Biodegradation **1**: 283–290, 1990.

Identification of metabolites from degradation of naphthalene by a *Mycobacterium* sp.

Ingrid Kelley, James P. Freeman & Carl E. Cerniglia*
National Center for Toxicological Research, Food and Drug Administration, Jefferson, AR 72079, USA
*(*requests for offprints)*

Received 23 August 1990; accepted 7 January 1991

Key words: bioremediation, metabolites, microbial degradation, naphthalene

Abstract

A *Mycobacterium* sp. isolated from oil-contaminated sediments was previously shown to mineralize 55% of the added naphthalene to carbon dioxide after 7 days of incubation. In this paper, we report the initial steps of the degradation of naphthalene by a *Mycobacterium* sp. as determined by isolation of metabolites and incorporation of oxygen from $^{18}O_2$ into the metabolites. The results indicate that naphthalene is initially converted to *cis*- and *trans*-1,2-dihydroxy-1,2-dihydronaphthalene by dioxygenase and monooxygenase catalyzed reactions, respectively. The ratio of the *cis* to *trans*-naphthalene dihydrodiol isomers was approximately 25 : 1. Thin layer and high pressure liquid chromatographic and mass spectrometric techniques indicated that besides the *cis*- and *trans*-1,2-dihydroxy-1,2-dihydronaphthalene, minor amounts of ring cleavage products salicylate and catechol were also formed. Thus the formation of both *cis* and *trans*-naphthalene dihydrodiols by the *Mycobacterium* sp. is unique. The down-stream reactions to ring cleavage products proceed through analogous dioxygenase reactions previously reported for the bacterial degradation of naphthalene.

Introduction

Naphthalene is the most frequently reported polycyclic aromatic hydrocarbon contaminant at hazardous waste sites (Grimmer & Pott 1983). Exposure to naphthalene has been implicated in hemolytic anemia in people with glucose-6-phosphate dehydrogenase deficiency and in newborn infants (Sitting 1985). Naphthalene is considered to be a primary irritant, and the US Environmental Protection Agency (EPA) has classified it as a 'priority toxic pollutant' (US-EPA, 1980a, b).

The microbial metabolism of naphthalene has been studied over a period of many years, and several reviews have summarized these earlier investigations (Cerniglia 1984; Gibson & Subramanian 1984; Heitkamp & Cerniglia 1989). These studies have indicated that naphthalene is rapidly mineralized by pure bacterial cultures and microbial consortia in sediments and aquatic ecosystems (Davies & Evans 1964; Groenewegen & Stolp 1976; Heitkamp & Cerniglia 1989; Heitkamp et al. 1987). The bacterial degradation of naphthalene is initiated by the introduction of both atoms of molecular oxygen via a dioxygenase to form (+) *cis*-1R, 2S dihydroxy-1,2-dihydronaphthalene (*cis*-naphthalene dihydrodiol) with subsequent dehydrogenation to form 1,2-dihydroxynaphthalene (Jerina et al. 1971; Jeffrey et al. 1975). The dihydroxylated derivative serves as a substrate for ring fission enzymes, and is oxygenatively cleaved to form salicylate and catechol and ultimately carbon dioxide

(Davies & Evans 1964). Eucaryotic microorganisms use cytochrome P-450 monooxygenase and epoxide hydrolase catalyzed reactions to form a naphthalene dihydrodiol with a *trans* configuration (Cerniglia et al. 1978, 1983; Cerniglia & Gibson 1977, 1978).

We recently isolated a *Mycobacterium* sp. from oil-contaminated estuarine sediments that has the ability to degrade polycyclic aromatic hydrocarbons (Heitkamp & Cerniglia 1988; Heitkamp et al. 1988). This bacterium has been shown to mineralize naphthalene, phenanthrene, pyrene, fluoranthene, 1-nitropyrene, 6-nitrochrysene, and 3-methylcholanthrene (Heitkamp & Cerniglia 1988; Heitkamp et al. 1988; Kelley & Cerniglia 1990) demonstrating its potential for the bioremediation of polycyclic aromatic hydrocarbons in the environment. In this report we describe two alternative modes of initial enzymatic attack on naphthalene by the *Mycobacterium* and identify the intermediates in the naphthalene degradation.

