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The metabolism of fluoranthene by a species of *Mycobacterium*

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SUMMARY

A *Mycobacterium* sp., which was previously isolated from oil-contaminated estuarine sediments, mineralized the polycyclic aromatic hydrocarbon fluoranthene. When supplemented with an alternative carbon source, the organism was able to mineralize up to 78% of the added [3-^{14}C]fluoranthene to $^{14}CO_2$ after 5 days of incubation, with relatively little accumulation of intermediate metabolites. The distribution of the C-14 label was monitored throughout the mineralization process. The *Mycobacterium* degraded in excess of 95% fluoranthene within a 24 hour period following an initial 6–12 h lag phase. At that point approximately 53% of the radioactivity was located in the ethyl acetate extractable fraction, 31.8% in CO_2, and 14.7% in the aqueous phase. Incubation of the *Mycobacterium* sp. with soil and river water, in the presence of fluoranthene, enhanced mineralization of fluoranthene by 92.7% over the indigenous biota. These results, in conjunction with previously reported studies, suggest the potential application of this *Mycobacterium* sp. for the bioremediation of polycyclic aromatic hydrocarbon contaminated wastes in the environment.

INTRODUCTION

Fluoranthene (Fig. 1) is commonly found as a major polycyclic aromatic hydrocarbon (PAH) contaminant in the environment, originating from petrogenic and pyrogenic sources [13]. Fluoranthene (FA) occurs in high concentrations in urban environments, as a product of incomplete combustion of fossil fuels and in diesel engine exhaust, mainstream and sidestream cigarette smoke, coal tar products, creosote, grilled and smoked food products, and kale [13,22]. FA has been shown to be as mutagenic as the potent carcinogen benzo[a]pyrene (B[a]P) in the *Salmonella* forward mutation assays [5,22,24,27,28] and in a diploid human lymphoblast cell line [29]. Interestingly, in 1983, the International Agency for Research on Cancer (IARC) classified FA as non-carcinogenic based on negative results obtained from mouse-skin initiation-promotion assays and a subcutaneous injection assay [4,22]. However, other evidence from a newborn mouse lung adenoma bioassay indicates that FA is a potent tumorigen [6]. Synergistic action of FA and B[a]P doubles the incidence of papillomas and carcinomas in mice over that of the same dose of B[a]P alone [22,31]. In addition, in vivo and in vitro prenatal FA toxicity to rats and mice has been demonstrated [23]. Fluoranthene has also been shown to be phototoxic to several different aquatic species [25], and to bacteria in the presence of near ultraviolet light [30].

Since PAHs such as FA have been shown to be cytotoxic, mutagenic and carcinogenic, and are ubiquitous in the environment, there is current interest in the environmental fate of PAHs.

Several microorganisms that degrade PAHs containing less than 4 rings have been isolated [12]. Considerably less is known about the ability of microorganisms to metabolize the more recalcitrant molecules such as FA and B[a]P. Although most studies of FA metabolism focus on eukaryotic systems [1,2], bacteria capable of PAH degradation, including some that are able to oxidize or co-oxidize FA, have been isolated in several laboratories [3,11,14,16–19]. Oxygen-dependent degradation of FA was demonstrated in culture with *Pseudomonas* species [3]. Additionally, co-oxidation was enhanced by the presence of cyanide in *Pseudomonas* strain NCIB 9816 [3]. A glucose grown *Pseudomonas* species HL7b at resting stage transformed FA and produced metabolites with absorption maxima of 352 nm, 407 nm, and 490 nm [11]. Microbial degradation of FA and other PAHs in soil-percolation units has also been reported [14]. To date, no metabolites from bacterial degradation of FA have been identified.

The recent discovery and characterization of a PAH-degrading *Mycobacterium* sp. [17–20] has led to the elucidation of the microbial metabolism of pyrene. Preliminary results have shown that, in pure culture, this

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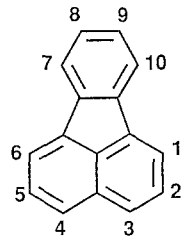

Fig. 1. The chemical structure of fluoranthene.

