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Anaerobic phenanthrene mineralization by a carboxylating sulfate-reducing bacterial enrichment

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Information on the susceptibility of higher molecular weight polynuclear aromatic hydrocarbons to anaerobic biodegradation is relatively rare. We obtained a sulfate-reducing bacterial enrichment capable of phenanthrene metabolism from a hydrocarbon-contaminated marine sediment. Phenanthrene degradation was in stoichiometric agreement with the theoretically expected amount of sulfate reduction and inhibited by molybdate. Mineralization of ^{14}C -phenanthrene by the enrichment was confirmed by the recovery of the expected amount of $^{14}\text{CO}_2$. Stable isotope studies with protonated or deuterated phenanthrene resulted in the detection of the correspondingly labeled phenanthrene carboxylic acid by gas chromatography–mass spectrometry. Comparison of the metabolite profile with a synthesized standard confirmed that the parent molecule was carboxylated at the C-2 position. Incorporation of ^{13}C -bicarbonate into the carboxyl group implicated a direct carboxylation of phenanthrene as a likely key initial reaction. Denaturing gradient gel electrophoresis analysis of the enrichment showed only two major bands and 16S rRNA sequences obtained by cloning clustered with known hydrocarbon-degrading sulfate-reducing δ -proteobacteria, indicating their possible involvement in the anaerobic oxidation of phenanthrene via carboxylation as the initial activation reaction.

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Introduction

Polynuclear aromatic hydrocarbons (PAHs) are chemically stable and sparingly water-soluble molecules that are known mutagens and carcinogens that can bioaccumulate in food chains leading to ecological and human health risks. As frequent environmental contaminants, however, they can be susceptible to biodegradation, even under anaerobic conditions. Indeed, the anaerobic degradation of 2–5-ringed PAHs has been demonstrated in soil and sediment incubations under various electron-accepting conditions (Coates *et al.*, 1996, 1997; Rockne and Strand, 1998; Rothermich *et al.*, 2002; Meckenstock *et al.*, 2004). Studies with the two-ring PAHs naphthalene and 2-methylnaphthalene yielded pure cultures and enrichments that have shed light on the

metabolic pathways for the degradation of these compounds. Based on these findings (reviewed in Meckenstock *et al.*, 2004; Suflita *et al.*, 2004), PAH activation was initially reported to be via carboxylation for unsubstituted polyaromatic compounds, such as naphthalene and phenanthrene, and via fumarate addition reaction for methylated species, such as 2-methylnaphthalene. However, a more recent study revealed that, at least for one naphthalene-degrading sulfate-reducing enrichment, 2-naphthoic acid was not a direct product of naphthalene activation. Rather, naphthalene was initially methylated to 2-methylnaphthalene before degradation via a fumarate addition and eventually converted into 2-naphthoic acid (Safinowski and Meckenstock, 2006).

Information on the anaerobic metabolism of larger molecular weight PAHs such as phenanthrene is more scarce. In the metabolic studies performed with the only well-documented sulfate-reducing enrichment culture capable of phenanthrene metabolism, incorporation of labeled ^{13}C -bicarbonate into phenanthrene suggested direct carboxylation as a key initial reaction but the carboxylation position

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remained unknown (Zhang and Young, 1997). Here, we present evidence of another sulfate-reducing enrichment capable of phenanthrene mineralization. Results obtained by stable isotope studies indicate that the culture likely activated phenanthrene by direct carboxylation at the C-2 position.

