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Review

Bacterial Degradation and Bioremediation of Polycyclic Aromatic Hydrocarbons

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Abstract

Many polycyclic aromatic hydrocarbons (PAHs) are known to be toxic and carcinogenic for humans, and their contamination of soils and aquifers is of great environmental concern. Some microorganisms, mainly from the genera *Pseudomonas* and *Mycobacterium*, were found to be capable of transforming and degrading PAHs. These abilities may be useful in removal of PAHs from the environment. The successful application of bacteria to the bioremediation of PAH-contaminated sites requires a deeper understanding of how microbial PAH degradation proceeds. In this review, the bacteria involved and the metabolic pathways for the degradation of many PAHs are summarized and the biological aspects of PAH bioremediation are discussed.

Keywords: bacteria, PAHs, metabolic pathways, degradation, bioremediation

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are important pollutants found in air, soil and sediments. These compounds enter the environment in many ways. PAHs and their derivatives are widespread products of incomplete combustion of organic materials arising, in part, from natural combustion such as forest fires and volcanic eruptions, but for the most part by human activities. In recent decades the major source of PAH pollution is industrial production, transportation, refuse burning, gasification and plastic waste incineration.

The fate of polycyclic aromatic hydrocarbons in nature is of great environmental concern due to their toxic, mutagenic, and carcinogenic properties. For example, phenanthrene is known to be a human skin photosensitizer and mild allergen [1]. It has also been found to be an inducer of sister chromatid exchanges and a potent inhibitor of gap-junction intercellular communications [2]. PAHs can sorb to organic-rich soils and sediments, accumulate in fish and other aquatic organisms, and may be transferred to humans through seafood consumption [3].

Because many PAHs are so toxic there is interest in understanding the physicochemical processes and microbial degradation reactions that affect the mobility and fate of these compounds in groundwater and soil sediment system. The biodegradation of PAHs can be considered on one hand to be part of the normal processes of the carbon cycle, and on the other as the removal of man-made pollutants from the environment. The use of microorganisms for bioremediation of PAH-contaminated environments seems to be an attractive technology for restoration of polluted sites.

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Chemical and Physical Properties of Selected PAHs

Polycyclic aromatic hydrocarbons (PAHs) are a group of compounds containing carbon and hydrogen, composed of two or more fused aromatic rings in linear, angular, and cluster arrangements (Fig. 1).

Many PAHs contain a "bay-region" and a "K-region". The bay- and K-region epoxides, which can be formed metabolically, are highly reactive both chemically and biologically. Phenanthrene is the simplest aromatic hydrocarbon which contains these regions. The bay-region of phenanthrene is a sterically hindered area between carbon atoms 4 and 5 and the K-region is the 9, 10 double bond, which is the most oleinic aromatic double bond with high electron density (Fig. 1). According to the Schmidt-Pullman electronic theory, K-region epoxides should be more carcinogenic than the parent hydrocarbon.

Low-molecular weight (LMW) PAHs (two or three rings) are relatively volatile, soluble and more degradable than are the higher molecular weight compounds. High-molecular weight (HMW) PAHs (four or more rings) sorb strongly to soils and sediments and are more resistant to microbial degradation. Because of solid state, high molecular weight and hydrophobicity, expressed as its log P value between 3 and 5, PAHs are very toxic to whole cells [4]. Some properties of selected PAHs are presented in Table 1.

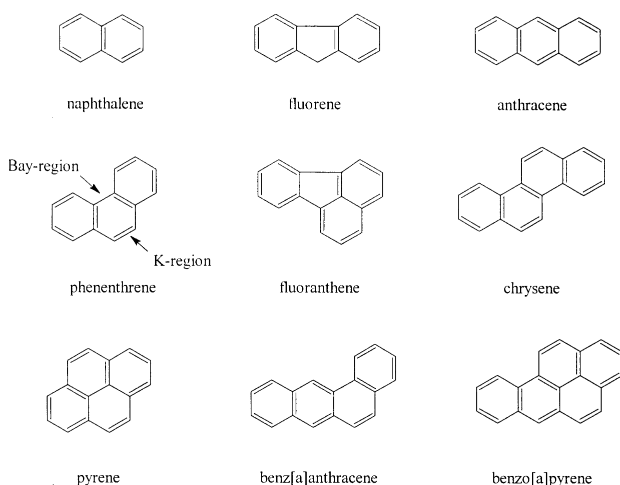

Fig.1. Structures of representative PAHs.

Table 1. Chemical and physical properties of selected PAHs.

Compound	No. C atoms	Molecular weight	Melting point (°C)	Boiling point (°C)	Solubility in water (mg/l)
Naphthalene	10	128.2	80.2	218.0	30.6
Acenaphthalene	12	154.2	96.0	278.0	3.9
Phenanthrene	14	178.2	100.0	339.0	1.2
Anthracene	14	178.2	217.0	340.0	0.7
Pyrene	16	202.26	150.4	393.0	0.145
Fluoranthene	16	202.26	108.8	383.0	0.262
Chrysene	18	228.29	253.8	431.0	0.003

Bacterial Degradation and Transformation of Selected PAHs

Naphthalene

Naphthalene biodegradation is the best studied of the PAHs because it is the simplest and most soluble PAH, and naphthalene-degrading microorganisms are relatively easy to isolate. Bacterial strains that are able to degrade aromatic hydrocarbons have been repeatedly isolated, mainly from soil. These are usually gram-negative bacteria, most of them belong to the genus *Pseudomonas*. The biodegradative pathways have also been reported in bacteria from the genera *Mycobacterium*, *Corynebacterium*, *Aeromonas*, *Rhodococcus,* and *Bacillus* [5-8].

