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METABOLITE-PROFILING TO ASSESS *IN SITU* ANAEROBIC MICROBIAL HYDROCARBON DEGRADATION IN DIVERSE ENVIRONMENTS

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METABOLITE-PROFILING TO ASSESS *IN SITU* ANAEROBIC MICROBIAL HYDROCARBON DEGRADATION IN DIVERSE ENVIRONMENTS

A DISSERTATION APPROVED FOR THE DEPARTMENT OF BOTANY AND MICROBIOLOGY

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Dedication

I dedicate this work to my parents, Kim and Don Broomhall. I would not be half the person I am today, without your unconditional love and support.

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Preface

Anaerobic hydrocarbon biodegradation is an important ecological process in petroleum-laden environments. While the characterization of the metabolic mechanisms involved in this process has just begun, it is known that oxygen-independent processes play key roles during *in situ* bioremediation of contaminated sites, geochemical changes in petroliferous formations, influence biocorrosion, and can impact the quality of oils extracted from petroleum reservoirs. Laboratory studies have demonstrated the mineralization of several hydrocarbon compound classes under anaerobic conditions, and a small number of enrichment cultures and isolates have been described. Currently, we understand that diverse hydrocarbon classes are activated under anaerobic conditions by the addition of the molecule to the double-bond of fumarate, and this seems to be a predominant mechanism employed by anaerobic hydrocarbon degrading organisms. The genes and enzymes responsible for the fumarate-addition mechanism to alkylated benzenes, such as toluene, have been identified. In addition, the gene involved in the addition of fumarate to alkanes was recently characterized at the nucleic acid level. It has become increasingly evident that other anaerobic mechanisms such as carboxylation and hydroxylation are employed by microorganisms to activate hydrocarbons for decay. Most importantly, the intermediates of the mechanisms described above for anaerobic hydrocarbon degradation can be detected to some extent *in situ*, and can be used to provide unequivocal evidence that biological mineralization of hydrocarbons is occurring.

This dissertation is comprised of four chapters and one appendix, each

describing how evidence for anaerobic hydrocarbon degradation can be used to assess microbial metabolism or functioning of microbial communities, through the detection of metabolic indicators (intermediates). The first chapter discusses the field and laboratory evidence for the anaerobic biodegradation of contaminating petroleum at a former refinery site in Casper, WY. In Chapter Two (collaborative) and Three, I examine *in situ* microbial hydrocarbon consumption in petroleum reservoirs on the North Slope of Alaska. Chapter four describes the potential for an alternate mechanism of oxygen-independent activation for methane, and the appendix consists of a collaborative project for which investigated the presence or absence of metabolites consistent with the anaerobic oxidation of ethane, propane and pentane in terrestrial enrichments under sulfate-reducing and methanogenic conditions.

Chapter one was written in the format for the journal *Microbial Biotechnology*. In this manuscript, I examined the *in situ* anaerobic biodegradation of hydrocarbons in a contaminated aquifer underlying a former refinery site. The spilt petroleum consisted of several compounds of regulatory interest (contaminants of concern), and I proposed through field and laboratory studies that select hydrocarbons would be susceptible to degradation due to precedence in the literature for their amenability to decay. The confirmation of the proposed hypothesis would support the use of natural attenuation as a remedial option for the former refinery site. Contrary to the hypothesis proposed, I determined the contaminants of concern were not metabolized, but with deference to other hydrocarbons present in the spilt petroleum mixture. The concept of field metabolomics was used for the first time to

describe the functioning of microbial communities at a hydrocarbon spill site when compared to a background (uncontaminated) sample.

My primary contributions to this collaborative manuscript were, the organic extraction and metabolite analysis, synthesis of metabolic intermediates (specifically 1-ethyl-3-methylbenzene), analytical analysis of hydrocarbon loss, measurement of terminal electron-acceptors, extraction of nucleic acids, prepping samples for phospolipid fatty acid analysis, and finally for writing the manuscript.

In Chapter two, I interrogated samples collected on the North Slope of Alaska for evidence of anaerobic hydrocarbon biodegradation via metabolite-profiling. This was a collaborative effort written for *Environmental Science and Technology*. My contribution to this paper included the organic extraction and analysis of samples collected from the field. I also produced the standard curves to measure the concentrations of low molecular weight alkylsuccinates and the subsequent tables and supplementary figures. I was involved in the planning and execution of the study and I co-authored the manuscript with particular attention to the metabolite-profiling methods, results and discussion.

Given the collective findings regarding anaerobic hydrocarbon metabolism, I hypothesized that several metabolic intermediates could be detected in oil production and processing facilities throughout the north slope oil fields. Furthermore, I predicted that the microbial oxidation of the petroleum components in the reservoir could potentially contribute to the biocorrosion observed on and around the oil field infrastructure. I expected to observe the presence of metabolites associated with several water-soluble hydrocarbons typically present in petroleum mixtures (i.e.,

toluene, ethylbenenze, xylene), but this was not the case. I did identify low molecular weight alkylsuccinates in many of the samples collected, suggesting the oxygen-independent activation of methane, ethane, propane and butane. This finding was consistent with the practice of the field operators to use miscible gas injectate (C_1-C_4 alkanes) to pressurize the reservoirs as part of oil recovery operations over the last 30 years. Aside from the role of such bioconversions in pipeline corrosion processes, an important fundamental inference was that methane could be activated in a manner similar to other alkanes via the addition of the parent molecule to fumarate. This inference is more completely detailed below and in chapter 4.

The findings detailed Chapter 2 prompted a return to the North Slope oil fields for a more extensive survey of the older field originally investigated relative to newer field. The third chapter represents a more extensive interrogation of these two North Slope oil fields, (Field B and Field A, respectively) for evidence of anaerobic hydrocarbon biodegradation. Chapter 3 is also written in the style of *Environmental Science and Technology.* I hypothesized there would be differences within the two oil fields with respect to anaerobic hydrocarbon degradation, given their age and varied geochemistry of the oils. Chapter 3 details how the inter-field comparisons were much greater then the comparable intra-field analysis of putative metabolites. The analysis also revealed that anaerobic hydrocarbon metabolism by diverse mechanisms was a prominent feature in both oil fields. The approach helps lend insight on the functioning of microbial communities in oil fields and also suggests that hydrocarbon biodegradation processes are involved in oil deterioration and potentially biocorrosion activities as well. To my knowledge, this report is the first

to use putative anaerobic hydrocarbon biodegradation metabolites as variables to characterize microbial community function in an operational oil-production facility.

The samples collected from the major oil production operations on the North Slope were part of a large collaborative effort that will result in multiple publications focusing on specific aspects of anaerobic microbial ecology. My contribution to this overall effort was the collection, preservation, GC-MS analysis and interpretation of >50 samples for the presence of polar organic compounds that are differentially associated with anaerobic hydrocarbon decay. My associate and friend, Mr. Will Beasley (University of Oklahoma) introduced me to and performed some of the statistical analyses.

As indicated above, evidence for the anaerobic oxidation of methane was obtained during my analysis of the North Slope samples. However, the mechanistic details are quite different from the presumed dogma associated with methane metabolism. Therefore, such an observation has many fundamental scientific implications. Chapter 4 is a more extensive description of an alternate hypothesis for the anaerobic oxidation of methane. The chapter was written in the style of a short communication for the *International Society for Microbial Ecology Journal*. This journal encourages short speculative reports on important topics in microbial ecology. I was responsible for the organic extractions, analysis and composing of the manuscript. Dr. Christopher House (Penn State University) and his student, Emily Beal, provided the Eel River Basin samples for my analysis and Dr. Mark Nanny (University of Oklahoma) did the ^{13}C-NMR analysis.

Metabolite extraction and analysis of oil field samples (see Chapters two and

three) revealed methylsuccinate, a low molecular weight alkylsuccinate that may indicate the anaerobic oxidation of methane. I hypothesized that methane may be activated in a similar manner to n-alkanes by addition to fumarate. I constructed several incubations using North Slope production waters as inocula to search for anaerobic methane oxidizing microorganisms. I also interrogated several natural gas and coalfields (not included in this dissertation) as well as the former refinery site in Casper, WY (Chapter 1) and identified methylsuccinate through metabolite analysis. To obtain positive control incubations, I also received samples from the known anaerobic methane-oxidizing Eel River Basin for comparative purposes and these samples reliably consumed $^{13}CH_4$ in the absence of oxygen. The oil field incubations have not currently demonstrated anaerobic methane-oxidizing activity. The Eel River samples yielded ^{13}C-intermediates that are at least consistent with a fumarate-addition methane activation mechanism, but more conclusive finding are needed for a more complete description of this fascinating process.

The Appendix represents a collaborative effort to probe the mechanism of low molecular weight alkanes (ethane, propane and pentane) utilization by anaerobic microorganisms form Zodletone spring in Kiowa County, Oklahoma. The spring is located at a faultline in the Anadarko basin and gaseous short-chain alkanes emanate into a highly sulfidic spring. The manuscript is written in the style of *FEMS Microbiology Ecology* and Dr. Kristen Savage-Ashlock (now at Oklahoma State University) spearheaded this project. The hypothesis that alkane substrates would be oxidized in a manner established with other hydrocarbon-degrading anaerobes (addition to fumarate or carboxylation) was explored. I helped demonstrate that

propane oxidation by the requisite culture occurred via fumarate addition through the identification of propyl- and isopropylsuccinic acids metabolites. I also analyzed samples for the presence of a carboxylation intermediate of pentane metabolism and performed organic extractions on the sulfate-reducing and methanogenic ethane degrading cultures (not included in the manuscript).

Abstract

It is evident that complete mineralization of hydrocarbons and related compounds can occur under anaerobic conditions. A large variety of hydrocarbon classes have been shown to be amenable to decay by enriched microbial consortia and to a lesser extent, pure cultures. With the exception of anaerobic methane oxidation, significant advances have been made towards understanding the mechanistic nature of anaerobic hydrocarbon activation and mineralization over the last 20 years. The ability to detect metabolic intermediates formed by biodegradation of petroleum-related compounds (alkylbenzene, polycyclic aromatic, *n*-alkane and alicyclic) during cultivation of single isolates, enrichments, and most importantly from *in situ* field experiments has become a valuable tool for garnering real-time data about the microbial processes occurring in hydrocarbon laden environments, and assessing the loss of specific contaminants in complex mixtures. For this thesis, field and laboratory approaches were used to evaluate metabolic processes that contribute to the loss of hydrocarbons in diverse environments. The petroleum constituents were either concentrated via anthropogenic activities or naturally occurring (e.g. reservoirs, methane hydrates), and were analyzed to assay for signature metabolites that represent evidence of fundamental pathways for anaerobic hydrocarbon metabolism.

At a former refinery site in Casper, WY, a combination of field and laboratory approaches were used to determine the feasibility of natural attenuation as a remediation strategy for the surrounding hydrocarbon-contaminated aquifer and sediments. The application of field metabolomics revealed several metabolic

intermediates, from a variety of hydrocarbon compound classes, within the petroleum-laden aquifer and substantiated the hypothesis that biodegradation is occurring within the environment. However, laboratory incubations using hydrocarbon-saturated sediments demonstrated a relative recalcitrance of contaminants of regulatory interest (benzene, ethylbenzene, 1,2,4- and 1,3,5-trimethylbenzene and 2-methylnaphthalene) that have been previously shown to be susceptible to anaerobic biodegradation. These findings were not due to the toxicity of the hydrocarbons (as validated by PLFA analysis), nor a limitation imposed by the terminal electron-accepting conditions (as indicated by continual sulfate-reduction). In addition, it was suggested that the persistence of benzene was due to the lack of endogenous microorganisms capable of anaerobic biodegradation of the aromatic compound. Thus, a precedent in the literature for the biodegradation of individual hydrocarbons does not necessarily deem these processes viable in all environmental systems. It is not only important to understand the activity of the microbial populations in the aquifer, but also to understand the abiotic processes that may limit or obscure the detection of biological processes in heterogeneous hydrocarbon mixtures.

Biocorrosion is a process that is potentially fueled by the turnover of hydrocarbons into organic acids and carbon dioxide under anaerobic conditions. Samples were collected from the Alaskan North Slope (ANS) oil fields in 2006 and 2008. Two sampling campaigns in Field B revealed alkysuccinic acids in the range of C_1-C_4, suggesting the addition of methane, ethane, propane and butane to fumarate, an analogous reaction that also occurs with higher molecular weight *n*-

alkanes. Hyperthermophilic communities that may be involved in the production of low molecular weight alkylsuccinates were comprised of several types of Bacteria (syntrophs, fermentative, sulfate/sulfur reducing, iron-reducing) and methanogenic and sulfate-reducing Archaea. In 2008, an analysis of more than 50 samples from the A and B oil fields revealed akylbenzylsuccinates, benzoate associated intermediates, monoaromatic metabolites, naphthoic acids and ring-reduced naphthoates, alkylsuccinates and alkanoic acids. There were differences in Field A and B, specifically in Field B there were a greater number of metabolites, and similar metabolites from sample to sample. While fumarate addition has been widely accepted as the predominant mechanism for anaerobic hydrocarbon decay, the detection of metabolites related to carboxylation and hydroxylation reactions suggest that these processes should be further investigated, and that their metabolic importance in the functioning of microbial communities in petroleum reservoirs should not be discounted.

Methane is the shortest *n*-alkane, and is the most abundant hydrocarbon in the Earth's atmosphere. Though reverse methanogenesis is the current dogma for the anaerobic oxidation of methane (AOM), I proposed that methane is activated by addition to fumarate in a manner similar to that of ethane, propane, butane and longer *n*-alkanes. Previous investigations in petroleum-laden environments revealed the presence of methylsuccinate, particularly in oil field reservoirs and hydrocarbon-contaminated sediments. Laboratory incubations with sediments known to oxidize methane under anaerobic conditions revealed a suite of ^{13}C -labeled downstream metabolic intermediates, suggesting the addition of methane to fumarate. The

evidence for downstream metabolites does not refute reverse methanogenesis as the mechanism for AOM, nor does it provide direct evidence for the addition of methane to fumarate. However, this finding suggests that multiple pathways for the cycling of methane in anaerobic environments may exist, and that additional research should be conducted to characterize the biochemistry of this process

CHAPTER 1

Field metabolomics and laboratory assessments of anaerobic intrinsic bioremediation of hydrocarbons at a petroleum-contaminated site.

Abstract

Field metabolomics and laboratory assays were used to assess the *in situ* attenuation of hydrocarbons in a contaminated aquifer underlying a former refinery. Benzene, ethylbenzene, 2-methylnaphthalene, 1,2,4-, and 1,3,5-trimethylbenzene were targeted as contaminants of greatest regulatory concern (COC) whose intrinsic remediation has been previously reported. Metabolite profiles associated with anaerobic hydrocarbon decay revealed the microbial utilization of alkylbenzenes including the trimethylbenzene COC, PAHs and several *n*-alkanes in the contaminated portions of the aquifer. Anaerobic biodegradation experiments designed to mimic *in situ* conditions showed no loss of exogenously amended COC; however, a substantive rate of endogenous electron acceptor reduction was measured (55 ± 8 μM SO_4•d^{-1}). An assessment of hydrocarbon loss in laboratory experiments relative to a conserved internal marker revealed that non-COC hydrocarbons were being metabolized. Purge and trap analysis of laboratory assays showed a substantial loss of toluene, *m*-and *o*- xylene, as well as several alkanes (C_6-C_{12}). Multiple lines of suggest that benzene is very persistent at this site: we could find no metabolic

intermediates (phenol or benzoate), benzene was relatively recalcitrant in laboratory experiments, and we found low copy numbers of *Desulfobacterium*, a genus recently implicated in benzene degradation under strongly anaerobic (methanogenic and sulfidogenic) conditions. This study shows albeit with notable exception, that there was a reasonable correlation between field and laboratory findings, and that other hydrocarbons were preferentially degraded to the COC, despite precedence for the intrinsic remediation of most of the COC at other sites.

Introduction

The release of petroleum components to the terrestrial subsurface is recognized as a pervasive environmental and human health problem requiring environmental remediation (USEPA, 1999). Monitored natural attenuation is a relatively low cost remedial option that has become more widely accepted since its efficacy has been repeatedly demonstrated since the early 1990s (Borden et al., 1995; Reinhard et al., 1997; Chapelle, 1999; Gieg et al., 1999; Phelps and Young, 1999; USEPA, 1999; Beller, 2002; Field, 2002; Roling and van Verseveld, 2002; Essaid et al., 2003; Griebler et al., 2004; Maurer and Rittmann, 2004; Rittmann, 2004; McKelvie et al., 2005). Natural attenuation involves multiple mechanisms of contaminant removal (e.g. volatilization, sorption, advection, dispersion) but relies on biodegradation of the contaminants of greatest regulatory concern (COC) by the indigenous microflora (NRC, 1993; USEPA, 1999). However, the COC are typically part of complex chemical mixtures consisting of hundreds or even thousands of other co-contaminants. Even though the individual COC may be inherently susceptible to biodegradation, it is important to determine if this potential is realized under the

prevailing environmental conditions.

Many studies have demonstrated the complete destruction of individual hydrocarbons catalyzed by isolated organisms or enriched microbial consortia under a variety of electron-accepting conditions. Recent literature attests to the importance of anaerobic biodegradation of mono- and polycyclic aromatic, alicyclic, alkene and alkane hydrocarbons in diverse ecosystems (Reinhard et al., 1997; Gieg et al., 1999; Phelps and Young, 1999; Spormann and Widdel, 2000; Elshahed et al., 2001; Widdel and Rabus, 2001; Gieg and Suflita, 2002; Phelps et al., 2002; Martus and Puttmann, 2003; Chakraborty and Coates, 2004; Meckenstock et al., 2004b; Townsend et al., 2004; Gieg and Suflita, 2005; Young and Phelps, 2005; Callaghan et al., 2006; Safinowski et al., 2006; Widdel et al., 2006. Prince and Suflita, 2007). The direct detection of signature metabolites produced during the anaerobic biodegradation of hydrocarbons is indicative of anaerobic biodegradation of a corresponding parent hydrocarbon.

Not only does the detection of signature metabolites in a hydrocarbon-contaminated environment describe the parent hydrocarbon compound undergoing anaerobic degradation, it reveals the consequence of microbial activity and provides us with a snapshot of ecosystem function. Traditionally, the term metabolomics has been defined as the study of the metabolism of both endogenous and exogenous metabolites present in biological systems (Dunn, 2005). From a microbial standpoint, metabolomics has been applied to the profiling of all metabolites in pure culture system under a given set of perturbations (van der Werf et al., 2005). When interrogating complex ecosystems with a variety of potential substrates and stressors, field metabolomics can be used in an attempt to describe the *in situ* functioning of

microbial communities.

Field metabolite profiling is a fast and interpretationally direct method that can make use of existing infrastructure such as monitoring wells at contaminated areas (Gieg and Suflita, 2005) to identify signature metabolites. Other methods have been used to describe anaerobic hydrocarbon degradation in petroleum-contaminated environments such as compound specific isotope analysis (CSIA) and quantitative PCR (qPCR) of functional genes associated with fumarate addition. Though it remains an emerging field technique to identify biologically catalyzed contaminant loss from the environment, CSIA requires large concentrations of the contaminant compound of interest, and the majority of the parent compound to have been consumed (80-90%) (Meckenstock et al., 2004a) to determine which of the compounds have been consumed faster (Beller et al., 2008). Gene based methods (qPCR and reverse-transcriptase qPCR (RT-qPCR) for hydrocarbon-degrading functional genes are evolving rapidly, but are limited to field studies with *bssA* (the gene involved in fumarate addition to toluene and xylenes) (Winderl et al., 2007). Furthermore, the identification of *bssA* genes has the potential to create false positive results with regards to anaerobic hydrocarbon degradation unless coupled to the observation of a signature metabolite (Beller et al., 2008).

Several methods are available to investigate anaerobic hydrocarbon degradation, but a field metabolomics approach combined with laboratory assessments can assist in elucidating microbial community function or phenotype *in situ*. There is no compelling reason to presume that the same metabolic potential is present in all environments, and addressing interpretational limits of a single technique by using several lines of evidence can shed light upon the bioremediation

potential of complex petroleum-contaminated sites.

We investigated whether a series of COC (benzene, ethylbenzene, 1,2,4-trimethylbenzene, 1,3,5-trimethylbenzene, and 2-methylnaphthalene) were being metabolized in an aquifer underlying a former refinery site in Casper, WY. During the almost eight decades of refinery operations, hydrocarbon contamination could be traced to a variety of releases (WDEQ, 2001). Despite the complex nature of the contaminant mixture, most regulatory attention focused on benzene. Benzene was present throughout the site at concentrations exceeding 50 μM (4000 ug/L) (Brubaker, 2003). Field metabolomics and laboratory biodegradation assays were used to garner evidence for the intrinsic remediation of all the COC. We found that while *in situ* microbial hydrocarbon metabolism was evident in the aquifer, not all COC were susceptible to anaerobic decay despite expectations from the literature. Generally, good agreement between the field and laboratory indications of anaerobic biodegradation was obtained. Several reasons for the relative recalcitrance of the COC are suggested.

Results

Metabolite profiling. A variety of signature metabolites associated with anaerobic hydrocarbon biodegradation were detected in groundwater from monitoring wells at the former refinery site, but not in samples from a background well (Table 1). Alkylbenzylsuccinic acid metabolites associated with the biodegradation of the xylene and C_3-alkylbenzene isomers (Table 1, 2) were detected in 7 of 8 monitoring wells within the refinery area and one of the wells on the north side of the river where hydrocarbons were stored. Toluene degradation was also evident by the

presence of benzylsuccinic acid on a single sampling occasion (Table 1). However, unlike the trimethylbenzene COC, no evidence for anaerobic ethylbenzene decay was obtained with the field metabolite profiling.

Metabolites associated with the anaerobic biodegradation of substituted naphthalenes were also detected at the site. The presence of the partial ring reduction metabolite 5,6,7,8-tetrahydro-2-naphthoic acid (rather than other isomeric components) was revealed by GC-MS in 3 of the 10 monitoring wells (Table 1). Another well indicated the presence of the unsubstituted naphthoic acid, while residues associated with methyl- and dimethyl naphthoic acids isomers could be found in MW-345. There was no evidence for the more reduced decahydronaphthoic acid in any of the wells, but most had a putative naphthalene metabolite with mass spectral features consistent with the presence of tetrahydronaphthoic acids. However, the mass spectral profiles exhibiting these features were not associated with retention times for the authentic standards for 1,2,3,4- or 5,6,7,8-tetrahydro-2-naphthoic acid. Notably, anaerobic naphthalene metabolism was not indicated in samples obtained from the monitoring well chosen for the biodegradation assays (MW-439).

Field evidence for the anaerobic biodegradation of pentane, hexane and a variety of C_5-C_9 unsaturated hydrocarbons (alkenes or alicyclic) was implicated based on the detection of the corresponding fumarate addition metabolites (Table 1). All wells, except MW-345, harbored one or more of these metabolites and a similar suite of compounds were observed in well MW-439 on both sampling occasions.

Sulfate reduction and hydrocarbon metabolism. Sulfate reduction could be measured in all aquifer incubations regardless of COC amendment (Figure 2). Since replicate incubations varied, the rates were averaged with extremes (highest and

lowest values) indicated. Given that hydrocarbon metabolism can sometimes require long incubation periods (Caldwell and Suflita, 2000), sulfate was replenished (134 d) when the concentration of this anion fell to approximately 100 μM. The sulfate reduction rates in the COC-amended incubations were not significantly different before or after the sulfate replenishment or the substrate-unamended control. Thus, the hydrocarbon addition did not inhibit background microbial activity. The average rate of sulfate loss for all incubations (excluding toluene) was 55.0 ± 8.0 $\mu M \bullet d^{-1}$ (Figure 2). Toluene, the positive control, stimulated sulfate reduction above the substrate-unamended control (Figure 2).

The steady depletion of sulfate over the 250 d incubation suggested that other forms of organic matter were being consumed by the aquifer microflora. A purge and trap GC-MS analysis (Townsend et al., 2004; Prince and Suflita, 2006) of the residual hydrocarbons in non-sterile incubations was compared with those in sterile controls (Figure 3) at the end of the experiment. Given the variation encountered in the sulfate depletion assays (above), quadruplicate incubations are depicted (Figures 3). This determination revealed that *n*-alkanes, ranging from C_6-C_{12}, were reduced or depleted by biodegradation in the aquifer incubations. Similarly, significant losses were evident for toluene and *o*-xylene in all incubations but there was no evidence for the removal of benzene or ethylbenzene. All but one of the four replicates showed a remarkably specific loss of 1,3-dimethyl and 1-ethyl-3-methyl-benzenes (Figure 3). Additional 1,3-disubstituted alkylbenzene biodegradation was noted with 1-methyl-3-propylbenzene but not 1-methyl-3-isopropylbenzene. Consistent with the sulfate reduction assay (above), there was no evidence for the biodegradation of any of the COC. Collectively, these findings argue that the

anaerobic biodegradation of some of the endogenous hydrocarbons accounted for the background levels of sulfate consumption.

General and specific biomass determination. We questioned if the chronic exposure of the resident microflora to hydrocarbons limited their population size and thus their ability to respond to the COC amendments. General biomass levels were determined by PLFA analysis on three samples that were COC-amended (benzene, 1,2,4-trimethylbenzene or 1,3,5-trimethylbenzene) and a fourth that was substrate-unamended. All samples harbored between 1.23×10^7 and 1.86×10^7 total microbial cells per gram (Table 3) and there was no significant difference in the levels of eukaryotes, sulfate-reducing bacteria, Proteobacteria, Firmicutes, or general heterotrophs (not shown). The physiological status of the microflora in the samples was indicated by the ratio of *trans*- to *cis* monoenoic fatty acids as well as the cyclopropyl fatty acids to their monoenoic precursors (White et al., 1996; Pinkart, 1997; White and Ringelberg, 1997; Green and Scow, 2000). Gram-negative microorganisms typically increase their *trans* fatty acids in the cell membrane when physiologically stressed with exposure to a toxic compound or starvation (Green and Scow, 2000). In the four samples analyzed, the *trans*- to *cis*- ratios (Table 2) were in the range of 0.05-0.09 (16:1ω7t/16:1ω7c) and 0.13 to 0.16 (18:1ω7t/11:1ω7c). Ratios for *trans*- and *cis*- fatty acids of 0.1 indicate that the organisms are generally healthy and values in the range we observed are typical of contaminated sites (MacNaughton et al., 1999; Green and Scow, 2000). The ratios of cyclopropyl fatty acids/monoenoic precursors (Table 3), were < 1.0 (cy17:0/16:1ω7c) with the exception of the substrate-unamended sample (1.36), and 0.16-0.22 for cy19:/18:1ω7c. These values are typical of contaminated sites (Green and Scow,

2000), and do not suggest any dramatic toxic effects on the microbiota.

In addition, a molecular assay for the presence of *Desulfobacterium* cells was conducted as members of this genus (clone OR-M2, GenBank AY118142) have been implicated in anaerobic benzene metabolism (Da Silva and Alvarez, 2007)(Table 2). Real-time quantitative PCR analysis revealed relatively low copy numbers of the 16S rRNA gene sequence associated with this putative benzene-degrading sulfate reducing bacterium ($\approx 1 \times 10^3$ cells per gram of soil). In fact the concentration of benzene-degrading bacteria determined by this method was less than one percent of the total bacterial population in the sample and comparable to negative control samples in the reported assay (Da Silva and Alvarez, 2007).

Discussion

Field and laboratory evidence was used to assess the intrinsic remediation of selected hydrocarbons in a petroleum-contaminated aquifer underlying a closed refinery. The detection of signature metabolites in ground water samples is compelling evidence that hydrocarbon biodegradation was an ongoing process. Moreover, the identification of the metabolites implicates the parent hydrocarbons undergoing biotransformation (Elshahed et al., 2001; Gieg and Suflita, 2002; Wilkes et al., 2003; Gieg and Suflita, 2005). We detected a variety of putative metabolites at multiple locations in contaminated portions of the aquifer, but none were found in the background well (Table 1). Based on the identity of the metabolites, we infer that several alkylbenzenes, naphthalenes, alkanes and potentially alicyclic hydrocarbons were undergoing anaerobic biodegradation in the shallow aquifer on both sides of the river. Most metabolite signatures were found on the south side of the river, the area

where refinery operations were centered. The lack of alkylsuccinic acid metabolites in water samples taken from the north side of the river probably reflects the different hydrocarbons present in that area, the differential attenuation of alkane/alicyclic hydrocarbons, or both. In one well, an almost identical suite of metabolites was detected at multiple samplings over different times of the year (Table 1, MW-439 a,b) suggesting that seasonal fluctuations did not substantially influence ongoing microbial metabolism in the aquifer.

