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ORIGINAL PAPER

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Metabolism of fluoranthene by mycobacterial strains isolated by their ability to grow in fluoranthene or pyrene

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Abstract *Mycobacterium* sp. strains CP1, CP2, CFt2 and CFt6 were isolated from creosote-contaminated soil due to their ability to grow in pyrene (CP1 and CP2) or fluoranthene (CFt2 and CFt6). All these strains utilized fluoranthene as a sole source of carbon and energy. Strain CP1 exhibited the best growth, with a cellular assimilation of fluoranthene carbon of approximately 45%. Identification of the metabolites accumulated during growth in fluoranthene, the kinetics of metabolites, and metabolite feeding studies, indicated that all these isolates oxidized fluoranthene by the following two routes: the first involves dioxygenation at C-1 and C-2, *meta* cleavage, and a 2-carbon fragment excision to produce 9-fluorenone-1-carboxylic acid. An angular dioxygenation of the latter yields *cis*-1,9*a*-dihydroxy-1-hydrofluorene-9-one-8-carboxylic acid, which is further degraded via 8-hydroxy-3,4-benzocoumarin-1-carboxylic acid, benzene-1,2,3-tricarboxylic acid, and phthalate; the second route involves dioxygenation at C-2 and C-3 and *ortho* cleavage to give *Z*-9-carboxymethylenefluorene-1-carboxylic acid. In addition, the pyrene-degrading strains CP1 and CP2 possess a third route initiated by dioxygenation at positions C-7 and C-8, which—following *meta* cleavage, an aldolase reaction, and a C_1-fragment excision—yields acenaphthenone. Monooxygenation of this ketone to the corresponding quinone, and its subsequent hydrolysis, produces naphthalene-1,8-dicarboxylic acid. The results obtained in this study not only complete and confirm the three fluoranthene degradation routes previously proposed for the pyrene-degrading strain *Mycobacterium* sp. AP1, but also suggest that such routes represent general microbial processes for environmental fluoranthene removal.

Keywords Biodegradation · Fluoranthene · Polycyclic aromatic hydrocarbon · Degradation pathway

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Introduction

The biochemical processes involved in the environmental degradation of high molecular weight (HMW) polycyclic aromatic hydrocarbons (PAHs) are of great interest due to the environmental persistence and toxicity of these compounds [18]. Fluoranthene, together with pyrene, is one of the most abundant HMW PAHs present in coal derivatives (e.g., creosote) [6] and, consequently, is found at many contaminated sites [10]. Although microbial degradation represents the major route for the ecological recovery of such sites, the success of bioremediation in removing HMW PAHs has been limited.

Recent research on the biodegradation of HMW PAHs has led to the isolation of a number of bacterial strains that utilize pyrene and/or fluoranthene as sole sources of carbon and energy, and main biodegradation routes have been proposed [11]. Bacteria isolated for their ability to degrade pyrene are mainly nocardioforms (Gram-positive), which, in addition to pyrene, often degrade phenanthrene and fluoranthene [2, 3, 9, 12, 20, 24]. In pyrene-degrading mycobacteria, two alternative routes for fluoranthene degradation have been proposed: the first involves dioxygenation at C-7 and C-8, *meta* cleavage, and subsequent C_1 or C_2 fragment release to form acenaphthenone; the second involves dioxygenation at C-1 and C-2, *meta* cleavage, and formation of 9-fluorenone-1-carboxylic acid, which is decarboxylated to 9-fluorenone. These routes were first described for the *Mycobacterium* sp. strain PYR-1 [12].

A number of bacteria isolated for their ability to degrade fluoranthene are Gram-negative. Although these fluoranthene-degraders also utilize phenanthrene

and other 2- or 3-ring PAHs [9], in general they do not degrade pyrene. While most of the recently reported bacterial strains belong to the genera *Sphingomonas* [9, 15], fluoranthene metabolic pathways have likewise been proposed for other genera (e.g., *Alcaligenes denitrificans*) [26]. Fluoranthene-degrading Gram-negative bacteria seem to follow either the C-1, C-2 [7, 22]; the C-7, C-8 [15, 27]; or both dioxygenation routes [9] for fluoranthene metabolism.

To our knowledge, the only Gram-positive bacterial strain isolated due to its ability to grow in fluoranthene, and for which a fluoranthene-degrading pathway has been proposed, is *Mycobacterium* sp. strain KR20 [21]. This strain, which does not appear to utilize other PAHs, oxidizes fluoranthene by a third route, initiated by a dioxygenasic attack at C-2, C-3, and *ortho* cleavage to form the novel metabolite *Z*-9-carboxymethylenefluorene-1-carboxylic acid. The authors of the latter work proposed that a subsequent loss of two carbon units would produce 9-fluorenone-1-carboxylic acid. This in turn would undergo an angular dioxygenasic attack before further degrading via benzene-1,2,3-tricarboxylic acid.

In a recent study, our group examined the metabolism of fluoranthene using the pyrene-degrading strain *Mycobacterium* sp. AP1 [14]. The kinetics of metabolite accumulation, as well as metabolite feeding experiments, indicated a three-branched pathway involving a new set of reactions for certain of the previously identified metabolites. The first branch coincides with the 7,8-dioxygenation route producing 1-acenaphthenone, which is here further oxidized to naphthalene-1,8-dicarboxylic acid, benzene-1,2,3-tricarboxylic acid, and phthalate. While the second branch is initiated by 1,2-dioxygenation, instead of producing 9-fluorenone, the 9-fluorenone-1-carboxylic acid formed is oxidized via benzene-1,2,3-tricarboxylic acid, thereby merging with the first branch. A third branch involves 2,3-dioxygenation and *ortho* cleavage to generate *Z*-9-carboxymethylene-fluorene-1-carboxylic acid, further degradation of which remains unclear. Growing in fluoranthene or pyrene as sole carbon sources, strain AP1 proved inefficient in substrate removal, its growth halting with a large portion of the substrate still remaining in the medium.

In an attempt to complete the fluoranthene degradation routes proposed for strain AP1, and to determine whether they might serve as common routes for fluoranthene removal in polluted environments, we examined the metabolism of this PAH in four new mycobacterial isolates. Detailed growth studies permit comparison of the efficiency of fluoranthene removal using these new isolates with respect to strain AP1.