Materials and methods

Chemicals

[1, 4, 5, 8-^{14}C]naphthalene (10.3 mCi mmol^{-1}) with a radiochemical purity of >98% was purchased from Sigma Chemical Co., St. Louis, Mo. Non-labeled naphthalene was purchased from Aldrich Chemical Co., Milwaukee, Wis. *Cis*-1,2,-Dihydroxy-1,2-dihydronaphthalene and *trans*-1,2-dihydroxy-1,2-dihydronaphthalene were kindly provided by David T. Gibson, University of Iowa, Iowa City, Iowa. Catechol, 1-naphthol, and salicylic acid were purchased from Chemical Service, Media, Pa. Bacterial media and reagents were purchased from Difco Laboratories, Detroit, MI. All solvents and chemicals used were of the highest purity available.

Bacterial strain and culture conditions

Mycobacterium cultures were grown in 125 ml Erlenmeyer flasks containing 30 ml minimal basal salts medium (MBS) supplemented with 250 μg ml^{-1} each of peptone, yeast extract and soluble starch, and 0.5 μg ml^{-1} fluoranthene dissolved in *N,N*-dimethylformamide (DMF). Cultures were grown in the dark and shaken (150 rpm) at 24° C. Fluoranthene appears to be a better inducer of oxygenase activity than naphthalene, thus for starter cultures, bacterial cells were subcultured at log phase from stock cultures into 30 ml of supplemented MBS medium containing low levels of fluoranthene (50 μg 30 ml^{-1} in DMF) for enzyme induction.

Naphthalene degradation conditions

Naphthalene mineralization ($^{14}CO_2$ evolution) was monitored in flowthrough microcosm test systems (Heitkamp & Cerniglia 1986; Huckins et al. 1984) containing 200 ml supplemented MBS medium with 1,4,5,8-[^{14}C]naphthalene (1.45 μCi) and 1.5 mg non-labeled naphthalene dissolved in DMF. Four ml aliquots (absorbance at 500 nm, 0.29) of bacterial cells were transferred into each of 5 replicate microcosms. Optical densities were measured at 500 nm (OD_{500} abs 0.05 was 1.49×10^6 cells per ml), using a Beckman DU-7 spectrophotometer (Beckman Instruments Inc., Irvine, CA). Samples were taken at 24 h intervals for 10 days.

$^{18}O_2$ Experiments

Experiments with $^{18}O_2$ were conducted using 125 ml flasks containing 30 ml of supplemented MBS medium and 8.47×10^6 *Mycobacterium* cells. The flasks were covered with rubber septa, and then evacuated five times with a vacuum pump and filled to atmospheric pressure with argon before evacuation and filling with 1 atmosphere of $^{18}O_2$ (99.8 atom %; Mound Facility, Miamisburg, Ohio). Each flask received 500 μg of naphthalene dissolved in DMF. The ratio of $^{18}O_2$ to $^{16}O_2$ in the air space was determined by mass spectrometry at the beginning and the conclusion of the experiment and was found to be approximately 97%. Flasks were incubated for 5 days.

