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UNIVERSITY OF OKLAHOMA
GRADUATE COLLEGE

PERIPHERAL AND CENTRAL PATHWAYS INVOLVED IN THE BIODEGRADATION OF MONOAROMATIC COMPOUNDS UNDER ANAEROBIC CONDITIONS

A Dissertation
SUBMITTED TO THE GRADUATE FACULTY
in partial fulfillment of the requirements for the
degree of
Doctor of Philosophy

By
Mostafa S. Elshahed
Norman, Oklahoma
2001

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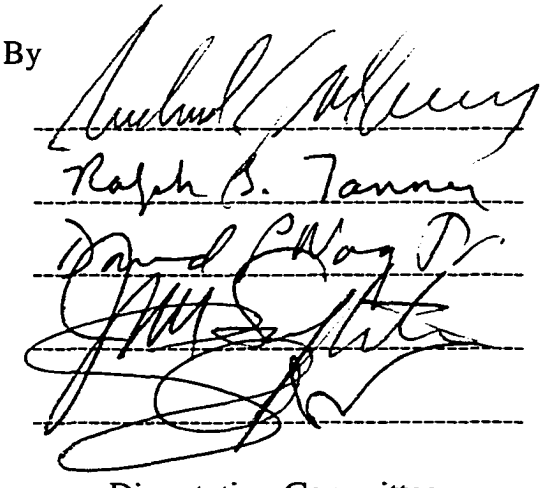
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Peripheral and central pathways involved in the biodegradation of monoaromatic compounds under anaerobic conditions

A Dissertation

Approved for the department of Botany and Microbiology

By



Dissertation Committee

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Acknowledgments

Many people had made my journey bearable, and even enjoyable, in the last five years. I am greatly indebted to my family back home for their everlasting emotional and financial support. I also owe a special gratitude for my uncle, professor M.Y. Elibiary, and my aunt, Laila, for being my family here in Norman, and for constant support and encouragement as well as endless challenging and entertaining Sunday conversations. Next I would like my to express my greatest gratitude for my advisor, Dr. Michael J. McInerney. Mike has been a great mentor from which I learned the true meaning of being a scientist. His great knowledge in microbiology is in stark contrast to his humbleness and appreciation to his students opinion. My thanks to other members of my advisory committee especially for Dr. Joseph M. Suflita, Dr. Ralph S. Tanner, and Dr. David P. Nagle for their excellent advice in various aspects of anaerobic microbiology. Special thanks to Dr. Lisa M. Gieg for teaching me almost everything I know about anaerobic biodegradation and mass spectroscopy as well as for endless editorial help. I would also like to thank Neil Wofford , Luis A. Rios-Hernandez, Chris L. Musselwhite, Dr. Glenn A. Ulrich, and Dr. Kevin G. Kropp for showing me my way around as well as for a lot of fruitful, and repetitive, lunch discussions in science and otherwise.

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Preface

Work in the previous two decades has indicated that anaerobic microorganisms channel the large number of aromatic compounds into few central intermediates. Benzoate (or benzoyl-CoA) is the most important central intermediate in the anaerobic biodegradation of monoaromatic hydrocarbons since a large number of these compounds, such as phenol, aromatic alcohols, aromatic amino acids, benzene, toluene, ethylbenzene, and xylenes are converted to benzoyl-CoA prior to ring cleavage.

Although the central pathway for benzoate metabolism under anaerobic conditions has been extensively studied in the nitrate-reducing bacterium *Thauera aromatica* and the phototrophic bacterium *Rhodopseudomonas palustris*, little is known about benzoate degradation in other groups of anaerobic bacteria such as sulfate-reducers, iron-reducing, and syntrophic microorganisms. The main body of work presented in this dissertation focuses on understanding the pathway involved on the degradation of toluates, a specific group of compounds of which very little is known about their anaerobic biodegradation as well as the central pathway for benzoate metabolism in syntrophic microorganisms, a process that is not as well understood as benzoate metabolism in denitrifiers and phototrophs.

Chapter one focuses on the fate and mechanisms involved in the biodegradation of toluic acids (methyl benzoic acids) under methanogenic and sulfate-reducing conditions by microorganisms derived from a hydrocarbon-contaminated aquifer sediments. Toluates have been identified as intermediates in the aerobic and anaerobic biodegradation of xylenes yet little is known about the

mechanisms involved in their biodegradation in anaerobic environments. The work presented here indicates that all toluic acid isomers are susceptible to biodegradation under both sulfate-reducing and methanogenic conditions, indicating that these compounds are not dead-end products of xylenes metabolism. Moreover, some of the metabolites that transiently accumulated in anaerobic toluates incubations were observed in the groundwater at the hydrocarbon-contminated site from which sediments were obtained, indicating that the biodegradation of these compounds is occurring in situ. Detection of metabolites in groundwater was performed by Dr. Lisa M. Gieg. The chapter was written by the style recommended by the journal "Biodegradation" and part of this work is published in the journal "Environmental Science and Technology".

In Chapter two, the pathway involved in benzoate metabolism in the syntrophic bacterium *Syntrophus aciditrophicus* was investigated by identifying and quantifying the metabolites detected during its growth on benzoate, testing the ability of *S. aciditrophicus* to metabolize putative metabolites of benzoate degradation, and measuring key enzymes postulated to be involved in benzoate metabolism. Results indicate that ring reduction steps in *S. aciditrophicus* occurs via a novel mechanism that involves the production of cyclohexane carboxylate or its CoA derivative as an intermediate. The nature of metabolites detected as well as enzyme studies suggested that the ring reduction steps in *S. aciditrophicus* is different than that observed with *T. aromatica* and *S. aciditrophicus*. This difference is probably imposed by the strict energetic constrains encountered during syntrophic benzoate metabolism. Enzyme studies in this chapter were

performed by Dr. Vishvesh Bhupathiraju, ^{13}C NMR work was conducted by Dr. Mark Nanny, and chemical synthesis of 2-hydroxycyclohexane carboxylate was done by Neil Wofford. This work is published in the journal " Applied and Environmental Microbiology".

Chapter 3 reports on the ability of *S. aciditrophicus* to grow in pure culture on benzoate in absence of hydrogen-utilizing partners by using part of the original substrate as an electron acceptor, reducing it to cyclohexane carboxylate. This benzoate fermentation ability has never been previously reported and is of ecological significance since it may affect our view concerning the need for interspecies hydrogen transfer in electron donor-rich, inorganic electron acceptor-deficient environments. This work is written according to the guidelines outlined for the journal "Applied and Environmental Microbiology" and has been submitted for publication.

In chapter 4, a protocol was developed to determine whether interspecies hydrogen transfer is required for the biodegradation of aromatic hydrocarbons under sulfate-reducing conditions, using toluene as a model compound. This protocol is based on fundamental aspects of syntrophic metabolism -such as independence of substrate turnover on terminal electron-accepting reaction, sensitivity of syntrophic metabolism to high levels of hydrogen, and ability of hydrogenase enzyme inhibitors to block syntrophic metabolism- rather than phylogenetic identification of bacteria. The chapter is written according to the guidelines and is published in the journal " FEMS Microbiology Ecology"

ABSTRACT

The anaerobic degradation of monoaromatic compounds was studied under a variety of terminal electron-accepting conditions to test the susceptibility to and possible metabolic pathways involved in toluic acids degradation under methanogenic and sulfate-reducing conditions, to determine the pathway for benzoate degradation in the syntrophic bacterium *Syntrophus aciditrophicus*, as well as to determine whether toluene degradation under sulfate-reducing conditions is dependent on interspecies hydrogen transfer. All toluic acid isomers were degraded under sulfate-reducing conditions, with *m*-toluate degradation occurring at the fastest rate. Benzoate, isophthalate, and *m*-carboxybenzylsuccinate were detected as transient intermediates in *m*-toluate-degrading, sulfate-reducing enrichments. All three toluate isomers were also degraded under methanogenic conditions with phthalic, isophthalic and terephthalic acids transiently accumulating in the culture supernatants of *o*-, *m*- and *p*-toluate methanogenic cultures, respectively. *o*-Carboxybenzaldehyde was detected in the culture supernatant of *o*-toluate methanogenic enrichments. These results show that the biodegradation of toluic acids is initiated at the methyl group under both electron-accepting conditions and that the formation of *m*-carboxybenzylsuccinic acid is an early step in *m*-toluate degradation under sulfate-reducing conditions.

The metabolism of benzoate, cyclohex-1-ene carboxylate and cyclohexane carboxylate by *Syntrophus aciditrophicus* in coculture with hydrogen-using microorganisms was studied. Cyclohexane carboxylate, cyclohex-1-ene

carboxylate, pimelate, glutarate (or their Co-A derivatives) transiently accumulated during growth with benzoate. Cyclohexane carboxylic acid accumulated to a concentration of 260 μM, accounting for about 18% of the initial benzoate added. Cocultures of *S. aciditrophicus* and *Methanospirillum hungatei* readily metabolized cyclohexane carboxylate and cyclohex-1-ene carboxylate at a rate slightly faster than that for benzoate. These results suggest that a unique ring-reduction mechanism operates in *S. aciditrophicus* which is probably imposed by energetic constraints encountered during syntrophic benzoate metabolism.

S. aciditrophicus also metabolized benzoate in pure culture in the absence of hydrogen-utilizing partners or terminal-electron acceptors. *S. aciditrophicus* produced approximately 0.5 mol of cyclohexane carboxylate and 1.5 mol of acetate per mol of benzoate when grown in pure cultures compared to 3 mol of acetate and 0.75 mol of methane per mol of benzoate in coculture with *M. hungatei*. Cyclohexane carboxylate was metabolized by *S. aciditrophicus* only in coculture with a hydrogen-user, but not by pure cultures of *S. aciditrophicus*. Cyclohex-1-ene carboxylate was incompletely degraded by *S. aciditrophicus* pure cultures when a net free energy change ($\Delta G'$) of –9.2 kJ/mol was reached (-4 kJ/mol for the hydrogen producing reaction). These results suggest that benzoate fermentation by *S. aciditrophicus* in absence of hydrogen-users proceeds via a dismutation reaction in which the hydrogen (or reducing equivalents) produced during oxidation of one benzoate molecule to acetate and carbon dioxide is used to reduce another benzoate molecule to cyclohexane carboxylate.

A novel protocol that is based on fundamental aspects of syntrophic metabolism was developed to determine whether the degradation of toluene under sulfate-reducing conditions by microbial populations in hydrocarbon-contaminated aquifer sediments depends on interspecies hydrogen transfer. Although the outcome of the experiments failed to support the hypothesis that toluene degradation under sulfate-reducing conditions involved interspecies hydrogen transfer, the protocol will be useful to determine the role of interspecies hydrogen transfer in the degradation of different compounds in environmental samples. Overall, the work in this dissertation contributes to our understanding of anaerobic biodegradation pathways, the role of syntrophic interactions in substrates degradation, and demonstrates a novel benzoate fermentation ability in *S. aciditrophicus*.

CHAPTER 1

Biodegradation of Toluic Acids Under Sulfate-Reducing and Methanogenic Conditions

ABSTRACT

The susceptibility to and possible metabolic pathways involved in the biodegradation of *o*-, *m*, and *p*-toluic acids under sulfate-reducing and methanogenic conditions were investigated using sediments from a gas condensate-contaminated site. Under sulfate-reducing conditions, *m*-toluate degradation occurred at the fastest rate (17.2 nmol g^{-1} $sediment^{-1}$ day^{-1}) and after the shortest lag time (28 days) compared to lag times of 84 days and rates of 10.6 and 9.3 (nmol g^{-1} $sediment^{-1}$ day^{-1}) for *o*- and *p*-toluate degradation, respectively. Benzoate and isophthalate were detected as transient intermediates in *m*-toluate-degrading, sulfate-reducing enrichments together with a third compound identified as *m*-carboxybenzylsuccinic acid. Identification was based on comparison of the retention time and mass spectrum of the trimethylsilyl derivative of the metabolite with an authentic standard that was chemically synthesized. Sequential adaptation and substrate competition experiments suggested that neither methylhydroxylation nor decarboxylation was the initial step in *m*-toluate degradation under sulfate-reducing conditions. Under methanogenic conditions, all three toluic acid isomers were degraded after a lag period of 84 days and at rates of 10.4, 8.5 and 12.1 (nmol g^{-1} $sediment^{-1}$ day^{-1}) for *o*-, *m*- and *p*-toluate, respectively. Phthalic, isophthalic and terephthalic acids were detected in the culture supernatants of *o*-, *m*- and *p*-toluate-degrading methanogenic cultures, respectively. *o*-Carboxybenzaldehyde was also detected in *o*-toluate-degrading methanogenic enrichments. These results show that the biodegradation of toluic acids is initiated at the methyl group under both

electron-accepting conditions and that the formation of *m*-carboxybenzylsuccinic acid is an early step in *m*-toluate degradation under sulfate-reducing conditions. Further, putative toluate metabolites identified in enrichment cultures were also identified in contaminated groundwater at the site.

Key words: Biodegradation , methanogenic, sulfate-reducing, toluic acids

INTRODUCTION

Knowledge of how different aromatic compounds are metabolized is essential for the development of effective treatment strategies for produced wastes and to determine suitable remediation approaches for contaminated sites. This study focuses on the fate and the pathways involved in the biodegradation of toluic acids under anaerobic conditions. Toluic acids are of environmental significance because they are often identified as intermediates (Krieger et al. 1999) or dead-end products (Jørgensen et al. 1995) in the anaerobic degradation of different xylene isomers. In numerous field studies (Cozzarelli et al. 1990 & 1995; Gieg et al. 1999; Godsy et al. 1992; Wilson et al. 1986 & 1990), one or more of the toluate isomers was detected in the groundwater at sites contaminated withbenzene, toluene, ethylbenzene, and xylenes (BTEX). Also, *p*-toluate is a by-productin the production of terephthalate, an important precursor in the plastic industry (Macarie et al. 1992).

While the anaerobic degradation of the xylene isomers has been demonstrated under different electron-accepting conditions (Heider & Fuchs 1997a and 1997b; Lovley 1997; Harms et al. 1999), little is known about the pathway for the oxidation of toluic acids under anaerobic conditions. Studies investigating xylene metabolism have usually focused on the initial step of xylene oxidation to toluic acids (Krieger et al. 1999; Jørgensen et al. 1995). We are aware of only one report focusing on toluate degradation under anaerobic conditions in which *o*-toluate was degraded by methanogenic enrichments derived from sewage sludge. In this study by Londry & Fedorak (1993a), a ring hydroxylation reaction was suggested as the first step in *o*-toluate degradation.

Here, we determined the ability of sediment-associated anaerobes to utilize toluic acid isomers under sulfate-reducing and methanogenic conditions. We present evidence that all toluic acid isomers are susceptible to degradation by

microorganisms derived from a hydrocarbon-contaminated site under both electron-accepting conditions, that the degradation of all toluate isomers involves the formation of the respective phthalic acid, and that *m*-toluate degradation under sulfate-reducing conditions involves the formation of *m*-carboxybenzylsuccinate as a metabolic intermediate.

MATERIALS AND METHODS

Chemicals. *o*-, *m*-, and *p*- Toluic acids were purchased from Fluka Chemical Corp. (Milwaukee, WI). Phthalic, isophthalic, terephthalic, and benzoic acids, *o*- and *m*-carboxybenzaldehyde, toluene, and *tert.* butyl peroxide were from Aldrich Chemical Co. (Milwaukee, WI). Methyl *m*-toluate and maleic anhydride were from Acros Organics (NJ, USA). All chemicals were of the highest purity available (≥ 97%).

Sampling. Samples were collected from a methanogenic/ sulfate-reducing zone at a gas condensate-contaminated site near Ft. Lupton, CO (Gieg et al. 1999). The gas condensate is comprised of 18% BTEX hydrocarbons (wt/wt); of this, the *o*-xylene and *m*- and *p*-xylene fractions account for 8% and 40% (wt/wt), respectively. Sediments were collected anaerobically by the hollow-stem auger method (Fredrickson et al. 1997) and stored under nitrogen in a gas-tight container. Groundwater for microcosm preparation was collected in pre-sterilized glass bottles that were previously flushed with nitrogen gas. Other groundwater samples were collected from various locations within the contaminated portion of the aquifer and acidified to pH<2 for putative metabolite analysis.

Microcosm preparation. Initial microcosms were prepared in an anaerobic glove box (Coy Laboratory Products, Inc. Ann Arbor, MI.). Twenty -five grams of sediment and 55 ml of groundwater were added to 120-ml serum bottles, which were closed with butyl rubber stoppers and crimped with aluminum seals. After preparation, microcosms were removed from the anaerobic chamber and the headspace was exchanged with 80:20 N_2:CO_2 (Balch & Wolfe 1976). Ferrous sulfate was added to the sulfate-reducing microcosms from a sterile, anoxic 100 mM stock solution. Toluates were added to a final concentration of 750 μM from a sterile, anoxic stock solution of 50 mM sodium toluate. Controls in this experiment included microcosms containing 350 μM benzoate with and without sulfate

(included as positive controls), microcosms without toluate addition (to determine the amount of sulfate reduction and methane production from endogenous substrates), and heat-killed controls for every toluate isomer under sulfate-reducing and methanogenic conditions. Triplicate microcosms were prepared for each treatment, except for the heat-killed controls, which were prepared in duplicate. The microcosms were incubated at room temperature. Enrichments were propagated by transferring 30% (vol/vol) inoculum into a defined mineral medium (McInerney et al. 1979) with the substrate added at a concentration of 0.5 mM and sodium sulfate added at a concentration of 7 mM for sulfate-reducing enrichments. The same defined medium was also used to test for the degradation of putative metabolites and for substrate competition experiments.

Synthesis of *m*-carboxybenzylsuccinic acid. *m*-Carboxybenzylsuccinic acid was synthesized according to a previously published procedure (Kousaka et al. 1968). In brief, methyl *m*-toluate (20 mol), maleic anhydride (1 mol), and *tert*-butyl peroxide (0.007 mol) were refluxed at 150°C for 5 hours. Excess methyl *m*-toluate was then removed by distillation and the residue was esterified by refluxing with excess methanol and 2 ml of concentrated sulfuric acid for 5 hours. The resulting trimethyl ester was hydrolyzed by refluxing with 15 ml of 10% sodium hydroxide solution for 30 hours. Upon acidifying with HCl to pH 3-4 and cooling under ice, *m*-carboxybenzylsuccinic acid was obtained as a white precipitate. The product was filtered, dried, and semi-purified by recrystallization in hot water.

Analytical methods. Toluic acids and all other aromatic compounds, except toluene, were analyzed by high performance liquid chromatography (HPLC) with a reversed-phase C_{18} column (250 mm by 4.6 mm, particle size 5 μm, Alltech Inc., Deerfield, IL). The isocratic mobile phase consisted of methanol: water: phosphoric acid in proportions of 60:40:0.5, respectively. A variable-wavelength UV absorbance detector at 254 nm was used to detect substrates and putative metabolites. Toluene

was analyzed in culture headspaces on a Hewlett Packard (HP) 5890 gas chromatograph (GC) equipped with a flame ionization detector and a 30 m long Carbograph VOC capillary column (Alltech Inc., Deerfield, IL), operated isothermally at 150°C. The carrier gas was helium and the flow rate was 16 ml/min. Methane was analyzed by GC as previously described (Jenneman et al. 1986). Sulfate concentrations were determined by ion chromatography (Londry et al. 1997).

To detect and quantify metabolites by gas chromatography-mass spectrometry (GC-MS), cultures were alkali-treated (pH> 12) for 30 min to cleave CoA thioester bonds on putative metabolites, acidified (pH< 2), then extracted three times with ethyl acetate. Groundwater samples for putative metabolite analysis were subjected only to the acid treatment prior to extraction. The organic extracts were filtered through anhydrous sodium sulfate, concentrated, and then derivatized with *N,O*-bis-(trimethylsilyl)-triflouroacetamide (Pierce Chemicals, Rockford, IL). The concentrated, derivatized extracts were analyzed on a HP 5890 GC equipped with a mass spectrometer and a 30 m long DB-5 fused silica capillary column (J&W Scientific, Folson, CA), using helium as a carrier gas. The oven temperature was held at 40°C for 2 min, then raised at a rate of 4°C/min to 240°C where it was held for 8 min.

RESULTS

Biodegradation of toluic acids in initial incubations. The results of the initial incubations with sediments and groundwater in the presence of sulfate are shown in Fig.1.1. *m*-Toluate degradation occurred at the fastest rate (17.2 nmol g^{-1} $sediment^{-1}$ day^{-1})and with the shortest lag time (28 days) compared to lag times of approximately 84 days and rates of 10.6 and 9.3 nmol$\cdot g^{-1}$sediment$\cdot day^{-1}$ for the *o*- and *p*- isomers, respectively. The rate of *m*-toluate degradation was slower than that of benzoate (25.6 nmol g^{-1} $sediment^{-1}$ day^{-1}), which was included in the experiment as a positive control. Upon reamending the enrichments with substrate, *m*-toluate was metabolized without a lag and at a faster rate (52.6 nmol g^{-1} $sediment^{-1}$ day^{-1}). The *m*-toluate-degrading organisms were subcultured into mineral medium and the activity was maintained for over two years. Attempts to maintain the *o*- or *p*-toluate degrading activity by reamending the enrichments or by subculturing into mineral medium were unsuccessful.

All toluic acid isomers were also degraded under methanogenic conditions after a lag time of 84 days (Fig. 1.2). The rate of *p*-toluate degradation (12.1 nmol g^{-1} $sediment^{-1}day^{-1}$) was slightly faster than the rates for *o*- and *m*-toluate degradation (10.4 and 8.5 nmol g^{-1} $sediment^{-1}day^{-1}$ for *o*- and *p*-toluate, respectively) but still slower than the rate of benzoate degradation under methanogenic conditions (16.5 nmol g^{-1} $sediment^{-1}day^{-1}$). We were able to subculture the degradation activities in mineral medium, but in contrast to the *m*-toluate-degrading sulfate-reducing cultures, lag times ranging between 24 and 53 days were evident and the degradation rates were only slightly enhanced upon subculturing.

Figure 1.1: Biodegradation of *o*-, *m*-, and *p*- toluic acids under sulfate-reducing conditions. -□- Toluate, live. -◊- Toluate, autoclaved. -o- Sulfate reduced in substrate-amended microcosms . -Δ- Sulfate reduced in substrate-unamended microcosms. Arrows indicate where substrate was reamended to the cultures.

