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14. ABSTRACT
Microbial cultures, capable of anaerobic hydrocarbon degradation, were obtained from the anaerobic marine sediments in San Diego Bay (Paleta Creek sediments). Using n-alkanes (from C_6 to C_{12}), alkyl-benzenes (toluene, ethylbenzene, o-, m-, and p-xylene isomers), and alkyl-cycloalkanes (ethylcyclopentane), in conjunction with ^{13}C nuclear magnetic resonance spectroscopy and gas chromatography-mass spectromerty (some of the hydrocarbon compounds were labeled with either ^{2}H and ^{13}C-nuclei), anaerobic biodegradation reaction pathways and metabolite structures were identified and characterized.

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FINAL REPORT

GRANT #: N00014-99-1-0076

PRINCIPAL INVESTIGATOR: Mark A. Nanny (PI), Joseph M. Suflita (CoPI), R. Paul Philp (CoPI)

INSTITUTION: University of Oklahoma

GRANT TITLE: Intrinsic Anaerobic Bioremediation of Hydrocarbons in Contaminated Marine Sediments

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OBJECTIVES: The long-term goals are to: 1) identify and assess intrinsic anaerobic bioremediation in contaminated marine sediments, 2) determine the environmental parameters that facilitate intrinsic remediation, 3) deter-mine biochemical mechanisms of anaerobic hydrocarbon biodegradation, 4) identify measurable, quantitative parameters such as the formation and concentration of critical metabolites, and 5) use this data to monitor the rate and extent of intrinsic anaerobic bioremediation in the field.

Further, the objectives of this research are to: 1) investigate the fate of individual hydrocarbons degraded under anaerobic conditions, 2) determine reaction pathways, rates, and extent of natural attenuation of hydrocarbons in contaminated marine sediments, 3) develop appropriate analytical methodologies for the assessment of intrinsic anaerobic bioremediation of hydrocarbons in contaminated marine sediments, 4) determine critical metabolites which can be used as signature biomarkers, and 5) detect signature biomarkers in sampling sites as an in situ evidence of biodegradation.

APPROACH: Microbial cultures, capable of anaerobic hydrocarbon degradation, were obtained from the anaerobic marine sediments in San Diego Bay (Paleta Creek sediments). Using *n*-alkanes (from C_6 to C_{12}), alkyl-benzenes (toluene, ethylbenzene, *o*-, *m*-, and *p*-xylene isomers), and alkyl-cycloalkanes (ethylcyclopentane), in conjunction with ^{13}C nuclear magnetic resonance spectroscopy and gas chromatography-mass spectrometry (some of the hydrocarbon compounds were labeled with either ^{2}H and ^{13}C-nuclei), anaerobic biodegradation reaction pathways and metabolite structures were identified and characterized. Individual participants in this work:
Irene A. Davidova, Ph.D - alkane biodegradation, cultural work;
Kevin G. Kropp, Ph.D - alkane biodegradation, GC-MS analysis;
Lisa M. Gieg, Ph.D - alkyl benzenes biodegradation, GC-MS analysis, cultural work
Luis A. Rios-Hernandez -ethylcyclopentane biodegradation (grad student)

CONCLUSIONS: The metabolites formed as the first initial step in biodegradation of H_{26}- and D_{26}-*n*-dodecane by a sulfate-reducing enrichment culture proved to be fatty acids, that likely resulted from C-H and C-D addition across the double bond of fumarate to give dodecylsuccinic acid derivatives containing all the protons or deuteriums of the original alkane. Fumarate addition reactions may represent a common mechanism for anaerobic alkane oxidation and doesn't depend on alkyl chain length. We followed a pattern of an alkylsuccinate formation in course of 10 weeks of culture growth on deuterated decane and demonstrated a transient accumulation of decylsuccinic acid. Transient nature of alkylsuccinates formation argues that they represent the first step in the degradation

pathway rather than a dead end product of side metabolism. Further in the pathway of hexane degradation we detected 4-methyloctanoic acid. It was positively identified by GC-MS in the organic extracts of ^{13}C-hexane-amended culture supernatants. The GC retention time and MS pro-file of the metabolite were identical to those of the authentic standard. NMR and GC-MS studies of the culture incubated with ^{13}C-n-hexane demonstrated that labeled hexylsuccinic acid formed in the experiment contained up to three ^{13}C-atoms, suggesting that the ^{13}C-label of the n-hexane substrate ultimately produced fumarate, which was recycled back into the metabolic process three times. This observation supports a fumarate regeneration pathway occurring during the anaerobic decay of hexane recently proposed in the literature. In vitro experi-ments on hexylsuccinic acid formation in cell-free extracts also showed that product formation doesn't depend on external fumarate. The lack of ^{13}C incorporation from given labeled fumarate suggested that pre-formed fumarate from internal pool was used preferentially for addition reaction, what indirectly supports a hypothetical model for co-substrate regeneration.

A 16S rRNA genes from sulfate-reducing alkane-degrading cultures ALDC and Lake were cloned and sequenced. A phylogenetic analysis showed that sulfate-reducing bacteria from ALCD and Lake cultures were very close (99.3% of similarity), though not identical. They were closely related to *Desulfacinum sp.*with 93 to 94% identity and *Syntrophobacter sp.* with 92 to 95% identity. However, they were only distantly related to other described alkane-degrading sulfate-reducers with similarity ranging from 85 to 88%. A phylogenetic primers 67F and 1023R specific to ALCD-Lake cluster were designed. The primers were highly specific and didn't form products with DNA of any other SRB, including the closest relatives *Desulfacinum sp* or *Syntrophobacter sp.* They can be used as a phylogenetic probe for qualitative and quantitative assessment of this group of alkane-degrading bacteria in the environments.

In our studies on ethylcyclopentane (ECP) degradation by sulfate-reduc-ing bacterial enrichments we detected transient accumulation of several metabolites. Mass spectral similarities to analogous authentic standards allowed us to identify these metabolites as ethylcyclopentyl-succinic acids, ethylcyclopentylpropionic acid, ethylcyclopentyl-carboxylic acid, and ethylsuccinic acid.

We have looked for metabolites indicative of anaerobic alkylbenzene, alkane, and PAH degradation in two hydrocarbon-impacted marine sedi-ments, including samples from harbour near Norfolk, VA (USA) and in the North Sea (Norway). Such metabolites included the signature fumarate addition metabolites (benzyl- and alkyl-succinates), carboxy-PAH and ring-reduced carboxy-PAH from anaerobic PAH decay, and simpler aromatic and alkanoic acids, which could feasibly result during hydrocarbon degradation.

Upon examining hydrocarbon-impacted samples collected from the Norfolk cruise (February 2002), including both the interstitial waters and sedi-ments, we did *not* find any signature metabolites of anaerobic hydrocarbon decay. What we did detect, however, were simple dicar-boxylic acids, such as oxalic acid (ethanedioic acid), propanedioic acid, succinic acid (butanedioic acid), adipic acid (hexanedioic acid), and azelaic acid (nonanedioic acid). Octanoic, nonanoic, decanoic, dodecanoic acids were also present. We also found some hydroxylated monocarboxylic acids, such as 2-hydroxypropanoic acid. In some samples we found benzoic acid, hydro-xybenzoic acid and p-hydroxycinnamic acid. Phthalic acid was also identi-

fied (for example, 1,4-benzenedicarboxylic acid). Some of these aromatic compounds could feasibly be metabolites of aromatic hydrocarbons, however, they are also commonly associated with sediment matter, thus one cannot conclude that they are a result of bacterial hydrocarbon metabolism. Similarly, the alkanoic acids and alkanedioic acids which were found are also commonly associated with sediment matter. In drilling mud-impacted sediments obtained from beneath an oil drilling platform in the the North Sea, we detected similar mono- and di-carboxylic acids as were seen in the Norfolk sediments including octanoic, nonanoic, decanoic, dodecanoic, hexdecanoic, octadecanoic, hexanedioic, octanedioic, nonanedioic, decanedioic, and undecanedioic acids. We also detected benzoic acid, which may be an aerobic or anaerobic metabolite of aromatic hydrocarbon decay. However, in these samples we did detect some metabolites of benzoate decay only known to be produced under anaerobic conditions including cyclohexanecarboxylic acid, pimelate (C7 di-acid), and glutarate (C5 di-acid). Further, in oil-based drilling mud samples, we detected a putative anaerobic fumarate addition metabolite from long-chain alkane. The mass spectral profile of this metabolite contained fragment ions which were similar to those observed for other alkylsuccinates (Kropp et al., 2000; Gieg and Suflita, 2002), with an $(M-15)^+$ ion suggestive of a C17 alkylsuccinate. In water-based drilling mud samples, we positively identified methylsuccinic acid and propylsuccinic acid based on matching gas chromatographic and mass spectral matches with authentic standards. Although not demonstrated in the literature, we can speculate that these latter metabolites may be produced during the anaerobic decay of the smallest hydrocarbons including methane and propane, respectively. We have also found signature metabolites with mass-spectral fragment typical for alkylsuccinates in a variety of other geographically and geologically-distinct hydrocarbon-impacted locations (Gieg and Suflita, 2002). Further, we detected naphthoic acids and tetrahydronaphthoic acids, recently identified metabolites produced during the anaerobic decay of naphthalenes in the groundwaters of several of the hydrocarbon-laden aquifers (Gieg and Suflita, 2002).

We have completed the following work:

1. Accumulation of alkylsuccinic acid metabolites was transient.
2. We detected and identified 4-methyloctanoic acid as a subsequent metabolite further in the pathway of alkane biodegradation.
3. NMR and GC-MS studies of the culture incubated with ^{13}C-n-hexane demonstrated that labeled hexylsuccinic acid formed in the experiment contained up to three ^{13}C-atoms, suggesting that the ^{13}C-label of the n-hexane substrate ultimately produced fumarate, which was recycled back into the metabolic process three times.
4. We detected and identified ethylcyclopentylsuccinic acids, ethylcyclopentylpropionic acid, and ethylsuccinic acid as metabolites in ethylcyclopentane degradation pathway.
5. Based on phylogenetic analysis of alkane-degrading cultures ALDC and Lake highly specific phylogenetic probes 67F and 1023R were designed.
6. Signature metabolites indicative of anaerobic biodegradation of hydrocarbons of different classes (benzyl- and alkyl-succinates, carboxy-PAH and ring-reduced carboxy-PAH from anaerobic PAH decay, and simpler aromatic and alkanoic acids) were detected *in situ* in a variety of geographically and geologically distinct hydrocarbon-impacted sites.

SIGNIFICANCE: Our studies on anaerobic pathway of alkane biodegradation showed that alkylsuccinates formed during the initial step can undergo further degradation with transient formation of fatty acids, such as 4-

methyloctanoic acid. Metabolic pathway of alkane degradation most probably is arranged as a cycle, where fumarate is regenerated from a parent alkane. *In vitro* experiments proved that the process is self-sufficient and doesn't depend on internal pool of fumarate.

Our technique of detection of signature metabolites was useful and sensitive tool to prove the occurrence of *in situ* anaerobic biodegradation of wide range of hydrocarbons in various geographical and geological conditions. Signature metabolites of anaerobic hydrocarbon metabolism along with phylogenetic probe specific for alkane-degrading SRB can be used for evaluation of intrinsic bioremediation in hydrocarbon-contaminated sites.

Our studies along with the research performed by other laboratories demonstrated critical importance of sulfate-reducing bacteria for intrinsic bioremediation of anoxic marine sites, as this group of bacteria can degrade a broad range of hydrocarbons with sulfate as an electron acceptor.

PUBLICATIONS AND ABSTRACTS:

L.M. Gieg and J.M. Suflita. Detection of Anaerobic Metabolites of Saturated and Aromatic Hydrocarbons in Petroleum-Contaminated Aquifers. 2002. Environ.Sci. Technol. 36:3755-3762

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Detection of Anaerobic Metabolites of Saturated and Aromatic Hydrocarbons in Petroleum-Contaminated Aquifers

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Recent investigations have demonstrated that several classes of petroleum hydrocarbons are susceptible to anaerobic decay, including alkanes and mono- and polycyclic aromatic compounds. In previous work, benzylsuccinates were shown to be useful indicators of in situ anaerobic alkylbenzene metabolism. In the present study, we sought to determine whether metabolites of alkanes and naphthalenes could similarly be used as indicators of the intrinsic decomposition of these compounds in petroleum-contaminated aquifers. Such metabolites include succinate derivatives of *n*-alkanes, cyclic alkanes, and alkylaromatic hydrocarbons as well as naphthoic acids. Using gas chromatography–mass spectrometry (GC–MS), we analyzed trimethylsilyl-derivatized organic extracts from six hydrocarbon-contaminated groundwaters for MS fragment ions indicative of such anaerobic metabolites. Geochemical indicators in these aquifers suggested the prevalence of anaerobic processes. In the groundwaters of the contaminated sites, we found compounds whose MS profiles suggested that they were indeed alkylsuccinic acids, ranging from C_3 to C_{11} succinates. Propyl-, hexyl-, octyl-, and decylsuccinic acids were positively identified in the groundwaters by GC–MS matches with chemical or biologically produced standards. In two of the aquifers, we also detected components whose MS profiles matched with authentic standards of naphthoic acids and tetrahydronaphthoic acids. Metabolites were detected in nanomolar concentrations. The finding of these putative anaerobic metabolites of alkanes and naphthalenes signifies the in situ biodegradation of these hydrocarbons and attests to their value as indicators of intrinsic bioremediation.

Introduction

Every year, the United States alone consumes approximately 250 billion gal of petroleum (*1*). Despite such reliance on fossil fuels, only a fraction of a percent (0.0001%) of petroleum hydrocarbons are accidentally released worldwide (*2*). However, these releases can do a differential amount of damage to various ecosystems with many petroleum hydrocarbons residing in environments that are oxygen-limited. Within the last two decades, it has become evident that anaerobic microorganisms can biodegrade different classes of hydrocarbons associated with such contamination. These include the relatively water-soluble BTEX compounds (benzene, toluene, ethylbenzene, and xylenes) and the less water-soluble saturated and polycyclic aromatic hydrocarbon (PAH) components of crude oil and other petroleum mixtures (*3*).

Knowledge of the mechanisms of anaerobic hydrocarbon biodegradation can be garnered from studies of alkylbenzene decay. Specifically, investigations with toluene have shown that anaerobes employ a novel enzymatic mechanism, distinct from that used by aerobes, to catalyze the initial activation of such hydrocarbons. Metabolic studies with nitrate-reducing species of *Thauera* and *Azoarcus* have shown that this mechanism involves a glycyl radical enzyme, benzylsuccinate synthase, which catalyzes the addition of the methyl group of toluene to the double bond of fumarate yielding benzylsuccinic acid (*4*–*7*). Benzylsuccinate is then thioesterified to its CoA derivative by a succinyl-CoA-dependent CoA transferase (*8*) and further transformed to (*E*)-phenylitaconic acid (or its CoA thioester). The latter compound presumably undergoes modified β-oxidation yielding benzoyl-CoA (*9*). This mechanism of toluene decay has also been reported in other studies with anaerobes incubated under sulfate-reducing (*10, 11*), methanogenic (*12*), and anoxic phototrophic conditions (*13*). An analogous fumarate addition reaction to a hydrocarbon has been shown for *m*-xylene conversion to 3-methylbenzylsuccinic acid by a denitrifying organism (*14*). The transient accumulation of the respective benzylsuccinic acid analogues of ethylbenzene, *o*-, *m*-, and *p*-xylene metabolism in sulfate-reducing enrichments further attests to this enzymatic reaction (*15*).

