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NOVEL ASPECTS OF BENZOATE AND CROTONATE METABOLISM BY THE STRICTLY ANAEROBIC BACTERIUM SYNTROPHUS ACIDITROPHICUS STRAIN SB.

A DISSERTATION

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Degree of

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By

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Novel aspects of benzoate and crotonate metabolism by the strictly anaerobic bacterium *Syntrophus aciditrophicus* strain SB.

A DISSERTATION APPROVED FOR THE
DEPARTMENT OF BOTANY AND MICROBIOLOGY

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amino acid sequences of Na^+-specific F_1F_0-ATPase of *Propionigenium modestum* and *Fusobacterium nucleatum*.

PREFACE

The main goal of this research is to investigate benzoate metabolism by the strictly anaerobic bacterium, *Syntrophus aciditrophicus*. In anaerobic ecosystems, a wide variety of aromatic compounds converge to benzoyl-CoA prior to ring reduction and cleavage. Therefore, metabolism of benzoyl-CoA is of great importance in the global carbon cycle. Although the study of the benzoyl-CoA pathway has been well described for facultative anaerobes, benzoate metabolism by strict anaerobes remains unclear.

In chapter 1, the degradation pathway of crotonate by the strictly anaerobic *S. aciditrophicus* was investigated by identifying the metabolites formed during growth on crotonate. The quantification of metabolites by high pressure liquid chromatography and the use of ^{13}C-labeled substrates coupled to a combination of GC-MS and ^{13}C-NMR as analytical tools identified a number of metabolites that are also found in the benzoate degradation pathway. Cyclohexane carboxylate, acetate, and traces of benzoate were formed from crotonate fermentation. Results indicated that cyclohexane carboxylate formation involved the condensation of two-carbon units rather than the direct use of the four-carbon skeleton of crotonate. Dr. Mark A. Nanny performed ^{13}C-NMR studies. This work is published in the journal "Applied and Environmental Microbiology".

Chapter 2 describes a new fate for benzoate metabolism, the use benzoate as an electron acceptor by *S. aciditrophicus*. Depending on growth conditions, *S.*

aciditrophicus has the ability to respire, ferment or syntrophically metabolize benzoate. An increase in cell yield occurred when benzoate was used as an electron acceptor in the presence of crotonate as an electron donor, suggesting that the organism receives energy value from the process. When crotonate to benzoate ratio decreased to less than one, *S. aciditrophicus* shifts its physiology to the fermentation of each substrate. From a practical standpoint, the addition of crotonate as a co-substrate might facilitate anaerobic degradation of aromatic compounds. Dr. Mark A. Nanny performed ^{13}C-NMR studies. This chapter was written according to the guidelines recommended by the journal "Applied and Environmental Microbiology".

In chapter 3, monosubstituted benzoate analogs were used to provide information on the initial steps of benzoate metabolism in the strictly anaerobic *S. aciditrophicus.* 2-Hydroxybenzoate and 3-fluorobenzoate were both transformed by *S. aciditrophicus* in the presence of crotonate. In both cases, benzoate transiently accumulated indicating a reductive elimination of the substituent. Moreover, in the presence of 3-fluorobenzoate, a 3-fluorocyclohex-diene carboxylate was detected as metabolite, indicating that the anaerobic benzoyl-CoA reduction is similar in strict and facultative anaerobes and involves a two-electron reduction step leading to the formation of a diene intermediate. This chapter was written according to the style recommended for submission to "Applied and Environmental Microbiology" journal.

Chapter 4 described the proteomic analyses of *S. aciditrophicus* cultures grown with crotonate or crotonate and benzoate. A total of 356 gene products were identified belonging to all functional groups with 25 being differentially abundant

between growth with crotonate or crotonate and benzoate. Among them, proteins involved in the early steps of benzoate metabolism were identified including benzoyl-CoA ligases (two were identified) and a putative novel benzoyl-CoA reductase annotated as tungsten-containing aldehyde: ferredoxin oxidoreductase that is highly similar than the predicted one identified in *G.metallireducens*. Furthermore, gene products with high similarities to proteins involved in benzoyl-CoA metabolism of *T. aromatica* and *Azoarcus* sp. were identified, including the cyclohex-1,5-diene carboxyl-CoA hydratase. Proteins involved in energy conservation and electron transport were identified, bringing new insights into the function of a microorganism leaving in strict energy constraints. The work in this chapter was done in collaboration with Dr. Robert Gunsalus, Dr. Rachel R. Ogorzalek Loo, and Dr. Yanan Yang. The 2D-gel electrophoresis and LC/MS/MS analyses were performed by Dr. R. Ogorzalek Loo, and Dr. Y. Yang. This chapter was written according to the style recommended for submission to "Journal of Bacteriology" journal.

The work presented in Appendix 1 to 3 described my early project in MEOR, that describes the use of microbes in the enhancement of oil recovery. Appendix 1 reports the effectiveness of *Bacillus* strain JF-2 and its biosurfactant for oil mobilization from sand packed columns. Appendix 2 reports the growth and biosurfactant synthesis of *Bacillus* strain JF-2 in anoxic, sand packed columns. In appendix 3 the competition and the ability to produce biosurfactant anaerobically by *Bacillus* strain JF-2 in the presence a natural population was tested. I provide evidence that the addition of glucose, high levels of nitrate and proteose peptone

enhanced the growth and biosurfactant production of strain JF-2. This is important because it shows that we can stimulate the growth and activity of *Bacillus* stain JF-2 in oil reservoirs. Dot blot analysis was performed by Dr. S. O. Han. The work was done in collaboration with Dr. R. Knapp and was reported to the Department of Energy as required by the contract.

ABSTRACT

In anoxic environments, many aromatic compounds are transformed to benzoyl-CoA, prior to reductive dearomatization and ring cleavage. Due to the resonance stability of the aromatic ring, aromatic compounds are difficult to degrade in the absence of oxygen. Benzoate degradation in methanogenic ecosystems is mediated by syntrophic association of a fermentative bacterium coupled to a hydrogen/formate-using methanogen. Three *Syntrophus* species are known to syntrophically metabolize benzoate in coculture and to grow in pure culture with crotonate.

The metabolism of crotonate in the strictly anaerobic bacteria *Syntrophus aciditrophicus* was studied. *S. aciditrophicus* produced 1.4 ± 0.24 moles of acetate and 0.16 ± 0.02 moles of cyclohexane carboxylate per mole of crotonate degraded. [U-^{13}C] Crotonate was metabolized to [1, 2-^{13}C] acetate and [1, 2, 3, 4, 5, 7-^{13}C] cyclohexane carboxylate. Cultures grown with unlabeled crotonate and [^{13}C] sodium bicarbonate formed [6-^{13}C] cyclohexane carboxylate. Cyclohex-1-ene carboxylate, benzoate, pimelate, glutarate, 3-hydroxybutyrate, and acetoacetate were detected as intermediates. These are the same intermediates as that detected during syntrophic or fermentative benzoate metabolism by *S. aciditrophicus.* When *S. aciditrophicus* was grown with [1, 2-^{13}C] acetate and unlabeled crotonate, the m/z-15 of TMS-derivatized 3-hydroxybutyrate, acetoacetate, and glutarate each increased by +0, +2, and +4 mass units, and the m/z-15 of TMS-derivatized pimelate, cyclohex-1-ene carboxylate,

benzoate, cyclohexane carboxylate, and 2-hydroxycyclohexane carboxylate each increased by +0, +2, +4 and +6 mass units. The data are consistent with a pathway for cyclohexane carboxylate formation involving the condensation of two-carbon units derived from crotonate degradation with CO_2 addition, rather than the use of the intact four-carbon skeleton of crotonate.

S. aciditrophicus was shown to ferment benzoate to acetate and cyclohexane carboxylate via a dismutation process where reducing equivalents produced during benzoate oxidation to acetate and CO_2 are used to reduce benzoate to cyclohexane carboxylate. The ability to ferment benzoate suggests that benzoate could serve as an electron acceptor if a suitable electron donor was present. To test whether benzoate can be respired, *S. aciditrophicus* was grown with crotonate and benzoate. Benzoate was stoichiometrically reduced to cyclohexane carboxylate while crotonate was oxidized to acetate. Cultures grown with [ring-^{13}C]-benzoate and unlabeled crotonate formed ring-labeled ^{13}C-cyclohexane carboxylate. No [^{13}C]-labeled acetate was detected. The molar growth yield was 22.7 ± 2.1 g (dry wt) cell per mol of crotonate compared to 14.0 ± 0.1g per mol of crotonate when crotonate was used as a sole substrate. Furthermore, benzoate fermentation occurred only if traces amounts of crotonate were present.

Phototrophic and denitrifying bacteria couple the hydrolysis of two ATP molecules to reduce benzoyl-CoA to cyclohex-1,5-diene carboxyl-CoA. The use of such an energy intensive reaction by fermentative bacteria such as *S. aciditrophicus* has been questioned since it is not clear how net ATP synthesis would occur. Rather,

a four- or six-electron reduction which thermodynamic calculations indicate is exergonic under standard conditions , or hydroxylation of the ring prior to its reduction have been proposed.

Fluorobenzoates and hydroxybenzoates were tested as substrates for *S. aciditrophicus* in order to detect potential intermediates of interest. The utilization of 3-fluorobenzoate allowed the detection of a metabolite, which had a mass ion increase of 2 units greater than the parent compound, or 3 or 4 units greater than the parent compound when deuterated water was used. These results were consistent with the formation of a fluorinated diene intermediate. The transient accumulation of benzoate when 2-hydroxybenzoate was the substrate showed that hydroxylation of the ring was not required for ring reduction. The metabolites detected with fluoro- and hydroxy-benzoates are consistent with the hypothesis that benzoyl-CoA reduction involves a two-electron reduction forming a diene intermediate, rather than a four- or six-electron reduction.

Proteomic analysis of *S. aciditrophicus* grown with crotonate or crotonate and benzoate allowed the identification of gene products involved in benzoate metabolism. Two benzoyl-CoA ligases and a possible novel benzoyl-CoA reductase, a tungsten/molybdenum-containing aldehyde ferredoxin oxidoreductase associated with heterodisulfide reductase components similar to the benzoate-induced proteins found in *G. metallireducens* were identified. Cyclohex-1,5-diene carboxyl-CoA hydratase and the enzymes needed to form 3-hydroxypimelyl-CoA from the diene were also detected. The detection of subunits of ATP synthase, cytoplasmic and

periplasmic formate dehydrogenases, a sodium-translocating glutaconyl-CoA, and sodium-driven membrane-bound NADH:ferredoxin oxidoreductase indicates that *S. aciditrophicus* has the potential to create and use both sodium and proton gradients. ATP synthesis from acetyl-CoA appears to occur by an archaeal-like acetyl-CoA synthetase (ADP-forming) rather than the typical bacterial phosphotransacetylase-acetate kinase system.

Although enzyme activities and metabolites detected in *S. aciditrophicus* indicated that benzoate was degraded by the pathway found in *Rhodopseudomonas palustris,* proteomic data detected the gene products homologous to that found in *Thauera aromatica*. Such observation supports the hypothesis of two routes for benzoate degradation exist in *S. aciditrophicus,* one involved in benzoate degradation to acetate and CO_2 and the other involved in cyclohexane carboxylate formation from benzoate.

CHAPTER 1

Cyclohexane carboxylate and benzoate formation from crotonate in *Syntrophus aciditrophicus*

ABSTRACT

The anaerobic, syntrophic bacterium, *Syntrophus aciditrophicus,* grown in pure culture, produced 1.4 ± 0.24 moles of acetate and 0.16 ± 0.02 moles of cyclohexane carboxylate per mole of crotonate metabolized. [U-^{13}C] Crotonate was metabolized to [1, 2-^{13}C] acetate and [1, 2, 3, 4, 5, 7-^{13}C] cyclohexane carboxylate. Cultures grown with unlabeled crotonate and [^{13}C] sodium bicarbonate formed [6-^{13}C] cyclohexane carboxylate. Trimethylsilyl (TMS) derivatives of cyclohexane carboxylate, cyclohex-1-ene carboxylate, benzoate, pimelate, glutarate, 3-hydroxybutyrate, and acetoacetate were detected as intermediates by comparison of retention times and mass spectral profiles to authentic standards. With [U-^{13}C] crotonate, the m/z-15 of TMS-derivatized glutarate, 3-hydroxybutyrate, and acetoacetate each increased by +4 mass units, and the m/z-15 of TMS-derivatized pimelate, cyclohex-1-ene carboxylate, benzoate, and cyclohexane carboxylate each increased by +6 mass units. With [^{13}C] sodium bicarbonate and unlabeled crotonate, the m/z-15 of TMS-derivatives of glutarate, pimelate, cyclohex-1-ene carboxylate,

benzoate, and cyclohexane carboxylate each increased by +1 mass unit, suggesting that carboxylation occurred after the synthesis of a four-carbon intermediate. With [1, 2-^{13}C] acetate and unlabeled crotonate, the m/z-15 of TMS-derivatized 3-hydroxybutyrate, acetoacetate, and glutarate each increased by +0, +2, and +4 mass units, and the m/z-15 of TMS-derivatized pimelate, cyclohex-1-ene carboxylate, benzoate, cyclohexane carboxylate, and 2-hydroxycyclohexane carboxylate each increased by +0, +2, +4 and +6 mass units. The data are consistent with a pathway for cyclohexane carboxylate formation involving the condensation of two-carbon units derived from crotonate degradation with CO_2 addition, rather than the use of the intact four-carbon skeleton of crotonate.

Key words: syntrophy, cyclohexane carboxylate, crotonate, methanogenesis, alicyclic ring

INTRODUCTION

Syntrophus aciditrophicus strain SB is a gram-negative bacterium that belongs to the δ-subdivision of the Proteobacteria (21). *S. aciditrophicus* metabolizes various saturated and unsaturated fatty acids, methyl esters of butyrate and hexanoate, and benzoate in coculture with hydrogen/formate-using microorganisms. The anaerobic degradation of saturated fatty acids and aromatic acids in the absence of terminal electron processes necessitates the presence of a hydrogen-consuming organism to maintain a hydrogen partial pressure low enough for these reactions to be thermodynamically favorable (9, 28, 29, 34). This metabolic interdependence has made the study of the syntrophic microorganisms difficult. However, a number of fatty acid- and aromatic acid-degrading syntrophic bacteria grow in pure culture by fermenting crotonate (6, 24, 46, 48). More recently, *S. aciditrophicus* was shown to grow in pure culture by benzoate fermentation (15).

Crotonate was shown to be dismutated to acetate and butyrate in some clostridia species (2, 42), *Eubacterium oxidoreducens* (23), *Ilyobacter polytropus* (38), and other syntrophic microorganisms such as *Syntrophomonas wolfei* (6), and *Syntrophus buswellii* (46). Jackson et al. (21) reported that crotonate-grown pure cultures of *S. aciditrophicus* produced acetate, butyrate, caproate and hydrogen. However, recently, no butyrate or caproate were detected as end products of crotonate fermentation by gas chromatography or high-pressure liquid chromatography.

In this paper, we report the results of gas chromatography-mass spectrometry and ^{13}C nuclear magnetic resonance to elucidate the pathway for crotonate oxidation and cyclohexane carboxylate formation in *S. aciditrophicus*.

MATERIALS AND METHODS

Media and cultivation conditions.

Pure cultures of *Syntrophus aciditrophicus* strain SB (ATCC 700169) were maintained in the basal medium described by McInerney et al. (27) without rumen fluid and with 20 mM of crotonate (6, 14). The media and stock solutions were prepared according to the anaerobic techniques described by Balch and Wolfe (3). The headspace was pressurized to 172 kPa with a mixture of N_2/CO_2 (80:20 v/v) and the cultures were incubated at 37°C without shaking. Cells were harvested at mid-log phase of growth by centrifugation (14,300 × *g*, 20min, 4°C). The cell pellet was washed twice with 50 mM phosphate buffer (pH 7.2) with resazurin (10 mg/l) and cysteine·HCl (0.5 g/l) and Na_2S (0.5 g/l). The final pellet was resuspended in basal medium without rumen fluid lacking substrate and was used as the inoculum for the different experiments. The culture purity was checked daily by microscopic examination and inoculation of a thioglycolate medium known not to support *S. aciditrophicus* growth.

The molar growth yield was determined in triplicate 50-ml serum bottles containing basal medium without rumen fluid supplemented with 20 mM crotonate. One-ml samples were taken immediately after inoculation and at mid-log phase of growth to determine the protein concentration. One-ml samples were taken daily from the same cultures to measure growth by monitoring change in optical density at 600

nm (27) and to measure substrate depletion and product formation to determine the mass balance.

To determine the end products of crotonate fermentation, duplicate 500-ml cultures of *S. aciditrophicus* were grown with 20 mM crotonate. Samples were taken immediately after inoculation and at mid-log phase for gas chromatography-mass spectroscopy (GC-MS) analysis. A heat-killed control and a substrate-unamended control were also analyzed. To confirm that the metabolites were made from crotonate, *S. aciditrophicus* was grown in 150-ml cultures containing 5.2 mM [U-^{13}C] crotonate or unlabeled crotonate. Samples (75 ml) were taken initially and when 94% of the original substrate was depleted. A substrate unamended culture and a 100-ml heat-killed culture containing 3.5 mM [U-^{13}C] crotonate were included as negative controls.

To study the formation of cyclohexane carboxylate, a 300-ml culture of *S. aciditrophicus* was grown with 3.8 mM [U-^{13}C] crotonate and 3.8 mM crotonate. Samples (75 ml) for GC-MS and NMR analyses were taken at three time points, immediately after inoculation, when 80% of the initial substrate was used, and when 99% of the initial substrate was used. In another experiment, *S. aciditrophicus* was grown with 20 mM crotonate and 40 mM unlabeled sodium acetate or 40 mM [1, 2-^{13}C] acetate. Triplicate 50-ml cultures in serum bottles with unlabeled sodium acetate and crotonate were used to test for the effect of acetate addition on growth. To detect intermediates and study the labeling pattern of cyclohexane carboxylate, 300-ml cultures were used. One set of cultures contained 20 mM crotonate with 40 mM

sodium acetate and another contained 20 mM crotonate and 40 mM [1, 2-^{13}C] acetate. A 100-ml heat-killed culture containing 20 mM crotonate and 40 mM [1, 2-^{13}C] acetate was included as a negative control. Samples (50 ml) for GC-MS analysis were taken immediately after inoculation and after one, two, and four days of incubation time.

The involvement of a carboxylation step in cyclohexane carboxylate formation was tested by growing *S. aciditrophicus* with 10 mM crotonate and 41 mM [^{13}C] sodium bicarbonate. Samples (50 ml) were taken for GC-MS and NMR analyses at three time points, initially, when 70% of the initial substrate was used, and when 95% of the initial substrate was used.

