).

Fungi catabolism of polycyclic aromatic hydrocarbons

Several studies have shown that diverse fungi are capable of PAH mineralization. These fungi can be classed into two

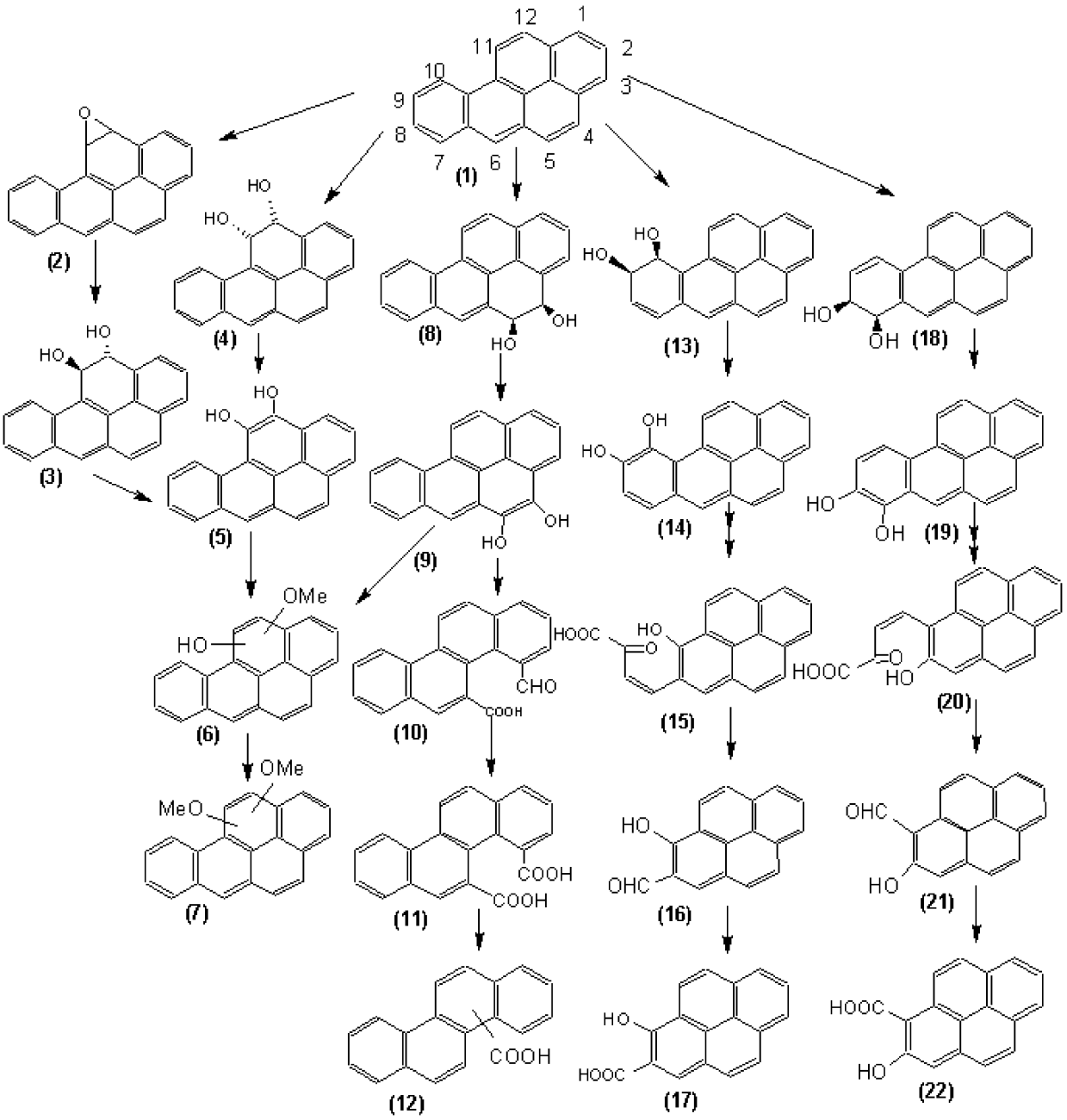

Fig. 8. Proposed catabolic pathways of benzo[α]pyrene by aerobic bacteria. The compounds are benzo[α]pyrene (1), benzo[α]pyrene-11,12-epoxide (2), *trans*-benzo[α]pyrene -11,12-dihydrodiol (3), *cis*-benzo[α]pyrene-11,12-dihydrodiol (4), 11,12-dihydroxy-benzo[α]pyrene (5), hydroxymethoxybenzo[α]pyrene (6), dimethoxybenzo[α]pyrene (7), *cis*-benzo[α]pyrene-4,5-dihydrodiol (8), 4,5-dihydroxy-benzo[α]pyrene (9), 4-formylchrysene-5-carboxylic acid (10), 4,5-chrysene-dicarboxylic acid (11), chrysene-4(5)-carboxylic acid (12), *cis*-benzo[α]pyrene-9,10-dihydrodiol (13), 9,10-dihydroxybenzo[α]pyrene (14), *cis*-4-(8-Hydroxypyrene-7-yl)-2-oxobut-3-enoic acid (15), pyrene-8-hydroxy-7-aldehyde (16), pyrene-8-hydroxy-7-carboxylic acid (17), *cis*-benzo[α]pyrene-7,8-dihydrodiol (18), 7,8-dihydroxy-benzo[α]pyrene (19), *cis*-4-(7-hydroxypyrene-8-yl)-2-oxobut-3-enoic acid (20), pyrene-7-hydroxy-8-aldehyde (20), and pyrene-7-hydroxy-8-carboxylic acid (22).

