APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1988, p. 2549–2555
0099-2240/88/102549-07$02.00/0

Vol. 54, No. 10

Microbial Metabolism of Polycyclic Aromatic Hydrocarbons: Isolation and Characterization of a Pyrene-Degrading Bacterium

MICHAEL A. HEITKAMP,† WIRT FRANKLIN, AND CARL E. CERNIGLIA*

National Center for Toxicological Research, Food and Drug Administration, Jefferson, Arkansas 72079

Received 19 April 1988/Accepted 19 July 1988

Microbiological analyses of sediments located near a point source for petrogenic chemicals resulted in the isolation of a pyrene-mineralizing bacterium. This isolate was identified as a *Mycobacterium* sp. on the basis of its cellular and colony morphology, gram-positive and strong acid-fast reactions, diagnostic biochemical tests, 66.6% G+C content of the DNA, and high-molecular-weight mycolic acids (C_{58} to C_{64}). The mycobacterium mineralized pyrene when grown in a mineral salts medium supplemented with nutrients but was unable to utilize pyrene as a sole source of carbon and energy. The mycobacterium grew well at 24 and 30°C and minimally at 35°C. No growth was observed at 5 or 42°C. The mycobacterium grew well at salt concentrations up to 4%. Pyrene-induced *Mycobacterium* cultures mineralized 5% of the pyrene after 6 h and reached a maximum of 48% mineralization within 72 h. Treatment of induced and noninduced cultures with chloramphenicol showed that pyrene-degrading enzymes were inducible in this *Mycobacterium* sp. This bacterium could also mineralize other polycyclic aromatic hydrocarbons and alkyl- and nitro-substituted polycyclic aromatic hydrocarbons including naphthalene, phenanthrene, fluoranthene, 3-methylcholanthrene, 1-nitropyrene, and 6-nitrochrysene. This is the first report of a bacterium able to extensively mineralize pyrene and other polycyclic aromatic hydrocarbons containing four aromatic rings.

Polycyclic aromatic hydrocarbons (PAHs) occur as natural constituents and combustion products of fossil fuels (24, 39) and are widespread environmental contaminants (21, 26, 27). There is toxicological concern about the presence, persistence, and disposition of PAHs in the environment; some low-molecular-weight PAHs are acutely toxic (12, 44), most higher-molecular-weight PAHs are genotoxic (38), and there is a potential for their bioaccumulation into food chains (34, 37). Chronic exposure to PAHs has been associated with cancerous diseases in aquatic animals (36) and enhanced mutagenicity of sediments (41). Because of their toxicity, carcinogenicity, and ubiquitous distribution the U.S. Environmental Protection Agency has listed 16 PAHs as priority pollutants (28).

PAHs may enter aquatic ecosystems by numerous routes and, because of their hydrophobicity (33), are usually bound and transported by fine particles which undergo sedimentation (21, 34, 37). Although PAHs may undergo volatilization, chemical oxidation, photodecomposition, and microbial degradation while in open waters, most sediment deposition occurs below the photolytic zone, and the primary factor affecting the persistence of deposited PAHs is microbial degradation (14). For this reason, sediments serve as natural repositories for PAHs in aquatic ecosystems, where they may persist, undergo resuspension, or be degraded by complex natural communities of bacteria and fungi (6, 7, 15; C. E. Cerniglia and M. A. Heitkamp, *in* U. Varanasi, ed., *Metabolism of Polycyclic Aromatic Hydrocarbons in the Aquatic Environment*, in press).

Chronic exposure to aromatic hydrocarbons has been reported to enhance PAH degradation in some soils and sediments, and several environmental isolates have been reported to degrade PAHs containing two or three aromatic rings (reviewed by Cerniglia and Heitkamp [in press]). The metabolic pathways, enzymatic reactions, and genetic control of the catabolism of lower-molecular-weight PAHs have been well documented (45). We recently confirmed the occurrence of known microbial metabolites of naphthalene in microcosms containing natural freshwater and estuarine sediments (19). To date, little is known about the ability of pure bacterial cultures to completely degrade PAHs containing more than three fused benzene rings.

In a previous study, we reported the effects of chronic chemical exposure and adaptation of sediment microbial populations on the mineralization rates of six PAHs in both estuarine and freshwater ecosystems (17). We found that relative differences in PAH mineralization among ecosystems were related to chemical structure, hexadecane mineralization rates, the occurrence and concentration of aromatic hydrocarbon residues in sediments, and elevated populations of hydrocarbon-degrading microorganisms. We are now presenting the results of subsequent microbiological investigation of sediments collected from sites chronically exposed to petrogenic chemicals within the watershed of Redfish Bay, Tex. (18). We isolated a pyrene-degrading bacterium from these sites and identified it as belonging to the genus *Mycobacterium*. To our knowledge, this investigation is the first report of the successful isolation and characterization of a bacterium able to completely degrade pyrene, a PAH containing four fused benzene rings, in pure culture.

MATERIALS AND METHODS

Chemicals. Radiolabeled PAHs, specific activities (millicuries per millimole), and sources were as follows: [1,4,5,8-^{14}C]naphthalene (5.10), Amersham Corp., Arlington Heights, Ill.; [9-^{14}C]phenanthrene (19.3), Amersham; [3-^{14}C]fluoranthene (54.8), Chemsyn Science Laboratories, Lenexa, Kans.; [4-^{14}C]pyrene (30.0), Midwest Research Institute, Kansas City, Mo.; 1-nitro-[4,5,9,10-^{14}C]pyrene (57.4), Chemsyn; 3-[6-^{14}C]methylcholanthrene (13.4), Dupont, NEN Research Products, Boston, Mass.; 6-nitro-[5,6,11,12-^{14}C]chrysene (57.4), Chemsyn. Nonlabeled pyrene was pur-

* Corresponding author.
† Present address: Monsanto Co., St. Louis, MO 63167.

chased from Chemical Service, Media, Pa. Chemical analyses by high-pressure liquid chromatography and gas chromatography-mass spectrometry showed that the purity of all PAH stocks exceeded 99%. Chloramphenicol was purchased from P-L Biochemicals, Inc., Milwaukee, Wis. Bacterial media and reagents used to culture the *Mycobacterium* sp. were purchased from Difco Laboratories, Detroit, Mich. All solvents and chemicals were of the highest purity available.