^{13}C-NMR spectroscopy and GC-MS.

Samples were withdrawn at the time mentioned above from *S. aciditrophicus* cultures that were grown with crotonate, [U-^{13}C] crotonate, [^{13}C] sodium bicarbonate with crotonate, and crotonate with [1, 2-^{13}C] acetate or non-labeled sodium acetate and were analyzed by GC-MS. The samples were adjusted to a pH of 12 with 1 N NaOH addition to hydrolyze possible thioester bonds (14). Then, samples were acidified to pH< 2 with 12N HCl, extracted with ethyl acetate three times, and filtered through anhydrous sodium sulfate to remove water. The samples were concentrated under vacuum at 47°C to a volume of 1 to 2 ml, and split into 2 sub-samples before they were evaporated to dryness under a stream of nitrogen gas. One was used for GC-MS analysis and the other was used for NMR analysis (except for the samples from the cultures grown with [1, 2-^{13}C] acetate and crotonate and the samples from

the cultures grown on non-labeled substrates). One sub-sample was resuspended in 200 μl ethyl acetate and derivatized with 100 μl of *N,O*-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) for GC-MS analysis and the other sub-sample was resuspended in 1 ml of deuterated chloroform for NMR analysis.

The derivatized samples were analyzed with an Agilent Technologies 6890N Network GC systems series gas chromatograph equipped with an Agilent Technologies 5973 Network Mass Selective Detector mass spectrometer and a HP-5MS capillary column (Agilent 19091S-433), 0.25 mm x 30 m x 0.25 µm (Willmington, DE). The mass spectrometer was operated at 400 Hz. Helium is used as the carrier gas with a flow of 1.2 ml/min. The oven temperature was held at 40°C for 5 minutes, then, increased at a rate of 4°C/ min until it reached a temperature of 250°C where it was held for 5 minutes. The metabolites were identified by comparison to the retention times and the mass spectra of BSTFA-derivatized chemical standards (14, 31).

Liquid ^{13}C WALTZ-16 proton-decoupled NMR experiments were performed using 5mm Wilmad Glass NMR tubes on a Varian Mercury 300 MHz spectrometer. The one-dimensional spectra were obtained by using a ^{13}C-frequency of 75.45 MHz, a tip angle of 45.0^{o}, a spectral width of 18868 Hz, and an acquisition time of 1.815s with a relaxation delay of 1.000s. The ^{13}C-^{13}C COSY spectra included 64 repetitions of 384 increments using a spectral width of 18868 Hz, an acquisition time of 0.217 s and a relaxation delay of 1.000s.

Analytical procedures.

The concentrations of crotonate, cyclohexane carboxylate, benzoate, cyclohex-1-ene carboxylate, and acetate were determined by high performance liquid chromatography with a Prevail Organic acid column (250 by 4.6 mm; particle size 5 μm; Alltech Inc, Deerfield, Ill.). The isocratic mobile phase consisted of 25 mM KH_2PO_4 (pH 2.5) at a flow rate of 1 ml/min to measure acetate concentrations. To quantify crotonate, cyclohexane carboxylate, benzoate, and cyclohex-1-ene carboxylate, the mobile phase consisted of 60% (v/v) KH_2PO_4 (25 mM, pH 2.5) and 40% (v/v) acetonitrile. The UV absorbance detector was set at 210 nm to detect acetate and cyclohexane carboxylate, and 254 nm for crotonate, cyclohexane carboxylate, benzoate, and cyclohex-1-ene carboxylate.

Protein concentrations were determined by the method of Bradford (8) using the protocol and reagent from Pierce (Rockford, Il.) with bovine serum albumin as the standard.

Thermodynamic calculations.

The $\Delta G°'$ were calculated by using the $\Delta G°_f$ available in Thauer et al. (43). The values of the $\Delta G°_f$ of cyclohexane carboxylate and heptanoate were calculated by the group contribution method of Mavrovouniotis (26).

Chemicals.

Stable isotopes of crotonic acid (U-^{13}C, 99%), acetic acid (1, 2-^{13}C, 99%), sodium bicarbonate (^{13}C, 99%), and chloroform-D (D, 99.8%) were obtained from Cambridge Isotope Laboratories, Inc. (Andover, Mass). *N, O-*

bis(Trimethylsilyl)trifluoroacetamide (BSTFA) was purchased from Pierce (Rockford, Il.).

RESULTS

End products of crotonate fermentation.

Syntrophus aciditrophicus grew in pure culture with crotonate with a specific growth rate of 0.025 ± 0.003 h^{-1}. Approximately 6.6 ± 0.05 g of protein was obtained per mole of crotonate consumed. Assuming that 47% of the cell dry-mass was protein (18), this corresponds to a cell yield of 14.0 ± 0.1 g dry weight per mole of crotonate. If the ATP yield is 10.5 g of biomass per mole of substrate (40), about 1.33 moles of ATP would be formed per mole of crotonate degraded.

Initial experiments recovered approximately 70 % of the carbon and reducing equivalents as acetate from crotonate. Longer chain fatty acids such as hexanoate or caproate were not detected by HPLC. A small amount of butyrate (0.112 mM) was detected by HPLC. However, its concentration did not change over the course of the experiment. To determine the fate of the missing crotonate carbon and reducing equivalents, cultures of *S. aciditrophicus* grown with crotonate were extracted, derivatized, and analyzed by gas chromatography-mass spectrometry. A large peak was detected in samples from crotonate-amended cultures and was not present in samples from the unamended or heat-killed controls. This peak had the same retention time (21.58 min) and the same mass fragmentation pattern as the TMS-derivative of authentic cyclohexane carboxylate.

To confirm that cyclohexane carboxylate was made from crotonate, *S. aciditrophicus* was grown with 5.2 mM [U-^{13}C] crotonate. The growth rate was

similar to that observed with unlabeled crotonate (see above). Since a methyl group is easily lost from the TMS moiety of TMS-derivatized compounds, the total mass ion peak is often absent or very small and the m/z-15 ion is often used for identification purposes (31). The m/z-15 ion of the TMS-derivatized metabolite of the culture increased by +6 (185 to 191) mass units relative to the metabolite detected in the culture grown with unlabeled crotonate and to the TMS-derivatized authentic standard of cyclohexane carboxylate, showing that the metabolite was made from crotonate (Fig. 1. 1; Table 1. 1). The major m/z fragments of the ^{13}C-labeled metabolite were 58, 73, 87, 118, 132, 147, 161, 191, and 206 compared to 55, 73, 82, 117, 129, 145, 155, 185, and 200 for the TMS-derivative of authentic cyclohexane carboxylate and to the peak detected in the culture fluid of cells grown with unlabeled crotonate (Fig. 1. 1).

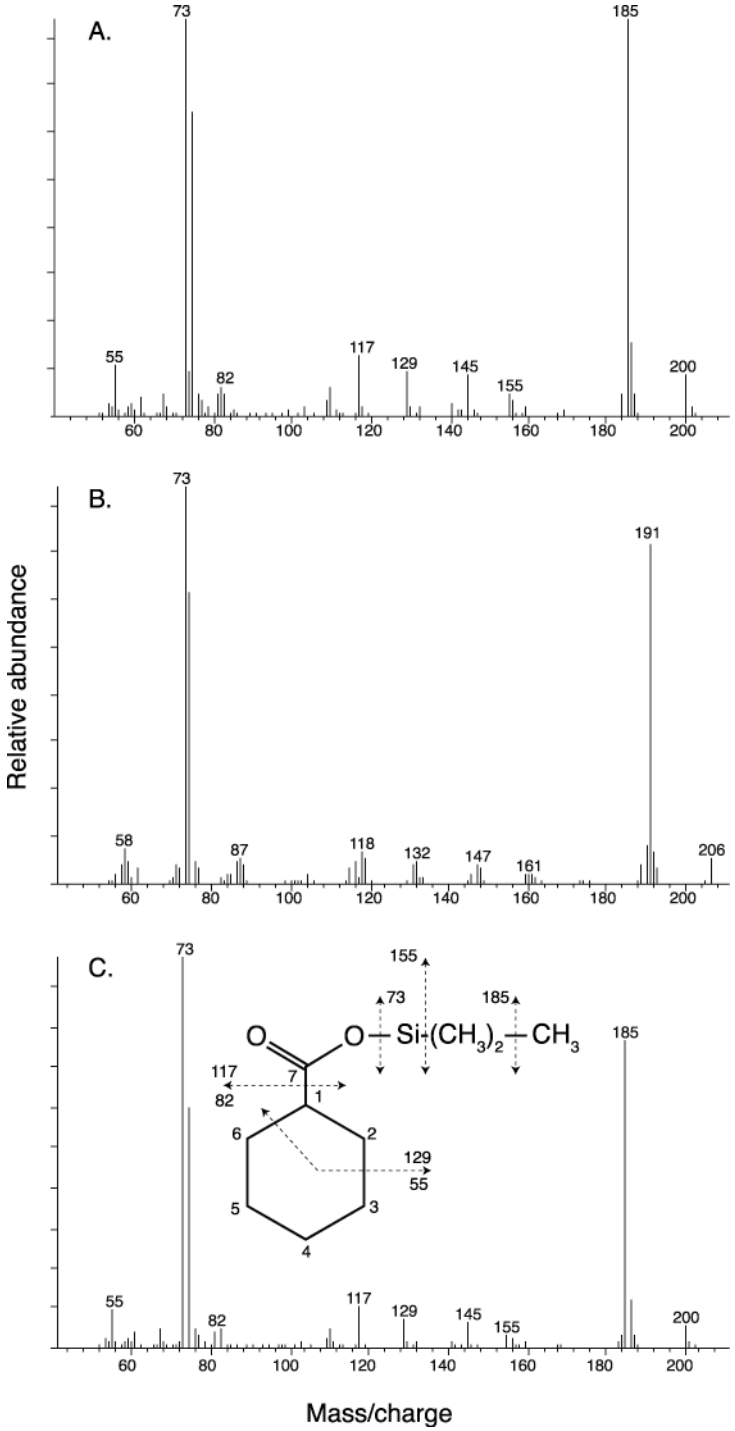
^{13}C-NMR analysis of the cell-free culture fluid of *S. aciditrophicus* grown with [U-^{13}C] crotonate detected peaks that were consistent with the presence of cyclohexane carboxylate. Initially, the ^{13}C-NMR spectrum had four peaks at 18.0, 122.1, 147.7, and 171.5 ppm, which correspond to the four carbons of [U-^{13}C] crotonate (Table 1. 2). When 94% of the substrate was depleted, the culture fluid was extracted and analyzed. The ^{13}C-NMR spectrum had 7 peak clusters including the deuterated chloroform peak. The C-7 corresponding to the carboxyl moiety of cyclohexane carboxylate was coupled to C-1 with a coupling constant of 55.2 Hz. The C-1 corresponding to the peak at 43.0 ppm, a doublet of doublets, was further coupled to C-2 with a coupling constant of 33.2 Hz. The values of these coupling

constants were in agreement with published values (11) and the correlations were further confirmed by off-diagonal peaks in the two-dimensional spectrum (data not shown). The integration of the one-dimension spectrum showed that the partially resolved peaks near 25 ppm accounted for 3 carbons, C-3, C-4 and C-5, and for one carbon for each of the other peaks, C-1, C-2, and C-7. The coupling constants for C-3, C-4, and C-5 could not be resolved (39). No peak was detected for the C-6 position of the cyclohexane carboxylate. If the C-6 carbon was labeled and the cyclohexane carboxylate was fully labeled, the integration of the peak area at 29.0 ppm would indicate two carbons, corresponding to the C-2, and the C-6 position, which was not observed. Moreover, the coupling pattern of C-1 would contain additional coupling resulting from the C-6 being ^{13}C-labeled. Since the C-6 was not ^{13}C-labeled, this indicates that a carboxylation step might be involved in the formation of cyclohexane carboxylate.

To test this, *S. aciditrophicus* was grown with unlabeled-crotonate and [^{13}C] sodium bicarbonate. The mass of the m/z-15 ion of the TMS-derivative of cyclohexane carboxylate increased by +1 mass unit from 185 to 186 relative to the TMS-derivative of the authentic standard (Table 1. 1). Moreover, the ^{13}C-NMR spectrum of the culture fluid displayed a single, uncoupled peak at 28.9 ppm, which corresponded to the C-6 position of cyclohexane carboxylate (Table 1. 2).

In addition to cyclohexane carboxylate, acetate was the only other major product detected when *S. aciditrophicus* was grown on [U-^{13}C] crotonate (Table 1. 2). The ^{13}C-NMR spectrum contained two peaks at 177.4 ppm and 21.0 ppm,

Figure 1. 1. Mass spectrum profiles of the TMS-derivatized metabolite detected at the retention time of 21.58 min (A), from a culture fluid of *S. aciditrophicus* grown with unlabeled crotonate; (B), from a culture fluid of *S. aciditrophicus* grown with [U-^{13}C] crotonate; and (C), from TMS-derivatized cyclohexane carboxylate authentic standard. The dashed arrows represent the fission sites of the different fragments of cyclohexane carboxylate.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117
129
145
155
200
60
80
100
120
140
160
180
200
Mass/charge

Table 1. 1. Comparison of the m/z-15 ions of TMS-derivatized metabolites to their corresponding TMS-derivatized authentic standard [a].

Metabolites	GC RT[b] (min)	m/z -15 ions					
		Crotonate	[$^{13}C_4$] Crotonate	[^{13}C] Sodium bicarbonate	[$^{13}C_4$] Crotonate + crotonate	[$^{13}C_2$] Acetate + crotonate	Authentic standards[c]
Acetoacetate	22.1	231	235	231	231,233,235	231,233,235	231
3-Hydroxybutyrate	20.5	233	237	233	233,235,237	233,235,237	233
Glutarate	28.8	261	265	261,262	261,263,265	261,263,265	261
Pimelate	34.8	289	295	289,290	289,291,293,295	289,291,293,295	289
Cyclohex-1-ene carboxylate	24.4	183	189	183,184	183,185,187,189	183,185,187,189	183
Cyclohexane carboxylate	21.6	185	191	185,186	185,187,189,191	185,187,189,191	185
Benzoate	23.3	179	185	179,180	179,181,183,185	179,181,183,185	179
2-Hydroxycyclohexane carboxylate	30.0	ND[d]	ND	ND	ND	273,275,277,279	273

[a] The m/z-15 ions of TMS-derivatized metabolites detected by GC-MS in culture fluids of *Syntrophus aciditrophicus* grown under different conditions. Metabolites were not detected in heat-killed or substrate-unamended controls.
[b] Retention times of TMS-derivatized chemical authentic standards and the detected metabolites.
[c] Chemical standards were commercially available.
[d] Not detected.

which correspond to the carboxyl carbon and the methyl carbon of acetate, respectively. ^{13}C-labeled metabolites were not detected in heat-killed or unamended controls. Except for acetate and cyclohexane carboxylate, no other compound was detected by ^{13}C-NMR analysis.

Now that the identity of the products of crotonate metabolism is known, the time course of crotonate metabolism and the carbon balance was determined by HPLC (Fig. 1. 2). The pure culture reached a maximum absorbance of 0.41 within 8 days of incubation at 37°C. *S. aciditrophicus* metabolized crotonate to 1.41 ± 0.24 moles of acetate and 0.16 ± 0.02 mole of cyclohexane carboxylate per mole of crotonate degraded, giving a 99.4% carbon recovery and 95.8 % hydrogen recovery. The above values are consistent with the following stoichiometry for crotonate fermentation (equation 1):

$$6C_4H_5O_2^- + HCO_3^- + 5H_2O \rightarrow 9C_2H_3O_2^- + C_7H_{11}O_2^- + 3H^+ \quad (1)$$

Metabolites detected during crotonate degradation.

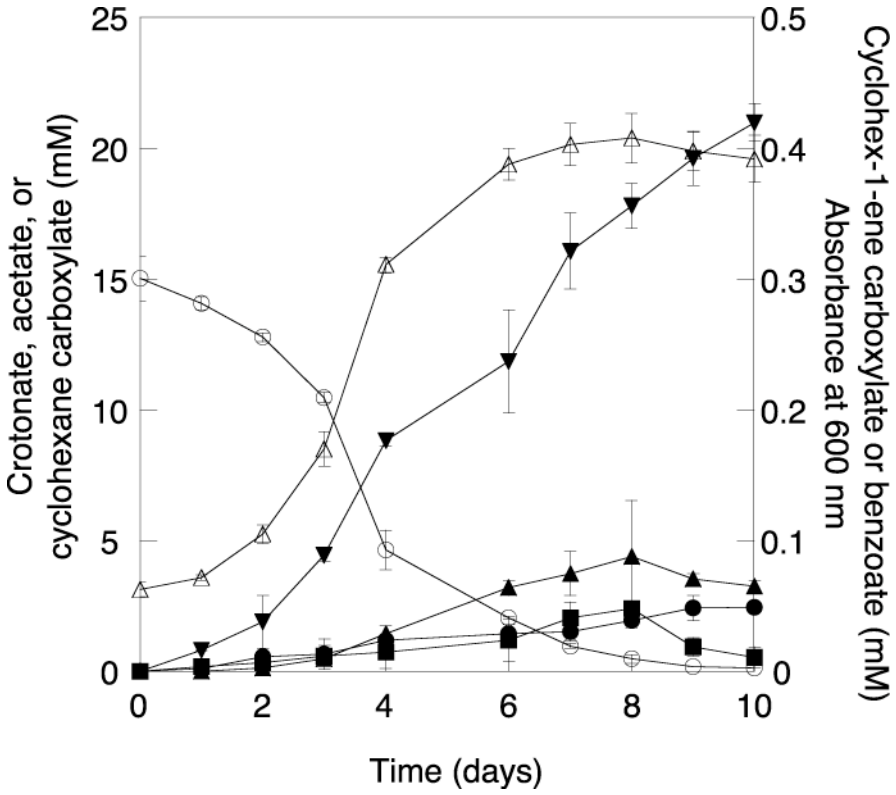
During growth of *S. aciditrophicus* with crotonate, GC-MS analyses of culture samples detected the presence of the TMS-derivatives of cyclohex-1-ene carboxylate, benzoate, pimelate, glutarate, 3-hydroxybutyrate, and acetoacetate in addition to cyclohexane carboxylate. Each metabolite eluted at the same retention time and mass spectral profile as the TMS-derivative of their respective authentic standard. None of these compounds were detected in substrate unamended or heat-killed controls. To

Table 1. 2. NMR data for compounds detected in cultures of *Syntrophus aciditrophicus* grown in pure culture with [U-^{13}C] crotonate or with crotonate with [^{13}C] sodium bicarbonate.

