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REVIEW

# Bacterial metabolism of polycyclic aromatic hydrocarbons: strategies for bioremediation

**Archana Chauhan · Fazlurrahman · John G. Oakeshott · Rakesh K. Jain**

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**Abstract** Polycyclic aromatic hydrocarbons (PAHs) are compounds of intense public concern due to their persistence in the environment and potentially deleterious effects on human, environmental and ecological health. The clean up of such contaminants using invasive technologies has proven to be expensive and more importantly often damaging to the natural resource properties of the soil, sediment or aquifer. Bioremediation, which exploits the metabolic potential of microbes for the clean-up of recalcitrant xenobiotic compounds, has come up as a promising alternative. Several approaches such as improvement in PAH solubilization and entry into the cell, pathway and enzyme engineering and control of enzyme expression etc. are in development but far from complete. Successful application of the microorganisms for the bioremediation of PAH-contaminated sites therefore requires a deeper understanding of the physiology, biochemistry and molecular genetics of potential catabolic pathways. In this review, we briefly summarize important strategies adopted for PAH bioremediation and discuss the potential for their improvement.

A. Chauhan · Fazlurrahman · R. K. Jain
Institute of Microbial Technology,
Sector-39A, Chandigarh, India

J. G. Oakeshott
CSIRO Entomology,
GPO Box 1700,
Canberra, ACT 2601, Australia

R. K. Jain (✉)
Tel: +91 / 172 / 2636680; Fax: +91 / 172 / 2690632;
e-mail: rkj@imtech.res.in

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## Introduction

PAHs are a class of toxic pollutants that have accumulated in the environment due to both natural and anthropogenic activities [1–4]. They are mainly produced from incomplete combustion of organic materials, fossil fuels, petroleum product spillage and various industrial activities, and partly also from natural processes such as forest fires and volcanic eruptions. Human exposure to PAHs can take place through multiple routes including air, soil, food, water and occupational exposure [5–6]. The adverse health and environmental effects of PAH compounds are widely known [7–9]. They have detrimental effects on the flora and fauna of affected habitats, resulting in the uptake and accumulation of toxic chemicals in food chains (biomagnification) and in some instances, serious health problems and/or genetic defects in humans [10, 11, 12]. Based on their ecotoxicity, the US Environmental Protection Agency (US EPA) has listed 16 PAHs as priority pollutants for remediation [10]. The IARC (International Agency for Research on Cancer) has identified 15 PAHs including 6 of the 16 USEPA-regulated PAHs, as potential carcinogens. The ubiquitous occurrence of these carcinogens represents an obvious health risk and therefore public concern as to their fate and removal from the environment is on the increase.

## Important properties and persistence of PAHs

PAHs consist of two or more fused benzene and/or pentacyclic rings in linear, angular or cluster arrangements [1, 2,

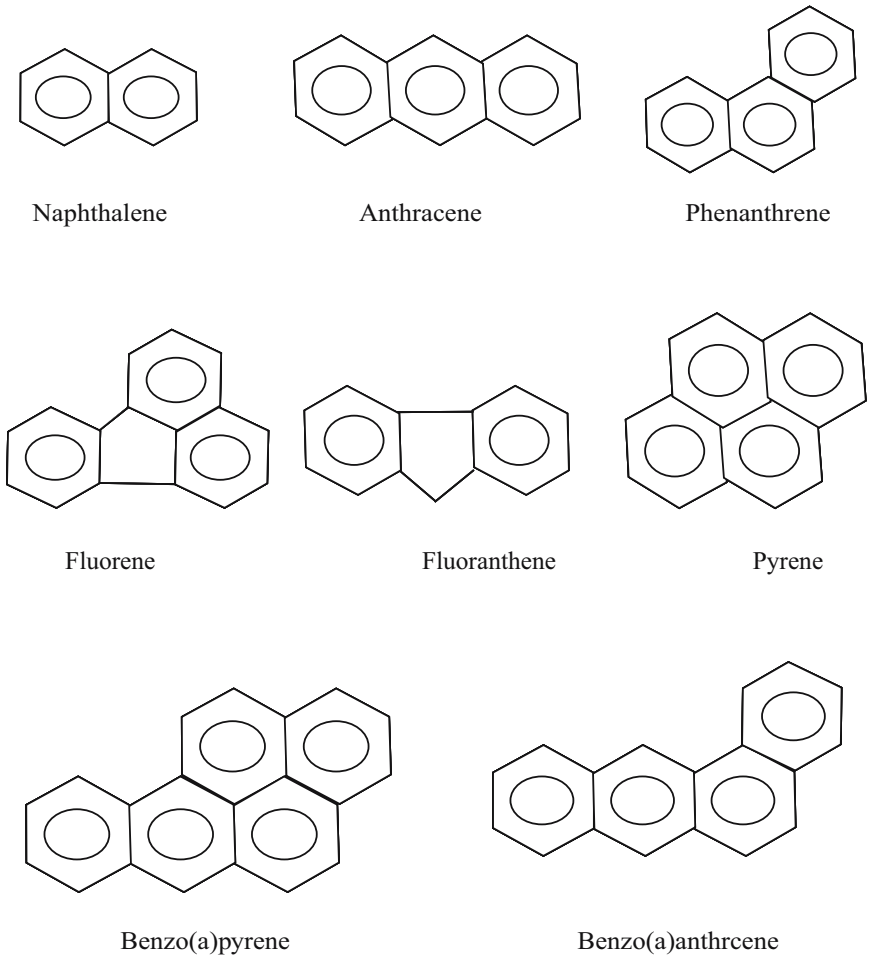


**Fig. 1** Different types of PAHs.

13]. Several hundred PAHs are known today. Some representative PAHs are shown in Figure 1. Depending on the number of rings present, PAHs have been classified into two categories, i.e. Low Molecular Weight (LMW, containing three or fewer rings) and High Molecular Weight (HMW, containing four or more rings) PAHs. The high hydrophobicity and chemical stability of PAHs make them persist in the environment. Their hydrophobicity generally increases with increasing molecular mass, with aqueous solubility declining from the low mg/l range for LMW PAHs to about 1μg/l for HMW PAHs [13]. Because of their hydrophobic properties, low volatility and high affinity for sediment particles, PAHs are readily adsorbed to surfaces in aquatic environments [14] or to soil and dust particles, which could get evenly distributed through air [11]. Many PAHs contain A, B, Bay, K and L-regions, which can be metabolised to highly reactive epoxides (Figure 2). Carcinogenicity has been demonstrated for some of these epoxides [15]. PAHs are rarely encountered alone in the environment and many interactions occur within a mixture of PAHs whereby the potency of known genotoxic and carcinogenic PAHs can be enhanced [16]. For example, 1-nitropyrene, a nitrated PAH, is produced during reactions between ketones in products of burning automobile fuel and airborne nitrogen oxides that take place on the surface of hydrocarbon particles in diesel exhaust. In the Ames assay (*Salmonella typhimurium*), 1-nitropyrene was found to be highly mutagenic and carcinogenic, whereas the parent compound, pyrene, is non-carcinogenic and only weakly mutagenic [17].