Mycobacterium is also capable of significant FA mineralization [17,19] following enzymatic induction by pyrene. In this study, we describe in more detail the ability of this *Mycobacterium* sp. to extensively degrade fluoranthene.

MATERIALS AND METHODS

Chemicals. 3-[^{14}C]FA (54.8 mCi/mmol) with a radiochemical purity of >98% was purchased from Chemsyn Science Laboratories, Lenexa, KS. Unlabeled FA was purchased from Fluka AG, CH-9470 Buchs, Switzerland. Bacterial media and reagents were purchased from Difco Laboratories, Detroit, MI; all solvents and chemicals used were of the highest purity available.

Organism and growth conditions. Mycobacterium sp. cultures were grown in 125 ml Erlenmeyer flasks containing 30 ml minimal basal salts medium (MBS) supplemented with 250 μg/ml each of peptone, yeast extract and soluble starch, and 0.5 μg/ml pyrene or FA dissolved in *N,N*-dimethylformamide. Cultures were grown in the dark and shaken (150 rpm) at 24 °C. For starter cultures, bacterial cells were subcultured at log phase from stock cultures into 30 ml of supplemented MBS medium containing low levels of FA and/or pyrene (50 μg/30 ml in dimethylformamide) for enzyme induction.

Actively growing cells were pelleted by centrifugation (8500 × *g*), rinsed in 15 ml fresh MBS medium, pelleted again, and re-suspended in 15 ml supplemented MBS medium. Three ml aliquots (absorbance at 500 nm, 0.66) were transferred into each of 8 replicate microcosms [21]. Growth rates were established by measuring optical densities at 500 nm, using a Beckman DU-7 spectrophotometer (Beckman Instruments Inc., Irvine, CA), and related to the values reported by Heitkamp et al. [19] (2.97×10^6 cells/ml at an absorbance of 0.10, at 500 nm).

Fluoranthene degradation conditions. FA mineralization ($^{14}CO_2$ evolution) was monitored in flowthrough microcosm test systems [16,21] containing 200 ml supplemented MBS medium with 3-[^{14}C]FA (0.021 μCi/ml) and 2.75 μg/ml non-labeled FA dissolved in 2.75 μl dimethylformamide. Volatile organic compounds were trapped with columns containing polyurethane foam and 500 mg of Tenax GC. The $^{14}CO_2$ was trapped in 50 ml of monoethanolamine-ethylene glycol (3 : 7, v/v). Two microcosms without FA and two sterile microcosms (autoclaved) were used as controls to correct for abiotic decomposition and adsorption of FA to cells and glass. Microcosms were shaken manually 3 times daily. Trapping agents were analyzed as reported below.

Following incubation for 0, 24, 48, 72, 96, 144, 216, and 336 h, bacteria and media were extracted with three equal volumes of ethyl acetate, dried with anhydrous NA_2SO_4, and evaporated in vacuo at 37 °C to dryness. Aqueous phase samples were acidified to pH 2.5 and extracted in the same manner. The residues were re-dissolved in 100 μl methanol for HPLC analysis. The amount of radioactivity remaining in the aqueous phase was measured by counting 0.5 ml aliquots in 7.5 ml Scintisol (Isolab Inc., Akron, OH) using a Packard 2000CA Tri-Carb Liquid Scintillation Analyzer (Packard Instrument Co., Downers Grove, IL).

Physical and chemical analysis. Reversed-phase high performance liquid chromatographic analysis was performed with a Beckman model 100A HPLC (Beckman Instruments Co., Berkeley, CA) equipped with a 5-μm C_{18} Ultrasphere ODS column (4.6 mm by 25 cm) and a Hitachi model 100-40 spectrophotometer (Hitachi Scientific Instruments, Mountain View, CA), using a water-methanol (35–95%, v/v, 40 min) gradient system at a flow rate of 1.0 ml/min. UV absorbance was measured at 254 nm and peak areas were calculated with an Altex model C-R1A integrator (Shimadzu Scientific Instruments, Columbia, MD).

Radioactive fractions were collected at 30-s intervals in scintillation vials, mixed with 7.5 ml of Scintisol, and analyzed by liquid scintillation spectrometry. Ultraviolet-visible absorption spectra of isolated compounds were determined in methanol on a Beckman model DU-7 spectrophotometer.