Materials and methods

Sulfate-reducing enrichment culture

A phenanthrene-degrading culture was enriched from hydrocarbon-contaminated marine sediments from the Paletta Creek sampling site in San Diego Bay, CA, USA. Sediment incubations were established in an anaerobic glove box. Sterile serum bottles (160 ml) received 50 ± 0.5 g sediments and 75 ml of reduced bicarbonate-buffered basal sea water mineral medium, pH 7.2–7.3 (Widdel and Bak, 1992). Phenanthrene (9 mg) dissolved in 3 ml of 2,2,4,4,6,8,8-heptamethylnonane (HMN) was supplied to serum bottle incubations as an overlay. The incubations were closed with Teflon-lined stoppers, secured with aluminum crimp seals and incubated under a $N_2:CO_2$ (80:20) headspace at 31°C. Incubations showing significant loss of the electron acceptor (sulfate) relative to the substrate-unamended and sterile controls were used for further enrichment. After repeated transfers (30%, by vol) in the same mineral medium, a stable sediment-free enrichment culture capable of phenanthrene degradation was obtained. For some experiments, phenanthrene- d_{10} (Isotec, Miamisburg, OH, USA) was amended to the HMN overlay. Strict anaerobic technique was used for all culture manipulations as well as for media and substrate preparations (Hungate, 1969; Balch *et al.*, 1979). Sulfate was analyzed by ion chromatography as described by Caldwell *et al.* (1998).

Phenanthrene measurements

The concentration of phenanthrene in the incubations was measured by periodic sampling of the HMN overlay and injection of a small portion (1 μ l) directly into an Agilent Technologies 5973 Network gas chromatograph (GC) equipped with a HP5-MS column (30 $\times$ 0.25 mm, 0.25 μ m film) and a mass selective (MS) detector. The oven temperature was held at 160°C for 2 min, then increased at 15°C/min to 220°C and held for 6 min. The injector temperature was 250°C. Helium was used as a carrier gas at a flow rate of 1.2 ml/min.

Mineralization of radiolabeled 9- ^{14}C -phenanthrene

Mineralization of radiolabeled (ring- ^{14}C (U), 9- ^{14}C) (ARC, St Louis, MO, USA) phenanthrene was examined in the cultures containing a mixture of labeled and unlabeled phenanthrene in the HMN overlay. After 28 weeks of incubation, the amount of radioactivity in the HMN layer was quantified

by analyzing a portion with a liquid scintillation counter. Mineralized ^{14}C -phenanthrene was accounted for as the $^{14}CO_2$ in the gas phase plus the radioactivity associated with $^{14}CO_2/H^{14}CO_3^-$ in the liquid phase. Radioactivity in the gas phase was measured by injecting a headspace sample in a GC (Varian 3300, Varian Inc., Palo Alto, CA, USA) equipped with gas proportional counter (GC-RAM, IN/US Systems Inc., Tampa, FL, USA). Radioactivity associated with $^{14}CO_2/H^{14}CO_3^-$ was quantified by liquid scintillation counting as the difference between total radioactivity in the liquid phase and the residual label remaining after acidification. Specifically, total radioactivity in the liquid phase was counted in an aliquot injected in a closed scintillation vial containing 0.5 M NaOH. Residual radioactivity (dissolved phenanthrene and potential metabolites) in the liquid phase was quantified on another aliquot placed in a closed scintillation vial and acidified (6 N HCl) to convert labeled bicarbonate into $^{14}CO_2$. An aliquot was then placed in 0.5 M NaOH and the residual radioactivity was determined.

[^{13}C]Bicarbonate incorporation

Biomass from a 100-ml active enrichment culture was collected by centrifugation under anaerobic conditions. The pellet was washed and resuspended in the same mineral medium as described above, except that 25 mM HEPES (pH 7.2–7.4) was used as the buffer instead of HCO_3^-/CO_2 . The cell suspension was distributed as 5-ml portions into serum vials that were closed with butyl rubber stoppers, and gas phase in the vials was exchanged to N_2 (100%). Experimental incubations received 6 mg phenanthrene in 2 ml HMN as a substrate and 2 mg/ml $NaH^{13}CO_3$ (Cambridge Isotope Laboratories Inc., Andover, MA, USA) from an anoxic stock solution. The bottles were incubated for 22 weeks at 31°C and subsequently extracted for metabolites as described below.