The persistence of PAHs in the environment depends on a number of factors such as the physical and chemical characteristics of both the PAHs and the medium, along with the concentration, dispersion and bioavailability of the PAHs. In general the higher the molecular weight of a PAH molecule, the higher is its toxicity and the longer its environmental persistence. The average half-life of the tricyclic phenanthrene ranges from 16 to 126 days in soil, whereas for the five ringed HMW PAH benzo[a]pyrene,

the half-life may range from 229 to 1500 days [18]. Biotic factors are also important because certain components of the soil microflora can degrade many PAHs. Thus factors including the production of toxic or dead-end metabolites, metabolic repression, presence of preferred substrates such as aliphatic hydrocarbons and lack of co-metabolite or inducer substrates can also prolong the residence time of PAHs in the environment. Various physico-chemical factors such as soil type and structure, pH, temperature and oxygen, nutrient and water levels also determine the survival and activity of the degrading strains in the polluted environments, thereby determining the persistence of PAHs in such environments [19].

## General aspects of bacterial degradation of PAHs

During the past thirty years, several different remediation technologies have been tested in efforts to remove these environmental contaminants. Among them, bioremediation is showing particular promise as a safe and cost-effective option [20]. In spite of their xenobiotic properties, a variety of genera of gram-positive and -negative bacteria, fungi and algae have been isolated and characterized for their ability to utilize PAHs. A thorough list of such organisms is provided by Muller *et al* [21]. Microorganisms have been found to degrade PAHs either via metabolism or cometabolism. Cometabolism is especially important for the degradation of mixtures of PAHs. Although anaerobic metabolism is also well documented, most attention has been paid to the aerobic metabolism of PAHs and the common metabolic pathways for this, their rates of degradation and the enzymatic and genetic regulation involved are quite well understood [22–24].

The first step in aerobic PAH catabolism mainly involves the action of dioxygenase/monoxygenase, which incorporates atoms of molecular oxygen into the aromatic nucleus, resulting in the oxidation of the aromatic ring [23–25]. Depending on the substituents on the original molecule, the two hydroxyl groups may be positioned either *ortho* (as in catechol and protocatechuate) or *para* to each other (as in gentisate and homogentisate). The cis-dihydrodiols formed in this reaction are further oxidized, first to the aromatic dihydroxy compounds (catechols), and then through the ortho- or meta cleavage pathways [26, 27]. Further reactions lead to the precursors of tricarboxylic acid cycle (TCA) intermediates. Although all the PAHs follow this same scheme of degradation, the kinetic efficiency of the pathway and the type of reaction intermediates produced depend on the number of the aromatic rings.

PAHs are also common contaminants of anaerobic environments such as aquifers [28–33], marine sediments [34, 34] and anaerobic zones within substantially aerobic contaminated soils [32]. In the absence of molecular oxygen, alternative electron acceptors such as nitrate, ferrous and sulphate ions can be used to oxidize aromatic compounds and recent studies have shown the anaerobic degradation of PAHs under both denitrifying and sulphate reducing conditions [35–38].

The general mechanisms of aerobic and anaerobic degradation are summarized in Figure 3, by taking naphthalene as a representative model for all PAHs [19, 30, 34]. Aerobic degradation of naphthalene involves the incorporation of molecular oxygen into one of the aromatic rings by naphthalene dioxygenase, leading to the formation of cis-1,2-naphthalene dihydrodiol. The latter undergoes a number of further degradative steps and finally gets metabolised to carbon dioxide through salicylic acid. On the contrary, anaerobic degradation of naphthalene involves the formation of 2-naphtholic acid by carboxylation of one of the aromatic rings, which gets further metabolized to carbon dioxide.

Several genes encoding PAH catabolic enzymes have been characterized. These genes are organized into operons which may be localized on chromosomal DNA or large, self-transmissible, catabolic plasmids [39–43] (Table 1). Analysis of the PAH catabolic genes is of interest in both fundamental and applied contexts. Fundamental knowledge of the catabolic genes in different species of bacteria can give useful information about the evolution of the encoded enzymes' sequence-structure function relationships and the evolution and diversity of the catabolic genes via horizontal gene transfer, transposition events, DNA fusion, point mutation etc [44]. This would help us to understand the molecular mechanisms by which bacteria adapt to the xenobiotics. In applied terms, the genetic information would help us to monitor the bacterial populations that degrade PAHs in the contaminated sites, and to engineer bacteria for developing bioremediation strategies.

However, most of the information about metabolic pathways, enzymes and genes has been restricted to LMW PAHs [39–41]. Much less information is available on the metabolism of HMW PAHs, albeit research in the past decade on the bacterial degradation of four ring PAHs such as flouranthene, pyrene, benz[a]anthracene etc has advanced significantly. A number of strains such as *Stenotrophomonas maltophilia* strain VUN 10003, *Mycobacterium* sp., *Gardona* sp., *Rhodococcus* sp., *Pseudomonas aeruginosa, Burkholderia cepacia, Flavobacterium* sp. and *Cycloclasticus* sp. etc. have been found to utilize benz[a]anthracene, chrysene, flouranthene and pyrene [45–47] as a source of carbon and energy (Table 1). Most of these bacteria are gram-positive, suggesting that these organisms play a more important role than gram-negative bacteria in the environmental degradation of HMW PAHs.

