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THE UNIVERSITY OF OKLAHOMA
GRADUATE COLLEGE

THE ANAEROBIC BIODEGRADATION OF ETHYLCYCLOPENTANE AND INTERMEDIATES OF BENZOATE METABOLISM BY MICROORGANISMS FROM A HYDROCARBON-CONTAMINATED AQUIFER

A Dissertation
SUBMITTED TO THE GRADUATE FACULTY
in partial fulfillment of the requirements for the
degree of
DOCTOR OF PHILOSOPHY

By
Luis A. Rios-Hernandez
Norman, Oklahoma
2003

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THE ANAEROBIC BIODEGRADATION OF ETHYLCYCLOPENTANE AND INTERMEDIATES OF BENZOATE METABOLISM BY MICROORGANISMS FROM A HYDROCARBON-CONTAMINATED AQUIFER

A Dissertation APPROVED FOR THE
DEPARTMENT OF BOTANY AND MICROBIOLOGY

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PREFACE

Most of the energy produced worldwide today comes from the burning of fossil fuels in the form of coal or petroleum hydrocarbons. This energy generation plays an important role in the technological advances that our society appreciates and enjoys. Unfortunately, such petroleum utilization also plays a key role in the contamination of our natural resources. In the United States, approximately half a million underground fuel tanks have been found leaking their contents into the environment in the past 20 years. This fuel is a complex mixture of hydrocarbons containing n-alkanes, aromatics, alicyclics, and their substituted analogs. Of these, the biodegradation of the aromatic hydrocarbon group has been relatively well-studied. In contrast, the alicyclic hydrocarbons that represent up to 12% (w/w) of most hydrocarbon mixtures, has only rarely been addressed under aerobic conditions, and to our knowledge, has not been documented in the absence of molecular oxygen.

In Chapter 1, I examine 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. This is the first report of an alicyclic hydrocarbon that can be biodegraded under anaerobic conditions. Furthermore, I propose a pathway for the degradation of this hydrocarbon based on the chemical identity of the metabolites detected in spent culture fluids. I also briefly describe one of the organisms obtained from the culture that does not degrade ECP. This isolate belongs to the genus *Syntrophobacter* as indicated by partial sequence of the 16S rRNA gene. This chapter was written in the format required by the journal *Applied and Environmental*

Microbiology.

Another group of hydrocarbons that are typical contaminants from leaking underground fuel tanks are the monoaromatic compounds. This group contains the compounds of greatest regulatory concern due to their toxicity and cancer-inducing ability. These compounds are known as the BTEX, and include benzene, toluene, ethylbenzene and the three isomers of xylene. Under anaerobic conditions, these compounds can be metabolized producing benzoate as an intermediate. Benzoate has been found to be a key intermediate formed during the anaerobic metabolism of many aromatic substrates. Benzoate is readily degraded under anaerobic conditions by at least two known pathways that result in the formation of different suites of intermediates depending on the organism involved. Key benzoate intermediates are cyclohexane carboxylate and cyclohex-1-ene carboxylate. These compounds are produced as free-acids in the presence or absence of molecular oxygen, do not require a syntrophic partnership for degradation under sulfate-reducing conditions, and are less volatile and more water-soluble than their presumed hydrocarbon predecessors.

I hypothesize (Chapter 2) that cyclohexane carboxylate and cyclohex-1-ene carboxylate could be useful as substrates for the isolation and characterization of bacteria capable of degrading presumed aromatic precursor compounds (BTEX). I characterize a microbial isolate that was capable of degrading cyclohexane carboxylate and cyclohex-1-ene carboxylate but was not able to degrade benzoate or BTEX hydrocarbons using sulfate as terminal electron acceptor. Furthermore, I determine that this isolate was a new species of the genus *Desulfomonile* based on morphology, physiology and 16S rRNA gene sequence. This isolate is only the third described species of this genus and I propose the

name *Desulfomonile liminquinati* that describes the origin of the organism, from hydrocarbon-contaminated sediment. This chapter was written in the format required by the journal *Archives of Microbiology*.

Of the BTEX compounds, toluene is the most susceptible to biodegradation in anaerobic environments. Typically, this aromatic hydrocarbon is degraded by the addition of fumarate to the methyl group producing the intermediate benzylsuccinate that is further metabolized to benzoate, which is subsequently mineralized. This pathway has been described in the literature by various researchers using pure or mixed cultures and with nitrate, sulfate or CO_2 as electron acceptors. A research group using a nitrate-reducing pure culture proposed a more controversial pathway for anaerobic toluene decay that involved the addition of two molecules of a two-carbon fragment; presumably acetate.

The Appendix describes collaborative a study with Dr. Matthew E. Caldwell examining the anaerobic biodegradation of toluene by a stable microbial enrichment that used nitrate as terminal electron acceptor. We were able identify various key intermediates using ^{13}C- and d_3-labeled parent substrate that allowed us to conclude that there are at least two pathways for the anaerobic degradation of toluene. One pathway involves the addition of two C2 molecules (presumably, acetate) directly to toluene forming benzylsuccinate, the other forms benzylsuccinate in one step utilizing fumarate as a reactant. The genetic characterization of the consortium confirmed the presence of an *Azoarcus sp.* based on two independent 16S rDNA genes sequences. In addition, the presence of the gene for subunit A of the benzylsuccinate synthase was confirmed by PCR using specific primers. This is the first time that evidence supporting the importance of both pathways is collected using a microbial consortium and indicates that fumarate is not the only

substituent group addition formed during the anaerobic metabolism of this important hydrocarbon.

ABSTRACT

In this thesis, I examine the microbial degradation of an alicyclic hydrocarbon and the intermediates of benzoate decay under anaerobic conditions. Contrary to popular belief, anaerobic environments are not difficult to find nor are they particularly extreme. An environment typically becomes anaerobic whenever the influx of degradable organic material exceeds the available molecular oxygen required for the hetrotrophic mineralization of the carbon. The most frequent scenario would be a leaking underground storage tank containing petroleum hydrocarbons. Typically, this mixture of hydrocarbons can be separated based on their chemical structures into groups such as aromatic, *n*-alkane, branched alkanes, and alicyclic. The best-studied groups of hydrocarbons are the aromatics, especially the BTEX compounds (benzene, toluene, ethylbenzene, and isomers of xylene) due to their physical properties, abundance in the mixtures and their harmful effects on humans. In general, the BTEX hydrocarbons are susceptible to microbial attack and degradation under anaerobic conditions with benzene being the most recalcitrant. In the past few years, a wide variety of hydrocarbons including toluene, isomers of xylene, hexane, dodecane, and naphthalene have been shown to be degraded under anaerobic conditions. This occurs by the addition to the double bond of fumarate, producing a succinate derivative of the attacked hydrocarbon. These anaerobic reactions have emerged as the favored mechanism among the scientific community for the bioconversion of these compounds in the absence of molecular oxygen. Another group of compounds that has been ignored due to low solubility and physical properties, despite its relative abundance (12% w/w in petroleum) is the alicyclic hydrocarbons. In Fort Lupton, my study site, ethylcyclopentane was chosen as a model

alicyclic hydrocarbon because GC analysis of contaminated core samples suggested that ECP was depleted relative to other hydrocarbons present in the gas condensate (a hydrocarbon mixture). This observation suggested that ECP might have been biologically attenuated in this aquifer in a manner similar to that previously demonstrated for BTEX hydrocarbons by other researchers. I obtained a bacterial enrichment which could mineralize ECP coupled to the reduction of sulfate in stoichiometrically expected amounts. In addition, I was able to propose a pathway for the degradation of this alicyclic hydrocarbon based on the identification of various transient metabolites produced during biodegradation. Furthermore, I describe the organisms in the ECP-degrading enrichment by denaturing gradient gel electrophoresis (DGGE) using a portion of the 16S rDNA gene. This study 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.

Using the same hydrocarbon-impacted sediments known to harbor microorganisms able to degrade aromatic hydrocarbons via benzoate, I isolated a cyclohexane carboxylate-degrading, sulfate-reducing bacterium. The isolate can grow heterotrophically with short chain fatty acids and two key intermediates in the degradation of benzoate, but not with benzoate itself or with BTEX hydrocarbons. Autotrophic growth was observed with various sulfur oxyanions and fumarate as terminal electron acceptors. The bacterium is a Gram-negative, non-sporeforming, non-motile, non-dehalogenating large rod with a collar-like morphology. I propose a degradation pathway for the decay of cyclohexane carboxylate by identifing transient metabolites

formed during degradation. The morphology, physiology and 16S rRNA gene partial sequence suggests that this isolate is a new species of the genus *Desulfomonile*. Unlike the other known *Desulfomonile* species, this organism was not able to dehalogenate 3-chlorobenzoate. We proposed that this isolate be named *Desulfomonile liminquinati*.

Chapter 1.

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

ABSTRACT

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 (GC-MS), three of which had identical MS 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 in samples collected from wells located upgradient of the gas condensate spill. Microbial

community analysis of the ECP-degrading enrichment by denaturing gradient gel electrophoresis (DGGE) revealed the presence of at least three different organisms using universal Eubacterial primers targeting 550bp 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 (CFB) 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.

INTRODUCTION

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% (w/w) of the total hydrocarbons (1, unpublished data). In crude oil, this fraction can represent up to 12% (w/w), depending on the origin of the petroleum formation (21). Not surprisingly, complex petroleum mixtures containing alicyclic hydrocarbons find their way into the environment and pollute aquifers and various water bodies. As of 2001, over 418,000 underground fuel tanks were found to be leaking hydrocarbons into the environment within the U.S. (32).

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 has only rarely been addressed under aerobic conditions (29, 30), and to our knowledge, has not been documented in the absence of molecular oxygen. In contrast, the anaerobic biodegradation of petroleum contituents such as aromatic and normal paraffin hydrocarbons has been demonstrated under nitrate-reducing (2, 7, 11, 18, 20, 27, 28, 36), sulfate-reducing (3, 8, 19, 27), and methanogenic conditions (5, 38). Under anaerobic conditions these hydrocarbons are activated by a novel enzymatic mechanism that employs the common TCA cycle intermediate fumarate (2, 3, 5, 7, 12, 14, 18, 19, 22, 27, 28, 36). 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 (4, 20) 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 (4). 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 (4). In contrast, the activation of alkanes by this strategy does not require the formation of the alkene prior to fumarate addition (19, 36).

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 (12, 14). Ethylcyclopentane was chosen as a model compound because 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 result suggested that ECP might have been biologically attenuated in this aquifer in a manner similar to that previously demonstrated for BTEX hydrocarbons (14). 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-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 Ft. Lupton, CO 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 (14). 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 samples for DNA extractions were preserved on ice while the samples for metabolites were acidified in the field with sulfuric acid to pH < 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 neat, 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

(0.1ml/10ml of a 1.25% solution), autoclaved pristine groundwater were placed in sterile 40 mL serum bottles while all materials were inside an anaerobic glovebox containing 5% H_2 in N_2. The bottles were sealed with sterile composite stoppers (37) and aluminum crimp seals. After the bottles were removed from the glovebox the headspace 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 (8). Ethylcyclopentane concentrations in the incubations were monitored by headspace analysis on a Hewlett Packard Model 5890 GC equipped with a flame ionization detector (FID). Ten-microliter headspace samples were injected at 250°C using a gas-tight glass syringe onto a Carbograph-VOC column (30 m x 0.25 mm i.d., Alltech, Deerfield, IL) held isothermally at 150°C with the FID 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 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 (19). 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)trifluoroacetimide (BSTFA; Pierce, Rockford, IL) 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, WI.), while *n*-propylsuccinic acid was a gift from the laboratory of Dr. 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 x 0.25 mm i.d. and 0.1 μm thickness; Alltech, Deerfield, IL). The temperature program for the oven began at 65°C, was held for 3 min, then 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, 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 capped tubes (2 ml) containing 1 g of 0.1 mm zirconia beads (BioSpec Products, Bartlesville, OK). The sample was centrifuged at 18,000 x g for 5 min and 750 μl of the supernatant was discarded. The tubes were then stored at -20°C until DNA extraction could be performed. After thawing the samples, 300 μl of each of the following liquids were added: 100 mM phosphate buffer (pH 8), lysis buffer (100 mM NaCl, 500 mM Tris pH 8, 10% SDS), and chloroform-isoamyl alcohol (24:1). The cells were physically disrupted in a mini-bead beater at 3800 rpm for one minute (BioSpec Products, Bartlesville, OK) (23). The DNA was isolated by phenol, chloroform-isoamyl alcohol (24:1) extraction, then precipitated with sodium acetate (3 M, pH = 7)

and 100% cold ethanol and centrifuged at 18,000 x 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 10X PCR buffer B (Fisher Scientific, Fair Lawn, NJ), 10 µl of $MgCl_2$ (25 mM), 2.5 U of Taq Polymerase (Fisher Scientific, Fair Lawn, NJ), and sterile water were mixed to a final volume of 100 µl. The samples were amplified using a touchdown PCR protocol (9). This was carried out as follows: the samples were first denatured (94°C/4 min), then subjected to 35 cycling steps of denaturing (94°C/45 sec), annealing (45 sec), 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 (9). The two universal eubacterial primers GM5F and DS907R were used for amplification (24). These primers amplify a 550 base pair fragment within the 16S rRNA gene (bp 341-907, *E. 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 (BioRad, Hercules, CA) as described previously (24). 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 1X TAE (40 mM Tris [pH=7], 20 mM acetate, 1 mM EDTA). The gels were loaded with 12 to 20 µl of PCR product and electrophoresis was performed at a constant voltage of 130 volts and a temperature of 60°C for 6 hours or at 60 volts for 16 hours at the same temperature. After electrophoresis, gels were stained with ethidium bromide for 5 to 10 minutes and destained for 10 minutes 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 base pair fragments of interest were excised from the DGGE gels using a sterile razor blade and pipette tip, immediately placed in a 200 µl sterile polypropylene tubes containing 36 µl of sterile water, then stored at 4°C overnight. One microliter of supernatant was used as the DNA template in a PCR re-amplification 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, WI), UltraClean 15 (Mo Bio), or UltaFree-MC 30,000 NMWL Filter Unit (Millipore) following the manufacturer

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 instructions (Invitrogen, Carlsbad, CA).

Once the fragments were deemed pure, they were sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction (PE Applied Biosystems, Foster City, CA) according to the manufacturer instructions. The primers used in the sequencing reactions were GM5F, DS907R, and the M13 vector primers (forward, GTAAAACGACGGCCAG and reverse, CAGGAAACAGCTATGAC both 5' to 3'); this resulted in the 2-fold 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 (NCBI).

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 were 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 equation 1:

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

After correction for sulfate reduction in the substrate-unamended controls, the amount 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.

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 (Fig. 2, peak 4) in nonsterile incubations exposed to ECP (Fig. 2). These peaks were not observed in the corresponding controls. The metabolites denoted as peak 5 in Fig. 2 eluted as a cluster of three peaks that shared identical mass spectral profiles (Fig. 3). 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 (26). 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. 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) (19). Thus, we propose that these ECP metabolites are ethylcyclopentylsuccinic acids. The GC cluster of three peaks could be further resolved into 5 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.

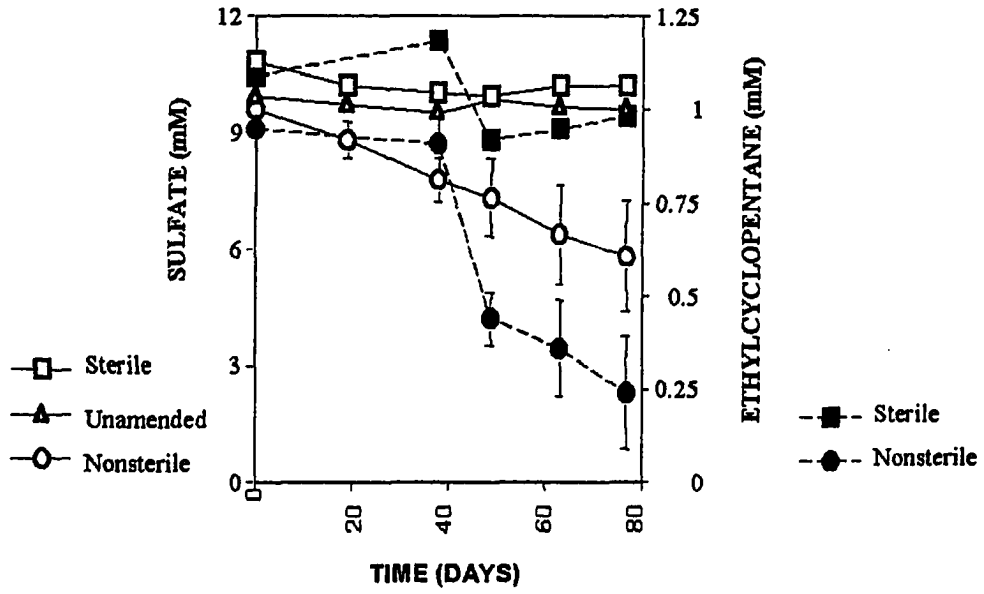

Figure 1. Ethylcyclopentane degradation by a sediment-free, sulfate-reducing microbial enrichment derived from sediments impacted with gas condensate hydrocarbons. Sulfate (open symbols), ethylcyclopentane (closed symbols).

