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Degradation of pyrene by *Mycobacterium flavescens*

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Abstract A strain of *Mycobacterium flavescens* was isolated from polluted sediments. It was capable of utilizing pyrene as a sole source of carbon and energy. When pyrene was supplied as a suspension at 50 μg/ml, the generation time was 9.6 h and the rate of pyrene utilization was 0.56 $\mu g\,ml^{-1}\,day^{-1}$. In addition to pyrene, the strain could mineralize phenanthrene (17.7%) and fluoranthene (17.9%), but failed to mineralize naphthalene, chrysene, anthracene, fluorene, acenaphthene and benzo[*a*]pyrene, as determined by recovery of radiolabeled CO_2 in incubations conducted for 2 weeks under growth conditions. Metabolites produced during growth on pyrene were detected and characterized by HPLC and GC-MS. The product of initial ring oxidation, 4,5-dihydroxy-4,5-dihydropyrene was identified, as well as ring-fission products including 4-phenanthroic acid, phthalic acid, and 4,5-phenanthrenedioic acid.

Introduction

Polynuclear aromatic hydrocarbons (PAH) are ubiquitous contaminants in nature, being present in fossil fuels or produced during the combustion of fossil fuels. They are also present in tobacco smoke. Their physical properties vary with the number of rings and hence their molecular mass. As the number of rings increases, their solubility in water decreases and their adsorption potential increases. Hence the higher-molecular-mass PAH tend to be associated with sediments in fresh and salt water environments or with soil in the terrestrial environment (Cerniglia 1992). Their distribution varies with distance from sources of industrial pollution; concentrations of 198–232 000 μg/l are typical in contaminated environments (Johnson and Larsen 1985).

Many of the individual chemicals present in environmental samples have been identified as genotoxicants in short-term mutagenicity assays such as the *Salmonella*/mammalian microsome test and as carcinogens in long-term rodent bioassays (Ames 1975; Dipple et al. 1990). Owing to their widespread distribution in the environment, they may represent a potential threat to human health. In mammals, PAH are metabolically activated by cytochrome-*P*-450-dependent monooxygenases, which leads to the formation of carcinogenic metabolites, dihydrodiol epoxides, which are capable of forming PAH-DNA adducts (Klaassen et al. 1986).

Microbial degradation represents one of the major routes by which PAH can be removed from the environment. Considerable attention has been devoted to the study of the naphthalene degradation pathway; the enzymology of various metabolic steps has been elucidated and the underlying genetic and regulatory characteristics of the pathway have been determined (Yen and Serdar 1988; Zylstra 1995). However, just as mutagenicity increases with increasing molecular mass, so does resistance to biodegradation. It has generally been found that while two- and three-ringed PAH are readily biodegradable, four- and five-membered rings are more difficult to biodegrade (Cerniglia 1992). Recently it has been shown that nocardioform actinomycetes of the genera *Mycobacterium*, *Rhodococcus* and *Gordona* isolated from PAH-contaminated sites were able to mineralize PAH with up to four aromatic rings (Kastner et al. 1994). Various pure cultures have been reported to cometabolize or utilize pyrene as the source of carbon and energy (Heitkamp et al. 1988; Walter

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et al. 1991; Grosser et al. 1991; Boldrin et al. 1993; Kastner et al. 1994; Kelley and Cerniglia 1995). However, less is known about the bacterial catabolic pathway for pyrene. It is, therefore, of considerable interest to isolate and characterize microorganisms capable of growth and/or utilization of high-molecular-mass PAH and elucidate the metabolic pathway. The research reported here describes the isolation and characterization of a strain of *Mycobacterium flavescens* that is capable of growth on the four-membered PAH, pyrene. We present data on the initial metabolites of pyrene formed by *M. flavescens*.

