Metabolism of Phenanthrene by the Marine Cyanobacterium *Agmenellum quadruplicatum* PR-6

MARTHA L. NARRO,^{1†} CARL E. CERNIGLIA,^{2*} CHASE VAN BAALEN,^{1‡} AND DAVID T. GIBSON³

National Center for Toxicological Research, Food and Drug Administration, Jefferson, Arkansas 72079²;
Port Aransas Marine Laboratory, University of Texas Marine Science Institute, Port Aransas,
Texas 78373¹; and Department of Microbiology, University of Iowa, Iowa City, Iowa 52242³

Received 21 November 1991/Accepted 27 January 1992

Under photoautotrophic growth conditions, the marine cyanobacterium *Agmenellum quadruplicatum* PR-6 metabolized phenanthrene to form *trans*-9,10-dihydroxy-9,10-dihydrophenanthrene (phenanthrene *trans*-9,10-dihydrodiol) and 1-methoxyphenanthrene as the major ethyl acetate-extractable metabolites. Small amounts of phenanthrols were also formed. The metabolites were purified by high-pressure liquid chromatography and identified from their UV, infrared, mass, and proton magnetic resonance spectral properties. *A. quadruplicatum* PR-6 formed phenanthrene *trans*-9,10-dihydrodiol with a 22% enantiomeric excess of the (–)-9S,10S-enantiomer. Incorporation experiments with ¹⁸O₂ showed that one atom of oxygen from O₂ was incorporated into the dihydrodiol. Toxicity studies, using an algal lawn bioassay, indicated that 9-phenanthrol and 9,10-phenanthrenequinone inhibit the growth of *A. quadruplicatum* PR-6.

Polycyclic aromatic hydrocarbons are a large class of ubiquitous environmental pollutants that exhibit toxic, mutagenic, or carcinogenic properties (4, 20, 24, 27, 36); consequently, there is considerable interest in the microbial metabolism and detoxification of this group of chemicals. Bacteria and fungi are known to play important roles in the degradation of polycyclic aromatic hydrocarbons in the environment (13, 23). However, the role of cyanobacteria (blue-green algae) has not been thoroughly investigated despite their wide distribution in aquatic ecosystems (50). Marine cyanobacteria oxidize naphthalene, biphenyl, and 1- and 2-methylnaphthalene to form 1-naphthol, 4-hydroxybiphenyl, and 1- and 2-hydroxymethylnaphthalene, respectively, as the major metabolites (10–12, 14, 15). These metabolites are similar to those formed by mammals and fungi (13, 43).

Phenanthrene, a tricyclic aromatic hydrocarbon, is widely distributed in the environment as a result of pyrolytic processes (4). Although phenanthrene is not mutagenic or carcinogenic, it has been shown to be toxic to aquatic organisms (3, 24, 30, 37, 38, 41). Interestingly, since it is the smallest aromatic hydrocarbon to have a “bay-region” and a “K-region,” phenanthrene is often used as a model substrate for studies on the metabolism of carcinogenic polycyclic aromatic hydrocarbons (7, 36).

The bacterial degradation of phenanthrene has frequently been reported over the last 60 years (19, 21, 23, 26, 28, 42, 47). The initial attack proceeds via the action of a dioxygenase to form *cis*-3,4-dihydroxy-3,4-dihydrophenanthrene (phenanthrene *cis*-3,4-dihydrodiol). The resultant dihydrodiol is then converted to 3,4-dihydroxyphenanthrene by dehydrogenases. The diphenol is then ring cleaved to form 1-hydroxy-2-naphthoic acid, which can be subsequently degraded via the naphthalene pathway to catechol or via *o*-phthalic acid to protocatechuic acid, depending upon the bacterial species (19, 21, 28). A *Beijerinckia* sp. also forms a

small amount of phenanthrene *cis*-1,2-dihydrodiol (26). The actinomycete *Streptomyces flavovirens* oxidizes phenanthrene at the K-region to form phenanthrene *trans*-9,10-dihydrodiol as the major metabolite (45). The metabolism of phenanthrene by *S. flavovirens* is similar to that reported for mammalian and fungal enzyme systems (9, 16, 17, 32, 35, 44, 46). Fungi utilize monooxygenases and epoxide hydrolases to catalyze the oxidation of phenanthrene to *trans*-1,2-, 3,4-, and 9,10-dihydrodiols (9, 16, 46). Glucoside conjugates of phenanthrols have also been identified as major metabolites of fungi (9, 46). An alternative mode of enzymatic attack reported for white-rot fungi is via lignin peroxidase and yields CO₂ and water-soluble metabolites (8, 33).

We now report the oxidation of phenanthrene by the marine cyanobacterium *Agmenellum quadruplicatum* PR-6 and describe the isolation and identification of the metabolites that were formed.

MATERIALS AND METHODS

Organisms and growth conditions. Stock cultures of the marine unicellular cyanobacterium *A. quadruplicatum* PR-6 were maintained in the light on slants of modified ASP-2 medium containing 8 µg of vitamin B₁₂ liter⁻¹ (39, 50). Cultures were grown photoautotrophically at 30°C in Pyrex test tubes (175 by 22.5 mm) containing 20 ml of medium. Air containing 1.0% CO₂ was bubbled continuously through the cultures, and illumination was provided by two fluorescent lamps (F20T12-D) placed 8.5 cm from the tubes. Cells for large-scale biotransformations were grown in test tubes (40 by 8 cm) containing 250 ml of medium. Illumination was provided by two fluorescent lamps. Four lamps were used as the cultures became more dense.

