

## Review article

# Advances in the field of high-molecular-weight polycyclic aromatic hydrocarbon biodegradation by bacteria

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## Summary

Interest in understanding prokaryotic biotransformation of high-molecular-weight polycyclic aromatic hydrocarbons (HMW PAHs) has continued to grow and the scientific literature shows that studies in this field are originating from research groups from many different locations throughout the world. In the last 10 years, research in regard to HMW PAH biodegradation by bacteria has been further advanced through the documentation of new isolates that represent diverse bacterial types that have been isolated from different environments and that possess different metabolic capabilities. This has occurred in addition to the continuation of in-depth comprehensive characterizations of previously isolated organisms, such as *Mycobacterium vanbaalenii* PYR-1. New metabolites derived from prokaryotic biodegradation of four- and five-ring PAHs have been characterized, our knowledge of the enzymes involved in these transformations has been advanced and HMW PAH biodegradation pathways have been further developed, expanded upon and refined. At the same time, investigation of prokaryotic consortia has furthered our understanding of the capabilities of microorganisms functioning as communities during HMW PAH biodegradation.

## Introduction

Research in regard to prokaryotic biodegradation of high-molecular-weight polycyclic aromatic hydrocarbons (HMW PAHs) has continued to advance worldwide. Although some of the first reports of HMW PAH biotransformations by bacteria were published in 1975 (Barnsley, 1975; Gibson *et al.*, 1975) and 1984 (Jerina *et al.*, 1984), it was from the late 1980s that research in this field began to proceed more quickly when studies that documented HMW PAH-utilizing bacteria and ring fission products of bacterial HMW PAH biodegradation were reported (Heitkamp and Cerniglia, 1988; Heitkamp *et al.*, 1988a,b; Mahaffey *et al.*, 1988; Mueller *et al.*, 1989). By the late 1990s, a diverse number of studies had been published that probed the field through different scientific approaches and that led to the isolation of HMW PAH-degrading and HMW PAH-utilizing bacterial strains, characterization of HMW PAH biodegradation metabolites and proposals for mechanisms of HMW PAH biodegradation. These studies were later the subject of reviews pertaining specifically to the field of HMW PAH biodegradation by bacteria (Kanaly and Harayama, 2000) or as part of excellent wider reviews that also covered HMW PAH biodegradation by eukaryotic organisms and biodegradation of other classes of hydrocarbon pollutants (Juhász and Naidu, 2000; Van Hamme *et al.*, 2003). Since that time, advances in molecular biology and more recently, high-throughput technologies, such as whole-genome sequencing, proteomics and metabolomics, are equipping the field with new tools that are facilitating even more rapid advancements. Research pertaining to bacterial biodegradation of HMW PAHs has indeed expanded worldwide and is the subject of this review.

## HMW PAH properties and occurrence

The PAHs that possess more than three aromatic rings have been referred to as HMW PAHs in the environmental microbiology literature. The physical and chemical properties of HMW PAHs are such that they generally appear to be persistent in the environment and may pose risks to

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human and ecological health in parent molecule form or after biological and/or chemical transformations (Lundstedt *et al.*, 2007). As is widely known, HMW PAHs are sparingly soluble in water, are electrochemically stable and may be acutely toxic, genotoxic, immunotoxic (Burchiel and Luster, 2001) or act as agents of hormone disruption (Van de Wiele *et al.*, 2005) depending upon circumstances and the mode of exposure. Due to their elevated octanol–water partition coefficients ( $K_{ow}$ ), HMW PAHs may partition into organic phases, soil and sediment organic matter and membranes of living organisms, and are candidates for bioconcentration, bioaccumulation and sometimes biomagnification through trophic transfers in terrestrial and marine food webs (Neff, 2002; Meador, 2003). The chemical structures of 12 HMW PAHs whose biodegradation is discussed within this review are shown in Fig. 1.

HMW PAH molecular stability, hydrophobicity and low water solubility appear to be some of the main factors that contribute to their persistence in the environment and these factors may also be correlated with the size of the molecule or the total number of aromatic rings (Cerniglia, 1992). HMW PAHs are formed by pyrogenic processes during the incomplete combustion of organic material through pyrolysis and pyrosynthesis (Mastral and Callén, 2000), are generally present in relatively high concentrations in fossil fuels and in products of fossil fuel refining (petrogenic origin; Quann, 1998) and are produced biologically. In areas of concentrated HMW PAH pollution

caused by anthropogenic releases they may co-occur in complex non-polar aqueous phase liquid (NAPL) mixtures, such as in the case of creosote, a coal tar distillate used as a wood preservative. However, HMW PAHs may originate from many different types of sources, both anthropogenic and non-anthropogenic (Van Metre *et al.*, 2000; Hylland, 2006; Johnsen and Karlson, 2007; Li *et al.*, 2007; Achten and Hofmann, 2009) and their levels of occurrence in the environment are dependent upon numerous factors including the degree of industrial development and proximity to point and/or non-point sources combined with the effects of regional and global transport phenomena. The environmental levels of HMW PAHs vary widely, they appear to occur just about everywhere and their occurrence has been studied in the atmosphere (Baek *et al.*, 1991; Lang *et al.*, 2008), in air (Finlayson-Pitts and Pitts, 1997; Han and Naeher, 2006), in soil (Wilcke, 2000; Nam *et al.*, 2009), in freshwater and marine sediments (Cornelissen *et al.*, 1998; Zakaria *et al.*, 2002), in ice cores (Kawamura and Suzuki, 1994), in the deep ocean (Ohkouchi *et al.*, 1999) and in numerous other media ranging from vegetation to food (Wagrowski and Hites, 1997; Phillips, 1999; Howsam *et al.*, 2000; Fimes *et al.*, 2002). As a class, PAHs are currently considered to be the most abundant free organic molecules in space (Ehrenfreund and Charnley, 2000) and detection of HMW PAHs in interstellar regions indicates that their ubiquity transcends planet earth (Ehrenfreund and Sephton, 2006).

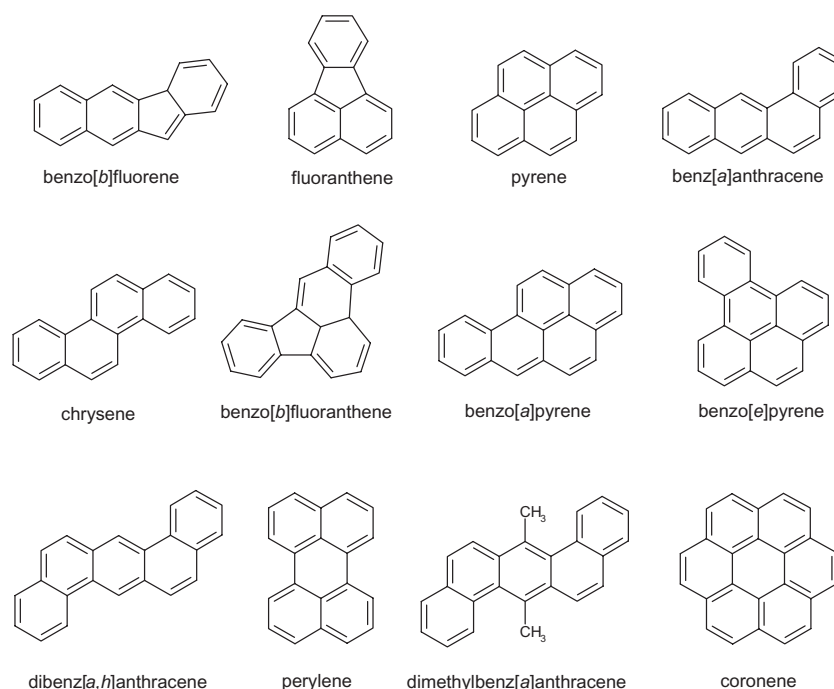


Fig. 1. Structures of representative HMW PAH molecules discussed in this review.

On earth there is interest to understand the environmental fates of these chemicals because such information may prove beneficial for controlling their concentrations in the environment and for limiting exposure. At the same time, demand for optically pure chemical products created by microorganisms is increasing and biological transformation of HMW PAHs may be advantageous for the production of regioselective and stereoselective organic chemicals in some settings.

### **A complete pyrene biotransformation pathway and *Mycobacterium vanbaalenii* PYR-1**

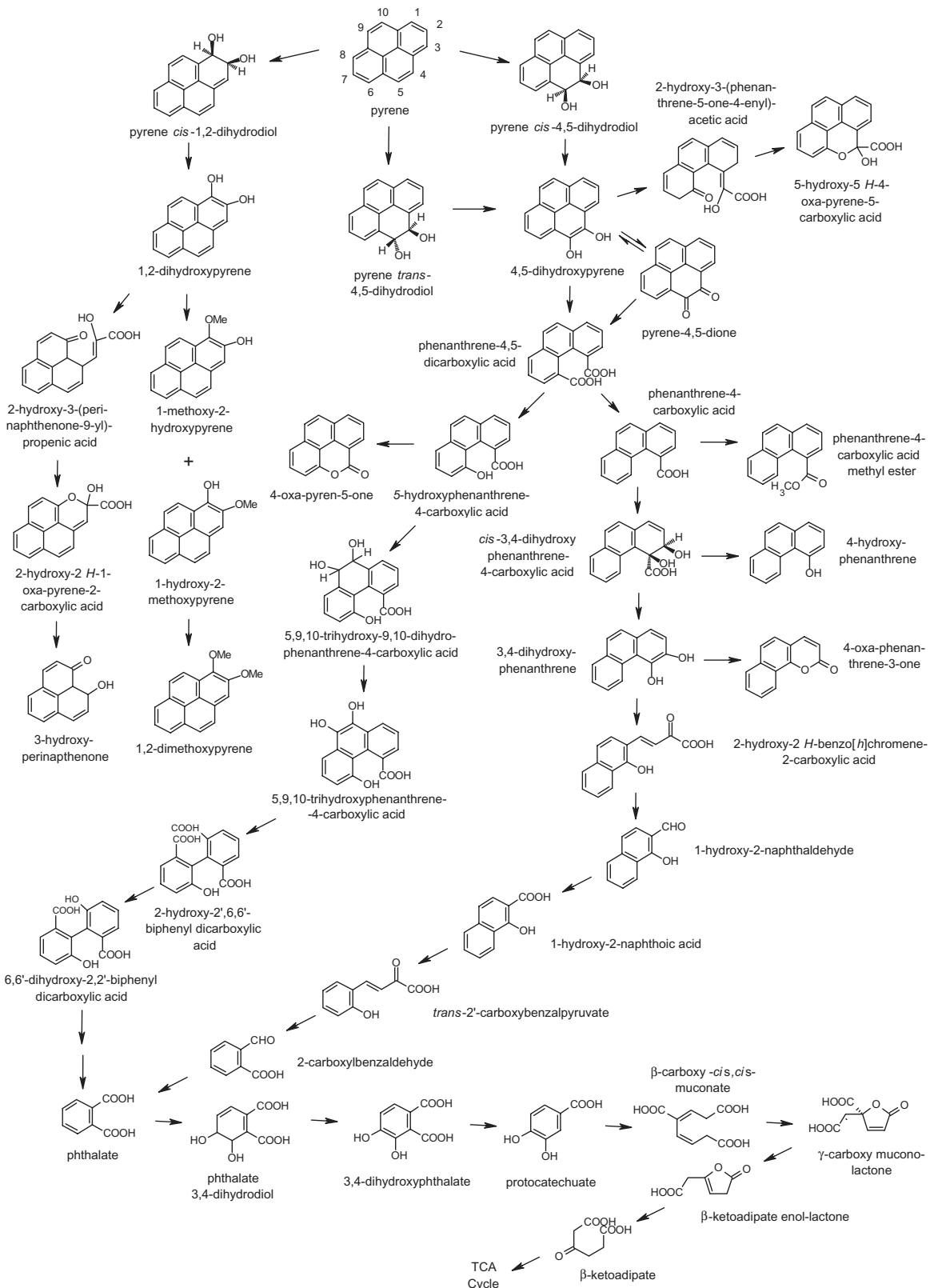
Research in regard to the biotransformation of the four-ring peri-condensed PAHs pyrene and fluoranthene by different genera of bacteria has been significantly advanced. Biodegradation pathways for these PAHs have been further developed since the first reports of metabolites and biodegradation pathways for these compounds were published for actinomycetes and Gram-negative organisms (Heitkamp *et al.*, 1988b; Mueller *et al.*, 1990; Weissenfels *et al.*, 1991; Cerniglia, 1992). In the last 10 years many new bacteria that possess HMW PAH biodegradation capabilities for pyrene and fluoranthene have been isolated from different environments throughout the world and the characteristics of these bacteria have been examined to various degrees and shall be discussed in this review. At the same time, comprehensive, rigorous investigations of previously discovered isolates, such as *Mycobacterium* sp. strain PYR-1 (Heitkamp and Cerniglia, 1988), now known as *M. vanbaalenii* PYR-1 (Khan *et al.*, 2002), have continued and are significantly expanding our understanding of prokaryotic HMW PAH biodegradation.

