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Benzo[*a*]pyrene degradation by *Sphingomonas yanoikuyae* JAR02

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Benzo[a]pyrene degradation and mineralization by Sphingomonas yanoikuyae JAR02 was stimulated with salicylate, and novel ring-cleavage metabolites were identified.

Abstract

Batch experiments were conducted to characterize the degradation of benzo[*a*]pyrene, a representative high molecular weight (HMW) polycyclic aromatic hydrocarbon (PAH), by *Sphingomonas yanoikuyae* JAR02. Concentrations up to the solubility limit (1.2 μg l^{-1}) of benzo[*a*]pyrene were completely removed from solution within 20 h when the bacterium was grown on salicylate. Additional experiments with [^{14}C]7-benzo[*a*]-pyrene demonstrated 3.8% mineralization over 7 days when salicylate was present is solution, and one major radio-labeled metabolite was observed that accounted for ~10% of the initial radio-label. Further characterization of the radio-labeled metabolite using HPLC/MS and HPLC/MS/MS identified radio-labeled pyrene-8-hydroxy-7-carboxylic acid and unlabeled pyrene-7-hydroxy-8-carboxylic acid as novel ring-cleavage metabolites, and a benzo[*a*]pyrene degradation pathway was proposed. Results indicate that biostimulation of HMW PAH degradation by salicylate, a water-soluble, non-toxic substrate, has significant potential for in situ bioremediation.

Keywords: Salicylate; Biostimulation; Biodegradation; Mineralization

1. Introduction

Benzo[*a*]pyrene (BaP), a representative high molecular weight (HMW) polycyclic aromatic hydrocarbon (PAH) and persistent organic pollutant, is of environmental concern due to its known carcinogenicity (NTP, 2002) and bioaccumulation potential (McElroy et al., 1989). Limited success of physical, chemical, and biological treatment of contaminated soils and sediments is often attributed to contaminant physical properties, such as high K_{ow} and low vapor pressure. A high K_{ow} suggests strong contaminant sorption to soil organic matter and low bioavailability (Cerniglia, 1992) and a low vapor pressure limits volatilization. The current trend towards developing in situ bioremediation strategies for economical contaminant cleanup requires an understanding of the factors that limit or enhance microbial degradation, and several reviews are available for HMW PAH biodegradation (Juhasz and Naidu, 2000; Kanaly et al., 2000b). However, limited information is available describing microbial benzo[*a*]pyrene degradation pathways that are stimulated by substrates suitable for in situ bioremediation, such as salicylate.

Recently, three studies have described bacterial metabolites from different benzo[*a*]pyrene degradation pathways (Table 1). Moody et al. (2004) used resting *Mycobacterium vanbaalenii* PYR-1 cultures induced with phenanthrene (Moody et al., 2003) to produce several dihydrodiols and one ring-cleavage product, 10-oxabenzo[*def*]chrysene-9-one, from benzo[*a*]pyrene. Additional ring-cleavage products were identified when cultures were initially fed benzo[*a*]pyrene-*cis*-4,5-dihydrodiol. Schneider et al. (1996) observed benzo[*a*]pyrene-7,8-dihydrodiol and three ring-cleavage products from growing

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Table 1
Benzo[*a*]pyrene metabolites resulting from bacterial degradation

Metabolite	Bacterium
Benzo[*a*]pyrene-7,8-dihydrodiol	*S. yanoikuyae* B8/36[a] *Mycobacterium* RJGII-135[b]
cis-Benzo[*a*]pyrene-9,10-dihydrodiol	*S. yanoikuyae* B8/36[a]
trans-Benzo[*a*]pyrene-11,12-dihydrodiol	*M. vanbaalenii* PYR-1[c]
cis-Benzo[*a*]pyrene-11,12-dihydrodiol	*M. vanbaalenii* PYR-1[c]
cis-Benzo[*a*]pyrene-4,5-dihydrodiol	*M. vanbaalenii* PYR-1[c]
10-Oxabenzo[*def*]chrysene-9-one	*M. vanbaalenii* PYR-1[c]
cis-4-(8-Hydroxypyrene-7-yl)-2-oxobut-3-enoic acid or *cis*-4-(7-hydroxypyrene-8-yl)-2-oxobut-3-enoic acid	*Mycobacterium* RJGII-135[b]
4,5-Chrysene-dicarboxylic acid	*Mycobacterium* RJGII-135[b]
7,8-Dihydro-pyrene-7-carboxylic acid or 7,8-dihydro-pyrene-8-carboxylic acid	*Mycobacterium* RJGII-135[b]
Pyrene-8-hydroxy-7-carboxylic acid	*S. yanoikuyae* JAR02[d]
Pyrene-7-hydroxy-8-carboxylic acid	*S. yanoikuyae* JAR02[d]

[a] Gibson et al., 1975.
[b] Schneider et al., 1996.
[c] Moody et al., 2004.
[d] This study.

Mycobacterium sp. strain RJGII-135 cultures where pyrene was used to maintain PAH degradation. Gibson et al. (1975) reported the oxidation of benzo[*a*]pyrene to dihydrodiols by *Sphingomonas yanoikuyae* B8/36 (formerly *Beijerinckia* B-836; Gibson, 1999), a strain that can be induced with biphenyl, *m*-xylene, or salicylate (Mahaffey et al., 1988), but no ring-cleavage products were observed. None of these reports demonstrated detoxification or reduced carcinogenicity of the described metabolites.

Benzo[*a*]pyrene mineralization (conversion of ^{14}C-labeled 7-carbon to $^{14}CO_2$), which has been investigated with numerous bacterial cultures, is likely to reduce carcinogenicity by disrupting the bay-region structure. *Pseudomonas saccharophila* P15 induced with salicylate mineralized 20% of BaP (Chen and Aitken, 1999) and *Sphingomonas paucimobilis* EPA505 grown on fluoranthene mineralized 28% of the radiolabel (Ye et al., 1996), each in 2 days. *Stenotrophomonas maltophilia* VUN 10,010 mineralized 30% over 30 days when pyrene was supplied for growth (Boonchan et al., 2000); polar metabolites were observed using HPLC with UV detection. Kanaly et al. (2000a) reported 33–65% benzo[*a*]pyrene mineralization in 16 days by a bacterial consortium grown using different concentrations of diesel fuel. Of these studies, only Ye et al. (1996) demonstrated reduced mutagenicity (per the *S. typhimurium* assay) of benzo[*a*]pyrene degradation products.