Recent investigations have demonstrated that this initial activation mechanism is not limited to anaerobic alkylbenzene decay. During sulfidogenic *n*-dodecane decay, Kropp et al. (*16*) detected two closely eluting metabolite peaks by GC–MS that were not present in sterile controls. The mass spectra of these peaks were identical and consistent with the structure of dodecylsuccinic acid (trimethylsilyl (TMS) ester). Although the metabolites had the same MS profile as an authentic standard of *n*-dodecylsuccinate (TMS ester), they eluted at different GC retention times, confirming that the succinyl portion of the molecule was present at a subterminal position on the dodecyl moiety. Indeed, such a molecule has two chiral centers and could exist as diastereomers that were likely chromatographically resolved. This finding suggested that anaerobic microorganisms enzymatically attack alkanes by fumarate addition in a manner analogous to that shown for toluene. Subsequent studies with a denitrifying, *n*-hexane-utilizing isolate (*17*) demonstrated that fumarate is indeed added to the alkane molecule at a subterminal position, forming diastereomers of hexylsuccinic acid. Evidence also suggested the involvement of a radical in the initial reaction of *n*-hexane metabolism (*17*) not unlike that demonstrated for anaerobic toluene decay (*18*). Recent work with this *n*-hexane-utilizing denitrifier demonstrated that hexylsuccinate ([1-methylpentyl]succinate) is further converted to 4-methyloctanoate, which is subsequently metabolized to central metabolic intermediates (*19*).

The identification of naphthyl-2-methyl- and naphthyl-2-methylenesuccinic acid in the supernatants of a sulfate-reducing enrichment culture incubated with 2-methylnaphthalene (*20*) demonstrates that a similar addition reaction can also occur for alkylated PAHs. Such a reaction is not known for unsubstituted aromatic hydrocarbons, such as benzene or naphthalene. Under sulfate-reducing conditions, naphthalene decay is initiated by carboxylation, forming 2-naphthoic acid (*21, 22*), which then undergoes ring reduction yielding tetrahydro-, octahydro-, and decahy-

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TABLE 1. Features of the Hydrocarbon-Contaminated (HC) Sites Examined in This Study

site designation	location	contaminants	geochemical details
CO	Weld County, CO	gas condensate; up to 20 mg/L BTEX and 23 mg/L TPH[a]	sandy aquifer; water table at 1.7 m; SO_4^{2-} depleted (0–100 mg/L) and CH_4 accumulated (up to 15 mg/L) in HC area relative to background[b] (200 mg/L SO_4^{2-}, no CH_4)
TX	Wise County, TX	natural gas liquids; 1.3–7.4 mg/L C_3–C_9 alkanes	sulfide accumulation (26–292 mg/L) in HC area relative to background (no S^{2-})
KS	Sedgwick County, KS	gasoline range organics; 6–27 mg/L BTEX; 18–50 mg/L TPH[c]	SO_4^{2-} (2–28 mg/L) and NO_3^- (0 mg/L) depleted and CH_4 accumulated (13–43 mg/L) in HC area relative to background (25–38 mg/L NO_3^-, 66–167 mg/L SO_4^{2-}, no CH_4)
AB1	southwest Alberta	diesel; 1 mg/L BTEX/TPH[d]; 1–30 mg/L TEH[e]	fractured sandstone; water table at 30 m; SO_4^{2-} depleted (0–20 mg/L) in HC area relative to background (40 mg/L)
AB2	west central Alberta	variable hydrocarbon mixture underlying a former flare pit; 0.5–10 mg/L TPH[f]; 0.5–60 mg/L TEH[e]	silt-clay till over sandstone; water table at 1-2 m; SO_4^{2-} depleted (1 mg/L) and some CH_4 produced (0.3 mg/L) in HC area relative to background (8 mg/L SO_4^{2-}, no CH_4)
AB3	east central Alberta	gas condensate; 0.01–0.025 mg/L BTEX; 0.2–2.1 mg/L TPH; 0.6 mg/L TEH[e]	sand-silt till; water table at 5-6 m; NO_3^- (<1 mg/L) and SO_4^{2-} (1–5 mg/L) depleted, Fe^{2+} (20 mg/L), Mn^{2+} (3 mg/L) elevated in HC area relative to background (300 mg/L NO_3^- [g], 20–40 mg/L SO_4^{2-}, <0.1 mg/L Fe^{2+} or Mn^{2+})

[a] These average values have been previously reported (*15, 42*); TPH, total petroleum hydrocarbons ranging from C_6 to C_{20}. [b] Background refers to hydrogeologically related uncontaminated groundwater. [c] TPH analysis includes C_5–C_{12} hydrocarbons, including alkanes and naphthalene. [d] TPH analysis includes C_3–C_{10} hydrocarbons. [e] TEH, total extractable hydrocarbons ranging from C_{11} to C_{30}. [f] TPH measurements include BTEX. [g] From nitrate impact.

dronaphthoic acids (*22, 23*) in a manner similar to anaerobic benzoate metabolism (*24*). These reduced products, also observed in 2-methylnaphthalene-degrading cultures (*20*), have recently been shown to undergo further ring cleavage to simpler products that can then funnel into central metabolic pathways (*25*).

The discovery of such novel anaerobic metabolites can be applied to evaluate the prospects for the in situ bioremediation of petroleum-contaminated environments. Given the large number of U.S. sites impacted by fuel components (418 000 as of September 2001; *26*), relying on natural biodegradation processes can be an attractive, low-cost approach to site cleanup. Evidence for the intrinsic bioremediation of hydrocarbons can be attained by collecting multiple lines of evidence to document geochemical and microbiological differences between contaminated and uncontaminated regions of the same environment. Recent advances in anaerobic metabolic pathways for alkylbenzenes and the detection of signature metabolites in situ have been particularly useful for demonstrating intrinsic BTEX biodegradation (*15, 27–29*).

However, a comparable effort has not been undertaken for other hydrocarbons. The regulatory focus on aquifer contamination with BTEX hydrocarbons is understandable given the relatively high aqueous solubility of these compounds and their potential for human health impact. While BTEX components comprise a substantial fraction of most petroleum products, saturated hydrocarbons are often quantitatively more important (*30, 31*). While the latter components are considered of lesser health concern relative to BTEX hydrocarbons, exposure to straight and branched alkanes can negatively impact a variety of organisms, including humans (*32–34*). For example, *n*-hexane is a known neurotoxin (*34, 35*). Furthermore, even though aliphatic and alicyclic fuel components are less water-soluble that BTEX, many are soluble enough to create concern when they contaminate groundwaters (*31*). Indeed, the levels of these compounds are closely monitored and regulated alongside BTEX hydrocarbons as part of soil and groundwater cleanup standards in much of the United States (*36*). Polycyclic aromatic hydrocarbons, prevalent in many fuel mixtures, have long been on the U.S. EPA list of priority pollutants (*37*) and are also regulated as groundwater contaminants in many States (*36*). In fact, the acceptable groundwater target level for naphthalene, one of the most water-soluble PAHs, is usually lower than that for the TEX hydrocarbons (*36, 38*).

Thus, in this study, we investigated whether metabolites of hydrocarbons other than BTEX could also be detected in fuel-impacted environments and be potentially useful for deducing if in situ biodegradation of these compounds was occurring. We found that many such putative metabolites are present in a variety of geologically and geographically distinct hydrocarbon-impacted aquifers and thus offer evidence that a variety of hydrocarbon classes are amenable to in situ metabolism.

Materials and Methods

Environmental Sampling and Analysis. To search for putative anaerobic hydrocarbon metabolites, groundwater samples were collected from six petroleum-impacted aquifers. Site designations, locations, and characteristics suggesting the in situ activity of anaerobic microorganisms are summarized in Table 1. In all cases, 1-L groundwater samples were collected into clean glass bottles and acidified immediately to pH < 2 with 50% HCl or H_2SO_4 to preserve putative acidic metabolites. Samples were collected from contaminated and uncontaminated groundwater wells associated with each aquifer. The 1-L groundwater samples were extracted with ethyl acetate, dried over anhydrous sodium sulfate, and concentrated by rotary evaporation and under a stream of N_2. Organic extracts were subsequently reacted with BSTFA (*N,O*-bis(trimethylsilyl)trifluoroacetimide; Pierce Chemical Co., Rockford, IL) to form trimethylsilyl (TMS) esters. To confirm the presence of metabolites, selected groundwater extracts were also reacted with a freshly prepared ethereal solution of diazomethane to form methyl esters. Diazomethane was prepared from *N*-methyl-*N*-nitroso-*N*-nitroguanidine (Aldrich, Milwaukee, WI). Unless otherwise noted, however, all metabolites discussed herein were analyzed and identified as TMS derivatives.

Derivatized components were separated on a Hewlett-Packard (HP) 5890 GC equipped with a DB-5 capillary column (30 m × 0.25 mm i.d., J&W Scientific, Folsom, CA). The oven temperature was held at 40 °C for 5 min, then increased at 4 °C per min to 300 °C, and held for 5 min. A HP 5970 mass selective (MS) detector acquired data in the scan mode, from 25 to 600 mass units. The "extracted ion chromatograms" function in HP G1034C MS ChemStation Software (version 3.0) was used to probe total ion chromatograms for the presence of components having specific fragment ions used to identify metabolites.

Identification and Quantification of Metabolites. Putative hydrocarbon metabolites in the environmental extracts were positively identified by matching GC retention times and MS profiles with those of known standards. Methylsuccinic acid, 1,2,3,4-tetrahydro-2-naphthoic acid, benzylsuccinic acid, and 1- and 2-naphthoic acids were acquired from Aldrich. Authentic *n*-propylsuccinic acid was a gift from the laboratory of Dr. Frank Abbott, Faculty of Pharmaceutical Sciences, University of British Columbia. Tentative identifications of some metabolites were made by comparison with published MS profiles or by analogy with the MS profiles of authentic standards. To determine the GC retention times and MS profiles of alkylsuccinates resulting from the anaerobic biodegradation of a variety of alkanes, a sulfidogenic *n*-dodecane-degrading culture was incubated with *n*-hexane, *n*-octane, or *n*-decane as previously described (*16*). The GC retention times and MS profiles of the resulting TMS-derivatized metabolites produced from each substrate were used as standards with which to compare putative metabolites in groundwater samples. Such biologically produced metabolites have been used previously to identify alkylbenzylsuccinates in environmental samples when chemical standards were not commercially available (*27*). Alkylbenzylsuccinic acids were identified based on matching GC retention times and MS profiles with authentic standards synthesized as previously described (*15*).

To determine the approximate concentrations of putative anaerobic alkane metabolites in selected groundwater samples, calibration curves were prepared from TMS-derivatized methylsuccinic acid or *n*-octylsuccinic acid. Methylsuccinic acid was used to determine the concentrations of putative alkane metabolites up to C_4, whereas *n*-octylsuccinic acid calibrations were used to quantify putative metabolites of C_5 alkanes or greater. This latter authentic standard was prepared by reacting *n*-octylsuccinic anhydride (TCI America, Portland, OR) with NaOH in methanol in a manner analogous to that described by Kropp et al. (*16*). *n*-Octylsuccinic acid was purified (mp 85–87 °C, lit. mp 87 °C; *39*) before using to prepare a calibration curve. To quantify putative anaerobic metabolites of naphthalenes, calibration curves were prepared from the TMS esters of 2-naphthoic acid and 1,2,3,4-tetrahydro-2-naphthoic acid. The use of these limited standards to determine concentrations of putative metabolites in environmental samples assumes that all compounds have a comparable response factor to GC–MS analysis, so the reported concentrations are only approximate. Alkylbenzylsuccinates were quantified as indicated previously (*15*).

Results

Anaerobic Alkane Metabolites Produced in Laboratory Enrichments. A dodecane-degrading, sulfate-reducing enrichment culture amended with *n*-hexane, *n*-octane, or *n*-decane utilized each of these alkanes, producing hexylsuccinic, octylsuccinic, or decylsuccinic acid intermediates, respectively (Figure 1). For each metabolite, two peaks with identical MS profiles eluted. Identifications were based on MS fragment ion analogies with dodecylsuccinic acid produced during dodecane degradation by this enrichment culture (*16*). When derivatized as TMS esters, these novel alkane metabolites were characterized by similar and distinctive mass spectral features. For example, the MS profiles of the alkylsuccinates resulting from *n*-C_6, *n*-C_8, and *n*-C_{10} degradation all have the same fragments ions at *m/z* 73, 147, 172, 217, and 262 (Figure 1), seen also in the MS profile of the TMS ester of dodecylsuccinate (*16*). Specifically, the fragment ion at *m/z* 262 was previously identified as being the most distinctive for TMS-derivatized alkylsuccinates as it represents the succinyl portion of the molecule as a result

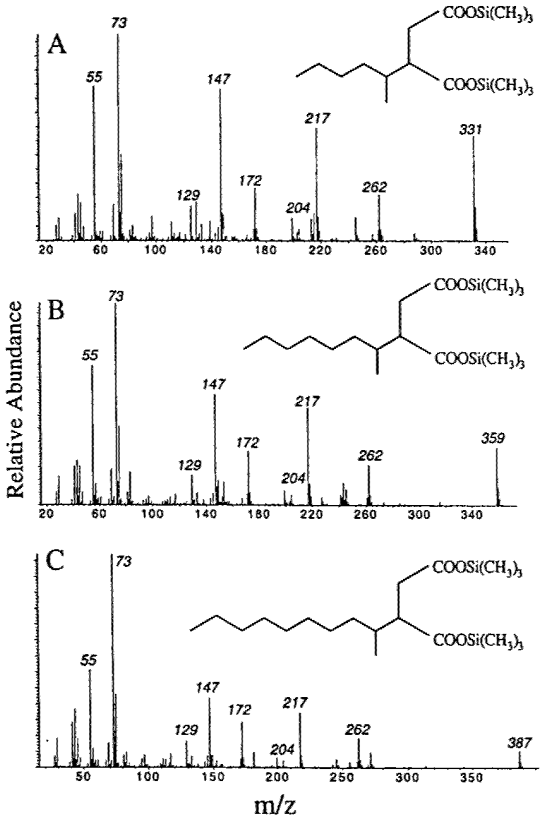

FIGURE 1. Mass spectral profiles of putative alkylsuccinic acids (analyzed as TMS esters) produced during the decay of (A) *n*-hexane, (B) *n*-octane, and (C) *n*-decane by a sulfate-reducing enrichment culture. The succinyl moiety is drawn on the C-2 position of the alkane based on previous studies (*16, 17, 41*).