Due to the enormous amount of high-quality research that has been conducted on *M. vanbaalenii* PYR-1 it most likely needs to be addressed as the subject of its own review; however, these authors will attempt to discuss some of the results pertaining to this organism herein. *M. vanbaalenii* PYR-1 was originally isolated from estuarine sediments near an oil field (Heitkamp and Cerniglia, 1988) and was shown at that time to biodegrade various HMW PAHs, including the four-ring PAHs pyrene and fluoranthene by fortuitous metabolism. Over 20 years later, this organism has come to be one of the most extensively studied organisms in the field of prokaryotic HMW PAH biodegradation research and PAH biodegradation research in general. Building upon many previous studies that have involved this organism, investigations have continued over the last 10 years, which have resulted in further development and refinement of the HMW PAH catabolic pathways for pyrene, fluoranthene, benz[a]anthracene, 7,12-dimethylbenz[a]anthracene and even benzo[a]pyrene in addition to many other comprehensive studies.

The proposed pathways for pyrene biodegradation by *M. vanbaalenii* PYR-1 have been continuously developed since its isolation and it was originally determined that oxidation by this organism occurred by both dioxygenation and monooxygenation (Heitkamp *et al.*, 1988b). Within dioxygenation-initiated pyrene metabolism, the predominant pathway was shown to occur via dioxygenation at the 4,5 positions of pyrene and resulted in *cis*-4,5-dihydroxy-4,5-dihydropyrene (pyrene *cis*-4,5-dihydrodiol), which after multiple transformations ultimately led to the tricarboxylic acid cycle (TCA) cycle. At least a second pathway that began with initial dioxygenation at the 1,2 positions to form *O*-methylated derivatives of pyrene *cis*-1,2-dihydrodiol appeared to serve as a detoxification process. In 2007, Kim and colleagues (2007) constructed the first complete pathway for pyrene biotransformation by *M. vanbaalenii* PYR-1 to TCA intermediates by utilizing a combination of metabolite identification, genomic and proteomic analyses that indicated that pyrene was metabolized through the  $\beta$ -ketoacid pathway. Twenty-seven enzymes that included 14 responsible for the degradation of pyrene to phthalate, six responsible for the degradation of phthalate to protocatechuate and seven responsible for the lower pathway from protocatechuate to acetyl-coenzyme A and succinyl coenzyme A were identified in addition to three enzymes responsible for the detoxification pathway of pyrene to 1,2-dimethoxypyrene. It was reported that all genes involved in the pyrene metabolic pathways were identified through these analyses and that alternative routes of pyrene degradation were not detected based upon genome sequence screening. The proposed pathways for pyrene biodegradation by mycobacteria, including the complete pathway proposed for *M. vanbaalenii* PYR-1 to the TCA cycle, are shown in Fig. 2, with further discussion in regard to pyrene biodegradation by other organisms discussed in later sections of this review.

### **Genes relevant to HMW PAH metabolism in *M. vanbaalenii* PYR-1**

To construct the complete pyrene pathway for *M. vanbaalenii* PYR-1 by such a biosystems approach (and for the fluoranthene biodegradation pathways discussed below), results were required from earlier studies where the enzymatic functions of various genes involved in PAH biodegradation processes were determined from *M. vanbaalenii* PYR-1 (Khan *et al.*, 2001; Kim *et al.*, 2004b; Stingley *et al.*, 2004a), *Mycobacterium* sp. strain 6PY1 (Krivobok *et al.*, 2003) and from phenanthrene biodegradation by *Nocardioides* sp. strain KP7 (Iwabuchi and Harayama, 1998a,b; Saito *et al.*, 2000) for example. In 2001, Khan *et al.* was the first to characterize the genes encoding a polycyclic aromatic ring dioxygenase in *M. vanbaalenii* PYR-1 whereby an aldehyde dehydrogenase



**Fig. 2.** Pathways proposed for the biodegradation of pyrene by mycobacteria, including the complete pathway proposed for *M. vanbaalenii* PYR-1 to the TCA cycle (Heitkamp *et al.*, 1988b; Walter *et al.*, 1991; Cerniglia, 1992; Dean-Ross and Cerniglia, 1996; Schneider *et al.*, 1996; Rehmann *et al.*, 1998; Vila *et al.*, 2001; Krivobok *et al.*, 2003; Habe *et al.*, 2004; Kim *et al.*, 2005b; 2007; Liang *et al.*, 2006; Zhong *et al.*, 2006).

(*nidD*), the dioxygenase small ( $\beta$ -)subunit (*nidB*) and the dioxygenase large ( $\alpha$ -)subunit (*nidA*) were cloned and sequenced and found to be arranged in a sequence different from genes found in other bacterial dioxygenase systems. These genes were later shown by Brezna and colleagues (2003) to have conserved homologues in four other distantly related *Mycobacterium* species through comparisons of *Mycobacterium* sp. strain RJGII-135, (Schneider *et al.*, 1996), *M. flavescens* PYR-GCK, (Dean-Ross and Cerniglia, 1996) *M. frederiksbergense* FAN9<sup>T</sup> (Willumsen *et al.*, 2001) and *M. gilvum* BB1 (Boldrin *et al.*, 1993; Böttger *et al.*, 1997). *M. austroafricanum* ATCC 33464, which did not show high PAH-degrading activity, did not possess the *nidA* gene even though it was reported to be almost identical to *M. vanbaalenii* PYR-1 at the 16S rDNA level.

Results from previous studies on PAH biodegradation by *M. vanbaalenii* PYR-1 and the three-ring compounds anthracene and phenanthrene suggested that this organism might possess multiple copies of different dioxygenase genes or at least possess relaxed dioxygenase specificity because the organism was capable of biotransforming a broad range of aromatic substrates (Moody *et al.*, 2001). Indeed, in 2004, Stingley and colleagues (2004a) focused on characterizing additional genes in the *nidDBA* region of *M. vanbaalenii* PYR-1 and identified and characterized a putative phthalate operon in a *Mycobacterium* species for the first time that was located approximately 12–19 kb upstream of the *nidA* large subunit gene. They showed that a putative regulatory protein (*phtR*) was encoded divergently with five tandem genes: phthalate dioxygenase large subunit (*phtAa*), small subunit (*phtAb*), phthalate dihydrodiol dehydrogenase (*phtB*), phthalate dioxygenase ferredoxin subunit (*phtAc*) and phthalate dioxygenase ferredoxin reductase (*phtAd*). When cloned into *Escherichia coli*, the authors concluded that phthalate was converted to 3,4-dihydroxyphthalate based upon the fragmentation pattern of the trimethylchlorosilylated metabolite derivative recovered by GC-MS analyses. Using PCR and Southern hybridization analyses, it was shown that homologues to the *pht* operon region were present in PAH-degrading *Mycobacterium* spp. that were assayed previously for the *nidA* gene by Brezna and colleagues (2003), with the exception of *Mycobacterium* sp. strain RJGII-135. *M. austroafricanum* GTI-23 (Bogan *et al.*, 2003) and a *Rhodococcus* sp. (Dean-Ross *et al.*, 2001) were also assayed and found to possess the *pht* operon.

Further investigations with *M. vanbaalenii* PYR-1 resulted in the characterization of ring-hydroxylating dioxygenase genes Orf25 and Orf26 that encoded putative aromatic oxygenase large and small subunits (Stingley *et al.*, 2004b). This was the first time that genes in the phthalate pathway were identified in a *Mycobac-*

*terium* sp. and confirmed previous reports by Moody and colleagues (2001) in their biodegradation studies with phenanthrene. Depending upon one's perspective, the main (4,5) pathway for the biodegradation of pyrene enters into the phenanthrene pathway through the formation of 3,4-dihydroxyphenanthrene during biodegradation to phthalate and the TCA cycle. By using a proteomics approach that consisted of protein profiling by two-dimensional electrophoresis analyses (2-DE) combined with liquid chromatography electrospray ionization tandem mass spectrometry, Kim and colleagues (2004a) documented the upregulation of multiple proteins in *M. vanbaalenii* PYR-1 cultures that were induced with PAH compounds. These included a catalase-peroxidase, putative monooxygenase, dioxygenase small subunit, a naphthalene-inducible dioxygenase small subunit and an aldehyde dehydrogenase. The dioxygenase small subunit ( $\beta$ -subunit) was a 20 kDa protein that showed a sevenfold increase when *M. vanbaalenii* PYR-1 was exposed to fluoranthene compared with other induction substrates. Further investigation by PCR with degenerate primers that were designed based upon *de novo* peptide sequencing combined with a series of plaque hybridizations were carried out to screen the genomic library of *M. vanbaalenii* PYR-1 and the genes *nidA3B3* were cloned and sequenced (Kim *et al.*, 2006). In cloned *E. coli* cells, *nidA3B3* converted the HMW PAHs fluoranthene, pyrene and benz[a]anthracene to *cis*-dihydrodiols with the aid of ferredoxin (PhdC) and ferredoxin reductase (PhdD) genes. *nidA3B3* represented the fourth copy of a terminal dioxygenase gene identified from *M. vanbaalenii* PYR-1.

In addition to the terminal dioxygenase genes documented in *M. vanbaalenii* PYR-1, a catalase-peroxidase gene, the *katG* gene was cloned, expressed and characterized when it was induced by pyrene. It was the first gene characterized in *M. vanbaalenii* PYR-1 and it was postulated that the enzyme served to protect dioxygenases from oxidative inactivation by exogenous oxidants or that the enzyme was induced to remove endogenous hydrogen peroxide that may have been generated as an intermediate during PAH metabolism (Wang *et al.*, 2000). The cytochrome P450 genes *cyp151* (*pipA*), *cyp 150* and *cyp 51* were characterized in *M. vanbaalenii* PYR-1 by Brezna and colleagues (2006) and represented the first evidence of functional cytochrome P450 genes coexisting with aromatic ring-hydroxylating dioxygenase in a HMW PAH-utilizing organism. These data corroborated data from previous work by Moody and colleagues (2001; 2003; 2004; 2005) that indicated monooxygenase attacks on phenanthrene, benz[a]anthracene, 7,12-dimethylbenz[a]anthracene and at the 11,12 position of benzo[a]pyrene for example (see below). In the case of pyrene, *M. vanbaalenii* PYR-1 monooxygenation was

discussed as early as 1988 (Heitkamp *et al.*, 1988b). After gene cloning, *pipA* and *cyp 150* were partially expressed in *E. coli* and resulted in the production of mono-oxygenated products of the HMW PAHs pyrene and 7-methylbenz[a]anthracene. In the case of pyrene, 1-, 2- and 4-hydroxypyrene were produced and it was noted that due to the symmetry of pyrene these were the only possible isomers. Further screening of 13 mycobacterium isolates for *pipA*, *cyp 150* and *cyp 51* indicated that there was little correlation between PAH-degrading ability and the presence of these genes and this was in contrast to assays for *nidA* and *nidB* carried out in this study (Brezna *et al.*, 2006) and in a previous study (Brezna *et al.*, 2003) discussed above.