Materials and methods

Organism and growth conditions

The *Mycobacterium flavescens* was isolated by enrichment culture using pyrene as the sole source of carbon and energy and sediment from the Grand Calumet River in northwestern Indiana as inoculum. For these and subsequent growth experiments, a mineral salts medium was used (Cohen-Bazire et al. 1957), with solid pyrene added prior to autoclaving. Isolation and purification of a culture able to degrade pyrene were achieved by procedures outlined in Cerniglia and Heitkamp (1990). In order to correlate growth with pyrene utilization, sufficient pyrene to provide a final concentration of 50 μg/ml was weighed and dissolved in dimethylsulfoxide, which was added to the mineral salts medium with stirring to disperse the insoluble pyrene. Duplicate flasks were inoculated with the *M. flavescens* and incubated at 24°C on a gyratory shaker (150 rpm) in the dark. At intervals, samples were taken for direct counting using acridine orange fluorescence microscopy (Dean-Ross and Mills 1989) and for extraction to determine the amount of pyrene remaining in suspension (Cerniglia and Heitkamp 1990). Recovery of pyrene under the extraction conditions was 89.4%.

Mineralization of PAH

In order to determine the ability of the *M. flavescens* to utilize a series of PAH, mineralization studies were conducted as described in Dean-Ross (1989). Vials were inoculated with 1 ml (containing 2.2×10^7 cells) of a growing culture of *M. flavescens*, and supplemented with 5 μl solution containing 50 000 dpm ^{14}C-labeled PAH and sufficient unlabeled PAH to bring the total concentration in the flask to 3 μg/ml. After 2 weeks incubation, absorbed $^{14}CO_2$ was determined by liquid scintillation counting (Packard Tri-Carb liquid scintillation analyzer, model 2000A; Downers Grive, Ill.).

Identification of metabolites

In order to detect and identify metabolites, 50-ml portions of medium were prepared and supplemented with 3 μg/ml pyrene and 1 μCi radiolabeled pyrene. In some cases, the incubations were conducted in flasks containing a center well, to which 5 ml 1M NaOH was added. The medium was inoculated with 1–5 ml (1.4×10^8–3.5×10^8 cells/ml) of a culture of *M. flavescens* grown under conditions of pyrene excess. At intervals, the contents of the flasks were subjected to two extractions as described above, one on the neutral culture and one on the culture after acidification with HCl to bring the pH to 4. The neutral and acidified extracts were analyzed for metabolites by HPLC. To verify that the transformations were microbially mediated, flasks prepared as described above were autoclaved prior to incubation and extraction. In cases where the incubations were conducted in flasks containing center wells, 1-ml aliquots were taken from the center well, added to 10 ml liquid scintillation cocktail and counted by liquid scintillation counting as described above to determine the extent of mineralization of the added pyrene.

Analytical methods

HPLC was conducted with a Hewlett Packard series 1050 pump system using a gradient of 50%–95% methanol in water over 40 min at a flow rate of 1.2 ml/min. Detection of metabolites was achieved by an HP diode array model 1040A set at 254 nm and a Radiomatic A-500 radio-chromatography detector connected in series. The HPLC column was a Spherisorb ODS-2 5-μm column, 4.6 × 250 mm (MetaChem Technologies, Torrance, Calif.).

Quantification of pyrene for the growth experiments was performed by GC using a model 8500 gas chromatograph (Perkin-Elmer Co, Inc.) equipped with a flame ionization detector and using a DB5 fused-silica capillary column, 30 m long with an internal diameter of 0.24 mm (J&W Scientific, Inc., Rancho Cordova, Calif.). The column temperature was held at 40°C for 4 min and then increased to 205°C at a rate of 3°C/min. The injector and detector temperatures were 300°C. Quantification was achieved using phenanthrene as an internal standard. The limit of detection was 2 μg/ml original mineral salts medium.

Identification of metabolites was performed by GC-MS as previously described (Heitkamp et al. 1987). Neutral extracts were subjected to acetylation with acetic anhydride and pyridine while acid extracts were methylated with diazomethane prior to analysis by GC-MS (Heitkamp et al. 1987).

