Metabolism of anthracene by a *Rhodococcus* species

Deborah Dean-Ross a,*, Joanna D. Moody b, James P. Freeman c, Daniel R. Doerge d, Carl E. Cerniglia b

a Department of Biology, Indiana University–Purdue University, Fort Wayne, IN 46805, USA
b Division of Microbiology, National Center for Toxicological Research, U.S. Food and Drug Administration, Jefferson, AR 72079, USA
c Division of Chemistry, National Center for Toxicological Research, U.S. Food and Drug Administration, Jefferson, AR 72079, USA
d Division of Biochemical Toxicology, National Center for Toxicological Research, U.S. Food and Drug Administration, Jefferson, AR 72079, USA

Received 4 June 2001; received in revised form 20 August 2001; accepted 20 August 2001

First published online 28 September 2001

Abstract

A *Rhodococcus* sp. isolated from contaminated river sediment was investigated to determine if the isolate could degrade high molecular mass polycyclic aromatic hydrocarbons. The *Rhodococcus* sp. was able to utilize anthracene (53%), phenanthrene (31%), pyrene (13%), and fluoranthene (5%) as sole source of carbon and energy, but not naphthalene or chrysene. In a study of the degradation of anthracene by a *Rhodococcus* sp., the identification of ring-fission products indicated at least two ring-cleavage pathways. One results in the production of 6,7-benzocoumarin, previously shown to be produced chemically from the product of meta cleavage of 1,2-dihydroxyanthracene, a pathway which has been well established in Gram-negative bacteria. The second is an ortho cleavage of 1,2-dihydroxyanthracene that produces 3-(2-carboxyvinyl)naphthalene-2-carboxylic acid, a dicarboxylic acid ring-fission product. This represents a novel metabolic pathway only identified in Gram-positive bacteria. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are significant contaminants in environments receiving wastes from coal- and fuel-processing industries, as well as being products of pyrolysis of organic chemicals [1,2,3]. PAHs are of concern to human health because high molecular mass PAHs have been found to exert genotoxic and carcinogenic effects. Anthracene, a tricyclic PAH, is found in high amounts in PAH-contaminated environments [4–6]. It is not itself genotoxic or carcinogenic [7], but it does represent a threat to the environment due to its toxicity to aquatic life, particularly via photo-induced toxicity [8].

Bacterial degradation represents a significant pathway for the removal of PAHs from the environment. Considerable attention has focused on the metabolic pathways and genetics of degradation of low molecular mass PAHs, such as naphthalene, phenanthrene and anthracene, by Gram-negative bacteria, particularly of the genera *Pseudomonas* and *Sphingomonas* [1,9–11]. In these bacterial genera, the pathway of anthracene metabolism has been shown to proceed by an initial hydroxylation of the aromatic ring to form 1,2-dihydroxyanthracene, followed by cleavage of the ring in the meta position to yield 4-(2-hydroxynaphth-3-yl)-2-oxobut-3-enolic acid [12]. This compound is converted abiologically to 6,7-benzocoumarin or can be further metabolized to 2-hydroxy-3-naphthoic acid. Subsequent metabolism proceeds via a pathway analogous to that of naphthalene metabolism to yield salicylate and catechol [12].

Less attention has been devoted to the degradation of PAHs by Gram-positive bacteria. Members of the genus *Mycobacterium* have been frequently reported to be capable of growth on and utilization of pyrene [13–19]. Growth on anthracene has been demonstrated in *Mycobacterium* and *Rhodococcus* strains [20,21]. Details of metabolism have been reported in *Mycobacterium* [20,22]. A novel pathway for the degradation of naphthalene by a thermophilic species of *Bacillus* has recently been reported [23]. In the course of studies on the simultaneous metabolism of
anthracene and fluoranthene by a strain of *Rhodococcus*, evidence for two ring-fission pathways was obtained. This represents the first report of a dual pathway in the Nocardiiforms, a group of organisms of significance for the biodegradation of PAHs.

2. Materials and methods

2.1. Chemicals

Unlabelled PAHs were obtained from Aldrich Chemical Company (Milwaukee, WI, USA) and were greater than 98% pure. Several radiolabelled chemicals were obtained from Chemsyn Science Laboratories (Lenexa, KS, USA): [4,5,9,10,14C]pyrene (55 mCi/mmol); [9,10-14C]anthracene (58 mCi/mmol); [5,6,11,12-14C]chrysene (54.4 mCi/mmol); [3-14C]fluoranthene (50 mCi/mmol). The following PAHs were supplied by Sigma: [1-14C]naphthalene (10.3 mCi/mmol); and [9,14C]phenanthrene (13.1 mCi/mmol).