of a McLafferty rearrangement (*16*). Furthermore, each alkylsuccinate was characterized by a unique $(M - 15^+)$ fragment ion (molecular mass less 15 mass units, a common fragment observed in TMS esterifications due to the loss of a methyl group from the TMS moiety; *40*), depending upon the carbon length of the alkane. Thus, we observed that the TMS-derivatized hexylsuccinate has an $(M - 15)^+$ fragment ion of *m/z* 331, whereas that for octyl- and decylsuccinate was 359 or 387, respectively (Figure 1). The MS profiles of the metabolites produced during *n*-octane degradation matched that of authentic *n*-octylsuccinic acid but did not match in GC rentention time. This confirms that the succinyl moiety is not attached at the terminal methyl group but rather must be attached subterminally as reported previously (*16, 17*). In Figure 1, the succinyl groups are drawn on the carbon atom in the second position of the alkyl moiety, based on previous observations (*16, 17, 41*). To confirm the identity of these putative metabolites, the culture extracts were also reacted with diazomethane to form methyl esters and analyzed by GC–MS (not shown). Again, common fragment ions were present in the MS profiles of the different alkylsuccinates. These included the fragment ions observed at *m/z* 146 (loss of $H_3COOC\text{-}CH_2\text{-}CH_2\text{-}COOCH_3$, analogous to the McLafferty rearrangement ion identified for TMS esters) and at *m/z* 114 (loss of CH_3OH from the *m/z* 146 ion). The culture metabolites also had different $(M - 31)^+$ ions (loss of $-OCH_3$, common with methyl esters) and $(M - 73)^+$ ions (loss of $-CH_2COOCH_3$) depending on the length of the alkyl moiety. The mass spectrum of the methyl ester of the hexylsuccinic acid was

TABLE 2. Alkane, Naphthalene, and Alkylbenzene Metabolites Detected in Hydrocarbon-Impacted Aquifers Examined in This Study[a]

putative alkylsuccinate from	characteristic $(M-15)^+$ ion[c]	metabolite detected at site[b]					
		CO[d]	TX	KS	AB1	AB2	AB3
C3	289		+				
C4	303		+				+
C5	317		+	+		+	
C6	331	+	+			+	+
C6 (with unsaturation)	329	+	+	+			+
C7	345	+				+	
C7 (with unsaturation)	343	+				+	+
C8	359	+				+	+
C8 (with unsaturation)	357	+					
C9	373	+				+	
C9 (with unsaturation)	371						+
C10	387					+	
C11	401					+	

putative naphthalene metabolite	characteristic *m/z*	metabolite detected at site[b]					
		CO[d]	TX	KS	AB1	AB2	AB3
2-naphthoic acid	127, 155, 185, 229 $(M-15)^+$, 244			+	+		
1-naphthoic acid	same as above			+	+		
C1-naphthoic acid	141, 169, 199, 243 $(M-15)^+$, 258			+	+		
C2-naphthoic acid	155, 183, 213, 257 $(M-15)^+$, 272				+		
1,2,3,4-tetrahydro-2-naphthoic acid	130, 233 $(M-15)^+$, 248				+		
5,6,7,8-tetrahydro-2-naphthoic acid	159, 189, 233 $(M-15)^+$, 248			+	+		

putative alkylbenzyl-succinate from[e]	metabolite detected at site[b]					
	CO[d]	TX	KS	AB1	AB2	AB3
xylene	+ (*m*-, *p*-)		+ (*o*-, *m*-)	+ (*m*-)		
ethylbenzene	+				+	+

[a] The characteristic fragment ions used to probe total ion chromatograms of TMS-derivatized groundwater extracts are included. [b] Indicated with a plus sign (+). [c] All alkylsuccinic acid metabolites derivatized as trimethylsilyl esters had the fragment ions at *m/z* 73, 147, 172, 217, and 262 in their mass spectral profiles in addition to the characteristic $(M-15)^+$ fragment shown. [d] Site designations are denoted in Table 1. [e] Characteristic fragment ions for alkylbenzene metabolites derivatized as trimethylsilyl esters have been previously reported (*15*); benzylsuccinic acid (from toluene) was not detected at any of the sites.

identical to that of the metabolite previously produced by a *n*-hexane-utilizing denitrifying bacterium (*17*).

Anaerobic Alkane Metabolites Detected In Situ. We postulated that the distinctive mass spectral features associated with anaerobic alkane metabolism could be used to examine contaminated environments for evidence of in situ decomposition of these hydrocarbons. Initially, we tested this by analyzing groundwater samples from a well-characterized gas condensate-impacted aquifer (designated CO in Table 1) where we have previously detected alkylbenzylsuccinic acids resulting from in situ anaerobic alkylbenzene metabolism (*15, 28*). While the gas condensate spilt into this aquifer contains approximately 25% (w/w) BTEX, it is composed mainly of nonaromatic hydrocarbons (68% w/w) including *n*-alkanes (29.9% w/w), branched alkanes (26.5% w/w), and cycloalkanes (11.6% w/w). Although these classes of hydrocarbons are generally less environmentally mobile than BTEX, many components dissolve in groundwater (*31*) and contaminate aquifers. Thus, if the indigenous bacterial populations were contributing to the anaerobic decay of these hydrocarbons as well as the alkylbenzenes, the total ion chromatograms of solvent-extracted, TMS-derivatized groundwater samples from this site could be probed for the presence of putative metabolites possessing the McLafferty rearrangement ion (*m/z* 262) like the corresponding intermediates found in the laboratory cultures. Indeed, we found many compounds within the groundwater extracts possessing this mass spectral feature (Figure 2). An examination of the MS profiles of these compounds revealed the presence of the other characteristic fragment ions common to alkylsuccinates including *m/z* 73, 147, 172, and

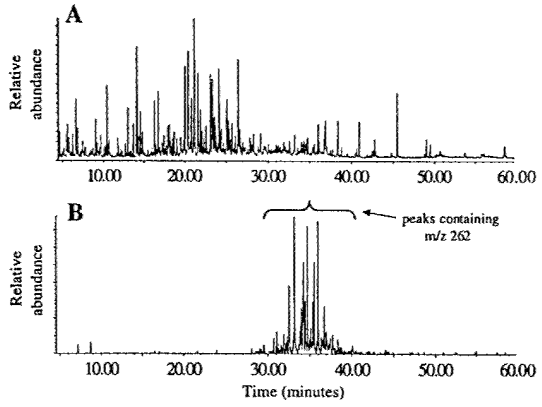

FIGURE 2. (A) Total ion chromatogram of extracted gas condensate-contaminated groundwater (from site CO) derivatized with TMS and (B) selected ion chromatogram showing components within this extract that have a fragment ion at *m/z* 262, indicative of the succinyl moiety of alkylsuccinic acids derivatized with TMS.

217. On the basis of the differing $(M-15)^+$ ions, we tentatively identified alkylsuccinates originating from C_6 to C_9 alkanes in the groundwater samples from this gas condensate-contaminated aquifer (Table 2). Figure 3A,B shows the MS profiles of alkylsuccinic acids thought to originate from C_6 and C_8 decay in situ. When the total ion chromatogram was examined for the presence of $(M-15)^+$ ions particular to an alkylsuccinate of a given carbon chain length, we observed a cluster of peaks (usually greater than two peaks) having the

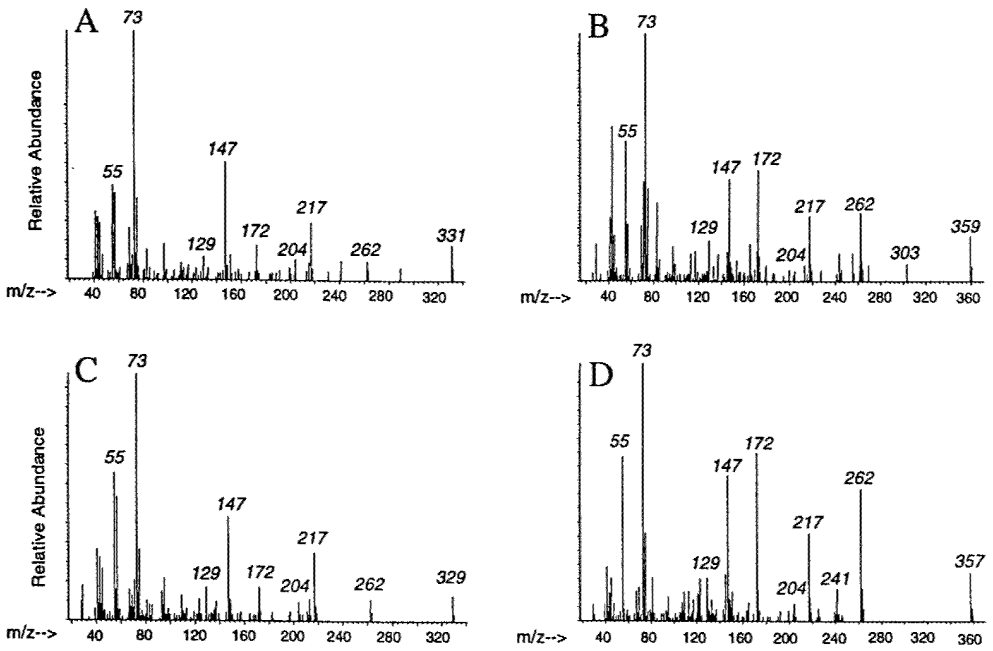

FIGURE 3. Typical mass spectral profiles of putative anaerobic alkane metabolites detected in gas condensate-impacted groundwater. All analyses were as TMS derivatives. (A) Tentatively identified hexylsuccinic acid ($M^+ - 15 = 331$). (B) Tentatively identified octylsuccinic acid ($M^+ - 15 = 359$). (C) Possible metabolite of a cyclic C_6 hydrocarbon ($M^+ - 15 = 329$). (D) Possible metabolite of a cyclic C_8 hydrocarbon ($M^+ - 15 = 357$). The GC retention times of the metabolites in panels A and B matched those produced by a sulfate-reducing enrichment culture incubated with *n*-hexane or *n*-octane, respectively.

same MS profile. At least one of the peaks having an MS profile indicative of a C_6 or C_8 succinate matched in GC retention time with those metabolites detected in the alkane-degrading bacterial culture.

For some of the putative metabolites detected at the CO site, an $(M - 15)^+$ ion with 2 mass units less than that predicted for a straight-chain alkylsuccinate was observed, suggesting that the metabolite resulted from a compound with one degree of unsaturation, such as an alkene or a cyclic alkane (Table 2). For example, the $(M - 15)^+$ ion of a straight-chain C_8 succinate would be at m/z 359 (Figure 1B), whereas that for an unsaturated compound would be at m/z 357. Straight-chain alkenes were not quantitatively important in the spilt gas condensate, but cyclic alkanes comprised about 12% (w/w) of this hydrocarbon mixture. Thus, we presume that the metabolites were more likely due to the anaerobic metabolism of alicyclic hydrocarbons. Figure 3C,D shows mass spectral profiles of such presumed succinates characterized by $(M - 15)^+$ ions having 2 mass less units than their straight-chain alkane counterparts (Figure 3A,B). Positive identifications of these compounds await synthesis of authentic standards. None of these putative alkane metabolites was detected in uncontaminated groundwater samples.

The finding of such putative anaerobic alkane metabolites at this well-characterized contaminated aquifer prompted us to similarly examine other petroleum-impacted areas. The sites were those where geochemical indicators suggested that anaerobic biodegradation may be occurring (Table 1). For example, at an aquifer in Texas (site TX), groundwater wells impacted with relatively low concentrations of natural gas liquids (comprised predominantly of C_1–C_9 alkanes) contained elevated levels of sulfide relative to uncontaminated wells (Table 1). The GC–MS analyses of such groundwater samples revealed compounds containing the McLafferty rearrangement ion (m/z 262), other distinctive ions (m/z 73, 147, 172, and 217; *16*), and $(M - 15)^+$ ions associated with alkylsuccinic acid derivatives from *n*-alkanes ranging from C_3 to C_6 (Table 2). In contrast, these fragment ions were not detected in sulfide-free groundwater extracts. Of the two peaks having identical MS profiles with an $(M - 15)^+$ ion of m/z 289, one (second peak indicated in Figure 4A) matched in both GC retention time and MS profile with that of an authentic standard of *n*-propylsuccinic acid (not shown). The other peak observed may be a structural isomer of this compound in which the succinyl group was added at a subterminal carbon. As was observed with the CO site, when the total ion chromatogram was probed for a particular $(M - 15)^+$ ion, at least two chromatographically resolved components eluted that had identical MS profiles (Figure 4A).

Multiple GC peaks with similar MS profiles were also observed in extracts of highly contaminated groundwater lying below a former flare pit (Figure 4B). In the hydrocarbon-impacted groundwater at this site (AB2), sulfate consumption and methane production were observed relative to uncontaminated groundwater samples (Table 1). At site AB2, alkylsuccinate metabolites of alkanes ranging from C_5 to C_{11} were detected (Table 2). Putative metabolites of C_6, C_8, and C_{10} detected in the groundwater matched in both retention time and MS profile with those produced by the alkane-degrading bacterial culture. However, still other peaks eluting at later retention times (i.e., after 32 min) had the same MS profile as biologically produced hexylsuccinic acid (Figure 4B). The presumed hexylsuccinic acids detected in two different groundwater samples (TX and AB2) eluted at the same GC retention time (Figure 4). A variety of putative alkylsuccinates were also detected at sites KS and AB3, as indicated in Table 2.