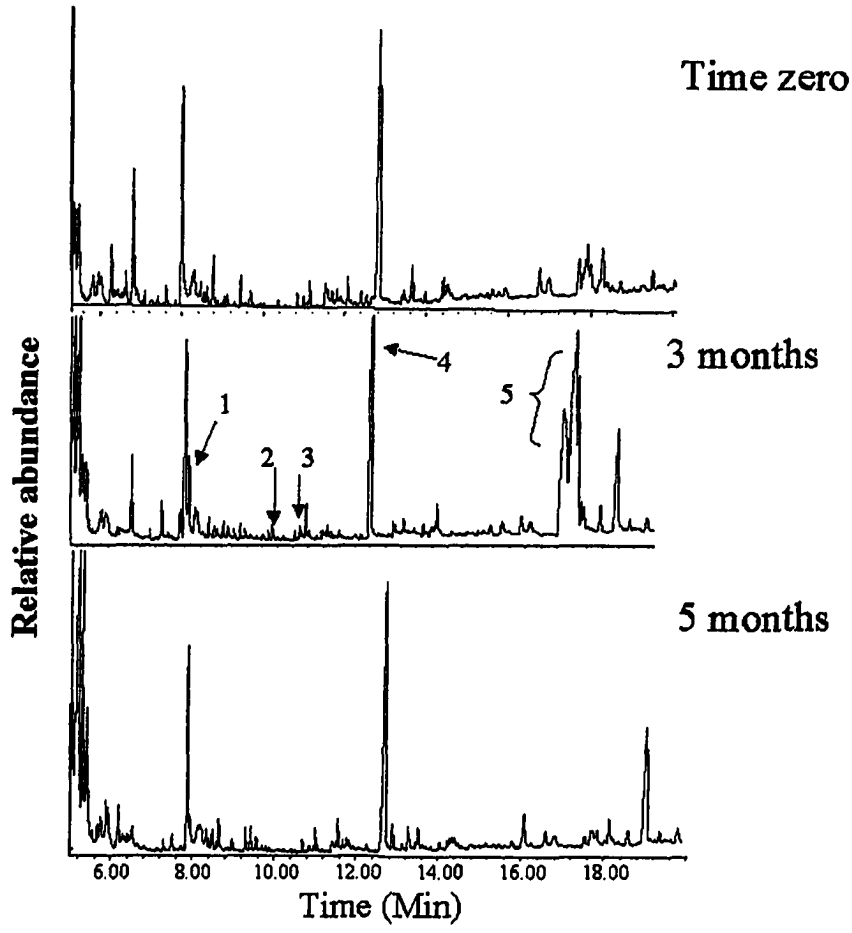

Figure 2. Gas chromatograms showing transient metabolites produced by a microbial enrichment actively degrading ECP. The samples were taken at time 0 and after 3, and 5 months of incubation. Transient peaks designation: 1) ethylcyclopentylcarboxylic acid; 2) ethylsuccinic acid; 3) ethylcyclopentylpropionic acid; 4) Hexadecane (internal standard); 5) ethylcyclopentylsuccinic acids (see text for details).

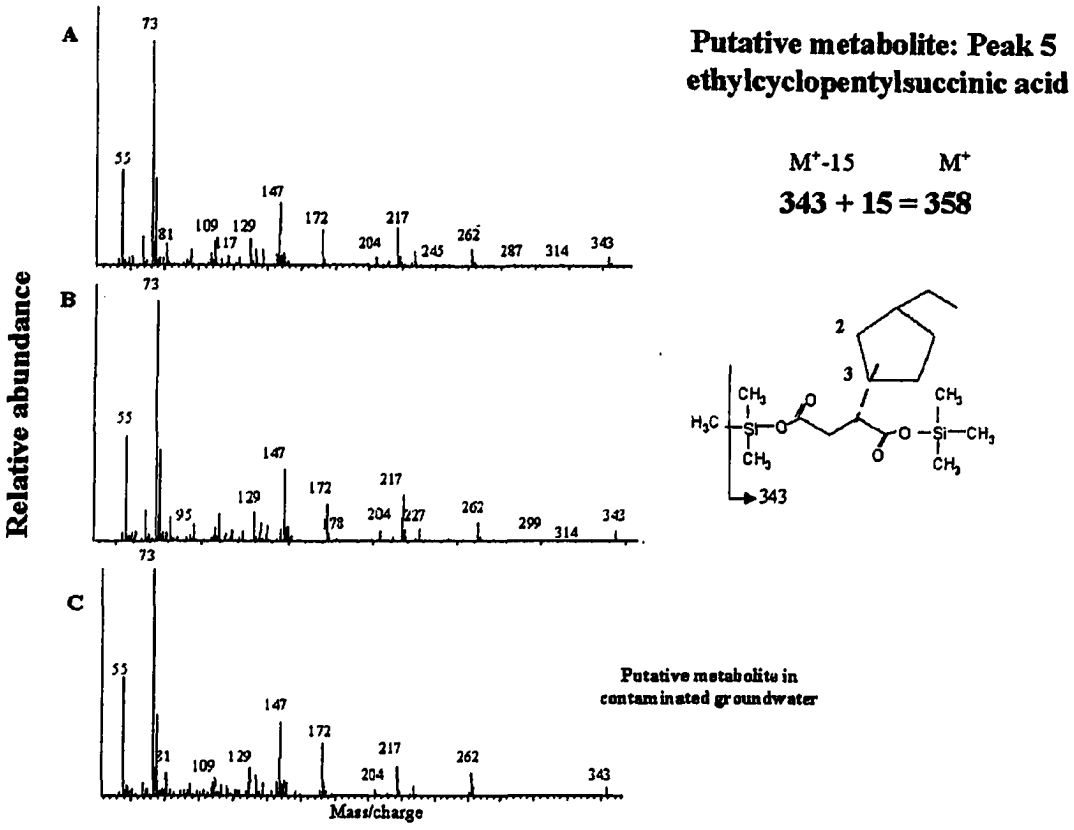

Figure 3. The mass spectral profiles of presumed ethylcyclopentylsuccinic acid isomers (analyzed as TMS esters) **A** and **B**: Mass profiles of two of the three metabolites found in laboratory enrichment culture degrading ECP; **C**: Putative metabolite found in all contaminated groundwater samples analyzed having the same GC retention time as one of the metabolite peaks in the microbial enrichment.

Another putative metabolite found in the ECP culture extracts (Fig. 2, peak 3) had a presumed M^+-15 of m/z 227, suggesting a molecular weight of 242 (Fig. 4A). 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. 4B). 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 vs. 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 (Peak 1, Fig. 2). 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. 5A). The mass spectrum of this metabolite was compared with that of the authentic standard cyclopentanecarboxylic acid (Fig. 5B). 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 vs. m/z 171) than the standard. Finally, the fourth metabolite was tentatively identified as ethylsuccinic acid (Fig. 2, peak 2). 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. 6A). The mass spectrum of this metabolite was compared with that of an authentic standard of *n*-propylsuccinic acid (Fig. 6B), and was identical except for the M^+-15 ion that 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 (19).

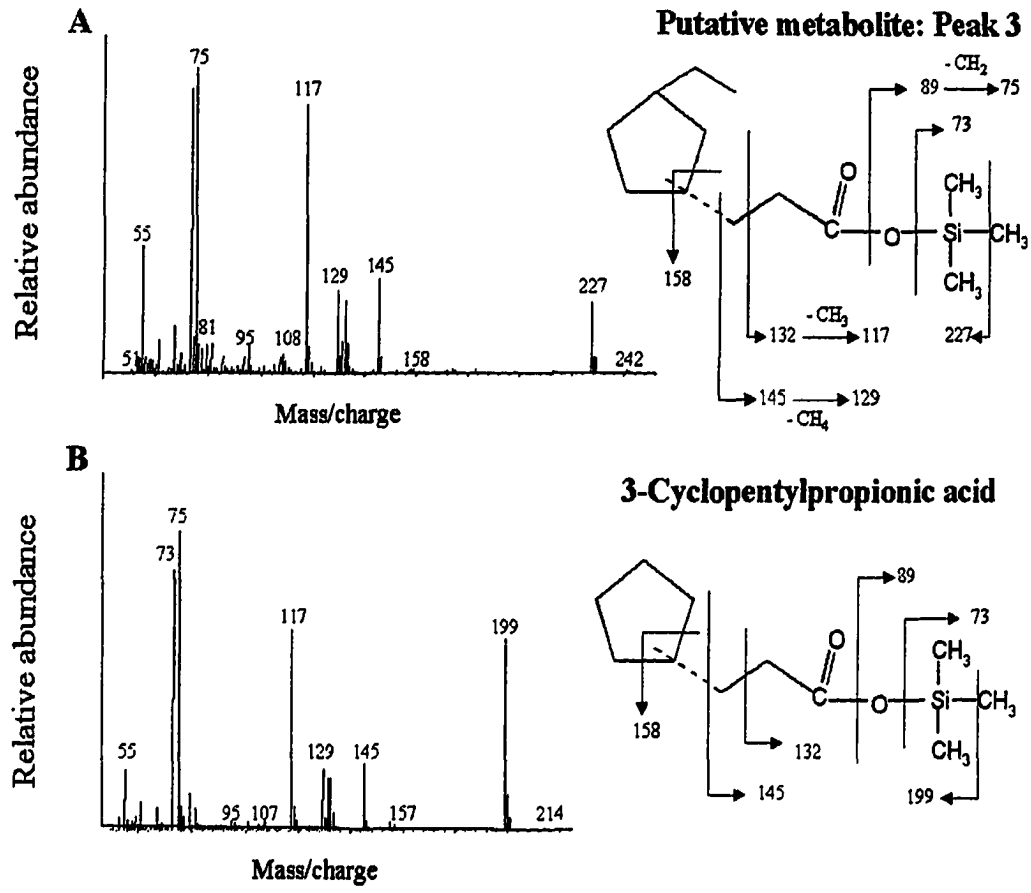

Figure 4. **A.** Mass spectrum of a metabolite tentatively identified as ethylcyclopentylpropionic acid (as TMS esters) in ECP-degrading cultures. **B.** Mass spectrum of an authentic standard of 3-cyclopentylpropionic acid (as TMS esters).

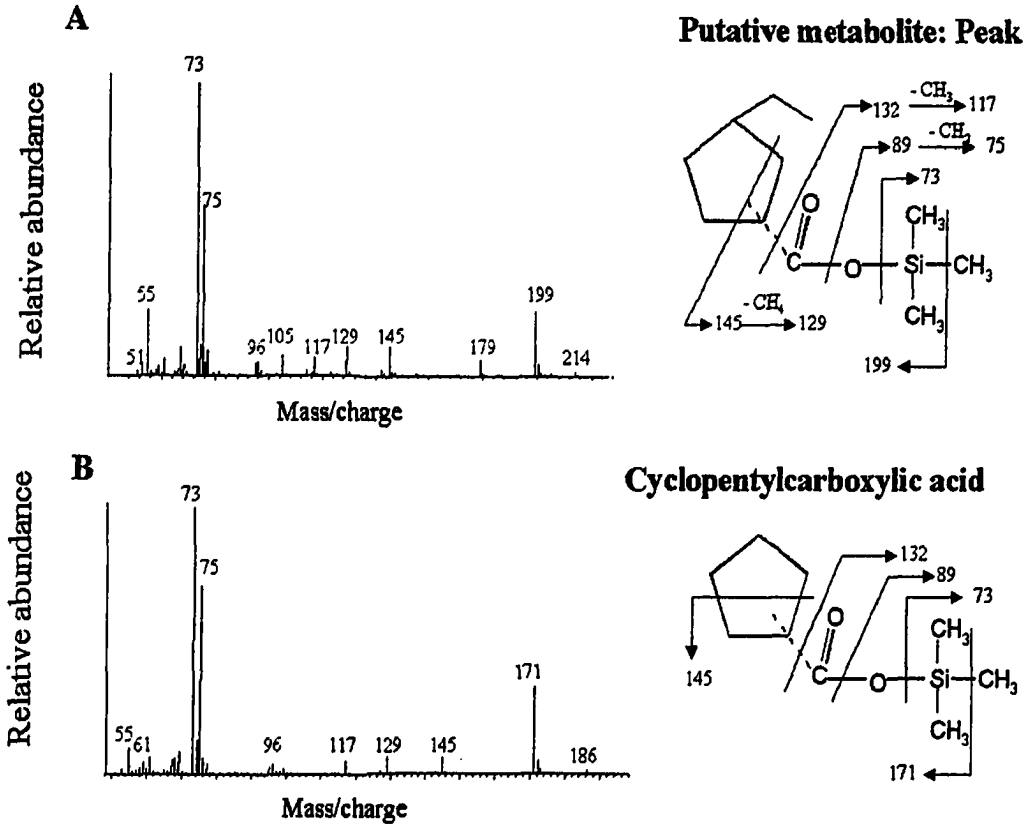

Figure 5. **A.** Mass spectrum of a metabolite tentatively identified as ethylcyclopentylcarboxylic acid (as TMS esters) in ECP-degrading cultures. **B.** Mass spectrum of an authentic standard of cyclopentylcarboxylic acid (as TMS esters).

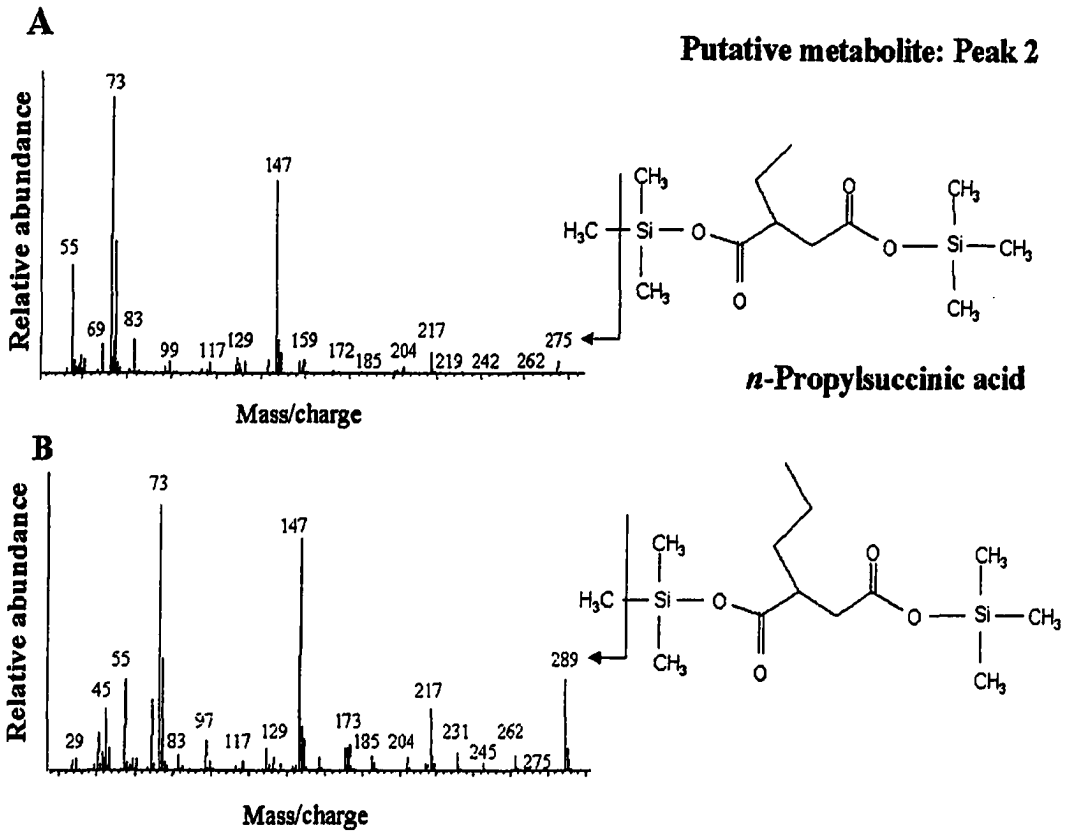

Figure 6. A. Mass spectrum of a metabolite tentatively identified as ethylsuccinic acid (as TMS esters) in ECP-degrading cultures. **B.** Mass spectrum of an authentic standard of *n*-propylsuccinic acid (as TMS esters).

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. 3C). These metabolites could not be detected in the groundwater samples from the background location, but were detected in all five contaminated locations tested.

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 3 predominant bands present in this microbial culture (Fig. 7). One of the bands showed 97% sequence homology to the uncultured eubacterial clone OCG6 (Accession # AB047117) in the CFB group (Fig. 7). 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 # AB074935.1) and *Syntrophobacter* (92%) (Accession # X82875) species (Fig. 7).