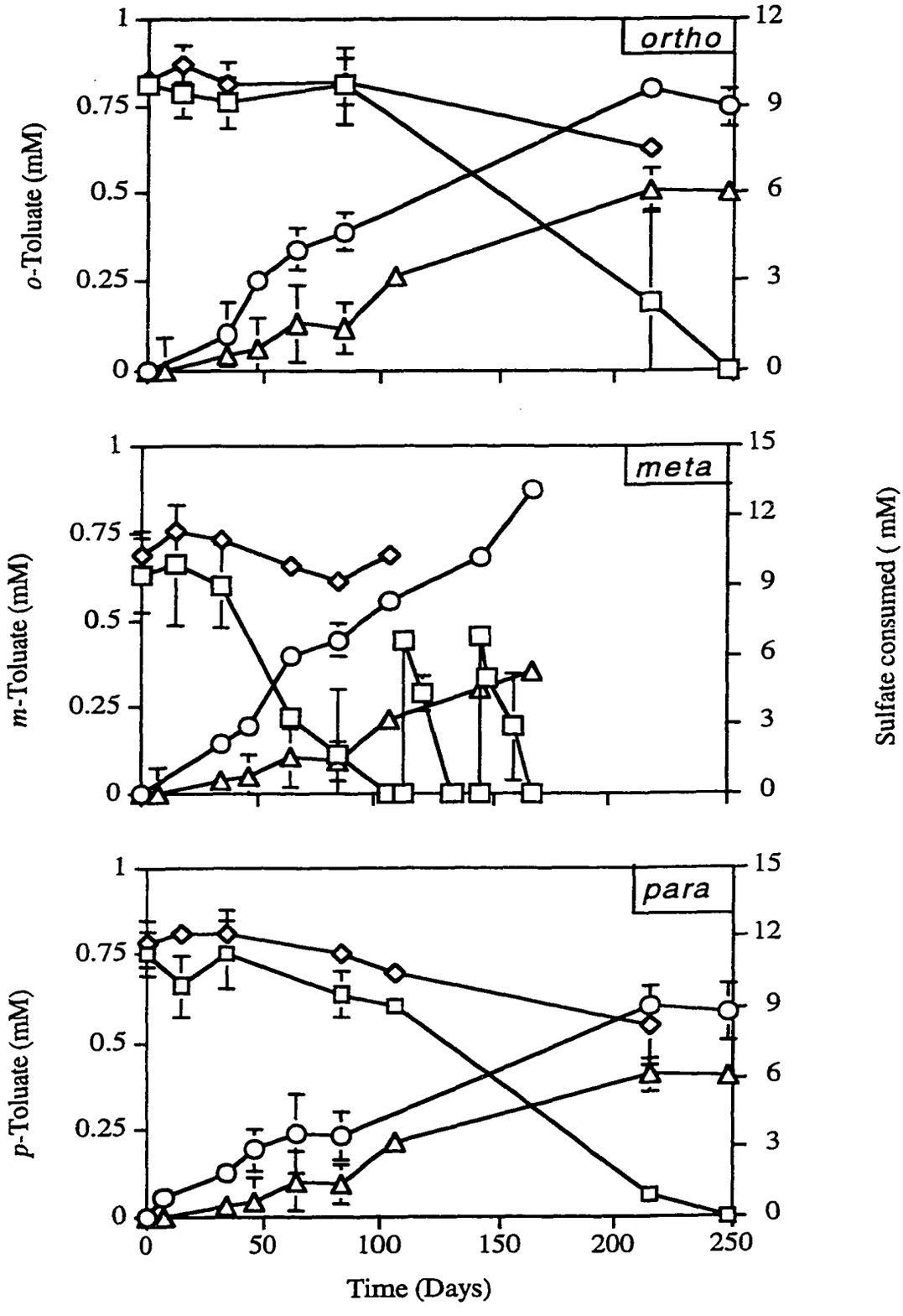
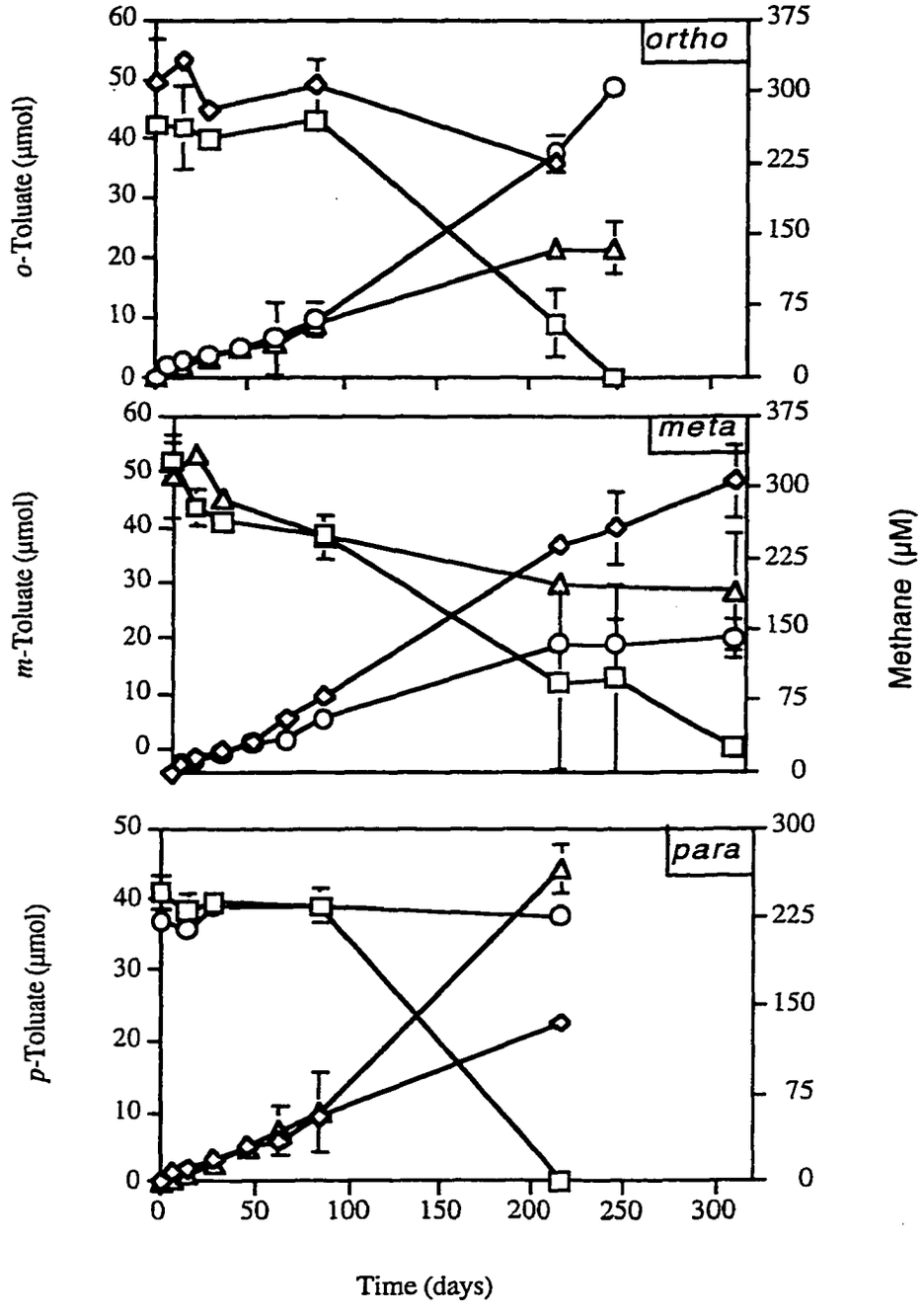

ortho
meta
para
o-Toluate (mM)
m-Toluate (mM)
p-Toluate (mM)
Sulfate consumed (mM)
Time (Days)
0
0.25
0.5
0.75
1
3
6
9
12
15
50
100
150
200
250

Figure 1.2: Biodegradation of *o*-, *m*- and *p*- toluic acids under methanogenic conditions. -□- Toluate, live. -◊- Toluate, autoclaved. -o- Methane produced in substrate-amended microcosms. -Δ- Methane produced in substrate-unamended microcosms.


ortho
meta
para
o-Toluate (µmol)
m-Toluate (µmol)
p-Toluate (µmol)
Methane (µM)
Time (days)
0
10
20
30
40
50
60
75
150
225
300
375
0
50
100
150
200
250
300

Stoichiometry of electron donor : electron acceptor. The theoretical equations describing toluic acid degradation under sulfate-reducing and methanogenic conditions (excluding biomass considerations) are: $C_8H_8O_2 + 4.5\ SO_4^{2-} \rightarrow 8\ CO_2 + 4.5\ S^{2-} + 4\ H_2O$ and $C_8H_8O_2 + 5\ H_2O \rightarrow 3.5\ CO_2 + 4.5\ CH_4$, respectively. The observed ratios for mol of electron acceptor consumed per mol of toluate degraded were in general agreement with these theoretical values (Table 1.1). In the original *m*-toluate-degrading, sulfate-reducing enrichments, the sulfate/ substrate ratio was 7.9:1. However, the values were closer to theoretical values upon reamending the enrichments (Table 1.1).

Metabolites detected in m-toluate-degrading, sulfate-reducing enrichments. We focused our metabolic studies on the sulfate-reducing, *m*-toluate-degrading enrichment cultures because of their ease of maintenance and relatively fast rate of degradation. While monitoring the course of *m*-toluate degradation by HPLC, we noticed the appearance of two peaks, usually after a significant amount (>50 %) of *m*-toluate was consumed. These two peaks matched the retention times of and co-eluted with standard solutions of benzoate and isophthalate using two different mobile phase systems (Table 1.2).

Using GC-MS, three compounds were detected in the organic extracts of active *m*-toluate-degrading culture supernatants, but not in the substrate-unamended or heat-killed controls. The trimethylsilyl (TMS) derivatives of two of the compounds had mass spectral profiles and GC retention times identical to those of TMS-derivatized isophthalate and benzoate, respectively (Table 1.2), confirming the HPLC results. The other compound was identified as *m*-carboxybenzylsuccinic acid. This identification was based on the identical GC retention times and mass spectral profiles of the TMS-derivatized culture metabolite and the TMS-derivatized

Table 1.1 Sulfate-reduction and methane production in toluate-degrading microcosms.

Enricm-ent	Iso-mer	Toluate degraded (μmol)[a]	Methane produced or sulfate reduced (μmol)[a]	Methane produced or sulfate reduced in substrate unamended controls (μmol)[a]	Net methane produced or sulfate consumed (μmol)	mol electron acceptor/ mol substrate
Methan-ogenic	*o*-	42.2±2.5	304±29	135±27	169	4.00
	m-	52.3±3.9	307±38	135±27	172	3.29
	p-	40.2± 2.9	266±21	135±27	131	3.26
Sulfate-reducing	*o*-	44.6±2.6	521±37	338±52	183	4.10
	m-[b]	24.1±0.8	191±23	78±23	113	4.69
	p-	41.3±4.3	500±81	338±52	162	3.92

[a] Values shown are expressed in average of triplicates ± standard deviation.
[b] The values shown for *m*-toluate consumption and sulfate-reduction were obtained from the second refeeding of the enrichments (days 144-166).

Table 1.2. Aromatic acids detected in toluate-degrading enrichments

Compound	A[a]	B[a]	C[a]	Characteristic mass spectrum m/z (% of base peak)	Detected in
Metabolite 1[b] Standard	4.71	5.51	16.2	194(10), 179(100), 135(6), 105(76), 77(51),51(16), 45(13)	*o*-, *m*-, *p*-toluate methanogenic enrichments and *m*-toluate sulfate-reducing enrichments.
benzoate	4.71	5.51	16.2	194(10), 179(100), 135(9), 105(76), 77(52),51(21), 45(15)	
Metabolite Standard	3.34	3.92	30.6	310(3), 295(23), 221 (12), 147 (100), 73(38), 45(14)	*o*-toluate methanogenic enrichments.
phthalate	3.34	3.92	30.6	310(3), 295(27), 221 (11), 147 (100), 73(38), 45(14)	
Metabolite 3[c] Standard	3.90	4.39	32.2	310(12), 295(100), 279 (18), 221(16), 205(13), 177(8), 135(6), 103(12), 73(26)	*m*-toluate methanogenic and sulfate-reducing enrichments.
Isophthalate	3.90	4.39	32.2	310(10), 295(100), 279 (20), 221(16), 205(14), 177(7), 135(3), 103(12), 73(26)	
Metabolite 4	3.75	4.38	33.1	310(7), 295(100), 251(15), 221(30), 103(19), 73(41)	*p*-toluate methanogenic enrichments.
Standard Terphthalate	3.75	4.38	33.1	310(7), 295(100), 251(16), 221(27), 103(19), 73(41)	

[a] (A) Retention times in HPLC analysis using methanol : water proportion of 60:40. (B) Retention times in HPLC analysis using methanol: water proportion of 50:50. C) Retention times by GC-MS program applied.

[b] The m/z ions shown were obtained from the metabolite in *p*-toluate methanogenic enrichments

[c] The m/z ions shown are obtained from the metabolite in *m*-toluate sulfate-reducing enrichments

synthesized chemical standard (Fig. 1.3a and 1.3b, respectively). In the course of the experiments where metabolite concentrations were monitored by GC-MS, *m*-carboxybenzylsuccinate, isophthalate, and benzoate transiently accumulated during the degradation of *m*-toluate (Figure 1.4). *m*-Carboxybenzylsuccinic acid accumulated to about 5 μM when approximately 80% of the *m*-toluate was degraded. Thus, the appearance and subsequent consumption of *m*-methylbenzylsuccinic acid, *m*-carboxybenzylsuccinic acid, *m*-toluate, and isophthalate suggest that these compounds are intermediates rather than dead-end metabolites.

Several putative metabolites of *m*-toluate decay were tested for degradation by the active sulfate-reducing population (Table 1.3). *m*-Toluate-adapted sulfate-reducing enrichments were capable of degrading isophthalate but not *m*-carboxybenzaldehyde (a proposed intermediate if *m*-toluate degradation is initiated by methylhydroxylation) (Table 1.3) even when the experiment was repeated using a lower concentration (25 μM, data not shown). Furthermore, toluene (a proposed intermediate if *m*-toluate degradation is initiated by decarboxylation) was not metabolized by *m*-toluate-adapted, sulfate-reducing enrichments. Substrate competition experiments in which *m*-toluate-adapted enrichments were challenged with *m*-toluate and either *m*-carboxybenzaldehyde or toluene showed that neither compound had an inhibitory effect on *m*-toluate degradation (Fig. 1.5).

Metabolites detected in methanogenic enrichments. Metabolite peaks matching in retention times and co-eluting with phthalate (in *o*-toluate cultures), benzoate and isophthalate (in *m*-toluate cultures), and benzoate and terephthalate (in *p*-toluate cultures) were identified during HPLC analysis of the methanogenic toluate-amended enrichments (Table 1.2). GC-MS analysis confirmed the presence of benzoate and the respective phthalic acid isomer in culture extracts (Table 1.2),

indicating that toluate metabolism under methanogenic conditions is also initiated at the methyl group. Using GC-MS, *o*-carboxybenzaldehyde was also detected in *o*-toluate-amended methanogenic enrichments (Fig. 1.3c & d) but the analogous benzaldehydes were not detected in the *m*- or *p*-toluate-amended incubations. No ring hydroxylation intermediates (hydroxymethyl benzoates or cresols) or carboxybenzylsuccinate intermediates were detected in any of the methanogenic culture extracts.

Putative toluic acid metabolites in BTEX-contaminated groundwater. Previously, we detected several putative metabolites from BTEX decay in the groundwater at the contaminated site from which these toluate-degrading enrichments were derived , including the toluic acids themselves (Gieg et al., 1999). Given the results of the current study, groundwater extracts were further examined for the presence of putative toluic acid metabolites including carboxybenzylsuccinic and phthalic acids, and carboxybenzaldehydes. Of these, isophthalic and terephthalic acids were detected in several of the groundwater extracts. The GC retention times and mass spectral profiles of these acids in groundwater matched those of authentic standards (Table 2). In addition, a peak eluting at the same GC retention time as 2-carboxybenzaldehyde was detected, but the mass spectral profile was more suggestive of *m*- or *p*-carboxybenzaldehyde, thus positive identification could not be made with certainty. None of these putative metabolites were detected in uncontaminated groundwater from the site.

Figure 1.3: Mass spectra and GC retention times of (A) TMS derivative of a metabolite detected in *m*-toluate degrading, sulfate-reducing culture extracts; (B) TMS derivative of an authentic chemical standard of *m*-carboxybenzylsuccinic acid; (C) TMS derivative of a metabolite detected in *o*-toluate-degrading methanogenic culture extracts; (D) TMS derivative of an authentic chemical standard of *o*-carboxybenzaldehyde.

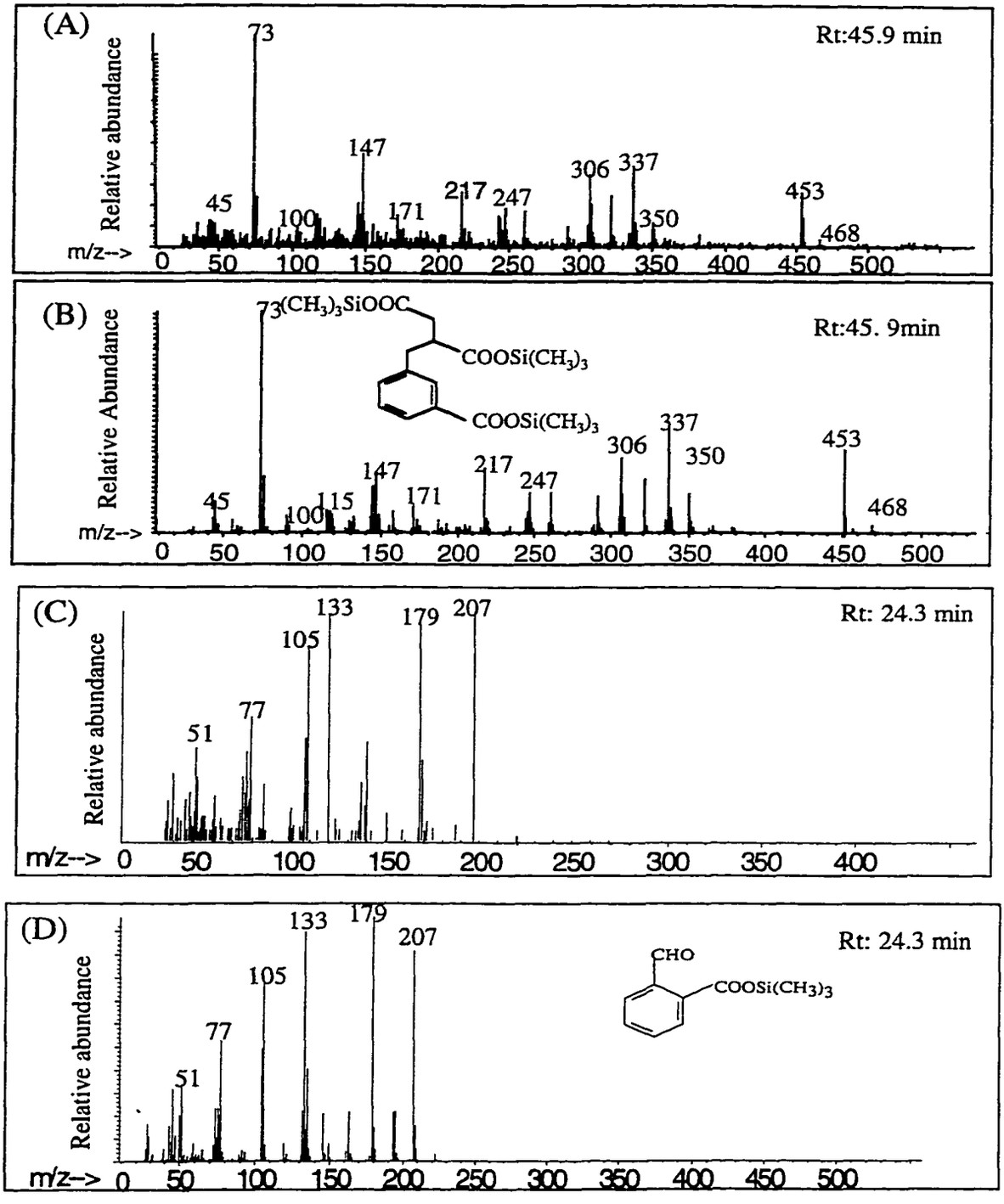

(A)
Rt:45.9 min
Relative abundance
m/z-->
73
147
45
100
171
217
247
306
337
350
453
468
0 50 100 150 200 250 300 350 400 450 500
(B)
Rt:45. 9min
Relative Abundance
m/z-->
73
(CH3)3SiOOC
COOSi(CH3)3
COOSi(CH3)3
147
45
100
115
171
217
247
306
337
350
453
468
0 50 100 150 200 250 300 350 400 450 500
(C)
Rt: 24.3 min
Relative abundance
m/z-->
133
179
207
105
77
51
0 50 100 150 200 250 300 350 400
(D)
Rt: 24.3 min
Relative abundance
m/z-->
133
179
207
105
77
51
CHO
COOSi(CH3)3
0 50 100 150 200 250 300 350 400 450 500

Figure 1.4: Metabolites detected during *m*-toluate degradation under sulfate-reducing conditions. - - *m*-Toluate. -◊- Benzoate. -o- Isophthalate. -Δ- *m*-Carboxybenzylsuccinate.

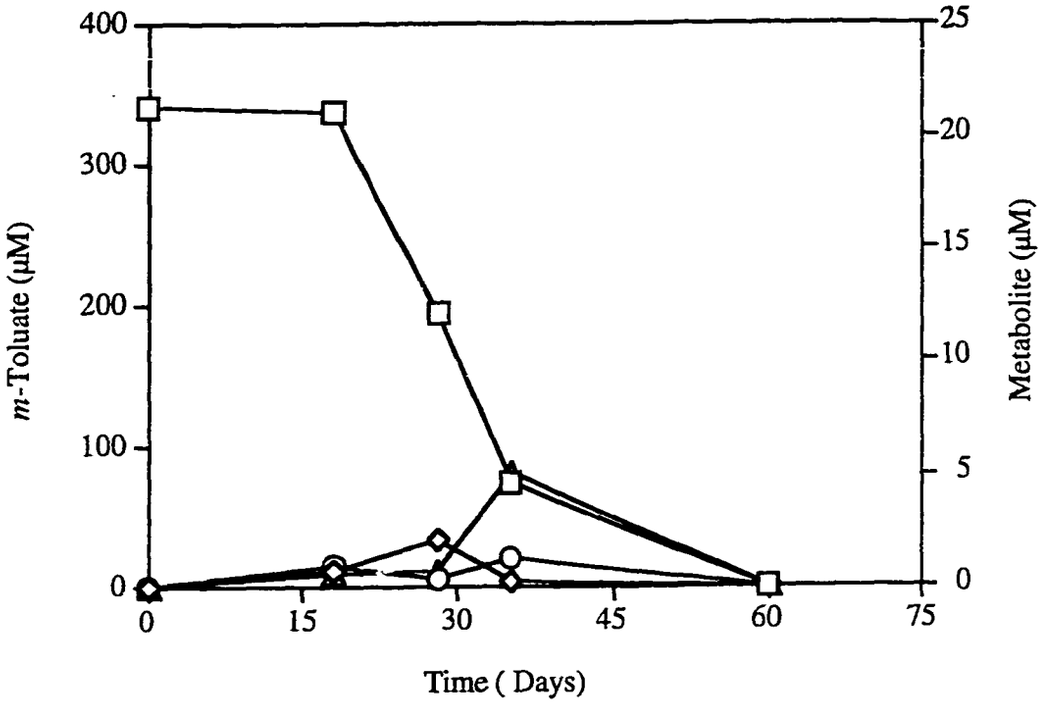
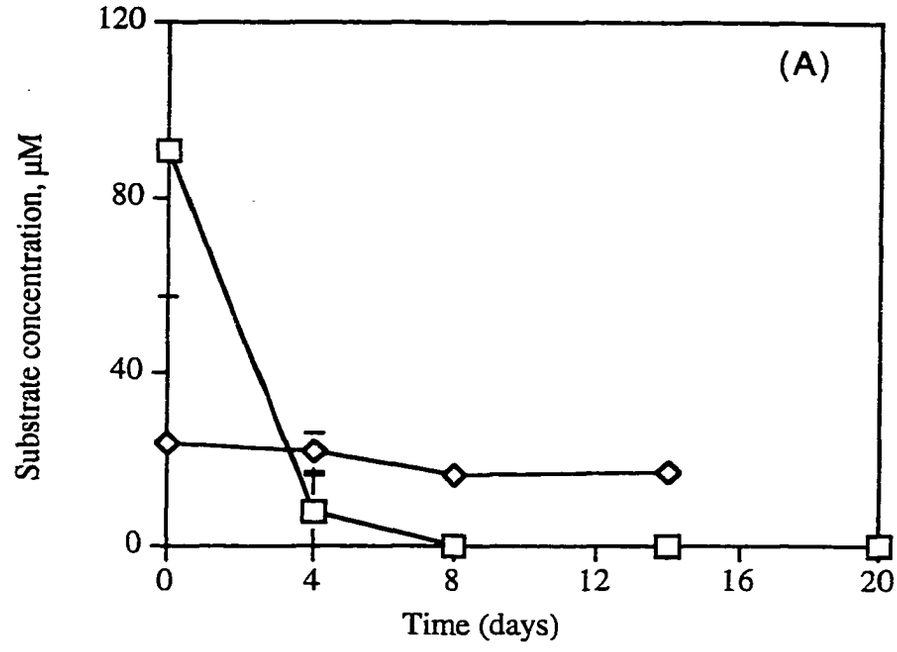

400
300
200
100
0
m-Toluate (μM)
25
20
15
10
5
0
Metabolite (μM)
0
15
30
45
60
75
Time (Days)

Figure 1.5: Selective degradation of *m*-toluate by *m*-toluate-adapted, sulfate-reducing enrichments amended with *m*-toluate and either *m*-carboxybenzaldehyde (A) or toluene (B). (- -) *m*-Toluate, live. (-■-) *m*-Toluate, autoclaved. (-◊-) *m*-Carboxybenzaldehyde. (-●-) toluene, live, (-o-) toluene, autoclaved.


(A)
120
80
40
0
Substrate concentration, µM
0
4
8
12
16
20
Time (days)

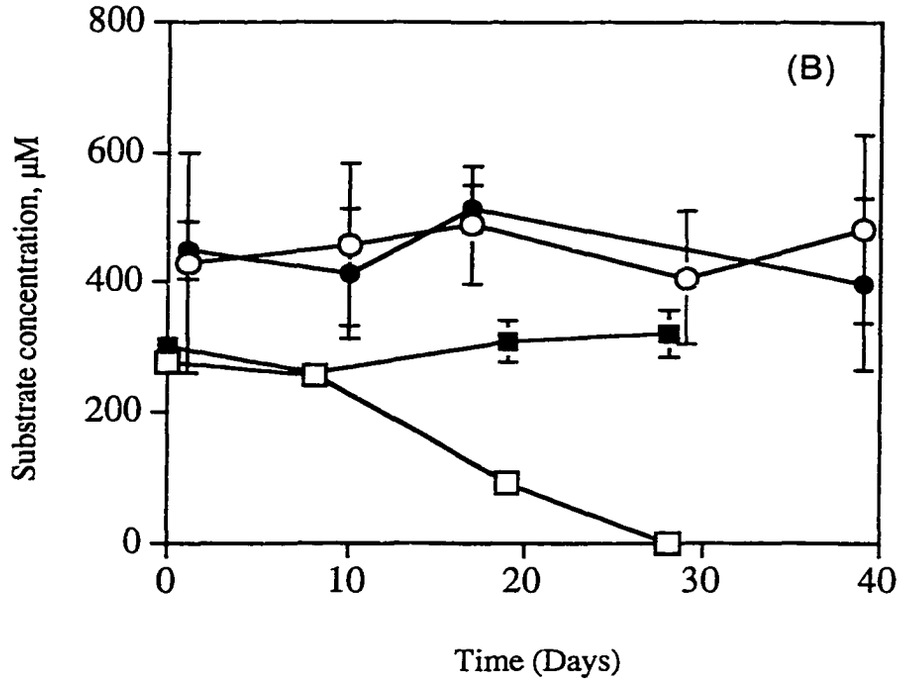

(B)
800
600
400
200
0
Substrate concentration, µM
0
10
20
30
40
Time (Days)

Table 1.3. Metabolism of different putative intermediates of toluate degradation by *m*-toluate adapted sulfate-reducing enrichments.