groups: ligninolytic and nonligninolytic fungi (Cerniglia, 1997). At least two mechanisms are involved in PAH biodegradation: one uses the cytochrome P-450 system and the other uses the soluble extracellular enzymes of lignin catabolism, including lignin peroxidase (LiP), manganese peroxidase (MnP) and laccases (Tortella & Diez, 2005). These enzymes are nonspecific and oxidize a wide variety of organic compounds. These enzymes lack selectivity because

lignin contains a variety of aromatic structures formed in plant cell walls by oxygen-radical coupling reactions of 4-hydroxy cinnamyl, 3-methoxy-4-hydroxy cinnamyl and 3,5-dimethoxo-4-hydroxy cinnamyl, resulting in a variety of intermonomer linkages (Hammel, 1992). In nonligninolytic fungi, cytochrome P450 monooxygenase enzymes catalyze the oxidation of PAHs to arene oxides, which are initial products of PAH metabolism.

The extracellular enzymes of lignin catabolism in ligninolytic fungi

Because of their advantage of being able to diffuse to the immobile PAHs, the fungal extracellular enzymes of lignin catabolism appear to be more likely than bacterial intracellular enzymes to make the initial attack on PAHs in soil. White-rot fungi are ubiquitous in nature in their ability to degrade and mineralize recalcitrant plant polymer lignin (Otjen & Blanchette, 1986; Martínez *et al.*, 2005). Several reports have shown that white-rot fungi are capable of degrading PAHs and have potential in the bioremediation of PAH-polluted soils and sediments. Large quantities of mycelia of several species of white-rot fungi are used to increase the extent of PAH bioremediation in soil. The best-characterized strain – *Phanerochaete chrysosporium* – can oxidize pyrene, anthracene, fluorine and benzo[α]pyrene to the corresponding quinines by lignin peroxidase and manganese peroxidase (Bogan *et al.*, 1996a, b). In contrast to *P. chrysosporium*, the metabolism of PAHs with *Phanerochaete laevis* is faster and more extensive (Bogan & Lamar, 1996).

Lignin peroxidase and manganese peroxidase can catalyze the oxidation of the recalcitrant nonphenolic lignin to form a high redox potential oxo-ferryl intermediate during the reaction of the heme cofactor with hydrogen peroxide (H_2O_2) (Jensen *et al.*, 1996; Gold *et al.*, 2000; Pérez *et al.*, 2002) (Fig. 9a). Two features in the molecular structure confer ligninolytic peroxidases with distinctive catalytic properties: (1) a heme group, conferring high redox potential to the oxo-ferryl complex and (2) the existence of specific-binding sites for oxidation of their characteristic substrates, including nonphenolic aromatics in the cases of LiP, manganous iron in the case of MnP (Reddy & D'souza, 1994; Reddy *et al.*, 2003; Martínez *et al.*, 2005).The heme group is sandwiched between an N-terminal and a C-terminal helix and rests at the bottom of the space formed by the surfaces of both structures. The iron coordination and the residues involved in the active site are conserved among most peroxidases. The iron is pentacoordinated to the four pyrrole nitrogens of the heme and to the nitrogen in the imidazole group of the proximal histidine. Another distal histidine, assisted by an asparagine residue, takes part in the transfer of the oxidizing equivalents from H_2O_2 to the heme. It is suggested that the length of the Fe–imidazolic