2.2. Organism and growth conditions

The bacterial strain was isolated by enrichment culture using anthracene as the sole source of carbon and energy. The source of inoculum was contaminated sediment from the Grand Calumet River (Gary, IN, USA). A mineral salts medium [24] was used for enrichment and subsequent growth experiments. Solid anthracene was added to the medium prior to autoclaving. After several transfers, the enrichment culture was plated on nutrient agar and sprayed with an acetone solution of anthracene to produce a solid film on the plate. Anthracene-utilizing cultures were identified by the presence of a clear zone around the colony, indicating utilization of the anthracene [25]. The culture was screened for the ability to mineralize PAHs by a previously described method [15]. Growth of the bacterial strain on anthracene was determined by measuring an increase in protein using the bicinchoninic acid method [26]. Concomitant utilization of anthracene was determined by gas chromatography analysis of culture extracts as described by Dean-Ross and Cerniglia [15].

2.3. Biodegradation of anthracene

While conducting studies on the simultaneous utilization of anthracene and fluoranthene by *Rhodococcus* sp., metabolites from anthracene were isolated and identified. For these studies, anthracene-grown cells were added to mineral salts medium to which was added a sufficient quantity of anthracene and fluoranthene in dimethyl sulfoxide (DMSO) to make a final concentration of 3 μg mℓ⁻¹ of each PAH. After 24 h growth, contents of the flask were extracted with three equal volumes of ethyl acetate, dried over anhydrous sodium sulfate, and concentrated under reduced pressure at 34°C using a Buchi 011 rotary evaporator (Brinkmann Instruments, Westbury, NY, USA). In order to isolate acidic metabolites, the aqueous fraction after extraction was acidified with concentrated HCl to pH 2 and extracted again with three equal volumes of ethyl acetate. The residues were dissolved in 3 ml methanol and concentrated using a model SS21 Savant Speed-Vac system (Savant Instruments, Holbrook, NY, USA) for analysis by reversed-phase high-performance liquid chromatography (HPLC).

2.4. Analytical methods

Anthracene and its metabolites were separated by HPLC using a Hewlett-Packard model 1050 pump system (Hewlett-Packard, Palo Alto, CA, USA) with a Hewlett-Packard photo-diode array model 1040A detector at 254 nm. The compounds were eluted using a linear gradient of 40 to 95% methanol/water over 40 min at 1 ml min⁻¹ with a 4.6 x 250 mm 5 μm C₁₈ Inertsil ODS-3 column (MetaChem Technologies, Torrance, CA, USA). Ultraviolet (UV) absorbance spectra were acquired on line.

For collection of sufficient quantities of metabolites to perform nuclear magnetic resonance (NMR) analysis, a Beckman model 100A dual-pump system equipped with a Beckman model 160 absorbance detector (Beckman Instruments, Inc., Fullerton, CA, USA), a Waters 486 tunable UV absorbance detector (Waters Corp., Milford, MA, USA), and a 10.0 x 250 mm 5 μm C₁₈ Inertsil ODS-3 column were used. The mobile phase was the same as above, but at a flow rate of 5 ml min⁻¹.

Liquid chromatography/mass spectrometry (LC/MS) analyses were performed using a Platform single quadrupole instrument (Micromass, Manchester, UK) equipped with an atmospheric pressure chemical ionization (APCI) interface. The total LC column effluent was delivered into the atmospheric pressure ion source through a heated nebulizer probe (450°C) using nitrogen as the probe and bath gas (275 l h⁻¹) with an ion source temperature of 150°C. Positive or negative ions were acquired in full scan mode (m/z 100–400, 1.0 s cycle time). At low cone voltage (15–20 V), the positive and negative ion mass spectra of the PAH metabolites consisted of predominantly the protonated and deprotonated molecules, respectively. When further fragmentation was required, a higher cone voltage was used (60 V). PAH metabolite sample extracts, dissolved in starting mobile phase and prepared as described above, were injected into the LC/MS system.

Electron ionization mass spectrometry (EI-MS) was performed using a Finnigan TSQ 700 triple quadrupole instrument (ThermoFinnigan, San Jose, CA, USA) equipped with a direct exposure probe (DEP). Isolated samples were dissolved in methanol and 1 μl of solution applied to the DEP wire. After evaporation of the solvent, the probe was inserted into the mass spectrometer and the DEP current was increased linearly at 5 mA/s. The ion source temper-
ature was 150°C and the electron energy was 170 V. The quadrupole was scanned from \( m/\Delta z \) 50–550 with a 0.5 s cycle time.