Sample sites and monitoring mineralization of pyrene. Composite sediment and water samples were collected in July 1986 from three sample sites (designated A, B, and C) located proximal to the Harbor Island oil tank farm in the watershed of Redfish Bay near Port Aransas, Tex. Site A samples were collected near a drainage pond located near the Harbor Island oil tank farm. Site B samples were collected from a salt-water pool alongside the Aransas ship channel, below the Harbor Island oil tank farm. Site C samples were collected from a downstream site alongside the edge of Redfish Bay where an oil spill had occurred 9 years earlier. A detailed description of Redfish Bay has been previously presented (17, 19). Mineralization of pyrene was monitored in a flowthrough microcosm test system that allowed continuous measurement of $^{14}CO_2$ evolution (23). Microcosms from each sample site contained 20 g of homogenized moist sediment and 180 ml of collected water. Microcosms were exposed to 0.92 μCi of [4-^{14}C]pyrene and 100 μg of pyrene dissolved in 30 μl of dimethylformamide. Mineralization was monitored at 7-day intervals for 8 weeks at 24°C.

Isolation of pyrene-degrading bacteria. Sediment subsamples (1 g) were removed after 3.5 weeks from microcosms from all three sample sites. This time point was chosen since rapid pyrene mineralization occurred after an initial 2- to 3-week lag phase observed in microcosms from all three sites. The sediment samples were serially diluted and assayed for the presence of pyrene-degrading microorganisms by a method modified from that of Kiyohara et al. (29). Growth medium consisted of a 2% agar mixture of minimal basal salts (MBS) medium (43) containing low levels (250 μg/liter) of Bacto-Peptone (Difco), yeast extract, and soluble starch. The surfaces of the agar plates were sprayed with a 2% pyrene solution in acetone-hexane (1:1, vol/vol) and dried overnight at 35°C. This treatment resulted in a visible and uniform surface coat of pyrene on the agar plates.

Inocula (100 μl) from the 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} dilutions of microcosm sediments were spread with sterile glass rods onto the agar surfaces of the petri dishes and incubated for 3 weeks at 24°C in clear plastic bags to conserve moisture. Although many heterotrophic bacteria were capable of growth on this medium, pyrene-degrading bacteria were distinguished as colonies surrounded by clear zones due to pyrene uptake and utilization. These pyrene-degrading bacterial colonies were aseptically removed from the mixed-culture plates and grown in pure culture on MBS agar and broth containing low levels of organic nutrients and pyrene (0.5 μg/ml).

Maintenance, identification, and characterization of a pyrene-degrading microorganism. The pyrene-degrading isolate was routinely cultured on pyrene-supplemented MBS liquid medium as described above and was inoculated into growth tubes containing the standard diagnostic substrates and reactants listed in Table 1. In addition, the cells were Gram and acid-fast stained. The isolate grew well on pyrene-supplemented MBS medium in the laboratory and, after extensive subculturing, maintained its growth rate and pyrene-degrading activity.

Freshly grown pyrene-degrading bacterial cells were strongly acid fast. Since acid-fast staining is indicative of bacterial cell walls containing mycolic acids, thin-layer chromatography (TLC) and mass spectral analyses of mycolic acids in whole-cell methanolysates were performed by the methods of Minnikin et al. (35) and Collins et al. (10), respectively. TLC was performed with 500 M silica gel GF plates (Analtech, Newark, Del.). Direct probe mass spectral analyses of extracts from TLC spots were performed on a Finnigan MAT model 4023 (Finnigan MAT Corp., San Jose, Calif.) quadrupole mass spectrometer. Direct probe analyses were conducted with a platinum wire probe and a Vacumetrics model DCI current programmer. The mass spectrometer was operated in 70-V electron impact and methane chemical ionization modes, and the ion-source temperature was maintained at 270°C. The mass spectra were collected as the probe was inserted and heated to 3 A over a ramp time of 120 s.

The ability of the cells to utilize pyrene as a sole source of carbon and energy was assayed by the inoculation of replicate tubes of MBS broth containing no supplemented organic substrate and pyrene concentrations ranging from 0.001 to 1.0 g/ml. These tubes were incubated at 30°C for 6 weeks and visually examined for turbidity.

The salt tolerance of the isolate was tested in brain heart infusion broth and nutrient-supplemented MBS broth containing NaCl concentrations ranging from 0.1 to 10%. Each tube was inoculated with a small sample of a pure culture of the pyrene-degrading isolate and incubated for 6 weeks at 24°C. The tubes were mixed three times each week and visually examined for turbidity.

Agar plates containing individual pyrene-degrading colonies and surrounding clear zones were examined by light microscopy. Photomicrographs of the pyrene-MBS agar spread plates were taken through a green filter under low magnification (×12.5) with an American Optical Series 20 Advanced Microstar light microscope equipped with an Expostar automatic shutter control and a model 1053 35-mm camera (American Optical Corp., Buffalo, N.Y.) by using Kodak Panatomic-X film (ASA 32; Kodak Chemical Co., Rochester, N.Y.). Bacterial samples for transmission electron microscopy were prepared by the method of Cole and Popkin (9). Transmission electron microscopy was performed on a Philips model EM301 electron microscope (Eindhoven, The Netherlands).