The co-metabolic degradation of benzo[*a*]pyrene and other HMW PAHs requires additional carbon sources for energy and growth, as well as an inducer to maintain or express PAH-degrading enzymes. Another important role of the primary substrate is to provide electrons to replenish NADH coenzymes that are needed for the functions of oxygenase enzymes that initiate aerobic PAH degradation. Of studies examining degradation pathways or mineralization, those examining *S. yanoikuyae* (Gibson et al., 1975; Gibson, 1999; Mahaffey et al., 1988) and *P. saccharophilia* P15 (Chen and Aitken, 1999) utilized salicylate as an inducer. Other studies used phenanthrene, fluoranthene, or pyrene, low molecular weight (LMW) PAHs, to achieve degradation and would not be feasible for stimulating in situ bioremediation; each of these organisms may have additional substrates that would serve as inducers that have not been described. For improved in situ bioremediation, identification of pathways that can be induced by water-soluble, non-toxic substrates would be beneficial.

In order to better understand bacterial degradation of benzo[*a*]pyrene, we isolated a phenanthrene-utilizing bacterium and studied salicylate supplemented cultures. The objectives of this study were to (i) identify a PAH-degrading bacterium using carbon source utilization and 16S rDNA phylogeny, (ii) determine the extent of benzo[*a*]pyrene mineralization by the isolate, and (iii) identify novel metabolites of benzo[*a*]-pyrene degradation. This is the first report demonstrating (i) benzo[*a*]pyrene ring-cleavage products from a bacterium other than *Mycobacterium*, (ii) production of *o*-hydroxyaromatic acids from benzo[*a*]pyrene, and (iii) an HPLC/MS method for the detection of *cis*-benzo[*a*]pyrene-dihydrodiols.

2. Materials and methods

2.1. Chemicals

Benzo[*a*]pyrene, phenanthrene, and acetonitrile were purchased from Sigma, (St. Louis, MO) and were HPLC grade or better. [^{14}C]7-Benzo[*a*]pyrene was purchased from Sigma (St. Louis, MO) with a radiochemical purity of ~98.0% ($n = 4$). Benzo[*a*]pyrene-*cis*-4,5-dihydrodiol and benzo[*a*]pyrene-*cis*-7,8-dihydrodiol were obtained from the NCI Chemical Resource Repository (Kansas City, MO). All other reagents were ACS grade or better.

2.2. Isolation, maintenance, and identification of *Sphingomonas yanoikuyae JAR02*

L9 minimal media and LB rich media were previously described (Rentz et al., 2004). L9 media plates were solidified with 1.9% Noble (Difco) agar and LB media plates were solidified with 1.9% Bacto (Difco) agar. JAR02 was isolated from soil collected at the Iowa City oil disposal facility using selective enrichment procedures with phenanthrene as a sole carbon and energy source. JAR02 was maintained on L9 media plates with phenanthrene supplied in the vapor phase (30 °C). Sublimation of phenanthrene was improved by spraying a 10% (wt per vol) acetone solution onto filter paper and letting the acetone evaporate. Filter papers with fine particles of phenanthrene were placed in plate lids and plates were incubated upside down.

Isolate JAR02 was examined using the BIOLOG identification system according to the manufacturer's protocol (Haywood, CA). Briefly, isolates were grown on BUGM plates overnight and transferred to GN/GP inoculating fluid to a percent transmittance of 52 ± 3%. A GN2 MicroPlate was then inoculated with 150 µl per well and incubated at 30 °C for 20 h. An automated reader was used to evaluate plates using the GN-NENT strain type.

For phylogenic characterization, isolate JAR02 was grown on LB media overnight and total genomic DNA was extracted using the Qiagen DNeasy Tissue Kit (Valencia, CA). 16S rDNA was amplified from isolate DNA through a polymerase chain reaction (PCR) using a primer set specific for bacterial 16S rDNA. The forward primer, 27F (5′-AGG GTT TGA TCC TGG CTC AG-3′), and reverse primer, 1522R (5′-AAG GAG GTG ATC CAR CCG CA-3′), were obtained from Integrated DNA Technologies (Coralville, IA) and diluted to 8 µmol upon receipt. The PCR Master Mix using the Qiagen

PCR kit contained (per 100 μl reaction) 10× Qiagen buffer (10 μl), dNTP (2 μl), 27F primer (5 μl), 1522R primer (5 μl), Taq polymerase (0.5 μl), and water (27.5 μl). Each PCR also contained 50 μl template DNA from isolates and test strains. Reactions were incubated using an Eppendorf Mastercycler with an initial denaturing period of 2 min at 94 °C, 35 cycles of 1 min at 94 °C, 1 min at 54.5 ± 1.5 °C, and 2 min at 72 °C, and an end cycle of 10 min at 72 °C. PCR products were cleaned using the QIAquick PCR Purification kit prior to Sanger-based fluorescent sequencing by the DNA Facility at the University of Iowa. Nearest neighbors were found using a BLAST search (http://www.ncbi.nlm.nih.gov/blast), and percent similarity values were calculated using BioEdit (http://jwbrown.mbio.ncsu.edu/BioEdit/bioedit.html).