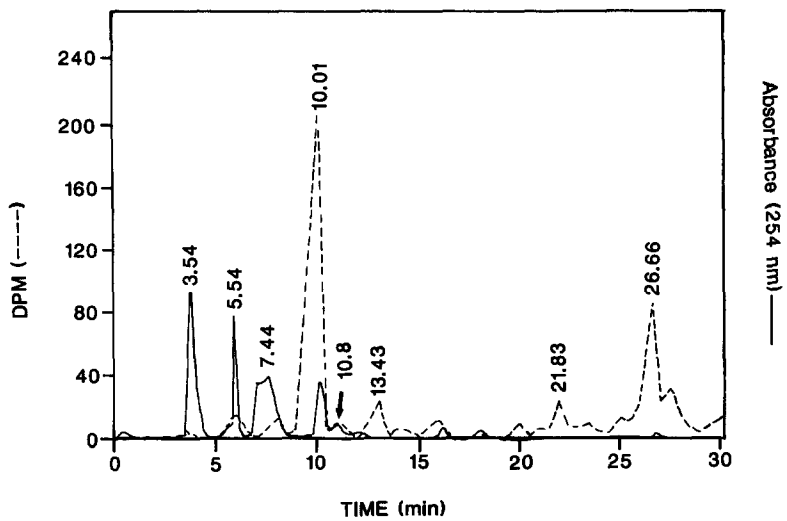

Fig. 1. HPLC profiles of neutral ethyl acetate extractable metabolites obtained when the *Mycobacterium* sp. was incubated with naphthalene for 72 h. The solid line indicates the UV absorbance at 254 nm and the dotted line indicates percent [^{14}C]naphthalene analogs. The numbers over the peaks represent retention times.

Physical and chemical analysis

Metabolites and residual naphthalene were extracted from the cells and medium with 3 equal volumes of ethyl acetate, dried with anhydrous Na_2SO_4, and evaporated to dryness under water aspirated vacuum.

The aqueous phase samples were then acidified to pH 2.5 with 6N HCl and re-extracted in the same manner. The residues were re-dissolved in 100 μl methanol for HPLC analysis. The amount of radioactivity remaining in the aqueous phase was measured by counting 0.5 ml aliquots in 7.5 ml Scintisol (Isolab Inc., Akron, OH). Naphthalene metabolites contained in the ethyl-acetate residues were dissolved in a small amount of methanol, and resolved by thin layer chromatography (TLC; 500 μm silica gel GF plates), using a benzene-hexane (1 : 1, vol/vol), benzene-ethanol (9 : 1, vol/vol), and chloroform-acetone (8 : 2, vol/vol) solvent system. Thin layer plates were scanned with a Varian Aerograph model 6000 2-pi Thin Layer Scanner to locate the bands containing ^{14}C metabolites. To isolate the radioactive spots the TLC plates were scraped and eluted with methanol.

Neutral ethyl-acetate extractable residues were derivatized by acetylation with acetic anhydride and acidified ethyl acetate extractable residues were derivatized by acetylation followed by methylation with diazomethane (Holder et al. 1985) for GCMS analysis.

Reversed-phase high performance liquid chromatographic analysis was performed using two Beckman model 100A HPLC pumps (Beckman Instruments Co., Berkeley, CA), a 5-μm C_{18} Ultrasphere ODS column (4.6 mm by 25 cm). Separation was achieved with a methanol-water linear gradient (35–95% methanol over 30 min) with 1% acetic acid at a flow rate of 1.0 ml min^{-1}. UV absorbance was measured at 254 nm with a Hitachi model 100-40 UV-visible absorbance detector (Hitachi Scientific Instruments, Mountain View, CA), and peak areas were calculated with an Altex Shimadzu model C-R1A integrator (Shimadzu Scientific Instruments, Columbia, MD).

Radioactive fractions of the HPLC effluent were collected at 30 sec intervals in scintillation vials, mixed with 7.5 ml of Scintisol, and analyzed by liquid scintillation spectrometry, using a Packard 2000CA Tri-Carb Liquid Scintillation Analyzer (Packard Instruments Co., Downers Grove, IL). Ultraviolet-visible absorption spectra of isolated compounds were determined in methanol using a Beckman model DU-7 spectrophotometer.

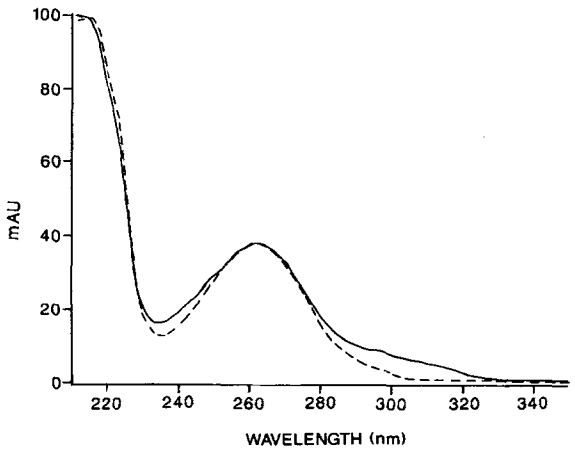

Fig. 2. Ultra-violet absorption spectra of (---) the authentic *cis*-1,2-dihydroxy-1,2-dihydronaphthalene and (———) the metabolite seen at R.T. 10.0 min in Fig. 1.