Materials and methods

Chemicals

PAHs and analogues were purchased from Aldrich (Milwaukee, WI). Diazomethane was generated by alkaline decomposition of Diazald (*N*-methyl-*N*-nitroso-*p*-toluenesulfonamide) [1]. Media and reagents were purchased from Panreac Química (Barcelona, Spain) or Merck (Darmstadt, Germany). Solvents were obtained from J.T. Baker (Deventer, The Netherlands). All chemicals and solvents were of the highest purity available.

Media and supply of PAHs

The mineral salts medium used in this study has been described previously [8]. To prepare liquid mineral medium with pyrene or fluoranthene, these hydrocarbons were added to sterile medium in acetone solution (5%) to obtain a final concentration of 0.1 or 0.2 g/L. Flasks were shaken at 200 rpm at a temperature of 30°C before inoculation to permit acetone removal. Solid medium with pyrene or fluoranthene was prepared by adding the hydrocarbon in acetone solution to sterile medium at 45°C to a final concentration of 0.1 or 0.2 g/L. The identified metabolites were added to sterile liquid medium in crystal form to obtain a final concentration of 5 mM, except for benzene-1,2,3-tricarboxylic acid, which was added in acetone solution. Hexadecane and pristane were sterilized separately and added to sterile mineral medium. Luria-Bertani (LB) medium was prepared with mineral salts medium and supplemented with glucose (2 g/L).

Isolation and identification of pyrene- and fluoranthene-degrading strains

A soil sample highly contaminated with creosote was obtained from Andújar, Spain. A 2 g soil suspension in 8 mL mineral medium was used to inoculate 250 mL-Erlenmeyer flasks, with 50 mL mineral medium and pyrene or fluoranthene (0.2 g/L) as sole carbon source. After several transfers (every 2 weeks over a 6-month period), the enrichment cultures were used to inoculate mineral medium plates with pyrene or fluoranthene, respectively. Plates were incubated at 25°C for 30 days. Colonies of fluoranthene- and pyrene-degrading strains obtained under these conditions were purified in LB medium supplemented with glucose. Bacteriological, growth, and biochemical tests were performed using standard methods [4].

Partial 16S ribosomal DNA gene sequences (approximately 1,400 nucleotides) of the isolates were obtained by direct sequencing of PCR-amplified 16S rDNA. Genomic DNA extraction, PCR-mediated amplification of 16S rDNA, and purification of PCR products were carried out as described by Rainey et al. [19]. The universal primers used were those described by Weisburg et al. [25]. Purified DNA products from strains CP1 and CP2 were sequenced using a Big Dye terminator cycle sequencing kit V. 2.0 (Applied Biosystems, Foster City, CA) in accordance with the manufacturer's instructions. PCR products from strains CFt2

and CFt6 were sequenced using a Big Dye terminator cycle sequencing kit V. 3.1 (Applied Biosystems). Sequence reaction mixtures were analyzed using an ABI PRISM 3700 DNA sequencer.

Utilization of other hydrocarbons by isolated fluoranthene- and pyrene-degrading strains

Growth in, or transformation of, other PAHs was tested in mineral medium plates with each PAH serving as the sole carbon source. Several colonies of each strain, grown in plates of LB medium, were sequentially transferred to replicate mineral medium plates. Each of these plates was sprayed with an acetone solution (5%) of one hydrocarbon [13] and incubated at 25°C. After 30 days of incubation, growth was evident by a significant increase in bacterial biomass on test plates compared with non-sprayed control plates. Transformation was demonstrated by clearing zones around the bacterial mass, accompanied by accumulation of colored metabolites. As an exception, naphthalene was supplied to the inoculated mineral medium plates as crystals in the lid, and its transformation was evident only by the accumulation of diffusible yellow-colored metabolites around the bacterial mass.

Growth in the aliphatic hydrocarbons hexadecane and pristane was tested in liquid mineral medium with each compound (5 mM). Following 15 days of incubation, the protein content of test cultures was compared with that of a control without a carbon source.

Growth in fluoranthene and identification of accumulated metabolites

The ability of each isolate to grow at the expense of fluoranthene was verified by an increase in cell density [measured as absorbance at 600 nm (A_{600})], as well as by the accumulation of metabolites in liquid cultures with mineral medium and fluoranthene. Metabolite accumulation was determined by HPLC analysis of culture fluids. Several colonies grown on LB plates for 6 days were suspended in mineral medium ($A_{600} = 1$), this suspension then being used as inoculum (2%). Replicate batch cultures were grown in 250-mL Erlenmeyer flasks containing 50 mL mineral medium and fluoranthene (0.2 g/L). Incubation was conducted at 25°C with rotary shaking (200 rpm). Uninoculated flasks, and flasks without fluoranthene, served as controls. To determine the increase in culture absorbance (A_{600}), 1 mL samples were removed at 36- or 48-h intervals and filtered through glass wool to eliminate any remaining fluoranthene crystals. Accumulated metabolites were detected and tentatively identified by HPLC analysis of the supernatant following centrifugation (3,000 *g*; 3 min) to remove cells.

At the end of the incubation period, whole cultures were solvent-extracted [14], with extracts then analyzed by HPLC and gas chromatography coupled to mass spectrometry (GC-MS). Prior to GC-MS analysis, the acidic extracts were treated with ethereal diazomethane to obtain methyl ester derivatives from potential acid metabolites.

Metabolite identity was established by comparing retention times (HPLC and GC), as well as UV-vis and MS spectra to those obtained for authentic products. Authentic *Z*-9-carboxymethylenefluorene-1-carboxylic acid was isolated from cultures of *Mycobacterium* sp. strain AP1 in mineral salts medium with fluoranthene [14], while 8-hydroxy-3,4-benzocoumarin was isolated from cultures of *Pseudomonas* sp. strain F274 [5].