2.3. Co-metabolism of benzo[a]pyrene

Fifty milliliter flasks contained 15 ml of 2× L9 media and 30 μl of a 1630 μg l^{-1} benzo[*a*]pyrene solution (acetonitrile). Filter sterilized succinate and salicylate stock solutions (100×) were used, and sterile DI water was added to bring the final solution volume to 30 ml. A killed control contained 750 μl of 20% H_3PO_4. Flasks were inoculated using an overnight culture of JAR02 in LB medium that was centrifuged at 8000 rpm (14,000 × *g*) for 8 min and resuspended in L9 media. Each growth condition examined was tested in triplicate. Cultures were incubated with shaking at 30 °C in a dark incubator (to limit photo-oxidation). Two samples were removed from the cultures every 4 h using sterile pipettes. An 800 μl sample was placed in an autosample vial, culture growth was halted with 25 μl 20% H_3PO_4, and 200 μl acetonitrile was added to improve analysis of benzo[*a*]pyrene loss. An additional 1.0 ml sample was transferred to a cuvette and OD_{600} was measured.

2.4. Benzo[a]pyrene mineralization

Amber bottles (250 ml) contained 25 ml of 2× L9 media and 1 ml of a 1.0 μCi ml^{-1} [^{14}C]7-benzo[*a*]pyrene solution in *N*,*N*-dimethylformamide (NNDMF). Filter sterilized succinate and salicylate stock solutions (100×) were used. Sterile DI water was added to bring the final solution volume to 50 ml. A killed control contained 1.25 ml of 20% H_3PO_4. Each bottle contained a culture tube with 5 ml of 1 N NaOH solution to serve as a CO_2 trap. Triplicate cultures were used for each growth condition examined. Bottles were inoculated using an overnight culture of JAR02 in LB medium that was centrifuged at 8000 rpm (14,000 × *g*) for 8 min and resuspended in L9 media. Cultures were incubated with shaking at 30 °C in a dark incubator (to limit photo-oxidation).

Aqueous samples (700 μl) were removed from the culture to assess growth (OD_{600}). A 100 μl sample was removed from the NaOH trap, transferred to a scintillation vial with 10.0 ml Ultima gold liquid scintillation liquid, and analyzed on a Beckman (Fullerton, CA) model LS 6000IC liquid scintillation system. At the conclusion of the experiments, radio-labeled products were recovered with ethyl acetate extraction (2 vols.) of acidified solutions (pH ~2.5). Ethyl acetate extracts were dried over anhydrous sodium sulfate and evaporated under vacuum at 30 °C in the dark. Pelleted cells (neutral fraction) were extracted with 30 ml of acetone and these extracts were evaporated under vacuum at 30 °C in the dark. Samples were dissolved in acetone or acetonitrile and stored at 4 °C prior to analysis.

2.5. Analytical methods

Benzo[*a*]pyrene concentrations were analyzed using an Agilent 1100 series HPLC equipped with an Agilent 1100 FLD. Chemical separation was achieved using a Supelcosil PAH 5 micrometer column (150 mm × 4.6 mm), a mobile phase of 10% water and 90% acetonitrile, a 1.0 ml/min flow rate, and an injection volume of 100 μl. The fluorescent detector was set with excitation at 264 nm and emission at 412 nm. The detection limit for benzo[ ]pyrene was approximately 32.0 ppt (32.0 ng l^{-1}).

^{14}C-Labeled metabolites of benzo[*a*]pyrene degradation were observed using Agilent 1100 series HPLCs. Chemical separation was achieved using an Alltech 150 mm × 2.1 mm Zorbax 80A extend C-18 (5 μm) column guarded with a 12.5 mm × 2.1 mm Agilent C-18 guard column (5 μm). The MeOH/water mobile phase at a flow rate of 0.3 ml/min contained 2 mM ammonium acetate with the following solvent ratios (MeOH/H_2O, minutes): 47.5/52.5, 0; 95/5, 20; 95/5, 33; 47.5/52.5, 38 (linear gradients were used). A 50 μl sample was injected for ^{14}C analysis that was detected using a Packard (Meridian, CT) model Radiomatic™ 525TR flow scintillation analyzer. Ultima-FLO scintillation liquid was used at a 1.0 ml min^{-1} flow rate. Data collection and processing were completed with Packard FLO-ONE software. A 20 μL sample was injected for mass spectrometry using an 1100 series Agilent LC/MSD SL (quadrupole). The mass spectrometer was operated in negative-ion electrospray mode with the following spray chamber parameters: drying gas, 12.0 l min^{-1}; nebulizer pressure, 35 psig, drying gas temperature, 350 °C; capillary voltage, 3 kV. Mass spectrometer detection (MSD) parameters were: low *m/z*, 100; high *m/z*; 400; fragmentor, 70; gain, 1.0; threshold, 150. Agilent Chemstations software was used for instrument control and data collection.

3. Results and discussion

3.1. Sphingomonas yanoikuyae JAR02

Isolate JAR02 was identified as *Sphingomonas yanoikuyae* based on comparison of 16S rDNA sequences and BIOLOG characterization. The 16S rDNA sequence was 99.2% similar to both *Sphingomonas yanoikuyae* (AB109749) and *Sphingomonas yanoikuyae* B1 (U37524) over 862 nucleotides. BIOLOG characterization provided corroborating evidence with a similarity index value (SIM) greater than 0.5 (Table 2) that suggested a good match at the species level (Solit, 1999). *S. yanoikuyae* JAR02, a Gram-negative rod, grew on salicylate, 1-hydroxy-2-naphthoic acid, naphthalene, biphenyl, and phenanthrene as sole carbon and energy sources (Rentz, 2004). Similar to *S. yanoikuyae* B1, JAR02 showed broad reactivity towards polyaromatic compounds (Gibson, 1999; Khan et al., 1996), including the ability to degrade benzo[*a*]pyrene (Fig. 1).

Sphingomonas yanoikuyae B1, an organism isolated for its ability to grow on biphenyl (Gibson et al., 1973), has been studied intensively as a model organism for dioxygenase enzyme systems (Eaton et al., 1996). Biphenyl, *m*-xylene, naphthalene, anthracene, or phenanthrene were all used as a sole carbon and energy source for growth (Kim et al., 1997). Oxidation of additional substrates, including, benz[*a*]anthracene, benzo[*a*]pyrene, acenaphthylene, and dibenzofuran by *S. yanoikuyae* has also been observed (Eaton et al., 1996; Gibson, 1999; Khan et al., 1996).