Proton (\(^1\text{H}\)) NMR spectra were obtained on a Bruker AM500 spectrometer (Bruker Instruments, Billerica, MA, USA). Each metabolite was dissolved in 0.5 ml deuterated DMSO or deuterated methanol (99.96 atom% \(^2\text{H}\)) for analysis. Chemical shifts are reported on the \( \delta \) scale (ppm) by assigning the residual solvent peak to 2.49 or 3.30 ppm, respectively. Typical data acquisition parameters were: data size, 32000; sweep width, 7042 Hz; filter width, 8900 Hz; acquisition time, 2.33 s; flip angle, 90°; relaxation delay, 0 s; temperature, 298.5 K. For the spectra recorded under quantitative conditions, a 10- to 2-s relaxation delay was used. For measurement of coupling constants, the free-induction decay was zero-filled to 64000, resulting in a final data point resolution of 0.215 Hz per point. Coupling constants reported are first order. Those that are non-first order and those of overlapping resonances are omitted. A proton-decoupled carbon (\(^1\text{H}\)) NMR spectrum was obtained at 125.77 MHz for one metabolite. The sample was dissolved in deuterated methanol with the methyl resonance assigned as 49.00 ppm. Assignments were made via homonuclear-decoupling experiments, nuclear Overhauser effect (NOE) experiments, integration, analysis of substituent effects, and comparison to spectra of authentic compounds.

3. Results and discussion

3.1. Isolation and characterization of the anthracene-degrading bacterial strain

Colonies that showed clearing of anthracene from solid media were isolated in pure culture and characterized. All anthracene-degrading colonies exhibited similar colony morphology, characterized by production of a yellow, water-insoluble pigment. The bacterial strain was identified as a Gram-positive organism based on its colony morphology, Gram reaction and fatty acid profile (Microbial ID Inc., Newark, DE, USA) as a \textit{Rhodococcus} sp. Neither the fatty acid profile nor results of physiological tests permitted identification to the species level. The results of the screening assay indicated that in addition to mineralizing anthracene (53%), this strain could also mineralize phenanthrene (31.0%), pyrene (13.6%), and fluoranthene (4.7%). Since this is a test designed to screen bacterial strains for relative ability to mineralize PAHs, the partial utilization observed in the case of pyrene and fluoranthene is considered positive for utilization, although at a slower rate than the utilization of anthracene and phenanthrene. It could not mineralize naphthalene or chrysene under conditions of the assay. Because of its ability to mineralize several three- and four-ringed PAHs, \textit{Rhodococcus} sp. was selected for additional study. Growth and utilization of

---

![Graph](https://academic.oup.com/femsle/article-abstract/204/1/205/632518)
anthracene are shown in Fig. 1. The culture gave a generation time of 28.8 h, and utilized anthracene at a rate of 0.024 µg anthracene h⁻¹ µg protein⁻¹.

3.2. Isolation and identification of neutral metabolites

HPLC analysis of the neutral extract revealed several metabolites that did not accumulate and were not present in sufficient quantities to perform MS or NMR analyses. However, one metabolite having a retention time of 23.0 min, gave an UV spectrum with λmax of 204, 252, 296, and 306 nm, similar to that of cis-1,2-dihydroxy-1,2-dihydroanthracene [20,27,28].

3.3. Isolation and identification of acid metabolites

HPLC analysis of the acidic extract of anthracene metabolism revealed the presence of two anthracene metabolites. Having retention times of 27.6, and 28.2 min, they are identified as peaks I, and II, respectively (Fig. 2).

Peak II produced an EI mass spectrum (Fig. 3B) with a molecular ion at m/z 196 [M⁺] and significant fragment ions at m/z 168 [M—CO]⁺ and 139 [M—C_2H_4O_2]⁺, and 70 [C_3H_2O_2]⁺. Peak II was dissolved in deuterated acetone for ¹H NMR analysis. The assignments and coupling constants are: 6.47 (H3; J_3,4 = 9.7 Hz), 8.12 (H4; J_3,4 = 9.7 Hz), 8.03 (H5; J_5,6 = 8.4 Hz), 7.54 (H6; J_5,6 = 7.7, J_6,7 = 1.3 Hz), 7.62 (H7; J_6,7 = 8.6, J_7,8 = 1.3 Hz), 8.00 (H8), 7.79 (H9), 8.27 (H10). The ¹H NMR spectrum (Fig. 3B) showed eight aromatic resonances with a coupling pattern indicative of substitution at the C-1 and C-2 positions. Assignment of key resonances was made possible from NOE experiments: irradiation of the singlet at 8.35 ppm (H10) produced NOEs to the doublet at 8.20 ppm (H11).