The biochemical sequence and enzymatic reactions leading to the degradation of naphthalene were first presented by Davies and Evans [9]. Further studies have indicated that initially bacteria oxidize naphthalene by incorporating both atoms of molecular oxygen into the aromatic molecule to form *cis*-1,2-dihydroxy-1,2-dihydronaphthalene. Naphthalene dioxygenases from *Pseudomonas* sp. NCIB 9816 and *Pseudomonas putida* ATCC 17 484 act as multicomponent enzyme systems which are responsible for naphthalene *cis*-dihydrodiol formation [5, 10]. These consist of three protein components: a flavoprotein (reductase$_{NAP}$), a two-iron, two sulphur ferredoxin$_{NAP}$, and terminal oxygenase ISP$_{NAP}$. Naphthalene dioxygenase is very unstable and for studying its activity rapid purification is necessary. Terminal dioxygenase has a molecular weight of 158 kD and is composed of two subunits which were established to be 55 and 20 kD. A naphthalene oxygenase has also been isolated from cells of *Corynebacterium renale,* which was able to use naphthalene as a main source of carbon and energy [5]. The enzyme has a molecular weight of about 99 kD and formed *cis*-1,2-dihydroxy-1,2-dihydronaphthalene as a predominant metabolite.

The second step in bacterial oxidation of naphthalene is the conversion of *cis*-1,2-dihydroxy-1,2-dihydronaphthalene to 1,2-dihydroxynaphthalene. This reaction is

catalyzed by naphthalene(+)-*cis*-dihydrodiol dehydrogenase and requires NAD^+ as an electron acceptor. The enzyme has a molecular weight of approximately 102 kD, consists of four subunits of 25,5 kD and is highly stereoselective for (+)-isomer *cis*-1,2-dihydroxy-1,2-dihydronaphthalene. In *Pseudomonas* sp. the next step leads to the enzymatic cleavage of 1,2-dihydroxynaphthalene to *cis*-2-hydroxybenzalpyruvate, which is then converted *via* series of dioxygenases to salicylate and pyruvate. Salicylate is oxidized by salicalate hydroxylase to catechol, which can undergo either *ortho* or *meta* fission depending upon bacterial metabolism [11, 12]. The proposed pathways for naphthalene oxidation by some bacteria from the genus *Pseudomonas* are illustrated in Fig. 2. A similar pathway has been reported for naphthalene metabolism by *Aeromonas* sp. [14]. Different salicylate metabolism has been observed in *Pseudomonas testosteroni*. These bacteria converted salicylate to gentisic acid (Fig. 2). Metabolism of naphthalene by a strain of *Mycobacterium* sp. involves both monooxygenation and dioxygenation with the formation of both *cis*- and *trans*-1,2-dihydrodiols (the ratio of *cis*- to *trans*- diol 25:1). The reaction is catalized by cytochrome P-450 monooxygenase that forms naphthalene 1,2-oxide which is further converted to the *trans*-diol by an epoxide hydrolase enzyme. The reaction scheme is

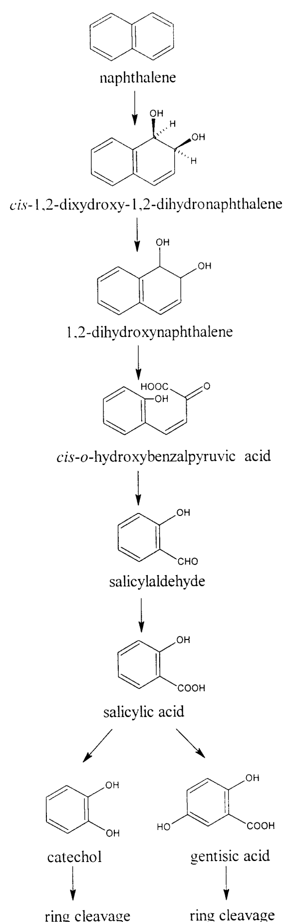

Fig. 2. The proposed pathway for naphthalene oxidation by some bacteria from the genus *Pseudomonas* [5, 13].

common in eukaryotic cells but it was first described in bacteria [6]. A different naphthalene degradation pathway has been described recently in the thermophilic bacterium *Bacillus thermoleovorans*. Apart from typical metabolites of naphthalene degradation known from mesophiles, intermediates such as 2,3-dihydroxynaphthalene, 2-carboxycinnamic acid, phthalic and benzoic acid were identified in the pathway of this bacterium [8].