Compound[a]	Percentage degraded[b]
m- toluate	100
Benzoate	100
Isopthalate	100
Toluene	0
m- Carboxybenzaldehyde	22
Benzaldehyde	100
Benzyl alchol	100

[a] Starting concentrations for all compounds ranged between 0.09 to 0.15 mM except for benzoate (0.35mM)

[b] All compounds were tested in triplicates, substrate concentrations were analyzed after 54 days of incubation

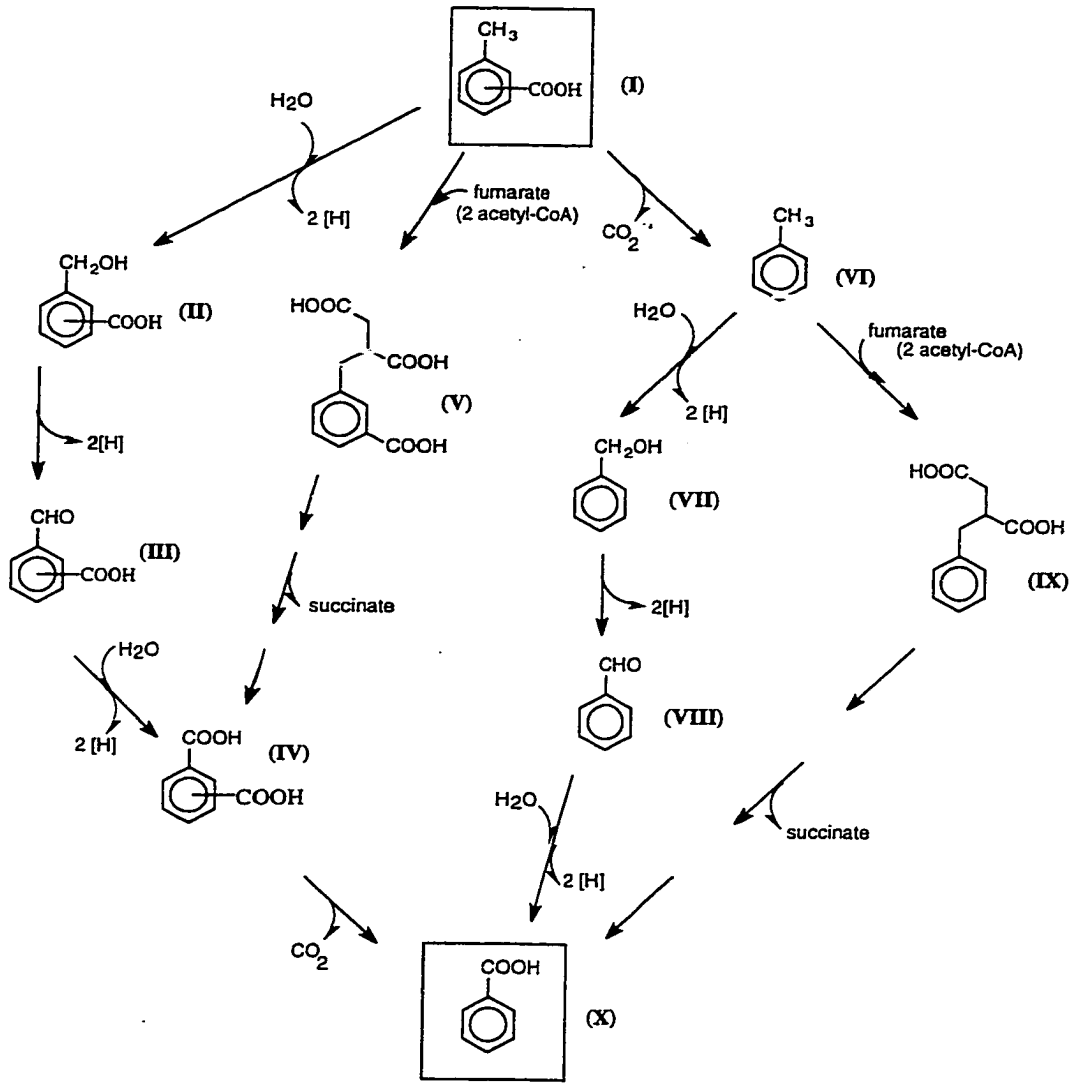
DISCUSSION

All toluic acid isomers were susceptible to biodegradation under sulfate-reducing and methanogenic conditions by sediment-associated populations obtained from a BTEX-contaminated site where toluic acids were detected as putative metabolites from xylene decay (Gieg et al., 1999). Under sulfate-reducing conditions, the onset of *m*-toluate decay was most rapid, with degradation beginning after a 28-day lag. In contrast, the other isomers under sulfate-reducing conditions, and all isomers under methanogenic conditions were not observed to biodegrade until after an 84-day lag period.

m-Toluate-degrading activity under sulfate-reducing conditions was easy to maintain, and thus permitted more in-depth studies of the mechanism of anaerobic toluate decay. However, attempts to study the *o*- and *p*-toluate degradation pathways under sulfate-reducing conditions were hampered by the fastidious nature of these enrichments. The reason for selective degradation of a certain isomer of a compound is an important and yet unanswered question. At the site from which these enrichments were derived, *m*-xylene, (along with *p*-xylene) is the most abundant xylene isomer present,which may select for in situ enrichment of populations capable of degrading this isomer over the others. It is interesting to note that *m*-xylene is also the preferred isomer for sediment-associated microorganisms at this site incubated under sulfate-reducing conditions (Gieg et al. 1999) as well as in another study under nitrate-reducing conditions (Kuhn et al. 1988). Previous studies that compared the rate of degradation of cresol, another isomeric monoaromatic hydrocarbon, concluded that *o*-cresol was the most recalcitrant isomer and that *p*-cresol was the preferred substrate (Goerlitz et al. 1985; Heider & Fuchs 1997b; Smolenski & Suflita 1987). Although these results collectively argue that isomeric preference occurs for cresols and xylenes regardless of the terminal electron-

Figure 1.6: Possible pathways for toluic acid (I) metabolism to benzoic acid (X). carboxybenzylalcohols (II), carboxybenzaldehydes (III), phthalic acids (IV), carboxybenzylsuccinic acids (V), toluene (VI), benzylalcohol (VII), benzaldehyde (VIII), benzylsuccinic acid (IX). Metabolites detected in *m*-toluate-degrading sulfate-reducing enrichments are marked as (S) and those detected in methanogenic enrichments are marked as (M).

Fig6


CH3
COOH
(I)
H2O
2 [H]
fumarate
(2 acetyl-CoA)
CO2
CH3
(VI)
CH2OH
COOH
(II)
HOOC
COOH
COOH
(V)
H2O
2 [H]
fumarate
(2 acetyl-CoA)
2[H]
CHO
COOH
(III)
succinate
CH2OH
(VII)
HOOC
COOH
(IX)
2[H]
CHO
(VIII)
H2O
2 [H]
COOH
COOH
(IV)
succinate
H2O
2 [H]
CO2
COOH
(X)

accepting process involved, this did not seem to be the case in our enrichments as the preferential degradation of *m*-toluate in sulfate-reducing enrichments was not observed in methanogenic enrichments. Further work is needed to determine whether isomeric preferences are due to differences in the initial reaction steps or to some other factor.

In this study, isophthalate was clearly identified as a metabolite in *m*-toluate degrading enrichments using a variety of techniques. This suggests that toluate metabolism under sulfate-reducing conditions is initiated at the methyl group rather than by decarboxylation to toluene (Fig. 1.6). Moreover, the detection of *m*-carboxybenzylsuccinic acid (Fig. 1.3) in culture extracts suggests that an early step in *m*-toluate degradation involves the addition of four carbon atoms to the methyl group of *m*-toluate to form this metabolite. This addition product may then be further metabolized to isophthalate (Fig. 1.6). The inability to detect *m*-carboxybenzaldehyde and *m*-carboxybenzylalcohol in culture extracts, the inability of the enrichments to metabolize *m*-carboxybenzaldehyde, and the failure of *m*-carboxybenzaldehyde to inhibit *m*-toluate degradation argue against a methylhydroxylation mechanism for *m*-toluate oxidation to isophthalate under sulfate-reducing conditions, similar to that described for cresol metabolism (Dangel et al. 1991; Häggblom et al. 1990; Londry et al. 1997 and 1999). An initial decarboxylation reaction is also unlikely in these enrichments because in the substrate degradation experiments, toluene was not readily metabolized by *m*-toluate-adapted enrichments and in substrate competition experiments, toluene did not inhibit *m*-toluate degradation. Furthermore, previously identified toluene degradation intermediates such as benzylsuccinic acid, phenylacetic acid, cinnamic acid, benzaldehyde, or benzyl alcohol (Heider & Fuchs 1997a and b) were not detected in the culture extracts. Another transformation reaction that we considered, but found no evidence for, was the hydroxylation of a toluate isomer to any of the

four possible hydroxymethyl benzoate isomers (Londry & Fedorak 1993a). Given the mixed nature of our enrichments, however, we cannot rule out the possibility of these other mechanisms of toluate decay but our data strongly argue in favor of addition product formation.

The formation of benzylsuccinates has been previously reported as an anaerobic activation strategy with nitrate-reducing and sulfate-reducing isolates and enrichments metabolizing monoaromatic hydrocarbons such as toluene and xylene (Evans et al. 1992; Biegert et al. 1996; Beller & Spormann 1997a and 1997b; Krieger et al. 1999) but not with oxygenated compounds such as toluates. With the exception of two recent report (Müller et al. 1999, Müller et al. 2001), the metabolism of the methyl group in oxygenated compounds occurs either by methylhydroxylation, demonstrated under methanogenic, sulfate-reducing, and nitrate-reducing conditions (Bossert & Young 1986; Dangel et al. 1991; Londry et al. 1997 and 1999; Suflita et al. 1989) or by demethylation, demonstrated under methanogenic conditions (Londry & Fedorak 1993b). Further studies are required to determine how widely this addition reaction is used to metabolize diverse aromatic compounds under different electron-accepting conditions.

Several lines of evidence suggest that *m*-carboxybenzylsuccinic acid is a transient intermediate and not a dead-end product formed by benzylsuccinate synthase activity involved in toluene metabolism (Beller & Spormann 1999). Toluene was not readily metabolized by *m*-toluate-adapted, sulfate-reducing enrichments. In time-course experiments where metabolites were monitored by GC-MS, *m*-carboxybenzylsuccinic acid was never detected at the start of the experiment nor after *m*-toluate was depleted (Fig.1.4). The presumed *m*-carboxybenzylsuccinic acid cleavage product, isophthalate, was detected in the enrichments. The good agreement between the actual and theoretical electron donor to electron acceptor ratio also argues against a dead-end product being formed, at least in significant amounts.

Finally, evidence precluding other pathways argues in favor of placing *m*-carboxybenzylsuccinic acid as an intermediate, rather than a dead-end product in the pathway.

Experiments with methanogenic enrichments suggest that toluate degradation also starts at the methyl group under this electron-accepting condition. *o*-Carboxybenzaldehyde was detected in *o*-toluate-degrading methanogenic enrichments, suggesting an initial methylhydroxylation reaction similar to that observed for cresol metabolism (Bossert & Young 1986; Dangel et al. 1991; Londry et al. 1997 & 1999; Suflita et al. 1989). The fact that *m*-carboxybenzylsuccinate was detected in *m*-toluate-degrading sulfate-reducing enrichments argues against methodological problems as the reason why benzylsuccinate analogues were not detected in the *o*-toluate-degrading, methanogenic enrichment. Moreover, benzylsuccinate derivatives have not been reported as intermediates in the methanogenic degradation of aromatic hydrocarbons. All of the above lines of evidence favor a methyl hydroxylation route for *o*-toluate metabolism under methanogenic conditions.

The detection of putative toluic acid metabolites in the contaminated groundwater at the site suggests that *in situ* degradation of these acids have occurred. Isophthalic and terephthalic acids were identified in *m*- and *p*-toluate-degrading enrichment cultures, respectively, and were detected in several samples of gas condensate-contaminated groundwater. Despite the detection of *o*-toluate in contaminated groundwater and its isomeric breakdown products in laboratory incubations, 2-carboxybenzaldehyde and phthalic acid were not detected in the groundwater. These results may be expected given that the *m*- and *p*-isomers comprise the majority of the xylene fraction of the gas condensate.

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CHAPTER 2

Metabolism of benzoate, cyclohex-1-ene carboxylate and cyclohexane carboxylate by *Syntrophus aciditrophicus* strain SB in syntrophic association with H_2-using microorganisms

ABSTRACT

The metabolism of benzoate, cyclohex-1-ene carboxylate and cyclohexane carboxylate by *Syntrophus aciditrophicus* in coculture with hydrogen-using microorganisms was studied. Cyclohexane carboxylate, cyclohex-1-ene carboxylate, pimelate, glutarate (or their Co-A derivatives) transiently accumulated during growth with benzoate. Identification was based on comparison of retention times and mass spectra of trimethylsilyl derivatives to authentic chemical standards. ^{13}C-Nuclear magnetic resonance (NMR) spectroscopy confirmed the production of cyclohexane carboxylate and cyclohex-1-ene carboxylate from [*ring* $^{13}C_6$] labeled benzoate. None of the above metabolites were detected in substrate-unamended or heat-killed controls. Cyclohexane carboxylic acid accumulated to a concentration of 260 μM, accounting for about 18% of the initial benzoate added. This compound was not detected in culture extracts of *Rhodopseudomonas palustris* grown phototrophically or *Thauera aromatica* grown under nitrate-reducing conditions. Cocultures of *S. aciditrophicus* and *Methanospirillum hungatei* readily metabolized cyclohexane carboxylate and cyclohex-1-ene carboxylate at a rate slightly faster than that for benzoate. In addition to cyclohexane carboxylate, pimelate, and glutarate, 2-hydroxycyclohexane carboxylate was detected in trace amounts in cocultures grown with cyclohex-1-ene carboxylate. Cyclohex-1-ene carboxylate, pimelate, and glutarate were detected in cocultures grown with cyclohexane carboxylate at levels similar to those found in benzoate-grown cocultures. Cell extracts of *S.*

aciditrophicus grown in coculture with *Desulfovibrio* sp. strain G11 with benzoate or in pure culture with crotonate contained the following enzyme activities: an ATP-dependent, benzoyl-CoA ligase, cyclohex-1-ene carboxyl-CoA hydratase, 2-hydroxycyclohexane carboxyl-CoA dehydrogenase, as well as pimelyl-CoA dehydrogenase, glutaryl-CoA dehydrogenase, and the enzyme activities required for the conversion of crotonyl-CoA to acetate. 2-Ketocyclohexane carboxyl-CoA hydrolase activity was detected in cell extracts of *S. aciditrophicus* - *Desulfovibrio* sp. strain G11, benzoate-grown cocultures, but not in crotonate-grown, pure cultures of *S. aciditrophicus*. These studies are consistent with the hypothesis that ring reduction during syntrophic benzoate metabolism involves a 4 or 6 electron reduction step and that once cyclohex-1-ene carboxyl-CoA is made, it is metabolized in a similar manner as found in *R. palustris*.

INTRODUCTION

Biodegradation of aromatic compounds is an important component of the carbon cycle in various anoxic environments. Despite the large number of natural and synthetic homocyclic aromatic compounds, anaerobic microorganisms initially channel all aromatic substrates into few central intermediates prior to ring cleavage (20). Benzoyl-CoA is the most important of these intermediates since a large number of compounds such as chloro-, nitro-, and aminobenzoates, aromatic hydrocarbons, and phenolic compounds are initially converted to benzoyl-CoA prior to ring reduction and cleavage (18). The central pathways for benzoate and benzoyl-CoA metabolism under anaerobic conditions have been primarily studied in two microorganisms, the phototrophic, purple non-sulfur, bacterium *Rhodopseudomonas palustris* and the nitrate-reducing bacterium, *Thauera aromatica* (18, 19). After activation of benzoate to benzoyl-CoA (1, 17), benzoyl-CoA is reduced to cyclohex-1,5-diene carboxyl-CoA by a benzyl-CoA reductase, which had been purified from *T. aromatica* (8, 9, 26). Based on DNA sequence homology, it is believed that a similar reductive reaction occurs in *R. palustris* (14). After ring reduction, the pathways diverge in the two organisms. In *T. aromatica*, cyclohex-1,5-diene carboxyl-CoA is hydrated to 6-hydroxycyclohex-1-ene carboxyl-CoA (28). The later compound is oxidized to 6-ketocyclohex-1-ene carboxyl-CoA, which is then hydrolytically cleaved to 3-hydroxypimelyl-CoA (29). The pathway in *R. palustris* is similar except that cyclohex-1,5 diene carboxyl-CoA is most probably reduced to cyclohex-1-ene carboxyl-CoA. This latter compound is metabolized to 2-ketocyclohexane carboxyl carboxyl-CoA, which is hydrolytically cleaved to pimelyl-CoA. The C_7 ring cleavage products then undergo β-oxidation to yield three molecules of acetate and one molecule of CO_2.

Benzoate degradation also occurs under methanogenic conditions (37, 49). Tarvin and Buswell (49) observed the degradation of benzoate in anoxic sediments with the production of carbon dioxide and methane as final end-products. The discovery that methanogenic benzoate degradation to carbon dioxide and methane is mediated by a consortium of a fermentative (syntrophic) microorganism together with hydrogen- and acetate-utilizing methanogens (15) and the subsequent isolation of the syntrophic partners (36) allows the opportunity to study the pathway for benzoate degradation under methanogenic conditions. So far, three species that syntrophically metabolize benzoate have been isolated (22, 36, 51), all of which belong to the genus *Syntrophus*. Benzoate degradation under syntrophic conditions is not as thoroughly investigated as benzoate degradation under nitrate-reducing and phototrophic conditions due to the relatively slow growth rates and low cell yields of these organisms (3). However, recent studies showed that benzoate is activated to benzoyl CoA by an ATP-dependent ligase as the first step in benzoate metabolism (4, 46). Also, enzyme activities for glutaryl-CoA metabolism to acetate and CO_2 were detected in cell extracts of *Syntrophus gentianea* (46) and glutaryl-CoA dehydrogenase as well as the enzyme activities corresponding to crotonyl-CoA metabolism to acetate were detected in *Syntrophus buswellii* strain GA (2). The ring reduction and cleavage steps required for syntrophic benzoyl-CoA metabolism have not been investigated thus far.

In this study, we investigated the pathway for syntrophic benzoate metabolism in *Syntrophus aciditrophicus* strain SB by identifying and quantifying metabolites produced during its growth with benzoate, cyclohexane carboxylate and cyclohex-1-ene carboxylate in coculture with hydrogen-utilizing partners and by measuring the key enzyme activities postulated to be involved in benzoate metabolism. We report the transient production of cyclohex-1-ene carboxylate and relatively larger amounts of cyclohexane carboxylate during benzoate degradation.

This is consistent with the hypothesis that benzoyl-CoA reduction during syntrophic benzoate metabolism may involve a 4 or 6 electron reduction (46, 47). We hypothesize that this variation in benzoyl-CoA metabolism from that observed in *R. palustris* and *T. aromatica* may be due to the energetic constrains imposed by syntrophic metabolism of aromatic substrates.

MATERIALS AND METHODS

Microorganisms and media. *Syntrophus aciditrophicus*, strain SB (ATCC # 700169^T) was isolated from a sewage treatment plant in Norman, OK (22). *Methanospirillium hungatei* strain JF1 and *Desulfovibrio* sp. strain G11 were obtained from the culture collection of M.P. Bryant (Urbana, IL., USA). All media and stock solutions were prepared anaerobically according to the techniques described by Balch and Wolfe (6). These organisms were grown in a basal medium (33) with the omission of rumen fluid. To grow *S. aciditrophicus* in pure culture, crotonate (40 mM) was added to the basal medium (7) and the headspace was pressurized to 172 kPa by a gas mixture of 80% N_2-20% CO_2. *M. hungatei* and *Desulfovibrio* sp. strain G11 were grown in the basal medium containing 2 mM sodium acetate with 243 kPa of 80% H_2-20% CO_2. Sodium sulfate (15 mM) was included in the medium when *Desulfovibrio sp.* strain G11 was present. *M. hungatei* and *Desulfovibrio* sp. strain G11 cultures were incubated in a shaking incubator (100 rpm). Cocultures of *S. aciditrophicus* and *M. hungatei* or *S. aciditrophicus* and *Desulfovibrio sp.* strain G11 were established by the addition of a 15-20% (vol/vol) inoculum of each microorganism to the basal medium containing 1.2-1.5 mM of sodium benzoate, sodium cyclohexane carboxylate, or sodium cyclohex-1-ene carboxylate as substrates and a headspace of 80% N_2-20% CO_2 (172 kPa). All inoculations were performed using sterile disposable plastic syringes and needles that were degassed by oxygen-free nitrogen gas. All cultures were incubated at 37^{0}C. *Thauera aromatica* was obtained from the Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany, DSM 6984) and cultured anaerobically at 28^{0}C in a benzoate and nitrate medium (50). *Rhodopseudomonas*

palustris strain CGA009 was kindly provided by Dr. Caroline S. Harwood and cultured as previously described (17).

Detection and quantification of metabolites by gas chromatography-mass spectrometry. Cocultures of *S. aciditrophicus* with either *M. hungatei* or *Desulfovibrio* sp. strain G11 were grown in basal medium with 1.4 mM sodium benzoate in 600-ml volumes to detect metabolites of benzoate metabolism. Samples (60 ml) were withdrawn from the cultures at various time intervals. The pH of the samples was brought to above12 for 30 minutes by stepwise addition of 1 N NaOH to hydrolyze putative thioester bonds. Each sample was then acidified to pH < 2 with 12 N HCl. The samples were then extracted 3 times with 25-ml aliquots of ethyl acetate. The ethyl acetate extracts were filtered through anhydrous sodium sulfate to remove water, combined, and then concentrated to 2 to 3 ml under vacuum. The concentrated ethyl acetate extract was then quantitatively transferred to 6-ml vials and evaporated to dryness under a stream of nitrogen gas. The dried ethyl acetate extract was then redissolved in 0.3 ml of ethyl acetate and derivatized with *N, O*-bis-(trimethylsilyl) triflouroacetamide (BSTFA) (Pierce chemicals, Rockford, IL). Each concentrated and derivatized extract was analyzed with a Hewlett Packard 5890 series II gas chromatograph (GC) equipped with a Hewlett Packard 5970 series mass spectrometer and a 30 m DB-5 fused silica capillary column (J&W scientific, Folson, CA). Helium was used as a carrier gas at a flow rate of 0.8 ml/minute. The oven temperature was held at 70°C for 5 minutes, raised at a rate of 10°C/ min to 220°C and then held at that temperature for 5 minutes. Controls for this experiment included heat-killed cocultures of *S. aciditrophicus* and substrate unamended cocultures to differentiate between metabolites formed due to benzoate metabolism and metabolites present in the inoculum of crotonate-grown *S. aciditrophicus* cultures or H_2-grown *M. hungatei* and *Desulfovibrio* strain G11 cultures. All treatments were performed in triplicate. The metabolites were identified by

comparing their retention times and mass spectral profiles with TMS-derivatized chemical standards and quantified by comparison to standard curves constructed with the TMS derivative of the compound of interest. The detection limits ranged between 0.07 μM (pimelic acid) to 0.14 μM (cyclohexane carboxylic acid) under the experimental conditions used. Benzoate concentrations calculated by GC-MS analysis at different time intervals were within ±10% of the benzoate concentrations determined by high performance liquid chromatography (HPLC) analysis. *R. palustris* and *T. aromatica* cultures were grown with similar benzoate concentrations and the samples were processed as described above. A similar protocol was used for the detection of metabolites in cyclohexane carboxylate or cyclohex-1-ene carboxylate-grown *S. aciditrophicus- M. hungatei* cocultures.

Nuclear magnetic resonance spectroscopy. Cocultures of *S. aciditrophicus-M. hungatei* were grown with 1.5 mM of [*ring* $^{13}C_6$] labeled benzoate. Samples (100 ml) were withdrawn from the cultures at various time intervals and acidified to a pH < 2 by dropwise addition of 12N HCl. Each sample was extracted three times with ethyl acetate, concentrated under vacuum and dried under N_2 atmosphere as described above. The dried samples were then dissolved in deuterated chloroform ($CDCl_3$). Substrate unamended and heat-killed controls were included as described above. A coculture of *S. aciditrophicus-M. hungatei* with unlabeled benzoate was used to ensure that none of the observed peaks in the NMR spectra were due to ^{13}C impurities in the solvents used. The NMR spectra of the organic solvent-extracted samples were obtained on a Unity INOVA 400 MHz NMR spectrometer (Varian) with a ^{13}C resonance frequency of 100.573 MHz. The ^{13}C spectra were obtained at 30^0C using a standard inverse-gated pulse sequence. The experimental parameters used were: 24,140 Hz sweep width, 1.00 sec acquisition time, and 1.5 sec recycle delay. The number of scans ranged from 500 to 36,000, depending upon the sample concentration. The data were processed with 1-Hz line broadening.

Other analytical procedures. Protein was determined as described previously (10). Growth was monitored by measuring absorbance at λ_{600}. Benzoate was analyzed by HPLC as described before (21). Methane was analyzed by GC (23) and sulfate was analyzed by ion chromatography (32). Enzyme activities were determined on either a Beckman DU-64 spectrophotometer or a Schimadzu 2101-PC dual-beam spectrophotometer.

Preparation of cell extracts. Cells were harvested by centrifugation (12000 x g; 20 min; 4^0C) and washed by resuspending and recentrifuging the cell pellets three times in anoxic 100 mM Tris-HCl buffer (pH 7.8). The final cell pellet was suspended in the same buffer (0.2 g cells/ml) containing 1 mM $MgCl_2$, 2 mM dithiothreitol (DTT) and 0.2 mg/ml DNAse. Cells were broken under anaerobic conditions by two passages through a chilled French pressure cell at 110,400 kPa. Unbroken cells and cell debris were removed by centrifugation at 27,200 x g for 20 minutes at 4^0C. The resulting supernatant, termed the cell extract, was used immediately in enzyme assays or stored anaerobically in liquid nitrogen until used.

Enzyme assays. Acyl-CoA ligase was assayed by measuring the amount of AMP formed in the CoA ligase reaction in a coupled enzyme assay (4). The reaction was initiated by the addition of 10 to 50 µl of the cell extract after which the oxidation of NADH was followed at 340 nm. The formation of one mole of AMP corresponded to the oxidation of two moles of NADH. Substrates tested include benzoate, 2-, 3-, and 4- chlorobenzoate, 2-, 3-, and 4- fluorobenzoate, 4-hydroxybenzoate, picolinic acid, phenyl acetate, crotonate, *n*-butyrarte, isobutyrate, heptanoate, and hexanoate. The formation of benzoyl-CoA from benzoate and coenzyme-A in cell extracts was confirmed by using [*phenyl* ^{14}C] labeled benzoate (56.9 mCi/mmol) as a substrate according to a previously described procedure (17). The reaction was stopped after 2 minutes and the assay mixture was extracted twice with ethyl acetate.

CoA-transferase activity was measured by using a procedure modified from Schref and Bückel (43). Benzoyl-CoA was used as the CoA donor and acetate was used as the CoA acceptor. The reaction mixture contained 100 mM phosphate buffer (pH 7.0), 0.2 M sodium acetate, 1 mM oxaloacetate, 1 mM 5,5'-dithio-bis-(2-nitrobenzoate), 0.1 mM benzoyl-CoA, and 4 U of citrate synthase, in a total volume of 1 ml. The reaction was initiated by the addition of 5 to 50 µl of cell extract. The CoA liberated by action of citrate synthase reacts with 5,5'-dithio-bis-(2-nitrobenzoate) to form a yellow thiophenolate anion. The initial rates were determined by measuring the formation of this anion at 412 nm.