The ubiquity of *S. yanoikuyae* within natural environments, particularly the root zone of plants (White et al., 1996), has been previously demonstrated. In addition, Takeuchi et al. (1995) reclassified *Chromobacterium lividum*, isolated from the roots of *Psychotria nairobiensis* and *Ardisia crispa*, as *S. yanoikuyae* following introduction of the *Sphingomonas* genus by Yabuuchi et al. (1990).

Table 2
BIOLOG characterization for isolate JAR02

Match	Genus species	Probability	SIM
1	*Sphingomonas yanoikuyae*	100	0.694
2	*Sphingomonas paucimobilis* B	0	0.000

SIM, similarity index value.

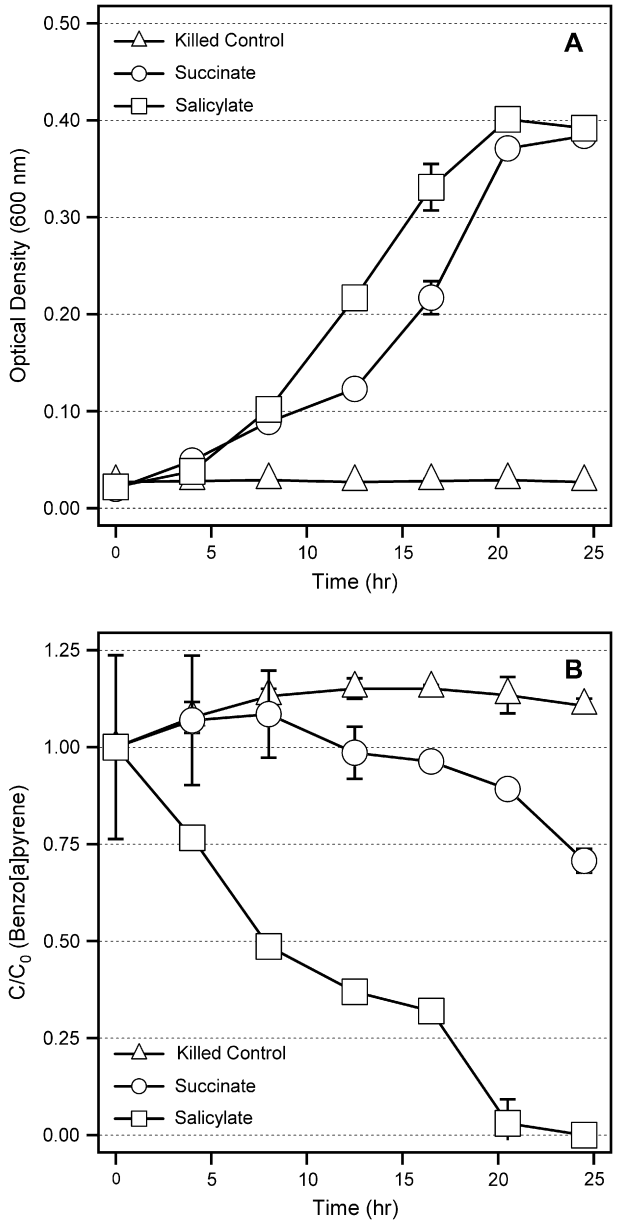

Fig. 1. (A) Growth of *S. yanoikuyae* JAR02 and concurrent (B) removal of benzo[*a*]pyrene from solution. Initial succinate and salicylcate concentrations were 2.0 and 2.5 mM, respectively. Bars represent one standard deviation from the mean of three replicate cultures and are not shown if smaller than symbol. Initial benzo[*a*]pyrene concentrations (C_0) were $\sim$1.3 μg l^{-1} (solubility limit), and the large initial variability (C_0) was for the salicylate grown cells.

3.2. Co-metabolism of benzo[a]pyrene

Sphingomonas yanoikuyae JAR02 removed benzo[*a*]pyrene from solution during growth on 2.0 mM succinate or 2.5 mM salicylate (Fig. 1). An acid (H_3PO_4) killed control exhibited no growth and no benzo[*a*]pyrene loss. For this test condition, minimal sorption of benzo[*a*]pyrene to bacterial cells occurred, suggesting the loss was the result of biodegradation. Benzo[*a*]-pyrene removal was significantly greater for salicylate grown cells than for succinate grown cells, indicating catabolic enzymes were induced by salicylate. This observation was expected, because salicylate is a known inducer of catabolic PAH genes (Shamsuzzaman and Barnsley, 1974) and was previously shown to induce phenanthrene degradation in *S. yanoikuyae* JAR02 (Rentz, 2004). Thirty percent degradation by *S. yanoikuyae* JAR02 during growth on succinate suggested that PAH-degrading enzymes were constitutively expressed at a low level for the culture conditions examined here.

Stimulation of benzo[*a*]pyrene degradation through addition of salicylate or other inducers of PAH catabolic pathways may have significant potential for in situ bioremediation applications. Providing native PAH-degrading microorganisms, previously selected by the presence of contaminants, a competitive advantage will increase relative population density, in addition to increasing catabolic enzyme expression on a per cell basis. The use of salicylate is particularly promising because it is water soluble, non-toxic, and completely biodegradable. A limitation would be the need for continuous or intermittent dosing due to consumption of the inducer as a carbon and energy source for co-metabolism.

