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MICROORGANISMS FROM ANAEROBIC, GAS CONDENSATE-CONTAMINATED SEDIMENTS THAT DEGRADE ACETATE, BUTYRATE, AND PROPIONATE UNDER METHANOGENIC AND SULFATE-REDUCING CONDITIONS

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MICROORGANISMS FROM ANAEROBIC, GAS CONDENSATE-CONTAMINATED SEDIMENTS THAT DEGRADE ACETATE, BUTYRATE, AND PROPIONATE UNDER METHANOGENIC AND SULFATE-REDUCING CONDITIONS

A DISSERTATION APPROVED FOR THE
DEPARTMENT OF BOTANY AND MICROBIOLOGY

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TABLE OF CONTENTS

Acknowledgements……..IV

Table of Contents…….VI

List of Tables……..VIII

List of Illustrations……...IX

ABSTRACT…….....X

CHAPTER 1. Literature Review

Text……..1

References……..16

CHAPTER 2. Evidence for Aceticlastic Methanogenesis in the Presence of Sulfate in a Gas Condensate-Contaminated Aquifer

Abstract……..23

Introduction……24

Materials and Methods……...28

Results……37

Discussion……..43

References……..47

CHAPTER 3. Syntrophic Metabolism Under Sulfate-Reducing Conditions in a Hydrocarbon-Contaminated Aquifer.

Abstract……...52

Introduction…….54

Materials and Methods……59

Results…….72

Discussion……...90

References……...96

APPENDIX 1. Propionate Degradation by Sulfate-Reducing Bacteria in a Gas Condensate-Contaminated Aquifer.

Abstract…….102

Introduction……...104

Materials and Methods……..109

Results……...119

Discussion…….131

References…….137

LIST OF TABLES

Page

Table 1.1 Isolated and characterized microorganisms that are capable of syntrophic growth on acetate, butyrate, and propionate. 4

Table 1.2 Isolated and characterized sulfate-reducing bacteria that utilize acetate, butyrate, and propionate in pure culture. 5

Table 2.1 Summary of MPN results obtained using acetate as a substrate. 38

Table 2.2 Fate of acetate carbon in microcosms from the contaminated site. 40

Table 3.1 Gibbs free energy changes for idealized reactions involved in the degradation of butyrate under sulfate-reducing and methanogenic conditions. 56

Table 3.2 Most probable number of different types of butyrate-degraders in Fort Lupton aquifer sediments. 73

Table 3.3 Butyrate degradation in microcosms with and without the addition of sulfate, 2-bromethanesulfonic acid, and molybdate. 74

Table 3.4 OTU sequences from clone libraries prepared with DNA from MPNs inoculated with Fort Lupton Sediment. 86

Table 3.5 OTU sequences from clone libraries prepared with DNA from Fort Lupton contaminated sediment. 89

Table A1.1 Summary of MPN results obtained using propionate as a substrate. 120

Table A1.2 Fate of propionate in microcosms from the contaminated sediment. 127

LIST OF ILLUSTRATIONS

Page

Figure 2.1 Evolutionary distance tree showing the relationship of each OTU found in contaminated sediments and DGGE band 1 from acetate enrichments with other members of the *Archaea*. 41

Figure 3.1 Restoration of butyrate degradation in BESA-inhibited microcosms with the addition *Desulfovibrio vulgaris* G11 and sulfate. 77

Figure 3.2 Effect of CO on butyrate metabolism in sulfate-reducing microcosms. 79

Figure 3.3 Effect of N_2 on butyrate metabolism in sulfate-reducing microcosms. 81

Figure 3.4 Denaturing gradient gel electrophoresis analysis of MPN tubes with sulfate from Ft. Lupton contaminated sediments. 87

Figure A1.1 The 16S rRNA gene sequence of CS7 125

Figure A1.2 PCR amplification of DNA from MPNs, microcosms, and contaminated sediment with *Desulfobulbus* specific 16S rRNA primers, DBB121 and DBB1237. 127

ABSTRACT

Acetate, butyrate, and propionate, are important intermediates that are produced as a result of the anaerobic degradation of organic matter in methanogenic and sulfate-reducing ecosystems. Despite the importance of these compounds in methanogenic and sulfate-reducing ecosystems, it is often unclear what populations of microorganisms are involved in the degradation of acetate, butyrate, and propionate. In order to understand the microbial populations involved, the anaerobic metabolism of these fatty acids was studied using sediments and groundwater from a gas condensate-contaminated aquifer near Denver, Colorado. This particular site was chosen for this study because previous work showed that methanogenesis and sulfate reduction were important terminal-electron accepting processes at this site.

Most probable number (MPN) dilutions with acetate indicated that there was no significant difference in the number of acetate degraders under methanogenic and sulfate-reducing conditions at this site. Acetate loss was coupled to methane production in all MPN dilution tubes regardless of whether sulfate was present or not. Higher quantities of $^{14}CH_4$ than $^{14}CO_2$ were observed in microcosms that contained either $^{14}CH_3COOH$ or $^{14}CH_3{}^{14}COOH$ in the presence or absence of sulfate. This $^{14}CH_4$ accounted for 70-100% of the total labeled gas in these [^{14}C] acetate microcosms regardless of whether sulfate was present or not. Denaturing gradient gel electrophoresis (DGGE) of the acetate microcosms both

with and without sulfate using *Archaea*-specific primers showed that identical predominant bands, which had 99% sequence similarity to acetate-degrading methanogens from the family *Methanosaetaceae,* were present in all of these microcosms. Analysis of clone libraries of archaeal 16S rRNA gene sequences amplified from sediments collected in the contaminated portion of the aquifer showed that 180 of the 190 sequenced clones were similar to acetate-using methanogens from the family *Methanosaetaceae*.

The most probable number of syntrophic butyrate-degraders (MPNs that were amended with *Methanospirillum hungatei* or *Desulfovibrio vulgaris* strain G11) was similar to the number of sulfate-reducing, butyrate-degraders (MPNs with sulfate but without a hydrogen-user). Butyrate loss was coupled to methane production in butyrate-amended microcosms without sulfate, and to sulfate reduction in microcosms amended with butyrate and sulfate. The addition of 2-bromoethanesulfonic acid (BESA) inhibited butyrate degradation in methanogenic microcosms, which was restored upon the addition of a hydrogen-using sulfate reducer and 5 mM sulfate, but not when only 5 mM sulfate was added. The addition of carbon monoxide, which inhibits hydrogenases, to the headspace of sulfate-reducing microcosms inhibited butyrate metabolism and caused the hydrogen partial pressure to increase to levels that would make syntrophic butyrate degradation thermodynamically unfavorable (-5 to +3 kJ mol^{-1} of butyrate). Inhibition of butyrate metabolism was not observed in control microcosms with butyrate and sulfate that were amended with nitrogen gas. Approximately thirty

percent of the 16S rRNA gene sequences in clone libraries from the MPN cultures grouped with members of the *Syntrophomonadaceae*. DGGE analysis of butyrate enrichments with sulfate detected an identical predominant band whose sequence was closely related to butyrate-degraders from the family *Syntrophaceae*. 16S rRNA sequences related to the *Syntrophaceae* were also present in clone libraries prepared from the contaminated sediment. 16S rRNA sequences related to *Desulfovibrio* accounted for 75% of the total number of sequences affiliated with sulfate reducers in clone libraries from MPN cultures.

Propionate was indirectly degraded to acetate and carbon dioxide in anoxic sediments and groundwater from a hydrocarbon-contaminated aquifer where geochemical evidence implicated sulfate reduction and methanogenesis as the predominant terminal electron-accepting processes. The most probable number of propionate-degraders from hydrocarbon-contaminated sediments was significantly higher ($p > 0.05$) in cultures with propionate and sulfate that contained hydrogen-using microorganisms compared to cultures with propionate and sulfate but without the hydrogen-user added, suggesting that syntrophic propionate degraders were more numerous than sulfate-reducing propionate degraders. However, propionate degraders were not detected in MPNs that contained propionate and a hydrogen-using methanogen, but were not amended with sulfate. A new propionate-degrading, sulfate-reducing bacterium, with less than 96% sequence similarity to all described *Desulfobulbus* spp., was isolated from MPN enrichments that contained propionate and sulfate. Propionate loss by the pure culture and in

microcosms with propionate and sulfate was coupled to sulfate loss and acetate accumulation. Acetate was converted to methane by aceticlastic methanogens in microcosms with propionate and sulfate. 16S rRNA gene sequences related to propionate-degrading, sulfate reducers from the genus *Desulfobulbus* were detected in all MPNs with sulfate, all propionate-degrading microcosms except those with molybdate added, and the contaminated sediments by using group specific PCR primers. *Desulfobulbus* sequences accounted for approximately four percent of the total 16S rRNA genes sequences in clone libraries prepared with DNA from the contaminated sediment. Sequences related to microorganisms capable of syntrophic propionate degradation were not detected in sediment clone libraries. This work shows that sulfate reduction was the dominant fate of propionate at this site and suggests that a new species of *Desulfobulbus* was involved in propionate degradation at this site.

The results of the work presented in this dissertation showed that aceticlastic methanogenesis and syntrophic metabolism can occur in sulfate-reducing ecosystems. These results are surprising since kinetic and thermodynamic comparisons of isolated species of aceticlastic methanogens, syntrophic microorganisms, and sulfate-reducing bacteria suggest that sulfate reducers should dominate in sulfate-reducing ecosystems. However, this kinetic and thermodynamic information is based on a relatively small number of isolates. The results of this study suggest that this kinetic and thermodynamic information cannot

always be used to predict what microorganisms are involved in the degradation of acetate, butyrate, and propionate in contaminated aquifers.

CHAPTER 1

Literature Review

Methanogenesis and sulfate reduction are important terminal electron-accepting processes in many anaerobic ecosystems including freshwater sediments, marine sediments, digesters, and petroleum-contaminated aquifers (2, 10, 11, 29, 35, 38, 39, 44, 46, 48, 56). Previous work has shown that fatty acids are produced in methanogenic and sulfate-reducing ecosystems as a result of the anaerobic degradation of organic matter (2, 29, 33, 35, 56). Acetate, butyrate, and propionate are typically the dominant fatty acids produced in methanogenic and sulfate-reducing ecosystems (2). In freshwater sediments, marine sediments, and digesters complex polymeric substrates such as proteins, polysaccharides, lipids, and nucleic acids are hydrolyzed and fermented to acetate, butyrate, and propionate by fermentative bacteria (29, 33, 35, 56). The exact source of these fatty acids in petroleum-contaminated aquifers is less clear, but the results of several studies suggest that acetate, butyrate, and propionate are produced as a result of the *in situ* biodegradation of hydrocarbons (10, 11, 53). Microorganisms from several different metabolic groups have been isolated and described that are capable of degrading these fatty acids under methanogenic and sulfate-reducing conditions

(13, 17, 20, 32, 42, 61, 62). To date, most of our knowledge concerning the microbial processes and microorganisms that are involved in the degradation of these fatty acids in methanogenic and sulfate-reducing ecosystems is based on information obtained from these isolates.

Several groups of microorganisms play a role in the degradation of acetate, butyrate, and propionate in methanogenic ecosystems (13, 17, 20, 32, 61, 62). Syntrophic bacteria have been isolated and described that are capable of degrading butyrate and propionate to acetate, CO_2, H_2, and formate in methanogenic ecosystems (Table 1.1) (32). The degradation of butyrate and propionate by syntrophic microorganisms alone is thermodynamically unfavorable due to H_2 and formate production (32, 33). Therefore, interspecies hydrogen/formate transfer from hydrogen/formate-producing syntrophic microorganisms to hydrogen/formate-using methanogens is required in order for the degradation of butyrate and propionate to become energetically favorable (32, 33). Syntrophic butyrate- and propionate-degraders are phylogenetically diverse and group with members of the low G+C Gram positives, the phylum *Synergistetes*, and the *Deltaproteobacteria* (23, 32). Syntrophic bacteria from the genera *Syntrophobacter* and *Desulfobacterium* can function as propionate degrading-sulfate reducers in ecosystems with sulfate, but are also capable of growing in syntrophic association with hydrogen-using methanogens in the absence of sulfate (5, 32, 40). Acetate that is produced in methanogenic ecosystems can be converted to CH_4 and CO_2 by a couple of different groups of microorganisms. Pure cultures

of aceticlastic methanogens from the genera *Methanosaeta* and *Methanosarcina* have been described, that are capable of degrading acetate to CH_4 and CO_2 in methanogenic ecosystems (13, 17, 61). Acetate can also be degraded to CH_4 and CO_2 in these ecosystems by consortia of hydrogen-producing syntrophic acetate-degraders (Table 1.1) and hydrogen-using methanogens (20).

Several pure cultures of sulfate-reducing bacteria have been described that degrade propionate and butyrate either completely to CO_2 or incompletely to acetate and CO_2 when sufficient sulfate is available (Table 1.2) (35, 42, 59). Pure cultures of sulfate reducers have also been described that degrade acetate to CO_2 in sulfate-reducing ecosystems (Table 1.2) (35, 42, 59). The results of these pure culture studies suggest that sulfate-reducing bacteria should be able to completely mineralize acetate, butyrate, and propionate to CO_2 in sulfate-reducing ecosystems (35, 42, 48, 59). These fatty acid-degrading, sulfate-reducing bacteria will compete with aceticlastic methanogens and syntrophic acetate-, butyrate-, and propionate-degraders for available acetate, butyrate, and propionate in ecosystems where sulfate is present (35, 39, 43, 49, 50, 54, 55). Several studies have compared the kinetic and thermodynamic properties of pure cultures of acetate-using sulfate-reducing bacteria and acetate-using methanogens (39, 49, 50). This work showed that acetate-using sulfate reducers had a lower k_m, a higher v_{max}, and a lower threshold for acetate than aceticlastic methanogens (39, 49, 50). Studies have also compared the kinetic properties of sulfate reducers and syntrophic microorganisms

TABLE 1.1. Described microorganisms that are capable of syntrophic growth with acetate, butyrate, and propionate. [1]

Genus	Acetate	Propionate	Butyrate
Syntrophomonas	- [3]	-	+ [3]
Thermosyntropha	-	-	+
Syntrophothermus	-	-	+
Algorimarina	-	-	+
Syntrophus	-	-	+
Syntrophobacter[2]	-	+	-
Desulfotomaculum[2]	-	+	-
Smithella	-	+	+
Pelotomaculum	-	+	-
Clostridium	+	-	-
Thermoacetogenium	+	-	-
Candidatus Contubernalis	+	-	-

[1]All of the data presented in this table was obtained and modified from McInerney *et al* (32).
[2]Syntrophic microorganisms from these genera can function as sulfate reducers in the presence of sulfate.
[3]Symbols: +, positive for growth; -, negative for growth.

TABLE 1.2. Described sulfate-reducing bacteria that utilize acetate, butyrate, and propionate in pure culture.

Genus	Acetate	Propionate	Butyrate	Reference
Desulfobulbus	- [1]	+ [1]	-	42
Desulfobacter	+	-	-	42
Desulfobacterium	+	+	+	42
Desulfococcus	+	+	+	42
Desulfosarcina	+	+	+	42
Desulfonema	+	+	+	42
Desulfobotulus	-	-	+	42
Desulfoarculus	+	+	+	42
Desulfotomaculum	+	+	+	42
Desulforhopalus	-	+	NT [2]	42
Desulforhabdus	+	+	+	42
Desulfofustis	+	+	+	42
Desulfocella	-	-	+	42
Desulfobacca	+	-	-	42
Desulfuromusa	+	+	+	42
Desulfobacula	+	-	-	42
Desulfosporosinus	-	-	+	42
Desulfoglaeba	-	-	+	15
Desulfatirhabdium	+	+	+	3
Desulfoluna	-	-	+	51
Desulfohalobium	-	+	+	22
Desulfatiferula	-	-	+	12
Desulfotignum	+	NT	+	37
Thermodesulforhabdus	+	-	+	42
Desulfacinum	+	+	+	42
Desulfurispora	-	-	+	24
Desulfovirgula	-	-	+	25
Desulfofaba	-	+	+	42

[1] Symbols: +, positive for growth; -, negative for growth
[2] Abbreviations: NT, compound not tested as a growth substrate.

that degrade butyrate and propionate (39, 49, 50). These studies showed that most butyrate- and propionate-degrading sulfate-reducing bacteria had faster growth rates on butyrate or propionate compared to syntrophic microorganisms (39, 49, 50). Based on these findings it is expected that fatty acid-degrading sulfate-reducing bacteria will consume acetate, butyrate, and propionate in ecosystems where sulfate is available (39, 43, 49, 50).

Numerous studies have monitored the fate of acetate, butyrate, and propionate in ecosystems where sulfate was present (2, 4, 6, 21, 30, 38, 44, 48, 60). The majority of these studies showed that sulfate reduction was the dominant fate of acetate, butyrate, and propionate in these ecosystems (2, 4, 6, 30, 44, 48). These findings along with the data from the kinetic and thermodynamic comparisons of sulfate-reducing bacteria, syntrophic bacteria, and aceticlastic methanogens described above, has led to the assumption that sulfate-reducing bacteria are responsible for the degradation of acetate, butyrate, and propionate in sulfate-reducing ecosystems. However, the results from several studies suggest that sulfate-reducing bacteria may not always be responsible for the consumption of these fatty acids in sulfate-reducing ecosystems (21, 38, 44, 49, 55, 60). A few studies that monitored the fate of acetate in anaerobic digesters with excess sulfate showed that acetate metabolism was coupled to methanogenesis rather than sulfate reduction (21, 38, 55, 60). Molecular studies have also observed 16S rRNA sequences related to aceticlastic methanogens from the genus *Methanosaeta* in

many anaerobic ecosystems where sulfate is present including digesters, hydrocarbon-contaminated sediments, brackish sediments, marine sediments, and oil storage tanks (1, 16, 38, 41, 57). The results suggest that aceticlastic methanogenesis may be an important process in many sulfate-reducing ecosystems.

Several studies have observed butyrate and propionate loss coupled to sulfate loss or sulfide production in a variety of sulfate-reducing ecosystems (2, 4, 6, 37, 44, 48). Most of these studies have used process-based measurements, such as sulfate loss and sulfide production measurements, or sodium molybdate, which inhibits sulfate-reducing bacteria, to show that sulfate reduction was involved in the degradation of butyrate and propionate in these ecosystems (2, 4, 6, 38, 44, 48). Even though these methods can be used to show that sulfate reduction plays an important role in the degradation of butyrate and propionate in sulfate-reducing ecosystems, they do not provide definitive proof that butyrate- and propionate-degrading sulfate reducers are involved in the degradation of butyrate and propionate. Many sulfate reducers that utilize hydrogen or formate have also been isolated and described (35, 42, 59). Thus, it is possible that syntrophic consortia of hydrogen- and formate-producing fatty acid degraders and hydrogen/formate-using sulfate reducers could catalyze butyrate and propionate degradation in sulfate-reducing ecosystems. Such an observation may not be surprising since hydrogen/formate-using sulfate reducers have commonly been used as the syntrophic partner to obtain syntrophic butyrate- and propionate-degraders in monoxenic culture (5, 34). Sulfate loss measurements, sulfide production

measurements, and molybdate cannot be used in sulfate-reducing ecosystems to distinguish between butyrate or propionate degradation that is carried out directly by fatty acid-degrading sulfate reducers, or indirectly by consortia of syntrophic butyrate- or propionate-degraders and hydrogen-using sulfate reducers, since the results will be similar. Therefore, it is not clear if syntrophic butyrate- and propionate-degrading consortia play a major role in the degradation of butyrate or propionate in sulfate-reducing ecosystems.

Several studies have provided indirect evidence, which suggests that syntrophic metabolism may play an important role in the degradation of butyrate and propionate in sulfate-reducing ecosystems. Most probable number (MPN) enumeration studies observed similar numbers of butyrate-degraders in the presence and absence of sulfate in UASB reactors that contained excess sulfate (55). Butyrate loss was coupled to sulfate loss in a hydrocarbon-contaminated aquifer where sulfate was present (28). However, only a portion of the butyrate that was lost at this site could be attributed to sulfate reduction, which is surprising since sulfate reduction is typically the dominant terminal electron-accepting process in hydrocarbon-contaminated aquifers (28, 58). Elevated levels of methane were observed in the portion of the aquifer that was sampled, which suggested that syntrophic butyrate metabolism was occurring (28). The microorganisms that were responsible for butyrate degradation in this hydrocarbon-contaminated aquifer were not identified (28). However, molecular studies from other hydrocarbon-contaminated aquifers have observed 16S rRNA sequences that are related to

Syntrophus spp., which are capable of degrading butyrate in syntrophic association with hydrogen-using sulfate reducers (1, 16, 27). Studies conducted with propionate-degrading syntrophs from the genus *Syntrophobacter* suggest that syntrophic propionate metabolism may also occur in ecosystems where sulfate is present. These studies showed that *Syntrophobacter* spp., which are capable of growing on propionate and sulfate in pure culture, prefer to grow syntrophically with hydrogen-using sulfate reducers from the genus *Desulfovibrio* when propionate and sulfate are present (49). The results from molecular studies carried out in digesters that were used to treat papermill wastewater also suggest that syntrophic consortia of *Syntrophobacter* and *Desulfovibrio* may be involved in the degradation of propionate in sulfate-reducing ecosystems (38, 44). Analysis of this wastewater indicated that it was sulfate-rich and contained high concentrations of propionate (38, 44). Enrichment cultures that actively coupled the degradation of propionate to the reduction of sulfate were obtained from this digester (44). Clone libraries from these enrichment cultures were dominated by 16SrRNA sequences related to *Syntrophobacter* spp., but also contained sequences related to *Desulfovibrio* spp. (44). However, it was never determined if propionate degradation was carried out directly by *Syntrophobacter* spp. or indirectly by syntrophic consortia of *Syntrophobacter* spp. and *Desulfovibrio* spp. in these digesters (44).