Naphthalene metabolites from the $^{18}O_2$ experiments were analyzed with a GC-MS model 4023 (Finnigan Instruments, San Jose, Ca.) equipped with a quadrupole, mass filter and a DB5 capillary column (0.25 mm dia by 15 m; J & W Scientific, Rancho Cordova, Ca.), operated in an electron impact mode with an electron energy of 70 eV; the column temperature was held isothermally at 60° C for 2 min, and then programmed to rise to 225° C, at 10° C min^{-1}. Naphthalene metabolites were also analyzed with a Finnigan model TSQ 70 instrument equipped with a BP-1 column (15 m). The column temperature was held isothermally at 60° C for 2 min, and then allowed to rise to 250° C at 10° C min^{-1}.

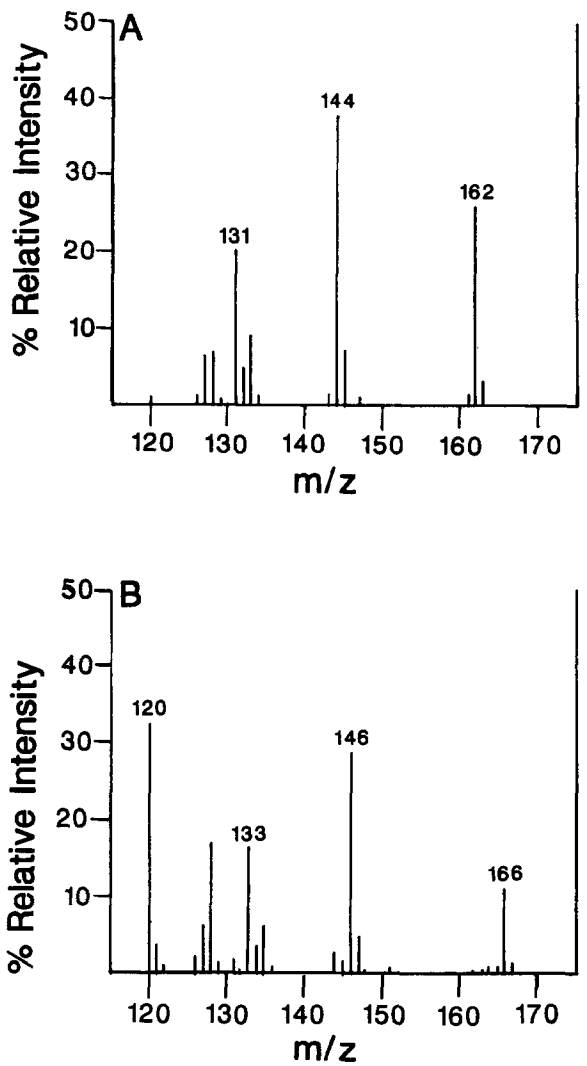

Fig. 3. Mass spectra obtained by GC/MS of *cis*-1,2-naphthalene dihydrodiols produced by the *Mycobacterium* sp. grown in naphthalene (A) under $^{16}O_2$, (B) under $^{18}O_2$.

Results and discussion

Recent investigations in this laboratory indicated that pyrene-induced *Mycobacterium* cultures degraded 55% of the initial naphthalene added to CO_2 and the remainder was distributed between cells and the aqueous culture filtrate (Heitkamp & Cerniglia 1988). Approximately 3.7% of the total radioactivity added was converted to ethyl-acetate extractable metabolites. In this study, we investigated the identification of the metabolites soluble in the culture medium to determine whether the catabolism was similar to the classic bacterial naphthalene degradative pathway (Davies & Evans 1964).