Nanomolar concentrations of alkylsuccinic acids were measured in the various groundwater samples (Figure 4). With methyl- or *n*-octylsuccinic acid as representative calibration standards, the estimated concentrations of alkylsuccinates ranged from approximately 10 to 170 nM (Figure 4). For some of the groundwater extracts (e.g., site AB3), it

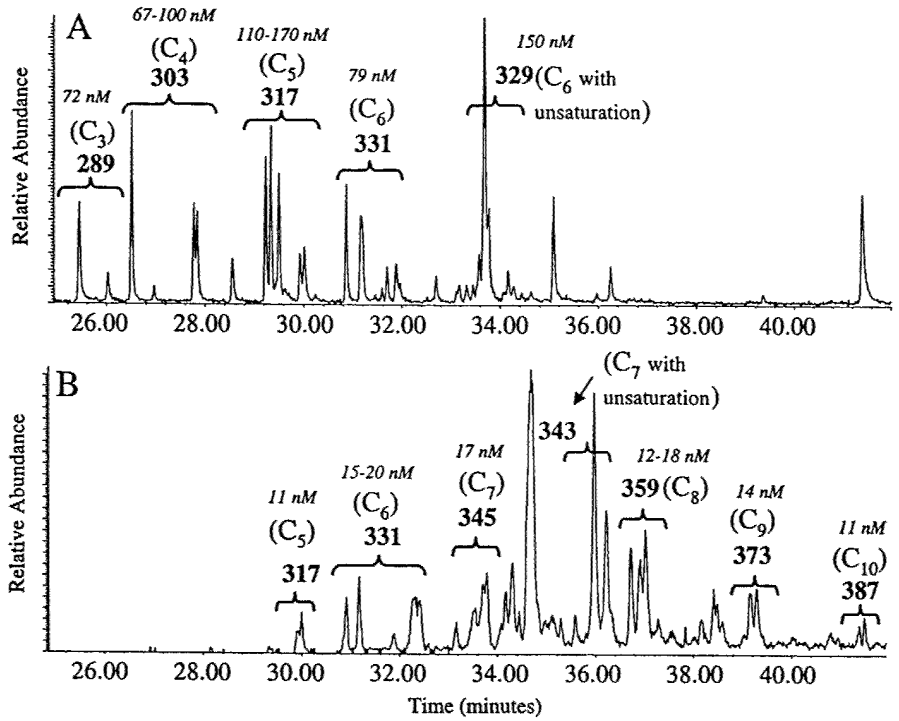

FIGURE 4. Portions of total ion chromatograms examined for the *m/z* 262 fragment ion from TMS-derivatized extracts of (A) groundwater impacted with natural gas liquids (C_1–C_5) and (B) groundwater underlying a flare pit contaminated with a variety of hydrocarbons. Bracketed peaks indicate those eluted components that have the same MS profile, including the $(M - 15)^+$ fragment. The bracketed letter/number above indicates the alkyl chain length from which the alkylsuccinate is presumably derived. The italicized numbers indicate the estimated concentration(s) of the alkylsuccinates in the groundwater.

was difficult to quantify a few of the alkylsuccinates because the GC peaks were barely above the baseline and near the detection limit. Such low concentrations attest to the extremely low concentrations of these metabolites generally found in natural environments. Clearly, however, they are present in sufficient amounts to be qualitatively detected and identified in contaminated, but not in uncontaminated, environments.

Extraction and reaction of replicate groundwater samples from the TX and AB2 sites with diazomethane confirmed the presence of alkylsuccinic acids in the groundwaters. The resulting MS profiles of the metabolites contained fragments at *m/z* 146 and 114 but were characterized by differing $(M - 31)^+$ and $(M - 73)^+$ ions, consistent with the results described above and in previous reports (*16, 17*). Overall, we found that TMS esterification resulted in more thorough reactions and sharper peak resolutions for the eluting alkylsuccinates in the environmental samples (data not shown).

Anaerobic Metabolites of Naphthalenes and Alkylbenzenes Detected In Situ. Extraction of groundwater from another anaerobic, diesel-contaminated site (AB1, Table 1) revealed no alkane metabolites despite the presence of such hydrocarbons in the petroleum mixture (specific data not shown). Instead, we detected numerous putative anaerobic metabolites of naphthalene and/or alkylnaphthalenes (Table 2). For example, we positively identified 2-naphthoic acid by GC–MS comparison with an authentic standard (Figure 5A,B). This compound has been reported as a metabolite produced during anaerobic naphthalene and 2-methylnaphthalene decay (*20–23*). We also detected components in these extracts whose mass spectra contained fragment ions which were 14 or 28 mass units higher than an authentic standard of TMS-derivatized 2-naphthoic acid, suggesting that these compounds may be methyl- or dimethylnaphthoic acids, respectively (Figure 5C,D). Furthermore, 1,2,3,4-tetrahydro-2-naphthoic acid was positively identified (Figure 5 E) on the basis of matching GC retention time and mass spectrum with an authentic standard (not shown). A metabolite in this diesel-impacted groundwater whose MS profile was identical to a published spectrum of a TMS-esterified standard of 5,6,7,8-tetrahydronaphthoic acid (*23*) was also found (Figure 5 F). No further ring reduction products, such as octa- or decahydronaphthoic acids, were detected, but cyclohexanecarboxylate was positively identified (data not shown). It has been postulated that this latter compound may formed following the cleavage of decahydronaphthoic acid (*22*). In the groundwaters of site KS, we also positively identified putative naphthalene metabolites, including 1- and 2-naphthoic acids, and tentatively identified 5,6,7,8-tetrahydronaphthoic acid (*23*).

As with the alkylsuccinic acids, nanomolar concentrations of the naphthalene metabolites were measured. 2-Naphthoic acid was detected at the highest concentration, from 28 to 30 nM. Putative methylnaphthoic and dimethylnaphthoic acid peaks ranged from 8 to 27 nM and from 5 to 10 nM, respectively, while tetrahydronaphthoic acids were present at 5–13 nM. Portions of the groundwater extracts from the AB1 and KS sites derivatized with diazomethane confirmed the presence of 2-naphthoic acid and 5,6,7,8-tetrahydronaphthoic acid. The resulting MS profiles matched those previously published (*22*).

In addition to the detection of alkylbenzylsuccinates previously reported for the CO site (*15*), these signature anaerobic metabolites of alkylbenzene decay were also detected in four of the other five sites examined in this study (Table 2). The concentrations detected ranged from as low as 10–45 nM (sites AB2 and AB3) to as high as 1 mM (sites

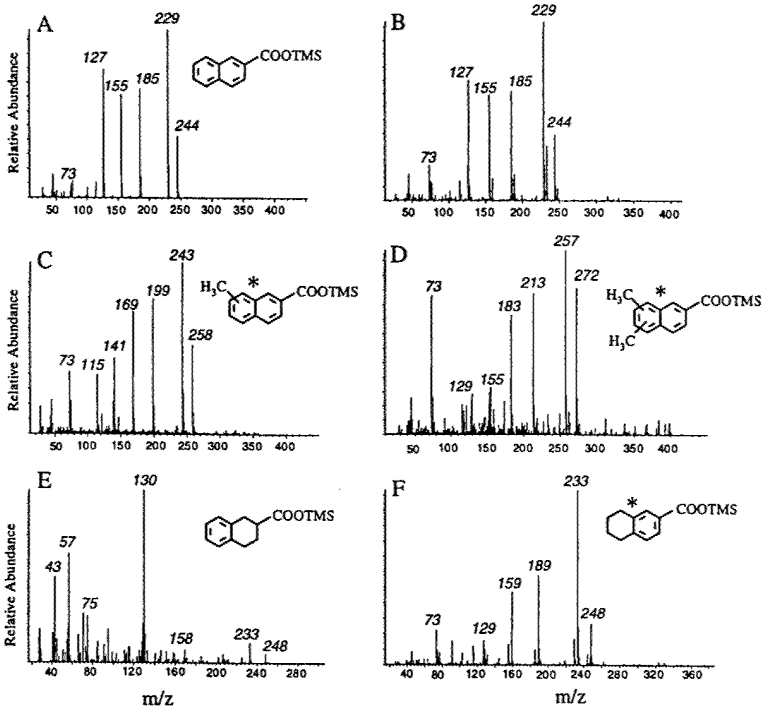

FIGURE 5. Mass spectra of anaerobic naphthalene metabolites detected in diesel-contaminated groundwater at site AB1 (analyzed as TMS derivatives). Structures marked with an asterisk are tentative. (A) Authentic standard of 2-naphthoic acid. (B) Metabolite detected in groundwater having the same GC retention time and MS profile as authentic 2-naphthoic acid. (C) Metabolite detected in groundwater having fragment ions that are 14 mass units higher than authentic 2-naphthoic acid, tentatively identified as methylnaphthoic acid. (D) Metabolite detected in groundwater having fragment ions that are 14 mass units higher than metabolite in panel C and 28 mass units higher than an authentic standard of 2-naphthoic acid, tentatively identified as dimethylnaphthoic acid. (E) Metabolite in groundwater having the same GC retention time and MS profile as authentic 1,2,3,4-tetrahydro-2-naphthoic acid. (F) Metabolite detected having the same MS profile as a 5,6,7,8-tetrahydronaphthoic acid standard published by Zhang et al. (*23*).

KS and AB1) and are similar to previously reported values (*15, 29*).

Discussion

The finding of putative anaerobic metabolites of alkanes and naphthalenes in anaerobic hydrocarbon-contaminated aquifers suggests that such metabolites may serve as indicators of the in situ biodegradation of these classes of hydrocarbons. This work extends the previous observations of anaerobic fumarate addition metabolites of alkylbenzenes that serve as indicators of the in situ decay of the TEX hydrocarbons (*15, 27, 29*). Indeed, we observed alkylbenzylsuccinic acids in the geographically distinct sites examined in this study, suggesting that such reactions are relatively common in anaerobic hydrocarbon-impacted environments. Furthermore, putative metabolites were found in sites with both relatively low (sites TX and AB3) and high hydrocarbon concentrations (sites CO, KS, AB1, and AB2) (Table 1), demonstrating that their detection is not limited to the latter aquifers.

Given the limitations of mass spectrometry, it is feasible that the fragment ion at m/z 262, which we found to be most diagnostic of silylated alkylsuccinates, could be present in the MS profiles of non-succinic acid-type compounds. However, only the components whose mass spectra contained m/z 262 and each of m/z 73, 147, 172, and 217 were identified as TMS-derivatized alkyl- or cycloalkylsuccinic acids in this study. Even though we used methyl esterification to confirm metabolite identifications, we found trimethylsilylation to be a better method for derivatizing the complex environmental samples analyzed because of improved GC separation of peaks and many useful diagnostic fragment ions. Both methods gave distinctive McLafferty rearrangement ions (m/z 262 or 146) and subsequent fragmentations of these (m/z 172 or 114) as well as predicted $(M - 15)^+$ or $(M - 31)^+$ ions. However, in the silylation reactions, the TMS moiety participates in much of the fragmentation chemistry within the mass spectrometer and gives rise to more ions diagnostic of dicarboxylic acids (*43*). For example, the fragments observed at m/z 73 and 147 indicate di-TMS compounds (*40*), m/z 129 is characteristic of all TMS-derivatized carboxylic acids, and stable ions commonly observed at m/z 204 and 217 signify further fragmentation of the McLafferty rearrangement product as the result of a TMS migration between two carboxylic acid groups (see mass spectra in Figures 1 and 3; *43*).

In previous laboratory experiments examining alkane decay under anaerobic conditions, diastereomer formation was proposed (*16, 17*). The formation of diastereomers is unprecedented since enzymes are usually stereospecific with regards to their substrate, as is the case with benzylsuccinate synthase (*7, 8*). Thus, our finding of at least two chromatographically different peaks in groundwater extracts that had the same MS profiles suggests that diastereomeric alkylsuccinates may be formed in situ. Of course, other explanations for this observation are possible. The multiple peaks can result from the addition of fumarate to multiple methylene carbons on a given alkane. Indeed, Rabus et al. (*17*) observed the formation of such structural isomers of hexylsuccinic acid during anaerobic hexane decay. Another explanation for the observation of multiple peaks having the same MS profiles may indicate that alkylsuccinates resulted from the

anaerobic attack of branched alkanes (i.e., 2-methylpentane vs *n*-hexane decay). Such isomeric alkanes are substantial components of petroleum mixtures (*31*) and are also susceptible to anaerobic decay (G. T. Townsend and J.M.S., unpublished results). It may also be argued that the detected putative alkylsuccinates were merely formed during anabolic reactions (e.g., of fatty acids) in bacterial cells, although to our knowledge, no such reactions have been reported. In fact, Rabus et al. (*17*) only detected fumarate addition metabolites when a nitrate-reducing isolate was incubated with *n*-hexane but not with hexanoic acid.

Of the putative naphthalene metabolites detected, 2-naphthoic acid was the most abundant; this compound has been reported during anaerobic naphthalene and 2-methylnaphthalene decay (*20*–*23*). However, it must be noted that 2-naphthoic acid is also a metabolite of aerobic 2-methylnaphthalene degradation (*44*). Thus, the possibility exists that this compound and possibly the methyl- or dimethylnaphthoic acids were produced aerobically. However, the highly anaerobic nature of the contaminated aquifers where these metabolites were produced argues against this possibility. Furthermore, tetrahydronaphthoic acids (ring reduction products known to form only under anaerobic conditions) were also detected, implying anaerobic decay in the sites examined. Given that naphthoic acids may be formed anaerobically or aerobically, reduced ring products, such as the tetrahydronaphthoic acids detected here, may have more use as indicators of the in situ anaerobic decay of naphthalenes.

Although sought, we did not detect putative anaerobic fumarate addition metabolites of methylnaphthalenes in any of the groundwater samples examined. The inability to find such metabolites may be due to analytical constraints such as detection limits of the instrumentation or the sample preparation methods used. However, the indigenous microorganisms at the sites where putative naphthalene metabolites were found had the ability to activate aromatic hydrocarbons via fumarate addition since alkylbenzylsuccinates were also detected (Table 2). It was also interesting to note that at a diesel-impacted site (site AB1), where both naphthalenes and alkanes are contaminants, only naphthalene metabolites were detected. It is possible that alkanes are being anaerobically metabolized at this site but were present below detection limits. Such an observation, though, demonstrates the importance of monitoring for metabolites from different classes of hydrocarbons as they become known. Thus, along with the known usefulness of benzylsuccinates as indicators of in situ anaerobic alkylbenzene decay (*15*, *27*, *29*), we propose that the identification of alkylsuccinates and anaerobic naphthalene metabolites in hydrocarbon-impacted groundwaters can also be used to indicate that anaerobic biotransformation of alkanes and naphthalenes occurs in situ.

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Biodegradation of an Alicyclic Hydrocarbon by a Sulfate-Reducing Enrichment from a Gas Condensate-Contaminated Aquifer

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We used ethylcyclopentane (ECP) as a model alicyclic hydrocarbon and investigated its metabolism by a sulfate-reducing bacterial enrichment obtained from a gas condensate-contaminated aquifer. The enrichment coupled the consumption of ECP with the stoichiometrically expected amount of sulfate reduced. During ECP biodegradation, we observed the transient accumulation of metabolite peaks by gas chromatography-mass spectrometry, three of which had identical mass spectrometry profiles. Mass-spectral similarities to analogous authentic standards allowed us to identify these metabolites as ethylcyclopentylsuccinic acids, ethylcyclopentylpropionic acid, ethylcyclopentylcarboxylic acid, and ethylsuccinic acid. Based on these findings, we propose a pathway for the degradation of this alicyclic hydrocarbon. Furthermore, a putative metabolite similar to ethylcyclopentylsuccinic acid was also found in samples of contaminated groundwater from the aquifer. However, no such finding was evident for samples collected from wells located upgradient of the gas condensate spill. Microbial community analysis of the ECP-degrading enrichment by denaturing gradient gel electrophoresis revealed the presence of at least three different organisms using universal eubacterial primers targeting 550 bp of the 16S rRNA gene. Based on sequence analysis, these organisms are phylogenetically related to the genera *Syntrophobacter* and *Desulfotomaculum* as well as a member of the *Cytophaga-Flexibacter-Bacteroides* group. The evidence suggests that alicyclic hydrocarbons such as ECP can be anaerobically activated by the addition to the double bond of fumarate to form alkylsuccinate derivatives under sulfate-reducing conditions and that the reaction occurs in the laboratory and in hydrocarbon-impacted environments.