PAH mineralization by induced and noninduced cells. Since several aromatic hydrocarbons are known to be degraded by inducible catabolic enzymes encoded on plasmids containing both structural and regulatory genes (45), the effects of enzyme induction and protein synthesis on pyrene mineralization were examined. Induced starter cultures were grown in the presence of pyrene as described above, whereas noninduced starter cultures were grown for 2 weeks in the absence of pyrene. The purity of all starter cultures was determined by Gram staining and visual examination after 48 h of growth at 30°C. Experimental cell cultures were grown at 24°C with constant stirring in 500-ml microcosm chambers containing 200 ml of MBS broth supplemented with low levels of organic nutrients, 0.92 μCi of [4-^{14}C]pyrene, and 0.5 μg of pyrene per ml. The optical density at 500 nm (OD_{500}) of each starter culture was measured, and each of the experimental cultures was inoculated at a level to give an OD_{500} of 0.05 (1.49×10^6 cells per ml). Mineralization of pyrene was monitored for 72 h by measuring the evolution of

TABLE 1. Growth, biochemical, and morphological characteristics of a pyrene-degrading *Mycobacterium* sp. isolated from microcosms exposed to pyrene[a]

Characteristic	Results
Colony morphology	0.5- to 1.0-mm circular, smooth with a convex surface, butyrous consistency, yellow pigmented scotochromogens
Cellular morphology	Rods with a straight axis, length of 0.7 to 1.4 μm, width of 0.4 to 0.7 μm, multiple dark-staining granules
Gram stain	Positive in fresh cultures, variable in older cultures
Acid-fast stain	Strongly acid fast
G+C content of DNA	66.6%
Carbon-chain length of mycolic acids	C_{58} to C_{64}
Utilization of carbohydrates[b]	
Dextrose	− (NC)
Xylose	− (NC)
Mannitol	− (Alk)
Lactose	− (Alk)
Sucrose	− (NC)
Maltose	− (Alk)
10% Dextrose	A
10% Lactose	Alk
Nutrient broth	+
Simmons citrate	+
Urea	+
Nitrate reduction	+
Triple sugar iron	Alk slant Alk butt
H_2S production	− (triple sugar iron butt paper) + (lead acetate paper)
Litmus milk	Alk

[a] The bacterium showed negative growth or reactions with adonitol, arabinose, cellobiose, dulcitol, galactose, inositol, malonate, melezitose, melibiose, raffinose, rhamnose, salicin, sorbitol, sorbose, salmonella-shigella agar, pseudosel agar, indole production (heart infusion broth), starch, trehalose, gelatin, motility, esculin, lysine decarboxylase, arginine dehydrolase, and ornithine decarboxylase.

[b] Fermentative and oxidative-fermentative (within parentheses) utilization is indicated as follows: +, growth or positive reaction; −, no growth or negative reaction; NC, no change; Alk, alkaline; A, acid.

$^{14}CO_2$ as described above for sediment-water microcosms. Sterile control cultures were included to detect abiotic degradation of pyrene. Protein synthesis was inhibited in both induced and noninduced cultures by the addition of 25 μg of chloramphenicol per ml at 0 h.

The biodegradation of [1,4,5,8-^{14}C]naphthalene, [9-^{14}C]phenanthrene, [3-^{14}C]fluoranthene, 1-nitro-[4,5,9,10-^{14}C]pyrene, 3-[6-^{14}C]methylcholanthrene, 6-nitro-[5,6,11,12-^{14}C]chrysene, and [7,10-^{14}C]benzo[*a*]pyrene was determined as described above for pyrene mineralization. The pyrene-degrading bacterium was incubated in the flowthrough microcosm test system and exposed in triplicate to 0.92 μCi of ^{14}C-labeled PAH and 50 μg of unlabeled PAH. Total mineralization was the sum of $^{14}CO_2$ evolved from each culture during the incubation period.

RESULTS

Pyrene mineralization. Pyrene was readily mineralized to $^{14}CO_2$ after initial 2- to 3-week lag phases in sediment-water microcosms from all three sample sites (Fig. 1). The highest initial rate of pyrene mineralization was observed with sediment and water from site A, which is closest to the Harbor Island oil tank farm. In these samples, over 50% of the pyrene was mineralized to $^{14}CO_2$ during weeks 3 through 5 after an initial 2-week lag phase. Site B, located immediately below the tank farm, showed the next highest rate of pyrene mineralization with over 25% of the pyrene mineralized during weeks 3 through 5. However, total mineralization of pyrene in these samples reached a maximum of 36% after 8 weeks. Samples from site C, located further downstream alongside Redfish Bay, showed a 3-week lag phase and the slowest initial rates of pyrene mineralization. However, pyrene mineralization rates increased sharply after 4 weeks in samples from site C, and over 50% of the pyrene was mineralized during the last 4 weeks of the study, resulting in a total mineralization of 67.5%.

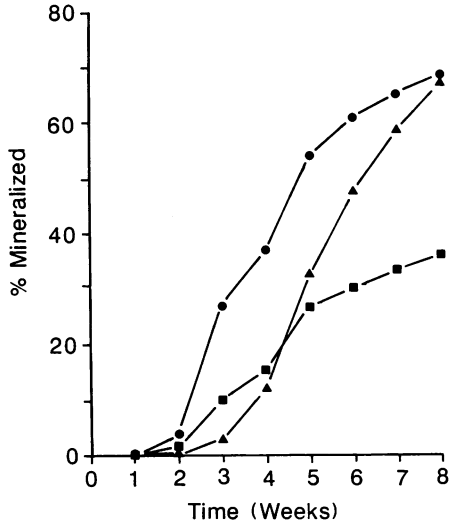

FIG. 1. Mineralization of pyrene in microcosms containing sediment and water from sites A (●), B (■), and C (▲) proximal to the Harbor Island oil tank farm near Port Aransas, Tex.