There was general agreement between the field metabolic profiling and the loss (or recalcitrance) of the parent hydrocarbons in laboratory incubations (Table 2). Nevertheless, there were also notable differences. For instance, the alkylsuccinate metabolites detected in the field suggested that pentane and hexane were biodegraded, while the alkane loss patterns in laboratory biodegradation assays showed that higher molecular weight hydrocarbons (i.e., C_7-C_{12}) were preferentially utilized by the aquifer microflora (Figure 3). Several reasons can be advanced to account for these observations. First, the lack of consistent pentane or hexane loss indications in the laboratory assay most likely reflects the analytical limit of the purge and trap analysis. Secondly, if the lower molecular weight hydrocarbons were preferentially utilized by the microflora relative to the higher molecular weight *n*-alkanes, a steady re-supply of the former from the NAPL known to be in the aquifer may differentially impact metabolism of the latter compounds. However, as the C_5-C_6 hydrocarbons get depleted from the laboratory incubations, the higher molecular weight *n*-alkanes could then be more amenable to microbial attack.

Similarly, while field and laboratory assays suggest that the COC were largely recalcitrant there are also subtle differences. Metabolites associated with the

anaerobic biodegradation of benzene, ethylbenzene, and 2-methylnaphthalene have been reported in a number of studies (Rabus and Heider, 1998; Caldwell and Suflita, 2000; Kniemeyer et al., 2003; Meckenstock et al., 2004b; Ulrich et al., 2005; Safinowski et al., 2006; Safinowski and Meckenstock, 2006). The benzene metabolites benzoate and phenol were not found in this study but benzylsuccinic acid was detected in at least one well (Table 1) implying that toluene was anaerobically metabolized. This conclusion was supported when toluene was used as a positive control in the laboratory biodegradation assays (Figure 2). Recent evidence suggests that toluene may be a putative metabolite in anerobic benzene degradation (Ulrich et al., 2005). However, since toluene is a frequent component in petroleum, its detection cannot be reliably construed as evidence for benzene metabolism.

The detection of dimethylbenzylsuccinic acids (or isomeric counterparts) in most of the wells (Table 2) suggests that C_3-alkylbenzenes were anaerobically transformed in the aquifer. To see if these signals could be attributed to the metabolism of 1,3,5 - or 1,2,4-trimethylbenzene COC, we synthesized the corresponding fumarate addition products. The same was done for 1-ethyl-3-methylbenzene since it was the only C_3-alkylbenzene implicated in the laboratory biodegradation assay. Gas chromatographic analysis revealed a characteristic suite of peak(s) with identical mass spectral profiles. Comparison of the GC-MS characteristics of the synthesized standards to the field metabolites allowed us to confirm that the two COC as well as 1-ethyl-3-methylbenzene were metabolized in the field. However, only the latter could be confirmed in the laboratory assay (Figure 3). Those peaks showing the same mass spectral profile but different GC retention times that could not be matched to 1,2,4- and 1,3,5-trimethylbenzene or 1-ethyl-3-

methylbenzene were identified as unassigned C_3-alkylbenzylsuccinates. Following the synthesis of the authentic C_3-alkylbenzylsuccinic acids, we were able to positively identify a putative fumarate addition metabolite resulting from the degradation of 1,2,4-trimethylbenzene in MW-439 during the first, but not the second sampling, while the opposite temporal appearance of a fumarate-addition metabolite was observed for 1,3,5-trimethylbenzene.

Of course, failure to detect a metabolite does not necessarily mean that the parent substrate is recalcitrant. However, the general lack of COC metabolism was also confirmed by the laboratory biodegradation assays. These compounds were added at 50 ppmC (500-600 μM) so that their anaerobic biodegradation would easily be manifest by an increased level of electron acceptor consumption relative to the substrate-unamended controls. However, none of the COC amendments or the COC mixture stimulated sulfate reduction above the COC-free controls (Figure 2). The rate of sulfate loss, while variable, was not substantially different in any of the incubations suggesting that the addition of the COC amendments was not inhibitory to the resident microflora.

The background sulfate respiration in all incubations suggested that some form of organic matter other than the COC was being metabolized over the course of the experiment. This was confirmed by purge and trap GC showing that a variety of alkanes and alkylated aromatic hydrocarbons were depleted from COC-unamended incubations (Figure 3). This result suggests that there may be some preferential utilization of hydrocarbons at the former refinery site and that the COC, while inherently amenable to intrinsic remediation may simply not be attacked in deference to other substrates.

Mechanistically, the lack of COC biodegradation may be a result of metabolic interference by other compounds in the complex hydrocarbon mixture in the aquifer. Previous studies have demonstrated a significant lag or inhibition in the degradation of benzene, toluene, ethylbenzene or xylene when one or more of those hydrocarbons were added to laboratory incubations degrading a single BTEX component under a variety of electron-accepting conditions (Evans et al., 1991; Edwards and Grbic-Galic, 1994; Meckenstock et al., 2004c). It has also been shown that once degradation occurred, the microbial enrichments could be inhibited by the presence of other organic acids and alcohols (Edwards and Grbic-Galic, 1994), or co-metabolites as demonstrated with polycyclic aromatic hydrocarbons (Safinowski et al., 2006). Thus, the incomplete biodegradation of various hydrocarbons or the presence of their metabolic breakdown products could potentially interfere with the biodegradation of the COC.

This point notwithstanding, the biomass levels measured in COC-amended and unamended samples (Table 3) were comparable to what has been found in hydrocarbon-contaminated aquifers (Green and Scow, 2000), and not significantly different at the end of the experiment. The physiological status of the organisms in this study suggests the Gram-negative microorganisms may be slightly stressed, presumably by the hydrocarbon contamination. However, laboratory incubations have also been shown to elicit similar stress responses (Green and Scow, 2000). The results of the PLFA analyses suggest there were active microbial populations in the samples and that the COC amendments were unlikely to be more inhibitory than the background hydrocarbons.

Yet another possible reason for the recalcitrance of the COC could be low

numbers or the inherent lack of specific types of catalytic microorganisms. (Ulrich et al.,2005; Da Silva and Alvarez, 2007). A recent study demonstrated an increase in the copy number of the 16S rRNA gene representing *Desulfobacterium* sp. clone OR-M2 and thus this organism has been implicated in the anaerobic biodegradation of benzene via sulfidogenic or methanogenic conditions (Ulrich and Edwards, 2003; Da Silva and Alvarez, 2007). Due the lack of benzene loss in our biodegradation assays, we investigated our samples for the suspected benzene degrading organism *Desulfobacterium* sp. clone OR-M2. We found very low copy numbers of the 16S rRNA gene, relative to the total numbers of organisms in the sample. If this organism is indeed responsible for anaerobic benzene degradation, it was virtually absent in the aquifer sediment incubations. Elucidating the reasons for such a low concentration of this putative benzene degrader was beyond the scope of this study. Of course, anaerobic benzene metabolism has been documented under several terminal electron-accepting conditions including sulfate reduction, and other organisms are undoubtedly capable of catalyzing the destruction of this compound. Recently a nitrate-reducing, benzene-degrading *Azoarcus* was isolated from a BTX-contaminated aquifer (Kasai, 2006). However, nitrate is not known to be an important potential electron acceptor in the aquifer and when nitrate was used as an experimental variable in our experiments, not only was benzene loss not evident, background hydrocarbon loss ceased (data not shown).

In summary, we observed excellent corroboration between field and laboratory findings (Table 3) in terms of both the types of hydrocarbons that were subject to intrinsic remediation and those that were not. The COC, while known to be susceptible to anaerobic biodegradation in other systems, proved largely

recalcitrant in laboratory assays, and positive field indications, while present, were transitory. The study suggests that the extrapolation of anaerobic biodegradation information from one site to another must be made with caution, and whenever possible, existing literature information must be supplemented with direct experimental evidence. Based on our current findings, we would not predict that the COC will be removed by natural attenuation without further manipulations at the site.

Experimental Procedures

Field Description. The former oil refinery is located south of the North Platte River in Casper, WY (Figure 1). The site is underlain by shale bedrock approximately 9-12 m below the ground surface. The subsurface is composed of highly permeable Quaternary alluvium of medium to coarse sand, with some gravel and cobble, while the surface is dominated by silt and clay. The water table is located between 1.5 and 4.5 m below the surface and hydraulic conductivity is between 61-106 m day^{-1}. Petroleum releases into the subsurface included fuel gas, liquid propane gas, motor/aviation gasoline, fluid cracking unit coke, heavy fuel oil, kerosene and distillates, asphalt, and other residual components that resulted in a relatively uniform distribution of non-aqueous phase liquid over approximately 90% of the site. Residual hydrocarbons spanned depths of 0.6 m above to 1.2 m below the water table surface.

The hydrocarbon plume migrated with the prevailing ground water flow toward the river (Figure 1). Over 460,000 m^3 of contaminated sediment and more than 50 million liters of non-aqueous phase liquid have been removed to date. A

>2,600 m sheet pile wall was installed on the south bank of the river as a contaminant migration barrier. Monitoring wells were selected along the groundwater flow for sampling purposes (Figure 1).

Historical groundwater geochemistry indicated that the steady state dissolved hydrogen values in plume areas were 1-2 nM and that sulfate concentrations decreased from 25 mM at the southern edge of the site to 3-4 mM along transects (Figure 1). These data suggested that sulfate reduction was a dominant terminal electron-accepting process at the site.

Metabolite profiling. Groundwater samples were collected from hydrocarbon-impacted and background wells in February and October 2005 and analyzed for signature anaerobic metabolites as previously described (Elshahed et al., 2001; Gieg and Suflita, 2002, 2005). The collected groundwater was immediately acidified in the field with 50% HCl to a pH < 2 and kept at 4°C until analyzed. Groundwater samples (1 L) were extracted with ethyl acetate, dried over anhydrous Na_2SO_4, concentrated by rotary evaporation under a flow of N_2, derivatized with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Pierce Chemical Co., Rockford, IL) and analyzed by gas chromatography-mass spectrometry (GC-MS)(Elshahed et al., 2001). Anaerobic metabolites were identified using derivatized authentic standards (Elshahed et al., 2001; Gieg and Suflita, 2002, 2005).

To resolve the alkylbenzylsuccinate isomers,1,2,4-, 1,3,5-trimethylbenzene and 1-ethyl-3-methylbenzene were synthesized and similarly derivatized as before (Elshahed et al., 2001). Increased chromatographic resolution was achieved by using a different column (DB-5ms 20 m x 0.18 mm i.d., 0.18 μm film, Agilent

Technologies, Foster City, CA) and the GC oven temperature was held at 45°C (5 min) then increased to 140°C (20°C/min), then to 180°C (1°C/min), to a final temperature of 270°C (30°C/min).

Laboratory assessments. Sediments and groundwater were collected from a single location at the site (MW-439, Figure 1) to construct aquifer sediment incubations. Approximately 50 ± 1.0 g of sediment collected from a depth of 6 m (within the hydrocarbon smear zone) and 75 ± 1.0 ml of groundwater were dispensed into sterile 160 ml serum bottles in an anaerobic chamber (5% H_2 in N_2). Groundwater was reduced prior to use with Na_2S (0.005%) and amended with resazurin (0.001%) as a redox indicator (Townsend et al., 2003). The serum bottles were closed with butyl rubber stoppers, crimped with aluminum seals and given an N_2 headspace. The aquifer incubations were stored in the dark at room temperature for approximately 30 d to equilibrate and to allow for the removal of endogenous levels of electron acceptors before being amended with COC, sulfate, or other treatments as indicated.

Hydrocarbon and Sulfate Analyses. Substrate-amended aquifer incubations received 500-600 µM (50ppm carbon) of benzene, ethylbenzene, 1,2,4-trimethylbenzene, 1,3,5-trimethylbenzene, 2-methylnaphthalene, or a mix of these compounds. Toluene (50ppm carbon) was added as a positive control. All substrates were added to the incubations as neat compounds with the exception of 2-methylnaphthalene which was dissolved in methanol. Heat-killed controls were autoclaved for 20 min on three consecutive days prior to substrate addition. For most laboratory incubations, hydrocarbon loss was monitored by headspace GC analysis (50 µl, 45ºC (5 min), to 90ºC (4°C/min). Utilization of 2-methylnaphthalene

was analyzed by HPLC equipped with a reversed-phase C_{18} column (250mm 4.6 mm, 5 µm particle size; Alltech, Deerfield, IL) and a UV detector (260 nm). The mobile phase (1 mL/min) consisted of acetonitrile:phosphoric acid (70:30) Losses of non-COC hydrocarbons from several incubations was assessed by purge and trap GC-MS as previously reported with 2,2,4-trimethylpentane as the conserved internal marker (Townsend et al., 2004; Prince and Suflita, 2006).

Sodium sulfate (4 mM) was added to the aquifer slurries at the start of the experiment and re-amended when levels were at or below 100 µM. Initial concentrations were selected to mimic *in situ* sulfate levels. Sulfate reduction activity was quantified by ion chromatography (DX-500, AS4A anion exchange column, DIONEX, Sunnyvale, CA) as previously described (Townsend et al., 2003).

Total and benzene-degrading biomass. Following 250 d, 60 ml samples were analyzed for total biomass by phospholipid fatty acid (PFLA) determination (Microbial Insights, Inc., Rockford, TN). DNA extracted from microcosm sediment (1 ± 0.5 g) was used to detect and quantify total bacteria 16S rRNA and bacteria that are closely associated with benzene degradation under strongly anaerobic (methanogenic and sulfidogenic conditions) as described by Da Silva and Alvarez (2007).

Supplemental materials are available. Figure S1 respresents fumarate addition product(s) of 1,2,4-trimethylbenzene, 1,3,5-trimethylbenzene, or 1-ethyl-3-methylbenzene. This information can be found in Appendix B.

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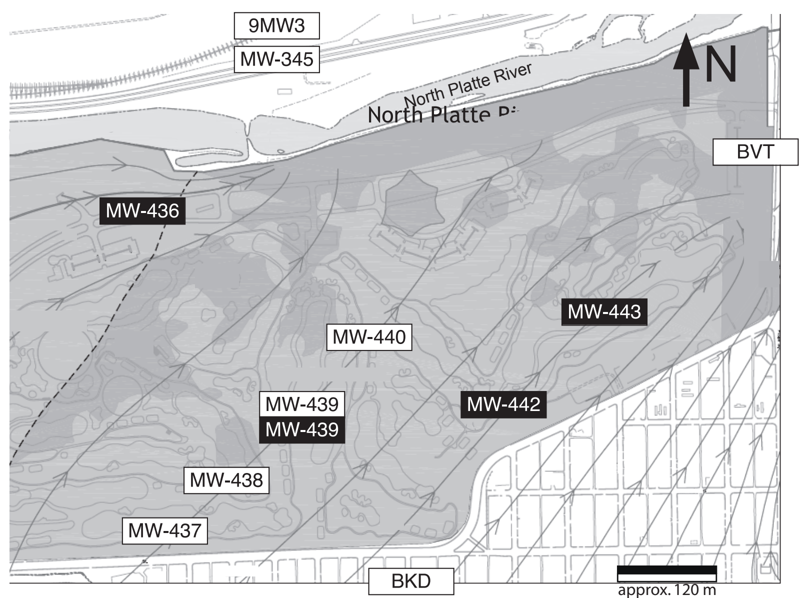

Figure. 1. Monitoring well locations (boxes) used for groundwater sampling at the former refinery site near the North Platte River in Casper, WY. Groundwater flow was in a northeastern direction, towards the river. Samples were collected in February (white) and October (black) 2005 along transects throughout the site. Darker shaded regions represent active remediation zones and BVT represents a well used for bio-venting.

Table 1. Signature anaerobic metabolites of microbial hydrocarbon decay detected in groundwater monitoring wells as indicated by a closed circle (●). Monitoring well (MW) 439 was sampled in February (MW-439a) and October (MW-439b). † Indicates matching mass spectral profiles were found, however retention times did not match.

Parent Compound	Metabolite	Bkgd	436	437	438	439(a)	439(b)	440	442	443	BVT	9MW-3	345
	Alkylbenzylsuccinic acids												
Toluene	benzylsuccinic acid					●							
Ethylbenzene	ethylbenzylsuccinic acid												
m- or *o*-Xylene	methylbenzylsuccinic acids				●	●	●		●		●		
p-Xylene	methylbenzylsuccinic acids			●	●	●	●	●	●		●		
C3 Alkylbenzenes													
1,2,4-Trimethylbenzene	dimethylbenzylsuccinic acids			●	●	●			●				
1,3,5-Trimethylbenzene	dimethylbenzylsuccinic acids				●		●	●	●				
1-Ethyl-3-methylbenzene				●	●	●	●						
Unassigned C3 Alkylbenzenes				●	●	●	●		●			●	
	Naphthoic acids												
Naphthalene or 2-methylnaphthalene	1- or 2-naphthoic acid									●			●
	methylnaphthoic acid												●
	dimethylnaphthoic acid												●
	1,2,3,4-tetrahydro-2-naphthoic acid												
	5,6,7,8-tetrahydro-2-naphthoic acid		●						●				●
	Unassigned tetrahydro-naphthoic acids†		●	●	●			●	●	●	●	●	●
	Alkylsuccinic acids												
Alkanes (C_n)													
	C5				●	●	●						
	C6				●			●	●	●			
Unsaturated Alkanes (C_{n-2})													
	C5					●	●	●	●	●			
	C6		●	●	●	●	●				●	●	
	C7		●			●	●				●		
	C8		●	●					●	●			
	C9		●						●	●			

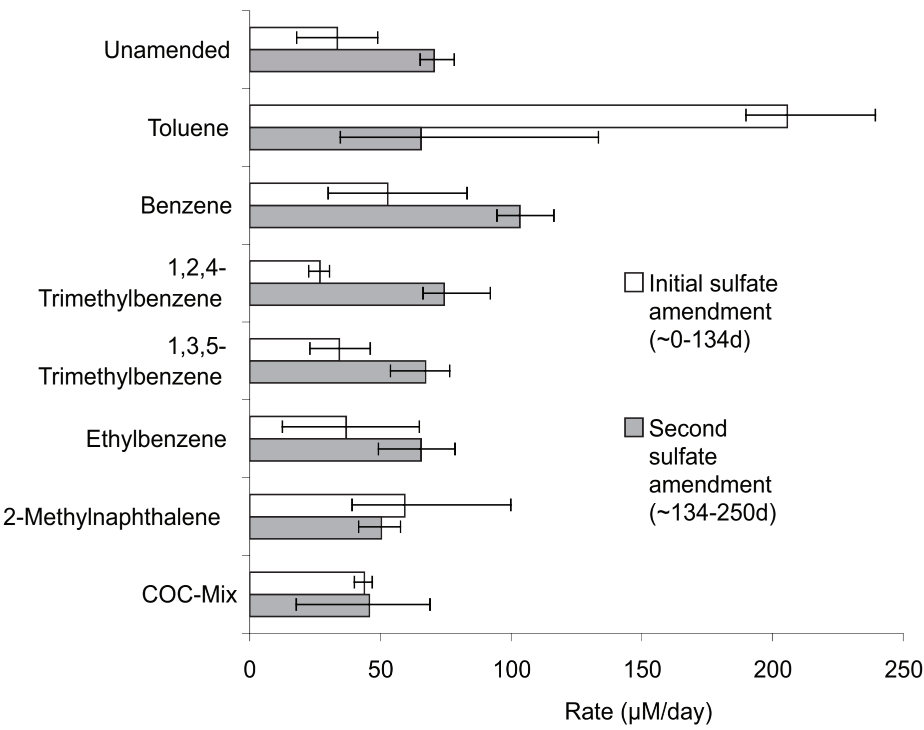

Figure 2. Rates of sulfate reduction in aquifer incubations. The average rate of sulfate reduction for the first sulfate amendment occurred at time zero until approximately 134 d (white bars). The second sulfate addition occurred at 134 d and average rates were calculated through 250 d (gray bars). Black marker bars demonstrate variability between samples, representing the highest and lowest rates of sulfate reduction observed amongst replicates.

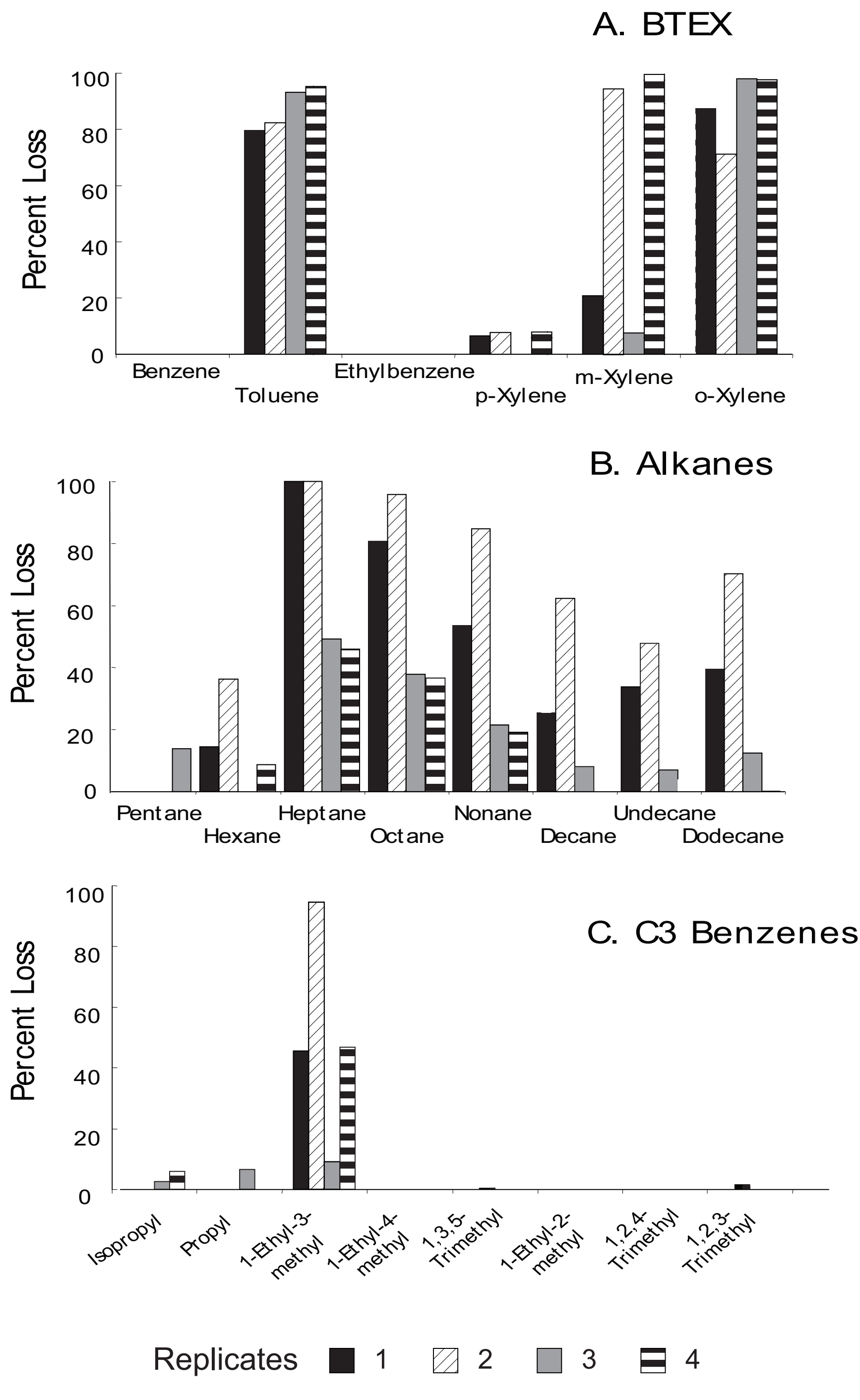

Figure 3. Select hydrocarbon loss in substrate-unamended incubations by GC-MS analysis (after a 250 d incubation). Four replicates are represented for each analysis. Hydrocarbon loss other than COC is depicted as a percentage total loss.

Table 2. Microbial biomass levels and community physiological status from *trans*- to *cis*- fatty acid ratios as well as and cyclopropyl fatty acids to their monoenoic precursors in selected incubations as determined by PLFA analysis.

Sample	1	2	3	4
Amendement	Benzene	None	1,2,4-Trimethylbenzene	1,3,5-Trimethylbenzene
pmol PLFA/ml	929	659	720	613
Total Biomass (cells g^{-1})	1.86×10^7	1.32×10^7	1.44×10^7	1.23×10^7
Physiological Status				
cy17:0/16:1ω7c	0.65	1.36	0.72	0.69
cy19:0/18:1ω7c	0.22	0.22	0.19	0.16
16:1ω7t/16:1ω7c	0.05	0.09	0.07	0.06
18:1ω7t/18:1ω7c	0.15	0.13	0.16	0.15

Table 3. Relationship between field profiling for metabolites, endogenous hydrocarbon decay and COC amended incubations for MW-439. Positive results reflecting degradation are indicated by a closed circle (●). No activity observed is represented by an open circle (O). The addition of toluene served as a positive control and degradation was observed by all experimental methods. No amendment (NA) indicates no amendment of hydrocarbon because it was not a COC.

Hydrocarbon	Field Profiling	Endogenous Activity	Hydrocarbon Amendment
Benzene	○	○	○
Toluene (positive control)	●	●	●
Ethylbenzene	○	○	○
Xylene	●	●	**NA**
1,2,4 - Trimethylbenzene	●	○	○
1,3,5 - Trimethylbenzene	●	○	○
1- Ethyl-3-methylbenzene	●	●	**NA**
2 - Methylnaphthalene	●	○	○
Various alkanes (saturated)	●	●	**NA**
Various alkanes (unsaturated)	●	–	**NA**

CHAPTER 2

Biocorrosive Thermophilic Microbial Communities in Alaskan North Slope Oil Facilities

Abstract

Corrosion of metallic oilfield pipelines by microorganisms is a costly but poorly understood phenomenon, with standard treatment methods targeting mesophilic sulfate-reducing bacteria. In assessing biocorrosion potential at an Alaskan North Slope oil field, we identified thermophilic hydrogen-using methanogens, syntrophic bacteria, peptide- and amino acid-fermenting bacteria, iron reducers, sulfur/thiosulfate-reducing bacteria and sulfate-reducing archaea. These microbes can stimulate metal corrosion through production of organic acids, CO_2, sulfur species, and via hydrogen oxidation and iron reduction, implicating many more types of organisms than are currently targeted. Micromolar quantities of putative anaerobic metabolites of C_1-C_4 *n*-alkanes in pipeline fluids were detected, implying that these low molecular weight hydrocarbons, routinely reinjected into reservoirs for oil recovery purposes, are biodegraded and can provide biocorrosive microbial communities with an important source of nutrients.

Introduction

The U.S. possesses a network of over 2.3 million miles of pipelines that transmit about 75% of the nation's crude oil and 60% of refined products (*1*). Despite this importance, pipelines are not regularly considered in assessments of

societal infrastructure needs (*2*), but there is little doubt that these facilities are vulnerable and can deteriorate over time. Through-wall breaches due to corrosion are expensive problems in the oil industry that can result in explosions, product interruptions, hazardous chemical releases and environmental damage. Such was the case in the August 2006 Prudhoe Bay release on Alaska's North Slope (ANS) *(3)*. The metabolic activities of microorganisms were implicated in this and other incidents of pipeline failure. In fact, it has long been known that microbes contribute to corrosion by multiple mechanisms *(4-6)*, yet biocorrosion is not a well-understood process. There is no consensus on the identity of specific microorganisms responsible for corrosion or how they function to catalyze such incidents, resulting in poorly targeted efforts to monitor and combat biocorrosion.

Following the pipeline breach at Prudhoe Bay, we obtained samples from an ANS field to assess the potential for biocorrosion via metabolic indicators and microbial community analysis. The geology and geochemistry of ANS fields have previously been described *(7)* and subsurface conditions are well within the range for microbial communities to thrive *(8)*. The facility produces oil, gas and water from multiple hot, anaerobic (average temperature 68°C) reservoirs and is typical of ANS oilfields that collectively produced up to 16% of the U.S. domestic oil requirements for over 30 years (*9*). Fluids and gases from multiple production wells are collected in a central facility, from which oil is channeled to the Trans-Alaska Pipeline System. At the facility, low molecular weight hydrocarbons (mostly methane, with lesser amounts of C_2-C_4 *n*-alkanes) and water are reinjected into the oil-bearing formations to maintain pressure and facilitate oil recovery. Most pipelines are above

ground and thermally insulated, so conditions inside the pipelines and processing facilities are anaerobic and hot (Table S1). As required, seawater is treated with biocide and added to maintain formation pressures or during oil processing, thus introducing seawater chemistry, a lower temperature, and potentially marine microorganisms into oil reservoirs. To assess biocorrosion potential in the ANS field, we obtained fluid samples from production well heads, from a water reinjection well following oil processing activities in a central facility (CF), 2 locations within the CF, from a pipeline carrying fluids and gas to the CF, treated seawater, and fluids and solids scraped from the inner surface of the pipeline carrying treated seawater. (Table S1). We sought to conduct a molecular survey of the predominant microbial communities in these facilities and to gain insight on the metabolic activities that might sustain these organisms.