Evidence for constitutive catechol-*O*-methyl transferase and PAH-quinone reductase was also provided from studies with *M. vanbaalenii* PYR-1. It was discussed that catechol-*O*-methyl transferase may play an important role in detoxifying non-K- and non-bay-region PAH catechols that are produced inside the cell during PAH metabolism (Kim *et al.*, 2004b). These compounds are not considered to serve as carbon and energy sources for *M. vanbaalenii* PYR-1 and it has been postulated that quinone reductases might be necessary for protecting the cell against cytotoxicity and to provide a PAH catechol supply during metabolism. One year before, Kim and colleagues (2003b) also reported on the recovery of two *o*-quinone reductases from the cell extracts of German soil isolate *Mycobacterium* sp. strain PYR100, which was isolated on pyrene as a sole source of carbon and energy and which also degraded fluoranthene and phenanthrene. Comparative analysis of the 16S rDNA and 16S-23S intergenic spacer sequences of *Mycobacterium* sp. strain PYR100 and *M. vanbaalenii* PYR-1 later revealed that strain PYR100 was an intergenic spacer sequence sequevar variant of *M. vanbaalenii* PYR-1 (Kim *et al.*, 2005a). Furthermore, the activity of *M. vanbaalenii* PYR-1 to metabolize pyrene under two different pH conditions was reported in 2005 in addition to a then updated version of the pyrene biodegradation pathway by this organism (Kim *et al.*, 2005b). Interestingly, a decrease in one pH unit, from 7.5 to 6.5, resulted in a fourfold faster degradation rate of 0.5 mM pyrene by a resting cell suspension of *M. vanbaalenii* PYR-1. Higher levels of pyrene were detected in cytosolic fractions and it was concluded that the acidic pH facilitated increased cell permeability to PAHs. However, *M. vanbaalenii* PYR-1 produced relatively higher levels of *O*-methylated derivatives of non-bay-region and non-K-region pyrene-diols in the cytosol at pH 6.5 compared with the presence of metabolic intermediates in the culture fluid at pH 7.5, and the authors concluded that acidic pH may become a significant burden to cells when toxic metabolites accumulate in the cytosol.

### Mycobacterial biodegradation of pyrene and fluoranthene

Many new pyrene- and fluoranthene-degrading mycobacteria have been isolated throughout the world in the last 10 years. In 1998, pyrene utilization as a sole source of carbon and energy and metabolite production by *Mycobacterium* sp. strain KR2, isolated from a German gas works soil, was documented by Rehmann and colleagues (1998). When the organism was fed 500 mg l<sup>-1</sup> pyrene, approximately 60% was metabolized in 8 days. Eight metabolites were recovered and contributed to our understanding of the pyrene catabolic pathways, including 2-carboxybenzaldehyde, 1-hydroxy-2-naphthoic acid and *cis*-3,4-phenanthrene dihydrodiol-4-carboxylic acid that were recovered for the first time (Fig. 2). The identity of *cis*-3,4-phenanthrene dihydrodiol-4-carboxylic acid was postulated from the identification of 4-phenanthrol in acidified media. *Mycobacterium* sp. strain KR2 continued to biodegrade pyrene even though the organism was maintained on nutrient agar slants without pyrene addition and the authors discussed that this was supportive evidence for chromosomal location of the degradative genes and further noted that *ortho*-cleavage pathways generally appeared to be chromosomally encoded (van der Meer *et al.*, 1992). Attempts to detect plasmids in strain KR2 were unsuccessful. In 1999, pyrene-utilizing *Mycobacterium* sp. strain MR-1 was documented after isolation from PAH-contaminated sediments from the Buffalo River, New York, USA by Molina and colleagues (1999). Cross-induction of pyrene mineralization by phenanthrene was demonstrated for strain MR-1 and an enrichment culture from the same sediments whereby it was shown that new protein synthesis was not required for [4,5,9,10-<sup>14</sup>C]pyrene mineralization after pre-acclimation on phenanthrene.

*Mycobacterium* sp. strain AP1 was isolated from crude oil-contaminated sand in Spain on enrichment cultures that consisted of 1 g l<sup>-1</sup> pyrene in mineral salts medium (Vila *et al.*, 2001). The organism also utilized fluoranthene, phenanthrene and hexadecane but not anthracene for example, as sole sources of carbon and energy. Investigation of the biodegradation mechanisms of *Mycobacterium* sp. strain AP1 during growth on pyrene showed that initiation of attack occurred by either mono- or dioxygenase activity at the 4,5 positions that resulted in either *trans*- or *cis*-4,5-dihydroxy-4,5-dihdropyrene metabolites in addition to other metabolites that further confirmed the pathways proposed for pyrene biodegradation. Vila and colleagues (2001) also identified the novel metabolite, 6,6'-dihydroxy-2,2'-biphenyl dicarboxylic acid whose presence indicated that a new branch of the proposed pathways for pyrene occurred by two sequential cleavages of both central K-region rings. This branch of the

pathway is also indicated in Fig. 2. They reported that the new metabolite was formed after dioxygenation at the 4,5 and 9,10 positions with subsequent cleavage of both central rings and that *meta*- and *ortho*-cleavage reactions followed by one- or two-carbon excisions might be possible. The metabolite was also found to increase in the culture medium and it was suggested that it might therefore be a dead-end-product. Interestingly, the UV-visible spectrum for 6,6'-dihydroxy-2,2'-biphenyl dicarboxylic acid was similar to an unidentified metabolite (metabolite IV) reported by Rehmann and colleagues (1998) during growth of *Mycobacterium* sp. strain KR2 on pyrene.

In 2003, Krivobok and colleagues (2003) identified two pyrene-induced proteins from *Mycobacterium* sp. strain 6PY1, originally isolated from PAH-contaminated soil that grew on pyrene as a sole source of carbon and energy. In  $^{14}\text{C}$ [PAH] assays, cells that were grown on pyrene or phenanthrene exhibited high levels of catabolic activity, but cells that were grown on acetate did not and this situation was exploited by examining protein extracts via 2-DE combined with *in vivo* protein-labelling assays. Pyrene-induced proteins were tentatively identified by peptide sequence analyses and the genes encoding the terminal components of the two enzymes were successfully cloned. These two new terminal dioxygenase components, Pdo1 and Pdo2, were reported to be similar in other actinomycetes, including *M. vanbaalenii* PYR-1 and *Nocardioides* strain KP7, and it was concluded that they were regulated differentially. *Mycobacterium* sp. strain 6PY1 was also utilized in pyrene biodegradation investigations in freshwater sediments whereby 15 mg of pyrene and radiolabelled pyrene was applied in 300 g of wet sediment (Jouanneau *et al.*, 2005). It was concluded that [4,5,9,10- $^{14}\text{C}$ ]pyrene mineralization was enhanced by sediment inoculation, 8.8%  $^{14}\text{CO}_2$  in 61 days, and that inoculation plus planting with the reed *Phragmites australis* resulted in twofold less enhancement, 4.4%  $^{14}\text{CO}_2$  over the same time period. The authors also reported that they recovered the water-soluble metabolites, 4,5-phenanthroic acid and 4-phenanthroic acid from their microcosms and isolated two pyrene-degrading strains most closely related to *M. austroafricanum* and *Stenotrophomonas maltophilia*. *Mycobacterium* sp. strain 6PY1 was also employed in experiments designed to investigate the effects of organic and inorganic materials on pyrene removal (Cottin and Merlin, 2008).

New *Mycobacterium* spp. strains JLS, KMS and MCS were isolated from creosote- and pentachlorophenol-contaminated soil from Montana, USA that was undergoing bioremediation through a prepared-bed land treatment unit process. All three isolates mineralized pyrene as a sole source of carbon and energy in radiolabel studies and were shown to possess dioxygenase *nid* genes, *nidA* and *nidB* (Miller *et al.*, 2004). They were employed in a

comparative study with *M. gilvum* PYR-GCK and *M. vanbaalenii* PYR-1 whereby various biochemical and genetic differences among the strains were documented (Miller *et al.*, 2007). *Mycobacterium* sp. strain JLS was the subject of synchrotron radiation-based Fourier transform infrared spectromicroscopy investigations where it was shown that the presence of humic acids significantly shortened the lag time for pyrene biodegradation by this strain from 168 to 2 h when it was studied on a magnetite chip (Holman *et al.*, 2002). These strains were also investigated in relation to their abilities to associate with plant roots when inoculated onto germinating barley seeds whereby strain KMS in the presence of diluted root wash (Child *et al.*, 2007a) or barley root colonization by strain KMS resulted in enhanced pyrene mineralization by radiotracer analyses (Child *et al.*, 2007b).

In other studies that involved *Mycobacterium* sp. strain KMS, the effects of varying soil humic acid concentrations on pyrene mineralization in liquid culture were investigated (Liang *et al.*, 2007) in addition to pyrene metabolite humification studies where it was incubated with [4- $^{13}\text{C}$ ]- or [4,5,9,10- $^{14}\text{C}$ ]pyrene with and without a soil humic acid standard to characterize the nature of the produced residues (Nieman *et al.*, 2007). Results of the [ $^{13}\text{C}$ ]/[ $^{14}\text{C}$ ]pyrene investigations indicated that most of the pyrene carbon was incorporated into cellular material and that direct coupling of extracellular pyrene metabolites to soil organic matter was not a primary fate mechanism under the experimental conditions. These results were in agreement with previous [4,9- $^{13}\text{C}$ ]pyrene-sediment investigations using flash-pyrolysis combined with  $^{13}\text{C}$  NMR where sequestered pyrene was found to be non-covalently associated with sediment organic matter and was not transformed (Guthrie *et al.*, 1999).

In 2006, *Mycobacterium* sp. strain KMS was the subject of a proteomics study (Liang *et al.*, 2006) whereby three metabolites, including pyrene-4,5-dione, were identified. Initial attack by *Mycobacterium* sp. strain KMS on pyrene was reported to occur by mono- or dioxygenation at the 4,5 positions based upon metabolite and protein identification and a pathway for this organism was proposed that confirmed previous biodegradation pathways proposed for other mycobacteria (Fig. 2). Although the metabolite *trans*-4,5-pyrene dihydrodiol was not recovered in this study, the gene coding an epoxide hydrolase was identified and, therefore, monooxygenation was included in the proposed pathway for strain KMS. It was noted that this was the first study to show transformation of pyrene to pyrene-4,5-dione by a *Mycobacterium* species. Interestingly, strain KMS metabolized pyrene-4,5-dione when it was added directly to culture media, indicating that it may have unique abilities to deal with pyrene-4,5-dione toxicity. The accumulation of pyrene-4,5-dione had been previously shown by Kazunga and Aitken (2000) in *M.*

*vanbaalenii* PYR-1 when it was incubated with *cis*-4,5-pyrene dihydrodiol and as a pyrene metabolite of *Sphingomonas yanoikuyae* R1 (isolated from a gas manufacturing plant; Aitken *et al.*, 1998). In whole-sediment incubations, the presence of pyrene-4,5-dione had been shown by Guthrie-Nichols and colleagues (2003) for the first time during studies designed to examine the effects of ageing on pyrene transformation in sediments. *cis*-4,5-Pyrene dihydrodiol had been documented in sediments in 1996 by Li and colleagues (1996). Additionally, diones of fluoranthene, fluoranthene-2,3- and -1,5-dione were documented for the first time from bacteria by Kazunga and colleagues (2001) by studying four strains that produced these compounds during fluoranthene metabolism. Fluoranthene-2,3-dione inhibited the mineralization of other HMW PAHs by some of the strains and, although the inhibition mechanism appeared to occur through cytotoxicity, results indicated that other less straightforward mechanisms also appeared to be possible.

Further isolation of pyrene and fluoranthene-degrading strains of mycobacteria was accomplished by different research groups and in 2003, Bogan and colleagues (2003) reported on the characterization of *M. austroafricanum* strain GTI-23, isolated from a manufactured gas plant site soil in Iowa, USA. Strain GTI-23 grew on pyrene and fluoranthene as sole sources of carbon and energy and also degraded benzo[*a*]pyrene. Inhibition was shown to occur when mixed with phenanthrene or pyrene. Interestingly, GTI-23 grew on both HMW PAHs and the aliphatics dodecane and hexadecane. As more studies document such occurrences, it appears that aromatic and aliphatic hydrocarbon degradative capacities within a single strain may not be as unusual as previously thought. In pyrene-contaminated soil inoculation studies that employed *M. austroafricanum* strain GTI-23, increased pyrene contact time resulted in increased sequestration and reduced biodegradation (Bogan and Sullivan, 2003). Zhong and colleagues (2006) have reported on the degradation of pyrene and PAH mixtures by *Mycobacterium* sp. strain A1-PYR that was isolated from mangrove sediments. It utilized pyrene as a sole source of carbon and energy and some pyrene metabolites were identified. Pyrene- and fluoranthene-degrading *Mycobacterium* sp. strains HH1 through HH3 were also isolated from mangrove sediments in the Ho Chung basin in Hong Kong (Zhou *et al.*, 2006; 2008) and the authors reported on fluoranthene biodegradation by a *Terrabacter* sp. strain. *Terrabacter* species were previously shown to metabolize low-molecular-weight PAHs but not HMW PAHs (Habe *et al.*, 2005). In 1999, *Mycobacterium* sp. strain CH1 was isolated from PAH-contaminated freshwater sediments in the USA by Churchill and colleagues (1999) and was shown to mineralize pyrene and fluoranthene with pyrene

as a sole carbon and energy source. Strain CH1 was also capable of utilizing branched- and *n*-alkanes as sole carbon and energy sources. Alkaliphilic *Mycobacterium* sp. strain MHP-1 was isolated from soil in Chiba, Japan (Habe *et al.*, 2004) and utilized pyrene as a sole source of carbon and energy via the 4,5 biodegradation pathway. Optimum growth pH was 9 and the strain was found to possess the aromatic ring hydroxylase gene *nidAB*.