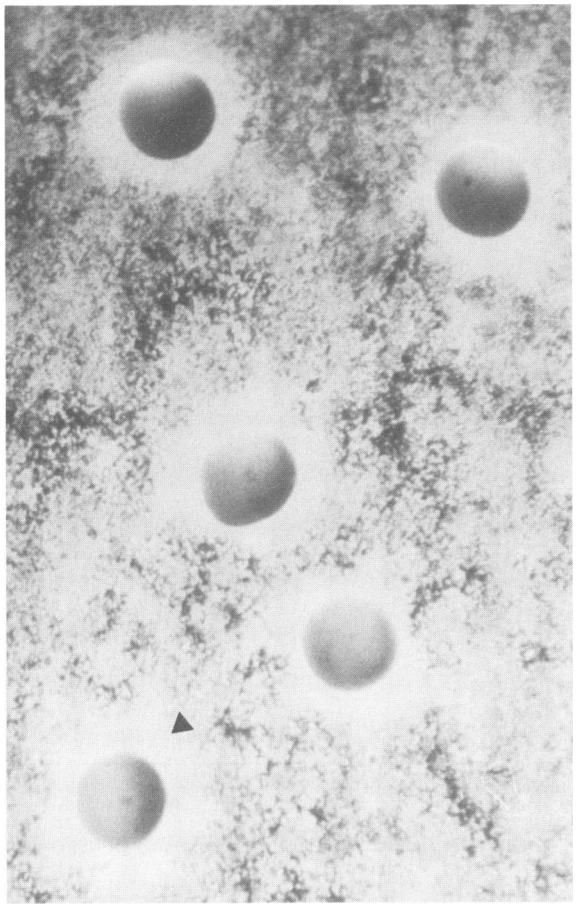

FIG. 2. Photograph of individual pyrene-degrading bacterial colonies and clear zones of pyrene utilization on MBS agar containing low-level nutrients and coated with pyrene (magnification, ×12.5).

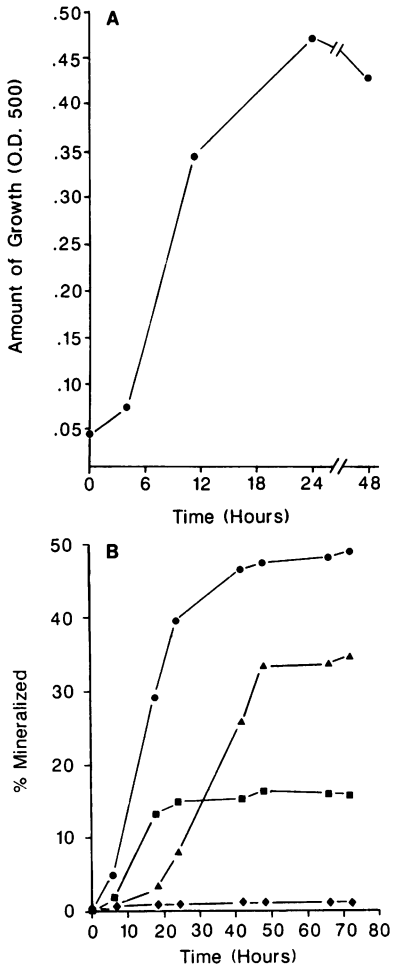

FIG. 3. (A) Growth curve of an *Mycobacterium* sp. cultured in MBS broth containing pyrene and low levels of nutrients. (B) Mineralization of pyrene by noninduced (▲) and pyrene-induced cultures (●) of *Mycobacterium* sp. Replicate pyrene-induced (■) and noninduced (◆) cultures were dosed with 25 μg of chloramphenicol per ml at 0 h.

Isolation and characterization of a pyrene-mineralizing bacterium. Since a rapid increase in pyrene mineralization was observed in microcosms containing sediment and water from site A, sediment samples were taken at 3.5 weeks and assayed for the presence of pyrene-degrading bacteria. Numerous small colonies of pyrene-degrading bacteria were observed on pyrene-supplemented MBS agar plates from the 10^{-3} and 10^{-4} dilutions of sediments from site A. These colonies were all identical in colony and cellular morphology and produced similar clear zones due to pyrene degradation. Individual pyrene-degrading colonies grown on MBS agar containing low-level nutrients appeared as circular colonies and produced clear zones of pyrene utilization ranging from 0.5 to 2.0 mm (Fig. 2). Although similar colonies probably occurred on plates from the 10^{-1} and 10^{-2} dilutions of sediment from site A, they were not clearly observable because of heavy overgrowth of non-pyrene-degrading heterotrophic bacteria. Pyrene-degrading bacteria were not isolated from sediments collected from sites B and C.

Pyrene-degrading colonies were aseptically removed with a sterile transfer loop and streaked via spread plating onto fresh pyrene-supplemented MBS agar containing organic nutrients. The isolate was also subcultured in pyrene-supplemented MBS broth containing low levels of organic nutrients. Bacterial cell density was monitored by measuring the OD_{500}. Serial dilutions and plating of cultures at known OD_{500} values determined a relationship of 2.97×10^6 cells per ml at an OD_{500} of 0.100 for actively growing cells. This relationship was linear at OD_{500} values ranging from 0.050 to 0.500.