Extraction and metabolite analysis

All preparations were acidified to pH < 2 with H_2SO_4 and the HMN overlays were removed before extraction with ethyl acetate. Organic extracts were concentrated by rotary evaporation and then reacted with *N,O*-bis(trimethylsilyl)trifluoroacetamide (Pierce Chemical Co., Rockford, IL, USA) to form trimethylsilyl derivatives. Derivatized metabolites were separated by gas chromatography–mass spectrometry using a previously published protocol (Gieg and Suflita, 2002). Metabolites were identified based on matching GC retention times and mass spectral profiles with authentic standards, or by comparison with previously published mass spectra. Phenanthrene-2-carboxylic acid was synthesized by refluxing a mixture of 2-acetylphenanthrene (Sigma-Aldrich, St Louis, MO, USA) with

a 5% solution of sodium hypochlorite for 24 h according to the procedure of Dixon and Neiswander (1960). Phenanthrene-4-carboxylic acid was a gift from Dr A Callaghan (Rutgers University) and originally supplied from Sigma Chemical Co. (Milwaukee, WI, USA). Phenanthrene-9-carboxylic acid synthesis involved refluxing phenanthrene-9-carboxaldehyde (Sigma-Aldrich, St Louis, MO, USA) with silver nitrate in an alkaline 50% ethanol solution (Burtner and Cusic, 1943).

Cloning and phylogenetic analysis

Genomic DNA was isolated with the FastDNA Spin Kit for Soil (QBiogene, Carlsbad, CA, USA). Approximately 1450 bp fragments of 16S rDNA were obtained by PCR amplification with 'universal' eubacterial primers 27F and 1492R. Cloning of the PCR products was performed with the TOPO TA kit (Invitrogen, Carlsbad, CA, USA). Five clones were randomly chosen to sequence. Cloned DNA was directly amplified from transformed cells using flanking M13 vector sequences and purified with Millipore Ultrafree-MC 30 000 NMWL Filter Devices. Sequencing of the purified cloned PCR products was performed at the University of Oklahoma DNA Sequencing Facility on an ABI Model 377 automated sequencer, using the ABI Prism Dye Terminator Kit (PE Applied Biosystems Inc., Foster City, CA, USA). Sequencher (Gene Codes Corp., Ann Arbor, MI, USA) was used to assemble the fragments. The assembled sequences were compared to those in NCBI databases using BLASTN. Of the five cloned sequences, two were most similar to those of sulfate-reducing δ -proteobacteria (Phe4A and Phe4C), one to an uncultured *Bacteroidetes* (95%), one to an uncultured *Firmicute* (92% similarity) and the fifth was a chimera. The Phe4A and Phe4C clones were chosen for further analysis and aligned together with selected sequences from the Genbank database using CLUSTALX (version 1.81) (Thompson *et al.*, 1997). Dendrograms were constructed using the neighbor-joining method in PAUP (Swofford, 2000), and 1000 bootstrap replicates were performed to estimate the support for each branch (Felsenstein, 1985). Denaturing gradient gel electrophoresis (DGGE) was performed as described previously (Muyzer *et al.*, 1993; Duncan *et al.*, 2003), using 'universal' eubacterial primers GM5F and D907R for 16S rRNA that amplify approximately 550 bp.

Results

After several transfers in a mineral medium with phenanthrene as the sole carbon source and electron donor, a sediment-free enrichment culture was obtained. The culture was able to couple phenanthrene degradation with sulfate reduction. Substrate loss was in stoichiometric agreement with the theoretically expected loss of sulfate, assuming complete phenanthrene mineralization (Table 1). Phenanthrene biodegradation was sulfate-dependent and inhibited by 20 mM sodium molybdate (data not shown). Mineralization of phenanthrene was confirmed with ^{14}C -radiolabeled substrate. In all experiments, the amount of radioactivity recovered as $^{14}\text{CO}_2$ / $\text{H}^{14}\text{CO}_3^-$ in the gas and liquid phases was in good agreement with phenanthrene loss as measured by GC in parallel non-radiolabeled incubations (Table 2). After 28 weeks of incubation, mineralization of phenanthrene ranged from approximately 20–45% relative to controls. Sterile controls produced low amounts of $\text{H}^{14}\text{CO}_3^-$ in the liquid phase that could be due to dissolved ^{14}C -phenanthrene or some impurities.