Utilization of fluoranthene as sole source of carbon and energy by *Mycobacterium* sp. strain CP1

A time-course experiment using liquid cultures in mineral medium with fluoranthene was set up to determine the kinetics of fluoranthene utilization, metabolite accumulation, and bacterial growth. Fluoranthene removal and metabolite accumulation were quantified by HPLC analysis of culture extracts [14], with growth measured by the increase in protein concentration. Several colonies of strain CP1 grown on LB for 5 days were resuspended in mineral medium ($A_{600} = 1.0$) and used as inoculum (1 mL). Replicate batch cultures were grown in 100-mL Erlenmeyer flasks containing 20 mL mineral medium and fluoranthene (0.1 g/L). Uninoculated and non-fluoranthene flasks served as controls. The concentration of fluoranthene and protein in cultures was measured at 96-h intervals over the first 12 days, and then at 2-day intervals until the end of incubation (20 days). Controls were analyzed at 0, 12, and 20 days. Additional flasks were used to study the underlying cause of the cessation of growth observed after 15 days incubation. At this point, 0.05 g/L extra fluoranthene was added to six replicate cultures. Residual fluoranthene was determined at 15 (immediately after this addition), 20, and 30 days.

Feeding experiments with fluoranthene metabolites

The ability of strains CP1 and CFt6 to further oxidize some of the identified metabolites was tested in mineral salts medium, with each metabolite serving as the sole source of carbon and energy [14]. Cells grown in mineral medium with casamino acids (5 g/L) and fluoranthene (1 g/L) were used as inoculum (5%) for 250 mL-Erlenmeyer flasks containing 50 mL mineral medium and each of the substrates (5 mM). Uninoculated flasks and flasks lacking a carbon source served as controls. At the time of inoculum, and following 15 days of incubation, whole contents of duplicate cultures and corresponding controls were solvent-extracted and analyzed by HPLC to determine the concentrations of the remaining substrate and accumulated oxidation products. The identity

of these products was confirmed by GC-MS analysis. Protein concentration was determined simultaneously using duplicates of separate cultures and controls.

Analytical methods

Reverse-phase HPLC was performed with a Hewlett-Packard model 1050 chromatograph equipped with an HP-1040 M diode array UV-visible detector set at 254 nm. Separation was achieved on a Chromspher C_{18} (Chrompack) (25 cm ×4.6 mm, 5-μm particle size) column, applying a linear gradient of methanol [10–95% (v/v) in 20 min] in acidified water (0.6% H_3PO_4). Flow was maintained at 1 mL/min. The injection volume for supernatant fluids was 100 μL. In cultures of strain CP1, the concentrations of residual substrate and oxidation products were calculated for duplicate cultures from the peak areas obtained by injections of 5–50 μL extract, using standard calibration curves for each chemical.

GC-MS analyses were conducted using a Hewlett-Packard 5890 series II with a 5989 mass selective detector. Compounds were separated on an HP-5 capillary column [30 m ×0.25 mm (internal diameter)] with 0.25 μm film thickness, and helium as the carrier gas. The column temperature was maintained at 50°C for 1 min, and then raised to 310°C at a rate of 10°C/min. The mass spectrometer was operated at 70 eV of electron ionization energy. Injector and analyzer temperatures were set at 290 and 315°C, respectively.

Results

Isolation and characterization of pyrene- and fluoranthene-degrading strains

Strains CP1 and CP2 were isolated from an enrichment culture established in pyrene-mineral medium inoculated with creosote-contaminated soil. Strains CFt2 and CFt6 were obtained from a similar culture in which the carbon source was fluoranthene. After 15 days of incubation in either pyrene- or fluoranthene–mineral medium plates, the isolates produced colonies (about 1–2 mm) surrounded by clearing zones indicative of substrate degradation. In LB medium, the pyrene-degrading strains CP1 and CP2 formed orange colonies (1 and 2 mm, respectively) in 5 days. Colonies from CP1 were characteristically dry and irregular, while those from CP2 were rounded and creamy. The fluoranthene-degrading strains CFt2 and CFt6 formed yellow colonies (2 mm and 1 mm, respectively). Strain CFt2 colonies were dry and irregular while those of CFt6 were creamy and round. All the strains were short and rod-shaped, Gram-positive, aerobic, non-motile and non-fermentative bacteria. Analysis of partial 16S rRNA gene sequences placed the four strains within the genus *Mycobacterium*. Strains CP1 and CP2 presented identical sequences, exhibiting 99.8% similarity to that reported for a PAH-degrading strain of *Mycobacterium vaccae* (GenBank accession number AF480591). Strains CFt2 and CFt6 also shared the same sequence, displaying less than 99% similarity compared to all *Mycobacterium* strains in the database.

Utilization of other PAHs by *Mycobacterium* sp. strains CP1, CP2, CFt2 and CFt6

Strains CP1 and CP2, isolated by their ability to grow in pyrene, were also able to grow in anthracene, phenanthrene, fluoranthene, and the heterocycle dibenzothiophene (Table 1). PAHs with lower molecular weights such as naphthalene, fluorene and acenaphthene were transformed but did not support growth. *Mycobacterium* sp. strain CP1 grew in the aliphatics hexadecane and pristane, while strain CP2 grew only in hexadecane. The fluoranthene-degrading strains were less versatile. In addition to fluoranthene, *Mycobacterium* sp. strain CFt2 utilized only pristane for growth, while CFt6 utilized anthracene, hexadecane, and pristane. Strains CFt2 and CFt6 were unable to transform the remaining hydrocarbons included in this study.

Growth in fluoranthene and accumulation of metabolites by pyrene- and fluoranthene-degrading *Mycobacterium* strains

The ability of the isolates to utilize fluoranthene as a sole source of carbon and energy was confirmed by an increase in cell mass (measured as absorbance) and accumulation of metabolites in fluoranthene–mineral medium (Fig. 1). Following 18 days of incubation, all strains attained maximum growth with a subsequent rapid decrease in culture absorbance (A_{600}). Strain CP1 exhibited the highest cell density (maximum $A_{600} = 0.28$), followed by strain CFt6 (maximum $A_{600} = 0.23$). All strains demonstrated better growth in fluoranthene than *Mycobacterium* sp. AP1 (maximum $A_{600} = 0.1$; Z. López, unpublished results). During early incubation, the culture fluids acquired a characteristic yellow color that gradually changed to brown. This color evolution was observed previously in fluoranthene enrichments and in cultures of *Mycobacterium* sp. strain AP1 with fluoranthene [14]. HPLC analysis of culture fluids revealed the accumulation of three metabolites with a kinetic profile very similar for all strains. Two of those metabolites, *Z*-9-carboxymethylenefluorene-1-carboxylic acid (**I**) and 9-fluorenone-1-carboxylic acid (**II**), had been previously identified by our group as degradation products of fluoranthene by *Mycobacterium* sp. strain AP1 [14]. Metabolite **II** appeared immediately following inoculation, reaching maximum concentration at the beginning of the exponential growth period (4–8 days, depending on the strain), and decreasing immediately thereafter to undetectable concentrations by day 15. The concentration of **I** increased progressively from the start of the exponential growth period, attaining a maximum