The bacterium grew well at 24 and 30°C and showed minimal growth at 35°C and no growth at 5 or 42°C. Exponential-phase cultures incubated at 24°C routinely showed generation times ranging from 2.7 to 4.0 h (Fig. 3A). No cell growth was observed in anaerobic incubations. Table 1 shows the growth and biochemical and morphological characteristics of fresh cultures of the bacterium. Salt-tolerance assays showed that the bacterium grew well at salt concentrations up to 3%, marginally at salt concentrations of 4 to 5%, and not at salt concentrations exceeding 5%.

Morphology and identification. Table 1 presents a summary of colony and cellular morphology and staining characteristics of the bacterium. The cells appeared singly, paired, and clustered with little pleomorphism. No spores were detected. Figure 4 is a transmission electron micrograph of thin-sectioned log-phase bacterial cells at ×114,000 magnification which shows internal cellular morphology. The ultrastructural details and occurrence of mesosomes in *Mycobacterium* spp. have been previously reviewed (2, 16, 42).

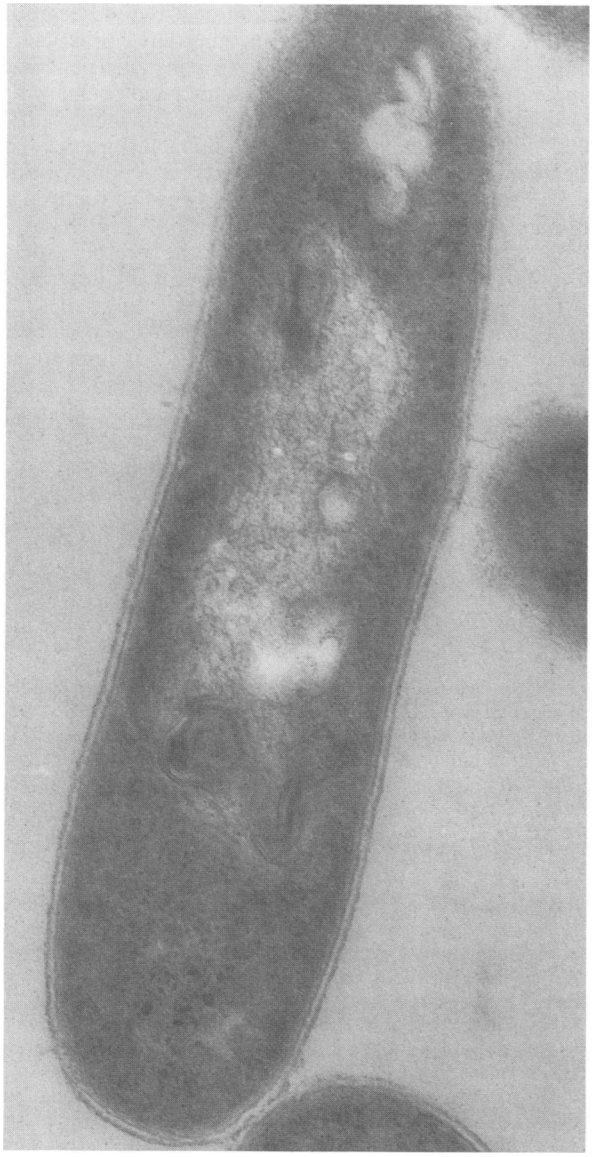

FIG. 4. Transmission electron micrograph of thin-sectioned *Mycobacterium* cells showing internal cellular morphology (magnification, ×114,000).

It is difficult to distinguish between species of *Corynebacterium*, *Mycobacterium*, *Nocardia*, and *Rhodococcus* solely on the basis of acid fastness, G+C content of DNA, and morphological characteristics. Mycolic acid composition of cell walls has been used as a definitive criterion to differentiate *Mycobacterium* from other related taxa. *Mycobacterium* strains are strongly acid fast and have mycolic acids which contain hydroxyl, keto, or carboxylic acid moieties causing a multiple spot pattern when whole-cell methanolysates are analyzed by TLC with a petroleum ether-diethyl ether (85:15, vol/vol) solvent system (35). In contrast, *Corynebacterium* spp. are non-acid-fast and *Nocardia* spp. are weakly acid-fast, gram-positive strains which contain mycolic acids which produce single spots in similar TLC analyses. The pyrene-degrading bacterium isolated in this study was strongly acid fast, and TLC analyses of methyl esters of mycolic acids from this bacterium produced three major and three minor spots on TLC with R_fs ranging from 0.1 to 0.65. All major and minor spots were immobile on TLC with a second methanol-water (5:2, vol/vol) solvent system, which is indicative of mycolic acid methyl esters (35). Furthermore, direct-probe mass spectral analyses of organic extracts from the mycolic acid spots on the TLC plate showed methyl esters of mycolic acids producing molecular ions ranging from 856 to 946 *m/z*, which are indicative of carbon-chain lengths ranging from C_{58} to C_{64}. Similar results were obtained from TLC and mass spectral analyses of a methylated mycolic acid standard obtained from *Mycobacterium tuberculosis*. Carbon-chain lengths ranging from C_{60} to C_{90} have been reported for mycolic acids in *Mycobacterium* spp. (1, 10).

This pyrene-degrading bacterium was identified as a *Mycobacterium* sp. on the basis of its cellular and colony morphology, Gram-staining reaction, strong acid-fast staining, mycolic acid analyses, and biochemical reactions. The complete characteristics of *Mycobacterium* spp. have been extensively reviewed by Barksdale and Kim (2) and Kubica and David (31). The assignment of this isolate to the genus *Mycobacterium* is further supported by the DNA G+C content of 66.6%, which falls midway in the known range of 62 to 70% for *Mycobacterium* spp. (31).