$^{14}CO_2$ was measured at 24-h intervals by removing 1 ml aliquots of the trapping solution and counting in a mixture of 15 ml Scintisol and 4 ml methanol. Polyurethane plugs were cut in half, immersed in 15 ml Scintisol, and counted in a Packard 2000CA Tri-Carb liquid scintillation analyzer. Radioactivity of organic residues adsorbed to Tenax GC was determined by liquid scintillation counting following combustion to CO_2 in a Packard model B306 Tri-Carb (Packard Instrument Co., Downers Grove, IL) sample oxidizer. Burn efficiency was determined to be 97%. Radioactivity of the sample was counted in 8 ml of Carbo-sorb and 10 ml Permafluor (United Technologies, Packard Instrument Co.).

Fluoranthene mineralization in soil and river water. Soil and river water samples were freshly collected at Lock and Dam no. 5 on the Arkansas River in Jefferson

County, Arkansas. Forty g of moist sandy soil were placed in sterile microcosms and either 160 ml of river water or 160 ml of supplemented MBS medium was added to each microcosm and mixed thoroughly. FA induced cells of the *Mycobacterium* sp. were rinsed in sterile MBS medium, and then in filter sterilized river water. Three ml of cell suspension (OD_{500}, 0.65) were added to five of the microcosms. FA, 355 μg (0.24 μCi), labeled and non-labeled was added to each of the microcosms. Two autoclaved controls, and one non-sterile control without FA were also included. Carbon dioxide trapping solution was prepared and analyzed as reported above, and replenished after 16 days (10 sampling times).

Kinetics of fluoranthene mineralization. Seven 250 ml biometer flasks were fitted with 12.7 cm and 22.9 cm long syringe needles with Luer locks, for both the side arm and the main flask, respectively. Each flask received 50 ml of supplemented MBS medium and 20 ml of monoethanolamine-ethylene glycol (3 : 7, v/v) trapping solution. Controls consisted of 1 flask without FA with cells, and 2 flasks with FA minus cells. FA, 500 μg (non-labeled) and 10 μl labeled (1.57 μCi) was added to each of 6 flasks. All 6 experimental flasks and 1 control were inoculated with 1 ml of exponentially growing FA and/or pyrene-induced bacterial cells. Syringe openings were plugged with rubber stoppers and covered with aluminum foil. The columns of the flasks were packed with a layer of glass wool, 500 mg of Tenax GC, and one styrofoam plug; all were capped with cotton and aluminum foil.

Samples were taken at 0, 6, 48, 72, 144, and 312 h. At each sampling time, 1 ml of trapping solution was removed with a 2.5 ml disposable glass syringe and counted for radioactivity as described above using Aquasol (NEN Research Products, Boston, MA) as the scintillator. Two-ml aliquots were taken from the medium, divided into two 1.5 ml nylon centrifuge tubes, and centrifuged for 10 min at 8500 × *g*. Supernatants of half of the samples were decanted into scintillation vials and counted in 15 ml Scintisol, and the remaining half were stored in culture vials at 4 °C for direct injection, into the HPLC for analysis of metabolites. Pellets were rinsed in 1 ml distilled H_2O, pelleted again and resuspended in distilled H_2O. Absorption was measured as described above at 285, 500 and 600 nm. The cellular fraction of radioactivity was determined using pre-dried, cellulose acetate filters (Millipore 22 μm, 45 mm diameter; Millipore Co. Bedford, MA). The 1 ml aliquots of cell suspensions were filtered, rinsed twice with 2 ml distilled H_2O, dried for 1 h at 60 °C, and cooled in a desiccator. Filters were dissolved completely in 1.5 ml of dimethylformamide and counted in 15 ml Scintisol.

RESULTS

Fluoranthene mineralization.

In supplemented MBS medium FA was rapidly transformed by the *Mycobacterium* sp. after a 6–12 h lag phase (Fig. 2). The highest rate of biotransformation occurred between 24 and 72 h following inoculation, resulting in a maximum transformation rate of 4.31 μg FA per hour (Fig. 3). The highest growth rate occurred after the FA had almost completely disappeared; however, FA did not inhibit growth at even the highest concentrations (510 μg/30 ml) used in these experiments (Fig. 4), and was non-toxic to the bacteria at 1000 μg/30 ml (data not shown). No change in spectrophotometric absorbance was observed in sterile controls. No significant difference in bacterial growth rate or FA utilization was noted between pyrene-induced and FA-induced cultures.