3.3. Benzo[a]pyrene mineralization

Mineralization of [^{14}C]7-benzo[*a*]pyrene was observed by *S. yanoikuyae* JAR02 that was grown in the presence of both succinate (2.0 mM) and salicylate (2.5 mM) over 7 days, but not in incubations grown on succinate alone (Fig. 2). The total amount of $^{14}CO_2$ evolved was 3.7 ± 0.8% and was comparable to mineralization observed within the first week for the bacterium *Stenophomonas maltophilia*, grown on pyrene

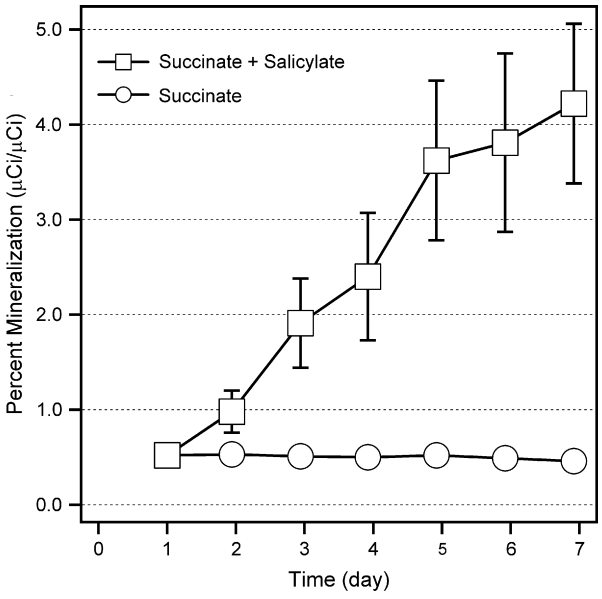

Fig. 2. Mineralization of [^{14}C]7-benzo[*a*]pyrene by *Sphingomonas yanoikuyae* JAR02 cultures exposed to salicylate, a known inducer of PAH degradation. Initial succinate and salicylcate concentrations were 2.0 and 2.5 mM, respectively. Initial applied radioactivity was 1.0 μCi per 50 ml (168 μg l^{-1} benzo[*a*]pyrene) and the benzo[*a*]pyrene radiochemical purity was 98.0%. Bars represent one standard deviation from the mean of three replicate cultures and are not shown when smaller than the symbol.

(Boonchan et al., 2000) and the white rot fungus *Bjerkandera* sp. strain BOS55 (Kotterman et al., 1998).

Benzo[*a*]pyrene co-mineralization was observed here by *S. yanoikuyae* JAR02 grown with salicylate as a primary substrate. Boonchan et al. (2000) also showed benzo[*a*]pyrene co-mineralization with *S. maltophilia* VUN 10,010 grown on pyrene. Other researchers, using resting, pre-induced cells demonstrated the utility of salicylate (Chen and Aitken, 1999) and fluoranthene (Ye et al., 1996) for stimulating benzo [*a*]pyrene mineralization. Neither study, however, demonstrated co-mineralization. The applicability of salicylate as a primary substrate and inducer would be greater than for pyrene or fluoranthene, two slightly soluble PAHs of environmental concern.

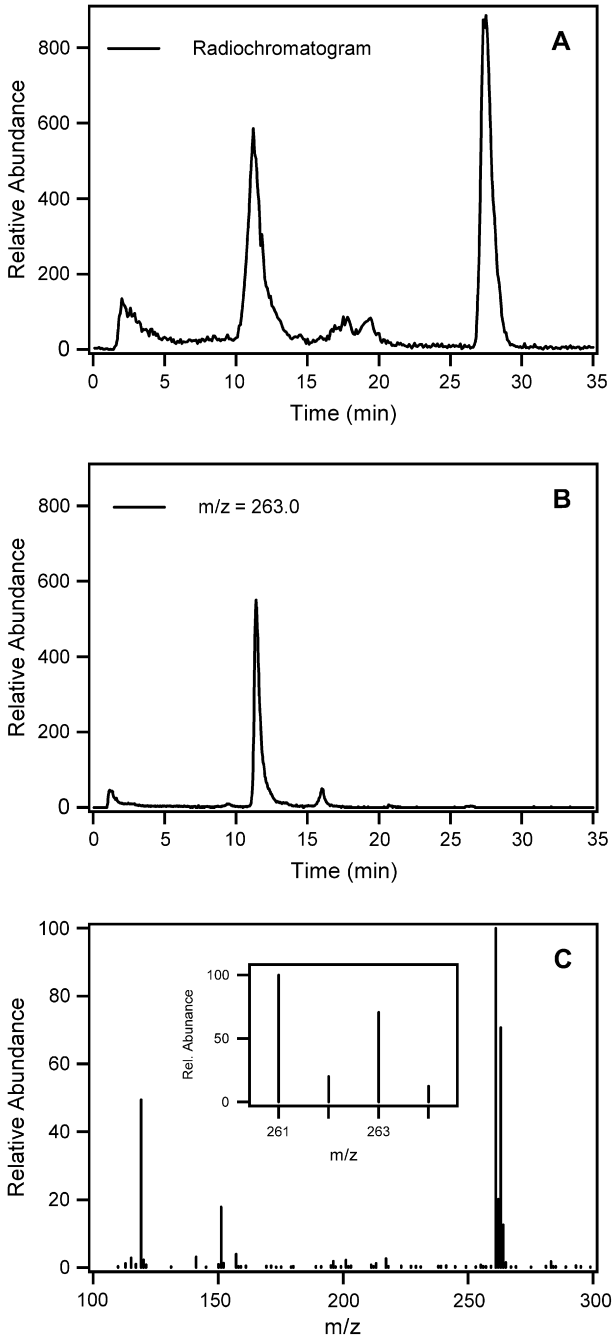

Fig. 3. HPLC characterization of polar metabolites formed during [^{14}C]7-benzo[*a*]pyrene degradation. (A) Radiochromatogram showing a major ^{14}C-labeled metabolite at 12 min; remaining ^{14}C-labeled benzo[*a*]pyrene eluted at 27 min. (B) Mass spectroscopy trace (*m/z* 263.0) showing a peak at 12 min that corresponded with the major ^{14}C-labeled metabolite. (C) Full scan mass spectra (apex of 12 min peak) showing the abundance of ions with *m/z* of 261.0 and 263.0; inset shows zoom view.