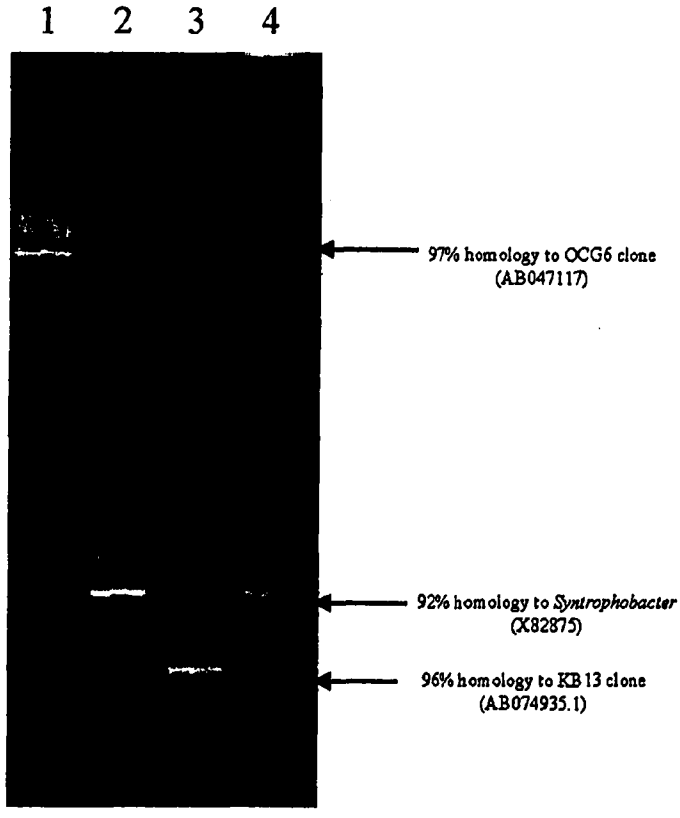

Figure 7. Denaturing Gradient Gel Electrophoresis of PCR-amplified 16S rRNA genes and phylogenetic characterization of the microorganisms amplified from the ECP-degrading culture using the partial sequence of the 16S rRNA gene. Lanes: (1) 97% similar to a uncultured Eubacterial clone OCG6 (AB047117); (2) 92% similar to a syntrophic propionate-oxidizing bacteria KoProp1 (X82875), (3) 96% similar to a clone KB13 (AB074935.1), (4) ECP degrading culture.

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 species within 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 (2-4, 7, 18, 20, 27) straight chain alkanes (19, 28), and for methylnaphthalene (22) occurring under nitrate and/or sulfate-reducing conditions and for toluene under methanogenic conditions (5). 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 succinyl moiety (m/z 172, 217, and 262) (Fig. 3A). These mass spectral features were also prominent in TMS-derivatized dodecylsuccinic acid, a metabolite associated with anaerobic dodecane biodegradation (19). The fact that we observed up to 5 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. 3). The presumed addition of fumarate to ECP at either of these positions would result in a metabolite with 3 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 (4, 20). The production of the 3 chiral centers within the diTMS ester of ethylcyclopentylsuccinic acid would theoretically result in the formation of 8 possible stereoisomers ($M=2^n$), producing 4 pairs of enantiomers which results in 4 diastereomers. The latter could be resolved with our GC conditions since they have different physical properties, there by producing 4 distinct peaks with identical MS profiles (although we observed 5 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 4 stereoisomers and 2 diastereomers from each position. This could feasibly produce 4 distinct GC peaks that correspond to the 4 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 3 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 co-migrating peak does not indicate that the other 2 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 the metabolism 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 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 (6, 12, 15). 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 (12) and most recently, from alkane degradation (15). 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 this hydrocarbon-impacted site.

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. 4 and 5). The common ion observed at m/z 145 (Fig. 4, 5) is produced by a rearrangement of the derivatized propyl tail which undergoes another rearrangement to lose a methane molecule (16 mass units) producing the ion at m/z 129 (31). The abundant ion at m/z 117 is thought to be the result of a methyl group loss from ion m/z 132 (10). The ion at m/z 73 indicates that a functional group has been derivatized as a TMS ester (26), 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 (31).

Ethylsuccinic acid (Fig. 6) 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 (15, 19). 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. 8).

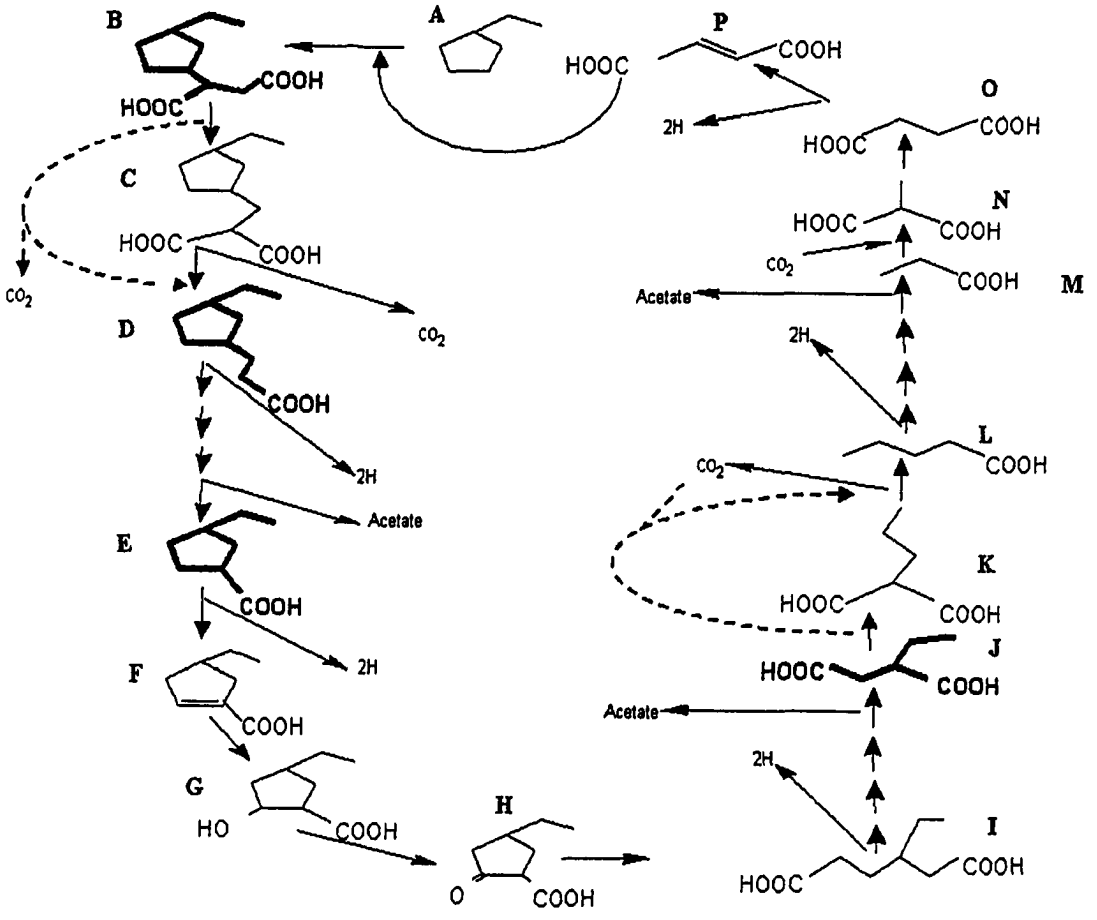

Figure 8. Proposed pathway for the anaerobic biodegradation of the hydrocarbon ethylcyclopentane by a sulfate-reducing enrichment culture. In the following list of depicted compounds, those that have been identified as TMS esters are followed by corresponding peak numbers (in parentheses) from Fig. 2 and there structures are in bold. A, ethylcyclopentane; B, ethylcyclopentylsuccinic acid (5); C, ethylcyclopentylmethylmalonic acid; D, ethylcyclopentylpropionic acid (3); E, ethylcyclopentanecarboxylic acid (1); F, ethylcyclopen-1-enecarboxylic acid; G, 2-hydroxyethylcyclopentanecarboxylic acid; H, 2-oxoethylcyclopentanecarboxylic acid; I, β-ethyladipic acid; J, ethylsuccinic acid (2); K, propylmalonic acid; L, valerate; M, propionate; N, methylmalonic acid; O, succinic acid; P, fumarate.

This pathway is analogous to the pathway outlined by Wilkes et. al. (36) 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 co-substrate fumarate (P, Fig. 8) for the activation of ECP (A, Fig. 8) to ethylcyclopentylsuccinic acid (B, Fig. 8).

In the pathway, ethylcyclopentylsuccinic acid is presumably decarboxylated to produce the ethylcyclopentylpropionic acid (D, Fig. 8). We do not know if the removal of the CO_2 is directly from the succinyl moiety or if a carbon rearrangement producing ethylcyclopentylmethylmalonic acid (C, Fig. 8) is necesary prior to the loss of CO_2 as proposed by Wilkes et. al. (36). The next metabolite identified in our proposed pathway is ethylcyclopentanecarboxylic acid (E, Fig. 8). 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 (F, Fig. 8), and be sequentially oxidized to an alcohol (G, Fig. 8) and a ketone (H, Fig. 8) followed by ring cleavage producing the proposed metabolite β-ethyladipic acid (I, Fig. 8). These series of reactions are similar to the well-characterized reactions carried out by the phototrophic anaerobe *Rhodopseudomonas palustris* and *Syntrophus acidotrophicus* when grown on benzoate (13, 25). β-Ethyladipic acid (I, Fig. 8), 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 (J, Fig. 8). 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 (L, Fig. 8). Again, this decarboxylation could occur dirrectly from the succinyl derivative or after a carbon rearrangement by an unknown mechanism analogous to the decarboxylation of ethylcyclopentylsuccinic acid (Fig. 8, steps B to D). Thus, valerate may be produced directly, or via the presumed metabolite propylmalonic acid (K, Fig. 8). Valerate may then undergo β-oxidation to produce propionate (M, Fig. 8), which may be carboxylated to methylmalonic acid (N, Fig. 8) and further metabolized to succinic acid (O, Fig. 8). These series of reactions would be similar to the reactions used by propionate oxidizers represented by the genera *Desulfobulbus* (17) and *Syntrophobacter* (34). Finally, succinic acid is then dehydrogenated, regenerating fumarate.

The DGGE profile of the sediment-free ECP-degrading culture shows the presence of 3 predominant bands representing the dominant amplifiable eubacterial microorganisms in the culture (Fig. 7). The associated sequences suggest that the organisms are phylogenetically related to the genus *Desulfotomaculum* (AF529223) within the Bacillus-Clostridium group, the genus *Syntrophobacter* (AF529225) within the δ subdivision of the Proteobacteria, and to the members of the *Bacteroidaceae* (AF529224) within the CFB group (Fig. 7). The sum of these organisms, represented by their sequences, constitutes the predominant members of the ECP-degrading culture. The sequence belonging to the *Bacteroidaceae* was 97% homologous to an uncultured clone obtained from groundwater contaminated with oil from an underground storage cavity (Accession # 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*, organisms which are best known for their ability to degrade fatty acids and other simple organic molecules by syntrophic association or using sulfate as terminal electron acceptor (16, 33, 34). Interestingly, various metabolites that might serve as substrates for these organisms are proposed in the ECP pathway (Fig. 8). 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 co-culture 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 (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 (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 (35). The fact that ECP degradation is coupled to sulfate reduction in our enrichment and the inability of the *Syntrophobacter* sp. isolate to degrade ECP supports

our working hypothesis that the *Desulfotomaculum* sp. is reponsible for the degradation of ECP.

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Chapter 2.

Desulfomonile liminquinati sp. nov., a cyclohexane carboxylate-degrading sulfate-reducing bacterium isolated from a gas condensate-contaminated aquifer.

Abstract

An anaerobic, cyclohexane carboxylate-degrading, sulfate-reducing bacterium was isolated from a gas condensate-contaminated aquifer. The bacterium is a Gram-negative, non-sporeforming, non-motile, non-dehalogenating large rod with a collar-like morphology. The isolate can grow autotrophically with sulfate, sulfite, thiosulfate or fumarate as terminal electron acceptors and hydrogen as electron donor. Heterotrophic growth was achived with lactate, propionate, crotonate, cyclohexane carboxylate and cyclohex-1-ene carboxylate as electron donor with sulfate as electron acceptor. During cyclohexane carboxylate degradation, we identified transient metabolites by comparing their gas chromatographic retention times and mass spectral profiles with authentic standards. The metabolites included cyclohex-1-ene carboxylate, 2-hydroxycyclohexane carboxylate, pimelic acid, and glutaric acid. The presence of these metabolites suggests that cyclohexane carboxylate is degraded by this isolate using a pathway similar to *Rhodopseudomonas palustris*. The morphology and 16S rRNA gene partial sequence

suggests that this isolate is a new species of the genus *Desulfomonile*, within the delta subdivision of the Phylum *Proteobacteria*. Unlike the *Desulfomonile* species, this organism was not able to dehalogenate 3-chlorobenzoate. We propose that this isolate be named *Desulfomonile liminquinati*.

Introduction

Under anaerobic conditions, a wide variety of aromatic compounds are metabolized, producing benzoate as an intermediate (Grbic-Galic and Vogel 1987, Young et al. 1995, Gibson and Harwood 1995, Kleerebezem et al. 1999, Elshahed et al. 2000). Benzoate is therefore a key intermediate formed during the anaerobic metabolism of aromatic hydrocarbons normally found in petrolume products (Gibson and Harwood 2002). Under sulfate-depleted conditions, the oxidation of benzoate usually requires a syntrophic partnership (Elshahed et al. 2001), although a variety of pure cultures have been shown to mineralize benzoate in the presence of electron acceptors like sulfate, nitrate, or iron (Coates et al. 2001, DeWeerd et al. 1990, Pelletier and Harwood 1998, Sun et al. 2001). *Rhodopseudomonas palustris*, a benzoate-mineralizing bacterium, produces cyclohex-1-ene carboxylate as a transient intermediate during benzoate and cyclohexane carboxylate metabolism (Harwood and Perrota 1994, Küver et al. 1995). Similarly, cyclohexane carboxylate is produced during the fermentation of benzoate by *Syntrophus acidotrophicus* strain SB when this microorganism is grown in pure culture (Elshahed and McInerney 2001) or when grown syntrophically with a hydrogenotrophic partner (Elshahed et al. 2001). Under the latter condition, up to 18% of the total carbon in the co-culture was found to transiently accumulate as cyclohexane carboxylate. Moreover, this intermediate was found as a free acid in the culture fluids suggesting that it may be released to the environment. Likewise, in a phenol-degrading methanogenic consortium, up to 28% of the total carbon was found to accumulate as cyclohexane carboxylate, which was also found as a free acid in the culture (Jones et al. 1989).

Cyclohexane carboxylate and cyclohex-1-ene carboxylate were also formed as intermediates during the aerobic metabolism of n-alkyl-substituted cycloparaffins (Dutta and Harayama 2001).

The oxidation of cyclohex-1-ene carboxylate or cyclohexane carboxylate to acetate, CO_2, and hydrogen in the absence of terminal electron acceptors is thermodynamically unfavorable with a $\Delta G^{\circ\prime}$ of 93.7 kJ/mol and 165.3 kJ/mol respectively (Elshahed and McInerney 2001). In the presence of sulfate the same process becomes favorable with a $\Delta G^{\circ\prime}$ of -96.2 kJ/mol in the case of cyclohex-1-ene carboxylate and -62.6 kJ/mol in the case of cyclohexane carboxylate. Since both of these compounds are known intermediates of aromatic substrates, but do not require a syntrophic partnership for degradation under sulfate-reducing conditions, are less volatile and are more water-soluble than presumed hydrocarbon precursors, we hypothesized that these compounds could be useful carbon sources for the isolation of bacteria capable of degrading the presumed parent compounds. We used sediments and groundwater from a natural gas production site near Ft. Lupton, CO (Gieg et al. 1999) to enrich and isolate microorganisms for their ability to degrade cyclohexane carboxylate and cyclohex-1-ene carboxylate. The same inoculum source was used to study the anaerobic biodegradation of ethylcyclopentane (Rios-Hernandez et al. 2003), isomers of toluic acids (Elshahed et al. 2000) and BTEX (benzene, toluene, ethylbenzene, and isomers of xylene) hydrocarbons (Gieg et al. 1999). In addition, multiple lines of evidence showed that BTEX hydrocarbons were being biologically attenuated with the production of benzoate at this location (Gieg et al. 1999).

In this study, we obtained an isolate capable of degrading cyclohexane carboxylate and cyclohex-1-ene carboxylate, but it was unable to metabolize benzoate or any of the BTEX hydrocarbons using sulfate as electron acceptor. The 16S rRNA gene sequence analysis suggests that this isolate is a member of the *delta* sub-division of the Phylum *Proteobacteria* and belongs to the genus *Desulfomonile*. This conclusion is further supported by the presence of a morphologically distinct collar, a feature that was originally used to help characterize this genus (DeWeerd et al. 1990). However it does not have the ability to metabolize *meta*-substituted chlorinated aromatic compounds, an ability shared by the other two members of the genus *Desulfomonile* (Deweerd et al. 1990 and Sun et al. 2001). We propose the name *Desulfomonile liminquinati* for this isolate.