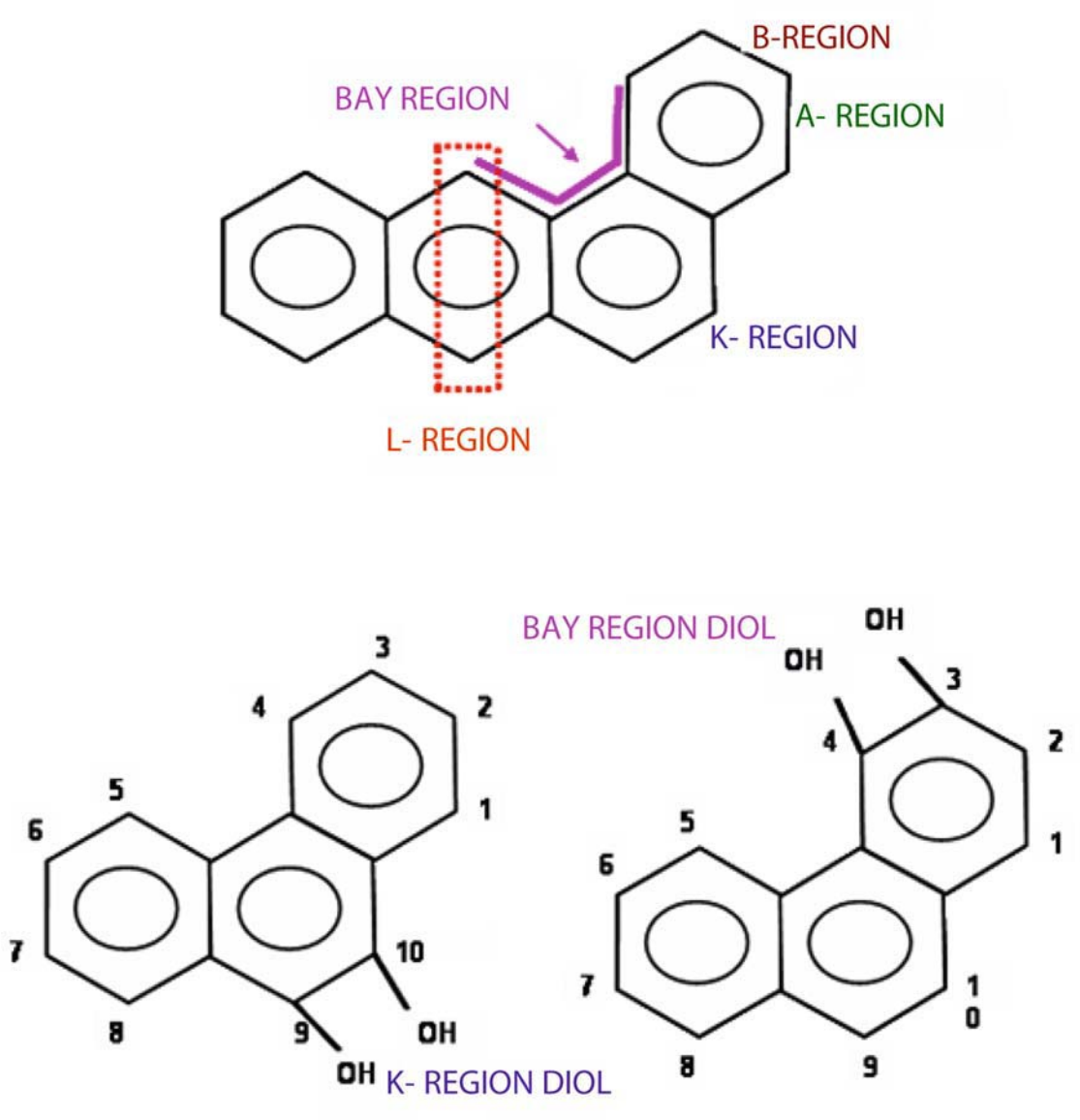


**Fig. 2** A, B, Bay, K and L regions of PAHs involved in the formation of metabolically active epoxides.

Genetic and biochemical data on HMW PAH degradation are relatively scarce, in part because gram-positive bacteria, particularly mycobacteria, are less amenable to study (due to their slow growth rate, cell clumping and extremely resistant cell wall etc) [48]. Nevertheless, just as *nah*, *pah* and *phn* genes encoding LMW PAHs dioxygenases have been found in gram-negative bacteria, *nid* and *pdo* genes encoding HMW PAH dioxygenases are also being found in gram positive bacteria [49–59]. The biochemical and genetic information on these genes and the encoded enzymes should help us to enhance the performance of the PAH degrading bacteria as bioremediators.

## Limiting factors and strategies

Only very limited success has so far been achieved in the bioremediation of PAHs [19, 22, 60, 61]. The reasons are not thoroughly understood but could involve failures at any of the four component steps in the process, namely a) the solubilization of the PAHs, b) their transport into the cell, c) the expression of the degradative genes, and d) the enzymatic breakdown of the PAHs (see also Figure 4). The following section therefore describes the current knowledge of these events and the strategies that might be developed to modify them and enhance the prospects for successful bioremediation of PAHs.

**Table 1** Chromosomally and plasmid-encoded PAHs degrading gene clusters found in bacteria (Adopted from Ref 44 and 149; n, not determined)

| Strain(s) | Location | Substrate(s) | Gene(s) | Encoded protein or function(s) |
|---|---|---|---|---|
| *Pseudomonas putida* strains | Plasmid | Naphthalene (upper pathway | *nahAa* | Reductase |
| | | | *nahAb* | Ferredoxin |
| | | | *nahAc* | Iron sulfur protein large subunit |
| | | | *nahAd* | Iron sulfur protein small subunit |
| | | | *nahB* | *cis*-Naphthalene dihydrodiol dehydrogenase |
| | | | *nahF* | Salicyaldehyde dehydrogenase |
| | | | *nahC* | 1,2-Dihydroxynaphthalene oxygenase |
| | | | *nahE* | 2-Hydroxybenzalpyruvate aldolase |
| | | | *nahD* | 2-Hydroxychromene-2-carboxylate isomerase |
| | | Salicylate | *nahG* | Salicylate hydroxylase |
| | | (lower pathway) | *nahT* | Chloroplast-type ferredoxin |
| | | | *nahH* | Catechol oxygenase |
| | | | *nahI* | 2-Hydroxymuconic semialdehyde dehydrogenase |
| | | | *nahN* | 2-Hydroxymuconic semialdehyde dehydrogenase |
| | | | *nahL* | 2-Oxo-4-pentenoate hydratase |
| | | | *nahO* | 4-Hydroxy-2-oxovalerate aldolase |
| | | | *nahM* | Acetaldehyde dehydrogenase |
| | | | *nahK* | 4-Oxalocrotonate decarboxylase |
| | | | *nahJ* | 2-Hydroxymuconate tautomerase |
| | | Regulator for both operons | *nahR* | Induced by salicylate |
| *P. putida* G7 | NAH7 | Naphthalene | *nah* | Encoded protein for both upper and lower pathway |
| | | | | Of naphthalene degradation |
| *P.aeruginosa* PaK1 | Chromosome | Naphthalene | *pah* | Encoded same proteins as in *Pseudomonas putida* OUS82 |
| *P.putida* BS202 | NPL-1 | Naphthalene | *nah* | encoding upper catabolic enzyme for naphthalene |
| *P. putida* | NCIB9816 | Plasmid Naphthalene | *ndoA* | Naphthalene-dioxygenase genes |
| | | (pDTG1 and pWW60-1) | | (these 3 genes correspond to NahAb,-c,and-d listed above) |
| | | | *NdoB* | |
| | | | NdoC | |
| *P.* sp. strain C18 | Plasmid | Dibenzothiophene | *doxA* | Naphthalene dioxygenase |
| | Naphthalene | | *doxB* | DoxA, -B, -D correspond to NahAb, -c, and-d listed above |
| | | & Phenanthrene | *doxD* | |
| | | | *doxE* | *cis*-Naphthalene dihydrodiol dioxygenase |