In microcosms, mineralization of up to 78% of the total estimated amount added was observed in 5 days, with an average mineralization of 66% for all 7 microcosms (Fig. 2), whereas an average of only 42% was observed in biometer flasks (Fig. 4). Lower mineralization rates in biometer flasks may be explained by reduced gas exchange in those containers. Additionally, in both cases CO_2 measurements were limited by the capacity of the trapping agent. Least square (linear regression) analysis of data from carbon dioxide evolution experiments from microcosms yielded correlation coefficients of 0.90–0.98 over the first 7 sampling times.

Visual evidence of FA utilization in MBS medium included color changes from clear to pale yellow to orange and back to light yellow. Changes in color were not observed in sterile controls or in controls without FA. Although a slight color change was observed in flasks containing only supernatant from stationary phase cultures and FA (500 μg, 0.089 μCi), significant mineralization of FA occurred only in the presence of cells.

Distribution of radioactivity.

Allocation of radioactivity, based on the total counts recovered from extracted cultures, is presented in Fig. 3. The relatively low solubility of FA in MBS medium prevented satisfactory time-zero measurements of extracts and freshly inoculated medium, in both the microcosms and the biometer flasks. Recovery of the carbon-14 label in active cultures was directly related to cell density and mineralization rates (Fig. 4). FA readily adsorbed to glass and cell surfaces; however, recovery of radioactivity increased with time and as mineralization proceeded (Figs. 3, 4). Only 25% of the initial radioactivity was recovered from extracts at time zero. At time zero, more than 99% of the counts were in the neutral ethyl acetate extractable fraction, and about 80% of this was located

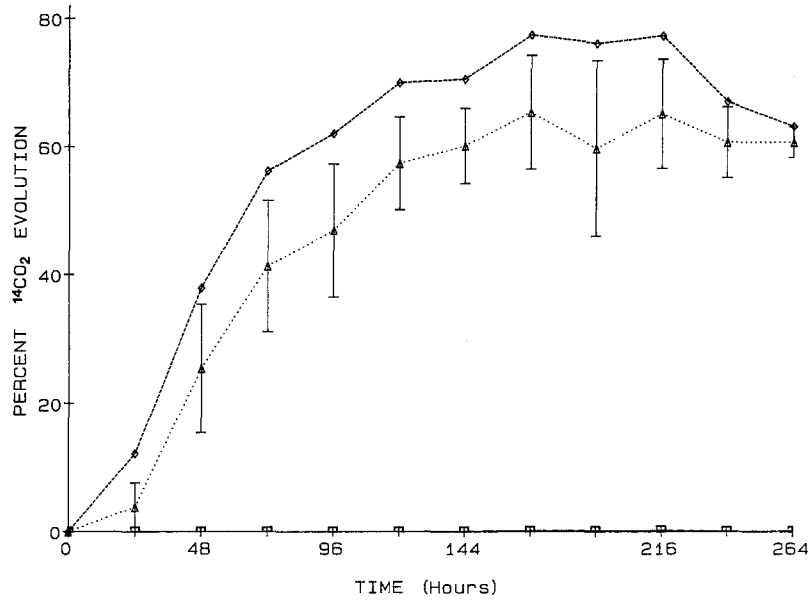

Fig. 2. Mineralization of FA by the *Mycobacterium* sp. in microcosms. (□) Sterile controls with FA. (△) The average mineralization rate of FA from 8 microcosms; bars indicate S.D. (◇) Maximum mineralization of FA.