When the neutral ethyl-acetate extractable material was analyzed by HPLC, several radioactive peaks were detected at 10.0, 10.8, 13.4, 21.8, and 26.6 min (Fig. 1). HPLC analysis and the radioactivity measured from collected fractions corresponding to UV absorbance at wavelength 254 nm indicated that only one metabolite (10.0 min) accumulated to an appreciable extent during the miner-

alization process (Fig. 1). This metabolite reached a maximum concentration of approximately 1.5% of the total radioactivity.

The two peaks seen at 10.0 and 10.8 min (Fig. 1) from the neutral ethyl acetate-extractable fraction co-chromatographed with and were later identified as *cis*- and *trans*-naphthalene-1,2-dihydrodiols, respectively. The compounds eluting at 13.4 and 21.8 min had retention times identical to authentic salicylate and 1-naphthol respectively. The compound at 26.6 min was undegraded naphthalene. Extracts from sterile controls also showed a single radioactive peak, corresponding to non-metabolized naphthalene.

Further identification of the naphthalene-1,2-dihydrodiols was obtained from comparison of their UV/visible absorbance spectra maxima at 262 nm (Fig. 2), and mass spectra of underivatized samples with authentic naphthalene *cis*- (Fig. 3) and *trans*-dihydrodiols. The UV/visible absorption and mass spectra of the authentic *cis* and *trans* naphthalene dihydrodiol isomers were identical to those of the metabolites. The capillary column GC retention time for the underivatized dihydrodiols [M^+] at m/z 162, and m/z 144 (M^+-H_2O((Fig. 3A) was 9 : 56 min. The GC-MS retention time was 12 : 07 for the diacetylated *cis* isomer, and 12 : 12 for the *trans* isomer (Table 1). Molecular ions [M^+] at m/z 246, and fragment ions at m/z 186 [M-60]$^+$, and m/z 144 [M-102]$^+$ were observed for both derivatized isomers (Table 1). These losses probably correspond to a CH_3COOH loss, and a CH_3COOH and CH_2 = C = O loss, respectively. Fragmentation patterns were similar for the *cis* and *trans* isomers, but the *trans* dihydrodiol had a much weaker molecular ion at m/z 246. Capillary column-gas-chromatography-mass spectrometry (GC-MS), and radioactivity from HPLC fractions, as well as TLC indicated that the ratio of the diacetylated *cis* to *trans* isomer was approximately 25 : 1, respectively. Trace amounts of 1-naphthol, probably caused by spontaneous dehydration of the *cis*-dihydrodiol was observed by HPLC and mass spectrometry in the neutral frac-

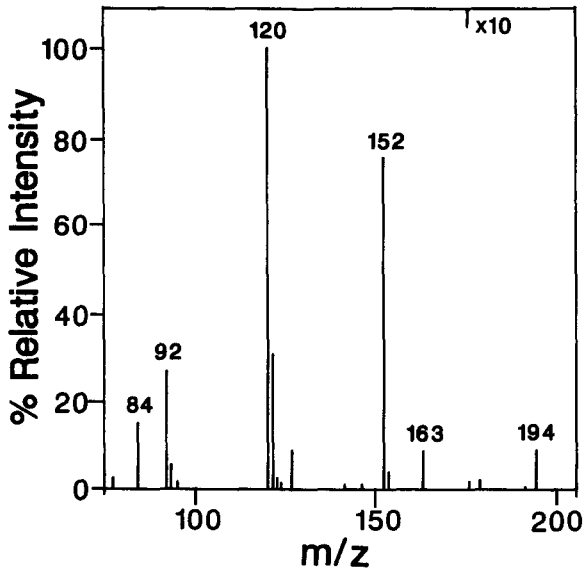

Fig. 4. Mass spectrum of acetylated and methylated derivative of salicylic acid.

Table 1. Gas liquid chromatography retention times and mass spectral data of naphthalene metabolites formed by the *Mycobacterium* sp.