**Table 1** *(Continued)*

| Strain(s) | Location | Substrate(s) | Gene(s) | Encoded protein or function(s) |
|---|---|---|---|---|
| | | | *doxF* | Salicylaldehyde dehydrogenase |
| | | | *doxG* | 1,2-Dihydroxynaphthalene dioxygenase |
| | | | *doxH* | Isomerase (interchangeable with *doxJ*?) |
| | | | *doxI* | Hydratase-aldolase |
| | | | *doxJ* | Isomerase |
| *P.* sp. strain U2 | Plasmid | Naphthalene | *nagAa* | Ferredoxin reductase |
| | | | *nagG* | Subunit of salicylate 5-hydroxylase with Rieske-type iron-sulfur centre |
| | | | *NagH* | Subunit of salicylate 5-hydroxylase |
| | | | *NagAb* | Ferredoxin |
| | | | *NagAc* | Large dioxygenase subunit |
| | | | *nagAd* | Small dioxygenase subunit |
| | | | *nagB* | Naphthalene *cis*-dihydrodiol dehydrogenase |
| | | | *nagF* | Salicylaldehyde dehydrogenase |
| *P. putida* | Chromosome | Naphthalene | *pahA* | ferredoxin reductase |
| OUS82 | | Phenanthrene | *pahAb* | ferridoxin |
| | | A variety of homo-hetero-, and monocyclics converted to phenols | | |
| | | | *pahAc* | Large subunit of iron-sulfur protein |
| | | | *pahAd* | Small subunit of iron-sulfur protein |
| | | | *pahB* | *cis*-Dihydrodiol dehydrogenase |
| | | | *pahC* | Dioxygenase |
| | | | *pahD* | Isomerase |
| | | | *pahE* | Hydratase-aldolase |
| | | | *pahF* | Dehydrogenase |
| *P. stutzeri* | Chromosome | Naphthalene | *nahG* | Salicylate 1-hydroxylase |
| AN10 | | 2-Methylnaphthalene | *nahW* | Salicylate 1-hydroxylase (outside *meta*-cleavage transcriptional unit) |
| *Comamonas testosterone* GZ39 | n | Naphthalene | *phd* | similar to *Pseudomona sp* & Phenanthrene |
| C. testosteroni *GZ42* | n | *Naphthalene & Phenanthrene* | nah | *encoding same enzyme as by strain NCIB9816 But two more by this strain* |
| | | | nahAa | *ferredoxin reductase* |
| | | | nahAb | *ferredoxin* |

| | | | | |
|---|---|---|---|---|
| *C. testosteroni* H | n | Naphthalene & Phenanthrene | *pah* | encoded protein same as by nag gene from *Ralstonia* sp. U21 |
| *Ralstonia sp.* U2 | Plasmid | Naphthalene | *nagAa* | ferredoxin reductase |
| | | | *nagAb* | ferredoxin |
| | | | *nagAc* | α subunit of ISP(iron sulfur protein) |
| | | | *nagAd* | β subunit of ISP |
| | | | *nagB* | cis-dihydrodiol dehydrogenase |
| | | | *nagF* | aldehyde dehydrogenase |
| *Burkholderia sp.* RP007 | n | Naphthalene, Phenanthrene & Anthracene | *phnAc* | ISP α subunit of initial dioxygenase |
| | | | *phnAd* | ISP β subunit of initial dioxygenase |
| | | | *phnB* | dihydodiol dehydrogenase |
| | | | *phnC* | extradiol dioxygenase |
| | | | *phnD* | isomerase |
| | | | *phnE* | Hydratase-aldolase |
| | | | *phnF* | aldehyde dehydrogenase |
| | | | *phnR* | regulatory protein |
| | | | *phnS* | regulatory protein |
| *Alcaligenes faecalis* | Plasmid | Phenanthrene | *phnAa* | ferredoxin reductase |
| AFK2 | | | p*hnAc* | α subunit of NDO(naphthalene dioxygenase) |
| | | | *phnAd* | β subunit of NDO |
| | | | *phnB* | cis-dihydrodiol dehydrogenase |
| | | | *phnC* | dihydroxyphenanthrene dioxygenase |
| | | | *phnD* | isomerase |
| | | | *phnE* | hydratase-aldolase |
| | | | *phnF* | 1-hydroxy-2-naphtholdehyde dehydrogenase |
| | | | *phnG* | 1-hydroxy-2-naphthoate dioxygenase |
| | | | *phnH* | trans-2-carboxybenzaldehyde dehydrogenase |
| | | | *phnI* | 2-carboxybenzaldehyde dehydrogenase |
| | | | *gst* | glutathione-S-transferase |
| *Rhodococcus sp.* | n | Naphthalene | *narAa* | α subunit of ISP |
| NCIMB12038 | | | *narAb* | β subunit of ISP |
| | | | *narB* | *cis*-naphthalene dihydrodiol dehydrogenase |
| *Rhodococcus* sp. 124 | Chromosome | Naphthalene | *nidA* | Naphthalene-inducible dioxygenase system |
| | | Toluene | *nidB* | Dioxygenase small subunit |

**Table 1** *(Continued)*

| Strain(s) | Location | Substrate(s) | Gene(s) | Encoded protein or function(s) |
|---|---|---|---|---|
| | | Indene | *nidC* | *cis*-Dihydrodiol dehydrogenase |
| | | | *nidD* | Putative aldolase |
| *Nocardiodes* sp. KP7 | Chromosome | Phenanthrene | *phdA* | Alpha subunit of dioxygenase |
| | | | *phdB* | Beta subunit of dioxygenase |
| | | | *phdC* | Ferredoxin |
| | | | *phdD* | Ferredoxin reductase |
| | | | *phdK* | 2-Carboxybenzaldehyde dehydrogenase |
| *Mycobacterium* sp. PYR-1 | Chromosome | Anthracene, Phenanthrene | *nidD* | Aldehyde dehydrogenase |
| | | Fluoranthene | *nidB* | Small subunit of dioxygenase |
| | | Pyrene, benzo [a]pyrene, 1-nitropyrene | *nidA* | Large subunit of dioxygenase |
| *Sphingomonas paucimobilis* var.EPA505 | | Phenanthrene | *pbhA* | Ring fission dioxygenase |
| | | Anthracene,Benzo[b]fluoranthene | *pbhB* | Rieske-type ferridoxin subunit of multicomponent dioxygenase |
| | | Naphthalene | *pbhC* | Hydratase-aldolase |
| | | Fluroanthene, Pyrene | *pbhD* | Pyruvate phosphate dikinase Intermediate catabolites |

## Solubilization of PAHs

Factors like hydrophobicity, aqueous solubility and polarity have a large influence on the bioavailability of pollutants. Contaminated soils often contain a separate non-aqueous phase liquid (NAPL) that may be present as droplets or films on soil surfaces. Many pollutants, especially those that are hydrophobic, are virtually insoluble in water and remain adsorbed in the NAPL. [62] PAHs have been found to persist in the NAPL due to their low water solubility and high octanol-water partition coefficients [63, 64, 65]. However, the intracellular localization of the PAH degrading enzymes implies that the PAHs have to be solubilized and must enter into the cytoplasm before they can be metabolized. Therefore, for biodegradation to occur, bacteria must have access to the target compounds, either by dissolution of the target compounds in the aqueous phase or by adhesion of the bacteria directly to the NAPL-water interface.