The characterization of the new species *Mycobacterium pyrenivorans* was documented in 2004 by Derz and colleagues (2004) following isolation from PAH-contaminated soil at a former coking plant site in Übach-Palenberg, Germany. *Mycobacterium pyrenivorans* was found to grow on the HMW PAHs pyrene and fluoranthene as a sole source of carbon and energy but not benzo[*a*]pyrene. Sho and colleagues (2004) identified two pyrene degradation gene clusters in *Mycobacterium* sp. strain S65 which was isolated from a jet fuel-contaminated site in Quebec, Canada. Using Southern hybridization, *nidA* homologues were shown to occur in two separate loci in the strain. The organism used pyrene, fluoranthene and phenanthrene as sole carbon and energy sources and pyrene mineralization was enhanced by the addition of less water-soluble HMW PAHs, such as benzo[*a*]pyrene or benz[*a*]anthracene. *M. frederiksbergense* FAn9<sup>T</sup> was isolated from a coal tar-contaminated former gas works site in Frederiksberg, Denmark and it was reported to use the HMW PAH fluoranthene and pyrene as sole sources of carbon and energy (Willumsen *et al.*, 2001). In 2000, Bastiaens and colleagues (2000) compared two different procedures for the isolation of PAH-utilizing bacteria and showed clear differences in the types of organisms isolated by each mode of isolation. They reported that liquid enrichment selected for *Sphingomonas* species and a membrane method selected for *Mycobacterium* species. A pyrene-utilizing strain LB208 was determined to be a *Mycobacterium* species that was 100% similar to pyrene-degrading strain *Mycobacterium gilvum* strain BB1 (Boldrin *et al.*, 1993; Böttger *et al.*, 1997). *M. frederiksbergense* strain LB501T was recovered in this study and although it has not been shown to biodegrade HMW PAHs, it has been the subject of various comprehensive investigations that have contributed to our understanding of PAH biodegradation (Wick *et al.*, 2001; 2002; 2003a,b; van Herwijnen *et al.*, 2003a).

*Mycobacterium gilvum* strain VF1 and *Gordonia*-like strain BP9 – the etymologically correct *Gordonia* was proposed in 1997 to replace *Gordona* (Stackebrandt *et al.*, 1997; Arenskötter *et al.*, 2004) – was originally isolated from hydrocarbon-contaminated soil and was capable of utilizing fluoranthene and pyrene as sole carbon and energy sources (Kästner *et al.*, 1994). It was demonstrated to grow on pyrene microcrystals formed by sonication with and without silicone oil (Mutnuri *et al.*,



2005) and under these conditions biodegradation was enhanced. Pyrene and fluoranthene biodegradation by strain B9 were also assessed in soil under different conditions including variable pH (Kästner *et al.*, 1998).

*Mycobacterium* sp. strain LP1 was isolated from enrichment culture produced from a control agricultural soil, Uppsala, Sweden, which was part of a previous study (Pizzul *et al.*, 2006) and was shown to grow on pyrene resulting in greater than 90% degradation in 2 weeks when incubated at a concentration of 50 mg l<sup>-1</sup> (Pizzul *et al.*, 2007). Incubation with a mixture of PAHs resulted in less than 25% pyrene biodegradation. After growth of *Mycobacterium* sp. strain SNP11, isolated from a grassland soil near Metz, France, on pyrene, fluoranthene and other PAHs, metabolite screening for acute, chronic and genotoxic effects was carried out and indicated significant reductions in all cases (Pagnout *et al.*, 2006). Genes encoding the  $\alpha$ -subunit of two ring-hydroxylating dioxygenases Nid and Pdo2 were detected in this organism (Pagnout *et al.*, 2007) and the *pdoA2* gene region (called *phdA* in their study) was cloned and sequenced. Expression of PAH-degrading genes in *Mycobacterium smegmatis* mc<sup>2</sup>155 was carried out for the first time and broad ring-hydroxylating substrate specificity was revealed.

### Biotransformation pathways of fluoranthene by actinomycetes

Although there have been numerous reports of both Gram-negative and Gram-positive bacteria specifically capable of degrading fluoranthene, with approximately 50 bacterial isolates reported by Rehmann and colleagues (2001), characterization of fluoranthene metabolites has occurred with less frequency. In the last 10 years however, various key studies that employed both classical and new approaches have resulted in the documentation of new metabolites and new proposals for pathways of fluoranthene biodegradation by actinomycetes. The currently proposed pathways for the biodegradation of fluoranthene by actinomycetes are given in Fig. 3 and are discussed below.

In 2001, an alternative pathway for fluoranthene biodegradation through carbon positions 2,3 by *Mycobacterium* sp. strain KR20 was proposed by Rehmann and colleagues (2001). This new information complemented the existing proposed pathways for fluoranthene biodegradation by *Alcaligenes denitrificans* (Weissenfels *et al.*, 1991), *M. vanbaalenii* PYR-1 (Kelley *et al.*, 1991; Cerniglia, 1992; Kelley *et al.*, 1993) and *Pasteurella* sp. strain IFA (Sepic *et al.*, 1998) that showed 1,2 position- and 7,8 position-initiating attacks on fluoranthene but for which the corresponding *cis*-1,2-fluoranthene and *cis*-7,8-fluoranthene dihydrodiols had yet to be recovered. *Mycobacterium* sp. strain KR20, isolated

from a PAH-contaminated German gas works site, was grown on 500 mg l<sup>-1</sup> fluoranthene as a sole source of carbon and energy, and seven metabolites were documented. Five metabolites, *cis*-2,3-fluoranthene dihydrodiol, Z-9-carboxymethylene-fluorene-1-carboxylic acid, 4-hydroxybenzochromene-6-one-7-carboxylic acid (indicated as 9-carboxymethylene-9*H*-fluorene-1-carboxylic acid and 8-hydroxy-3,4-benzocoumarin-1-carboxylic acid respectively in Fig. 3), *cis*-1,9a-dihydroxy-1-hydrofluorene-9-one-8-carboxylic acid and benzene-1,2,3-tricarboxylic acid, were identified by NMR and MS spectroscopy and a new pathway was proposed. At that time, the possibility of initial dioxygenation at the 2,3 positions for *M. vanbaalenii* PYR-1 and *Pasteurella* sp. strain IFA was also discussed even though comparison of downstream fluoranthene metabolites among the strains indicated that pathway divergence was likely. This was later confirmed in *M. vanbaalenii* PYR-1 in 2007 when Kim and colleagues (2007) published a comprehensive investigation of fluoranthene metabolites for this organism and explained that the 2,3 pathway was most likely the main mechanism of fluoranthene biodegradation.

*Mycobacterium holderi* was isolated from PAH-contaminated soil and was reported to grow on fluoranthene and co-oxidize pyrene in the presence of fluoranthene. It produced 29 metabolites during fluoranthene biodegradation and the *cis*-2,3-fluoranthene dihydrodiol was the only metabolite reported to be identified; however, evidence was not provided (Kleespies *et al.*, 1996). In 2002, Dean-Ross and colleagues (2002) documented biodegradation of fluoranthene via fortuitous metabolism by a *M. flavescens* strain and a *Rhodococcus* sp. strain. Both strains acted on fluoranthene through the 1,2 pathway and *meta* cleavage; however, it was also proposed that the *Rhodococcus* sp. strain produced 9-(carboxymethylene)fluorene-1-carboxylic acid (9-carboxymethylene-9*H*-fluorene-1-carboxylic acid) by *ortho*-cleavage of 2,3-dihydroxy-fluoranthene similar to *Mycobacterium* sp. strain KR20.

Metabolites of fluoranthene biodegradation by various mycobacteria were characterized by López *et al.* in two reports from 2005 and 2006 whereby *Mycobacterium* sp. strains CP1, CP2, Cft2 and Cft6 and *Mycobacterium* sp. strain AP1, which was described above in the context of pyrene biodegradation (Vila *et al.*, 2001), were investigated under conditions where fluoranthene was administered as the sole source of carbon and energy (López *et al.*, 2005; 2006). In the 2005 report, strains CP1, CP2, Cft2 and Cft6, isolated from creosote-contaminated soil in Andújar, Spain were all found to oxidize fluoranthene by dioxygenation at the 1,2 positions followed by *meta* cleavage and a two-carbon excision to produce 9-fluorenone-1-carboxylic acid and by dioxygenation at the 2,3 positions followed by *ortho*-cleavage to yield



9-carboxymethylene-9H-fluorene-1-carboxylic acid. Additionally, strains CP1 and CP2 metabolized fluoranthene by dioxygenation at the 7,8 positions followed by *meta* cleavage and eventual transformation to acenaphthone. These three routes of fluoranthene biodegradation were also shown to occur in *Mycobacterium* sp. strain AP1 when it was grown on fluoranthene as a sole source of carbon and energy (López *et al.*, 2006).

In 2007, through the use of genomic, metabolomic and proteomic approaches, Kweon and colleagues (2007) reported that there were 53 potential enzymes likely to be involved in the biodegradation of fluoranthene by *M. vanbaalenii* PYR-1. They proposed a comprehensive pathway that included four possible metabolic routes that could be initiated by either mono- or dioxygenation reactions. They explained that most of the enzymes used in the pyrene degradation pathway were identified in fluoranthene-induced cultures and that similar to pyrene degradation in *M. vanbaalenii* PYR-1, all of the routes channelled through phthalate and the  $\beta$ -keto adipate pathway with the exception of detoxification pathways. Also in 2007, fluoranthene metabolism was characterized by proteomic methods using *Mycobacterium* sp. strain JS14, which was isolated from PAH-contaminated soil in Hilo, Hawaii, USA along with other strains, including *Mycobacterium* sp. strain JS19b1, which grew on fluoranthene and pyrene (Lee *et al.*, 2007; Seo *et al.*, 2007). Sixteen metabolites of fluoranthene biodegradation generated by strain JS14 were detected following 2 weeks of incubation with 200 mg l<sup>-1</sup> fluoranthene. Comparison of glucose-fed and fluoranthene-fed cultures by 1-D PAGE or 2-DE and nano-LC-MS/MS revealed upregulation of 25 proteins related to fluoranthene biodegradation including catalase and superoxide dismutase. In strain JS14, initial dioxygenation at the 1,2-, 2,3-, 7,8- and 8,9-positions were revealed and pathways for fluoranthene biodegradation by strain JS14 were proposed. Strain JS14 was also shown to transform the organophosphorous pesticides chlorfenvinphos and diazinon (Seo *et al.*, 2007).