To probe the metabolic pathway of phenanthrene metabolism, the cultures were grown in the presence of either protonated (H_{10})- or fully deuterated (d_{10})-phenanthrene. Biodegradation experiments resulted in the formation of the corresponding unlabeled and labeled phenanthrene-2-carboxylic acid (Figures 1b and c). The unlabeled metabolite was identified based on a matching GC retention time (43.60 min) and mass spectral profile with an authentic standard (Figure 1a). Other standards (phenanthrene-9- and -4-carboxylic acids) were also tested as putative metabolites, but their GC retention times (42.35 and 40.35 min, respectively) did not coincide with that of the metabolite. The labeled metabolite was 9 mass units heavier than the corresponding unlabeled metabolite, accounting for the nine remaining deuterium atoms in the phenanthrene parent substrate (Figure 1c).

To assess whether carboxylation was a possible mechanism of phenanthrene metabolism, enrichment cells were incubated with ^{13}C -bicarbonate in a HEPES-buffered medium. Mass spectral analysis of culture extracts revealed the presence of a metabolite (Figure 1d) containing several fragments (m/z 206, 280 and 295) that were 1 mass unit larger than the corresponding features in the authentic silylated phenanthrene-2-carboxylic acid standard

Table 1 Stoichiometry of phenanthrene degradation coupled to sulfate reduction

Phenanthrene mineralization $\text{C}_{14}\text{H}_{10} + 8.25\text{SO}_4^{2-} + 9\text{H}_2\text{O} \rightarrow 14\text{HCO}_3^- + 4.125\text{HS}^- + 4.125\text{H}_2\text{S} + 1.625\text{H}^+$				
Incubation time	Phenanthrene loss (μmol)	Expected sulfate loss (μmol)	Observed sulfate loss (μmol)	% of expected
33 weeks	23.1 ± 1.15	190 ± 9	208 ± 13	109 ± 6

Standard deviations were determined based on four replicates.

Table 2 Mineralization of radiolabeled (ring- ^{14}C (U); 9- ^{14}C) phenanthrene by a sulfate-reducing enrichment culture

Incubation	Radioactivity in HMN overlay (dpm)	Radioactivity in aqueous ^a phase (dpm) $^{14}\text{CO}_2/\text{H}^{14}\text{CO}_3^-$	Radioactivity in headspace (dpm) $^{14}\text{CO}_2$	Total radio-activity recovered (dpm)	Radioactivity recovery (%)	Substrate degradation (aqueous and headspace gas) (%)	Substrate loss (by GC) ^b (%)
Culture 1	229 600	213 500	7 500	450 600	105	48.9	49
Culture 2	305 600	132 500	12 900	451 000	105	32.3	32.5
Culture 3	345 200	104 000	10 650	459 850	107	25	19.3
Sterile	377 600	25 000	Not detected	402 600	94	6.6	8
Applied radioactivity	427 260						

Abbreviation: GC, gas chromatography.

^aWhen the aqueous phase of nonsterile incubations was acidified and flushed with nitrogen, radioactivity decreased by about 90% indicating the label was essentially mineralized end product ($^{14}\text{CO}_2/\text{H}^{14}\text{CO}_3^-$).^bSubstrate loss was determined by GC in three replicate cultures that were divided at the start of the experiment. One portion of the divided cultures received ^{14}C -phenanthrene, while the other did not. Radiochemical determinations were made on the ^{14}C -amended cultures, while GC analysis was on the corresponding ^{14}C -free incubations.