Many investigations have indicated that genes that encode for naphthalene oxidation in pseudomonads are found on plasmids. There are three known plasmids that determine the degradation of naphthalene: NAH7, NPL1 and pND[13], but the plasmid which has been studied most intensively is NAH7. This plasmid specifies a complete degradative pathway for the degradation of naphthalene, with formation of salicylate as a main intermediate [15]. Plasmid NAH7 is 83-kB, and consists of two clusters, which form *nah*1 and *nah*2 operons (*nah*A-F = *nah*1 and *nah*G-M = *nah*2 = *sal*). The *nah*1 operon includes *nah*A-F genes coding for the conversion of naphthalene to salicylate and the *nah*2(sal) operon includes *nah*G-M coding for the metabolism of salicylate to pyruvate and acetaldehyde. The *sal* operon (56 MD in size) specifies a complete salicylate degradation including a functional *meta* pathway [16].

In contrast *Pseudomonas stutzeri* strain AN10 is a naphthalene-degrading strain whose dissimilatory genes are chromosomally-encoded [17, 18]. Genetic organization of its naphthalene-catabolic pathway is similar to that found in other well-characterized naphthalene-degradation pathways such as the archetypal plasmid NAH7 from *P. putida* G7 [15, 19, 20] and the NAH plasmid pWW60-1 from *P. putida* NCIB9816 [21, 22]. The naphthalene-degradation pathway of *P. stutzeri* AN10 is encoded by 19 genes distributed within two operons: *upper* and *lower* pathways and shows high degrees of similarity of homologous catabolic genes. Such events accelerate the evolution of modern catabolic pathways, providing new genetic material within the environment and resulting in an enhanced natural bioremediation potential.

Fluorene

Recent studies have shown that during fluorene transformation or biodegradation by bacteria several different intermediates occurred. *Pseudomonas* sp. strain F274 produced six major metabolites. Five of them were identified as 9-fluorenol, 9-fluorenone, 1,1-dihydroxy-1-hydro-9-fluorenone, 8-hydroxy-3,4-benzocoumarin and phthalic acid. Identification of 8- hydroxy-3,4-benzocoumarin and phthalic acid suggests that the five-membered ring of the angular diol is opened first and that the resulting 2-carboxy derivative of 2,3-dihydroxy-biphenyl is catabolized by analogous reactions to those of biphenyl degradation, leading to the formation of phthalic acid [23]. Alternatively in some bacteria the initial oxygenation may take place to produce 3,4-dihyxydroxyfluorene followed by extradiol ring fission and further degradation to 3,4- dihydrocoumarin. *Pseudomonas*

cepacia strain F297 degraded fluorene *via* 3,4-dihydroxyfluorene and extradiol fission to 1-indanone as the terminal metabolite [7].

A new fluorene catabolic pathway has recently been found in *Brevibacterium* sp. strain DPO1361 in which hydroxylation at C-9 of fluorene generated 9-fluoroenol, which was then dehydrogenated to 9-fluorenone [24]. This intermediate then undergoes dioxygenation at an angular site to form 1,10-dihydro-11,10-dihydroxyfluorene-9-one, the five-membered ring of which is subsequently cleaved to generate a substituted biphenyl.

Another fluorene degradation pathway by *Staphylococcus auriculans* DBF63 was described by Monna and co-workers [25]. The formation of 9-fluorenol, 9-fluorenone, 4-hydroxy-9-fluorenone, 1-hydroxy-9-fluorenone and accumulation of 1,1-dihydroxy-1-hydro-9-fluorenone was observed when this strain grew on fluorene.

During fluorene degradation by *Arthrobacter* sp. strain F101 new metabolites were identified: 3-hydroxy-1-indanone, 1-indanone, 2-indanone, 3-(2-hydroxyphenyl) propionate and compound tentatively identified as a formyl indanone. 2-indanone may be transformed to the aromatic lactone, 3-isochromanone [26, 27].

Anthracene and Phenanthrene

These tricyclic aromatic hydrocarbons are widely distributed throughout the environment. They have been used as model substrates in studies on the environmental degradation of PAHs, since both structures are found in carcinogenic PAHs such as benzo[a]pyrene, benz[a]anthracene and 3-methylcholanthrene.

Pure cultures and mixed cultures of bacteria isolated from fresh-water and marine environments have the ability to metabolize anthracene and phenanthrene as the sole source of carbon. Anthracene can be completely mineralized by *Pseudomonas*, *Sphingomonas, Nocardia*, *Beijerinckia, Rhodococcus* and *Mycobacterium* with the initial oxygenated intermediate being a dihydriol [28-30]. Apart from *Mycobacterium*, these species oxidize anthracene in the 1,2 positions to form *cis*-1,2-dihydroxy-1,2-dihydroanthracene and then convert it to 1,2-dihydroxyanthracene via NAD^+-dependent dihydrodiol dehydrogenase. In the next step these bacteria oxidize 1,2-dihydroxyanthracene to the ring fission product *cis*-4-(2-hydroxynaphth-3-yl)-2-oxobut-enoic acid with subsequent conversion to 2-hydroxynaphthoic acid. The ring fission product is metabolized to salicylate and catechol through 2,3-dihydroxynaphthalene. Catechol is degraded to simple aliphatic compounds by a similar pathway to catechol conversion in the naphthalene degrading pathway [5, 28]. Various *Mycobacterium* species metabolize phenanthrene at different sites of the molecule, presumably *via* both dioxygenase and monooxygenase attack on the aromatic nucleus. The resulting anthracene *cis*-1,2-dihydrodiol is dehydrogenated to 1,2-dihydroxyanthracene. The accumulation of 1-methoxy-2-hydroxyanthracene provides further evidence for the dioxygenation of anthracene. This is a novel metabolite in anthracene biodegradation studies; however, methylation of a dihydroxylated PAH intermediate was found previously during the metabolism of fluoranthene. The proposed pathway for the degradation of anthracene by *Mycobacterium* sp. PYR-1 is shown in Fig. 3.