Materials and Methods

Molecular analysis. Samples (Table S1) from two production wells (2P, 2L), two locations in a central facility (CF, 1st stage separator [PS] and coalescer [CO]), and from a seawater line prior to exposure to oil (SW) were collected in 2006 from an oil field complex on the North Slope of Alaska. The seawater line sample consisted of fluids and solids from a pigging operation, whereas the other samples were fluids. Two samples (PS and SW), 150 mL each) were filtered (0.45 µm) and preserved in the field by the addition of DNAzol® Direct (Molecular Research Center, Inc., Cincinnati, OH) to the filter then extracted upon return to our laboratories using a bead-beating protocol (UltraClean™ Mega Soil DNA Isolation, MO BIO

Laboratories, Inc., Carlsbad, CA). The cells and DNA from lysed cells in the remaining samples (20 ml) were first concentrated by centrifugation at 6,000xg for 10 minutes at 4°C after the addition of ½ volume frozen absolute ethanol and the pellet resuspended in PCR-grade water before DNA purification using the PowerSoil™ DNA Isolation Kit (MO BIO). 16S rRNA gene primers for eubacteria (27F, 1391R;*10*), and 27F and 1492R for the seawater line *(11)*, ARC333F and 958R for archaea *(11)*, *dsrAB* (dsr1F, dsr4R, *12*), and thermal cycling conditions as described in the references above (*11-12*) were used to obtain PCR products to create clone libraries (see Table S1) using the TOPO® TA Cloning Kit (Invitrogen Corp., Carlsbad, CA). Primer sets used to create each library and the number of non-chimeric sequences obtained are listed in Tables S2A and S2B. The DOE Joint Genome Institute (Lawrence Livermore Laboratory, Walnut Creek, CA) sequenced the libraries using M13F and M13R primers complementary to regions on the vector flanking the PCR product insert. Two bacterial 16S rRNA gene libraries, one using primers 27F and 1492R, the other using primers 27F and 1391R were created from 2P to compare the effect of using the 1492R versus the 1391R primer. Both libraries contained the same dominant *Petrobacter* sequence (87.7% versus 90.1% of total sequences) and had low sequence diversity. Data from the library created with primers 27F and 1391R are listed in Tables S1, S2A and S4. Results for the *dsrAB* libraries are briefly referred to in this work and will be reported in detail later (manuscript in preparation).

Primer binding sites were identified using the "Motifs" function in Sequencher (version 4.7, Gene Codes, Ann Arbor, MI) as a guide to trim the

sequences to homologous regions, approximately 1250 bp for bacterial 16S rRNA gene sequences and 600 bp for archaea. The sequences in the clone libraries were aligned using the greengenes NAST-aligner *(13)* and examined for chimeric sequences using the Bellerophon program *(14)*. Potential chimeric sequences identified by Bellerophon were further examined by Pintail *(15)*, and comparing separate regions of the sequences by BLASTN *(16)*. Distance matrices *(17*) were created from each library after it had been purged of chimeras. The Lane mask filter *(18)* was applied to limit distance matrix calculations to conserved portions of the aligned sequences. DOTUR *(19)* used the distance matrix values to produce OTUs at the 97% level of similarity and calculate the Chao and ACE estimates of species richness and Shannon-Weaver and Simpson measures of diversity reported in Table S3. Percentage library coverage at the 97% level of similarity was estimated by the method of Good *(20)*. One representative sequence was chosen from each OTU, its taxonomic affiliation determined by the RDP Classifier *(21)* and closest match to sequences in the GenBank database by BLASTN (16; Tables S2A and S2B). Distance matrices were constructed from the pooled representative sequences originating from all libraries of the same type (e.g. 5 bacterial libraries pooled into one, 3 archaeal libraries pooled into one) and DOTUR applied to produce pooled-sample OTUs at the 97% level of similarity. Correct assignment of pooled-sample OTU membership for each individual representative sequence within a pooled-sample OTU was confirmed by inspection of the taxonomic affiliation and BLASTN matches previously determined for the representative sequence.

Representative bacterial sequences were deposited in GenBank under accession

numbers FJ269280-FJ269403; representative archaeal sequences were assigned accession numbers FJ446497-FJ446523.

Enrichments and isolation. Heterotrophs were enumerated in anaerobic half-strength tryptic soy broth (Becton, Dickinson and Co.) plus 1% NaCl under a N_2:CO_2 atmosphere in a MPN assay *(22)*. Hydrogen oxidizers were enumerated in an anaerobic, reduced basal medium under a H_2:CO_2 atmosphere *(22- 23)*. SRB were enumerated in a medium designed for rapid quantitation *(22, 24)*.. MPN enumerations of microorganisms from the seawater pig envelope were incubated at room temperature (22°C). MPN enumerations of microorganisms from the 1st stage separator were incubated at 50°C. Strains isolated from the MPN enumerations were identified by analysis of partial 16S rRNA gene sequences (*18*).

Metabolite-profiling. Fluids (1 L) from the North Slope oil field were collected from a central facility, production wells, a pipeline carrying fluids and gas to the central facility, a water reinjection well and seawater lines (biocide-treated seawater "TS" as well as fluids and solids scraped from the inner surface of the pipeline carrying treated seawater "SW") and immediately preserved in the field with 50% HCl (pH < 2) for metabolite analysis (see Table S1 for a complete list of samples). Samples TS and SW were used to provide background values as they do not contain hydrocarbons. Acidified samples were kept at room temperature until they were extracted with ethyl acetate, dried over anhydrous Na_2SO_4, and concentrated by rotary evaporation and under a stream of N_2 to a volume of 100 μl. Concentrated extracts were derivatized with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Pierce Chemical Co., Rockford, IL) to add trimethylsilyl groups prior to analyses of

the resulting compounds on an Agilent 6890 model gas chromatograph (GC) coupled with a Agilent model 5973 mass spectrometer (MS). Derivatized components were separated on a HP-5ms capillary column (30 m x 0.25 mm i.d. x 0.25 μm film, J&W Scientific, Folsom, CA, USA) with a starting oven temperature of 45°C (held 5 min) increasing at 4°C/min to 270°C (held 10 min) before mass spectral analysis. To confirm the presence of low molecular weight alkylsuccinates (C_1-C_4), samples were re-derivatized with BSTFA and analyzed with a different chromatographic column (a DB-5-ms capillary column; 30 m x 0.25 mm i.d. x 1.0 μm film, J&W Scientific, Folsom, CA, USA) using the same temperature program. All metabolite identifications were made by comparison with the GC-MS features of authentic standards or with previously reported MS profiles (*24-26)*. Methylsuccinic acid was available commercially (Sigma Aldrich, St. Louis, MO, USA), whereas ethylsuccinic, 2-propylsuccinic, and 2- butylsuccinic acids were custom synthesized (Key Synthesis LLC, Philadelphia, PA, USA). Branched and linear fatty acids (butanoic, pentanoic, 4-methylpentanoic, 4-methylhexanoic, and hexanoic acids) were also purchased (Sigma Aldrich, St. Louis, MO, USA). Metabolite concentrations were determined by comparison with external calibration curves. The instrument detection limit for the majority of metabolites was just under 20 μM and samples (typically 1 L) were concentrated 10,000 fold prior to analysis.

Results and Discussion

Bacterial Community Profiling

Despite oil production from several major reservoirs with different geological

histories, the facility-wide bacterial community profiles at the ANS field showed striking similarities for three of the high temperature sites. Bacterial communities from production well 2L, the 1st stage separator (PS) and the coalescer (CO) exhibited a high degree of class-level similarity (Fig. 1; Tables S1, S2A), and greater levels of genetic diversity and species richness (Table S3) than did the archaeal or the bacterial libraries from production well 2P and the seawater pig envelope sample. Ten "core" taxa (defined as OTUs with 97% nucleotide sequence similarity) were found at all three sites and represented over 87% of the bacterial 16S rRNA gene sequences (Fig. 2; S4). Figure 2 also illustrates the taxa and number of sequences shared between any two of the sites as well as those unique to each site. The most abundant of the core sequences (2L: 83%, PS: 57%, and CO: 31%) has 97-99% identity to that of *Thermovirga lienii (27)*. *T. lienii* is a thermophilic anaerobe isolated from a North Sea oil well and described as a member of the Firmicute family Syntrophomonadaceae. However, it has also been designated a member of the candidate division Synergistes *(28)* and sequences similar to those of *T. lienii* will be designated as "Synergistes" here. The type strain of *T. lienii* has an optimum growth temperature of 58°C and ferments certain amino acids, proteinaceous substrates and organic acids, producing ethanol, acetate, propionate, isovalerate/2-methylbutyrate, H_2, and CO_2 *(27)*. It can also reduce cystine and elemental sulfur to H_2S. Synergistes-associated sequences were also abundant in an extensively biodegraded mesophilic ANS oil reservoir *(29)* but the majority were more similar to uncultured *Thermovirga* clones than to the type strain, suggesting the presence of more than one *Thermovirga* species in ANS

reservoirs.

The most abundant delta proteobacterial 16S rRNA gene sequences (2L: 4%, PS: 7%, CO: 18%) were similar to that of *Desulfomicrobium thermophilum (30)*, a sulfate-reducing bacterium isolated from a hot spring. Sulfate-reducing bacteria (SRB) have been routinely monitored by the oil industry because of their ability to produce H_2S from sulfate. However, many other core sequences were similar to those of organisms that produce sulfide through the reduction of elemental sulfur, thiosulfate, sulfate, or other sulfur oxyanions (e.g. *Thermosipho africanus/ T. geolei, Pelobacter carbinolicus, Desulfacinum subterraneum,* and *Thermodesulfobacterium commune*). Clone libraries based on *dsrAB* genes that code for an essential enzyme for sulfate reduction (dissimilatory (bi)sulfite reductase) were heavily dominated by sequences similar to those of the archaeal sulfate-reducer *Archaeoglobus fulgidus* (2P: 60%, PS and CO: >99%). These findings suggest that bacterial sulfate reduction makes only a minor contribution to sulfide production at the facility, although careful studies of microbial sulfate reduction activity will be required to test this hypothesis.

Core sequences similar to *Thermoanaerobacter pseudethanolicus* (and other *Thermoanaerobacter* species)*, Thermacetogenium phaeum*, or *P. carbinolicus* indicate the possible importance of iron-reduction and/or syntrophic metabolic interactions *(31)*. Anaerobic iron-reducing thermophiles in deep subsurface petroleum reservoirs have been previously demonstrated *(32)*. However, thermophilic strains of *Pelobacter* are not yet known, so its detection as a core taxon awaits further exploration.

In comparison to the 2L and CF samples, the production well 2P bacterial community had much lower diversity and species richness (Fig 1; Table S3). The dominant (90%) bacterial 16S rRNA gene sequence is similar (97-99%) to that of moderately thermophilic, organic acid-utilizing, nitrate-reducing *Petrobacter* species *(33)*. The second-ranked (4.7%) sequence type is similar to *T. lienii* and sequences similar to the core taxa *T. pseudethanolicus* and Thermotogales were present in low abundance (Table S2A). The specific factors responsible for the differences between well head 2P and 2L are unknown; well head temperatures were similar (avg. 48°C and 49°C respectively) with little variation for 9 months prior to sampling, however the wells draw from different formations. Collectively, all well and CF samples strongly resemble anaerobic thermophilic oil reservoir and well communities (*29, 31-33*).

Archaeal Community Profiling

Sequences similar to those of hyperthermophilic Archaea, notably sulfate-reducing *Archaeoglobus* species, methane-producing *Methanothermobacter thermautotrophicus,* and H_2S-producing *Thermococcus* were abundant in the PS, CO, and 2P samples. More than 90% of the archaeal 16S rRNA gene sequences fell into the corresponding three families of Euryarchaeota (Fig. 3, Tables S2B and S5). Crenarchaeota were only detected in the PS sample, which also exhibited the highest diversity and species richness of the three archaeal libraries (Table S3). All three Euryarchaeota groups have frequently been detected in hot oil reservoirs and production fluids *(8)*. *Archaeoglobus* and *Thermococcus* enriched from a North Sea oil field grew at high temperatures on the water-washed fraction of crude oil as the

sole source of carbon and nutrients *(34)*. The same study also found *Archaeoglobus*-like cells in hyperthermophilic cultures enriched from ANS reservoirs.

Sequences of methanogens (approximately ¼ of the archaeal 16S rRNA gene sequences) were less abundant than those of fermentative and sulfate-reducing archaea. Most methanogenic sequences were related to those of hydrogen-utilizing *Methanothermobacter* species. Hot oil reservoirs typically contain hydrogen-utilizing methanogens *(35)*. Approximately 12% of methanogenic sequences were 99.8% similar to that of "*Methermicoccus shengliensis*" (DQ787474, Methanosaetaceae). "*M. shengliensis*" strain ZC-1 *(36)* was isolated from oil-production water and has optimal growth at 65°C. Strain ZC-1 is not an acetoclastic methanogen, unlike other members of the Methanosaetaceae. In contrast to our results, which found CO_2-reducing methanogens to prevail, acetoclastic methanogens were by far the most abundant archaea in a heavily biodegraded mesophilic North Slope oil reservoir *(29)*.

Seawater Pig Envelope Community Profiling

The seawater pig envelope sample (SW) community profile was quite different from that of the archaea-rich production wells and the CF, primarily consisting of sequences similar to those of mesophilic and psychrophilic marine bacteria. We were unsuccessful in obtaining a small subunit ribosomal archaeal RNA gene library with archaeal primers although a bacterial 16S rRNA gene sequence library was successfully obtained from the same DNA sample. The most abundant DNA sequence from the seawater 16S rRNA gene library was most similar to the Gammaproteobacteria, *Pseudomonas stutzeri* and/or related species (Fig. 1, Table

S2A). In accord with the 16S rRNA library results, no *dsrAB* sequences similar to those of *Archaeoglobus* were obtained from the SW sample (Table S1). 16S rRNA gene sequences similar to those of the deep-sea genera *Sulfurimonas* and *Arcobacter* (epsilon proteobacteria, Table S2A) were abundant in the SW sample but were not found in the production wells or CF samples. Only two sequences from the other four bacterial libraries, one from the PS and one from well 2L (both *Pseudomonas*) were as much as 97% similar to any of the sequences from the SW sample. Thus, mesophilic and psychrophilic microorganisms originating from seawater seem unlikely to be responsible for biocorrosion problems at high temperature oilfield production facilities. However, seawater can contribute sulfate and other nutrients that might stimulate increased corrosive microbial activity. For example, it was noted that H_2S was not detected in one ANS oilfield until after seawater flooding was initiated *(37)*.

Targeted Cultivation from the 1st Stage Separator and Seawater Pig Envelope

In agreement with the molecular analysis, *M. thermautotrophicus* was isolated as the numerically dominant (2.3 cells/mL) hydrogen-using prokaryote from the 1st stage separator. A thermophilic *Anaerobaculum* sp. was the numerically dominant heterotroph cultured from the same sample *(38)*. Members of the genus *Anaerobaculum* ferment organic acids and peptides but also reduce thiosulfate, sulfur, and cysteine to H_2S *(39)* and therefore have the potential to be involved in corrosion processes. However, all populations of culturable bacteria screened (SRB, anaerobic/facultative heterotrophs, hydrogen-users) were found in low numbers in the separator (2-4 cells/mL), implying that these organisms would be missed in most

routine MPN screening procedures.

Populations of culturable bacteria from the seawater pig envelope sample were 10^3 to 10^6 /mL, with the numerically dominant organism *Pseudomonas stutzeri* and/or related species, as was found for the seawater 16S rRNA gene library. The numerically dominant culturable hydrogen-user was *Acetobacterium*. An *Acetobacterium* species was previously isolated from marine environments *(40)*. Sulfate-reducing bacteria were estimated at 2.4 x 10^6/mL.

Cell numbers were estimated from visual quantification of the DNA extracts electrophoresed on an agarose gel and compared with molecular weight standards (assuming 9 fg DNA/cell; 41). Cell numbers based on DNA quantification were estimated to be 9×10^4 cells/mL in the 1st stage separator and 2×10^6 cells/mL in the seawater pig envelope sample. Higher cell numbers were estimated by DNA quantification than MPN determinations for the 1st stage separator, but comparable cell numbers were estimated by the two methods for the seawater sample. These results reemphasize that current screening protocols are especially likely to miss dominant thermophiles.

Metabolite Profiling

It is well established that biocorrosive organisms form complex surface assemblages where cells are imbedded in a matrix of biologically-produced extracellular polymeric substance that forms a protective microenvironment *(3)*. However, the carbon source(s) supporting the formation of such surface-associated communities remain enigmatic. Clearly, the largest potentially available source of

carbon to support microbial activity is the oil itself. Since hydrocarbons are known to be suitable substrates for anaerobes *(42)*, we suspected that the more water-soluble oil components, like benzene, toluene, ethylbenzene, and xylene isomers (BTEX) might be preferentially metabolized to support the diverse microbial communities detected at the facility. This prospect was explored by assaying for the signature metabolites associated with anaerobic oil biodegradation *(25-26)*. The identification of these intermediates implicates the parent hydrocarbons being metabolized.

Contrary to expectations, there was no evidence for the biodegradation of the most water-soluble BTEX components, as characteristic anaerobic metabolites such as alkylbenzylsuccinates were below detection limits. However, putative low molecular weight alkylsuccinate metabolites associated with anaerobic *n*-alkane biodegradation were found facility-wide. Six of the eight central facility and production well samples collected contained 0.8-2.2 μM concentrations of low molecular weight (C_1-C_4) alkylsuccinates (Table 1). The identity of these metabolites was confirmed by comparison of the GC-MS profiles to authentic standards analyzed using two different GC columns. The mass spectral profiles of the trimethylsilylated ethyl-, 2-propyl, and 2-butylsuccinic acids (both authentic standards and those detected in the oil field samples) showed fragment ions (m/z 262, 217, 172, 147, 73) characteristic of higher chain length alkylsuccinates (Figures S1-S4; *20*). No signature hydrocarbon metabolites were detected in the hydrocarbon-free seawater samples. The identification of methyl-, ethyl-, 2-propyl- and 2-butylsuccinate suggested that the hydrocarbons routinely reinjected during

normal oil recovery operations were being biologically oxidized by a fumarate addition reaction in a manner analogous to higher molecular weight *n*-alkanes (Table 1) *(25)*. The recycling of these gases to help maintain formation pressures occurred throughout the decades-long production history of the formation and we suspect that the requisite organisms were enriched over this long time period.

If the analogy to higher molecular weight *n*-alkane anaerobic metabolism is accurate, we predict the formation of a series of downstream branched and straight-chain fatty acid metabolites formed as a result of the presumed carbon skeleton rearrangement and subsequent decarboxylation of the alkylsuccinate intermediates *(43)*. Indeed, the expected downstream metabolites were also found in the same samples from the central facility, but were not found in the seawater samples (Table 1).

The detection of methylsuccinate in conjunction with the other low molecular weight alkylsuccinates is particularly evocative. This finding suggests that fumarate addition may represent an alternative to previously described mechanisms such as reverse methanogenesis for the anaerobic oxidation of methane *(44)*. Consistent with an alternate hypothesis, the methanogen and Deltaproteobacterial sequences were dissimilar to the genera described in other environments undergoing anaerobic methane oxidation *(45)*.

Implications for Biocorrosion

Our results suggest that temperature and hydrocarbon utilization are contributing factors governing microflora species composition at the ANS facility.

Contrary to the emphasis placed on mesophilic bacterial SRB by standard biocorrosion monitoring procedures, many of the organisms detected using molecular techniques and targeted isolation are thermophilic bacteria capable of reducing various sulfur oxyanions or hyperthermophilic sulfate-reducing archaea that produce H_2S. Methanogenic, fermentative, H_2-producing and H_2-utilizing physiologies were also common, unlikely to be detected using industry standard assay procedures, and could likewise stimulate corrosion. The similarity of core taxa in these samples and those from other thermophilic oil reservoirs and wells suggests that hydrocarbon-degrading, potentially corrosive microbes found in oil reservoirs will readily inoculate and proliferate in oil production facilities maintained at compatible temperatures. Such similarities also imply that pipeline integrity management programs might be able to differentially target a relatively few core taxa.

The detection of putative low molecular weight alkane metabolites throughout the hot oilfield facilities suggests that anaerobic hydrocarbon biodegradation is inherent and likely involved in supporting biocorrosive biofilms. Indeed the formation of relatively high concentrations of alkylsuccinates (Table 1; μM vs nM concentrations more typically found in fuel-contaminated aquifers, *25*) allows us to postulate that such acidic intermediates can directly contribute to biocorrosion processes. While such relatively high concentrations of alkylsuccinates may suggest that they are "dead-end" metabolites, the detection of the predicted downstream branched and linear fatty acids argues against this prospect. The subsequent metabolism of the fatty acids would eventually form acetate and CO_2,

microbial products known to exacerbate corrosion of pipeline surfaces *(6, 46)*. Thus, these findings support the hypothesis that anaerobic hydrocarbon biodegradation processes in the oilfield environment can be an important factor in microbial influenced corrosion.

Supporting Information Available. Tables S1–S5; Figures S1–S4; References not cited in main text. Supporting information can be found in Appendix B, Chapter 2.

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Parent compound	Fumarate addition metabolite§	Mean metabolite concentration (μM ± s.d.)	Downstream metabolite (rearrangement)
Methane (CH_4)	Methylsuccinate	2.08 ± 1.10	Butanoic acid
Ethane (C_2H_6)	Ethylsuccinate	1.77 ± 1.54	Pentanoic acid
Propane (C_3H_8)	Subterminal addition: Terminal addition: Propylsuccinate*	2.18 ± 0.20	4-Methylpentanoic acid; Hexanoic acid
Butane (C_4H_{10})	Butylsuccinate*	0.76 ± 0.11	4-Methylhexanoic acid; Heptanoic acid

Table 1. Metabolites associated with the anaerobic biodegradation of C_1-C_4 hydrocarbons in Alaskan North Slope (ANS) oil field samples. Alkylsuccinates were detected in processing facility and production well samples (n = 6), but not in seawater or in a pipeline transporting seawater, suggesting the anaerobic oxidation of the parent compounds methane, ethane, propane or butane. The mean concentrations of metabolites were in the μM range. Downstream metabolites resulting from the predicted carbon skeleton rearrangement and subsequent decarboxylation of the alkylsuccinate were also found in ANS samples. For *n*-alkanes C_3 or greater, a terminal and subterminal addition of fumarate (denoted with *) are possible, resulting in two possible downstream metabolites (branched or straight chain). §Mass spectral profiles and retention times for the authentic standards for C_1-C_4 alkylsuccinates including their identification in sample 2K-28 are provided as Supplementary Information (Figures S1-S4).

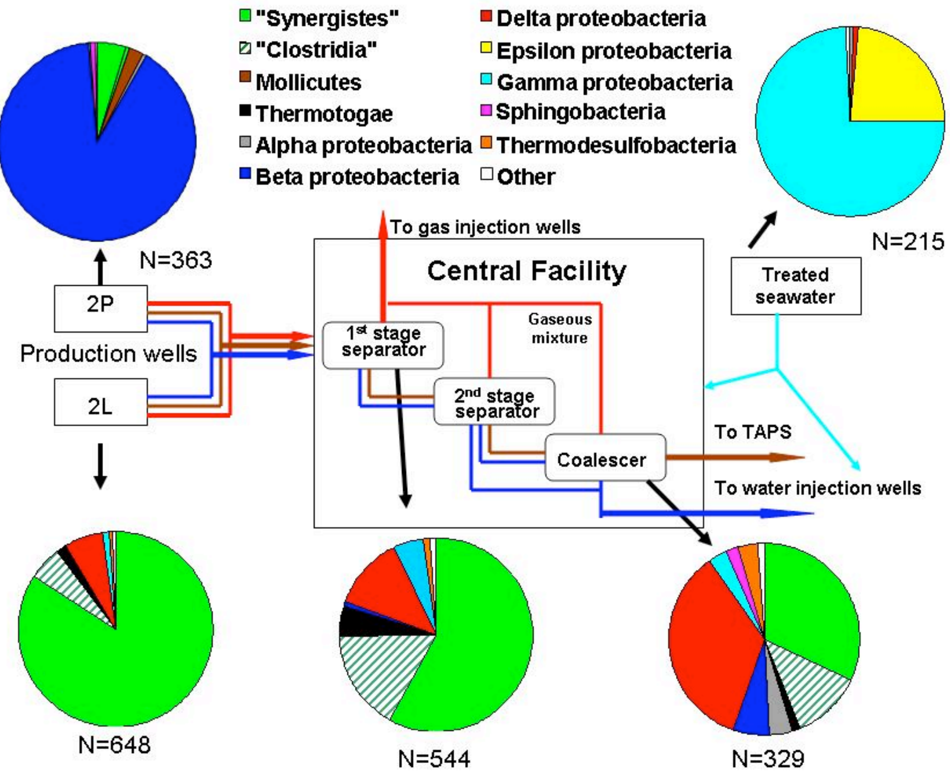

Figure 1. Relative abundances of sequences from five bacterial 16S rRNA gene libraries. The RDP Classifier tool (*21*) was used to assign representative sequences (97% similarity) to the higher-level taxonomic groups shown, except for sequences affiliated with *Thermovirga lienii* (referred to as "Synergistes" in this figure), which currently are classified under "Clostridia", Incertae sedis XV by RDP Classifier. N represents the total number of sequences in a library, after exclusion of chimeric sequences. Origin of samples: production wells 2P and 2L (sampled at wellhead), outflow from the 1st stage separator (PS) and coalescer (CO) units in a central facility, and the "pig envelope" (e.g. the scraped inner surface of the pipeline) of a pipeline transporting treated seawater (SW) from the Arctic Ocean to the central facility. "TAPS" indicates the Trans-Alaska Pipeline System. Table S2A contains the accession number of the closest match, affiliation, and the relative abundance (as a percentage of the total library) of each representative sequence.

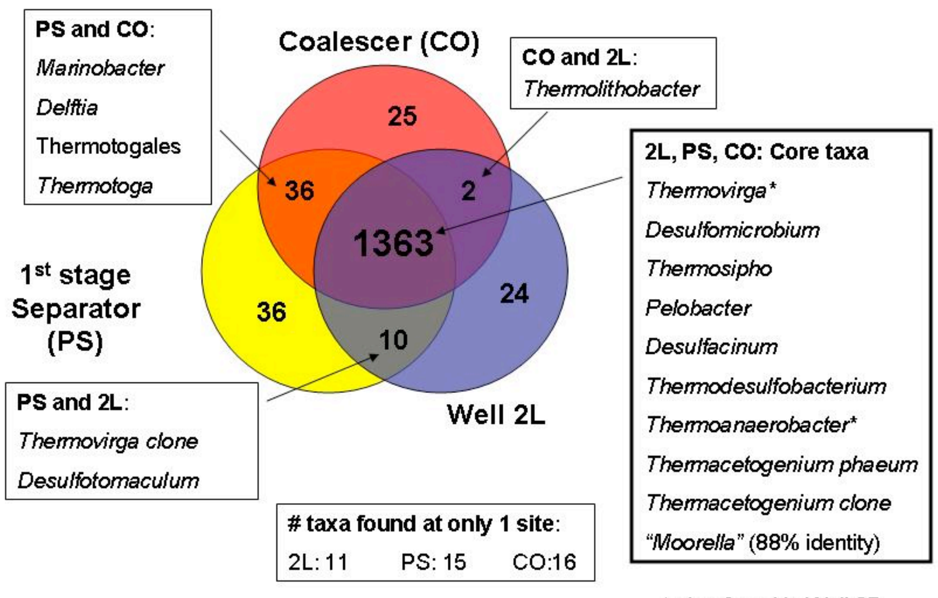

Figure 2. Distribution of bacterial sequences from 2L, PS, and CO illustrating the number of sequences from taxa found in all three libraries ("2L, PS, CO: Core taxa"), 2 libraries ("PS and 2L", "CO and 2L", "PS and CO") or unique to one sample

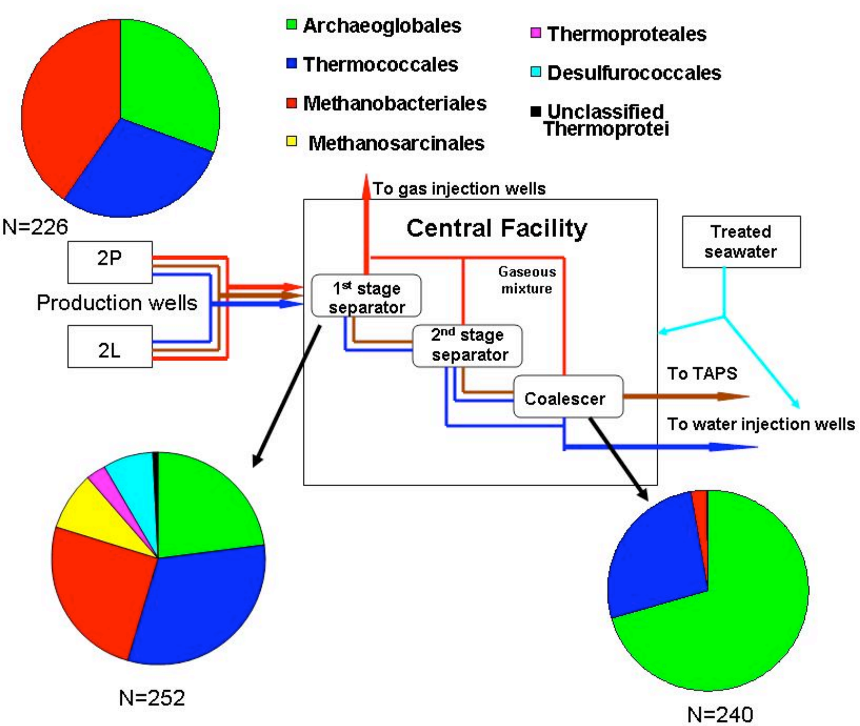

Figure 3. Relative abundances of sequences from three archaeal 16S rRNA gene libraries. The RDP Classifier tool (*21*) was used to assign representative sequences (97% similarity) to the higher-level taxonomic groups shown. N represents the total number of sequences in a library, after exclusion of chimeric sequences. Origin of samples: production well 2P (sampled at wellhead), outflow from the 1st stage separator (PS) and coalescer (CO) units in a central facility. Table S2B contains the accession number of the closest match, affiliation, and the relative abundance (as a percentage of the total library) of each representative sequence.