Metabolic experiments. All transformation experiments were conducted under photoautotrophic growth conditions. In studies on the metabolism of [9-¹⁴C]phenanthrene, *A. quadruplicatum* PR-6 was grown to a density of approximately 0.3 mg ml⁻¹ (dry weight) and 30 ml of the culture was transferred to a closed growth system in a 500-ml Nephalo flask (12, 14, 15). The flask was clamped to an oscillating bar and incubated in a 30°C water bath. Cultures were illumi-

* Corresponding author.

† Present address: Department of Biochemistry, University of Arizona, Tucson, AZ 85721.

‡ Deceased 20 January 1986.

nated by two fluorescent lamps (Westinghouse F48T-12/CWX/HO) placed 29 cm below the flasks. A yellow Plexiglas filter (Rohm and Haas no. 2422; 3.18 mm thick) with no transmission below 530 nm was placed between the cultures and the light source to prevent any direct photochemical effects on the substrate or metabolites. Cells were incubated for 24 h in the presence of [^{14}C]phenanthrene (443 nmol in 20 μl of 95% ethanol; specific activity, 5.65 mCi mmol $^{-1}$). Carbon dioxide was introduced into the flask via a hole in the sidearm cap; the CO_2 concentration was maintained between 0.5 and 1.0% throughout the experiments. Control experiments with [^{14}C]phenanthrene were conducted in the absence of organisms and with boiled cultures.

Large-scale experiments were conducted in low-form culture flasks (Corning no. 4422) containing 1-liter cultures of *A. quadruplicatum* PR-6 (0.3 mg ml $^{-1}$ [dry weight]). Organisms were illuminated by four fluorescent lamps and incubated for 24 h in the presence of phenanthrene (56.1 μmol in 500 μl of 95% ethanol) as described above. After incubation, cells were removed by centrifugation and the supernatant was extracted with 3 volumes of ethyl acetate. The organic extract was dried over anhydrous Na_2SO_4 , and the solvent was removed in vacuo. Phenanthrene metabolites were analyzed by high-pressure liquid chromatography (HPLC) and quantitated by liquid scintillation counting.

$^{18}\text{O}_2$ experiments. The incorporation of $^{18}\text{O}_2$ into phenanthrene was determined by incubating a 100-ml culture of *A. quadruplicatum* PR-6 (0.3 mg ml $^{-1}$ [dry weight]) in a 500-ml closed flask containing $^{18}\text{O}_2$ in the headspace (14). The isotope composition of the oxygen in the atmosphere of the flask, as measured by mass spectrometry, was 25% ^{16}O - ^{16}O and 75% ^{18}O - ^{18}O at the beginning of the experiment. Cells were incubated for 12 h in the presence of phenanthrene (28.1 μmol) under the conditions described above. The phenanthrene *trans*-9,10-dihydrodiol formed was extracted with ethyl acetate and purified by HPLC, and its isotopic composition was determined by mass spectrometry. The isotopic abundance of the dihydrodiol was calculated from the relative intensities of the molecular ions at m/z 212 (two ^{16}O 's), 214 (one ^{16}O and one ^{18}O), and 216 (two ^{18}O 's). The values were corrected for the average $^{18}\text{O}_2$ content of the gas phase in the flask.

Enantiomeric purity of *trans*-9,10 dihydrodiol. The phenanthrene dihydrodiol formed by *A. quadruplicatum* PR-6 was dissolved in chloroform and analyzed by polarimetry to determine its optical activity. The enantiomeric purity of the metabolically formed dihydrodiol was determined as described by Thakker et al. (48). ^{14}C -labeled phenanthrene ($\pm$)-*trans*-9,10-dihydrodiol formed by *A. quadruplicatum* PR-6 (30-ml cultures, 0.3 mg ml $^{-1}$ [dry weight]) was derivatized with (–)-methoxytrifluoromethylphenylacetic acid [(–)-MTPA], and the resulting diastereomeric bis-MTPA diesters of the dihydrodiol were separated by HPLC. The enantiomeric purity of the metabolically formed phenanthrene *trans*-9,10-dihydrodiol was determined from the radioactivity in each diastereomer (48).

Analytical methods. UV absorption spectra were recorded on a Beckman model 25 recording spectrophotometer. Infrared spectra were obtained on a Nicolet model 7000 instrument operating in the Fourier transform mode. Proton magnetic resonance (^1H NMR) spectra were obtained on Nicolet NT-200, NT-360, and Bruker WM 500 spectrometers with tetramethylsilane as an internal standard. Low-resolution mass spectra were obtained on a DuPont 21-491 double-focusing electron impact spectrometer coupled to an Inco data system. Samples were introduced by using a direct inlet

FIG. 1. HPLC analysis of phenanthrene metabolites formed by *A. quadruplicatum* PR-6. Separation was achieved by using a Zorbax octyldecyl silane column with a programmed linear gradient of methanol-water (50 to 95% methanol, 60 min) at a flow rate of 1 ml min $^{-1}$. Metabolites were monitored at 254 nm, and 1-ml fractions were collected. (A) Radioactive metabolites; (B) phenanthrene metabolites formed in large-scale biotransformation experiments; (C) synthetic phenanthrene derivatives ([a] *trans*-9,10-dihydroxy-9,10-dihydrophenanthrene, [b] 9,10-phenanthrenequinone, [c] 9-phenanthrol, [d] phenanthrene, and [e] 9-methoxyphenanthrene).

probe with a source temperature of 220°C and an ionization beam of 70 eV. Polarimetry was performed on a Perkin-Elmer 241MC polarimeter. Radiolabeled samples were counted in 10 ml of Beckman Ready-Solv EP liquid scintillation cocktail in a Beckman model LS 6800 liquid scintillation counter.

