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Transformation and mineralization of benzo[*a*]pyrene by microbial cultures enriched on mixtures of three- and four-ring polycyclic aromatic hydrocarbons

J Dries[1,a] and BF Smets[1,2]

[1]*Environmental Engineering Program, University of Connecticut, Storrs, CT 06269-2037, USA;* [2]*Microbiology Program, University of Connecticut, Storrs, CT 06269-2037, USA*

Microorganisms originating from a soil contaminated by low levels of polycyclic aromatic hydrocarbons (PAHs) were enriched with three- and four-ring PAHs as primary substrates in the presence of benzo[*a*]pyrene (BaP). Most enrichment cultures, isolated in the presence or absence of a sorptive matrix, significantly transformed BaP. Evidence of BaP mineralization was obtained with cultures enriched on phenanthrene and anthracene. Our findings supplement literature data suggesting the wide occurrence of microbial activity against BaP.
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Introduction

Polycyclic aromatic hydrocarbons (PAHs) form a class of highly toxic organic compounds consisting of two or more fused benzene rings. PAHs are components of coal tar, creosote and crude oil, and are formed by the incomplete combustion of organic chemicals [13]. PAHs with more than four rings, such as benzo[*a*]pyrene (BaP) are of specific concern because of their mutagenic and carcinogenic nature. Bacteria readily mineralize PAHs up to four rings under well-defined laboratory conditions [6,13], but biodegradation of the five-ring PAH benzo[*a*]pyrene is more problematic due to its resonance energy and extremely low aqueous solubility [6,9]. The main route of microbial transformation of BaP identified thus far is via cometabolism [16]. Primary substrates for BaP-degrading microorganisms can range from single PAHs to complex hydrocarbon mixtures [3,13,16-18].

The objectives of this study were (i) to assess the potential of soil bacteria originating from a contaminated soil to cometabolize BaP when supplied with several simple mixtures of PAHs, and (ii) to determine the degree of BaP mineralization by cultures enriched on three- and four-ring PAHs.

Materials and methods

Chemicals and media

Phenanthrene (PHE), anthracene (ANT), pyrene (PYR), fluoranthene (FLT) and benzo[*a*]pyrene (BaP) were purchased from Sigma-Aldrich (St. Louis, MO). [7,10-^{14}C]Benzo[*a*]pyrene (specific activity=54.0 mCi/mmol) was purchased from Amersham (Piscataway, NJ). Enrichments and cultivations were performed in a minimal salts medium (MSM) with the target PAHs as sole organic carbon source, both NH_4^+ and NO_3^- as N sources, and containing all required macro- and micronutrients in appropriate ratios [8]. Amberlite XAD-1180 beads (Sigma, St. Louis, MO), rinsed by repetitive washes in 50 mM phosphate buffer, were used as sorptive matrix.

Enrichment of BaP degraders

Top-soil samples (2–4 cm below surface) originated from a former manufactured gas plant site in Rockville, CT. The soil contained low levels of PAHs (approx. 45 mg total PAH/kg dry weight). The indigenous bacterial population was extracted from 50 g of sieved (2 mm) wet soil using a solution of 3 g/l of pyrophosphate and 0.5 g/l of polyvinyl pyrrolidone in demineralized water [8]. This suspension containing eluted bacterial cells was washed, concentrated, resuspended in MSM, and kept at 4°C.

Six triplicate enrichment sets were initiated in the presence of BaP, starting from the extracted indigenous bacterial population. A classical enrichment procedure was applied in the first three sets, where the PAHs were supplied as free crystals in liquid media. A novel enrichment procedure was applied in the remaining three sets, where the PAHs were presorbed to a hydrophobic matrix, i.e., XAD-1180 beads. In the parallel enrichment sets 1 and 4, 2 and 5 and 3 and 6 the primary substrates for BaP degradation were, respectively, a combination of the three-ring compounds PHE and ANT, the four-ring PAHs PYR and FLT, and all four PAHs. Blanks were set up without PAHs. Sodium azide-killed abiotic controls (at 100 mg/l) were supplied with a combination of all five PAHs.

All PAHs were supplied at a concentration of 100 mg/l, except BaP, which was supplied at 10 mg/l. The PAHs were delivered from individual stock solutions (in methylene chloride), to triplicate 120-ml glass serum bottles. In the sets with XAD (at 10 g/l), the methylene chloride was injected directly on the beads. Autoclaved MSM (49 ml) was added to every bottle after all solvent had evaporated. All serum bottles were equilibrated for 5 days in the dark before 1 ml of the soil bacterial suspension was

Correspondence: Dr BF Smets, Environmental Engineering, University of Connecticut, Storrs, CT 06269-2037, USA
[a]Current address: VITO, Flemish Institute for Technological Research, Department of Environmental Technology, Boeretang 200, 2400 Mol, Belgium.
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added. The serum bottles were capped with Teflon lined rubber septa and incubated on a shaker (150 rpm) in a temperature-controlled incubator (20°C) in the dark.