Numerous studies have monitored the fate of acetate, butyrate, and propionate in methanogenic ecosystems (4, 8, 9, 19, 45, 46, 47). Since syntrophic

microorganisms are responsible for the degradation of butyrate and propionate in methanogenic ecosystems and require the presence of hydrogen/formate-using methanogens, the observation of butyrate or propionate loss coupled to methane production has frequently been used to show that syntrophic butyrate or propionate metabolism occurred in these ecosystems (4, 8, 9, 47). Studies have also used 2-bromoethanesulfonic acid, which inhibits methanogens, to show that syntrophic butyrate- or propionate-degradation occurred in methanogenic ecosystems (45). Even though these methods are useful for showing that syntrophic butyrate- or propionate-degradation occurs at a given study site, they have revealed very little information about the identities of the microorganisms that are responsible for syntrophic butyrate and propionate degradation in methanogenic ecosystems. The highly fastidious nature of syntrophic butyrate- and propionate-degraders makes it difficult to isolate these microorganisms (19, 31). To date, there is also a lack of suitable molecular markers available for studying syntrophic butyrate- and propionate-degraders in anaerobic ecosystems (19, 31). Therefore, our knowledge of the ecology and diversity of syntrophic butyrate- and propionate-degraders is based on a limited number of pure cultures (Table 1.1).

In several studies that monitored the fate of acetate in methanogenic ecosystems, it was unclear if acetate was degraded directly to CH_4 and CO_2 by aceticlastic methanogens or indirectly by consortia of hydrogen/formate producing acetate-degrading syntrophs and hydrogen/formate-using methanogens (7, 46, 47). Many of these studies observed acetate loss coupled to methane production or

showed that acetate-degradation was inhibited upon the addition of BESA (7, 46, 47). Although these approaches are useful for showing that methanogens play a role in the degradation of acetate in methanogenic ecosystems, they cannot be used to distinguish between acetate-degradation that is carried out by acetate-degrading syntrophs or aceticlastic methanogens, since the results will be similar. Subsequent studies have begun to use [2-^{14}C] acetate ($^{14}CH_3COOH$) to distinguish between acetate degradation that is carried out directly by aceticlastic methanogens or indirectly by syntrophic acetate-degraders (20, 26, 36, 62). Acetate degradation that is carried out directly by aceticlastic methanogens will result in 100% label recovery from $^{14}CH_3COOH$ as $^{14}CH_4$, whereas acetate degradation by syntrophic acetate-degraders will result in 100% label recovery from $^{14}CH_3COOH$ as $^{14}CO_2$ (20, 26, 36, 62). This use of labeled acetate has allowed for the differentiation of acetate-degradation that is carried out by aceticlastic methanogens or syntrophic acetate-degraders in methanogenic ecosystems. However, these studies have revealed very little information about the identities of the microorganisms that are responsible for aceticlastic methanogenesis or syntrophic acetate-degradation in methanogenic ecosystems. Only a few species of acetate-using methanogens have been described from the genera *Methanosaeta* and *Methanosarcina* (13, 17, 61). Even less is known about the populations of microorganisms that carry out syntrophic acetate-degradation in methanogenic ecosystems. To date, only a limited number of microorganisms have been isolated and described that are capable of degrading acetate syntrophically in methanogenic ecosystems (Table 1.1) (20, 32).

The work presented in this dissertation was carried out in order to gain a better understanding of the populations of microorganisms that are involved in the degradation of acetate, butyrate, and propionate in methanogenic and sulfate-reducing ecosystems. To date, the microbial communities that are involved in the degradation of these fatty acids under methanogenic and sulfate-reducing conditions are often poorly understood and have commonly been referred to as a black box (14, 44). This work presented in this dissertation was carried out using sediment and groundwater samples that were collected from a shallow aquifer, which overlies an active natural gas production field in Fort Lupton, Colorado (18, 52). The aquifer was contaminated with gas condensate (96% w/w C_5-C_{15} hydrocarbons, including 18% w/w BTEX) in the 1970's as a result of a leaking storage sump that was used to store liquids produced during natural gas recovery (18, 52). The geochemistry of this particular site has been thoroughly characterized over a period of several years (18). Geochemical data from this site indicated that sulfate concentrations in the contaminated portion of the aquifer were always depleted, relative to uncontaminated sediments (18). Spikes in the sulfate concentration were often observed in the contaminated portion of the aquifer, which indicated that sulfate was periodically replenished at this site (18). High concentrations of methane, relative to uncontaminated sediment, were observed in the contaminated portion of the aquifer (18). The steady state hydrogen concentrations in the contaminated portion of the aquifer suggested that sulfate reduction and methanogenesis were the dominant terminal electron-accepting

processes at this site (18). Previous work at this site also suggested that active populations of microorganisms including sulfate-reducing bacteria, syntrophic microorganisms, and methanogens, which are commonly associated with sulfate-reducing and methanogenic ecosystems, were present at this site (18, 52). All of these findings made this site ideal for studying the populations of microorganisms that are involved in the degradation of acetate, butyrate, and propionate in methanogenic and sulfate-reducing ecosystems.

The work presented in chapter two focused on determining what populations of microorganisms were involved in the degradation of acetate under methanogenic and sulfate-reducing conditions at this site. Acetate metabolism was coupled to methane production in the presence or absence of sulfate. 16S rRNA sequences, related to aceticlastic methanogens from the family *Methanosaetaceae*, were detected in both methanogenic and sulfate reducing enrichments. These findings showed that aceticlastic methanogenesis was the dominant fate of acetate under methanogenic and sulfate-reducing conditions at this site. The occurrence of aceticlastic methanogenesis in the presence of sulfate was surprising since pure culture studies and kinetic studies predict that sulfate-reducing bacteria will metabolize acetate when sulfate was available. This chapter is published in *Applied and Environmental Microbiology*.

Chapter three focused on determining what populations of microorganisms were involved in the degradation of butyrate under methanogenic and sulfate-reducing conditions at this site. Butyrate loss was coupled to methane production

under methanogenic conditions and to sulfate reduction under sulfate-reducing conditions. The exact microorganisms that were responsible for butyrate degradation at this site were not identified. However, microorganisms from several genera that are capable of syntrophic butyrate metabolism, including members of the *Syntrophomonadaceae* and *Syntrophaceae* were observed in methanogenic and sulfate-reducing enrichments. The majority of sulfate reducers that were present in sulfate-reducing enrichments were related to hydrogen-using sulfate reducers, rather than butyrate-degrading sulfate reducers. Butyrate-degradation was inhibited by BESA in methanogenic enrichments, and by carbon monoxide, which inhibits hydrogen-using microorganisms, in sulfate-reducing enrichments. These findings suggested that syntrophic butyrate-degradation was the dominant fate of butyrate under methanogenic and sulfate-reducing conditions at this site. The results of the work presented in this chapter suggest that syntrophic microorganisms are not confined to methanogenic ecosystems and are more ecologically diverse than previously thought. This chapter is written in the style recommended by the *ISME Journal*.

The work presented in the appendix focused on determining what populations of microorganisms were responsible for propionate metabolism under methanogenic and sulfate-reducing conditions at this site. Propionate-degradation was only observed in MPN dilutions and microcosms that contained sulfate. Propionate-degradation was coupled to sulfate loss in MPNs and microcosms that contained sulfate. A propionate-degrading, sulfate-reducing bacterium that was

96% similar to *Desulfobulbus propionicus* was isolated from MPNs with propionate and sulfate. Molecular studies at this site also suggested that propionate was degraded by microorganisms that are related to propionate-degrading sulfate reducers from the genus *Desulfobulbus.* These findings suggest that sulfate-reducing bacteria, rather than syntrophic propionate-degraders, were responsible for propionate degradation at this site.

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CHAPTER 2

Evidence for Aceticlastic Methanogenesis in the Presence of Sulfate in a Gas Condensate-Contaminated Aquifer

ABSTRACT

The anaerobic metabolism of acetate was studied in sediments and groundwater from a gas condensate-contaminated aquifer in an aquifer where geochemical evidence implicated sulfate reduction and methanogenesis as the predominant terminal electron accepting processes. Most probable number (MPN) tubes containing acetate and microcosms containing either [2-^{14}C] acetate or [U-^{14}C] acetate produced higher quantities of CH_4 compared to CO_2 in the presence or absence of sulfate. $^{14}CH_4$ accounted for 70-100% of the total labeled gas in the ^{14}C-acetate microcosms regardless of whether sulfate was present or not. Denaturing gradient gel electrophoresis (DGGE) of the acetate enrichments both with and without sulfate using *Archaea* specific primers showed identical predominant bands that had 99 % sequence similarity to members of *Methanosaetaceae*. Clone libraries containing *Archaeal* 16S rRNA gene sequences amplified from sediment from the contaminated portion of the aquifer showed that 180 of the 190 clones sequenced belonged to the *Methanosaetaceae*. The production of methane and the high frequency of sequences from the *Methanosaetaceae* in acetate enrichments

with and without sulfate indicate that aceticlastic methanogenesis was the predominant fate of acetate at this site even though sulfate-reducing bacteria would be expected to consume acetate in the presence of sulfate.

INTRODUCTION

Redox reactions in anaerobic environments play a pivotal role in the fate of organic compounds in contaminated aquifers (17, 19). The populations of microorganisms that are involved in the degradation of acetate, as well as other organic compounds in contaminated anaerobic sediments, depend on many factors, but the availability of electron acceptor at the particular site is an important governing factor (17, 19). Typically electron acceptors with a higher redox potential such as NO_3^- will be utilized first, followed by Fe (III), SO_4^{2-}, and CO_2 (19).

Relatively few studies have focused on the fate of acetate in hydrocarbon-contaminated environments. The majority of these studies that have been conducted in hydrocarbon-contaminated environments used culture-independent molecular approaches to describe the microbial community, and infer the putative function of the different phylotypes present (7, 8, 14, 39, 41). One recent study showed that sulfate reduction accounted for the degradation of petroleum hydrocarbon constituents in approximately 70% of all sites studied in a survey of 38 petroleum impacted sites (14, 42). Kinetic studies have demonstrated that sulfate-reducing bacteria have a lower k_m value for acetate (34, 40,44) and are capable of acetate utilization at lower threshold concentrations than aceticlastic methanogens (34, 40, 44). These findings along with the ability of the sulfate-reducing bacteria to completely mineralize a wide variety of hydrocarbon

contaminants including alkanes, aromatic hydrocarbons, and a variety of fatty acids including acetate (44) suggests that sulfate-reducing bacteria should be responsible for acetate utilization in hydrocarbon-contaminated sites.

We used a combination of cultivation and molecular approaches to test the hypothesis that aceticlastic methanogenesis was the predominant fate of acetate in a hydrocarbon-contaminated aquifer where both methanogenesis and sulfate reduction have been implicated as the predominant electron-accepting processes (TEAP) (12). In the 1970's, the site was contaminated with gas condensate, which contains a mixture of C_5-C_{15} hydrocarbons (20 percent of the gas condensate is composed of a mixture of benzene, toluene, ethylbenzene, and the xylene isomers) that was co-produced with the natural gas (12). Dissolved oxygen and nitrate were depleted in the contaminated portion of the aquifer with respect to uncontaminated sediments (12). Fe (III) was undetectable in the contaminated portion of the aquifer, but was present at significantly higher levels in uncontaminated sediment (12). Geochemical data along with microcosm studies, which showed that the degradation of several compounds including benzene, toluene, *o*-xylene, *m*-xylene, *p*-xylene, and ethylbenzene was accompanied by sulfate loss, suggested that sulfate-reduction is the predominant TEAP in the contaminated portion of this aquifer (12). However, dissolved methane within the contaminated portion of the aquifer, ranged from 5-17 milligrams/liter (12), which suggested a role for methanogenesis within this site. Our study suggests that acetate is an important intermediate in hydrocarbon-contaminated aquifers where sulfate reduction occurs

even though one would predict that sulfate-reducing bacteria should completely mineralize the hydrocarbons.

MATERIALS AND METHODS

Sample collection. Sediments and groundwater were collected from a shallow aquifer that lies just above a natural gas field that is located approximately 40 miles northeast of Denver, Colorado. Samples were collected in October 1998 and August 2000. Contaminated sediments and groundwater from well number 37 (12) were collected at a distance of approximately 10 meters downgradient from a sump that leaked hydrocarbons in the 1970's (12). Contaminated sediments were collected by hand boring to a depth of 1.5 meters below the surface and placed in sterile 1 L mason jars. Samples were kept anaerobic by filling the jars to capacity with sediment and groundwater. Groundwater and sediments from an uncontaminated portion of the aquifer were collected as above from well 18 (12), which was located approximately 10 meters upgradient from the original source of contamination. All sediment and groundwater samples were stored on ice until they were delivered to the laboratory. The samples were stored at 4 ^{0}C upon arrival at the laboratory.

Microorganisms and Media. The hydrogen-using organisms *Methanospirillum hungatei* strain JF-1 (DSM 864) and *Desulfovibrio vulgaris* strain G11 (DSM 7057) were grown with an 80% H_2: 20% CO_2 gas phase (69 kPa) under strictly ananaerobic conditions (2) using a previously described basal

medium (21). The basal medium was amended with 10 mM acetate when growing JF-1 and with 10 mM acetate and 30 mM sulfate when growing G11.

The basal medium (21) with 10 mM acetate was used for most probable number analysis (MPN) and contained 10 mM sulfate for the growth of sulfate reducers. The MPN medium was prepared anaerobically (2) and each tube contained either 6 ml of medium in MPN's that contained either JF-1 or G11 medium or 9 ml of medium in MPN's that did not contain a hydrogen-using organism. The headspace of all the MPN tubes was replaced with an atmosphere containing 80% N_2 and 20% CO_2 (34 kPa) gas phase (2). MPN's tubes were incubated at room temperature without shaking.

Most probable number (MPN) analysis. To test for the presence of different metabolic groups involved in acetate degradation, a three-tube MPN analysis was conducted using sediments from the contaminated and uncontaminated portions of the aquifer. Sterile sodium pyrophosphate solution (pH 7) was prepared by adding 1 g/L sodium pyrophosphate to the basal medium (21) without rumen fluid. The sterile, anaerobically prepared (2), sodium pyrophosphate solution was taken into an anaerobic chamber where the stoppers and seals were removed. Three tubes of sodium pyrophosphate per MPN set were each amended with 1g (wet wt.) of sediment from the appropriate location, stoppered, sealed, removed from the anaerobic chamber, and used to inoculate the appropriate MPN set. Each of the three tubes was mixed by hand for 30 sec and 1 ml of each solution was removed aseptically and transferred into 9 ml of the

appropriate MPN medium using needles and syringes flushed with 100% N_2. This procedure was repeated using these first three tubes of inoculated MPN medium and continued until each tube of the dilution series was inoculated.

Three different series of MPN's were conducted using 10 mM acetate as the substrate. The first series contained no additional sulfate and 3 ml of a hydrogen-using methanogen *M. hungatei* strain JF-1. The second series contained an additional 10 mM sulfate. The final series contained an additional 10 mM sulfate and 3 ml of a hydrogen-using sulfate-reducing bacterium, *D. vulgaris* strain G11. The hydrogen-users were added to each tube of the dilution series to enrich for syntrophic bacteria capable of degrading acetate. Individual MPN tubes were scored positive if more than 50 percent of the acetate was metabolized after 120 days. As controls, MPN analysis was conducted using the basal medium without added acetate.

Preparation of ^{14}C-acetate amended microcosms. Microcosms were prepared in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI) using sterile, 40-ml serum bottles, which were left in the chamber overnight prior to inoculation. Thirteen grams of sediment from the contaminated portion of the aquifer were added to each serum bottle. Groundwater from a well located upgradient of the contaminated area was added to bring the final volume of each microcosm to 20 ml. The microcosms were stoppered, sealed inside the chamber, and the gas phase was exchanged three times by evacuation with vacuum and repressurization with 100% N_2 (2). One set of microcosms received between 1.7 x

10^4 and 3.3 x 10^4 becquerels (Bq) of [2-^{14}C] acetate; another set of microcosms received a similar amount of [U-^{14}C] acetate. The labeled acetate was added by injecting 0.2 ml of either [2-^{14}C] acetate or [U-^{14}C] acetate stock solutions, which contained 1.13x 10^5 Bq/ml and 9.93 x 10^4 Bq/ml, respectively. Unlabeled acetate was added to all of the microcosms to bring the final acetate concentration to approximately 500 μM. Each set of microcosms contained three replicates of each of the following treatments: sediment and acetate alone, acetate and an inhibitor of methanogenesis (7.5 mM 2-bromoethanesulfonic acid (BESA)), acetate with 7.5 mM sulfate, and acetate and an inhibitor of sulfate reduction (5 mM sodium molybdate). Heat-killed controls were run in duplicate for each of the above treatments. The heat-killed controls were autoclaved at 121^{0}C for 20 minutes. All of the microcosms were incubated for 18 days at room temperature.

Analytical Methods. Non-labeled acetate loss was measured by high-pressure liquid chromatography (16). The mobile phase was 25 mM KH_2PO_4 (pH 2.5) at a flow rate of 1 ml min^{-1}. This HPLC was equipped with a radioisotope detector, which was used to quantify radioactive acetate loss. The radioisotope detector was calibrated by comparing its response to that of a scintillation counter. Standards of both [2-^{14}C] and [U-^{14}C] acetate were prepared from the same stock solutions that were used to amend the microcosms. One hundred microliters of standard solutions ranging from 1.67 x 10^3 Bq to 1.67x 10^4 Bq were run on the radioisotope detector. The same volume of each standard was also placed into 5 ml of scintillation cocktail and counted using a scintillation counter. Each standard

and unamended scintillation cocktail were counted by using a scintillation counter. Quenching of standards during liquid scintillation counting was corrected by autocalibration using an unquenched ^{14}C-standard, and through the use of both an H# monitor and a random coincidence monitor.

CH_4 and CO_2 production were measured by using a gas chromatograph (GC) equipped with a thermal conductivity detector. The GC had a 3.05 m x 0.004 m Carbosphere 80/100 column (Altech Inc, Deerfield, IL). Helium was the carrier gas at 2 ml min^{-1}. The injector and the column were set at 175°C and the detector was set at 81 ^{o}C. The gas chromatograph was connected to a gas proportional counter (Insus Systems Incorporated, Fairfield, NJ). Standards containing $^{14}CO_2$ were prepared from a stock solution containing 1.6 x 10^5 Bq/ml of $H^{14}CO_3$. This solution was then diluted to concentrations ranging from 1.3 x 10^2 to 8.02 x 10^4 Bq/ml by adding the appropriate volume of stock solution to enough 0.1N NaOH to bring the final volume of each standard to 20 ml. Each standard was then acidified with 1 ml of 12 N HCl. A 0.2 ml aliquot of the headspace of each standard was then injected into the GC. Also, 0.2 ml aliquots of each standard were slowly bubbled into 0.8 ml of 0.1N NaOH and 0.45 ml of the solution was added to 5 ml of scintillation cocktail and counted using the same procedure described for the ^{14}C-acetate standards. The retention time of CH_4 was determined through the use of non-labeled methane standards, which were detected with the thermal conductivity detector. An enrichment culture that degraded [U-^{14}C] methyl tert-butyl ether (MTBE) was provided by the laboratory of Dr. Joseph M. Suflita. This

enrichment culture, which was known to produce $^{14}CH_4$, was used to verify the retention time of $^{14}CH_4$.

The pH of the individual microcosms was measured with Color pHast Indicator Strips (EM Science, Gibbstown, NJ) at the end of the 18-day incubation period. The final pH of each microcosm and the amount of $^{14}CO_2$ (obtained by GC) was used to calculate the amount of $H^{14}CO_3$ that was dissolved in the liquid phase of the microcosms by using the Henderson-Hasselbach equation (equation 1):

Final pH of microcosm= $6.35 + \log ([H^{14}CO_3]/[^{14}CO_2])$

The amount of $H^{14}CO_3$ determined with equation 1 was added to the amount of $^{14}CO_2$ measured by gas chromatography to obtain the total amount of $^{14}CO_2$ produced in each microcosm.

Sulfate concentrations were determined by ion chromatography (16) and methane was quantified by gas chromatography (13).

Molecular analysis. DNA was extracted from enrichments that were prepared by inoculating MPN medium with 1 ml aliquots of sediment and groundwater from microcosms containing [2-^{14}C] acetate, [2-^{14}C] acetate with sulfate, [U-^{14}C] acetate, and [U-^{14}C] acetate with sulfate. These enrichments were transferred three times prior to being used as a source of material for DNA extraction. Two milliliters of each enrichment was added to sterile 2 ml polypropylene screw-cap tubes that contained 1 gram of 0.1 mm zirconia/silica beads (Biospec Products, Bartlesville, OK). Samples were centrifuged for 5 min at

14,000 x *g* to pellet the cells and any remaining supernatant was discarded. DNA was also extracted directly from sediments by using approximately 1 gram of sediment (wet weight) that was added directly to a 2 ml polypropylene screw-cap tube containing zirconia/silica beads. DNA was extracted from enrichments and sediments using a bead beating protocol as previously described (28)

DNA extracted from contaminated aquifer sediments was used as a template to screen for the presence of different groups of sulfate-reducing bacteria. Five sets of group specific 16S rRNA gene primers were used to screen for members of the *Desulfobulbus, Desulfobacterium, Desulfovibrio, Desulfobacter,* and *Desulfotomaculum* (5). DNA from *Desulfobulbus propionicus, Desulfobacterium autotrophicum, Desulfovibrio vulgaris* strain G11, *Desulfobacter curvatis,* and *Desulfotomaculum nigrificans* was used as a positive control to ensure that each set of primers amplified the 16S rRNA gene of the appropriate group. PCR reactions and cycling conditions were set up and carried out as previously described (5).