Table 1 Growth and/or transformation of several hydrocarbons by *Mycobacterium* sp. strains CP1, CP2, CFt2 and CFt6[a]

Substrate	CP1	CP2	CFt2	CFt6
Naphthalene	T	T	-	-
Biphenyl	-	-	-	-
Fluorene	T	T	-	-
Acenaphthene	T	-	-	-
Dibenzothiophene	G/T	G/T	-	-
Anthracene	G/T	G/T	-	G/T
Phenanthrene	G/T	G/T	-	-
Fluoranthene	G/T	G/T	G/T	G/T
Pyrene	G/T	G/T	-	-
Hexadecane	G/T	G/T	-	G/T
Pristane	G/T	-	G/T	G/T

[a]*G* Growth on plates with mineral medium and the specific hydrocarbon as a sole carbon source, *T* transformation as demonstrated by clearing zones surrounding the colonies, - negative for both growth and transformation

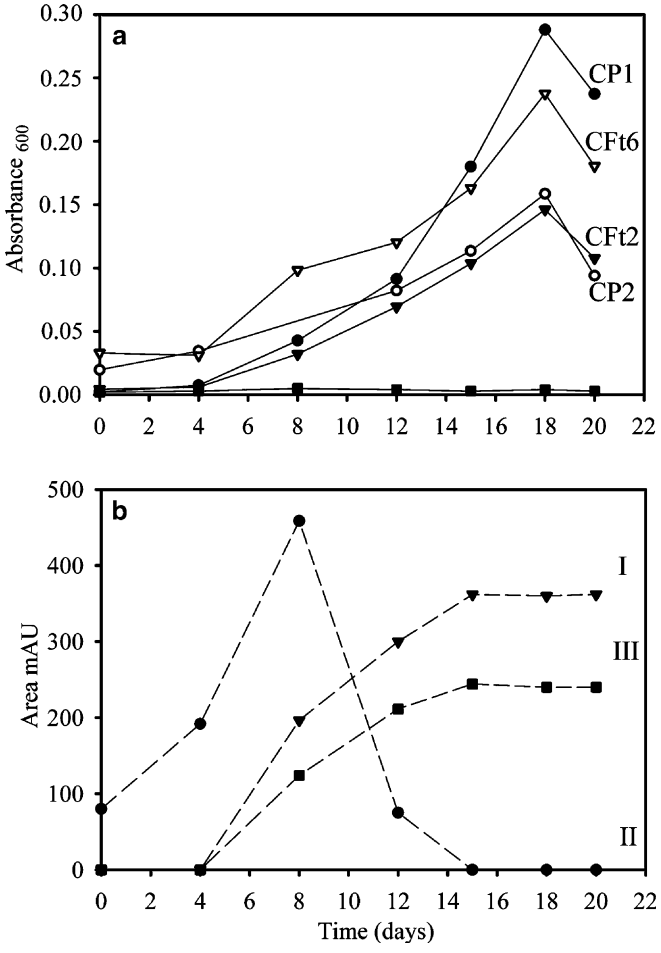

Fig. 1 Growth of *Mycobacterium* sp. strains CP1, CP2, CFt2 and CFt6 (**a**) and accumulation of metabolites *Z*-9 carboxymethylene-fluorene-1-carboxylic acid (**I**), 9-fluorenone-1-carboxylic acid (**II**), and *cis*-1,9*a*-dihydroxy-1-hydrofluorene-9-one-8-carboxylic acid (**III**) by strain CP1 (**b**), in mineral medium with fluoranthene as a sole source of carbon and energy

value several days later than **II** (days 8–14). This value remained relatively constant until the end of the incubation period. Evolution of the third metabolite [*cis*-1,9*a*-dihydroxy-1-hydrofluorene-9-one-8-carboxylic acid (**III**); R_t 11.7 min] was similar to that of **I**.

Identification of fluoranthene metabolites

After 20 days of incubation, the liquid cultures used to determine growth of the strains in fluoranthene (see above) were halted, and their entire content extracted with ethyl acetate. HPLC analyses of neutral extracts revealed the presence of a single peak corresponding to fluoranthene. The acidic extracts displayed six major peaks (Fig. 2), four of which were accumulated by all strains: metabolites **I**; **III**; benzene-1,2,3-tricarboxylic acid (**V**—previously identified during the degradation of fluoranthene by *Mycobacterium* sp. AP1 [14]); and a new metabolite, designated as **IV** (R_t 14.9 min), the identity of which could not be established on the basis of HPLC data. In addition to these, *Mycobacterium* sp. strains CP1 and CP2 accumulated naphthalene-1,8-dicarboxylic acid (**VIII**), also identified during the degradation of fluoranthene by strain AP1 [14], and metabolite **VII** (R_t 19.0 min). Metabolite **II**, observed in HPLC analyses of supernatants during the time course study of metabolite accumulation, was not detected in extracts due to its low concentration at day 20.