(Figure 1a). The ions at m/z 235 and 176/177 were the same as the standard owing to the loss of the derivatized $-\text{COO}$ group during mass spectral fragmentation. The silylated ^{13}C -labeled phenanthrene carboxylic acid metabolite eluted at the same retention time as the silylated authentic standard of phenanthrene-2-carboxylic acid (43.60 min). The incorporation of the heavy isotope into the phenanthrene-2-carboxylic acid molecule from $\text{H}^{13}\text{CO}_3^-$ suggested the direct carboxylation of the parent molecule.

Molecular analysis of the phenanthrene-degrading enrichment revealed a low diversity with the DGGE profile of the enrichment consisting of only two bands (not shown). 16S Ribosomal DNA analysis of cloned sequences (Phe4A GenBank accession no. EF467179 and Phe4C GenBank accession no. EF467180) revealed that the enrichment contained δ -Proteobacteria that were phylogenetically similar, but not identical to known anaerobic hydrocarbon-degrading organisms and uncultured clones obtained from hydrocarbon-degrading communities (Figure 2).

Discussion

Although the anaerobic biodegradation of PAHs is now accepted, relatively little is known about the organisms responsible for such bioconversions or the mechanism(s) associated with substrate transformation. In this work, we obtained a highly enriched sediment-free sulfidogenic phenanthrene-degrading culture and sought to elucidate the initial steps involved in the destruction of this three-ringed PAH. DNA sequence analysis revealed just a few clones of δ -Proteobacteria in the culture, which clustered together with other known hydrocarbon degraders and uncultured clones obtained from hydrocarbon-degrading communities. One clone (Phe4C) from the phenanthrene-degrading enrichment was 99% similar to the 16S rDNA sequence of the alkane-degrading sulfate-reducing bacterium strain Hxd3 (proposed new species: *Desulfococcus oleovorans*). The distinguishing feature of the strain Hxd3 is its ability to attack *n*-alkanes by an initial carboxylation reaction (Aeckersberg *et al.*, 1991; So *et al.*, 2003; Callaghan *et al.*, 2006). In contrast, Phe4A is phylogenetically distant from other known sulfate-reducing bacteria, but most similar (91%) to *Desulfotribacter oceanense* (not shown). However, *D. oceanense* is not known to degrade hydrocarbons. The family *Desulfobacteraceae* includes Phe4A, Phe4C and the naphthalene-degrading sulfate-reducing bacterium strain NaphS2, but 16S similarities among the three are 90% or less. With specific regard to phenanthrene, *dsr* sequences cloned from consortia (Pérez-Jiménez *et al.*, 2001) were also related to the *Desulfobacteraceae*, to other families and to various Gram-positive bacteria, suggesting that a fairly diverse group of microorganisms are

To date, two possible mechanisms for anaerobic PAH activation have been reported, with most work focused on the metabolism of two-ring compounds such as naphthalene and 2-methylnaphthalene (Sullivan *et al.*, 2001; Annweiler *et al.*, 2000, 2002). Only one study has helped clarify the metabolism of phenanthrene (Zhang and Young, 1997). In that study, a marine enrichment culture was found to initially attack both phenanthrene and naphthalene by direct carboxylation from an inorganic source, confirmed by the incorporation of ^{13}C -labeled bicarbonate in both parent molecules (Zhang and Young, 1997). Another naphthalene-degrading sulfate-reducing enrichment obtained from an oil-contaminated aquifer initially showed the same pattern (Annweiler *et al.*, 2000; Meckenstock *et al.*, 2000). However, a more recent study with the same culture and deuterated naphthalene demonstrated that the initial attack on the parent substrate was methylation to 2-methylnaphthalene, which was subsequently oxidized to 2-naphthoic acid via a fumarate addition reaction (Safinowski and Meckenstock, 2006). The report of naphthalene methylation resembles the recently described methylation of benzene (Ulrich *et al.*, 2005). For benzene, however, methylation is not the only possible activation