Alicyclic hydrocarbons can comprise a substantial fraction of organic molecules in petroleum mixtures, such as gas condensate, gasoline, and crude oil. In the former two mixtures, alicyclic hydrocarbons typically represent 11 to 12% (wt/wt) of the total hydrocarbons (certificate of analysis of unleaded gasoline, 1991 [American Petroleum Institute]; also unpublished data). In crude oil, this fraction can represent up to 12% (wt/wt), depending on the origin of the petroleum formation (20). Not surprisingly, complex petroleum mixtures containing alicyclic hydrocarbons find their way into the environment and pollute aquifers and various water bodies. As of 2001, more than 418,000 underground fuel tanks were found to be leaking hydrocarbons into the environment within the United States (31).

Despite the frequency of environmental contamination with petroleum mixtures and the quantitative importance of alicyclic hydrocarbons, the biological fate of the latter group of compounds under aerobic conditions has only rarely been addressed (28, 29) and, to our knowledge has not been documented in the absence of molecular oxygen. In contrast, the anaerobic biodegradation of petroleum constituents such as aromatic and normal paraffin hydrocarbons has been demonstrated under nitrate-reducing (1, 6, 10, 17, 19, 26, 27, 35), sulfate-reducing (2, 7, 18, 26), and methanogenic conditions (4, 36). Under anaerobic conditions these hydrocarbons are activated by a novel enzymatic mechanism that employs the common tricarboxylic acid cycle intermediate fumarate (1, 2, 4, 6, 11, 13, 17, 18, 21, 26, 27, 35). This fumarate addition mechanism produces a succinyl derivative of a hydrocarbon, such as benzylsuccinic acid from toluene, dodecylsuccinic acid from dodecane, and (1-methylpentyl)-succinic acid from hexane. In the case of toluene, the addition across the double bond of fumarate results in the production of near-optically pure (*R*)-(+)-benzylsuccinic acid (2, 19) and is catalyzed by the enzyme benzylsuccinate synthase. Furthermore, partially purified benzylsuccinate synthase catalyzed the addition of fumarate to the methyl groups of xylenes and 1-methyl-1-cyclohexene but failed to react with 4-methyl-1-cyclohexene or methylcyclohexane (3). These results led researchers to conclude that an aromatic ring or a conjugated double bond was necessary to stabilize the methyl radical produced during the fumarate addition reaction (3). In contrast, the activation of alkanes by this strategy does not require the formation of the alkene prior to fumarate addition (18, 27, 35).

We studied the anaerobic biodegradation of ethylcyclopentane (ECP), a model alicyclic hydrocarbon, under sulfate-reducing conditions and enriched for a bacterial consortium capable of mineralizing this substrate. The enrichment was obtained from a gas condensate-contaminated aquifer where the intrinsic bioremediation of hydrocarbons under sulfate reducing conditions was previously documented (11, 13). Ethylcyclopentane was chosen as a model compound because gas chromatographic (GC) analysis of contaminated core samples from the aquifer suggested that it was depleted relative to other hydrocarbons present in the gas condensate (unpublished results). This suggested that ECP might have been biologically attenuated in this aquifer in a manner similar to that

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previously demonstrated for BTEX hydrocarbons (13). In sediment-free laboratory enrichments, various putative metabolites were identified during the course of ECP degradation. Based on the identification of these metabolites, we propose a pathway for the degradation of ECP. In addition, GC-mass spectrometry (MS) analysis of extracted and derivatized groundwater samples suggested that the same metabolic strategy was used to activate alicyclic hydrocarbons in situ. To our knowledge, this is the first report of alicyclic hydrocarbon biodegraded under anaerobic conditions.

MATERIALS AND METHODS

Sample collection. Sediments collected from a gas condensate-contaminated aquifer near Fort Lupton, Colo., were used to evaluate the anaerobic biodegradation of ECP. Uncontaminated and hydrocarbon-laden sediments were collected at the water table in jars that were filled to capacity, sealed without a headspace, stored on ice, and transported back to the laboratory. One-liter samples of groundwater were collected in sterile glass bottles from five monitoring wells within the hydrocarbon-impacted aquifer and from another well located upgradient of the contamination (13). The latter sample served as a background comparison and as incubation medium. The groundwater samples were used for DNA extraction or to search for putative hydrocarbon metabolites. The former samples were preserved on ice, while the latter samples were acidified in the field with sulfuric acid to a pH of <2. Aquifer sediments and groundwater were used for experiments immediately upon return to the laboratory.

Biodegradation experiments. Background and hydrocarbon-contaminated sediments and groundwater were amended with ethylcyclopentane (1 μl undiluted, 7.8 μmol or ~1 mM) or benzoate (1 mM) as electron donors and 10 mM sulfate as the electron acceptor. Benzoate was used as a positive control, since this compound is readily degraded under anaerobic conditions. Typically, 10 g of sediment and 20 ml of Na_2S-reduced (0.1 ml/10 ml of a 1.25% solution), autoclaved pristine groundwater were placed in sterile 40-ml serum bottles while all materials were inside an anaerobic glove box containing 5% H_2 in N_2. The bottles were sealed with sterile composite stoppers (15a) and aluminum crimp seals. After the bottles were removed from the glove box, the headspaces of the incubations were exchanged with 20% CO_2 in N_2. Substrate-unamended and autoclaved samples served as controls. All incubations were at 30°C in the dark. The rate of sulfate depletion was monitored by ion chromatography as previously reported (7). Ethylcyclopentane concentrations in the incubations were monitored by headspace analysis on a Hewlett Packard model 5890 GC equipped with a flame ionization detector. Ten-microliter headspace samples were injected at 250°C using a gas-tight glass syringe onto a Carbograph-VOC column (30 m by 0.25 mm; Alltech, Deerfield, Ill.) held isothermally at 150°C with the flame ionization detector heated at 250°C. Helium was used as the carrier gas at a flow of 0.8 ml/min. Once ECP degradation was evident, 10% transfers of the culture into sulfate-medium containing ECP as the sole carbon and energy source were made repeatedly until a sediment-free enrichment was obtained.

Ethylcyclopentane metabolites. To assay for putative ECP metabolites in the sediment-free enrichments, 30 ml of culture fluids were periodically taken and treated with base and then acid as previously reported (18). The pooled extracts were dried over anhydrous Na_2SO_4, and an internal standard (hexadecane) was added before concentration on a rotary evaporator and subsequently under a flow of N_2 gas. The extracts were derivatized with *N,O*-bis(trimethylsilyl)trifluoroacetamide (Pierce, Rockford, Ill.) to form trimethylsilyl (TMS) esters. Groundwater samples, acidified on-site with sulfuric acid, were extracted with ethyl acetate, dried over Na_2SO_4, concentrated, and derivatized in the same manner. Authentic standards of 3-cyclopentylpropionic acid and cyclopentanecarboxylic acid were acquired from Aldrich (Milwaukee, Wis.), while *n*-propylsuccinic acid was a gift from the laboratory of Frank Abbott, Faculty of Pharmaceutical Science, University of British Columbia. These authentic standards were analyzed as TMS esters.

One microliter of the derivatized culture or groundwater extracts was analyzed using a HP Model 5890 GC connected to a HP Model 5970 MS detector. Injector and detector temperatures were held at 250°C. Compounds were separated using a DB-5 column (30 m by 0.25 mm; thickness, 0.1 μm; Alltech). The temperature program for the oven began at 65°C, was held for 3 min, and then was increased by 10°C/min to 155°C and held for 5 min. The oven temperature was further increased by 5°C/min to 185°C and then ramped by 20°C/min to 220°C with a final hold time of 5 min. Helium was used as the carrier gas at a flow of 0.8 ml/min.

DNA extraction. Samples of the enrichment culture (1 to 2 ml) were placed in sterile polypropylene screw-cap tubes (2 ml) containing 1 g of 0.1-mm zirconia beads (BioSpec Products, Bartlesville, Okla.). Samples were centrifuged at 18,000 × *g* for 5 min, and 750 μl of the supernatants was discarded. The tubes were then stored at −20°C until DNA extraction could be performed. After the samples were thawed, 300 μl of each of the following liquids was added: 100 mM phosphate buffer (pH 8), lysis buffer (100 mM NaCl, 500 mM Tris (pH 8), 10% sodium dodecyl sulfate), and chloroform-isoamyl alcohol (24:1). The cells were physically disrupted in a mini-bead beater at 3,800 rpm for 1 min (BioSpec Products, Bartlesville, Okla.) (22). The DNA was isolated by phenol, chloroform-isoamyl alcohol (24:1) extraction and then precipitated with sodium acetate (3 M; pH = 7) and 100% cold ethanol and centrifuged at 18,000 × *g* for 20 min. The pellet was washed twice, first with 70% and then with 100% cold ethanol. The resulting DNA was resuspended in 20 μl of TE buffer (10 mM Tris [pH 8], 1 mM EDTA) or sterile water, and the nucleic acid concentration was estimated by density in an ethidium bromide-stained, 0.8% agarose gel.

The cells present in the groundwater samples were collected by centrifugation, resuspended in 1 ml of TE buffer (10 mM Tris [pH 8], 1 mM EDTA), and serially diluted prior to DNA extraction. The DNA was extracted using the procedure described above.

PCR amplification of 16S rRNA gene. PCR amplifications were performed with a Techne Genius temperature cycler (Techne, Cambridge, United Kingdom). Approximately 25 ng of purified genomic DNA, 10 pmol each of the appropriate primers, 250 μmol of each deoxyribonucleoside triphosphate, 10 μl of 10× PCR buffer B (Fisher Scientific, Fair Lawn, N.J.), 10 μl of $MgCl_2$ (25 mM), 2.5 U of *Taq* polymerase (Fisher Scientific), and sterile water were mixed to a final volume of 100 μl. The samples were amplified using a touchdown PCR protocol (8). This was carried out as follows: the samples were first denatured (94°C/4 min) and then subjected to 35 cycling steps of denaturing (94°C/45 s), annealing (45 s), and elongation (72°C/2 min). The process was completed following one final elongation step (72°C/10 min). The touchdown protocol started with an annealing temperature of 10°C above the expected annealing temperature (63°C) and was decreased by 1°C every two cycles until 53°C, the temperature at which 15 additional cycles were carried out (8). The two universal eubacterial primers GM5F and DS907R were used for amplification (23). These primers amplify a 550-bp fragment within the 16S rRNA gene (bp 341 to 907, *Escherichia coli*). All PCR product sizes were confirmed by agarose-gel electrophoresis, stained with ethidium bromide, and visualized by UV transillumination.

DGGE analysis. The PCR products were separated by denaturing gradient gel electrophoresis (DGGE) using a D-code universal mutation detection system (Bio-Rad, Hercules, Calif.) as described previously (23). The PCR samples were loaded directly onto a 6% polyacrylamide gel with a 30 to 60% built-in denaturant gradient (100% is equal to 7 M urea and 40% formamide) in 1× TAE (40 mM Tris [pH = 7], 20 mM acetate, 1 mM EDTA). The gels were loaded with 12 to 20 μl of PCR sample, and electrophoresis was performed at a constant voltage of 130 V and a temperature of 60°C for 6 h or at 60 V for 16 h at the same temperature. After electrophoresis, gels were stained with ethidium bromide for 5 to 10 min and destained for 10 min in nanopure water. The gels were then photographed under UV transillumination (302 nm) using a Kodak DC120 digital camera and analyzed with the NucleoTech GelExpert-Lite software.

Sequencing of fragments. The 550-bp fragments of interest were excised from the DGGE gels using a sterile razor blade and pipette tip, immediately placed in 200-μl sterile polypropylene tubes containing 36 μl of sterile water, and then stored at 4°C overnight. One microliter of supernatant was used as the DNA template in a PCR reamplification using the same primers, which was then run in a DGGE gel following the protocols described above. Once the purity of a given fragment was confirmed by DGGE, the PCR product was cleaned and concentrated by one of the following methods: Wizard PCR Preps DNA purification system (Promega, Madison, Wis.), UltraClean 15 (Mo Bio), or UltraFree-MC 30,000 NMWL filter unit (Millipore) following the manufacturer's instructions. If the fragment could not be purified by the above method, it was used as a template for cloning using the TOPO TA Cloning kit following manufacturer's instructions (Invitrogen, Carlsbad, Calif.).

Once the fragments were deemed pure, they were sequenced using the BigDye terminator cycle sequencing ready reaction kit (PE Applied Biosystems, Foster City, Calif.) according to the manufacturer's instructions. The primers used in the sequencing reactions were GM5F, DS907R, and the M13 vector primers (forward, GTAAAACGACGGCCAG; reverse, CAGGAAACAGCTATGAC; both 5′ to 3′); this resulted in the twofold coverage of the sequenced area.

Sequence analysis. The sequences were aligned and corrected using the computer program Sequencer. Related sequences were obtained from the GenBank database by using the Blastn search program of the National Center for Biotechnology Information.

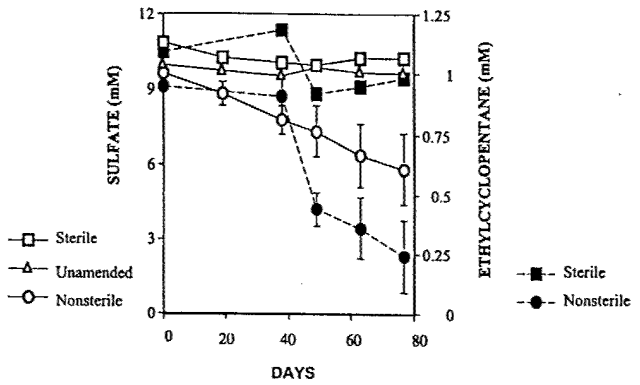

FIG. 1. Ethylcyclopentane degradation by a sediment-free sulfate-reducing microbial enrichment derived from sediments impacted with gas condensate hydrocarbons. Open symbols, sulfate; closed symbols, ethylcyclopentane.

RESULTS

Response of microflora to substrate exposure. In sediment-containing incubations amended with ECP and sulfate, the initial amount of ECP added was consumed in 150 days with an approximate lag period of 50 days. Sulfate consumption paralleled the ECP degradation activity observed in these incubations (data not shown). Ethylcyclopentane decay was not observed in the incubations consisting of uncontaminated sediments, although a positive control substrate (benzoate) was metabolized (data not shown). Neither ECP nor benzoate decay was detected in the autoclaved controls. Subsequent additions of ECP to the sediment-containing incubations were utilized without a lag phase and at higher rates of degradation. This sulfidogenic, ECP-degrading activity was transferable to sediment-free medium, since upon transfer the culture continued to consume ECP and sulfate (Fig. 1).

The amount of sulfate consumption theoretically expected, assuming complete ethylcyclopentane mineralization, was determined according to the following equation:

$$C_7H_{14} + 5.25\ SO_4^{2-} \rightarrow 7HCO_3^- + 5.25\ HS^{1-} + 1.75H^+$$

After correction for sulfate reduction in the substrate-unamended controls, the concentration of sulfate reduced in the sediment-free incubations was 3.51 mM. This amount represents 94% of the theoretically expected amount, assuming the complete mineralization of ECP.