Induction of pyrene mineralization. The relationship between enzyme induction and pyrene mineralization was examined with *Mycobacterium* cultures grown in the presence and absence of pyrene and chloramphenicol. The *Mycobacterium* cultures grown with constant exposure to pyrene were highly induced for pyrene degradation (Fig. 3B). These cultures mineralized 5% of the pyrene within 6 h at 24°C and reached a maximum of 48% mineralization within 72 h. Chloramphenicol, an inhibitor of bacterial protein synthesis, was added initially to cultures pregrown with or without pyrene to determine whether pyrene-degrading enzymes were constitutive or inducible. Cells grown for 2 weeks in the absence of pyrene showed an 18-h lag phase in mineralization when exposed to pyrene and plateaued at 34% mineralization within 72 h. Induced cultures that were dosed with chloramphenicol at 0 h mineralized 15% of the pyrene during the first 24 h of exposure. However, further pyrene mineralization by these cultures was not observed after 24 h. In contrast to induced cultures, the addition of chloramphenicol at 0 h to noninduced cultures inhibited pyrene mineralization to less than 1% in 72 h. In both experiments, no abiotic mineralization of pyrene was detected in sterile controls.

Mineralization of other PAHs. Pure cultures of the *Mycobacterium* sp. mineralized other PAHs containing two, three, or four fused aromatic rings as well as several nitrated or alkylated PAHs. Tests in the microcosm system described above showed mineralization (evolution of $^{14}CO_2$) of the following PAHs (total percentage) after 2 weeks of incubation: naphthalene (59.5%), phenanthrene (50.9%), fluoranthene (89.7), 1-nitropyrene (12.3), 3-methylcholanthrene (1.6%), and 6-nitrochrysene (2.0%).

DISCUSSION

Microorganisms play an important role in the biodegradation of chemicals in natural ecosystems. The degradation of xenobiotics may result from catabolism by individual strains of microorganisms or from combined metabolism by microbial communities. Furthermore, degradation may be enhanced at some sites because of chemical-induced selection or adaptation of microorganisms resulting from chronic exposure to chemicals. In this study, samples were collected

from sites located proximal to Harbor Island oil tank farm, a point source for petrogenic chemicals entering Redfish Bay, Tex. Since we had previously reported relatively high rates of PAH degradation and elevated populations of hydrocarbon-degrading microorganisms in sediment and water from Redfish Bay (17–19), we speculated that the mild climate, high inorganic and organic nutrient levels, and long history of exposure to high concentrations of petroleum hydrocarbons would favor these sites as possible sources of bacteria able to degrade higher-molecular-weight PAHs. In this paper, we report the isolation of a mycobacterium that readily degraded pyrene as well as other PAHs. Although pyrene has been shown to be degraded in soil (5), this study is the first report of a bacterial pure culture able to mineralize pyrene.

Pyrene is a highly symmetrical PAH containing four fused aromatic rings and is one of 16 PAHs included in the list of 129 priority pollutants compiled by the U.S. Environmental Protection Agency (28). This PAH, encountered regularly in environmental samples (21), is not genotoxic but has a chemical structure found in several carcinogenic PAHs such as benzo[*a*]pyrene, indeno-(1,2,3-*cd*)-pyrene, and 1-nitropyrene, and it has been used as a model compound in PAH metabolism studies (8, 25).

Pyrene mineralization rates in all three sample sites located near the Harbor Island oil tank farm were significantly higher than those we previously reported for Redfish Bay (17). Although total amounts of pyrene mineralization in microcosms from sites A and C were similar after 8 weeks, the initial rates of pyrene mineralization observed in microcosms from all three sample sites during the first 3 weeks of the experiment were inversely proportional to the distance from the point source of aromatic hydrocarbon contamination. These results support the hypothesis that a chemical concentration gradient exists below point sources of contamination, and optimal microbial adaptation may occur at a point on this gradient where chemical concentrations are subtoxic but are still above some threshold level necessary for enzyme induction and possible utilization as sources of carbon and energy. Several previous attempts in our laboratory with similar enrichment methods to isolate a pyrene-degrading bacterium from Redfish Bay sediments were not successful. The pyrene-degrading bacterium in this study was isolated from site A, closest to the Harbor Island oil tank farm.

The pyrene-degrading bacterium isolated in this study was identified as a *Mycobacterium* sp. on the basis of its cellular and colony morphology, gram-positive and strong acid-fast staining, mycolic acids, diagnostic biochemical reactions, and nucleic acid contents. *Mycobacterium* spp. are common in the environment (2) and have been reported to degrade *n*-butane and 2-butanone (40), several gaseous unsaturated hydrocarbons (13), cycloparaffinic hydrocarbons (4), *n*-alkyl-substituted cycloparaffins (3), and methyl ketone (32). The oxidation of aliphatic hydrocarbons and other hydrocarbons by *Mycobacterium* spp. has been reviewed by Hou (22).

Inducible enzymes appear to be responsible for pyrene catabolism in this *Mycobacterium* sp. since lag phases in pyrene mineralization were observed in cultures grown in the absence of pyrene and no pyrene mineralization was observed in noninduced cultures dosed with chloramphenicol at time zero. Inducible pyrene-degrading enzymes in this mycobacterium may be either chromosome or plasmid mediated. Crawford and Bates (11) have isolated plasmid DNA from several strains of *Mycobacterium avium-M. intracellulare*. The isolation and identification of a pyrene-degrading plasmid in this *Mycobacterium* sp. may have practical application for future cloning studies to construct genetically engineered bacteria able to degrade higher-molecular-weight PAHs and warrants further investigation. The inducibility of pyrene-degrading enzymes in this mycobacterium is consistent with the observed inducibility of hydrocarbon-degrading enzymes encoded on plasmids containing both structural and regulatory genes in other bacteria (30, 45).