Growth conditions and sampling time	Compound detected	Position	δ C^{13} (ppm)		J_{cc} (Hz)[c]
			Expected [a]	Observed	
[U-^{13}C] Crotonate at initial time point	Crotonate	C-1	172.4	171.4	77.7 (1-2)
		C-2	122.4	122.1	77.7 (2-1), 69.9 (2-3), 7.0 (2-4)
		C-3	147.6	147.7	69.9 (3-2), 42.0 (3-4)
		C-4	18.1	18.0	
[U-^{13}C] Crotonate at final time point[b]	Cyclohexane carboxylate	C-7	181.0	182.4	55.2 (7-1)
		Ring C-1	41.5	43.0	33.2 (1-2), 55.2 (1-7)
		Ring C-2	26.6	29.0	33.2 (2-1, 2-3)
		Ring C-6	26.6		Not observed
		Ring C-4	27.1	25.0	Unresolved peaks
		Ring C-3, and C-5	24.3	25.0	Unresolved peaks
	Acetate	Carboxyl	177.0	177.4	56.7 (1-2)
		Methyl	20.7	21.0	56.7 (2-1)
[^{13}C] sodium bicarbonate at final time point	Cyclohexane carboxylate	C-6	29.0	28.9	

[a] The predicted chemical shifts (δ C^{13}) were obtained from ChemNMR (Upstream Solutions).
[b] The culture was analyzed when 94% of the crotonate was degraded
[c] Jcc, coupling constants

Figure 1. 2. Metabolism of crotonate by *S. aciditrophicus* pure cultures. Symbols: ○, crotonate; ▼, acetate; ●, cyclohexane carboxylate; ▲, cyclohex-1-ene carboxylate; ■, benzoate; △, absorbance at 600 nm. The data are averages ± standard deviations of triplicate microcosms.


Crotonate, acetate, or cyclohexane carboxylate (mM)
Cyclohex-1-ene carboxylate or benzoate (mM)
Absorbance at 600 nm
Time (days)
0
5
10
15
20
25
0
0.1
0.2
0.3
0.4
0.5
0
2
4
6
8
10

confirm that these metabolites were made from crotonate, samples from cultures grown with [U-^{13}C] crotonate were analyzed. The m/z-15 ion of TMS-derivatives of the metabolites corresponding to cyclohexane carboxylate, cyclohex-1ene carboxylate, pimelate, and benzoate increased by +6 mass units while those corresponding to glutarate, 3-hydroxybutyrate, and acetoacetate increased by +4 mass units relative to their respective authentic TMS-derivatized standards, confirming that these compounds are metabolites of crotonate degradation (Table 1. 1).

3-Hydroxybutyrate and acetoacetate were detected only after alkaline hydrolysis of cells. Neither compound was detected when the samples were filtered to remove cells. This suggests that the above metabolites were intracellular and present as their CoA derivatives. However, pimelate, glutarate, cyclohex-1-ene carboxylate, benzoate, and cyclohexane carboxylate were detected whether or not the cells were alkaline hydrolyzed, and whether or not the sample was filtered to remove cells. These data imply that the latter compounds were excreted into the medium as their free acids.

The time course experiment showed that cyclohex-1-ene carboxylate and benzoate were transiently produced and degraded (Fig. 1. 2). Each was detected at much lower concentrations (maximum concentration of 0.088 and 0.048 mM, respectively) than acetate and cyclohexane carboxylate. Pimelate, glutarate, 3-hydroxybutyrate, and acetoacetate were not detected by HPLC (concentrations <0.05mM). Nevertheless, GC-MS analysis showed that the peak areas corresponding

to each compound increased and decreased during growth supporting their role as intermediates.

Pathway for cyclohexane carboxylate formation.

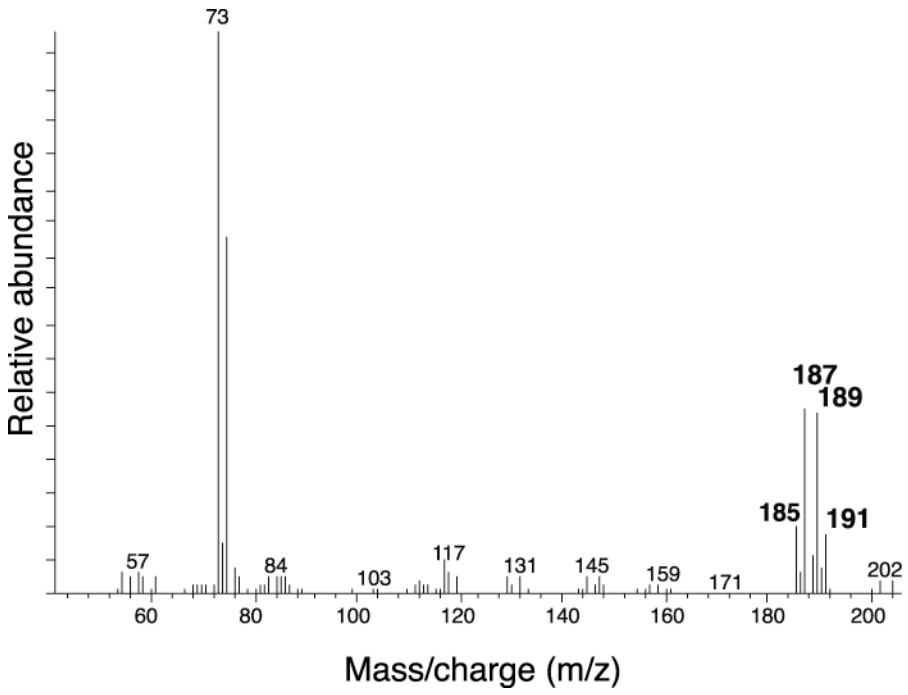
To resolve the major steps in the formation of cyclohexane carboxylate, we performed a series of experiments in which ^{13}C-labeled compounds were used. In order to determine when the carboxylation step occurred, *S. aciditrophicus* was grown in basal medium with 20 mM of unlabeled crotonate and 41 mM of [^{13}C] sodium bicarbonate. The m/z-15 ion of the TMS-derivatives of cyclohexane carboxylate, benzoate, cyclohex-1-ene carboxylate, pimelate and glutarate increased by +1 mass unit relative to the respective TMS-derivatized standards (Table 1. 1). However, peaks corresponding to a potential three-carbon metabolite such as TMS-derivative of malonate were not detected by GC-MS. The m/z-15 ion of the TMS-derivatives of 3-hydroxybutyrate and acetoacetate remained unchanged. These results are consistent with a carboxylation of a four-carbon intermediate.

To determine whether cyclohexane carboxylate is formed by the condensation of a 4-carbon unit derived from crotonate without carbon-carbon bond cleavage and a 2-carbon unit or by the condensation of three 2-carbon units, *S. aciditrophicus* was grown with equimolar concentrations of [U-^{13}C] crotonate and non-labeled crotonate as substrates. In the first model, part of the [U-^{13}C] labeled and non-labeled crotonate is oxidized to form a mix of [1, 2-^{13}C] acetate and non-labeled acetate. If we assume that a 4-carbon skeleton remains intact to form cyclohexane carboxylate, and that no

isotopic discrimination occurs, four possible labeling patterns to form cyclohexane carboxylate are possible. The U-^{13}C four-carbon intermediate could condense with a U-^{13}C-labeled or a non-labeled two-carbon intermediate to give isotopomers of cyclohexane carboxylate with an increase of +6 or +4 mass units, respectively. The non-labeled four-carbon intermediate could condense with a U-^{13}C-labeled or a non-labeled two-carbon intermediate to give isotopomers with an increase of +2 or +0 mass units, respectively. Each isotopomer occurs with the same probability. In the second model, a mix of [1, 2-^{13}C] acetate and non-labeled acetate are formed and eight possible labeling patterns for cyclohexane carboxylate formation are possible resulting in isotopomers with a m/z-15 ion increase of +0, +2, +4, and +6 mass units. The probability of having the m/z-15 ion of the TMS-derivatized cyclohexane carboxylate increased by +2 or +4 mass units would each be 0.375, and that by +0 or +6 mass units would each be 0.125, assuming that no isotopic discrimination occurred.

The mass spectrum profile of the TMS-derivatized cyclohexane carboxylate formed when *S. aciditrophicus* grown with equal amounts of labeled and unlabeled crotonate showed a mixture of differently labeled cyclohexane carboxylate, with a m/z-15 mass ion increases of +0, +2, +4 and +6 units (Table 1. 1). The relative abundance of those with the mass ion increases of +2 and +4 units (m/z-15 of 187 and 189) are around 2.9 times higher than those with mass increases of +0 or +6 units (m/z-15 of 185 and 191) (Fig. 1. 3). These data are consistent with the model that

Figure 1. 3. Mass spectrum profile of the TMS-derivatized metabolite detected at a retention time of 21.58 min from cultures of *S. aciditrophicus* grown on an equimolar mixtures of [U-^{13}C] crotonate and unlabeled crotonate.


73
187
189
185
191
57
84
103
117
131
145
159
171
202
60
80
100
120
140
160
180
200
Relative abundance
Mass/charge (m/z)

cyclohexane carboxylate formation occurs by the condensation of three two-carbon units.

Further evidence to support the hypothesis that the formation of cyclohexane carboxylate occurs by the condensation of three two-carbon units was obtained by growing *S. aciditrophicus* in the presence of [1, 2-^{13}C] acetate and crotonate. Acetate does not support *S. aciditrophicus* growth in the absence of crotonate. The presence of 40 mM sodium acetate with 20 mM crotonate in the medium partially inhibited the growth of *S. aciditrophicus* compared to that with 20 mM crotonate alone. The growth rate was 0.006 ± 0.002 h^{-1} in the presence of 40 mM sodium acetate compared to 0.025 ± 0.003 h^{-1} without sodium acetate addition (data not shown). When *S. aciditrophicus* was grown with 40 mM [1, 2-^{13}C] acetate and 20 mM crotonate, samples of culture fluids were taken at different time points, derivatized, and submitted for GC-MS analysis. The metabolites detected by GC-MS when *S. aciditrophicus* was grown under these conditions were those detected previously (e.g., the TMS-derivatives of cyclohexane carboxylate, cyclohex-1-ene carboxylate, benzoate, pimelate, glutarate, 3-hydroxybutyrate, and acetoacetate) along with one additional metabolite, the TMS-derivative of 2-hydroxycyclohexane carboxylate. Multiple m/z-15 ions were detected for each metabolite. The m/z-15 ion of the TMS derivatives of 3-hydroxybutyrate, acetoacetate, and glutarate each showed increases of +0, +2, and +4 mass units relative to the TMS derivatives of their respective authentic standards. The m/z-15 ion of TMS derivatives of pimelate, benzoate, cyclohex-1-ene carboxylate, 2-hydroxycyclohexane carboxylate, and cyclohexane

carboxylate each showed increases of +0, +2, +4, and +6 mass units relative to the TMS derivatives of their respective authentic standards (Table 1. 1). The cyclohexane carboxylate formed in cultures after 24 hours of incubation had a mass spectrum profile with a mixture of m/z-15 ion of 185, 187, 189, and 191 corresponding to a mass increase of +0, +2, +4, and +6 units, respectively. The relative abundance of the 191 ion was higher than the other ions. To form this ion, cyclohexane carboxylate must have been formed by the condensation of three molecules of [1, 2-^{13}C] labeled acetate. Over the course of the experiment, the relative abundance of the different m/z-15 ions changed and, at the end of the experiment, the m/z-15 ion with a mass of 185 increased in abundance. This was probably due to the utilization of nascent, non-labeled acetate derived from crotonate oxidation for cyclohexane carboxylate formation. These data confirm that cyclohexane carboxylate is formed from the condensation of two-carbon units.

DISCUSSION

The conversion of crotonate to acetate and cyclohexane carboxylate by a microorganism has never been reported before. A number of anaerobes including syntrophic bacteria are known to dismutate crotonate to 1 mole of acetate and 0.5 mole of butyrate (2, 6, 10, 24, 32, 42, 48, 49). Some organisms will also form caproate in addition to butyrate. Jackson et al. (21) reported that *S. aciditrophicus* degraded 1 mole of crotonate to 0.1 mole of butyrate, 0.13 mole of caproate and 1.4 moles of acetate, which differs from the results presented here. This discrepancy may be due to variations in the growth conditions of *S. aciditrophicus.* However, since *S. aciditrophicus* has been repetitively transferred in liquid culture for a number of years, it is possible that a genetic change occurred that resulted in this change in physiology.

Our results obtained from GC-MS, ^{13}C-NMR, and HPLC analyses demonstrated the synthesis of a cyclic molecule from a straight chain, unsaturated fatty acid (Fig. 1. 4). Approximately two thirds of the crotonate present is degraded to acetate. The rest of the crotonate (probably as acetyl Co-A) is used to form cyclohexane carboxylate and reoxidize the reducing equivalents generated during crotonate oxidation to acetate. The labeling patterns observed in cyclohexane carboxylate when *S. aciditrophicus* was grown with [1, 2-^{13}C] acetate or [U-^{13}C] crotonate in the presence of non-labeled crotonate are consistent with the synthesis of cyclohexane carboxylate by the condensation of two-carbon units derived from

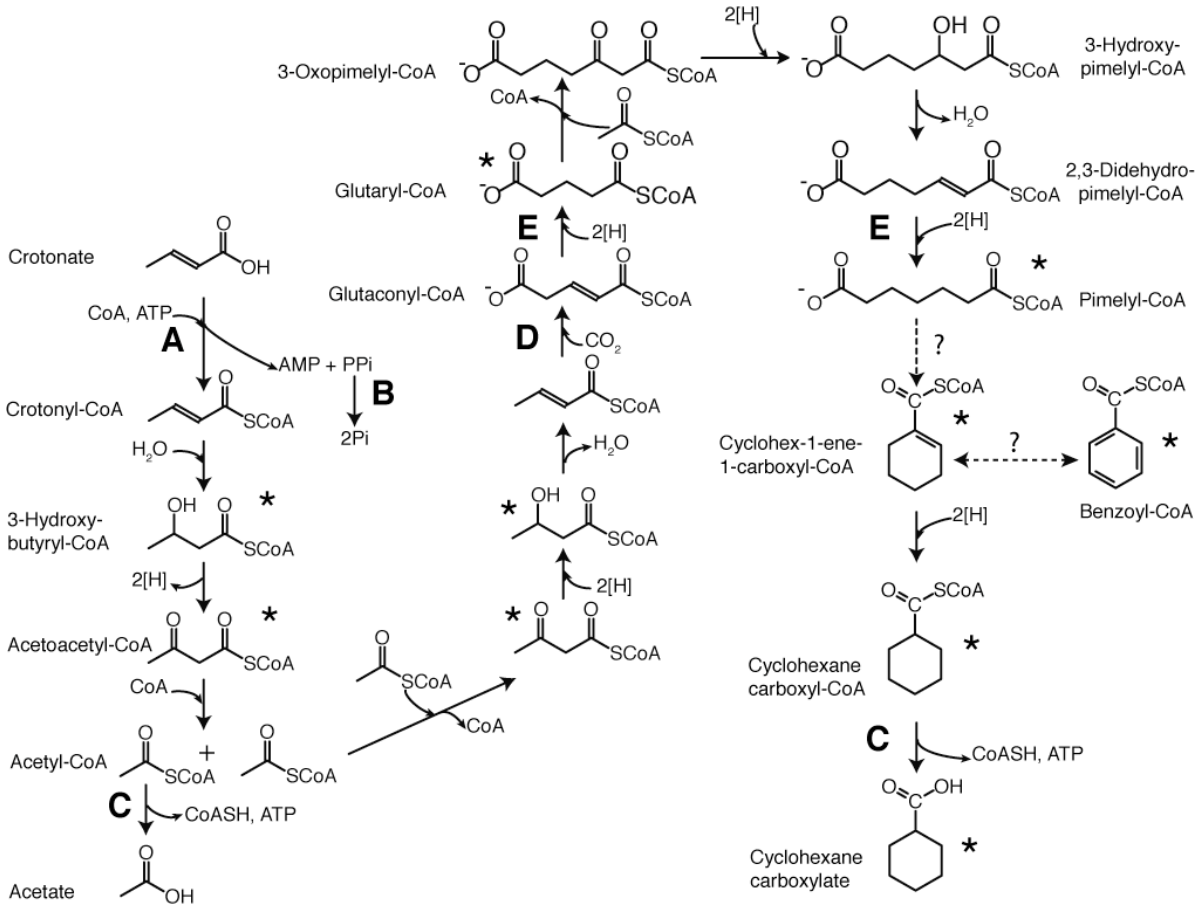
crotonate rather than the use of the intact four-carbon skeleton of crotonate (Table 1. 1; Fig. 1. 3). Thus, cyclohexane carboxylate formation probably serves an analogous function as the formation of butyrate and caproate by *Clostridium kluyveri* (4, 37, 42). In the case of C. *kluyveri*, the reducing equivalents generated during ethanol oxidation to acetate are used to reduce two or three equivalents of acetate to form butyrate or caproate.

The carboxylation step probably occurs after the four-carbon intermediate is made since the mass units of TMS derivatives of glutarate, pimelate, and cyclohex-1-ene carboxylate each increase by one relative to the respective TMS-derivatized standards (Table 1. 1) when *S. aciditrophicus* was grown with [^{13}C] sodium bicarbonate. In the biosynthesis of membrane lipids, the fatty acid elongation step involves the use of malonyl-acyl carrier protein (ACP), which is decarboxylated during the transfer of the acetyl-group to the growing fatty acid molecule (25). One possible pathway for cyclohexane carboxylate formation would be the synthesis of a five-carbon intermediate by the condensation of malonyl-CoA and acetyl-CoA (or as their ACP derivatives). However, no labeled malonyl-CoA was detected when *S. aciditrophicus* was grown in the presence of unlabeled crotonate and [^{13}C] sodium bicarbonate. Also, the m/z-15 ion of the TMS-derivatives of 3-hydroxybutyrate and acetoacetate increase by +0, +2, and +4 mass units relative to the TMS-derivatives of their respective standards when *S. aciditrophicus* was grown in the presence of [1, 2-^{13}C] acetate (Table 1.1), consistent with their formation from two-carbon intermediates (Fig. 1. 4).

GC-MS analysis allowed the identification of acetoacetate, 3-hydroxybutyrate, glutarate, pimelate, 2-hydroxycyclohexane carboxylate, and cyclohex-1-ene carboxylate as intermediates of cyclohexane carboxylate formation from crotonate. With the exception of the TMS derivative of 3-hydroxybutyrate and acetoacetate, all of the above compounds were detected in low amounts in cell-free culture broth. The GC peak areas increased and then subsequently decreased during growth, consistent with their role as intermediates in crotonate metabolism. Given that the enzymes involved in benzoate and cyclohexane carboxylate metabolism in *S. aciditrophicus* and *Rhodopseudomonas palustris* (14, 19) use CoA derivatives as their substrates, it is likely that cyclohexane carboxylate formation does as well.