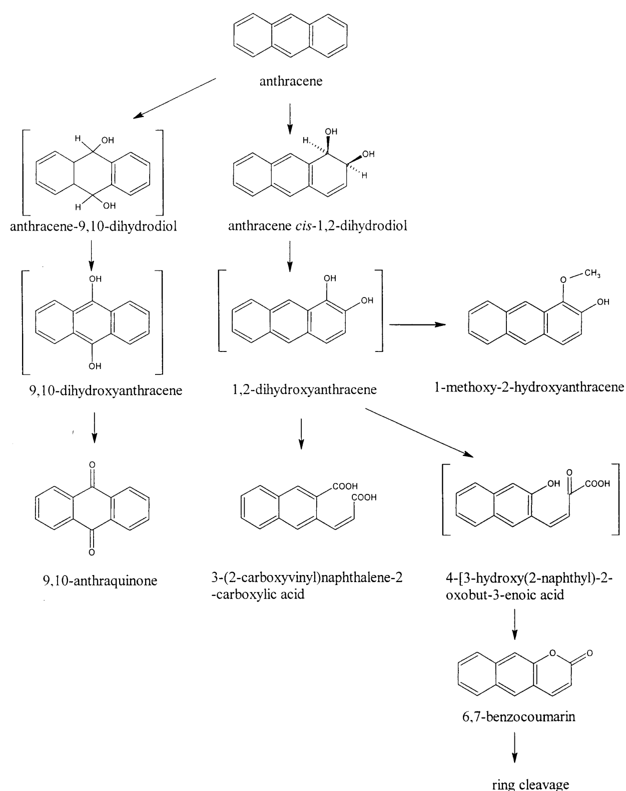

Fig. 3. The proposed pathway for the degradation of anthracene by *Mycobacterium* sp. strain PYR-1 [30].

The pathways of phenanthrene metabolism by bacteria have been reported by several investigators [13, 31-33]. Studies have been conducted on many strains from *Pseudomonas*, *Arthrobacter*, *Aeromonas*, *Sphingomonas*, *Acidovorax*, *Brevibacterium*, *Mycobacterium* and *Nocardia* [5, 14, 34-37].

In general bacteria from the genus *Pseudomonas* initially oxidize phenanthrene in the 1,2 and 3,4 positions to form *cis*-1,2-dihydroxy-1,2-dihydrophenanthrene or *cis*-3,4-dihydroxy-3,4-dihydrophenanthrene. The phenanthrene-*cis*-3,4-dihydrodiol is the predominant isomer which is converted to 3,4-dihydroxyphenanthrene. The ring cleavage product is further metabolized to 1-hydroxy-2-naphthoic acid, which is subsequently oxidatively decarboxylated to 1,2-dihydroxynaphthalene and then subjected to *meta*-cleavage to form salicylic acid [13, 28, 38]. Salicylic acid can also be further degraded *via* the formation of either catechol or gentisic acid. Both catechol and gentisic acid undergo ring fission to form TCA-cycle intermediates [39]. Kiyohara and Nagao [14] found

that a strain of *Aeromonas* and some vibrios utilize an alternative pathway for phenanthrene metabolism. They found that *Aeromonas* strain converted phenanthrene to 1-hydroxy-2-naphthoic acid; however, it was not able to catalyze the decarboxylation of 1-hydroxy-2-naphthoate to 1,2-dihydroxynaphthalene. Alternatively, *Aeromonas* converted 1-hydroxy-2-naphthoate *via* an intradiol cleavage to form *o*-phthalic acid. This was then hydroxylated and decarboxylated to protocatechuate, which underwent either *ortho* or *meta* cleavage to pyruvic acid, which ultimately entered the tricarboxylic acid cycle [40].

A new intermediate, 1-naphthol was detected during utilization of phenanthrene by *Brevibacterium* sp. HL4 and *Pseudomonas* sp. DLC-P11. *Brevibacterium* sp. HL4 degraded phenanthrene *via* 1-hydroxy-2-naphthoic acid, 1-naphthol and salicylic acid, whereas *Pseudomonas* sp. DLC-P11 degraded phenanthrene *via* the formation of 1-hydroxy-2-naphthoic acid, 1-naphthol and *o*-phthalic acid (Fig. 4). Both transformations are novel and have not been reported previously in the literature [36].

Pinyakong *et al.* [37] described and characterized metabolites in the degradation of phenanthrene by the newly isolated phenanthrene utilizing strain *Sphingomanas* sp.