There is a paucity of information on the microbial degradation of pyrene. The chemical pathway for the degradation of pyrene by this *Mycobacterium* sp. is reported in the accompanying paper (20). Since PAHs are widespread environmental contaminants and occur at relatively high concentrations in some freshwater and marine sediments (24), there is interest in the use of PAH-degrading microorganisms for the bioremediation of PAHs in the environment. The *Mycobacterium* sp. reported in this study readily degraded pyrene in pure culture under optimal conditions. The survival, performance, and overall ability of this *Mycobacterium* sp. to enhance the degradation of pyrene or other higher-molecular-weight PAHs in natural ecosystems warrant further investigation.

ACKNOWLEDGMENTS

We thank Warren Pulich for assistance in the collection of environmental samples, Jerome Perry for G+C analyses of DNA, Shirley Hunter for mycolic acid standards, James P. Freeman for mass spectral analyses of mycolic acids, Annette Andrews for electron micrographs of the *Mycobacterium* sp., and Rebecca Robertson for providing excellent technical assistance in the laboratory.

LITERATURE CITED

1. **Apajalahti, J. H. A., P. Karpanoja, and M. S. Salkinoja-Salonen.** 1986. *Rhodococcus chlorophenolicus* sp. nov., a chlorophenol-mineralizing actinomycete. Int. J. Syst. Bacteriol. **36**:246–251.
2. **Barksdale, L., and K. Kim.** 1977. Mycobacterium. Bacteriol. Rev. **41**:217–327.
3. **Beam, H. W., and J. J. Perry.** 1974. Microbial degradation and assimilation of *n*-alkyl-substituted cycloparaffins. J. Bacteriol. **118**:394–399.
4. **Beam, H. W., and J. J. Perry.** 1974. Microbial degradation of cycloparaffinic hydrocarbons via co-metabolism and commensalism. J. Gen. Microbiol. **82**:163–169.
5. **Bossert, I. D., and R. Bartha.** 1986. Structure-biodegradability relationships of polycyclic aromatic hydrocarbons in soil. Bull. Environ. Contam. Toxicol. **37**:490–495.
6. **Cerniglia, C. E.** 1984. Microbial metabolism of polycyclic aromatic hydrocarbons. Adv. Appl. Microbiol. **30**:31–71.
7. **Cerniglia, C. E.** 1984. Microbial transformation of aromatic hydrocarbons, p. 100–128. *In* R. Atlas (ed.), Petroleum microbiology. Macmillian Publishing Co., New York.
8. **Chen, F. M.** 1983. Binding of pyrene to DNA, base sequence specificity and its implications. Nucleic Acids Res. **11**:7231–7250.
9. **Cole, R. M., and T. J. Popkin.** 1981. Electron microscopy, p. 34–51. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), Manual of methods for general microbiology. American Society for Microbiology, Washington, D.C.
10. **Collins, M. D., M. Goodfellow, and D. E. Minnikin.** 1982. A survey of the structures of mycolic acids in *Corynebacterium* and related taxa. J. Gen. Microbiol. **128**:129–149.
11. **Crawford, J. T., and J. H. Bates.** 1979. Isolation of plasmids from *Mycobacteria*. Infect. Immun. **24**:979–981.
12. **Darville, R. G., and J. L. Wilhm.** 1984. The effect of naphthalene on oxygen consumption and hemoglobin concentration in *Chironomus attenuatus* and on oxygen consumption and life cycle of *Tanytarus dissimilis*. Environ. Toxicol. Chem. **3**:135–