HPLC was performed on a Beckman component system,

consisting of a model 112 solvent delivery system, a model 160 absorbance detector, a model 421 controller, an Altex model 210 injector, and a Spectra-Physics SP4270 integrator. The ethyl acetate-soluble phenanthrene metabolites were first fractionated on a DuPont Zorbax octyldecyl silane column (10 μ m, 6.2 mm by 25 cm) and eluted with a programmed linear methanol-water gradient (50 to 95% methanol, 60 min) at a flow rate of 1 ml min⁻¹. Dihydrodiols were purified by further chromatography on a Waters μ Bondapak CN column (10 μ m, 3.9 mm by 30 cm) with hexane-ethanol (97:3, vol/vol) at a flow rate of 1 ml min⁻¹ as the eluting solvent. Methoxyphenanthrenes were purified by further chromatography on a DuPont Zorbax octyldecyl silane column (10 μ m, 6.2 mm [inside diameter] by 25 cm) with methanol-water (80:20, vol/vol) at a flow rate of 2 ml min⁻¹ as the eluant. The MTPA esters of phenanthrene *trans*-9,10-dihydrodiol were separated by HPLC on a Spherisorb CN column (5 μ m, 4.6 mm by 25 cm) with hexane-dioxane (98.9:1.1, vol/vol; 1 ml min⁻¹) as the eluant.

Toxicity evaluation. The toxicity of phenanthrene and several phenanthrene derivatives to *A. quadruplicatum* PR-6 was tested by an algal lawn technique described previously (10). Approximately 10⁵ CFU was added to 20 ml of sterile ASP-2 medium containing 1% Bacto Agar at 42°C and then poured into sterile plastic petri dishes (15 by 100 mm). Test compounds were applied to water-washed cellulose disks (12.7-mm diameter) which were placed in the center of the petri dishes. Stock solutions of the compounds were made in 95% ethanol. Petri dishes were sealed with transparent tape and incubated under 60-W tungsten lamps at 30 to 34°C. The plates were examined visually and microscopically for colony size and pigmentation and measured for zones of inhibition.

Chemicals. [9-¹⁴C]phenanthrene (19.3 mCi mmol⁻¹, 95% radiochemical purity; Amersham Searle Corp., Arlington Heights, Ill.) was diluted with unlabeled phenanthrene (Aldrich Chemical Co., Milwaukee, Wis.) to a specific activity of 5.65 mCi mmol⁻¹ and purified by thin-layer chromatography prior to use in metabolic studies. 9-Phenanthrol and 9,10-phenanthrenequinone used as analytical standards were purchased from Aldrich Chemical Co. Phenanthrene *cis*-9,10-dihydrodiol was synthesized by oxidation of phenanthrene with osmium tetroxide in pyridine (22). Phenanthrene 9,10-oxide was synthesized by oxidation of phenanthrene with sodium hypochlorite, using tetrabutylammonium hydrogen sulfate as a phase transfer catalyst (29). Phenanthrene *trans*-9,10-dihydrodiol was prepared by hydration of phenanthrene 9,10-oxide with pig liver microsomal epoxide hydride (51). Pig liver microsomes were kindly provided by D. M. Ziegler of the University of Texas at Austin. Phenanthrene *cis*-3,4-dihydrodiol was prepared from phenanthrene by using *Beijerinckia* sp. strain B-836 as previously described (26). Phenanthrene *trans*-3,4-dihydrodiol was kindly provided by D. M. Jerina, National Institutes of Health, Bethesda, Md. 9-Methoxyphenanthrene was synthesized from 9-phenanthrol with diazomethane as the alkylating agent (22). (–)- α -Methoxy- α -trifluoromethylphenylacetic acid was purchased from Sigma Chemical Co., St. Louis, Mo. ¹⁸O₂ (99 atom%) was obtained from Prochem, Summit, N.J. All solvents were of glass-distilled grade.

RESULTS

Isolation and identification of metabolites. When *A. quadruplicatum* PR-6 was grown under photoautotrophic conditions in the presence of [¹⁴C]phenanthrene, two major ethyl

FIG. 2. HPLC analysis of phenanthrene dihydrodiol formed by *A. quadruplicatum* PR-6. Separation was achieved on a Bondapak CN column by using a hexane-ethanol (97:3, vol/vol) isocratic solvent system at a flow rate of 1 ml min⁻¹. Metabolites were monitored at 254 nm, and 1.0 ml fractions were collected. (A) Radiolabeled phenanthrene dihydrodiol formed by *A. quadruplicatum* PR-6; (B) phenanthrene dihydrodiol formed in large-scale biotransformations; (C) synthetic phenanthrene dihydrodiols ([a] *trans*-9,10-dihydroxy-9,10-dihydrophenanthrene, [b] *trans*-1,2-dihydroxy-1,2-dihydrophenanthrene, [c] *cis*-9,10-dihydroxy-9,10-dihydrophenanthrene, [d] *cis*-3,4-dihydroxy-3,4-dihydrophenanthrene, and [e] *trans*-3,4-dihydroxy-3,4-dihydrophenanthrene).