Both chemically and biologically derived surfactants (biosurfactants) have the potential to increase the bioavailability of PAHs via mechanisms such as emulsification of NAPLs, enhancement of the apparent solubility of the PAHs or mobilization of PAHs adsorbed to the soil [66]. For example, the addition of the surfactant Tergitol NP-10 increased the dissolution rate of solid-phase phenanthrene and resulted in an overall increase in the growth of a strain of *Pseudomonas stutzeri* [67]. A similar effect was obtained by the addition of Tween-80 to two *Sphingomonas* strains in which the rate of fluoranthene mineralization was almost doubled [68,69]. Recently, it has been demonstrated that a biosurfactant also increases the apparent solubilities of PAHs 5–20-fold and significantly increases their rate of biodegradation [70, 71]. Biosurfactants are preferred over synthetic surfactants because they are more cost-effective, less toxic and easily biodegradable.

While some research groups have found that the presence of surfactants enhances biodegradation of various pollutants including PAHs [66, 72–78], others have found that the presence of surfactants can actually inhibit biodegradation [75, 76, 79–81]. For example, Tween-80 was found to inhibit the rate of fluoranthene mineralization by two strains of *Mycobacterium* [68]. No stimulation was observed in other studies using several surfactants [69, 82, 83]. This may be due to the entrapment of PAHs within the surfactant micelles, so it may be necessary to use different surfactant-utilizing and PAH-degrading strains together, or preferably to use a single organism which can simultaneously produce biosurfactants as well as degrade PAHs. Recently, García-Junco *et al.* [84] indeed isolated a biosurfactant-producing strain, *Pseudomonas aeruginosa* 19SJ, which also had the capability to degrade phenanthrene.

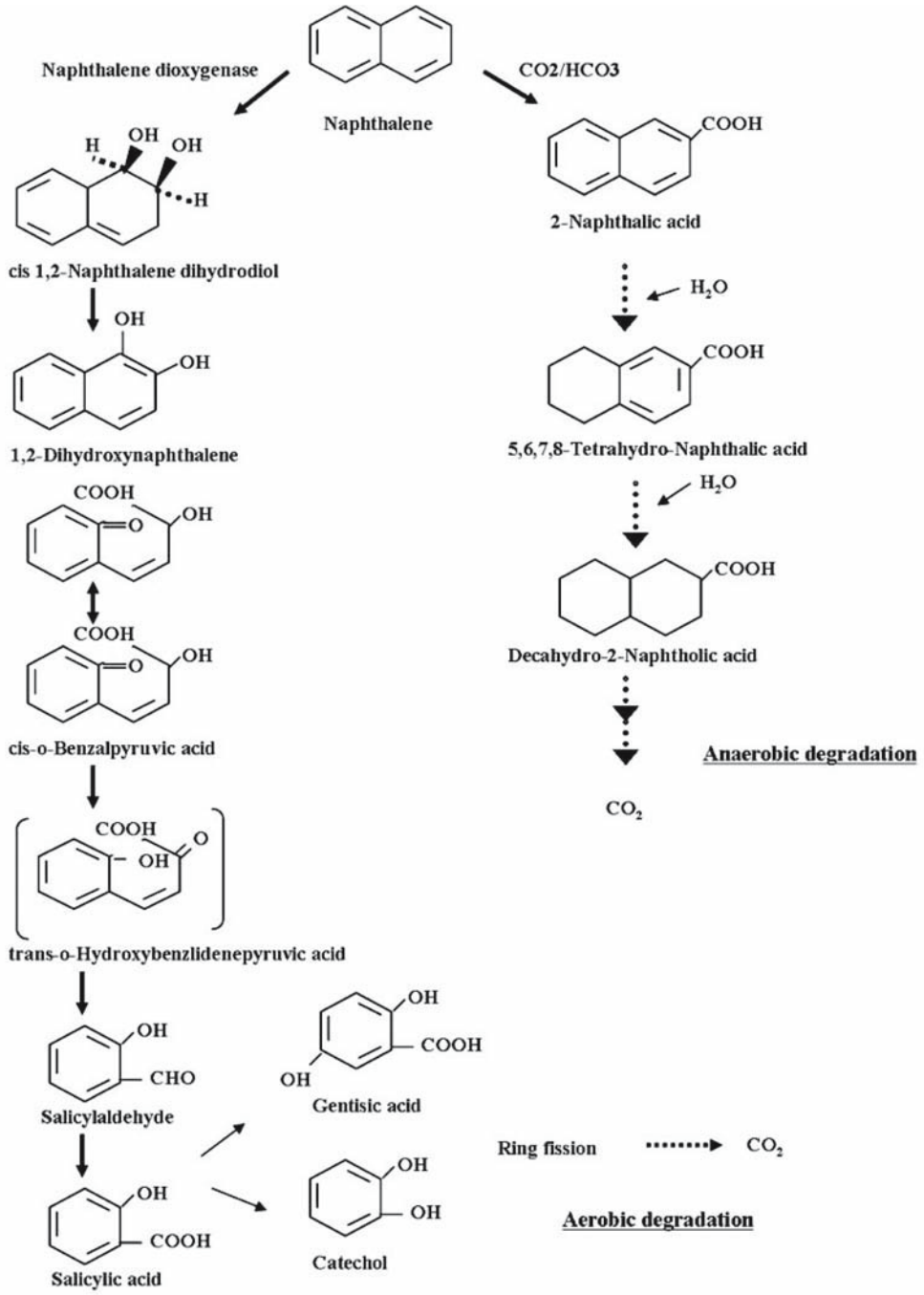


**Fig. 3** Aerobic and anaerobic bacterial degradation pathways of naphthalene (Adapted from ref.19).

Isolation of such strains or their development through modern genetic technologies would be a breakthrough in PAH bioremediation. There would of course be significant regulatory issues to address before transgenic bacterial bioremediants could be released into the open environment. However modern technology also allows many non-transgenic genetic manipulations to be made, for some species at least.

Transport of PAHs into the cell

Due to their low water solubility and high octanol-water partition coefficients, organic compounds such as PAHs tend to partition into cell wall structures. Such movement is generally brought about by passive transport down a concentration gradient from the environment into the cell [85–87]. Such transport, however, depends on a number

of factors, crucial amongst which are concentration and the bioavailability of contaminants from the surrounding medium. The higher are the concentration and bioavailability of a PAH, the higher is its transport into the cell. While some bacteria can utilize this transport to achieve rapid degradation of PAHs, with concomitant growth, the rapid accumulation of some more toxic PAHs may lead to disruption of the cell membrane or inhibition of the membrane proteins etc., ultimately causing cell death [88].

At low PAH concentrations and low bio-availability the passive diffusion process above may not function, potentially decreasing the efficiency of the degrading microorganism and therefore the bioremediation process. However some bacteria seem to have adapted to low bio-availability and low concentrations of PAHs. Mechanisms of the adaptation can involve the direct contact with solid-phase PAHs [89–91] and biosurfactant excretion to facilitate desorption (as discussed earlier). A third type of adaptation was reported by Wick *et al.* [65, 91] who observed that suspended cells of *Mycobacterium* sp. strain LB501T do not produce biosurfactant but still have a strong ability to degrade low concentrations of aqueous-phase anthracene. The specific affinity of strain LB501T cells for aqueous anthracene was found to be higher than the specific affinities of other cultures reported elsewhere [91–93]. Wick *et al.* [91] proposed the presence of a high-affinity uptake system in strain LB501T to account for these observations.