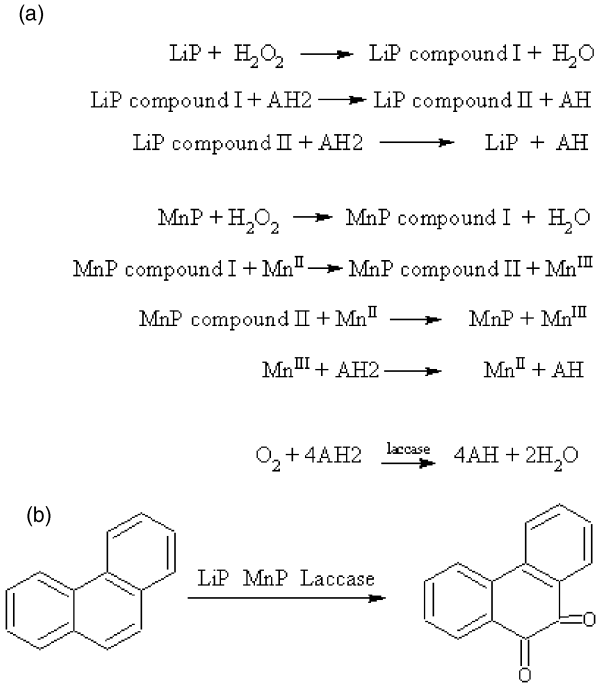

Fig. 9. Catalysis reaction of polycyclic aromatic hydrocarbons by the fungal lignin peroxidase, manganese peroxidase, and laccase. (a) The mechanism of reaction is described in equation. AH2 is the reducing substrate. AH is the reducing substrate after one electron oxidation. (b) Oxidation of polycyclic aromatic hydrocarbons by ligninolytic fungi. Lignin peroxidase, manganese peroxiase, and laccase are capable of oxidizing PAHs to the corresponding quinines.

nitrogen (Fe–Nε2) bond is related to the redox potential of the different peroxidases (Piontek *et al.*, 1993; Poulos *et al.*, 1993; Sundaramoorthy *et al.*, 1997). Laccases catalyze a one-electron oxidation concomitantly with the four-electron reduction of molecular oxygen to water using a range of phenolic compounds as hydrogen donors (Fig. 9a). The catalysis is carried out by the presence of different copper centers, which were arranged in a trinuclear cluster with one type-1 (T1), one type-2 (T2) and two type-3 (T3) copper ions. The presence of the four cupric ions, each co-ordinated to a single polypeptide chain, is an absolute requirement for optimal activity (Messerschmidt, 1997; Solomon *et al.*, 2001; Baldrian, 2006).

Various researches have revealed that the mechanism of oxidation of PAHs by fungi ligninolytic enzymes is similar to the degradation of nonphenolic lignin. A correlation has been found between the ionization potential (IP) of PAHs and the specific activity of MnP and LiP. Aromatic substrates with a lower IP were oxidized by the two ligninolytic enzymes and the lower the IP, the faster the oxidation rate. A threshold value of IP was found for each enzyme: LiP oxidizes PAHs with an IP $\leq$ 7.55 eV (Vazquez-Duhalt *et al.*, 1994), while MnP oxidizes PAHs with an IP up to 8.2 eV. For

example, MnP isolated from *Irpex lacteus* was able to efficiently degrade three- and four-ringed PAHs with an IP higher than 7.8 eV (Bogan & Lamar, 1995; Bogan *et al.*, 1996a, b). Despite the possibility of direct electron transfer from the aromatic substrate to the heme environment, long-range electron transfer has also been detected in LiP from *P. chrysosporium* (Schoemaker, 1994). Thus, extracellular LiPs can directly oxidize condensed PAHs to produce unstable aryl cation radicals, whereas MnPs catalyze the oxidation of PAHs in a reaction that requires Mn^{2+}, oxygen, and unsaturated lipids (Baborová *et al.*, 2006; Eibes *et al.*, 2006). A novel manganese–lignin peroxidase (MnLiP) hybrid enzyme produced by *Bjerkandera adusta* can efficiently oxidize Mn(II) to Mn(III) and can also carry out Mn(II)-independent activity on aromatic substrates (Wang *et al.*, 2003). The role of laccase in the oxidation of nonphenolic lignin structures is similar to LiP. All laccases can be divided into three groups according to their redox potential (E°) at the T1 copper site (Cambria *et al.*, 2008). It has been observed that the different activities of various laccases toward substrates can mainly be attributed to differences in their redox potential. PAHs show E° higher than that of laccase (500–800 mV), and so the PAH degradation by laccase needs to be aided by small mediators (Johannes *et al.*, 1996; Majcherczyk *et al.*, 1998). The most common mediators used are 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (Bourbonnais *et al.*, 1995) and –N–OH-type mediators such as 1-hydroxybenzotriazole (HBT) and violuric acid (VA). The E° of ABTS, HBT, and VA are 1.09, 1.12, and 0.91 V, respectively (Xu *et al.*, 2000, 2001). A greater ΔE° could create a more favorable transitional energy state for the interaction between molecular orbitals of the mediator and the enzyme, resulting in faster electron transfer. Laccase efficiently oxidized [^{14}C]phenanthrene in the presence of HBT and Tween 80. 73% of initially added phenanthrene was degraded within 182 h to yield phenanthrene-9,10-quinone and 2,2′-diphenic acid as the major products (Fig. 9b). The addition of the redox mediator ABTS to the reaction mixture increased oxidation of phenanthrene by *c.* 40% (Han *et al.*, 2004). However, the use of these synthetic mediators implies high added costs and the possible generation of toxic oxidized species. Some of the natural compounds, such as ρ-coumaric acid, strongly promoted the removal of PAHs by laccase. This compound was a better laccase mediator than ABTS and was similar to HBT, attaining 95% removal of anthracene and benzo[α]-pyrene and around 50% removal of pyrene within 24 h (Cañas *et al.*, 2007).