The first phase of the enrichment ended after 95 days. Samples were taken for protein assay, and selected serum bottles were sacrificed for PAH analysis. Aliquots from the aqueous phase were concentrated by centrifugation, and the resulting pellets were resuspended in MSM. These suspensions were used to inoculate duplicate serum bottles for a second similar enrichment phase, which lasted about 80 days. The final cell density in the second enrichment phase was approx. 1 mg protein/l.

BaP mineralization and transformation assay

An experiment was set up to validate the potential of selected enrichment cultures to transform and/or mineralize BaP (at 10 mg/l) in the presence of other PAHs (at 100 mg/l). Mixtures of PHE, ANT, BaP and [7,10-^{14}C]BaP or PYR, FLT, BaP and [7,10-^{14}C]BaP in methylene chloride were added to duplicate 25-ml biometer flasks (Kontes Glass, Vineland, NJ). After evaporation of the solvent, 4 ml of autoclaved MSM was added. The flasks were capped with rubber stoppers. A polypropylene center well was filled with 200 μl NaOH (0.2 N) to trap evolved $^{14}CO_2$.

The inocula (1 ml), derived from the second enrichment phase, were injected through the septum of the sidearm of the biometer flasks, to obtain a final inoculum density of approx. 1 mg protein/l. The flasks were then incubated on a shaker in the dark, at room temperature. The caustic solution in the center well was sampled and replaced at regular time intervals. At the end of the experiment, 100 μl of sulfuric acid (5 N) was injected into the medium to release all aqueous carbonate species as CO_2.

An analogous experiment, using 40-ml VOC vials, was set up without radiolabeled BaP to allow for GC-MS analysis of residual BaP. Abiotic control sets were prepared with sodium azide added (at 100 mg/l NaN_3).

Analyses

Residual PAHs, whether dissolved in the aqueous phase, sorbed to the glass walls of the serum bottles or VOC vials, or sorbed to the XAD beads, were extracted three consecutive times with methylene chloride. PAHs were quantified via GC-MS on a Hewlett Packard gas chromatograph, model 5890A, and Hewlett Packard mass selective detector, model 5970B, with helium as the carrier gas, according to method 8270 C [Semivolatile Organic Compounds by Gas Chromatography/Mass Spectrometry] from the standard EPA SW-846 procedures [Test Methods for Evaluating Solid Waste, Physical/Chemical Methods]. The protein assay was performed with the bicinchoninic acid protein assay kit (Sigma-Aldrich) according to Sigma procedure no. TPRO-562. The serum bottle gas phase was sampled at certain time intervals with a gastight Hamilton syringe (50 μl) and injected into a HP-5891 gas chromatograph equipped with a thermal conductivity detector (GC-TCD) to monitor oxygen consumption. Target gases were separated over an Alltech gas chrome MP-1 packed column, with helium as reference gas. Samples for radioactivity analysis were collected in scintillation vials to which 10 ml of scintillation cocktail (biodegradable counting scintillant NBCS104, Amersham) was added. The samples were analyzed on a Beckmann LS 5000TD liquid scintillation counter.

Results

Enrichment of BaP degraders

Growth, oxygen consumption and PAH transformation was observed in most enrichment sets (Table 1). The only exception was the set supplied with all five PAHs without XAD, where the high total concentration of mixed PAHs (450 mg/l) possibly inhibited bacterial activity. This inhibition was not observed in the presence of XAD probably because it lowered the directly available PAH concentration, as shown for naphthalene [19] and phenanthrene [8]. Bacterial activity, in terms of growth and respiration, was most significant in the enrichments without XAD (not shown). BaP transformation ranged from 11% to 55% relative to the killed controls. Phenanthrene and anthracene, or pyrene and fluoranthene, or all four PAHs, seemed appropriate primary substrate mixtures for BaP cometabolism (Table 1).

BaP mineralization and transformation assay

After 60 days incubation, BaP transformation was significant in most reaction flasks (Table 2). A low but significant degree of [7,10-^{14}C]BaP mineralization occurred only when phenanthrene and anthracene were supplied as primary substrates, by a culture enriched on three-ring PAHs without XAD. After a long lag time

Table 1 Summary of the growth, PAH respiration and removal data, and the degree of BaP transformation after two enrichment phases

Enrichment condition	Primary substrates	Growth[a]	Respiration[b]	PAH removal[c] of				
				PHE	ANT	PYR	FLT	BaP
−XAD	PHE+ANT	+	+	+	+			11%
	PYR+FLT	+	+			+	+	47%
	All 4 PAHs	−	−	−	−	−	−	0%
+XAD	PHE+ANT	+	+	+	+			22%
	PYR+FLT	+	+			+	+	55%
	All 4 PAHs	+	+	+	+	+	+	36%

[a]Net growth as indicated by an increase in the protein concentration vs. inoculated blank sets without PAHs: −, no net growth; +, net growth.
[b]Net respiration as indicated by oxygen consumption in the headspace vs. inoculated blank sets without PAHs: −, no net oxygen consumption; +, net oxygen consumption.
[c]Net relative PAH removal vs. killed controls: −, no removal; +, distinct removal.