CHAPTER 3

Detection of polar compounds as putative metabolic intermediates as *in situ* evidence for petroleum biodegradation in Alaskan North Slope oil reservoirs

Abstract

Anaerobic hydrocarbon biodegradation has been well described in laboratory studies, but little is known about the extent of such processes in reservoirs, pipelines and processing facilities. To assess *in situ* activity, metabolite profiling was used to assay for a suite of polar compounds that are differentially associated with hydrocarbon biodegradation in two Alaska North Slope oil fields designated as Field A and B. Samples collected from production wells and processing facilities revealed a suite of metabolites suggesting the anaerobic microbial biodegradation of alkylbenzene, polycyclic aromatic, alkane and alicyclic compounds. Fumarate addition intermediates were rarely detected, with the exception of n-C_1-C_5 metabolites. This observation suggested that the small molecular weight *n*-alkanes, routinely recycled in both fields for oil production efforts, were subject to anaerobic biodegradation processes. The majority of the polar compounds detected throughout Field B were associated with the hydroxylation and/or carboxylation of an array of aromatic compounds. The detection of benzoic acid, phenol and various isomers of toluic acid, cresol, and hydroxybenozate suggested the anaerobic microbial

biodegradation monoaromatic hydrocarbons including benzene, toluene and xylene isomers. Relatively fewer monoaromatic compounds and less metabolites overall were detected in Field A compared to Field B. Based on the patterns of polar compounds detected, most of the samples from either field co-varied with each other, but not with the other field. This observation suggests a "smearing" effect as fluids are moved and re-injected throughout individual oil fields. The nature of the polar compounds further implicates anaerobic hydrocarbon biodegradation as a process occurring in the reservoirs, pipelines and processing facilities.

Introduction

Increases in global energy demand as well as the corrosion of oil pipelines and facilities have fueled interest in both the microbial communities and their functioning in petroleum reservoirs. As early as 1926, anaerobic bacteria were isolated from production well fluids *[1]* and provided the first support for a microbial presence in subsurface oil reservoirs. Since then, significant deterioration of oil within reservoirs has been linked to microbial biodegradation of saturated and aromatic hydrocarbons. While physical processes (e.g. water-washing, and evaporative fractionation) contribute to compositional alterations of oil, it is widely accepted that the biological oxidation of hydrocarbons within the reservoir results in increases in oil viscosity, sulfur content and total acid number *[2-4]*. The recovery of such bio-deteriorated oil increases production costs, and is deemed economically unfavorable *[5]*. Additionally, hydrocarbon consumption by microorganisms produces polar acidic intermediates and end products such as naphthenic and low molecular weight organic

acids and carbon dioxide. The appearance of polar compounds in oil processing facilities and transport pipelines increases their vulnerability to corrosion *[6]* and requires additional prevention and maintenance costs.

The aerobic microbial consumption of hydrocarbons was the long-standing explanation for alteration of oil constituent and production of polar compounds in reservoirs. It was thought that an influx of meteoric waters brought with it a replenishing supply of oxygen to the deep subsurface. However, these waters do not carry enough oxidative potential to be the primary reactant for hydrocarbon mineralization by aerobic microbial consortia *[3, 4, 7]*. In addition, the presence saline water within reservoirs argues against subsurface mixing with fresh oxygenated meteoric waters. Despite the apparent lack of oxidative potential, biodegradation of petroleum components can occur in deep reservoirs and many studies have provided conclusive evidence for the anaerobic decay of hydrocarbons under methanogenic, sulfate-, iron- and nitrate-reducing conditions (as reviewed in refs; *[8-10]*). Petroleum reservoirs typically have abundant free sulfate, show evidence for iron availability *[3, 4]* or produce methane with biological signatures ($\delta^{13}C$ -45‰ to -55‰)[3]. Therefore, it seems reasonable to presume that the alteration of oil and the production of polar compounds may be driven by oxygen-independent microbial metabolism. Evidence to support the susceptibility of crude oil components to microbial alteration with concurrent sulfate reduction or methane production has been shown *[11-14]*.

Anaerobic biodegradation of oil is a slow process and estimates of significant reservoir alteration *in situ* are on the order of 10^{-4} kg hydrocarbons m^{-2} yr^{-1} for

reservoirs at 40-70°C *[3, 15]* and larger scale deterioration occurs over millions of years. However it may be possible to assess current mechanisms of *in situ* degradation in petroleum reservoirs via the identification of polar metabolites that are differentially associated with the anaerobic biodegradation of aliphatic and aromatic hydrocarbons *[10, 16-19]*. Subsurface microbial consortia employ multiple mechanisms, including addition to fumarate, hydroxylation and carboxylation to activate petroleum components for biodegradation *[8, 10, 20-23]*.

In this study we investigated two oil fields (A and B) on the North Slope of Alaska that service several reservoirs and pools containing oils of varying degrees of deterioration *[4, 24]*. The geology and petroleum geochemistry of the fields differ within each field and between the two fields with respect to API gravity of the oils, sulfur content and temperatures *[4, 24, 25]*. Current reservoir temperatures for the two oil fields were below 80°C, well within the range to support microbial activity *[3, 7]* and we collected fluid samples at well heads and processing facilities ranging from 16°C upward to 60°C. The two oil fields receive chemical treatments (unknown) to prevent and treat corrosion processes. Water recovered during oil separation processes and miscible gas injections (C_1- C_5 *n*-alkanes) are routinely used to pressurize the oil formations and assist in oil recovery operations. These fluids are occasionally augmented with seawater or freshwater from nearby facilities. It has been previously shown that Field B has a microflora comprised of sulfur/thiosulfate-reducing bacteria, sulfate-reducing archaea, synthrophic bacteria, peptide- and amino acid-fermenting bacteria and hydrogen-utilizing thermophilic methanogens *[26]* that are likely involved in anaerobic hydrocarbon biodegradation

and corrosion processes.

We profiled the two oil fields for the presence of polar compounds that are differentially associated with anaerobic hydrocarbon biodegradation. The metabolic profiling represents the first extensive survey of the functioning of hydrocarbonoclastic microbial communities on the North Slope of Alaska.

Experimental Procedures

Site description. Oil fields A and B are located on Alaska's North Slope, approximately 400 kilometers north of the Arctic Circle and within the 160 km that separates the National Petroleum Reserve from the Arctic National Wildlife Refuge. Field B facilities serviced multiple reservoirs and/or pools [27] while Field A was associated with a single reservoir *[4, 24, 25, 27-32]*. Oil in Field B had an average reservoir temperature at discovery of 68°C [4] an average API gravity of 24°, and sulfur contents ranging from 1.4-2% [4]. The comparable measures from Field A oil were 71°C at discovery, a 40° average API gravity and relatively low sulfur content *[25, 27]*. Table 1 details several of the salient geochemical parameters including oil viscosity (as an indicator of the extent of biodegradation) for the samples investigated.

Metabolite profiling. Fluids (1 L) were collected from production and injection wells, and processing facilities. In total, 34 samples were collected from Field B and 16 from Field A (Table 1). Upon collection, the fluid samples were acidified (pH < 2) with 50% HCl to protonate acid intermediates and halt microbial activity. Acidified samples were kept at room temperature in the dark until extraction with

ethyl acetate. When thick oil/water emulsions were encountered, field samples were either i) treated with a commercial emulsion-breaking reagent (Emulstron®, Champion Technologies, Inc., Houston, TX, USA) or ii) water extracted, and in some instances, both methods were employed. For water extractions, hot water (65°C, 1 L) was added to oily samples and shaken overnight at room temperature. The water and emulsion breaker were subject to organic extraction respectively, to rule out the introduction contaminants from either method to the sample. Extracts were dried over anhydrous Na_2SO_4, and concentrated by rotary evaporation and under a stream of N_2 to a volume of 100 µl (10,000 fold concentration). All concentrated extracts were derivatized with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Sigma Aldrich, St. Louis, MO) prior to analysis. Derivatized extracts were analyzed with an Agilent 6890 model gas chromatograph (GC) coupled with a model 5973 mass spectrometer (MS) and separated with a DB-5ms capillary column (30 m x 0.25 mm i.d. x 1.0 µm film, J&W Scientific, Folsom, CA, USA). The starting oven temperature was 45°C (held 5 min), increased 4°C/min to 270°C, and held for 10 min before mass spectral analysis *[26]*.

Data analysis. All putative metabolite identifications were made by comparison to authentic derivatized standards (purchased commercially or synthesized) or with previously reported MS profiles of nearly 100 compounds implicated in anaerobic hydrocarbon metabolism (see Table 2). The chromatographic peaks of positively identified metabolites were analyzed with respect to their integrated area using the MS ChemStation (G1701DA D.01.00) Software (Agilent Technologies, Santa Clara CA). Standard curves were prepared with select representative compounds from the

metabolites listed in Table 1 (Supplementary Figures S1 and S2). For most compounds, the limit of detection for the GC-MS analysis was approximately 20 μM *[26]*.

Statistical analyses. The relationship between the putative metabolites detected at the wellheads in Fields A and B were analyzed using CLUSTER 3.0 (http://bonsai.ims.utokyo.ac.jp/~mdehoon/software/cluster/software.htm) *[33]* and Java Tree View (http://jtreeview.sourceforge.net) *[34]* open source software packages for dendrogram and heat map creation. The hierarchical clustering analysis for the two oil fields used Euclidean distances and average linkages to describe the relationship among all samples. The relationship between the spatial locations of points sampled in Field B and the profile of putative metabolites (Figure 3) were analyzed using the open source statistical program R (http://www.r-project.org).

Results

Each oil field sample was assayed for nearly 100 polar compounds, many of which were differentially associated with the anaerobic biodegradation of petroleum constituents (Table 3). Collectively we were able to identify polar compounds (Figure 1) within the seven classes of metabolites, suggesting the *in situ* alteration of alkylbenene, polycyclic aromatic (PAH), and alkane components.

Alkylbenzylsuccinate intermediates

Evidence for the *in situ* biodegradation of alkylaromatic hydrocarbon metabolism was evident in only three of the 50 samples interrogated (Figure 2). In

two samples (B26 and B27) from the northern section of Field B, benzylsuccinate, the fumarate addition product formed during anaerobic toluene decay, was identified (Figure 3). Similarly, ethylbenzylsuccinate, the anaerobic metabolite associated with ethylbenzene biodegradation was also found in B27. One sample in Field A was positive for benzylsuccinate, *o*-methyl- and *p*-methylbenzylsuccinate. The latter two compounds are known intermediates formed during anaerobic xylene metabolism. The sample (A3) was collected from a drain on a pipeline dead-leg that had been subjected to corrosion. The detection of alkylbenzylsuccinates constituted less than one percent of all polar compounds detected in the oil fields (Figure 1).

Benzoate associated intermediates

Benzoate is an intermediate that can be formed during the anaerobic biodegradation of a wide variety of aromatic compounds. The subsequent metabolism of benzoate results in the formation of numerous polar metabolites [35, 36] (refs; Table 2). The detection of such polar compounds in oil field samples is at least consistent with their formation as putative benzoate metabolites. We detected benzoate and the putative downstream metabolites cyclohexane-1-carboxylate, cyclohex-1-ene carboxylate, glutarate and pimelate. These compounds were found throughout Field B (Figure 3, benzoate associated), suggesting widespread metabolism of aromatic substrates. Field A was also positive for benzoate, cyclohexane-1-carboxylate, cyclohex-1-ene carboxylate, glutarate and pimelate (Figure 2). The putative intermediate 3,5-dimethylbenzoate, was detected throughout Field B samples (Figure 2), indicating 1,3,5-trimethylbenzene biodegradation *[37]*.

Benzoate associated metabolites comprised 10 % and 2% of the integrated peak area of all polar compounds detected in Field B and Field A respectively (Figure 1). The widespread occurrence of benzoate, its downstream metabolites, and 3,5-dimethylbenzoate (Figures 2, 3) in samples spanning the *in situ* temperature range suggest that the anaerobic biodegradation of aromatic substrates is common in these reservoirs.

Other aromatic polar compounds

Monoaromatic compounds are potential hydrocarbon degradation intermediates (Table 3) and were abundant throughout Field B, and were present in Field A to a lesser extent (Figures 1-3). These compounds represented just over half of the putative metabolic intermediates found in the two oil fields (Figure 1). When the integrated area from the GC-MS is compared, monoaromatic polar compounds are the prominent signal in Field B samples, and just under one-third of the total signal in Field A (Figure 2). Phenol as well as the isomers of both toluic acid and cresol was in greatest abundance relative to other polar monoaromatic compounds (Figure 1). Monoaromatic compounds were found throughout the temperature range of samples from Field B and Field A (Table 1).

The identification of several putative naphthalene or alkyl naphthalene intermediates in eight Field B wells provides evidence consistent with the anaerobic microbial biodegradation of polycyclic aromatic hydrocarbons. That is, 1- or 2-naphthoic and methylnaphthoic acids were found in four samples in the northern section of Field B, three samples in the western area, and in one well in the

easternmost section (Figure 2, 3). The integrated area associated with these putative intermediates was less than 1% of the total (Figure 2).

Alkylsuccinic, alkanoic, and dicarboxylic acid intermediates

Alkylsuccinic acids were detected throughout Field B (Figure 1, 3) and in five Field A samples (Figure 1) and represented just over 10% and 7% percent of total polar organic compounds detected, respectively (Figure 1). The integrated area of the total alkysuccinates in the two oil fields was low relative to the other compounds in this class of constituents. The most abundant alkylsuccinates were in the range of *n*-C_1-C_4 (Figure 2), a finding consistent with the anaerobic oxidation of methane, ethane, propane and butane. More rarely, we detected putative intermediates associated with the anaerobic oxidation of pentane and hexane.

Alkanoic acids are the second most abundant group of potential hydrocarbon-associated compounds detected in Field B and Field A (Figure 1, 2). These polar compounds constituted nearly one-quarter of the total detected in the two fields. The integrated area associated with the alkanoic acids was 38% of the total in Field B and up to 63% in Field A. Interestingly, the response factor for acetate is slightly less than order of magnitude lower than benzylsuccinate (Figure S2), yet the integrated area for alkanoic acids dominate the total area for metabolites detected in Field A. Alkanoic acids can be formed as intermediates in either aerobic or anaerobic hydrocarbon biodegradation pathways. The most abundant alkanoic acid detected was butyrate (Figure 1) followed by pentanoate. Acetate, a known contributor to pipeline corrosion, was detected in many of the wells throughout the two oil fields as

were several short-chained branched fatty acids. Larger molecular weight fatty acids (C_{14}-C_{22} in chain length) were more rarely detected in both Field A and B (Figure 1). Dicarboxylic acids were not readily detected in either field.

Discussion

Fifty samples from two oil fields on the North Slope of Alaska servicing multiple reservoirs with different ambient temperatures and oil viscosity characteristics were selected for metabolite profiling (Table 1) to gain insight on the functioning of the resident microflora. We hypothesized that there were extant microbial communities in the reservoirs, and that they were able to biodegrade petroleum hydrocarbons and produce a characteristic suite of metabolites. The identity of the intermediates would provide insight into the predominant *in situ* community functioning since there are distinct fundamental differences in the metabolites formed during hydrocarbon biodegradation under aerobic or anaerobic conditions. However, in either case, the resulting metabolites are oxidized and more polar than the parent substrates. Therefore, we assayed a wide variety of reservoir facilities for polar organic compounds using GC-MS and found that these chemicals could be grouped into seven categories, the alkylbenzylsuccinic acids, benzoate and associated metabolites, other monoaromatic, naphthalene or phenanthrene-associated components, alkylsuccinic acids, alkanoic acids and dicarboxylic acids (Table 3). These classes of compounds are often differentially associated with the microbial metabolism of specific parent hydrocarbons like benzene, alkylbenzenes, polycyclic aromatic hydrocarbons, and normal or cyclic alkanes.

The detection and nature of the polar organic compounds found in both North Slope oil fields is consistent with the interpretation that anaerobic hydrocarbon biodegradation is a widespread and ongoing process associated with the resident microbial communities. Previously, metabolite-profiling has been used to help characterize the intrinsic remediation of hydrocarbons in contaminated anoxic aquifers *[17, 19, 37-43]*. The detection of diagnostic intermediates in contaminated portions of the subsurface, compared to uncontaminated background areas provides unequivocal evidence for *in situ* anaerobic hydrocarbon mineralization. Samples from the North Slope of Alaska were assayed in the same manner, but we compared our findings with seawater (sample A7) as a background control since it has occasionally been used during oil recovery operations *[26]*. The current effort is a far more extensive survey of polar organic compounds in North Slope oil fields. A previous and more limited assay was used to help infer a potential role for anaerobic hydrocarbon biodegradation in pipeline biocorrosion processes *[26]*.

Distinct differences can be detected in the polar compounds identified in the various fields (Figures 1-, 2), but the intra-field differences between samples are generally more subtle. That is, when the oil field samples are compared statistically based on the presence or absence of a particular metabolite (Figure 1), samples from the same field tend to cluster together. The presence of a consistent array of putative metabolites throughout Field B suggests there may be a “smearing” of polar compounds due to the decades-long practice of processing and re-injecting production fluids into the reservoirs of this field during routine oil extraction operations (Figure 3). In contrast, the different polar organic compounds signals in

Field A likely reflect the lower diversity of oil reservoirs serviced by that facility.

While there is no doubt that many of the polar organic compounds detected in Field A and B are characteristic metabolites associated with anaerobic hydrocarbon decay, Field B is dominated by carboxylated and hydroxylated aromatic constituents (Figures 1-3). That is, *in situ* activity in Field B is associated with the anaerobic biodegradation of benzene, toluene, ethylbenzene and xylene isomers (the BTEX hydrocarbons) *[8, 17, 44, 45]*. Typically, moderately biodegraded oils have major alterations in BTEX constituents *[3]*, but some areas of Field B get a secondary gas condensate amendment that includes these hydrocarbons *[4]*. Thus, it is not surprising that BTEX metabolites were detected in our assay.

In consistent fashion, toluic acids were found and are known intermediates of anaerobic xylene biodegradation *[39]*. Similarly, phenol is commonly found in oil fields *[7, 46]* and was readily detected in both oil fields. Phenol has been previously implicated in anaerobic benzene biodegradation *[47-49]*. Thus, the carboxylated and hydroxylated polar compounds are also consistent with the anaerobic biodegradation of the more water-soluble BTEX hydrocarbons. Our assay procedures are capable of detecting catechol and related metabolites associated with aerobic hydrocarbon biodegradation. However, we were unable to find signature aerobic metabolites including catechol or methylcatechol derivatives. Not only is the presence of the detected intermediates consistent with the anaerobic biodegradation of petroleum components but phenols, dimethylphenols and cresols are known to be readily metabolized by microbial consortia in the absence of oxygen [50-54]

Similarly, naphthoic acids were detected in Field B, again suggesting the

microbial biodegradation of naphthalene and related alkylated naphthalenes. Such compounds have been previously detected in oil samples from around the world *[18]* as well as the putative downstream ring reduction intermediates. The latter compounds were not detected in our assay of polar organic compounds found in aqueous oil field fluids.

We have previously demonstrated the presence of low molecular weight alkylsuccinates (C_1-C_5) in Field B [26] and we now report them in Field A. The presence of these compounds suggests the potential for the anaerobic oxidation of methane, ethane, propane, butane and pentane within the reservoirs and/or pipeline facilities, from both fields. Previously reported isotopic signatures for propane in Field B suggests that the microbial oxidation of this C_3 compound is an important fate process [4, 24]. Mounting evidence for the oxidation of low molecular weight alkanes by fumarate addition to produce the corresponding succinic acid derivatives *[55, 56]* also supports our findings. The presence of methylsuccinate suggests that methane may also be anaerobically oxidized in the same manner *[57]*. Methane is abundant in Field B, and in addition to its presence in miscible gas injectate, carbon isotopic signatures in indicate that much of the C_1 hydrocarbon with the reservoirs is biogenic in origin *[4, 24]*. Therefore it is possible that both the production and consumption of methane is occurring within the oil fields on the North Slope.

Alkanoic acids were relatively abundant in both fields and their presence in petroleum reservoirs has been heavily documented. They were thought to be produced through abiotic oxidation by minerals or hydrolytic disproportionation, but carbon isotopic studies argued against these prospects *[58]*. Given what is known

about the microbial oxidation of *n*-alkanes under anaerobic conditions, several downstream reactions yield straight chain and branched fatty acids subsequent to the initial activation of the parent substrate by fumarate-addition *[20, 23]*. Interestingly, alkanoic acids may be suitable substrates for syntrophic bacteria *[59]* previously shown to reside within Field B reservoirs, pipelines and processing facilities *[26]*.

In summary, we detected a suite of distinctive putative polar metabolites associated with ongoing hydrocarbonoclastic activities of microbial communities in two major oil fields on the North Slope of Alaska. Further, the identity of the polar organic compounds suggests that fumarate-addition reactions are certainly not the only mechanism for the anaerobic biodegradation of hydrocarbons in these fields. Rather, this analysis confirms that hydroxylation and carboxylation of aromatic compounds in particular can also represent important fate processes for hydrocarbons in oil reservoirs. The significance of such observations not only lends insight into how microbial communities may function in petroliferous deposits, but includes how such bioconversions contribute to the gradual deterioration of energy resources and their potential to contribute to biocorrosion processes as recently suggested *[26]*.

Supporting materials are available as Figures S1 and S2 with legends in Appendix B, Chapter 3.

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Table 1. Description and characteristics of samples collected from oil Field A. Samples were collected from well heads and processing facilities. Temperatures were directly recorded from the sampling point.

Sample	Type of sample	Temperature (°C)	Oil Gravity (°API)[27]
A1	Processing Facility	35	
A2	Processing Facility	65.56	
A3	2" drain on CD-1	35	39
A4	Processing Facility	23.72	
A5	Processing Facility	23.72	
A6	Processing Facility	23.33	
A7	Processing Facility	16.67	
A8	Gas injection?	36.67	39
A9	Producer	40.56	39
A10	Producer	43.33	39
A11	Producer	41.11	39
A12	Producer	37.22	39
A13	Producer	32.78	39
A14	Producer	24.44	39
A15	Producer	36.11	39
A16	Producer	46.67	39

Table 2. Description and characteristics of samples collected from oil Field B. Samples were collected from well heads and processing facilities. Temperatures were directly recorded from the sampling point.

Sample	Type of sample	Temperature (°C)	Oil Gravity (°API) [27]
B1	Primary Separator	54.44	
B2	Gas -lifted producer	30	24
B3	Water injector	43.89	24
B4	Water injector	50.06	24
B5	Gas lifted producer (Manifold)	39.44	24
B6	Gas lifted producer	48.67	24
B7	Primary separator	54.44	
B8	Producer	54.44	24
B9	Producer	51.11	24
B10	Producer	44.44	24
B11	Producer	52.22	24
B12	Producer	61.67	24
B13	Producer	59.44	24
B14	Producer	53.89	24
B15	Producer	51.11	24
B16	Producer	21.11	37
B17	Producer	31.67	37
B18	Producer	33.33	37
B19	Producer	22.22	37
B20	Producer	40	37
B21	Producer	41.67	37
B22	Producer	40	37
B23	Producer	50.06	16
B24	Producer	40.11	16
B25	Primary separator	51.67	
B26	Producer	51.72	24
B27	Water injector/PW	38.89	24
B28	Water injector/PW	49.44	24
B29	Producer	40	24
B30	Producer	56.06	24
B31	Water injector/PW	57.56	24
B32	Producer	38.33	24
B33	Producer	15.33	24
B34	Producer	50.61	24

Table 3. List of polar organic compounds differentially associated with the anaerobic biodegradation of hydrocarbons. Seven classes of putative metabolites are represented (in bold, italics and gray), based on the hydrocarbon type most likely metabolized. Standards are commercially available or synthesized in the laboratory. Those compounds identified by mass spectral fragmentation patterns are indicated by an (*).

Alkylbenzylsuccinates

Benzylsuccinic acid
Ethylbenzylsuccinic acid
o-Methylbenzylsuccinic acid
m-Methylbenzylsuccinic acid
p-Methylbenzylsuccinic acid
Dimethylbenzylsuccinic acid (from 1,3,5-TMB)
Dimethylbenzylsuccinic acid (from 1,2,4-TMB)
1-Ethyl-3-methylbenzylsuccinic acid
Propylbenzylsuccinic acid

Benzoate and benzoate associated anaerobic metabolites

Benzoate
Cyclohexane-1-carboxylate
Cyclohex-1-ene-1-carboxylate
2-Hydroxycyclohexane-1-carboxylate
Glutarate
Pimelate
Benzyl alcohol
3,5-Dimethylbenzoate

Other Putative Monoaromatic Metabolites

o-Toluic acid
m-Toluic acid
p-Toluic acid
o-Phthalic acid
m-Phthalic acid
p-Phthalic acid
o-Carboxybenzaldehyde
m-Carboxybenzaldehyde
p-Carboxybenzaldehyde
o-Tolylacetic acid
m-Tolylacetic acid
p-Tolylacetic acid
Acetophenone
1-Phenylethanol
4-Phenylvaleric acid
m-Hydroxybenzoate
p-Hydroxybenzoate
3-Hydroxy-4-methylbenzoate
Various hydroxymethylbenzoate isomers*
Phenol
o-Cresol
m-Cresol
p-Cresol
4-Propylphenol
3-Propylphenol
3,5-Dimethylphenol
4-Propylphenol
3-Propylphenol
3,5-Dimethylphenol

Putative naphthalene/phenanthrene metabolites:

Naphthylsuccinic acid
1- or 2-Naphthoic acid
Methylnaphthoic acid*
Dimethylnaphthoic acid*
1,2,3,4-Tetrahydro-2-naphthoic acid
5,6,7,8-Tetrahydro-2-naphthoic acid
Decahydronaphthoic acid*
Phenanthrene carboxylic acid

Alkylsuccinic acids

C_1 (Methylsuccinate)
C_2 (Ethylsuccinate)
C_3 (Propylsuccinate)
C_4
C_5
$C_{5(n-2)}$
C_6
$C_{6(n-2)}$
C_7
C_8
$C_{7(n-2)}$
$C_{8(n-2)}$
C_9
$C_{9(n-2)}$
C_{10}
C_{11}
C_{12}
C_{16}

Alkanoic acids

Acetic acid
Propionic acid
Butanoic acid
Pentanoic acid
2-Methypentanoic acid
4-Methylpentanoic acid
Hexanoic acid
4-Methylhexanoic acid
Heptanoic acid
4-Methylheptanoic acid
Octanoic acid
4-Methyloctanoic acid
Nonanoic acid
Decanoic acid
Dodecanoic acid
Tetradecanoic acid
Hexadecanoic acid
Heptadecanoic acid
Octadecanoic acid
Henicosanoic acid
Docosanoic acid
Henicosanoic acid
Docosanoic acid

Dicarboxylic acids

Succinic acid
Adipic Acid

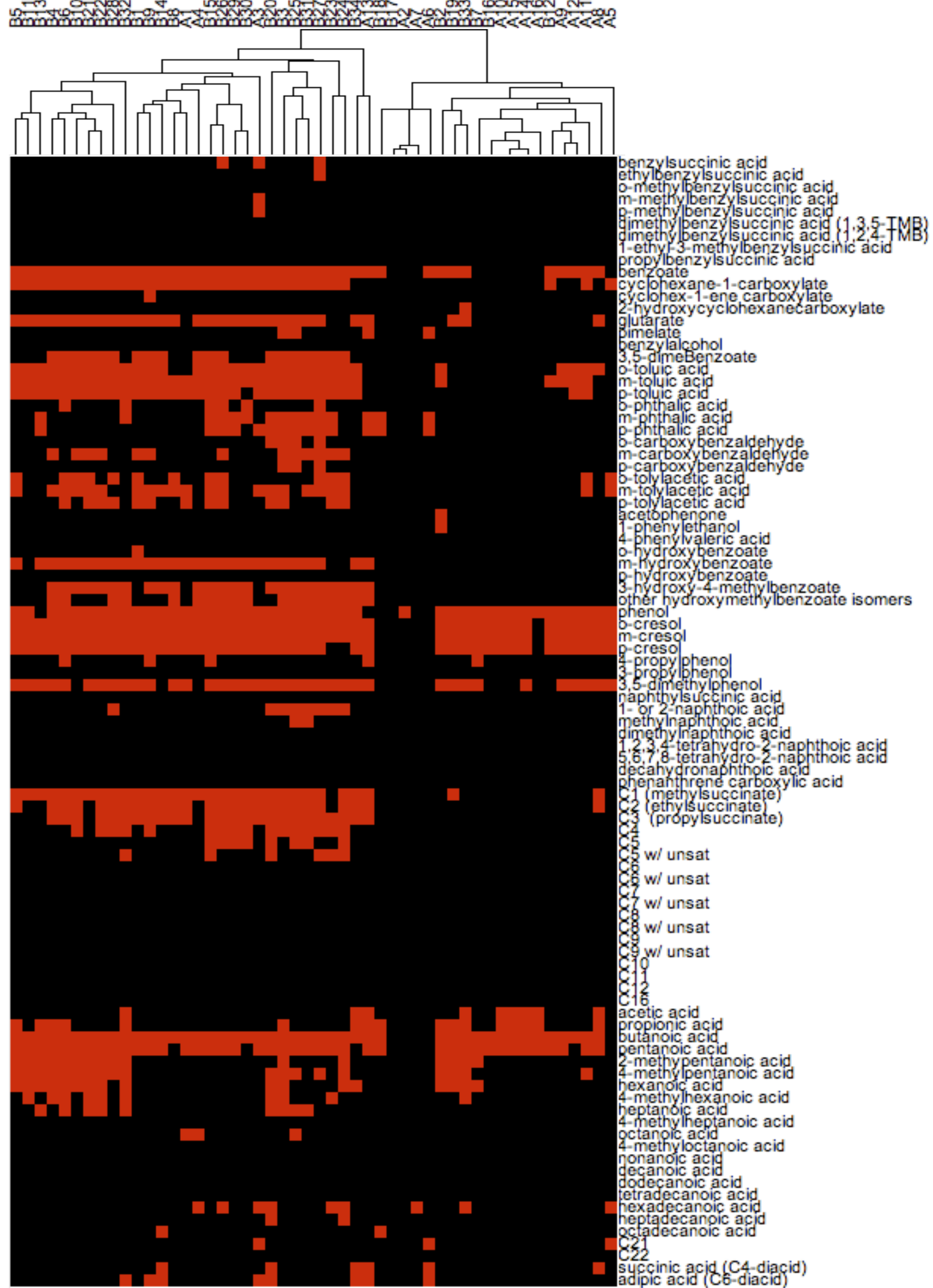

Figure 1. Heat map and dendrogram of metabolite analysis for oil Field A and B. The presence or absence of a metabolite was computed as a Euclidean distance and average linkage in CLUSTER 3.0 then visualized in Java Tree View. The dendrogram at the top depicts the relative relationship of each well to another based presence/absence of each metabolite (right column and Table 2). Positive detection of metabolites are indicated as red squares, no detected metabolite is represented in black.