Cyclohex-1-ene carboxyl-CoA hydratase and 2-hydroxycyclohexane-carboxyl-CoA dehydrogenase were assayed as previously described (40). Cyclohex-1-enecarboxyl-CoA hydratase activity was determined as the combined cyclohex-1-ene carboxyl-CoA hydratase and 2-hydroxycyclohexane carboxyl-CoA dehydrogenase activities assayed in the forward direction using cyclohex-1-ene carboxyl-CoA as the substrate. 2-Hydroxycyclohexanecarboxyl-CoA dehydrogenase was assayed in the reverse direction using 2-ketocyclohexane carboxyl-CoA as the substrate. Both reactions were initiated by the addition of 1 to 5 µl of cell extract and followed by measuring the oxidation of NADH at 340 nm. 2-Ketocyclohexane carboxyl-CoA hydrolase activity was assayed by using a procedure modified from Perrota and Harwood (40). The reaction mixture contained 100 mM Tris-HCl buffer (pH 7.0), 100 mM $MgCl_2$, 1 mM DTT, and 1 mM 2-ketocyclohexane carboxyl-CoA in a total reaction volume of 50 µl. The reaction was initiated by the addition of 1-5 µl of the cell extract and followed by measuring the decrease in absorbance of the magnesium enolate complex at 314 nm.

Acyl-CoA dehydrogenase activity was assayed by the ferricenium-hexafluorophosphate method (30). The reaction mixture contained 100 mM Tris-HCl buffer (pH 7.5), 0.1 mM ferriceniumhexafluorophosphate and 0.05 mM

substrate in a total reaction volume of 1 ml. The reaction was initiated by the addition of 5 to 50 µl of the cell extract. The enzyme activity was determined by following the initial decrease in absorbance at 300 nm upon reduction of the ferricenium ion. For the oxidation of 1 mole of an acyl-CoA substrate, 2 moles of ferricenium ions were required. Substrates examined included glutaryl-CoA, pimelyl-CoA, butyryl-CoA, octanoyl-CoA, and palmitoyl-CoA.

Enoyl-CoA hydratase was assayed indirectly by using a coupled assay containing L-(+)-3-hydroxyacyl-CoA dehydrogenase (52). The reaction was initiated by the addition of crotonyl-CoA and followed by measuring the reduction of NAD. L-(+)-3-Hydroxyacyl-CoA dehydrogenase was determined by measuring the oxidation of NADH coupled to the reduction of S-acetoacetyl-CoA to 3-hydroxybutyryl-CoA (52). The reaction was initiated by the addition of S-acetoacetyl-CoA. 3-Ketoacyl-CoA thiolase activity was determined by following the CoA-dependent, acetoacetyl-CoA cleavage (52). The reaction was initiated by the addition of CoA and followed by measuring the decrease in absorbance at 303 nm.

Phosphotransacetylase activity was assayed by measuring the formation of acetyl-CoA from acetyl-phosphate (52). The reaction was initiated by the addition of 10 to 50 µl of cell extract and followed by measuring the appearance of the thioester bond at 233 nm. Acetate kinase was assayed by the hydroxamate method (41). The reaction mixture contained 770 mM sodium acetate, 50 mM Tris-HCl buffer (pH 7.4), 1 mM $MgCl_2$, 10 mM ATP, 10% hydroxylamine hydrochloride, and cell extracts in a total volume of 1 ml. After incubation for two minutes at room temperature, the reaction was stopped by the addition of 1 ml of 10 % trichloroacetic acid. The absorbance was measured at 540 nm against a blank that contained all reagents except ATP.

Enzyme assays were performed aerobically at room temperature unless otherwise stated. Activities were corrected for the endogenous activity present in the cell extracts and were proportional to protein concentration. Controls using boiled extracts or lacking the substrate were performed for each assay. Enzyme assays were performed using cell extracts prepared from at least two or three different cell batches and the coefficient of variation between replicate cultures was less than 15%.

Chemical Synthesis. 2-Hydroxycyclohexane carboxylic acid was synthesized by reducing ethyl 2-cyclohexanone carboxylate with sodium borohydride in 95% ethanol as previously described (40). 2-Ketocyclohexane-carboxylic acid was also synthesized from ethyl-2-cyclohexanone (12,40). 2-Ketocyclohexane carboxyl-CoA, cyclohex-1-ene- carboxyl-CoA, pimelyl-CoA, and glutaconyl-CoA were synthesized by reacting cyclohex-1-ene carboxylic acid, pimelic acid, and glutaconic acid, respectively, with free coenzyme-A using a procedure modified after Merckel et al. (35) and Callus and Schink (16). The crude preparations of the CoA thioesters were purified using C_{18} reversed-phase cartridges (Sep-Pak Plus, Millipore Corp., Midford, MA) as previously described (40). Ferricenium hexafluorophosphate was synthesized from ferrocine and sodium hexafluorophosphate (30).

Chemicals. Sodium benzoate was purchased from Sigma Chemical Co. (St. Louis, MO), cyclohexane carboxylic acid was purchased from Acros Organics (New Jersey), and cyclohex-1-ene carboxylic acid were purchased from Aldrich Chemical Co. (Milwaukee, WI). [*Ring* $^{13}C_6$] labeled benzoate and $CDCl_3$ was purchased from Cambridge Isotope Laboratories (Andover, MA). S-Acetoacetyl-CoA, benzoyl-CoA, glutaryl-CoA, butyryl-CoA, octanoyl-CoA, coenzyme A (sodium salt), ferrocine, sodium hexafluorophosphate, phosphoenol pyruvate, pyruvate kinase, myokinase, lactate dehydrogenase, citrate synthase, NADH, NAD^+, crotonase, L-(+)-3-hydroxyacyl-CoA dehydrogenase, acetyl phosphate, and ATP were purchased from

Sigma Chemical Co. All other chemicals used in this study were obtained from Sigma, Aldrich, or Fluka (Milwaukee, WI).

RESULTS

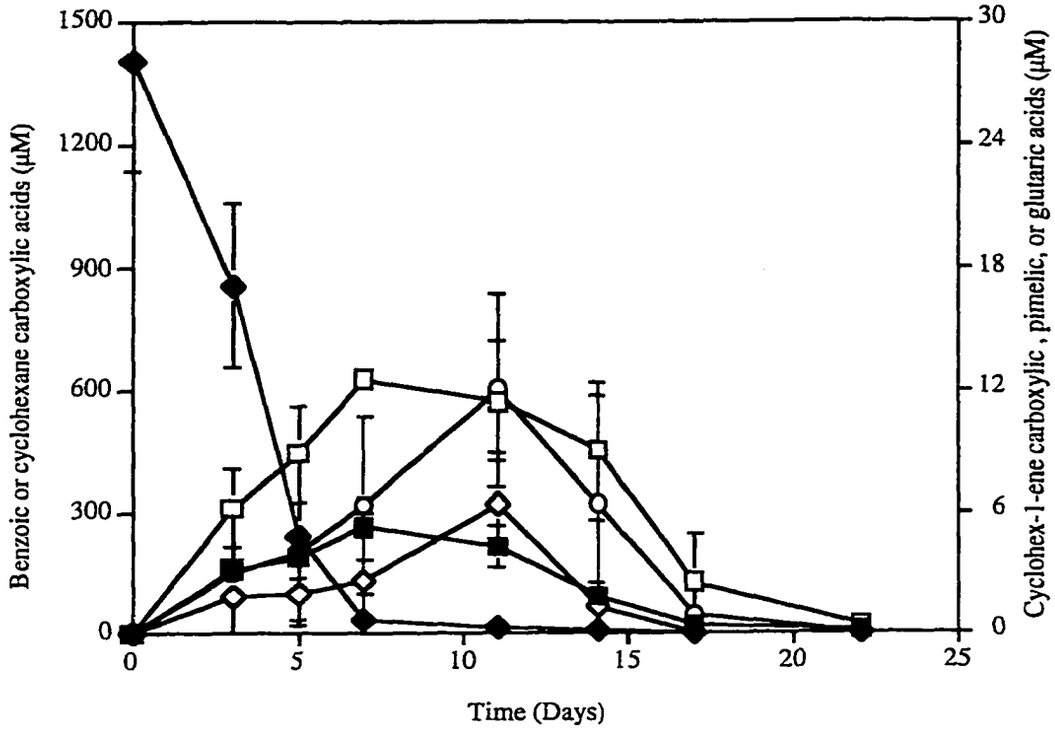
Metabolites produced during benzoate degradation. During growth of the *S. aciditrophicus-M. hungatei* cocultures with benzoate, cyclohexane carboxylate, cyclohex-1-ene carboxylate, pimelate, and glutarate were detected as their TMS derivatives. The TMS spectrum of each compound had an identical retention time and mass spectrum compared to the TMS derivatives of the authentic chemical standard (Fig.2.1). None of these compounds were detected in substrate unamended or heat-killed controls. Cyclohexane carboxylate (Fig.2.1a) was transiently produced and accumulated to a maximum concentration of 260 μM in culture fluids (Fig. 2.2). The maximum concentration of cyclohexane carboxylate occurred when about 97.5% of benzoate was consumed (Fig. 2.2). Cyclohex-1-ene carboxylate, pimelate, and glutarate (Fig. 2.1b-d) were also transiently produced and consumed, but these compounds were detected at much lower concentrations (Fig. 2.2). The maximum concentrations observed were 12.5, 6.4, and 11.3 μM for cyclohex-1-ene-carboxylate, pimelate, and glutarate, respectively, which represented 0.45-0.9% of the initial amount of benzoate. These four compounds were detected at similar concentrations regardless of whether the alkaline hydrolysis step was included or not and whether the entire culture, or the cell-free culture fluid was analyzed.

In addition, another metabolite with identical GC retention time and mass spectrum to the TMS spectrum of 3-hydroxybutyrate was identified in culture fluids of *S. aciditrophicus- M. hungatei*. However, this compound was also detected in substrate-unamended and heat-killed controls, suggesting that 3-hydroxybutyrate was present in the cells used as the inoculum. 3-Hydroxybutyrate is an intermediate in crotonate metabolism by syntrophic bacteria (34, 52).

Figure 2.1. Mass spectra of trimethylsilyl derivatives (TMS) of metabolites detected in *Syntrophus aciditrophicus* - *Methanospirillium hungatei* benzoate-grown cocultures (upper panels) compared to TMS derivatives of authentic chemical standards (lower panels). (A) cyclohexane carboxylate -TMS; (B) cyclohex-1-ene carboxylate-TMS. (C) pimelate -TMS. (D) glutarate-TMS.

A
Relative Abundance
m/z
73
185
200
117
129
145
COOSi(CH3)3
B
Relative Abundance
m/z
73
75
108
139
156
183
198
C
Relative Abundance
m/z
73
125
155
173
186
217
231
245
262
289
(CH3)3SiO
OSi(CH3)3
D
Relative Abundance
m/z
73
147
158
186
204
233
261
(CH3)3SiO
OSi(CH3)3

Figure 2.2: Transient detection of cyclohexane carboxylate (-■-), cyclohex-1-ene carboxylate (-□-), pimelate (-◊-), and glutarate (-o-) in *S. aciditrophicus -M. hungatei* cocultures grown with benzoate (-◆-). Each compound was detected and quantified as its TMS derivative .


1500
1200
900
600
300
0
30
24
18
12
6
0
0
5
10
15
20
25
Time (Days)
Benzoic or cyclohexane carboxylic acids (μM)
Cyclohex-1-ene carboxylic , pimelic, or glutaric acids (μM)

Cyclohexane carboxylate, cyclohex-1-ene carboxylate, pimelate and glutarate were detected as their TMS derivative in cocultures of *S. aciditrophicus* with *Desulfovibrio* sp. strain G11 at concentrations similar to that of the methanogenic cocultures (data not shown). Thus, the identity and quantity of metabolites produced during syntrophic growth on benzoate is independent of the hydrogen-utilizing partner.

Time-course experiments relating methane production to benzoate consumption in *S. aciditrophicus-M. hungatei* cocultures indicated that the amount of methane produced per mol of benzoate consumed was much less than theoretically predicted (0.75 moles of methane per mole of benzoate) during the initial stages of benzoate metabolism. After 3 days, 72.7 % of the initial benzoate present was consumed and the ratio of methane produced per benzoate consumed was 0.3. After 5 days, 96 % of the benzoate was consumed and the methane to benzoate ratio was 0.49. Only after 100 % of benzoate was consumed (day 11) was the methane to benzoate ratio (0.76) close to the theoretical value of 0.75. Thus, it appears that part of the electrons produced during benzoate metabolism was used to reduce benzoyl-CoA to cyclohexane carboxylate (or its CoA derivative).

^{13}C NMR analysis of culture extracts provided further evidence that cyclohexane carboxylate and cyclohex-1-ene carboxylate (or their CoA derivatives) were intermediates in benzoate metabolism by *S. aciditrophicus*. The ^{13}C NMR spectrum of the day 0 sample from *S. aciditrophicus - M. hungatei* cocultures contained two peak clusters centered at 133.5 and 129.5 ppm resulting from C-1 and C-2 through C-6, respectively, of [*ring*-$^{13}C_6$] benzoic acid. This was confirmed by comparison to the [*ring* $^{13}C_6$] benzoic acid standard in $CDCl_3$. The ^{13}C-labeled compounds detected during the experiment are listed in Table 2.1. Cyclohexane carboxylic acid was produced at the highest concentration of all compounds detected. It slowly decreased over the experimental period and was no longer

Table 2.1. NMR data for metabolites produced by *S. aciditrophicus- M. hungatei* cocultures grown on [*ring*-^{13}C] benzoic acid.

Compound	Position	δ ^{13}C (ppm)	J_{CC} (Hz)
acetic acid[1,2]	carboxyl	177.0	55.7
	methyl	20.7	56.9
benzoic acid[1,2]	C-1	133.5	53.6, 10.5
	C-2 – C-6	127.7 – 130.7	
cyclohexane carboxylic acid[1,2,]	C-1	42.8	32.0
	C-2, C-6	28.7	31.9
	C-3, C-5	25.7	
	C-4	25.4	
cyclohex-1-ene carboxylic	C-1	142.6	70.3, 41.0
acid[4]	C-2	147.5	69.8, 41.1
	C-3 – C-6	18.0 – 25.0	

[1,2,3] Chemical shifts and coupling constants were obtained from references (11,42,48)

[4] Chemical shifts and coupling constants were obtained from reference (26)

detectable by day 11. Coupling constants were not measurable for C-3, C-4, and C-5 of cyclohexane carboxylic acid since the difference in their resonance frequencies was less than the carbon-carbon coupling constant frequency (J_{CC}), thus the n+1 coupling rule no longer held (48). Benzoic acid was detected at low concentrations up to day 9. Signals from cyclohex-1-ene carboxylic acid were detected from day 3 through day 9. The concentration of acetic acid increased over the 11-day incubation period and acetate was the only detectable compound by day 11. Both of the peaks for the methyl and the carboxyl groups of acetate were doublets overlaid with a singlet (data not shown). The singlet at 20.8 ppm represented 36 to 40% of the total acetic acid detected in the methyl group and results from acetic acid molecules that were ^{13}C-labeled only at the methyl group. The presence of a carboxyl singlet indicated that some of the acetic acid molecules had ^{13}C-labeled atoms only at the carboxyl position.

Time course experiments were conducted to identify and quantify metabolites of benzoate metabolism by *R. palustris* and *T. aromatica* grown with a similar benzoate concentration (about1.5 mM) and analyzing similar sample volumes (about 60 ml). In both cases, none of the above mentioned compounds or any other potential intermediate of benzoate metabolism was detected. This indicated that intermediates of benzoate degradation in *R. palustris* and *T. aromatica* were produced at much lower concentrations than was the case with *S. aciditrophicus*.

Metabolism of cyclohexane carboxylate and cyclohex-1-ene carboxylate by *S. aciditrophicus- M. hungatei* cocultures. Cyclohex-1-ene carboxylate and cyclohexane carboxylate were metabolized without a lag by *S. aciditrophicus-M. hungatei* cocultures at rates slightly faster than that for benzoate (Table 2.2). Cocultures of *S. aciditrophicus* and *M. hungatei* grown with cyclohex-1-ene

carboxylate and cyclohexane carboxylate were analyzed by GC-MS as described above. In cyclohex-1-ene carboxylate-grown cocultures, pimelate and glutarate were

Table 2.2: Metabolism of benzoate, cyclohex-1-ene carboxylate, and cyclohexane carboxylate by *S. aciditrophicus* - *M. hungatei* cocultures.

Compound	Substrate added[a] (μmol)	Methane produced[a] (μmol)	Ratio of methane to substrate (mol/mol)[b]	Degradation rate (day^{-1})
Benzoate	108.5[b]	88.9	0.82	0.26
Cyclohex-1-ene carboxylate	115.5	166.2	1.44	0.35
Cyclohexane carboxylate	116.6	193.8	1.66	0.35

[a] Values shown are average of duplicates.

[b] Theoretical values for the amount of methane produced per substrate degraded (mol/mol) are: 0.75 for benzoate, 1.25 for cyclohex-1-ene carboxylate, and 1.5 for cyclohexane carboxylate.

Figure 2.3: Mass spectum of a TMS derivative of a metabolite detected in *S. aciditrophicus* - *M. hungatei*, cyclohex-1-ene carboxylate grown cultures (upper panel) compared to a synthesized TMS derivatized standard of 2-hydroxycyclohexane carboxylic acid.

Relative abundance
m/z
40
45
73
80
81
109
120
129
147
160
170
183
198
200
240
257
273
280
Relative abundance
m/z
40
45
73
80
81
109
120
129
147
160
170
183
198
200
240
257
273
280
COOSi(CH3)3
OSi(CH3)3

detected as their TMS derivatives at levels comparable to that found in benzoate-grown cultures. The maximum concentrations measured were 6.6 and 20.7 μM, respectively. Although cyclohexane carboxylate was detected (as the TMS derivative) in cyclohex-1-ene carboxylate-grown cultures, it was not produced at as high of a concentration as observed in benzoate-grown cocultures. Instead, it was detected at levels comparable to that of pimelate and glutarate (e.g. maximum concentration of 4.2 μM). In addition, 2-hydroxycyclohexane carboxylate (Fig. 2.3) was detected as its TMS derivative in trace amounts (< 0.5 μM). In cyclohexane carboxylate-grown cocultures, cyclohex-1-ene carboxylate, pimelate, and glutarate were detected. The maximum concentrations of these compounds in cyclohexane carboxylate-grown cultures were also comparable to those measured in benzoate-grown cocultures, 8.9, 6.1, and 15.7 μM for cyclohex-1-ene carboxylate, pimelate, and glutarate, respectively. None of the above mentioned compounds were detected in heat-killed or substrate-unamended controls.

Enzyme activities detected in cell extracts of *S. aciditrophicus*. Cell extracts of benzoate-grown cocultures of *S. aciditrophicus* and *Desulfovibrio* sp. strain G11 contained an ATP-dependent benzoyl-CoA ligase activity (Table 2.3). The formation of benzoyl-CoA from benzoate was confirmed by the isotopic assay using [*phenyl* ^{14}C] labeled benzoate (17). The results showed that 84 % of ^{14}C benzoate added was converted to benzoyl-CoA after 2 minutes. In addition to benzoate, 2-,3-, and 4-fluorobenzoate as well as 3-hydroxybenzoate were also served as substrates. No activity was detected with 4-hydroxybenzoate, 2-, 3-, or 4-chlorobenzoate, picolinic acid, phenylacetate, 4-hydroxyphenyl acetate, crotonate, *n*-butyrate, isobutyrate, heptanoate, and hexanoate. No acyl-CoA ligase activity was detected in the cell extracts of the pure cultures of *Desulfovibrio* sp. strain G11 (data not shown). Similar levels of acyl-CoA activities were detected in crotonate-grown pure cultures of *S. aciditrophicus*, suggesting that this activity was constitutively present in *S.*

aciditrophicus. Cell extracts of benzoate-grown cocultures contained very low levels of a benzoyl-CoA transferase activity. This activity was not detected in cell extracts of *Desulfovibrio* sp. strain G11 or pure cultures of *S. aciditrophicus*.

Cell extracts of benzoate-grown cocultures of *S. aciditrophicus* and *Desulfovibrio* sp. strain G11 contained many of the enzyme activities required for the conversion of cyclohex-1-ene carboxyl-CoA to pimelyl-CoA (Table 2.3). The presence of cyclohex-1-ene carboxyl CoA hydratase was measured as the combined cyclohex-1-ene-carboxyl-CoA hydratase and 2-hydroxycyclohexane carboxyl-CoA dehydrogenase. The enzyme activity was present at levels similar to that reported for *R. palustris* (40). The hydratase activity was not detected in cell extracts of *Desulfovibrio* sp. strain G11, but low levels of activity were detected in crotonate-grown cultures of *S. aciditrophicus*. The activity was 28-fold higher in benzoate-grown cells than in crotonate-grown cultures of *S. aciditrophicus*. The presence of 2-hydroxycyclohexanecarboxyl-CoA dehydrogenase was measured in the reverse direction using 2-ketocyclohexanecarboxyl-CoA as the substrate. Benzoate-grown *S. aciditrophicus* and *Desulfovibrio* sp. strain G11 cocultures contained high levels of the dehydrogenase activity. The specific activity of this enzyme was several-fold higher than the cyclohex-1-ene carboxyl-CoA hydratase, a pattern similar to that reported for *R. palustris* (40). This enzyme activity was not detected in the pure cultures of *Desulfovibrio* sp. strain G11 and was 8-fold higher in benzoate grown cocultures than in crotonate-grown pure cultures of *S. aciditrophicus* (Table 3). Cell extracts of benzoate-grown cocultures of *S. aciditrophicus-Desulfovibrio* sp. strain G11 also contained 2-ketocyclohexanecarboxyl-CoA hydrolase at levels similar to that of 2-hydroxycyclohexanecarboxyl-CoA dehydrogenase. This activity was not detected in the pure cultures of *Desulfovibrio* sp. strain G11. The hydrolase activity was present at a level several fold lower than that reported for *R. palustris* (40). The addition of 1 mM exogenous CoA did not stimulate the hydrolase activity,

Table 2.3. Enzyme activities detected in cell extracts of benzoate-grown cocultures of *S. aciditrophicus* -*Desulfovibrio* strain G11 and in crotonate-grown pure cultures of *S. aciditrophicus*.

Enzyme[a]	Specific Activity [nmol/min/mg protein]	
	Benzoate-grown coculture	Crotonate-grown pure culture
Acyl-CoA Ligase		
Benzoate	141	103
2-Fluorobenzoate	70	107
3-Fluorobenzoate	28	108
4-Fluorobenzoate	102	129
3-Hydroxybenzoate	36	6
Benzoyl CoA:Acetate CoA transferase	3	-[d]
Cyclohex-1-ene-1-carboxyl-CoA hydratase	57	2
2-Hydroxycyclohexane carboxyl-CoA dehydrogenase		317
2-ketocyclohexane carboxyl-CoA hydrolase	391	-[d]

Acyl-CoA Dehydrogenase		
Pimelyl-CoA	1144	581
Glutaryl-CoA	630	395
Butyryl-CoA	-[b]	257
Octanoyl-CoA	-	622
Palmitoyl-CoA	-	256
Enoyl-CoA Hydratase	18780	23880
L-3-Hydroxyacyl-CoA dehydrogenase	1244	1488
3 Ketoacyl-CoA Thiolase	287	323
Phosphotransacetylase	128	-
AcetateKinase[c]	21	10

[a] Unless otherwise noted, the activity was not detected in cell extracts of pure culture of *Desulfovibrio* strain G11.

[b] Activity not determined in pure culture of *Desulfovibrio* strain G11

[c] Specific activity was 41 $nmol^{-1}$ min^{-1} mg^{-1} protein in cell extracts of the pure culture of *Desulfovibrio* strain G11.

[d] Not detectable.

consistent with the ring cleavage reaction being hydrolytic rather than thiolytic. Unlike *R. palustris,* the 2-ketocyclohexane carboxyl-CoA hydrolase activity in benzoate-grown cocultures was detectable only after the inclusion of dithiothreitol in the reaction mixture, or when the reaction mixtures were processed in the anaerobic chamber. This suggested that the 2-ketocyclohexanecarboxyl-CoA hydrolase in *S. aciditrophicus* may be oxygen-sensitive. Also, no hydrolase activity was detected in crotonate-grown pure cultures of *S. aciditrophicus*, which suggested that the 2-ketocyclohexanecarboxyl-CoA hydrolase activity in *S. aciditrophicus* was induced by growth on benzoate.

Cell extracts of benzoate-grown cocultures of *S. aciditrophicus* and *Desulfovibrio* sp. strain G11 contained high levels of pimelyl-CoA dehydrogenase, the first enzyme in the reaction sequence leading to the formation of glutaryl-CoA from pimelyl-CoA. The specific activity of this enzyme was two-fold higher in benzoate-grown cocultures than in crotonate-grown pure cultures of *S. acidotrophicus* (Table 2.3). No activity was detected in cell extracts of pure cultures of *Desulfovibrio* sp. strain G11. Benzoate- grown cocultures of *S. aciditrophicus-Desulfovibrio* sp. strain G11 did not contain detectable acyl-CoA dehydrogenase activity with butyryl-CoA, octanoyl-CoA, or palmitoyl-CoA as substrate. Also, glutaryl-CoA dehydrogenase activity was detected in cocultures as well as in crotonate-grown pure cultures of *S. aciditrophicus*. This activity was two-fold higher in benzoate-grown cocultures than in crotonate-grown pure cultures. In addition to pimelyl and glutaryl-CoA dehydrogenases, cell extracts of crotonate-grown pure cultures of *S. aciditrophicus* contained high levels of butyryl-CoA dehydrogenase, crotonyl-CoA dehydrogenase, and palmitoyl-CoA dehydrogenase activities (Table 2.3).

All of the enzymes required for the conversion of crotonyl-CoA to acetyl-CoA and then to acetate were present in cell extracts of benzoate-grown *S.*

aciditrophicus- *Desulfovibrio* sp. strain G11 as well as in crotonate-grown pure cultures of *S. aciditrophicus*. The specific activities were more or less the same regardless of the substrate used for growth (Table 2.3).