The cytochrome P450 system

The biological functions of cytochrome P450 monooxygenases, such as detoxification of xenobiotics and steroidogenesis, are based on its ability to catalyze the insertion of oxygen into a wide variety of compounds. It was well known that mammalian P450 plays key roles not only in the detoxification of PAHs but also in the activation of PAHs (Shimada, 2006). The pathways leading to the formation of PAH diol-epoxides have been extensively studied in rat liver P450s and epoxide hydrolase using BαP as a prototype (Fig. 10a). Traditionally, a K-region oxide, B[α]P-4,5-oxide, was thought to be an oxidation product of BαP by P450s. The metabolite was found to be readily hydrolyzed to an inactive B[α]P-4,5-diol by epoxide hydrolase (Conney, 1982). Subsequent examination of several BαP metabolites revealed that CYP1A1 and CYP1B1 enzymes can activate PAHs to toxic and carcinogenic metabolites in collaboration with epoxide hydrolase by the formation of B[α]P-7,8-diol, which were again oxidized to the highly reactive bay region oxides, B[α]P-7,8-diol-9,10-epoxide (Levin *et al.*, 1976; Pelkonen & Nebert, 1982; Shimada & Fujii-Kuriyama, 2004).

Fungi metabolize PAH compounds to metabolites similar to those formed by mammalian enzymes. The predominant pathway in the initial oxidation of PAHs is a cytochrome P450 monooxygenase/epoxide hydrolase that catalyzes the reaction that forms *trans*-dihydrodiols (Mueller *et al.*, 1995; Cerniglia, 1997) (Fig. 10b). These metabolic steps are found in nonligninolytic fungi, such as *Cunninghamella elegans*, and ligninolytic fungi, such as *Pleurotus ostreatus*. For example, *C. elegans* metabolized fluoranthene into fluoranthene *trans*-2,3-dihydrodiol, 8- and 9-hydroxyfluoranthene *trans*-2,3-dihydrodiol (Tortella & Diez, 2005), whereas *P. ostreatus* metabolized pyrene into pyrene *trans*-4,5-dihydrodiol and anthracene into anthracene *trans*-1,2-dihydrodiol and 9,10-anthraquinone (Bazalel *et al.*, 1996a, b). The cytochrome P450 can also oxidize numerous PAHs to phenols that are subsequently conjugated with sulfate, glucoronic acid, or glucose (Cerniglia *et al.*, 1982, 1986; Casillas *et al.*, 1996; Pothuluri *et al.*, 1996). *Cunninghamella elegans* metabolizes benzo[e]pyrene to 10-hydroxy-3-benzo[e]pyrenylsulfate (Cerniglia *et al.*, 1989; Pothuluri *et al.*, 1996) and the fungus *A. niger* SK9317 metabolizes pyrene to 1-pyrenylsulfate and 1-hydroxy-8-pyrenylsulfate (Wunder *et al.*, 1994; Capotorti *et al.*, 2005).

The fungus cytochrome P450 monooxygenases are a superfamily of heme-thiolate proteins that are associated with membranes in the endoplasmic reticulum (Degtyarenko, 1995; Yadav *et al.*, 2003). Whole-genome sequencing of the white-rot basidiomycete *P. chrysosporium* has revealed the presence of the largest P450 monooxygenases in fungi comprising *c.* 150 P450 genes, which could be classified into 12 families and 23 subfamilies and under 11 fungal P450 clans. The amino acid sequence of these P450 monooxygenase proteins is extremely diverse, with levels of identity as low as 16% in some cases, but their structural fold has