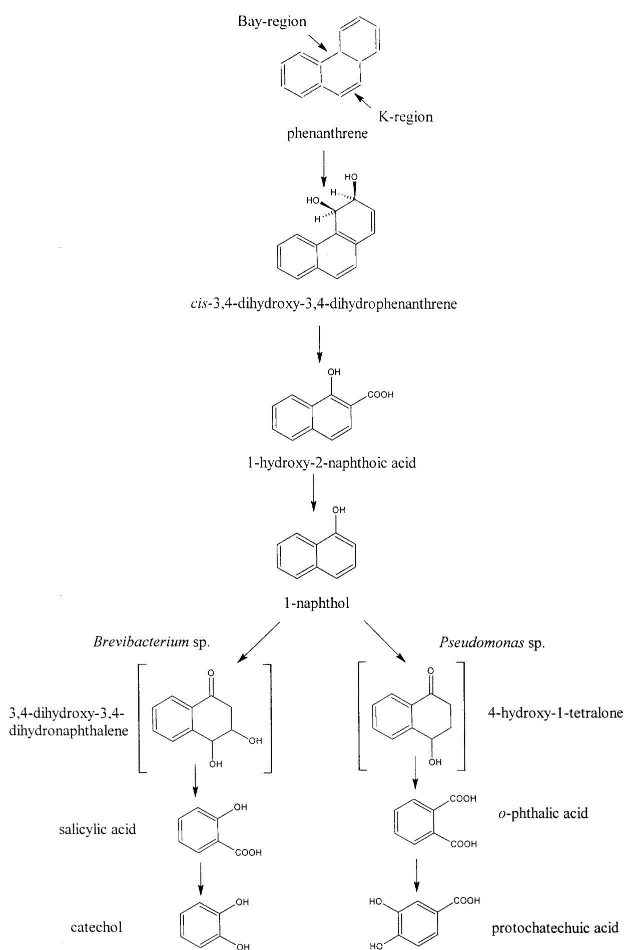

Fig. 4. The proposed pathways for phenanthrene metabolism by *Brevibacterium* sp. HL4 and *Pseudomonas* sp. DLC-P11 [36].

strain P2. They suggested that this strain can degrade phenanthrene *via* dioxygenation at both 1,2 - and 3,4-positions followed by *meta*-cleavage. As ring cleavage products are unstable, *cis*-4-(1-hydroxynaph-2-yl)-2-oxobut-3-enoic acid, *cis*-4-(2-hydroxynaph-1-yl)-2-oxobut-3-enoic acid and *cis*-o-hydroxybenzylidene-pyruvic acid can be readily decarboxylated and oxidized to 7,8-benzocoumarin, 5,6-benzocoumarin and coumarin, respectively.

Results obtained by PCR suggest that PAH-degradation genes are arranged in a polycistronic operon in the chromosome or a plasmid. Phenanthrene degradation genes were found in plasmids in different strains of *Comamonas testosteroni*, *Beijerinckia* sp. and *Alcaligenes facealis* AFK2 [41-43]. Although these plasmids have been found to be involved in phenanthrene degradation it was unable to obtain any cured derivatives. More studies are needed to determine the role and structure of these plasmids in the phenanthrene-degradation pathway. Goyal and Zystra [44] showed that *Comamonas testosteroni* strain has the genes responsible for the degradation of this compound on the chromosome. Using the whole *nah* operon as a probe they did not observe any hybridization signals with total DNA isolated from this strain. These results suggest that the gene sequence was different from that on the *nah* operon.

Other Polycyclic Aromatic Hydrocarbons

The biodegradation of PAHs containing more than three aromatic rings is not as well understood as the utilization of di- and tricyclic aromatic hydrocarbons. This may be due to the large size and extreme insolubility of such PAHs as fluoranthene, benz[a]anthracene, benzo[a]pyrene and pyrene [5, 6]. Special attention has been paid to the initial oxidation of PAHs, enzymes involved in the biodegradation, and the identification of intermediates. Still, little is known about complete metabolic pathways and genetic control of the biodegradation processes.

Fluoranthene

Fluoranthene is mainly degraded by bacteria from the genera *Mycobacterium* and *Alcaligenes.* Degradation by *Mycobacterium* sp. strain PYR-1 involves dioxygenation at both the 1,2- and 7,8 positions, producing fluorene-9-one or acenaphthene-7-one after ring fission. In a strain of *Alcaligenes denitrificans* the latter is further oxidized by an oxydation to 3-hydroxymethyl-3,4-dihydro-benzocoumarin [7]. In another strain *Mycobacterium* sp. KR20 at least seven metabolites were produced during fluoranthene metabolism. Five of them were identified using NMR and MS spectroscopic techniques as: *cis*-2,3-fluoranthene dihydriol, 9-carboxymethylene-fluorene-1-carboxylic acid, *cis*-1,9-dihydroxy-1-hydro-fluorene-9-one-8-carboxylic acid, 4-hydroxybenzochromene-6-one-7-carboxylic acid, and benzene-1,2,3-tricarboxylic acid [45].