Fig. 2. HPLC chromatogram of acidic metabolites after 25 h exposure to an anthracene-fluoranthene mixture.

Fig. 3. EI mass spectrum (A) and ¹H NMR spectrum (B) of metabolite II identified as 6,7-benzocoumarin.

Fig. 4. APCI mass spectrum (A) and ¹H NMR spectrum (B) of metabolite I identified as 3-(2-carboxyvinyl)naphthalene-2-carboxylic acid.
(H4) and 8.05 ppm (H5). Irradiation of the other singlet, H9, produced only one NOE to H8 at 8.00 ppm. The other assignments were made from homonuclear-decoupling experiments. The chemical shifts of H3 and H4 are consistent with those of coumarin [29] and the metabolite was identified as 6,7-benzocoumarin.

The 1H NMR spectrum of peak I (Fig. 4B) was similar to that of 6,7-benzocoumarin with eight aromatic resonances, two of which were overlapped, having a coupling pattern consistent with either substitution or ring cleavage at the C-1 and C-2 positions. Assignments of proton resonances 3 through 10 were made from decoupling and NOE measurements as described above. The 1H NMR assignments and coupling constants in deuterated DMSO are: 6.56 (H3, $J_{3,4} = 16.1$ Hz), 8.9 (H4), 7.84 (H5), 7.44 (H6,7), 7.81 (H8), 7.96 (H9), 8.10 (H10). The proton-decoupled 13C NMR spectrum of the metabolite had 12 aromatic resonances; four of the resonances were from quaternary carbons. The resonances at 170.33 and 177.59 ppm are consistent with the chemical shifts of carbons in carboxylic acid groups. The metabolite eluted with a retention time of 26.2 min using APCI/MS detection of diagnostic fragment ions in addition to molecular species. The negative ion mass spectrum acquired at 25 V (Fig. 4A) contained ions corresponding to the deprotonated molecule (M−H)$^-$ at m/z 241. Other diagnostic ions present were (M−CO$_2$−H)$^-$ at m/z 197 and (M−2CO$_2$−H)$^-$ at m/z 153, indicating sequential losses of the acid moieties. Based on the NMR and APCI/MS data, this compound was identified as 3-(2-carboxyvinyl)naphthalene-2-carboxylic acid.

Metabolism of anthracene by Gram-negative organisms has been demonstrated to proceed via an initial hydroxylation of the aromatic ring to produce cis-1,2-dihydroxy-

![Fig. 5. Pathway proposed for anthracene metabolism by Rhodococcus sp.](https://academic.oup.com/femsle/article-abstract/204/1/205/632518)
1,2-dihydroanthracene which is subsequently oxidized to 1,2-dihydroxyanthracene. The next step in the metabolism of anthracene is the cleavage of the aromatic ring in the meta position to produce cis-4-(2-hydroxynaphth-3-yl)-2-oxobut-3-enoic acid. This compound is unstable and will spontaneously rearrange to form 6,7-benzocoumarin [12]. The presence of a metabolite with a UV spectrum identical to that of cis-1,2-dihydroxy-1,2-dihydroanthracene, along with the identification of 6,7-benzocoumarin attests to the presence of a similar pathway in the Rhodococcus species. This pathway has also been found to occur in a Mycobacterium species [20], suggesting that it may be a common pathway for the initial degradation of anthracene in both Gram-negative and Gram-positive organisms.

In addition to metabolites of the meta cleavage pathway, the present work supports the existence of an ortho ring-cleavage pathway, as evidenced by the identification of 3-(2-carboxyvinyl)naphthalene-2-carboxylic acid in the culture extracts. The identification of both ring-fission products in culture extracts indicates that ortho and meta cleavage of 1,2-dihydroxyanthracene are proceeding simultaneously. This is a novel reaction that has only been identified in Gram-positive organisms [20]. A similar reaction has been reported in the degradation of naphthalene by Bacillus thermoleovorans [23]. The proposed pathway for metabolism of anthracene by Rhodococcus sp. is shown in Fig. 5.

Acknowledgements

The authors wish to acknowledge partial support of this research by a grant from the National Oceanographic and Atmospheric Administration through the Illinois–Indiana Sea Grant College Program. Additional support was provided by the Oak Ridge Institute for Science and Education Faculty Research program at the National Center for Toxicological Research, Jefferson, AR, under the sponsorship of C.E.C. The authors thank Thomas M. Heinze and Claude L. Holder for valuable advice and assistance.

References