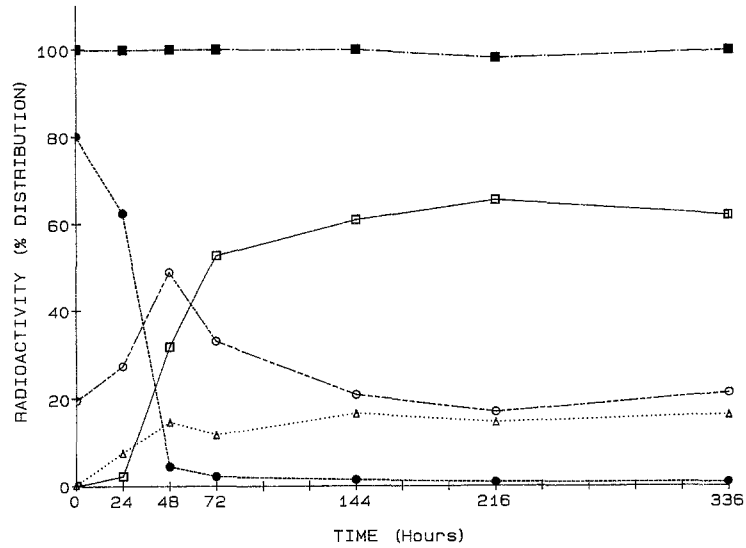

Fig. 3. Distribution of [3-^{14}C] FA residues over two weeks of incubation with the *Mycobacterium* sp. in microcosms. (■) Total radioactivity recovered. (●) Non-utilized [^{14}C]FA remaining. (△) [^{14}C]FA residues remaining in the aqueous phase. (○) Kinetic analysis of combined acid and neutral ethyl acetate extractable FA metabolites. (□) $^{14}CO_2$ formation.

in the FA peak (Fig. 3). Radioactivity recovered from the autoclaved control added up to 71% of the initial amount, and approximately 94% of the counts were in the FA peak. Disregarding the zero time point, recovery values from 8 microcosms averaged about 89%, and had a S.D. of 17.7%. Radioactivity from polyurethane plugs never exceeded 0.20%, and adsorption to Tenax GC yielded less than 0.002% of the total counts added (data not shown), indicating that volatile organic compounds were probably not formed. Due to rapid mineralization, metabolites did not accumulate to any large extent.

Fluoranthene was recovered mainly from the neutral ethyl acetate extractable fraction, and eluted at approximately 42.5 min. The earliest metabolites appeared in the

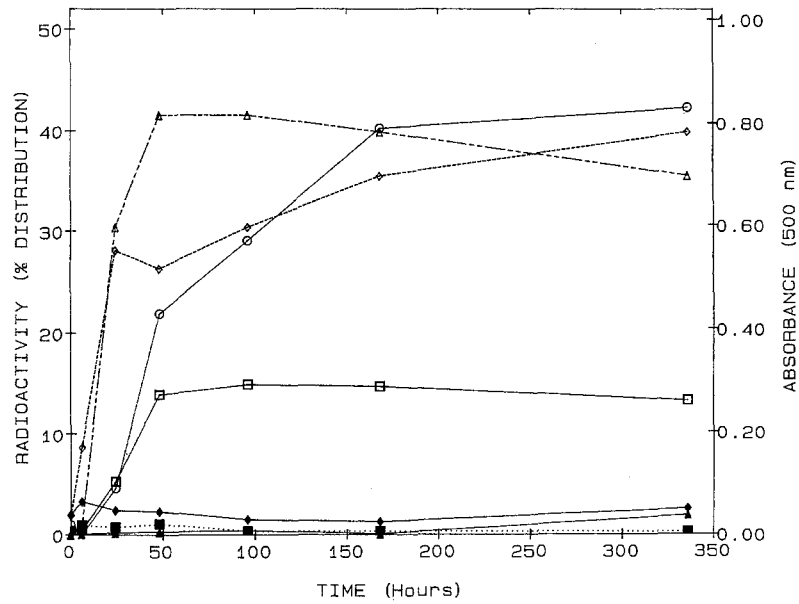

Fig. 4. Distribution of [3-^{14}C] FA over two weeks of incubation with the *Mycobacterium* sp. in biometer flasks. (□) Percent radioactivity associated with cells. (◇) Percent radioactivity in the culture medium. (△) Percent $^{14}CO_2$ evolution. (▲, ■, ◆) Controls. (○) Growth curve of *Mycobacterium* sp. dosed with 510 μg FA.