PCR amplification of the *Archaeal* 16S rRNA gene sequences were amplified using a previously described touchdown PCR protocol (28) and 5 pmol of GM5F (5' CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGG GCGTACGGGAGGCAGCAG 3') (22) and 20 pmol of the Archaea specific primer Arc 958r (5' YCCGGCGTTGAMTCCATTT 3') (27). Denaturing gradient gel electrophoresis (DGGE) was performed with PCR amplified products of DNA from enrichments using GM5F and Arc 958R (22). Predominant bands were

excised from the gel, reamplified by the above touchdown PCR protocol, checked for purity by DGGE, and then sequenced.

For constructing archaeal 16S rRNA gene clone libraries from aquifer sediments, 16S rRNA genes were amplified from the DNA extracted from the sediments by using the GM5F and Arc 958r primers. The PCR product obtained was cloned into the TOPO 2.1 cloning vector (Invitrogen Corp., Carlsbad, CA) according to the instructions of the manufacturer. Randomly picked clones (190 total) were sequenced at the Advanced Center for Genome Technology at the University of Oklahoma. Details of the sequencing protocols applied were described previously (10) and can be found at: (http://www.genome.ou.edu/ds_seq_template_isol_hydra.html).

The 16S rRNA gene sequences were initially screened with the Basic Local Alignment Search Tool (1) to determine their rough phylogenetic affiliation. Sequences with greater than 98% similarity were grouped into the same operational taxonomic unit (OTU) using Sequencher (Gene Codes Corp., Ann Arbor, MI). Sequences from this study and Genbank downloaded sequences were aligned using the Clustal X program version 1.83 (37). The alignment obtained by Clustal X was also manually checked for errors. The aligned sequences were exported from Clustal X and loaded into Phylogenetic Analysis Using Parsimony (PAUP) version 4.0 beta 10 (Sinauer Associates, Sunderland, MA). Evolutionary distance trees were constructed using the Neighbor Joining algorithm with Jukes-Cantor corrections. Bootstrap support values are based on 1000 replicates.

Nucleotide sequence accession numbers. The 16S rRNA sequences of the excised DGGE band and the OTU's from the sediment clone libraries have the following GenBank accession numbers: AY894806 for DGGE band 1, AY894807 for OTU1, and AY894808 for OTU2.

RESULTS

Most probable number analysis. There was no significant ($p < 0.05$) difference in the number of acetate degraders (as defined by acetate depletion) in MPN's amended with either *Methanospirillum hungatei* JF-1 or *Desulfovibrio vulgaris* G11 relative to MPN's containing only acetate and sulfate (Table 2.1). Acetate consumption was coupled to methane production and no sulfate loss was observed in any of the acetate MPN's inoculated with sediments from the contaminated portion of the aquifer. Substrate unamended MPN's that contained sulfate and were inoculated with contaminated sediments produced up to 50 micromoles of methane in the 10^{-5} dilutions after 120 days. Acetate MPN's inoculated with uncontaminated sediments showed no acetate loss, no methane production, and no sulfate loss after 120 days.

^{14}C-Acetate amended microcosms. The quantities of both $^{14}CH_4$ and $^{14}CO_2$ produced as a result of the degradation of either [U-^{14}C] acetate or [2-^{14}C] acetate are shown in Table 2.2. The majority of the label (between 70 and 90 %) from [U-^{14}C] acetate was recovered as $^{14}CH_4$ rather than $^{14}CO_2$ in the active bottles regardless of whether or not sulfate was present. The addition of BESA caused this ratio to shift such that approximately 80% of the label appeared as $^{14}CO_2$ in bottles with BESA compared to bottles without BESA. The addition of molybdate did not affect the fate of carbon from [U-^{14}C] acetate compared to active bottles with

TABLE 2.1. Summary of MPN results obtained using acetate as a substrate

Treatment	Contaminated	95% Confidence Limits[a]		Uncontaminated
		Lower	Upper	
Acetate + JF-1[b]	2.4×10^{6}[c]	5.8×10^{6}	9.9×10^{7}	BDL[b]
Acetate + sulfate	1.1×10^{6}	3.6×10^{5}	3.5×10^{6}	BDL
Acetate + sulfate + G11[b]	2.1×10^{6}	7.9×10^{5}	5.6×10^{6}	BDL

[a] 95% upper and lower confidence intervals used for three tube MPN analysis

[b] Abbreviations: JF-1; *Methanospirillum hungatei* strain JF-1; G11, *Desulfovibrio vulgaris* strain G11; BDL, below detection limit.

[c] MPN values are per gram of sediment.

acetate alone or with both acetate and sulfate (Table 2.2). The heat-killed controls containing [U-^{14}C] acetate showed only a minor decrease in the quantities of labeled acetate with respect to the active bottles. However, no accumulation of either $^{14}CH_4$ or $^{14}CO_2$ was observed. The addition of [2-^{14}C] acetate produced similar results when compared to microcosms containing [U-^{14}C] acetate (Table 2.2).

Molecular Analyses. Clone libraries of 16S rRNA genes amplified from DNA extracted from contaminated sediments contained only two OTUs (Fig. 2.1). Sequences from OTU 2 (180 out of 190 clones) were most similar to clone SSADM_AG7 (100% similar based on a total of 564 bases sequenced). The sequence of OTU 2 was also 99.8 % similar (563/564 bases were identical) to the sequence of the most intense DGGE band (Fig. 2.1) that was common to all microcosm enrichments regardless of whether sulfate was present or not (data not shown). OTU 1 (10 out of 190 clones) grouped with hydrogen-using methanogens from the genus *Methanobacterium.*

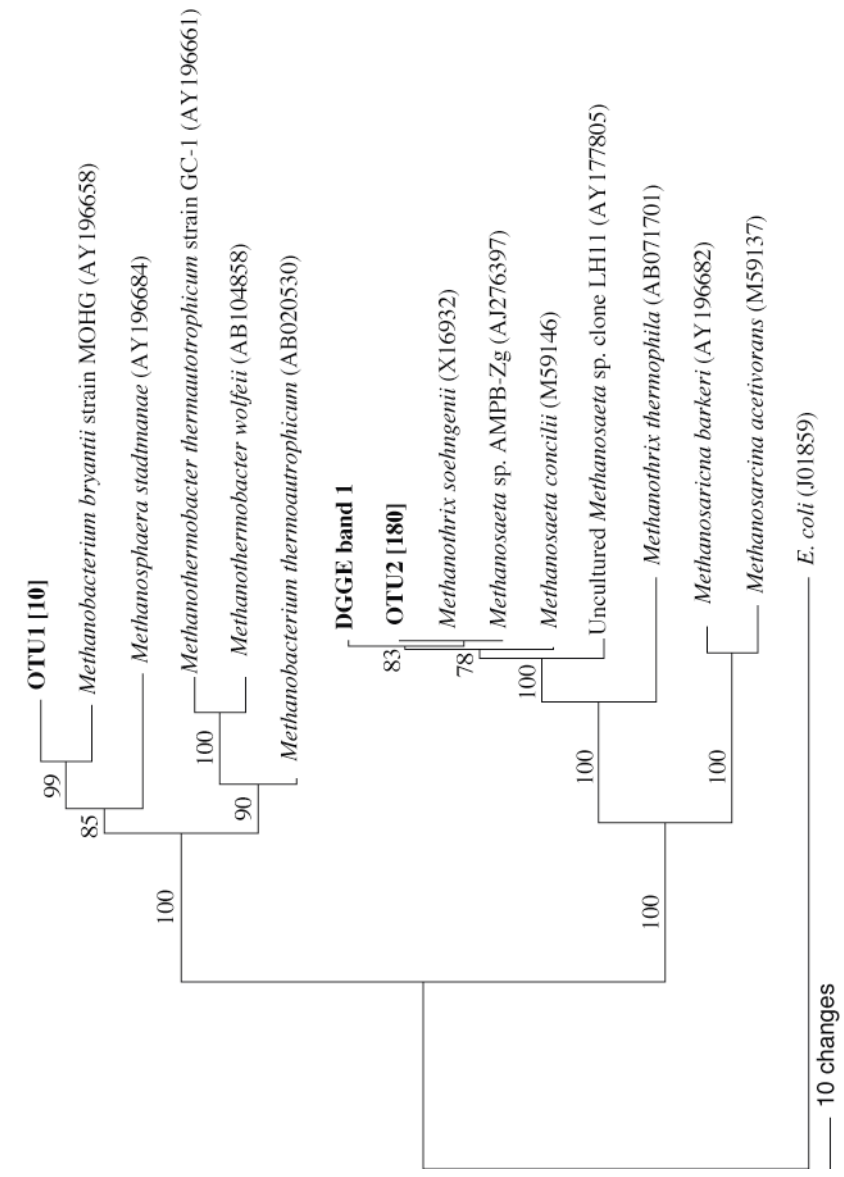
PCR products were observed in reactions containing primers specific for members of the *Desulfobulbus, Desulfovibrio, Desulfotomaculum,* and the *Desulfobacter*. PCR product was not observed in PCR reactions that contained primers specific for members of the *Desulfobacterium*.

TABLE 2.2. Fate of acetate carbon in microcosms from the contaminated site

Treatment	Acetate Lost (Bq x 10^4)	$^{14}CH_4$ Produced (Bq x 10^4)	$^{14}CO_2$ Produced (Bq x 10^4)
[2-^{14}C] acetate	1.7 ± 0.28[a]	1.4 ± 0.05	0.15 ± 0.1
[2-^{14}C] acetate HK	0	0	0
[2-^{14}C] acetate + BESA	1.1 ± 0.11	0.06 ± 0.01	0.4 ± 0.03
[2-^{14}C] acetate + BESA HK	0.03-0.4[c]	0	0
[2-^{14}C] acetate + sulfate	1.3 ± 0.27	1.2 ± 0.08	0.11 ± 0.02
[2-^{14}C] acetate + sulfate HK	0	0	0
[2-^{14}C] acetate + sulfate + molybdate	1.8 ± 0.27	1.9 ± 0.01	0.17 ± 0.04
[2-^{14}C] acetate + sulfate + molybdate HK	0.53-0.92	0	0
[U-^{14}C] acetate	1.4 ± 0.23	0.78 ± 0.1	0.15 ± 0.07
[U-^{14}C] acetate HK	0.013-0.24	0	0
[U-^{14}C] acetate + BESA	0.77 ± 0.09	0.05 ± 0.02	0.4 ± 0.03
[U-^{14}C] acetate + BESA HK	0.025-0.4	0	0
[U-^{14}C] acetate + sulfate	0.93 ± 0.15	0.82 ± 0.08	0.1 ± 0.02
[U-^{14}C] acetate + sulfate HK	0	0	0
[U-^{14}C] acetate + sulfate + molybdate	1.7 ± 0.28	1.1 ± 0.1	0.17 ± 0.03
[U-^{14}C] acetate + sulfate + molybdate HK	0-0.23	0	0

[a]Mean ± standard deviation
[b]HK indicates heat-killed samples.
[c]Range of values obtained for duplicate heat-killed samples.

FIGURE 2.1: Evolutionary distance tree showing the relationship of each OTU found in contaminated sediments and DGGE band 1 from acetate enrichments with other members of the *Archaea*. The phylogenetic tree was constructed using the neighbor-joining algorithm. Bootstrap values that are greater than 50% are shown at each clade, and are based on 1000 replicates. Accession numbers are listed in parentheses. The frequency of occurrence of specific OTUs in clone libraries from contaminated sediments is listed in brackets.


OTU1 [10]
Methanobacterium bryantii strain MOHG (AY196658)
Methanosphaera stadtmanae (AY196684)
Methanothermobacter thermautotrophicum strain GC-1 (AY196661)
Methanothermobacter wolfeii (AB104858)
Methanobacterium thermoautrophicum (AB020530)
DGGE band 1
OTU2 [180]
Methanothrix soehngenii (X16932)
Methanosaeta sp. AMPB-Zg (AJ276397)
Methanosaeta concilii (M59146)
Uncultured Methanosaeta sp. clone LH11 (AY177805)
Methanothrix thermophila (AB071701)
Methanosaricna barkeri (AY196682)
Methanosarcina acetivorans (M59137)
E. coli (J01859)
99
85
100
100
90
83
78
100
100
100
100
10 changes

DISCUSSION

This work showed that aceticlastic methanogenesis was the predominant fate of acetate in an aquifer where geochemical evidence implicates both sulfate reduction and methanogenesis as important terminal electron accepting processes (12). Anaerobic acetate degradation can occur by iron reduction (4, 29), sulfate reduction (6, 18, 25, 26, 30, 32, 35), aceticlastic methanogenesis (6, 18, 23, 32, 33, 36), or syntrophic acetate degradation (31). Iron reduction did not appear to play an important role in contaminated sediments (12). The high quantities of methane observed in MPN's, the high ratios of $^{14}CH_4/^{14}CO_2$ in microcosms containing ^{14}C-acetate, the lack of sulfate consumption in MPN's amended with sulfate, and the high frequency of sequences from the family *Methanosaetaceae* in enrichments and clone libraries from contaminated sediments lead to the conclusion that aceticlastic methanogenesis rather than sulfate-reduction is responsible for acetate consumption at this site. The possibility of syntrophic acetate metabolism seems unlikely since one methane was produced per acetate consumed in all MPN tubes and all label from the microcosms amended with [2-^{14}C] acetate was recovered as $^{14}CH_4$ as expected for acetoclastic methanogenesis (11). The lack of sulfate consumption in MPN's with sulfate and G11 would also suggest that syntrophic metabolism of acetate is not occurring at this site. The ratios of $^{14}CH_4/^{14}CO_2$ observed in some of the microcosms amended with [U-^{14}C] acetate were higher than the expected 1/1

ratio, which may indicate that hydrogenotrophic methanogenesis is also an important process in the contaminated sediments.

These results of this work raise the question: Why do aceticlastic methanogens control the fate of acetate in an aquifer where geochemical evidence indicates that sulfate-reduction is an important TEAP? Several studies have shown that a number of different factors including pH (38), sulfide toxicity (38), substrate specificity (26), sulfate limitation (15), and kinetic factors including k_m, v_{max}, and acetate threshold concentration (24, 26, 34, 40, 43) control whether acetate is utilized by methanogens or sulfate-reducing bacteria in anaerobic environments. The microcosms used in this study had a pH range from around 7.0 to 7.4 throughout the experiment and sulfide concentrations ranged from 1 mg/L to 10 mg/L in the contaminated portion of the aquifer. These pH values and sulfide concentrations have been shown to be favorable for the growth of both aceticlastic methanogens as well as acetate-utilizing sulfate-reducing bacteria (38). The predominance of members of the family *Methanosaetaceae* at this site, which are only capable of using acetate (26, 33), is interesting since sulfate-reducing bacteria are known to completely mineralize the hydrocarbons in petroleum contaminated environments where sulfate was present (14, 42, 44) and group specific PCR indicates the presence of a potential acetate-using sulfate reducer, *Desulfobacter* sp. From the specific radioactivity of ^{14}C-acetate and the bequerels present, we estimate that the ^{14}C-acetate concentration after 18 days was approximately 0.3 μM in microcosms containing ^{14}C-acetate, ^{14}C-acetate with sulfate, and ^{14}C-acetate with

sulfate and molybdate. By HPLC analysis the final concentration of acetate was below the detection limit of 50 μM. These results show that the final acetate concentration in the microcosms was between 0.3 μM and 50 μM, which is consistent with previously described acetate threshold concentrations in *Methanosaeta* (20, 26). These findings, along with those describing similar kinetic properties (k_m, V_{max}, and acetate threshold concentration) in two acetate utilizing sulfate-reducers and *Methanosaeta soehngenii* (26), suggest that some members of the *Methanosaeta* may be able to compete with sulfate-reducers for acetate. Low levels of sulfate in the contaminated region of the aquifer may favor acetate degradation by methanogenesis rather than by sulfate reducers since the acetate user, *Desulfobacter postgatei*, was a less successful competitor for limiting sulfate than two other sulfate-reducers (15).

While it is clear that acetate is an important intermediate in this hydrocarbon-contaminated site, the source of acetate in the contaminated portion of this aquifer is unclear. Bacterial clone libraries prepared with DNA from acetate enrichments and contaminated sediments contained a large number of both clostridial and *Cytophaga-Flavobacter-Bacteroides* sequences (data not shown), indicating that fermentative metabolism could be a source of acetate. Another possibility is the incomplete metabolism of the BTEX hydrocarbons, which are major components of gas condensate. Dolfing (9) suggests that the incomplete metabolism of benzoate, which is known to be an important intermediate in the anaerobic biodegradation of the BTEX hydrocarbons, to acetate is more

energetically favorable than its complete mineralization to CO_2 under methanogenic conditions (P_{H2} > 2 Pa). Thus, under sulfate-limiting conditions, it is likely that incomplete BTEX hydrocarbon degradation is occurring, which results in acetate excretion and creates a niche for the aceticlastic methanogens to function in this hydrocarbon-contaminated environment.

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CHAPTER 3

Syntrophic Metabolism Under Sulfate-Reducing Conditions in a Hydrocarbon-Contaminated Aquifer

ABSTRACT

Butyrate metabolism was studied in anoxic sediments and groundwater from a hydrocarbon-contaminated aquifer where geochemical evidence implicated sulfate reduction and methanogenesis as the predominant terminal electron-accepting process. The most probable number (MPN) of syntrophic butyrate-degraders (e.g., cultures amended with *Methanospirillum hungatei* or *Desulfovibrio vulgaris* strain G11) was similar to the number of sulfate-reducing, butyrate-degraders (cultures with sulfate but without a hydrogen-user added). Butyrate loss was coupled to methane production in microcosms without sulfate and to sulfate reduction in microcosms amended with sulfate. The addition of 2-bromoethanesulfonic acid (BESA) inhibited butyrate degradation in methanogenic microcosms, which was restored with the addition of a hydrogen-using sulfate reducer and 5 mM sulfate, but not when only 5 mM sulfate was added. The addition of carbon monoxide, which inhibits hydrogenases, to the headspace of sulfate-reducing microcosms inhibited butyrate metabolism and caused the hydrogen partial pressure to increase to levels that would make syntrophic butyrate degradation thermodynamically unfavorable (-5 to +3 kJ mol^{-1} of butyrate).

Approximately thirty percent of the 16S rRNA gene sequences in clone libraries from the MPN cultures grouped with members of the *Syntrophomonadaceae* and DGGE analysis detected an identical predominant band whose sequence was closely related to members of the genus *Syntrophus* in the sulfate-reducing MPN cultures. 16S rRNA gene sequences related to *Desulfovibrio* accounted for 75% of the total number of sequences affiliated with sulfate reducers in clone libraries from MPN cultures. 16S rRNA sequences related to syntrophic butyrate-degraders from the genus *Syntrophus* were present in clone libraries prepared from the contaminated sediment. Our findings showed that syntrophic metabolism occurs under sulfate-reducing conditions and suggests that syntrophic metabolism may be more prevalent than previously thought.

INTRODUCTION

Sulfate reduction is an important terminal electron-accepting process in marine sediments, petroleum reservoirs, and other ecosystems where sulfate is plentiful (Jørgensen, 1982; Muyzer and Stams, 2008). Sulfate-reducing bacteria oxidize a wide variety of organic compounds including alcohols, fatty acids, amino acids, sugars, and hydrocarbons completely to CO_2 or to acetate and CO_2 coupled to the reduction of sulfate without the need for interspecies transfer of hydrogen or formate (Jørgensen, 1982; Muyzer and Stams, 2008; Widdel and Pfennig, 1977; Balk *et al*., 2008). Microorganisms capable of syntrophic metabolism use many of the same substrates that sulfate-reducing bacteria use including fatty acids (McInerney *et al*., 2008). However, kinetic studies showed that butyrate-degrading sulfate reducers grew faster than cocultures of syntrophic butyrate-degraders and hydrogen-using microorganisms (Oude Elferink *et al*., 1994; Stams *et al*., 2003). Based on the work with pure cultures, it is believed that the degradation of fatty acids and hydrocarbons in sulfate-reducing ecosystems does not involve interspecies hydrogen and formate transfer (Widdel, 1988; Jorgenson, 1982; Oude Elferink *et al*., 1994; Stams *et al*., 2003; Muyzer and Stams, 2008) while interspecies hydrogen and formate transfer is required to degrade these compounds in methanogenic ecosystems (McInerney and Gieg, 2004; Schink, 1997).