Chemicals

Unlabeled PAH were obtained from Aldrich Chemical Company (Milwaukee, Wis.) and were all more than 98% pure. Several radiolabeled chemicals were obtained from Chemsyn Science Laboratories (Lenexa, Kan.): [4,5,9,10-^{14}C]pyrene (55 mCi/mmol); [9,10–^{14}C]anthracene (58 mCi/mmol) [5,6,11,12-^{14}C]chrysene (54.4 mCi/mmol) [3-^{14}C]fluoranthene (50 mCi/mmol); [7-^{14}C]-benzo[*a*]pyrene (50 mCi/mmol). The following PAH were supplied by Sigma (St. Louis): [9-^{14}C]fluorene (14.2 mCi/mmol), [1-^{14}C]-naphthalene (10.3 mCi/mmol) and [9-^{14}C]phenanthrene (13.1 mCi/mmol). [1, 8-^{14}C]Acenaphthene (15.7 mCi/mmol) was supplied by Dynapol (Palo Alto, Calif.).

Results

Growth on pyrene

The culture consisted of gram-positive, acid-fast rods and was identified as *Mycobacterium flavescens* on the basis of the fatty acid profile (Microbial ID Inc., Newark, Del.). This culture has been placed in the American Type Culture Collection with the accession number ATCC 700033. In order to correlate pyrene utilization with growth, *M. flavescens* was inoculated into mineral salts media containing 50 μg/ml pyrene (Fig. 1). After an initial decrease in cell numbers, as determined by direct counting, numbers increased as pyrene was utilized by the culture. The generation time

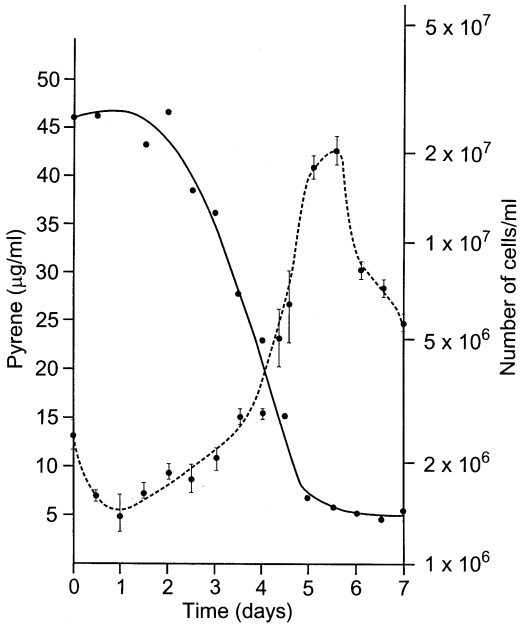

Fig. 1 Growth on (---) and utilization of (——) pyrene (50 µg/ml) by *Mycobacterium flavescens*

under these conditions was 9.6 h and the rate of pyrene utilization was 0.56 $\mu g\,ml^{-1}\,h^{-1}$.

Utilization of PAH

The culture had the ability to utilize several PAH during the 2-week incubation period as determined by the conversion of ^{14}C-labeled substrates into $[^{14}C]CO_2$. In addition to pyrene (38.8%), fluoranthene (17.9%) and phenanthrene (17.7%) were also mineralized. Chrysene, naphthalene, fluorene, anthracene, benzo[*a*]pyrene and acenaphthene were not utilized to a significant extent.

Identification of metabolites

In a preliminary experiment to determine the time course of phenanthrene metabolism for the purpose of detection of metabolites, aliquots of NaOH were removed from the center well of the incubation flasks at 6, 12 and 24 h. Mineralization of pyrene as determined by release of $[^{14}C]CO_2$ was determined to be 58.4%, 60.9% and 62.6% at the respective assay times. No radiolabeled CO_2 was recovered from flasks that were autoclaved prior to incubation.

Examination of the neutral extract of the culture medium after 24 h incubation indicated the presence of a metabolite having a retention time (15.1 min) and a UV spectrum (Fig. 2A) identical to that of 4,5-dihydroxy-4,5-dihydropyrene. The metabolite was not present in sufficient quantity to confirm its identification by GC-MS.