FIG. 3. NMR spectra of the phenanthrene dihydrodiol formed by *A. quadruplicatum* PR-6 (A), authentic *trans*-9,10-dihydroxy-9,10-dihydrophenanthrene (B), and authentic *cis*-9,10-dihydroxy-9,10-dihydrophenanthrene (C). Spectra were recorded on a Nicolet NT-200 spectrometer.

acetate-extractable products were detected (Fig. 1). The radioactive products coeluted with synthetic phenanthrene *trans*-9,10-dihydrodiol (retention time = 19.8 min) and 9-methoxyphenanthrene (retention time, 58 min) when analyzed by reversed-phase HPLC. In addition, small amounts of phenanthrols (retention time, 37 min) were formed. *A. quadruplicatum* PR-6 metabolized approximately 2.2% of the added [^{14}C]phenanthrene to organic-soluble products. The ratio of organic- to water-soluble products was 9:1. No products were detected in controls with boiled cultures or in the absence of cells.

Since the reversed-phase chromatographic system did not separate the isomers of phenanthrene dihydrodiols, a normal-phase system was utilized to separate the fractions containing radiolabeled metabolites that cochromatographed with phenanthrene dihydrodiol in the reversed-phase chromatography. Under normal-phase conditions, the radioac-

FIG. 4. Separation of MTPA diastereomers formed from phenanthrene *trans*-9,10-dihydrodiol. The dihydrodiol formed from [^{14}C]phenanthrene by *A. quadruplicatum* was derivatized with (-)-MTPA, and the resulting diastereomers were separated by HPLC on a Spherisorb CN column. Hexane-dioxane (98.9:1.1, vol/vol) at a flow rate of 1.0 ml/min was used as the eluting solvent. (A) Radioactivity in diastereomers formed from phenanthrene by *A. quadruplicatum* PR-6; (B) diastereomers detected by measuring the A_{254} ; (C) diastereomers formed from synthetic phenanthrene *trans*-9,10-dihydrodiol.

FIG. 5. Mass spectrum of the 1-methoxyphenanthrene formed by *A. quadruplicatum*.

tive metabolite had the same retention time (8.1 min) as authentic phenanthrene *trans*-9,10-dihydrodiol.

To obtain sufficient quantities of phenanthrene metabolites for chemical characterization, biotransformation experiments were conducted using 1-liter cultures of *A. quadruplicatum* PR-6. The organic extracts from 20 1-liter biotransformation experiments were pooled and analyzed by reversed-phase HPLC. Metabolites in fractions with the same retention time as synthetic phenanthrene *trans*-9,10-dihydrodiol (20 min) were pooled, concentrated, and analyzed by normal-phase chromatography to separate dihydrodiol isomers (Fig. 2). The phenanthrene dihydrodiol isolated from cultures of *A. quadruplicatum* PR-6 (1.48 mg of white crystals, UV maximum at 267 nm) had the same retention time (8.1 min) as authentic phenanthrene *trans*-9,10-dihydrodiol. The molecular weight of the dihydrodiol was confirmed by mass spectral analysis ($M^+ = m/z$ 212, with fragment ions at m/z 194 because of a loss of water, m/z 181 because of a loss of H_2O and CH , and m/z 165 because of a loss of H_2O and CHO). In addition, the infrared spectrum of the metabolite showed a strong, broad absorption band at 3,100 to 3,400 cm^{-1} , characteristic of hydroxyl group stretching, and was identical with the spectrum of synthetic phenanthrene *trans*-9,10-dihydrodiol.

The 200-MHz 1H NMR spectra of the dihydrodiol formed by *A. quadruplicatum* and synthetic phenanthrene *trans*-9,10-dihydrodiol were identical (Fig. 3A and B). Signals were observed at 4.62 s, 2H (s, H_9 , H_{10}); 7.37 s, 4H (m, H_2 , H_3 , H_6 , H_7); 7.72 s, 2H (d, H_1 , H_8); and 7.81 s, 2H (d, H_4 , H_5). Coupling constants were $J_{3,4} = J_{5,6} = 6.3$ Hz and $J_{1,2} = J_{7,8} = 6.5$ Hz. The vicinal coupling constant for the carbinol protons ($J_{9,10}$) was 10.0 Hz, as determined from the ^{13}C -satellite bands observed in a 360-MHz 1H NMR spectrum. The 1H NMR spectrum of phenanthrene *cis*-9,10-dihydrodiol (Fig. 3C) showed signals at 4.75 s, 2H (s, H_9 , H_{10}); 7.37 s, 4H (m, H_2 , H_3 , H_6 , H_7); 7.58 s, 2H (d, H_1 , H_8); and 7.85 s, 2H (d,

H_4 , H_5). Coupling constants were $J_{3,4} = J_{5,6} = 7.4$ Hz and $J_{1,2} = J_{7,8} = 6.7$ Hz.

The phenanthrene *trans*-9,10-dihydrodiol formed by *A. quadruplicatum* PR-6 was analyzed by polarimetry to determine whether it was optically active. While there was an excess of the (–)-enantiomer, it was not possible to accurately determine the magnitude of the rotation since only a small amount of the phenanthrene *trans*-9,10-dihydrodiol was available. Therefore, the enantiomeric purity of the phenanthrene *trans*-9,10-dihydrodiol formed by *A. quadruplicatum* PR-6 was determined by forming radiolabeled diastereomeric bis-MTPA diesters of the dihydrodiol. The radiolabeled MTPA esters were separated by normal-phase HPLC, and the radioactivity in each diastereomer was determined (Fig. 4). The (–)-MTPA-(+)-diol eluted at 23.7 min, whereas the (–)-MTPA-(–)-diol eluted at 26.8 min. The radioactivity in the two peaks indicated that *A. quadruplicatum* PR-6 formed phenanthrene *trans*-9,10-dihydrodiol with a 22% enantiomeric excess of the (–)-9*S*,10*S*-enantiomer.