DISCUSSION

By GC-MS analysis, we identified cyclohexane carboxylate, cyclohex-1-ene carboxylate, pimelate, and glutarate in benzoate-grown cocultures of *S. aciditrophicus- M. hungatei* based on comparison on retention times and mass spectra of their TMS derivatives to authentic chemical standards (Fig.2.1, 2.2). In addition to pimelate and glutarate, we also detected cyclohexane carboxylate and 2-hydroxycyclohexane carboxylate (Fig. 2.3) in cyclohex-1-ene carboxylate-grown cocultures and cyclohex-1-ene carboxylate in cyclohexane carboxylate-grown cocultures. These compounds were transiently produced and were not detected in heat-killed or substrate-unamended controls, providing strong evidence that these compounds, probably as their CoA derivatives, are intermediates in the metabolism of benzoate, cyclohexane carboxylate and cyclohex-1-ene carboxylate. ^{13}C NMR spectroscopy confirmed that cyclohexane carboxylate and cyclohex-1-ene carboxylate were produced from benzoate. The fact that cyclohexane carboxylate, cyclohex-1-ene carboxylate, pimelate, and glutarate were detected when the alkaline hydrolysis step was omitted and were found in whole culture broth (cells plus medium) and in cell-free culture broth suggests that *S. aciditrophicus* may excrete potential intermediates of benzoate and alicyclic acid metabolism. Several studies have detected free acids corresponding to potential intermediates in phenol (5) and benzoate (13) metabolism. Since *S. aciditrophicus* cocultures and pure cultures contain enzymatic activities that metabolize the various alicyclic and aliphatic CoA intermediates of benzoate metabolism (Table 2.3), this suggests that the CoA derivatives of the above compounds and not the free acids are the functional intermediates involved in benzoate and alicyclic acid metabolism.

Cyclohexane carboxylate was originally thought to be an intermediate in benzoate metabolism by *Rhodopseudomonas palustris* (13). However, subsequent

investigations of cyclohexane carboxylate metabolism in this microorganism (27) as well as a better understanding of the biochemistry and genetics of benzoyl-CoA metabolism in *R. palustris* (38-40) and *T. aromatica* (28, 29) led to the exclusion of cyclohexane carboxylate from the benzoyl-CoA pathway in both microorganisms. It was suggested that the transient production of cyclohexane carboxylate in *R. palustris* is a physiological response to excess reducing equivalents or is a mechanism to maintain appropriate intracellular levels of free CoA (27). Some of the earlier studies on benzoate degradation by methanogenic consortia from sewage sludge, rumen fluid, or anaerobic mud (15, 24, 37) also suggested cyclohexane carboxylate as a benzoate degradation intermediate based on co-chromatography and ability of the consortium to degrade cyclohexane carboxylate without a lag period. Given the number of different organisms that will be present in such a consortium, it is difficult to make definitive conclusions regarding potential intermediates involved in the degradation of benzoate in these studies. However, we clearly show that relatively large amounts of cyclohexane carboxylate, accounting for about 18% of the benzoate carbon, are formed and consumed by *S. aciditrophicus* during growth with benzoate. These data suggest that cyclohexane carboxylate may serve as a repository for reducing equivalents generated during benzoate metabolism.

The detection of cyclohexane carboxylate, as well as cyclohex-1-ene carboxylate, pimelate, and glutarate at levels not encountered in *R. palustris* or *T. aromatica* suggests that the pathway for benzoate metabolism in syntrophic bacteria is different from that of phototrophs and nitrate-reducers. This difference is not surprising for two reasons. First, members of the genus *Syntrophus*, which belong to the δ-subgroup of proteobacteria, *T. aromatica*, which belongs to the β-subgroup of protobacteria, and *R. palustris*, which belongs to the α-subgroup of the protobacteria, are phylogeneticallty distinct. Second, is the energy constraints

encountered in syntrophic aromatic metabolism (45-47) that would not be encountered by organisms that can obtain energy from respiration and photosynthesis. It is obvious that syntrophic microorganisms can gain energy for growth on benzoate yet it is not clear how net ATP production occurs if benzoate activation and ring reduction during syntrophic benzoate metabolism occurs as found in benzoate-degrading phototrophs and denitrifiers (18, 46, 47). This study as well as others (4, 46) demonstrates benzoate activation proceeds by a benzoyl-CoA ligase reaction which uses two energy-rich bonds. We also detected a benzoate: acetyl-CoA transferase reaction, similar to succinyl-CoA: benzylsuccinate transferase reaction suggested for benzylsuccinate activation in *T. aromatica* (31), which would only use the equivalent of one energy-rich bond for benzoate activation. However, the activity of this enzyme is very low in benzoate-grown cocultures and is probably not sufficient to account for benzoate metabolism. While our evidence to date cannot exclude a 2-electron reduction of benzoyl-CoA to cyclohex-1,5-diene carboxylate, the transient production of large amounts of cyclohexane carboxylate suggests that ring reduction may differ in syntrophic metabolism. Thermodynamic calculations indicate that if the product of benzoyl-CoA reduction is cyclohex-1-ene-carboxyl-CoA or cyclohexane carboxyl-CoA instead of cyclohex-1,5-diene carboxyl-CoA, the product observed in *T. aromatica* (9), then the reaction could proceed without ATP investment (44, 46). The detection of cyclohexane carboxylate and cyclohex-1-ene carboxylate in cell extracts is consistent with a 4- or 6- electron reduction step rather than a 2-electron reduction step.

Although our work demonstrates the production and consumption of cyclohexane carboxylate by benzoate-grown cultures of *S. aciditrophicus*, the role of this compound (or its CoA derivative) in syntrophic benzoate degradation is unclear. Cyclohexane carboxyl-CoA might be the product formed by a 6-electron

reduction of benzoyl-CoA. Cyclohexane carboxyl-CoA could then be oxidized to cyclohex-1-ene carboxyl-CoA by cyclohexane carboxyl-CoA dehydrogenase and the later compound metabolized in a manner similar to that observed in *R. palustris* (Fig. 2.4). However, this does not explain why cyclohexane carboxylate is produced at a level far higher than other metabolites. Another possible explanation is that cyclohexane carboxylate is produced in a dismutation reaction where the reducing equivalents produced during oxidation of one benzoyl-CoA molecule is used to reduce another benzoyl-CoA molecule to cyclohexane carboxyl-CoA (Fig.2.4). This scheme would require two distinct enzymes to reduce benzoyl-CoA, one to reduce benzoyl-CoA to cyclohex-1-ene carboxyl-CoA and another to reduce benzoyl-CoA to cyclohexane carboxyl-CoA. Benzoate dismutation (simultaneous oxidation/reduction of benzoate) has been recently suggested as an alternative mechanism for hydrogen removal in methanogenic, benzoate-degrading enrichments where the electron flow to methanogenesis was inhibited by bromoethanesulfonic acid (BESA) (25). However, if such a reaction occurs in *S. aciditrophicus*, it is not clear what factors determine whether the reducing equivalents produced during benzoate metabolism will form hydrogen or be used to reduce benzoyl-CoA to cyclohexane carboxyl-CoA. A third possibility is that benzoyl-CoA is first reduced to cyclohex-1-ene carboxyl-CoA then a dismutation reaction occurs in which the oxidation of one molecule of cyclohex-1-ene carboxyl-CoA is coupled to the reduction of three molecules of cyclohex-1-ene carboxyl-CoA (Fig. 2.4).However, the fact that cyclohex-1-ene carboxylate-grown cocultures do not accumulate cyclohexane carboxylate in levels comparable to benzoate-grown cultures argues against this possibility.

Enzyme studies using *S. aciditrophicus* cell-free extracts indicate that the organism metabolizes cyclohex-1-ene carboxyl-CoA by a pathway similar to that observed in *R. palustris* . It could be argued that the same enzymes could also

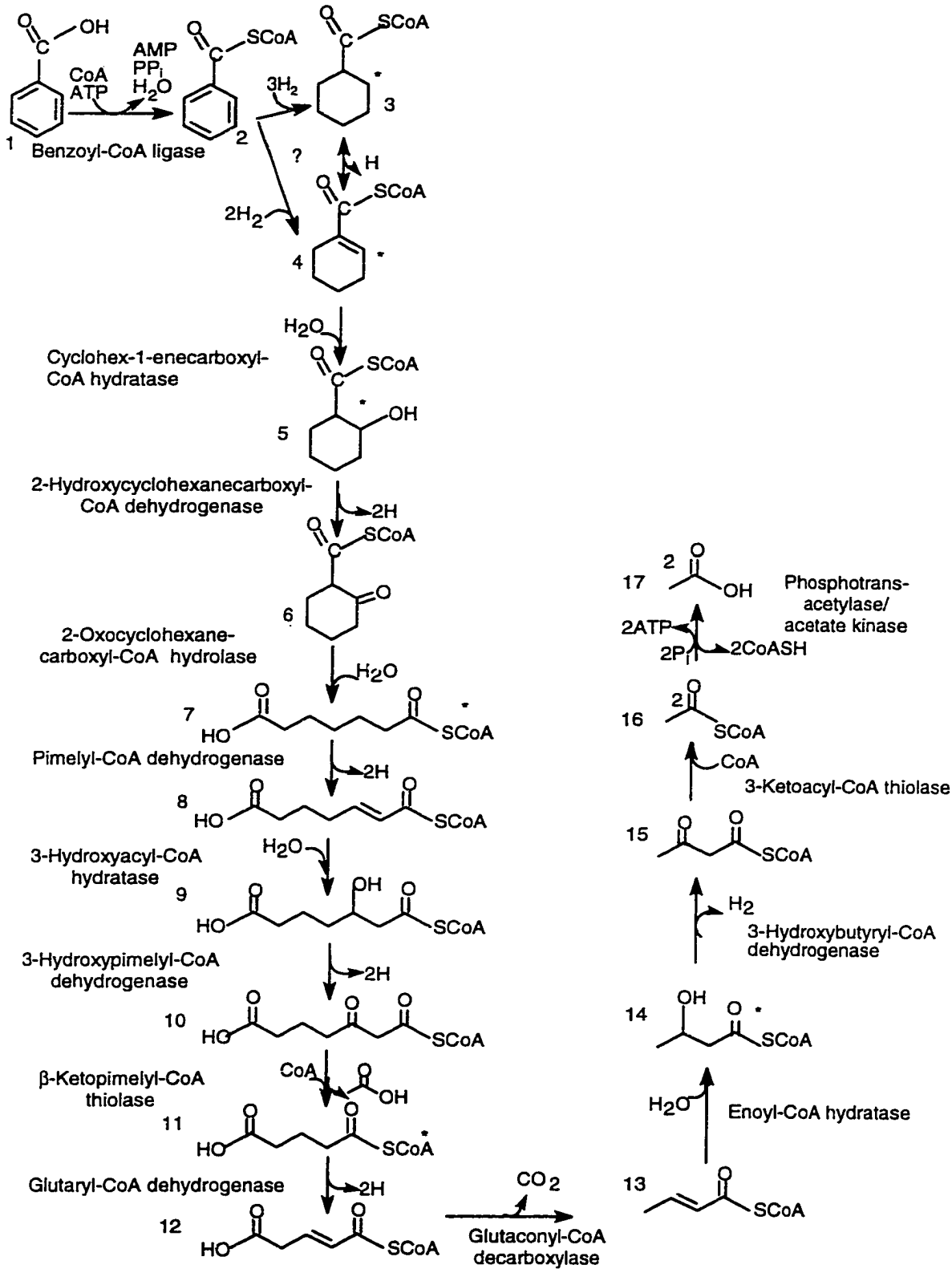
catalyze cyclohex-1,5-diene transformation to 6-hydroxycyclohex-1-ene carboxylate, 2-keto-6-hydroxycyclohex-1-ene carboxyl-CoA and 3-hydroxypimelyl-CoA (19) since the later substrates were not tested in our enzyme assays due to their commercial unavailability. However, the detection of cyclohex-1-ene carboxylic acid, 2-hydroxycyclohexane carboxylic acid, and pimelic acid in culture extracts are consistent with a pathway similar to *R. palustris*. Also, the 2-fold higher level of pimelyl-CoA dehydrogenase in benzoate-grown cells than in crotonate-grown cells is consistent with the production of pimelate as the ring cleavage product (40). This activity was specific for pimelyl-CoA since benzoate-grown cells of *S. aciditrophicus* lacked detectable activity with butyryl-CoA, octanoyl-CoA, and palmitoyl-CoA as substrates. Thus, it is unlikely that the enzyme activity detected in benzoate-grown cells was due to the presence of a non-specific, acyl-CoA dehydrogenase. The pimelyl-CoA dehydrogenase activity, demonstrated for the first time in a benzoate-grown anaerobic microorganism, was not detected in a nitrate-reducing isolate when grown with benzoate (16) probably because benzoate degradation in that isolate proceeds via the transformation of cyclohex-1,5-diene carboxyl-CoA to 3-hydroxypimelate without the formation of pimelyl-CoA. The dehydrogenase activity was detected, however, when the same isolate was grown on pimelate (16). It could also be argued that the observed cyclohex-1-ene carboxyl-CoA hydratase activity is due to the action of an enoyl-CoA hydratase, which acts primarily on short-chain unsaturated fatty acids (28). However, the detection of cyclohex-1-ene carboxyl-CoA hydratase at 28-fold higher levels when grown with benzoate compared to crotonate and the detection of enoyl-CoA hydratase at similar activity levels in benzoate- and crotonate-grown cells indicates that a specific cyclohex-1-ene carboxyl-CoA hydratase activity is present in *S. aciditrophicus*.

An intriguing observation is the presence of both ^{12}C and ^{13}C carbons in the carboxyl and methyl moieties of acetate. Acetate molecules with ^{13}C only in

the methyl group most likely arose from pimelate in which one of the carboxyl groups is the original unlabeled carboxyl group of benzoate. The presence of acetate molecules that have ^{13}C only in the carboxyl group is less easily understood since the use of [*ring* $^{13}C_6$] should give acetate molecules with both carbons ^{13}C labeled, except for the situation discussed above. It is possible that the glutaconyl-CoA decarboxylation reaction is in equilibrium with the bicarbonate pool and that this reaction allows the exchange of ^{12}C and ^{13}C atoms to occur.

In conclusion, although syntrophic benzoate metabolism follows the main themes observed in nitrate-reducers and phototrophs, it appears that a third variant of the benzoyl-CoA pathway where cyclohexane carboxylic acid is produced from benzoate may operate in microorganisms that syntrophically degrade benzoate (Fig 2.4). The variation is probably imposed by the strict energy constraints of syntrophic metabolism. Detailed investigations are still required to determine the exact function of cyclohexane carboxylic acid in syntrophic benzoate metabolism and to determine how syntrophic microorganisms are able to obtain net ATP production to support growth on benzoate. The fact that three variants of the benzoyl-CoA pathway have been detected in the three groups of microorganisms that have been studied so far raises the question of how similar or different benzoate metabolism is in other physiological groups of microorganisms such as sulfate reducing and iron-reducing bacteria that have yet to be studied.

Figure 2.4: Proposed pathway for benzoate metabolism is *S. aciditrophicus*. Enzyme activities detected in *S. aciditrophicus* cell-free extracts are indicated in bold. Asterisks indicate compounds detected as their TMS derivative in *S. aciditrophicus - M. hungatei* culture extracts. (1) Benzoate. (2) Benzoyl-CoA. (3) Cyclohexane carboxyl-CoA (4) Cyclohex-1-ene carboxyl-CoA. (5) 2-Hydroxycyclohexane carboxyl-CoA. (6) 2-Ketocyclohexane carboxyl-CoA. (7) Pimelyl-CoA. (8) 2-heptenedioc acid-1-CoA (9) 3-Hydroxypimelyl-CoA. (10) 3-Ketopimelyl-CoA. (11) Glutaryl-CoA. (12) Glutaconyl-CoA. (13) Crotonyl-CoA. (14) 3-Hydroxybutyryl-CoA. (15) Acetoacetyl-CoA. (16) Acetyl-CoA. (17) Acetate. ? indicates that the exact benzene ring reduction mechanism in *S. aciditrophicus* is not yet completely understood.


Benzoyl-CoA ligase
Cyclohex-1-enecarboxyl-CoA hydratase
2-Hydroxycyclohexanecarboxyl-CoA dehydrogenase
2-Oxocyclohexane-carboxyl-CoA hydrolase
Pimelyl-CoA dehydrogenase
3-Hydroxyacyl-CoA hydratase
3-Hydroxypimelyl-CoA dehydrogenase
β-Ketopimelyl-CoA thiolase
Glutaryl-CoA dehydrogenase
Glutaconyl-CoA decarboxylase
Enoyl-CoA hydratase
3-Hydroxybutyryl-CoA dehydrogenase
3-Ketoacyl-CoA thiolase
Phosphotrans-acetylase/ acetate kinase

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CHAPTER 3

Benzoate fermentation by the anaerobic bacterium *Syntrophus aciditrophicus* in absence of hydrogen-utilizing microorganisms

ABSTRACT

The anaerobic bacterium *Syntrophus aciditrophicus* metabolized benzoate in pure culture in absence of hydrogen-utilizing partners or terminal-electron acceptors. The pure culture of *S. aciditrophicus* produced approximately 0.5 mol of cyclohexane carboxylate and 1.5 mol of acetate per mol of benzoate while the coculture of *Syntrophus aciditrophicus* with the hydrogen-using methanogen *Methanospirillium hungatei* produced 3 mol of acetate and 0.75 mol of methane per mol of benzoate. The growth yield of *S. aciditrophicus* in pure culture was 6.9 g (dry weight) per mol of benzoate metabolized, compared to 11.8 g (dry weight) per mol benzoate for the *S. aciditrophicus-M. hungatei* coculture. Cyclohexane carboxylate was metabolized by *S. aciditrophicus* only in coculture with a hydrogen-user but not by *S. aciditrophicus* pure cultures. Cyclohex-1-ene carboxylate was incompletely degraded by *S. aciditrophicus* pure cultures until a net free energy change ($\Delta G'$) of –9.2 kJ/mol was reached (-4 kJ/mol for the hydrogen producing reaction). Cyclohex-1-ene carboxylate, pimelate, and glutarate transiently accumulated in micromolar levels during growth of *S. aciditrophicus* in pure culture with benzoate. High hydrogen (10.1 kPa) and acetate (60 mM) levels inhibited benzoate metabolism by *S. aciditrophicus* pure cultures. These results suggest that benzoate fermentation by *S. aciditrophicus* in absence of hydrogen-users proceeds via a dismutation reaction in which the reducing-equivalents produced during oxidation of one benzoate molecule to acetate and carbon dioxide are used to reduce

another benzoate molecule to cyclohexane carboxylate, which is not further metabolized by the pure culture. Thermodynamic calculations indicate that benzoate fermentation to acetate, CO_2, and cyclohexane carboxylate is thermodynamically favorable and that substrate metabolism can proceed at free energy values more positive than –20 kJ/mol, the postulated minimum free energy value for substrate metabolism.

INTRODUCTION

Anaerobic metabolism of benzoate to acetate, CO_2, and hydrogen or formate in absence of light or terminal electron acceptors is thermodynamically unfavorable and proceeds only if the hydrogen produced during benzoate oxidation is continuously maintained at a low concentration by a hydrogen-using microorganism (12, 24). This kind of mutual cooperation between two different species to degrade a single substrate is called syntrophism. In syntrophic cocultures, hydrogen-utilizing bacteria serve only to maintain low hydrogen levels and are not directly involved in the metabolism of the original substrate. Also, many syntrophic microorganisms can grow in absence of hydrogen-utilizing partners on unsaturated substrate analogues by dismutation reactions where part of the original substrate is used as an electron acceptor (24).

The syntrophic benzoate degrader, *Syntrophus aciditrophicus*, metabolizes benzoate in cocultures with hydrogen-using methanogens or sulfate-reducers to acetate, hydrogen, and CO_2. *S. aciditrophicus* can also grow in pure culture on crotonate (12). Recent studies on benzoate metabolism in *S. aciditrophicus-M. hungatei* cocultures showed that cyclohexane carboxylate transiently accumulated during benzoate metabolism up to a level of 18% of the original benzoate concentration (8). The large concentration of cyclohexane carboxylate, together with the observation that the amount of methane produced per mol of benzoate consumed in *S. aciditrophicus-M. hungatei* benzoate-grown cocultures was less than the theoretically-predicted ratio (0.75) during the initial stages of benzoate metabolism, led us to hypothesize that a portion of the electrons produced during benzoate oxidation to acetate and CO_2 is used to reduce benzoate to cyclohexane carboxylate (8). Benzoate reduction to cyclohexane carboxylate could thus provide an alternative mechanism for the disposal of reducing equivalents

produced during benzoate oxidation instead of interspecies hydrogen transfer. Thermodynamic calculations indicate that while benzoate oxidation to acetate, carbon dioxide, and hydrogen is thermodynamically unfavorable (equation 1, Table 3.1), benzoate reduction to cyclohexane carboxylate is an exorgenic reaction (equation 2, Table 3.1), which makes the overall fermentation reaction of benzoate to acetate, HCO_3^-, and cyclohexane carboxylate exorgenic ($\Delta G^{0\prime}$ of –12.0 kJ/mol) (equation 3, Table 3.1). We here present evidence that *S. aciditrophicus* is able to grow and metabolize benzoate in absence of hydrogen-utilizing partners by oxidizing about half the benzoate to acetate and CO_2 and reducing the other half of the benzoate to cyclohexane carboxylate (equation 3, Table 3.1).

Table 3.1: $\Delta G^{o\prime}$ of different oxidation/reduction reactions involved in benzoate, cyclohexane carboxylate and cyclohex-1-ene carboxylate metabolism[a]

	Reaction	Equation	$\Delta G^{o\prime}$ (kj/mol)
1	Benzoate oxidation	$C_7H_5O_2^- + 7\ H_2O$ ---> $3\ CH_3COO^- +$ $HCO_3^- + 3H_2 + 3H^+$	+ 70.5
2	Benzoate reduction	$C_7H_5O_2^- + 3H_2$---> $C_7H_{11}O_2^-$	-94.5
3	Benzoate fermentation	$2\ C_7H_5O_2^- + 7\ H_2O$ ---> $C_7H_{11}O_2^- + 3$ $CH_3COO^- + HCO_3^- + 3H^+$	-12.0
4	Cyclohex-1-ene carboxylate oxidation	$C_7H_9O_2^- + 7\ H_2O$ ---> $3\ CH_3COO^- +$ $HCO_3^- + 5H_2 + 3H^+$	+93.7
5	Cyclohex-1-ene carboxylate reduction	$C_7H_9O_2^- + H_2$---> $C_7H_{11}O_2^-$	-71.3
6	Cyclohex-1-ene carboxylate fermentation	$6C_7H_9O_2^- + 7\ H_2O$ ---> $3\ CH_3COO^- +$ $HCO_3^- + 5\ C_7H_{11}O_2^- + 3H^+$	-43.8
7	Cyclohexane carboxylate oxidation	$C_7H_{11}O_2^- + 7\ H_2O$ ---> $3\ CH_3COO^- +$ $HCO_3^- + 6H_2 + 3H^+$	+ 165.3

8	Benzoate metabolism by *S. aciditrophicus-* *M. hungatei* coculture	$C_7H_5O_2^- + 4.75\ H_2O$ ---> $3\ CH_3COO^- + 0.25\ HCO_3^- + 0.75\ CH_4 + 2.25H^+$	-31.2
9	Cyclohex-1-ene carboxylate metabolism by *S. aciditrophicus- M. hungatei* coculture	$C_7H_9O_2^- + 3.25\ H_2O + 0.25\ HCO_3^-$ --- > $3\ CH_3COO^- + 1.25\ CH_4 + 1.75\ H^+$	-75.5
10	Cyclohexane carboxylate metabolism by "*S. aciditrophicus*"- *M. hungatei* coculture	$C_7H_{11}O_2^- + 2.5\ H_2O + 0.5\ HCO_3^-$ ---> $3\ CH_3COO^- + 1.5\ CH_4 + 1.5\ H^+$	-38.1

[a] The ΔG_f of benzoate (-225 kJ/mol) was obtained from Kaiser and Hanselmann (15). The ΔG_f of cyclohexane carboxylate was estimated as –319.5 kJ/mol based on ΔG_f of benzoate and the energy of benzene ring reduction (22). The ΔG_f of cyclohex-1-ene carboxylate was estimated as –248.2 kJ/mol based on the reported $E^{o\prime}$ value of –350 mV for the benzoate/cyclohex-1-ene carboxylate redox pair (26). All other values were obtained from Thauer et al. (27).

MATERIALS AND METHODS

Microorganisms and Media. *Syntrophus aciditrophicus* SB^T (ATCC 700169^T) (12) and *Methanospirillium hungatei* strain JF1 were obtained from our culture collection. All media and stock solutions were prepared anaerobically according to the techniques described by Balch and Wolfe (3). Pure cultures of *S. aciditrophicus* and *M. hungatei* were maintained in the basal medium but lacking rumen fluid as described earlier (8, 18). Co-cultures of *S. aciditrophicus- M. hungatei* were established and grown in sulfate-free, benzoate basal medium (sulfate concentration < 5 μM) as described earlier (8). When *S. aciditrophicus* was tested for its ability to grow with aromatic or alicyclic substrates, basal medium containing 1.2 to 1.7 mM of the test substrate was inoculated with crotonate-grown, pure cultures in stationary phase (inoculum size of 15 to 20 %). All cultures were incubated at 37^0 C. The cultures were routinely checked for purity by microscopical observation as well as by inoculation of thioglycolate medium. Methane production was routinely checked in pure cultures of *S. aciditrophicus* growing with benzoate, cyclohexane carboxylate, and cyclohex-1-ene carboxylate to ensure the absence of any methanogenic activity.