Many sulfate reducers use hydrogen or formate as electron donors for sulfate reduction (Muyzer and Stams, 2008). Thus, it is possible that fatty acid

degradation in sulfate-reducing environments could be catalyzed by syntrophic consortia of hydrogen- and formate-producing fatty acid degraders and hydrogen/formate-using sulfate reducers. In fact, it is common to use a hydrogen-using sulfate reducer as the syntrophic partner to obtain syntrophic metabolizers in monoxenic culture (McInerney *et al.*, 1979; Boone and Bryant, 1980). Thermodynamically, the Gibbs free energy changes for the oxidation of butyrate to CO_2 coupled to sulfate reduction ($\Delta G^{o'}$ of -6.1 kJ per electron transferred) or to methane production ($\Delta G^{o'}$ of -4.1 kJ per electron transferred) are nearly identical (McInerney and Beaty, 1988) (Table 3.1). Thus, there does not appear to be an energetic preference whether the oxidation of the fatty acids occurs syntrophically or directly by sulfate reducers.

Several studies implicate a role for syntrophic metabolism in sulfate-reducing ecosystems. Most probable number (MPN) enumeration studies found similar numbers of butyrate-degraders in the presence and absence of sulfate in UASB reactors (Roest *et al*., 2005; Visser *et al.*, 1993). Molecular inventories of the 16S rRNA gene sequences present at other hydrocarbon-contaminated sites detected sequences related to *Syntrophus* spp. (Allen *et al*., 2007; Doijka *et al*., 1998; Kasai *et al*., 2005), which are capable of degrading fatty and aromatic acids in syntrophic association with either hydrogen-using sulfate reducers or methanogens (Jackson *et al*., 1999). In one region of a hydrocarbon-contaminated

TABLE 3.1. Gibbs free energy changes for idealized reactions involved in the degradation of butyrate under sulfate-reducing or methanogenic conditions[a]

Reaction	$\Delta G^{o\prime}$ (kJ/electron)
[1] $CH_3CH_2CH_2COO^- + 2.5\ SO_4^{2-} \Leftrightarrow 4\ HCO_3^- + 2.5\ HS^- + 0.5\ H^+$	-6.1
[2] $CH_3CH_2CH_2COO^- + 2.5\ H_2O \Leftrightarrow 2.5\ CH_4 + 1.5\ HCO_3^- + 0.5\ H^+$	-4.1

[a]Modified from McInerney and Beaty (1988).

aquifer where 156 μM sulfate was detected in the groundwater, butyrate metabolism was coupled to the reduction of sulfate (Kleikemper *et al*., 2002a). A push pull test conducted at the different region of the same hydrocarbon-contaminated site showed that only a portion of the butyrate loss could be attributed to sulfate reduction (Kleikemper *et al*. 2002b). The elevated levels of methane observed in this region of the aquifer suggested that butyrate was syntrophically metabolized (Kleikemper *et al*., 2002b). However, the microorganism(s) that were responsible for butyrate degradation were not identified in either study (Kleikemper *et al*., 2002a; Kleikemper *et al*., 2002b).

Here, we test whether the butyrate is syntrophically metabolized in sediments and groundwater from a hydrocarbon-contaminated aquifer using a combination of cultivation-dependent and cultivation-independent techniques. The study site is located near Denver, Colorado and is used for the production of natural gas (Gieg *et al*., 1999; Townsend *et al*., 2003). The aquifer was contaminated with gas condensate (96% wt/wt C_5-C_{15} compounds, of which 20 % wt/wt is BTEX compounds) in the late 1970s as a result of corrosion and the subsequent leakage of an underground storage sump that was used to store liquids produced during the recovery of natural gas (Gieg *et al*., 1999; Rios-Hernandez *et al*., 2003; Struchtemeyer *et al*., 2005; Townsend *et al*., 2003). Geochemical data showed decreased levels of sulfate and increased levels of both sulfide and methane in the contaminated portion of this aquifer compared to uncontaminated zones and showed that sulfate was periodically replenished at the site (Gieg *et al*., 1999).

Steady state hydrogen measurements implicated both sulfate reduction and methanogenesis as important terminal electron-accepting processes in the contaminated portion of this aquifer (Gieg *et al*., 1999). Microcosms containing sediments and groundwater from this site degraded benzene, ethylbenzene, ethylcyclopentane, and the xylene isomers only under sulfate-reducing conditions (Gieg *et al*., 1999; Rios-Hernandez *et al*., 2003) while the metabolism of toluene and a number of crude oil hydrocarbons occurred either under methanogenic or sulfate-reducing conditions (Gieg *et al*., 1999; Townsend *et al*., 2003). We found direct evidence for the involvement of syntrophic consortia in the degradation of butyrate both in the presence and absence of sulfate at this site.

MATERIALS AND METHODS

Sample collection. Sediment and groundwater were collected from a shallow aquifer, located approximately 40 miles northeast of Denver, Colorado in August 2000. The aquifer lies above an active natural gas production field and was contaminated in the 1970s with gas condensate as a result of leakage from an underground storage sump that was used to store liquids produced during natural gas recovery (Gieg *et al*., 1999; Townsend *et al*., 2003). The majority (96 wt % /wt) of hydrocarbons associated with this gas-condensate ranged from C_5 to C_{15} in length. Hydrocarbon-contaminated sediment and groundwater were collected from well number 37 (Gieg *et al*., 1999), which is located approximately 10 m downgradient from the source of contamination. Contaminated sediment was collected by hand boring to a depth of 1.5 m below the surface (Townsend *et al*., 2003). The sediment was placed in sterile 1-liter Mason jars, which were filled to capacity in order to keep the samples anaerobic. Groundwater samples were collected in sterile 2 L Schott bottles, which were also filled to capacity. Uncontaminated sediment and groundwater were also collected as described above from well 18, which is located approximately 10 m upgradient from the source of contamination (Gieg *et al*., 1999).

Sediment samples were also collected from a Duck Pond located on the campus of the University of Oklahoma. Sediments were collected approximately 5-6 cm below the sediment surface of the pond where large quantities of sulfide

were observed. Samples were collected in sterile 50 ml centrifuge tubes, which were filled to capacity to ensure that the tubes remained anaerobic. Sediment was also collected from a sulfate-reducing pond located within the Great Salt Plains National Park located in Alfalfa County, OK. These samples were provided to us by Dr. Ralph S. Tanner and were collected in 50 ml centrifuge tubes that were filled to capacity. All sediment and groundwater samples used in this study were stored on ice until they were delivered to the laboratory. The samples were stored at 4^0C upon arrival at the laboratory.

Microorganisms and Media. *Syntrophomonas wolfei* strain Goettigen (DSM 2245B), *Syntrophus aciditrophicus* strain SB (ATCC 700169), *Desulfovibrio vulgaris* strain G11 (DSM 7057), and *Methanospirillum hungatei* strain JF-1 (DSM 864) were obtained from our culture collection. These microorganisms were grown in a previously described basal medium that lacked rumen fluid (McInerney *et al.*, 1979). This medium was prepared under strictly anaerobic conditions by using the techniques described by Balch and Wolfe (1976). Pure cultures of *Syntrophomonas wolfei* and *Syntrophus aciditrophicus* were grown in basal medium (McInerney *et al*., 1979) amended with 0.2% crotonate and were incubated at 37^0C without shaking. The hydrogen-using microorganism *Desulfovibrio vulgaris* strain G11 was grown in basal medium (McInerney *et al*., 1979) amended with 10 mM acetate and 10 mM sulfate. *Methanospirillum hungatei* strain JF-1 was grown in basal medium (McInerney *et al*., 1979) amended with 10 mM

acetate. Cultures of both G11 and JF-1 were grown with an 80% H_2-20% CO_2 gas phase (69 kPa) and were incubated at 37^0C with shaking (100 rpm).

Most Probable Number Analysis. A three-tube most probable number (MPN) assay was used to test for and quantify different metabolic groups that could be responsible for butyrate degradation. MPN analysis was carried out using sediments from both the contaminated and uncontaminated portion of the Fort Lupton aquifer. Sediment was first added into a sterile, anaerobic sodium pyrophosphate solution in order to separate cells from the sediment. The pyrophosphate solution was prepared by adding 1 g l^{-1} sodium pyrophosphate into basal medium without rumen fluid and adjusting the pH to 7. This solution was then taken into an anaerobic glove bag where the stoppers and seals were removed. Three tubes of sodium pyrophosphate solution per MPN set were each amended with 1 g (wet weight) of sediment from the appropriate location, stoppered, sealed, and removed from the anaerobic glove bag. Each of these three tubes was then mixed by hand for approximately 30 s by hand inversion, and 1 ml of each solution was removed aseptically and transferred into 9 ml of the appropriate MPN medium using needles and syringes flushed with 100% N_2. This procedure was repeated using the first three tubes of inoculated MPN medium and continued until each tube of the dilution series was inoculated.

Basal medium with rumen fluid was prepared as previously described (McInerney *et al.*, 1979), amended with 10 mM butyrate, and used in three different MPN series. The first MPN series contained 6 ml of medium and 3 ml of

the hydrogen-using methanogen, *M. hungatei* strain JF-1. The second series contained 6 ml of medium amended with 10 mM sulfate and 3 ml of the hydrogen-using, sulfate reducer *D. vulgaris* strain G11. The third series contained 9 ml of medium amended with 10 mM sulfate. The addition of hydrogen-using organisms into MPN tubes was done to allow growth of bacteria that are capable of degrading butyrate syntrophically. Individual MPN tubes were scored positive if more than 50% of the butyrate was metabolized after 60 days. As controls, MPN analysis was carried out using basal medium both with and without sulfate to quantify background levels of methane production and sulfate reduction.

Effect of BESA and molybdate on butyrate degradation. Microcosms were prepared in an anaerobic glove box using sterile 40 ml serum bottles. These serum bottles were placed in the glove box 24 hours prior to inoculation to ensure that no oxygen was present. Thirteen grams of sediment from the contaminated portion of the Fort Lupton aquifer was added to individual serum bottles. Each serum bottle also contained 12 ml of a basal medium that lacked rumen fluid. Following the addition of basal medium, the microcosms were stoppered, sealed, and removed from the anaerobic glove box. The headspace of the microcosms was then exchanged three times by evacuation with a vacuum and repressurization with a gas mixture that contained 80% N_2 and 20% CO_2 (Balch and Wolfe, 1976). To determine if butyrate degradation coupled to methanogenesis could be shifted to sulfate reduction, twelve microcosms were amended with 5 mM butyrate from a sterile anoxic stock solution that contained 100 mM sodium butyrate. Three of the

twelve microcosms did not receive any additional amendments. Six microcosms were amended with 2-bromoethanesulfonic acid (BESA), which inhibits methanogenesis (Gunsalus *et al*., 1978), once butyrate loss was observed. BESA was added from a sterile, anoxic 100 mM stock solution to a final concentration of 5 mM. Three of the six microcosms that contained BESA were also amended with 10 mM sulfate to determine if butyrate degradation could be coupled to sulfate reduction. The other three microcosms that contained BESA were also amended with 10 mM sulfate and 5 ml of an actively growing culture the hydrogen-user *Desulfovibrio vulgaris* strain G11 to determine if syntrophic butyrate degradation occurred. Three microcosms served as heat-killed controls and were autoclaved at 121^0C for 20 minutes. Three additional microcosms were prepared without butyrate in order to quantify background levels of methane that were produced from endogenous electron donors found in the sediments.

Another set of microcosms was prepared to determine if butyrate degradation coupled to sulfate-reduction could be shifted to methanogenesis. Twelve microcosms were amended with 5 mM butyrate and 10 mM sulfate. Six of these microcosms were amended with 5 mM sodium molybdate, which inhibits sulfate reduction (Taylor and Oremland, 1979), when butyrate loss was first observed. Three of the six microcosms that contained molybdate received no additional amendments to determine if butyrate degradation would couple with methanogenesis. The other three microcosms that contained molybdate were amended with 5 ml of an actively growing culture of the hydrogen-user

Methanospirillum hungatei strain JF-1 to stimulate syntrophic butyrate-degraders. Three microcosms that contained butyrate and sulfate were amended with 5 mM sodium chloride, which served as a control for ionic strength. Three microcosms that contained butyrate and sulfate served as heat-killed controls and were autoclaved at 121^0C for 20 minutes. Three additional microcosms were prepared and amended only with 10 mM sulfate to quantify sulfate-reduction that was coupled to endogenous electron donors. All of the microcosms described above were incubated at room temperature without shaking.

The effect of carbon monoxide of butyrate degradation. A second series of microcosms was prepared in order to determine whether butyrate degradation under sulfate-reducing conditions was linked to interspecies hydrogen transfer. Microcosms were prepared as described above with twenty-five grams of the appropriate sediment and 50 milliliters of basal medium that lacked rumen fluid (McInerney *et al.*, 1979) and was amended with 5 mM sodium butyrate and 10 mM sodium sulfate. Six microcosms were prepared with sediments from each of the following sites: Fort Lupton, the Norman duck pond, and a sulfate-reducing pond at the Great Salt Plains State Park. These microcosms were incubated at room temperature until butyrate depletion was observed. Once butyrate degradation occurred, three microcosms from each site were amended with 10% CO, which has been shown to inhibit hydrogen-users in a previous study (Elshahed and McInerney, 2001). The other three microcosms were amended with an equivalent volume of pure N_2. All microcosms were then transferred to a room temperature

shaking incubator set at 90 rpm. Triplicate microcosms amended only with 10 mM sulfate were prepared for each sampling site to correct for sulfate-reduction that occurred due to endogenous electron donors. Triplicate heat-killed controls were also prepared for each sampling site.

Analytical Methods. Butyrate loss was measured using a high-pressure liquid chromatograph equipped with a UV detector set at 214 nanometers and a 150 x 4.6 mm Prevail™ organic acid column (Alltech Inc, Deerfield, IL). The mobile phase contained 60% 25 mM KH_2PO_4 (pH 2.5) and 40% acetonitrile and was run at a flow rate of 1 ml min^{-1}. Headspace methane concentrations were measured as previously described by using a gas chromatograph equipped with a flame ionization detector (Jenneman *et al*., 1986). Headspace hydrogen concentrations were measured with a gas chromatograph equipped with a reducing gas detector and a 1 m by 0.32 cm Spherocarb 60/80 column (Trace Analytical, Menlo Park, CA). The hydrogen-detecting gas chromatograph was run as previously described (Mormile *et al*., 1996) with the exception that the column and detector temperatures were 44^0C and 271^0C respectively. Sulfate depletion was monitored using ion chromatography (Londry *et al*., 1997).

Thermodynamic calculations. The $\Delta G'$ for syntrophic butyrate degradation from Fort Lupton microcosms amended with either N_2 or CO was calculated using the measured concentrations of acetate, butyrate and equation 1:

$$\Delta G' = \Delta G^{0'} + RT \ln \frac{[\text{Acetate}]^2 (pH_2)^2}{[\text{Butyrate}]} \qquad (1)$$

where (pH_2) is the hydrogen partial pressure, R is the universal gas constant (0.00831 kJ K^{-1} mol^{-1}), T is the temperature (degrees Kelvin), and the values listed in brackets are the molar concentrations of the respective compounds. The $\Delta G^{0'}$ value for butyrate degradation was calculated according to Thauer *et al.* (Thauer *et al*., 1977).

Molecular Analysis. DNA was extracted from the highest MPN dilutions that were positive for butyrate depletion and from liquid cultures of *S. aciditrophicus.* Two milliliters of these highest positive MPN dilutions and liquid cultures of *S. aciditrophicus* were added to 2-ml polypropylene, screw-cap tubes that contained 1g of 0.1-mm zirconia-silica beads (Biospec Products, Bartlesville, OK). The 2 ml aliquots were centrifuged at 14,000 x g for 5 minutes to pellet the cells, and the remaining supernatant was discarded. DNA was also extracted directly from Fort Lupton contaminated sediments by weighing 1 g of sediment (wet weight), which was then placed directly in a 2-ml polypropylene screw-cap tube that contained 1 g of zirconia-silica beads. DNA was extracted from enrichments and sediments by using a mini-bead beater as previously described (Rios-Hernandez *et al*., 2003) with the exception that 300 microliters of TE buffer (10 mM Tris [pH 8] and 1 mM EDTA) was added to the cell pellet prior to bead beating rather than 300 microliters of phosphate buffer. Agarose (0.8%) gels were used to confirm the presence of DNA in all extraction preparations and to estimate the concentration of DNA that was present. All agarose gels were stained with ethidium bromide and viewed with UV light.

DNA that was extracted from Fort Lupton MPN dilutions was used as template in PCR reactions that amplified the majority of the 16S rRNA gene. This amplification of the 16S rRNA gene was carried out with the universal eubacterial primers 27F (5' AGAGTTTGATCCTGGCTCAG 3') (Lane, 1991) and 1492R (5'GTGCCAGCMGCCGCGGTAA 3') (Lane, 1991). PCR reactions and cycling conditions were carried out as previously described (Lane, 1991). PCR products obtained above were then used to create clone libraries.

DNA from MPN dilutions, contaminated Fort Lupton sediments, and pure cultures of *Syntrophus aciditrophicus* was also used as a template in PCR reactions that amplified a 550 bp portion of the eubacterial 16S rRNA. PCR products obtained using this reaction were either used for denaturing gradient gel electrophoresis (DGGE) or the preparation of clone libraries from contaminated sediments. Individual PCR reactions that were used for DGGE contained one of the following as template; DNA from the butyrate and sulfate MPN's, the butyrate, sulfate, and G11 MPNs, or DNA from a pure culture of *Syntrophus aciditrophicus.* PCR products for DGGE were amplified by using a previously described touchdown PCR protocol (Rios-Hernandez *et al*., 2003) with the universal eubacterial primers GM5F-GC (5'CGCCCGCCGCGCGCGGCGGGCGGGGC GGGGGCACGGGGGGCGTACGGGAGGCAGCAG 3') (Santegoeds *et al*., 1999) and D907R (5'CCGTCAATTCCTTTGAGTT T 3') (Santegoeds *et al*., 1999). PCR reactions were also prepared with DNA from contaminated sediment as a template. These PCR reactions were prepared as previously described (Rios-

Hernandez *et al*., 2003) with the exception that the GMF5 primer was modified (5' CGTACGGGAGGCAGCAG 3') and did not have the GC clamp attached. PCR products were obtained from these reactions by using a previously described touchdown PCR protocol (Rios-Hernandez *et al*., 2003). The products from this reaction were then used to create the contaminated sediment clone library.

PCR was also conducted to test for the presence of specific groups of sulfate-reducing bacteria in the contaminated sediments from Fort Lupton. Five sets of group-specific 16S rRNA primers were used to screen for the following genera: *Desulfobacterium, Desulfobulbus, Desulfobacter, Desulfovibrio-Desulfomicrobium,* and *Desulfotomaculum*. DNA from *Desulfobacterium autotrophicum, Desulfobulbus propionicus, Desulfobacter curvatis, Desulfovibrio vulgaris* strain G11, and *Desulfotomaculum nigrificans* were used as positive controls to ensure that each set of primers amplified the 16S rRNA gene from the appropriate group. PCR reactions and cycling conditions were prepared and carried out as previously described (Daly *et al.*, 2000). Upon completion of all PCR cycles described, reaction products were subjected to agarose gel electrophoresis to check for the presence of PCR product and to ensure that the correct product size was obtained. Agarose gel electrophoresis was carried out as described above.

PCR products amplified using the primers GM5F-GC and D907R were analyzed by denaturing gradient gel electrophoresis (DGGE) using the D-Code Universal Mutation System (BioRad, Hercules, CA). PCR products were analyzed by DGGE through the use of 6% polyacrylamide gels that were prepared as

previously described (Muyzer *et al.*, 1995) and contained a gradient of denaturant ranging from 30% to 60%. Each well used for DGGE was loaded with 15-25 microliters of PCR product and electrophoresis was carried out at 65V for 16 hours. Following electrophoresis, the polyacrylamide gels were stained with ethidium bromide for 5 minutes. This staining procedure was immediately followed by a 10 minute destaining procedure in 500 ml of nanopure water. The gels were then viewed using a UV transilluminator (302 nm), photographed using a Kodak DC120 camera, and analyzed with NucleoTech GelExpert-Lite Software (Nucleotech, San Mateo, CA). Bands obtained during DGGE analysis were excised, placed in sterile water, and reamplified with the GM5F primer (no clamp) and the D907R primer as previously described (Rios-Hernandez et al., 2003). Once the bands were successfully reamplified they were sent for sequencing at the Oklahoma Medical Research Foundation Sequencing Facility (http://www.omrf.org/OMRF/Core/DNASEQ/) located in Oklahoma City, OK.

Bacterial 16S rRNA gene clone libraries were constructed from Fort Lupton most probable number dilutions and contaminated sediment. Clone libraries for most probable number dilutions were prepared using PCR products from reactions that contained DNA from each MPN series, which was amplified with the 27F and 1492R primers as described above. Clone libraries for contaminated sediment libraries were prepared with PCR products from reactions that contained sediment DNA, which was amplified with the GMF5 (no clamp) and D907R as described above. In both cases the PCR products that were obtained were cloned into the

TOPO 2.1 cloning vector (Invitrogen Corp., Carlsbad, CA) according to the instructions of the manufacturer. Approximately 100 randomly picked clones from MPN dilutions and an additional 100 clones from Fort Lupton contaminated sediment were sequenced at the Advanced Center for Genome Technology at the University of Oklahoma. Details of the sequencing protocols applied were described previously (Elshahed *et al*., 2003) and can be found at: http://www.genome.ou.edu/ds_seq_template_isol_hydra.html.