GC-MS analyses of the methylated acidic extracts revealed eight major chromatographic peaks (Table 2), three of which were present in the organic extracts from the cultures of the four strains: metabolites **V** and **I**, whose GC retention times and mass spectra confirmed their previous identification by HPLC, and a new product (R_t 23.9 min). The latter exhibited a mass spectrum with a molecular ion at *m*/*z* 284 (M^+) and initial fragments indicative of easy loss of a methylated carboxylic group [225 ($M^+ - 59$)]. Further fragmentation was very similar to that observed for the methylated derivative of 8-hydroxy-3,4-benzocoumarin, a fluorene metabolite identified in a previous study by one of us [5]. On this basis, the product with a GC R_t of 23.9 min was identified as the methylated derivative of 8-hydroxy-3,4-benzocoumarin-1-carboxylic acid. This identification was confirmed by the presence of a product (**IV**) in HPLC analyses with a UV-vis spectrum very similar to that reported by Rehmann et al. [21] for this same compound. While those authors also reported GC-MS data for 8-hydroxy-3,4-benzocoumarin-1-carboxylic

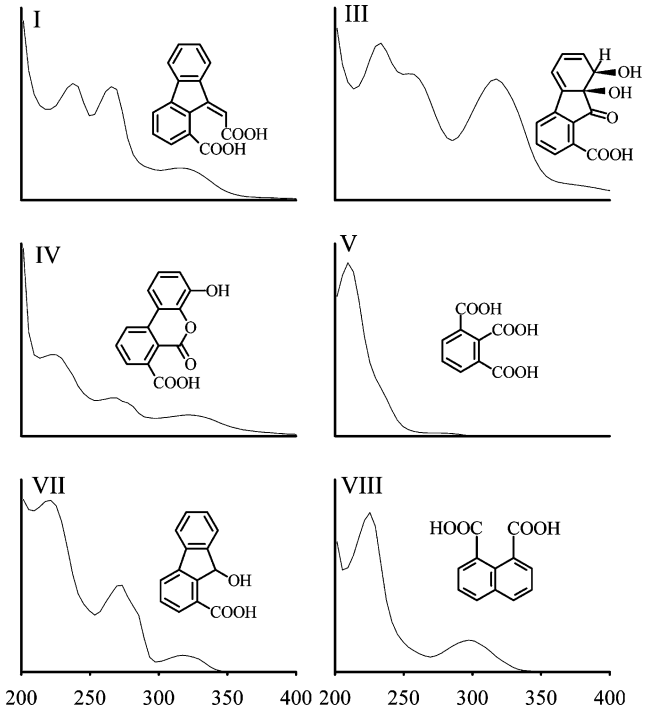

Fig. 2 UV-vis spectra of six major metabolites formed from fluoranthene by *Mycobacterium* sp. strains CP1, CP2, CFt2 and CFt6, as obtained by HPLC analysis of culture extracts. Retention times (R_t): **I** 18.2, **III** 11.7, **IV** 14.9, **V** 16.6, **VII** 19.0, **VIII** 14.4

acid (**IV**), they analyzed the TMS derivative instead of the methylated derivative.

GC-MS analyses of the acidic extracts from cultures of strains CP1 and CP2 showed three additional products, two of which were identified as naphthalene-1,8-dicarboxylic acid (**VIII**) (previously observed in HPLC) and its anhydride (**VIIIa**). The third metabolite (R_t 20.1 min) exhibited a mass spectrum and GC retention time identical to those of authentic 9-hydroxyfluorene-1-carboxylic acid, whose UV-vis spectrum and HPLC retention time were identical to those of metabolite **VII**. In addition to benzene-1,2,3-tricarboxylic acid (**V**), *Z*-9-carboxymethylenefluorene-1-carboxylic acid (**I**), and 8-hydroxy-3,4-benzocoumarin-1-carboxylic acid (**IV**), GC-MS analysis of acidic extracts from cultures of strains CFt2 and CFt6 revealed two products, one of which (R_t 21.7 min) exhibited a mass spectrum consistent with a hydroxylated derivative of 9-fluorenone-1-carboxylic acid. According to published data [5], this compound could be a byproduct of the thermal decomposition of *cis*-1,9*a*-dihydroxy-1-hydrofluorene-9-one-8-carboxylic acid during GC-MS analysis. In fact, the UV-vis spectrum obtained for metabolite **III** is very similar to that reported by Rehmann et al. [21] for this acid. Accordingly, metabolite **III** was identified as *cis*-1,9*a*-dihydroxy-1-hydrofluorene-9-one-8-carboxylic acid, while the second product (R_t 12.4 min) was identified as phthalic acid (**VI**).

Table 2 Gas chromatography (GC) retention times (R_t) and electron ionization (EI) mass spectral properties of major compounds detected in acidic extracts from cultures of strains CP1, CP2, CFt2 and CFt6 in mineral medium with fluoranthene. Acidic compounds were analyzed as methyl ester derivatives. *ME* Methyl ester, *diME* dimethyl ester, *trME* trimethyl ester

R_t (min)	m/z of fragment (% relative intensity)	Identification	Product number	Strains			
12.4	194(M^+, 9), 163(100), 135(9), 104(6), 92(10), 76(4), 51(3)	Phthalic acid (diME)[a]	**VI**			CFt2	CFt6
16.6	252(M^+, 2), 221(100), 193(2), 175(6), 149(11), 120(7), 104(23), 77(7), 51(2)	Benzene-1,2,3-tricarboxylic acid (trME)[a]	**V**	CP1	CP2	CFt2	CFt6
18.7	244 (M^+, 30), 213(100), 198(8), 185(30), 170(39), 154(19), 126(29), 114(27), 77(8), 63(14)	Naphthalene-1,8-dicarboxylic acid (diME)[a]	**VIII**	CP1	CP2		
19.0	198(M^+, 57), 154(87), 126(100), 98(10), 74(23), 63(33)	Naphthalic anhydride[a]	**VIIIa**	CP1	CP2		
20.1	240(M^+, 39), 224(5), 208(59), 192(3), 180(100), 152(79), 126(6), 77(11), 63(17), 51(10)	9-Hydroxyfluorene-1-carboxylic acid (ME)[a]	**VII**	CP1	CP2		
21.7	268(M^+, 5), 255(22), 238(69), 223(50), 207(100), 195 (18), 180(49), 151(96), 139(55), 127(22), 103(20), 75(47)	*cis*-1,9*a*-Dihydroxy-1-hydrofluorene-9-one-8-carboxylic acid (ME)[b]	**III**			CFt2	CFt6
22.9	308(M^+, 21), 276(42), 232(51), 217(100), 189(88), 163(16), 124(15), 94(15), 59(26)	*Z*-9-Carboxymethylenefluorene-1-carboxylic acid derivative (diME)[a]	**I**	CP1	CP2	CFt2	CFt6
23.9	284 (M^+, 100), 253 (87), 241(22), 225 (37), 210(6), 207(14), 196(9), 182(7), 170(11), 168(8) 154(14), 139(36), 126(54), 114(13), 100(1)	8-Hydroxy-3,4-benzocoumarin-1-carboxylic acid (ME)[b]	**IV**	CP1	CP2	CFt2	CFt6