Materials and Methods

Enrichment, Media and Isolation

Contaminated sediments from a hydrocarbon-impacted site near Ft. Lupton, CO (Gieg et al. 1999) were used as inoculum source and amended with benzoate, cyclohex-1-ene carboxylate, or cyclohexane carboxylate as electron donors, while sulfate (10 mM) served as the electron acceptor. Typically, 10 g of sediment and 20 ml of autoclaved Na_2S-reduced (0.1ml/10ml of a 1.25% solution) groundwater, were placed in sterile 40 ml serum bottles. All manipulations were done inside an anaerobic glovebox containing 5% H_2 in N_2. The bottles were sealed with sterile butyl rubber stoppers and aluminum

crimps. After removal from the glovebox, the headspace of the incubations were exchanged with 20% CO_2 in N_2. Substrate-unamended controls without added hydrocarbon were used to account for background levels of sulfate reduction. Once the degradation of the substrate and the reduction of sulfate were observed, active incubations were used in attempts to isolate the responsible microorganisms using roll tubes. The roll tubes contained the same medium and carbon source as the slurried incubations, except that 2% agar noble (Difco Laboratories, Detroit, MI) was added. Colonies were picked from roll tubes while inside an anaerobic chamber and transferred to tubes with 5 mL of the original medium to confirm substrate metabolism. A subsample of the culture was used to extract its DNA followed by PCR amplification and Denaturing Gradient Gel Electrophoresis to confirmed the purity of the isolate (Rios-Hernandez et al. 2003).

A defined medium for the routine cultivation of the isolate consisted of minerals, trace metals, and vitamins with rezasurin as the redox indicator as previously described (Tanner et al. 1989). The pH of the medium was adjusted to 7.2 with NaOH, and then boiled. The medium was then cooled while being sparged with a N_2:CO_2 (80:20) gas mixture. Sodium bicarbonate was added (3 g/L) when the medium reached room temperature and the culture vessel was sealed. Finally, the medium was reduced with sodium sulfide or cysteine-sulfide to a initial concentration of 1 mM. The final gas phase was N_2 : CO_2 (80:20) over pressurized to 34 kPa. Typically, sulfate was added to an initial concentration of 10 mM. In dechlorinating experiments, the above medium was modified by replacing the sulfate with 3-chlorobenzoate (1 mM) and adding 10 ml of a supplemental solution containing nicotinamide, 1,4-naphthoquinone, lipoic acid, hemin,

and thiamine (5 mg, 2 mg, 1.2 mg, 0.5 mg, and 0.5 mg respectively, in 100 ml) (Sun et al. 2000).

The optimum temperature for growth was determined using the above medium with 1.27 mmoles of hydrogen (68.9 kPa) as the electron donor. The pH optimum was determined using the same medium but containing 10 mM each of MES (2-[N-morpholino] ethane-sulfonic acid), PIPES (Piperazine-N,N'-bis-[2-ethanesulfonic acid]) or HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) instead of sodium bicarbonate as the buffer. The ability of this organism to grow with various aromatic compounds, alicyclic acids, fatty acids, alcohols, tricarboxylic acid intermediates, pyruvate, lactate, crotonate or hydrogen was tested with sulfate as electron acceptor. The concentration of all test substrates was approximately 1 mM, except when toluene, benzene, m-xylene, butylbenzene and 1-methylcyclohexene were used as substrates that 1 μL of neat substrate was added to 50 ml of liquid medium resulting in the following concentrations 0.132 mM, 0.157 mM, 0.115 mM, 0.070 mM, 0.029 mM respectively. Similarly, the ability to use different electron acceptors including sulfate, sulfite, thiosulfate, nitrate, fumarate, tetrachloroethylene and 3-chlorobenzoate was tested using the same medium with hydrogen as the electron donor. In addition, propionate, crotonate, lactate, cyclohex-1-ene carboxylate and cyclohexane carboxylate were used as electron donors to test for the ability to dechlorinate 3-chlorobenzoate.

Cyclohexane carboxylate metabolites

To assay for putative metabolites in the pure culture, 30 ml of culture fluids were periodically taken, and treated with base and then acid as previously reported (Kropp et al. 2000). The 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 (BSTFA; Pierce, Rockford, IL) to form trimethylsilyl (TMS) esters prior to gas chromatography mass spectroscopy (GC-MS) analysis.

Microscopy

Cellular morphology was determined by phase contrast and electron microscopy on mid-log phase cells grown autotrophically or heterotrophically with cyclohex-1-ene carboxylate and sulfate. Scanning and transmission electron microscopy was performed as previously described (DeWeerd et al. 1990).

DNA Extraction and 16S rRNA gene sequence

The cells were collected by centrifugation, and the DNA was extracted as previously described (Rios-Hernandez et al. 2003). The DNA was amplified by the polymerase chain reaction (PCR) using universal Eubacterial primers 27F and 1492R (Herrick et al.

1993). The 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 10X PCR buffer B (Fisher Scientific, Fair Lawn, NJ), 10 µl of $MgCl_2$ (25 mM), 2.5 U of Taq Polymerase (Fisher Scientific, Fair Lawn, NJ), and sterile water were mixed to a final volume of 100 µl. The samples were amplified using the following PCR protocol (Herrick et al. 1993). This PCR product was purified using the Wizard PCR Preps DNA Purification System (Promega, Madison, WI). Once purified, the DNA was sequenced using 4 different primers, 27F, 1492R, 926F, and 1100R (Johnson 1994) with the BigDye Terminator Cycle Sequencing Ready Reaction (PE Applied Biosystems, Foster City, CA) according to the manufacturer instructions. The four individual sequences were aligned in the computer program Sequencer and a consensus strand was produced. All PCR product sizes were confirmed by agarose-gel electrophoresis, stained with ethidium bromide, and visualized by UV transillumination. The gels were then photographed under UV transillumination (302 nm) using a Kodak DC120 digital camera and analyzed with the NucleoTech GelExpert-Lite software.

Related sequences were obtained from the GenBank database by using the Blastn search program of the National Center for Biotechnology Information (NCBI) (Altschul 1997). These sequences were aligned using ClustalX (Thompson et al. 1997) and their phylogenetic relationship was examined using the evolutionary distance method with the corrections of Jukes and Cantor with the computer program PAUP (Swofford 2000).

Analytical Methods

Growth was measured in culture tubes as absorbance at 600 nm using a Bausch and Lomb Spectronic 21 spectrophotometer. Substrate decay was tested in duplicate cultures and compared against uninoculated and substrate-unamended controls. The cultures were incubated in the dark without agitation at 28°C and analyzed for the disappearance of electron acceptors by ion chromatography (Caldwell et al. 1998). The disappearance of the electron donor in the culture was confirmed with a Beckman HPLC equipped with a Prevail Organic Acid column (Alltech, Deerfield, IL.) with UV detection at 214 nm. The mobile phase for the analysis of the aromatic compounds and alicyclic acids was composed of 70% potassium or sodium phosphate (25 mM, pH = 2.5) and 30% acetonitrile with a flow rate of 1 ml/min. The fatty acids were analyzed similarly but the mobile phase was 100% potassium or sodium phosphate (25 mM, pH = 2.5). The BTEX and other volatile hydrocarbons were analyzed by gas chromatography (GC) as previously described (Gieg et al. 1999). Tetrachloroethylene was also analyzed as described above by GC with the exception that the oven temperature was held at 40°C.

Chemicals

All halogenated and aromatic compounds as well as 1,4-naphthoquinone were obtained from Aldrich Chemical Co., Milwaukee, WI. All other chemicals used were obtained from Sigma Chemical Co., St. Louis, MO with the exception of 2-hydroxycyclohexane carboxylate; a gift from the laboratory of Dr. Michael J. McInerney at the University of Oklahoma.

Results

Isolation of strain IA6

Individual colonies were visible in the roll tubes after 4 weeks of incubation with cyclohexane carboxylate as the sole carbon and energy source. Well isolated, whitish colonies were picked from the roll tubes after 8 to 10 weeks and transfered to liquid medium (5 ml) with 1 mM cyclohexane carboxylate and 10 mM sulfate. The culture volume was gradually increased with repeated transfers (~25%) to bigger volumes of fresh media following 3 to 6 weeks of incubation. Cultures were subjected to a round of serial dilution and inoculation to roll tubes as described above for a second time. Colonies were picked and inoculated into tubes containing the medium previously described. The isolate was checked for purity by phase contrast microscopy, by assay on a thioglycolate medium (Difco Laboratories, Detroit, MI, USA.) which did not support the growth of the isolate and by molecular analysis (DGGE). This isolate was called strain IA6.

Cellular morphology

Strain IA6 cells stained gram-negative and are non-motile, non-spore-forming, large rods (8 to 10 μM x 0.6 to 0.8 μM) with a collar-like morphology (Figure 1). The collar is apparent in electron micrographs (Fig. 1A, B). Interestingly, the polar end of the bacterium had a rough surface while the rest of the cell was smooth (Fig. 1C). The morphology of the cell did not change when grown with different electron donors, but at late stationary-phase a large amorphous phase-bright area in the center of the cell was observed by phase contrast microscopy (Fig. 1 E).

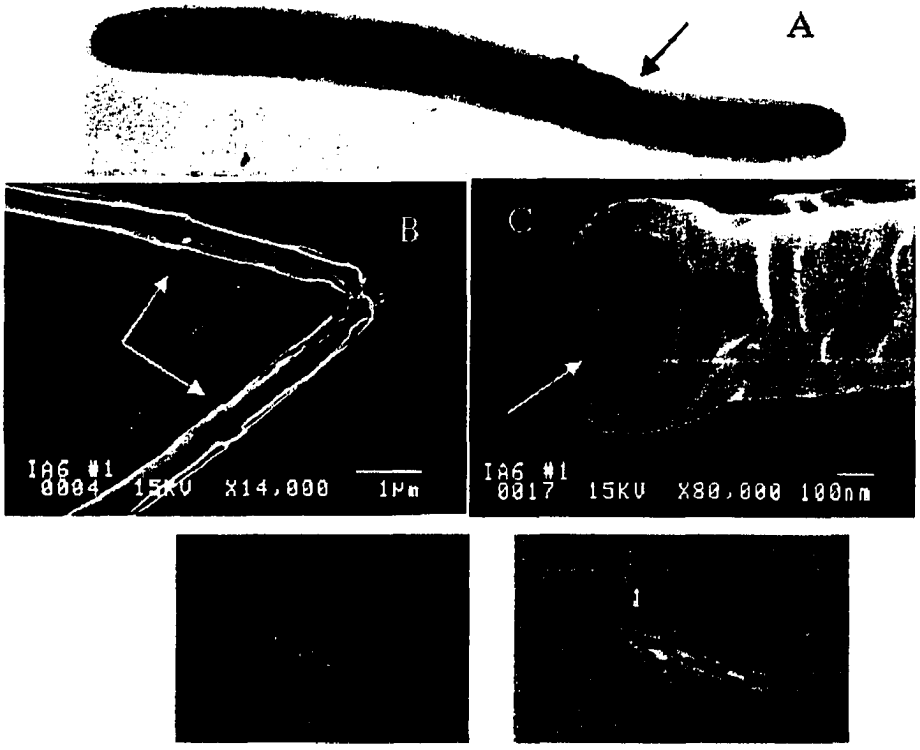

Figure 1. A) Transmission electron micrograph of isolate *Desulfomonile liminquinati* (arrow indicating collar). B) Scanning electron micrograph of cells (arrow indicating collar). C) Polar end of daughter cell inside collar of mother cell. D) Phase contrast photograph of cells at mid-log phase. E) Phase contrast photograph of cells at late stationary-phase.

Growth substrates

Strain IA6 is a strictly anaerobic, sulfate reducer capable of using H_2/CO_2, lactate, propionate, crotonate, cyclohexane carboxylate, or cyclohex-1-ene carboxylate as electron donors. Propionate, lactate and crotonate were incompletely oxidized to acetate with sulfate as electron acceptor while the others were mineralized. This bacterium was unable to use formate, acetate, isovalerate, pyruvate, fumarate, succinate, adipate, pimelate, methanol, ethanol, phenol, benzoate, p-hydroxybenzoate, m-hydroxybenzoate, t-cinnamic acid, hydroxycinnamic acid, toluene, m-xylene, benzene, phenylpyruvate, 4-phenylbutyric acid, butylbenzene, 1-methylcyclohexene, methylcyclohexane, or syringate as electron donors. Strain IA6 used sulfate, sulfite, thiosulfate and fumarate as electron acceptors but not nitrate, Fe(III)-NTA, Fe $(OH)_3$ or polysulfides. Molybdate (5 mM) inhibited the growth of IA6 with H_2/CO_2 and either sulfate or thiosulfate as the electron acceptor. The optimum pH for growth was 7.2. Strain IA6 did not grow at pH less than 6.0 or at pH's above 7.5. IA6 grew fastest at 28 °C, but it can grow at temperatures ranging from 25 °C to 32 °C.

In our hands, this isolate did not halorespire 3-chlorobenzoate or tetrachloroethylene in the presence or absence of sulfate with pyruvate, cyclohexane carboxylate, cyclohex-1-ene carboxylate, propionate, crotonate, lactate, or hydrogen as ethe lectron donor. In addition, the isolate did not degrade benzoate, cyclohexane carboxylate or propionate syntrophically when grown in co-culture with the methanogen *Methanospirillum hungateii* strain JF-1.

Cyclohexane carboxylate metabolism

During the degradation of cyclohexane carboxylate by strain IA6, we detected and identified several transient metabolites by GC-MS analysis of culture fluids. The first metabolite identified by comparison to an authentic standard was cyclohex-1-ene carboxylate (Table 1). Similarly, 2-hydroxycyclohexane carboxylate was identified by comparison of its mass spectral profile to that of an authentic standard (Table 1). The next metabolites identified are the ring cleavage products of cyclohexane carboxylate oxidation, namely pimelate (Table 1) and glutarate (Table 1). These intermediates were not observed in inoculated culture fluids amended with propionate as electron donor or while the cell were growing using hydrogen as electron donor and CO_2 as carbon source. The identification of metabolites produced from the mineralization of cyclohexane carboxylate were consistant with the degradation pathway of *Rhodopseudomonas palustris* (Küver et al. 1995) (Fig. 2).

Table 1. Comparison of GC-MS characteristics of TMS-Derivatized metabolites detected in culture fluids of cells actively degrading cyclohexane carboxylate and their corresponding TMS-Derivatized authentic standards.

		characteristic mass spectrum m/z (% of base ion)	
Metabolite	**GC RT** (Min)	**In culture fluids**	**Standards**
2-hydroxycyclohexane carboxylate	21.9	**73**(98), **81**(31), **129**(19), **147**(100), **170**(10), **183**(8), **198**(5), **273**(16),	**73**(77), **81**(29), **129**(17), **147**(100), **170**(10), **183**(8), **198**(5), **273**(17),
cyclohex-1-ene carboxylate	16.5	**73**(85), **75**(98), **81**(31), **108**(47), **139**(18), **156**(5), **183**(100), **198**(18)	**73**(64), **75**(80), **81**(20), **108**(36), **139**(21), **156**(7), **183**(100), **198**(21)
pimelate	15.4	**73**(100), **75**(77), **97**(9), **125**(26), **129**(12), **147**(29), **155**(23), **173**(16), **186**(6), **204**(6), **217**(7), **289**(9)	**73**(100), **75**(88), **97**(15), **125**(40), **129**(13), **147**(31), **155**(43), **173**(24), **186**(6), **204**(6), **217**(11), **289**(25)
glutarate	21.0	**73**(100), **75**(66), **97**(15), **116**(13), **129**(18), **147**(82), **158**(22), **186**(9), **204**(7), **233**(4), **261**(17)	**73**(71), **75**(41), **97**(11), **116**(9), **129**(12), **147**(100), **158**(22), **186**(6), **204**(6), **233**(6), **261**(31)

Cyclohexane carboxylate intermediates were detected by injecting 1 μL of the TMS-derivatized culture fluid extracts into a HP Model 5890 GC connected to a HP Model 5970 MS detector. Compounds were separated using a DB-5 column (30 m x 0.25 mm ID and 0.1 μm thickness; Alltech, Deerfield, IL). The injector and detector temperatures were held at 250°C. The temperature program for the oven was described previously (Elshahed 2001). Helium was used as the carrier gas at a flow of 0.8 mL / min. All of the authentic standards were commercially available with the exception of 2-hydroxycyclohexane carboxylate.