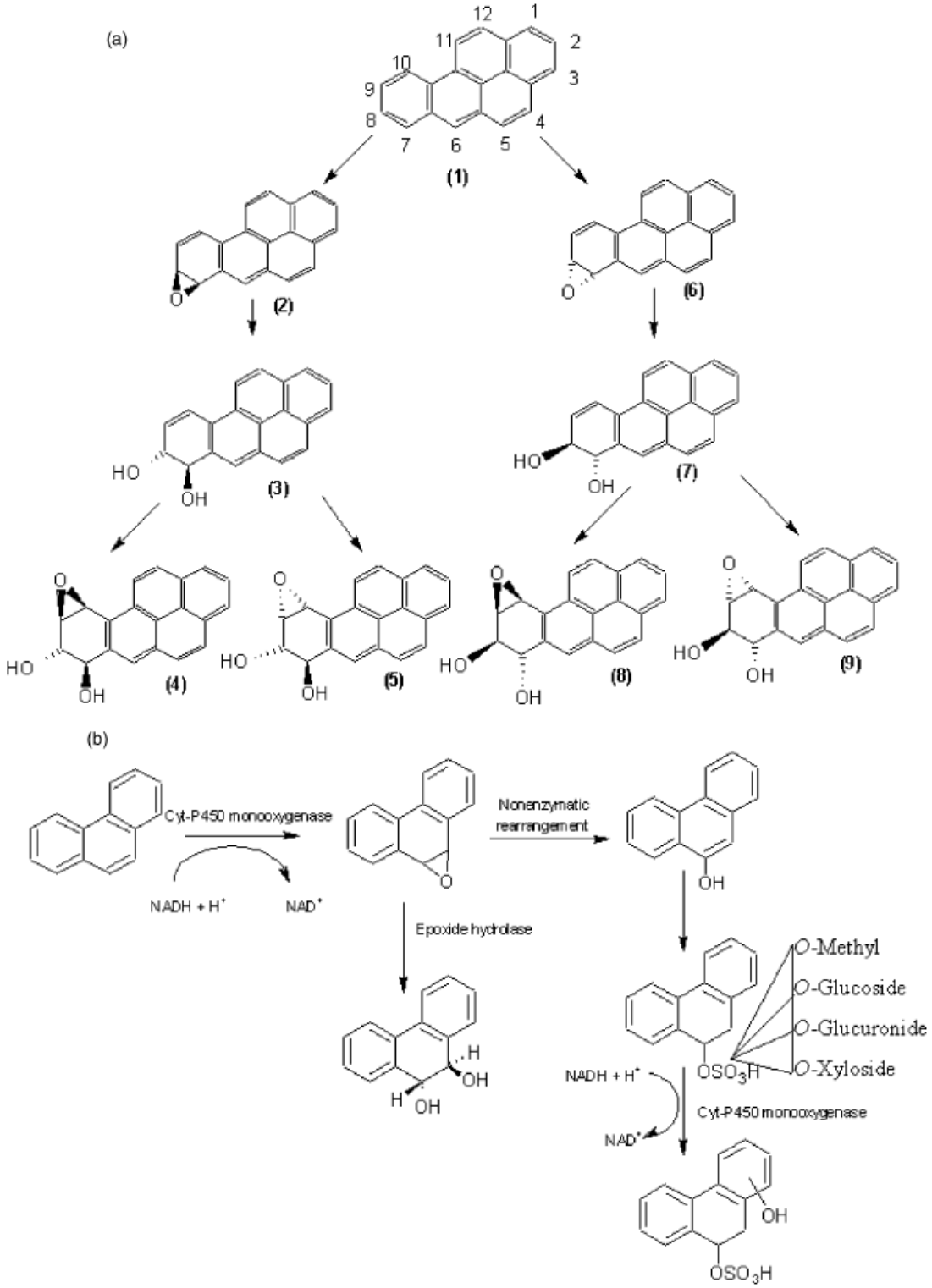

Fig. 10. Similar metabolic pathways of PAHs by mammals and fungi P450s monooxygenase. (a) Proposed metabolic pathways for activation of benzo[α]pyrene by mammalian P450s. The compounds are benzo[α]pyrene (1), (+)benzo[α]pyrene-7,8-epoxide (2), (−)benzo[α]pyrene-7,8-diol (3), (−)benzo[α]pyrene-7,8-diol-9,10-epoxide-1 (4), (+)benzo[α]pyrene-7,8-diol-9,10-epoxide-2 (5), (−)benzo[α]pyrene-7,8-epoxide (6), (+)benzo[α]pyrene-7,8-diol (7), (−)benzo[α]pyrene-7,8-diol-9,10-epoxide-2 (8), and (+)benzo[α]pyrene-7,8-diol-9,10-epoxide-1 (9). (b) Proposed pathway for phenanthrene degradation by fungi. A metabolic pathway of fungi for PAHs degradation is similar to that of mammals, which is considered to be the formation of diol-epoxides by P450s and epoxide hydrolase. Another metabolic pathway of fungi for PAHs degradation involving: hydroxylation by a monooxygenase, conjugation with sulfate ion (methyl, glucoside, glucuronide, and xyloside), followed by a further hydroxylation to hydroxylarylsulfates (methyl, glucoside, glucuronide, and xyloside) compounds.