[a]Identified by comparison (of R_t and mass spectrum) with authentic material
[b]Identification supported by other studies

Utilization of fluoranthene as a sole source of carbon and energy by *Mycobacterium* sp. strain CP1

The results described above suggested CP1 as the strain with the best growth yields in fluoranthene mineral medium. CP1 was therefore selected for a more accurate study of fluoranthene utilization. A time-course experiment was set up to quantify the removal of fluoranthene from cultures in fluoranthene–mineral medium (0.1 g/L), as well as the concomitant increase in bacterial protein (Fig. 3). During the exponential growth period (1–15 days), the fluoranthene concentration decreased from 100 to 14.5 μg/mL, while bacterial protein increased from 9.8 to 53.0 μg/mL. Given that protein accounts for approximately 50% of cell dry weight, and that approximately 50% of cell dry weight is carbon [17], a cellular assimilation of depleted fluoranthene of approximately 45% occurred under these conditions. Although 20% of the initial fluoranthene remained in the medium at day 15, growth halted abruptly and a cellular lysis effect was observed.

HPLC analyses of acidic extracts from the cultures confirmed accumulation of most of the metabolites previously identified (**I**–**V** and **VII**), exhibiting accumulation kinetics consistent with those displayed in Fig. 1b. The commercial availability of some metabolites (**II**, **V**, **VII**), or their isolation from culture (**I**), permitted calculation of a partial mass balance for the fluoranthene utilized. At the end of exponential growth period, approximately 15% of the fluoranthene removed by strain CP1 (390 nmol/mL) was recovered as benzene-1,2,3-tricarboxylic acid (**V**), 9-fluorenone-1-carboxylic acid (**II**), *Z*-9-carboxymethylenefluorene-1-carboxylic acid (**I**), and 9-hydroxyfluorene-1-carboxylic acid (**VII**) (Table 3). Only about 3% of the fluoranthene was accumulated as *Z*-9-carboxymethylenefluorene-1-carboxylic acid (**I**).

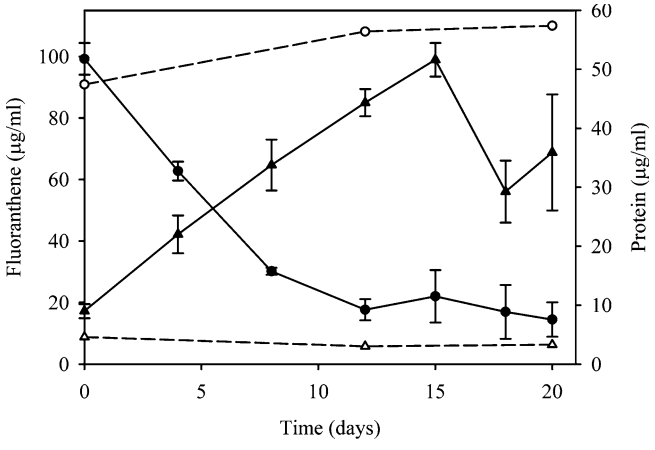

Fig. 3 Utilization of fluoranthene as sole source of carbon and energy by *Mycobacterium* sp. strain CP1 in liquid mineral medium at 25°C and 200 rpm. Growth is shown as an increase of cell protein in cultures (*closed triangles*) and in controls without carbon source (*open triangles*). Fluoranthene concentration was determined by HPLC analyses of organic extracts from cultures (*filled circles*) and uninoculated controls (*open circles*)

As mentioned above, growth of strain CP1 in fluoranthene–mineral medium stopped abruptly after day 15, at which time 20% of the initial fluoranthene remained in the medium. This fluoranthene was not further utilized, its concentration remaining constant until the end of the incubation period. This halt in growth and fluoranthene degradation may have resulted from toxic effects (e.g., accumulation of toxic metabolites), or from a reduced bioavailability of the residual fluoranthene. To rule out the possibility of toxicity, 0.05 g/L fresh fluoranthene was added to replicate cultures at day 15 (final concentration = 0.065 g/L). These cultures were then incubated and solvent-extracted at days 15, 20 and 30. HPLC analyses of the neutral extracts revealed a rapid removal of fluoranthene (results not shown), thereby demonstrating that the halt in fluoranthene uptake, and consequently in growth, was due to the low availability of the residual fluoranthene.

Feeding experiments with fluoranthene metabolites

To determine whether 9-fluorenone-1-carboxylic acid (**II**), naphthalene-1,8-dicarboxylic acid (**VIII**), and benzene-1,2,3-tricarboxylic acid (**V**) were further metabolized, and to identify their possible oxidation products, mineral medium containing each metabolite (5 mM) was inoculated with strains CP1 or CFt6. Following 15 days of incubation, none of the cultures of strain CFt6 showed an increase in cell protein content or accumulation of oxidation products. Identical results were obtained for cultures of strain CP1 with metabolites **V** and **VIII**. Cultures of strain CP1 with compound **II** exhibited a significant increase in bacterial protein (from 16.3 to 72 μg/mL), and accumulation of several oxidation products. HPLC analyses of culture extracts revealed that strain CP1 depleted 97% of metabolite **II** (from 133 μg/mL to 4 μg/mL), which corresponds to a cellular assimilation of 9-fluorenone-1-carboxylic acid carbon of approximately 43%. The three oxidation products present in the acidic extracts were 9-hydroxyfluorene-1-carboxylic acid (**VII**), detected previously as a fluoranthene metabolite in strains CP1 and CP2, *cis*-1,9*a*-dihydroxy-1-hydrofluorene-9-one-8-carboxylic acid (**III**), and benzene-1,2,3-tricarboxylic acid (**V**), detected during the biodegradation of fluoranthene by strains CP1, CP2, CFt2 and CFt6. GC-MS analyses detected only metabolites **V** (19% abundance) and **VII** (81%).