mechanism under anaerobic conditions (Coates *et al.*, 2002; Chakraborty and Coates, 2004; Ulrich *et al.*, 2005). The detection of unlabeled, d_9 -labeled and ^{13}C -labeled phenanthrene-2-carboxylic acid implies that carboxylation is an important initial activation reaction by our marine enrichment culture. In cases where PAHs were activated by other mechanisms and subsequently metabolized, no label was found in carboxylated metabolites after incubation with ^{13}C -bicarbonate. For instance, 2-methylnaphthalene-degrading enrichments produced 2-naphthoic acid, but did not incorporate ^{13}C in the carboxyl group of the metabolite when incubated with labeled bicarbonate (Sullivan *et al.*, 2001). Similarly, a sulfidogenic consortium capable of benzene mineralization did not incorporate ^{13}C -carbon in the carboxyl group of benzoate when the cells were supplemented with ^{13}C -bicarbonate, suggesting that the carboxyl group in the metabolite was not a product of direct carboxylation (Phelps *et al.*, 2001). The positive identification of a metabolite consistent with carboxylation at the C-2 position was not unexpected since the net atomic charge of the carbons at the C-2 and C-3 positions of phenanthrene are most negative and thus most reactive (Hites and Simonsick, 1987). However, since standards of phenanthrene-1- and -3-carboxylic acid were not available for gas chromatography-mass spectrometry comparison, these compounds cannot be conclusively ruled out as possible phenanthrene metabolites.

Although the mass spectral evidence supports carboxylation as a mechanism of phenanthrene oxidation, we cannot rule out other possibilities, such as methylation, that might be simultaneously occurring in the marine enrichment and escape our detection. However, if we suppose that the carboxylic acid group containing ^{13}C that we detected was formed after methylation and subsequent oxidation via fumarate addition, in a manner analogous to naphthalene metabolism (Safinowski and Meckenstock, 2006), we would then have to postulate that an isotopically heavy $^{13}\text{CH}_3$ group be formed from the labeled bicarbonate. This prospect is at least theoretically possible. In bacteria, reduction of a carboxyl equivalent to a methyl group can occur in the energy-requiring methyl branch of the acetyl-CoA pathway (Wood pathway) (Drake, 1994; Ljungdahl, 1994). The initial reduction of CO_2 is catalyzed by formate dehydrogenase. The resulting formate undergoes further reduction to the methyl level via the tetrahydrofolate pathway. Then, the methyl group of CH_3 -tetrahydrofolate is transferred to the cobalt active site of a corrinoid protein. Another molecule of CO_2 is fixed in the carbonyl branch of acetyl-CoA pathway. Finally, the methyl and carbonyl carbon equivalents and CoA are assembled into acetyl-CoA by carbon monoxide dehydrogenase (CODH), the key enzyme in the acetyl-CoA pathway (Ragsdale, 1991, 1994). The acetyl-CoA pathway can work in reverse and some

sulfate-reducing bacteria can oxidize acetate in this manner (Schauder *et al.*, 1986; Zinder, 1994). However, regardless of the direction of this pathway, methyl formation from inorganic carbon would be strongly dependent on CODH and formate dehydrogenase, and require corrinoid and tetrahydrofolate enzymes. At the moment, no strong experimental evidence is available supporting this prospect in anaerobes that degrade PAHs. In their study on naphthalene methylation, Safinowski and Meckenstock (2006) detected CODH in cell extracts; however, the level of activity was quite low. Our metabolic studies did not show any mass spectral evidence suggesting the methylation of phenanthrene or methylphenanthrene succinate formation, although we cannot rule out this possibility. Most probably, both pathways are important for anaerobic PAH metabolism and attest to the metabolic diversity of the requisite bacteria.

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