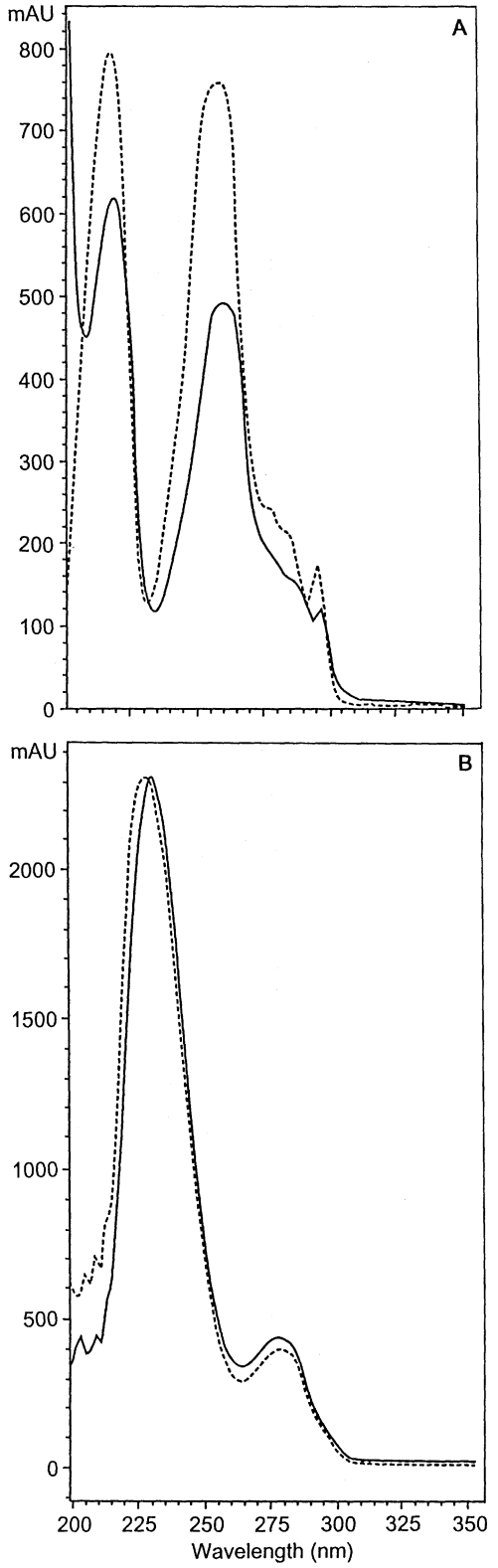

Fig. 2A, B UV spectra of metabolites in spent culture medium (——) compared with that of authentic standards (---). **A** UV spectrum of metabolite identified as 4,5-dihydroxy-4,5-dihydropyrene. **B** UV spectrum of metabolite I identified as phthalic acid

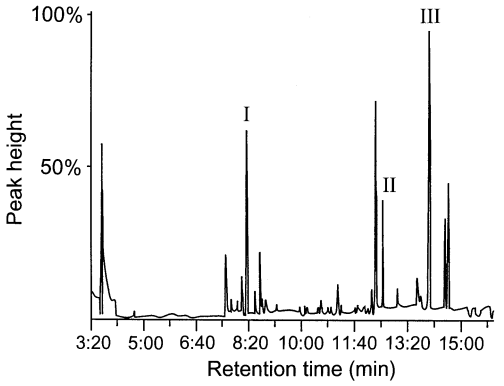

Fig. 3 Gas chromatogram of methylated organic-extractable pyrene residue under acid conditions after 24 h exposure to pyrene

Examination of the acid extracts revealed the presence of substantial amounts of several metabolites using both UV and radiochemical detection, indicating that the compounds were produced from the radiolabeled pyrene. Of particular interest was a metabolite (metabolite I) that had a HPLC retention time of 3.4 min and a UV spectrum identical to that of authentic phthalic acid (Fig. 2B), with absorption maxima at 230 nm and 280 nm. Incubations were repeated using unlabeled pyrene for the purpose of obtaining metabolites for GC-MS. The acid extract was methylated and analyzed by GC-MS (chromatogram illustrated in Fig. 3), which revealed the presence of a compound with a molecular ion M^+ at *m/z* 194 and fragmentation ions at *m/z* 163 and *m/z* 133, corresponding to loss of $-OCH_3$ and $-OCH_2$, consistent with it being dimethylated phthalic acid (Fig. 4A).