The above intermediates are the same as those detected during syntrophic (14) and fermentative benzoate metabolism (15) by *S. aciditrophicus*, suggesting that cyclohexane carboxylate formation may involve the same pathway as used for benzoate degradation. However, analysis of the genome sequence of *S. aciditrophicus* (GenBank CP000252) (McInerney et al., submitted for publication; 33) suggests that benzoate is degraded to 3-hydroxypimelyl-CoA by a pathway involving cyclohex-1,5-diene-1-carboxyl-CoA, 6-hydroxycyclohex-1-ene-1-carboxyl-CoA, and 6-oxocyclohex-1-ene-1-carboxyl-CoA as intermediates rather than those shown in Fig. 1. 4. The *S. aciditrophicus* genome contains three genes (SYN1653-SYN1655) whose gene products have high sequence similarity (>47% identity) to cyclohex-1,5-diene-1-carboxyl-CoA hydratase, 6- hydroxycyclohex-1-ene-1-carboxyl-CoA

Figure 1. 4. Proposed pathway for the formation of acetate and cyclohexane carboxylate from crotonate by *S. aciditrophicus*. Asterisks indicated ^{13}C-labeled intermediates that were detected. Letters A through E indicate potential sites of energy conservation or use as discussed in the text.


3-Oxopimelyl-CoA
2[H]
3-Hydroxy-pimelyl-CoA
CoA
SCoA
H2O
Glutaryl-CoA
2,3-Didehydro-pimelyl-CoA
E
2[H]
Crotonate
Glutaconyl-CoA
Pimelyl-CoA
CoA, ATP
D
CO2
A
AMP + PPi
B
2Pi
Crotonyl-CoA
?
Cyclohex-1-ene-1-carboxyl-CoA
Benzoyl-CoA
3-Hydroxy-butyryl-CoA
Acetoacetyl-CoA
Cyclohexane carboxyl-CoA
Acetyl-CoA
C
CoASH, ATP
Cyclohexane carboxylate
Acetate

dehydrogenase, and 6-oxocyclohex-1-ene-1-carboxyl-CoA hydrolase of *Azoarcus* strain CIB (5). *S. aciditrophicus* may use separate pathways to degrade cyclohexane carboxylate and benzoate to 3-hydroxypimelyl-CoA. Cyclohexane carboxylate formation (Fig. 1. 4) may occur by the reversal of the pathway used to degrade cyclohexane carboxylate, which is similar to the pathway used by *R. palustris* to degrade benzoate and cyclohexane carboxylate (19). The pathway in each organism has several identical intermediates and cell-free extracts of syntrophically-grown *S. aciditrophicus* contain the enzyme activities for the conversion of cyclohex-1-ene carboxyl-CoA to pimelyl-CoA (14). Interestingly, genes homologous to those involved in benzoate and cyclohexane carboxylate degradation in *R. palustris* (13, 19) were not detected in the *S. aciditrophicus* genome (<30% amino acid sequence identity). The unfavorable carboxylation of crotonyl-CoA to glutaconyl-CoA could be driven by a sodium gradient since membrane-bound decarboxylation enzymes have been shown to be reversible (12, 36).

There are several possible mechanisms for the formation of the alicyclic ring of cyclohexane carboxylate. The ring formation from short chain fatty acids is already well established for polyketide biosynthesis (20). Here, the correct alignment of carbonyl and methylene groups of poly-β-ketone intermediates leads to cyclization. The formation of cyclohexane carboxylate may be analogous to the synthesis of 1,4-dihydroxy-2-naphthoyl-CoA from O-succinylbenzyoyl-CoA (OSB) by 1,4-dihydroxy-2-naphthoyl-CoA synthase (MenB) in menaquinone biosynthesis (44). This is essentially the back reaction of 2-oxocyclohexane-1-carboxyl-CoA hydrolase

in *R. palustris* (30) and both enzymes have conserved Asp and Tyr at the active site (44). As mentioned above, a gene homologous to the *R. palustris* gene was not detected in the *S. aciditrophicus* genome.

The transient accumulation of benzoate (probably as its CoA derivative) from crotonate is surprising. However, previous studies have shown the formation of aromatic compounds from alicyclic compounds such as cyclohexane carboxylate or 4-oxocyclohexane carboxylate in animals and microorganisms. Enzymes purified from the liver of guinea pigs can convert cyclohexane carboxyl-CoA to benzoyl-CoA with cyclohex-1-ene carboxylate as an intermediate (1). Cyclohexane carboxylate can be converted to *p*-hydroxybenzoate aerobically by *Arthrobacter* sp. (7) or *Alcaligenes* strain W1 (41). *Corynebacterium cyclohexanicum* is able to convert 4-oxocyclohexane carboxylate to 4-hydroxybenzoate (22). The *S. aciditrophicus* genome contains two gene clusters with high similarity (>50% identity at the amino acid level) to the benzoate-induced gene cluster in *Geobacter metallireducens* (47), but lacks two of the four genes needed to encode for the ATP-dependent, benzoyl-CoA reductase found in denitrifiers and anaerobic phototrophs (19). The presence of two putative benzoyl-CoA reductase systems is consistent with the benzoate dismutation hypothesis (15) where one reductase functions in a pathway that oxidizes benzoate to acetate and carbon dioxide and the other reductase functions in a pathway that forms cyclohexane carboxylate.

Thermodynamic considerations show that the formation of cyclohexane carboxylate and acetate from crotonate is favorable, with a standard free energy of -

48.1 kJ/mole (Table 1. 3). The free energy change for cyclohexane carboxylate formation is similar to the standard free energy changes for butyrate, caproate, or heptanoate formation from crotonate fermentation (Table 1. 3). The molar growth yield suggests that about 1.33 moles of ATP are made per mole of crotonate. The formation of 1.41 moles of acetate and 0.16 moles of cyclohexane carboxylate per mole of crotonate could result in the formation of 1.57 moles of ATP by substrate-level phosphorylation reactions involving the CoA derivatives of these compounds (reaction C, Fig. 1. 4). However, energy must be used to activate crotonate to crotonyl-CoA. No acetyl-CoA: crotonyl-CoA transferase activity was detected when *S. aciditrophicus* was grown with crotonate (14), suggesting that a CoA ligase reaction is used (reaction A, Fig. 1. 4). Crotonyl-CoA ligase activity has been detected in crotonate-grown cells (N. Q Wofford and M. J. McInerney, unpublished data). This reaction forms AMP and pyrophosphate in addition to crotonyl-CoA (16, 17) and would consume 2 ATP equivalents if pyrophosphate is hydrolyzed. These calculations suggest that *S. aciditrophicus* must also use the energy in ion gradients for growth. Energy could be conserved by a membrane-bound, proton-translocating pyrophosphatase as shown in *Syntrophus gentianae* (35) (reaction B, Fig. 1. 4). In addition, the redox reactions leading to the formation of cyclohexane carboxylate may be energy-yielding. During benzoate oxidation to acetate, the production of hydrogen (E°’ of -414 mV) from high redox electrons derived during pimelyl-CoA and glutaryl-CoA oxidation (E°’ of -10 mV) is coupled to a reverse electron transport and probably consumes the equivalent of two-thirds of an ATP (36, 45). Since glutarate

and pimelate were detected as intermediates, the redox reactions involved in the formation of their respective CoA substrates may be energy yielding as well (reaction E, Fig. 1. 4).

Table 1. 3. Standard free energy changes for various crotonate fermentations.

Equation no.	End product	Equation	ΔG°' (kJ/mol)[a]
1	Butyrate	$2C_4H_5O_2^- + 2H_2O \rightarrow 2C_2H_3O_2^- + C_4H_7O_2^- + H^+$	-51.1
2	Caproate	$4C_4H_5O_2^- + 4H_2O \rightarrow 5C_2H_3O_2^- + C_6H_{11}O_2^- + 2H^+$	-51.1
3	Heptanoate	$7C_4H_5O_2^- + HCO_3^- + 7H_2O \rightarrow 11C_2H_3O_2^- + C_7H_{13}O_2^- + 4H^+$	-52.1
4	Cyclohexane-carboxylate	$6C_4H_5O_2^- + HCO_3^- + 5H_2O \rightarrow 9C_2H_3O_2^- + C_7H_{11}O_2^- + 3H^+$	-48.1

[a]Calculated from the $\Delta G°_f$ listed in the study by Thauer et al. (43) except that the $\Delta G°_f$ results for cyclohexane carboxylate and heptanoate were calculated as -281.2 and -330.9 $kJ.mol^{-1}$, respectively, based on the group contributions method of Mavrovouniotis (26).

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CHAPTER 2

Reduction of benzoate by *Syntrophus aciditrophicus* grown in pure culture with crotonate.

ABSTRACT

Benzoate and its coenzyme A derivative are key intermediates in the anaerobic degradation of aromatic compounds. Pure cultures of *Syntrophus aciditrophicus* simultaneously degraded crotonate and benzoate when both substrates were present. The growth rate was 0.08 h^{-1} when crotonate and benzoate were present compared to 0.025 h^{-1} with crotonate alone. After eight days of incubation, 4.12 ± 0.50 mM of cyclohexane carboxylate and 8.40 ± 0.61 mM of acetate were formed and 4.0 ± 0.04 mM of benzoate and 4.8 ± 0.5 mM of crotonate were consumed. The molar growth yield was 22.7 ± 2.1 g (dry wt) cell per mol of crotonate. Cultures grown with [ring-^{13}C] benzoate and unlabeled crotonate initially formed ring-labeled ^{13}C-cyclohexane carboxylate. No [^{13}C] labeled acetate was detected. In addition to cyclohexane carboxylate, ^{13}C-labeled cyclohex-1-ene carboxylate was detected as TMS-derivatized intermediate that was six mass units greater than the authentic standard. Once almost all of the benzoate was gone, cyclohexane carboxylate with a ^{13}C-atom at C-7 and m/z-15 ions with mass increases of 0 to 6 mass units relative to

the TMS-derivative of the authentic standard was observed. Glutarate and pimelate were also detected at this time and each had m/z-15 ions of their TMS-derivatives with mass of 0 to 5 or 0 to 7 mass units greater, respectively, than the TMS-derivative of the respective authentic standard. The increase in molar growth yield with crotonate and benzoate and the formation of [ring-^{13}C] cyclohexane carboxylate from [ring-^{13}C] benzoate in the presence of crotonate is consistent with benzoate serving as an electron acceptor.

Key words: Benzoate respiration, *Syntrophus aciditrophicus*, crotonate.

INTRODUCTION

Aromatic and fatty acids are important intermediates in the degradation of organic matter in anaerobic ecosystems (31). Aromatic compounds are naturally produced as derivatives of secondary metabolism of plants (22) and as by-products of biological and chemical cleavage of lignins (3, 26). As a consequence, the degradation of aromatic compounds plays an important role in the global carbon cycle (14, 18). The degradation of the vast majority of aromatic substrates converges to a central intermediate, benzoate or its coenzyme A thioesters, prior to ring cleavage (15, 17).

In the absence of electron-accepting processes, the degradation of benzoate is thermodynamically difficult and necessitates the presence of hydrogen- and formate-using partners to maintain hydrogen and formate concentrations low enough to allow the overall reaction to be thermodynamically favorable (Table 2. 1, eq. 1 and 2). The observation that cyclohexane carboxylate accumulated in phthalate-degrading, methanogenic enrichments (27) suggested that benzoate could be dismutated, i.e. some of the benzoate reduced and the rest oxidized. This was confirmed with pure cultures of *Syntrophus aciditrophicus*, which fermented benzoate in the absence of a hydrogen-using microorganism to 0.5 mol of cyclohexane carboxylate and 1.5 mol of acetate (13).

The accumulation of cyclohexane carboxylate up to 250 μM during syntrophic benzoate metabolism and as an end product during benzoate fermentation

suggested that *S. aciditrophicus* used part of the benzoate as an electron acceptor (12). To test this hypothesis, we investigated the fate of benzoate when *S. aciditrophicus* was grown in the presence of both benzoate and crotonate. Interestingly, we did not detect butyrate production as would be predicted if crotonate served as an electron acceptor (1, 36). Instead, we found the concomitant degradation of benzoate and crotonate with the formation of cyclohexane carboxylate and acetate as end products consistent with the use of benzoate as the electron acceptor.

Table 2. 1. Standard Gibbs free energy of chemical reactions for the different possible fates of benzoate.

Eq. #	Reaction	Equation	ΔG°' (kJ/reaction)[a]
1	Benzoate oxidation	$C_7H_5O_2^- + 7\ H_2O \rightarrow 3\ C_2H_3O_2^- + HCO_3^- + 3\ H_2 + 3\ H^+$	+70.5
2	Benzoate metabolism with *M. hungatei*	$C_7H_5O_2^- + 4.75\ H_2O \rightarrow 3\ C_2H_3O_2^- + 0.25\ HCO_3^- + 0.75\ CH_4 + 2.25\ H^+$	-31.2
3	Crotonate fermentation	$6C_4H_5O_2^- + HCO_3^- + 5H_2O \rightarrow 9C_2H_3O_2^- + C_7H_{11}O_2^- + 3H^+$	-48.1
4	Benzoate fermentation	$2\ C_7H_5O_2^- + 7\ H_2O \rightarrow C_7H_{11}O_2^- + 3\ C_2H_3O_2^- + HCO_3^- + 3\ H^+$	-24.0
5	Benzoate as an electron acceptor	$C_7H_5O_2^- + 3\ C_4H_5O_2^- + 6\ H_2O \rightarrow C_7H_{11}O_2^- + 6\ C_2H_3O_2^- + 3\ H^+$	-121.5

[a]Values represent calculated ΔG°' for benzoate fermentation, benzoate used as an electron acceptor, or benzoate used as an electron donor if crotonate was the reducible cosubstrate (reduced to butyrate).The $\Delta G°_f$ values are the one listed in Thauer et al. (44) except for the $\Delta G°_f$ of cyclohexane carboxylate which was calculated to be -281.2 $kJ.mol^{-1}$ based on the group contributions method of Mavrovouniotis (30).

MATERIALS AND METHODS

Media and cultivation conditions.

Pure cultures of *Syntrophus aciditrophicus* strain SB (ATCC 700169) were grown anaerobically in a basal medium with 20 mM crotonate as described previously (35). The media and stock solutions were prepared according to the anaerobic techniques described by Balch and Wolfe (5). All cultures were incubated at 37°C without shaking. The culture purity was checked daily by microscopic examination and inoculation of a thioglycolate medium.

To determine whether benzoate and crotonate were degraded simultaneously, triplicate serum bottles with 50 ml of medium containing 20 mM crotonate and 5 mM benzoate were inoculated with five ml of *S. aciditrophicus* culture grown with the same two substrates. One-ml samples were taken daily to measure growth, substrate depletion and product formation. When the molar growth yield was determined, one-ml samples were taken at initial time and after seven days of incubation to determine protein concentration, substrate depletion and product formation.

To establish the fate of the carbons derived from benzoate when used as a cosubstrate with crotonate, 300 ml cultures of *S. aciditrophicus* were grown with 20 mM crotonate and 5 mM [ring-^{13}C] benzoate, or 5 mM unlabeled benzoate. Samples (50 ml) were taken for GC-MS and ^{13}C-NMR analyses immediately after inoculation, after one day (when 30 % of benzoate and 5 % of crotonate were utilized), and after 7

days of incubation (when 99.8 % of benzoate and 98.6 % of crotonate were utilized). A heat-killed control and a substrate-unamended control were also analyzed as above.

To test the dependence of benzoate metabolism on the presence of crotonate, a pure culture of *S. aciditrophicus* was grown in a 1-liter of the basal medium with 20 mM crotonate. When the culture reached mid-log phase of growth, the cells were harvested by centrifugation at 14,300 × *g* at 4°C for 15 minutes. Cells were washed twice by suspending the pellet in anoxic phosphate buffer (25 mM, pH 7.0) with cysteine-HCL (0.5g/l) and Na_2S (0.5g/l) and centrifuged as above. The final pellet was resuspended in 50 ml medium lacking crotonate and benzoate. One ml of concentrated cells was used to inoculate 50 ml medium containing 3 mM benzoate and crotonate concentration ranging from 0 to 10 mM. Duplicate serum bottles were used for each condition. One set of triplicate bottles containing medium with 10 mM crotonate and without benzoate served as the positive control.

The degradation of benzoate by *S. aciditrophicus* in pure culture in the presence of electron donors other than crotonate was tested in 10-ml triplicate cultures. Initially, to remove traces of crotonate in the inoculum, cells grown with crotonate were collected by centrifugation nd washed as described above. The final pellet was resuspended in the basal medium lacking substrate and used as an inoculum. Each cosubstrate was added to an approximate concentration of 5 mM with 5 mM benzoate. The following cosubstrates were tested and added aseptically and anaerobically from stock solutions: succinate, formate, fumarate, malate, methylbutyrate, methylhexanoate, hexanoate, butyrate, stearate, and palmitate.

Growth was monitored and benzoate concentration was measured at initial time, after two weeks, and 6 weeks of incubation at 37°C. Growth with benzoate alone, and crotonate and benzoate were used as controls.

Preparation of culture fluids for ^{13}C NMR spectroscopy and GC-MS analyses.

Each 50-ml sample from *S. aciditrophicus* grown with 20 mM crotonate and 5 mM benzoate or 20 mM crotonate and 5mM [ring-^{13}C] benzoate was extracted with ethylacetate as described previously (12, 35). The samples were concentrated under vacuum and split into two sub-samples and each sub-sample was evaporated to dryness under a stream of nitrogen gas. One of the sub-samples was resuspended in 200 µl ethyl acetate, derivatized with 100 µl of *N,O*-bis-(trimethylsilyl)trifluoroacetamide (BSTFA), and used for GC-MS analysis. The other sub-sample was resuspended in one milliliter of deuterated methylene chloride and used for NMR analysis.

Analytical procedures.

Metabolite analyses by ^{13}C NMR spectroscopy and GC-MS were conducted as described previously (35). Concentrations of the substrates, products and metabolites were determined by high performance liquid chromatography as described previously (35). Growth was determined by monitoring change in optical density at 600 nm (32). Protein concentrations were determined by the method of Bradford (7) using the protocol and reagent from Pierce (Rockford, Il.) with bovine serum albumin as standard.

Chemicals.

Stable isotopes of benzoic acid (ring-^{13}C, 99%) and chloroform-D (D, 99.8%) were obtained from Cambridge Isotope Laboratories, Inc. (Andover, Mass). *N, O*-bis(Trimethylsilyl)trifluoroacetamide (BSTFA) was purchased from Pierce (Rockford, Il.).

Thermodynamic calculations.

The $\Delta G^{\circ\prime}$ were calculated by using the ΔG°_{f} available in Thauer et al. (44). The value of the ΔG°_{f} of cyclohexane carboxylate was calculated by the group contribution method of Mavrovouniotis (30).

RESULTS

Growth of *S. aciditrophicus* with benzoate and crotonate.