141.
13. **DeBont, J. A. M., S. B. Primrose, M. D. Collins, and D. Jones.** 1980. Chemical studies on some bacteria which utilize gaseous unsaturated hydrocarbons. J. Gen. Microbiol. **117**:97–102.
14. **Gibson, D. T., V. Mahadevan, D. M. Jerina, H. Yagi, and H. J. C. Yeh.** 1975. Oxidation of the carcinogens benzo[a]pyrene and benz[a]anthracene to dihydrodiols by a bacterium. Science **189**:295–297.
15. **Gibson, D. T., and V. Subramanian.** 1984. Microbial degradation of aromatic hydrocarbons, p. 181–252. *In* D. T. Gibson (ed.), Microbial degradation of organic compounds. Marcel Dekker, New York.
16. **Greenawalt, J. W., and T. L. Whiteside.** 1975. Mesosomes: membranous bacterial organelles. Bacteriol. Rev. **39**:405–463.
17. **Heitkamp, M. A., and C. E. Cerniglia.** 1987. Effects of chemical structure and exposure on the microbial degradation of polycyclic aromatic hydrocarbons in freshwater and estuarine ecosystems. Environ. Toxicol. Chem. **6**:535–546.
18. **Heitkamp, M. A., and C. E. Cerniglia.** 1988. Mineralization of polycyclic aromatic hydrocarbons by a bacterium isolated from sediment below an oil field. Appl. Environ. Microbiol. **54**:1612–1614.
19. **Heitkamp, M. A., J. P. Freeman, and C. E. Cerniglia.** 1987. Naphthalene biodegradation in environmental microcosms: estimates of degradation rates and characterization of metabolites. Appl. Environ. Microbiol. **53**:129–136.
20. **Heitkamp, M. A., J. P. Freeman, D. W. Miller, and C. E. Cerniglia.** Pyrene degradation by a *Mycobacterium* sp.: identification of ring oxidation and ring fission products. Appl. Environ. Microbiol. **54**:2556–2565.
21. **Hites, R. A., R. E. Laflamme, and J. G. Windsor.** 1980. Polycyclic aromatic hydrocarbons in marine/aquatic sediments: their ubiquity, p. 289–311. *In* L. Petrakis and F. T. Weiss (ed.), Petroleum in the marine environment. Advances in chemistry series. American Chemical Society, Washington, D.C.
22. **Hou, C. T.** 1982. Microbial transformation of important industrial hydrocarbons, p. 81–107. *In* J. P. Rosazza (ed.), Microbial transformations of bioactive compounds, vol. 1. CRC Press, Boca Raton, Fla.
23. **Huckins, J. N., J. D. Petty, and M. A. Heitkamp.** 1984. Modular containers for microcosm and process model studies on the fate and effects of aquatic contaminants. Chemosphere **13**:1329–1341.
24. **International Agency for Research on Cancer.** 1983. Polynuclear Aromatic Compounds. Part 1, chemical, environmental and experimental data, p. 95–451. *In* IARC Monographs on the evaluation of the carcinogenic risk of chemicals to humans. World Health Organization, Lyon, France.
25. **Jacob, J., G. Grimmer, G. Raab, and A. Schmoldt.** 1982. The metabolism of pyrene by rat liver microsomes and the influence of various mono-oxygenase inducers. Xenobiotica **12**:45–53.
26. **Jacob, J., W. Karcher, J. J. Belliado, and P. J. Wagstaffe.** 1986. Polycyclic aromatic compounds of environmental and occupational importance. Fresenius Z. Anal. Chem. **323**:1–10.
27. **Johnson, A. C., and D. Larsen.** 1985. The distribution of polycyclic aromatic hydrocarbons in the surficial sediments of Penobscot Bay (Maine, USA) in relation to possible sources and to other sites worldwide. Mar. Environ. Res. **15**:1–16.
28. **Keith, L. H., and W. A. Telliard.** 1979. Priority pollutants I—a perspective view. Environ. Sci. Technol. **13**:416–423.
29. **Kiyohara, H., K. Nagao, and K. Yana.** 1982. Rapid screen for bacteria degrading water-insoluble, solid hydrocarbons on agar plates. Appl. Environ. Microbiol. **43**:454–457.
30. **Kiyohara, H., M. Sugiyama, F. J. Mondello, D. T. Gibson, and K. Yano.** 1983. Plasmid involvement in the degradation of polycyclic aromatic hydrocarbons by a *Beijerinckia* species. Biochem. Biophys. Res. Commun. **111**:939–945.
31. **Kubica, G. P., and H. L. David.** 1980. The mycobacteria, p. 1693–1730. *In* A. C. Sonnenwirth and L. Jarett (ed.), Gradwohl's clinical laboratory methods and diagnosis, vol. 2. The C. V. Mosby Co., St. Louis, Mo.
32. **Lukins, H. B., and J. W. Foster.** 1963. Methyl ketone metabolism in hydrocarbon-utilizing *Mycobacteria*. J. Bacteriol. **85**:1074–1087.
33. **Mackay, D., and W. Y. Shiu.** 1977. Aqueous solubility of polynuclear aromatic hydrocarbons. J. Chem. Eng. Data **22**:399–402.
34. **Means, J. C., S. G. Ward, J. J. Hassett, and W. L. Banwart.** 1980. Sorption of polynuclear aromatic hydrocarbons by sediments and soils. Environ. Sci. Technol. **14**:1524–1528.
35. **Minnikin, D. E., L. Alshamaony, and M. Goodfellow.** 1975. Differentiation of *Mycobacterium*, *Nocardia*, and related taxa by thin-layer chromatographic analysis of whole-organism methanolysates. J. Gen. Microbiol. **88**:200–204.
36. **Mix, M. C.** 1986. Cancerous diseases in aquatic animals and their association with environmental pollutants: a critical literature review. Mar. Environ. Res. **20**:1–141.
37. **Morehead, N. R., B. J. Eadie, B. Lake, P. D. Landrum, and D. Berner.** 1986. The sorption of PAH onto dissolved organic matter in Lake Michigan waters. Chemosphere **15**:403–412.
38. **Mortelmans, K., S. Haworth, T. Lawlor, W. Speck, B. Tainer, and E. Zeiger.** 1986. Salmonella mutagenicity tests. II. Results from the testing of 270 chemicals. Environ. Mutagen. **8**(Suppl. 7):1–119.
39. **National Academy of Sciences.** 1983. Polycyclic aromatic hydrocarbons: evaluation of sources and effects. National Academy Press, Washington, D.C.
40. **Phillips, W. E., and J. J. Perry.** 1974. Metabolism of *n*-butane and 2-butanone by *Mycobacterium vaccae*. J. Bacteriol. **120**:987–989.
41. **Sato, T., T. Momma, Y. Ose, T. Ishikawa, and K. Kato.** 1983. Mutagenicity of Nagara River sediment. Mutat. Res. **118**:257–267.
42. **Shinohara, C., K. Fukushi, and I. Suzuki.** 1957. Mitochondrium-like structures in ultrathin structures of *Mycobacterium avium*. J. Bacteriol. **74**:413–415.
43. **Skerman, V. B. D.** 1967. A guide to the identification of the genera of bacteria, 2nd ed. The Williams & Wilkins Co., Baltimore.
44. **Struble, V. G., and H. J. Harmon.** 1983. Molecular basis for inhibition of mitochondrial respiration by naphthalene. Bull. Environ. Contam. Toxicol. **31**:644–648.
45. **Williams, P. A.** 1981. Genetics of biodegradation, p. 97–130. *In* T. Leisinger, R. Hutter, A. M. Cook, and J. Nuesch (ed.), Microbial degradation of xenobiotics and recalcitrant compounds. Academic Press, Inc., New York.