Benz[a]anthracene

Pathways for the partial degradation of degradation of benz[a]anthracene has been described for the bacteria *Beijerinckia* and *Mycobacterium*. Initial studies showed that mutant strains of *Beijerinckia* sp. B836 and *Beijerinckia* sp. B1 involved dioxygenation at the 1,2-, 8,9- or 10, 11-positions with production of three distinct diols after induction by growth on biphenyl [5, 46]. The major dihydrodiol isomer formed was *cis*-1,2-dihydroxy-1,2-dihydrobenz[a]anthracene. These diols were further degraded to 1- hydroxy-2-carboxyanthracene or the corresponding phenanthrenes. On the other hand, *Mycobacterium* sp. strain RJGII-135 formed the 5,6-dihydrodiol as the predominant metabolite and secondarily, 8, 9- and 10, 11-dihydrodiols [47].

Pyrene and Benzo[a]pyrene

The bacterial degradation of pyrene, a pericondensed PAH, has been reported by many investigators and some of them have identified metabolites and proposed pathways. Interestingly, mycobacteria have been repeatedly isolated as bacteria that are able to degrade pyrene and benzo[a]pyrene. Although these bacteria are known for their comparatively slow growth, their growth on PAHs is faster than other bacteria: for example, the growth rate of *Mycobacterium* sp. BB1 on pyrene was twice faster than *Rhodococcus* sp. UW1 [48]. *Mycobacterium* sp. mineralized pyrene when grown in mineral salt medium supplemented with organic nutrients. Seven metabolites of pyrene metabolism were detected by HPLC [48]. Three products of ring oxidation, *cis*-4,5-pyrenedihydrodiol, *trans*-4,5-pyrenedihydrodiol and pyrenol, and four products of ring fission 4-hydroxyperinaphthenone, 4-phenanthroic acid, phthalic acid and cinnamic acid were identified by MS, NMR and GC. 4-phenanthroic acid was the major product. The detection of both *cis*- and *trans*-4,5-dihydrodiols suggested multiple pathways for the initial oxidative attack of pyrene. Key metabolites of the proposed pathway were confirmed for *Mycobacterium* sp. strain RJGII-135 [49], *M. flavescens* [50] and *Mycobacterium* sp. KR2 [51]. Similar intermediates were demonstrated for *Rhodococcus* sp. UW1, which is capable of utilizing pyrene and chrysene as sole sources of carbon and energy [52]. As well as of the described major metabolites, *Mycobacterium* sp. strain AP1 is able to form a novel metabolite identified as 6,6-dihydroxy-2-2-biphenyl dicarboxylic acid, which demonstrates a new pathway that involves the cleavage of both central rings of pyrene [53]. The schematic pathway proposed for the degradation of pyrene by *Mycobacterium* sp. strain AP1 is shown in Fig. 5.

In a recent study it has been shown that *Sphingomonas yanoikuyae* strain R1 can transform pyrene to *cis*-4,5-dihydro-4,5-dihydroxypyrene and pyrene-4,5-dione. This strain R1 and *Pseudomonas saccharophila* P15 transformed *cis*-4,5-dihydro-4,5-dihydroxypyrene to pyrene-4,5-dione nearly stoichiometrically, suggesting that pyrene-4,5-dione is formed by oxidation of *cis*-4,5-dihydro-4,5-dihydroxypyrene to 4,5-dihydroxypyrene and subsequent autoxidation of this metabolite [54].

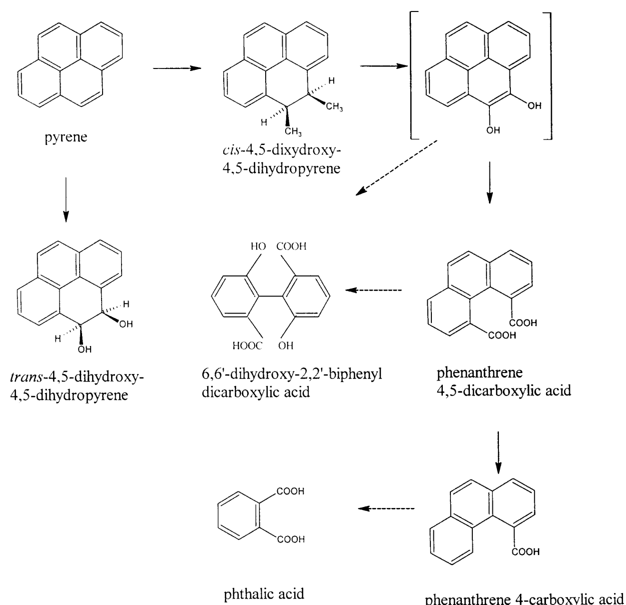

Fig. 5. The schematic pathway proposed for the degradation of pyrene by *Mycobacterium* sp. AP1 [53].

Mycobacterium sp. strain RJGII-135 has been found to mineralize not only pyrene but also a benzo[a]pyrene [55]. An analysis of degradation products shows that benzo[a]pyrene was transformed by dioxygenation at the 4,5- , 7,8- and 9,10- positions. Chrysene-4,5-dicarboxylate was presumably formed by intradiol fission of the 4,5-dihydroxy[a]pyrene, while the 7,8- and 9,10- dihydrodiols underwent extradiol fission to dihydropyrene carboxylates [7]. The proposed pathway for the degradation of benzo[a]pyrene by *Mycobacterium* sp. RJGII-135 (based on isolated metabolites) is illustrated in Fig. 6.