Limited research has been carried out on the cellular binding and transport of PAHs in aqueous phase by bacteria. Bugg *et al.* [91] demonstrated the presence of an active efflux mechanism in *Pseudomonas fluorescens* LP6a for the transportation of phenanthrene, fluoranthene, and anthracene. In contrast, Whitman *et al.* [95] proposed the involvement of a specific, energy-dependent transport system for incorporation of naphthalene in *P. fluorescens* Uper-1. Miyayta *et al.* [87] also suggested that both passive diffusion and a saturable transport system(s) may contribute to the utilization of phenanthrene by *Mycobacterium* sp. strain RJGII-135 in the aqueous phase. Recent studies have proposed the presence of facilitated or active transport systems for uptake of different hydrophobic compounds such as toluene [96] and *m*-xylene [97], alkanes such as *n*-hexadecane [98-100] and dibenzothiophene [101]. Moreover, some long-chain fatty acids such as oleic acid are also known to be bound to bacterial cells and transported actively [102, 103]. While the area still needs much more attention, these studies suggest that various active or facilitated transport systems could be exploited in future to develop more efficient bioremediators.

## Expression of the degradative genes

Once the PAHs have entered into the cell, the next step is the transcription of the degradative genes to produce the required enzymes. Generally the degradative genes have been found to be inducible, being expressed under certain conditions only (Figure 4). The inducer molecule is often the pathway substrate and/or a pathway intermediate but some structural analogues of the natural effectors (gratuitous inducers) can also induce the pathway even if they are not themselves substrates for the corresponding catabolic enzymes. The regulator may act as a transcriptional activator in the presence of the inducer or as a transcriptional repressor in the absence of the inducer.

However, the transcription of the degradative genes is not just dependent on the performance of their specific regulatory and inducer signals but also relies on overimposed mechanisms that connect the activity of individual promoters to the metabolic and energetic status of the cell [105]. This superimposed regulation is mediated by global regulatory factors, such as the integration host factor (IHF), cAMP receptor protein (CRP), alternative sigma factors (σ), the PtsN (IIANtr) protein and the alarmone (p)ppGpp, that interact with different targets (*cis*-acting elements, regulatory proteins) in the transcriptional machinery. Intermediates or products of the TCA cycle may control the transcriptional flow by acting directly as anti-inducers of the specific regulator protein or by determining the energy status of the cell that governs the overimposed regulation [104].

Different regulatory mechanisms have been identified in different PAH degrading bacteria (Table 1). Both the upper and lower operons of the well characterized naphthalene catabolic plasmid NAH7 are regulated by a *trans*-acting positive control regulator encoded by the *nahR* gene (which is localized between the two operons). The NahR protein is needed for high levels of expression of the *nah* genes and their induction by salicylate [106–108]. However in *Burkholderia* sp. strain RP007, the *phnR* and *phnS* regulatory genes encoding a two component regulatory system regulate the degradation of naphthalene and phenanthrene degradation [109]. Both types of regulatory proteins in this system belong to the LysR family. The precise binding and the activation mechanisms of the various regulatory proteins in PAH catabolic operons are subjects of further investigation.

As noted earlier, only very limited success has so far been achieved in the application of bioremediation technologies to PAH contaminated sites [110–112] and this is partly due to the lack of induction of the catabolic enzymes [113, 114]. External addition of the inducers is generally neither a cost-effective nor an ecofriendly alternative.

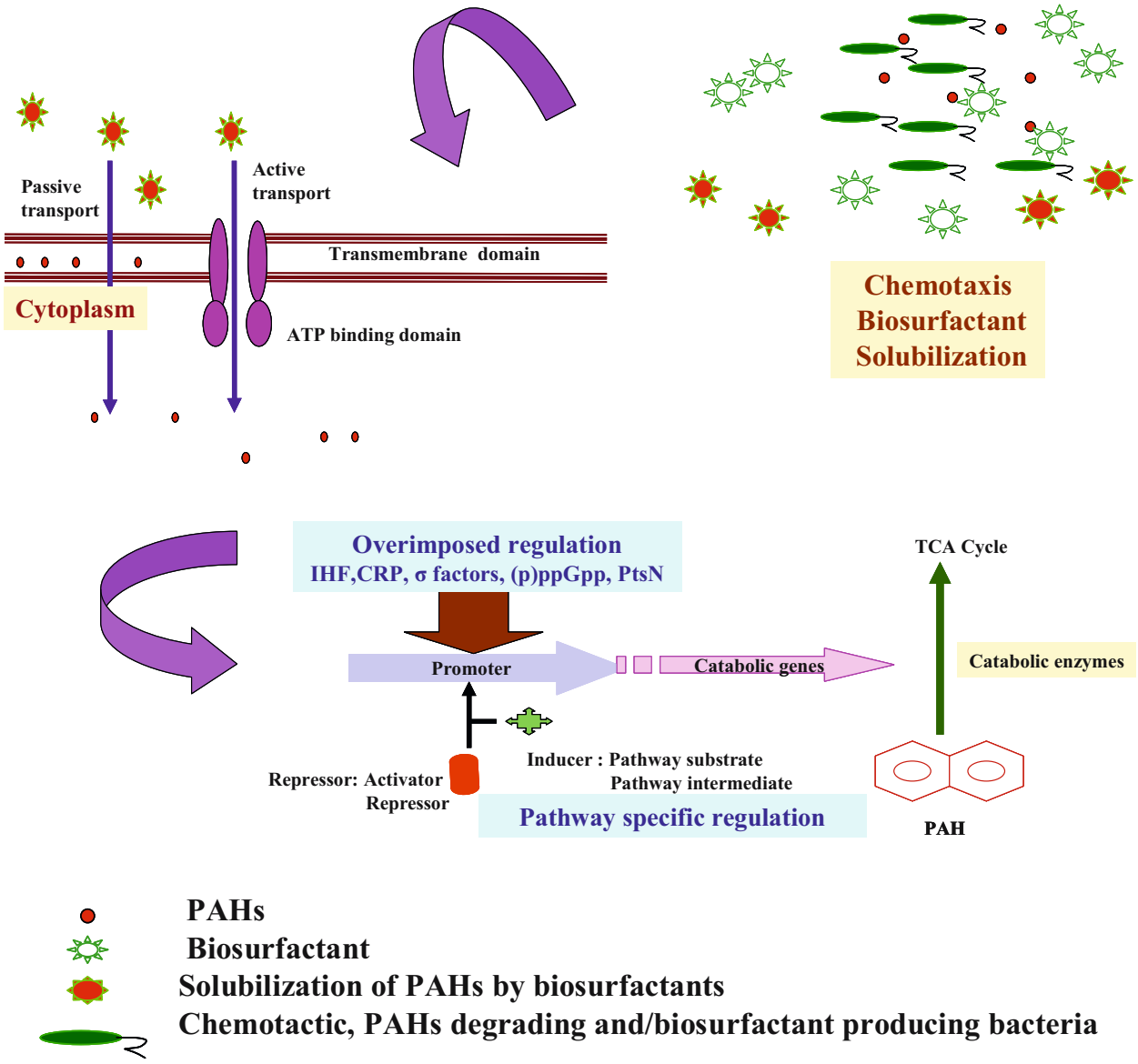


**Fig. 4** A general model depicting the major strategies adapted by bacteria for PAHs degradation.

Interactions between LMW and HMW PAHs and their metabolites have been reported to play an important role in the induction of the catabolic enzymes and such interactions can be either synergistic or antagonistic [115, 116]. In the former case, the metabolites produced in the degradation of LMW PAHs in one strain may enhance the induction of catabolic enzymes of other LMW or HMW PAHs in other strain(s) (cross-induction and co-metabolism) [95]. In the latter case LMW/HMW PAHs or their metabolites may inhibit degradation due to substrate competition or microbial toxicity [116].