#### **Biotransformation pathways of benz[a]anthracene by *Mycobacterium* spp.**

Reports that document the biodegradation of katabolized HMW PAHs occur with less frequency in the biodegradation literature, even though reports of their biodegradation were published as early as 1947 when it was shown that a marine consortium biodegraded 47% and 13% of 25 mg each of benz[a]anthracene and dibenz[a,h]anthracene through the detection of carbon dioxide (Sisler and ZoBell, 1947). Since the 1980s, reports that documented the biodegradation of the four-ring benz[a]anthracene by bacterial isolates representing the genera *Sphingobium* (Jerina *et al.*, 1984; Mahaffey

*et al.*, 1988; Ye *et al.*, 1996; Boyd *et al.*, 2006), *Sphingomonas* (Demanèche *et al.*, 2004; Jouanneau *et al.*, 2006), *Stenotrophomonas* (Juhász *et al.*, 1996; 1997; 2000b), *Alcaligenes* (Weissenfels *et al.*, 1991), *Pseudomonas* (Caldini *et al.*, 1995; Chen and Aitken, 1999) and *Mycobacterium* (Schneider *et al.*, 1996) were published and documentations of metabolites under various circumstances were published for *Sphingobium yanoikuyae* strains B8/36 and B1 (Jerina *et al.*, 1984; Mahaffey *et al.*, 1988; Boyd *et al.*, 2006), *Sphingomonas* sp. strain CHY-1 (Demanèche *et al.*, 2004; Jouanneau and Meyer, 2006; Jouanneau *et al.*, 2006) and *Mycobacterium* sp. strain RJGII-135 (Schneider *et al.*, 1996). In 2005, Moody and colleagues (2005) characterized multiple metabolites from cultures of *M. vanbaalenii* PYR-1 exposed to 120 mg l<sup>-1</sup> benz[a]anthracene applied in dimethylformamide. HPLC, UV-visible absorption, GC-MS and NMR analyses revealed that *M. vanbaalenii* PYR-1 attacked benz[a]anthracene at the 1,2-, 5,6-, 7,12-, and 10,11-positions and it was concluded that the 10,11-pathway was the predominant route of metabolism. *ortho*- and *meta*-ring cleavages resulted in various benz[a]anthracene biodegradation metabolites and multiple pathways for biodegradation by *M. vanbaalenii* PYR-1 were proposed (Fig. 4). Previously, only the *cis*-5,6- and 10,11-dihydrodiols had been documented from mycobacteria (Schneider *et al.*, 1996).

Regio- and stereo-selective biodegradation of 7,12-dimethylbenz[a]anthracene was also documented in *M. vanbaalenii* PYR-1 whereby mono- and dioxygenation reactions at the 5,6 positions and monooxygenation of the C-7 methyl group were shown to occur (Moody *et al.*, 2003).

#### **Biotransformation pathways of benzo[a]pyrene by *Mycobacterium* spp.**

Benzo[a]pyrene is currently not known to be utilized as a sole source of carbon and energy by any bacteria and significant biodegradation appears to require bacterial cooperation although the mechanisms are still unclear. At the same time, some isolates have been shown to biotransform benzo[a]pyrene, metabolites have been characterized and our understanding of biodegradation pathways has been advanced. In 2004, Moody and colleagues (2004) showed that *M. vanbaalenii* PYR-1 metabolized benzo[a]pyrene delivered in dimethylformamide, 0.48 mM final benzo[a]pyrene concentration, in cultures that consisted of 500 ml of basal salts medium supplemented with 0.38 g ml<sup>-1</sup> each of peptone, yeast extract and soluble starch. Phenanthrene in dimethylformamide, 100  $\mu$ l of a 70 mM solution, was also added for enzyme induction. After 96 h, a total of nine metabolites were identified by UV-visible, mass, NMR

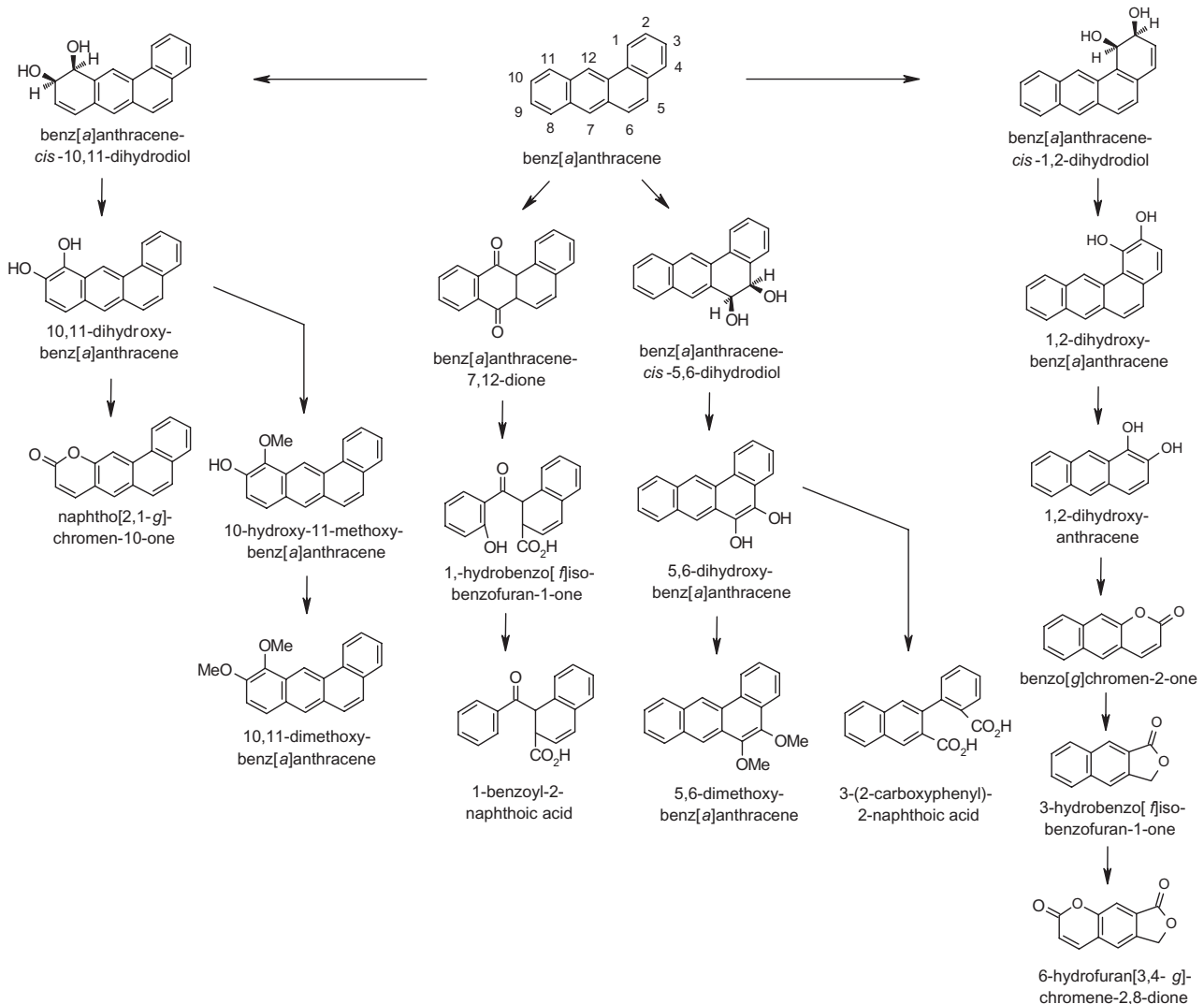


Fig. 4. Pathways proposed for the biodegradation of benz[a]anthracene by mycobacteria (Schneider *et al.*, 1996; Moody *et al.*, 2005).

and circular dichroism spectral analyses: *cis*-4,5-dihydro-4,5-dihydroxybenzo[a]pyrene (benzo[a]pyrene *cis*-4,5-dihydrodiol), *cis*-11,12-dihydro-11,12-dihydroxybenzo[a]pyrene (benzo[a]pyrene *cis*-11,12-dihydrodiol), *trans*-11,12-dihydro-11,12-dihydroxybenzo[a]pyrene (benzo[a]pyrene *trans*-11,12-dihydrodiol), 10-oxabeno[*def*]chrysen-9-one, and dimethoxy and hydroxymethoxy derivatives of benzo[a]pyrene. Included in their findings were three *ortho*-ring fission metabolites that originated from cultures that were fed benzo[a]pyrene *cis*-4,5-dihydrodiol: 4-formylchrysen-5-carboxylic acid, 4,5-chrysen-9-dicarboxylic acid and a monocarboxylated chrysen product. Initial dioxygenation of benzo[a]pyrene by *M. vanbaalenii* PYR-1 at the 4,5 positions, K region, was consistent with findings from other studies on mycobacteria that degrade pyrene such as *M. flavescens* (Dean-Ross and Cerniglia, 1996), *Mycobac-*

*terium* sp. strain KR2 (Rehmann *et al.*, 1998) and *Mycobacterium* sp. strain AP1 (Vila *et al.*, 2001).

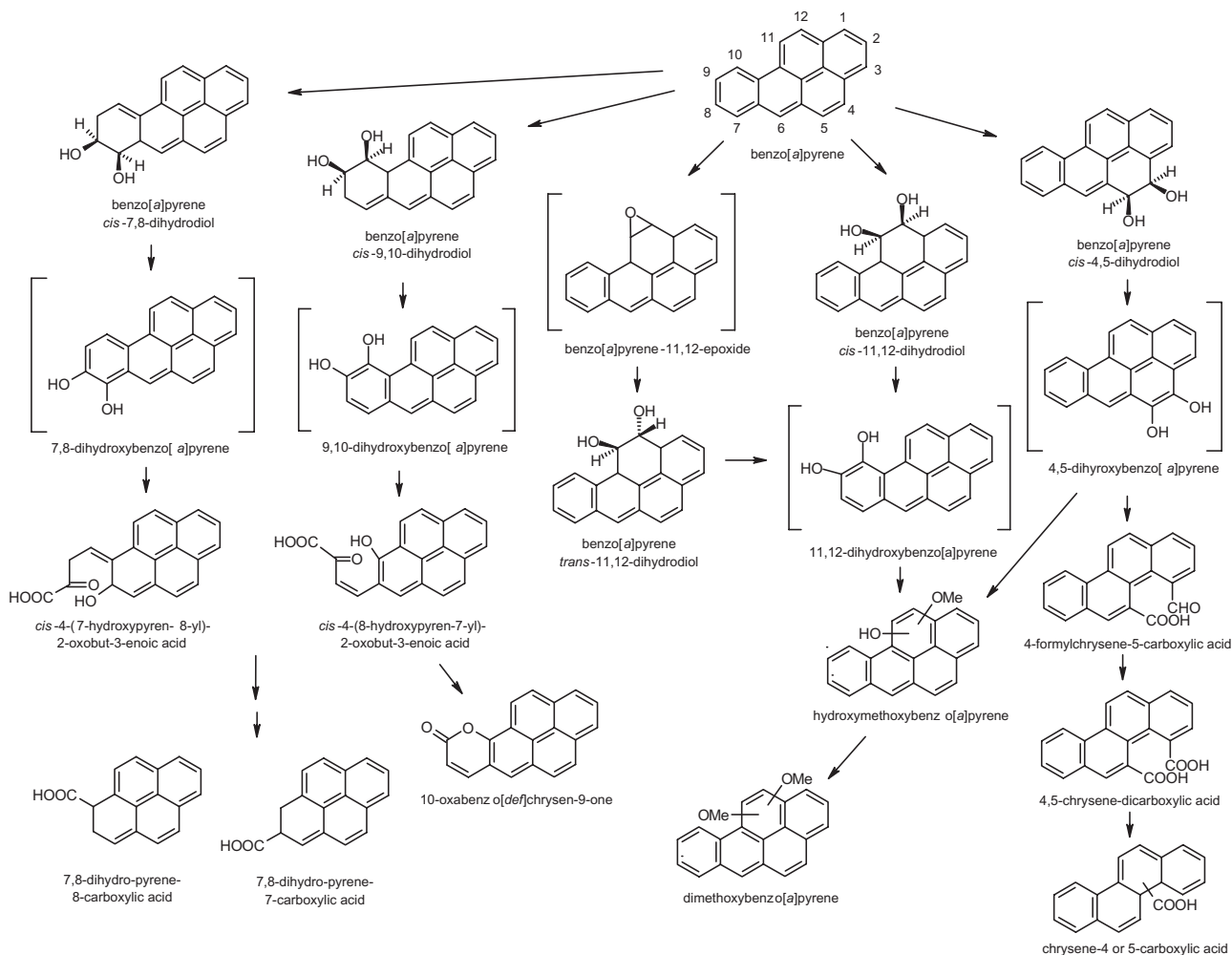
These results were significant because they expanded our understanding of the enzymatic capabilities of bacteria to biodegrade benzo[a]pyrene and built upon a previous study whereby enzymatic attack on benzo[a]pyrene by *Mycobacterium* sp. strain RJGII-135 at the 4,5-, 7,8- and 9,10- positions was proposed based upon the identification of four metabolites and included the first documentation of ring fission products of benzo[a]pyrene metabolism by a bacterium (Schneider *et al.*, 1996). At that time, fluorescence and mass analyses were used to identify benzo[a]pyrene *cis*-7,8-dihydrodiol plus the downstream products of either 7,8- or 9,10-dioxygenase attack, *cis*-4-(8-hydroxypyren-7-yl)-2-oxobut-3-enoic acid or *cis*-4-(7-hydroxypyren-8-yl)-2-oxobut-3-enoic acid and 7,8-dihydro-pyrene-7-carboxylic acid or 7,8-dihydro-pyrene-8-

carboxylic acid. Because the *meta* fission products through the 7,8-bond or the 9,10-bond were not distinguishable, there were two possible identities for each of the two of the metabolites and it was noted that direct evidence for 9,10 initial dioxygenation was lacking. In 2004, 10-oxabenz[*def*]chrysen-9-one was identified by Moody and colleagues (2004) and this has provided further evidence to the 9,10 pathway. The *cis*-9,10-dihydrodiol (and the *cis*-7,8-dihydrodiol) had been documented previously only from *S. yanoikuyae* strain B8/36 discussed below.