Sequences from excised DGGE bands, the contaminated sediment clone library, and MPN clone libraries were checked for chimeras using the Bellerophon-based chimera check program that is available through Greengenes (http://greengenes.lbl.gov) (DeSantis *et al*., 2006b; Huber *et al*., 2004). Chimera-checked sequences from the clone libraries were imported into Greengenes (http://greengenes.lbl.gov/) (DeSantis *et al*., 2006b) and aligned using the NAST alignment tool (DeSantis *et al*., 2006a). The NAST alignment files were then used to create distance matrices on Greengenes. These distance matrices were then exported to DOTUR (Schloss and Handelsman, 2005), which was used to group the sequences into OTUs. Sequences contained in the individual OTU's from this study were grouped together based on 97% sequence similarity.

The phylogenetic affiliation of 16S rRNA sequences obtained from excised DGGE bands and each OTU from the clone libraries was determined using the Classifier program that is available through the Ribosomal Database Project (http://rdp.cme.msu.edu/classifier/classifier.jsp) (Wang et al., 2007).

Nucleotide sequence accession numbers. The 16S rRNA sequences of the OTUs from MPN dilutions and sediments clone libraries have been assigned the following GenBank accession numbers: EU552837-EU552873 for sediment clones and EU552874-EU552910 for MPN clones.

RESULTS

Most-probable-number analysis. Low numbers of butyrate-degraders were observed in most probable number (MPN) dilutions under all conditions tested (Table 3.2). There was no significant ($P < 0.05$) difference in the number of butyrate-degraders (defined by butyrate depletion in MPN tubes) in MPNs amended with the hydrogen-users *Desulfovibrio vulgaris* strain G11 or *Methanospirillum hungatei* strain JF-1 relative to MPNs that contained only butyrate and sulfate. Butyrate consumption in MPN tubes amended with JF-1 was coupled to acetate and methane production. Butyrate consumption in MPNs, that either contained butyrate and sulfate alone or butyrate, sulfate, and G11 was coupled to acetate production and sulfate reduction. Butyrate MPNs that were inoculated with uncontaminated sediments showed no butyrate loss, no methane production, and no sulfate loss after 120 days.

Evidence for syntrophic butrate degradation. The fate of butyrate was monitored in microcosms that contained contaminated sediment and basal medium amended with either butyrate alone or butyrate and sulfate (Table 3.3). Butyrate consumption in microcosms that contained only butyrate was coupled to acetate and methane production. BESA was added to an additional set of microcosms that only contained butyrate and were actively degrading butyrate. The addition of BESA to these microcosms inhibited butyrate metabolism. Acetate production and

TABLE 3.2. Most probable number of different types of butyrate-degraders in Fort Lupton aquifer sediments.

Treatment	Contaminated	95% Confidence Limits[a] Lower	Upper	Uncontaminated
Butyrate + sulfate + G11 [b]	9.2×10^{1}[c]	2.3×10^{1}	3.7×10^{2}	BDL[b]
Butyrate + sulfate	2.3×10^{2}	7.4×10^{1}	7.2×10^{2}	BDL
Butyrate + JF-1 [b]	2.1×10^{6}	1.6×10^{2}	1.1×10^{3}	BDL

[a] 95% upper and lower confidence intervals used for three tube MPN analysis
[b] Abbreviations: JF-1; *Methanospirillum hungatei* strain JF-1; G11, *Desulfovibrio vulgaris* strain G11; BDL, below detection limit.
[c] MPN values are per gram of sediment.

TABLE 3.3. Butyrate degradation in microcosms with and without the addition of sulfate, 2-bromoethanesulfonic acid and molybdate.

Microcosm	Butyrate Consumed (μmoles)	Acetate Produced (μmoles)	Methane Produced (μmoles)	Sulfate Consumed (μmoles)
Butyrate	94 ± 4 [b]	218 ± 11	41 ± 0.1	0 ± 0
Butyrate + BESA [a]	25 ± 6	41 ± 6	8 ± 0.71	0 ± 0
Butyrate SO_4 + NaCl	90 ± 8	229 ± 26	0 ± 0	58 ± 16
Butyrate + SO_4 + Molybdate	48 ± 4	119 ± 8	2.5 ± 0.8	25 ± 9
Butyrate HK [a]	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Butyrate SO_4 HK	0 ± 0	0 ± 0	0 ± 0	0 ± 0

[a] Abbreviations: BESA, 2-bromoethanesulfonic acid; HK, heat-killed sample.
[b] Mean ± standard deviation of triplicate microcosms.

sulfate reduction were observed in microcosms amended with butyrate and sulfate. Molybdate was added to an additional set of microcosms that contained butyrate and sulfate, and were actively degrading butyrate. The addition of molybdate to these microcosms inhibited butyrate metabolism. No butyrate metabolism was observed in heat-killed controls that contained either butyrate alone or butyrate and sulfate.

Butyrate metabolism was restored in BESA-inhibited microcosms when sulfate and the hydrogen-using sulfate reducer G11 were added (Figure 3.1), showing that the butyrate degraders in these sediments can couple their metabolism with hydrogenotrophic sulfate reducers. The addition of only sulfate to butyrate microcosms inhibited by BESA did not restore butyrate metabolism (Figure 3.1). The addition of the hydrogen-user JF-1 did not restore butyrate metabolism in molybdate-inhibited microcosms (data not shown).

Inhibition of butyrate degradation under sulfate-reducing conditions by CO. To avoid the potential bacteriocidal action of molybdate, we used CO, a known inhibitor of hydrogenases (Adams, 1990), to test if syntrophic metabolism occurred when sulfate was in excess. The addition of 10% CO to microcosms with butyrate and sulfate that contained contaminated sediment from Fort Lupton inhibited butyrate and sulfate consumption and caused acetate accumulation to stop and the hydrogen partial pressure to increase (Figure 3.2A). The $\Delta G'$, which ranged from -15 to -20 kJ mol^{-1} prior to the addition of CO, increased to between + 3 to -5 kJ mol^{-1} during the 4-day period that butyrate metabolism was inhibited (Figure

3.2B). After 8 days, CO was no longer detectable in the headspace most likely due to its consumption by CO-oxidizing microorganisms. Once CO was gone, butyrate degradation recommenced and was coupled to sulfate reduction, acetate accumulation, and the return of the hydrogen partial pressure to levels observed prior to CO amendment (Figure 3.2A). The $\Delta G^{'}$ values returned to values observed prior to CO addition, about -13 and -15 kJ mol^{-1} (Figure 3.2B). Butyrate metabolism was not inhibited in control microcosms that contained Fort Lupton contaminated sediment, butyrate, sulfate, and were amended with N_2 (Figure 3.3A). The addition of N_2 to these microcosms did not result in an increase in the H_2 partial pressure, which ranged from 2-5 Pa throughout the experiment and acetate concentrations steadily increased as butyrate was degraded. Values of $\Delta G^{'}$ in the N_2 amended microcosms ranged between -12.5 and -16 kJ/mol (Figure 3.3B). Butyrate metabolism was coupled to sulfate-reduction in both CO and N_2-amended microcosms, which consumed 1.4 mM (± 0.2 mM) and 1.9 mM (± 0.3 mM) sulfate respectively.

To test whether CO inhibited butyrate metabolism at other sites that actively reduce sulfate, microcosms were prepared with sediments either from a duck pond that had large quantities of sulfide or from the Great Salt Plains that contained high concentrations of sodium chloride and sulfide. Butyrate metabolism continued in microcosms from each site after the addition of CO at rates nearly identical to that observed in control microcosms that were amended with N_2 (data not shown). Butyrate metabolism was coupled to the reduction of sulfate in all of these

FIGURE 3.1. Restoration of butyrate degradation in BESA-inhibited microcosms with the addition *Desulfovibrio vulgaris* G11 and sulfate. All microcosms were reamended with butyrate and received either 10 mM sulfate and the hydrogen-user *Desulfovibrio vulgaris* G11 (♦) or 10 mM sulfate alone (•). Points are the mean and error bars are the standard deviations of triplicate microcosms.

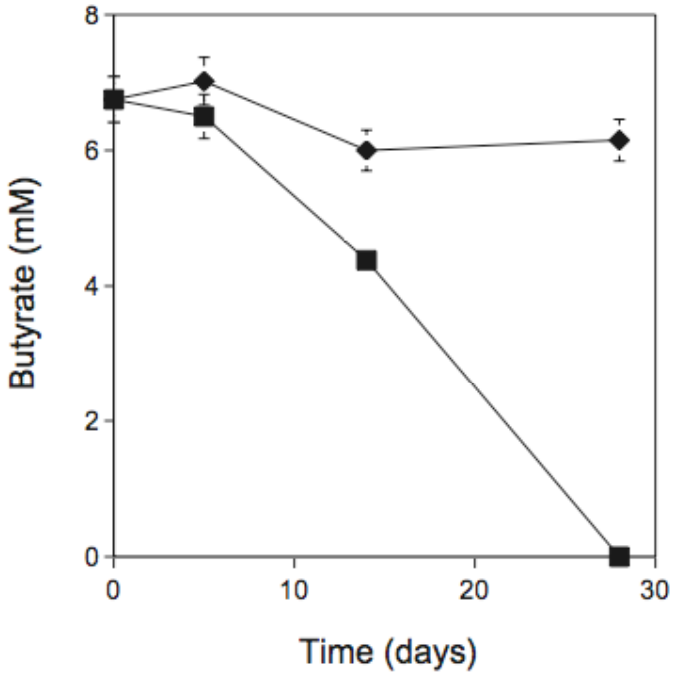
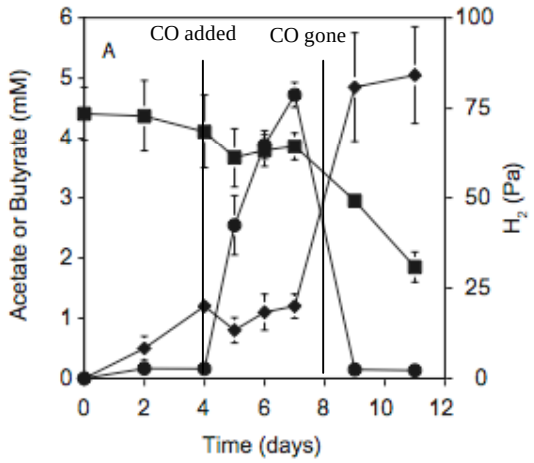

Butyrate (mM)
8
6
4
2
0
0
10
20
30
Time (days)

FIGURE 3.2. Effect of CO on butyrate metabolism in sulfate-reducing microcosms. Butyrate loss (■), acetate production (●), and hydrogen partial pressure (◆) in microcosms with butyrate, sulfate, and CO (A). The ΔG' value for butyrate degradation in microcosms amended with CO (B). Data points are the mean and error bars are the standard deviations of triplicate samples. The ΔG' for butyrate degradation from Fort Lupton microcosms amended with CO was calculated using the measured concentrations of acetate, butyrate and the equation below:

$$\Delta G' = \Delta G^{0'} + RT \ln \frac{[\text{Acetate}]^2 (pH_2)^2}{[\text{Butyrate}]}$$

where (pH_2) is the hydrogen partial pressure, R is the universal gas constant (0.00831 kJ K^{-1} mol^{-1}), T is the temperature (degrees Kelvin), and the values listed in brackets are the molar concentrations of the respective compounds. The $\Delta G^{0'}$ value for butyrate degradation was calculated according to Thauer *et al.* (Thauer *et al.*, 1977).


A
CO added
CO gone
Acetate or Butyrate (mM)
H_2 (Pa)
Time (days)
0
1
2
3
4
5
6
0
25
50
75
100
0
2
4
6
8
10
12

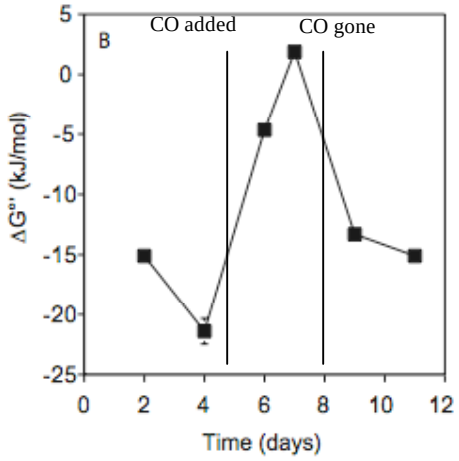
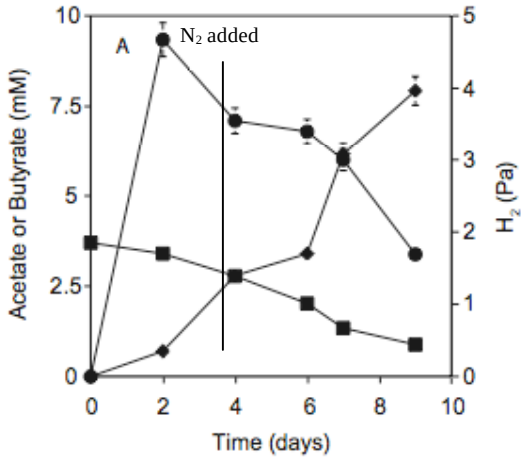
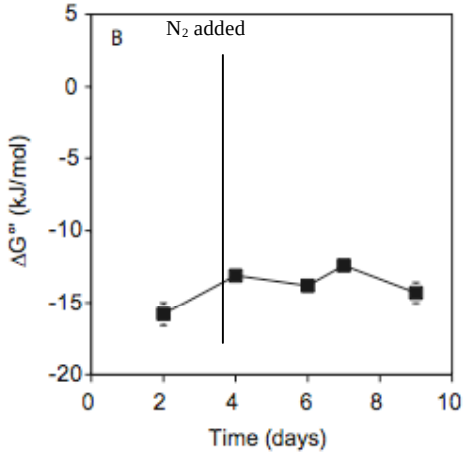

B
CO added
CO gone
ΔG'' (kJ/mol)
Time (days)
5
0
-5
-10
-15
-20
-25
0
2
4
6
8
10
12

FIGURE 3.3. Effect of N_2 on butyrate metabolism in sulfate-reducing microcosms. Butyrate loss (■), acetate production (●), and hydrogen partial pressure (◆) in microcosms with butyrate, sulfate, and N_2 (A). The $\Delta G'$ value for butyrate degradation in microcosms amended with N_2 (B). Data points are the mean and error bars are the standard deviations of triplicate samples. The $\Delta G'$ for butyrate degradation from Fort Lupton microcosms amended with N_2 was calculated using the measured concentrations of acetate, butyrate and the equation below:

$$\Delta G' = \Delta G^{0'} + RT \ln \frac{[\text{Acetate}]^2 \, (pH_2)^2}{[\text{Butyrate}]}$$

where (pH_2) is the hydrogen partial pressure, R is the universal gas constant (0.00831 kJ K^{-1} mol^{-1}), T is the temperature (degrees Kelvin), and the values listed in brackets are the molar concentrations of the respective compounds. The $\Delta G^{0'}$ value for butyrate degradation was calculated according to Thauer *et al.* (Thauer *et al.*, 1977).


A
N2 added
Acetate or Butyrate (mM)
H2 (Pa)
Time (days)
10
7.5
5
2.5
0
5
4
3
2
1
0
0
2
4
6
8
10


B
N2 added
ΔG'' (kJ/mol)
Time (days)
5
0
-5
-10
-15
-20
0
2
4
6
8
10

microcosms (data not shown). Hydrogen partial pressures did increase following the addition of CO (data not shown).

Molecular Analyses. Molecular studies provided further evidence for syntrophic butyrate metabolism in Ft. Lupton sediments. 16S rRNA genes sequences from clone libraries prepared with DNA extracted from each most probable number dilution series are shown in Table 3.4. A total of 12 OTUs, which contained 20 of the 66 total sequences (30%) obtained from these MPN cultures, were most similar to members of the family *Syntrophomonadaceae*. Sequences that were most similar to members of the *Syntrophomonadaceae* accounted for 5 of the 19 total sequences (26%) obtained from MPN tubes with butyrate and the hydrogen-user JF-1, 13 of the 24 total sequences (54%) obtained from MPN tubes with butyrate and sulfate, and 2 of the 23 total sequences (9%) obtained from MPN tubes with butyrate, sulfate, and the hydrogen-user G11.

A total of 11 OTUs, which contained 20 of the 66 total sequences (30%) obtained from these MPN tubes were affiliated with sulfate-reducing bacteria. Of the 20 total sequences affiliated with sulfate-reducing bacteria from MPNs, 2 (10%) were most similar to members of the family *Desulfobulbaceae* and accounted for 1 of the 23 total sequences (4%) from the butyrate, sulfate, and G11 MPNs and 1 of the 24 total sequences (4%) from the butyrate and sulfate MPNs. Three of the 20 total sequences (15%) affiliated with sulfate-reducing bacteria were most similar to members of the family *Desulfobacteriaceae* and accounted for 3 of the 23 total sequences (13%) from butyrate, sulfate, and G11 MPN's. Fifteen of

the 20 total sequences (75%) of the sequences that were most similar to sulfate-reducing bacteria were affiliated with the family *Desulfovibrionaceae* and accounted for 1 of the 19 total sequences (5%) from butyrate and JF-1 MPNs, 6 of the 24 total sequences (25%) from butyrate and sulfate MPNs, and 8 of the 23 total sequences (35%) from butyrate, sulfate, and G11 MPNs. Sequences related to *Desulfovibrio* species were expected in MPN tubes that were amended with G11.

DNA from the MPN tubes that contained sulfate was also used as a template for DGGE analysis. A very intense band was present in tubes both from the butyrate and sulfate MPNs and from the butyrate, sulfate, and G11 MPNs (Figure 3.4). This band migrated to the same position in the gel in both MPN sets and co-migrated with DNA from a pure culture of *Syntrophus aciditrophicus* (Figure 3.4). A BLAST search was conducted using the sequences of the predominant bands (highlighted with arrows in Figure 3.3) from the two MPN cultures. These sequences were 100% similar (551/551 bases were identical) to clone WCHB 1-12 from a jet fuel contaminated aquifer (Doijka *et al.*, 1998). The phylogenetic affiliation of these sequences was determined using the Classifer program from the Ribosomal Database project. The results obtained for the Classifier program indicated that the sequences were affiliated with members of the family *Syntrophaceae*.

16S rRNA gene sequences from clone libraries that were prepared using DNA extracted directly from contaminated sediment and amplified with the universal eubacterial primers are shown in Table 3.5. A total of 4 OTUs, which

contained 9 of the 85 total sequences (11%) obtained from contaminated sediment, were affiliated with members of the family *Syntrophaceae*. Four out of the 85 total sequences (5%) obtained from contaminated sediment were affiliated with sulfate-reducing bacteria. One of these 4 sequences was affiliated with the family *Desulfobacteriaceae*, while the other 3 sequences were affiliated with the family *Desulfobulbaceae*.

Screening of the contaminated sediment with primers sets that are specific for several genera of sulfate-reducing bacteria showed that members of the genera *Desulfobulbus*, *Desulfovibrio-Desulfomicrobium*, *Desulfotomaculum*, and *Desulfobacter* were present. No PCR products were observed in reactions mixtures that contained DNA from the contaminated sediment and primers that were specific for the genus *Desulfobacterium*.

TABLE 3.4. OTU sequences from clone libraries prepared with DNA from MPNs inoculated with Fort Lupton sediment.