Several strategies could be employed to improve the induction of PAH catabolic operons under field conditions. One strategy could be the identification of a cheap and non-toxic inducer, as demonstrated by Gilbert and Crowley [117] for PCB degrading *Arthrobacter sp.* B1B. Another strategy could be the expression of the PAH degrading genes under the control of a constitutive promoter. For this the novel approach of promoter implantation by homologous recombination developed by Ohtsubo *et al.* [118] for biphenyls and PCBs could be exploited (Fig. 5). This would, however, require screening of candidate promoters and selection of the one with the best performance. Catabolic promoters generally show remarkably little specificity (regulatory noise) with respect to the signals to which they respond. This allows them to evolve and to be recruited to

control novel pathways. This property could also be exploited further for developing bioremediation systems with broad substrate ranges.

## Enzymatic breakdown of PAHs

Once the enzymes are expressed the next step is their catalysis of PAH degradation. As stated earlier, ring-hydroxylating dioxygenases (RHDs) catalyze the initial and most crucial oxidation step of PAHs, generating *cis*-dihydrodiols. So far, only a few RHDs have been purified and extensively characterized, including phthalate dioxygenase [119, 120], naphthalene dioxygenases [121, 122] and biphenyl dioxygenase [123]. None of these enzymes is able to oxidize substrates with more than three fused rings but Jouanneau *et al.* [124] recently isolated a new naphthalene dioxygense from *Sphingomonas* CHY-1 which has the ability to attack PAHs composed of up to five rings. The use of such strains having broad substrate specificities is one strategy to improve the degradation of PAHs. Genetic engineering of well characterised enzymes is another strategy for the creation of the enzymes with the desired properties.

During the past years, several oxidoreductases such as laccases and cytochrome P450 monooxygenases (CYPs) isolated from different bacterial and fungal sources have been extensively exploited for the enzymatic degradation of PAHs [125]. Laccases catalyze the oxidation of a wide variety of phenolic compounds including PAHs. Being fungal in origin, laccase is difficult to express in non-fungal systems and knowledge of structure–function relations underlying the key functional properties of laccase is limited [126]. Hence, directed evolution holds exciting potential for improving the performance of the enzyme. In a study undertaken by Bulter *et al.* [127] the laccase gene from *Myceliophthora thermophia*, was transformed into *Saccharomyces cerevisiae* and subjected to directed evolution. After 10 rounds of directed evolution, a laccase with a 170 fold increase in total activity and high thermal stability was obtained. The evolved enzyme was able to work at the elevated temperatures needed to increase the solubility of highly recalcitrant PAHs [126]. As another approach to the solubility issue, Alcalde *et al.* [128] recently used five rounds of sequential error-prone PCR, *in vivo* shuffling and saturation mutagenesis to evolve an already thermophilic laccase into one which showed several fold improvement in turnover rates at high concentrations of organic solvents (acetonitrile and ethanol). Given the findings of the previous section, another worthwhile property to engineer into a laccase would be activity in the absence of inducers/mediators.

CYPs are one of the largest known enzyme superfamilies and are expressed in most living organisms. PAHs can be

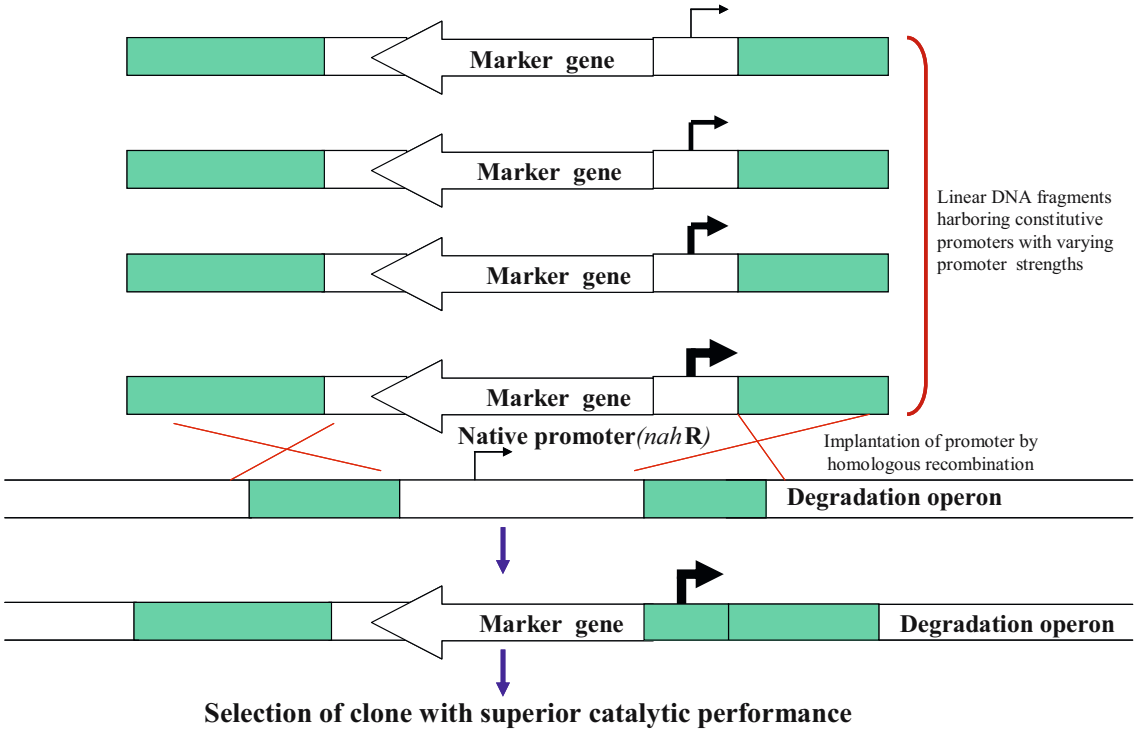


**Fig. 5** Promoter implantation strategy for optimization of the promoter activities (Adapted from ref.118).

oxidized by CYP enzymes to form catechols, which are then degraded by other enzymes, including catechol dioxygenases, to harmless products which can be incorporated into the TCA cycle. Wild-type CYP101 ($P450_{cam}$) from *Pseudomonas putida* has been shown to have an inherently low activity (<0.01 $min^{-1}$) towards various PAH substrates such as phenanthrene, fluoranthene, pyrene and benzo[a]pyrene. Therefore, certain CYP enzymes with known crystal structures have been subjected to rational design mutagenesis to enhance their catalytic performance [126]. For example, selective mutations were introduced into the active site residues F87 and Y96 of the CYP101 enzyme [129]. The absolute oxidation rates (approximately 1 $min^{-1}$) of the mutants Y96A, Y96F, F87A/Y96A and F87L/Y96F were increased by two to three orders of magnitude relative to the wild-type enzyme for all PAHs substrates studied.