The effect of benzoate addition on the growth and metabolism of crotonate by pure cultures of *S. aciditrophicus* was determined. The growth rate of *S. aciditrophicus* in medium with 20 mM crotonate and 5 mM benzoate decreased compared to that of cultures grown with 20 mM crotonate alone (0.08 h^{-1} versus 0.025 h^{-1}, respectively) (Fig 2. 1). The maximum optical density obtained by the cultures under each growth condition was similar (about 0.40 units) although cultures grown with 20 mM crotonate and 5 mM benzoate required 16 days to reach this value compared to eight days for cultures grown with 20 mM crotonate alone (Fig 2. 1).

The substrate depletion pattern showed that both crotonate and benzoate were degraded simultaneously (Fig 2.2). The benzoate depletion rate was 1.01±0.05 mM d^{-1}, and the crotonate depletion rate within this same time frame was 1.39±1.8 mM d^{-1}. Once approximately 96 % of the benzoate was consumed, corresponding to seven days of incubation, the crotonate depletion rate increased to 2.0±0.35 mM d^{-1}. Acetate and cyclohexane carboxylate accumulated as benzoate and crotonate were used (Fig. 2. 2). No other fatty acid other than acetate was detected indicating that crotonate was not used as an electron acceptor as shown earlier for *Syntrophus buswellii* (2). After eight days of incubation, when about 99.3% of the benzoate was depleted, 4.0 ± 0.04 mM of benzoate and 4.8 ± 0.5 mM of crotonate were consumed and 4.12 ± 0.50 mM

Figure 2. 1. Growth of *S.aciditrophicus* in the presence of 20 mM crotonate (■) or 20 mM crotonate and 5 mM benzoate (●). The data are averages ± standard deviations of triplicate microcosms.

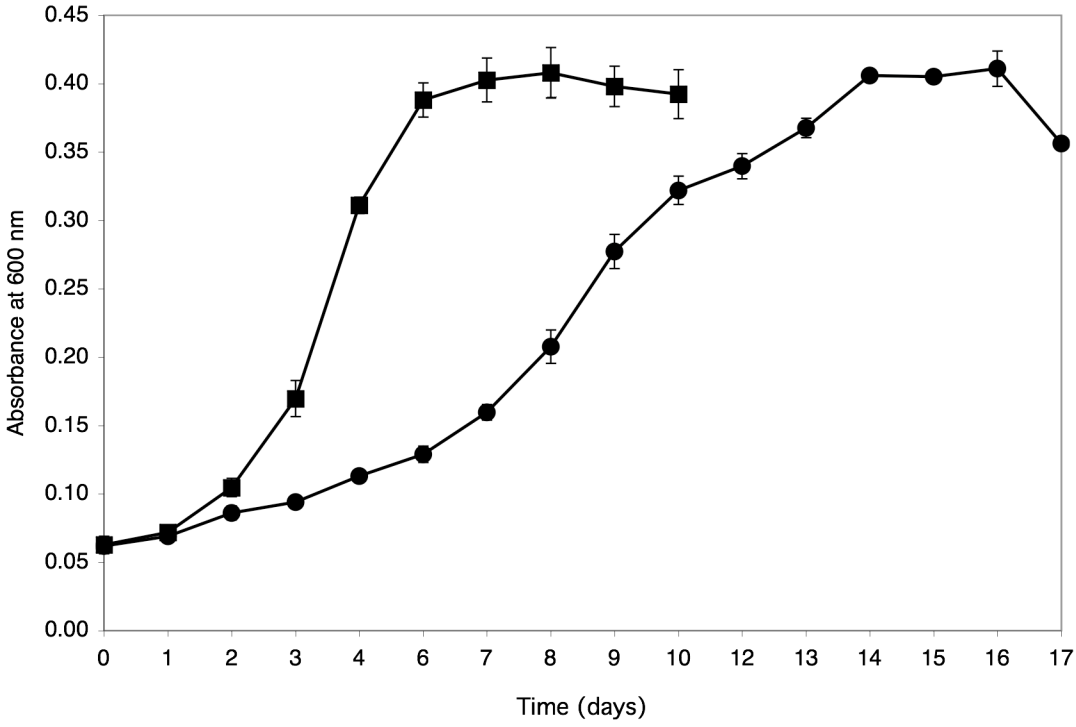
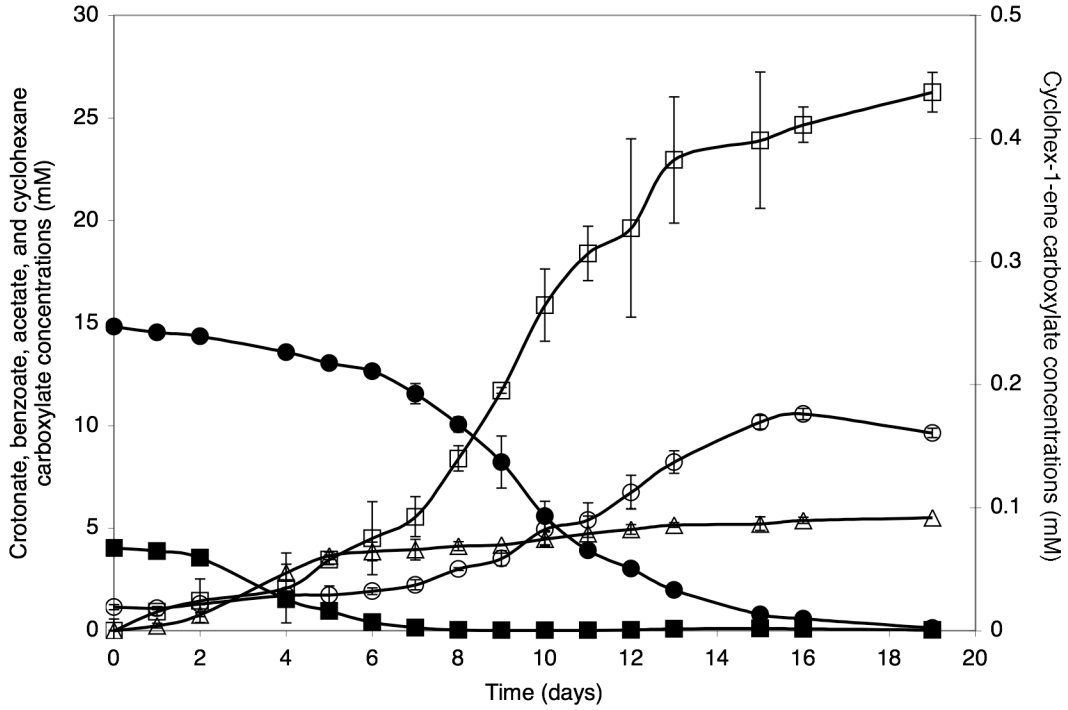

0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Absorbance at 600 nm
0
1
2
3
4
6
7
8
9
10
12
13
14
15
16
17
Time (days)

Figure 2. 2. Metabolism of crotonate in the presence of benzoate as an electron acceptor by *S. aciditrophicus*. Symbols: ●, crotonate; ■, benzoate; □, acetate; △, cyclohexane carboxylate; ○, cyclohex-1-ene carboxylate. The data are averages ± standard deviations of triplicate microcosms.


Crotonate, benzoate, acetate, and cyclohexane carboxylate concentrations (mM)
Cyclohex-1-ene carboxylate concentrations (mM)
Time (days)
0
5
10
15
20
25
30
0
0.1
0.2
0.3
0.4
0.5
0
2
4
6
8
10
12
14
16
18
20

of cyclohexane carboxylate and 8.40 ± 0.61 mM of acetate were formed (Fig. 2. 2). The carbon recovery was 97.0 % and the hydrogen recovery was 102.2 % at the eight-day time point. About 1.1 mol of cyclohexane carboxylate was made per mol benzoate used and about and 1.75 mol of acetate was made per mol of crotonate. These data are close to the theoretical ratios predicted for benzoate reduction coupled to crotonate oxidation (Table 2. 1, eq. 5). However, the ratio of crotonate oxidized to benzoate reduced (1.2) was much lower than the expected ratio of 3 (Table 2. 1, eq. 5). In other experiments, the ratio of crotonate oxidized to benzoate reduced varied from 1.2 to 2.4.

The amount of cellular protein made after seven days (e.g., when >97% of the benzoate was used) was 35.3 ± 1.1 g l^{-1}, which corresponded to 75.2 ± 2.3 g (dry wt) l^{-1} of cells assuming that 47% of the cell dry weight is composed of protein (16). In this experiment, 3.3 ± 0.25 mM of crotonate and 3.90 ± 0.10 mM of benzoate were consumed. The molar growth yield was 22.7 ± 2.1 g (dry wt) cell per mol of crotonate.

Substrate fate determined by GC-MS and ^{13}C-NMR.

Because cyclohexane carboxylate and acetate are end products of benzoate fermentation (13) and crotonate fermentation (35) by *S. aciditrophicus* grown in pure culture, the fate of the benzoate carbons was followed by using [ring-^{13}C] benzoate. Two major peaks were detected by the GC-MS chromatography in *S. aciditrophicus* cultures with 5 mM [ring-^{13}C] benzoate and 10 mM unlabeled crotonate immediately

after inoculation. The first peak had the same retention time (11.1 min) and mass spectrum profile (m/z-15 ion mass of 143) as the TMS-derivative of the authentic crotonate standard. The second peak had the same retention time (23.3 min) but with a mass spectrum profile with a 6 mass unit increase (m/z-15 ion of 185 compared to m/z-15 ion of 179) than the TMS-derivative of the benzoate authentic standard. These data are consistent with the TMS-derivative of [ring-^{13}C] labeled benzoate as the second peak.

After one day of incubation, 30 % of benzoate and 5 % of the crotonate were consumed and a third major peak was detected by GC-MS. The metabolite had the same retention time as the TMS-derivative of cyclohexane carboxylate (21.6 min). The m/z-15 ion of the metabolite was a single peak with a mass of 191 (Fig 2. 3B), which is 6 mass units greater than the TMS-derivative of the cyclohexane carboxylate authentic standard (m/z-15 of 185) (Fig 2. 3A). No peak with a mass ion of 185, which would correspond to unlabeled, TMS-derivatized cyclohexane carboxylate, was detected by GC-MS after one day of incubation (Fig 2. 3B).

To confirm that the cyclohexane carboxylate was made from [ring-^{13}C] benzoate and not from unlabeled crotonate, the cell-free culture fluid of *S. aciditrophicus* grown with 5 mM [ring-^{13}C] benzoate and 10 mM crotonate was analyzed by ^{13}C-NMR spectroscopy. Immediately after inoculation, the NMR spectrum contained two peak clusters centered around 129.4 and 133.8 ppm, which were multiplets, corresponding to C-1 and C-2 to C-6 of [ring-^{13}C] benzoate, respectively (Table 2. 2), and is similar to that previously data (12). After one day of

Figure 2. 3. Mass spectral profile of the TMS-derivatized A) cyclohexane carboxylate authentic standard; B) metabolite detected at the same GC retention time as the cyclohexane carboxylate authentic standard from the culture fluid from *S. aciditrophicus* grown with [ring-^{13}C] benzoate and unlabeled crotonate when 30 % of the ^{13}C-benzoate and 5% of the crotonate were consumed; and C) when 99.8 % of benzoate and 98.6 % of crotonate were consumed.

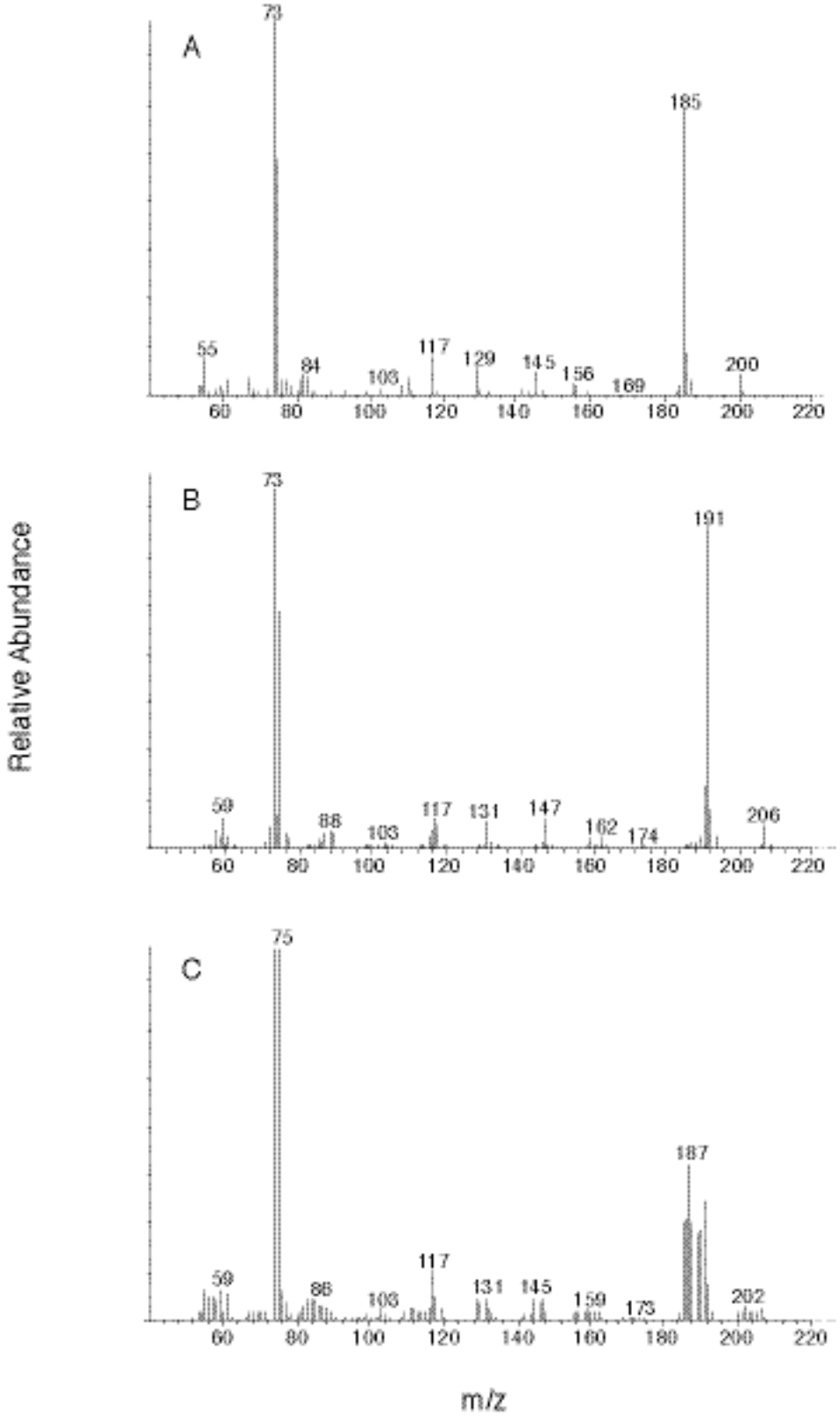

A
73
185
55
84
103
117
129
145
156
169
200
B
73
191
59
88
103
117
131
147
162
174
206
C
75
187
59
86
103
117
131
145
159
173
202
60
80
100
120
140
160
180
200
220
Relative Abundance
m/z

Table 2. 2. NMR data for compounds detected in culture of *Syntrophus aciditrophicus* grown in pure culture with 5 mM [ring-^{13}C] benzoate and 10 mM crotonate.

Compound detected	Position	δC^{13} Expected (ppm)[a]	δC^{13} Observed (ppm)	J_{cc} (Hz)
Benzoate	C-1	129.4	127.0	unlabeled
	C-2 to C-6	133.8	134.0	multiplets
Cyclohexane carboxylate	C-7	181.0	182.4	55.2 (7-1)[b]
	Ring C-1	41.5	43.0	33.2 (1-2), 55.2 (1-7)[b]
	Ring C-2, 6	26.6	29.0	33.2 (2-1, 2-3, 6-1, 6-5)
	Ring C-4	27.1	~25.0	unresolved peaks
	Ring C-3, 5	24.3	~25.0	unresolved peaks

[a] The predicted chemical shifts were obtained from ChemNMR (Upstream Solutions)

[b] The C-7 of cyclohexane carboxylate becomes ^{13}C-labeled after 7 days of incubation, whereas this peak does not exist after one day of incubation time.

incubation, the NMR spectrum of the culture fluid contained the above peak clusters corresponding to [ring-^{13}C] benzoate and a new set of peaks that were consistent with the presence of ^{13}C-labeled cyclohexane carboxylate (Table 2. 2). The unresolved peaks around 25.0 ppm corresponded to the C-3, C-4, and C-5 of cyclohexane carboxylate (12). The peak at 29.0 ppm corresponded to C-2, and C-6 of cyclohexane carboxylate. The values of these coupling constants were in agreement with published values (10) . These data show that the cyclohexane carboxylate that was formed after one day of incubation was ring-labeled and derived mainly from [ring-^{13}C] benzoate. No ^{13}C-labeled acetate or other metabolites were detected in the culture fluid after one day of incubation by ^{13}C-NMR analysis.

After seven days of incubation, when more than 98% of [ring-^{13}C] benzoate and crotonate were consumed, the mass spectral profile of the metabolite corresponding to TMS-derivatized cyclohexane carboxylate had a mixture of m/z-15 ions with masses ranging from 185 to 192, corresponding to an increase of 0 to 7 mass units (Fig 2. 3C) relative to the TMS-derivative of authentic cyclohexane carboxylate (Fig 2. 3A). These results indicated that the cyclohexane carboxylate formed after seven days was derived not only from [ring-^{13}C] benzoate but also from other labeled and non-labeled metabolites such as acetate or CO_2.

Consistent with this conclusion, ^{13}C-NMR analysis showed that, after seven days of incubation, C-7 of cyclohexane carboxylate was labeled and C-1 and C-2 displayed complex couplings. The complex coupling pattern was probably due to the mixture of cyclohexane carboxylate molecules with different numbers of ^{12}C and ^{13}C

atoms. No other metabolites including ^{13}C-labeled acetate were detected by ^{13}C-NMR at this time.

Intermediates detected during benzoate reduction to cyclohexane carboxylate.

In cultures of *S. aciditrophicus* grown with [ring-^{13}C] benzoate and unlabeled crotonate, after one day of incubation time, when approximately 70% of the benzoate was still present, the only metabolite detected by GC-MS in addition to cyclohexane carboxylate was a compound with the same retention time as TMS-derivatized cyclohex-1-ene carboxylate (24.4 min). The m/z-15 ion of this metabolite was 189, corresponding to a mass increase of 6 units compared to the authentic TMS-derivatized cyclohex-1-ene carboxylate (m/z-15 of 183). After seven days of incubation when over 98 % of the benzoate was degraded, the m/z-15 ion of metabolite corresponding to TMS-derivatized cyclohex-1-ene carboxylate had a mixture of peaks ranging from 183 to 190, corresponding to a mass increase of 0 to 6 or 7 mass units.