Metabolite	Retention time (min : sec)	Prominent fragment ions (m/z) (% relative intensity)
Catechol diacetate	7 : 47	194(10.0)[M^+] 152(24.6)[M-42] 110(100.0)[M-42-42] 81(6.9)[M-42-42-29]
Salicylic acid, methylated & acetylated	8 : 08	194(0.7)[M^+] 163(4.4)[M-31] 152(74.4)[M-42] 121(31.6)[M-42-31] 120(100.0) [M-31-43] 92(24.0) 84(11.2)
1-Naphthol acetylated	10 : 01	186(9.6)[M^+] 168(4.0) 144(100.0)[M-42] 126(5.6)[M-60] 116(18.8) 115(34.4) 100(21.6) 86(14.4)
Naphthalene *cis*-1,2-dihydrodiol diacetate	12 : 07	246(2.0)[M^+] 186(11.8)[M-60] 161(7.3) 144(100)[M-60-42] 127(14.6) 115(25.4)
Naphthalene *trans*-1,2-dihydrodiol diacetate	12 : 12	246(0.1)[M^+] 186(9.2)[M-60] 156(7.2) 144(100.0)[M-60-42] 127(11.2) 115(11.2)

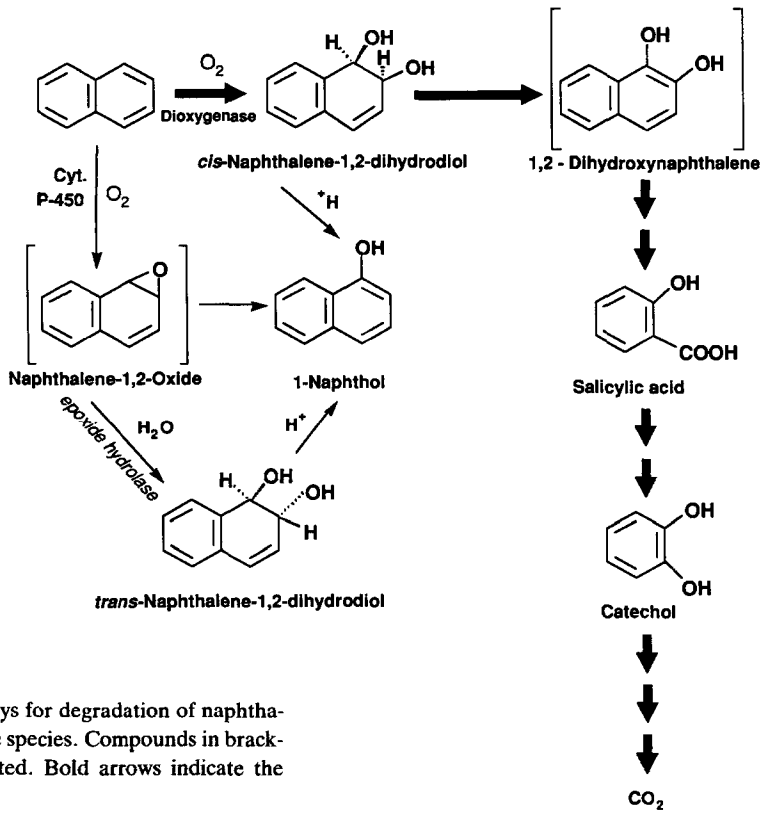

Fig. 5. Proposed pathways for degradation of naphthalene by a *Mycobacterium* species. Compounds in brackets have not been isolated. Bold arrows indicate the predominant pathway.

tion. Separation of underivatized *cis* and *trans* isomers was also achieved by TLC. The respective RF values were 0.21 and 0.14, and all chemical and spectral properties were identical to the authentic compounds.

One metabolite, obtained from the acidified ethyl acetate extractable fraction was found to be identical with salicylic acid (Table 1). The acetylated and methylated metabolite, and the similarly treated authentic salicylic acid, had a GC-MS retention time of 8: 08 min, and a $[M^+]$ at m/z 194, with fragment ions at m/z 163 $[M\text{-}31]^+$, m/z 152 $[M\text{-}42]^+$ and m/z 92, and a base peak at m/z 120 (Fig. 4). These fragments may represent losses of CH_3O, $CH_2 = C = O$ the combined loss of ketene and CH_3OH.