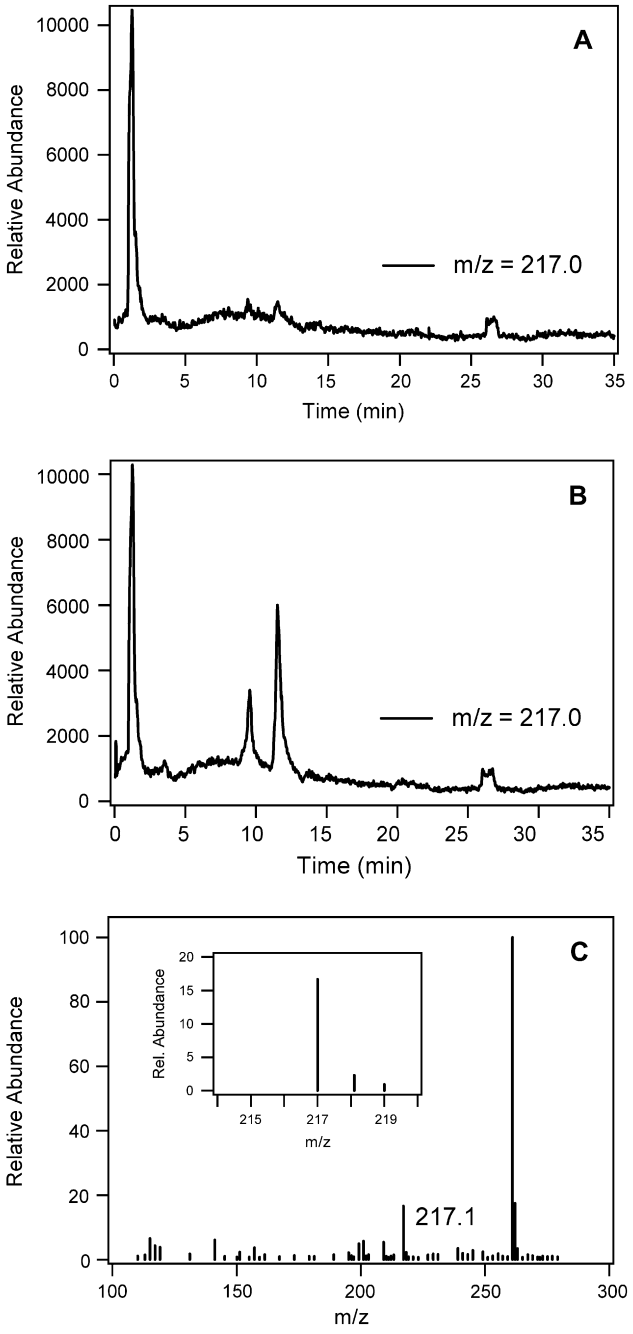

Fig. 4. Formation of a fragment ion with a *m/z* 217.0 from the ^{14}C-labeled metabolite that eluted at 12 min (Fig. 3) using elevated collision energy. (A) Mass spectroscopy trace (*m/z* 217.0) without elevated collision energy and (B) mass spectroscopy trace (*m/z* 217.0) with elevated collision energy. (C) Full scan mass spectra (apex of 12 min peak from (B)) showing the abundance of ions with *m/z* of 217.0 and the absence of ions with a *m/z* of 215.0 or 219.0; inset shows zoom view.

3.4. HPLC/MS detection of benzo[a]pyrene metabolites

High performance liquid chromatography (HPLC) analysis with radioactive detection demonstrated the presence of one polar metabolite (12 min elution) from the degradation of [^{14}C]7-benzo[*a*]pyrene by salicylate biostimulated *S. yanoikuyae* JAR02 cultures (Fig. 3A); the metabolite accounted for ~10% of initial applied radioactivity. While benzo[*a*]pyrene was completely removed from solution within 24 h for the co-metabolism studies described above, a significant portion remained in solution at the end of these mineralization studies. This occurred because the amount of [^{14}C]7-benzo[*a*]pyrene initially fed to reactors for the mineralization study was approximately 129 times greater than the aqueous solubility limit (the approximate initial concentration for the degradation study). When averaged over the 7 days of the mineralization experiment, the rate of benzo[*a*]pyrene degradation was 0.96 μg l^{-1} day^{-1}, a value that was similar to 1.3 μg l^{-1} day^{-1} observed during the co-metabolism study.

The ^{14}C-labeled metabolite did not correspond to authentic 4,5-benzo[*a*]pyrene-dihydrodiol (data not shown) or 7,8-benzo[*a*]pyrene-dihydrodiol (Fig. 6A), two known metabolites of benzo[*a*]pyrene degradation by *S. yanoikuyae* B8/36 (Gibson et al., 1975). Rather, mass spectrometry analysis showed