Table 2 Relative degree of [7,10-^{14}C]BaP mineralization and BaP transformation vs. killed controls by enriched inocula growing on three- or four-ring PAHs (average±SD; $n=2$)

Enrichment condition of inoculum	Primary substrates during enrichment phase	Primary substrates in mineralization and transformation assay	% BaP transformation after 60 days	% [7,10-^{14}C]BaP mineralization after 60 days	% [7,10-^{14}C]BaP mineralization after 250 days
Without XAD	PHE+ANT	PHE+ANT	37±3[b]	6±3	9±1
	PYR+FLT	PYR+FLT	32±5[b]	0.21±0.02	0.51±0.05
With XAD	PHE+ANT+PYR+FLT	PHE+ANT	13±1	0.19±0.01	0.46±0.01
	Mixed[a]	PHE+ANT	15.66±0.04[b]	0.39±0.01	7±1
	PHE+ANT+PYR+FLT	PYR+FLT	27±5[b]	0.28±0.01	0.58±0.01
	Mixed[a]	PYR+FLT	21±23	0.23±0.02	0.48±0.01

[a]Inoculum was a mixture of cultures enriched with XAD on PHE+ANT or PYR+FLT.
[b]BaP transformation was significantly different from the killed abiotic control (paired t-test, $P=0.05$).

(>150 days, not shown), mineralization of [7,10-^{14}C]BaP, in the presence of phenanthrene and anthracene, also occurred in the biometer flasks inoculated with an XAD enriched culture (Table 2). The latter culture was a mixture of enrichments supplied with phenanthrene and anthracene, or pyrene and fluoranthene as primary substrates (Table 2).

Discussion

PAHs always appear in complex mixtures in contaminated soils. The presence of other PAHs may influence the degradation potential of individual PAHs in the mixture, both positively and negative [4,14,15]. The results of the present study indicate that mixed cultures originating from soils with low contaminant levels are able to transform benzo[*a*]pyrene to a significant degree when supplied with simple mixtures of three and/or four ring PAHs. Other researchers have also reported BaP transformation by bacteria growing in the presence of PAHs such as phenanthrene [1,7,15], pyrene [3,14,20], fluoranthene [23] and diesel fuel (containing PAHs) [17,18]. BaP degradation was significantly enhanced in the presence of PAHs with lower molecular weight [3,14]. It has been hypothesized that the structural similarities between PAHs favor the cometabolic degradation of BaP and other PAHs with more than four rings by the enzymes expressed for the attack of three- and/or four-ring PAHs [3,14,15,23].

The products of such fortuitous reactions may or may not be degraded further to carbon dioxide. Some of our enrichment cultures mineralized [7,10-^{14}C]BaP to some extent when phenanthrene and anthracene were supplied as primary substrates. Schneider and coworkers [20] identified several metabolites from BaP degradation by a *Mycobacterium* species pregrown on pyrene. These metabolites presumably resulted from initial ring fission reactions occurring in the 7,8,9,10-ring or the 4,5-ring. Intermediates such as 7,8-BaP-dihydriol and 9,10-BaP-dihydriol are more likely to yield radiolabeled CO_2 than 4,5-BaP-dihydriol with the employed [7,10-^{14}C]BaP [17,20]. These observations may explain the difference in the cultures' activity against BaP when measured in terms of mineralization and parent compound disappearance (Table 2).

Our findings supplement the few reports on BaP mineralization by cultures growing on three- or four-ring PAHs [1,3,7,23]. The mineralization rates reported in Table 2 were quite modest in comparison to the rapid BaP mineralization kinetics observed by Kanaly *et al* [18] with consortia growing on diesel fuel, by Ye *et al* [23] with high densities of fluoranthene grown cell suspensions, and by Boonchan *et al* [3] with pyrene-degrading consortia. Unfortunately, a direct comparison of kinetic data is not straightforward as it should take into account the varying experimental conditions (e.g., inoculum density and history; type, complexity and concentration of cosubstrate(s); BaP concentration; temperature) [18]. The results from these and other studies indicate that the microbiological potential to transform and mineralize benzo[*a*]pyrene is present in PAH-contaminated soils [5,9,10]. In order to obtain BaP degraders, it might be useful to set up enrichment cultures in the presence of BaP, as done in the present work. Extension of the enrichment phase with multiple transfers may yield fully adapted and more performant BaP-degrading cultures.

The major factor limiting the bioremediation of PAH contaminated sites, however, is most probably the bioavailability of the highly hydrophobic PAHs [6,13,22]. Interestingly, we observed a significant degree of BaP transformation in enrichments containing hydrophobic XAD beads. Recent studies involving isolation and enrichment procedures based on sorbed PAHs indicate that bacteria may possess or acquire mechanisms to deal with sequestered or sorbed substrates [2,11,21]. Such mechanisms may be the production of surface-active chemicals, or the development of hydrophobic cell walls promoting adherence to surfaces and stimulating pollutant desorption kinetics [2,11,12,21]. Although it remains unexplored, microorganisms enriched in the presence of sorptive matrices may prove to be effective PAH degraders in low-bioavailability environments typically encountered in PAH-contaminated soils.

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