Bioremediation of Polycyclic Aromatic Hydrocarbons

There has been increasing interest in the bioremedation of terrestrial and aquatic PAH-contaminated environments. Interest in the biodegradation mechanisms and environmental fate of the pollutants is prompted by their ubiquitous distribution and their potential deleterious effects on human health [56-58].

The concentration of polycyclic aromatic hydrocarbons in the environment varies widely, depending on the level of industrial development, proximity of the contaminated sites to the production source and the mode of PAH transport. Reported soil and sediment PAH concentrations range from 1 μg/kg to over 300 g/kg [58-60].

There are two reasons for limiting bioremediation of PAH-contaminated sites. First, biological methods have been successfully used to clean-up municipal and industrial

wastewater but their applications in land remediation is still in the stage of infancy. Second, because of their chemical structure and properties they are not easily degradable by bacteria and may persist for a long time in the environment. For example, half-lives in soils and sediments of the three-ring phenanthrene molecule may range from 16 to 126 days while for the five-ring benzo[a]pyrene this may range from 229 to 1400 days [61].

Considerable attention has been focused on the potential use of microorganisms to remediate soils contaminated with persistent organic pollutants [62, 63]. Since PAHs are hydrophobic compounds with low solubility in water they have a tendency to bind with organic matter or soil, limiting their availability to microorganisms. Despite these properties many bacterial strains have been isolated for their ability to transform, degrade and utilize PAHs as a source of carbon and energy [64, 65].

The biodegradation of PAHs by bacteria has been observed under aerobic and anaerobic conditions. Anaerobic biodegradation proceeds very slowly and the biochemical mechanism of this process has not yet been determined in detail [66]. Coates *et al.* [67] demonstrated that a wide variety of hydrocarbon contaminants can be degraded under sulphate-reducing conditions and they suggested that it may be possible to use sulphate reduction rather than aerobic respiration as a treatment strategy for hydrocarbon-contaminated dredged sediments. PAH degradation by bacteria occurs primarily under aerobic condition involving oxygenase-mediated ring oxidation and subsequent catabolite formation-ring fission and metabolism.

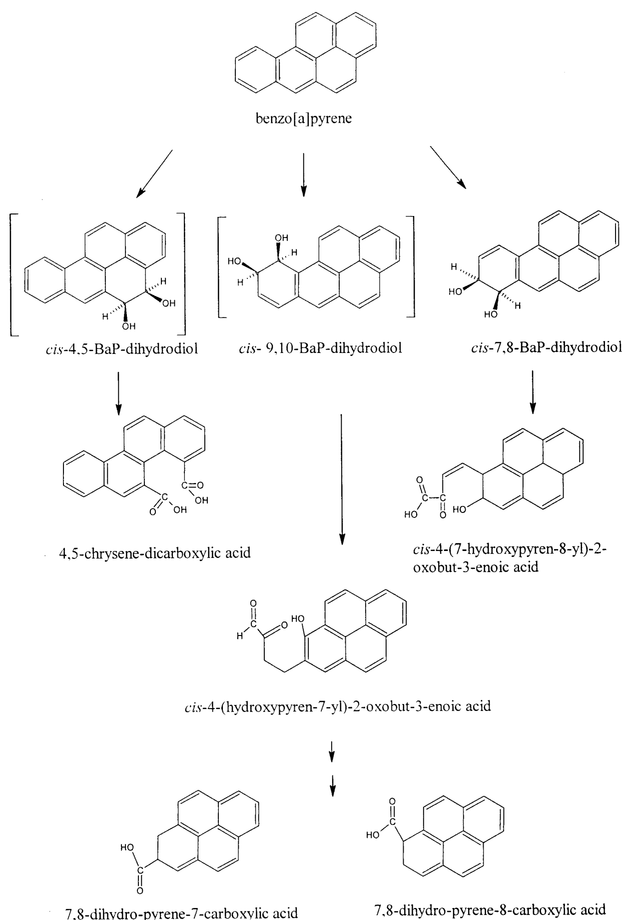

Fig. 6. The proposed pathways for the metabolism of benzo[a]pyrene by *Mycobacterium* sp. RJGII-135 [47].

To enhance biodegradation some researchers have used surfactants or organic solvents to improve the availability of PAHs to microorganisms [68-72]. The use of surfactants like SDS, TritonX-102, Brij 35, Marlipal 013/90 and Genapol X150 increases the concentration of hydrophobic compounds in the water phase by solubilization or emulsification. Solubilization occurs above a specific threshold of surficant, the critical micellar concentration (CMC) where surfactant molecules aggregate to micelles. Another important parameter describing physical properties of surfactants is the hydrophil-lipophil-balance (HLB) which is determined by the relationship of the hydrophilic and the hydrophobic parts of the surfactant molecule [73]. Although surfactants have been studied in complex water-soil systems, the effects are not well understood. In some experiments surfactants stimulated biodegradation whereas they worked as inhibitors in others [68]. Several hypotheses have been proposed to explain this inhibitory effect. One proposal is that if microorganisms do not have direct access to PAHs inside the micelles, the rate of mass transport between these micelles and the aqueous phase would limit biodegradation. The second hypothesis suggests that inhibition of biodegradation would occur if the surfactant is toxic or if it is preferentially used by microorganisms as source of carbon and energy. Moreover, several studies have also suggested that the use of surfactants above the critical micelle concentration (CMC) would decrease desorption and limit bioremediation [55]. All these suggest that the influences of surfactants on degradation processes depend on many factors like bacterial strain, chemical structures and PAH concentration, and types and concentrations of surfactants used. For example, under unsaturated (but moist) conditions, one surfactant, Witconcol SN70, stimulated pyrene mineralization, whereas the same surfactant inhibited pyrene mineralization in soil slurries [71].