The HPLC fractions that cochromatographed with 9-methoxyphenanthrene (retention time, 58 min) in the methanol-water gradient were pooled, concentrated, and purified by HPLC. An isocratic methanol-water solvent system was used in this experiment. The methoxyphenanthrene formed by *A. quadruplicatum* PR-6 was dissolved in methanol, and the UV absorption spectrum of the resulting solution had maxima at 212, 249, 292, and 304 nm. The molecular weight of the methoxyphenanthrene was confirmed by mass spectrometry ($M^+ = m/z$ 208, with fragment ions at m/z 193 because of a loss of CH_3 and m/z 165 because of a loss of CH_3 and CO) (Fig. 5). The metabolically formed methoxyphenanthrene was determined from the 1H NMR spectrum (500 MHz) and homonuclear decoupling experiments to be 1-methoxyphenanthrene. The proton assignments for 1-methoxyphenanthrene (acetone- d_6) are as fol-

FIG. 6. Mass spectrum of *trans*-9,10-dihydroxy-9,10-dihydrophenanthrene formed by *A. quadruplicatum* PR-6 incubated in the presence of $^{18}\text{O}_2$. The inset shows the spectrum of the dihydrodiol formed in the presence of air ($^{16}\text{O}_2$).

lows: 4.07 s, 3H (s, $-\text{OCH}_3$); 7.17 s, 1H (d, H_2); 7.65 s, 3H (m, H_3 , H_6 , H_7); 7.18 s, 1H (d, H_9); 7.98 s, 1H (d, H_8); 8.22 s, 1H (d, H_5). Observed coupling constants were $J_{2,3} = 7.7$ Hz, $J_{3,4} = 8.6$ Hz, $J_{5,6} = 8.6$, $J_{7,8} = 7.7$ Hz, and $J_{9,10} = 9.0$ Hz.

$^{18}\text{O}_2$ incorporation studies. The mass spectrum of phenanthrene *trans*-9,10-dihydrodiol formed by *A. quadruplicatum* PR-6 in the presence of $^{16}\text{O}_2$ revealed a parent ion peak (M^+) at m/z 212. During the $^{18}\text{O}_2$ incorporation experiment, the atmosphere of the flask contained 72, 75, and 64% $^{18}\text{O}_2$ at 0, 6, and 12 h, respectively. After correcting for an average $^{18}\text{O}_2$ content of 70% in the atmosphere, $84\% \pm 4\%$ of the phenanthrene *trans*-9,10-dihydrodiol formed by *A. quadruplicatum* PR-6 gave a parent ion at m/z 214 (Fig. 6). There was no ion at m/z 216, indicating that ^{18}O had not been incorporated into both hydroxyl groups.

Toxicity studies. The growth of *A. quadruplicatum* PR-6 was inhibited by 9-hydroxyphenanthrene and 9,10-phenanthrenequinone, with the former compound being slightly more toxic than the latter (Table 1). 1-Methoxyphenanthrene is not commercially available and was not tested for toxicity. However, 9-methoxyphenanthrene was slightly toxic at the highest concentration tested. Phenanthrene and phenanthrene *trans*-9,10-dihydrodiol did not inhibit growth under the test conditions. Aniline is included in the table for comparative purposes, since it is known to be extremely toxic to cyanobacteria (2, 11).

DISCUSSION

The marine cyanobacterium *A. quadruplicatum* PR-6 metabolized phenanthrene to form phenanthrene *trans*-9,10-dihydrodiol and 1-methoxyphenanthrene as the major metabolites. The dihydrodiol was identified as the *trans*-9,10-isomer on the basis of its UV, infrared, mass, and ^1H NMR spectral properties, which were identical with those of synthetic phenanthrene *trans*-9,10-dihydrodiol (18). The vic-

TABLE 1. Effect of phenanthrene, phenanthrene derivatives, and aniline on the growth of *A. quadruplicatum* PR-6

Compound	Growth inhibition (mm) ^a at:		
	10 $\mu\text{g}/\text{disk}$	100 $\mu\text{g}/\text{disk}$	500 $\mu\text{g}/\text{disk}$
Phenanthrene	0	0	0
9-Phenanthrol	12	30	33
9,10-Phenanthrenequinone	12	24	30
9-Methoxyphenanthrene	0	0	1
Phenanthrene <i>trans</i> -9,10-dihydrodiol	0	0	0
Aniline ^b	35	35	NT ^c

^a The algal lawn technique was described in Materials and Methods. Numbers represent the zone of growth inhibition in millimeters from the edge of the disk: 0 denotes no inhibition, and 36 denotes complete inhibition.

^b Data from reference 2.

^c NT, not tested.

inal coupling constant for the carbinol hydrogens ($J_{9,10}$) was 10.0 Hz, as determined from the ^{13}C -satellites of the 360-MHz ^1H NMR spectrum. This value is consistent with the value reported by Cobb et al. (18). Since 9,10-phenanthrene dihydrodiols (in acetone) exist mainly in the conformation in which the hydroxyl groups are quasi-equatorial, to maximize intramolecular hydrogen bonding, a vicinal coupling constant of 10.0 Hz for $J_{9,10}$ (26) is consistent with the *trans*-dihydrodiol in which the dihedral angle between the carbinol hydrogens is approximately 180° . In the *cis* isomer, a coupling constant ($J_{9,10}$) of 4.0 Hz (26) or less would be expected, since the dihedral angle between the carbinol hydrogens is 60° . In addition, each hydroxyl group is anti-periplanar to the adjacent carbinol hydrogen, which is the optimal geometry for the reduction of the coupling constant ($J_{9,10}$) due to the electronegative hydroxyl group (18, 26).