Analytical procedures: Benzoate, cyclohexane carboxylate, and cyclohex-1-ene carboxylate were analyzed by high performance liquid chromatography (HPLC) with a reverse phase C_{18} Econosphere column (250 mm by 4.6 mm, particle size of 5μm, Alltech Inc., Deerfield, IL). The isocratic mobile phase consisted of 75% phosphate buffer (25 mM sodium dihydrogen phosphate, pH 2.75) and 25% acetonitrile. A variable-wavelength UV absorbance detector set at 214 nm was used to detect substrates and metabolites. Benzoate was also occasionally determined on the same column by using an isocratic mobile phase consisting of 70% 50 mM sodium acetate buffer (pH 4.5) and 30 % acetonitrile at 254 nm (11). Gas

chromatography-mass spectroscopy (GC-MS) was used for identification and quantification of metabolites produced at μM levels as described before (8).

Acetate was quantified by ion chromatograhy using a Dionex system and an AS11A-SC column (4-mm particle size) (Dionex, Sunnyvale, CA) and 0.1% NaOH as the mobile phase. Butyrate and crotonate were analyzed by gas chromatography (GC) as described before (12). Methane was also analyzed by gas chromatography (14). Hydrogen was measured by a mercury vapor detector (12). Protein was quantified by the Bradford method (6) utilizing commercially available kits (Pierce chemical Co, Rockford, IL).

Thermodynamic calculations. At the end of every experiment, the concentration of substrate (benzoate, cyclohexane carboxylate, or cyclohex-1-ene carboxylate) and each of the products (acetate, hydrogen, cyclohexane carboxylate) was determined. The $\Delta G'$ for the hydrogen-producing reactions under experimental conditions were calculated according to the following equation:

$$\Delta G' = \Delta G^{o\prime} + RT \ln [CH_3COO^-]^3 [HCO_3^-] [H_2]^x / [\text{substrate}]$$

Where the number mol of hydrogen produced per mol substrate degraded (X) is 3 for benzoate, 5 for cyclohex-1-ene carboxylate, and 6 for cyclohexane carboxylate. The $\Delta G'$ for the overall syntrophic substrate degradation or substrate fermentations was calculated according to the general equation:

$$\Delta G' = \Delta G^{o\prime} + RT \ln [A]^a [B]^b / [C]^c [D]^d$$

Where [A], [B] represent the concentrations of products formed [C], [D] represent the concentration of the reactants in the reaction, and a, b, c, and d represent the number of mol produced or consumed for every compound. Equations describing benzoate, cyclohexane carboxylate, and cyclohex-1-ene carboxylate fermentation or syntrophic degradation as well as their $\Delta G^{o\prime}$ values used in these calculations are given in Table 1. R is the gas constant (0.008314 $kJ.mol^{-1}K^{-1}$) and T is the absolute temperature (310^0 Kelvin). Values used are molar concentrations, except hydrogen

and methane, which were measured in atm. (1 atm. = 101325 Pa). The HCO_3^{-1} concentration was assumed to be 0.0365 M in all experiments.

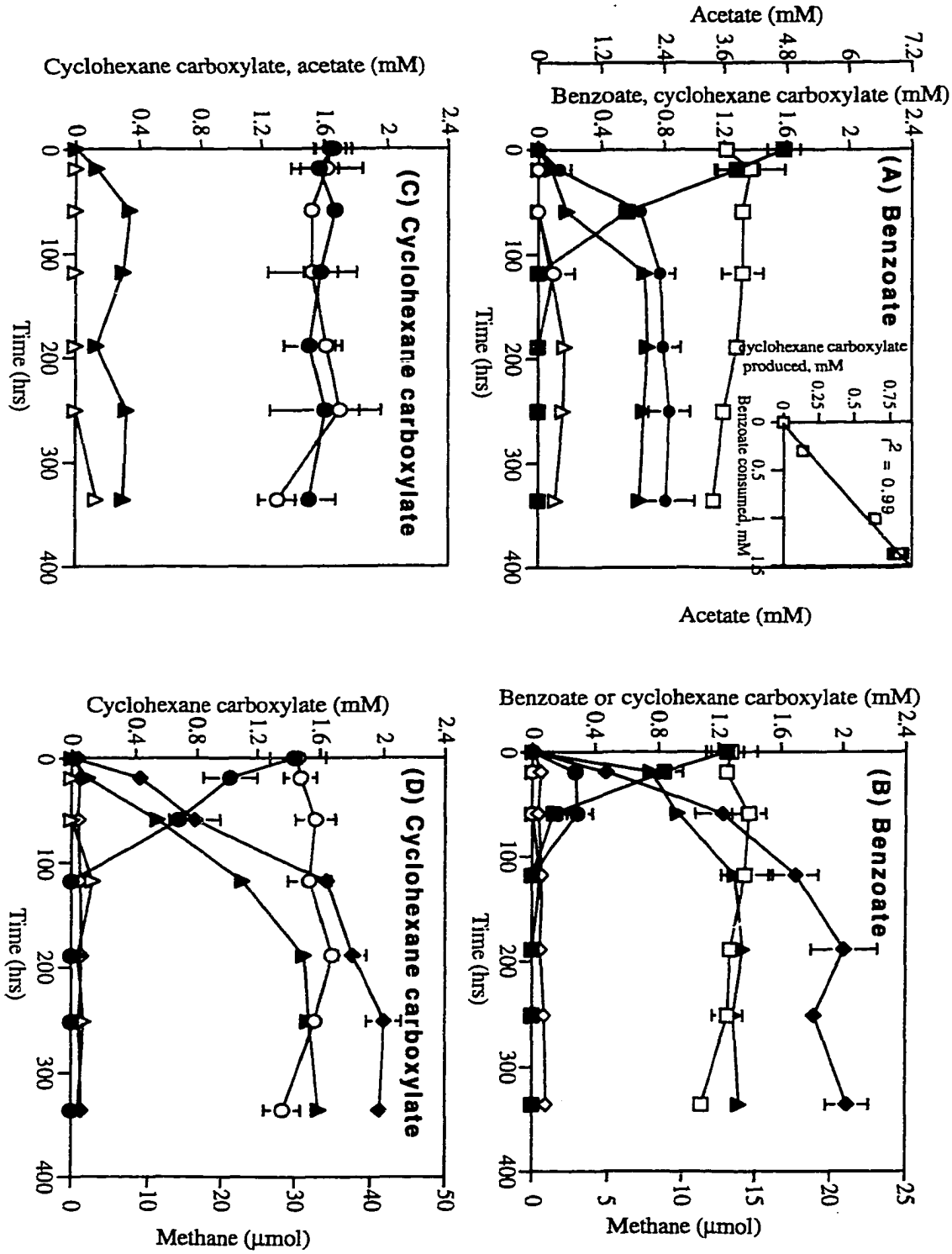
Chemicals. Sodium benzoate was purchased from Sigma Chemical Co. (St. Louis, MO), cyclohexane carboxylic acid was purchased from Acros Organics (Fair Lawn, New Jersey), and cyclohex-1-ene carboxylic acid was purchased from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals used in this study were obtained from Sigma, Aldrich, or Fisher (Pittsburgh, Pa).

RESULTS

Metabolism of benzoate and cyclohexane carboxylate by *S. aciditrophicus* in presence and absence of H_2-utilizing microorganisms. Pure cultures of *S. aciditrophicus* metabolized benzoate (Fig 3.1a) at a rate of 0.41 ± 0.04 day^{-1}. The pure culture of *S. aciditrophicus* produced 0.53 mol of cyclohexane carboxylate and 1.44 mol of acetate per mol benzoate degraded (Table 3.2). These values are close to the values predicted from the fermentation reaction (equation 3, table 3.1). A significant correlation ($r^2 = 0.99$) was observed between benzoate consumption and cyclohexane carboxylate production during the experiment (Fig. 3.1a, inset). No methane was detected during the course of the experiment. Microscopical observation revealed the presence of only the *S. aciditrophicus*-cell morphotype at the end of the experiment. Hydrogen concentrations increased from 38.5± 9.5 Pa at the start of the experiment to 97.9 ± 7.2 Pa at the end of the experiment (Table 3.2), indicating that a small portion of the electrons produced during benzoate oxidation were used to reduce protons to hydrogen. The initial crotonate concentration in these cultures was 120 μM due to carry over with the inoculum. The final crotonate concentration was 85 μM. Butyrate was not produced in detectable amounts (detection limit of about 0.5 μM) by the end of the experiment which indicates that crotonate did not act as a terminal electron acceptor in this experiment. At the end of the experiment, the $\Delta G'$ for benzoate fermentation (equation 3, table 3.1), and for hydrogen production from benzoate (equation1, table 1) was −15.4 and −3.3 kJ//mol, respectively.

The coculture of *S. aciditrophicus- M. hungatei* metabolized benzoate at a slightly faster rate (0.53 ± 0.13 day^{-1}) than *S. aciditrophicus* pure cultures (Fig. 3.1 b). The

Fig.3.1 Metabolism of benzoate by (A) pure cultures of *S. aciditrophicus* and (B) by cocultures of *S. aciditrophicus- M. hungatei* and metabolism of cyclohexane carboxylate by *S. aciditrophicus* pure cultures (C) and (D) by *S. aciditrophicus- M. hungatei* coculture. (-■-) Benzoate; (-●-) cyclohexane carboxylate; (-▲-) acetate; (-◆-) methane; (-□-) benzoate in autoclaved controls; (-O-) cyclohexane carboxylate in autoclaved controls; (-Δ-) acetate in autoclaved controls; and (-◊-) methane in autoclaved controls. Fig 1a (inset): Correlation between benzoate consumption and cyclohexane carboxylate production in *S. aciditrophicus* pure cultures.


(A) Benzoate
Acetate (mM)
Benzoate, cyclohexane carboxylate (mM)
Time (hrs)
cyclohexane carboxylate produced, mM
Benzoate consumed, mM
r² = 0.99
(C) Cyclohexane carboxylate
Cyclohexane carboxylate, acetate (mM)
Time (hrs)
Acetate (mM)
(B) Benzoate
Benzoate or cyclohexane carboxylate (mM)
Time (hrs)
Methane (µmol)
(D) Cyclohexane carboxylate
Cyclohexane carboxylate (mM)
Time (hrs)
Methane (µmol)

coculture produced 3.27 mol acetate and 0.84 mol methane per mol benzoate metabolized (Table 3.2). These numbers are consistent with the theoretical stoichiometry (equation 8, Table 3.1), as well as earlier reported values for benzoate degradation by *S. aciditrophicus* (8) and other syntrophic benzoate degraders (2, 19, 31) in coculture with hydrogen-using methanogens. Cyclohexane carboxylate transiently accumulated to a maximum concentration of 0.29 mM, representing 23% of the original benzoate concentration added.

Benzoate metabolism by pure cultures of *S. aciditrophicus* was accompanied by a net increase of 3.26 μg protein per μmol benzoate metabolized. Assuming that 47% of the cell dry-mass is protein (9), this indicates that *S. aciditrophicus* had a cell yield of about 6.9 g/mol of benzoate and hence the reaction would have produced about 0.66 mol ATP (net) per mol benzoate (assuming a Y_{ATP} value of 10.5 g biomass/ mol substrate). The coculture of *S. aciditrophicus-M. hungatei* had a cell yield of 11.8 g cells per mol of benzoate metabolized, indicating that about 1.1 mol ATP (net) is produced per mol benzoate metabolized. The benzoate fermentation activity by pure cultures of *S. aciditrophicus* could be repeatedly subcultured, with benzoate metabolism coupled to cyclohexane carboxylate production and an increase in protein concentration with every subculture (data not shown).

S. aciditrophicus metabolized cyclohexane carboxylate only in coculture with a hydrogen-using methanogen (Figure 3.1d). The coculture produced 3.27 mol acetate and 1.43 mol methane per mol cyclohexane carboxylate consumed. These values are consistent with the theoretical stoichiometry for cyclohexane carboxylate consumption by

Table 2: Stoichiometry of benzoate, cyclohex-1-enecarboxylate and cyclohexane carboxylate metabolism by *"S. aciditrophicus"* in pure culture and in coculture with *M. hungatei.*

Culture	Original substrate concentration (mM)	Final substrate concentration (μM)	Cyclohexane carboxylate (mM)	Initial acetate concentration (mM)	Final acetate concentration (mM)	Methane produced (μmol)	Final H_2 concentration (Pa)	Carbon recovery (%)[a]	Hydrogen recovery (%)[b]	$\Delta G'$ of hydrogen producing reaction (kJ/mol)	$\Delta G'$ of overall reaction (kJ/mol)
S. aciditrophicus with benzoate	1.58 ± 0.10	5.1 ± 1.1	0.84 ± 0.14	1.67 ± 0.22	3.95 ± 0.39	-	97.9 ± 7.2	96.8	98.9	-3.3	-15.4
S. aciditrophicus-M. hungatei with benzoate	1.25 ± 0.11	0.83 ± 0.13	-	2.18 ±0.23	6.27 ± 0.41	21.1	2.32 ± 0.7	109.3	109.8	-18.3	-35.6
S. aciditrophicus with cyclohexane carboxylate	1.65 ± 0.14	1510 ± 160	-	1.87 ± 0.06	2.19 ± 0.23	-	96.2 ± 10.2	ND[d]	ND[d]	ND[d]	ND[d]
S. aciditrophicus-M. hungatei with cyclohexane carboxylate	1.43 ± 0.05	BDL[c]	-	2.05 ± 0.18	6.73 ± 0.38	41.0	2.12 ± 0.5	105.6	104.7	-13.2	-44.4
S. aciditrophicus with cyclohex-1-ene carboxylate	1.58 ± 0.01	590 ± 20	1.01 ±0.22	1.95 ± 0.31	2.22 ± 0.18	-	839 ± 68.9	110.1	114.4	-4.7	-9.2
S. aciditrophicus-M. hungatei with cyclohex-1-ene carboxylate	1.71 ± 0.08	BDL[c]	-	1.82 ± 0.21	6.45 ± 0.78	44.3	2.6 ± 0.6	92.9	94.2	-54.4	-83.1

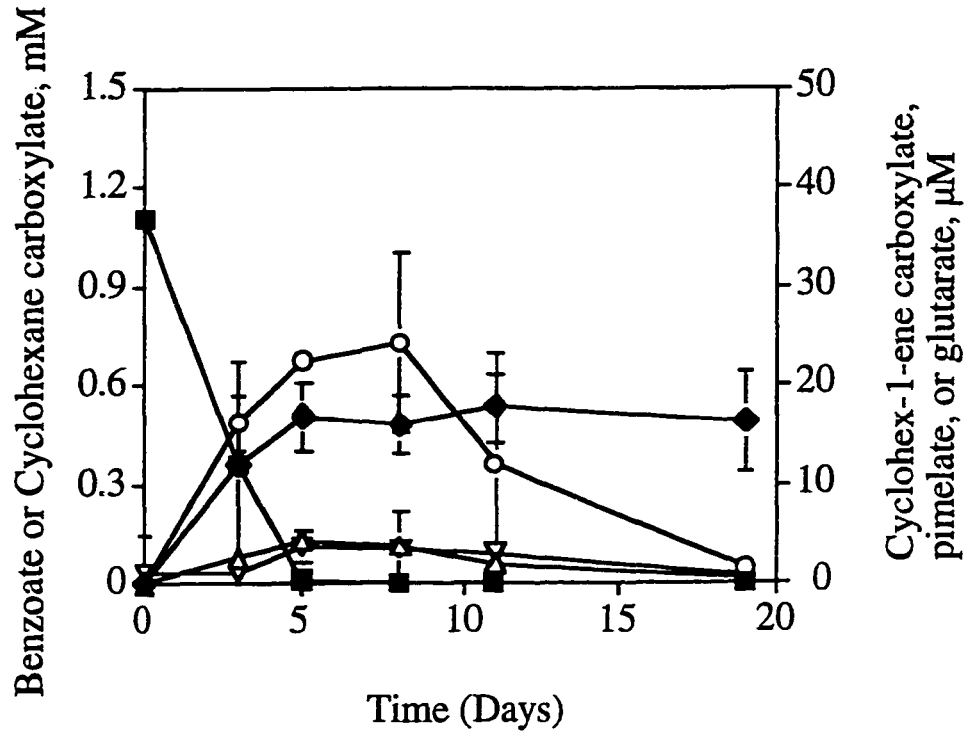
[a] Carbon recovery values were calculated excluding biomass. The concentration of HCO_3^- was not quantified and the bicarbonate concentrations were calculated assuming 1/3 mol HCO_3 was made for every acetate produced, corrected for the amount of HCO_3^- used to make CH_4 by the methanogenic cocultures.

[b] Hydrogen is 'available hydrogen' calculated by the method of Baker (5)

[c] Below detection limit. Detection limits were 5μM for cyclohexane carboxylate and 1.5 μM for cyclohex-1-ene carboxylate. In both cases, a value of 1 μM was assumed for residual $\Delta G'$ calculations.

[d] Not determined

Fig. 3.2 Production of cyclohexane carboxylate (-◆-), cyclohex-1-ene carboxylate (-O-), pimelate (-Δ-), and glutarate (-▽-) in *S. aciditrophicus* pure cultures grown with benzoate (-■-). Each compound was detected and quantified as its TMS derivative.


Benzoate or Cyclohexane carboxylate, mM
1.5
1.2
0.9
0.6
0.3
0
0
5
10
15
20
50
40
30
20
10
0
Cyclohex-1-ene carboxylate, pimelate, or glutarate, µM
Time (Days)

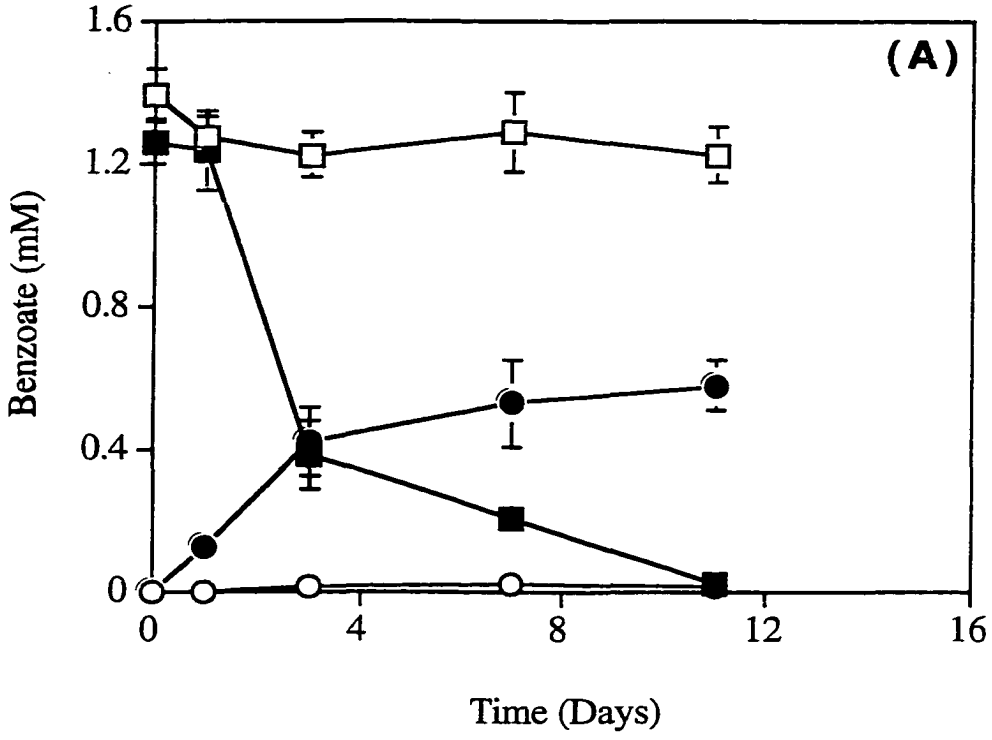
syntrophic cocultures of 3 mol acetate and 1.5 mol methane per mol cyclohexane carboxylate (equation 10, Table1).

GC-MS was used to detect and quantify metabolites produced during benzoate metabolism by pure cultures of *S. aciditrophicus*. Cyclohexane carboxylate accumulated to a final concentration of 0.53 mM, representing 48% of the original benzoate concentration added. Cyclohex-1-ene carboxylate, pimelate, and glutarate transiently accumulated to maximum concentrations of 24.2, 4.5, and 3.67 μM, respectively (Fig. 2).

Effect of hydrogen and acetate on benzoate metabolism by pure cultures of *S. aciditrophicus*. Experiments were conducted to evaluate the effect of hydrogen and acetate on benzoate metabolism by pure cultures of *S. aciditrophicus*. Hydrogen, at a concentration of approximately 10.1 kPa inhibited benzoate degradation (Fig 3a). Benzoate consumption was not inhibited in controls that received an equal amount of nitrogen. Benzoate metabolism in pure cultures of *S. aciditrophicus* that received 60 mM sodium acetate was inhibited, compared to controls that received 60 mM sodium chloride (Fig 3 b).

Metabolism of Cyclohex-1-ene carboxylate by pure cultures of *S.aciditrophicus*. We tested the ability of pure cultures of *S. aciditrophicus* to metabolize cyclohex-1-ene carboxylate. The theoretical stoichiometry for cyclohex-1-ene carboxylate fermentation indicated that 6 mol of cyclohex-1-ene carboxylate is metabolized to 3 mol of acetate, one mol of CO_2, and 5 mol of cyclohexane carboxylate (equation 6, Table 2). *S. aciditrophicus* in pure culture partially metabolized cyclohex-1-ene carboxylate with the concurrent production of cyclohexane carboxylate and small amounts of acetate. Only

Fig. 3.3 (A) Effect of hydrogen on benzoate metabolism by *S. aciditrophicus*. Benzoate utilization (-□-) and cyclohexane carboxylate production (-o-) in cultures receiving 10.1 kPa hydrogen. Benzoate production (-■-) and cyclohexane carboxylate production (-●-) in cultures receiving 10.1 kPa nitrogen. (B) Effect of acetate on benzoate metabolism by *S. aciditrophicus*. Benzoate utilization (-□-) and cyclohexane carboxylate production (-o-) in cultures receiving 60 mM sodium acetate. Benzoate production (-■-) and cyclohexane carboxylate production (-●-) in cultures receiving 60 mM sodium chloride.


(A)
1.6
1.2
0.8
0.4
0
Benzoate (mM)
0
4
8
12
16
Time (Days)

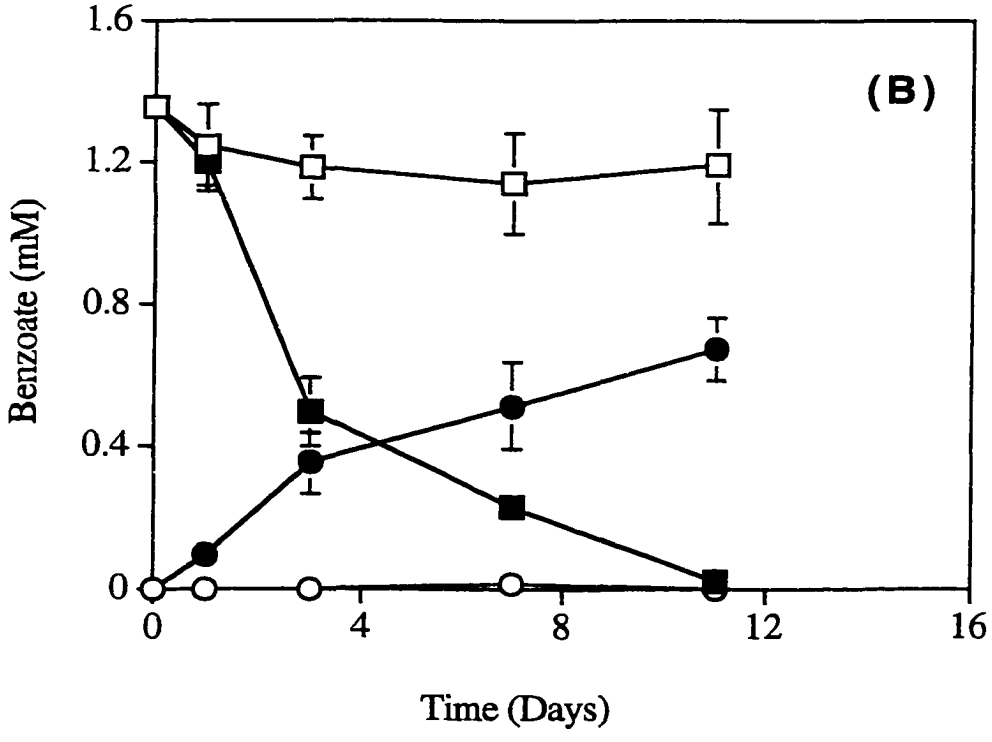
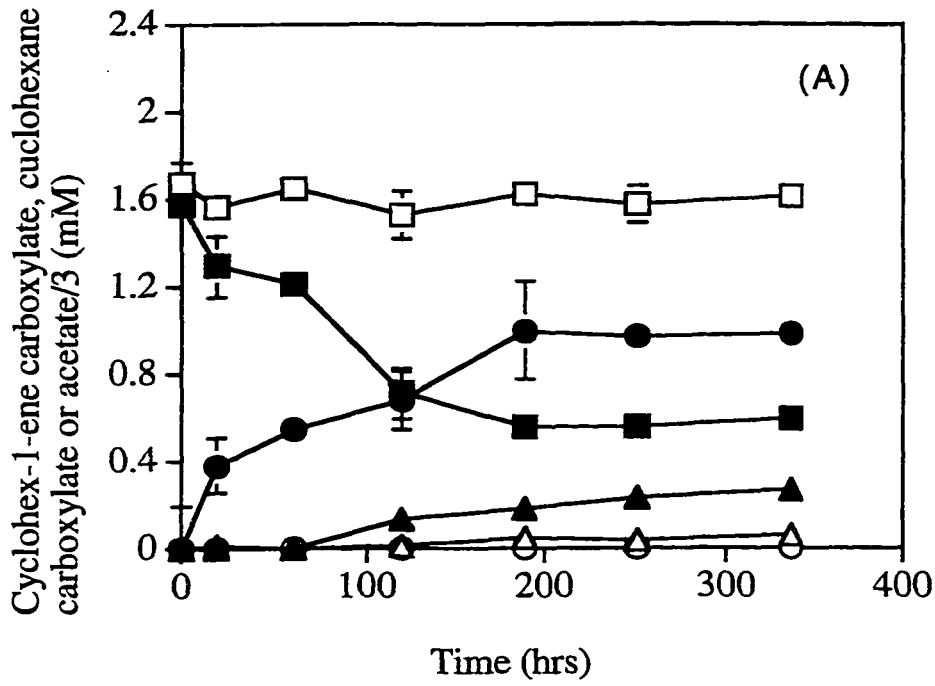
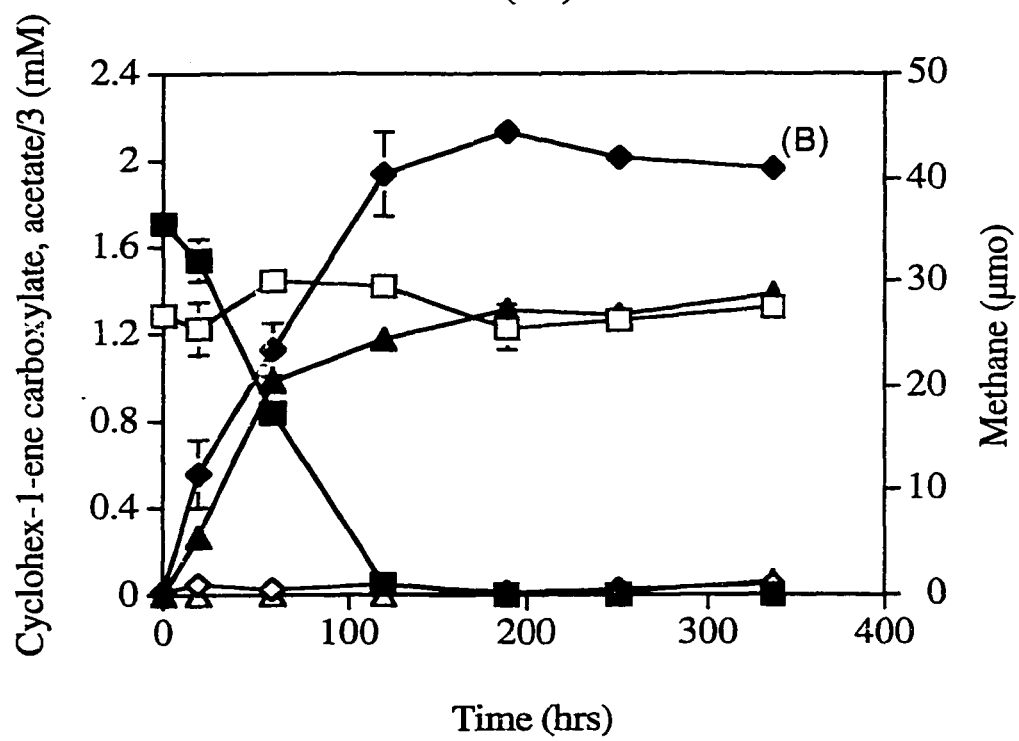

(B)
1.6
1.2
0.8
0.4
0
Benzoate (mM)
0
4
8
12
16
Time (Days)

Fig. 3.4 Metabolism of cyclohex-1-ene carboxylate by *S. aciditrophicus* pure cultures (A) and by *S. aciditrophicus- M. hungatei* cocultures (B). (-■-) Cyclohex-1-ene carboxylate; (-●-) cyclohexane carboxylate; (-▲-) acetate; (-◆-) methane; (-□-); cyclohex-1-ene carboxylate in autoclaved controls; (-O) cyclohexane carboxylate in autoclaved controls; (-Δ-) acetate in autoclaved controls; and (-◊-) methane in autoclaved controls.


