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 (Juhasz 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
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; Fismes 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.

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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-dihydriodiol), 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-dihydriodiol 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 β-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-dimethoxy pyrene. 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 (β)-subunit (nidB) and the dioxygenase large (α)-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. frederiksb ergense FAn97 (Willumsen et al., 2001) and M. gilvum BB1 (Baldrin et al., 1993; Böttger et al., 1997). M. astro africana 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 (phtBa), 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-dihydroxymaleate based upon the fragmentation pattern of the trimethyloxiranes 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. astro africana 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 Mycobacterium 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 monoxygenase, dioxygenase small subunit, a naphthalene-inducible dioxygenase small subunit and an aldehyde dehydrogenase. The dioxygenase small subunit (β-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-dihydriodiolis 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 cyph51 (pipA), cyph 150 and cyph 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) that indicated monooxygenase attacks on phenanthrene, benz[a]anthracene, 7,12-dimethylnz[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-methylbenzo[a]anthracene. In the case of pyrene, 1-, 2- and 4-hydroxy pyrene 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 nidaB 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⁻¹ 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 [4,5,9,10-¹⁴C]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⁻¹ 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-dihydropyrene 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 Rehm 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 14C[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 Nocardiooides 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 [4,5,9,10-14C]pyrene mineralization was enhanced by sediment inoculation, 8.8% 14CO2 in 61 days, and that inoculation plus planting with the reed Phragmites australis resulted in twofold less enhancement, 4.4% 14CO2 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 [4-13C]- or [4,5,9,10-14C]pyrene with and without a soil humic acid standard to characterize the nature of the produced residues (Nieman et al., 2007). Results of the [13C]/[14C]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 [4,9,13C]pyrene-sediment investigations using flash-pyrolysis combined with 13C 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 docu-
mented 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 bacte-
ria by Kazunga and colleagues (2001) by studying four
strains that produced these compounds during fluoran-
thenet 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. austroafric-
icanum 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[ap]pyrene. Inhibition was shown
to occur when mixed with phenanthrene or pyrene. Inter-
estingly, GTI-23 grew on both HMW PAHs and the ali-
phatics 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 de-
radiation of pyrene and PAH mixtures by Mycobacterium
sp. strain A1-PYR that was isolated from mangrove sedi-
ments. 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 man-
grove 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 Mycobacte-
rium 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]py-
rene. 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 benzo[a]anthracene. M. frederiksbergense FAn97 was
isolated from a coal tar-contaminated former gas works
site in Frederiksdberg, 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 iso-
lated 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. frederiksber-
genese 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 iso-
lated 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.,

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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\(^{-1}\) (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 Ned 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\(^{2}\)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-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\(^{-1}\) 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-9H-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-9H-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.
Fig. 3. Pathways proposed for the biodegradation of fluoranthene by bacteria (Kelley et al., 1991; 1993; Weissenfels et al., 1991; Cerniglia, 1992; Sepic et al., 1998; Rehmann et al., 2001; Dean-Ross et al., 2002; López et al., 2005; 2006; Kweon et al., 2007; Lee et al., 2007). Metabolites detected from fluoranthene, acenaphthene and acenaphthylene transformations are shown.
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 acenaphthene. 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 phthlate and the β-ketoadipate pathway with the exception of dehydrogenation 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⁻¹ 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 benzo[a]anthracene by Mycobacterium spp.**