In the presence of $^{18}\text{O}_2$, only one atom of ^{18}O was incorporated into the phenanthrene *trans*-9,10-dihydrodiol formed by *A. quadruplicatum* PR-6. This result indicates that the oxygenation of the aromatic ring is catalyzed by a monooxygenase rather than by a dioxygenase. In addition, the dihydrodiol was formed with a 22% enantiomeric excess of the (-)-9*S*,10*S*-enantiomer. Although the optical purity of the dihydrodiol formed by *A. quadruplicatum* PR-6 was relatively low, it is comparable to optical purity values reported from mammalian systems (1). Thakker et al. (48) reported that phenobarbital-induced rat liver microsomes formed phenanthrene *trans*-9,10-dihydrodiol with an optical purity of 26% when racemic phenanthrene 9,10-oxide had an optical purity of 60 to 70%. In both cases the (-)-9*S*,10*S*-enantiomer was in excess. Nordqvist et al. (34) reported formation of phenanthrene *trans*-9,10-dihydrodiol from phenanthrene with a 16% excess of the (-)-9*S*,10*S*-enantiomer. Recently, Sutherland et al. (45) reported that *S. flavovirens* forms phenanthrene *trans*-9,10-dihydrodiol with the (-)-9*S*,10*S* absolute configuration.

A. quadruplicatum PR-6 must possess enzymes that catalyze the enzymatic hydration of phenanthrene 9,10-oxide, since it forms optically active phenanthrene *trans*-9,10-dihydrodiol as a metabolite of phenanthrene. However, the possibility of nonenzymatic hydration of metabolically formed phenanthrene 9,10-oxide cannot be ruled out (6). The formation of an optically active *trans*-9,10-dihydrodiol in which only one atom of oxygen originates from O_2 strongly indicates that *A. quadruplicatum* PR-6 oxidizes phenanthrene to phenanthrene *trans*-9,10-dihydrodiol via a monooxygenase-epoxide hydrolase-catalyzed reaction (31, 40).

The metabolic formation of 1-methoxyphenanthrene from phenanthrene has not been reported previously. Daly et al. (20) reported that catechols may be methylated by catechol-*O*-methyltransferase in mammalian systems. The formation of 1-methoxyphenanthrene by *A. quadruplicatum* PR-6 may serve to detoxify any 1-phenanthrol that is formed. 1-Phenanthrol is probably toxic, since the hydroxylated aromatic compounds 9-phenanthrol, 1-naphthol, and 2-naphthol are known to be toxic to this organism (10). The algal lawn bioassay (Table 1) indicated that the phenolic and quinone derivatives of phenanthrene were more toxic than 9-methoxyphenanthrene.

The metabolism of phenanthrene by the marine cyanobacterium *A. quadruplicatum* PR-6 shows more similarities to the detoxification reactions catalyzed by mammalian liver microsomes (5, 25, 36) than to the catabolic reactions catalyzed by bacteria that utilize phenanthrene as a source of carbon and energy (13, 23). Further studies are necessary to

FIG. 7. Postulated pathways for phenanthrene metabolism by *A. quadruplicatum* PR-6. Brackets indicate hypothetical intermediates that have not been isolated.

characterize the enzymes involved in aromatic hydrocarbon oxidation by *A. quadruplicatum* PR-6.

The pathways postulated for the oxidation of phenanthrene by *A. quadruplicatum* PR-6 are shown in Fig. 7. A monooxygenase-catalyzed reaction could form phenanthrene 9,10- and 1,2-oxides. The 9,10-oxide is relatively stable and could undergo enzymatic hydration to yield optically active phenanthrene *trans*-9,10-dihydrodiol. In contrast, phenanthrene 1,2-oxide is unstable in solution and would undergo rapid isomerization to form mainly 1-phenanthrol (6). The latter compound could then serve as the substrate for a methyl transferase to yield 1-methoxyphenanthrene. Alternatively, 1-phenanthrol could be formed by a direct hydroxylation of the aromatic ring (49). The ability of marine cyanobacteria to oxidize aromatic hydrocarbons indicates that these organisms may play a role in determining the fate of aromatic pollutants in the marine environment.

ACKNOWLEDGMENT

This work was supported in part by grant GM29909 from the National Institute of General Medical Sciences to D.T.G.