In cultures of *S. aciditrophicus* with non-labeled crotonate and benzoate, the concentration of cyclohex-1-ene carboxylate was 37 ± 4 μM during the first phase of growth when benzoate was still present. Once benzoate was almost fully consumed and crotonate was the dominant substrate, cyclohex-1-ene carboxylate transiently accumulated to a concentration of up to 176 ± 4 μM (Fig. 2. 2). This value is higher than that observed when *S. aciditrophicus* was grown with crotonate alone (88 μM recorded by Mouttaki et al. (35))

Trace amounts of metabolites with the same retention time as TMS-derivatized pimelate and glutarate authentic standards were detected by GC-MS after seven days of incubation but not after 1 day of incubation in *S. aciditrophicus* cultures grown with [ring-^{13}C] benzoate and unlabeled crotonate. The mass of the m/z-15 ions of glutamate ranged from 261 up to 266 (a mass increase of 0 to 5 relative to the authentic standard) while the mass of the m/z-15 ion of pimelate ranged from 289 up to 296 (a mass increase of 0 to 7 relative to the authentic standard).

Crotonate dependence for benzoate degradation.

To determine whether the degradation of benzoate by *S. aciditrophicus* was dependent on the presence of crotonate, crotonate-grown cells were washed with a phosphate buffer to remove any traces of crotonate or other metabolites and then used to inoculate fresh media containing 3 mM of benzoate and a range of crotonate concentrations (Table 2. 3). When no crotonate was added, no benzoate depletion was detected after five weeks of incubation. Only a small amount of benzoate (7.7% of the initial amounts added) was used when the 0.1 mM crotonate was added. When 0.5 mM of crotonate or more was added, almost all of the benzoate was used within 12 days of incubation (Table 2. 3). Benzoate degradation proceeded even after most of the crotonate was exhausted corresponding to when the initial concentration of crotonate was between 0.5 and 1mM. However, trace amounts of crotonate were always detected, which is probably due to the fact that crotonate is an intermediate of benzoate degradation.

Table 2. 3. Amounts of benzoate depleted with various amounts of crotonate when crotonate and benzoate were used as cosubstrates.

	Percent benzoate depleted[a]	
Initial amount of crotonate	After 5 days of incubation[b]	After 12 days of incubation
none	0 %	0 %
0.1mM	0 ±%	7.7 ± 1.0%
0.2 mM	3.5 ± 7.0 %	30.0 ± 7.2 %
0.5 mM	21.6 ± 3.8 %	79.8 ± 2.7 %
1 mM	59.4 ± 4.4 %	99.9 ± 0.1 %
2 mM	72.9 ± 0.1 %	99.9 ± 0.1 %
5 mM	66.2 ± 5.1%	99.1 ± 1.2 %
10 mM	81.3 ± 1.5 %	99.7 ± 0.4 %

[a] The initial amount of benzoate was around 3 mM.

[b] The values are averages of duplicate ± the standard deviations.

When initial concentrations of benzoate were approximately 3 mM and crotonate concentrations were below 1 mM, the ratio of cyclohexane carboxylate formed per benzoate degraded was about 0.5 (Table 2. 4). This data is close to the theoretical ratio predicted for benzoate fermentation (Table 2. 1, eq. 4). However, the ratio increased as the crotonate concentration increased, and approached a ratio of 1 when the crotonate concentration was 5 mM or greater (Table 2. 4). A ratio of 1 is closer to the theoretical ratio predicted for the use of benzoate use as an electron acceptor (Table 2. 1, eq. 5).

The dependence of benzoate degradation on the presence of crotonate was also tested with washed cell suspensions of *S. aciditrophicus* to determine whether crotonate was needed for benzoate metabolism or for growth. Benzoate metabolism was observed only when crotonate was added and benzoate was completely degraded within 44 hours (data not shown). In the absence of crotonate, no benzoate loss was detected after three days.

A number of possible electron donors including hydrogen, formate, malate, succinate, butyrate, methylbutyrate, hexanoate, methyl-hexanoate, palmitate, stearate, were tested. However, benzoate degradation was observed only when crotonate was added (data not shown).

Table 2. 4. Amount of substrates degraded and products formed and the ratio of cyclohexane carboxylate to benzoate when the concentration of crotonate was varied[a].

Initial amounts of crotonate [b]	Amount of crotonate used (mM)	Amount of benzoate used (mM)	Amount of acetate formed (mM)	Amount of CHAC formed (mM)	CHAC/ Benzoate ratio
0.5 mM	0.24±0.10	0.58±0.13	1.20±0.14	0.31±0.17	0.53
1 mM	0.39±0.01	1.73±0.21	2.73±0.14	0.95±0.04	0.55
2 mM	0.89±0.02	2.15±0.05	4.14±0.0	1.49±0.01	0.70
5 mM	1.79±0.05	2.07±0.15	5.04±0.0	1.96±0.01	0.95
10 mM	3.59±0.01	2.43±0.07	6.8±0.05	2.49±0.29	1.02

[a] Values are average ± standard deviations of duplicate cultures taken after five days of incubation.
[b] The initial benzoate concentration was 3 mM.

DISCUSSION

Here, we reported another fate for benzoate, the use of benzoate as an electron acceptor, in addition to syntrophic benzoate degradation (34, 39)) and benzoate fermentation (13). Previous studies showed that crotonate rather than benzoate was used as an electron donor by *Syntrophus buswellii* (1) or *Sporotomaculum syntrophicum* (37) when both substrates were present. In the presence of crotonate, *S. buswellii* and *S. syntrophicum* oxidized benzoate to acetate and CO_2 and reduced crotonate to butyrate. We found that *S. aciditrophicus* degraded benzoate and crotonate simultaneously. In medium with 20 mM crotonate and 5 mM benzoate, the growth rate of *S. aciditrophicus* decreased (0.008 h^{-1} compared to 0.025 h^{-1}, respectively) and the degradation rate of crotonate decreased (1.39 day^{-1} and 2.4 day^{-1}, respectively) compared to cultures grown with 20 mM crotonate alone. Also, the benzoate degradation rates increased when crotonate was present (1.01 day^{-1}) compared to benzoate alone (0.41 day^{-1}) (13). These data show that the physiology of *S. aciditrophicus* changed when both substrates were present.

^{13}C-NMR and GC-MS analyses showed that benzoate was reduced to cyclohexane carboxylate because ring-labeled cyclohexane carboxylate was formed and no [^{13}C] labeled acetate was detected when *S. aciditrophicus* was grown with [ring-^{13}C] benzoate and 10 mM crotonate. However, the ratio of crotonate oxidized to benzoate reduced ranged from 1.2 to 2.4, much less than the expected ratio of 3. The amount of acetate produced per crotonate used (about 1.75) was less than the

predicted ratio (2.0) (Table 2. 1, eq. 5). The metabolism of acetate could have provided the needed reducing equivalents for benzoate reduction. Thermodynamically, there does not appear to be an advantage to couple crotonate oxidation to benzoate reduction (Table 2. 1, eq. 5) versus crotonate fermentation to acetate and cyclohexane carboxylate (Table 2. 1, eq. 3). The standard free energy change for the first reaction is -40.5 kJ per mol of crotonate compared to a $\Delta G^{o'}$ of – 48.1 kJ per mol of crotonate for the second reaction. The free energy change for benzoate respiration was -122 kJ per reaction in growing cultures of *S. aciditrophicus*.

Interestingly, the molar growth yield was 22.7 ± 2.1 g (dry wt) of cells per mol of crotonate when *S. aciditrophicus* was grown with both crotonate and benzoate compared to about 14.0 ± 0.1 g (dry wt) of cells per mol of crotonate when *S. aciditrophicus* was grown with crotonate alone (35). An increase in the molar growth yield for crotonate in the presence of benzoate is surprising because the initial reduction of benzoyl-CoA is energy intensive and requires two molecules of ATP (6). Thus, one would expect that the molar growth yield for crotonate would decrease if benzoate is being reduced. The fact that it increases suggests that there is an energetic value to *S. aciditrophicus* to couple crotonate oxidation to benzoate reduction, consistent with this process being a respiration.

After seven days when almost all of the benzoate was reduced, trace amounts of ^{13}C-labeled pimelate and glutarate were detected and the carbon-7 of cyclohexane carboxylate was ^{13}C-labeled when *S. aciditrophicus* was grown with [ring-^{13}C] benzoate and non-labeled crotonate. It may be that the remaining benzoate was

oxidized resulting in the formation of these intermediate (13). However, very little benzoate was present when these intermediates were detected. It is unlikely that they were formed from ^{13}C-labeled cyclohexane carboxylate because cyclohexane carboxylate metabolism has not been detected by pure cultures of *S. aciditrophicus* (13). It is possible that exchange reactions occurred between cyclohexane carboxylate and the above intermediates, suggesting that at least part of benzoate degradation pathway may be reversible. The above intermediates were detected during syntrophic benzoate metabolism by cocultures of *S. aciditrophicus* with a methanogen and during benzoate or crotonate fermentation by pure cultures of *S. aciditrophicus* (12, 35). *Thermacetogenium phaeum* in coculture with a methanogen was able to immediately switch from syntrophic acetate oxidation to homoacetogenic acetate formation indicating that the entire enzyme apparatus used by *T. phaeum* appears to operate in a reversible manner (23). The reversibility of the above metabolisms suggests that they operate close to the thermodynamic equilibrium.

The concomitant degradation of benzoate and crotonate by *S. aciditrophicus* might be described as co-metabolism when the crotonate to benzoate ratio is less than one (Table 2. 4). Dalton and Stirling (9) described co-metabolism as the "transformation of a non-growth substrate in the obligate presence of a growth substrate or another transformable compound." Previous work showed that the function of the cosubstrate was to provide energy, cofactors, or metabolites for the transformation of the non-growth substrate. Co-metabolism was demonstrated in *Methylococcus capsulatus* where the degradation of various compounds such as n-

alkanes, haloalkanes, alkenes, ethers and aromatic alicyclic and heterocyclic compounds did not occur unless formaldehyde was present to supply reducing power (8, 40-43). The use of electron donor to provide reducing equivalents to facilitate aromatic compounds degradation has been described previously (19, 20, 28, 33). The oxidation of the co-substrate provided reducing equivalents that allowed the reductive elimination of substituent groups from the aromatic ring. Crotonate may supply the reducing power or energy needed to initiate the reduction of the aromatic ring. Small amount of crotonate (about 100 μM) were present in pure cultures of *S. aciditrophicus* that fermented benzoate (13). When *S. aciditrophicus* cells were washed to remove any medium components and metabolites, no benzoate degradation occurred after 5 weeks of incubation (Table 2. 3). However, if the inoculum had small amounts of crotonate (13) or small amounts of crotonate were added (0.1 to 0.5 mM) (Table 2. 3), benzoate was metabolized. Our data are similar to those of Ramos et al. (38) who showed that a small amount of glucose was needed for citrate degradation by *Lactococcus lactis* (38). Once citrate degradation was initiated, it proceeded after glucose was completely exhausted. The degradation of citrate did not occur in the absence of glucose.

Although cyclohexane carboxylate is not an intermediate in the benzoate degradation by denitrifiers of photosynthetic bacteria (29), cyclohexane carboxylate is an important metabolite in the syntrophic and fermentative metabolism of benzoate (12, 13). Earlier studies implicated cyclohexane carboxylate and cyclohex-1-ene carboxylate as intermediates in the anaerobic degradation of aromatic compounds by

phototrophic and denitrifying microorganisms (11). Keith et al. (25) found that both compounds were metabolized without any lag phase and at the same rate as benzoate. Cyclohexane carboxylate was detected in methanogenic enrichments that degraded β-phenylpropionate (4), ferulate and other monomers of lignin (21, 24), phthalate (27), and phenol (Jones and Suflita, unpublished data). The reduction of benzoate to cyclohexane carboxylate reported here and the accumulation of cyclohexane carboxylate during syntrophic benzoate metabolism (12) argues that cyclohexane carboxylate is not an artifact of aromatic compound degradation as proposed by Evans (14). It may be a mechanism for some bacteria to dispose of reducing equivalents when syntrophy is not possible (13). Earlier, Küver et al. (29) proposed that cyclohexane carboxylate was a repository of reducing equivalents during benzoate degradation. Because cyclohexane carboxylate accumulates to high concentrations during syntrophic and fermentative benzoate metabolism (12, 13), the detection of cyclohexane carboxylate in environments contaminated with aromatic compounds may indicate that anaerobic degradation of these compounds is occurring. The addition of a co-substrate e.g. crotonate, to such environments might facilitate anaerobic biodegradation.

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CHAPTER 3

The metabolism of hydroxylated and fluorinated benzoates supports a two-electron reduction mechanism for anaerobic benzoate metabolism by *Syntrophus aciditrophicus.*

ABSTRACT

The transformation of hydroxybenzoate and fluorobenzoate isomers was investigated in the strictly anaerobic *Syntrophus aciditrophicus* to gain insight into the initial steps involved in benzoate metabolism. 2-Hydroxybenzoate was metabolized to methane and acetate by *S. aciditrophicus* and *M. hungatei* cocultures, and reduced to cyclohexane carboxylate by pure cultures of *S. aciditrophicus* when grown in the presence of crotonate. Under both conditions, transient accumulation of benzoate but not phenol was observed, indicating that dehydroxylation occurred prior to ring reduction. Pure cultures of *S. aciditrophicus* reductively dehalogenated 3-fluorobenzoate with the stoichiometric accumulation of benzoate and fluorine. A metabolite was detected in the 3-fluorobenzoate-degrading cultures that had a fragmentation pattern almost identical than that of 3-fluorobenzoate TMS-derivative, but with a mass increase of 2 units or 3 and 4 units when cells were incubated with deuterated water. The mass spectral data are consistent with the addition of two

hydrogen or deuterium atoms to 3-fluorobenzoate, probably forming a 3-fluorocyclohex-diene carboxylate, TMS-derivative. The transient accumulation of benzoate during 2-hydroxybenzoate metabolism and the accumulation of a diene intermediate during 3-fluorobenzoate metabolism supports the hypothesis that the initial step in benzoyl-CoA metabolism in *S. aciditrophicus* is a two-electron reduction to cyclohex-1,5-diene carboxyl-CoA.

Keywords: benzoate metabolism, diene intermediate, fluorinated analogs, 2-hydroxybenzoate

INTRODUCTION

In anaerobic environments, the biodegradation of aromatic compounds plays an important role in the cycling of carbon and the bioremediation of environmental contaminants (19, 20, 22, 31). The benzene ring is the main structural component of lignin, which comprises about 30% of plant material and is the second most abundant polymer in nature next to cellulose. A wide variety of complex mononuclear aromatic compounds are transformed by microorganisms though oxidation or reduction of substituent groups, carbon-carbon cleavage of substituents from the ring, decarboxylations, and the removal of O-methyl, sulfur, nitrogen and halogens from the ring (7, 23, 24, 26, 47, 49). The conversion of these aromatic compounds generally lead to the formation of benzoate or its coenzyme A (CoA). Then the benzoate pathway is the main route for aromatic ring degradation via ring reduction, ring cleavage and β oxidation (25).

The reduction of benzoyl-CoA represents a considerable energy barrier for anaerobic microorganisms because of the high resonance energy that stabilize the aromatic ring (6, 7, 26). In *Thauera aromatica*, to overcome this energy barrier, benzoyl-CoA reduction was shown to require the hydrolysis of two molecules of ATP per electron pair (5). In methanogenic environments, defined syntrophic cocultures of hydrogen/formate-producing microorganisms and hydrogen/formate-using methanogens degrade benzoate (28, 37, 43, 46). The degradation of benzoate to

acetate, carbon dioxide and hydrogen (equation 1) is unfavorable unless the hydrogen partial pressure is kept low, <1 Pa, by the methanogen (28, 38, 45, 46).

$$C_6H_5O_2^- + 7\ H_2O \rightarrow 3\ C_2H_3O_2^- + HCO_3^- + 3\ H^+ + 3\ H_2 \quad (1)$$

$\Delta G^{o\prime}$ = + 70.1 kJ/mole of benzoate (48)

If benzoyl-CoA is metabolized by the ATP-intensive, benzoyl-CoA reductase as found in denitrifiers and photosynthetic bacteria, then it is not clear how net energy production can occur during syntrophic benzoate metabolism. Benzoate activation to its CoA ester, benzoyl-CoA reduction and hydrogen production from acyl-CoA intermediates would require more ATP (>4 ATP per benzoate) than is produced by the known ATP-yielding reactions, i.e. substrate-level phosphorylation from acetate (3 ATPs), proton translocation by a membrane-bound pyrophosphatase (equivalent to 1/3 ATP per benzoate), and the decarboxylation of glutaconyl-CoA through a sodium-ion translocation (1/3 of an ATP per benzoate) (1, 5, 12-14, 46). This raises the question of whether ring reduction requires ATP or proceeds by another mechanism (41, 46).

Schöcke and Schink (46) propose that benzoate is reduced by a four- or six-electron reduction reaction. The reduction of benzoate to cyclohex-1-ene-1-carboxylate ($\Delta G^{o\prime}$ of -71.3 kJ/mol) or cyclohexane-1-carboxylate ($\Delta G^{o\prime}$ of -94.5 kJ/mol) is favorable (17, 46). The inability to detect an ATP-requiring, benzoyl-CoA reductase activity in syntrophic benzoate degraders (40, 46) or similar gene to the benzoyl-CoA reductase subunits *bad*DEFG of *Rhodopseudomonas palustris* within the genome of *Syntrophus aciditrophicus* (15, 35) and the transient accumulation of

large amounts of cyclohexane-1-carboxylate during syntrophic benzoate metabolism (16) support this hypothesis. Alternatively, Peters et al. (41) showed that benzoate metabolism by *Desulfococcus multivorans* required molybdenum and selenium. They proposed that a molybdenum-selenocysteine enzyme is involved in benzoate ring hydroxylation and that hydroxylation of the benzoate ring was needed prior to its reduction. Such addition will weaken the aromatic character of the ring and reductive dearomatization by electron donor such as ferredoxin would not need to be coupled to ATP hydrolysis (30, 44).

Halogenated compounds introduction to natural habitats has been of major concern due to their release in high concentrations (29). Fluorinated aromatic compounds are the results of the plastic, agricultural, and pharmaceutical industries (3, 50). A lot of attention has been given to microbial degradation of chlorinated compounds, however little was done on the degradation of organofluorine compounds. Fluorine atoms are in size 20 % bigger than hydrogen atom and due to their pronounced electro-negativity fluorine has a strong polarizing effect (18, 29). As a result, fluorinated analogs can be metabolized to a metabolic stage where the strong electronic influence of the fluorine substituent can slow or prevent further degradation of a metabolite and allow its temporary accumulation in a culture. For such reasons, fluorine analogs of aromatics has been frequently used to investigate the transformation of certain metabolites (18, 21, 34).