Another acetylated metabolite isolated from the neutral ethyl acetate fraction also had an m/z at 194. However, it had a capillary column GC-MS retention time of 7 : 47 min. Fragment ions were at m/z 152 $[M\text{-}42]^+$ and a base peak at m/z 110 $[M\text{-}84]^+$. The fragmentation pattern was indicative of a diacetylated catechol. However, not enough of this compound accumulated in the sample to confirm the identification. Salicylic acid appears to be the major ring fission product. Accumulation of catechol was too minor to be detected by HPLC. But presumably, once formed, it is quickly metabolized.

To investigate the mechanism of naphthalene dihydrodiol formation, $^{18}O_2$ experiments were conducted. Figure 3B shows the mass spectrum of *cis*-naphthalene-1,2-dihydrodiol (M^+ at m/z 166), produced by the *Mycobacterium* sp. grown with naphthalene in the presence of $^{18}O_2$. The shift of the molecular ion peak from m/z 162 (Fig. 3A) to m/z 166 (Fig. 3B) and other changes in the spectra indicated incorporation of both atoms of molecular oxygen into the *cis*-naphthalene dihydrodiol. In contrast, similar experiments indicated that the *trans*-naphthalene dihydrodiol contained one atom of molecular oxygen M^+ at m/z 164 (data not shown).

The $^{18}O_2$ experiments and identification of metabolites recovered from the degradation of naph-

thalene by this *Mycobacterium* sp. indicate that there are at least two pathways for naphthalene degradation. The mineralization pathway (Fig. 5) is similar to the classical naphthalene catabolism pathway (Davies & Evans 1964) and is the predominant pathway in the *Mycobacterium*. Similar metabolites were also detected by Heitkamp et al. (1987) for naphthalene degradation in natural sediments. The initial ring oxidation appears to be mediated, primarily, by 1,2-dioxygenase and results in the formation of *cis*-1,2-dihydroxy-1,2-dihydronaphthalene, with further metabolism to ring cleavage products. However, the fact that trace amounts of the *trans*-dihydrodiol were isolated, an observation previously reported for the fungal and mammalian oxidation of naphthalene (Cerniglia & Gibson 1977, 1978; Cerniglia et al. 1983; Cerniglia 1984), indicates that a minor pathway is involved (Fig. 5). Eucaryotic metabolism of naphthalene proceeds via initial epoxidation to form naphthalene-1,2-oxide which rapidly rearranges to 1-naphthol or is enzymatically hydrated to *trans*-naphthalene-1,2-dihydrodiol (Cerniglia 1984). To our knowledge aryl monooxygenase-epoxide hydrolase activity has not been described in bacteria. Either cytochrome P-450 monooxygenase-epoxide hydrolase activities as evidenced by the $^{18}O_2$ experiments and the identification of *trans*-naphthalene-dihydrodiol, or monooxygenation of the benzylic nucleus by the naphthalene dioxygenase and enzymatic hydration occurred. Recently, a stereospecific incorporation of a single oxygen molecule by toluene- and naphthalene dioxygenases was demonstrated by Wackett et al. (1988) with the oxidation of indan to 1-indanol in *Pseudomanas putida*. We previously reported that *Mycobacterium* sp. also formed a small amount of pyrene *trans*-dihydrodiol from pyrene oxidation (Heitkamp et al. 1988). Further studies on the characterization and properties of the oxygenases in the *Mycobacterium* sp. will allow for a better understanding of the metabolic pathways used in this bacterium, which holds promise for the bioremediation of the high molecular weight PAHs.

References

Cerniglia CE (1984) Microbial metabolism of polycyclic aromatic hydrocarbons. Advances Appl. Microbiol. 30: 31–71

Cerniglia CE, Althaus JR, Evans FE, Freeman JP, Mitchum RK & Yang SK (1983) Stereochemistry and evidence for an arene oxide-NIH shift pathway in the fungal metabolism of naphthalene. Chem.-Biol. Interactions 44: 119–132

Cerniglia CE & Gibson DT (1977) Metabolism of naphthalene by *Cunninghamella elegans*. Appl. Environ. Microbiol. 34: 363–370

— (1978) Metabolism of naphthalene by cell extracts of *Cunninghamella elegans*. Arch. Biochem. Biophys. 186: 121–127

Cerniglia CE, Herbert RL, Szaniszlo PJ & Gibson DT (1978) Fungal transformation of naphthalene. Arch. Microbiol. 117: 135–143