acid extractable fraction, and eluted at 14.5 and 24.5–25.0 min, followed by a peak at 27–27.5 min (Fig. 5). The metabolites at 23.8–24.0 min and 24.4–25 min had absorption maxima at 225, 259, 296, and 223, 266, and 316 nm, respectively, and are thought to be initial ring fission products. After reaching a maximum following 72 h of incubation these metabolites decreased, with a concomitant increase of a metabolite in the 19–19.5 min region. All radioactivity in fractions corresponded to UV absorbance peaks at wavelength 254 nm in the HPLC elution profile, and decreased after 72 h, with the exception of the 19–19.5 min peak (Fig. 5). Although less than 3% of the radioactivity was located at each 27–27.5 min and 19–19.5 min, these represented the largest amounts

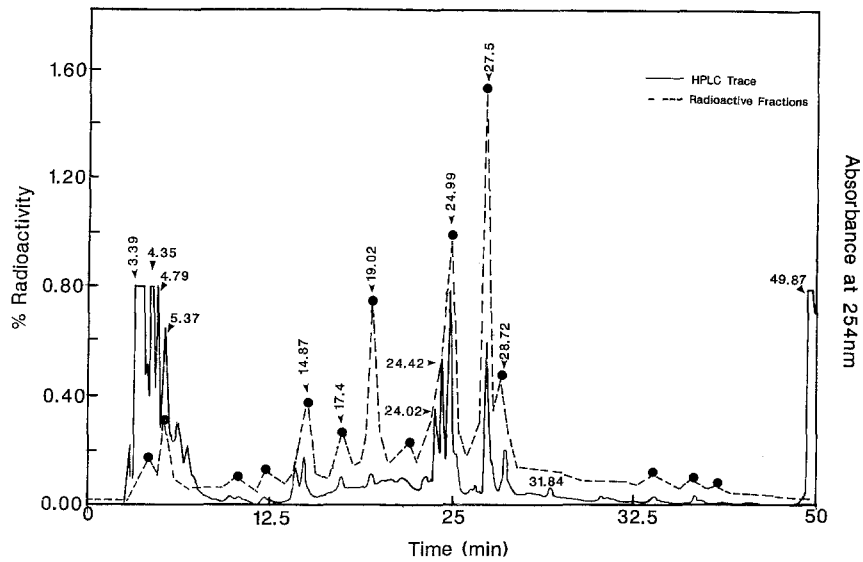

Fig. 5. HPLC profile of acidified ethyl acetate extractable metabolites obtained when the *Mycobacterium* sp. was incubated with FA for 72 h. The solid line indicates the UV absorbance at 254 nm and the dotted line indicates percent [^{14}C]FA residues.

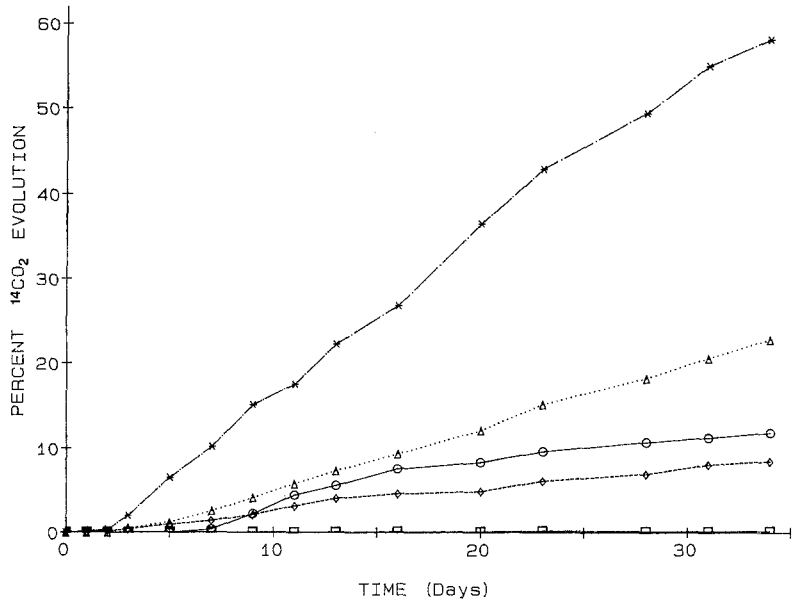

Fig. 6. Mineralization of FA in microcosms containing sediment and river water. (□) Autoclaved control. (○) Mineralization by the indigenous microbiota in river water and sediment. (△) Mineralization in river water and sediment spiked with *Mycobacterium*. (◇) Mineralization in sediment and supplemented MBS medium by the indigenous biota. (×) Mineralization in sediment and supplemented MBS medium spiked with *Mycobacterium*. Data represent averages of duplicate microcosms.

of radioactive metabolites recovered over the course of the experiment.