Mono-substituted hydroxybenzoate isomers are important intermediate metabolites of aerobic and anaerobic microbial degradative pathways of a wide range

of aromatic hydrocarbons (26). Under denitrifying, photosynthetic, and fermenting conditions, all three hydrobenzoate isomers were shown to be activated to their CoA derivative ester, followed by a reductive dehydroxylation leading to the formation of the benzoyl-CoA intermediate (25, 26, 40). Brauman et al. (10) have isolated a strictly anaerobic bacterium *Sporotomaculum hydroxybenzoicum* able to ferment 3-hydroxybenzoate to acetate, butyrate, and CO_2 in pure culture ($\Delta G^{o'}$ = - 38 kJ per mole of 3-hydroxybenzoate).

Here we use fluorinated and hydroxylated compounds to investigate the early metabolites formed during benzoate degradation in the presence of crotonate as a cosubstrate. GC-MS analyses were performed to detect such metabolites.

MATERIALS AND METHODS

Media and cultivation conditions.

Pure cultures of *Syntrophus aciditrophicus* strain SB (ATCC 700169) and *Methanospirillum hungatei* (ATCC 27890) were grown anaerobically as described previously (16). The media and stock solutions were prepared according to the anaerobic techniques described by Balch and Wolfe (2). All cultures were incubated at 37°C without shaking. The culture purity was checked daily by microscopic examination and inoculation of a thioglycolate medium.

To determine if pure cultures of *S. aciditrophicus* metabolize aromatic compounds other than benzoate when grown in the presence of crotonate, triplicate 10-ml cultures with 10 mM crotonate and 2 mM of the aromatic compound were each inoculated with one milliliter of a pure culture of *S. aciditrophicus* grown with 20 mM crotonate. The following aromatic compounds were tested: *o*-phthalate, 2-fluorobenzoate, 3-fluorobenzoate, 4-fluorobenzoate, *p*-aminobenzoate, 2-chlorobenzoate, 3-chlorobenzoate, 4-chlorobenzoate. 2-hydroxybenzoate, 3-hydroxybenzoate, 4-hydroxybenzoate, and *o*-toluate. Growth was monitored and compared to growth with crotonate alone or crotonate with benzoate. Additionally, samples were taken immediately after inoculation and after 16 days of incubation to measure substrate depletion.

The degradation of hydrobenzoate isomers by pure cultures and cocultures of *S. aciditrophicus* was also followed in triplicate 50-ml cultures with 10 mM crotonate

and 2 mM of the hydroxybenzoate. Each culture was inoculated with 5 ml of a culture of a pure culture of *S. aciditrophicus* grown with 20 mM crotonate. Cocultures were established by co-inoculation of the serum bottles with 5 ml of a pure culture of *M. hungatei*. Heat-killed controls were included for each condition. One ml samples were taken daily to measure substrate depletion and product formation. Methane formation by cocultures was measured by daily headspace analysis.

To test whether benzoate and 2-hydroxybenzoate were simultaneuously used, pure cultures of *S. aciditrophicus* was grown in duplicate, 500-ml cultures with 10 mM crotonate, 2 mM 2-hydroxybenzoate, and 2 mM [ring-^{13}C] benzoate. Samples (50 ml) were taken daily for GC-MS analyses as described previously (39).

The metabolism of fluorinated benzoate isomers by pure cultures of *S. aciditrophicus* was followed as described above. Each serum bottle contained 50 ml of medium with 10 mM crotonate and 0.5 mM of 2-fluorobenzoate, 3-fluorobenzoate, or 4-flurobenzoate. Heat- killed controls were included for each condition and served as negative controls. Growth in medium with 10 mM crotonate or with 10 mM crotonate and 3 mM benzoate served as positive controls. One-milliliter samples were taken daily to monitor growth, substrate depletion, and products formation. Hydrogen formation was measured in the headspace. Larger volume cultures (300 ml) were used to detect the formation of metabolites. Fifty-milliliter samples were taken initially and after 7 days of incubation for GC-MS analyses (39).

Metabolite formation by washed cell suspensions of *S. aciditrophicus* was also followed in the presence of deuterated water. The pure culture of *S.*

aciditrophicus was grown in one-liter of medium with 20 mM crotonate. At mid log phase of growth, cells of *S. aciditrophicus* were harvested by centrifugation at 14,300 g x 20 min x 4°C, and washed twice with 50 mM phosphate buffer pH 7.0 by resuspending the pellet and centrifuging as described above. The final cell pellet was resuspended in 10 ml of anoxic, phosphate buffer (pH 7.0) and 5 ml was used as an inoculum. Duplicate washed cell suspensions (50 ml final volume) each contained 45 ml of deuterated water, 2.5 ml each of Pfennig I (50 mg/l of K_2HPO_4), Pfennig II (33 mg/l of $MgCl_2$, 40 mg/l of NaCl, 40 mg/l of NH_4Cl, and 5 mg/l $CaCl_2$), resazurin (10 mg/liter), cysteine-HCL (0.5 g/liter), Na_2S (0.5 g/liter), 0.5 mM of 3-fluorobenzoate, and 10 mM crotonate. Twenty-five ml samples were taken initially and after 2 days of incubation at 37°C for GC-MS analyses (39).

Analytical procedures.

Growth was monitored by measuring optical density at 600 nm. Acetate was analyzed with a gas chromatography apparatus equipped with a flame ionization detector and a glass column (2 m × 2 mm) packed with 80/120 Carbopack B-DA/ 4 % Carbowax 20M. The carrier gas was nitrogen with a flow rate set at 24 ml/min. The isothermal column temperature was 155°C. The injector and the detector temperatures were 200°C. Each sample contained 30 mM oxalic acid. Fluoride was analyzed by ion-exchange chromatography with a Dionex system (Dionex Corporation, Sunnyvale, CA) equipped with an AS-4A column (4 mm particle size) and bicarbonate buffer as the mobile phase at a flow rate of 2 ml/min. The

concentrations of the other non-gaseous products and the substrates were analyzed by high performance liquid chromatography (HPLC) as described previously (39).

Hydrogen was quantified by gas chromatography equipped with a mercury vapor detector (RGA3 Reduction Gas Analyzer, Trace Analytical, Menlo Park, CA).

Methane was measured by gas chromatography with a flame ionization detection equipped with Poropak Q, 80/100 column (6 feet x 1/8 inch) (Supelco, Bellefonte, PA). The injector temperature was set at 100°C, the column at 100°C and the detector at 125°C. Helium was used as a carrier gas.

RESULTS

Transformation of aromatic compounds by *S. aciditrophicus*.

A number of aromatic compounds were tested to determine if they could be metabolized by pure cultures of *S. aciditrophicus* grown with crotonate. The growth of *S. aciditrophicus* in pure culture with crotonate was not affected by the addition of the following aromatic compounds: *o*-phthalate, 4-fluorobenzoate, para-aminobenzoate, 2-chlorobenzoate, 3-chlorobenzoate, 4-chlorobenzoate, 3-hydroxybenzoate, 4-hydroxybenzoate, and ortho-toluate (Table 3. 1). In each case, *S. aciditrophicus* reached a maximum absorbance at 600 nm of 0.22 and 0.26 units within ten days of incubation. Furthermore, no substrate depletion was detected after 16 days of incubation. However, when 2- fluorobenzoate, 3-fluorobenzoate, or 2-hydroxybenzoate was present, the growth of *S. aciditrophicus* with crotonate was inhibited (Table 3. 1) and transformation of the aromatic compound was observed (see below).

Degradation of 2-hydroxybenzoate in the presence of crotonate by *S. aciditrophicus* in pure culture or in coculture with *Methanospirillum hungatei*.

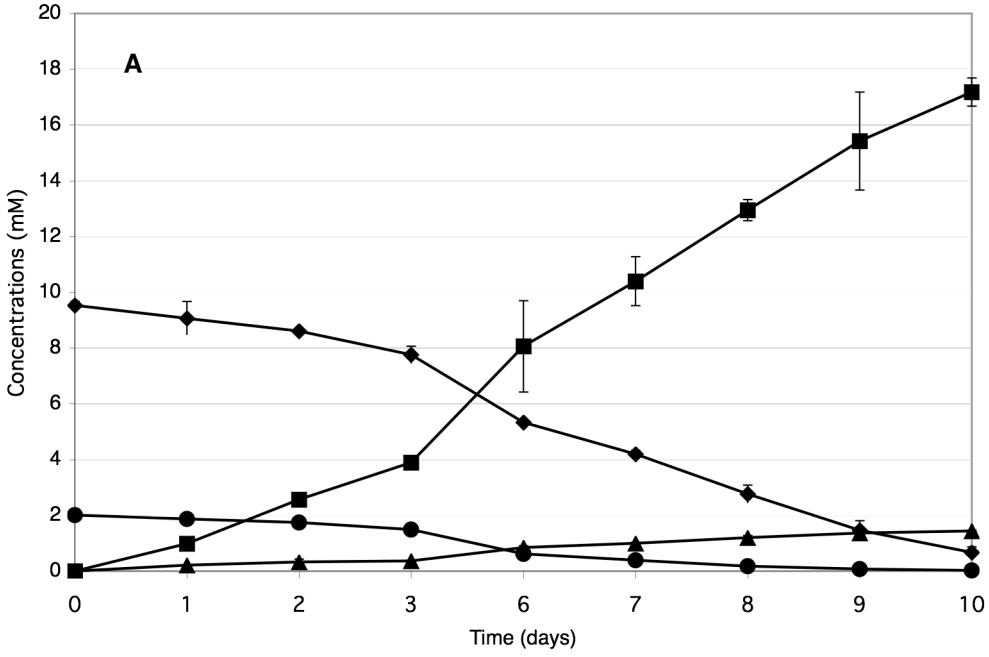
Pure cultures of *S. aciditrophicus* degraded crotonate and 2-hydroxybenzoate simultaneously (Fig. 3. 1). After ten days of incubation, 8.9 ± 0.11 mM of crotonate and

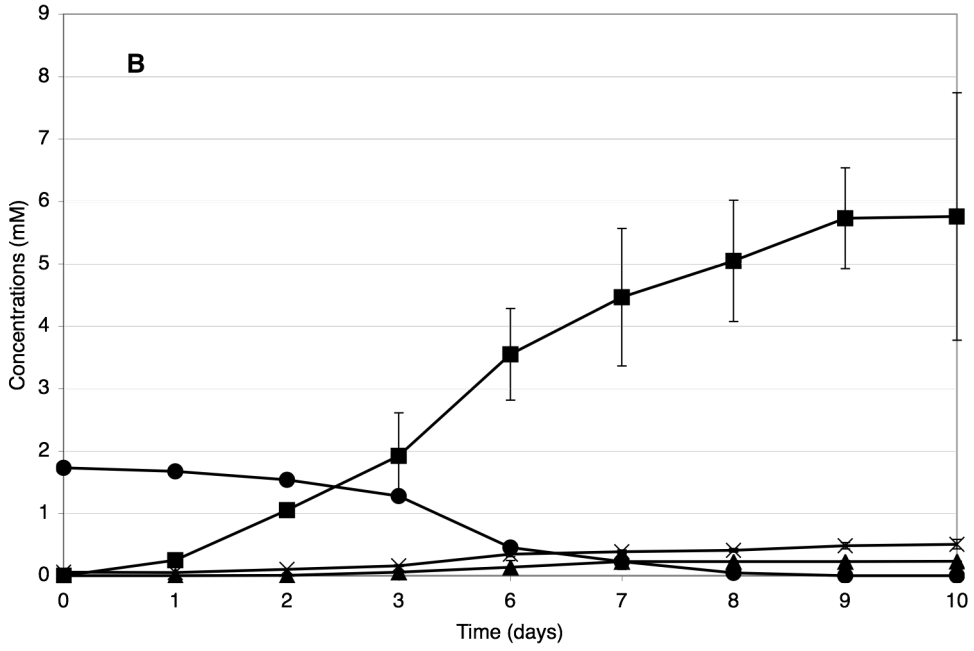
Table 3. 1. Growth of *S. aciditrophicus* with 10 mM crotonate and different cosubstrates.

Cosubstrates	Maximum absorbance reached after 10 days of incubation
None	0.257 ± 0.019
Benzoate	0.097 ± 0.001
o-Phthalate	0.239 ± 0.015
2-Fluorobenzoate	0.045 ± 0.005
3-Fluorobenzoate	0.044 ± 0.005
4-Fluorobenzoate	0.219 ± 0.018
p-Aminobenzoate	0.244 ± 0.008
2-Chlorobenzoate	0.247 ± 0.016
3-Chlorobenzoate	0.205 ± 0.007
4-Chlorobenzoate	0.236 ± 0.016
2-Hydroxybenzoate	0.225 ± 0.006
3-Hydroxybenzoate	0.253 ± 0.009
4-Hydroxybenzoate	0.263 ± 0.012
o-Toluate	0.260 ± 0.014

Data are mean ± std dev of triplicates

Figure 3. 1. Metabolism of 2-hydroxybenzoate by *S. aciditrophicus* A) in pure culture with crotontate as the cosubstrate, and B) in coculture with *M. hungatei*. The data are averages ± standards deviations of triplicate cultures. Symbols: ●, 2-hydroxybenzoate; ○, crotonate; ▲, cyclohexane carboxylate; ■, acetate; □, benzoate; ◆, methane.


A
Concentrations (mM)
Time (days)
0
1
2
3
6
7
8
9
10
0
2
4
6
8
10
12
14
16
18
20


B
Concentrations (mM)
Time (days)
0
1
2
3
6
7
8
9
10
0
1
2
3
4
5
6
7
8
9

2.0 ± 0.06 mM of 2-hydroxybenzoate were degraded and 17.2 ± 0.50 mM of acetate and 1.44 ± 0.04 mM of cyclohexane carboxylate were formed. Benzoate transiently accumulated to a concentration of 0.05 ± 0.02 mM, The carbon recovery was 90 % and the hydrogen recovery was 88 %. No degradation of the two substrates occurred in the heat-killed controls. Acetate, benzoate and cyclohexane carboxylate were the metabolites detected during crotonate and 2-hydroxybenzoate degradation. In the absence of crotonate, no degradation of 2-hydroxybenzoate by pure cultures of *S. aciditrophicus* was observed during the three-week incubation period (data not shown).

The above stoichiometry is consistent with a coupled process where crotonate acts as the electron donor (equation 2) and 2-hydroxybenzoate acts as the electron acceptor (equation 3); equation 4 gives the overall reaction:

Crotonate oxidation

$$C_4H_5O_2^- + 2\ H_2O \rightarrow 2\ C_2H_3O_2^- + H^+ + 2\ H \qquad (2)$$

($\Delta G°' = -21.8$ kJ per mole of crotonate) (48)

2-Hydroxybenzoate reduction

$$C_7H_5O_3^- + 8\ H \rightarrow C_7H_{11}O_2^- + H_2O \qquad (3)$$

($\Delta G°' = -96.7$ kJ per mole of hydrobenzoate)

Overall equation

$$C_7H_5O_3^- + 4\ C_4H_5O_2^- + 7\ H_2O \rightarrow C_7H_{11}O_2^- + 8\ C_2H_3O_2^- + 4\ H^+ \qquad (4)$$

($\Delta G°' = -183.9$ kJ per mole of hydrobenzoate)

S. aciditrophicus and *M. hungatei* cocultures formed methane and acetate from 2-hydroxybenzoate (Fig. 3. 1). After ten days of incubation, 1.7 ± 0.07 mM 2-hydroxybenzoate was consumed and 5.8 ± 2 mM of acetate and 0.5 ± 0.03 mM of methane accumulated. Benzoate and cyclohexane carboxylate transiently accumulated up to 0.02 ± 0.0004 mM. The percent carbon recovery was 101 % and the percent hydrogen recovery was 106 %. The overall equation of methanogenic degradation of 2-hydroxybenzoate can be expressed as following:

$$2\ C_7H_5O_3^- + 9\ H_2O \rightarrow 6\ C_2H_3O_2^- + 5\ H^+ + HCO_3^- + CH_4 \qquad (5)$$

($\Delta G^{\circ\prime}$= -38.1 kJ per mole of hydroxybenzoate)

The theoretically expected ratios of acetate or methane per 2-hydroxybenzoate are 3 and 1, respectively. The observed ratios of acetate or methane per 2-hydroxybenzoate were 3.4 and 0.3, respectively.

3-Hydroxybenzoate and 4-hydroxybenzoate were not degraded by *S. aciditrophicus* in pure culture with or without the presence of crotonate, or by cocultures *S. aciditrophicus* with *M. hungatei* (data not shown).

Reductive dehydroxylation of 2-hydroxybenzoate.

When pure cultures of *S. aciditrophicus* were grown with 15 mM crotonate. 2 mM [ring-^{13}C] benzoate, and 2 mM 2-hydroxybenzoate, crotonate and benzoate were degraded simultaneously while 2-hydroxybenzoate concentration remained unchanged (data not shown). Cyclohexane carboxylate and acetate were formed during benzoate and crotonate degradation as seen previously (Chapter 2). After one

day of incubation, the only ^{13}C-labeled metabolite detected was cyclohexane carboxylate. It had a m/z-15 ion of 191, which corresponds to a mass increase of +6 units compared to the authentic standard of TMS-derivatized cyclohexane carboxylate and is consistent with the formation of cyclohexane carboxylate from ^{13}C-labeled benzoate as shown in Chapter 2. ^{13}C-Labeled benzoate was completely depleted from the culture after four days of incubation. Once benzoate was depleted, 2-hydroxybenzoate started to be degraded. Peaks indicating the presence of phenol or 2-hydroxycyclohexane carboxylate were not detected by GC-MS analysis. However, the newly formed benzoate was not ^{13}C-labeled (the TMS-derivatized benzoate had a m/z-15 ion of 179 compared to 185 mass units). The ratio of the m/z-15 ion of ^{13}C-labeled versus that of non-labeled cyclohexane carboxylate decreased as 2-hydroxybenzoate was depleted from the culture medium. These results are consistent with the dehydroxylation of 2-hydroxybenzoate to benzoate (or its CoA derivative) prior to ring reduction. Once all the crotonate was gone, no further metabolism of 2-hydroxybenzoate was observed.

Reductive defluorination of 3-fluorobenzoate.

Growth and crotonate metabolism of pure cultures of *S. aciditrophicus* was inhibited when 0.5 mM of 2- fluorobenzoate or 3-fluorobenzoate was added (Table 3. 2). After ten days of incubation, pure cultures of *S. aciditrophicus* used 0.13 ± 0.01

Table 3. 2. Stoichiometry of crotonate metabolism alone or in the presence of benzoate or fluorobenzoate isomers by *S. aciditrophicus* grown in pure culture.