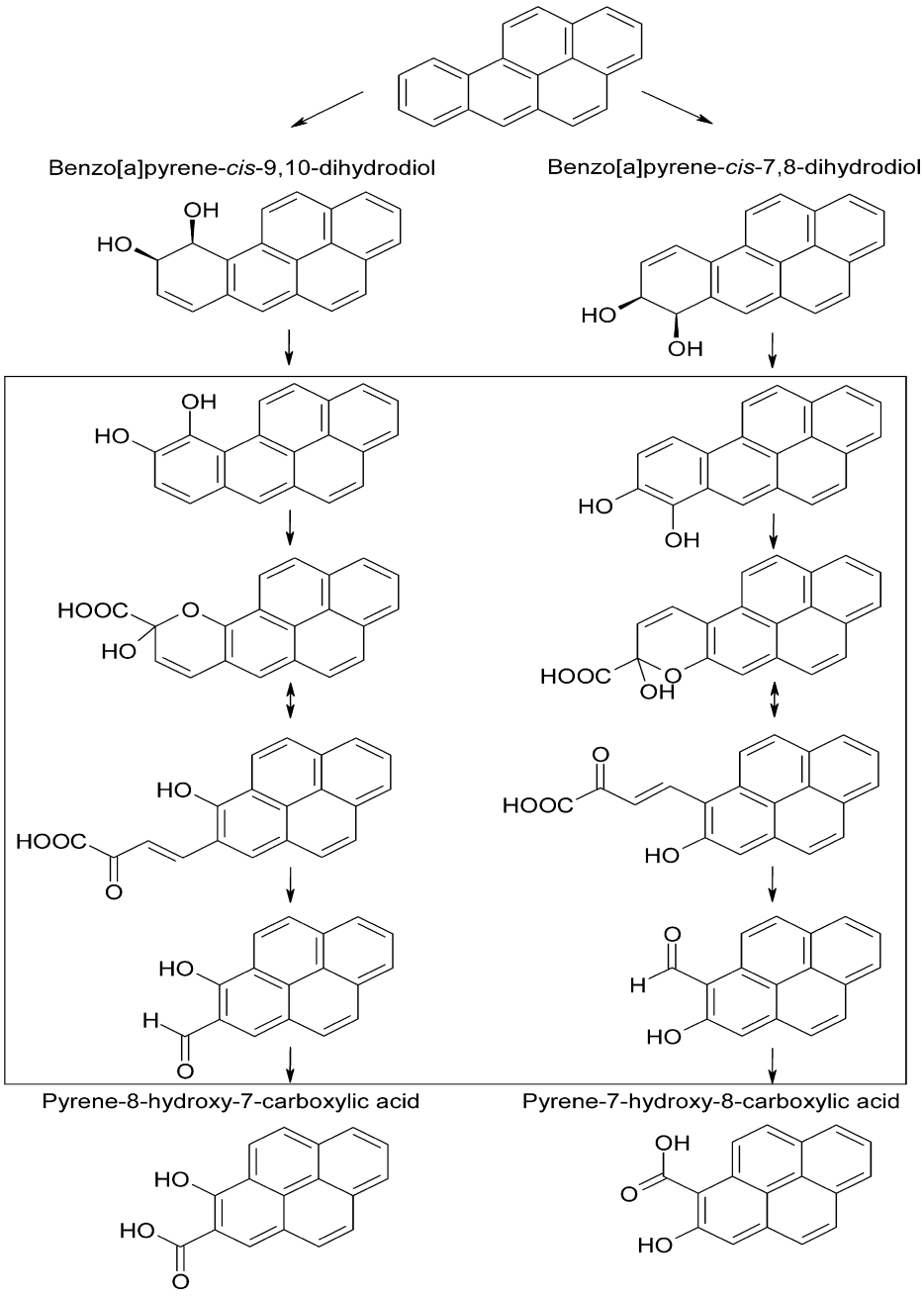

Fig. 5. Proposed pathway for degradation of benzo[*a*]pyrene by *S. yanoikuyae* JAR02 following phenanthrene degradation proposed by Kiyohara et al. (1994), Pinyakong et al. (2000), and Takizawa et al. (1994). Metabolites within the box have not been characterized, and the dihydrodiols were observed previously by Gibson et al. (1975).

a peak with a mass to charge ratio of 263.0 Da ($[M-1]^-$) corresponding to the radio-labeled peak (Fig. 3B). The most abundant ions within this peak had *m/z* 261.0 and *m/z* 263.0 ($[M-1]^-$; Fig. 3C), suggesting two metabolites may co-elute with similar structures differing by the radioactive ^{14}C-label. A second mass spectrometry analysis with elevated collision energies (nebulizer pressure, 55 psig; fragmentor, 100) produced a fragment with an *m/z* of 217.0 ($[M-44]^-$ or $[M-46]^-$) that corresponded to peaks for ions 261.0 and 263.0 (Fig. 4; also compare Fig. 3C to Fig. 4C). The loss of CO_2 (44 Da) or $^{14}CO_2$ (46 Da) from ions 261.0 and 263.0, respectively would produce a fragment ion with *m/z* 217.0. Radio-labeled benzo[*a*]pyrene-8-hydroxy-7-carboxylic acid and non-labeled benzo[*a*]pyrene-7-hydroxy-8-carboxylic acid were consistent with both ions *m/z* 263.0 and 261.0 producing a 217.0 fragment (Fig. 5), and the absence of ions with *m/z* 215.0 and 219.0 supports this determination (Fig. 4C). The structural similarity (chirality) of these metabolites may allow for identical retention times during HPLC as observed by Mahaffey et al. (1988) during the separation of 2-hydroxy-3-phenanthroic acid and 3-hydroxy-2-phenanthroic acid, metabolites of benz[*a*]anthracene degradation by *S. yanoikuyae* B1. Additionally, reports identifying *o*-hydroxyaromatic acids as metabolites from the degradation of naphthalene (salicylic acid), anthracene, and phenanthrene (Yang et al., 1994; Mahaffey et al., 1988), low molecular weight PAHs, supports the proposed structures. A degradation pathway for benzo[*a*]pyrene by *S. yanoikuyae* JAR02 was proposed (Fig. 5).

Benzo[*a*]pyrene-*cis*-4,5-dihydrodiol (not shown) and benzo[*a*]pyrene-*cis*-7,8-dihydrodiol (Fig. 6) were also observed using HPLC/MS. An acetate adduct ($[M+59]^-$) ion with an *m/z* of 345.1 produced similar chromatography compared to UV_{254} traces for both authentic standards. Parent ions ($[M-1]^-$) were also produced for the 4,5 (*m/z* 285) and 7,8 (*m/z* 285.1) isomers. The method used here was not optimized to produce a low detection limit because no radio-labeled peaks corresponded to these metabolites. The difference in time for the UV_{254} and MS (*m/z* 345.1) traces was used to align radio-labeled peaks to appropriate HPLC/MS peaks.

HPLC/MS analysis of benzo[*a*]pyrene metabolites may provide advantages over other analytical techniques, especially for the hydroxy-aromatic acids described here. First, the carboxylic acid moiety promotes formation of ions in solution that is required for LC/MS detection (Straub and Voyksner, 1993) with no methyl derivatization that GC/MS requires. Also, the same HPLC system used to separate metabolites with UV or radio-labeled detection could be used in-line with MS. Second, small quantities of metabolites can be examined without the need for purification, which is needed for NMR analyses. Within this report, only two of five reported dihydrodiols were examined, but the method could be transferred to others.