REFERENCES

- Balani, S. K., P. J. Van Bladeren, N. Shirai, and D. M. Jerina. 1986. Resolution and absolute configuration of K-region *trans*-dihydrodiols from polycyclic aromatic hydrocarbons. *J. Org. Chem.* 51:1773-1778.
- Batterton, J., K. Winters, and C. Van Baalen. 1978. Anilines:

- selective toxicity to blue-green algae. *Science* **199**:1068–1070.
3. Black, J. A., W. J. Birge, A. G. Westerman, and P. C. Francis. 1983. Comparative aquatic toxicology of aromatic hydrocarbons. *Fundam. Appl. Toxicol.* **3**:353–358.
 4. Blumer, M. 1976. Polycyclic aromatic hydrocarbons in nature. *Sci. Am.* **234**:34–44.
 5. Boyland, E., and G. Wolf. 1950. Metabolism of polycyclic compounds. 6. Conversion of phenanthrene into dihydroxydihydrophenanthrenes. *Biochem. J.* **47**:64–69.
 6. Bruice, P. Y., T. C. Bruice, P. M. Dansette, H. G. Selander, H. Yagi, and D. M. Jerina. 1976. Comparison of the mechanisms of solvolysis and rearrangement of K-region vs. non-K-region arene oxides of phenanthrene. Comparative solvolytic rate constants of K-region and non-K-region arene oxides. *J. Am. Chem. Soc.* **98**:2965–2973.
 7. Bucker, M., H. R. Glatt, K. L. Platt, D. Avnir, Y. Ittah, J. Blum, and F. Oesch. 1979. Mutagenicity of phenanthrene and phenanthrene K-region derivatives. *Mutat. Res.* **66**:337–348.
 8. Bumpus, J. A. 1989. Biodegradation of polycyclic aromatic hydrocarbons by *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **55**:154–158.
 9. Cerniglia, C. E., W. L. Campbell, J. P. Freeman, and F. E. Evans. 1989. Identification of a novel metabolite in phenanthrene metabolism by the fungus *Cunninghamella elegans*. *Appl. Environ. Microbiol.* **55**:2275–2279.
 10. Cerniglia, C. E., J. P. Freeman, J. R. Althaus, and C. Van Baalen. 1983. Metabolism and toxicity of 1- and 2-methylnaphthalene and their derivatives in cyanobacteria. *Arch. Microbiol.* **136**:177–183.
 11. Cerniglia, C. E., J. P. Freeman, and C. Van Baalen. 1981. Biotransformation and toxicity of aniline and aniline derivatives in cyanobacteria. *Arch. Microbiol.* **130**:272–275.
 12. Cerniglia, C. E., D. T. Gibson, and C. Van Baalen. 1979. Algal oxidation of aromatic hydrocarbons: formation of 1-naphthol from naphthalene by *Agmenellum quadruplicatum* strain PR-6. *Biochem. Biophys. Res. Commun.* **88**:50–58.
 13. Cerniglia, C. E., and M. A. Heitkamp. 1989. Microbial degradation of polycyclic aromatic hydrocarbons (PAH) in the aquatic environment, p. 41–68. In U. Varanasi (ed.), *Metabolism of polycyclic aromatic hydrocarbons in the aquatic environment*. CRC Press, Inc., Boca Raton, Fla.
 14. Cerniglia, C. E., C. Van Baalen, and D. T. Gibson. 1980. Metabolism of naphthalene by the cyanobacterium *Oscillatoria* sp., strain JCM. *J. Gen. Microbiol.* **116**:485–494.
 15. Cerniglia, C. E., C. Van Baalen, and D. T. Gibson. 1980. Oxidation of biphenyl by the cyanobacterium *Oscillatoria* sp., strain JCM. *Arch. Microbiol.* **125**:203–207.
 16. Cerniglia, C. E., and S. K. Yang. 1984. Stereoselective metabolism of anthracene and phenanthrene by the fungus *Cunninghamella elegans*. *Appl. Environ. Microbiol.* **47**:119–124.
 17. Chaturapit, S., and G. M. Holder. 1978. Studies on the hepatic microsomal metabolism of [¹⁴C]phenanthrene. *Biochem. Pharmacol.* **27**:1865–1871.
 18. Cobb, D. I., D. A. Lewis, and R. N. Armstrong. 1983. Solvent dependence of the conformation and chiroptical properties of *trans*-9,10-dihydroxy-9,10-dihydrophenanthrene and its monoglucuronides. *J. Org. Chem.* **48**:4139–4141.
 19. Colla, C., A. Fiecchi, and V. Treccani. 1959. Ricerche sul metabolismo ossidativo microbico dell'antracene e del fenantrene. Nota II. Isolamento e caratterizzazione del 3,4-diidro-3,4-diossifenantrene. *Ann. Microbiol. Enzimol.* **9**:87–91.
 20. Daly, J. W., D. M. Jerina, and B. Witkop. 1972. Arene oxides and the NIH shift: the metabolism, toxicity and carcinogenicity of aromatic compounds. *Experientia* **28**:1129–1149.
 21. Evans, W. C., H. N. Fernley, and E. Griffiths. 1965. Oxidative metabolism of phenanthrene and anthracene by soil pseudomonads: the ring-fission mechanism. *Biochem. J.* **95**:819–831.
 22. Fieser, L. F., and M. Fieser. 1967. Reagents for organic synthesis, p. 191. John Wiley & Sons, Inc., New York.
 23. 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, Inc., New York.
 24. International Agency for Research on Cancer. 1983. Polynuclear aromatic compounds. Part 1. Chemical, environmental and experimental data, p. 286. In IARC monographs on the evaluation of carcinogenic risk of chemicals to man, vol. 32. International Agency for Research on Cancer, Lyon, France.
 25. Jakoby, W. B., and J. H. Keen. 1977. A triple threat in detoxification: the glutathione S-transferases. *Trends Biochem. Sci.* **2**:229–231.