The increase of biodegradation rate has been observed in studies in which organic solvents were used to improve PAH bioavailability to bacteria. Lee *et al.* [65] using acetone and ethanol found that total PAH biodegradation rates for soils pretreated with these solvents were estimated to be about twice faster than soils without solvent pretreatment. Approximately 90% of total PAHs in the acetone and ethanol pretreated Vandalia (EXC) soil were removed within 17 days while 35 days were required to achieve the same removal percentage for non-pretreated soil.

Most studies concerning the biodegradation of PAHs were conducted by pure cultures of microorganisms or by purified enzyme systems, but less is known about the degradation and transformation of these compounds in soils and aquatic systems where mixed populations of bacteria are present. As reported by Tadros *et al.* [74] individual

strains could degrade several PAHs, but preferred one. Mixed culture, isolated from coastal sediments, degraded fluorene and naphthalene faster than single bacterial strains originating from the indigenous community of bacteria. Collective metabolism by mixed culture of microorganisms may result in enhanced PAH utilization since intermediatory biotransformation products from one microorganisms may serve as substrates for catabolism and growth by others [31, 75].

It is difficult to study the biodegradation of PAHs in natural environments because of a number of factors determining the degradation rate and extent of bacterial metabolism. Environmental factors include temperature, pH, oxygen concentration, salinity, light intensity, sediment type, the presence of co-substrates, and season. Moreover, the degree of degradation of polycyclic aromatic hydrocarbons significantly increases in the presence of other nutrients. For example, the addition of inorganic nitrogen and phosphorous stimulated the degradation of PAHs in topsoil and aquifer sand [76, 77]. Future research should focus on identifying the factors that enhance the degradation of PAHs by indigenous species of the soils and sediments.

Studies on PAH metabolism are entering a new era with the application of genetically engineered microorganisms (GEM)s for bioremediation processes. Modified microorganisms have shown potential for bioremediation of many chemical contaminants; however, the majority of studies have been conducted in laboratory scale [78, 79].

The University of Tennessee in collaboration with Oak Ridge National Laboratory has achieved the first field release of a GEM for bioremediation purposes. The GEM involved was a *Pseudomonas fluorescens,* strain HK44. The naphthalene-catabolic plasmid pUTK21 was introduced into this strain and additionally it contains a transposon-based *lux* gene fused within a promoter for the naphthalene catabolic genes [80-82]. In the presence of naphthalene, catabolic gene expression, naphthalene degradation and coincident bioluminescent response increased. Bioluminescence signaling is easy to detect and can be used as a good tool for *in situ* monitoring of the bioremediation process. The experiment was conducted in lysimeters containing soil with or without contaminant PAHs. The use of a lysimeter was due to research requirements for reproducibility and to ensure that the GEM remains within the experimental field-test area.

However, the future potential for GEMs in bioremediation in the field scale is limited by problems such as obtaining permission for the introduction of GEMs into soil and water, monitoring and control of the fate of released bacteria, and risk assessment for human health. Such studies are often difficult because GEM survival in the environment is unpredictable and there is a possibility of transfer of introduced genes to other organisms.

To study the fate and survival of introduced bacteria some detection systems based on molecular biology may be used. The ideal detection systems for PAH-degrading environmental isolates should include probes for genes present in the isolates, which encode PAH-degradative enzymes, or a PCR assay depending on these genes. These detection systems let Hamann and co-workers [83] show that genomic DNA from different strains of *Pseudomonas*, *Mycobacterium*, *Gordona*, *Sphingomonas*, *Rhodococcus* and *Xanthomonas* was hybridized with a fragment of ndoB, coding for the large iron sulfur protein (ISPa) of the naphthalene dioxygenase from *Pseudomonas putida* PaW736 (NCIB 9816). Using a polymerase chain reaction (PCR) approach, some *Pseudomonas* strains showed a PCR fragment of the expected size with ndoB- specific primers and additionally some strains of *Gordona*, *Mycobacterium*, *Pseudomonas*, *Sphingomonas* and *Xanthomonas* were detected with degenerate primer-pools specific for the ISPa [2Fe-2S]-rieske center of diverse aromatic hydrocarbon dioxygenases. This may be a basis for the isolation of new PAH-catabolic genes, which could be used to evaluate the PAH degradation potential of mixed populations.

In situ bioremediation is the application of biological treatment to the cleanup of hazardous chemicals present in the soil. The optimization and control of microbial transformations of polycyclic organic hydrocarbons require the integration of many scientific and engineering disciplines.

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