Fluoranthene mineralization in soil and river water.

FA mineralization in soil proceeded at a much slower rate than in pure culture. A 24–48 h lag period was observed for all microcosms. After 34 days, microcosms containing only soil, water, and the indigenous microbiota had mineralized 11.9% of FA. Microcosms supplemented with the *Mycobacterium* cells showed an average of 22.9% mineralization, which represents a 92.7% increase over the indigenous microbiota alone (Fig. 6). FA degradation was not observed in the controls. The addition of supplemented MBS medium to soil greatly enhanced the ability of the *Mycobacterium* sp. to utilize FA, but did not significantly increase the rate of mineralization by the naturally occurring microorganisms (Fig. 6).

DISCUSSION

The *Mycobacterium* sp. used in this research was isolated from a chronically polluted location. This bacterium has already been shown useful in the degradation of such compounds as pyrene, naphthalene, and phenanthrene, as well as minor degradation of 1-nitropyrene, 6-nitrochrysene, and B[a]P [17]. Previous experiments have also demonstrated its longevity in non-sterile environmental microcosms containing soil from polluted areas without any additional carbon source [18]. The data presented above indicate that although the indigenous microorganisms in unacclimated sediment degraded FA to some extent, the addition of the *Mycobacterium* greatly enhanced mineralization rates of fluoranthene. It was originally thought that this *Mycobacterium* sp. cometabolized PAHs [17,19], but the present results indicate that mineralization of FA also takes place in the absence of nutrient enrichment. However, the possibility of small molecular weight hydrocarbons and nitrogen-containing compounds occurring naturally in the sediments cannot be ruled out as a factor in FA degradation. Heitkamp and Cerniglia [18] reported that addition of organic nutrients in the form of glucose and peptone (0.01% of each) to sediments reduced mineralization of PAHs by the *Mycobacterium* sp., presumably because of competition for these nutrients by the indigenous microbiota. In the present study, enrichment of the soil with MBS medium supplemented with yeast extract peptone, and soluble starch, resulted in a nearly 7-fold increase in mineralization rate of FA by the *Mycobacterium* over that of the naturally occurring microorganisms (Fig. 6).

The ability of this *Mycobacterium* sp. to degrade FA completely and rapidly, with a relatively small accumulation of intermediate metabolites, makes it a very good candidate as a treatment method for bioremediation of PAHs. From a practical standpoint, since the bacterium was able to degrade FA and pyrene, hydrocarbons

containing four fused aromatic rings, it might be useful in the biodegradation of higher molecular weight PAHs and their substituted derivatives.

PAHs are ubiquitous in the environment. Many are known carcinogens and mutagens [15], acting singly or in concert with other PAHs, either by direct or indirect action. FA, because of its abundance, volatility, and wide distribution in the environment, poses a special risk to humans. Exposure to FA may be via skin contact, inhalation, or ingestion. Conventional bioassays tend to underestimate the risk of FA to human health and its toxicological importance [4,5,25,26,29] when combined with other PAHs such as B[a]P. Considering the synergistic relationship of FA and B[a]P, concerning tumor incidence, it is significant that this *Mycobacterium* sp. may also be capable of B[a]P degradation, or even mineralization, when in the presence of FA. Numerous studies have shown that metabolites of PAHs are frequently more toxic than the original compound [7–10]. Consequently it is important to understand the biological activity of PAHs and their derivatives, to determine the fate of these compounds in situ, and to screen organisms that will effect their detoxification. The identification of FA metabolites and the degradation pathway employed by this *Mycobacterium* sp. are now in progress in our laboratory.

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