In addition to expanding our understanding of the 4,5 and 9,10 pathways, Moody and colleagues (2004) also revealed two 11,12 pathways that occurred by both a monooxygenase and dioxygenase attack on benzo[*a*]pyrene that resulted in *trans*-11, 12- and *cis*-11,12-dihydrodiols respectively. For the first time, the absolute configurations of the benzo[*a*]pyrene *cis*- and *trans*-dihydrodiols were documented whereby the regio- and stereo-selective activities of the mono- and dioxygenases

of *M. vanbaalenii* PYR-1 were shown through the production of benzo[*a*]pyrene-*cis*-11,12-dihydrodiol (11*S*, 12*R*, 100% optically pure), *-trans*-11,12-dihydrodiol (equal mixture of 11*S*, 12*S* and 11*R*, 12*R* compounds) and the *-cis*-4,5-dihydrodiol (30% 4*S*, 5*R* and 70% 4*R*, 5*S* absolute stereochemistry). It was discussed that monooxygenation of benzo[*a*]pyrene was most likely carried out by cytochrome P450 enzymes and as discussed above, three cytochrome P450 genes were previously characterized from *M. vanbaalenii* PYR-1 (Brezna *et al.*, 2006). Hydroxymethoxy and dimethoxy metabolites of benzo[*a*]pyrene were also identified and corroborated a report that demonstrated a catechol-*O*-methyltransferase located in the soluble fraction of *M. vanbaalenii* PYR-1 cell extracts (Kim *et al.* 2004b). The pathways proposed for benzo[*a*]pyrene biodegradation by mycobacteria are given in Fig. 5.

Further study on the effects of benzo[*a*]pyrene biodegradation in the presence of co-occurring PAHs by



**Fig. 5.** Pathways proposed for the biodegradation of benzo[*a*]pyrene by mycobacteria (Schneider *et al.*, 1996; Moody *et al.*, 2004). Hypothetical intermediates are indicated by brackets.

*Mycobacterium* sp. strain RJGII-135 revealed that biodegradation was inhibited by anthracene, benz[a]anthracene and pyrene, but were stimulated by phenanthrene under the conditions tested (McLellan *et al.*, 2002). *Mycobacterium* sp. strain LP1, described above in the context of pyrene biodegradation, was also reported to mineralize approximately 10% of [7,10-<sup>14</sup>C]benzo[a]pyrene in liquid culture, 50 mg l<sup>-1</sup>, when incubated with a mixture of PAHs for 2 weeks (Pizzul *et al.*, 2006).

### ***Sphingobium yanoikuyae* strains B1 & B8/36, *Sphingobium* sp. strain EPA505 and HMW PAH biodegradation**

Reports of HMW PAH biodegradation by non-actinomycete bacteria appear to have occurred with less frequency when compared with the mycobacteria; however, since the late 1990s more numerous reporting has resulted in the documentation of isolates representing a range of non-actinomycete organisms in regard to the biodegradation of the four-ring PAHs, pyrene, fluoranthene, chrysene and benz[a]anthracene, the five-ring benzo[a]pyrene, perylene, dibenz[a,h]anthracene and the seven-ring coronene. During the same time, in-depth research in regard to previously isolated strains, such as *S. yanoikuyae* strains B1 and B8/36, and *Sphingobium* sp. strain EPA505, has been advanced.

The term sphingomonad is used in the proceeding sections as described by Stolz (2009) (*sensu lato*) in a new review that details the sphingomonads in relation to their xenobiotic-degrading capabilities. Indeed, member organisms of the sphingomonads are well known for their abilities to degrade a wide range of natural and xenobiotic compounds, including HMW PAHs in some cases, and the diverse catabolic capabilities of some sphingomonad isolates have been aptly demonstrated (Balkwill *et al.*, 2006; Stolz, 2009). Among these, a *Beijerinckia* species, strain B1, was first isolated in 1973 by Gibson and colleagues (1973) and, in 1975 a mutant strain, *Beijerinckia* species strain B8/36, was reported to biotransform benz[a]anthracene and benzo[a]pyrene (Gibson *et al.*, 1975; Jerina *et al.*, 1984). These archetypal xenobiotic-degrading organisms were reclassified in 1996 as *Sphingomonas yanoikuyae* strain B1; B8/36 (Khan *et al.*, 1996; Gibson, 1999) and more recently reclassified as *Sphingobium yanoikuyae* strain B1; B8/36 following the proposal of the new genus *Sphingobium* (Takeuchi *et al.*, 2001).

In rigorous chemical investigations of chrysene biotransformation, resting cells of *S. yanoikuyae* strain B8/36 were shown to oxidize chrysene to (+)-*cis*-(3*S*,4*R*)-dihydroxy-3,4-dihydrochrysene (Boyd *et al.*, 1997) with further investigations revealing that strain B8/36 produced (+)-*(3S,4R,9S,10R)*-3,4,9,10-tetrahydroxy-3,4,9,10-tetrahydrochrysene, a *bis-cis*-dihydrodiol that represents a

new class of metabolites derived from prokaryotic biotransformation of PAHs (Boyd *et al.*, 1999). Most recently, Boyd and colleagues (2006) characterized enantiopure *bis-cis*-dihydrodiols from the biotransformation of the HMW PAH benz[a]anthracene (and anthracene) by strain B8/36, thus adding two more members to this new family of tetraol metabolites. Other recent studies with *S. yanoikuyae* strain B1 that involved HMW PAHs have resulted in the purification and characterization of the three protein components of a biphenyl 2,3 dioxygenase system, encoded by the genes *bphA4*, *bphA3* and *bphA1f,A2f*. This system exhibited broad substrate specificity including dioxygenation of benzo[a]pyrene to benzo[a]pyrene *cis*-7,8- and benzo[a]pyrene *cis*-9,10-dihydrodiols following expression in an *E. coli* recombinant (Ní Chadhain *et al.*, 2007; Yu *et al.*, 2007). The large subunit BphA1f was found to share 90% identity with and was most closely related to the ring hydroxylating dioxygenase from *Sphingomonas* sp. strain CHY-1 discussed below (Demanèche *et al.*, 2004). The crystal structures of the ferredoxin and terminal oxygenase components of the biphenyl 2,3 dioxygenase were documented by Ferraro and colleagues (2007) and this was the first report of a structure of a Rieske oxygenase that oxidized substrates with five aromatic rings. It was determined that the ability to catalyse the oxidation of such large substrates was made possible by both a larger active site entrance combined with the ability of the active site to accommodate larger substrates.

*S. yanoikuyae* strain B1 and other sphingomonads including *Sphingobium* sp. strain EPA505 were employed in autecological investigations by Cunliffe and Kertesz whereby it was concluded that phenotypic characteristics that may allow for sphingomonads to dominate in PAH-contaminated soils did not appear to be necessary for their survival and propagation (Cunliffe and Kertesz, 2006a,b).

Examination of the versatile *Sphingobium* sp. strain EPA505 has also continued. Strain EPA505 was originally isolated from a creosote-contaminated soil in Pensacola, Florida, USA (Mueller *et al.*, 1989; 1990) and was shown to utilize or biotransform many HMW PAHs, including fluoranthene, benzo[b]fluorene, benz[a]anthracene, chrysene, pyrene, benzo[a]pyrene, benzo[b]fluoranthene and dibenz[a,h]anthracene under different circumstances (Ye *et al.*, 1996). It was used as a model organism in surfactant (Triton X-100, Tween 80), bioemulsifier (alasan) and sphingan biodegradation studies of the HMW PAHs fluoranthene and pyrene (Willumsen and Karlson, 1998; Barkay *et al.*, 1999; Willumsen and Arvin, 1999; Luning Prak and Pritchard, 2002; Johnsen and Karlson, 2004), including an investigation with phenanthrene that documented the preferential utilization of Tween surfactant hydrophobic fractions as a carbon source by this strain

(Kim and Weber, 2003) and the documentation of horizontal transfer of alasan to its cell surface (Osterreicher-Ravid *et al.*, 2000).

In 2001, Story and colleagues (2001) documented a biodegradation pathway for fluoranthene (and other PAHs) by *Sphingobium* sp. strain EPA505 using three classes of Tn5 mutant derivative strains that were defective for PAH degradation (Story *et al.*, 2000). Biochemical evidence suggested that more than one operon was involved in aromatic catabolism by strain EPA505 and that the biodegradation pathway for fluoranthene diverged from the naphthalene, phenanthrene and anthracene pathways for this organism. Fluoranthene biodegradation by fluoranthene-grown cultures of the wild-type strain was proposed to occur through oxidative attack at the 7,8 position of fluoranthene and proceed through acenaphthoquinone and 1,8-naphthalic anhydride similarly to the 7,8-pathway in mycobacterium species (see Fig. 3). Further investigation of the biodegradation capability range of strain EPA505 was carried out whereby it was confirmed that the strain did not grow on chrysene, pyrene or benzo[*b*]fluoranthene (Story *et al.*, 2004), but that oxidation products of pyrene (4,5-dihydroxypyrene, phenanthrene-4,5-dicarboxylic acid and 5-hydroxyphenanthrene-4-carboxylic acid; Fig. 3) and benzo[*b*]fluoranthene were detected by the wild-type strain and mutants respectively. The authors noted that based upon these oxidation results that they also expected initial oxidation of chrysene and it was discussed that the lack of chrysene biotransformation may have been attributable to strict dioxygenase substrate specificity in strain EPA505. Metabolites from fortuitous metabolism of pyrene by strain EPA505 had been previously documented by Ho and colleagues (2000) as part of a wider study aimed at characterizing fluoranthene- and pyrene-degrading bacteria from soils.

A new 16S rRNA gene-based primer set was developed for the detection and monitoring of strain EPA505 and related strains by Leys and colleagues (2005b) in PAH-contaminated soils (Leys *et al.*, 2004) and detection of strain EPA505 in four out of five contaminated soils but not in five uncontaminated soils was documented and provided further evidence for the importance of this strain in PAH pollutant biodegradation in nature. Examination of fluoranthene and pyrene biodegradation in soil slurries under low-nutrient conditions by strain EPA505 and *Mycobacterium* species strain VM552 (100% similar to *M. gilvum* by 16S rDNA sequence; Bastiaens *et al.*, 2000), respectively, were also carried out by Leys and colleagues (2005a) and among other findings indicated that PAH degradation was unaffected by such conditions by these organisms.

With the purpose of developing pollutant biodegradation models, biodegradability parameters were

reported for 20 out of 22 PAHs tested for *Sphingobium* sp. strain EPA505 (Dimitriou-Christidis *et al.*, 2007) and investigations into the biodegradation kinetics of 2-methylphenanthrene, fluoranthene and pyrene in binary and ternary mixtures were performed (Dimitriou-Christidis and Autenrieth, 2007). Strain EPA505, when combined with *Sphingomonas aromaticivorans* B0695 (Frederickson *et al.*, 1995; Balkwill *et al.*, 1997; now classified as a *Novosphingobium*, Takeuchi *et al.*, 2001) was reported to degrade HMW PAHs in mixtures when the PAHs were dissolved in dodecane or silicone oil in two-phase partitioning bioreactors (Daugulis and McCracken, 2003; Vandermeer and Daugulis, 2007). In UV-irradiation studies, little effect on the biodegradation of HMW PAH degradation in mixtures by strain EPA505 and *S. yanoikuyae* B1 were reported (Lehto *et al.*, 2003).