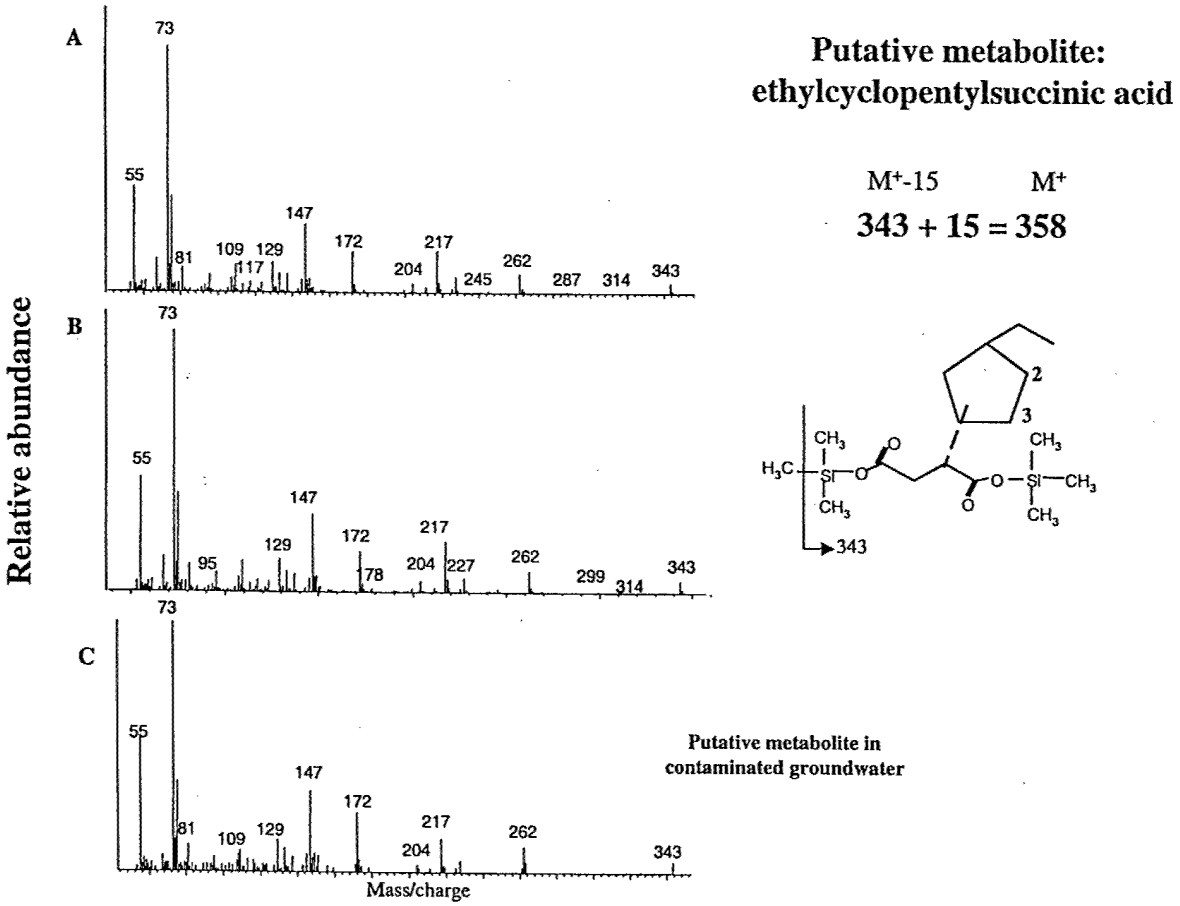

FIG. 2. Mass-spectral profiles of presumed ethylcyclopentylsuccinic acid isomers (analyzed as TMS esters). (A and B) Mass profiles of two of the three metabolites found in laboratory enrichment culture degrading ECP. (C) Putative metabolite found in all contaminated groundwater samples analyzed having the same GC retention time as one of the metabolite peaks in the microbial enrichment.

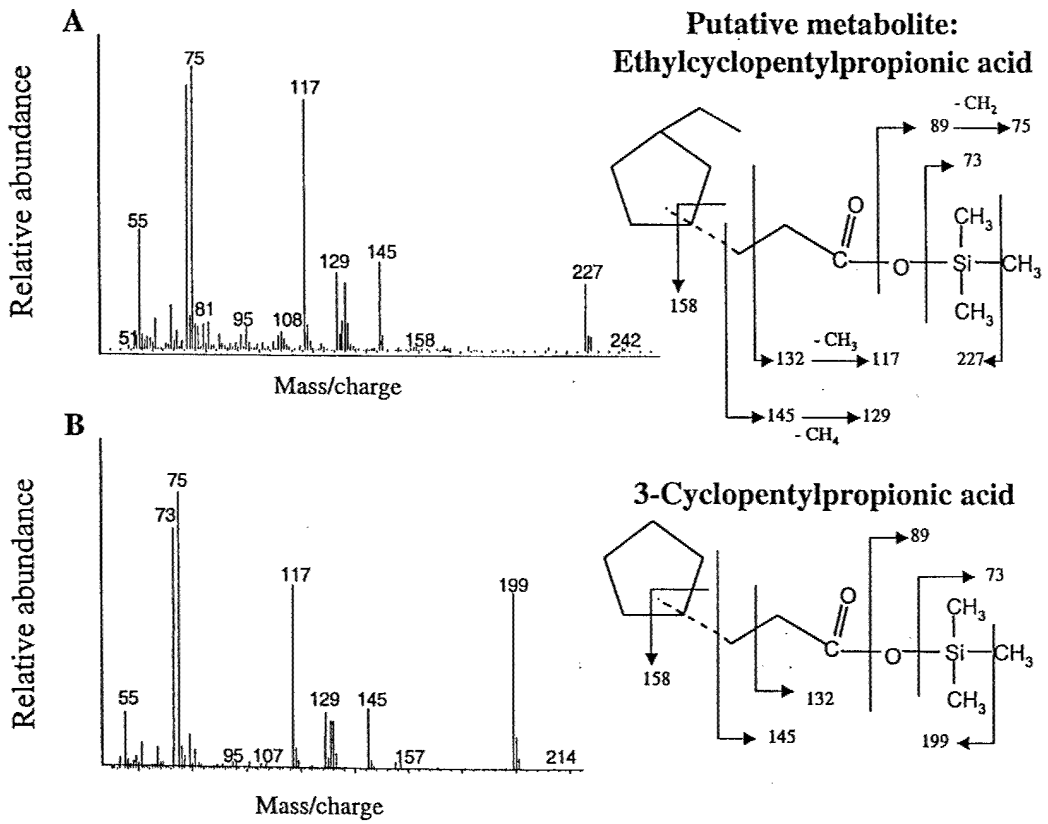

FIG. 3. (A) Mass spectrum of a metabolite tentatively identified as ethylcyclopentylpropionic acid (as TMS ester) in ECP-degrading cultures. (B). Mass spectrum of an authentic standard of 3-cyclopentylpropionic acid (as TMS ester).

Metabolites of ethylcyclopentane degradation. After extraction, derivatization and GC-MS analysis of the ECP-degrading culture fluids, we detected four transient metabolites relative to a hexadecane internal standard in nonsterile incubations exposed to ECP. These peaks were not observed in the corresponding controls.

The first transient metabolite eluted as a cluster of three peaks which shared identical mass spectral profiles (Fig. 2). The ion at *m/z* 343 likely represents the molecular ion less 15 mass units, a common fragment observed with TMS-derivatized compounds, suggesting the loss of a methyl group from one TMS substituent (25). Thus, the ECP metabolite presumably has a mass of 358, which is consistent with the structure of the diTMS ester of ethylcyclopentylsuccinic acid. In addition, the mass spectra contained the key fragment ions *m/z* 73, 117, 147, and 204, which are associated with a molecule containing two TMS-derivatized carboxylic acids (25). The other ions observed in the MS profile are distinctive of and have been used to characterize the presumed fumarate addition metabolite of dodecane, namely dodecylsuccinic acid (TMS ester; at *m/z* 262, 217, 172, 147, and 73) (18). Thus, we propose that these ECP metabolites are ethylcyclopentylsuccinic acids. The GC cluster of three peaks could be further resolved into five peaks (using a different temperature program), all of which had the same MS profile. This observation may be a reflection of the maximum number of stereoisomers resolved by our analytical method.

Another putative metabolite found in the ECP culture extracts had a presumed M^+-15 of *m/z* 227, suggesting a molecular weight of 242 (Fig. 3A). The mass spectrum of this metabolite showed predominant ions at *m/z* 145, 129, 117, 75, 73, and 55. This compound was tentatively identified as ethylcyclopentylpropionic acid by comparison of its MS profile to that of an authentic standard of 3-cyclopentylpropionic acid (Fig. 3B). The mass spectra of these two compounds were nearly identical with the exception that the M^+-15 ion of the metabolite was 28 mass units higher (*m/z* 227 versus *m/z* 199). This difference is consistent with the mass of the ethyl group, absent from the TMS-derivatized authentic standard. Similarly, a third metabolite was identified as ethylcyclopentylcarboxylic acid. This compound had an M^+-15 ion of *m/z* 199, suggesting a molecular weight of 214, and predominant ions at *m/z* 145, 129, 117, 96, 75, 73, and 55 (Fig. 4A). The mass spectrum of this metabolite was compared with that of the authentic standard cyclopentanecarboxylic acid (Fig. 4B). Again, the MS profiles of these two compounds were nearly identical with the exception that the M^+-15 ion of the formed metabolite was 28 mass units higher (*m/z* 199 versus *m/z* 171) than the standard. Finally, the fourth metabolite was tentatively identified as ethylsuccinic acid. This compound had an M^+-15 ion of *m/z* 275, suggesting a molecular weight of 290, and predominant ions at *m/z* 55, 73, 75, 117, 129, 147, 172, 204, 217, and 262 (Fig. 5A). The mass spectrum of this metabolite was compared with that of an authentic standard of *n*-propylsuccinic acid (Fig. 5B) and

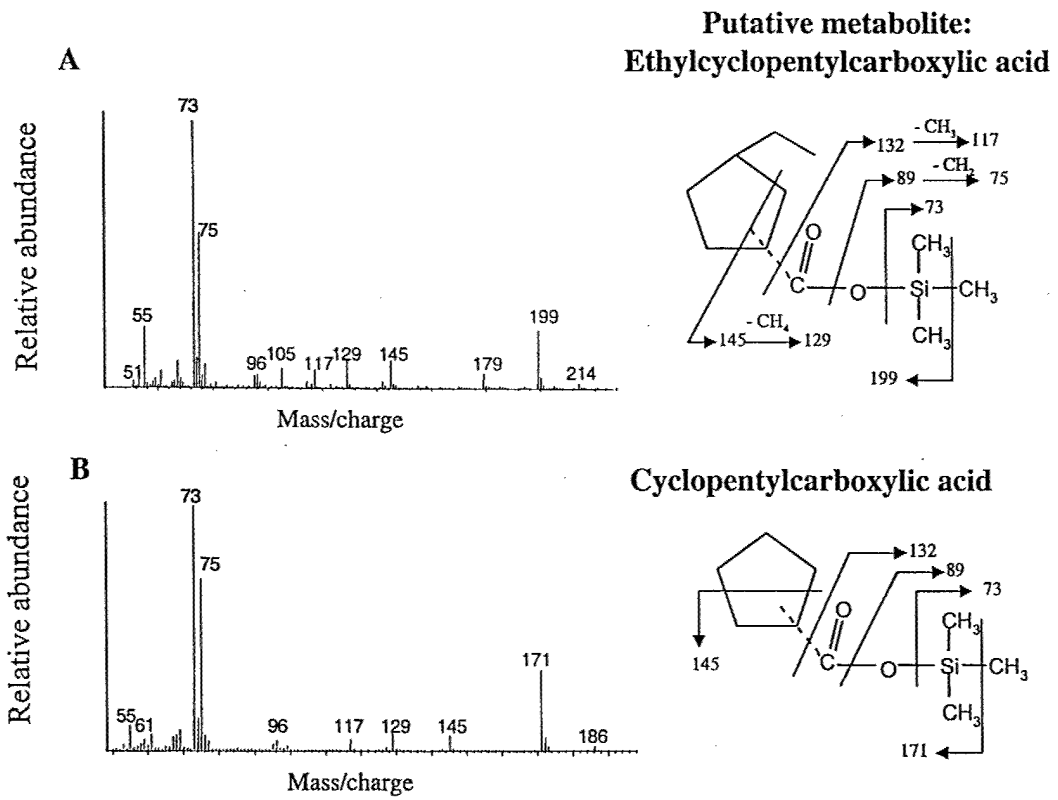

FIG. 4. (A) Mass spectrum of a metabolite tentatively identified as ethylcyclopentylcarboxylic acid (as TMS ester) in ECP-degrading cultures. (B) Mass spectrum of an authentic standard of cyclopentylcarboxylic acid (as TMS ester).

was identical except for the M^+-15 ion, which was 14 mass units less than that of the standard. In addition, this metabolite shared many of the predominant ions with the ethylcyclopentylsuccinic acids (Fig. 3) and dodecylsuccinic acid (18).

A putative hydrocarbon metabolite with the same GC retention time and MS features as one of the anaerobic fumarate addition intermediates produced by the ECP-degrading culture was also detected in groundwater from the gas condensate-contaminated aquifer from which the laboratory culture was derived (Fig. 2C). This metabolite could not be detected in the groundwater samples from the background location but was detected in all five contaminated locations.

DGGE findings. The amplifiable eubacterial microbial community in the sediment-free ECP-degrading enrichment was assayed by DGGE. We were able to purify and sequence three predominant bands present in this microbial culture (Fig. 6). One of the bands showed 97% sequence homology to the uncultured eubacterial clone OCG6 (accession no. AB047117) in the CFB group (Fig. 6). The other organisms in the enrichment were similarly sequenced to reveal the presence of organisms phylogenetically related to sulfate reducers belonging to the genera *Desulfotomaculum* (96%) (accession no. AB074935.1) and *Syntrophobacter* (92%) (accession no. X82875) species (Fig. 6).

DISCUSSION

To our knowledge, this is the first study to report that a saturated alicyclic hydrocarbon can serve as an electron donor for sulfate-reducing bacteria. The fact that this activity was observed with contaminated sediments and not with pristine sediments might suggest an adaptation by the microbial community to the selective pressure imposed by the gas condensate contaminants. The possibility that the organisms within the pristine sediments were killed or inactivated by the manipulation of the sample could be ruled out, since microorganisms from these sediments were able to degrade benzoate. This indicates that the microorganisms were viable and that the incubations were anaerobic, since sulfate was reduced in stoichiometrically expected amounts. However, it is also possible that the organisms responsible for the degradation of ECP have a lower tolerance to oxygen than the organisms responsible for the degradation of benzoate. If this is correct, then the highly reduced contaminated sediments may have protected the ECP degraders from the possible exposure to molecular oxygen, while the pristine sediments were incapable of providing the same protection.