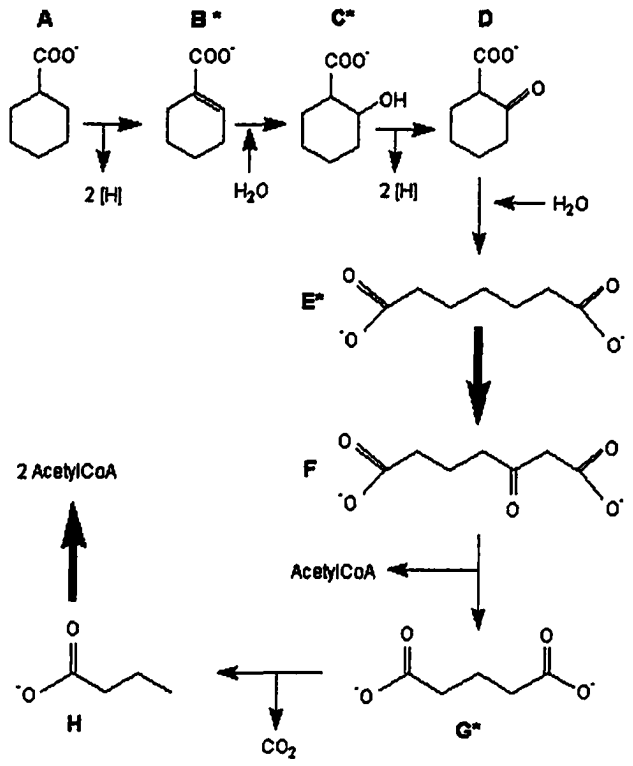

Figure 2. Proposed pathway for the anaerobic degradation of cyclohexane carboxylate by the sulfate-reducing isolate *Desulfomonile liminquinati*. In the following list of depicted compounds, those that have been identified as TMS esters in culture fluids are in boldface with an asterisks. A, cyclohexane carboxylate; B, cyclohex-1-ene carboxylate; C, 2-hydroxycyclohexane carboxylate; D, 2-ketocyclohexane carboxylate; E, pimelic acid; F, β-ketopimelic acid; G, glutaric acid; H, butyric acid. Bold arrows represent β oxidation steps.

Phylogeny

The phylogenetic relationship of this isolate to other sulfate-reducing bacteria and other species in the phylum *Proteobacteria* of the Bacterial kingdom is shown in Figure 3. Partial DNA sequence (1464 bp) of the 16S rRNA gene showed 93% homology to both of its closest relatives *Desulfomonile tiedjei* and *Desulfomonile limimaris*. These bacteria are members of the family *Desulfoarculaceae* within the order *Desulfobacteriales* in the class, *delta-Proteobacteria* .

Discussion

The morphological features of strain IA6 observed in the electron micrographs are consistent with those observed in other members of this genus (DeWeerd et al. 1990, Sun et al. 2001). The predominant feature in this organism is the collar-like morphology that may be involved in cell division (Mohn et al. 1990). In addition, the terminus of both cells, mother and daughter, appear to have a grainy surface while the rest of the cell is smooth (Fig. 1B and C).

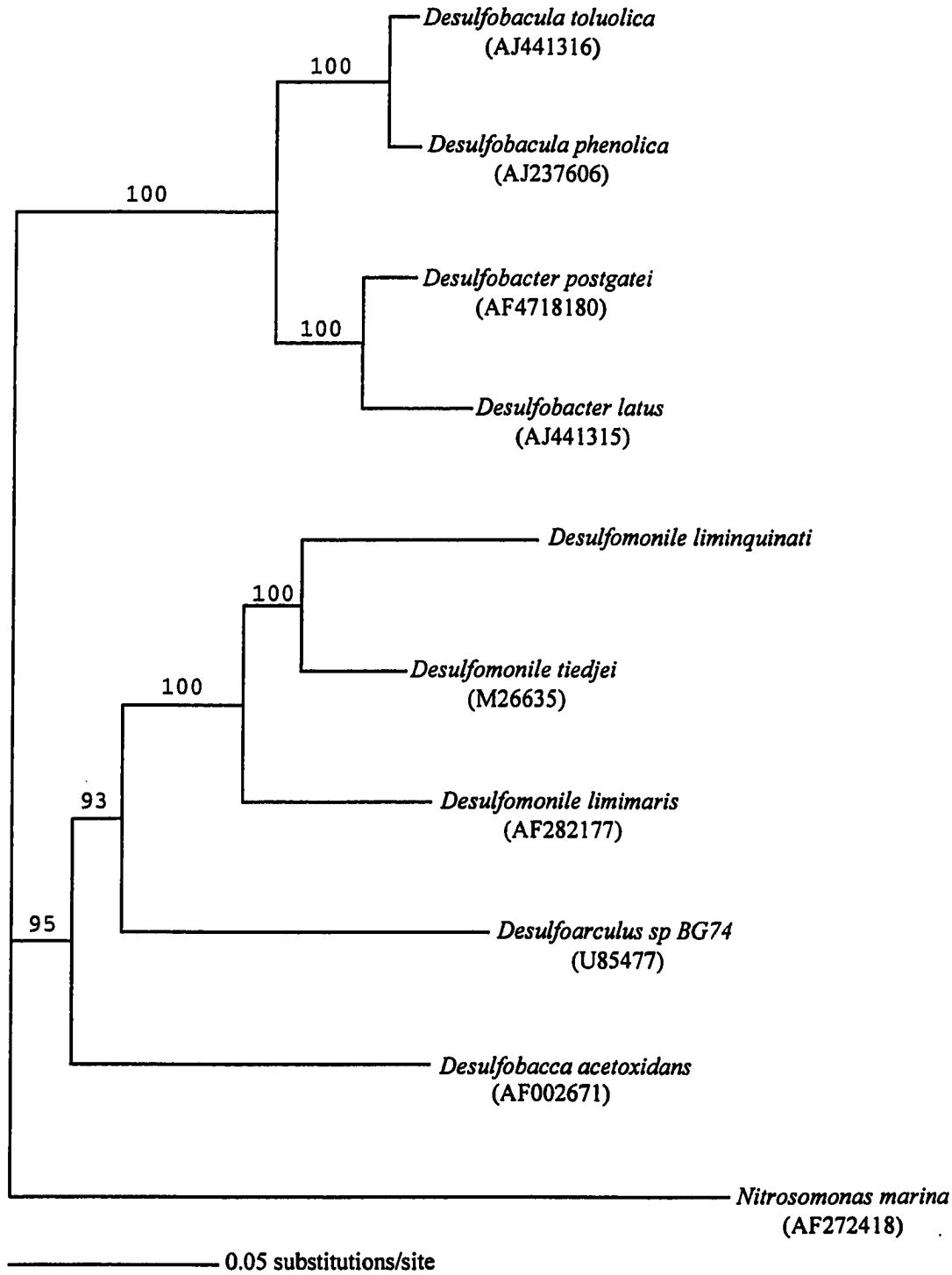

Figure 3. Evolutionary distance phylogenetic tree based on the 16S rDNA sequences of isolate *Desulfomonile liminquinati* and relatives within the *delta*-proteobacteria. Numbers at nodes represent the bootstrap values of 1000 replicates. In parenthesis, GenBank sequence accession numbers.

Strain IA6 is a sulfate-reducing bacterium that grows autotrophically on H_2/CO_2 and sulfate, sulfite, thiosulfate, or fumarate. Heterotrophic growth can be achieved by propionate, lactate, crotonate, cyclohexane carboxylate or cyclohex-1-ene carboxylate oxidation with sulfur oxyanions as electron acceptors. Propionate, lactate, and crotonate are incompletely oxidized to acetate, while cyclohexane carboxylate and cyclohex-1-ene carboxylate are mineralized. In comparison to known species of *Desulfomonile* (DeWeerd et al. 1990, Sun et al. 2001), our isolate shares the ability to utilize sulfur oxyanions as electron acceptors, with hydrogen or lactate as electron donors. Like *Desulfomonile limimaris*, our isolate can be differentiated from *Desulfomonile tiedjei* by its ability to oxidize propionate, use fumarate as electron acceptor and its inability to oxidize acetate. In contrast, *Desulfomonile tiedjei* and our isolate can be differentiated from *Desulfomonile limimaris* by the ability to grow in the absence of NaCl and their inability to use nitrate as electron acceptor. Furthermore, the IA6 isolate can be differentiated from both of these organisms by its inability to use benzoate, formate or pyruvate as electron donors as well as its inability to grow at 37 °C. In addition, IA6 is not able to use 3-chlorobenzoate as an electron acceptor in the presence of any of the electron donors that supported metabolism with sulfur oxyanions acceptors. Therefore, we must conclude that IA6 is a non-dehalogenating member of the *Desulfomonile* genus. However, it is possible that the electron donor required for dechlorinating activity is not utilized by IA6 under any other electron-accepting condition as is the case for *Desulfovibrio dechloracetivorans* (Sun et al. 2000). This organism only oxidizes acetate in the presence of 2-chlorophenol and not with any other electron acceptor (Sun et al. 2000).

Another difference between our isolate and the previously described *Desulfomonile* species is its ability to mineralize cyclohexane carboxylate, cyclohex-1-ene carboxylate, and oxidize crotonate. During cyclohexane carboxylate degradation we were able to identify four transient metabolites which allowed us to deduce the likely degradation route by which strain IA6 consumes this compound (Fig. 2). The metabolites have been identified previously in the cyclohexane carboxylate degradation pathway of *Rhodopseudomonas palustris* (Küver et al. 1995) and *Syntrophus acidotrophicus* (Elshahed and McInerney 2001). These organisms are better known for their ability to degrade benzoate. *Rhodopseudomonas palustris* metabolizes benzoate and cyclohexane carboxylate by different pathways since each compound requires different activating enzymes. Once the substrate is activated by different CoA ligases the pathways converge on cyclohex-1-ene carboxylate. In contrast, *Syntrophus acidotrophicus* will degrade both substrates utilizing the same pathway since cyclohexane carboxylate is an intermediate in the benzoate pathway (Elshahed et al. 2001). Our isolate does not degrade benzoate in the presence of sulfur oxyanions, suggesting a specific enzyme for the activation of cyclohexane carboxylate. Based on this evidence and the transient metabolites identified (Table 1) we conclude that the cyclohexane carboxylate degradative pathway of our isolate is similar to that of *Rhodopseudomonas palustris*. Furthermore, our isolate was not able to degrade benzoate in co-culture with a hydrogenotrophic partner like *Syntrophus acidotrophicus*. The environment from which strain IA6 was derived is consistant with the fact that this isolate is able to degrade cyclohexane carboxylate and cyclohex-1-ene carboxylate, but not benzoate. The hydrocarbon contamination at this location is dominated by aromatic compounds (26% of

total carbon; unpublished data) which are anaerobically degraded via benzoate which in turn is transformed into the free acid intermediates that represent electron donors for the isolate. This suggests that our isolate occupies a highly specialized niche within the microbial community at this location. Furthermore, strain IA6 was able to degrade cyclohexane carboxylate and reduce sulfate at comparable rates in groundwater whether or not the groundwater was tainted with gas-condensate (data not shown). This findings suggests that the organism is at least tolerant to hydrocarbons despite its inability to degrade gas condensate constituents.

The phylogenetic analysis supports the morphologic data that suggested that this isolate is a member of the genus *Desulfomonile*. Regardless of the marked physiological differences, the isolates in this genus form a monophyletic group within the family *Desulfoarculaceae* in the class *delta-Proteobacteria.*

Desulfomonile liminquinati sp. nov.

Desulfomonile liminquinati (li. min. qui. na.ti. L. n. limus mud; L. adj. n. inquinatum polluted; M. L. gen. liminquinati from polluted mud, referring to the organism from hydrocarbon-contaminated sediments).

Cells are gram-negative, non-motile, non-spore-forming, large rods (8 to 10 μM x 0.6 to 0.8 μM) with a collar-like morphology. Strict anaerobe, sulfate reducer capable of using H_2/CO_2, lactate, propionate, crotonate, cyclohexane carboxylate, and cyclohex-1-ene carboxylate as energy and carbon sources. Propionate, lactate and crotonate are incompletely oxidized to acetate. Sulfate, sulfite, thiosulfate and fumarate serve as

electron acceptors but nitrate, Fe(III)-NTA, Fe $(OH)_3$, polysulfides or 3-chlorobenzoate do not. The pH range for growth is 6.0-7.5 and optimum at 7.2. The temperature range is 25-32 °C and optimum is 28 °C.

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Appendix

Evidence for multiple routes of anaerobic toluene biodegradation in a nitrate-reducing consortium.

ABSTRACT

The metabolic fate of toluene was investigated with a nitrate-reducing microbial enrichment obtained from the terrestrial subsurface. Using ^{13}C-methyl-toluene as a substrate, labeled cinnamic acid, *E*-phenylitaconic acid, benzylsuccinic acid and benzoate were detected by ^{13}C-nuclear magnetic resonance spectroscopy (NMR) and confirmed with gas chromatography-mass spectroscopy (GCMS). Similarly, when d_3-ααα-toluene was used as a starting substrate we were able to detect the same metabolites by GCMS with the exception of benzylsuccinic acid that was found to have a mixed mass spectral profile with all three or no deuterium atoms in the molecule. Genetic characterization of the microbial enrichment by group-specific or universal Eubacterial 16S rRNA gene PCR primers and detection by denaturing gradient gel electrophoresis (DGGE) indicated the presence of a single strain of an *Azoarcus* sp., a genus known for the ability to metabolize toluene under nitrate-reducing conditions. Furthermore, we were able to amplify the α-subunit of the benzylsuccinate synthase gene from members of the enrichment using specific primers for this gene. Thus, we could minimally confirm the presence of a

metabolic type of bacterium known to catalyze the fumarate addition pathway. Collectively, these results support the hypothesis that the addition of a two-carbon fragment as well as the addition of a four-carbon fragment (e.g. fumarate) to the alkyl group of the parent molecule occurred simultaneously in the enrichment.

INTRODUCTION

Intrinsic bioremediation of hydrocarbons has become a preferred strategy for restoring gasoline-contaminated soils and sediments. The hydrocarbons of greatest regulatory concern are the mono-aromatic compounds found in gasoline, better known as the BTEX compounds (i.e. benzene, toluene, ethylbenzene, and the xylene isomers). In aerobic environments these mono-aromatic hydrocarbons are degraded via heterotrophic respiration and their fate has been well documented (23). In contrast, the anaerobic biodegradation of the BTEX hydrocarbons has not been thoroughly studied, although the environmental importance of these materials has stimulated increased investigative scrutiny over the last 15 years (as reviewed in 26, 27). Of the BTEX hydrocarbons, toluene appears to be the most susceptible to anaerobic decay under nitrate-reducing conditions as evidenced by the number of isolates known to metabolize this substrate (as reviewed in 22, 26, 27; 20, 43-45, 50). Anaerobic bacteria capable of toluene degradation coupled to the reduction of other electron acceptors are also known (25, 32, 37, 49). With increasing number of isolates available, studies of the metabolic fate of toluene has resulted in the identification of a diverse array of metabolites depending on how the parent substrate is initially attacked (as reviewed in 22, 27; 10, 11, 19, 38). Recently, the activation of toluene by substituent group addition to the methyl moiety of toluene has become of primary interest (Fig. 1). These uniquely anaerobic reactions have been studied using several nitrate- and sulfate-reducing bacterial isolates (4-7, 12, 15-17, 21, 28-30, 37). The addition of acetyl-CoA to the methyl group of toluene has been proposed to occur in a single nitrate-reducing microorganism (*Azoarcus tolulyticus* strain

Tol-4) but several key intermediates in the pathway have not been detected (Fig. 1) (15). Conversely, the addition of fumarate to the methyl group of toluene has been under intense scrutiny in recent years, and several metabolites and enzymes have been characterized in a variety of anaerobic bacteria (4-7, 12, 16, 17, 28-30), including an *Azoarcus* species, strain T (7). The fumarate addition pathway (Fig. 1) has been supported by the demonstration of a number of enzyme activities including a succinyl-CoA: benzylsuccinate CoA transferase (28, 30), that alleviates the requirement for ATP generally expected if a CoA ligase activity was involved. Furthermore, the enzymatic formation of benzylsuccinate from toluene and fumarate have been shown using permeabilized cells of nitrate-reducers as well as sulfate reducers (5). The addition pathways differ in the whether two C2 fragments or a single C4 molecule is added to toluene. However, both routes converge on the formation of benzoyl-CoA, an observation that is consistent with the frequent detection of benzoate as a central intermediate in the anaerobic biodegradation pathways of a wide variety of aromatic compounds (23, 24).