In a similar study, Carmichael and Wong [130] introduced two mutations into CYP102, R47L and Y51F, and found that the oxidation activity of the enzyme for phenanthrene and fluoranthene was increased by 40- and 10-fold, respectively. The double mutant was then used as a basis for further engineering of the active site. When the A264G mutation was introduced into the double mutant, NADPH turnover, PAH oxidation and the coupling efficiency of the enzyme were greatly improved. And another mutation, F87A, resulted in a larger space in the substrate binding pocket of the enzyme, leading to better accommodation of larger fluoranthene and pyrene molecules in the vicinity of the heme site, and hence a more efficient PAH oxidation. The most active mutants generated in this study showed more than a 200-fold increase in PAH oxidation activity compared to the wild-type enzyme. In another CYP102 study, Li *et al.* [131] created a triplet mutant, A74G/F87V/L188Q, with improved activity on naphthalene, fluoranthene, acenaphthalene, acenaphthylene and 9-methylanthracene. The F87V mutation alone improved activity toward the PAHs by two to three orders of magnitude. The L188Q mutation significantly increased activities towards all three-ring PAHs by as much as 30-fold. The A74G mutation increased NADPH consumption rates, and consequently activities towards all PAHs. In total, the activities of the triplet mutant towards all the PAHs studied were two to four orders of magnitude higher than those of the wild-type enzyme.

One of the main challenges facing the use of isolated CYP enzymes in bioremediation is the need to regenerate the expensive cofactor, NAD(P)H, which is consumed in the oxidation reaction. One approach to expand the practical utility of CYPs is to eliminate the cofactor requirement. Directed evolution has indeed been used to create CYP101 mutants that hydroxylated naphthalene in the absence of the cofactor NAD(P)H via the 'peroxide shunt' pathway [132]. This process yielded several mutants with 20-fold improvements in naphthalene hydroxylation activity relative to the wild-type enzyme. Previously, it has been difficult to improve the thermostability of the P450 enzymes by protein engineering, because it is a multicomponent enzyme that depends on thermolabile cofactors. However, use of the peroxide shunt pathway negates the need for a cofactor and a reductase domain and should also allow the thermostability of the P450 enzyme to be improved via directed evolution [133].

## Additional measures for improving PAH degradation

### Survival of the degrading strain

One of the problems in extrapolating laboratory bioremediation experiments to the field has been the poor survival of the degrading strains in the field environment [113–114]. The inoculated strains are affected by the predation of protists and competition with indigenous microorganisms for nutrients or electron acceptors. Therefore, selection of an appropriate strain is vital for successful *in situ* bioremediation. There is abundant empirical evidence that a strain derived from a population that is temporally and spatially prevalent in a specific type of habitat is more likely to persist as an inoculum when reintroduced than one that is transient or even alien to such a habitat [134]. Whilst the strain may need genetic modification in the laboratory to enhance its PAH degradative performance, it should still be competitive when re-introduced to its native habitat. This consideration also applies to the concept of rhizoremediation, where the degrading strain is also chosen for its ability to colonise the rhizosphere of the environment in question (see below).

### Bacterial Chemotaxis

Chemotaxis is defined as the migration of microorganisms under the influence of a chemical gradient. Some pollutant-degrading bacteria such as *Pseudomonas putida* G7, a naphthalene degrader, have been demonstrated to show chemotactic behaviour [134, 136]. Another naphthalene- and salicylate-degrading strain, RKJ1, also shows the chemotactic property [137] and the genes for both its catabolic and chemotactic properties are present on its plasmid, pRKJ1. It is presumed that the chemotactic properties make the toxic molecule more bioavailable to the degrading bacteria. Cells displaying chemotaxis can sense chemicals such as those adsorbed to soil particles in a particular niche and swim towards them; hence, the

mass-transfer limitations that impede the bioremediation process can be overcome. Although there is some information about the role of catabolic genes and the associated receptor, the precise molecular mechanisms underlying the chemotactic response are yet to be elucidated. Complete understanding of these mechanisms would help to engineer chemotactic PAHs degraders and exploit them for the degradation of more recalcitrant PAHs.

Plant Microbe associations

Plants forming part of the natural vegetation or used during phytoremediation have been reported to assist bioaugmented bacterial strains in degrading pollutants, especially those inhabiting the rhizosphere. The main reason for the improved degradation in the rhizoshere is presumably due to the increase in the number and the metabolic activity of the microbes. Both plant and the microbes are mutually benefited by this association. Plant exudates help to stimulate the survival and action of the bacteria, which results in a more efficient degradation of pollutants. The root system of plants can help to spread bacteria through soil and help to penetrate otherwise impermeable soil layers [138]. Similarly, the microbes benefit plants by producing phytohormones, solubilization of minerals, and through the synthesis of vitamins, siderophores and other growth stimulating compounds [139]. Such plant-microbes interactions have been exploited for bioremediation of heavy metals [140], toluene [141], polychlorinated biphenyl [142], naphthalene [143], and 2,4-dinitrotoluene [144]. Species of the genera *Pseudomonas*, *Comamonas* and *Burkholderia* are the most commonly used players [141, 145–147]. Successful application of plant–microbe systems for rhizoremediation relies on *in situ* establishment of a high level of competence of the introduced bacteria. The inoculation of pollutant-degrading bacteria on plant seed can be an important additive to improve the efficiency of phytoremediation or bioaugmentation. Recently, Child *et al.* [148] used five PAH degrading mycobacterial strains to explore their relationship with the plant roots and found that the mycobacterial cells were both associated with the root surfaces and distributed through the root. Such traits have great potential in the development of better bioremediation strategies.

## Conclusions

Significant advances have been made regarding the bioremediation of PAHs during the last decade. Several new microbes with bioremediation potential have been isolated and many new degradation pathways have been elucidated. Nevertheless, this knowledge is far from complete. The efficiency of PAH degradation can be significantly improved by addressing key issues such as tolerance to different PAHs, constitutive expression of the catabolic genes and the substrate specificity, kinetics and stability of the encoded enzymes. Moreover, bioavailability issues can be tackled by choosing chemotactic and biosurfactant producing PAH degrading strains which are also capable of symbiotic associations with the plant. However, in order to develop such bioremediation strategies in the near future, a deeper understanding of the physiology, biochemistry, molecular genetics and microbial ecology of PAH degrading strains is required.

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