OTU	Number of Clones B[a]	BS[a]	BSG[a]	Closest % match from NCBI	Accession Number of closest % match	Affiliation[b]
MPN1	0	9	0	Uncultured bacterium clone 50 (97%)[c]	EF644507	*Syntrophomonadaceae*
MPN8	0	1	0	Uncultured bacterium clone A3 (94%)	AY540494	*Syntrophomonadaceae*
MPN10	0	1	0	Uncultured bacterium clone 50 (95%)	EF644507	*Syntrophomonadaceae*
MPN12	0	1	0	Uncultured bacterium clone 50 (84%)	EF644507	*Syntrophomonadaceae*
MPN14	0	1	0	Uncultured bacterium clone 50 (93%)	EF644507	*Syntrophomonadaceae*
MPN19	0	0	1	Uncultured bacterium clone 30f08 (95%)	EF515595	*Syntrophomonadaceae*
MPN23	0	0	1	Uncultured bacterium clone TANB7 (100%)	AY667252	*Syntrophomonadaceae*
MPN31	1	0	0	Uncultured bacterium clone R4b14 (82%)	AF482440	*Syntrophomonadaceae*
MPN35	1	0	0	Uncultured bacterium clone R4b14 (84%)	AF482440	*Syntrophomonadaceae*
MPN38	1	0	0	*Syntrophomonas wolfei* strain Goettingen (98%)	CP000448	*Syntrophomonadaceae*
MPN42	1	0	0	Uncultured low G+C Gram positive cloneKB11 (86%)	AB074932	*Syntrophomonadaceae*
MPN47	1	0	0	*Syntrophomonas wolfei* strain Goettingen (96%)	CP000448	*Syntrophomonadaceae*
MPN3	0	2	0	Uncultured bacterium clone ASP-33 (94%)	EF679191	*Enterobacteriaceae*
MPN20	0	0	2	*Citrobacter* TNT5 (97%)	DQ229104	*Enterobacteriaceae*
MPN30	0	0	1	Uncultured bacterium clone L3T_005 (84%)	EF551897	*Enterobacteriaceae*
MPN4	0	1	0	Uncultured bacterium clone E449-6 (96%)	EU037964	*Desulfobulbaceae*
MPN29	0	0	1	Uncultured bacterium clone E449-6 (98%)	EU037964	*Desulfobulbaceae*
MPN5	0	2	1	*Desulfovibrio* sp. clone B4 (97%)	AJ133797	*Desulfovibrionaceae*
MPN6	0	1	0	Uncultured bacterium clone LS4-150 (96%)	AB234259	*Desulfovibrionaceae*
MPN11	0	2	0	*Desulfovibrio* sp. strain STL10 (97%)	X99502	*Desulfovibrionaceae*
MPN13	0	1	0	*Desulfovibrio* sp. clone B4 (96%)	AJ133797	*Desulfovibrionaceae*
MPN17	0	0	5	*Desulfovibrio desulfuricans* strain MB (97%)	AF192154	*Desulfovibrionaceae*
MPN24	0	0	1	*Desulfovibrio desulfuricans* strain MB (94%)	AF192154	*Desulfovibrionaceae*
MPN27	0	0	1	*Desulfovibrio desulfuricans* strain MB (95%)	AF192154	*Desulfovibrionaceae*
MPN40	1	0	0	*Desulfovibrio desulfuricans* strain MB (91%)	AF192154	*Desulfovibrionaceae*
MPN18	0	0	3	Uncultured *Desulfobacteraceae* clone D25_17 (92%)	EU266896	*Desulfobacteriaceae*
MPN26	0	0	1	Uncultured *Sedimentibacter* sp. clone VE117 (92%)	EF681724	*Peptostreptococcaceae*
MPN32	2	0	0	*Sedimentibacter* sp. JN_18_A14_H (96%)	DQ168650	*Peptostreptococcaceae*
MPN9	6	2	3	Firmicutes bacterium AD3-1 (98%)	DQ833381	Unclassified Firmicutes
MPN45	1	0	0	Uncultured Firmicute clone MN013 (89%)	AM157458	*Peptococcaceae*
MPN34	1	0	0	Uncultured bacterium clone M13_Pitesti (95%)	DQ378233	*Clostridiaceae*
MPN41	1	0	0	Uncultured bacterium clone AuSVC14 (97%)	DQ833326	*Clostridiaceae*
MPN21	0	0	1	Uncultured beta proteobacterium clone CF_8 (94%)	EF562569	*Oxalobacteriaceae*
MPN43	1	0	0	Uncultured bacterium clone TSACO1 (97%)	AB186804	*Campylobacteraceae*
MPN25	0	0	1	Uncultured bacterium clone YWB40 (93%)	AB294309	No affiliation
MPN44	1	0	0	Uncultured bacterium JN18_A7_F* (87%)	DQ168648	No affiliation
Total	19	24	23			

[a]Abbreviations for clone libraries: B, Clone library for MPNs amended with butyrate and hydrogen-user *Methanospirillum hungatei* strain JF-1; BS, clone library for MPNs amended with butyrate and sulfate; BSG, clone library for MPNs amended with butyrate, sulfate, and the hydrogen-user *Desulfovibrio vulgaris* G11.
[b]Affiliations listed are the closest family obtained from the Ribosomal Database Classifer program.
[c]Numerical values listed in parentheses are the percent similarity to the closest phylogenetic relative obtained from blast searches.

FIGURE 3.4. Denaturing Gradient Gel Electrophoresis analysis of MPN tubes with sulfate from Ft. Lupton contaminated sediments. Lane 1 contains sequences from MPNs that contained butyrate and sulfate. Lane 2 contains sequences from MPNs that contained butyrate, sulfate, and *Desulfovibrio vulgaris* strain G11. Lane 3 contains DNA from a pure culture of *Syntrophus aciditrophicus*. Arrows in lanes 1 and 2 indicate the predominant bands that were excised, reamplified, sequenced, and found to group with members of the family *Syntrophaceae*.

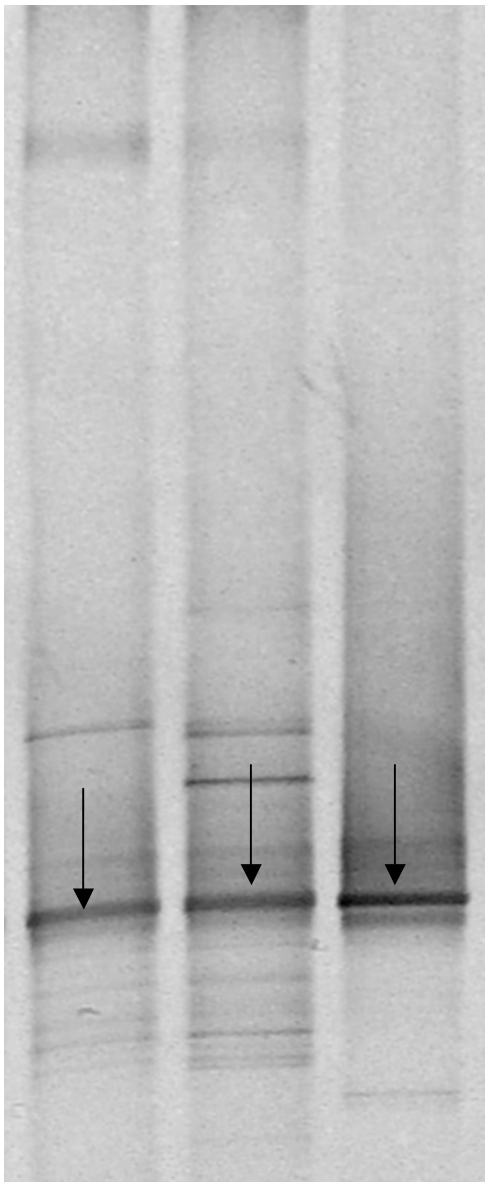


TABLE 3.5. OTU sequences from clone libraries prepared with DNA from Fort Lupton contaminated sediment.

OTU	Number Of Clones	Closest % match from NCBI	Accession Number of closest % match	Affiliation[b]
FLSED1	8	Uncultured bacterium clone E7-966 (99%)[b]	DQ200718	*Rhodocyclaceae*
FLSED2	1	Uncultured bacterium clone BSC14 (97%)	AB161272	*Hydrogenophilaceae*
FLSED3	1	Uncultured bacterium clone WCHB1-69 (99%)	AF050545	*Bacteroidaceae*
FLSED4	3	Uncultured bacterium clone RB353 (99%)	AB240349	*Bacteroidaceae*
FLSED8	6	Uncultured bacterium clone E45-1238 (99%)	DQ200782	*Bacteroidaceae*
FLSED5	6	Uncultured bacterium clone 5S27 (99%)	DQ664010	*Syntrophaceae*
FLSED20	1	Uncultured bacterium clone 5S27 (95%)	DQ664010	*Syntrophaceae*
FLSED31	1	Uncultured bacterium clone 30f10 (90%)	EF515596	*Syntrophaceae*
FLSED44	1	Uncultured bacterium clone HsB48fl (94%)	AB267042	*Syntrophaceae*
FLSED6	12	Uncultured bacterium clone ZZ9C12 (98%)	AY214177	*Comamonadaceae*
FLSED16	9	Uncultured beta proteobacterium clone RBE2CI-57 (99%)	EF111164	*Comamonadaceae*
FLSED11	1	Uncultured *Desulfobacteraceae* bacterium clone D25_41 (90%)	EU266914	*Desulfobacteraceae*
FLSED9	1	Uncultured bacterium clone E35-1238 (94%)	DQ200778	*Spirochaetaceae*
FLSED37	1	Uncultured bacterium clone SJA-102 (97%)	AJ009481	*Spirochaetaceae*
FLSED10	4	*Caulobacter* sp. BF03_Tho7 (99%)	DQ677873	*Caulobacteraceae*
FLSED15	1	Uncultured *Phenylobacterium* sp. clone AUVE_04G09 (99%)	EF651169	*Caulobacteraceae*
FLSED25	1	*Caulobacter* sp. BF03_Tho7 (86%)	DQ677873	*Caulobacteraceae*
FLSED27	1	*Acidithiobacillus ferroxxidans* strain DX-2 (99%)	DQ676506	*Acidithiobacillaceae*
FLSED17	1	Uncultured eubacterium clone WCHB1-08 (96%)	AF050573	*Microbacteriaceae*
FLSED18	2	Uncultured bacterium clone anG09 (99%)	EF034573	*Erysipelotrichaceae*
FLSED19	1	Uncultured bacterium clone CbR3s.15 (98%)	EF014654	*Desulfobulbaceae*
FLSED33	2	Uncultured bacterium clone WCHB1-67 (98%)	AF050536	*Desulfobulbaceae*
FLSED23	1	Uncultured *Clostridium* sp. clone ObedB-2D (99%)	EU073770	*Clostridiaceae*
FLSED24	1	Uncultured bacterium clone LCKS745B19 (99%)	EF201760	*Peptococcaeae*
FLSED30	1	Uncultured bacterium clone E449-8 (98%)	EU037965	*Peptococcaceae*
FLSED32	1	Uncultured bacterium clone MW2-66 (89%)	AY122599	*Peptococcaceae*
FLSED34	2	Uncultured Peptococcaceae bacterium clone D25_37 (98%)	EU266910	*Peptococcaceae*
FLSED29	4	Uncultured bacterium clone E18-966 (99%)	DQ200724	*Phyllobacteriaceae*
FLSED38	1	Uncultured delta proteobacterium clone AKYG1663 (99%)	AY921969	*Geobacteraceae*
FLSED39	1	Uncultured bacterium clone TANB142 (98%)	AY667270	*Geobacteraceae*
FLSED43	1	Uncultured bacterium clone ES3-48 (98%)	DQ463267	*Flavobacterium*
FLSED22	1	Uncultured bacterium clone 227b2 (98%)	EF459940	Uncultured CFB
FLSED26	1	Uncultured bacterium clone 227b2 (93%)	EF459940	Uncultured CFB
FLSED35	2	Uncultured bacterium clone MP16_L (96%)	AB290365	Uncultured CFB
FLSED41	1	Uncultured bacterium clone 5C95 (97%)	DQ663992	Uncultured *Actinobacteria*
FLSED42	1	Uncultured bacterium clone E7-966 (96%)	DQ200718	Uncultured ß-proteobacterium
FLSED28	1	Uncultured *Bacteroidetes* clone CD_05 (97%)	EF562561	Uncultured *Bacteroidetes*
Total	85			

[a] Affiliations listed are the closest family obtained from the Ribosomal Database Classifer program.
[b]Numerical values listed in parentheses are the percent similarity to the closest phylogenetic relative obtained from blast searches.

DISCUSSION

We used multiple lines of evidence including enumerations, CO inhibition, and molecular inventories to provide evidence for syntrophic metabolism under sulfate-reducing conditions. The numbers of butyrate degraders detected under syntrophic conditions were similar to the number detected under sulfate-reducing conditions (Table 3.2). Previous MPN studies found similar numbers of butyrate-degraders in the presence and absence of sulfate in UASB reactors (Roest *et al.*, 2005; Visser *et al*., 1993). Similar numbers of butyrate-degraders were also detected under syntrophic conditions and sulfate reducing conditions in MPN studies carried out in a portion of the Norman Landfill where sulfate reduction predominates (Sieber, 2004). The loss of butyrate coupled to methane production in tubes with butyrate and JF-1 showed that Fort Lupton sediments contained microorganisms capable of syntrophic butyrate metabolism, as *M. hungatei* cannot use butyrate. If butyrate degradation in these sediments occurred by sulfate reduction without hydrogen or formate transfer, then the numbers of butyrate degraders would have been significantly higher under sulfate-reducing conditions (MPN tubes that did not receive a hydrogen-user) compared to syntrophic conditions (MPN tubes that received a hydrogen-user).

Additional evidence for syntrophic butyrate metabolism came from microcosm studies, which showed that butyrate degradation in BESA-inhibited microcosms was restored by the addition of sulfate and a hydrogen-using sulfate

reducer but not by sulfate addition alone (Figure 3.1). This experiment shows that Fort Lupton sediments contained microorganisms capable of syntrophic butyrate metabolism that could couple their metabolism to that of hydrogenotrophic sulfate reducers and not just to hydrogenotrophic methanogens. The reciprocal experiment where a hydrogen-using methanogen was added to molybdate-inhibited microcosms did not restore butyrate metabolism. It is known that molybdate depletes adenylate pools (Taylor and Oremland, 1979) so it is possible that butyrate-degraders died before they could switch their metabolism to couple with the methanogen.

To avoid the potential bacteriocidal effects of molybdate, we used CO inhibition to test whether butyrate degradation required interspecies hydrogen transfer. Butyrate metabolism ceased during the time interval when hydrogen partial pressure was high and the $\Delta G'$ values for syntrophic butyrate metabolism ranged from -5 to +3 kJ mol^{-1} of butyrate (Figs. 3.2A and 3.2B). Gibb's free energy values in this range would be insufficient to support growth or metabolism (Schink, 1997; Jackson *et al*., 1999). Taken together, these data clearly support a role for interspecies hydrogen transfer for butyrate degradation under sulfate-reducing conditions. It is possible that CO inhibited butyrate-degrading, sulfate reducers present in the aquifer sediments. However, CO did not inhibit butyrate degradation in sediments from two other sulfate-reducing sites, indicating that not all butyrate-degrading, sulfate reducers are sensitive to CO. Hydrogen partial pressures remained low in the latter experiments as would be expected if interspecies

hydrogen transfer was not involved. Three days after its addition, CO was no longer detected in the headspace of the Fort Lupton microcosms and butyrate metabolism resumed accompanied by acetate accumulation, SO_4 loss, and a drop in hydrogen partial values to levels observed prior to CO-amendment (Fig. 3.2A). The exact cause of CO removal was not determined, but diverse microorganisms are present at the Fort Lupton site including methanogens, sulfate reducers and members of the *Clostridaceae* (Tables 3.4 and 3.5) that are capable of using CO (Daniels *et al*., 1977; Kim *et al*., 1984; Parshina *et al*., 2005). The results that we obtained from the CO inhibition experiment were similar to those obtained when BESA was added to a methanogenic butyrate-degrading coculture (Dwyer *et al*., 1988). Following BESA addition, butyrate degradation ceased, methane production ceased, hydrogen partial pressure increased, and the $\Delta G'$ values approached 0 kJ mol^{-1} (Dwyer *et al.*, 1988). The hydrogen partial pressure of the coculture was about 15 Pa prior to the addition of BESA and increased to approximately 95 Pa after CO addition (Dwyer *et al*., 1988), values very similar to what we observed (Fig. 3.2A).

Molecular analyses detected sequences related to microorganisms capable of syntrophic metabolism in contaminated sediments and MPN cultures (Tables 3.4 and 3.5). 16S rRNA sequences related to syntrophic butyrate-degraders from the families *Syntrophomonadaceae* and *Syntrophaceae* were observed in both the MPN and the contaminated sediment clone libraries. 16S rRNA sequences related to members of the *Syntrophomonadaceae* accounted for 30% of the sequences that

were obtained from MPN clone libraries and were present in each MPN condition that was analyzed. 16S rRNA sequences that were related to syntrophic butyrate degraders from the genus *Syntrophaceae* accounted for 11% of the total sequences in clone libraries from the contaminated sediment. The predominant band detected in DGGE gels from the two butyrate-degrading sulfate-reducing MPN conditions was related to the family *Syntrophaceae* (Fig. 3.4).

While 16S rRNA sequences related to hydrogen-using, sulfate-reducing bacteria from the family *Desulfovibrionaceae* were present in high numbers in MPN clone libraries, these sequences were not detected in clone libraries from the contaminated sediment. However, 16S rRNA sequences related to members of the *Desulfobulbaceae* were detected and accounted for about 4% of the total 16S rRNA sequences in the clone library from the contaminated sediment. *Desulfobulbus* spp. use propionate and lactate but not butyrate (Kuever *et al*., 2005). These organisms also use H_2 in the presence of acetate (Kuever *et al*., 2005). Acetate is most likely degraded by acetoclastic methanogens at the Fort Lupton site (Struchtemeyer *et al*., 2005). It is possible that organisms related to *Desulfobulbus* spp serve as hydrogen-users in the contaminated sediments. The primer set used for the construction of sediment clone libraries may have been biased for 16S rRNA sequences other than those of family *Desulfovibrionaceae* and just simply did not detect the *Desulfovibrio* sequences. However, we were able to detect *Desulfovibrio* sequences in Fort Lupton contaminated sediments when genus specific primer sets were used.

16S rRNA sequences related to the butyrate-oxidizing, sulfate reducer, *Desulfatirhabdium butyrativorans* (Balk *et al*., 2008) were observed in the contaminated sediments. *D. butyrativorans* was also the closest cultured relative to MPN 18 (92% 16S rRNA sequence similarity), which contained the only 3 sequences related to butyrate-degrading sulfate reducers in MPN tubes with butyrate and sulfate. *D. butyrativorans* completely oxidizes butyrate to CO_2 in the presence of sulfate (Balk *et al*., 2008). The accumulation of acetate that accompanied butyrate-degradation in MPN cultures and microcosms with sulfate argue that *D. butyrativorans*-like organisms did not play an important role in the metabolism of butyrate. *D. butyrativorans* has also been shown to use hydrogen when acetate is present (Balk *et al*., 2008), which may also explain the presence of related organisms in MPNs and microcosms.

The observation of syntrophic butyrate degradation under sulfate-reducing conditions is surprising because sulfate-reducing bacteria that can directly couple butyrate oxidation to sulfate reduction have faster growth rates than microorganisms capable of syntrophic metabolism (Oude Elferink *et al*., 1994). However, a number of studies implicate syntrophic metabolism in sulfate-reducing ecosystems. The numbers of butyrate-degraders in the presence and absence of sulfate were similar and sequences related to syntrophic butyrate-degraders from the genus *Syntrophomonas* were present in UASB reactors that contained butyrate and sulfate (Roest *et al*., 2005; Santegoeds *et al*., 1999; Visser *et al*., 1993). Sequences related to *Syntrophus* species were observed in hydrocarbon-

contaminated sites (Allen *et al.*, 2007; Doijka *et al.*, 1998; Kasai *et al*., 2005), providing evidence for the importance of *Syntrophus* spp. at hydrocarbon-contaminated sites. Studies with marine sediments also suggest that syntrophic bacteria may be present and active in areas where sulfate concentrations are high (Kendall *et al.*, 2006; Parkes *et al.*, 1990). High concentrations of methane were observed in marine sediments from Peru that contained non-limiting concentrations of sulfate (Parkes *et al.*, 1990). The syntrophic butyrate-degrader *Algorimarina butyrica* was obtained in coculture with hydrogen-using methanogens from psychrophilic marine sediments where sulfate is present (Kendall *et al*., 2006). Although studies have clearly shown that butyrate degradation is directly coupled to the reduction of sulfate in many sulfate-reducing environments (Banat and Newell, 1983), it is important to point out that many of these studies did not distinguish whether the loss of butyrate was coupled directly to sulfate reduction or if butyrate was degraded syntrophically with hydrogen-using sulfate reducers (Alphenaar *et al*., 1993; Visser *et al*., 1993). The results of the our study and previous studies show the need for a better understanding of both the microbial processes and microbial community members that are involved in the degradation of fatty acids in sulfate-reducing ecosystems.

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APPENDIX 1

Propionate Degradation by Sulfate-Reducing Bacteria in a Gas Condensate-Contaminated Aquifer

ABSTRACT

Propionate was indirectly degraded to acetate and carbon dioxide in anoxic sediments and groundwater from a hydrocarbon-contaminated aquifer where geochemical evidence implicated sulfate reduction and methanogenesis as the predominant terminal electron-accepting processes. The most probable number of propionate-degraders from hydrocarbon-contaminated sediments was significantly higher ($p > 0.05$) in cultures with propionate and sulfate that contained hydrogen-using microorganisms compared to cultures with propionate and sulfate but without the hydrogen-user added, suggesting that syntrophic propionate degraders were more numerous than sulfate-reducing propionate degraders. However, propionate degraders were not detected in MPNs that contained propionate and a hydrogen-using methanogen, but were not amended with sulfate. A new propionate-degrading, sulfate-reducing bacterium, with less than 96% sequence similarity to all described *Desulfobulbus* spp., was isolated from MPN enrichments that contained propionate and sulfate. Propionate loss by the pure culture and in microcosms with propionate and sulfate was coupled to sulfate loss and acetate

accumulation. Acetate was converted to methane by aceticlastic methanogens in microcosms with propionate and sulfate. 16S rRNA gene sequences related to propionate-degrading, sulfate reducers from the genus *Desulfobulbus* were detected in all MPNs with sulfate, all propionate-degrading microcosms except those with molybdate added, and the contaminated sediments by using group specific PCR primers. *Desulfobulbus* sequences accounted for approximately four percent of the total 16S rRNA genes sequences in clone libraries prepared with DNA from the contaminated sediment. Sequences related to microorganisms capable of syntrophic propionate degradation were not detected in sediment clone libraries. This work shows that sulfate reduction was the dominant fate of propionate at this site and suggests that a new species of *Desulfobulbus* was involved in propionate degradation at this site.