Reports that document the biodegradation of katanelated 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 benzo[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 fourring benzo[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 (Juhasz 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 RGGII-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⁻¹ benzo[a]anthracene applied in dimethylformamide. HPLC, UV-visible absorption, GC-MS and NMR analyses revealed that M. vanbaalenii PYR-1 attacked benzo[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 benzo[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 7,12dimethylbenzo[a]anthracene was also documented in M. vanbaalenii PYR-1 whereby mono- and dioxygenation reactions at the 5,6 positions and monoxygenation 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⁻¹ each of peptone, yeast extract and soluble starch. Phenanthrene in dimethylformamide, 100 μl 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-oxabenzo[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-formylchrysene-5-carboxylic acid, 4,5-chrysene-dicarboxylic acid and a monocarboxylated chrysene 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), Mycobacterium 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-hydroxyphenyl-7-yI)-2-oxobut-3-enoic acid or cis-4-(7-hydroxyphenyl-8-yI)-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-oxa benzo[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 (11S, 12R, 100% optically pure), -trans-11,12-dihydrodiol (equal mixture of 11S, 12S and 11R, 12R compounds) and the -cis-4,5-dihydrodiol (30% 4S, 5R and 70% 4R, 5S 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 [7,10-14C]benzo[a]pyrene in liquid culture, 50 mg l\(^{-1}\), 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-(3S,4R4)-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 enantio pure *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 tetracol 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 (Ni 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 hydroxylation 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]fluoranthene, benzo[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 benz[a]fluoranthene (Story et al., 2004), but that oxidation products of pyrene (4,5-dihydroxyxypyrone, phenanthrene-4,5-dicarboxylic acid and 5-hydroxyphenanthrene-4-carboxylic acid; Fig. 3) and benz[a]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 *Myco-bacterium* 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; Vandenmeir 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⁻¹ 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. [5,6,11,12-¹⁴C]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, PhnI, catalysed the oxidation of a wide range of PAHs including the HMW PAHs chrysene and benz[a]anthracene and furthermore that PhnI was required for PAH biodegradation 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-PhnI 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
HMW PAH biodegradation by bacteria

Sphingomonas sp. strain PheB4 was isolated from surface mangrove sediments in Shenzhen, China and was shown to biodegrade fluoranthene by fortuitous metabolism and a pathway was proposed. 9-Fluorenone-1-carboxylic acid 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, FinA1–FinA2, 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\(^{-1}\)) or a peptone/buffer 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, benzo[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 dihydrodial, 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\(^{-1}\) 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 VT1 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 (\(\gamma\)-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, benzo[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 *γ-Proteobacteria*, have been shown to biodegrade HMW PAHs in different studies. The *S. maltophilia* 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 (Juhasz 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 (Juhasz et al., 1997). When incubated with pyrene as a co-substrate, the strain was shown to biodegrade benzo[a]pyrene and dibenz[a]anthracene, 10–15 mg l$^{-1}$, over 56 days concomitant with near complete pyrene degradation (250 mg l$^{-1}$) 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 (Juhasz et al., 2002). Studies that documented decreased mutagenicity of PAHs after metabolism by *S. maltophilia* strain VUN 10,003 (Juhasz 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 (Juhasz et al., 1997). Another *S. maltophilia* 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. maltophilia* sp. strain C-7, isolated from freshwater sediments by Jouanneau and colleagues (2005), was capable of pyrene biodegradation and a *Pasteurella* strain (also *γ-Proteobacteria*) biodegraded fluoranthene (Sepic et al., 1997) and was employed in toxicity studies (Sepic et al., 2003).

*β-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* (*α-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. pumilus* 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 (Hartford-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, Feitenhauer 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[a]pyrene. At moderately thermophilic temperature, thermophilic *Nocardiopsis* 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\(^{-1}\) 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 sp. 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 Yeosuana 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; Hillyard 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-\(^{14}\)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, γ-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 α- and β-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-\(^{14}\)C]pyrene,
54%, and [7,10-14C]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⁻¹ pyrene in 60 days and showed that the members of the biofilm included members of the α-, β- and γ-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 montefiorensense (Uyttebroek et al., 2007).

HMW PAH degradation by consortia recovered from soils and sediments (Juhasz et al., 2000a; Yuan et al., 2000; Dries and Smet, 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[a]pyrene biodegradation by a moderately halophilic marine consortium and Cui and colleagues (2008) analyzed 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; Ni 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; Leonard 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 widespread 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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