Davies JI & Evans WC (1964) Oxidative metabolism of naphthalene by soil pseudomonads. Biochem. J. 91: 251–261

Gibson DT & Subramanian V (1984) Microbial degradation of aromatic hydrocarbons. In: Gibson DT (Ed) Microbial Degradation of Organic Compounds (pp 181–252). Marcel Dekker, New York

Grimmer G & Pott F (1983) Occurrence of PAH. In: Grimmer G (Ed) Environmental Carcinogens: Polycyclic Aromatic Hydrocarbons (pp 61–128). CRC Press, Boca Raton, FL

Groenewegen D & Stolp H (1976) Mikrobieller Abbau von polyzyklischen aromatischen Kohlenwasserstoffen. Zentralbl. Bakteriol. 162: 225–232

Heitkamp MA & Cerniglia CE (1989) Microbial degradation of polycyclic aromatic hydrocarbons in the aquatic environment. In: Varanasi U (Ed) Metabolism of Polycyclic Aromatic Hydrocarbons in the Aquatic Environment (pp 41–68). CRC Press, Boca Raton, Florida

— (1986) Microbial degradation of *tert*-butylphenyl diphenyl phosphate: A comparative microcosm study among five diverse ecosystems. Toxicity Assessment Bulletin 1: 103–122

— (1988) Mineralization of polycyclic aromatic hydrocarbons by a bacterium isolated from sediment below an oil field. Appl. Environ. Microbiol. 54: 1612–1614

— (1989) Polycyclic aromatic hydrocarbon degradation by a *Mycobacterium* sp. in microcosms containing sediment and water from a pristine ecosystem. Appl. Environ. Microbiol. 55: 1968–1973

Heitkamp MA, Franklin W & Cerniglia CE (1988) Microbial metabolism of polycyclic aromatic hydrocarbons: Isolation and characterization of a pyrene-degrading bacterium. Appl. Environ. Microbiol. 54: 2549–2555

Heitkamp MA, Freeman JP & Cerniglia CE (1987) Naphthalene biodegradation in environmental microcosms: Estimates of degradation rates and characterization of metabolites. Appl. Environ. Microbiol. 53: 129–136

Heitkamp MA, Freeman JP, Miller DW & Cerniglia CE (1988) Pyrene degradation by a *Mycobacterium* sp.: Identification of ring oxidation and ring fission products. Appl. Environ. Microbiol. 54: 2556–2565

Holder CL, Korfmacher WA, Slikker W, Thompson HC &

Gosnell AB (1985) Mass spectral characterization of doxylamine and its rhesus monkey urinary metabolites. Biomed. Mass Spectrometry 12: 151–158

Huckins JN, Petty JD & Heitkamp MA (1984) Modular containers for microcosm and process model studies on the fate and effects of aquatic contaminants. Chemosphere 13: 1329–1341

Jeffrey AM, Yeh HJC, Jerina DM, Patel TR, Davey JF & Gibson DT (1975) Initial reaction of naphthalene by *Pseudomonas putida*. Biochemistry 14: 575–584

Jerina DM, Daly JW, Jeffrey AM & Gibson DT (1971) *Cis*-1,2-Dihydroxy-1,2-dihydronaphthalene: A bacterial metabolite from naphthalene. Arch. Biochem. Biophys. 142: 393–396

Kelley I & Cerniglia CE (1991) The metabolism of fluoranthene by a species of *Mycobacterium*. J. Ind. Microbiol. 1: 19–26

Sitting M (1985) Handbook of Toxic and Hazardous Chemicals and Carcinogens (pp 630–632). Noyes Publications, New Jersey

U.S. Environmental Protection Agency (1980A) Naphthalene: Ambient Water Quality Criteria. Wash. D.C.

— (1980B) Naphthalene, Health and Environmental Effects Profile No. 131, Wash. D.C., Office of Solid Waste

Wackett LP, Kwart LD & Gibson DT (1988) Benzylic monooxygenation catalyzed by toluene dioxygenase from *Pseudomonas putida*. Biochem. 27: 1360–1367