Discussion

Enrichment cultures in mineral salts medium with pyrene, followed by plating in the same medium, led to the isolation of two *Mycobacterium* sp. strains designated CP1 and CP2. These pyrene-degrading strains were also

Table 3 Mass balance of products identified in organic extracts from cultures of *Mycobacterium* sp. strain CP1 in mineral medium and fluoranthene at the end of exponential growth

Products	μg/mL	nmol/mL
Initial fluoranthene	99.2 ± 5.1	496 ± 25.5
Remaining fluoranthene	22.0 ± 8.5	110 ± 42.5
Benzene-1,2,3-tricarboxylic acid (**V**)	2.2 ± 0.4	10 ± 1.9
9-Fluorenone-1-carboxylic acid (**II**)	2.7 ± 0.7	12 ± 3.0
Z-9-Carboxymethylenefluorene-1-carboxylic acid (**I**)	3.3 ± 1.4	12 ± 5.0
9-Hydroxyfluorene-1-carboxylic acid (**VII**)	5.2 ± 0.4	23 ± 1.7

able to grow in fluoranthene and phenanthrene, and to transform a number of other PAHs. The isolates obtained when fluoranthene was used as a carbon source, strains CFt2 and CFt6, also proved to belong to the genera *Mycobacterium*. These fluoranthene-degrading strains were more specific, being unable to act on any of the other PAHs studied with the exception of anthracene, which served as a growth substrate for strain CFt6. All the strains could grow in the presence of alkanes. Similar growth substrate spectra have been reported for other pyrene- and fluoranthene-degrading mycobacteria [2, 3, 9, 12, 21, 24]. The fact that all of the strains isolated were mycobacteria confirms the role of this genus in the removal of high molecular weight PAHs from contaminated sites.

Mycobacterium sp. strains CP1 and CFt6 exhibited the highest cell densities when grown in liquid mineral medium with fluoranthene crystals. Although strain CP1 assimilated approximately 45% of the fluoranthene carbon depleted from cultures, its cell growth halted abruptly when 20% of the initial fluoranthene remained in the medium. A similar effect was observed during growth of *Mycobacterium* sp. AP1 in fluoranthene, where carbon assimilation was only 30% and the amount of substrate remaining undegraded was 70% [14]. When fresh fluoranthene was added to cultures of strain CP1 in which growth and fluoranthene degradation had ceased, the extra substrate was removed in 2 days, demonstrating that the halt in degradation was due to the reduced bioavailability of the remaining fluoranthene. Mulder et al. [16] reported that during the growth of *Pseudomonas* sp. strain 8909 N in liquid medium with naphthalene, biofilm formation reduced the dissolution rate of the substrate by 90%, an effect that had a dramatic impact on biodegradation rates. Additionally, Wick et al. [28] demonstrated that the growth of *Mycobacterium* sp. strain LB501T was directly related to substrate dissolution from crystals and to uptake of substrate from solution by the microorganisms. Given that strain CP1 also forms biofilms on fluoranthene crystals, these biofilms are probably responsible for the decline in bioavailability.

During growth at the expense of fluoranthene, the pyrene- and the fluoranthene-degrading strains exhibited four metabolites in common: *Z*-9-carboxymethylenefluorene-1-carboxylic acid (**I**), *cis*-1,9*a*-dihydroxy-1-hydrofluorene-9-one-8-carboxylic acid (**III**), 8-hydroxy-3,4-benzocoumarin-1-carboxylic acid (**IV**), and benzene-1,2,3-tricarboxylic acid (**V**). In addition, the pyrene-degrading strains CP1 and CP2 accumulated 9-hydroxyfluorene-1-carboxylic acid (**VII**) and naphthalene-1,8-dicarboxylic acid (**VIII**), the latter also detected as its anhydride (**VIIIa**). The fluoranthene-degrading strains CFt2 and CFt6 accumulated phthalic acid (**VI**).

The metabolites formed from fluoranthene by *Mycobacterium* sp. strains CP1, CP2, CFt2 and CFt6, as well as their kinetics of accumulation, confirm the three routes for fluoranthene degradation proposed for *Mycobacterium* strain AP1 [14] (Fig. 4). Moreover, these findings suggest their potentially widespread distribution in soil mycobacteria and involvement in general processes of environmental fluoranthene removal.

The first route, involving dioxygenation at C-7 and C-8, *meta* cleavage, and acenaphthenone formation, and the second, involving dioxygenation at C-1 and C-2, *meta* cleavage, and formation of 9-fluorenone-1-carboxylic acid, were first proposed for *Mycobacterium* sp. strain PYR-1 [12], in which 9-fluorenone-1-carboxylic acid appears to be further degraded via 9-fluorenone. In *Mycobacterium* sp. AP1, the formation of benzene-1,2,3-tricarboxylic acid (**V**) from 9-fluorenone-1-carboxylic acid indicated an alternative route for the oxidation of this intermediate. Here, the results from the metabolite feeding experiments with strain CP1, as well as the identification of metabolites **III** and **IV** in all isolates, demonstrate that 9-fluorenone-1-carboxylic acid is attacked by an angular dioxygenase, thus generating *cis*-1,9*a*-dihydroxy-1-hydrofluorene-9-one-8-carboxylic acid (**III**). The latter is then dehydrogenated to 8-hydroxy-3,4-benzocoumarin-1-carboxylic acid (**IV**), further degradation of which, by reactions similar to those described for biphenyl, would explain the formation of benzene-1,2,3-tricarboxylic acid (**V**). This set of reactions is similar to that previously proposed for the oxidation of 9-fluorenone during the degradation of fluorene by *Pseudomonas* sp. strain F274 [5].