(A)
Cyclohex-1-ene carboxylate, cuclohexane carboxylate or acetate/3 (mM)
2.4
2
1.6
1.2
0.8
0.4
0
0
100
200
300
400
Time (hrs)


(B)
Cyclohex-1-ene carboxylate, acetate/3 (mM)
Methane (µmo)
2.4
2
1.6
1.2
0.8
0.4
0
50
40
30
20
10
0
0
100
200
300
400
Time (hrs)

about 63% of the initial amount of substrate was metabolized with about one mol cyclohexane carboxylate and 0.3 mol acetate was produced per mol cyclohex-1-ene carboxylate by *S. aciditrophicus* pure cultures. Hydrogen levels in pure cultures of *S. aciditrophicus* grown with cyclohex-1-ene carboxylate cultures increased from 51.7 ± 1.6 Pa to 839 ± 68 Pa, a value which is about 320 times of that observed in *S. aciditrophicus- M. hungatei* co-cultures grown with cyclohex-1-ene carboxylate and 9 times that observed in *S. aciditrophicus* pure cultures grown with benzoate. The net change in free energy ($\Delta G'$) observed for cyclohex-1-ene carboxylate oxidation (equation 4, table 3.1) at the end of the experiment was – 4.7 kJ, while the $\Delta G'$ observed for the overall cyclohex-1-ene carboxylate fermentation reaction (equation 6, Table 3.1) was –9.2 kJ/mol. Cocultures of *S. aciditrophicus- M. hungatei* completely metabolized cyclohex-1-ene carboxylate with the production of 2.71 mol of acetate and 1.30 mol of methane per mol of cyclohex-1-ene carboxylate. These values are consistent with the theoretical stoichiometry for cyclohex-1-ene carboxylate metabolism by syntrophic cocultures (equation 9, Table 3.2).

DISCUSSION

S. aciditrophicus in pure culture metabolized benzoate to cyclohexane carboxylate, acetate, and CO_2 (equation 3, table 3.1). This reaction supported the growth of *S. aciditrophicus* as indicated by our ability to successfully transfer the activity as well as by the growth yield experiments which indicated that 6.9 g of cells (dry weight) were made per mol benzoate. The redox potential of the benzoate/cyclohexane carboxylate electron pair (-244 mV) makes benzoate reduction to cyclohexane carboxylate exoergenic if hydrogen (-410 mV), NADH (-320 mV), or FADH (-220 mV) are the electron donors.

The ability of various syntrophically oxidizing microorganisms to grow in pure cultures with unsaturated substrates via dismutation was first reported in 1986 when Beaty and McInerney (4) reported the ability of *S. wolfei* to grow in pure culture with crotonate. This ability to dismutate crotonate has been demonstrated for other microorganisms that syntrophically oxidize butyrate and benzoate (2, 19, 27, 28, 30), including *S. aciditrophicus*, as has the ability of fumarate dismutation to support anaerobes that syntrophically metabolize propionate (10, 30). This work adds benzoate to the short list of compounds such as crotonate, unsaturated short chain volatile fatty acids (1), pyruvate, fumarate (10, 30), acetoin, acetaldehyde (7), and acetylene (23) that are capable of supporting growth of microorganisms that conduct syntrophic metabolism in pure cultures. Unlike all other dismutation reactions previously described, benzoate dismutation reaction involves aromatic ring reduction rather than olefinic bond reduction as a mechanism for hydrogen disposal.

In absence of light or inorganic terminal electron acceptors, monoaromatic hydrocarbons are usually incompletely metabolized to acetate, HCO_3^-, and hydrogen (24). Thermodynamic considerations usually determine whether hydrogen removal, and hence interspecies hydrogen transfer, is needed for the degradation of a certain

substrate. However, by using part of the original substrate as the electron acceptor, anaerobic microorganisms can degrade some aromatic compounds where the degradation of these compounds to acetate, hydrogen, and HCO_3^- would be thermodynamically unfavorable. *Sporotomaculum hydroxybenzoicum* degrades 3-hydroxybenzoate in absence of H_2-using microorganisms (20) by using the crotonyl-CoA produced during substrate degradation as an electron acceptor, resulting in the production of butyrate, acetate, and HCO_3^- as end products. Karlsson et al. (16) recently described an enrichment that is capable of fermenting phenol by using the reducing equivalents to reduce phenol to benzoate via a reductive elimination reaction. Benzoate reduction to cyclohexane carboxylate is therefore another novel strategy allowing fermentation of monoaromatic hydrocarbons without the need for interspecies hydrogen transfer.

The ability of *S. aciditrophicus* to ferment benzoate may be of ecological significance since benzoate (or benzoyl-CoA) is a central intermediate in anaerobic degradation of many natural and xenobiotic aromatic compounds. The transient accumulation of cyclohexane carboxylate at high concentration (maximum concentration of 140 μM, representing 28% of the original substrate concentration) has been previously observed in methanogenic phenol enrichments from landfill sediments (R. Jones and J.M. Suflita, unpublished work). Also, Kleerebezem et al. (17) observed the accumulation of cyclohexane carboxylate in anaerobic sewage sludge enrichments amended with benzoate and either of the three phthalate isomers when methanogenesis in these enrichments was inhibited by bromoethanesulfonic acid (BESA). This observation implies that cyclohexane carboxylate production could be a mechanism for hydrogen removal in anaerobic ecosystems, which is especially important since high level of hydrogen is shown to inhibit benzoate metabolism by *S. aciditrophicus* in pure culture (this report) as well as in coculture (12). Benzoate reduction to cyclohexane carboxylate may be hence regarded as a

survival mechanism utilized by syntrophic microorganisms for hydrogen removal and transient energy production until the development of optimum coupling with a hydrogen-user occurs. Once optimum coupling is achieved, both the aromatic substrate(s) and the cyclohexane carboxylate produced initially could be metabolized via interspecies hydrogen transfer.

Pure cultures of *S. aciditrophicus* grown with benzoate accumulated hydrogen up to 97.9 Pa, as compared to a value of 2.3 Pa observed for *S. aciditrophicus- M. hungatei* cocultures. The higher hydrogen value observed for *S. aciditrophicus* pure culture is probably responsible for the higher final benzoate concentration observed in the pure culture as compared to the coculture (Table 2). Previous studies showed that hydrogen levels influence the extent of benzoate degradation in syntrophic cocultures, with benzoate metabolism ceasing at higher thresholds in cultures with higher hydrogen levels compared with those with lower hydrogen levels (11, 13, 31, 32). Thermodynamic inhibition by hydrogen was further confirmed by the fact that high levels of hydrogen inhibited benzoate fermentation by *S. aciditrophicus* pure cultures growing on benzoate (Fig 3.3a) in a manner similar to that observed in *S. aciditrophicus* cocultures with a hydrogen-user. This inhibition suggests that hydrogen is produced as a free intermediate during benzoate oxidation by *S. aciditrophicus* pure cultures and hence its accumulation will inhibit the benzoate oxidation reaction. Production and consumption of hydrogen during substrate degradation by a single microorganism has been previously suggested during lactate metabolism by *Desulfovibrio sp.* (21) However, if this is true, it is not clear why *"S. aciditrophicus"* was not able to utilize exogenously provided hydrogen to reduce benzoate to cyclohexane carboxylate, similar to the observation of Karlesson et al (16) that hydrogen (0.5 atm) addition to a fermentative phenol enrichment resulted in an increase in phenol reduction to benzoate and a decrease in phenol oxidation to butyrate and acetate. Another

possibility is that hydrogen, although produced as a fermentation product in small amounts during benzoate metabolism (Table 3.2), is not the electron donor for the benzoate reduction to cyclohexane carboxylate reaction. Therefore, while high levels of hydrogen will inhibit the forward oxidation reaction due to thermodynamic considerations (Fig 3.3a), exogenously added hydrogen is not utilized by *S. aciditrophicus* to reduce benzoate to cyclohexane carboxylate. It is not clear however how *S. aciditrophicus* regulates the electron flow towards ring reduction or hydrogen production when grown in pure culture with benzoate. Detailed studies of the nature of the ring-reducing enzyme(s) as well as the localization of hydrogenases is necessary to understand the exact mechanism of ring-reduction in *S. aciditrophicus* when grown in pure culture and coculture on benzoate.

S. aciditrophicus in pure culture was able to metabolize cyclohex-1-ene carboxylate. Since cyclohex-1-ene carboxylate is more reduced than benzoate, more electrons (reducing equivalents) are produced per mol cyclohex-1-ene carboxylate oxidized while fewer electrons are disposed per mol cyclohex-1-ene carboxylate reduced. Therefore, while only one mol of benzoate is reduced per mol of benzoate oxidized to acetate and CO_2, 5 mol of cyclohex-1-ene carboxylate are needed to account for all of the reducing equivalents produced during the oxidation of one mol of cyclohex-1-ene carboxylate to acetate and CO_2. Therefore, much less energy is available per mol of cyclohex-1-ene carboxylate to support growth. Cyclohex-1-ene carboxylate fermentation stopped after only 62.7% of the initial cyclohex-1-ene carboxylate was degraded. This may be due to the accumulation of high levels of hydrogen (839 Pa), which may inhibit further oxidation of cyclohex-1-ene carboxylate to acetate and CO_2, as indicated by the low net $\Delta G'$ value of – 4.7 kJ for this reaction (equation 4, Table 3.1). The net free energy changes ($\Delta G'$) of about –3 to– 5 kJ/mol observed for oxidation of benzoate and cyclohex-1-ene carboxylate to acetate, CO_2, and hydrogen (equations 1 and 4, Table 3.1) by pure cultures of *S.*

aciditrophicus as well as the net $\Delta G'$ for the overall reaction involved in benzoate and cyclohex-1-ene carboxylate fermentation (equations 3 and 6, Table 3.2) by pure cultures of *S. aciditrophicus* (-15.4 and –9.2 kJ/mol, respectively) observed in this report indicates that biological reactions can proceed to values close to thermodynamic equilibrium ($\Delta G'$ of 0 kJ) rather than the previously suggested value of –20 kJ (24).

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CHAPTER 4

Is interspecies hydrogen transfer needed for toluene degradation under sulfate-reducing conditions?

ABSTRACT

Sediments from a hydrocarbon-contaminated aquifer, where periodic shifts between sulfate reduction and methanogenesis occurred, were examined to determine whether the degradation of toluene under sulfate-reducing conditions depended on interspecies hydrogen transfer. Toluene degradation under sulfate-reducing conditions was inhibited by the addition of 5 mM sodium molybdate, but the activity was not restored upon the addition of an actively growing, hydrogen-using methanogen. Toluene degradation was not inhibited in microcosms where hydrogen levels were maintained at a level theoretically sufficient to inhibit toluene degradation if the process proceeded via interspecies hydrogen transfer. Finally, the addition of carbon monoxide, a potent inhibitor of hydrogenase activity inhibited hydrogen but not toluene consumption in sulfate-reducing microcosms. These results suggest that toluene is degraded directly by sulfate-reducing bacteria without the involvement of interspecies hydrogen transfer. The sequence of experiments used to reach this conclusion could be applied to determine the role of interspecies hydrogen transfer in the degradation of a variety of compounds in different environments or under different terminal electron-accepting conditions.

INTRODUCTION

The metabolic interactions among species involved in anaerobic hydrocarbon degradation in natural environments are not well understood. The involvement of metabolic interactions such as interspecies hydrogen transfer would make the anaerobic degradation of aromatic hydrocarbons sensitive to fluctuations in environmental conditions such as the dissolved hydrogen concentration. These fluctuations could affect the rate and extent of degradation of these compounds. Thus, a greater understanding of the involvement of interspecies interaction will provide useful insights to the operation of anaerobic bioreactors and in the design of bioremediation efforts.

In methanogenic environments, interspecies hydrogen transfer is required for the degradation of a wide range of organic compounds including alcohols, fatty acids, amino acids, organic acids such as lactate, and benzoate and other aromatic acids (1). Thus, it is likely that the degradation of aromatic hydrocarbons such as toluene also requires interspecies hydrogen transfer (2). However, it is not clear whether similar metabolic interactions are operative under other terminal electron-accepting conditions. Several pure cultures of anaerobic bacteria have been characterized that can mineralize aromatic hydrocarbons such as toluene coupled to the reduction of sulfate (equation 1, Table 4.1) (3,4) or other electron acceptors (5-7). Recently, Meckenstock (2) found that a newly isolated sulfate reducer, strain TRM1, and the iron reducer, *Geobacter metallireducens*, could degrade toluene in the absence of their respective electron acceptor when grown in syntrophic association with a hydrogen-using bacterium. This study entertains the possibility that toluene degradation in sulfate- or iron-reducing environments could proceed by a syntrophic association where the hydrogen produced by the toluene-degrading organism (equation 2, Table 4.1) is rapidly used by a hydrogen-using, sulfate

reducer (equation 3, Table 4.1). The combined reaction (equation 4, Table 4.1) is thermodynamically favorable since hydrogen levels are maintained at a very low level by the hydrogen-using bacterium. If acetate is consumed by another sulfate-reducing bacterium (equation 5, Table 4.1), the overall stoichiometry for toluene degradation by a sulfate-reducing consortium (equation 1, Table 4.1) would be indistinguishable from that catalyzed by known pure cultures.

In support of this concept, it is now known that some bacteria capable of growth by anaerobic respiration can grow in the absence of their electron acceptor in syntrophic associations with hydrogen-using bacteria. For example, some sulfate-reducing bacteria can grow with ethanol or lactate in the absence of sulfate in syntrophic association with hydrogen-using methanogens (8, 9). Wu et al. (10) suggested the involvement of sulfate-reducing bacteria in propionate and ethanol degradation in methanogenic granules treating brewery wastewater. The iron-reducing bacterium, *Geobacter sulfurreducens*, can metabolize acetate in the absence of iron in syntrophic association with *Wollinella succinogenes*, a hydrogen-using nitrate reducer (11). Conversely, *Syntrophobacter* species, which were once considered to be obligate syntrophic bacteria, can use sulfate as the electron acceptor for the oxidation of propionate and other organic acids (12,13). These studies show that many anaerobic bacteria have diverse modes of energy metabolism and that their function in natural ecosystems cannot be inferred based only on their affiliation with a group of organisms that have been historically classified as having one mode of energy metabolism such as sulfate reduction.

We tested whether toluene degradation under sulfate reducing conditions in a shallow aquifer contaminated with gas-condensate hydrocarbons involved interspecies hydrogen transfer. Geochemical and dissolved hydrogen measurements indicated that sulfate reduction and methanogenesis were the dominant terminal electron-accepting processes and that these two processes varied temporally and

spatially at the site (14). We hypothesized that the periodic shifts in the terminal electron-accepting processes may have established conditions where syntrophic populations involved in toluene degradation under methanogenic conditions would be able to couple toluene degradation with hydrogen-using sulfate reducers when sulfate was periodically replenished at this site. While the outcome of the experiments failed to support the hypothesis that toluene degradation under sulfate-reducing conditions involves interspecies hydrogen transfer, the protocol that we used to test this hypothesis will be useful to determine the role of interspecies hydrogen transfer in the degradation of compounds in natural ecosystems.

Table 4.1 Different reactions possibly involved in anaerobic toluene degradation under sulfate-reducing conditions [a]

Reaction	$\Delta G^{0\prime}$ (kJ/mol)
(1) $C_7H_8 + 4.5\ SO_4^{2-} + 3H_2O$ ------> $7\ HCO_3^- + 2.5\ H^+ + 4.5\ HS^-$	-205 kJ/mol
(2) $C_7H_8 + 9H_2O$ ------> $HCO_3^- + 3\ CH_3COO^- + 4\ H^+ + 6\ H_2$	+166 kJ/mol
(3) $1.5\ SO_4^{2-} + 1.5\ H^+ + 6H_2$ ------> $1.5\ HS^- + 6\ H_2O$	-227 kJ/mol
(4) $C_7H_8 + 3\ H_2O + 1.5\ SO_4^{2-}$ ------> $3\ CH_3COO^- + HCO_3^- + 2.5\ H^+ + 1.5HS^-$	-61 kJ/mol
(5) $3CH_3COO^- + 3\ SO_4^{2-}$ ------> $6\ HCO_3^- + 3\ HS^-$	-143 kJ/mol

[a] Values obtained from (7, 34) or calculated according to ΔG_f values from (19)

MATERIALS AND METHODS

Microorganisms and media. *Methanospirillum hungatei* strain JF1 and *Desulfovibrio* strain G11 were obtained from the culture collection of M.P. Bryant (Urbana, IL, USA). *Syntrophus acidotrophicus* strain SB, a syntrophic microorganism that utilizes benzoate in the presence of hydrogen-using bacteria, was isolated in our laboratory from a sewage treatment plant in Norman, OK (15). A basal medium (16) was used for growing the above strains with the omission of rumen fluid. *M. hungatei* was grown in basal medium with a headspace pressurized to 140 kPa by a gas mixture of 80% H_2:20% CO_2. *Desulfovibrio* strain G11 was grown similarly, but in a basal medium containing 15 mM sodium sulfate. Both microorganisms were grown in a shaking incubator (100 rpm). *S. aciditrophicus* was grown in pure culture in a basal medium with crotonic acid (40 mM) as a substrate and a headspace of 80% N_2:20% CO_2. Cocultures of *S. aciditrophicus* and *M. hungatei* were cultured in a basal medium containing 2.5 mM benzoate as a substrate and a headspace of 80% N_2:20% CO_2. All microorganisms were incubated at 37°C.

Sampling and microcosms preparation. Sediment samples were obtained from a gas condensate-contaminated site near Ft. Lupton, CO (14). Microcosms to study the factors affecting toluene degradation were prepared in an anaerobic glove box (17) by combining 25 g of sediment with 40 ml of basal medium containing 6 mM sodium sulfate in 120 ml serum bottles. The bottles were sealed with a composite of a butyl rubber stopper (Bellco CO, Vineland, NJ) with a layer of teflon fused to the bottom of the stopper and crimped with aluminum seals. After preparation, the headspace of each microcosm was exchanged with 80% N_2:20% CO_2 (70 kPa). Toluene (2 μL) (99.8%, Aldrich chemical Co, Milwaukee, WI) was then added as a

pure liquid using a glass syringe to a final aqueous concentration of approximately 0.3-0.4 mM. All microcosms were incubated at room temperature in the dark without shaking unless otherwise stated.

Experimental design. To determine whether electron flow during toluene degradation could be shifted from sulfate reduction to methanogenesis, fifteen microcosms with about 0.4 mM toluene and 6 mM sulfate were prepared as described above. The microcosms were incubated until toluene degradation and sulfate-consumption commenced. After 16 days, sodium molybdate was added from a 100 mM sterile anoxic stock solution to six of the microcosms to a final concentration of 5 mM to inhibit sulfate reduction. The other three microcosms received NaCl from a 100 mM sterile anoxic stock solution to a final concentration of 5 mM and served as a positive control for ionic strength. After 70 days of incubation, 5 ml of an actively-growing culture of *M. hungatei* strain JF1 (OD_{600} 0.37) was added to three of the microcosms that received sodium molybdate to determine if toluene degradation could be coupled to methanogenesis. After 295 days of incubation, two of the three microcosms that received *M. hungatei* each received 5 ml of a coculture of *S. aciditrophicus* and *M. hungatei* and enough sodium benzoate to give a final concentration of ≈ 0.5 mM. The headspaces of the later three microcosms were then evacuated and flushed with 80% N_2:20% CO_2 and the concentrations of benzoate and methane were monitored. This treatment was done to show that syntrophic benzoate degradation could be coupled to methanogenesis in these microcosms. In addition to the above microcosms, three microcosms did not receive toluene to correct for sulfate reduction coupled to endogenous electron donors. Another three microcosms with about 0.4 mM toluene and 6 mM sodium sulfate were autoclaved (20 minutes, 121^{0}C) and served as heat-killed controls.

As a positive control to show that syntrophic metabolism could be shifted from one terminal electron-accepting process (methanogenesis) to another (sulfate reduction), an experiment similar to that described above was conducted with butyrate as the electron donor. Fifteen microcosms with 5 mM sodium butyrate were prepared as described above. Three of these microcosms were autoclaved (20 minutes, 121^{0}C) and served as heat-killed controls. Three other microcosms were unamended with substrate to correct for methane production from endogenous electron donors. After 14 days of incubation, bromoethanesulfonic acid (BESA) was added from a 100 mM sterile, anoxic stock solution to a final concentration of 5 mM to each of six microcosms to inhibit methanogenesis. The other three microcosms each received sodium chloride to give a final concentration of 5 mM and served as positive controls. After 44 days of incubation, three of the microcosms that received BESA each received 5 ml of an actively-growing culture of *Desulfovibrio* strain G11 (OD_{600} of about 0.34) and sodium sulfate to give a final concentration of 6 mM.

Effect of hydrogen and carbon monoxide on toluene degradation. To test the effect of hydrogen on toluene degradation under sulfate-reducing conditions, nine microcosms with about 0.4 mM toluene and 5 mM ferrous sulfate heptahydrate ($FeSO_4.7H_2O$) were prepared as described above. $FeSO_4.7H_2O$ was used to prevent the accumulation of potential toxic levels of sulfide (18) due to stimulation of sulfate reduction by repeated hydrogen additions. Three of these microcosms were autoclaved (121^{0}C, 20 minutes) to serve as heat-killed controls. After 28 days of incubation, three microcosms each received hydrogen ($2x10^{-2}$ atm) and the hydrogen concentration was monitored daily. Microcosms were reamended with hydrogen whenever the partial pressure of hydrogen fell below 10^{-2} atm. This value is approximately 100 times the hydrogen partial pressure needed to theoretically inhibit hydrogen production as calculated according to Thauer et al. (19) given the

concentrations of reactants and products present in the microcosms and equation 1 (Table 1) for toluene degradation. The other three replicates received equal volumes of 100% N_2 gas instead of hydrogen each time hydrogen was added to the above microcosms. Each gas was added to the microcosms using sterile syringes and that had been flushed with the respective gas at least 10 times (17). Microcosms were incubated within a shaker (100 rpm) to allow free hydrogen diffusion to the liquid phase.

To test the effect of CO on toluene degradation under sulfate-reducing conditions, experiments were first conducted to determine the minimum concentration of CO that inhibits hydrogen uptake. Duplicate microcosms with about 1.5 to 2% headspace concentration of hydrogen and 5 mM sodium sulfate were each amended with 0 to 10% CO. A concentration of 10% CO in the headspace completely inhibited hydrogen uptake (Fig. 4B) and hence was used in toluene-amended microcosms. Microcosms were prepared as mentioned above with about 0.4 mM toluene and 5 mM sodium sulfate. Toluene was consumed after 68 days. At day 69, toluene (0.4 mM) and sulfate (5 mM) were amended to all microcosms. Three microcosms also received CO at a concentration of 10% while the three control microcosms received a similar volume of N_2 instead of CO.