 26. Jerina, D. M., H. Selander, H. Yagi, M. C. Wells, J. F. Davey, V. Mahadevan, and D. T. Gibson. 1976. Dihydrodiols from anthracene and phenanthrene. *J. Am. Chem. Soc.* **98**:5988–5996.
 27. Jerina, D. M., H. Ziffer, and J. W. Daly. 1970. The role of the arene oxide-oxepin system in the metabolism of aromatic substrates. IV. Stereochemical considerations of dihydrodiol formation and dehydrogenation. *J. Am. Chem. Soc.* **92**:1056–1061.
 28. Kiyohara, H., K. Nagao, and R. Nomi. 1976. Degradation of phenanthrene through o-phthalate by an *Aeromonas* sp. *Agric. Biol. Chem.* **40**:1075–1082.
 29. Krishnan, S., D. G. Kuhn, and G. A. Hamilton. 1977. Direct oxidation in high yield of some polycyclic aromatic compounds to arene oxides using hypochlorite and phase transfer catalysts. *J. Am. Chem. Soc.* **99**:8121–8123.
 30. Kusk, K. O. 1981. Comparison of the effects of aromatic hydrocarbons on a laboratory alga and natural phytoplankton. *Bot. Mar.* **24**:611–613.
 31. Lu, A. Y. H., and G. T. Miwa. 1980. Molecular properties and biological functions of microsomal epoxide hydrolase. *Annu. Rev. Pharmacol. Toxicol.* **20**:513–531.
 32. Miura, R., S. Honmaru, and M. Nakazaki. 1968. The absolute configurations of the metabolites of naphthalene and phenanthrene in mammalian systems. *Tetrahedron Lett.* **50**:5271–5274.
 33. Morgan, P., S. T. Lewis, and R. J. Watkinson. 1991. Comparison of abilities of white-rot fungi to mineralize selected xenobiotic compounds. *Appl. Microbiol. Biotechnol.* **34**:693–696.
 34. Nordqvist, O., D. R. Thakker, K. P. Vyas, H. Yagi, W. Levin, D. E. Ryan, P. E. Thomas, A. H. Conney, and D. M. Jerina. 1981. Metabolism of chrysene and phenanthrene to bay-region diol epoxides by rat liver enzymes. *Mol. Pharmacol.* **19**:168–178.
 35. Pandov, H., and P. Sims. 1970. The conversion of phenanthrene-9,10-oxide and dibenz[a,h]anthracene into dihydrodiols by rat liver microsomal enzyme. *Biochem. Pharmacol.* **19**:299–303.
 36. Pelkonen, O., and D. W. Nebert. 1982. Metabolism of polycyclic hydrocarbons: etiologic role in carcinogenesis. *Pharmacol. Rev.* **34**:189–222.
 37. Pipe, R. K., and M. N. Moore. 1986. An ultrastructural study on the effects of phenanthrene on lysosomal membranes and distribution of the lysosomal enzyme β -glucuronidase in digestive cells of the periwinkle *Littorina littorea*. *Aquat. Toxicol.* **8**:65–76.
 38. Pipe, R. K., and M. N. Moore. 1986. Arylsulphatase activity associated with phenanthrene induced digestive cell deletion in the marine mussel *Mytilus edulis*. *Histochem. J.* **18**:557–564.
 39. Provasoli, L., J. J. McLaughlin, and M. R. Droop. 1957. The development of artificial media for marine algae. *Arch. Microbiol.* **25**:392–428.
 40. Sato, R., and T. Omura. 1978. Cytochrome P-450, p. 16–18. Academic Press, Inc., New York.
 41. Savino, J. F., and L. L. Tanabe. 1989. Sublethal effects of phenanthrene, nicotine, and pinane on *Daphnia pulex*. *Bull. Environ. Contam. Toxicol.* **42**:778–784.
 42. Shiaris, M. P. 1989. Seasonal biotransformation of naphthalene, phenanthrene, and benzo[a]pyrene in surficial estuarine sediments. *Appl. Environ. Microbiol.* **55**:1391–1399.
 43. Smith, R. V., and J. P. Rosazza. 1974. Microbial models of mammalian metabolism. Aromatic hydroxylation. *Arch. Biochem. Biophys.* **161**:551–558.
 44. Solbakken, J. E., and K. H. Palmork. 1981. Metabolism of phenanthrene in various marine animals. *Comp. Biochem. Physiol.* **70C**:21–26.
 45. Sutherland, J. B., J. P. Freeman, A. L. Selby, P. P. Fu, D. W. Miller, and C. E. Cerniglia. 1990. Stereoselective formation of a

- K-region dihydrodiol from phenanthrene by *Streptomyces flavovirens*. Arch. Microbiol. **154**:260–266.
46. Sutherland, J. B., A. L. Selby, J. P. Freeman, F. E. Evans, and C. E. Cerniglia. 1991. Metabolism of phenanthrene by *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **57**:3310–3316.
47. Tausson, W. O. 1928. Die Oxidation des Phenanthrens durch Bakterien. Planta **5**:239–273.
48. Thakker, D. R., H. Yagi, W. Levin, A. Y. H. Lu, A. H. Conney, and D. M. Jerina. 1977. Stereospecificity of microsomal and purified epoxide hydase from rat liver. J. Biol. Chem. **252**: 6328–6334.
49. Tomaszewski, J. E., D. M. Jerina, and J. W. Daly. 1975. Deuterium isotope effects during formation of phenols by hepatic monooxygenases. Evidence for an alternative to the arene oxide pathway. Biochemistry **14**:2024–2031.
50. Van Baalen, C. 1962. Studies on marine blue-green algae. Bot. Mar. **4**:129–139.
51. Westkaemper, R. B., and R. P. Hanzlik. 1980. A convenient reversed-phase liquid chromatographic assay for epoxide hydase. Anal. Biochem. **102**:63–67.