4. Conclusions

Concern about HMW PAH, especially benzo[*a*]pyrene, results from toxicity and carcinogenicity within mammals that requires in vivo activation. P450 enzymes oxidize benzo[*a*]pyrene sequentially to an epoxide, dihydrodiol, and dihydrodiol-epoxide (Cheng et al., 1989). The dihydrodiol-epoxide intermediates are highly reactive and form adducts with cellular DNA, RNA, and proteins. Present knowledge suggests the bulky benzo[*a*]pyrene-DNA adducts inhibit cellular repair by housekeeping enzymes leading to mutation and carcinogenicity (Perlow and Broyde, 2003). The oxidation of benzo[*a*]pyrene demonstrated here by *S. yanoikuyae* JAR02 did not accumulate dihydrodiols that may lead to possible DNA adducts,

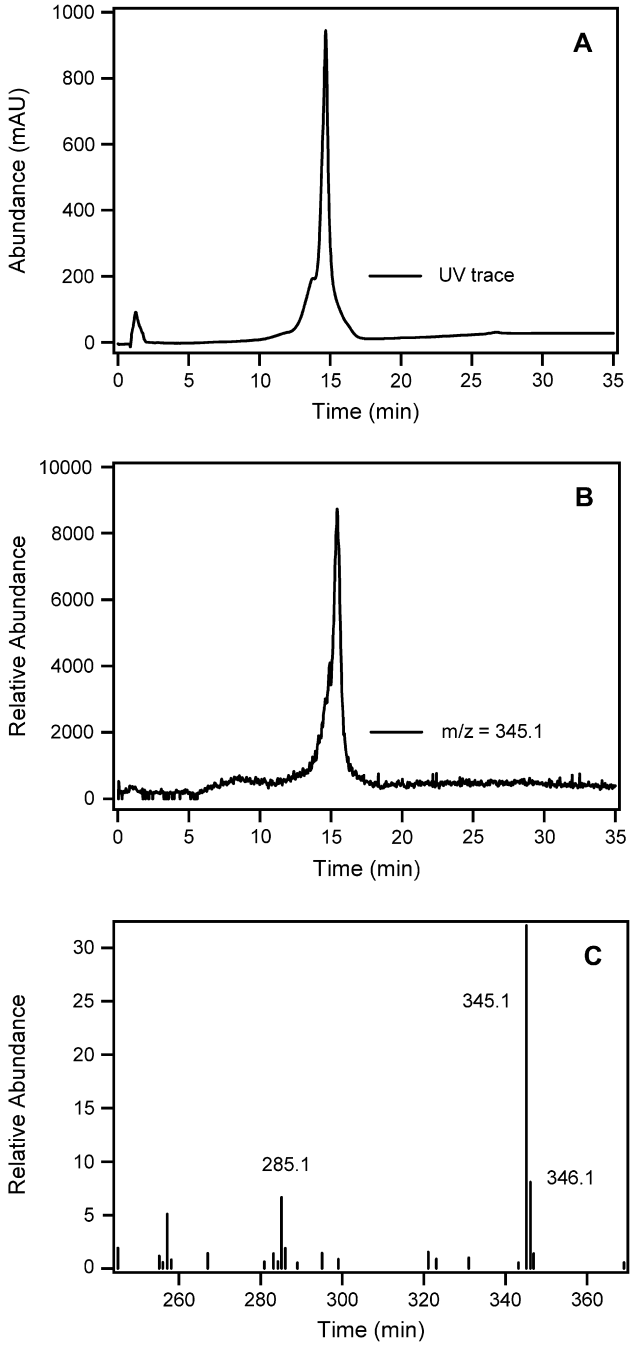

Fig. 6. HPLC characterization of benzo[*a*]pyrene-*cis*-7,8-dihydrodiol. (A) UV trace and (B) mass spectroscopy trace (*m/z* 345.1) showing elution at 15 min. (C) Full scan mass spectra (apex of 15 min peak) showing the abundance of the acetate adduct ion ($[M+59]^-$) with a *m/z* of 345.1 and the presence of parent ions ($[M-1]^-$) with a *m/z* of 285.1.

and bacterial degradation by this mechanism will reduce toxicity and carcinogenicity of benzo[*a*]pyrene. Production of $^{14}CO_2$ from the seven carbons of benzo[*a*]pyrene reported here and by others (Boonchan et al., 2000; Kotterman et al., 1998; Bezalel et al., 1996; Rafin et al., 2000) also provided evidence that the toxicity of benzo[*a*]pyrene was reduced by *S. yanoikuyae* JAR02.

To date, aerobic bioremediation of HMW PAH, including benzo[*a*]pyrene, must utilize co-metabolic degradation that requires a carbon/energy source, an inducer of catabolic enzymes, and oxygen. The pathway of degradation described here for *S. yanoikuyae* JAR02 utilized salicylate as an inducer, as well as a carbon and energy source. Previous studies also showed the ability of salicylate to stimulate HMW PAH mineralization, but no metabolites were identified and resting cells were used (Chen and Aitken, 1999). Co-metabolic degradation of benzo[*a*]pyrene has demonstrated metabolite formation (Schneider et al., 1996) or mineralization (Boonchan et al., 2000), but no reports used a water-soluble, non-toxic substrate suitable for stimulating in situ bioremediation, as we have in this study. Previous efforts have used toxic LMW PAHs that would not be suitable for use as a biostimulation strategy.

Within natural environments, multiple pathways have been described for the biodegradation of benzo[*a*]pyrene and other HMW PAH (Doddamani and Ninnekar, 2000), and it is unlikely that salicylate will stimulate all PAH-degrading organisms. Therefore, the efficacy of salicylate biostimulation for HMW PAH degradation must be examined at the field scale. Also, there is potential that additional water-soluble, non-toxic metabolites exist that could act as inducers and carbon/energy sources for HMW PAH co-metabolism.

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