Anaerobic ethylcyclopentane biodegradation under sulfate-reducing conditions appears to be mediated by an anaerobic fumarate addition mechanism. This activation mechanism was previously demonstrated for alkylbenzenes (1–3, 6, 17, 19, 26), straight chain alkanes (18, 27), and for methylnaphthalene (21) occurring under nitrate- and/or sulfate-reducing conditions and for toluene under methanogenic conditions (4). The mass spectra of the observed ECP metabolites formed by microorganisms in the sediment-free incubations (analyzed as TMS esters) contained key fragment ions associated with two TMS-derivatized functional groups (*m/z* 73, 117, 147, and 204) and a

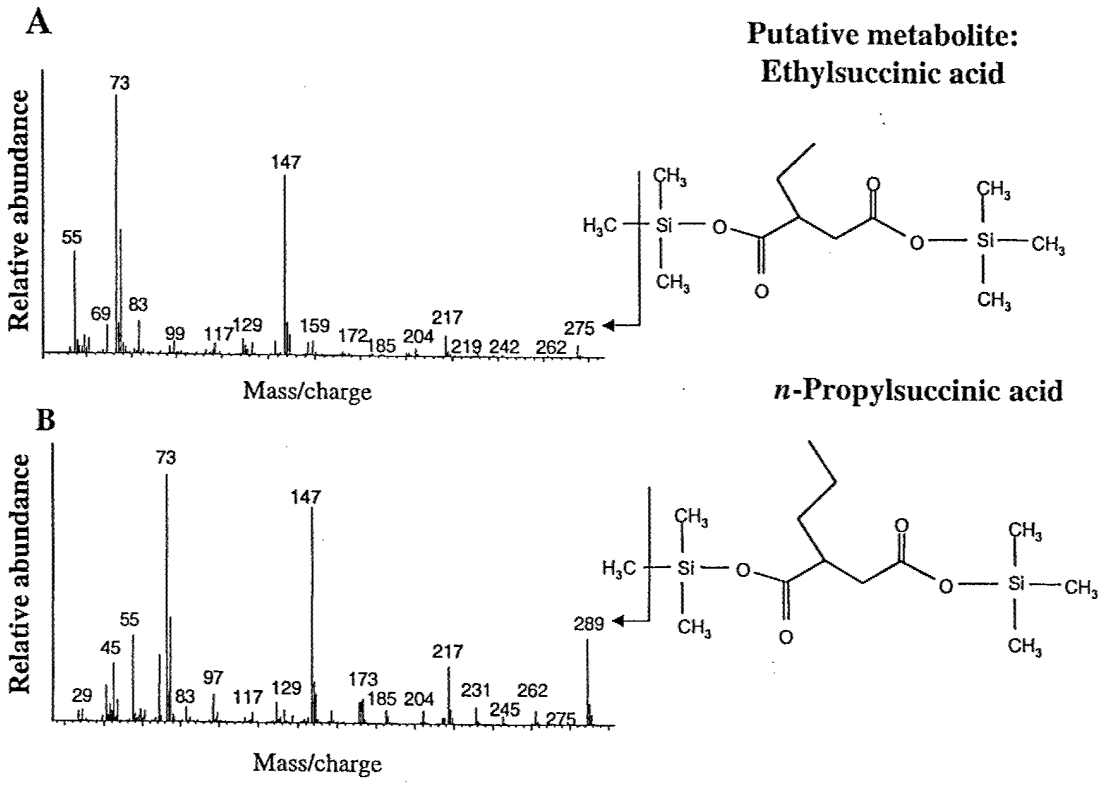

FIG. 5. (A) Mass spectrum of a metabolite tentatively identified as ethylsuccinic acid (as TMS ester) in ECP-degrading cultures. (B) Mass spectrum of an authentic standard of *n*-propylsuccinic acid (as TMS ester).

succinyl moiety (*m/z* 172, 217, and 262) (Fig. 2A). These mass-spectral features were also prominent in TMS-derivatized dodecylsuccinic acid, a metabolite associated with anaerobic dodecane biodegradation (18). The fact that we observed up to five chromatographic components with identical MS profiles suggests that the attack of ECP is most likely at positions 2 or 3 of the alicyclic ring with respect to the ethyl group (Fig. 2). The presumed addition of fumarate to ECP at either of these positions would result in a metabolite with three chiral centers, assuming that the enzyme responsible for such an addition reaction is not stereoselective. This assumption is in contrast to studies performed with benzylsuccinate synthase characterized from *Thauera aromatica* that has been shown to be highly stereoselective (2, 19). The production of the three chiral centers within the diTMS ester of ethylcyclopentylsuccinic acid would theoretically result in the formation of eight possible stereoisomers ($M = 2^n$), producing four pairs of enantiomers, which results in four diastereomers. The latter could be resolved with our GC conditions, since they have different physical properties, producing four distinct peaks with identical MS profiles (although we observed five peaks). The addition of fumarate at other positions within the ECP molecule would produce fewer chiral centers and correspondingly fewer diastereomers than we have successfully resolved. It is possible that more than one organism in the enrichment or the same organism could activate ECP at different positions (carbons 2 or 3 of the ring) with respect to the methine carbon in the succinyl moiety, producing four stereoisomers and two diastereomers from each position. This could feasibly produce four distinct GC peaks that correspond to the four diastereomers. However, the mass-spectral profile of these metabolites will most likely be different, due to the proximity of the succinyl moiety to the ethyl group (attack to carbon 2), not to mention the steric effect that this group will cause if both groups are positioned simultaneously to the front or back of the cyclopentane plane. At present, the lack of an authentic standard precludes a more rigorous identification of these metabolites.

Interestingly, we identified a similar putative metabolite (ethylcyclopentylsuccinic acid) in all contaminated groundwater samples obtained from the gas condensate impacted aquifer from which the ECP-degrading culture was derived. This metabolite had the same GC retention time and MS profile as one of the three peaks produced by the enrichment culture. Importantly, this metabolite was not found in the pristine portion of the aquifer. The fact that we can only identify one comigrating peak does not indicate that the other two peaks formed in the enrichment culture are not formed in situ; this observation may just reflect our analytical limitations. It is possible that these metabolites are formed and degraded simultaneously in situ by organisms that we were unable to culture in the laboratory. It is also possible that the putative metabolite found in native groundwater is an intermediate of another alicyclic compound, such as methylcyclohexane or dimethylcyclopentane, since both of these chemicals are present at higher concentrations within the gas condensate mixture (unpublished data). Nevertheless, these findings suggest that alicyclic hydrocarbons like ECP could be subject to biodegradation by anaerobic activation mechanisms only in the

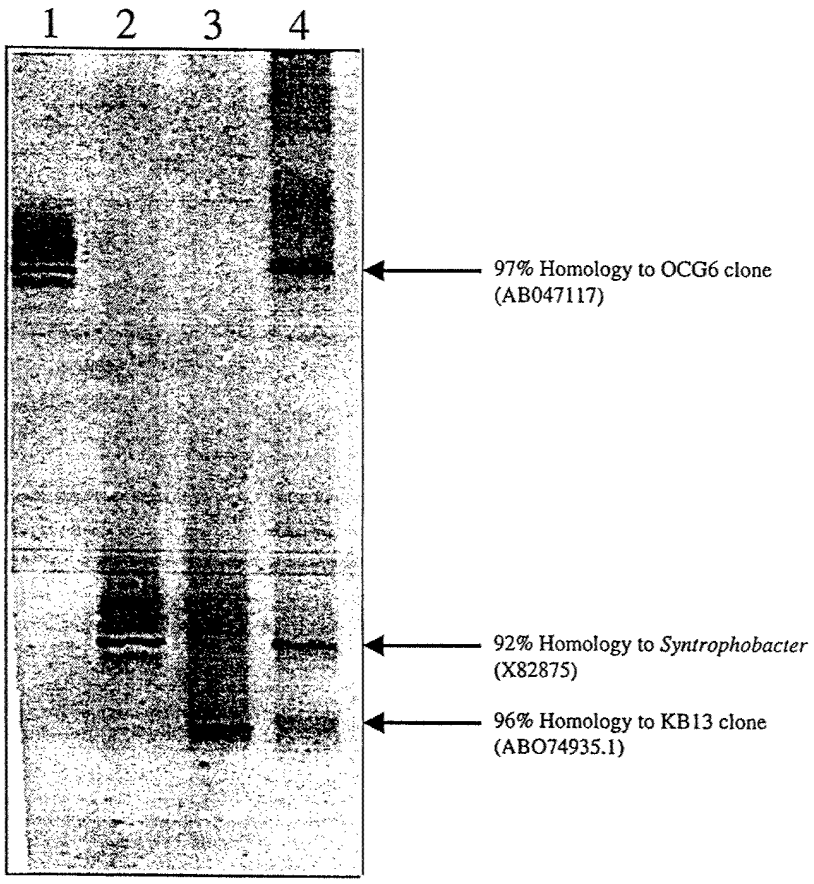

FIG. 6. DGGE of PCR-amplified 16S rRNA genes and phylogenetic characterization of the microorganisms amplified from the ECP-degrading culture using the partial sequence of the 16S rRNA gene. Lanes: 1, 97% similar to uncultured eubacterial clone OCG6; 2, 92% similar to a syntrophic propionate-oxidizing bacterium, KoProp1; 3, 96% similar to a clone, KB13; 4, ECP-degrading culture. Numbers in parentheses indicate GenBank accession numbers.

contaminated portion of the aquifer. This suggests that the anaerobic metabolites of alicyclic hydrocarbons could be used as indicators of in situ anaerobic biodegradation, as has been found with alkylbenzenes and alkanes (5, 11, 14). It should be noted that we have previously detected succinic acid analogs in this gas condensate-contaminated aquifer resulting from ethylbenzene and xylene decay under sulfate-reducing conditions (11) and, most recently, from alkane degradation (14). The finding of a putative anaerobic metabolite of an alicyclic compound at this site extends our knowledge of the biodegradation capabilities of the native microbial populations at hydrocarbon-impacted sites.

The other metabolites produced during ECP degradation were identified based on comparison with analogous authentic standards. Ethylcyclopentylpropionic acid and ethylcyclopentylcarboxylic acid were compared to their ethyl-free counterparts, differing only in their M^+ and M^+-15 fragments by 28 mass units (Fig. 3 and 4). The common ion observed at *m/z* 145 (Fig. 3 and 4) is produced by a rearrangement of the derivatized propyl tail, which undergoes another rearrangement to lose a methane equivalent (16 mass units), producing the ion at *m/z* 129 (30). The abundant ion at *m/z* 117 is thought to be the result of a methyl group loss from ion *m/z* 132 (9). The ion at *m/z* 73 indicates that a functional group has been derivatized as a TMS ester (25), while the ion at *m/z* 75 is the result of a rearrangement and the loss of a methylene group from an ion at *m/z* 89 (30).

Ethylsuccinic acid (Fig. 5) was identified by comparison to the authentic standard of *n*-propylsuccinic acid. In this particular case the standard was 14 mass units heavier than the metabolite, because it had an additional methylene (CH_2) group. Notably, the predominant ions present in the MS profile of ethylsuccinic acid were present in those from *n*-propylsuccinic acid, ethylcyclopentylsuccinic acid, and all alkylsuccinates derivatized with TMS (14, 18). The majority of the ions present in these profiles are highly indicative of a succinyl moiety.

Based on the metabolite evidence presented herein, we propose a pathway for the anaerobic biodegradation of ECP under sulfate-reducing conditions (Fig. 7). This pathway is analogous to the pathway outlined by Wilkes et al. (35) for the degradation of hexane by a denitrifying organism (HxN1) that uses fumarate addition to activate alkanes. Our pathway also includes the regeneration of the presumed cosubstrate fumarate (Fig. 7, P) for the activation of ECP (Fig. 7, A) to ethylcyclopentylsuccinic acid (Fig. 7, B).

In the pathway, ethylcyclopentylsuccinic acid is presumably decarboxylated to produce the ethylcyclopentylpropionic acid (Fig. 7, D). We do not know if the removal of the CO_2 is

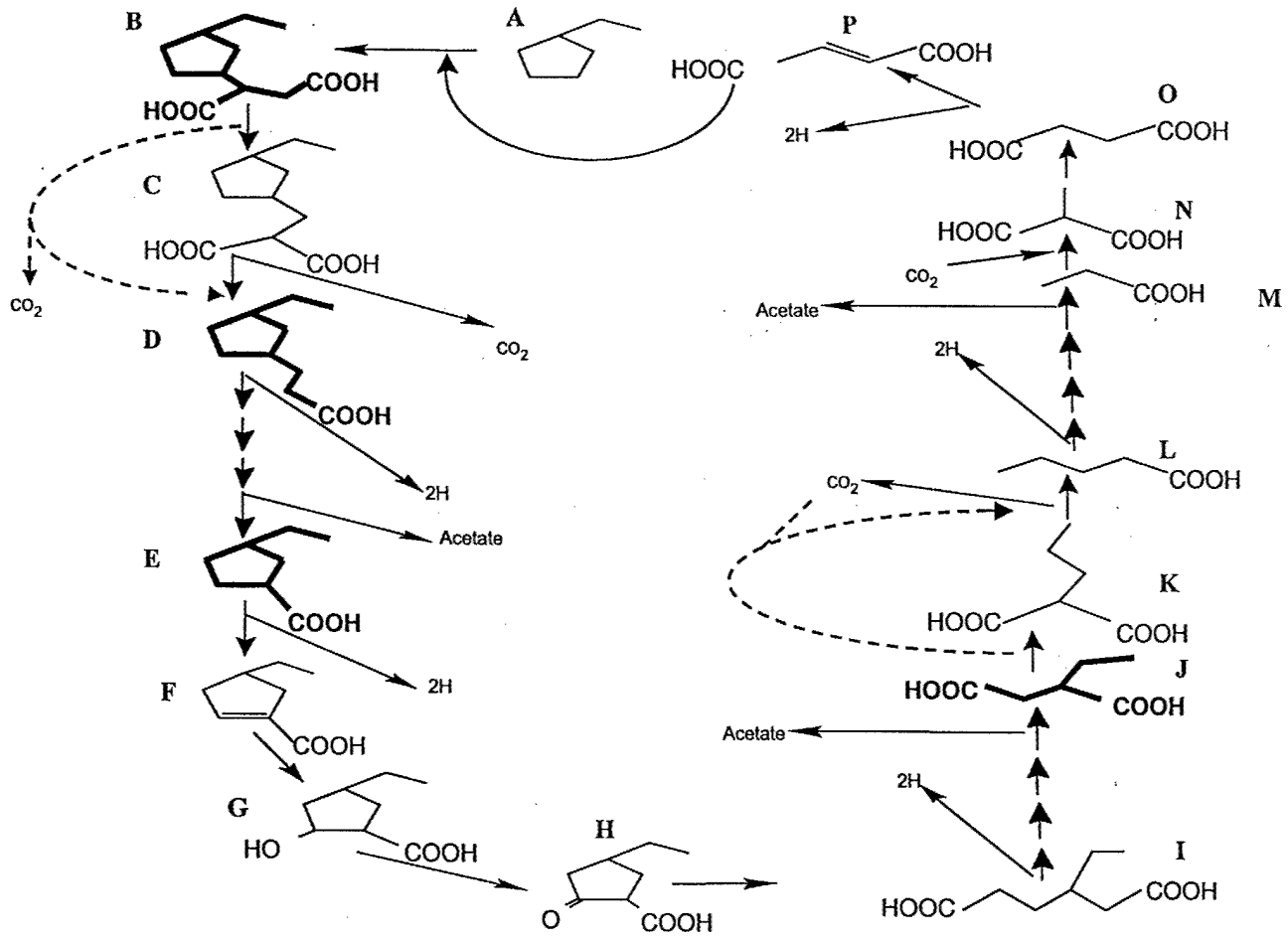

FIG. 7. Proposed pathway for the anaerobic biodegradation of the hydrocarbon ethylcyclopentane by a sulfate-reducing enrichment culture. In the following list of depicted compounds, those that have been identified as TMS esters in culture supernatants are in boldface. A, ethylcyclopentane; B, ethylcyclopentylsuccinic acid; C, ethylcyclopentylmethylmalonic acid; D, ethylcyclopentylpropionic acid; E, ethylcyclopentanecarboxylic acid; F, ethylcyclopent-1-enecarboxylic acid; G, 2-hydroxyethylcyclopentanecarboxylic acid; H, 2-oxoethylcyclopentanecarboxylic acid; I, β-ethyladipic acid; J, ethylsuccinic acid; K, propylmalonic acid; L, valerate; M, propionate; N, methylmalonic acid; O, succinic acid; P, fumarate. Dashed lines indicate alternate possible bioconversions.