Two other metabolites were identified by GC-MS of the methylated acid extract. One (metabolite II) yielded a GC peak that had a mass spectrum consistent with that of 4-phenanthroic acid (Fig. 4B): a molecular ion M^+ at *m/z* 236, corresponding to the molecular mass of monomethylated 4-phenanthroic acid, and fragmentation ions at *m/z* 221, *m/z* 205, *m/z* 177 and *m/z* 151, corresponding to losses of M^+-15, M^+-31, and M^+-59, which are characteristic of a methylated carboxylic acid. The mass spectral properties are identical to 4-phenanthroic acid produced by *Mycobacterium* sp. PYR-1 (Heitkamp et al. 1988).

A third metabolite (metabolite III) had a molecular ion M^+ at *m/z* 294, which is consistent with it being a dicarboxy derivative of phenanthrene (Fig. 4C). Fragmentation ions at *m/z* 263, *m/z* 235 and *m/z* 220 are consistent with the structure of an aromatic carboxylic acid, lending further support to the identification of this compound as a dicarboxy derivative of phenanthrene. On the basis of studies by Cerniglia (1992), it is likely to be 4,5-phenanthrenedioic acid.

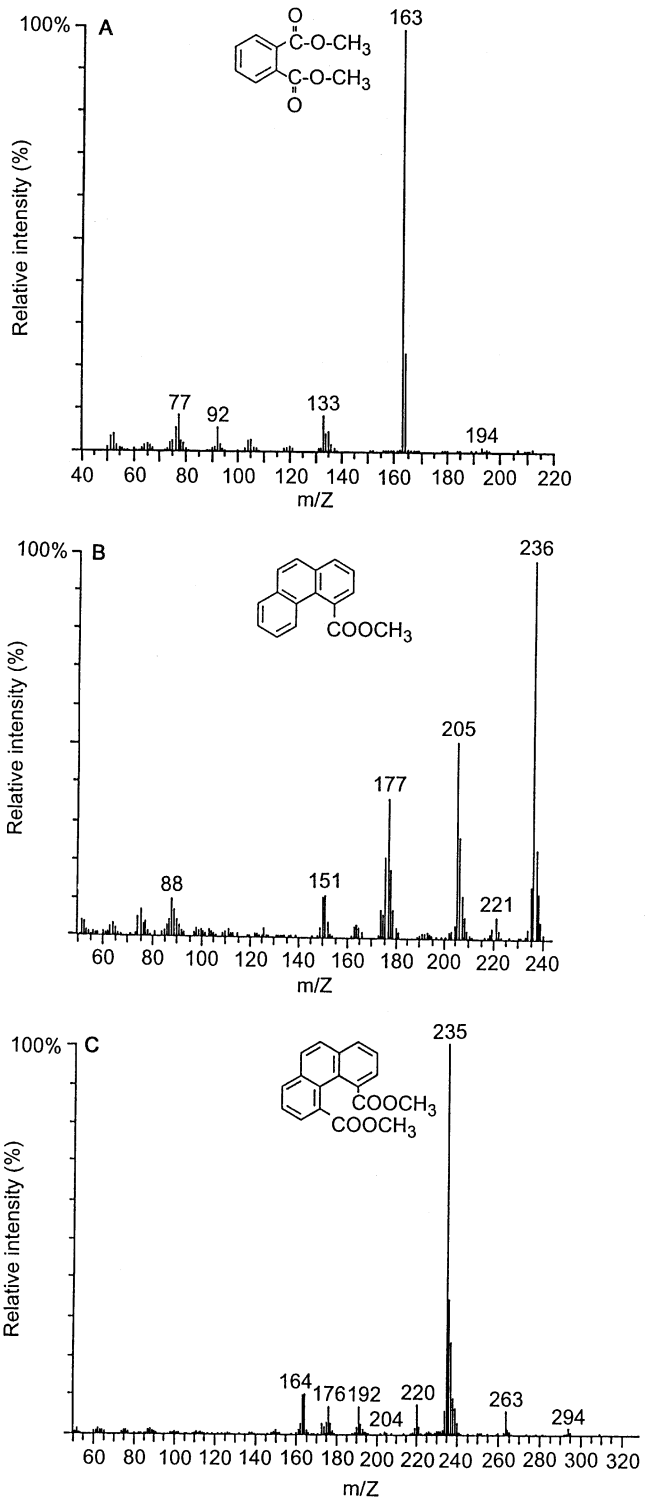

Fig. 4A–C Mass spectra of methylated derivatives of metabolite I identified as phthalic acid (**A**), metabolite II identified as 4-phenanthroic acid (**B**) and metabolite III identified as 4,5-phenanthrenedioic acid (**C**)

Discussion

Several researchers have reported the ability of bacteria to utilize pyrene. A *Mycobacterium* sp. PYR-1 was

isolated from oil-contaminated sediments and was found to be capable of mineralizing pyrene as well as several other PAH (Heitkamp et al. 1988) when growing on peptone, yeast extract and soluble starch. It was not capable of growth on pyrene as a sole source of carbon and energy. Another strain of *Mycobacterium* sp. was isolated from soil at an abandoned coal gasification site (Boldrin et al. 1993); this organism grew on phenanthrene, pyrene and fluoranthene and degraded fluorene cometabolically. Grosser and coworkers (1991) reported the mineralization of pyrene in soils collected from abandoned coal