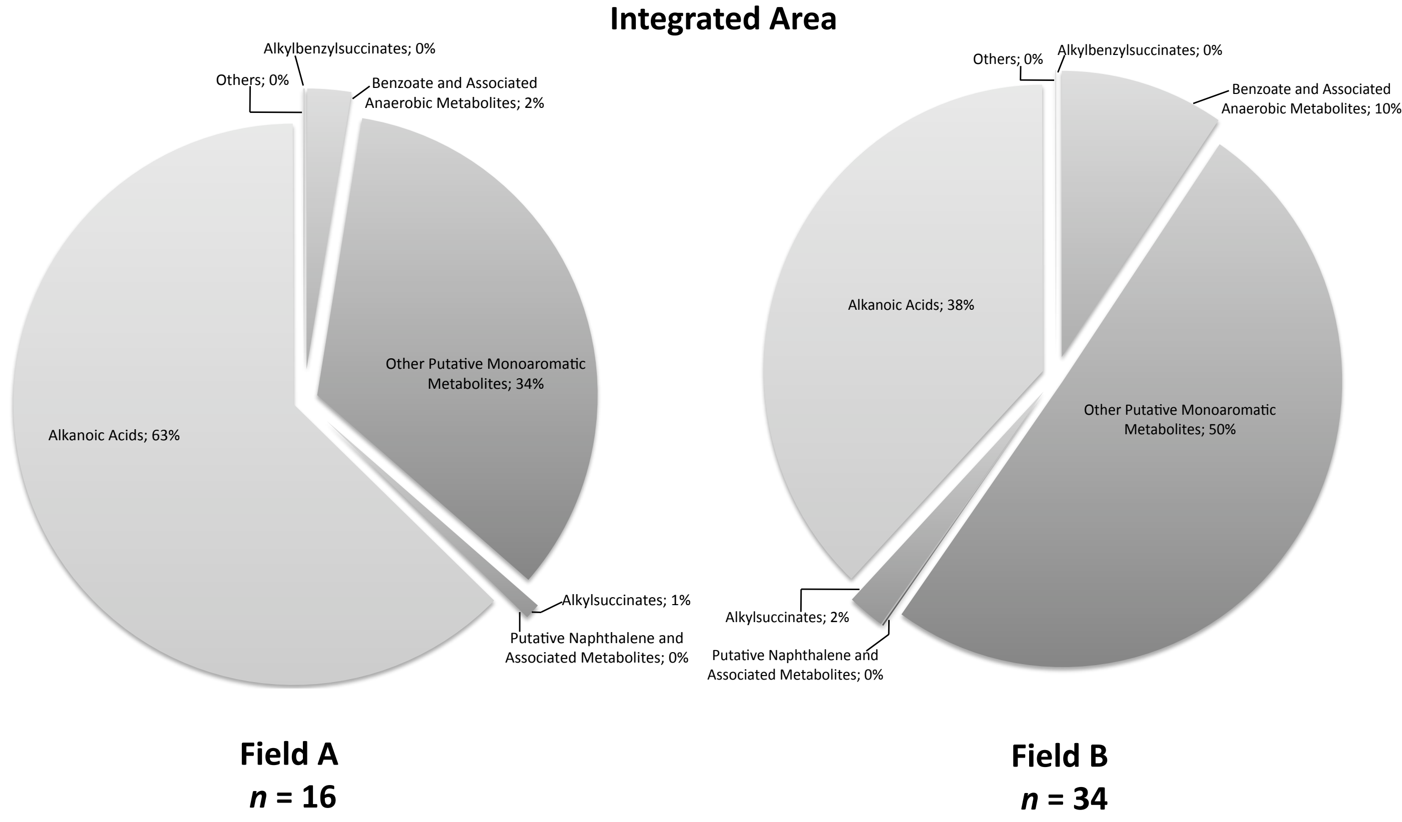

Figure 2. Integrated GC-MS areas for all samples collected in Field A and Field B. The seven classes of metabolites listed in Table 3 are represented by percent of total integrated area. The number of samples collected from each field is represented as (n).

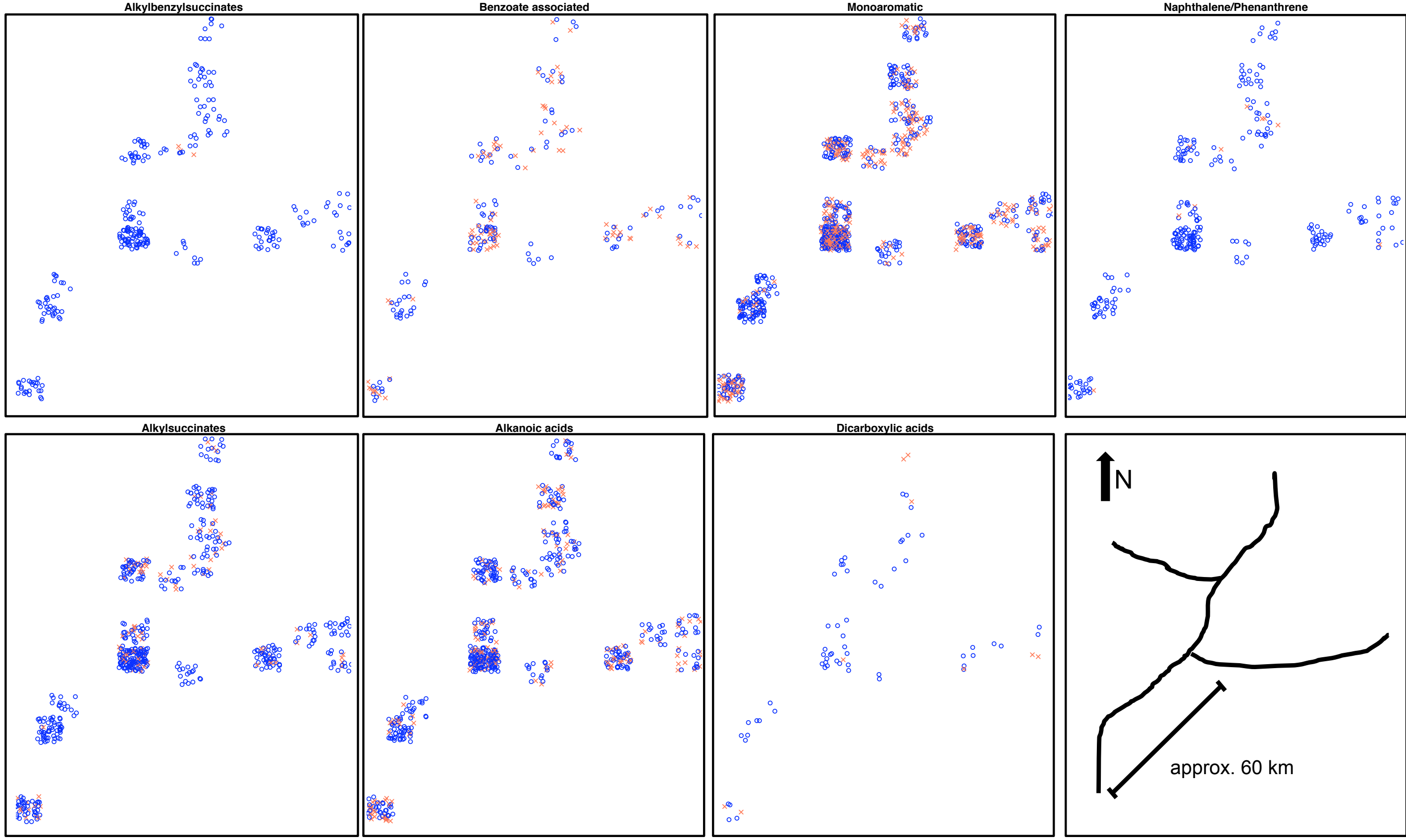

Figure 3. Spatial map of Field B showing sampling points and metabolites detected. The plots represent a map of Field B, with the relative locations of sample collection. The boxes represent the different metabolite classes in the study. All samples taken and the number of metabolites assayed for in each class are represented by a open blue circle (o). The presence of a metabolite is depicted with a red (x). The circles for the sampling points are “jittered” to avoid overlap with points from neighboring wells.

CHAPTER 4

An alternate hypothesis for the microbial oxidation of methane in the absence of oxygen

Abstract

Anaerobic methane oxidation (AOM) plays an important role in the global cycling of methane, but the requisite microorganisms and the metabolic pathway(s) associated with this bioconversion are not completely described. Herein, we suggest that methane is anaerobically oxidized in a manner analogous to higher molecular weight hydrocarbons. Such a mechanism involves a fumarate-addition reaction to form methylsuccinate as well as a series of intermediates predicted based on a presumed hydrocarbon-biodegradation pathway. Methylsuccinate was found in several environments where anaerobic methane oxidation was suspected. NMR analysis of anaerobic marine sediment incubations containing cells capable of metabolizing $^{13}CH_4$, revealed the presence of $^{13}CH_3$-labeled signals consistent with downstream metabolites of the hypothesized mechanism. These results suggest an alternate mechanism for the AOM that is quite distinct from the prevailing view of reverse methanogenesis.

Anaerobic methane oxidation (AOM) has been recognized as a major biogeochemical fate process influencing the cycling of carbon on the planet [1]. The prevailing paradigm for AOM involves a syntrophism between methane-oxidizing

archaea and sulfate-reducing bacteria wherein methane is oxidized by reverse methanogenesis, or the reversal of methyl-coenzyme M reductase (MCR) as the initial biochemical step [2, 3]. It is not known with certainty whether the entire process occurs in a single archaeon or through the biochemical linkage between syntrophic partners, however methanethiol transfer has recently been implicated [4]. The prospect of AOM linked to the consumption of alternate terminal electron acceptors has also been established [5]. Despite the recognition of AOM since the mid-1970's [6] a description of the underlying biochemical mechanism(s) is still undeveloped.

While investigating corrosion processes in oil facilities on the north slope of Alaska, we found alkylsuccinate metabolites that would be the expected intermediates if methane, as well as a series of other low molecular weight hydrocarbons, were anaerobically metabolized by fumarate addition reactions [7] This finding prompted us to also look for the putative methane metabolite, methylsuccinate, in other environments where AOM was known or suspected. To that end, water samples were collected, i) from a hydrocarbon-contaminated aquifer underlying a former refinery site in WY [8], ii) Beluga River natural gas field and iii) San Juan Basin coal field. Field samples were acidified upon collection to a pH ≤ 2 to preserve them and to protonate acidic metabolites. In addition, active AOM incubations by Beal et al. (2009) of marine cold-seep sediment from the Eel River Basin (ERB) containing ^{13}C-methane were also explored. Six-month incubations (July 2008 – January 2009) used ERB sediment collected September 2006 (R/V *Atlantis*). The pH of the ERB

incubations was initially adjusted with strong alkali (NaOH; 10N) to a pH$\geq$ 12 to cleave potential CoA thioester derivatives, and then acidified to a pH $\leq$ 2. All samples were then extracted with ethyl acetate, concentrated, derivatized with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide, and analyzed by gas chromatography-mass spectrometry (GC-MS) as previously described [8, 9]. Extracted and derivatized samples of the ERB incubations were also dried, re-suspended in deuterated chloroform and subject to ^{13}C-NMR as previously described [10].

Methylsuccinate was readily identified based on the GC-MS characteristics of the trimethysilane derivative (Table 1). The concentration of the putative metabolite ranged from about 20 nM to more than 2.0 µM in oilfield production waters from the North Slope of Alaska [11] Only a single sample (out of 6) was positive for methylsuccinate in the Belgua River natural gas field. Out of the 11 samples collected in the San Juan Basin, methylsuccinate was detected in only one sample. Methylsuccinate was initially detected ERB marine sediment incubations that were exposed to $^{13}CH_4$. However, the similar MS fragmentation pattern of the trimethylsilane derivatives and overlapping retention times of this putative metabolite made distinguishing between ethylmalonate and methysuccinate difficult.

Therefore, a different GC column and ^{13}C-NMR was employed to detect ^{13}C-methylsuccinate and other prospective intermediates that might be associated with AOM in the ERB incubations. The NMR spectra were obtained with a Unity INOVA 400-MHz NMR (Varian) with a ^{13}C resonance frequency of 100.530 MHz.

The proton-decoupled ^{13}C spectra were obtained at 25°C using a standard inverse gated pulse sequence. The following experimental parameters were employed: sweep width, 24,125 Hz; acquisition time, 1.300 s; recycle time, 1.000 s; and number of acquisitions, 23,500. The data were processed with 0.5 Hz line broadening. Chemical shifts were referenced relative to the center peak of the deuterated chloroform.

Six ^{13}C-NMR spectral features were observed between 10 and 35 ppm, indicating a series of compounds possessing a labeled methyl functional group (Table 2). A comparison of the ^{13}C-NMR spectral chemical shifts with predicted ^{13}C chemical shifts for methyl carbons expected to be labeled according to the pathway outlined by Wilkes et al., 2002 and Davidova et al., 2005 was consistent with the formation of several other downstream metabolites (Table 2; Figure 1) if methylsuccinate was indeed the activation mechanism. However, assignments labeled with an asterisk must be considered tentative since they could result from ethylacetate, labeled only with natural abundance ^{13}C, (peaks at 14.08, 20.88, 60.26 and 170 ppm) used to originally solvent extract the sample. In addition, there were two carboxylic acid signals in the spectrum between 161.44 and 161.80 ppm. Given that the recycle time (1.000 sec) employed was quite short relative to typical carboxylic acid T_1 relaxation times, resulting in a low sensitivity for such carbons, we suspect that these spectral features may indeed be ^{13}C-labeled and likely reflect an unidentified dicarboxylic acid.

We did not find any of the expected ^{13}C-NMR signals if methane was oxidized by

reverse methanogenesis, but we certainly cannot rule out this prospect as concentrations of such intermediates may be below detection or lost through volatilization. The detection of methylsuccinate in several field samples where AOM was suspected and the formation of multiple ^{13}C-labeled putative metabolites in anaerobic laboratory incubations amended with $^{13}CH_4$, however, allows us to suggest that methane metabolism may be initiated by adding across the double bond of fumarate in a manner analogous to higher molecular weight hydrocarbons. It therefore seems prudent to explore this process in other areas where AOM is evident.

Acknowledgements

We would like to thank Dr. Margaret Eastman of the Oklahoma State-wide NMR Facility for assistance with the ^{13}C NMR.

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Table 1. Methylsuccinate detected as a trimethylsilane derivative in various samples were obtained as part of another study of *Alaskan North Slope oil production facilities or †natural gas/ coal bed methane and will be reported soon.

Sample	Year Sampled	Number of samples	Methylsuccinate (approx. μM ± std. dev.)	Site Reference
Hydrocarbon-contaminated water	2005	1	0.04	[8]
Oil production waters	2006	6	2.08 ± 1.10	[7]
Oil production waters	2008	31	2.22 ± 1.14	*
Natural gas field (Beluga, AK)	2009	1	0.096	†
Coal field waters (San Juan Basin, NM)	2009	1	0.021	†

Table 2. Comparison of predicted ^{13}C-NMR shifts for putative AOM metabolites based on the pathway predicted in Figure 1 with those actually observed. ND=no observed signal in this region of the NMR spectrum. [1]ACD/C+H NMR Predictor v.10.05 * Tentative assignment. NM = No measurement possible with the column for gas chromatography.

Putative AOM intermediate	^{13}C-NMR predicted shifts (ppm)[1]	^{13}C-NMR observed shifts (ppm)	Detection of intermediate by GC-MS
Methylsuccinate	17.10	ND	No
Ethylmalonate	10.28	ND	Yes
Butyric acid	13.62	14.19*	Yes
2-butenoic acid	18.47	19.0	Yes
3-hydroxybutanoic acid	22.65	22.85	Yes
Acetoacetate	29.2	29.72	Yes
Acetaldehyde	30.9	30.72	NM
Acetic acid	20.81	21.03*	NM

Figure 1. Proposed pathway for anaerobic methane oxidation based on the pathway for anaerobic alkane biodegradation [10, 12]. Intermediates are represented as CoA derivates. The black circle (●) helps trace the presumed fate of the isotopically heavy carbon atom in the ERB incubations.

Appendix A

Biodegradation of low molecular weight alkanes under mesophilic, sulfate-reducing conditions: metabolic intermediates, and community patterns

Abstract

We evaluated the ability of the native microbiota in a low-temperature, sulfidic natural hydrocarbon seep (Zodletone) to metabolize short chain hydrocarbons. Propane and pentane were metabolized under sulfate-reducing conditions, and the sulfate-dependent propane and n-pentane oxidation activities were successfully maintained until sediment-free enrichments were obtained. Carbon isotope analysis of residual propane in active enrichments showed that propane became enriched in ^{13}C by 6.7 (±2.0) per mil, indicating a biological mechanism for propane loss. The detection of *n*-propylsuccinic, and isopropylsuccinic acids in active propane-degrading enrichments provides further evidence for biodegradation. 16S rRNA survey of bacterial communities showed that *Deltaproteobacteria*, *Firmicutes*, and *Chloroflexi* were the most abundant lineages within both enrichments. *Deltaproteobacteria* sequences were phylogenetically affiliated with previously described hydrocarbon-degrading sulfate-reducing strains of marine origin, volatile fatty acid metabolizers, hydrogen-users, and a novel *Deltaproteobacteria* lineage. *Firmicutes* sequences were detected mainly in propane-degrading enrichments, and

the majority were closely related to previously described clones from a marine, thermophilic, propane-degrading sulfate-reducing enrichment. These results document the potential for anaerobic short chain alkane metabolism for the first time in a terrestrial environment, provides evidence for fumarate addition mechanism for propane activation under these conditions, and reveals the microbial community associated with this process.

Introduction

Alkane mixtures of various lengths (C_1-C_{100}) are major components of various fossil fuel formations (Hunt, 1995, Philp, 2005) as well as of hydrocarbon seeps (Whelan*,* *et al.*, 1988, Clifton*, et al.*, 1990), and sub-seafloor gas hydrates (Kvenvolden, 1995). High molecular weight liquid and solid alkanes are present as the main constituents of crude oil, while lower molecular weight alkanes are mainly encountered in thermogenic and biogenic natural gas (Hunt, 1995). In addition, methane is biogenically produced in globally significant quantities in wetlands, rice fields, and rumina of herbivores (Wahlen, 1993, Kinnaman*, et al.*, 2007).

The aerobic degradation of alkanes with various chain lengths has been extensively studied and documented (Leahy & Colwell, 1990, Hanson & Hanson, 1996, Berthe-Corti & Fetzner, 2002, Van Hamme*, et al.*, 2003). This process involves the stepwise oxidation of the substrate into an alcohol, aldehyde and carboxylic acid prior to β-oxidation to produce intermediates that enter the tricarboxylic acid cycle. Anaerobic alkane degradation has received considerable attention in the last two decades (Reviewed in Heider*, et al.*, 1998, Widdel & Rabus,

2001, Boll, *et al.*, 2002, Heider, 2006), mainly to document occurrence of this metabolism in hydrocarbon-contaminated, oxygen-depleted environments and subsequently to develop strategies for the remediation of such ecosystems. As such, a handful of enrichments and isolates capable of liquid C_6-C_{16} *n*-alkanes have been described (Aeckersberg, *et al.*, 1991, Rueter *et al.*, 1994, So & Young, 1995, Caldwell, *et al.*, 1998, Ehrenreich *et al.,* 2000, Cravo-Laureau, *et al.*, 2005, Davidova, *et al.*, 2006, Callaghan, *et al.*, 2009,). The mechanisms by which such compounds are metabolized have also been actively researched during the last decade (So & Young, 1999, Kropp, *et al.*, 2000, So, *et al.*, 2003, Cravo-Laureau, *et al.*, 2005, Davidova, *et al.*, 2006). Most studies have shown that *n*-alkanes are activated by fumarate addition to the corresponding alkylsucccinic acids in the initial metabolic step (Kropp, *et al.*, 2000, Rabus, *et al.*, 2001, Cravo-Laureau, *et al.*, 2005, Callaghan, *et al.*, 2006), although some cultures clearly activate *n*-alkanes by a different mechanism (i.e. carboxylation) (So, *et al.*, 2003, Callaghan, *et al.*, 2009).

With the exception of anaerobic methane oxidation (Boetius, *et al.*, 2000, Raghoebarsing, *et al.*, 2006, Beal, *et al.*, 2009), the anaerobic metabolism of short chain alkanes (e.g. C_2-C_5) has received far less attention. Such low molecular weight alkanes are present in significant quantities in hydrothermal, as well as other marine vents (e.g. hydrocarbon seeps in Gulf of Mexico, Guaymas basin) (Whelan, *et al.*, 1988, Fonnoloa, *et al.*, 2004, Kniemeyer, *et al.*, 2007) and terrestrial habitats (e.g. natural gas fields of thermogenic origin) (Jarvie, *et al.*, 2007, Kinnaman, *et al.*, 2007, Mochimaru, *et al.*, 2007). Recently, Kniemeyer *et al*. (2007) isolated a mesophilic *Deltaproteobacterium* capable of anaerobic, sulfate-dependant propane and butane

metabolism, and described multiple sediment free enrichments dominated by *Deltaproteobacteria* or *Firmicutes* that are capable of propane and butane degradation at 12°C, and propane degradation at 60°C, respectively. These cultures, derived from natural marine hydrocarbon seeps, were shown to metabolize the gaseous hydrocarbons by fumarate addition (Kniemeyer*, et al.*, 2007). Herein we show the anaerobic biodegradation of propane and pentane in a terrestrial hydrocarbon seep and compare the microbial community within these hydrocarbon-degrading enrichments. The identification of alkylsuccinates indicative of the anaerobic metabolism of C_1 to C_4 alkanes in a hydrocarbon-contaminated aquifer or in oilfield fluids has also offered *in situ* evidence for this metabolism (Gieg & Suflita, 2002, Duncan*, et al.*, 2009). However, little information is currently available on the global occurrence of such phenomenon (e.g. in terrestrial as opposed to marine habitats), the rate of degradation of such hydrocarbons, whether specific hydrocarbons are preferentially degraded, and the phylogenetic and metabolic diversity of the microorganisms capable of mediating such a process.

In Zodletone Spring in southwestern Oklahoma, gaseous alkanes (methane, ethane, and propane) are continuously ejected from a deep underground formation, together with anaerobic, sulfide-rich ground water at the source of the spring. The spring source sediments thus represent an ideal habitat to examine the potential of non-marine microbial communities to metabolize short chain alkanes under anaerobic conditions. Here, we evaluated the ability of the native microbiota to degrade short chain alkanes under sulfate-reducing conditions. We provide quantitative, isotopic and metabolic evidence for the anaerobic degradation of short-

chain alkanes, and identify microbial community members presumably involved in the process using 16S rRNA gene based analysis.

Materials and Methods

Site Description. Zodletone Spring is located in Kiowa County in southwestern Oklahoma, USA. The spring flows for 20 m at a rate of approximately 8 L/min, where it then empties into nearby Saddle Mountain Creek (Younger, 1986). The spring is characterized by high concentrations of dissolved sulfide (8-10 mM at the source), which maintains anaerobic conditions throughout the spring. Sulfate is present at lower concentrations (about 50 μM) in source water. The source is a contained area (approximately 1 m^2) overlaid by water at a depth of about 50cm and filled with biomass and soft sediments to a depth of at least 15cm.. A detailed site description of the spring geochemistry and microbial community is found elsewhere (Havens, 1983, Sanders, 1998, Elshahed*, et al.*, 2003, Senko*, et al.*, 2004).

Enrichments set up. Enrichment cultures were set-up in an anaerobic glove bag (Coy, Grass Lake, MI, USA) in 40 ml serum bottles using 10 g of source sediment and 15 ml of spring water. The groundwater was buffered with sodium bicarbonate (0.04 M) and amended with 10 ml vitamins (Tanner, 1989), 10 ml minerals (Brandis & Thauer, 1981, Odom & Wall, 1987), and 15.0 mM ferrous sulfate per liter groundwater (to live sulfate-reducing enrichments only). Bottles were sealed with Teflon-lined stoppers, and the headspace was flushed with N_2/CO_2 (80/20) gas. The bottles where then amended with approximately 4.0 ml (0.160 mmol) of ethane, propane, or butane or 1.0 μl (8.7 μmol) of neat liquid pentane (Sigma-Aldrich,St.

Louis, MO, USA), and incubated at room temperature (22^0C, approximately similar to the *in-situ* temperature of Zodletone spring source) in the dark. Additional enrichments without the addition of a hydrocarbon substrate were maintained to account for background sulfate reduction and methane production. Enrichments showing alkane degradation were subcultured after approximately 80-90% of the substrate was lost by transferring 1.0 ml of liquid from the active enrichment into an anaerobic Balch tube containing 9.0 ml of anaerobically prepared (Bryant, 1972, Balch & Wolfe, 1976) mineral medium containing: (ml or g l^{-1}) NH_4Cl (0.27), KH_2PO_4 (0.03), NaCl (11.7), $MgCl_2.6H_2O$ (1.22), $CaCl_2.2H_2O$ (0.44), vitamin solution (6.25) (Tanner, 1989), mineral solution (6.25) (Brandis & Thauer, 1981, Odom & Wall, 1987), and 0.1% resazurin (0.5), as well as ferrous sulfate (final concentration 15mM) to prevent possible inhibition of alkane-degradation activity due to sulfide-toxicity (Beller & Reinhard, 1995). All bottles and tubes were monitored for sulfate and substrate loss as described below. Subcultured enrichments showing alkane loss were then serially diluted (up to 10^{-5}).

Isotope Fractionation. Biological degradation of organic compounds results in enrichment of the heavy carbon isotope ^{13}C in the residual substrate, expressed as δ^{13}C (Ahad*, et al.*, 2000, Meckenstock*, et al.*, 2004). This occurs as bonds between heavier isotopes are slightly more difficult to cleave (Hoefs, 2009). Enrichment of δ^{13}C can thus be used to definitively demonstrate the degradation of organic compounds. The experiments required appropriate controls. We determined the δ^{13}C value of propane gas used as a substrate (Sigma-Aldrich, St. Louis, MO, USA), and compared it to residual propane in propane-degrading enrichments, as well as the

propane from sulfate amended sterile controls and propane from live enrichments that were not amended with sulfate, and where no propane degradation was observed. $\delta\ ^{13}C$ Isotope ratios were determined using gas chromatography and isotope ratio mass spectrometry (GC-IRMS) (Finnigan MAT 252 IRMS) as previously described (Schoell, 1983, Sofer, 1984, Rodriguez Maiz & Philp, 2009).