remained the same throughout evolution (Rabinovich *et al.*, 2004; Doddapaneni *et al.*, 2005a, b). Under two distinct nutrient conditions, nutrient-limited (ligninolytic) and nutrient-sufficient (nonligninolytic), P450 monooxygenases proved to be a functional divergence in transcriptional profiling. P450 genes in this basidiomycetous fungus showed varying degrees of structural similarities to the P450 genes from different ascomycetous fungi such as *Aspergillus* and *Fusarium*, suggesting that *P. chrysosporium* has acquired these P450 families as a part of a vertical descent from a common ancestor. For instance, the CYP63 family of proteins of *P. chrysosporium*, which consists of seven members (pc-1 through pc-7), shows a structural resemblance to the CYP52 family of ascomycetous yeasts and has, therefore, been classified under the CYP52 clan (Doddapaneni & Yadav, 2004; Doddapaneni *et al.*, 2005a, b; Yadav *et al.*, 2006). All seven genes of the CYP63 family of proteins showed differential transcriptional induction with alkanes and their derivatives (Anzenbacher & Anzenbacherova, 2001). Moreover, their substrate specificity seems to extend beyond alkanes/substituted alkanes as they also showed induction in the presence of various aromatics, including polycyclic aromatic compounds like PAHs. For example, the gene of *pc-1* was induced by mono-aromatics (*m*-hydroxy benzoic acid, nitrophenol, and phenoxy acetic acid), polycyclic aromatic compounds (naphthalene, phenanthrene, pyrene, 3-methylcholanthrene, *p-p′*-biphenol and polychlorinated biphenyl), and alkyl-substituted aromatics (dodecylbenzene sulfonate, 1-phenyl dodecane and limonene). On the other hand, *pc-2* showed no induction in response to mono-aromatic compounds, but could be induced by polycyclic aromatic compounds. *pc-4* showed induction specifically in response to polycyclic aromatic compounds and alkyl-substituted aromatics. *pc-6* showed high levels of induction in response to some polycyclic aromatic compounds (1-naphthol and 1,2-benzanthracene) and several alkyl-substituted aromatic compounds (Yadav *et al.*, 2006).

Under optimized conditions, the ligninolytic enzymes LiP, MnP, and laccase are capable of oxidizing PAHs with an IP < 7.6. However, for PAHs with a higher IP, a cytochrome P450 monoxygenase activity is likely required (Bazalel *et al.*, 1996a, b). For example, the amount of CO_2 released from BαP oxidation decreased by 66% in 10 days of culture in the presence of the inhibitor of cytochrome P450, 1-aminobenzotriazole, suggesting that such an enzyme is involved in BαP degradation by *Fusarium solani* (Rafin *et al.*, 2000). In some fungi that degrade PAHs, extracellular laccase and peroxidase activities were not induced by the presence of PAHs. Moreover, the percentages of degradation did not seem to correlate with the ligninolytic enzyme activities (Gramss *et al.*, 1999; Verdin *et al.*, 2004). In certain fungi, it seems that a wide variety of PAH can be degradated by cytochrome P450 monoxygenase.

Although cytochrome P450-type monooxygenases might be involved in the oxidation of PAHs by several fungi, they oxidize PAHs via a pathway similar to the mammalian enzyme systems to form potent carcinogens – epoxides and dihydrodiols (Mueller *et al.*, 1995; Cerniglia, 1997). On the contrary, peroxidase-mediated extracellular oxidation of PAHs in cultures of white rot fungi results initially in quinines (Bumpus, 1989; Vyas *et al.*, 1994). Because quinones are less toxic than their respective PAH and dihydroxylated metabolites generated by cytochrome P450 monooxygenases, enzymatic oxidation of PAHs by ligninolytic oxidoreductases could be a more useful strategy in detoxification and bioremediation processes.

Degradation of environmental PAH mixtures

Several bacteria have the ability to degrade low- and high-MW PAHs. For example, the *Mycobacterium* sp. AP1 strain, for which pyrene and fluoranthene degradation pathways have been proposed, can also utilize phenanthrene as its sole source of carbon and energy (Vila *et al.*, 2001; López *et al.*, 2006). The kinetics of the disappearance of individual PAHs was in agreement with differences in their respective aqueous solubilities (Volkering *et al.*, 1992; Harms & Bosma, 1997). *Mycobacterium* sp. AP1 showed a significant depletion of naphthalene, phenanthrene, and anthracene when compared with uninoculated controls after 30 days. The decrease in the concentrations of fluoranthene, pyrene and benzo[α]pyrene with respect to the controls was not statistically significant, although typical ring-cleavage products from those compounds could be detected (Vila *et al.*, 2001; López *et al.*, 2007).

It was suggested that the simultaneous bacteria-mediated biodegradation of PAHs dissolving from a PAH mixture was strongly influenced by their different bioavailabilities. Bioavailability is considered to be a dynamic process that is determined by the rate of substrate mass transfer to microbial cells relative to their intrinsic catabolic activity. At high mass transfer rates, the overall biodegradation rate is controlled by the metabolic activity of the bacteria (Koch, 1990; Bosma *et al.*, 1997). The observed disappearance rates and extents of the PAH compounds are in agreement with their aqueous solubilities. When the growth-linked compounds phenanthrene, fluoranthene, and pyrene are all added together to a *Mycobacterium* sp. AP1 strain culture, they will reduce simultaneously and their reduction rates will reach maximum values during the first 168 h. However, the biodegradation rates of fluoranthene and pyrene are significantly lower than that of phenanthrene (López *et al.*, 2007).

Degradation of PAHs in contaminated soil will be also influenced by the diffusional release of PAHs from the

nonaqueous phase liquids (NAPLs) phase. The specific surface area of the NAPLs may negatively influence biodegradation because of kinetic restrictions to the dissolution process (Ortega-Calvo & Alexander, 1994). For bacteria growing on NAPL-dissolved PAHs in aqueous systems, production of bioemulsifiers would generate a larger surface area of the substrates and subsequently a higher substrate flux (Reddy *et al.*, 1982). sphingomonads and *Mycobacteria* growing on NAPL-dissolved PAHs can adhere strongly to the NAPL phase. Consequently, it appears that attachment to and biofilm formation on PAH sources is a widespread mechanism among bacteria to overcome mass-transfer limitations when growing on weakly soluble and strongly sorbed PAHs (Johnsena *et al.*, 2005).