gasification plants. One organism capable of mineralizing pyrene was also isolated in pure culture and identified as a *Mycobacterium* sp. PAH 135. It could grow on pyrene in mineral salts medium providing the medium was supplemented with cofactors, including yeast extract, peptone and soluble starch. The initial products of pyrene degradation and a pyrene catabolic pathway were not determined. A *Rhodococcus* species has been isolated from contaminated soils with the ability to use pyrene as sole source of carbon and energy (Walter et al. 1991). It is interesting to note that all of these strains, including the strain reported here, are gram-positive organisms; four out of the five are members of the genus *Mycobacterium*. A phylogenetic analysis of PAH-utilizing mycobacteria by 16S rRNA sequencing has recently been reported (Wang et al. 1995, 1996).

The strain of *M. flavescens* isolated as reported here has the ability to grow on pyrene as a sole source of carbon and energy. Under conditions in which the pyrene was supplied as a finely dispersed suspension, the generation time was found to be 9.6 h. Other researchers report generation times of 20 h observed for *Rhodococcus* (Walter et al. 1991) when growing on pyrene and 12–35 h for growth on various PAH by bacterial pure cultures (Weissenfels et al. 1990). These differences may reflect different intrinsic growth rates of the cultures in question or they may be due to differences in media composition and particle sizes of pyrene.

Of the studies cited above, two have attempted to characterize the metabolic pathway used by the bacterial strains to degrade pyrene. Walter et al. (1991) reported the presence of a metabolite that was subjected to mass-spectral analysis, and it was proposed that the metabolite is formed by the recyclization of the initial ring-cleavage product. They were unable to determine whether initial attack occurred at the 1,2 or 4,5 positions. A more complete elucidation of the pathway was achieved by Heitkamp et al. (1988), who confirmed the identity of *cis*-4,5-dihydroxy-4,5-dihydropyrene