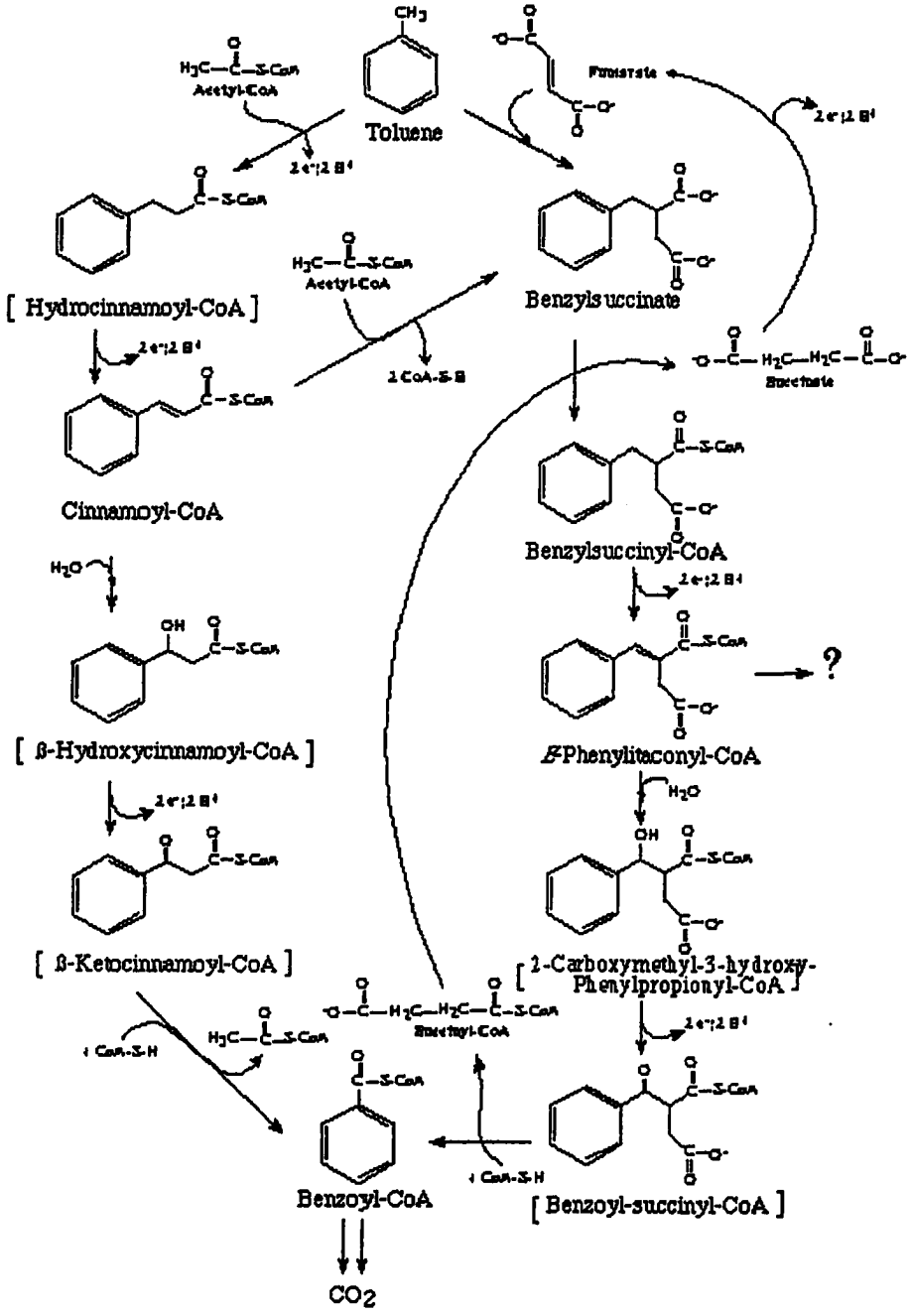

Figure 1. Proposed pathways for the anaerobic oxidation of toluene to benzoyl-CoA. The initial attack involves the addition of either a 2C fragment (like acetyl-CoA) or a single molecule of fumarate. Compounds shown in brackets have not been confirmed as either free acids or CoA derivatives.

To examine these potentially competing activation processes further, we studied the anaerobic degradation of toluene by a nitrate-reducing consortium obtained from an aquifer contaminated by landfill leachate. In sediment-free enrichments amended with either ^{13}C-labeled toluene (^{13}C-labeled at the methyl group) or d_3-ααα-toluene we observed several metabolites during the course of toluene degradation. The identification of the labeling patterns in these metabolites allowed us to conclude that toluene was simultaneously activated by both addition reactions. The analysis of the enrichment culture by denaturing gradient gel electrophoresis (DGGE) and sequence information on the resulting bands revealed the presence of a single *Azoarcus sp.* Furthermore, we were able to amplify the α-subunit of the benzylsuccinate synthase gene form our enrichment using specific primers for this gene. The work presented herein confirms the importance of the toluene degradation pathway proposed by Chee-Sanford *et. al.* and indicate that fumarate is not the only substituent group addition reaction involved during the anaerobic activation of toluene.

MATERIALS AND METHODS

A bacterial enrichment, capable of completely oxidizing toluene while reducing nitrate as an electron acceptor was obtained from an anaerobic leachate-impacted aquifer located in Norman, OK (2). Sediment-free cell suspensions were obtained by repeated toluene supplements and periodic transfers. Transfers were done by adding 5 mL of cell suspension to 45 mL of a minimal medium (47) supplemented with toluene (1 mM) and nitrate (4 mM), in sterile 120 ml serum bottles. The medium contained resazurin (0.0001

%) and 2.5% cysteine-sodium sulfide solution (0.5 mM), the latter two ingredients as redox indicator and reductant, respectively. All work was carried out in an anaerobic glovebox and bottles were sealed with a composite stopper (26a) and held in place with an aluminum crimp seal. The bottles were removed from the glovebox and the headspace exchanged to N_2:CO_2 (80:20). Benzoate (1mM) was used as a positive control substrate. Initial toluene utilization studies were performed in triplicate incubations, while autoclaved incubations served as sterile controls. Incubations were at room temperature in the dark.

The ^{13}C-NMR experiments were carried out by growing the enrichment in the same medium to a volume of 8 L. The culture was split into 4 L aliquots, one aliquot was amended with 1 mM of ^{12}C-toluene, and the other with the same concentration of ^{13}C-labeled toluene (^{13}C-labeled in the methyl group) (purity >99%; Cambridge Isotopes, Inc., Cambridge, MA). Nitrate was amended to a concentration of 4 mM. The incubations were placed in an anaerobic glovebox and 400 mL samples were periodically withdrawn, amended with 1 g NaOH, stirred for 30 min. and then acidified with 3 mL of H_2SO_4. The samples were then extracted immediately or placed in a refrigerator (4°C) for later analysis. Care was taken to ensure that separate glassware was used in the extraction procedure to avoid the prospect of isotopic cross contamination of the samples. Sample extraction was with diethylether. The ether extract was dried over anhydrous Na_2SO_4 and each extract was then placed under a stream of purified nitrogen and evaporated to dryness to remove the solvent and any residual toluene. The extracts were then dissolved in 1.5 mL of deuterated chloroform (Cambridge Isotope Laboratories) and placed into, HCl and solvent washed, 5 mm NMR tubes (Wilmad, Buena, NJ) for

analysis. After NMR analysis, extract components were derivatized by resuspending them in 150 μL of acetonitrile and 50 μL of bis-trifluoroacetamidetrimethylsilane BSTFA, incubating for 15 min. at 70°C and then concentrating under a stream of nitrogen prior to gas chromatography-mass spectrometry (GC-MS) analysis as previously described (14).

Toluene was analyzed by reverse phase HPLC (C18 column: 250 x 4.6 [i.d.] mm; Econosphere 5μm; Alltech Associates, Inc., Deerfield, Illinois) and detected by UV absorption at 254 nm as previously described (31). Intermediates of toluene decay were analyzed using a mobile phase of 85% 0.05 M acetate buffer (pH 4.5)-15% acetonitrile mixture with a flow rate of 1.0 ml/min. Nitrate was determined by ion chromatography using HPLC as previously reported (13, 42).

The NMR spectra were obtained on a Unity INOVA 400 MHz NMR spectrometer (Varian) with a ^{13}C resonance frequency of 100.573 MHz. The ^{13}C spectra were obtained at 303^0C using a standard inverse-gated pulse sequence. The experimental parameters were: (1) a sweep width of 24,140 Hz, (2) a 30^0 pulse width, (3) an acquisition time of 1.00 sec, and (4) a recycle delay of 1.5 sec. The data were processed with 1-Hz line broadening. ^{13}C-NMR spectra of saturated $CDCl_3$ solutions of ^{13}C-labeled benzoic acid (carboxylic acid labeled) (167.7 ppm), unlabeled cinnamic acid (130.7, 129.0, 128.3, 116.6, 111.4 ppm), and unlabeled hydrocinnamic acid (127.6, 127.1, 124.9, 35.1, 26.8 ppm) were obtained. No carboxylate or alkylated aromatic carbons were observed in the unlabeled cinnamic acid or unlabeled hydrocinnamic acid ^{13}C-NMR spectra due to the short recycle delay time used.

DNA Extraction and PCR Amplification: The DNA of the organisms present in our enrichment culture was extracted using a protocol previously described (40). The PCR amplifications were performed with a Techne Genius Temperature Cycler (Techne, Cambridge, United Kingdom) as follows: approximately 25 ng of purified genomic DNA, 10 pmol each of the appropriate primers, 250 μmol of each deoxyribonucleoside triphosphate, 10 μl of 10X PCR buffer B (Fisher Scientific, Fair Lawn, NJ), 10 μl of $MgCl_2$ (25 mM), 2.5 U of Taq Polymerase (Fisher Scientific, Fair Lawn, NJ), and sterile water were mixed to a final volume of 100 μl. The samples were amplified using a touchdown PCR protocol (18). This was carried out as follows: the samples were first denatured (94°C/4 min.), then subjected to 35 cycling steps; denaturing (94°C/45 sec), annealing (45 sec), and elongation (72°C/2 min.). The process is finished with 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, at which temperature 15 additional cycles were carried out (18). The two primers used for amplification were universal Eubacterial primers, GM5F and DS907R (35). These primers amplify a 550 base pair fragment within the 16S rRNA gene. A second pair of specific PCR amplification primers targeting a 600 base pair fragment of the 16S rRNA gene of *A. tolulyticus* was also used (51). The PCR reaction using this set of primers C and D (51) was performed as the above protocol with the following modifications, a single annealing temperature (45°C); elongation for 1.5 min.; a 3 mM final concentration of $MgCl_2$ and 14 pmol of each primer. The PCR amplification of the α-subunit of the benzylsuccinate synthase gene was performed using the primer set previously described (9). The PCR protocol was

performed as follows: 10 pmol each of the appropriate primers, 250 µmol of each deoxyribonucleoside triphosphate, 5 µl of 10X PCR buffer B (Fisher Scientific, Fair Lawn, NJ), 4 µl of $MgCl_2$ (25 mM), 2.5 U of Taq Polymerase (Fisher Scientific, Fair Lawn, NJ), and sterile water were mixed to a final volume of 50 µl. The initial denaturation step and final elongation were performed as described above. The samples were subjected to 30 cycling steps; denaturing (94°C/1 min), annealing (58°C /1min), and elongation (72°C/1 min.). All PCR product sizes were confirmed by agarose-gel electrophoresis, stained with ethidium bromide and visualized by UV transillumination.

DGGE analysis: The amplified 550 base pair fragments within the 16S rRNA gene were separated using Denaturant Gradient Gel Electrophoresis using a D-code Universal Mutation Detection System (BioRad, Hercules, CA.) as described previously (35). The PCR samples were loaded directly onto a 6% polyacrylamide gel with a 30% to 60% build-in denaturant gradient (100% is equal to 7 M urea and 40% formamide) in 1X TAE (40 mM Tris [pH=7], 20 mM acetate, 1 mM EDTA). The gels were loaded with 20 µl of PCR sample and electrophoresis was performed at a constant voltage of 130 volts and a temperature of 60°C for 6 hours. After electrophoresis, the gels were stained with ethidium bromide for 5 to 10 minutes and destained for 10 minutes in nanopure water. All 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 base pair fragments were excised from the DGGE gels using a sterile razor blade and pipette tip, immediately placed in a 200 µl sterile

polypropylene tube containing 36 µl of sterile water, then stored at 4°C overnight. One microliter of supernatant was used as the DNA template in a PCR re-amplification, then run in a DGGE gel following the protocols described above. Once the purity of the 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, WI), UltraClean 15 (Mo Bio) or UltaFree-MC 30,000 NMWL Filter Unit (Millipore) following the manufactures 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 the manufacturers instructions (Invitrogen, Carlsbad, CA).

Once the purity of the fragments in question was established, they were sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction (PE Applied Biosystems, Foster City, CA) following the manufacturer's instructions. The primers used in the sequencing reactions were the same ones used to generate the fragments in the PCR reaction. In all cases a minimum of three different primers were used, including the vector primers.

Sequence Analysis: The sequencing reactions were analyzed using the computer program Sequencer to generate homologous contigs from the sequences. Related sequences were obtained from the GenBank database by using the Blastn search program of the National Center for Biotechnology Information (NCBI) (1). These sequences were aligned using ClustalX (48) and their phylogenetic relationship was examined using the evolutionary distance method with the corrections of Jukes and Cantor with the computer program PAUP (46).

RESULTS

Anaerobic biodegradation of toluene, coupled with nitrate reduction, was established with cells enriched from the terrestrial subsurface (Fig. 2). Toluene degradation was observed only in the presence of nitrate (Fig. 2A). No loss of toluene was seen in sterile controls or in bottles not amended with nitrate. Similarly, nitrate depletion occurred only in bottles amended with toluene, with no loss observed in sterile or substrate-unamended incubations (Fig. 2B).

During the course of toluene decomposition, transient intermediates were detected (Fig. 3). The first observable intermediate was identified as *E*-phenylitaconic acid by comparing its HPLC retention time to an authentic standard (RT=6.1 min.). A second intermediate was similarly identified as benzoate (RT=4.3 min.) (Fig. 3).

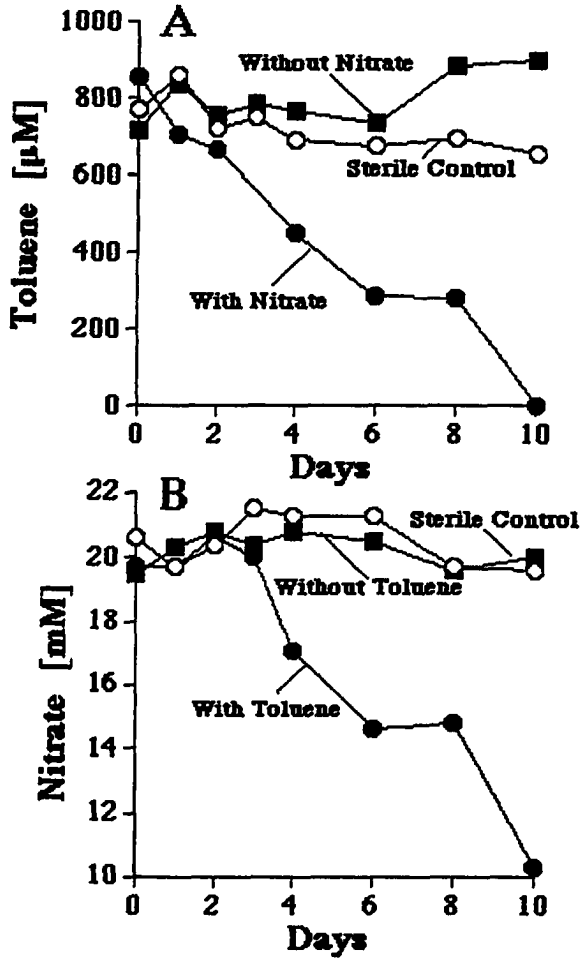

Figure 2. Toluene degradation linked to nitrate reduction by a sediment-free enrichment from a leachate-impacted terrestrial subsurface. **A**: Toluene loss only in the presence of nitrate. **B**: Nitrate loss only in the presence of toluene. No loss of toluene or nitrate is observed in sterile or substrate-unamended controls.

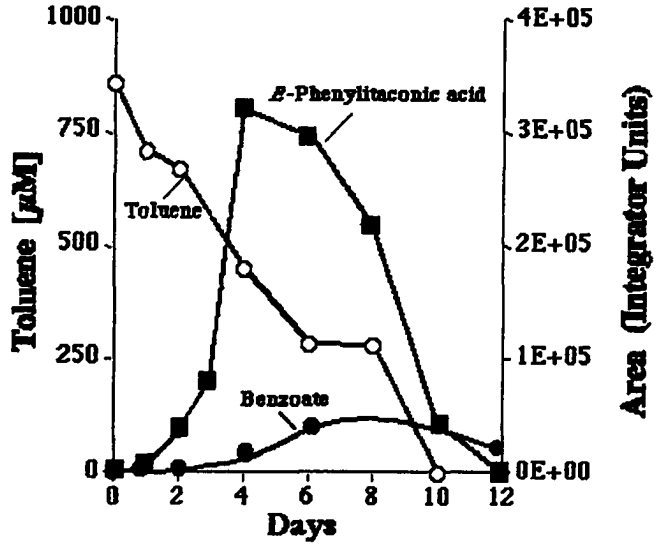

Figure 3. Transient accumulation of intermediates during toluene degradation by a nitrate-reducing microbial consortium. Closed squares indicate the metabolite identified as *E*-phenylitaconic acid. Closed circles denote benzoate as identified by HPLC retention time.