### Biodegradation of HMW PAHs by newly isolated sphingomonads

New HMW PAH-degrading sphingomonad isolates have been reported to varying degrees in the literature since the late 1990s. Notably, Willison (2004) documented *Sphingomonas* species strain CHY-1, isolated from PAH-contaminated soil at a coal-gasification site in the Rhône-Alpes region of France that grew on chrysene as a sole source of carbon and energy and this organism has been the subject of many comprehensive investigations. When chrysene was administered at an initial concentration of 500 mg l<sup>-1</sup> in silicone oil and an organic/aqueous phase ratio of 1:4, 50% of added chrysene was biodegraded in 5 days and greater than 97% after 35 days. [5,6,11,12-<sup>14</sup>C]Chrysene radiotracer studies showed that 40–60% mineralization occurred and that addition of naphthalene increased the rate of mineralization. Further investigation with strain CHY-1 through direct peptide analysis of PAH-induced proteins, followed by cloning and functional analyses of genes encoding target proteins revealed that a single ring-hydroxylating dioxygenase component, Phnl, catalysed the oxidation of a wide range of PAHs including the HMW PAHs chrysene and benz[*a*]anthracene and furthermore that Phnl was required for PAH catabolism by strain CHY-1 (Demanèche *et al.*, 2004). Benz[*a*]anthracene was reported to be converted into three compounds that most likely represented three isomeric dihydrodiol derivatives.

Jouanneau and colleagues (2006) separately purified and characterized the components of the CHY-1 dioxygenase and documented its broad substrate specificity, initiating attack on PAHs ranging from two to five rings. Component ht-Phnl was shown to convert chrysene and benz[*a*]anthracene to doubly dihydroxylated derivatives, the *bis-cis*-dihydrodiols that were documented by Boyd *et al.* in 1999 and 2006 for chrysene and

benz[a]anthracene, respectively (Boyd *et al.*, 1999; 2006). The crystal structure of Phnl oxygenase was determined and was reported to possess a substantially larger hydrophobic substrate binding pocket and residue flexibility at the pocket entrance (Jakoncic *et al.*, 2007b), including that molecular modelling studies revealed that the pocket was large enough to accommodate benzo[a]pyrene (Jakoncic *et al.*, 2007a). The authors concluded that the broad substrate specificity of strain CHY-1 oxygenase was influenced by these factors. A versatile *cis*-dihydrodiol dehydrogenase (PDDH) was also characterized from strain CHY-1 whereby gene *bphB* was cloned and overexpressed in *E. coli* and was shown to oxidize two- to five-ring PAH dihydrodiols (Jouanneau and Meyer, 2006). 3,4-Dihydroxy-3,4-dihydrochrysene, 2,3-dihydroxy-2,3-dihydrofluoranthene, 9,10-dihydroxy-9,10-dihydrobenzo[a]pyrene and the 1,2-, 8,9- and 10,11-dihydrodiol isomers of benz[a]anthracene were all converted to their corresponding catechols by PDDH. Interestingly, 4,5-dihydroxy-4,5-dihydropyrene was also converted by PDDH even though pyrene was not a substrate for Phnl. A three-component salicylate 1-hydroxylase was also characterized from strain CHY-1 (Jouanneau *et al.*, 2007).

*Sphingomonas* sp. strain LB126 isolated from PAH-contaminated soil (Bastiaens *et al.*, 2000) was shown to biodegrade fluoranthene by fortuitous metabolism and a pathway was proposed. 9-Fluorenone-1-carboxylic was identified and indicated that 1,2 or 2,3 initial dioxygenation followed by either *meta*- or *ortho*-ring cleavage was possible (van Herwijnen *et al.*, 2003b). A novel angular dioxygenase complex, FlnA1–FlnA2, was also characterized from strain LB126 and was shown to transform fluoranthene to monohydroxyfluoranthene (Shuler *et al.*, 2008). *Sphingomonas* sp. strain PheB4 was isolated from surface mangrove sediments in Shenzhen, China and was shown to biodegrade fluoranthene by fortuitous metabolism when phenanthrene (10 mg l<sup>-1</sup>) or a peptone/beef extract/NaCl/glucose mixture was added as co-substrate (Zhong *et al.*, 2007). When incubated together, phenanthrene biodegradation by strain PheB4 was found to be significantly inhibited by fluoranthene. *Sphingomonas* sp. strain VKM B-2434 was isolated from contaminated sediment at a coke wastewater plant in Mariupol, Ukraine and was shown to grow on fluoranthene as a sole source of carbon and energy, but was also capable of biodegrading many other PAHs, including the HMW PAHs pyrene, benz[a]anthracene, chrysene and benzo[a]pyrene. Various metabolites were reported for many of the PAH substrates and indicated that strain VKM B-2434 possessed broad substrate specificity for a range of compounds (Baboshin *et al.*, 2008). Using a soil isolate that was 99.2% similar to *S. yanoikuyae* isolates based upon 862 sequenced bases, Rentz and colleagues (2008)

reported that they identified *cis*-7,8 dihydrodiol, pyrene-8-hydroxy-7-carboxylic acid and pyrene-7-hydroxy-8-carboxylic acid by LC-MS analyses from benzo[a]pyrene biotransformation by this organism after induction with salicylate. In a previous study using the same organism, removal of approximately 1 mg l<sup>-1</sup> or less of benzo[a]pyrene was reported to occur in cultures that were supplemented with plant root extracts (Rentz *et al.*, 2005).

Among a group of sphingomonad strains isolated from the deep Atlantic coastal plain (Frederickson *et al.*, 1995), *Novosphingobium aromaticivorans* strain B0695 was shown to biodegrade various PAHs including fluoranthene in the presence of Tween 80 (Shi *et al.*, 2001). Isolated from an oil-refinery waste-contaminated sandpit, *Sphingomonas* sp. strain 107 was reported to grow on pyrene, produce a bioemulsifier and it was determined that its PAH degradation genes were plasmid-encoded (Dagher *et al.*, 1997). *Sphingomonas* sp. strain VTI was documented to mineralize low concentrations (below aqueous solubility) of various HMW PAHs (Aitken *et al.*, 1998) and *Sphingomonas paucimobilis* strain TNE12 was shown to utilize fluoranthene as a sole source of carbon and energy (Shuttleworth *et al.*, 2000). Using PAH-degrading *Sphingomonas xenophaga* strain AJ1 (Iwabuchi *et al.*, 1995), Navarro and colleagues (2008) reported on the removal of pyrene and benzo[a]pyrene from aqueous DNA solutions following extraction from PAH-contaminated soil (Navarro *et al.*, 2007).

#### Reports of HMW PAH biodegradation by bacteria other than mycobacteria and sphingomonads

Generally, the sphingomonads ( $\alpha$ -*Proteobacteria*) and actinomycetes, with specific emphasis on the mycobacteria, appear to be the most versatile groups of bacteria in the degradation of HMW PAHs; however, this may also be a result of enrichment and isolation methods and further research will be required (Bastiaens *et al.*, 2000; Daane *et al.*, 2001; Stach and Burns, 2002; Gaskin and Bentham, 2005). Whatever the case may be, new HMW PAH-metabolizing organisms representing diverse genera have been documented in the last 10 years and among the Gram-negative organisms, members of the  $\gamma$ -*Proteobacteria* and  $\beta$ -*Proteobacteria* have also been documented for their HMW PAH biodegradation capabilities. Members of the genus *Pseudomonas*, such as *Pseudomonas saccharophila* P15, isolated from creosote-contaminated soil, was shown to exhibit enhanced removal rates of fluoranthene, pyrene, benz[a]anthracene, chrysene and benzo[a]pyrene when salicylate was used as an inducer of PAH dioxygenase activity (Chen and Aitken, 1999). The authors concluded that *P. saccharophila* P15 expressed low-level constitutive metabolism that was inducible by phenanthrene to bring



about HMW PAH metabolism. *Pseudomonas alcaligenes* PA-10, isolated from a PAH-contaminated bioreactor soil, was shown to biodegrade fluoranthene through fortuitous metabolism, four metabolites were identified (Gordon and Dobson, 2001) and it utilized fluoranthene in the presence of surfactants (Hickey *et al.*, 2007). *Pseudomonas* strains, *P. putida* strain KBM-1 (from sediments with background PAH levels) and *P. stutzeri* strain SAG-R (from creosote-contaminated soil) were shown to biodegrade pyrene both aerobically and anaerobically under nitrate-reducing conditions and also represented the first documentation of HMW PAH degradation under such conditions (McNally *et al.*, 1998; 1999). Kazunga and Aitken (2000) also documented pyrene transformation by *P. stutzeri* strain P16.

Pyrene biodegradation in the presence of biosurfactant-producing strain *Pseudomonas fluorescens* strain 29L was demonstrated by Husain (2008) and in earlier studies, *P. fluorescens* strain P2a was reported to utilize chrysene and benz[a]anthracene as sole sources of carbon and energy (Caldini *et al.*, 1995; Cenci and Caldini, 1997). Recently, Das and Mukherjee (2006) documented two biosurfactant-producing strains of *Pseudomonas aeruginosa* isolated from a crude oil-contaminated soil in northeast India that grew on pyrene and enhanced its apparent solubility five- to sevenfold and Obayori and colleagues (2008) documented the isolation of pyrene-degrading *P. aeruginosa* strains LP5 and LP6 and *Pseudomonas* sp. strain LP1 that were isolated from differently polluted soils in Lagos, Nigeria.

*Stenotrophomonas* spp., also members of the  $\gamma$ -*Proteobacteria*, have been shown to biodegrade HMW PAHs in different studies. The *S. maltophila* strain VUN 10,003 was reported to remove various HMW PAHs from solution by fortuitous metabolism under differing growth conditions and documentation for the removal of fluoranthene, benz[a]anthracene, benzo[a]pyrene, dibenz[a]anthracene and coronene was demonstrated in different studies (Juhász *et al.*, 1996; 1997; 2000b; 2002). Strain VUN 10,003 (previously characterized as *Burkholderia cepacia*) was originally isolated from soil on an abandoned gas works site in Victoria, Australia and grew on phenanthrene and pyrene as sole sources of carbon and energy (Juhász *et al.*, 1997). When incubated with pyrene as a co-substrate, the strain was shown to biodegrade benzo[a]pyrene and dibenz[a,h]anthracene, 10–15 mg l<sup>-1</sup>, over 56 days concomitant with near complete pyrene degradation (250 mg l<sup>-1</sup>) by 3 weeks. Pyrene refeeding did not stimulate much additional biodegradation; however, various metabolites were recovered from the culture media and the authors concluded that metabolite repression may have occurred (Juhász *et al.*, 2002). Studies that documented decreased mutagenicity of PAHs after metabolism by *S. maltophila* strain VUN 10,003 (Juhász *et al.*, 2000b) and biodegradation of

five-ring PAHs during growth on phenanthrene by this strain and similar strains from the same site were also reported (Juhász *et al.*, 1997). Another *S. maltophila* strain, VUN 10,010 was reported to biodegrade a range of HMW PAHs through surfactant enhancement (Boonchan *et al.*, 1998) and in fungal-bacterial co-cultures (Boonchan *et al.*, 2000), including benzo[a]pyrene. In 2004, a second organism, *Mycobacterium* sp. strain B1 was also found to be present in this culture and was shown to biodegrade pyrene and fluoranthene but not benzo[a]pyrene, and it was discussed that the roles of these two organisms in the biodegradation of benzo[a]pyrene were still unclear (Dandie *et al.*, 2004). Mentioned previously, *S. maltophila* sp. strain C-7, isolated from freshwater sediments by Jouanneau and colleagues (2005), was capable of pyrene biodegradation and a *Pasteurella* strain (also  $\gamma$ -*Proteobacteria*) biodegraded fluoranthene (Sepic *et al.*, 1997) and was employed in toxicity studies (Sepic *et al.*, 2003).