Analytical methods. Substrates loss was monitored using a Shimadzu GC-14A GC (Shimadzu Scientific Instruments Columbia, MD, USA), equipped with a Chemipak C-18 column (Sigma-Aldrich St. Louis, MO, USA) and a flame ionization detector set at 125°C. The oven temperature was set at 30°C to measure ethane and propane and set at 100°C to measure butane and pentane. The injector temperature was kept at 100°C. Sulfate loss was monitored using Dionex DX500 ion chromatography system (Dionex Corp. Sunnyvale, CA, USA) with an AS4A column as previously described (Caldwell*, et al.*, 1998, Gieg*, et al.*, 1999).

Active subcultured enrichments were used for the detection of metabolic intermediates. Cultures (10.0 ml) were acidified to a $pH < 2$ using 12N HCl. Cultures were then extracted 3 times using 15 ml of ethyl acetate, and the combined organic layer was subsequently concentrated by rotary evaporation and under a stream of nitrogen to 0.2ml. The concentrated extract was then derivatized using 50 μl of *N, O*-bis(trimethylsilyl)trifluoroacetimide (BSTFA) according to the manufacturer's instructions (Pierce Chemicals, Rockford, IL, USA). Potential metabolic intermediates in the concentrated and derivatized samples were identified using gas chromatography-mass spectrometry GC-MS on an Agilent HP 6890 GC and an Agilent 5973 MS as previously described in detail (Duncan*, et al.*, 2009).

Peaks were identified by either comparing their mass spectral profiles to chemical standards, or by detailed examination of their mass spectral profile and characteristic fragmentation patterns as described previously (Gieg & Suflita, 2002, Rios-Hernandez*, et al.*, 2003).

Community Analysis. DNA was extracted from 1.5 ml of active diluted enrichments, using the MoBio Power Soil DNA Isolation Kit (MO BIO Laboratories Carlsbad, CA, USA) according to the manufacturer's instructions. Bacterial 16S rRNA gene was amplified using 8F 5'-AGAGTTTGATCCTGGCTCAG-3' and 805R 5'-GACTACCAGGGTATCTAATCC-3' primers as previously described (Elshahed*, et al.*, 2003). Amplified products were then cloned into *Escherichia coli* using a TOPO-TA cloning kit (Invitrogen Corp., Carlsbad, CA, USA) and sequenced at Washington University at St. Louis Genome Center (St. Louis, MO, USA)

Sequences obtained were initially compared to the GenBank nr database and checked using BLAST (Altschul*, et al.*, 1997). Sequences with more than 97% similarity were considered to be of the same operational taxonomic unit (OTU). Zodletone sequences and GenBank-downloaded sequences were aligned using the ClustalX program (Thompson*, et al.*, 1997). Phylogenetic trees were constructed using representatives of closely related reference sequences to highlight the phylogenetic affiliation of clones obtained in this study. Evolutionary distance trees were constructed using PAUP (Version 4.01b10; Sinauer associates, Sunderland, MA, USA).

Nucleotide sequences accession numbers. Sequences generated in this study were deposited in GenBank under accession numbers GU211106-GU211166.

Results and Discussion

Hydrocarbon biodegradation. Enrichments for ethane, propane, butane, and pentane degradation were constructed using Zodletone Spring source sediment and ground water under methanogenic and sulfate-reducing conditions. No appreciable or subculturable substrate-loss was observed in all methanogenic enrichments or in ethane and butane sulfate-reducing enrichments (data not shown). Significant loss (>70%) of substrate was observed only in propane and pentane amended enrichments.

Under sulfate-reducing conditions, propane degradation commenced after approximately 80 days of incubation, and the majority of added propane (>90%) was metabolized after 467 days (Fig. 1). Sulfate-reduction was observed in both substrate amended and substrate unamended controls (0.571 and 0.364 mmols, respectively, Fig 1a), and the net sulfate-reduction was only a fraction of the theoretical amount of expected from sulfate-dependant complete oxidation of propane to CO_2 according to the equation (Kniemeyer*, et al.*, 2007):

$$2C_3H_8 + 5SO_4^{2-} + 4H^+ \rightarrow 6HCO_3^- + 5H_2S + 2H_2O \qquad \textbf{Equation 1}$$

($\Delta G°_{pH7}$= -102 kj per mol propane)

Active propane enrichments were subcultured into anaerobic mineral media using a 10% v/v inoculum after 578 days of incubation. Upon subculturing approximately 70% (0.03 ± 0.01 mmol) of the propane added was degraded after 286 days of incubation. The stoichiometry of mol sulfate-reduced per mol propane lost (after correction for sulfate-reduction in substrate-unamended controls) was 3.0 (mmols of

sulfate/mmol propane), this value is 120% of the theoretical value Propane subcultures were further serially diluted up to 10^{-5}. After 124 days there was a loss of approximately 50% of the substrate in the 10^{-1} dilution, this sample was used for 16S rRNA community analysis.

Compared to propane, pentane degradation by Zodletone spring source sediments under sulfate-reducing conditions was relatively faster, with degradation commencing after 80 days and pentane was 90% metabolized in one enrichment after 301 days. Sulfate-reduction was observed in both substrate amended and substrate unamended control (0.432 and 0.364 mmols, respectively, Fig. 1b), and the net sulfate-reduction was greater than the theoretical amount of expected from sulfate-dependant complete oxidation of propane to CO_2 according to the equation

$$C_5H_{12} + 4SO_4^{2-} + 3H^+ \rightarrow 5HCO_3^- + 4H_2S + H_2O \quad \text{Equation 2}$$

($\Delta G°_{pH7}$= -191 kj per mol pentane)

Subcultured pentane enrichments (10% v/v) consumed 85% (3.9 ±1.2 μmols) of added pentane after 471 days of incubation (data not shown). The corrected mol sulfate / mol pentane stoichiometry was 7.2:1, which is in excess of the theoretically expected amount (4:1) according to equation 2. Serial dilution of pentane subcultures was conducted, and >80% of pentane was degraded in the 10^{-3} dilution, which was used for 16S rRNA gene community analysis.

Isotopic fractionation in propane-degrading sulfate-reducing enrichments. Due to the relatively long time period observed for complete propane degradation (approximately 1.3 years, Fig. 1), we sought to further ascertain the biological origin of propane degradation in sulfate-reducing enrichments. Active propane-degrading

enrichments showed that residual propane in subcultured enrichments were indeed enriched in the heavy carbon isotope (δ C^{13}= -16.03‰ (± 2.00)) compared with propane used as the substrate (δ ^{13}C = -22.75‰ (± 0.92)) (Table 1). Furthermore, analysis of the isotopic ratio of CO_2 after incubation of the active propane-degrading enrichment indicated that carbon dioxide was further enriched in ^{12}C. (Table 1), providing evidence that the propane was being mineralized to light carbon dioxide.

Activation of n-propane through the formation of alkylsuccinate intermediates. We tested for the presence of potential anaerobic intermediates of propane and pentane metabolism by GC-MS. Two compounds eluting within a minute of each other were detected in propane-degrading enrichments with mass spectral features consistent with those of *n*-propylsuccinic acid and isopropylsuccinic acid (Figure 2). These metabolites were not detected in substrate-unamended controls (data not shown). The MS profiles of both compounds showed nearly identical ion fragmentation patterns with a characteristic M-15 ion of 289 and other ions distinctive for alkylsuccinates (262, 217, 172, 147, 73) (Gieg & Suflita, 2002, Rios-Hernandez*, et al.*, 2003). The identity of *n*-propylsuccinic acid in the propane-amended culture extracts was confirmed by comparing its GC-MS profile to that of a TMS-derivatized standard. In a propane-degrading culture enriched from marine sediments, both isomers of propylsuccinic acid were also detected (Kniemeyer*, et al.*, 2007). These findings, coupled with the detection of propylsuccinic acid in other petroleium-laden environments (Gieg & Suflita, 2002, Duncan*, et al.*, 2009) suggest that fumarate addition is a key mechanism for activation of n-propane in disparate anaerobic environments.

Comparable pentylsuccinate formation was not found when the pentane-degrading enrichments were similarly analyzed by GC-MS. It is unclear if the formation of diagnostic metabolites was simply below the detection limits of the analytical procedures or whether pentane-utilization was by an alternate mechanism.

Community patterns in propane and pentane-degrading enrichments. 16S rRNA gene based analysis was conducted to identify the prominent bacterial taxa within the propane and pentane-degrading enrichments. A total of 63 and 69 clones were sequenced from the propane- and pentane-degrading enrichments respectively.

Members of the *Deltaproteobacteria* dominated both propane and pentane clone libraries (68.2 % and 79.8% of clones respectively), followed by members of *Chloroflexi*, *Firmicutes*, and candidate division MBMPE71 (Table 2, Fig. 3). Collectively, these four phyla constituted 92% and 95.6% of clones in the propane and pentane clone libraries, respectively (Table 2). The differences between the phylogenetic affiliations of OTUs observed in the propane-degrading versus the pentane-degrading enrichments, the stark difference between both clone libraries and the native bacterial community in Zodletone spring source sediments (described in detail in a previous publication (Elshahed*, et al.*, 2003)), and the fact that clone library construction was conducted after successful subculturing and serial dilutions of the original enrichment, argues that a majority of these OTUs have an active role in these hydrocarbon-degrading enrichments.

The *Deltaproteobacteria* OTUs identified in both enrichments could be assigned to one of four categories: Close relatives of known hydrocarbon degraders, volatile fatty acid (VFA) metabolizers, hydrogen metabolizers, and representatives of

a novel *Deltaproteobacterial* lineage. Within the propane-degrading enrichments, multiple OTUs sequences, designated "gaseous alkane associated" in Figure 3 belonged to the *Desulfococcus-Desulfonema-Desulfosarcina* cluster within the family *Desulfobacteraceae*. Specifically, these sequences formed a phylogenetically coherent lineage with a large number of clones identified in studies of globally distributed gaseous hydrocarbon-impacted marine ecosystems including hydrocarbon seeps in the Gulf of Mexico (GenBank accession nos. **AM745215, AM745148, AM745130**), gas hydrates off the Oregon Coast (Knittel*, et al.*, 2003), deep-sea mud volcanoes in the eastern Mediterranean (GenBank accession no. **AY592396**), and Japan trench (Inagaki*, et al.*, 2002), as well as hydrocarbon-degrading enrichments derived from marine sediments (Musat & Widdel, 2008) (Fig. 3). To our knowledge, this is the first report that identifies members of this lineage in a non-marine environment, and strongly implies the involvement of these microorganisms in global turnover of propane under sulfate-reducing conditions. In addition, sequences retrieved in this study were 88.2-95.2% similar to the 16S rRNA gene of isolate BuS5 (GenBank accession no. **EF077225**), the only pure culture capable of propane and butane degradation known to date, and 87.5-95.1% similar to clone Butane12-GMe, the most dominant sequence in a cold-adapted butane-degrading enrichment culture from the Gulf of Mexico (GenBank accession no. **EF077226**) (Kniemeyer*, et al.*, 2007) (Fig. 3).

Relatives of known hydrocarbon degraders in pentane-degrading enrichments were closely related to a different lineage within the *Desulfococcus-Desulfonema-Desulfosarcina* cluster in the family *Desulfobacteraceae*. The closest cultured

relative to these OTUs (designated “gaseous alkane associated” in Fig. 3) was the 16S rRNA gene of *Desulfococcus oleovorans* strain Hxd3 (95% sequence similarity), with the next closest relatives being < 88% similar. *Desulfococcus oleovorans* strain Hxd3 is the first described hydrocarbon-degrading sulfate-reducer, and is capable of degrading C_{12}-C_{20} n-alkanes (Aeckersberg*, et al.*, 1991, Aeckersberg*, et al.*, 1998). Interestingly, strain Hxd3 is one of the few hydrocarbon degrading isolates known to use carboxylation instead of fumarate addition to activate hydrocarbons (So*, et al.*, 2003). These results suggest the involvement of a potentially novel *Desulfococcus* species in anaerobic pentane metabolism in Zodletone spring pentane-degrading enrichments.

Similar to microorganisms potentially involved in alkane degradation in propane and pentane enrichments, the OTUs affiliated with VFA-metabolizing lineages within both enrichments were phylogenetically distinct. Within propane enrichments, relatives of the propionate degrading bacterium *Smithella propionica* (95%) were identified (order Syntrophobacterales, Fig. 3). *Smithella propionica* is a propionate utilizing bacterium that can grow syntrophically in co-culture with a hydrogen utilizing sulfate-reducer (Liu*, et al.*, 1999). Within the pentane-degrading enrichments, close relatives of *Desulfarculus baarsii* (order Desulfoarculales, Fog 3) were observed. *D. baarsii* is capable of incomplete degradation of higher fatty acids including long-chain fatty acids as well as pentanoic acid (Kuever*, et al.*, 2001). The enrichment of propionate-metabolizing specialists in the propane enrichments and long chain fatty acid specialists in the pentane enrichments argue for a direct or indirect involvement of these microorganisms in substrate turnover.

The remaining sequences identified in both enrichments were either members of the order *Desulfovibrionales* or belonged to novel lineages within the family *Desulfobacteraceae* within the *Deltaproteobacteria*. *Desulfovibrionales* are regarded as the main hydrogen-utilizing bacteria under sulfate-reducing conditions and are ubiquitous in nature (Kuever*, et al.*, 2005). The novel *Desulfobacteraceae* associated lineage (represented by OTUs Zod02-A03, Zod33-F06, Zod62-F03 and Zod69-H06, Novel Desulfobacterales, Fig. 3) has no pure culture representative, and Zodletone OTUs belonging to this lineage had 91-96% sequences similarity to clones identified from hydrocarbon-impacted marine ecosystems (Mussmann*, et al.*, 2005, Paissé*, et al.*, 2008). Members of this lineage were identified in both of the propane and pentane-degrading enrichments.

In addition to *Deltaproteobacteria*, members of the *Chloroflexi*, *Firmicutes*, and Candidate division MBMPE71 were encountered in both enrichments. Members of the phylum *Firmicutes* comprised 6.3% of the propane fed enrichments but only 1.6% of pentane fed enrichments. In general, the majority of clones belonged to the mostly sulfate-reducing *Desulfotomaculum-Pelotomaculum* group within the *Peptococcaceae*. Sequences from the propane-degrading enrichments belonging to this group e.g. Zod06-A09 (Fig. 3) were closely related (98% similar) to clones from an oil contaminant plume (Winderl*, et al.*, 2008) as well as clones from a benzene degrading enrichment (Laban*, et al.*, 2009). Interestingly, this OTU was also related to the dominant phylotype in a thermophilic propane-degrading enrichment, Propane60-GuB (Kniemeyer*, et al.*, 2007).

Chloroflexi OTUs identified in both enrichments were either close relatives

of known anaerobic fermenters e.g. *Bellilinea caldifistulae* (Yamada*, et al.*, 2007) and *Leptolinea tardivitalis* (Yamada*, et al.*, 2006) within the order *Anaerolineales* or belonged to uncultured bacterial lineage with clones originating from hydrocarbon-impacted environments (Elshahed*, et al.*, 2003) or hydrocarbon degrading enrichments (Ficker*, et al.*, 1999). Sequences belonging to candidate division MBMPE71, in which several OTUs from propane- and pentane-degrading enrichments were identified, are mainly from hypersaline microbial mats (Senbarger*, et al.*, 2008), marine sediments (Li*, et al.*, 2008), and deep mud volcano Mediterranean sediments (GenBank accession number **AY592413**).

This study documents the potential for propane and pentane metabolism in terrestrial sediments under sulfate-reducing conditions, shows that propane is activated by fumarate addition at the terminal and subterminal carbon, and identifies the microbial community members involved in such anaerobic biodegradation. Documenting the occurrence of such processes provide a microbiological explanation of various geochemical patterns and processes observed within hydrocarbon rich ecosystems (Kniemeyer*, et al.*, 2007) such as the detection of metabolites of gaseous alkane degradation in polluted aquifers (Gieg & Suflita, 2002), oil reservoir well heads (Duncan*, et al.*, 2009), and the differences in gas composition in thermogenic and biogenic natural gas reservoirs (Hunt, 1995, Mochimaru*, et al.*, 2007).

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Table 1. Differences in the carbon isotope ratios (δC^{13}) of propane and carbon dioxide in active propane-degrading, sulfate amended enrichments compared with the propane (from tank), propane-fed sterile control and a propane-fed enrichment without sulfate.

Enrichment Conditions	δC^{13} (‰)			
	Propane	**$\Delta\delta C^{13}$**	**Carbon Dioxide**	**$\Delta\delta C^{13}$**
Propane	-22.75 (±0.92)	-	-	-
Sterile Control	-23.28 (±0.54)	-0.53	-10.58 (±0.22)	-
Live Enrichment (-sulfate)	-22.35 (±0.35)	+0.40	-13.65 (±0.21)	-3.07
Live Enrichment (+ sulfate)	-16.03 (±2.00)	+6.72	-17.23 (±0.15)	-6.65

Table 2. Community composition of subcultured propane (10^1) and pentane (10^{-3}) degrading enrichments.

Putative Phylum Assignment	Propane-degrading Enrichment (% of total clones)		Pentane-degrading Enrichment (% of total clones)	
Deltaproteobacteria	68.2		79.8	
Desulfovibrionaceae		17.4		26.1
Desulfobacteraceae		27.0		34.8
Syntrophaceae		15.8		7.3
Desulfarculaceae		-		11.6
Desulfobulbaceae		3.2		-
Desulfuromonaceae		4.8		-
Firmicutes	7.9		2.8	
Clostridiaceae		1.6		-
Peptococcaceae		6.3		1.4
Thermoanaerobacterales		-		1.4
Chloroflexi	14.3		5.8	
Caldineaceae		14.3		5.8
Other	9.6		11.6	
MBMPE71		1.6		7.2
Gammaproteobacteria		3.2		1.4
Bacteroidetes		1.6		-
Deferribacteres		1.6		1.4
Planctomycetes		1.6		1.4

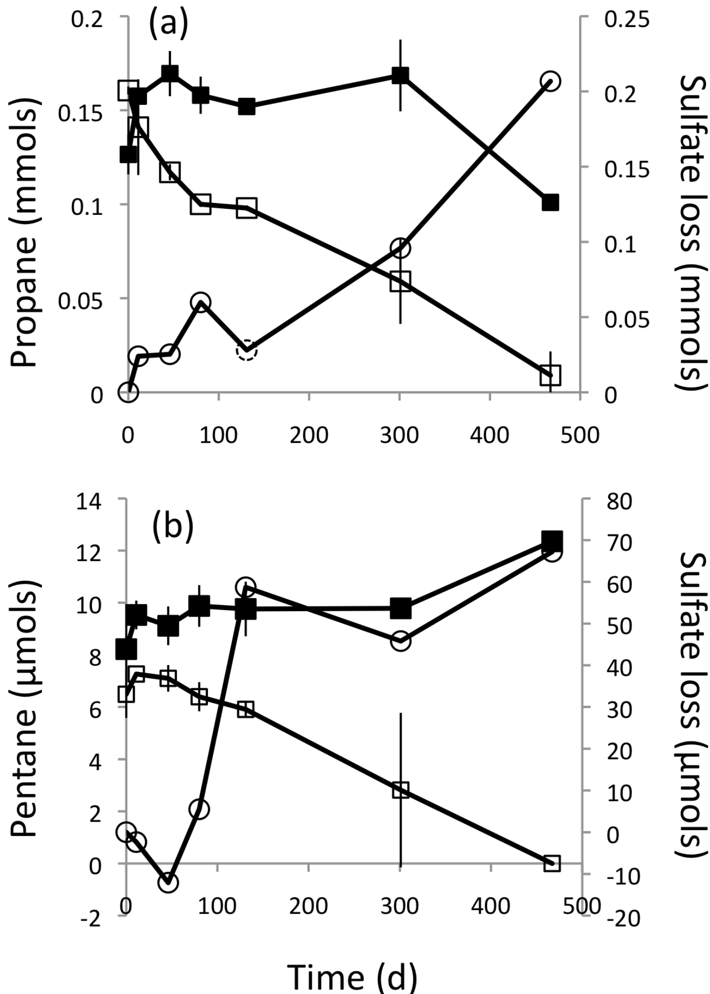


Fig 1. Propane and pentane degradation under sulfate-reducing conditions in enrichments from Zodletone spring source sediments and ground water. Live propane and pentane sulfate-reducing enrichments (-□- ; -O-); corresponding sterile incubations (-■- ; -●-).Values are presented as percentage loss of original hydrocarbon concentration: propane = 0.167 ± .01 mmol; pentane = 6.49 ± 0.91 µmol.

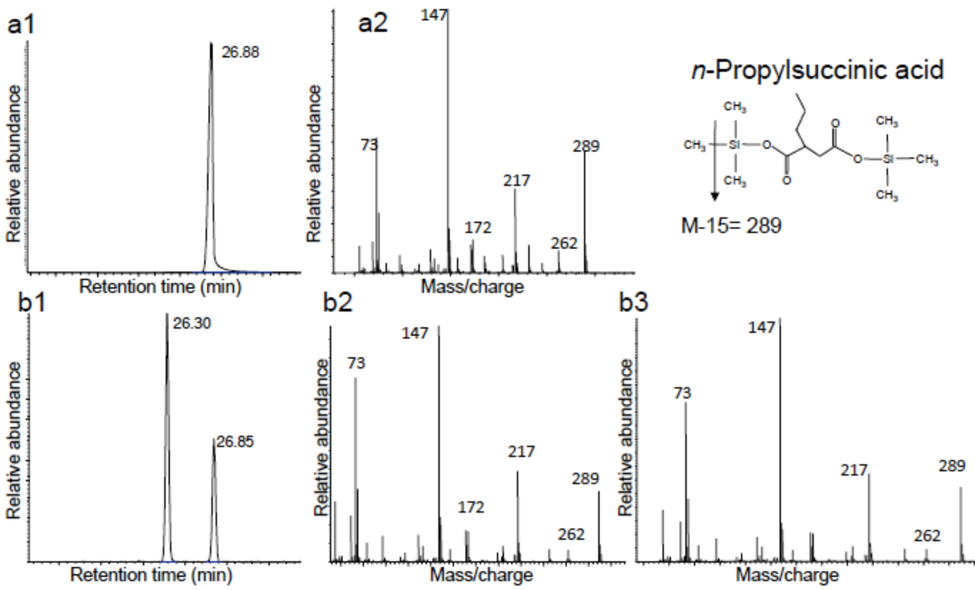


Fig. 2. GC-MS analysis of an *n*-propylsuccinic acid standard and of propane-degrading enrichment culture extracts. (a1) GC partial ion chromatogram following selection for the m/z 289 ion of a *n*-propylsuccinic acid standard, (a2) Mass spectrum of *n*-propylsuccinic acid (retention time, 26.88 min). (b1) GC partial ion chromatogram following selection for the m/z 289 ion in the propane-degrading culture extract, (b2) Mass spectrum for *n*-propylsuccinic acid in the culture extract (retention time, 26.85 min), (b3) mass spectrum for isopropylsuccinic acid in the culture extract (retention time, 26.30 min).

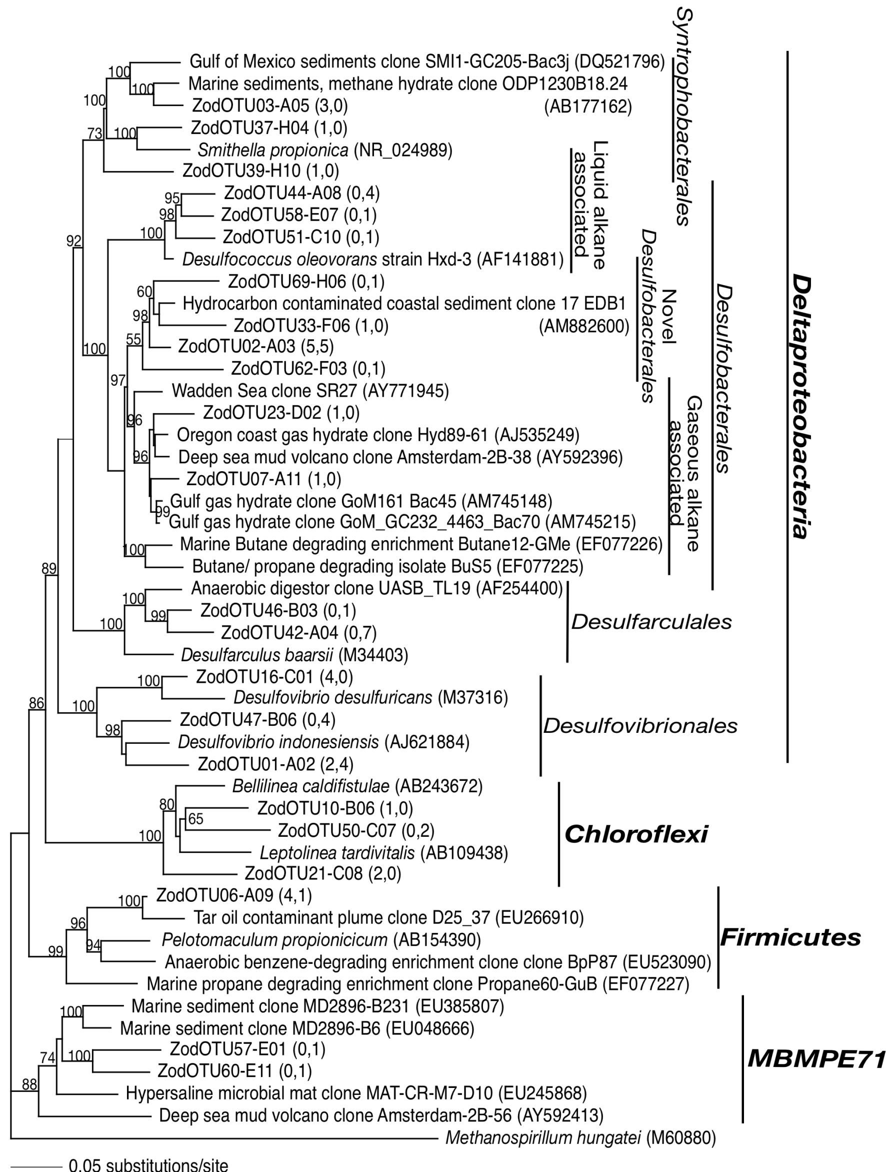


Fig. 3. Distance neighbor-joining tree based on the 16S rRNA sequences identified in both the propane and pentane-degrading sulfate-reducing enrichments from Zodletone spring sediments. Only selected OTUs are included in the tree, and a total inventory of all sequences identified are presented in table 2. Bootstrap values (expressed as percentages) are based on 1000 replicates and are shown for branches with more than 50% bootstrap support. Sequences retrieved in this study are labeled "Zod", and the frequency of occurrence of each OTU in the propane and pentane degrading enrichments, respectively are indicated in parenthesis (out of a total of 63 clones from the propane enrichment, and 69 clones in the pentane enrichment).

Appendix B

Supplementary Tables and Figures

CHAPTER 1

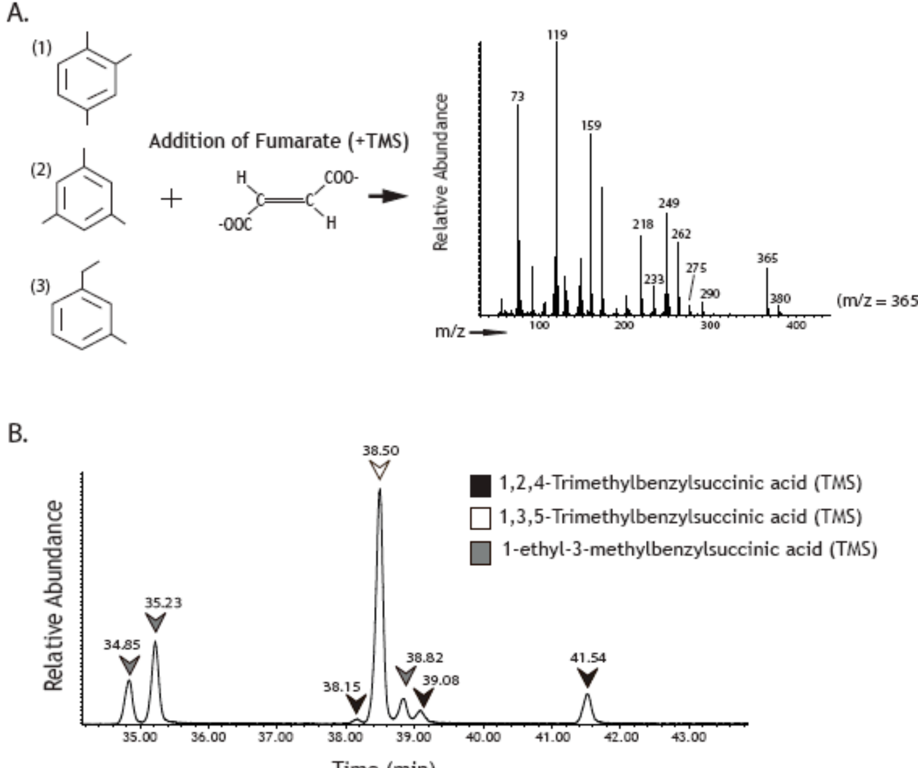


Figure S1. The fumarate addition product(s) of 1,2,4-trimethylbenzene, 1,3,5-trimethylbenzene, or 1-ethyl-3-methylbenzene were synthesized and exhibited similar mass spectral profiles when derivatized (TMS) and analyzed as indicated in the text (A). The resulting isomers could be distinguished based on the number of products and their respective gas chromatographic retention times (B). Peaks associated with the fumarate-addition product of 1,2,4-trimethylbenzene (black arrows), 1,3,5-trimethylbenzene (white arrow) and 1-ethyl-3-methylbenzene (gray arrows) are based on assignments made with individual C3-alkylbenzene parent compounds.