To investigate further the metabolic pathway of toluene decomposition by the consortium, ^{13}C-labeled toluene was used to identify and confirm the presence of transitory intermediates. Cultures amended with either ^{12}C-toluene or ^{13}C-toluene, were sampled over time, extracted and analyzed by ^{13}C-NMR (Fig. 4). At the start of the experiment (0 hr) only a singlet at 168.6 ppm was detected, corresponding to the carboxylate ion of benzoate (Bz) while the large triplet at 77.0 ppm is from the deuterated chloroform solvent (Fig. 4). No NMR signals were detected in the ^{12}C-toluene amended incubations or in samples of the ^{13}C-stock solution, other than the solvent signal (data not shown), demonstrating that the singlet at 168.6 ppm originated from toluene. After 2 hours, a singlet at 111.4 ppm appeared, which is characteristic of the alkene carbon atom bonded to the aromatic ring in cinnamic acid (Fig. 4). By 16 hours, the peak for cinnamic acid disappeared, and another singlet at 144.6 ppm was observed which coincides with the alkene carbon atom bonded to the aromatic ring in *E*-phenylitaconic acid. Migaud et al. measured the ^{13}C chemical shift of this carbon to be 141.8 ppm using deuterated acetone as solvent (33). Further observation at 27 h showed an increase in the intensity of the singlet at 144.6 ppm and the first appearance of a singlet at 37.3 ppm that most likely corresponds to the alkene carbon atom bonded to the aromatic ring in benzylsuccinic acid. Note that the chemical shift for the similar carbon atom in hydrocinnamic acid is 35.1 ppm. Two unidentified singlets appeared in the alkene region of the spectra at 139.9 and 146.9 ppm. The singlet at 29.5 ppm is also unidentified, however it is considered to be a background signal since it was also observed in spectra amended with ^{12}C -toluene after 27 hours of incubation (data not shown). When nitrate and nitrite were completely depleted in both incubations at 44 hours the sampling was

ended. The ^{13}C NMR spectrum at 44 hours displays several unidentified singlets with chemical shifts characteristic of carboxylic acids (178.3, 176.6, 176.1, 175.3, 171.5 ppm) and alkanes (29.7, 29.0, 27.2 ppm). Increase in the signal intensity of the *E*-phenylitaconic acid (144.6 ppm) and benzylsuccinic acid (37.3 ppm) at 44 hours is also evident. The 44 h NMR spectrum of the ^{12}C-toluene amended sample did not contain any signals other than that of the unidentified background peaks at 29.5 ppm. During this experiment, we observed the simultaneous depletion of the parent substrates and nitrate in the two incubations indicating no apparent disproportional use of ^{12}C- or ^{13}C-toluene (data not shown).

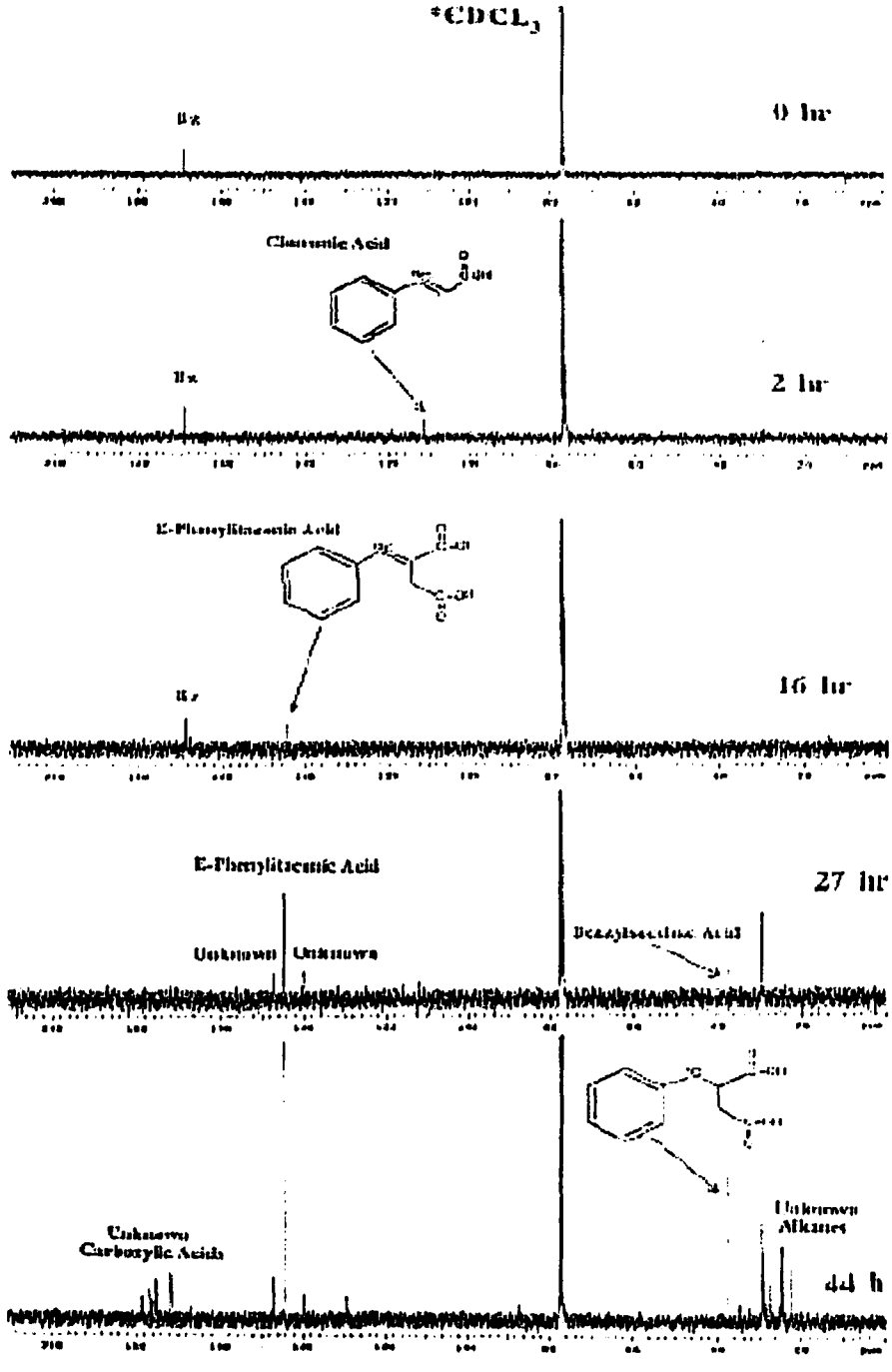

Figure 4. ^{13}C-NMR spectra of extracts sampled over time from the enrichment amended with ^{13}C-labeled toluene (^{13}C label at methyl group) in the presence of 4 mM nitrate. Arrows denote distinct ^{13}C-labeled intermediates during the course of toluene decay. *CD_3Cl = deuterated chloroform used as an internal standard; Bz = benzoate.

Using GC-MS of the trimethylsilyl derivatives of culture extracts and authentic standards, we were able to identify several metabolites detected by ^{13}C-NMR (Fig. 5). Figure 5a represents a mass spectral comparison of a ^{13}C-labeled metabolite and a cinnamic acid standard. The compounds possessed the same retention time. An increase of one mass unit in the major ion fragments is seen in the metabolite signal indicating that the ^{13}C-label of toluene was retained in the metabolite. When cultures amended with ^{12}C-toluene were similarly analyzed, the mass espectral profiles of the putative metabolite and authentic cinnamic acid were identical (data not shown).

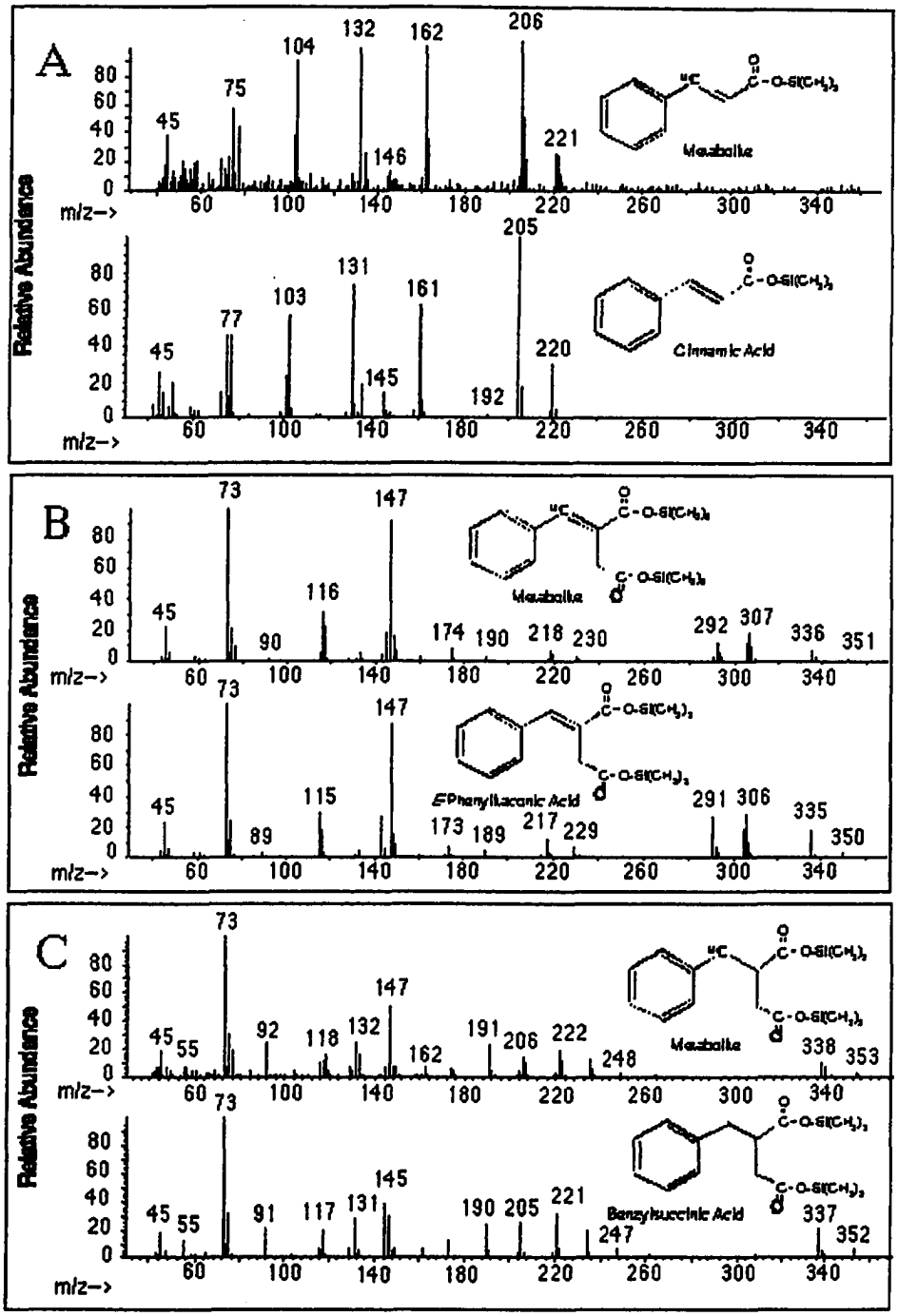

Figure 5. Mass spectral profiles of TMS-derivatized metabolites during anaerobic decomposition of ^{13}C-methyl-toluene and their respective TMS-dervatized authentic standards. (**A**) Metabolite and standard of cinnamic acid. (**B**) Metabolite and standard of *E*-phenylitaconic acid. (**C**) Metabolite and standard of benzylsuccinic acid.

Similarly, *E*-phenylitaconic acid was identified as an intermediate by comparison of the metabolite mass spectral profile with an authentic standard (Fig. 5B). Many mass spectral features found in the standard are increased by one mass unit in the metabolite profile, including the parent ion at m/z 350. The same was not true when the ^{12}C-toluene amended culture was similarly analyzed. Again, the original label of ^{13}C-toluene was retained in this metabolite. The same basis pattern was also observed for the mass spectral comparison of authentic benzylsuccinic acid, and the metabolite eluting at the corresponding GC retention time when the ^{13}C-toluene-amended culture was analyzed (Fig. 5C). No metabolites associated with ring oxidation, methyl group oxidation or ring reduction were detected in the extracts. Further, no metabolites of benzoate degradation were detected by mass spectral analysis.

To further investigate the anaerobic metabolism of toluene by the enrichment, deuterated toluene (d_3-$\alpha\alpha\alpha$-toluene) was supplied as a parent substrate. The intermediates, cinnamic acid, *E*-phenylitaconic acid, benzylsuccinic acid and benzoic acid were also observed. The GC-MS spectra obtained for cinnamic acid, *E*-phenylitaconic acid and benzoic acid indicated no retention of the deuterium atoms in these compounds from the microbial transformation of d_3-toluene (data not shown). However, the mass spectral profile obtained for benzylsuccinic acid confirmed the retention of the deuterated atoms in the resulting metabolite (Fig. 6).

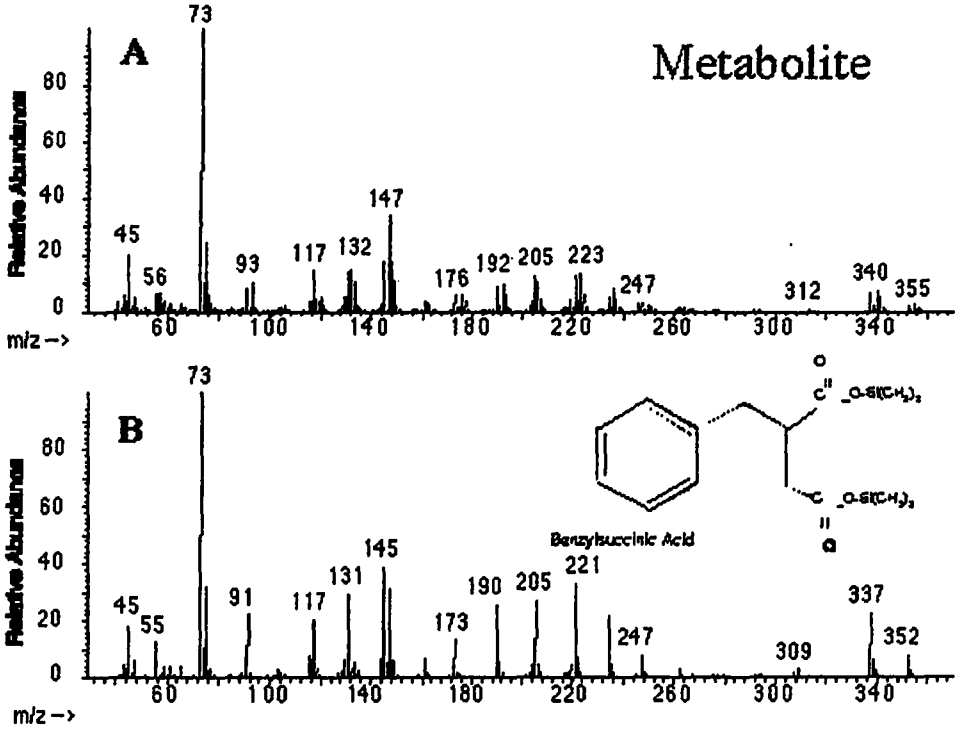

Figure 6. Mass spectral profile of the TMS-derivatized metabolite (A) observed in ααα-d_3-toluene amended incubations and (B) authentic benzylsuccinic acid.

The bacterial consortium was further characterized, using molecular techniques employing PCR-amplified DNA. Using universal primers for the 16S rRNA gene we were able to detect at least six microorganisms in the toluene-degrading consortium by DGGE (Fig. 7A). Neither, *Thauera aromatica* strain K172 (Fig. 7A, lane 1) nor *Azoarcus tolulyticus* strain Tol-4 (Fig. 7A, lane 3), were present in the consortium (Fig. 7A, lanes 5).

Excision of the DNA bands from the DGGE gels combined with isolation, re-amplification, and cloning of the DNA, allowed us to sequence the most prominent bands present in our enrichment. We were able to obtain two clones, MCOU and F1-3 (Fig. 7A, lanes 4 and 6). Sequence analysis of both clones suggested that clone MCOU, was a member of the genus *Azoarcus* within the beta subdivision of the Phylum *Proteobacteria.* The other clone F1-3, was 99% homologous to *Leptospira illini* (data not shown).

To investigate if more than one species of *Azoarcus* was present in the toluene-degrading consortium, group-specific primers were used (Fig. 7B). Using the species-specific C+D primer set developed to identify strains of *Azoarcus tolulyticus* based on 16S rRNA gene amplification (51), we were able to detect an amplification product from the consortium corresponding to the expected genetic size of 600 bp (Figure 7B, lane 3). No PCR amplification product was obtained when *Thauera aromatica* strain K172 was employed as a negative control (Fig. 7B, lane 2). Amplification from the positive control *Azoarcus tolulyticus* strain Tol-4, was easily detected (Fig. 7B, lane 4).