INTRODUCTION

The fate of propionate in methanogenic and sulfate-reducing ecosystems has been extensively studied (1, 3, 22, 28, 30, 31). However, very little is known about the populations of microorganisms that degrade propionate in these ecosystems (1, 3, 16, 26, 30, 31). Most of our knowledge about the microorganisms that degrade propionate in methanogenic ecosystems is based on a limited number of syntrophic propionate-degraders that have been isolated and described from the genera *Desulfotomaculum*, *Pelotomaculum*, *Syntrophobacter*, and *Smithella* (16, 17, 24). Previous work has shown that the degradation of propionate by syntrophic microorganisms alone is thermodynamically unfavorable due to H_2 and/or formate production (17, 18). Therefore, the degradation of propionate in methanogenic ecosystems requires interspecies hydrogen/formate transfer from hydrogen/formate-producing syntrophs to hydrogen/formate-using methanogens (17, 18). Several studies have shown that 2-bromoethanesulfonic acid, which inhibits methanogens, inhibits propionate degradation in methanogenic ecosystems (3, 30). This has led to the presumption that syntrophic metabolism is the major fate of propionate in methanogenic ecosystems (3, 30). While this method can be useful for determining whether syntrophic propionate metabolism occurs at a given study site, it does not reveal any information about the identities of the microorganisms responsible for syntrophic propionate metabolism. The

highly fastidious nature of syntrophic propionate degraders makes it difficult to isolate these microorganisms (16). To date, there is also a lack of suitable molecular markers to uniquely identify syntrophic propionate degraders in anaerobic ecosystems (16, 26). Therefore, our knowledge of the ecology and diversity of syntrophic propionate-degraders is limited (16).

It is also possible that syntrophic consortia of hydrogen- and formate-producing propionate-degraders and hydrogen/formate-using sulfate reducers could catalyze propionate degradation in sulfate-reducing ecosystems. Such an observation may not be surprising as hydrogen/formate-using sulfate reducers have commonly been used as the syntrophic partner to obtain syntrophic propionate-degraders in monoxenic culture (5). These syntrophic consortia will compete with sulfate-reducing bacteria for available propionate in sulfate-reducing ecosystems (20, 23). Several genera of sulfate-reducing bacteria have been described that degrade propionate either completely to CO_2 or incompletely to acetate and CO_2 (20, 25, 37). These microorganisms couple the degradation of propionate to the reduction of sulfate without the need for interspecies transfer of hydrogen or formate (20, 25, 37). Studies that compared the kinetic properties of propionate-degrading, sulfate reducers and syntrophic propionate degraders showed that propionate-degrading, sulfate reducers have faster growth rates with propionate than syntrophic-propionate degraders (23). Based on these findings, it is expected that propionate-degrading sulfate reducers will outcompete syntrophic propionate degraders for propionate in sulfate-reducing ecosystems (23). However, in many

studies that have monitored the fate of propionate in sulfate-reducing ecosystems, it was not possible to distinguish whether propionate degradation was carried out directly by propionate-degrading, sulfate reducers (i.e., without the involvement of interspecies hydrogen and/or formate transfer), or indirectly by consortia of syntrophic propionate-degraders and hydrogen-using sulfate reducers (1, 3, 30, 35). Therefore, the importance of syntrophic propionate metabolism in sulfate-reducing ecosystems is unclear.

Several studies have provided indirect evidence that suggests syntrophic metabolism may play an important role in the degradation of propionate in sulfate-reducing ecosystems (1, 3, 22, 28, 35). *Syntrophobacter* spp., which are capable of growing on propionate and sulfate in pure culture, prefer to grow syntrophically with hydrogen-using sulfate reducers from the genus *Desulfovibrio* when propionate and sulfate are present (32). Molecular analyses of the populations present in digesters that were used to treat sulfate-rich, papermill wastewater suggested that syntrophic consortia of *Syntrophobacter* and *Desulfovibrio* were involved in the degradation of propionate (22, 28). Enrichment cultures that actively coupled the degradation of propionate to the reduction of sulfate were obtained from this digester (28). Clone libraries from these enrichment cultures were dominated by 16S rRNA gene sequences related to *Syntrophobacter* spp., but also contained sequences related to *Desulfovibrio* spp (28). However, it was never determined if propionate degradation was carried out directly by *Syntrophobacter*

spp. alone or indirectly by syntrophic consortia of *Syntrophobacter* spp. and *Desulfovibrio* spp (28).

The goal of this work was determine the fate of propionate, e.g., sulfate reduction or syntrophic metabolism, in hydrocarbon-contaminated aquifer sediments and to characterize the populations of microorganisms involved. Sediments and groundwater samples from a gas condensate-contaminated aquifer located near Denver, Colorado were used (11). The aquifer was contaminated with gas condensate (96% w/w C_5-C_{15} hydrocarbons, including 18% w/w BTEX) in the 1970's as a result of a leaking storage sump that was used to store liquids produced during natural gas recovery (11, 34). Previous work has shown that fatty acids, including propionate, are important intermediates produced during the anaerobic degradation of hydrocarbons (6). This particular site was chosen for this study because the geochemistry of the aquifer has been thoroughly characterized over a period of several years (11). Geochemical data from this site indicated that sulfate concentrations in the contaminated portion of the aquifer were depleted relative to uncontaminated sediments (11). Spikes in the sulfate concentration were often observed in the contaminated portion of the aquifer, which indicated that sulfate was periodically replenished at this site (11). High concentrations of methane, relative to uncontaminated sediment, were observed in the contaminated portion of the aquifer (11). The steady state hydrogen concentrations in the contaminated portion of the aquifer suggested that sulfate reduction and methanogenesis were the dominant terminal electron-accepting processes at this site (11). Previous work at

this site also suggested that active populations of microorganisms including sulfate-reducing bacteria, syntrophic metabolizers, and methanogens were present at this site (11, 34). All of these findings made this site ideal for studying the role of syntrophic metabolism versus sulfate reduction in propionate degradation.

MATERIALS AND METHODS

Sample Collection. Sediment and groundwater samples were collected from a shallow aquifer, located near Fort Lupton, Colorado in August 2000. The aquifer lies above an active natural gas production field and was contaminated with gas condensate (96% wt/wt C_5-C_{15} hydrocarbons, including 18% wt/wt BTEX) in the 1970s as a result of a leaking sump that was used to store liquids produced during natural gas recovery (11, 34). Gas condensate-contaminated sediments and groundwater were collected from well 37 (11), which was located approximately 10 m downgradient from the original source of contamination. Contaminated sediment was collected by hand boring to a depth of 1.5 m below the surface (34). The sediment was placed in sterile 1-L Mason jars, which were filled to capacity to keep the samples anaerobic. Groundwater samples were collected in sterile 2 L Schott bottles, which were also filled to capacity. Uncontaminated sediment and groundwater samples were also collected as described above from well 18, which is located approximately 10 m upgradient from the source of contamination (11). All sediment and groundwater samples were stored on ice until they were delivered to the laboratory. The samples were stored at 4^0C upon arrival at the laboratory.

Microorganisms and Media. The hydrogen-using microorganisms *Methanospirillum hungatei* strain JF-1 (DSM 864) and *Desulfovibrio vulgaris*

strain G11 (DSM 7057) were obtained from our culture collection. These microorganisms were cultured in a previously described medium that lacked rumen fluid (19). This medium was prepared under strictly anaerobic conditions using the techniques described by Balch and Wolfe (2). The hydrogen-using, sulfate reducer *Desulfovibrio vulgaris* strain G11 was grown in basal medium (19) amended with 10 mM acetate and 10 mM sulfate. *Methanospirillum hungatei* strain JF-1 was grown in basal medium (19) amended with 10 mM acetate. Cultures of both G11 and JF-1 were grown with an 80% H_2-20% CO_2 gas phase (69 kPa) and were incubated at 37^0C with shaking (100 rpm).

Most Probable Number Analysis. A three-tube most probable number (MPN) dilution assay was used to quantify different metabolic groups that could be involved in propionate degradation. MPN assays were carried out using sediment from both the contaminated and uncontaminated portion of the Fort Lupton aquifer. Prior to MPN analysis, each sediment was added to a sterile, anaerobic sodium pyrophosphate solution in order to separate cells from the sediment. The pyrophosphate solution was prepared by adding 1 g L^{-1} sodium pyrophosphate to the basal medium without rumen fluid (19) (see below) and adjusting the pH to 7. The medium with pyrophosphate was then taken into an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI) where the stoppers and seals were removed. Three tubes of sodium pyrophosphate per MPN set were each amended with 1 g (wet weight) of sediment from the appropriate location, stoppered, sealed, and removed from the anaerobic chamber. Each of these three tubes was then mixed by

hand for approximately 30 s by hand inversion, and 1 ml of each solution was removed aseptically and transferred into 9 ml of the appropriate MPN medium using needles and syringes flushed with 100% N_2. This procedure was repeated using the first three tubes of inoculated MPN medium and continued until each tube of the dilution series was inoculated.

Basal medium with rumen fluid was prepared as previously described (19), amended with 10 mM propionate, and used in three different MPN series. The first MPN series contained 6 ml of medium and 3 ml of the hydrogen-using methanogen, *Methanospirillum hungatei* strain JF-1. The second series contained 6 ml of medium amended with 10 mM sulfate and 3 ml of the hydrogen-using sulfate reducer, *Desulfovibrio vulgaris* strain G11. The third series contained 9 ml of medium amended with 10 mM sulfate. The addition of hydrogen-using microorganisms into MPN tubes was done in order to enumerate for bacteria capable of syntrophic propionate degradation. Individual MPN tubes were scored positive if more than 50% of the propionate was metabolized after 60 days. As controls, MPN analysis was carried out using basal medium without propionate and either with or without sulfate to quantify background levels of sulfate reduction and methane production.

Isolation of propionate-degrading sulfate reducers from MPNs with sulfate. The highest MPN dilutions that were positive for propionate degradation and sulfate reduction were used to isolate propionate-degrading sulfate reducers. One milliliter of culture from tube 1 of the 10^{-3} dilution from MPNs with

propionate and sulfate was transferred to 9 ml of substrate unamended basal medium (19). This 1/10 dilution was mixed by hand inversion and one milliliter from this 1/10 dilution was transferred to a second tube containing 9 ml of substrate unamended basal medium. This process was repeated until the original culture was diluted by a factor of 10^6. One milliliter of culture from tube 1 of the 10^{-4} dilution from MPNs with propionate, sulfate, and G11 was diluted by a factor of 10^6 as described above. Approximately 0.5 ml from each dilution was then transferred into a Balch tube that contained 9 ml of roll tube medium. The roll tube medium consisted of a previously described basal medium (19) with 10 mM propionate, 10 mM sulfate, and 1.5% agar. After 0.5 ml of the appropriate dilution was added to 9 ml of roll tube medium, the entire volume of liquid was mixed by hand inversion. The Balch tube was then placed into a Belco tube roller (Belco Glass, Vineland, NJ). The tube was then covered with ice and spun until the agar solidified. This process was repeated until triplicate roll tubes were inoculated for each dilution. All roll tubes were incubated at room temperature for approximately 6 weeks.

Preparation of [^{14}C] propionate amended microcosms. Microcosms were prepared in an anaerobic chamber using sterile, 40-ml serum bottles, which were left in the chamber overnight prior to inoculation. Thirteen grams of sediment from the contaminated portion of the aquifer was added to each serum bottle. Groundwater from a well that was located upgradient from the contaminated area was added to bring the final volume of each microcosm to 20 ml. The microcosms were stoppered and sealed inside the chamber, and brought out of the chamber

where the gas phase was exchanged three times by evacuation and repressurization with 100% N_2 (2). The microcosms were then amended with approximately 3.3 x 10^4 Becquerels (Bq) of [3-^{14}C]-propionate. The labeled propionate was added by injecting 0.2 ml of a [3-^{14}C]-propionate stock solution that contained approximately 1.7 x 10^5 Bq/ml. Unlabeled propionate was added to all microcosms to bring the final propionate concentration to approximately 500 μM. Each set of microcosms contained three replicates of each of the following treatments: propionate alone; propionate and an 7.5 mM 2-bromethanesulfonic acid (inhibits methanogenesis) (12); propionate and 7.5 mM sulfate; and propionate, 7.5 mM sulfate, and 5 mM sodium molybdate (inhibits sulfate-reducing bacteria) (21). Heat-killed controls were run in duplicate for each of the above treatments. The heat-killed controls were autoclaved at 121^{0}C for 20 minutes. All of the microcosms were incubated for 18 days at room temperature.

Analytical methods. Non-labeled propionate loss was measured by high-pressure liquid chromatography (15). The mobile phase was 25 mM KH_2PO_4 (pH 2.5) at a flow rate of 1 ml min^{-1}. This HPLC was equipped with a radioisotope detector, which was used to quantify labeled propionate loss. The radioisotope detector was calibrated by comparing its response to that of a scintillation counter. Standards of [3-^{14}C] propionate were prepared from the same stock solutions that were used to amend the microcosms. One hundred microliters of standard solutions ranging from 2.0 x 10^3 Bq to 4.0 x 10^4 Bq were run on the radioisotope

detector. The same volume of each standard was also placed into 5 ml of scintillation cocktail and counted using a scintillation counter.

$^{14}CH_4$ and $^{14}CO_2$ production were measured using a gas chromatograph (GC) equipped with a thermal conductivity detector. The GC had a 3.05 m x 0.004 m Carbosphere 80/100 column (Altech Inc, Deerfield, IL). Helium was the carrier gas at 2 ml min^{-1}. The injector and the column were set at 175^0C and the detector was set at 81^0C. The gas chromatograph was connected to a gas proportional counter (Insus Systems Inc, Fairfield, NJ). Standards containing $^{14}CO_2$ were prepared from a stock solution containing 1.6 x 10^5 Bq/ml of $H^{14}CO_3$. This solution was diluted to concentrations ranging from 1.3 x 10^2 Bq/ml to 8.0x 10^4 Bq/ml by adding the appropriate volume of stock solution to enough 0.1N NaOH to bring the final volume of each standard to 20 ml. Each standard was then acidified with 1 ml of 12N HCl. A 0.2 ml aliquot of the headspace of each standard was then injected into the GC. Additionally, 0.2 ml aliquots of each standard were slowly bubbled into 0.8 ml of 0.1N NaOH and 0.45 ml of the solution added to 5 ml of scintillation cocktail and counted using the same procedure described above for the ^{14}C-propionate standards. The retention time of $^{14}CH_4$ was verified using a methanogenic enrichment culture that produced $^{14}CH_4$ as a result of the degradation of [U-^{14}C] methyl tert-butyl ether (MTBE).

The pH of the individual microcosms was measured with Color pHast Indicator Strips (EM Science, Gibbstown, NJ) at the end of the 18-day incubation period. The final pH of each microcosm and the amount of $^{14}CO_2$ (obtained by

GC) was used to calculate the amount of $H^{14}CO_3$ that was dissolved in the liquid phase of the microcosms by using the Henderson-Hasselbach equation (equation 1):

$$\text{Final pH of microcosm} = 6.35 + \log([H^{14}CO_3]/[^{14}CO_2]) \qquad \text{(eq. 1)}$$

The amount of $H^{14}CO_3$ determined with equation 1 was added to the amount of $^{14}CO_2$ measured by gas chromatography to obtain the total amount of $^{14}CO_2$ produced in each microcosm.

Sulfate concentrations were determined by ion chromatography (15) and the concentration of non-labeled methane was quantified using gas chromatography (14).

Molecular analysis. DNA was extracted from the highest MPN dilutions and microcosms that were positive for propionate depletion. Two milliliters of these MPNs and microcosms were collected and added directly to 2-ml polypropylene, screw-cap tubes that contained 1 g of 0.1 mm zirconia-silica beads (Biospec Products, Bartlesville, OK). These 2 ml aliquots were centrifuged at 14,000 x g for 5 minutes to pellet the cells, and the remaining supernatant was discarded. DNA was also extracted directly from Fort Lupton contaminated sediment by weighing 1 g of sediment (wet weight), which was then placed directly in a 2-ml polypropylene screw-cap tube that contained 1 g of zirconia-silica beads. DNA was extracted from MPNs, microcosms, and sediments by using a mini-bead beater as previously described (27) with the exception that 300 microliters of TE

buffer (10 mM Tris [pH 8] and 1 mM EDTA) was added to the cell pellet prior to bead beating, rather than 300 microliters of phosphate buffer. Agarose gels were used to confirm the presence of DNA in all extraction preparations and to estimate the concentration of DNA that was present. All agarose gels were stained with ethidium bromide and viewed with UV light.

DNA that was extracted from Fort Lupton MPN dilutions, microcosms, and contaminated sediment was used as a template in PCR reactions to test for the presence of propionate-degrading sulfate-reducing bacteria from the genus *Desulfobulbus*. The group specific 16S rRNA primers DBB121 (ATACCCSCW WCWCCTAGCAC) and DBB1237 (GTAGKACGTGTGTAGCCCTGGTC) were used to screen for members of the genus *Desulfobulbus* (7). DNA from *Desulfobulbus propionicus* was used as a positive control to ensure that this set of primers amplified 16S rRNA gene sequences from members of the genus *Desulfobulbus*. PCR reactions and cycling conditions were carried out as previously described (7).

PCR was also conducted to test for the presence of other groups of sulfate-reducing bacteria in the contaminated sediments from Fort Lupton. Four other sets of group-specific 16S rRNA primers were used to screen for the following genera: *Desulfobacterium*, *Desulfobacter*, *Desulfovibrio-Desulfomicrobium*, and *Desulfotomaculum*. DNA from *Desulfobacterium autotrophicum*, *Desulfobacter curvatis*, *Desulfovibrio vulgaris* strain G11, and *Desulfotomaculum nigrificans* were used as positive controls to ensure that each set of primers amplified the 16S

rRNA gene from the appropriate group. PCR reactions and cycling conditions were prepared and carried out as previously described (7).

DNA from the contaminated Fort Lupton sediments was also used as a template in PCR reactions that amplified a 550 bp portion of the eubacterial 16S rRNA. These PCR reactions and cycling conditions were carried out as previously described (27) with the exception that the GM5F primer was modified (5' CGTACGGGAGGCAGCAG 3') and did not have the GC clamp attached. Upon completion of all PCR reactions described above, the reaction products were subjected to agarose gel electrophoresis to check for the presence of PCR product and to ensure that the correct product size was obtained. Agarose gel electrophoresis was carried out as described above.

A bacterial 16S rRNA gene clone library was constructed from Fort Lupton contaminated sediments using PCR products from reactions that contained DNA from the contaminated sediment, which was amplified with the GM5F (no clamp) and D907 primers as described above. The PCR products that were obtained were cloned into the TOPO 2.1 cloning vector (Invitrogen Corp, Carlsbad, CA) according to the instructions of the manufacturer. Approximately 100 clones from the Fort Lupton contaminated sediment clone library were sequenced at the Advanced Center for Genome Technology at the University of Oklahoma. Details for the sequencing protocols applied were described previously (10) and can be found at http://www.genome.ou.edu/ds_seq_template_isol_hydra.html.

Sequences from the contaminated sediment clone library were checked for chimeras using the Bellerophon-based chimera check program that is available through Greengenes (http://greengenes.lbl.gov) (8, 13). Chimera-checked sequences from the clone library were then imported into Greengenes (8) and aligned using the NAST alignment tool (9). The NAST alignment files were then used to create distance matrices on Greengenes. These distance matrices were exported to DOTUR (29), which was used to group the sequences into OTUs. Sequences contained in the individual OTUs from this study were grouped together based on 97% sequence similarity. The phylogenetic affiliation of 16S rRNA sequences obtained from each OTU was determined using the Classifier program that is available through the Ribosomal Database Project (http://rdp.cme.msu.edu/classifier/classifier.jsp) (36).

RESULTS

Most probable number analysis. Propionate degraders were detected in all MPNs that were amended with sulfate, but not in MPNs without sulfate (Table A1.1). Based on the 95% confidence intervals, there was a statistically significant difference ($p > 0.05$) in the number of propionate-degraders (defined by propionate depletion in MPN tubes) in MPNs that were amended with propionate, sulfate, and *Desulfovibrio vulgaris* strain G11 relative to MPNs that were amended with only propionate and sulfate (Table A1.1). Propionate consumption was coupled to acetate production, methane production, and sulfate loss in propionate MPNs that were amended with sulfate and inoculated with sediment from the contaminated portion of the aquifer. Propionate MPNs that were inoculated with uncontaminated sediment showed no propionate loss, no methane production, and no sulfate loss after 120 days.

Isolation of propionate-degraders from sulfate-reducing MPNs. Numerous isolated black colonies were observed on roll tubes from the highest positive dilutions from MPN enrichments that contained propionate and sulfate or propionate, sulfate, and G11. One of the colonies that was isolated from MPNs with propionate and sulfate was chosen for further study, and labeled CS7. Colonies of CS7 were circular with an entire margin, slightly raised, and black.

TABLE A1.1 Summary of MPN results obtained using propionate as a substrate.

Treatment	Contaminated	95% Confidence Limits[a] Lower	Upper	Uncontaminated
Propionate + JF-1 [b]	BDL[b]	BDL	BDL	BDL[b]
Propionate + sulfate	2.4×10^{4}[c]	5.8×10^{3}	9.9×10^{4}	BDL
Propionate + sulfate + G11[b]	2.4×10^{5}	5.8×10^{4}	9.9×10^{5}	BDL

[a] 95% upper and lower confidence intervals used for three tube MPN analysis (4)

[b] Abbreviations: JF-1; *Methanospirillum hungatei* strain JF-1; G11, *Desulfovibrio vulgaris* strain G11; BDL, below detection limit.

[c] MPN values are per gram of sediment.