CHAPTER 2

Supporting tables and figures

Table S1. Summary information regarding samples collected and types of analyses performed. 16S Bac clone libraries (e.g. 16S rDNA bacterial gene sequence clone libraries) were constructed from PCR products amplified with primers 27F and 1391R (*10*), except for SW and a replicate 2P library, which used 27F and 1492R (*11*). 16S Arc clone libraries (e.g. 16S rDNA archaeal gene sequence clone libraries) were constructed after amplification with primers ARC333F and 958R (*11*). *dsrAB* clone libraries were constructed after amplification with primers dsr1 and dsr4 (*12*).
* The oil production facility draws seawater from the Beaufort Sea, part of the Arctic Ocean.
** clone library attempted, but amplification was unsuccessful
***clone library attempted, but only 10 sequences out of approx. 700 obtained matched most closely to *dsrAB*.

Sample ID	Sample type	Temperature	Clone libraries	Cultivation	Metabolite analysis
2P	Production well	48°C ±1.7 (1 SD)	16S Bac 16S Arc dsrAB	No	Yes
2L	Production well	avg. 49°C ±2.1 (1 SD)	16S Bac	No	Yes
2T	Production well	Unknown	No	No	Yes
2K	Water reinjection well	Unknown	No	No	Yes
2U	Pipeline to central facility	Unknown	No	No	Yes
PS	1st stage separator: Central facility	50-55°C	16S Bac 16S Arc dsrAB	Yes	Yes
CO	Coalescer: Central facility	59-78°C	16S Bac 16S Arc dsrAB	No	no
TS	Biocide-treated seawater	Arctic Ocean Seawater*	no	No	Yes
SW	Fluids and solids scraped from treated seawater pipeline	Arctic Ocean Seawater*	16S Bac 16S Arc** dsrAB***	Yes	Yes

Table S2A. Sequence similarity and taxonomic relationships of bacterial representative small subunit partial rRNA gene sequences (OTUs at 97% similarity).

OTU	% of total	Accession number	Most similar sequences* (accession no.)	%	Class**	Source
Well 2P: total # sequences =363, primers 27F, 1391R. 97% OTUs from DOTUR						
2P327SHNG718	90.1	FJ469286	*Petrobacter* sp. NFC7-F8 (EU250943) *Petrobacter* sp. DM-3 (DQ539621)	99 99	β proteo	50°C compost Dagang oil field
2P17SHNG539	4.7	FJ469280	*Thermovirga lienii* Cas60314 (DQ071273)	99	Synergistes	North Sea oil well
2P9SHNG554	2.5	FJ469288	Uncultured clone CK06-06_Mud_MAS1B-28 (AB369171)	99	Mollicutes	Offshore drilling mud fluid
2P3SHNG611	0.8	FJ469287	Uncultured clone B5_B4 (EF025213)	99	Sphingobacteria	Turkey intestine
2P2SHNG411	0.6	FJ469285	*Thermoanaerobacter pseudethanolicus* ATCC 33223 (CP000924)	99	"Clostridia"	Octopus Springs
2P2SHNG385	0.6	FJ469284	*Bradyrhizobium* sp. JR016 (EF221629)	99	α proteo	Root nodule
2P1SHNG397	0.3	FJ469281	Uncultured Thermotogales clone bh459.f1.4.b07 (AM184116)	99	Thermotogae	Low-temp enrichment degrading polychlorinated biphenyls
2P1SHNG452	0.3	FJ469282	*Stenotrophomonas* sp. ROi7 (EF219038)	99	γ proteo	Reverse osmosis membrane
2P1SHNG731	0.3	FJ469283	*Ralstonia pickettii* 12J (CP001069)	99	β proteo	
Well 2L: total # sequences = 648, primers 27F, 1391R. 97% OTU from DOTUR.						
2L474SGXO1136	73.1	FJ469308	*Thermovirga lienii* Cas60314 (DQ071273)	99	Synergistetes	North Sea oil well
2L65SGXO482	10.0	FJ469310	*Thermovirga lienii* Cas60314 (DQ071273)	99	Synergistes	North Sea oil well
2L27SGXO638	4.2	FJ469303	*Desulfomicrobium thermophilum* P6.2 (AY464939)	98	δ proteo	Hot spring in Colombia
2L14SGXO613	2.2	FJ469289	*Thermoanaerobacter pseudethanolicus* ATCC 33223 (CP000924)	99	"Clostridia"	Octopus Springs
2L9SGXO552	1.4	FJ469316	*Thermosipho africanus* (DQ647057) *Thermosipho* sp. TBA5 AF231727	99 99	Thermotogae	Shallow hydrothermal system North Sea oil field
2L7SGXO640	1.1	FJ469315	*Desulfacinum subterraneum* (AF385080)	97	δ proteo	High temp Vietnam oil field
2L6SGXO418	0.9	FJ469313	Uncultured *Thermovirga* sp. clone TCB169x (DQ647105)	95	Synergistetes	North Sea oil well
2L6SGXO1151	0.9	FJ469311	Uncultured organism clone ctg_NISA224 (DQ396164) *Shewanella* sp. IS5 (AY967729)	95 95	γ proteo	Deep-sea octacoral Diseased larval rock lobster cultures

2L6SGXO579	0.9	FJ469314	Uncultured clone Niigata-10 (AB243821) *Pelobacter carbinolicus* (CP000142)	99 97	δ proteo	Niigata (Japan) oil well
2L6SGXO407	0.9	FJ469312	*Thermacetogenium phaeum* strain PBT (AB020336)	98	"Clostridia"	Thermophilic anaerobic methanogenic reactor
2L5SGXO560	0.8	FJ469309	*Clostridium* sp. C9 (EU862317)	99	"Clostridia"	Off-shore oil well, India
2L3SGXO643	0.5	FJ469306	*Thermodesulfobacterium commune* DSM 2178 (AF418169)	99	Thermodesulfobacteria	Yellowstone thermal spring
2L3SGXO888	0.5	FJ469307	Uncultured clone Niigata-15 (AB243826)	99	"Clostridia"	Niigata (Japan) oil well
2L2SGXO601	0.3	FJ469304	*Dehalococcoides* sp. CBDB1 (AF230641)	99	Chloroflexi Dehalococcoides	Methanogenic enrichment from Saale river sediment
2L2SGXO622	0.3	FJ469305	Gram-positive thermophile strain ODP159-02 (AY704384)	94	"Clostridia"	Ocean ridge flank crustal fluid
2L1SGXO770	0.2	FJ469299	*Sulfurospirillum* sp. NO3A (AY135396)	99	ε proteo	Coleville (Canada) oil field
2L1SGXO762	0.2	FJ469298	*Thermolithobacter thermoautotrophicus* KA2b (AF282254)	98	Thermolithobacteria	Yellowstone Calcite Springs
2L1SGXO814	0.2	FJ469301	Uncultured *Natronoanaerobium* sp. clone SHBZ503 (EU639010) *Moorella thermoacetica* ATCC 39073 (CP000232)	88 88	"Clostridia"	Thermophilic microbial fuel cell Horse manure
2L1SGXO566	0.2	FJ469295	*Flexistipes* sp. vp180 (AF220344)	98	Deferribacteres	High temperature oil reservoir
2L1SGXO817	0.2	FJ469302	*Desulfotomaculum thermocisternum* strain ST90 (U33455)	99	"Clostridia"	Hot North Sea oil reservoir
2L1SGXO1041	0.2	FJ469292	*Pseudomonas stutzeri* strain 24a97 (AJ312172)	99	γ proteo	Soil beneath filling station
2L1 SGXO442	0.2	FJ469293	Uncultured bacterium clone PL-25B8 (AY570610) *Acetobacterium carbinolicum* (AY744449)	99 99	"Clostridia"	Low-temperature biodegraded Canadian oil reservoir
2L1SGXO751	0.2	FJ469297	Uncultured bacterium clone PL-38B5 (AY570590)	99	"Clostridia" (Anaerovorax , 100%)	Low-temperature biodegraded Canadian oil reservoir
2L1SGXO459	0.2	FJ469294	*Thermosipho africanus* strain Ob7 (DQ647057) *Thermosipho* sp. TBA5 (AF231727)	99 99	Thermotogae	North Sea oil field
2L1SGXO697	0.2	FJ469296	*Thermovirga lienii* Cas60314 (DQ071273)	97	Synergistetes	North Sea oil well
2L1SGXO811	0.2	FJ469300	Uncultured *Thermacetogenium* sp. clone B11_otu13 (DQ097678)	97	"Clostridia"	High temperature Dagang oil field (China)

2L1SGXO1021	0.2	FJ469290	*Thermosipho geolei* (AJ272022)	99	Thermotogae	Siberian oil reservoir
2L1SGXO1038	0.2	FJ469291	Uncultured Spirochaetaceae clone TCB129x (DQ647164) *Spirochaeta* sp. MET-E (AY800103)	99 99		North Sea oil field Congo oil field
1st stage separator: total= 544 sequences, primers 27F, 1391 R. 97% OTUs from DOTUR.						
PS313SGXI1055	57.7	FJ469337	*Thermovirga lienii* Cas60314 (DQ071273)	99	Synergistetes	North Sea oil well
PS74SGXI1247	13.6	FJ469348	*Thermoanaerobacter pseudethanolicus* ATCC 33223 (CP000924) *Thermoanaerobacter* strain X514(CP000923)	99 99	"Clostridia"	Octopus Springs Colorado deep subsurface, iron-reducing
PS39SGXI1143	7.2	FJ469338	*Desulfomicrobium thermophilum* P6.2 (AY464939)	99	δ proteo	Hot spring in Colombia
PS20SGXI1921	3.7	FJ469333	Uncultured bacterium clone Niigata-10 (AB243821) *Pelobacter carbinolicus* DSM 2380 (CP000142)	99 97	δ proteo	Niigata (Japan) oil well
PS10SGXI1101	1.8	FJ469317	*Thermosipho africanus* (DQ647057)	99	Thermotogae	Hot North sea oil field
PS9SGXI1270	1.7	FJ469351	*Thermacetogenium phaeum* (AB020336)	99	"Clostridia"	Thermophilic anaerobic methanogenic reactor
PS8SGXI1894	1.5	FJ469350	*Halomonas meridiana* strain aa-9 (EU652041)	99	γ proteo	Ocean sediment
PS8SGXI1020	1.5	FJ469349	*Halomonas* sp. A-07 (AY347310)	99	γ proteo	Tanzania soda lakes
PS6SGXI1029	1.1	FJ469345	*Thermodesulfobacterium commune* DSM 2178 (AF418169)	99	Thermodesulfo-bacteria	Yellowstone thermal spring
PS6SGXI904	1.1	FJ469347	*Thermotoga petrophila* RKU-1 (CP000702)	99	Thermotogae	Kubiki oil reservoir, Niigata, Japan
PS6SGXI1183	1.1	FJ469346	*Desulfacinum subterraneum* (AF385080)	97	δ proteo	High temp Vietnam oil field
PS5SGXI1002	0.9	FJ469344	Uncultured bacterium clone S25_271 (EF573927) *Marinobacter bacchus* strain FB3 (DQ282120)	99 99	γ proteo	Costa Rica island Evaporation pond of wine wastewater
PS4SGXI1172	0.7	FJ469341	Uncultured *Natronoanaerobium sp.* clone SHBZ503 (EU639010) *Moorella thermoacetica* AMP (AY884087)	87 87	"Clostridia"	Thermophilic microbial fuel cell Methanogenic sludge
PS4SGXI910	0.7	FJ469343	*Delftia acidovorans* SPH-1 (CP000884)	99	β proteo	Sewage treatment plant
PS4SGXI1530	0.7	FJ469342	*Thermotoga naphthophila* RKU-10 (AB027017)	87	Thermotogae	Kubiki oil reservoir, Niigata, Japan
PS4SGXI1065	0.6	FJ469339	*Marinobacter hydrocarbonoclasticus*	99	γ proteo	Middle Atlantic Ridge Sediment

			MARC4F (DQ768638)			
PS3SGXI1245	0.6	FJ469340	Uncultured clone MAT-CR-H3-B03 (EU245152)	85	unclassified	Hypersaline microbial mat, P.R.
PS2SGXI1098	0.4	FJ469335	*Petrotoga siberica* strain SL25T (AJ311702)	99	Thermotogae	Siberian oil reservoir
PS2SGXI1003	0.4	FJ469334	*Desulfotomaculum thermocisternum* (U33455)	99		Hot North Sea oil reservoir
PS2SGXI1381	0.4	FJ469336	Thermotogales TBF19.5.1 (EU980631)	99	Thermotogae	North Sea oil production fluid
PS1SGXI1064	0.2	FJ469318	*Thermosipho geolei.* DSM 13256 (AJ272022)	98	Thermotogae	Siberian oil reservoir
PS1SGXI1111	0.2	FJ469319	Uncultured bacterium clone Zplanct13 (EF602474)	93	unclassified	Zodletone Spring source sediments
PS1SGXI1133	0.2	FJ469320	Uncultured *Sulfurospirillum* sp. clone LA4-B52N (AF513952) *Sulfurospirillum carboxydovorans* (AY740528)	93 93	ε proteo	Hawaiian lake water North Sea sediment
PS1SGXI1244	0.2	FJ469321	*Geotoga aestuarianus* strain T3B (AF509468)	99	Thermotogae	Karst sink hole thiosulfate-reducer
PS1SGXI1249	0.2	FJ469322	*Burkholderia multivorans* strain LMG 13010^{T} (Y18703)	99	β proteo	Cystic fibrosis patient
PS1SGXI1260	0.2	FJ469323	Uncultured bacterium clone: HDBW-WB60 (AB237723)	99	"Clostridia"	Deep subsurface groundwater
PS1SGXI1272	0.2	FJ469324	*Thermovirga lienii* Cas60314 (DQ071273)	97	Synergistetes	North Sea oil well
PS1SGXI1281	0.2	FJ469325	Uncultured *Thermovirga* sp. clone TCB8y	97	Synergistetes	North Sea produced water
PS1SGXI1300	0.2	FJ469326	*Marinobacterium* sp. IC961 strain IC961	99	γ proteo	Carbazole-utilizing bacterium
PS1SGXI1313	0.2	FJ469327	Uncultured bacterium a2b00 (AF419657)	92	unclassifed	Hydrothermal sediments in the Guaymas Basin
PS1SGXI1413	0.2	FJ469328	Uncultured *Thermacetogenium* sp. clone B11_otu13 (DQ097678)	98	"Clostridia"	High temperature Dagang oil field (China)
PS1SGXI1848	0.2	FJ469329	*Pseudomonas putida* W619 (CP000949) *Pseudomonas* sp. OCR2 (AB240201)	100 99	γ proteo	Japan:Shizuoka, Sagara oil field
PS1SGXI1964	0.2	FJ469330	Uncultured *Thermovirga* sp. clone TCB169x (DQ647105)	96	Synergistetes	High temp North Sea oil field
PS1SGXI1984	0.2	FJ469331	Uncultured bacterium gene (AB195893)	96	Bacteroidetes	Anaerobic sludge
PS1SGXI995	0.2	FJ469332	*Desulfotignum balticum* DSM 7044 (AF418176)	99	δ proteo	Marine coastal sediment, Baltic Sea
Coalescer: total # sequences = 329. primers 27F, 1391 R.97% OTUs from DOTUR.						
CO105SHNF	31.9	FJ46935	*Thermovirga lienii*	99	Synergistetes	High temp North

404		2	Cas60314 (DQ071273) Uncultured *Thermovirga* sp. clone TCB8y (DQ647105)	99		Sea oil field High temp North Sea oil field
CO60SHNF483	18.2	FJ469383	*Desulfocaldus* sp. Hobo (EF442977) *Desulfomicrobium thermophilum* P6.2 (AY464939)	99 99	δ proteo δ proteo	Not specified Hot spring in Colombia
CO46SHNF563	14.0	FJ469378	Uncultured bacterium clone: Niigata-10 (AB243821) *Pelobacter carbinolicus* DSM 2380 (CP000142)	99 97	δ proteo	Niigata (Japan) oil well
CO26SHNF710	7.9	FJ469371	*Thermoanaerobacter pseudethanolicus* ATCC 33223(CP000924)	100	"Clostridia"	Octopus Springs
CO12SHNF562	3.6	FJ469354	Uncultured bacterium clone cc187 (DQ057384) Beta proteobacterium B7 AF035053	100 98	β proteo	Chicken intestine Drinking water system
CO11SHNF516	3.3	FJ469353	*Thermodesulfobacterium commune* DSM 2178 (AF418169)	99	Thermodesulfobacteria	Yellowstone thermal spring
CO7SHNF526	2.1	FJ469386	*Stenotrophomonas maltophilia* strain DN1.1 (EU034540) Uncultured bacterium clone Ana10UA-2 (EU499720)	99 99	γ proteo γ proteo	Not specified Freshwater sediment
CO6SHNF422	1.8	FJ469384	*Bradyrhizobium japonicum* strain SEMIA 6164 (AY904765)	99	α proteo	Acacia root nodule
CO6SHNF588	1.8	FJ469385	*Desulfacinum subterraneum* (AF385080)	98	δ proteo	High temp Vietnam oil field
CO5SHNF732	1.5	FJ469382	*Thermacetogenium phaeum* strain PBT (AB020336)	99	"Clostridia"	Thermophilic anaerobic methanogenic reactor
CO5SHNF565	1.5	FJ469380	*Ralstonia pickettii* 12J (CP001069) Uncultured bacterium clone P7X3b4E02 (EU491068)	100 100	β proteo β proteo	Seafloor lavas from the Loi'hi Seamount South Rift X3
CO5SHNF607	1.5	FJ469381	Uncultured clone B5_B4 (EF025213) *Sediminibacterium salmoneum* (EF407879)	100 96	Sphingobacteria	Turkey intestine Eutrophic reservoir
CO4SHNF461	1.2	FJ469379	*Mesorhizobium plurifarium*,strain LMG 10056 (Y14161)	99	α proteo	Tropical tree
CO3SHNF446	0.9	FJ469375	Uncultured *Natronoanaerobium* sp. clone SHBZ503 (EU639010) *Moorella thermoacetica*	88 88	"Clostridia"	Microbial fuel cell Horse manure

			ATCC 39073 (CP000232)			
CO3SHNF573	0.9	FJ469376	Beta proteobacterium A1040 (AF236008) Beta proteobacterium MB7 (AB013409)	99 99	β proteo	Not specified Soil isolate degrading aliphatic polyesters
CO3SHNF586	0.9	FJ469377	*Hyphomicrobium* sp. P2 (AF148858)	99	α proteo	Portuguese soil
CO2SHNF510	0.6	FJ469373	*Thermosipho africanus* (DQ647057) *Thermosipho* sp. TBA5 AF231727	99 99	Thermotogae	Shallow hydrothermal system North sea oil field
CO2SHNF508	0.6	FJ469372	*Solemya velum* symbiont (M90415)	99	γ proteo	Sulfur-oxidizing mollusk symbiont
CO2SHNF712	0.6	FJ469374	*Spirochaeta thermophila* (X62809)	98	Spirochaetes	Kuril Island hot springs
CO1SHNF389	0.3	FJ469355	Uncultured *Hydrogenothermus* sp. clone OPPB154 (AY861874) Aquificales bacterium YNP-SS1 (AF507961)	99 99	Aquificales	Yellowstone Obsidian Pool Yellowstone Calcite Springs
CO1SHNF407	0.3	FJ469356	*Desulfomicrobium norvegicum* strain DSM 1741T (AJ277897)	99	δ proteo	Oslo Harbour water
CO1SHNF410	0.3	FJ469357	*Marinobacter bacchus* strain FB3 (DQ282120)	99	γ proteo	Wine wastewater
CO1SHNF443	0.3	FJ469358	*Thermotoga elfii* strain SM-2 (EU276416)	99	Thermotogae	Not specified Oil-production water
CO1SHFN484	0.3	FJ469359	Uncultured bacterium clone rRNA082 (AY958855) *Solibium* sp. I-32 (AM990455)	98 97	Sphingobacteria	Human vaginal epithelium Ultra pure water
CO1SHNF528	0.3	FJ469360	Uncultured clone B5_F26 (EF025264) Flavobacteria bacterium KF030 (AB269814)	99 94	Sphingobacteria	Turkey intestine Freshwater lake
CO1SHNF536	0.3	FJ469361	Uncultured bacterium clone PS18 (DQ984666) *Syntrophomonas palmitatica* (AB274040)	93 93	"Clostridia"	Sulfate-reducing LCFA enrichment Methanogenic sludge
CO1SHFN544	0.3	FJ469362	Uncultured *Thermacetogenium* sp. clone B11_otu13 (DQ097678)	98	“Clostridia”	High temperature Dagang oil field (China)
CO1SHNF610	0.3	FJ469363	*Desulfovibrio aespoeensis* clone Aspo3 (EU680957) *Desulfovibrio aespoeensis* isolate Aspo2 (X95230)	98	δ proteo	Aespoe hard rock
CO1SHNF612	0.3	FJ469364	*Thermotoga petrophila* RKU-1 (AJ872269)	100	Thermotogae	Kubiki oil reservoir
CO1SHNF615	0.3	FJ469365	Uncultured Termite group 1 bacterium clone HAVOmat14 (EF032762)	99	candidate division TG1	Cyanobacterial mat in Hawaiian lava cave

CO1SHNF622	0.3	FJ469366	Thermotogales bacterium 2SM-2 (EU276414)	100	Thermotogae	Oil-production water
CO1SHNF639	0.3	FJ469367	*Thermolithobacter thermoautotrophicus* clone KA2b (AF282254)	99	Thermolithobacteria	Yellowstone Calcite Springs
CO1SHNF644	0.3	FJ469368	Thermoanaerobacteriaceae clone EV818FW062101BH4MD48 (DQ079638) *Moorella thermoacetica* strain AMP (AY884087)	95 95	"Clostridia"	Terrestrial subsurface fluid-filled fracture Methanogenic sludge
CO1SHNF669	0.3	FJ469369	Uncultured *Anaerovorax* sp. clone C14B-1H (EU073780) Clostridiaceae bacterium FH042 (AB298771)	97 96	"Clostridia"	Coal enrichment culture Anaerobic sludge of a methanogenic reactor
CO1SHNF695	0.3	FJ469370	*Desulfovibrio* sp. X (EF442979) *Desulfovibrio zosterae* (Y18049)	99 95	δ proteo	Not specified Roots of seagrass *(Zostera marina*)
Seawater pig envelope. Total # sequences = 215. primers 27F, 1492R. 97% OTUs from DOTUR						
SW76FGIT720	35.3	FJ469401	*Pseudomonas* sp. HZ06 (AY690706)	99	γ proteo	Rhizosphere soil of salt marshes
SW55FGIT591	25.6	FJ469399	*Pseudomonas stutzeri* strain aa-28 (EU652047)	99	γ proteo	Ocean sediment
SW35FGIT483	16.3	FJ469395	Uncultured proteobacterium clone B01R008 (AY197379)	97	ε proteo *Sulfurimonas*	Guaymas Basin hydrothermal vent sediments
SW12FGIT423	5.6	FJ469387	*Pseudomonas* sp. EP27 (AM403529)	98	γ proteo	Deep-sea sediments
SW9FGIT664	4.2	FJ469403	Uncultured *Arcobacter* sp. clone DS172 (DQ234254)	98	ε proteo *Arcobacter*	Mangrove
SW7FGIT497	3.3	FJ469402	Uncultured bacterium clone W26 (AY770966)	98	γ proteo *Pseudomonas*	Water injection well of Dagang oilfield
SW5FGIT667	2.3	FJ469400	Uncultured *Pseudomonas* sp. clone Lupin-1130m-2-MDA-pse3 (EF205269)	98	γ proteo	Lupin gold mine fracture water
SW3FGIT405	1.4	FJ469396	*Pseudomonas marincola* (AB301071)	96	γ proteo	Deep-sea brittle star
SW3FGIT554	1.4	FJ469397	Uncultured alpha proteobacterium clone 131582 (AY922182)	97	ε proteo *Arcobacter*	Grey whale bone, Pacific Ocean, depth 1674 meters
SW3FGIT592	1.4	FJ469398	Uncultured epsilon proteobacterium clone: NKB11 (AB013263)	96	ε proteo *Sulfurimonas*	Nankai Trough sediments
SW1FGIT389	0.5	FJ469388	"Gamma" proteobacterium IR (AF521582)	99	γ proteo /unclassified	Not specified "Diversity of marine humics-oxidizing bacteria"
SW1FGIT424	0.5	FJ469389	Uncultured bacterium clone B8S-8 (EU652615)	88	δ proteo	Yellow Sea sediment
SW1FGIT462	0.5	FJ46939	*Phaeobacter arcticus*	99	α proteo	Arctic marine

		0	strain 20188 (DQ514304)			sediment
SW1FGIT467	0.5	FJ46939 1	Uncultured bacterium clone GZKB9 (AJ853504)	97	ε proteo *Arcobacter*	Landfill leachate
SW1FGIT501	0.5	FJ46939 2	Uncultured delta proteobacterium clone d13 (AY062878)	98	δ proteo *Desulfuromonas*	Electrode surface
SW1FGIT563	0.5	FJ46939 3	Uncultured bacterium clone P9X2b3A09 (EU491225)	86	δ proteo /unclassified	Seafloor lavas
SW1FGIT660	0.5	FJ46939 4	Uncultured bacterium ARCTIC23_B_12 (EU795085)	99	Flavobacteria *Polaribacter*	Arctic

* most similar sequence and/or isolate in Genbank, as determined by BLASTN.
** Class affiliation, as determined by Classifier (RDP). One sequence from each operational taxonomic unit (OTU) at the 97% level of similarity as defined by DOTUR *(15)* was chosen from among the sequences in that OTU and its taxonomic affiliation and closest GenBank match was determined. Representative sequences are named with the first two letters indicating the sample origin, the following numerals designate the total number of sequences within that particular OTU. The final four letters and 3-4 numbers are the JGI code identifying the sample library and location within the 384-well plate.