Environmentally derived bacterial isolates often degrade only a narrow range of PAHs, and patterns of simultaneous degradation of PAH mixtures are complex. The observed pattern of utilization of PAHs is not only the result of bioavailability but also of metabolic interactions related to competition and cometabolism effects (Bouchez *et al.*, 1995). Cometabolism is an important feature of the degradation of PAHs. Cometabolism of one kind of PAH could have a synergistic effect increasing the degradation of another. Therefore, cometabolism can widen the range of PAH attacked by a defined strain, especially for the degradation of high-MW PAHs (van Herwijnen *et al.*, 2003a, b). During the incubation, a number of acid metabolites carried out by high-MW PAH-degrading *Mycobacteria* accumulated in the medium. Most of these metabolites were dicarboxylic aromatic acids formed as a result of the utilization of growth substrates (phenanthrene, pyrene, or fluoranthene) by multibranched pathways including *meta* and *ortho*-ring-cleavage reactions: phthalic acid, naphthalene-1,8-dicarboxylic acid, phenanthrene-4,5-dicarboxylic acid, diphenic acid, Z-9-carboxymethylenefluorene-1-carboxylic acid and 6,6′-dihydroxy-2,2′-biphenyl dicarboxylic acid. These partially oxidized compounds produced by one microbial strain could be degraded by others (López *et al.*, 2007).

A number of species of white-rot fungi can cometabolize PAHs with bacteria by improving the compound bioavailability. Fungi cannot utilize any high-MW PAH as their sole carbon and energy source, but can partially degrade these if cultured in a nutrient broth (Sutherland, 1992). Some fungi can oxidize PAHs rapidly to a higher aqueous solubility with their extracellular ligninolytic enzyme systems, resulting in higher rates of mineralization of these metabolites by bacteria. A study carried out with anthracene has confirmed that all detected oxidation products of this compound breakdown by white-rot fungi can be mineralized by indigenous bacteria (e.g. activated sludge) more rapidly than anthracene itself is mineralized (Meulenberg *et al.*, 1997). Addition of indigenous microbial communities to the white-rot fungus *Bjerkandera* sp. strain BOS55 resulted in an initially rapid increase in the level of CO_2 recovery from [^{14}C]BαP and the level of water-soluble label decreased to 16% of initial levels. This demonstrated that some polar metabolites of fungal benzo[α]pyrene oxidation were readily degraded by indigenous microorganisms (Kotterman *et al.*, 1998). Some fungi can use cytochrome P450 to transform PAHs and activate PAHs for further degradation by bacteria. When the nonligninolytic fungus *Penicillium janthinelum* VUO 10 201 and either its bacterial consortium VUN 10 009 or *S. maltophilia* VUN 10 010 were combined in one culture, 25% of the BαP was mineralized to CO_2 by these fungal–bacterial cocultures over 49 days, accompanied by the transient accumulation and disappearance of intermediates. Inoculation of fungal–bacterial cocultures into PAH-contaminated soil resulted in significantly improved degradation of some high-MW PAHs, such as chrysene, benzo[α]anthracene and dibenzo[α,h]anthracene (Boonchan *et al.*, 2000). Thus, it was suggested that PAH degradation in nature is a consequence of sequential breakdown by fungi and bacteria, with the fungi performing the initial oxidation step (Meulenberg *et al.*, 1997; Sack *et al.*, 1997; Kotterman *et al.*, 1998).

With mixtures of two individually degradable PAHs, metabolic competition took place whether cometabolism occurred or not (Bouchez *et al.*, 1995). Competition at the active site of enzymes, in particular the initial oxygenase, has been observed most commonly. For example, the presence of low-MW PAHs, such as phenanthrene and fluorene, can inhibit the degradation of fluoranthene and pyrene (Bouchez *et al.*, 2000; Dean-Ross *et al.*, 2002; Lotfabat & Gray, 2002). A second possible cause of inhibition is the dead-end products that result from cometabolic oxidation of nongrowth substrates, such as *o*-xylene and *p*-xylene, in a *Pseudomonas stutzeri* strain (Barbieri *et al.*, 1993; Stringfellow & Aitken, 1995).