The colonies were approximately 1.5 to 2 mm in diameter. Cells of CS7 were lemon-shaped and appeared as single cells or pairs of cells when viewed with a phase contrast microscope. CS7 incompletely oxidized 4.9 ± 0.2 mM propionate to 4.75 ± 0.3 mM acetate. The incomplete oxidation of propionate was coupled to the reduction of 3.5 ±0.3 mM sulfate. CS7 grew very poorly (final OD of around .05) on medium that contained propionate and sulfate, whereas *Desulfobulbus propionicus* grew to an OD of around 0.2 to 0.3 on the same medium. Cultures of CS7 took approximately 10-12 days to degrade 5 mM propionate, whereas *D. propionicus* took only 2-3 days to degrade 5 mM propionate. The 16S rRNA sequence of CS7 (Fig A1.1) was 95% similar to *Desulfobulbus propionicus* (1397/1457 bases were identical), 94% similar to *Desulfobulbus elongatus* (1375/1457 bases were identical), 93% similar to *Desulfobulbus rhabdoformis* (1352/1449 bases were identical), 92% similar to *Desulfobulbus mediterraneus* (1338/1448 bases were identical), and 91% similar to *Desulfobulbus japonicus* (1320/1450), which are the only described species from the *Desulfobulbus* genus. Attempts were made to further characterize CS7. However, the isolate was lost during the initial stages of these characterization studies.

[3-^{14}C] propionate-amended microcosms. Both $^{14}CH_4$ and $^{14}CO_2$ were produced as a result of the degradation of [3-^{14}C] propionate (Table A1.2). Microcosms that contained only [3-^{14}C] propionate produced nearly equivalent amounts of $^{14}CH_4$ and $^{14}CO_2$. This ratio shifted slightly in microcosms that contained [3-^{14}C] propionate and sulfate. $^{14}CH_4$ and $^{14}CO_2$ accounted for 60% and

40%, respectively, of the total labeled gas produced in these microcosms. The addition of BESA to microcosms with [3-^{14}C] propionate caused this ratio to shift such that $^{14}CH_4$ and $^{14}CO_2$ accounted for approximately 20% and 80%, respectively, of the total labeled gas produced in these microcosms. However, the addition of BESA had no effect on the amount of propionate that was consumed relative to microcosms that contained [3-^{14}C] propionate alone or [3-^{14}C] propionate and sulfate. The addition of molybdate to microcosms with [3-^{14}C] propionate and sulfate caused the ratio of labeled gas to shift such that $^{14}CH_4$ and $^{14}CO_2$ accounted for 70% and 30%, respectively, of the total labeled gas produced in these microcosms, compared to bottles with only propionate and sulfate, which produced nearly equivalent amounts of $^{14}CH_4$ and $^{14}CO_2$. The addition of molybdate to microcosms with [3-^{14}C] propionate also caused between a 62 and 67% reduction in the amount of propionate that was consumed relative to microcosms with [3-^{14}C] propionate alone, [3-^{14}C] propionate and sulfate, and [3-^{14}C] propionate and BESA. [^{14}C]-Acetate was produced in all live microcosms. Approximately 50% of the [3-^{14}C] propionate that was consumed in microcosms with [3-^{14}C] propionate alone and microcosms with [3-^{14}C] propionate and sulfate was recovered as [^{14}C]-acetate. Approximately 92% of the label from [3-^{14}C] propionate in microcosms with BESA was recovered as [^{14}C] acetate. In microcosms that contained [3-^{14}C] propionate, sulfate, and molybdate, approximately 70% of the label from [3-^{14}C] propionate was recovered as [^{14}C] acetate.

As expected, the consumption of propionate in microcosms with [3-^{14}C] propionate and sulfate was coupled to sulfate loss (0.5 ± 0.05 mM). It also appears that propionate loss was coupled to sulfate reduction in microcosms that contained [3-^{14}C] propionate alone and microcosms with [3-^{14}C] propionate and BESA. Sulfate measurements indicated that between 2 and 2.5 mM sulfate was present in the groundwater that was used in all microcosms. Approximately 0.4 ± 0.1 mM and 0.5 ± 0.1 mM sulfate were consumed in microcosms amended with only [3-^{14}C] propionate and [3-^{14}C] propionate and BESA, respectively. No sulfate loss was observed in microcosms that contained [3-^{14}C] propionate, sulfate, and molybdate. No propionate loss, sulfate loss, methane production, or acetate production was observed in any of the heat-killed microcosms that were amended with [3-^{14}C] propionate. The data from these radioactive microcosms are consistent with incomplete propionate degradation to acetate and CO_2 by sulfate reduction. The decrease in propionate metabolism and inhibition of sulfate reduction that was observed in microcosms with molybdate clearly showed that sulfate reduction was involved in propionate metabolism at this site. The addition of BESA to microcosms with propionate had no effect on propionate metabolism, but inhibited acetate consumption and methane production. These findings showed that acetate was metabolized to methane by aceticlastic methanogens, which is consistent with the work presented in Chapter 2.

Molecular analysis. 16S rRNA sequences related to propionate-degrading sulfate reducers from the genus *Desulfobulbus* were detected using the group

specific 16S rRNA primers DBB121 and DBB 1237 in all MPNs with sulfate, microcosms with only [3-^{14}C] propionate, microcosms with [3-^{14}C] propionate and BESA, microcosms with [3-^{14}C] propionate and sulfate, and the contaminated sediments (Figure A1.2). PCR product was not obtained with these group specific PCR primers in tubes that contained DNA from microcosms with [3-^{14}C] propionate, sulfate, and molybdate. PCR product was observed in reactions that contained the group specific 16S PCR primers and DNA from *Desulfobulbus*

FIGURE A1.1 The 16S rRNA gene sequence of CS7

GTGCTTAACACATGCAAGTCGAACGCGAAAGGGACTTCGGTCCTGAGT
AAAGTGGCGCACGGGTGAGTAACGCGTAGATAACCTGTCTTTATGTCTG
GAATAATACGCCGAAAGGGGTACTAATACCGGATATTCTTGCTTTATAT
AAGTTTTGCAAGCAAAGGTGGCCTCTGGCATAAGCTACTGCATGAAGA
GGGGTCTGCGTACCATTAGCTTGTAGGTGGGGTAATGGCCTACCTAGGC
GACGATGGTTAGCGGGTCTGAGAGGATGATCCGCCACACTGGCACTGG
AACACGGGCCAGACTCCTACGGGAGGCAGCAGTGAGGAATATTGCGCA
ATGGGGGCAACCCTGACGCAGCGACGCCGCGTGAGCGAGGAAGGCCTT
CGGGTCGTAAAGCTCTGTCAAAGGGAAAGAAATGTATAATGGTTAATA
CCTGTTATATTTGACGGTACCCTTAAAGGAAGCACCGGCTAACTCCGTG
CCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTGTTCGGAATTACT
GGGCGTAAAGGGCGCGTAGGCGGTTTGATAAGTCAGATGTGAAAGCCC
ACGGCTTAACTGTGGAAGTGCATTTGATACTGTCAGACTTGAGTACCAG
AGGGGAAAGTGGAATTCCCGGTGTAGAGGTGAAATTCGTAGATATCGG
GAGGAATACCGGTGGCGAAGGCGACTTTCTGGCTGGATACTGACGCTG
AGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCC
ACGCTGTAAACGATGTCAACTAGATGTAGGGGGTGTTGATCCCTTCTGT
GTCGCAGCTAACGCATTAAGTTGACCGCAGCTAACGCATTAAGTTGACC
GCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGG
GCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAG
AACCTTACCTGGTCTTGACATCCCAGAGATCCCTTGGAAACTTGGGAGT
GCTTCCATTAGGAAGAATCTGGAGACAGGTGCTGCATGGCTGTCGTCAG
CTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTG
CCTTTAGTTGCCAGCAGTTCGGCTGGGCACTCTAAAGGGACTGCCGGTG
TTAAACCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCTTTATG
ACCAGGGCTACACACGTACTACAATGGCCGATACAAAGGGCAGCGACA
TCGCGAGATGAAGCCAATCCCATAAATTCGGTCTCAGTCCGGATTGGAG
TCTGCAACTCGACTCCATGAAGTTGGAATCGCTAGTAATCGTGGATCAG
CATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACA
CCACGGGAGTCGGTTGTACCAGAAGTCAGTTGAGCTAACCGCAAGGAG
GC

TABLE A1.2. Fate of propionate carbon in microcosms from the contaminated site.

Treatment	Propionate lost (Bq [10^4])	T_f $^{14}CH_4$ produced (Bq [10^4])	T_f $^{14}CO_2$ produced (Bq [10^4])	T_f ^{14}C-acetate produced (Bq [10^4])
[3-^{14}C] propionate	2.3 ± 0.2 [a]	1.0 ± 0.2	0.92 ± 0.12	1.3 ± 0.33
[3-^{14}C] propionate HK [b]	0	0	0	0
[3-^{14}C] propionate + BESA[b]	2.0 ± 0.3	0.14 ± 0.06	0.52 ± 0.17	1.8 ± 0.29
[3-^{14}C] propionate + BESA HK	0	0	0	0
[3-^{14}C] propionate + SO_4	2.0 ± 0.3	0.57 ± 0.087	0.38 ± 0.058	0.92 ± 0.25
[3-^{14}C] propionate + SO_4 HK	0	0	0	0
[3-^{14}C] propionate + SO_4 + molybdate	0.76 ± 0.19	0.37 ± 0.013	0.18 ± 0.079	0.52 ± 0.15
[3-^{14}C] propionate + SO_4 + molybdate HK	0	0	0	0

[a] Mean ± standard deviation of triplicate microcosms.

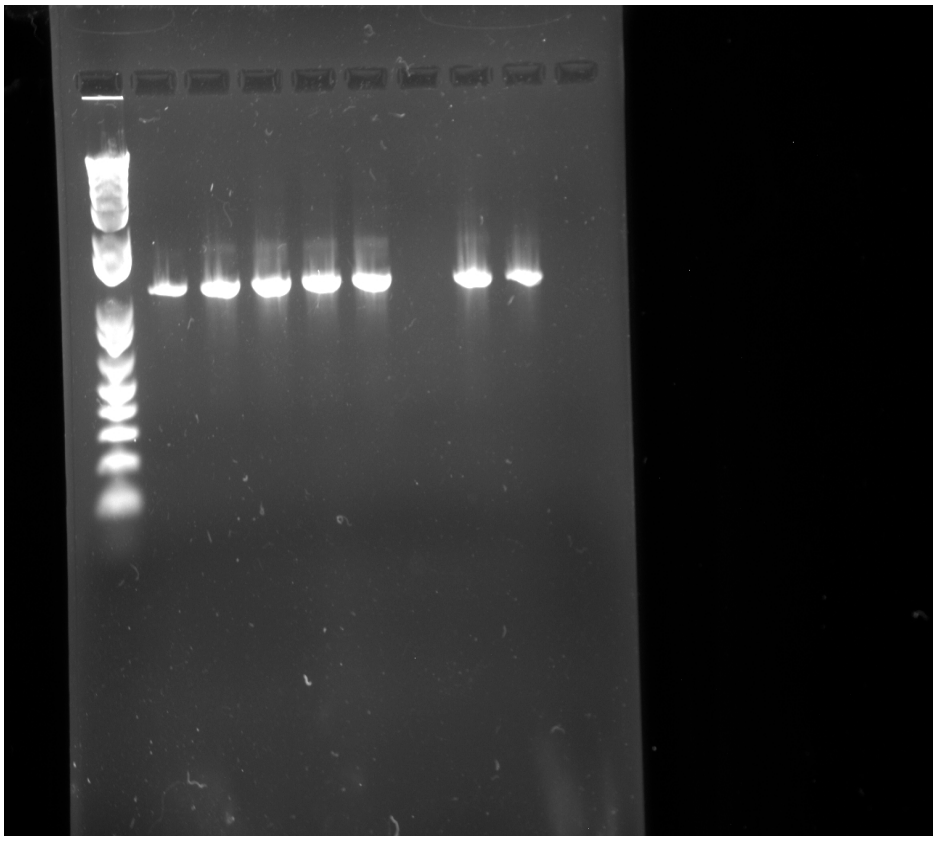
[b]Abbreviations: BESA, 2-bromoethanesulfonic acid; HK, heat-killed sample.

propionicus, which was the positive control (Figure A1.2). PCR product was not observed in the negative control reactions, which contained only the PCR master mix and water instead of DNA (Figure A1.2). These group specific PCR primers also amplified DNA from CS7 (data not shown).

Further screening of the contaminated sediment with primers that are specific for several other genera of sulfate-reducing bacteria showed that members of the *Desulfovibrio-Desulfomicrobium*, *Desulfotomaculum*, and *Desulfobacter* were also present. No PCR products were observed in reaction mixtures that contained DNA from the contaminated sediment and primers that were specific for the genus *Desulfobacterium*.

16S rRNA gene sequences from clone libraries prepared with DNA extracted directly from the contaminated sediment and amplified with the universal eubacterial primers GM5F and D907R are shown in Table 3.5. A total of 3 OTUs, which contained 4 of 85 total sequences (5%) obtained from the contaminated sediment, were affiliated with sulfate-reducing bacteria. Three of these 4 sequences were affiliated with propionate-degrading sulfate-reducing bacteria from the family *Desulfobulbaceae*. One of these 4 sequences was affiliated with members of the family *Desulfobacteraciaceae*.

FIGURE A1.2 PCR amplification of DNA from MPNs, microcosms, and contaminated sediment with the *Desulfobulbus* specific 16S rRNA primers, DBB121 and DBB1237. Lane 1 contains a 1 kb ladder. Lane 2 contains PCR product from propionate + sulfate MPNs. Lane 3 contains PCR product from propionate + sulfate + G11 MPNs. Lane 4 contains PCR product from microcosms with [3-^{14}C] propionate. Lane 5 contains PCR product from microcosms with [3-^{14}C] propionate + BESA. Lane 6 contains PCR product from microcosms with [3-^{14}C] propionate + sulfate. Lane 7 contains PCR product from microcosms with [3-^{14}C] propionate, sulfate, and molybdate. Lane 8 contains PCR product from the contaminated sediment. Lane 9 contains PCR product from *Desulfobulbus propionicus*. Lane 10 contains PCR product from reactions that contained PCR master mix and no added DNA.



DISCUSSION

Even though previous work implicated both sulfate reduction and methanogenesis as important terminal electron-accepting processes at this site (11), it appears that sulfate reduction was the dominant fate of propionate in this study. Propionate loss was only observed in MPN tubes that contained sulfate (Table A1.1). The degradation of propionate in these tubes was coupled to sulfate loss and acetate accumulation. The isolation of the propionate-degrading, acetate-producing, sulfate-reducing bacterium CS7 from MPNs that contained propionate and sulfate provided additional evidence that sulfate-reducing bacteria were involved in the degradation of propionate at this site. 16S rRNA sequence data indicated that this microorganism was less than 96% similar to all described species of propionate-degrading, sulfate reducers from the genus *Desulfobulbus*. The observation of numerous black colonies, which had similar characteristics to CS7, on roll tubes from the highest dilutions from MPNs with either propionate and sulfate or propionate, sulfate, and a hydrogen-using sulfate reducer suggested that these microorganisms were the dominant propionate degraders under sulfate-reducing conditions at this site.

Propionate loss was also coupled to sulfate loss and acetate accumulation in microcosms prepared with hydrocarbon-contaminated sediments (Table A1.2). The addition of molybdate to microcosms that contained [3-^{14}C] propionate and

sulfate resulted in a 62 to 67% reduction in the amount of [3-^{14}C] propionate that was consumed relative to microcosms with [3-^{14}C] propionate alone, [3-^{14}C] propionate and BESA, or [3-^{14}C] propionate and sulfate (Table A1.2). The addition of molybdate to microcosms with propionate and sulfate also inhibited sulfate reduction. The observation of decreased propionate and sulfate consumption in when molybdate was added clearly showed that sulfate-reducing bacteria played an important role in the degradation of propionate at this site.

The observation of propionate metabolism in the absence of added sulfate, e.g., in microcosms with [3-^{14}C] propionate alone and [3-^{14}C] propionate + BESA (Table A1.2), was surprising because propionate consumption was not observed in propionate MPNs without sulfate (Table A1.1). Sulfate measurements showed that higher than expected concentrations of sulfate were present in all of the microcosms from this study. Microcosms without sulfate amendment had 2 mM sulfate and those with sulfate amendments had around 10 mM sulfate rather than the expected 7.5 mM sulfate. These findings suggested that sulfate was present in the groundwater that was added to all of the microcosms in this study. Subsequent sulfate measurements showed that the groundwater contained between 2 and 2.5 mM sulfate. The amount of sulfate consumed in microcosms with [3-^{14}C] propionate alone, [3-^{14}C] propionate and BESA, and [3-^{14}C] propionate and sulfate (0.4 ± 0.1, 0.5 ± 0.1, and 0.5 ± 0.05 mM, respectively) was close to that expected if all of the propionate present (0.5 mM) was incompletely oxidized to acetate by sulfate-reducing bacteria according to the following equation:

$$CH_3CH_2COO^- + 0.75\ SO_4 \Leftrightarrow CH_3COO^- + HCO_3^- + 0.25\ H^+ + 0.75\ HS^-.$$

The addition of BESA to microcosms with [3-^{14}C] propionate had no effect on the amount of propionate that was consumed relative to microcosms with either [3-^{14}C] propionate alone or [3-^{14}C] propionate and sulfate (Table A1.2). These findings showed that methanogenesis was not required for propionate degradation to occur. However, the addition of BESA to microcosms with [3-^{14}C] propionate decreased the amount of label recovered as $^{14}CH_4$ relative to microcosms without BESA addition (Table A1.2). Nearly all the label from propionate in microcosms with [3-^{14}C] propionate and BESA was recovered as [^{14}C] acetate (Table A1.2). The observation of lower concentrations of [^{14}C] acetate and $^{14}CH_4$ in microcosms with [3-^{14}C] propionate and BESA, relative to microcosms with either [3-^{14}C] propionate alone or [3-^{14}C] propionate and sulfate, suggests that BESA inhibited the utilization of acetate by aceticlastic methanogens, which have been shown to be the dominant acetate-users at this site (33) (see chapter 2).

Molecular analyses also support a role for sulfate-reducing bacteria propionate degradation at this site. 16S rRNA sequences related to propionate-degrading sulfate reducers from the genus *Desulfobulbus* were detected using group specific 16S rRNA PCR primers in all MPNs with sulfate (Figure A1.2). *Desulfobulbus* sequences were also detected with these group specific primers in all active microcosms with the exception of those with propionate, sulfate, and molybdate (Figure A1.2). The absence of sequences related to *Desulfobulbus* spp.

in microcosms with molybdate coupled with the reduction in propionate consumption in these microcosms compared to that observed in all other active microcosms which contained *Desulfobulbus* spp. suggest that these microorganisms played an active role in propionate degradation of propionate at this site. The presence of 16S rRNA sequences related to *Desulfobulbus* spp. in sediment clone libraries (Table 3.5) also suggested that these sulfate-reducing bacteria were involved in the degradation of propionate at this site.

Even though 16S rRNA sequences related to hydrogen/formate-using sulfate reducers from the genus *Desulfovibrio* were detected in the contaminated sediments and the most probable number of propionate degraders was higher when when a hydrogen/formate-using sulfate reducer was added to MPN tubes, it is unlikely that propionate was degraded syntrophically under sulfate-reducing conditions. If syntrophic consortia of hydrogen/formate-producing propionate degraders and hydrogen/formate-using sulfate reducers were responsible for propionate consumption under sulfate-reducing conditions, then these hydrogen/formate-producing propionate degraders would also be expected to metabolize propionate syntrophically when coupled with a hydrogen/formate-using methanogen. However, no propionate loss was observed in MPNs with propionate and *Methanospirillum hungatei* strain JF-1 (Table A1.1). 16S rRNA gene sequences related to known syntrophic propionate degraders were not detected in clone libraries from the contaminated sediments, which also suggests that syntrophic propionate degradation was not an important process at this site.

The work described here and in chapter 2 showed that propionate was incompletely metabolized to acetate and carbon dioxide coupled to sulfate reduction and that acetate was then metabolized by aceticlastic methanogens. Propionate degradation coupled to sulfate reduction has been shown in a number of other sulfate-reducing ecosystems (1, 3, 22, 28). However, the results of this study suggest that new propionate-degrading sulfate reducers were present at this site. The 16S rRNA gene sequence of propionate-degrading sulfate reducer, strain CS7, isolated from MPNs with propionate and sulfate was less than 96% similar to all described species from the genus *Desulfobulbus*; a difference sufficient to support the assignment of strain CS7 to a new species in this genus. The observation of large numbers of colonies that were similar to those of CS7 on roll tubes from MPNs that contained propionate and sulfate suggests that microorganisms similar to CS7 were the dominant propionate degraders in sulfate-reducing MPNs and possibly the aquifer. The group specific primers for *Desulfobulbus* spp. also amplified DNA from CS7 and detected 16S rRNA gene sequences related to *Desulfobulbus* spp. in all sulfate-reducing MPNs, all active microcosms except those amended with molybdate, and the contaminated sediments. Blast searches showed that 3 of 85 16S rRNA gene sequences in sediment clone libraries were related to *Desulfobulbus* spp. (Table 3.5); all three sequences were all less than 97% similar to those of any cultured species of *Desulfobulbus*. Further work on the new propionate degrader was hampered with of the loss of CS7. However, several strains from sulfate-reducing MPNs are being revived in fresh medium with

propionate and sulfate. These cultures will be analyzed to determine if they are capable of degrading propionate and reducing sulfate and if so their 16S rRNA gene sequence will be determined in the hope of isolating strains of the novel *Desulfobulbus* species from the site.

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