Table S2B. Sequence similarity and taxonomic relationships of archaeal representative small subunit partial rRNA gene sequences (OTUs at 97% similarity)

OTU	% of total	Accession number	Most similar sequences (accession no.)	%	Orders	Source
Well 2P: total # sequences =226, primers ARC333 and 958R, 97% OTUs from DOTUR						
2P66FGIP571	29.2	FJ446503	Archaeon enrichment culture clone PW5.2A (EU573152) *Thermococcus alcaliphilus* DSM 10322 (AB055121)	100 100	Thermo-coccales	Ekofisk oil field
2P66FGIP425	29.2	FJ446502	Uncultured archaeon SSE_L4_E01(EU635 901) *Archaeoglobus fulgidus* strain L3 (DQ374392)	99 97	Archaeo-globales	Hot spring sediment
2P64FGIP517	28.3	FJ446501	*Methanothermobacter thermautotrophicus* strain JZTM (EF100758) *M. wolfeii* strain KZ24a (DQ657904)	99 99	Methano-bacteriales	Jiaozhou Bay sediment Dagang oil field
2P26FGIP436	11.5	FJ446499	Uncultured Methanobacteriaceae clone A1m_OTU 3 (DQ097668) *Methanothermobacter thermautotrophicus* strain JZTM (EF100758)	99 96	Methano-bacteriales	Dagang oil field Jiaozhou Bay sediment
2P2FGIP540	0.9	FJ446500	Archaeon enrichment culture clone PW30.6A (EU573155) *Archaeoglobus* sp. NI85-A (AB175518)	99 99	Archaeo-globales	Ekofisk oil field Deep-sea hydrothermal vent chimney
2P1FGIP407	0.4	FJ446497	Uncultured archaeon SSE_L4_E01(EU635 901) *Archaeoglobus fulgidus* strain L3 (DQ374392)	98 97	Archaeo-globales	Hot spring sediment
2P1FGIP710	0.4	FJ446498	Uncultured clone QHO-A15 (DQ785496) *Methanobacterium* sp. F (AB302952)	96 96	Methano-bacteriales	High temperature oil field in China Rice paddy soil
1st stage separator: total # sequences =252 , primers ARC333 and 958R, 97% OTUs from DOTUR						
PS70SGXN402	27.8	FJ446513	*Thermococcus mexicalis* strain GY 869 (AY099181) *Thermococcus sibiricus* (AJ238992)	99 99	Thermo-coccales	Hydrothermal deep-sea vents Siberian high-temperature oil reservoir
PS56SGXN497	22.2	FJ446512	*Archaeoglobus*	99	Archaeo-	

			fulgidus DSM 4304 (AE000782) *Archaeoglobus fulgidus* strain L3 (DQ374392)	99	globales	
PS35SGXN478	13.9	FJ446511	Uncultured archaeon clone NAK1-a1 (DQ867048) Uncultured bacterium clone QHO-A27 (DQ785508) *Methanothermobacter thermautotrophicus* strain JZTM (EF100758)	99 99 96	Methano-bacteriales	High-temperature natural gas field High-temperature petroleum reservoir Jiaozhou Bay sediment
PS29SGXN477	11.5	FJ446509	Uncultured archaeon clone NAK1-a1 (DQ867048) *Methanothermobacter thermautotrophicus* strain JZTM (EF100758)	99 99	Methano-bacteriales	High-temperature natural gas field Jiaozhou Bay sediment
PS22SGXN482	8.7	FJ446508	Methanogenic archaeon ZC-1 (DQ787474) "*Methermicoccus shengliensis*"	99	Methanosarcinales	Oil production water
PS11SGXN711	4.4	FJ446504	Uncultured Desulfurococcales YNP_SSp_A61 (DQ243776) *Staphylothermus achaiicus* (AJ012645)	100 94	Desulfuro-coccales (Cren-archeota)	Yellowstone hot springs Geothermal vents, Greece
PS8SGXN439	3.2	FJ446514	*Thermococcus acidaminovorans* strain DSM 11906 (AY099170) Archaeon enrichment culture clone EA3.5 (EU573147)	100 99	Thermo-coccales	Hydrothermal deep-sea vents, Italy Ekofisk oil field
PS8SGXN687	3.2	FJ446515	Uncultured archaeon clone SSE_L4_H05 (EU635920) *Thermosphaera aggregans* (X99556)	99 99	Desulfuro-coccales (Cren-archaeota)	Nevada hot spring sediment Yellowstone hot spring
PS8SGXN753	3.2	FJ446516	Uncultured archaeon G04b_L4_A09 (EU635911) *Vulcanisaeta distributa* strain IC-065 (AB063639)	99 87	Thermo-proteales (Cren-archaeota)	Nevada hot spring sediment Japan hot spring
PS2SGXN537	0.8	FJ446510	Uncultured crenarchaeote	99	Desulfuro-coccales	Canadian oil sands tailings

			WIP_20m_6B_A (EF420183) "*Desulfurococcus kamchatkensis*" (EU167539)	86	(Cren-archeota)	pond Kamchatka hot spring
PS1SGXN453	0.4	FJ446505	Archaeon enrichment culture clone PW30.6A (EU573155) *Archaeoglobus profundus* (AF297529)	99 98	Archaeo-globales	Ekofisk oil field
PS1SGXN470	0.4	FJ446506	*Thermococcus mexicalis* strain GY 869 (AY099181) *Thermococcus sibiricus* (AJ238992)	99 99	Thermo-coccales	Hydrothermal deep-sea vents Siberian high-temperature oil reservoir
PS1SGXN592	0.4	FJ446507	Archaeon enrichment culture clone PW15.7A (EU573156) *Archaeoglobus profundus* (AF297529)	99 90	Archaeo-globales	Ekofisk oil field
Coalescer: total # sequences = 240, primers ARC333 and 958R, 97% OTUs from DOTUR						
CO146FGIO3	60.8	FJ446519	Uncultured archaeon SSE_L4_E01(EU635901) Archaeon enrichment culture clone EA8.8 (EU573151) *Archaeoglobus fulgidus* strain L3 (DQ374392)	99 98 98	Archaeo-globales	Hot spring sediment Ekofisk oil field
CO64FGIO506	26.7	FJ446522	*Thermococcus alcaliphilus* DSM 10322 (AB055121) Archaeon enrichment culture clone PW5.2A (EU573152)	100 99	Thermo-cocales	 Ekofisk oil field
CO12FGIO387	5.0	FJ446518	Uncultured archaeon SSE_L4_E01(EU635901) *Ferroglobus placidus* (AF220166)	96 96	Archaeo-globales	Hot spring sediment
CO10FGIO395	4.2	FJ446517	Archaeon enrichment culture clone PW30.6A (EU573155) *Archaeoglobus profundus* (AF297529)	99 98	Archaeo-globales	Ekofisk oil field

CO6FGIO519	2.5	FJ446523	Uncultured *Methanothermobacter* sp. clone ARCA-3F (EU073827) *Methanothermobacter thermautotrophicus* strain JZTM (EF100758) *Methanothermobacter wolfeii* strain KZ24 (DQ657904)	99 99 99	Methano-bacteriales	Coal enrichment culture Jiaozhou Bay sediment Dagang oil field
CO1FGIO425	0.4	FJ446520	Archaeon enrichment culture clone PW30.6A (EU573155) *Ferroglobus placidus* (AF220166)	96 96	Archaeo-globales	Ekofisk oil field
CO1FGIO557	0.4	FJ446521	Uncultured crenarchaeote Clone MDS-r-E06 (AB353218) "*Desulfurococcus kamchatkensis*" strain 1221n (EU167539)	98 86	Desulfuro-coccales (Cren-archaeota)	Mesophilic digested sludge Kamchatka hot spring

Table S3. Measures of genetic diversity and species richness.

Sample code	# clones	# 99.9% OTUs (%)	# 97% OTUs (%)	S_{Chao1} (97%) (95% CI)	ACE (97%) (95% CI)	H (97%)	S (97%)	% library coverage (97%)
SWBAC	215	91	17	38 (22.1-103.3)	27.2 (19.4-59.8)	1.86 (1.71-2.01)	0.2205	97.2
2PBAC	334	35	9	10 (9.09-19.7)	13.9 (9.8-36.8)	0.47 (0.36-0.59)	0.8139	99.2
2LBAC	648	280	28	54 (34.8-127.2)	45.8 (33.7-83.7)	1.21 (1.08-1.34)	0.5474	98.0
PSBAC	544	267	35	61.3 (42.4-127.5)	53.2 (41.5-86.2)	1.76 (1.63-1.91)	0.3567	97.2
COBAC	329	60	35	65 (43.7-138.2)	56.1 (42.4-95.0)	2.36 (2.21-2.51)	0.1633	95.1
2PARC	226	82	7	7.5 (7.0-15.3)	10 (7.5-25.4)	1.41 (1.34-1.49)	0.2608	99.1
PSARC	252	83	13	14.5 (13.2-28.1)	15.9 (13.4-33.5)	2.00 (1.89-2.10)	0.1684	98.8
COARC	240	63	7	8 (7.07-20.8)	9.2 (7.3-26.5)	1.07 (0.95-1.20)	0.4418	99.2

SWBAC: Bacterial 16S rRNA library from seawater pig envelope sample.
2PBAC: Bacterial 16S rRNA library from well 2P sample.
2LBAC: Bacterial 16S rRNA library from well 2L sample.
PSBAC: Bacterial 16S rRNA library from 1st stage separator sample.
COBAC: Bacterial 16S rRNA library from coalescer sample.
2PARC: Archaeal 16S rRNA library from well 2P sample.
PSARC: Archaeal 16S rRNA library from 1st stage separator sample.
COARC: Archaeal 16S rRNA library from coalescer sample.
OTUs, SChao1 (97%), ACE (97%), H (Shannon-Weaver), S (Simpson's index) were estimated using DOTUR *(19)*.
% library coverage was estimated by the method of Good *(20)* for sequences at 97% similarity.
95% CI: 95% confidence interval values.

Table S4. Core and dominant bacterial OTUs (97% similarity) and physiologies.

OTU closest match (% similarity)	%*	Physiology of closest match	2L	2P	PS	CO	SW	*Total #*
Bacteria: Core 2L, PS, CO								
Thermovirga lienii (DQ071273)	99	[22***] Fermentative, H_2S from cystine/S^o	540**	17	313	105	0	*975*
Desulfomicrobium thermophilum (AY464939)	98	[25]SRB	27	0	39	60	0	*126*
Thermosipho africanus/geolei (DQ647057/AJ272022)	99	[SR1]H_2S from S^O	10	0	11	2	0	*23*
Uncultured clone/*Pelobacter carbinolicus* (AB243821/CP000142)	97	Fermentative, Syntroph, [SR1]H_2S from cystine/S^O, IR	6	0	20	46	0	*72*
Desulfacinum subterraneum (AF385080)	97	[SR2]SRB	7	0	6	6	0	*19*
Thermodesulfobacterium commune (AF418169)	98	[SR2]SRB	3	0	6	11	0	*20*
Thermoanaerobacter pseudethanolicus/X514 (CP000924/CP000923)	99	[SR3]Fermentative, IR (some strains), thiosulfate reduction	14	2	74	26	0	*116*
Thermacetogenium phaeum (AB020336)	98	[31]Acetogen, Syntroph, Sulfate/thiosulfate reduction	6	0	9	5	0	*20*
Uncultured *Thermacetogenium* clone B11_otu13 (DQ097678)	97	Uncultured	1	0	1	1	0	*3*
Moorella thermoacetica (AY884087)	86	Fermentative	1	0	4	3	0	*8*
Bacteria: dominant 2P								
Petrobacter sp. DM-3 (DQ539621)	99	[33]Fermentative NR	0	327	0	0	0	*327*
Bacteria: dominant SW								
Pseudomonas stutzeri/putida	*98*	*[SR4]NR- and/or HC-degrading (some)*	*1*	*0*	*1*	*0*	*143*	*145*

*: % similarity; **: number of sequences per sample; ***: reference; SRB: sulfate-reducing bacteria; IR: iron reduction; NR: nitrate reduction

Table S5. Dominant archaeal OTUs (97% similarity) and physiologies.

OTU closest match (% similarity)	%*	Physiology of closest match *(25)*	2P	PS	CO	Total #
Archaea: Core 2P, PS, CO						
Archaeoglobus fulgidus DSM 4304/L3 (AE000782/DQ374392)	98	SRA	66**	56	158	280
Thermococcus alcaliphilus/mexicalis/sibiricus (AB055121/AY099181/AJ238992)	99	Fermentative, H_2S from S^O, IR (*T. sibiricus*)	66	78	64	208
Methanothermobacter thermautotrophicus (EF100758)	99	Methanogen H_2-utilizing	64	29	6	99
Uncultured Methanobacteriaceae clone A1m_OTU 3 (DQ097668)	99	Uncultured, from Dang oil field	26	35	0	61
Archaeoglobus profundus (AF297529)	98	SRA	2	1	11	14

*: % similarity; **: number of sequences per sample; SRA: sulfate-reducing archaea; IR: iron reduction

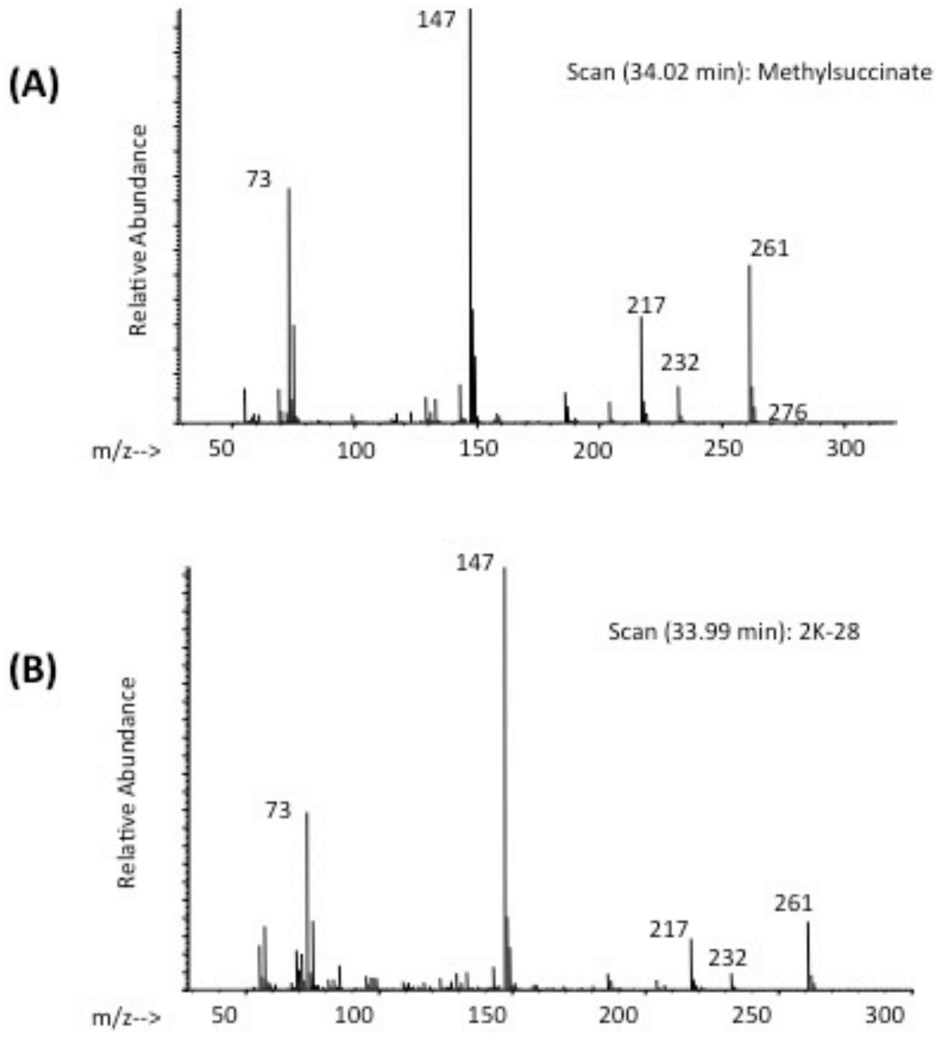


Figure S1. Mass spectral profile and retention time for the TMS-derivatized authentic standard of methylsuccinate (A) and a putative metabolite detected at the same retention time in sample 2K-28 (B). Features common to both mass spectra and used for identification include m/z 261 (loss of a methyl group or M^{+}-15), 232, 217, 147 and 73.

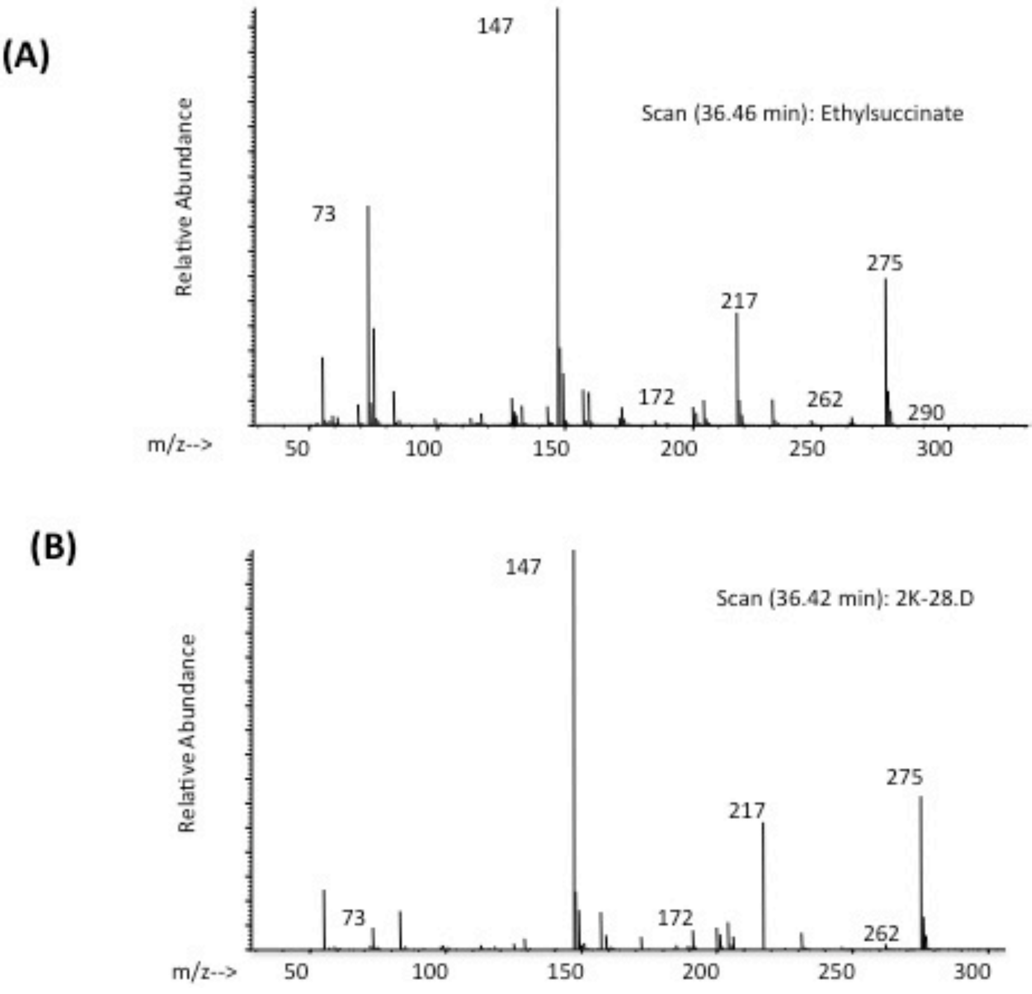


Figure S2. Mass spectral profile and retention time of the TMS-derivatized authentic standard ethylsuccinate (A) and a putative metabolite detected at the same retention time in sample 2K-28 (B). Features common to both mass spectra and used for identification include m/z 275 (M^+-15), 262, 217, 172, 147 and 73.

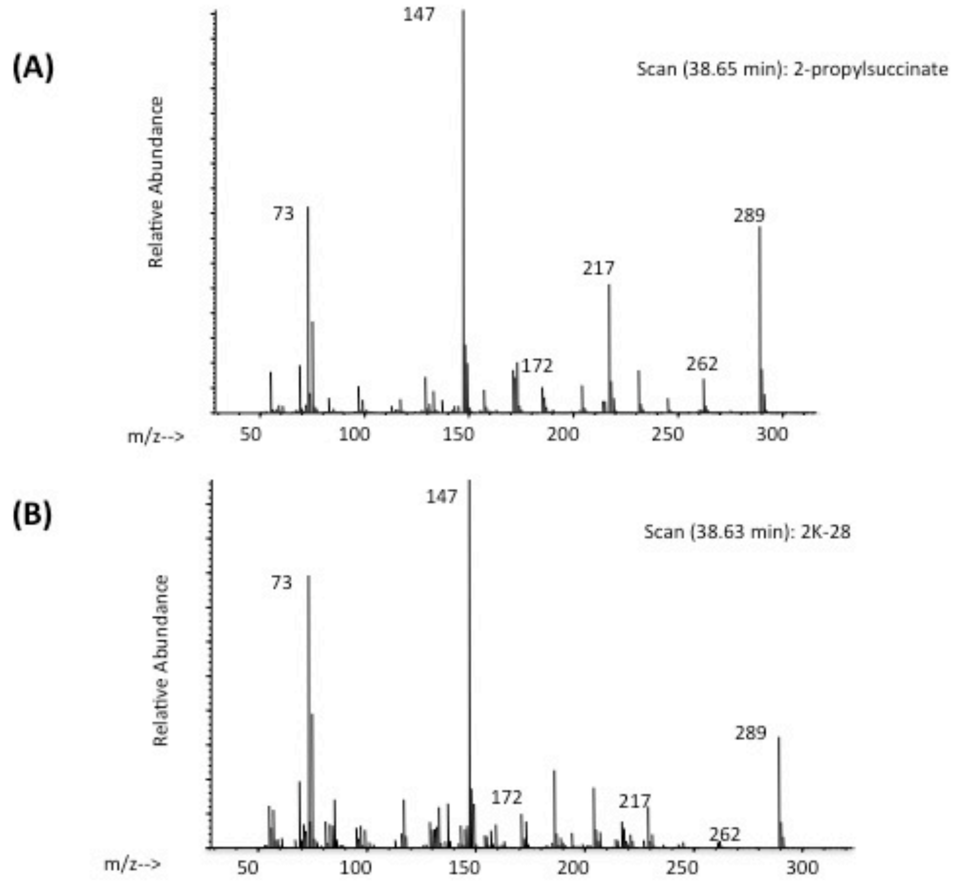
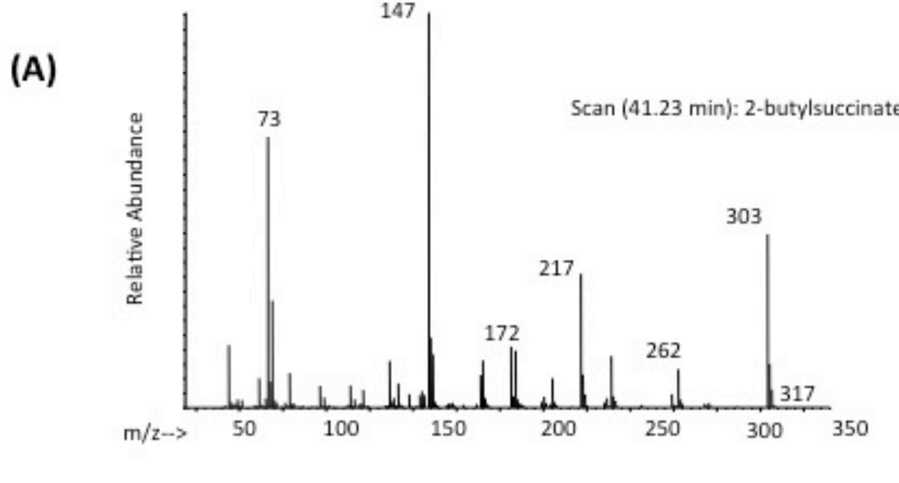


Figure S3. Mass spectral profile and retention time for the TMS-derivatized authentic standard 2-propylsuccinate (A) and a putative metabolite detected at the same retention time in sample 2K-28 (B). Features common to both mass spectra and used for identification include m/z 289 (M^+-15), 262, 217, 172, 147 and 73.



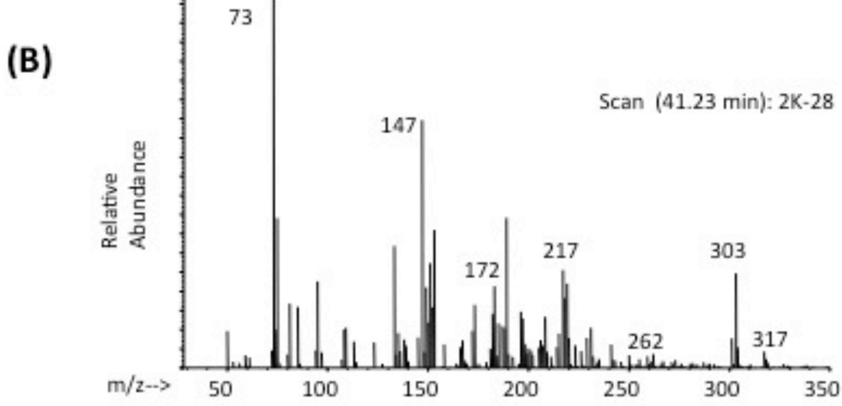


Figure S4. Mass spectral profile and retention time for the TMS-derivatized authentic standard 2-butylsuccinate (A) and a putative metabolite detected at the same retention time in sample 2K-28 (B). Features common to both mass spectra and used for identification include m/z 303 (M^{+}-15), 262, 217,172, 147 and 73.

Supplemental References (SR) Not Cited in Chapter 2

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CHAPTER 3

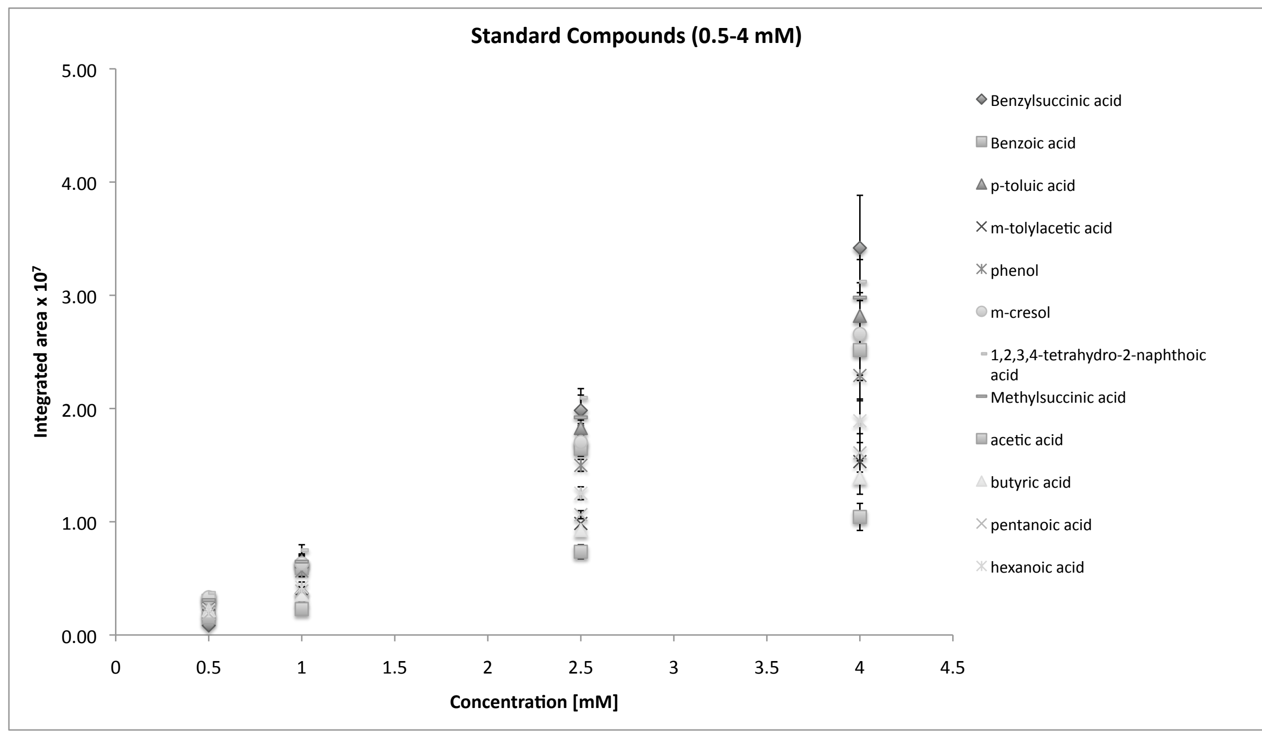

Figure S1. Standard curves for selected metabolic intermediates used in the metabolite-profiling assay. Injections for each standard concentration (0.5-4.0 mM) were performed in triplicate and reported as an average. Error bars on the y-axis represent the standard deviation.

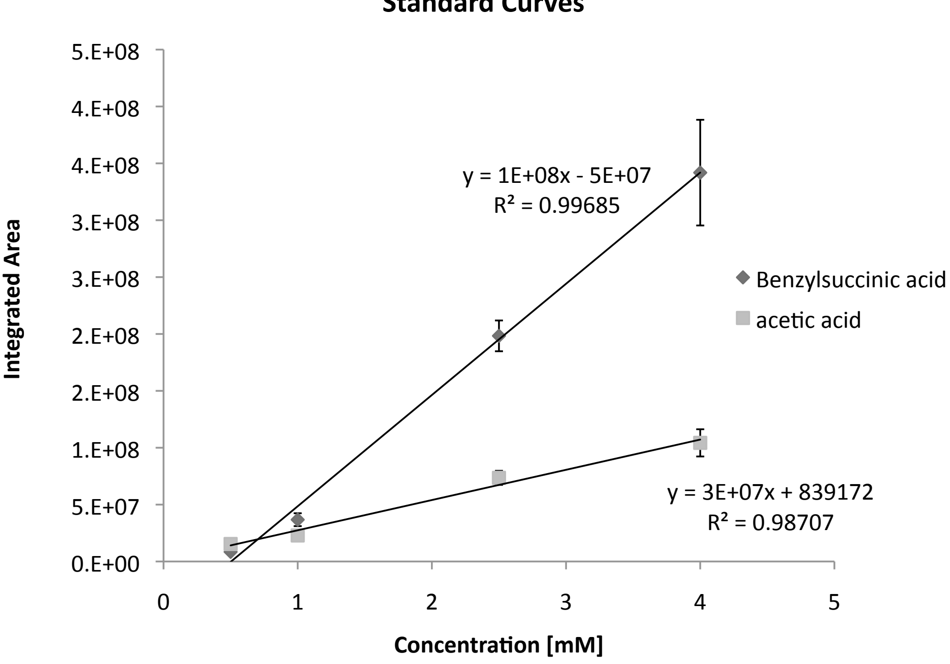

Figure S2. Standard curves for the selected metabolic intermediates showing linear regression and R^2 values. Benzylsuccinate and acetate were selected for their response factor in the GC-MS as the highest and lowest response respectively. Acetate is just slightly under an order of magnitude lower than benzylsuccinate.