$\beta$ -*Proteobacteria* isolates, *B. cepacia* strain 2A-12 (Kim *et al.*, 2003a) and *Burkholderia* sp. strain VUN10013 (Somtrakoon *et al.*, 2008) were both documented to biodegrade pyrene by fortuitous metabolism with yeast extract or phenanthrene, respectively, and a strain of *Alcaligenes faecalis* was recently reported to grow on fluoranthene (Toledo *et al.*, 2006). The first documentation of a *Paracoccus* ( $\alpha$ -*Proteobacteria*), strain Ophe1, was reported to have been isolated from polluted soil in Attica, Greece and utilized pyrene as a sole source of carbon and energy in addition to other HMW PAHs (Zhang *et al.*, 2004). *Bacillus* isolates, *Bacillus cereus* strain P21 (Kazunga and Aitken, 2000), *B. cereus* strain Py5, *B. megaterium* strain Py6 (both from Lin and Cai, 2008), *B. subtilis* strain DM-04 (Das and Mukherjee, 2006) and three *B. pumilis* strains (Toledo *et al.*, 2006) were recently reported to transform or grow on pyrene and the protein engineering of *B. megaterium* CYP102 for the purpose of accelerating PAH oxidation was also described (Harford-Cross *et al.*, 2000; Carmichael and Wong, 2001). *Paenibacillus* sp. strain PR-P1 and *Arthrobacter* sp. strain PR-P3, although unable to utilize pyrene in pure culture, were shown to facilitate pyrene degradation in sediment slurry microcosms (Daane *et al.*, 2001).

Based upon the notion that higher transfer rates, PAH solubility and therefore bioavailability may increase at higher temperatures, Feitkenhauer and colleagues (2003) examined the biodegradation of HMW PAHs under extreme thermophilic conditions (60–70°C) by *Bacillus* spp. and a *Thermus* sp. and documented the biodegradation of fluoranthene, pyrene and benzo[e]pyrene. At moderately thermophilic temperature, thermophilic *Nocardia* sp. strain TSH1 was shown to grow on pyrene at 50°C as a sole source of carbon and energy (Zeinali *et al.*, 2007). An organism belonging to a genus that represents

enteric bacteria, *Leclercia adecarboxylata* strain PS4040, was isolated from an oily sludge-contaminated soil at the Digboi oil refinery in northeastern India and was shown to grow on pyrene resulting in approximately 60% biodegradation of 200 mg l<sup>-1</sup> pyrene over 20 days (Sarma *et al.*, 2004). The actinomycete, *Saccharothrix xinjiangensis* sp. strain PYX-6, isolated from Tianchi Lake, Xinjiang Uygur Autonomous Region, China, was reported to grow on pyrene as a sole source of carbon and energy and represented the first HMW PAH-degrading organism from this genus (Hu *et al.*, 2004).

Investigations in marine environments have led to the discovery of previously unreported HMW PAH-degrading organisms as well. Geiselbrecht and colleagues (1998) recovered *Cycloclasticus* sp. strains from marine sediments that were shown to biodegrade low levels of pyrene or fluoranthene by fortuitous metabolism when phenanthrene was present. Recently, Wang and colleagues (2008) reported on the isolation of a HMW PAH-utilizing organism from the deep sea for the first time. *Cycloclasticus spirillensus* strain P1, which grew on pyrene as a sole source of carbon and energy was sampled from a depth of 2682 m from Pacific deep sea sediments approximately 300 km from the northeast coast of the Philippines and currently represents the only member of *Cycloclasticus* capable of growth on a HMW PAH. Two new Gram-negative marine strains, *Novosphingobium pentaromativorans* US6-1 (Sohn *et al.*, 2004) isolated from estuarine sediments in Ulsan Bay and *Yeosaura aromativorans* strain GW1-1 (Kwon *et al.*, 2006) isolated from estuarine sediments of the Korean Strait, Republic of Korea were described by the authors as HMW PAH-degrading organisms that were capable of metabolizing a range of PAHs including benzo[a]pyrene.

Additionally, comprehensive environmental screening surveys that have resulted in the isolation and identification of numerous HMW PAH-degrading bacteria representing different genera from diverse locations throughout the world have been conducted (Mueller *et al.*, 1997; Aitken *et al.*, 1998; Ho *et al.*, 2000; Zhou *et al.*, 2006; Johnsen *et al.*, 2007; Seo *et al.*, 2007; Hilyard *et al.*, 2008; Launen *et al.*, 2008; Zhou *et al.*, 2008).

### Prokaryotic consortia and HMW PAH biodegradation

Investigations of the catabolic capabilities of HMW PAH-degrading bacterial consortia have increased in the last 10 years and research aimed at understanding consortia-mediated biodegradation of HMW PAHs has been further enabled by advances in molecular biology and isolation-independent techniques, such as denaturing gradient gel electrophoresis (DGGE). In an early demonstration of DGGE application to investigate HMW PAH-biodegradation, Kanaly and colleagues

(2000) compared the NAPL-mediated mineralization of [7-<sup>14</sup>C]benzo[a]pyrene by a bacterial consortium recovered from cattle pasture soil (Kanaly *et al.*, 1997) to changes in consortium populations over time. Members of known HMW PAH-degrading genera *Sphingomonas*, *Mycobacterium*, *Alcaligenes* and *Burkholderia* were documented by sequencing the V3 region of their 16S rDNA genes and eight of the 10 sequence types corresponded to *Proteobacteria*. Later isolation of bacterial strains from the consortium combined with sequence analyses of their 16s rDNA genes confirmed results obtained by DGGE, and one isolate,  $\gamma$ -*Proteobacteria* member, *Rhodanobacter* sp. strain BPC1, was monitored in the consortium during NAPL and benzo[a]pyrene biodegradation by competitive PCR (Kanaly *et al.*, 2002). Related studies of this consortium revealed the necessity of hydrocarbon NAPLs to facilitate the mineralization of benzo[a]pyrene (Kanaly *et al.*, 2001; 2006) and the NAPL-enhanced mineralization of chrysene and 3-ring PAHs but not pyrene (Kanaly and Watanabe, 2004). Lafortune and colleagues (2009) used DGGE, construction of a 16S rDNA library and strain isolation to document the diversity of a consortium recovered from a creosote-contaminated soil that degraded HMW PAHs in aqueous-silicone oil two-liquid phase (TLP) culturing systems. Results indicated that members of the  $\alpha$ - and  $\beta$ -*Proteobacteria* were most dominant in the consortium and that the biodegradative abilities of the isolates were limited. In support of previous investigations (e.g. Bouchez *et al.*, 1999; Kanaly *et al.*, 2002; Viñas *et al.*, 2005a), their data indicated that the functionality of the consortium as a whole was more effective to perform the task of HMW PAH biodegradation when compared with organisms that were isolated from the community. In earlier studies using the same consortium in TLP systems, biodegradation of HMW PAHs including benzo[a]pyrene were documented (Marcoux *et al.*, 2000; Villemur *et al.*, 2000) and the activities of various isolates obtained from the consortium were also investigated (Gauthier *et al.*, 2003). TLP systems were shown to enhance pyrene degradation by *M. vanbaalenii* PYR-1 (MacLeod and Daugulis, 2003) through association with the organic phase (MacLeod and Daugulis, 2005).

Comparisons of consortia that were cultivated on PAHs including HMW PAHs in chemostat cultures were carried out by van Herwijnen and colleagues (2006) using DGGE and fluorescent *in situ* hybridization. Bioaugmentation was shown to have little effect on the PAH degradation-capable community and the addition of PAHs resulted in increases in sphingomonads. Derz and colleagues (2006) recovered a consortium from creosote-contaminated soil discussed above that contained *M. pyrenivorans* (Derz *et al.*, 2004) and demonstrated extensive mineralization of [4,5,9,10-<sup>14</sup>C]pyrene,

54%, and [7,10-<sup>14</sup>C]benzo[a]pyrene, 36%, in 3 and 10 weeks respectively when inoculated into soil slurries. Eriksson and colleagues (2002) prepared enrichment cultures that originated from a polychlorinated biphenyl-contaminated Arctic soil, Labrador, Canada, and grew biofilms on pyrene crystals. They reported that the biofilm culture degraded 20 µg ml<sup>-1</sup> pyrene in 60 days and showed that the members of the biofilm included members of the  $\alpha$ -,  $\beta$ - and  $\gamma$ -*Proteobacteria*. A pyrene-utilizing consortium that functioned at pH 3 was documented for the first time after sampling a highly acidic soil (pH 2.4) located at a manufactured gas plant site in Belgium and was the first report of HMW PAH biodegradation at such extreme pH. DGGE analyses indicated that a dominant consortium member was most closely related to a member of the slow-growing mycobacteria, *Mycobacterium montefiorensis* (Uyttebroek *et al.*, 2007).

HMW PAH degradation by consortia recovered from soils and sediments (Juhász *et al.*, 2000a; Yuan *et al.*, 2000; Dries and Smets, 2002; Sartoros *et al.*, 2005; Chang *et al.*, 2007) and from marine and brackish saline environments (Yamada *et al.*, 2003; Yu *et al.*, 2005; Luan *et al.*, 2006; Lin and Cai, 2008) have been documented in recent studies, including consortia that were psychrotolerant to at least 7°C (Eriksson *et al.*, 2003). Arulazhagan and Vasudevan (2009) have reported pyrene and benzo[e]pyrene biodegradation by a moderately halophilic marine consortium and Cui and colleagues (2008) analysed a consortium community from the deep sea by DGGE under different conditions that was originally enriched on a PAH mixture that included pyrene.

## Closing

In the last 20 years, our knowledge in regard to the bacterial biodegradation of HMW PAHs has been significantly advanced and interest in this field has continued to grow worldwide. In the last 10 years, many new HMW PAH-degrading bacterial isolates and bacterial consortia have been recovered from different types of contaminated and uncontaminated environments and studied. During this time, advances in our understanding of *in situ* HMW PAH biodegradation were also achieved, and although beyond the scope of this review, HMW PAH biodegradation studies in soils (Kanaly and Bartha, 1999; Haderlein *et al.*, 2001; 2006; Lottfabad and Gray, 2002; Fava *et al.*, 2004; Huesemann *et al.*, 2004; Macleod and Semple, 2006; Mohan *et al.*, 2006; Stroud *et al.*, 2007; Singleton *et al.*, 2008; Mohan *et al.*, 2009), including the use of electrokinetic methods (Shi *et al.*, 2001; Niqui-Arroyo and Ortega-Calvo, 2007), in sediments (Bach *et al.*, 2005; Boyd *et al.*, 2005; Lei *et al.*, 2005; Tian *et al.*, 2008), by monitoring prokaryotic HMW PAH biodegradative activities and populations in such

media through cultivation-independent techniques, such as DGGE, real-time PCR and stable isotope probing among others (Cheung and Kinkle, 2001; Ringelberg *et al.*, 2001; Leys *et al.*, 2004; 2005a,b; Viñas *et al.*, 2005b; Ní Chadhain *et al.*, 2006; Singleton *et al.*, 2006; Gomes *et al.*, 2007; Singleton *et al.*, 2007; Cébron *et al.*, 2008; Jones *et al.*, 2008), by the cooperative effects of bacteria with plants and fungal species (Johnson *et al.*, 2004; Liu *et al.*, 2004; Jouanneau *et al.*, 2005; Krutz *et al.*, 2005; Parrish *et al.*, 2005; Corgié *et al.*, 2006; Child *et al.*, 2007a,b; Kim and Lee, 2007; Mueller and Shann, 2007; Byss *et al.*, 2008; Leonardi *et al.*, 2008; Li *et al.*, 2008) and by the combined conditions of chemical treatment and biodegradation (Nam and Kukor, 2000; Zeng *et al.*, 2000; Lehto *et al.*, 2003; Piskonen and Itävaara, 2004) for example have been carried out. Indeed, combined knowledge from studies of bacterial isolates, bacterial consortia and *in situ* investigations will continue to shape our understanding of prokaryotic HMW PAH biodegradation.

As discussed in this review, studies involving newly isolated and previously isolated organisms have furthered our understanding of HMW PAH biodegradation pathways for four- and five-ring compounds, advanced our knowledge of the enzymes involved in these transformations and have demonstrated HMW PAH biodegradation over an expanding range of temperature and pH conditions. Recently, proteomic and metabolomic approaches have been applied to the study of HMW PAH biodegradation and mark the beginning of more wide use of these techniques in the future while consortia studies and the utilization of culture-independent techniques have begun to provide a view into the dynamics of the bacterial communities that utilize HMW PAHs. At the same time, much still needs to be investigated in regard to the complex ecological interactions of HMW PAH-degrading communities, the roles of biofilms in HMW PAH biodegradation, the mechanisms of HMW PAH uptake by bacteria and the biodegradation of HMW PAHs in mixtures including unresolved issues of fortuitous metabolism. Research in nanotechnology is now occurring at a brisk pace and to determine the biodegradability of 'ultimate' HMW PAHs such as fullerenes and carbon nanotubes will present new and exciting challenges for the field in the near future.

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