directly from the succinyl moiety or if a carbon rearrangement producing ethylcyclopentylmethylmalonic acid (Fig. 7, C) is necessary prior to the loss of CO_2 as proposed by Wilkes et al. (35). The next metabolite identified in our proposed pathway is ethylcyclopentanecarboxylic acid (Fig. 7, E). This metabolite would presumably result from β-oxidation of the propionic acid substituent, a step which would also produce acetate and reducing equivalents. The cyclopentyl ring of this metabolite may then become unsaturated (Fig. 7, F) and be sequentially oxidized to an alcohol (Fig. 7, G) and a ketone (Fig. 7, H), followed by ring cleavage, producing the proposed metabolite β-ethyladipic acid (Fig. 7, I). These series of reactions are similar to the well-characterized reactions carried out by the phototrophic anaerobe *Rhodopseudomonas palustris* as well as *Syntrophus acidotrophicus* when grown on benzoate (12, 24). β-Ethyladipic acid (Fig. 7, I) is postulated to then undergo classical β-oxidation of the longer fatty-acid chain to produce a second molecule of acetate, reducing equivalents, and the identified metabolite ethylsuccinic acid (Fig. 7, J). This metabolite is a key intermediate in this pathway since it would be produced regardless of the actual position (carbon 2 or 3) at which the initial attack of the fumarate takes place. Ethylsuccinic acid may be further decarboxylated to the fatty acid valerate (Fig. 7, L). Again, this decarboxylation could occur directly from the succinyl derivative or after a carbon rearrangement by an unknown mechanism analogous to the decarboxylation of ethylcyclopentylsuccinic acid (Fig. 7, steps B to D). Thus, valerate may be produced directly or via the presumed metabolite propylmalonic acid (Fig. 7, K). Valerate may then undergo β-oxidation to produce propionate (Fig. 7, M), which may be carboxylated to methylmalonic acid (Fig. 7, N) and further metabolized to succinic acid (Fig. 7, O). These series of reactions would be similar to those used by propionate oxidizers represented by the genera *Desulfobulbus* (16) and *Syntrophobacter* (33). Finally, succinic acid is then dehydrogenated, regenerating fumarate.

The DGGE profile of the sediment-free ECP-degrading culture shows the presence of three predominant bands representing the dominant amplifiable eubacterial microorganisms in the culture (Fig. 6). The associated sequences suggest that

the organisms are phylogenetically related to the genus *Desulfotomaculum* within the *Bacillus-Clostridium* group, the genus *Syntrophobacter* within the δ subdivision of the *Proteobacteria*, and to the members of the *Bacteroidaceae* within the CFB group (Fig. 6). The sum of these organisms, represented by their sequences, constitutes the predominant members of the ECP-degrading culture and have been deposited in GenBank as AF529223, AF529224, and AF529225, respectively. The sequence belonging to the *Bacteroidaceae* was 97% homologous to an uncultured clone obtained from groundwater contaminated with oil from an underground storage cavity (GenBank accession no. AB047117). It is very difficult to discuss the niche of this particular organism within our enrichment without knowing the metabolic ability of its closest relative, but their respective habitats are clearly related to hydrocarbon contamination. In contrast, we found a sequence that is related to the genus *Syntrophobacter*, comprised of organisms which are best known for their ability to degrade fatty acids and other simple organic molecules by syntrophic association or using sulfate as a terminal electron acceptor (15, 32, 33). Interestingly, various metabolites that might serve as substrates for these organisms are proposed in the ECP pathway (Fig. 7). Unfortunately, we were unable to detect the transient accumulation of fatty acids, such as acetate or propionate, in the cultures actively degrading ECP. It is possible that these fatty acids were consumed as rapidly as they were produced, maintaining their concentration lower than 10 μM, our detection limit for these compounds. Furthermore, we were able to isolate this organism, and it is not able to degrade ECP as a pure culture or in coculture with a hydrogen-consuming methanogen (data not shown). A brief characterization of its degradative range revealed its ability to degrade acetate, propionate, isovalerate, and crotonate under sulfate-reducing conditions (data not shown). These findings suggest that this organism might not be the ECP degrader in our ECP-degrading enrichment but might play an important role in the degradation of low-molecular-weight fatty acids. The other organism capable of using sulfate as a terminal electron acceptor in our enrichment based on our sequence data is an organism related to the genus *Desulfotomaculum* obtained from oil-contaminated groundwater (GenBank sequence accession no. AB074935.1). The organisms in this genus are not known for their ability to degrade hydrocarbons, but alcohols, fatty acids, other aliphatic monocarboxylic or dicarboxylic acids among other compounds are used as electron donors for dissimilatory sulfate reduction (34). The fact that ECP degradation is coupled to sulfate reduction in our enrichment and the inability of the *Syntrophobacter* sp. isolate to degrade ECP support our working hypothesis that the *Desulfotomaculum* sp. is responsible for the degradation of ECP.

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Project Title: Intrinsic anaerobic bioremediation of hydrocarbons in contaminated marine sediments

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In recent years, several classes of petroleum hydrocarbons contaminating marine environments have been found susceptible to anaerobic biodegradation using novel mechanisms entirely distinct from aerobic metabolic pathways. For example, the anaerobic decay of toluene can be initiated by the addition of the aryl methyl group to the double bond of fumarate, resulting in a benzylsuccinic acid metabolite. Our work has shown that an analogous mechanism also occurs with ethylbenzene and the xylene isomers, yielding 3-phenyl-1,2-butane dicarboxylic acid and methylbenzylsuccinic acid, respectively. These metabolites could also be detected in contaminated environments. Most recently, we have identified metabolites that result from the initial attack of H_{26}- or D_{26}-*n*-dodecane during degradation by a marine sulfate-reducing bacterial culture. Using GC-MS, these metabolites were identified as fatty acids that result from C-H or C-D addition across the double bond of fumarate to give dodecylsuccinic acids in which all 26 protons or deuteriums of the parent alkane were retained. Further, when this enrichment culture was challenged with hexane or decane, hexylsuccinic acid or decylsuccinic acid were identified as resulting metabolites. Similarly, the study of an ethylcyclopentane-degrading sulfate-reducing enrichment produced a metabolite, which is consistent with the addition of fumarate to the parent substrate. These novel anaerobic addition products are characterized by similar, distinctive mass spectral (MS) features (ions specific to the succinic acid portion of the molecule) which can potentially be used to probe contaminated environments for evidence of intrinsic remediation of hydrocarbons. Indeed, analyses of water extracts from two gas condensate-contaminated sites resulted in the tentative detection of alkyl- and cycloalkylsuccinic acids ranging from C3 to C9, including ethylcyclopentyl-succinic acid. In water extracts collected from an area underlying a petroleum production plant, MS profiles consistent with the addition products of methylcycloalkenes were observed. Similarly, interstitial water from hydrocarbon-contaminated drill cutting piles from the North Sea also contained a metabolite consistent with the structure of a C17-succinic acid derivative. Other marine incubations have been found to harbor bacteria that metabolize alkane and polycyclic aromatic hydrocarbon-containing fractions of crude oil in a sulfate-dependent fashion. Work is underway to determine if marine crude oil-degrading bacteria produce comparable anaerobic metabolites. This work helps attests to, 1) the extrapolatability of laboratory results to the field, 2) the unifying metabolic features for the anaerobic destruction of diverse types of hydrocarbons, and 3) how this information can be used to assess the intrinsic bioremediation processes in petroleum-contaminated environments.

The anaerobic oxidation of hydrocarbons: The role of fumarate addition reactions

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Petroleum hydrocarbons exhibit rather low chemical reactivity and were assumed to undergo biodegradation only in the presence of free oxygen. Since the late 1980s, this view has been revised since several petroleum components were found susceptible to anaerobic catalysis by an unprecedented biochemical mechanism. In the absence of oxygen, methyl-substituted aromatic compounds, including alkylbenzenes, cresols, and methylnaphthalenes, are activated by the addition of the methyl group to the double bond of fumarate to form aromatic-substituted succinic acids. Analogous metabolites were identified during the initial anaerobic catalysis of H_{26}- or D_{26}-*n*-dodecane. The metabolites proved to be fatty acids that resulted from C-H and C-D addition across the double bond of fumarate to give dodecylsuccinic acid derivatives containing all the protons or deuteriums of the original alkane. A synthesized authentic standard of *n*-dodecylsuccinic acid had the same mass spectral features as the metabolites, but a different chromatographic profile, so the succinyl moiety of the metabolites was not attached at the terminal C atom. A subterminal attachment point would result in a metabolite with two chiral C atoms and, assuming relaxed enzyme specificity for chirality at one of these two centers, exists as a pair of diasteromers that could be resolved by GC analysis. The formation of diasteromers is exceptional among enzymatic reactions. The corresponding alkylsuccinic acid metabolites were also detected when the culture catalyzed the decay of *n*-alkanes ranging in chain length from C_6 to C_{12}. Similarly, an ethylcyclopentane (ECP)-degrading culture exhibited four nearly co-eluting peaks with the same mass spectral profile that was consistent with the structure of ethylcyclopentylsuccinic acid. The multiplicity of peaks suggests that fumarate addition is either occurring at more than one C atom in ECP, or that it is occurring at one of the two methylene C atoms in the ring. Fumarate addition to either one of these positions would destroy the symmetry of ECP and give a metabolite with >2 chiral C atoms, and hence many possibilities for diastereomer formation. The environmental relevance of such catalytic reactions was manifest by the detection of fumarate addition metabolites with similar, distinctive mass spectral features (ions specific to the succinic acid portion of the molecule) in hydrocarbon-contaminated areas, but not in corresponding background locations. Our findings attest to the generalizing features associated with the catalysis of aromatic, saturated and alicyclic hydrocarbons by microorganisms in the absence of oxygen.

Abs.no.: 255

Anaerobic biodegradation of n-alkanes by sulfate-reducing bacterial cultures

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We enriched for alkane-degrading sulfate-reducing bacterial cultures from a marine sediment and an oil reservoir. These cultures were highly purified after numerous dilution series and contained cells of mostly similar morphology. The two cultures degraded n-alkanes with stoichiometric reduction of sulfate and each was inhibited by molybdate. The growth rate of the marine culture on C_{10} was 0.13 day^{-1} and the rate of decane consumption was 1.5 ±0.07 µmol x day^{-1}. In experiments with [1-^{14}C]-dodecane, the expected amount $^{14}CO_2$ was produced. Alkane degradation was not dependent on CO_2. Previous work confirmed that the attack on H26- and D26-dodecane was initiated by addition to the double bond of fumarate to give dodecylsuccinic acid derivatives containing all the protons or deuteriums of the original alkane. When the same culture was grown on C_6, C_8, C_{10}, the corresponding alkylsuccinic acid metabolites were detected. Also, in cell-free extracts hexylsuccinic acid was formed from D14-C_6 with full deuterium retention. When the oil reservoir culture metabolized hexane, hexylsuccinic acid was produced indicating a comparable mechanism of alkane activation. The metabolites were typically found in trace amounts under normal cultivation conditions, but could accumulate under sulfate-limiting conditions and be subsequently metabolized upon re-amendment with sulfate. The 16S rRNA genes from the sulfate-reducing alkane-degrading cultures were cloned and sequenced. DGGE of DNA extracted from both cultures revealed one dominant band, which co-migrated with that of the cloned sequences. Analysis showed that sulfate-reducing bacteria from marine and oil reservoir cultures shared a close but non-identical phylogenetic relatedness (99.3% similarity). The organisms are most closely related to Desulfacinum sp.(93 to 94% identity) and Syntrophobacter sp. (92 to 95% identity).

Abs.no.: 101

Anaerobic alkane bioedegradation and the detection of signature metabolites in petroleum-contaminated subsurface environments

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Petroleum hydrocarbons are common contaminants in the terrestrial subsurface and exhibit remarkably low chemical reactivity. In the past, aerobic biodegradation was assumed to be the only dominant processes influencing the fate of these contaminants in the subsurface. Since the late 1980s, this view has been substantially altered, as many petroleum components are now known to be susceptible to anaerobic metabolism by unprecedented mechanisms. In the absence of oxygen, alkylbenzenes, n-alkanes, cyclic alkanes and methylnaphthalenes, are activated by addition to the double bond of fumarate to form substituted succinic acid derivatives. With many hydrocarbons, the attachment point of the succinyl moiety results in intermediates with multiple chiral centers and diasteromers can be formed. The production of diasteromer intermediates is exceptional in biologically catalyzed reactions. Relative to other hydrocarbons, alkanes are quantitatively more important components in complex mixtures. Probing alkane biodegradation with 13C-NMR and a model hydrocarbonoclastic sulfate reducing bacterial culture revealed several subsequent metabolic steps. The environmental relevance of these reactions was evident by the detection of unique anaerobic metabolites in field samples. Gas chromatography-mass spectrometry, was used to analyze trimethylsilyl-derivatized organic extracts from six hydrocarbon-contaminated groundwaters. In the contaminated groundwaters, compounds whose mass spectral profiles suggested that they were alkylsuccinic acids, ranging from C3 to C11 succinates were found. In two of the aquifers, components whose mass spectral profiles matched with authentic standards of naphthoic acids and tetrahydronaphthoic acids derivatives were also detected. In-situ metabolites were detected in nanomolar concentrations and evidence for diastereomers was found. No evidence for hydrocarbon metabolites was found in corresponding background locations. Our findings attest to the generalizing features associated with the catalysis of aromatic, saturated and alicyclic hydrocarbons by microorganisms in the absence of oxygen. The finding of the putative anaerobic metabolites signifies the in situ biodegradation of the corresponding hydrocarbons and attests to the value of these intermediates as indicators of intrinsic bioremediation.