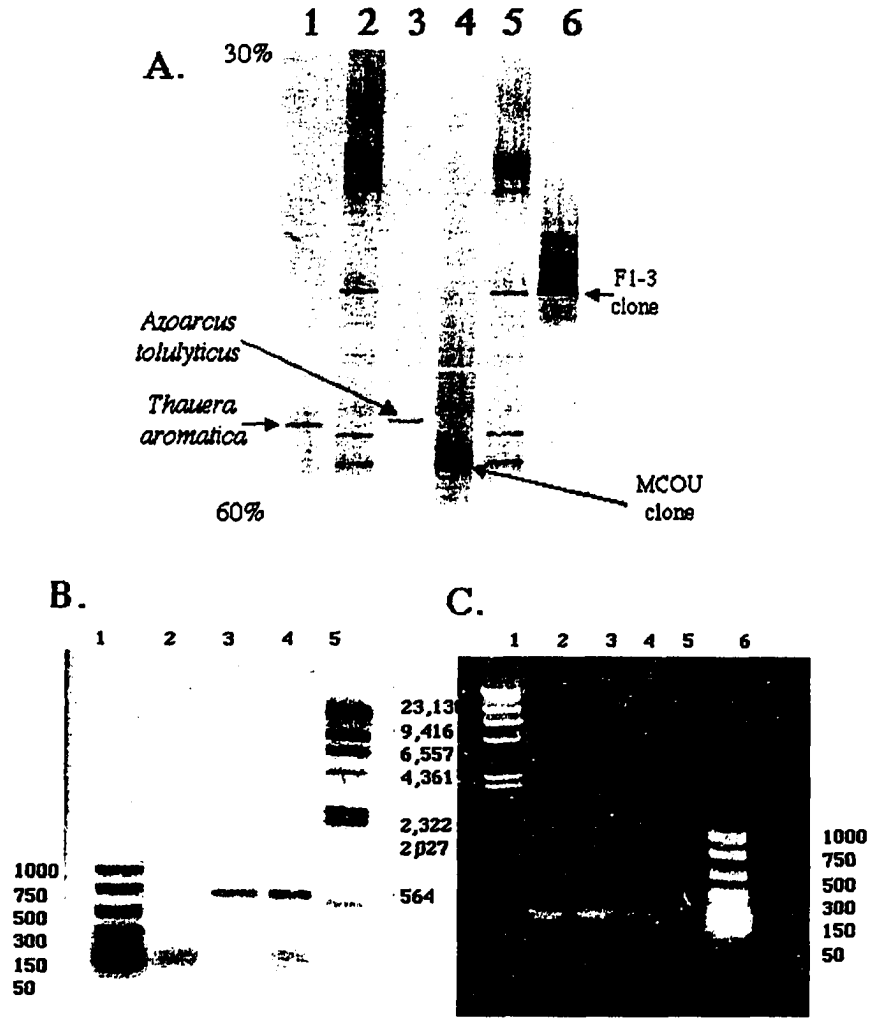

Figure 7. Molecular characterization of the toluene-degrading, nitrate reducing microbial enrichment. A) Denaturing Gradient Gel Electrophoresis of PCR-amplified 16S rDNA gene. Lanes: (1) *Thauera aromatica* K172; (2 and 5) Nitrate-reducing toluene-degrading microbial enrichment; (3) *Azoarcus tolulyticus* strain Tol-4; (4 and 6) clone from the nitrate-reducing toluene-degrading microbial enrichment. B) Agarose gel of PCR-amplified 16S rDNA gene using specific primers for *Azoarcus species*. Lanes: (1) PCR Markers; (2) *Thauera aromatica* K172; (3) Nitrate-reducing toluene-degrading microbial enrichment; (4) *Azoarcus tolulyticus* strain Tol-4; (5) Lambda Hind III molecular marker. C) Agarose gel of PCR-amplified α-subunit of the benzylsuccinate synthase gene. Lanes: (1) Lambda Hind III molecular marker; (2) *Azoarcus tolulyticus* strain Tol-4; (3) *Thauera aromatica* K172; (4) Nitrate-reducing toluene-degrading microbial enrichment; (5) No DNA control; (6) PCR Markers.

Isolation of the PCR product from the agarose gel, purification and re-amplification with the *Azoarcus* specific DGGE primers (addition of a GC-clamp to the 5' end of the forward primer) was performed and analyzed by DGGE (data not shown). These results indicated that only one bacterial species from the consortium was amplified. Sequencing of this band, confirmed the sequence obtained from the DGGE using universal primers. Furthermore, a phylogenetic analysis using evolutionary distance supported the phylogenetic placement of our cloned sequence as an *Azoarcus sp.* in the beta subdivision within the Phylum *Proteobacteria* (Fig. 8). In addition, we were able to obtain an amplification product using gene specific primers to amplify the α-subunit of the benzylsuccinate synthase indicating the presence of this gene in our enrichment as well as in the control organisms, *Azoarcus tolulyticus* strain Tol-4 and *Thauera aromatica* strain K172 (Fig. 7C).

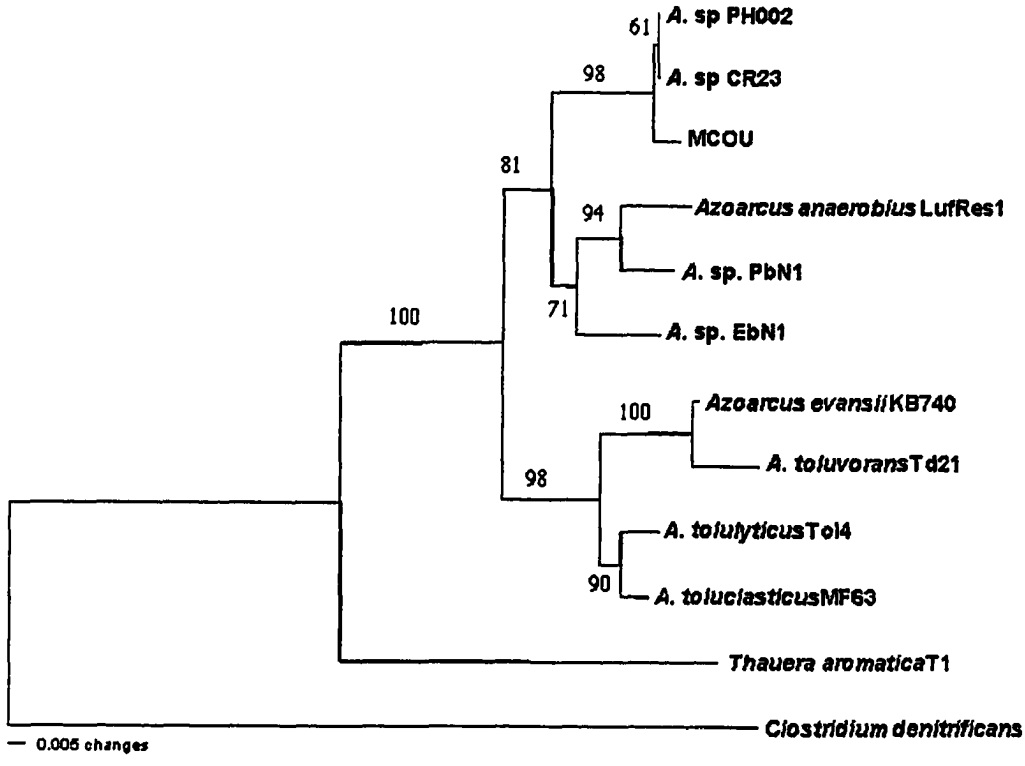

Figure 8. Evolutionary distance phylogenetic tree based on the partial sequence of the 16S rDNA gene of a clone from a nitrate-reducing toluene-degrading microbial enrichment and relatives within the genus *Azoarcus* in the beta subdivision of the Phylum *Proteobacteria*. Numbers at nodes represent the bootstrap values of 1000 replicates.

DISCUSSION

The metabolic fate of the mono aromatic hydrocarbon toluene was examined in a denitrifying microbial consortium enriched from an anoxic aquifer. Toluene degradation was coupled to the reduction of nitrate (Fig. 2) and the amount of nitrate consumed was slightly higher (~110%) than that theoretically expected based on the presumed stoichiometry: $C_7H_8 + 8.32\ NO_3^- \rightarrow 7\ HCO_3^- + 4.15\ N_2 + 4\ H_2O + 1.32\ H^+$. During the course of toluene degradation, two intermediates were detected by HPLC. One intermediate was identified as benzoate (Fig. 3) and later confirmed by GC-MS and NMR. The detection of benzoate is not unexpected, since benzoate is a key metabolite formed during the anaerobic biodegradation of many aromatic compounds (23a, 24, 25). A second intermediate was identified as *E*-phenylitaconic acid and also confirmed by GC-MS and NMR. The determination of *E*-phenylitaconic acid indicated that a substituent group addition reaction must have occurred during the course of anaerobic toluene metabolism.

Two mechanisms for the anaerobic activation of toluene by a substituent group addition have been suggested in the literature. Evans and colleagues first suggested that the nitrate reducing isolate, strain T1 (later designated *Thauera aromatica* strain T1) catalyzed the addition of acetyl-CoA to the methyl group of toluene to form phenylpropionyl-CoA (hydrocinnamyl-CoA or the free acid product hydrocinnamic acid) (21). Although evidence for this metabolite or its free acid was not obtained, the authors speculated that phenylpropionyl-CoA was precursor of the benzoyl-CoA formation since

the culture accumulated benzylsuccinic acid and benzylfumaric acid as dead end metabolites during the anaerobic toluene metabolism.

Likewise, the work of Chee-Sanford and co-workers (15) on the anaerobic degradation of toluene with the nitrate reducing strain *Azoarcus tolulyticus* strain Tol-4 suggested that the parent substrate was activated by the addition of acetyl-CoA to the methyl group of toluene to form hydrocinnamyl-CoA (15). They proposed that hydrocinnamyl-CoA could then be oxidized to cinnamyl-CoA, and that subsequent metabolism involved the addition of another molecule of acetyl-CoA to eventually form benzylsuccinate (Fig. 1) (15, 33). The further thioesterification and oxidation of latter metabolite would produce *E*-phenylitaconic acid, a structural isomer of the dead end metabolite benzylfumaric acid proposed by Evans et al (21). Supporting evidence for the suggested pathway included the identification of cinnamic acid as an intermediate in toluene grown cells and the observation of radiolabeled benzylsuccinic acid produced by *A. tolulyticus* strain Tol-4 incubated with *trans*-cinnamic acid and ^{14}C-acetate.

In contrast, evidence for toluene activation by adding across the double bond of fumarate has garnered much greater support (Fig. 1). Isolation of critical enzymes in the proposed pathway for fumarate addition to toluene as well as isolation of CoA derivatized intermediates convincingly substantiates this pathway (4-7, 12, 28-30). Furthermore, isolation of the genes involved in toluene catabolism also supports a fumarate addition mechanism (16, 17, 28, 29) as well as the production of specific primers designed to amplify the α-subunit of the benzylsuccinate synthase gene (8). Substantiation of fumarate addition as the dominant mechanism of anaerobic toluene decay was bolstered by the fact that the organism (strain T1) originally thought to be

involved in acetyl-CoA addition reaction, was later identified as a strain of *Thauera aromatica* (43). This organism was a close relative of *Thauera aromatica* strain K172 one of most intensively investigated bacteria involved in catalyzing anaerobic fumarate addition reactions (12, 28-30). Further, a strain of *Azoarcus* (strain T) was shown to possess the enzyme benzylsuccinate synthase (7). *Azoarcus* strain T is phylogenetically related to *Azoarcus tolulyticus* strain Tol-4, the organism in which acetyl-CoA addition to toluene has largely been based.

However, our evidence supports both pathways. First, the detection of ^{13}C-cinnamic acid early in the incubation (Fig. 4 & 5) suggest the addition of a two carbon fragment to the methyl group of toluene. Further characterization of the enrichment showed evidence for ^{13}C-labeled *E*-phenylitaconic acid and benzylsuccinic acid. The identity of these metabolites was confirmed by subsequent GC-MS analysis (Fig. 5). The pattern and order of appearance of these metabolites follows that suggested by Evans et al. (21) and Chee-Sanford et al. (15) which would involve an addition of acetyl-CoA to the methyl group of toluene to form hydrocinnamic acid (Fig. 1). However, the detection of these intermediates does not preclude the involvement of another pathway for toluene decay involving fumarate addition.

Previous work by Beller and Spormann (4, 6, 8) indicated that during the addition of fumarate to the d_3-methyl group of toluene, all three deuterium atoms were retained in the resulting benzylsuccinic acid product. This is an important finding for determining not only the enzymatic function of benzylsuccinate synthase but also for distinguishing between the different routes of anaerobic toluene metabolism as proposed in Fig. 1.

We used deuterated toluene ($C_6H_5CD_3$) to further clarify the pathway(s) for toluene decay by the consortium. Mass spectral evidence was obtained for each of the previously identified intermediates when the consortium was exposed to d_3-methyl-toluene. No deuterium label was associated with the cinnamic acid peak in the total ion chromatogram (data not shown). This finding is consistent with the acetyl-CoA addition mechanism proposed by Chee-Sanford *et.al.* for the formation of hydrocinnamoyl-CoA via an aryl cation. Such a reaction should result in the loss of two of the deuterium atoms originally associated with the parent substrate. Further oxidation and formation of the double bond to form cinnamoyl-CoA (or the free acid as detected in our study) would likely result in the loss of the remaining deuterium atom previously associated with the methylyl carbon of toluene.

The mass spectral results associated with benzylsuccinic acid production by consorted cells grown on d_3-methyl-toluene showed a mixed pattern. The mass spectrum obtained for this metabolite showed both the retention of all deuterium atoms as might be expected for a fumarate addition mechanism (4, 7), and no retention of any of the deuterium atoms suggesting the acetyl-CoA addition mechanism (Fig. 6). Such findings indicate that the formation of benzylsuccinic acid appears to be catalyzed by at least two distinct pathways. A mechanism supporting the addition of two acetyl-CoA molecules during the metabolism of toluene is also supported by the lack of deutirium retention in *E*-phenylitaconic acid produced by the consortium amended with deuterated toluene (data not shown). The retention of at least one deutirium atom would be expected if only the fumarate addition pathway was operating in the consortium. That is, the oxidation of benzylsuccinyl-CoA to *E*-phenylitaconyl-CoA should result in a molecule that would

have at least a single deuterium atom associated with the succinyl moiety as predicted by Beller and Spormann (6). Conceivably, the *E*-phenylitaconic acid produced during d_3-methyl-toluene metabolism by fumarate addition could be below the mass spectral detection level and escape verification.

During the time-course extractions of the ^{13}C-toluene amended incubation, a singlet peak was evident at the start of the experiment that corresponded with the carboxylate ion of benzoate (168.6 ppm) that did not appear in the time zero spectrum for the ^{12}C-toluene amended incubation (Fig. 4). A test run of the ^{13}C-labeled starting material indicated that the benzoate was not a contaminating species in the toluene. This result would clearly indicate that it was indeed ^{13}C-labeled and originated from toluene. During the course of the experiment, the first sampling and extraction of the ^{13}C-amended incubation was delayed shortly by the amending and mixing of the toluene, first to the ^{13}C-incubation and followed by amending of the ^{12}C-toluene to the other incubation. Sampling of both incubations and subsequent removal of the culture fluids from the glovebox may have added enough time for biological transformation of the substrate to benzoate before the first step of the extraction protocol occurred, possibly explaining this result. When this experiment was repeated and increased care was given between the time of ^{13}C-toluene amendment and the extraction's first step, no evidence of benzoate or any other labeled intermediate or contaminant was observed in the ^{13}C-NMR spectra (data not shown). But, evidence for both *E*-phenylitaconic acid and benzylsuccinic acid were detected and increased in intensity within the first 10 hours of incubation, as previously observed in Fig. 4, although no evidence for cinnamic acid was observed using ^{13}C-NMR during the repeated experiment.

Molecular evidence obtained by analysis of the microbial consortium indicated the presence of an *Azoarcus* species (Fig. 7A, 7B). These results were obtained by two independent assays using different primer sets. By reducing the annealing temperature of the strain specific PCR primers and adding a GC-clamp to the forward primer we were able to determine that our enrichment contained only a single species of *Azoarcus*, based on the DGGE profile (data not shown). The sequence associated with the clone MCOU clustered most closely with two species of *Azoarcus*, strains PH002 and CR23, that were originally isolated as anaerobic phenol degrading microorganisms (41). Though strains PH002 and CR23 were not able to anaerobically biodegrade toluene (41), many other *Azoarcus* species are known to metabolize aromatic hydrocarbons. These strains include two other *Azoarcus* species, strain PbN1 and EbN1 (as reviewed in 26, 50) that are also closely related to clone MCOU. The fact that we can amplify the α-subunit of the benzylsuccinate synthase gene in a member of the consortium supports our metabolite analysis since this enzyme catalyzes the addition of fumarate to toluene. Interestingly, the same gene could also be amplified in *Azoarcus tolulyticus* Tol-4, the isolate suggested to be capable of activating toluene via a two carbon addition reaction. Thus, this organism might have the genetic potential to activate toluene by two different reaction mechanisms and help explain our findings which implicate the simultaneous activation of toluene via a C2 and a C4 addition reaction. Whether this is catalyzed by a single or multiple organisms in our consortium is currently being investigated. In addition, we will assay *Azoarcus tolulyticus* Tol-4 for its ability to metabolizing d3-methyl-toluene by the two potential activating steps.

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