The moisture content, pH value, aeration condition, and availability of nutrients were determined to be the key factors in the bioremediation of contaminated soils (Roling-Wilfred *et al.*, 2002; Kim *et al.*, 2005). At early stages (0–45 days) of the bioremediation process, the addition of KNO_3 and K_2HPO_4 caused significant increases in both the heterotrophic and the PAH-degrading microbial populations, while soil stimulated only by aeration and optimum humidity exhibited smaller increases in the sizes of both populations and a marked delay in the increase in microbial PAH degraders. The proportion of the PAH-degrading microbial population compared with the heterotrophic population not treated with nutrients increased noticeably at 21 and 45 days, and reached 100% at 135 days, while lower proportions were observed for the populations treated with nutrients. Denaturing gradient gel electrophoresis analysis and principal component analysis confirmed that there was a remarkable shift in the composition of the

bacterial community due to both the biodegradation process and the addition of nutrients. At early stages of biodegradation, the *Alphaproteobacteria* group (genera *Sphingomonas* and *Azospirillum*) was the dominant group in all treatments. At later stages, the *Gammaproteobacteria* group (genus *Xanthomonas*), the *Alphaproteobacteria* group (genus *Sphingomonas*), and the *Cytophaga–Flexibacter–Bacteroides* group (*Bacteroidetes*) were the dominant groups that did not receive nutrients, while the *Gammaproteobacteria* group (genus *Xathomonas*), the *Betaproteobacteria* group (genera *Alcaligenes* and *Achromobacter*), and the *Alphaproteobacteria* group (genus *Sphingomonas*) were the dominant groups that received nutrients (Vinas *et al*., 2005). Monitoring the microbial community by terminal restriction fragment length polymorphism (TRFLP) analysis suggests that the aeration change stimulated by soil excavation would cause the change in the microbial community composition. The fragments show several ribotypes considerably increasing in aerobic organisms with a corresponding decrease in anaerobic organisms such as *Clostridia* sp. (Grant *et al*., 2007).

Conclusion

During the past decade, a variety of microorganisms have been isolated and characterized for their ability to degrade different PAHs. Furthermore, many metabolic enzymes for the degradation of different PAHs have been isolated from microorganisms and several novel pathways have been elucidated based on the identification of initial ring oxidation and ring cleavage products. The genes responsible for PAH catabolic pathways are always localized as gene clusters, and some gene clusters have been cloned and sequenced. An analysis of the gene organization and transcription patterns in the clusters of different species and an analysis of the homology of related loci led to the conclusion that genetic exchange and rearrangements played a major part in the evolution of the regions that play a role in PAH degradation. Genes that catalyze PAH degradation are believed to have adaptively evolved in nature by various genetic events. In bacteria, some aromatic ring hydroxylating dioxygenases share high homologies in structure and exhibit very relaxed substrate specificities, whereas some dioxygenases with small differences in their amino acid sequences display major differences in their enzymatic properties, such as substrate range and regiospecificity. In fungi, more than one isoenzyme of extracellular enzymes and P450 monooxygenases was found. These genes offer an insight into the evolution of the catalytic versatility of fungus in the detoxification and biodegradation of a broad range of xenobiotic chemicals.

In the natural environment, PAHs tend to interact with non-aqueous phases and soil organic matter and, as a consequence, become potentially unavailable for microbial degradation. Microorganisms have evolved different strategies to adapt to the PAH compounds present in the contaminated situation. Many microorganisms produce biosurfactants to increase the bioavailability of the poorly available substrates. Many microorganisms show chemotaxis towards PAH compounds, which could lead to improved degradation. Environmentally derived bacterial isolates often degrade only a narrow range of PAHs, and degradation of a PAH mixture becomes visible as a cooperative process involving a consortium of strains with complementary capacities. The ubiquitous coexistence of bacteria and fungi in soil and their catabolic cooperation suggests that fungal–bacterial interactions may be of importance for PAH degradation in the natural environment.

Combing genetic engineering tools such as gene conversion, gene duplication and transposition, we can produce novel strains with desirable properties for bioremediation applications according to the knowledge of PAH degradation by microorganisms. For example, the rates of PAH mineralization can be enhanced when NAH plasmids from different bacteria are hybridized using colony hybridization. Overexpression of the dioxygenase fusion system from the *Mycobacterium* sp. strain PYR-1 in other bacteria might improve its ability to degrade high-MW PAHs. Other technology designed according to the principles of microbial PAH-degradation in soil may also be useful for PAH bioremediation. For example, sequential treatment by bacterial consortia and fungi (e.g. white-rot fungi) or defined fungal–bacterial cocultures are proposed to improve the degradation of PAHs. Salicylate, acting as an inducer of genetic operons, can be used to enhance the degradation of PAH compounds. Addition of small amounts of biosurfactants to the PAH-containing soil may increase the compound's dispersion and bioavailability, whereas it appears to be a good strategy to improve emulsification of NAPL-dissolved PAH in aqueous systems with turbulent mixing.

Acknowledgements

This research was supported by 863 Program (2006AA06Z358; 2006AA10Z117; 2008AA10Z401); Shanghai Subject Chief Scientist (06XD14017); Shanghai Key laboratory and basic research project (06DZ19103-07dz22011); National and Shanghai Natural Science Foundation (06ZR14073-30670179); and Shanghai municipal committee of agriculture (No. 2006-2-1).

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