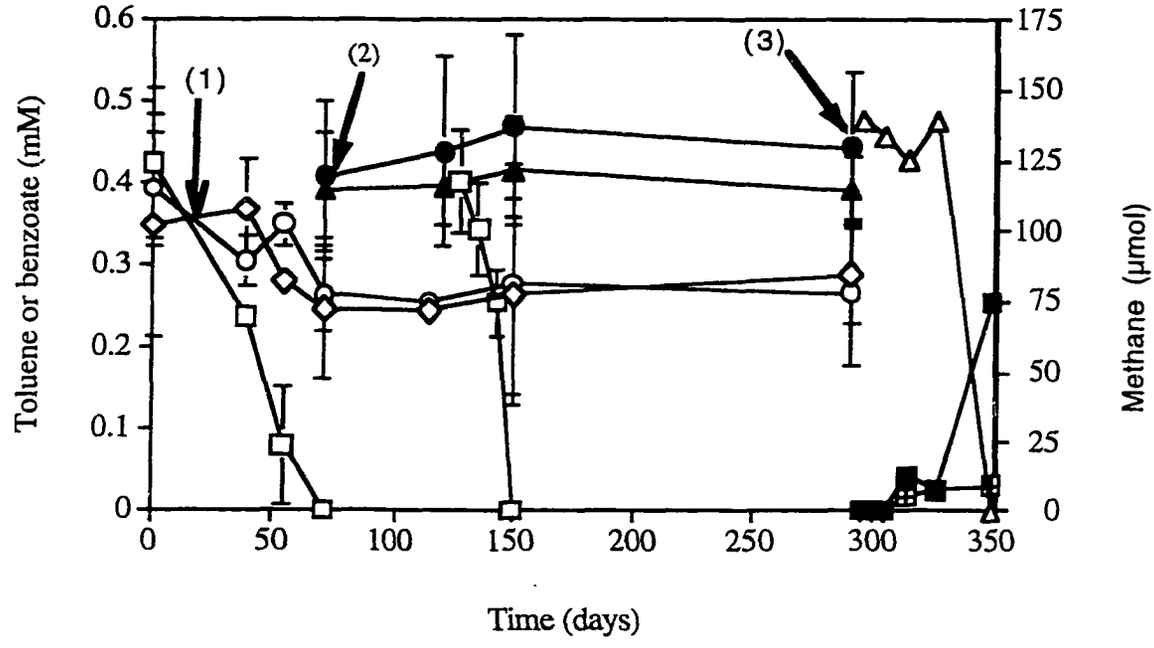
Analytical procedures. Toluene concentrations were determined by headspace analysis with a gas chromatograph (GC) equipped with a flame ionization detector and a 30 m Carbograph VOC capillary column (Alltech Inc., Deerfield, IL) held isothermally at 150^0 C. The carrier gas was helium at a flow rate of 16 ml/min. Hydrogen in the headspace was analyzed with a GC equipped with a mercury reduction vapor detector (14). Methane was also analyzed by GC (20) and sulfate was analyzed by ion chromatography (21). Benzoate concentrations were determined by high-performance liquid chromatography (HPLC) as described elsewhere (22).

RESULTS

Shifting of the terminal electron accepting process. We hypothesized that if toluene degradation under sulfate-reducing conditions was mediated by a syntrophic microorganism and a hydrogen-using sulfate-reducer, then toluene degradation would be restored in molybdate-inhibited, toluene-degrading enrichments by the addition of an actively-growing, hydrogen-using methanogen. The result of an experiment to test this hypothesis is shown in Figure 4.1. Microcosms that received toluene and sulfate but not molybdate completely degraded toluene to below the detection limit of our analysis after about 70 days of incubation. Toluene consumption occurred with concomitant consumption of nearly stoichiometric amounts of sulfate according to equation 1 (3.94 mols of sulfate per mol of toluene consumed, after correction for sulfate reduction in substrate-unamended microcosms, not shown). In contrast, sulfate reduction and toluene degradation were inhibited in the replicate microcosms that received 5 mM sodium molybdate at day 16. The addition of 5 ml of an actively growing culture of *M. hungatei* to three of the molybdate-inhibited microcosms at day 70 did not restore toluene degradation, even after prolonged incubations to 295 days (Fig. 4.1). The addition of 0.4 mM toluene at day 128 to microcosms that did not receive molybdate showed that these microcosms could still actively degrade toluene (Fig. 4.1)

Although molybdate is considered to be a specific inhibitor of sulfate-reduction (23), the concentration used in this experiment may have been high enough to inhibit microorganisms capable of syntrophic metabolism or the methanogens in the microcosms. To rule out this possibility, 5 ml of a syntrophic, methanogenic benzoate-degrading coculture of *S. aciditrophicus* and *M. hungatei* and 0.5 mM sodium benzoate (final concentration) were added, at day 295 to two of the microcosms that received molybdate and *M. hungatei*. In the two microcosms

Figure 4.1. Influence of the addition of a hydrogen-using methanogen (*M. hungatei*) on toluene degradation in molybdate-inhibited microcosms. Symbols: (-□-) Toluene utilization in microcosms amended with toluene, sulfate, and NaCl; (-o-) toluene utilization and methane production (black triangle) in microcosms amended with toluene, sulfate, and sodium molybdate; toluene utilization (-◊-) and methane production (-●-) in microcosms amended with toluene, sulfate, sodium molybdate, and *M. hungatei*; Benzoate utilization (-Δ-) and methane production (-■-) in duplicate microcosms receiving benzoate and the syntrophic coculture; and (cross) methane production in the microcosm that did not receive benzoate and the syntrophic coculture. Arrows: (1) addition of 5 mM sodium molybdate or sodium chloride; (2) Addition of 5 ml of *M. hungatei* to the three microcosms that received sodium molybdate; (3) Addition of 5 ml of a syntrophic, methanogenic benzoate-degrading coculture of *S acidotrophicus* and *M. hungetei* to two of the three microcosms that received sodium molybdate at day 16 and *M. hungatei* at day 70. Error bars represents ± standard deviation of triplicate microcosms.


0.6
0.5
0.4
0.3
0.2
0.1
0
Toluene or benzoate (mM)
175
150
125
100
75
50
25
0
Methane (µmol)
(1)
(2)
(3)
0
50
100
150
200
250
300
350
Time (days)

that received the syntrophic coculture, benzoate was consumed and methane was produced at levels far above those observed in the microcosms that did not receive benzoate and the syntrophic coculture (Fig. 4.1). These latter data suggest that neither syntrophic metabolism nor methanogenesis were likely to have been inhibited by the concentrations of sodium molybdate or toluene present in the microcosms, although it is possible that the toluene oxidizer in this system is sensitive to molybdate.

The ability of sediment syntrophic consortia to shift from one electron acceptor to another was confirmed in a separate experiment where methanogenic butyrate- degrading enrichments, which are dependent on interspecies hydrogen transfer, were used. The addition of 5 mM of BESA, a potent inhibitor of methanogenesis, after an initial 14 days of incubation inhibited butyrate degradation and methanogenesis (Fig. 4.2). Butyrate degradation coupled to sulfate reduction and acetate production was restored by the addition of 5 mM sulfate (final concentration) and 5 ml of a culture of *Desulfovibrio* strain G11 at day 44 (Fig. 4.2).

Effect of hydrogen on toluene degradation. In order for syntrophic toluene degradation to be thermodynamically favorable, the hydrogen partial pressure must be kept at a very low level (approximately10^{-5} atm). We maintained the hydrogen partial pressure at a value 100 times above the value theoretically needed to inhibit syntrophic toluene metabolism. The rate of toluene degradation in these hydrogen-amended microcosms was not different from that of control microcosms that received nitrogen (Fig. 4.3).

Effect of carbon monoxide on toluene degradation. Carbon monoxide is a potent inhibitor of the enzyme hydrogenase, which is required for interspecies hydrogen transfer. We compared toluene degradation in microcosms that received carbon monoxide to final concentration of 10% to controls that received an equal

volume of nitrogen gas. Figure 4.4 shows that toluene degradation occurred at similar rates regardless of whether CO was present or not.

Figure 4.2. Shifting butyrate degradation from methanogenesis to sulfate reduction. Symbols: (-□-) butyrate utilization in microcosms without inhibitor; butyrate utilization (-o-), acetate production (-♦-), and sulfate reduction (-■-) in microcosms receiving 5 mM BESA (after 14 days), sulfate and *Desulfovibrio* strain G11 (after 44 days); (-Δ-) sulfate-reduction in microcosms receiving 5 mM BESA and 5 mM sulfate but no butyrate; (-◊-) butyrate in heat-killed controls. Arrows: (1) addition of BESA at day 14; (2) addition of 5 ml of *Desulfovibrio sp.* and sulfate at day 44. Error bars represents ± standard deviation of triplicate microcosms.

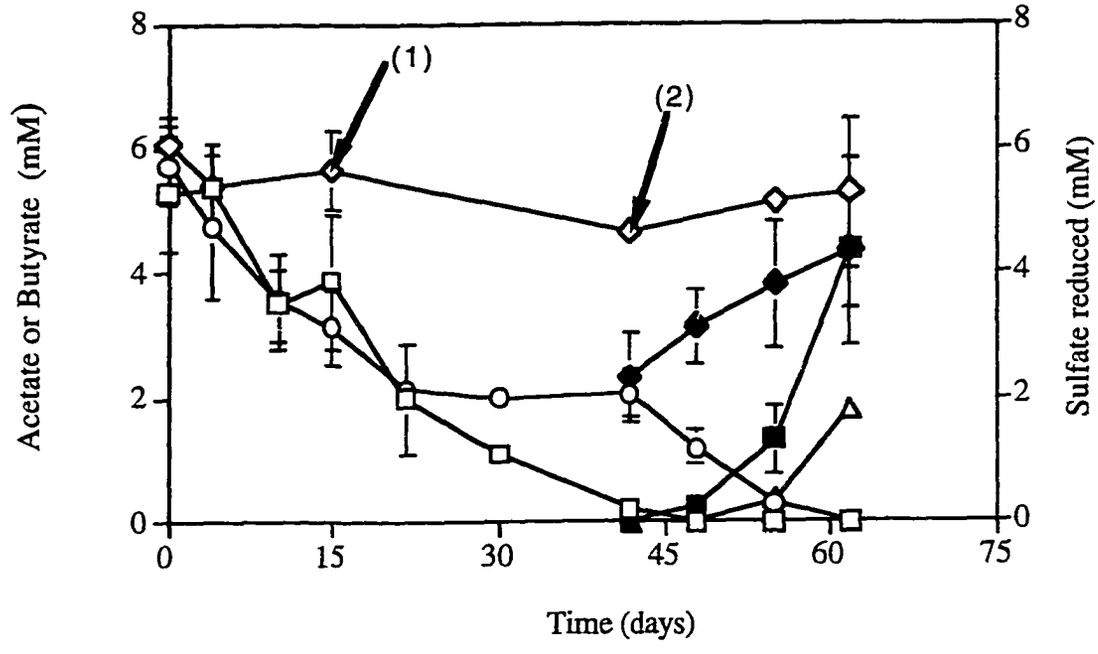

Acetate or Butyrate (mM)
Sulfate reduced (mM)
Time (days)
(1)
(2)
0
2
4
6
8
0
15
30
45
60
75

Figure 4.3. Effect of hydrogen on toluene metabolism under sulfate-reducing conditions. Symbols: (-□-) toluene plus nitrogen addition; (-Δ-) toluene plus hydrogen addition (-o-) toluene in autoclaved controls. Arrow indicates when hydrogen additions commenced. Error bars represents ± standard deviation of triplicate microcosms.

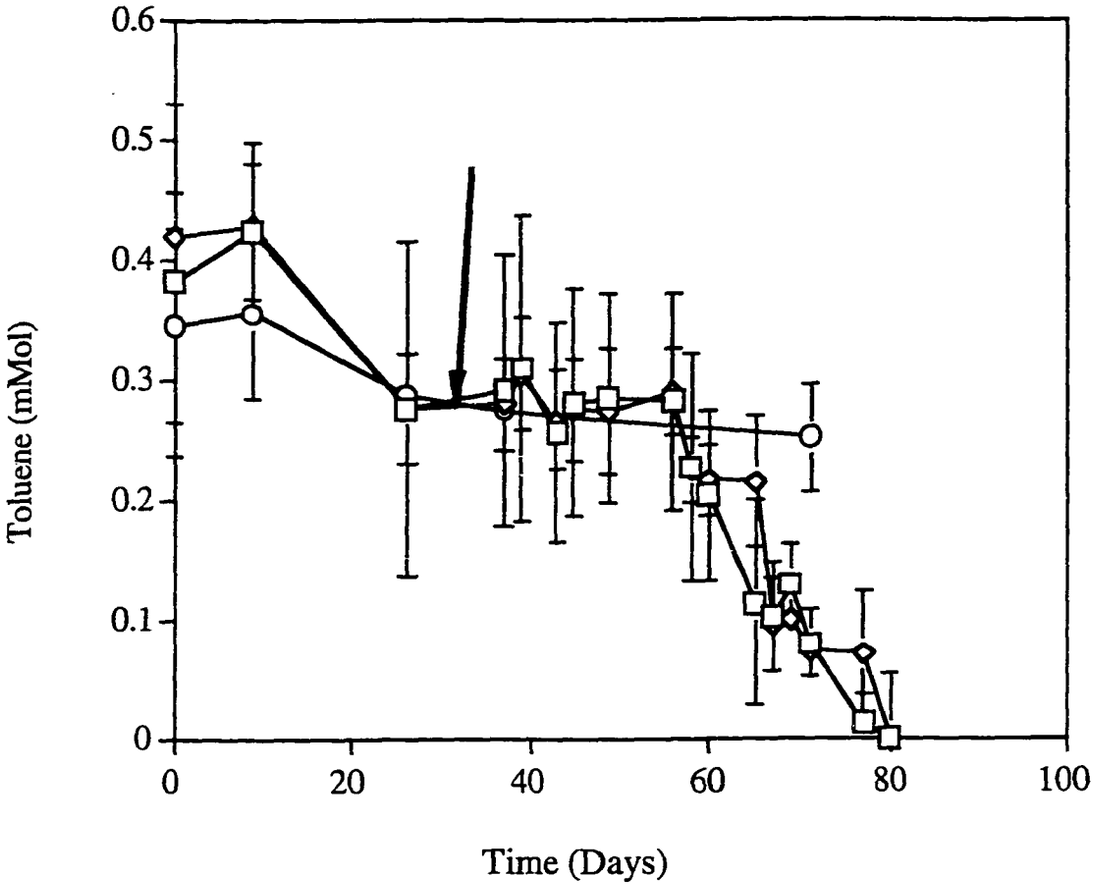
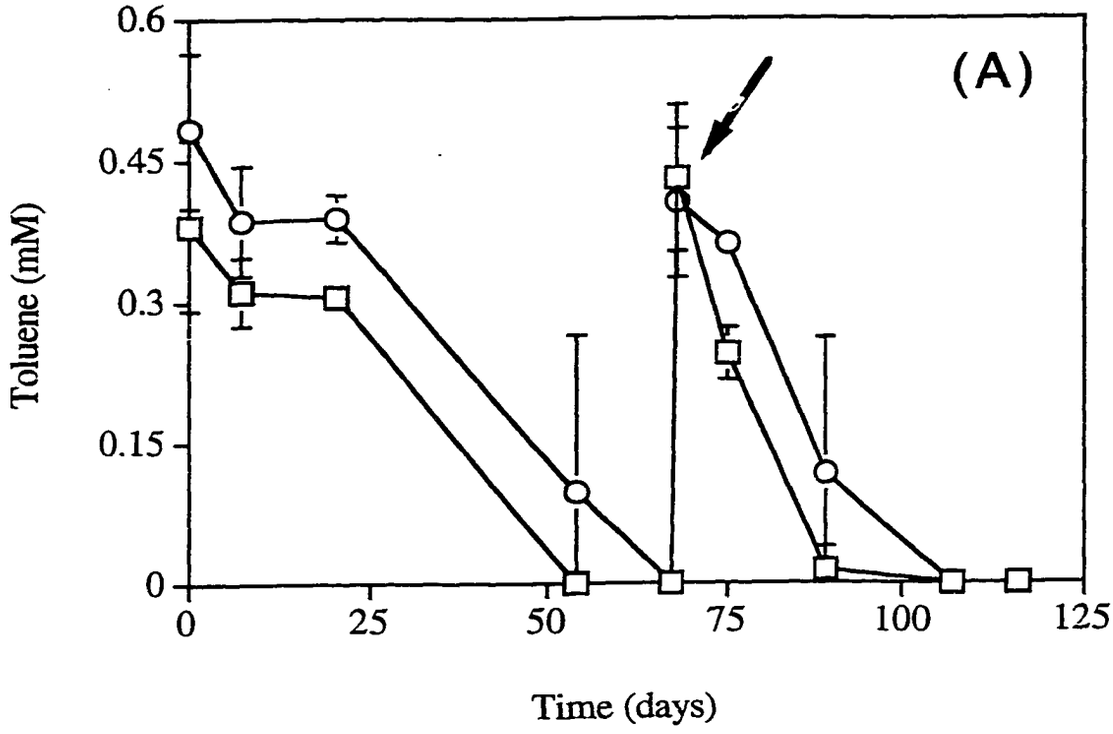
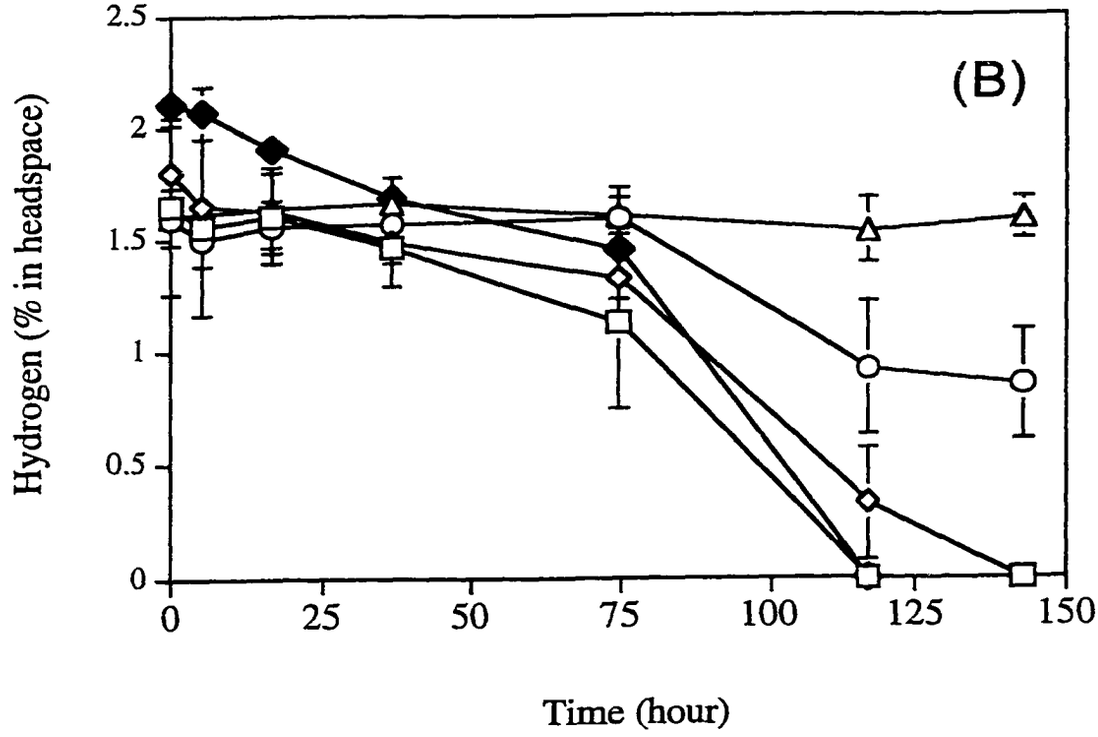

0.6
0.5
0.4
0.3
0.2
0.1
0
Toluene (mMol)
0
20
40
60
80
100
Time (Days)

Figure 4.4 (A) Effect of carbon monoxide on toluene degradation under sulfate-reducing conditions. Symbols: (-□-) toluene with nitrogen addition; (-o-) toluene with 10% Carbon monoxide. Arrow indicates where CO, N_2, toluene and sulfate were added to the microcosms.(B)Effect of various concentrations of carbon monoxide on hydrogen uptake in sulfate-reducing sediments. (-□-)0.1% CO; (-◊-) 1% CO; (-Δ-) 5 % CO; ,(-o-)10% CO; (-◆-) no carbon monoxide added. Error bars represents ± standard deviation of triplicate microcosms.


(A)
Toluene (mM)
0.6
0.45
0.3
0.15
0
0
25
50
75
100
125
Time (days)


(B)
Hydrogen (% in headspace)
2.5
2
1.5
1
0.5
0
0
25
50
75
100
125
150
Time (hour)

DISCUSSION

This work provides conclusive evidence that toluene degradation under sulfate-reducing conditions by bacteria derived from a hydrocarbon-contaminate aquifer does not depend on interspecies hydrogen transfer. However, we believe that the approach we used will be useful in determining the role of interspecies hydrogen transfer in the degradation of compounds in natural ecosystems. Our protocol for testing the role of syntrophic interactions in substrate degradation is based on fundamental characteristics of syntrophic metabolism such as independence of terminal electron-accepting reaction, sensitivity to high levels of hydrogen, and sensitivity to hydrogenase inhibitors.

First, by using an inhibitor of the terminal electron accepting process, we showed that toluene degradation directly depended on sulfate reduction. If sulfate reduction was needed only for hydrogen removal (i.e. if toluene degradation under sulfate-reducing conditions required interspecies hydrogen transfer), the addition of another hydrogen-user which is not inhibited by molybdate (e.g. a methanogen) should restore toluene degradation activity. To interpret this experiment correctly, we had to show that (a) molybdate did not inhibit methanogenic or syntrophic metabolic activities and that (b) sediment-associated syntrophic populations were capable of coupling with different terminal electron-accepting processes. This was done by showing that syntrophic benzoate degradation could occur in molybdate-inhibited microcosms and that syntrophic butyrate degradation could be coupled to methanogenesis or sulfate reduction. Our protocol is also based on the assumption that maintaining high hydrogen levels will inhibit substrate turnover in systems that depend on interspecies hydrogen transfer. We maintained hydrogen concentrations at levels much higher than needed to inhibit syntrophic metabolism but low enough to avoid stimulating hydrogen-utilizing microorganisms in the enrichments. This

necessitated the continuous monitoring and addition of hydrogen gas. A set of control microcosms that received only an inert gas (nitrogen) was needed to show that repeated additions of a gas did not inhibit substrate decay or stimulate aerobic metabolism by repeated addition of oxygen. It was also necessary to ensure that enough electron acceptor (sulfate in our case) was present since continuous hydrogen additions will consume sulfate and to prevent sulfide build-up, which can have inhibitory effects on microbial activities (18), by providing sulfate as $FeSO_4.7H_2O$. The presence of iron will remove any sulfide produced as ferrous sulfate precipitate.

It has been previously suggested that syntrophic and hydrogen-using microorganisms are closely positioned next to each other in flocs or microniches where hydrogen concentrations may not be in an equilibrium with the bulk aqueous hydrogen pool, i.e. the measured hydrogen concentrations (24). We used hydrogen concentrations that were roughly 100 times greater than that needed to inhibit syntrophic toluene degradation. Also, we used hydrogenase inhibitors to block hydrogen uptake and interspecies hydrogen-transfer. For valid interpretation of the results, controls should be run to ensure that the concentration of the inhibitor used blocks hydrogen uptake. The techniques used in all experiments described above are common to almost all anaerobic laboratories. Thus, this approach should be useful to determine the role of syntrophic metabolism in the degradation of a variety of compounds regardless of the ecosystem.

Few reports have examined the role of syntrophic metabolism in substrate turnover in the presence of sulfate. Lovley et al. (25) suggested that benzene degradation in marine sediments under sulfate-reducing conditions is mediated by a single microorganism rather than a microbial consortium. This conclusion was based on the inability to detect potential extracellular intermediates of benzene degradation (including acetate) in isotope trapping experiments utilizing

^{14}C benzene. It has been suggested that syntrophic propionate -degraders can effectively compete with propionate oxidizing sulfate-reducers, especially at lower concentrations of sulfate (12, 26). Schoelten & Stams (27) suggested that propionate and butyrate metabolism in freshwater sediments was mediated by sulfate-reducing bacteria. This conclusion was based on the observation that methane production was inhibited in propionate and butyrate-degrading enrichments by the addition of sulfate. However, it could be argued that the addition of sulfate allowed sulfate-reducing bacteria to outcompete methanogenic bacteria for hydrogen produced by syntrophic metabolism of these compounds. Balba & Nedwell (28) concluded that propionate and butyrate were degraded directly by sulfate-reducing bacteria in marine sediments, based on the observation that molybdate inhibited propionate and butyrate metabolism and that the addition of 80% hydrogen to the gas phase did not inhibit butyrate degradation. However, showing that molybdate inhibits substrate degradation is not sufficient in itself to conclude that syntrophic metabolism is not operative since the results will be similar. Also, the time span of such experiments must be long enough to determine if a shift of electron flow from sulfate-reduction to methanogenesis occurred. The use of high initial hydrogen concentrations to inhibit syntrophic metabolism is problematic since sulfate-reducing bacteria could consume all the hydrogen initially added and lower the hydrogen concentrations to levels low enough to allow interspecies hydrogen transfer to proceed. This may also deplete sulfate pools and lead to the potentially inhibitory concentrations of sulfide.

The identification of microorganisms by molecular techniques such as phospholipid fatty acid analysis, denaturing gradient gel electrophoresis (DGGE) analysis, or direct cloning and sequencing of 16s RNA genes of organisms has greatly added to our understanding of the microbial community structures in many environments (29-31). Although the benefits of these techniques are indisputable,

they do not provide direct information on the types of compounds that these organisms degrade. This information is usually inferred from the known physiological traits for organisms that have culturable representatives. However for organisms with diverse modes of metabolism such as the sulfate-reducing bacteria, the assignment of a physiological function based on phylogenetic identification is extremely difficult. Also, organisms with a close phylogenetic (e.g 16s RNA gene sequence similarity values of up to 98.7%) can have different substrate utilization patterns. (32, 33). The application of our approach together with molecular analysis would help clarifying the physiological roles of different groups of microorganisms in natural ecosystems.

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