The detection of naphthalene-1,8-dicarboxylic acid (**VIII**) in cultures of strains CP1 and CP2 indicates that, like *Mycobacterium* sp. AP1 [14], these pyrene-degrading strains degrade fluoranthene using the 7,8-dioxygenation route. The acenaphthenone produced via this route is likely mono-oxygenated in its methylenic carbon to give acenaphthenequinone. Hydrolysis of this quinone yields naphthalene-1,8-dicarboxylic acid (**VIII**), which is in equilibrium with its anhydride (**VIIIa**). These reactions have also been proposed for the oxidation of the acenaphthenone formed during growth in fluoranthene by *Sphingomonas paucimobilis* strain EPA 505 [23]. However, they have not been observed in other strains that initiate the attack on fluoranthene by 7,8-dioxygenation,

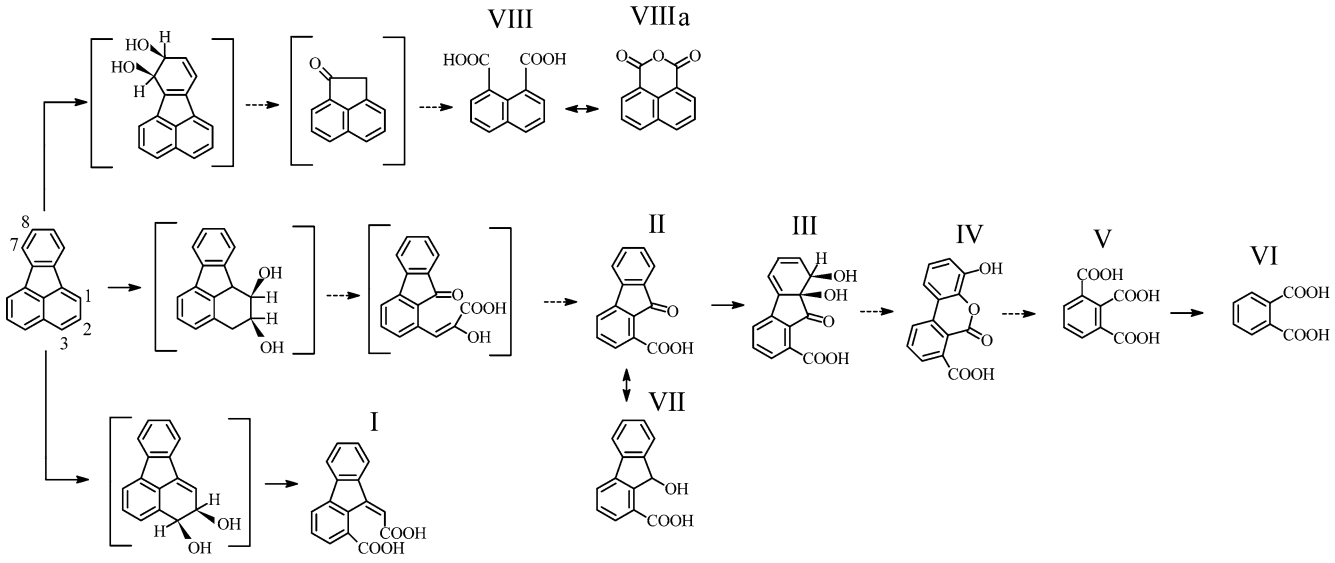

Fig. 4 Schematic pathways proposed for the degradation of fluoranthene by *Mycobacterium* sp. strains CP1, CP2, CFt2 and CFt6. Structures in *brackets* are proposed intermediates and have not been isolated. *Dashed arrows* indicate two or more successive reactions

such as *Mycobacterium* sp. strain PYR-1 [12], in which there is no evidence of further acenaphthenone oxidation, and *Alcaligenes denitrificants* WW1, which accumulates a product identified as 3-hydroxymethyl-4,5-benzocoumarine [27]. In *Mycobacterium* sp. strain AP1, naphthalene-1,8-dicarboxylic acid (**VIII**) is further degraded via benzene-1,2,3-tricarboxylic acid (**V**) and phthalic acid (**VI**). Here, although these compounds have been identified as fluoranthene metabolites, there is no evidence that they are formed from naphthalene-1,8-dicarboxylic acid (**VIII**).

The third route for degradation of fluoranthene, involving dioxygenation at C-1 and C-2, and *ortho* cleavage to form *Z*-9-carboxymethylenefluorene-1-carboxylic acid (**I**), has been proposed as a unique route for fluoranthene metabolism in *Mycobacterium* sp. KR20, a strain unable to act on other PAHs [21]. Based on metabolite identification, the authors proposed that the *Z*-9-carboxymethylenefluorene-1-carboxylic acid in strain KR20 is degraded to 9-fluorenone-1-carboxylic acid, which undergoes angular dioxygenation and further degradation to benzene-1,2,3-tricarboxylic acid. While our results do not provide evidence ruling out the possible formation of 9-fluorenone-1-carboxylic acid (**II**) from *Z*-9-carboxymethylenefluorene-1-carboxylic acid (**I**), the kinetics of metabolite accumulation observed in all isolates has shown this not to be the principal route. As is evident from Fig. 1b, the maximum concentration of 9-fluorenone-1-carboxylic acid (**II**) attained during exponential growth was not preceded by a corresponding maximum concentration of *Z*-9-carboxymethylenefluorene-1-carboxylic acid. Furthermore, the subsequent rapid utilization of 9-fluorenone-1-carboxylic acid is accompanied by an increase in the concentration of *Z*-9-carboxymethylenefluorene-1-carboxylic acid, which is inconsistent with the latter being a precursor of 9-fluorenone-1-carboxylic acid. This pattern in the kinetics of accumulation of 9-fluorenone-1-carboxylic acid and *Z*-9-carboxymethylenefluorene-1-carboxylic acid, which was previously observed in *Mycobacterium* sp. strain AP1 [14], is now confirmed for all the strains included in the present study. The further degradation of metabolite *Z*-9-carboxymethylenefluorene-1-carboxylic acid (**I**) remains unclear. The fact that it accumulated until the end of the incubation period, and that it was not utilized during metabolite feeding experiments, may well indicate that it is a dead-end product. However, these findings might also be explained by the lack of an uptake mechanism for the compound once it has left the bacterial cell. Although the fate of *Z*-9-carboxymethylenefluorene-1-carboxylic acid (**I**) remains unclear, it is important to note that we have identified this metabolite in all our isolates, which suggests that it may be a product frequently formed during the bacterial oxidation of fluoranthene.

As previously described for *Mycobacterium* sp. AP1, the pyrene-degrading strains isolated here, *Mycobacterium* sp. CP1 and CP2, possess all three routes for fluoranthene degradation described above. The fluoranthene-degrading strains *Mycobacterium* CFt2 and CFt6 appear to possess only the pathways initiated by dioxygenation at C-1, C-2 or C-2, C-3, which may explain their lower versatility in PAH utilization.

This work contributes to a further understanding of the general microbial processes at work in the removal of PAHs from contaminated sites. Current research is focused on determining the individual pathways involved during the environmental degradation of PAH mixtures.

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