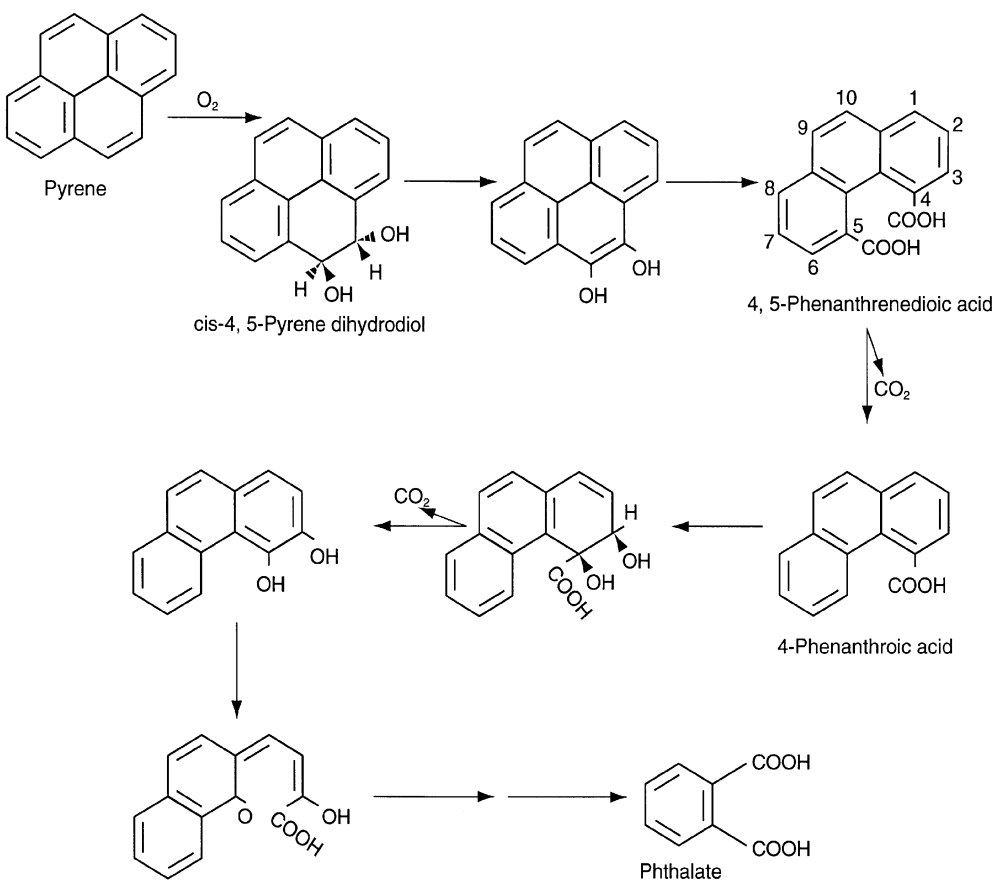

Fig. 5 Proposed pathway for the degradation of pyrene by *M. flavescens*

(*cis*-4,5-pyrenedihydrodiol) as the initial ring-cleavage product. In addition, they were able to identify 1-hydroxypyrene, 4-hydroxyperinaphthenone, 4-phenanthroic acid, cinnamic acid and phthalic acid as metabolites. On the basis of these identifications, Cerniglia (1992) proposed a pathway involving initial attack by a dioxygenase to form *cis*-4,5-pyrenedihydrodiol, which is subsequently cleaved to yield 4,5-phenanthrenedioic acid. Following loss of a carboxyl, 4-phenanthroate is formed. Subsequent metabolism of 4-phenanthroic acid via 1-hydroxy-2-naphthoic acid yields phthalic acid and cinnamic acid, indicating that two possible degradation pathways are present for the utilization of the proposed intermediate, 1-hydroxy-2-naphthoic acid. In addition, Cerniglia proposed that an alternative pathway was operating, leading to the formation of 4-hydroxyperinaphthenone, indicating that a dioxygenase capable of hydroxylating the pyrene at the 1,2 position was present; this is a dead-end pathway.

The present research confirms the essential features of the major pathway outlined by Cerniglia (1992) and illustrated in Fig. 5. *M. flavescens* grown on pyrene as sole source of carbon and energy initially attacks pyrene in the 4,5 position, presumably by dioxygenase action, to form 4,5-dihydroxy-4,5-dihydropyrene; this is followed by ring cleavage to produce 4,5-phenanthrenedioic acid, which is decarboxylated to form 4-phenanthroic acid. Phthalic acid has been identified, indicating that one of the two proposed pathways for 1-hydroxy-2-naphthoic acid degradation is operating in this strain. The presence of other metabolites identified by Cerniglia was not detected in *M. flavescens* cultures, which suggests that these alternative pathways may not be operating in this strain or simply that they were not produced in sufficient quantities for detection. The study lends further support for the importance of mycobacteria in the biodegradation of PAH.

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