Culture	Maximum OD_{600}[a]	Initial crotonate conc (mM)	Final crotonate conc (mM)	Initial cosubstrate conc (mM)	Final cosubstrate conc (mM)	Final acetate conc (mM)	Final CHAC conc (mM)	Final Fluoride conc (mM)	Benzoate formed (mM)	Final H_2 (Pa)
Crotonate alone	0.24±0.002	7.13±0.21	0.02±0.0	NA	NA	10.4±2.09	1.16±0.08	NA	0.036±0.004	9.4±1.2
Crotonate +benzoate	0.25±0.007	7.8±0.44	0.10±0.05	1.82±0.12	0.03±0.0	15.1±0.90	1.67±0.26	NA	NA	8.9±2.3
Crotonate + 2-Fluorobenzoate	0.09±0.003	8.56±0.48	6.37±0.49	0.48±0.03	0.42±0.04	2.19±2.05	0.17±0.009	0.06±0.004	0.040±0.002	87.4±7.8
Crotonate + 3-Fluorobenzoate	0.08±0.003	8.22±0.58	4.54±0.70	0.34±0.02	0.21±0.03	5.66±0.26	0.53±0.14	0.15±0.02	0.17±0.0	116.6±13.9
Crotonate + 4-Fluorobenzoate	0.25±0.004	6.74±0.95	0.02±0.002	0.35±0.05	0.34±0.02	9.9±1.80	1.3±0.20	0.005±0.14	0.042±0.012	7.8±0.8

[a]The maximum absorbance was reached within six days for the cultures grown with crotonate alone or crotonate and 4-fluorobenzoate, and within ten days for the cultures grown with crotonate and benzoate, 2-fluorobenzoate, or 3-fluorobenzoate.
The values are the averages ± standard deviations of triplicate cultures measured after ten days of incubation.
Abbreviations: OD, optical density; conc, concentration; CHAC, cyclohexane carboxylate; NA, not applicable.

mM of 3-fluorobenzoate and formed 0.15 ± 0.02 mM of fluoride and 0.17 ± 0.0 mM benzoate, indicating that dehalogenation of 3-fluorobenzoate occurred. Some dehalogenation of 2-fluorobenzoate was also observed since a loss of 0.06 ± 0.06 mM of 2-fluorobenzoate and the formation of 0.06 ± 0.04 mM of fluoride and 0.04 ± 0.002 mM benzoate was observed. The addition of 4-fluorobenzoate had no effect on growth or crotonate metabolism and little if any 4-fluorobenzoate degradation was observed after 17 days of incubation (Table 3. 2). The small amount of benzoate made in cultures with 4-fluorobenzoate could be due to crotonate metabolism (Mouttaki et al, 2007).

The final partial pressure of hydrogen was 87.4 ± 7.8 Pa and 116.6 ± 13.9 Pa when 2- or 3-fluorobenzoate were present, respectively, compared to final partial pressures of 7.8 ± 0.8 Pa, 9.4 ± 1.2 Pa, and 8.9 ± 2.3 Pa when crotonate and 4-fluorobenzoate, crotonate alone, and crotonate and benzoate, respectively were used as substrates (Table 3. 2).

Diene as an intermediate of benzoate metabolism.

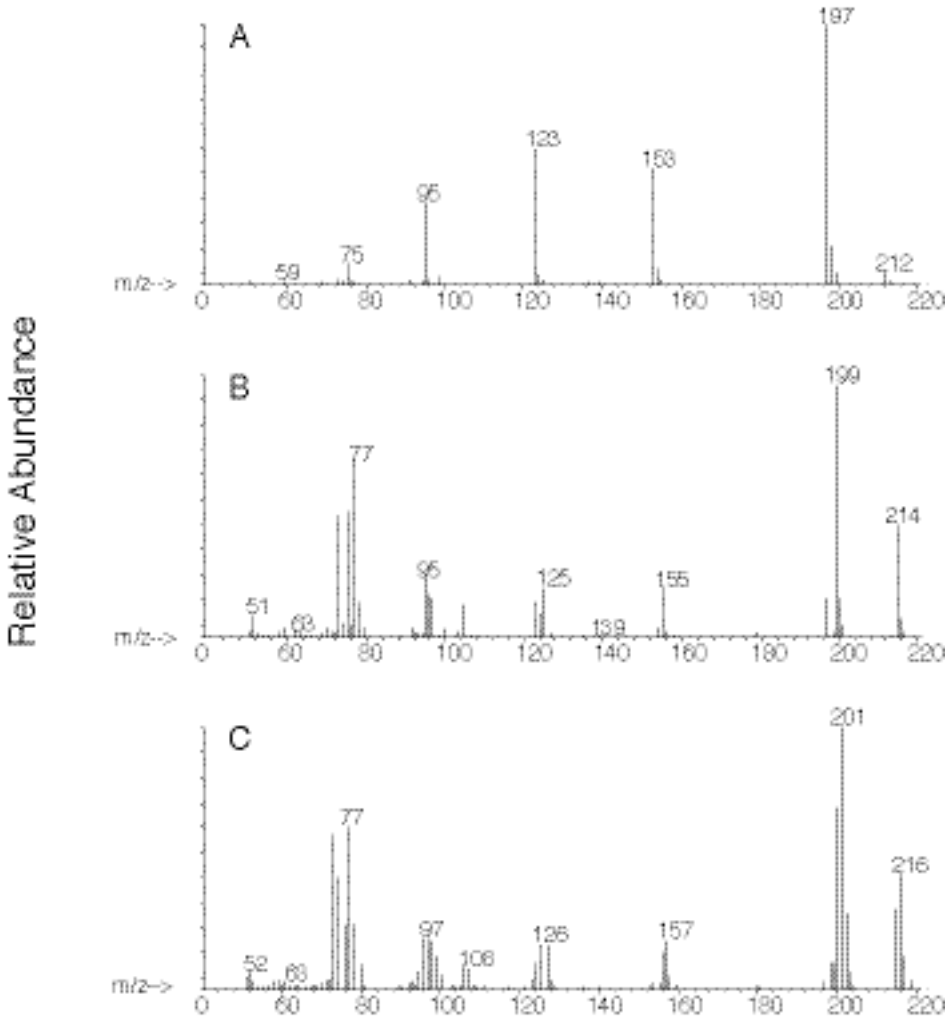
Isomers of hydroxylated and fluorinated benzoate were tested in order to detect metabolites of benzoate metabolism. Since 2-fluorobenzoate and 3-fluorobenzoate affected growth and metabolism of *S. aciditrophicus*, these substrates were used as cosubstrates in large volume cultures in order to detect benzoate metabolites. Initially, peaks with the same mass spectrum profiles and retention time as the TMS-derivative of the substrates (crotonate and 2-, 3-, or 4-fluorobenzoate),

cyclohexane carboxylate, benzoate, cyclohex-1-ene carboxylate, glutarate, and pimelate, were detected. The presence of the latter compounds was due to their presence in the inoculum of *S. aciditrophicus* grown with crotonate. After 12 and 26 days of incubation, no additional peaks were detected by GC-MS when either 2-fluorobenzoate or 4-fluorobenzoate were used as cosubstrates. However, in cultures with 3-fluorobenzoate and crotonate, the transient formation of a new metabolite was observed. The retention time of the TMS-derivative of 3-fluorobenzoate was 22.8 min and the masses of its m/z ion fragments were 75, 95, 123, 153, 197 (m/z-15), and 212 (total mass ion) (Fig. 3. 2A). After 12 days of incubation, a new peak with a retention time of 25.07 min was observed with major m/z ion fragments of 77, 95, 105, 125, 155, 199 (m/z-15), and 214 (total mass ion) mass units (Fig. 3. 2B). The fragmentation pattern was almost identical than that of 3-fluorobenzoate TMS-derivative, but with a mass increase of 2 units. This is consistent with the addition of two hydrogen atoms to 3-fluorobenzoate, probably forming a 3-fluorocyclohex-diene carboxylate, TMS-derivative. However, no chemical standard is available.

In order to obtain further evidence for the formation of a diene intermediate, the metabolism of crotonate and 3-fluorobenzoate by washed cell suspension of *S. aciditrophicus* was conducted in the presence of deuterated water. Initially, two peaks were observed by GC-MS, one corresponding to the TMS-derivative of crotonate and the second one corresponding to the TMS-derivative of 3-fluorobenzoate. After two days of incubation, several new peaks were detected by GC-MS with the same retention times and mass spectrum profiles as the TMS-derivatives of intermediates

of crotonate and benzoate metabolism, including pimelate, glutarate, cyclohexane carboxylate, cyclohex-1-ene carboxylate, 3-hydroxybutyrate, and benzoate, with benzoate being the major peak. One additional peak was present with a retention time of 25.07 min, the same as the TMS-derivative of the putative diene intermediate. The major m/z ion fragments were 77, 96, 106, 126, 156, 200 and 201 (m/z-15), and 215 and 216 (total mass ion) mass units (Fig. 3. 2C). This corresponds to an increase of 1 or 2 mass units compared to the putative diene intermediate detected, consistent with the addition of one or two deuterium atoms.

Figure 3. 2. Mass spectrum of the TMS derivative of A) 3-fluorobenzoate, B) the metabolite with a the retention time of 25.07 min detected in cultures grown with 3-fluorobenzoate and crotonate, and C) the metabolite detected in washed cell suspensions incubated in the presence of deuterated water with 3-fluorobenzoate and crotonate.


Relative Abundance
A
B
C
m/z-->
0
60
80
100
120
140
160
180
200
220
59
75
95
123
153
197
212
51
63
77
95
125
139
155
199
214
52
63
77
97
106
126
157
201
216

DISCUSSION

The anaerobic degradation of hydroxybenzoate isomers has been reported in anoxic aquifer slurries (32), where hydroxybenzoate isomers were degraded under methanogenic, sulfate-reducing, and denitrifying conditions, by a defined syntrophic coculture (49), and by a pure culture of *Sporotomaculum hydroxybenzoicum* (10). The conversion of hydroxybenzoate to acetate, hydrogen and CO_2 is endergonic under standard conditions,

$$C_6H_4(OH)COO^- + 6\ H_2O \rightarrow 3\ CH_3COO^- + 3\ H^+ + HCO_3^- + 2H_2O \quad (6)$$

($\Delta G^{\circ\prime}$= +29.7 kJ per mole of hydroxybenzoate) (48)

suggesting that the presence of a hydrogen/formate-using partner is needed for hydroxybenzoate degradation under methanogenic conditions. However, *S. hydroxybenzoicum* ferments 3-hydroxybenzoate to butyrate, acetate and CO_2 (10). The free energy change of 3-hydroxybenzoate degradation is favorable ($\Delta G^{\circ\prime}$= -38 kJ/mol) when butyrate is one of the end products (10). *S. aciditrophicus* did not metabolize 3- or 4-hydroxybenzoate in pure culture with or without the crotonate or in coculture with *M. hungatei*. Cocultures of *S. aciditrophicus* and *M. hungatei* did metabolize 2-hydroxybenzoate to acetate and methane. The free energy change under standard conditions for 2-hydroxybenzoate degradation under methanogenic conditions is favorable ($\Delta G^{\circ\prime}$= -38.1 kJ per mole of hydroxybenzoate) (equation 5) Interestingly, *S. aciditrophicus* formed acetate and cyclohexane carboxylate when

grown with crotonate and 2-hydroxybenzoate. In Chapter 2, I have shown that benzoate serves as an electron acceptor when *S. aciditrophicus* is grown with crotonate. *S. aciditrophicus* also uses 2-hydrobenzoate as an electron acceptor, reducing it to cyclohexane carboxylate as crotonate is oxidized to acetate according to equation 4. The free energy change under standard conditions for this reaction is favorable ($\Delta G^{\circ\prime} = -183.9$ kJ/reaction) (equation 4) (48).

Most likely, the reductive elimination of the hydroxyl moiety occurred prior to ring reduction because cultures grown with 2-hydroxybenzoate and crotonate transiently formed benzoate. 2–Hydroxybenzoate is probably activated to its CoA thioester prior to reductive dehydroxylation as shown for the degradation of other hydroxybenzoate isomers (36, 40). Bonting and Fuchs (9) showed that *Pseudomonas* strain S100 metabolized 2-hydroxybenzoate under denitrifying conditions in two steps. First, 2-hydroxybenzoate was activated to 2-hydroxybenzoyl-CoA by a CoA ligase, followed by the dehydroxylation of 2-hydroxybenzoyl-CoA by a 2-hydroxybenzoyl-CoA reductase to form benzoyl-CoA.

In *T. aromatica*, benzoyl-CoA is reduced to cyclohex-1, 5-diene-1-carboxyl-CoA by a mechanism analogous to the Birch reaction for the chemical reduction of the aromatic ring (5, 6, 8). The hydrolysis of two ATP molecules per pair of electrons is required (5). *R. palustris* contains *badDEFG* genes whose deduced amino acid sequences have high degree of identity to the BcrCBAD subunits of the benzoyl-CoA reductase of *T. aromatica* (11, 15). Several investigators have argued that syntrophic benzoate metabolism cannot involve a two-electron reduction mechanism because it

is difficult to envision how syntrophic benzoate metabolism could result in net ATP synthesis if such a large energy investment was needed to activate and reduce benzoate (41, 46). One alternative hypothesis is that a hydroxylation of the ring occurs prior to ring reduction (41) . In support of this hypothesis, Peters et al. (41) showed that benzoate metabolism by *Desulfococcus multivorans* required molybdenum and selenium. These authors reasoned that enzymes that catalyze hydroxylations contain Mo, thus explaining the dependence of benzoate metabolism on the presence of molybdenum in the medium. Although *S. aciditrophicus* was able to metabolize 2-hydroxybenzoate, it appears that the hydroxyl group was reductively removed prior to ring reduction.

The second hypothesis to explain syntrophic benzoate metabolism involves a four- or six-electron reduction mechanism (46). The redox potential of a four-electron ring reduction reaction was calculated to be about -350 mV for the benzene/cyclohexene couple compared to a redox potential of -620 mV for the benzene/cyclohexadiene couple (46). Therefore, these investigators reasoned that the formation of a monoene rather than a diene could occur with an electron donor at the potential of NAD^+/NADH without energy investment. Elshahed et al. (16) found that cyclohex-1-ene carboxylate and cyclohexane carboxylate (up to 250 μM) transiently accumulate when *S. aciditrophicus* is grown with benzoate in coculture with *M. hungatei*. The formation of cyclohex-1-ene carboxylate and cyclohexane carboxylate from benzoate would be consistent with either a four- or six-electron reduction mechanism.

However, the genomes of *S. aciditrophicus* (CP000252, (35)) and *Geobacter metallireducens* (CP000148) (a microorganism that oxidizes benzoate coupled to iron reduction) each contain a homolog with high similarity at the amino acid level to the cyclohex-1-5-diene carboxyl-CoA hydratase of *T. aromatica* or *Azoarcus* sp. EbN1 (42, 51). Cyclohex-1-5-diene carboxyl-CoA hydratase catalyzes the formation of 6-hydroxycyclohex-1-ene carboxyl-CoA from cyclohex-1,5-diene carboxyl-CoA (33). The product of $bamR_{Syn}$ (gi 85860872) of *S. aciditrophicus* is 47% identical to predicted gene product of *Azoarcus* sp. EbN1 (4) and the gene product of $bamR_{Geo}$ (gi 78223357) is 68% identical to cyclohex-1-5-diene carboxyl-CoA hydratase of *T. aromatica*. The respective genes from *S. aciditrophicus* and *G. metallireducens* were each heterologously expressed in *Escherichia coli* and the expressed gene products shown to catalyze the hydration of cyclohex-1,5-diene carboxyl-CoA to 6-hydroxycyclohex-1-ene carboxyl-CoA (42). The presence of a gene that encodes for cyclohex-1,5-diene carboxyl-CoA hydratase in *S. aciditrophicus* and *G. metallireducens* argues that cyclohex-1,5-diene carboxyl-CoA is the product of benzoyl-CoA reduction. That is that these organisms use a two-electron reduction mechanism to reduce benzoyl-CoA. Here, I showed that a diene intermediate is formed by *S. aciditrophicus* from 3-fluorobenzoate. This further supports the hypothesis that the mechanism for anaerobic benzoyl-CoA metabolism involves a two-electron reduction reaction.

Interestingly, the use of fluorinated benzoates to study the mechanism of benzoyl-CoA reduction allowed me to show that *S. aciditrophicus* has the ability to

defluorinate monosubstituted benzoates. *S. aciditrophicus* metabolized 2- and 3-fluorobenzoate with the stoichiometric release of fluoride. The transformation was only observed in the presence of crotonate as a cosubstrate, although the transformation was not tested in coculture with a hydrogen/formate-using microorganism. The complete degradation of fluorobenzoate isomers was previously observed in microcosms and the rate of their degradation increased with the addition of glucose as a cosubstrate (27). The metabolism of fluorinated benzoates by *S. aciditrophicus* was associated with high partial pressures of hydrogen (87 to 116 Pa) (Table 3. 2). The partial pressure of hydrogen was much lower (about 8 Pa) when *S. aciditrophicus* was grown with crotonate and 4-fluorobenzoate, which as not metabolized by *S. aciditrophicus* (Table 3. 2). These data show that the metabolism of 2- and 3-fluorobenzoatae affected the physiology of *S. aciditrophicus* leading to an accumulation of hydrogen. Although there was sufficient crotonate present to support benzoate reduction, 3-fluorobenzoate was stoichiometrically converted to benzoate by *S. aciditrophicus*, The high partial pressure of hydrogen might explain why benzoate degradation was not observed in cultures containing 3-fluorobenzoate (and 2-fluorobenzoate) because high levels of hydrogen have been shown to inhibit benzoate degradation (17).

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CHAPTER 4

Proteomic analysis of *Syntrophus aciditrophicus* strain SB grown on crotonate and benzoate indicates previously undescribed fermentative metabolism.

ABSTRACT

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and nanospray LC/MS/MS analyses of pure cultures of *Syntrophus aciditrophicus* grown with crotonate or with crotonate and benzoate allowed the identification of a total of 356 gene products that belonged to all functional classes. A number of proteins potentially involved in crotonate and benzoate metabolism were identified. The determination of spot intensities detected 25 gene products that were differentially abundant between the two growth conditions, crotonate compared to crotonate with benzoate. Identification of the differentially abundant proteins provided important clues concerning the initial steps of benzoate metabolism. The proteins included a benzoyl-CoA ligase and a set of proteins homologous to the benzoate-induced proteins identified in *Geobacter metallireducens*, which are postulated to be a novel benzoyl-CoA reductase, i. e. tungsten-containing aldehyde: ferredoxin oxidoreductase probably associated with membrane components. Proteomic analyses also detected

proteins involved in energy conservation and electron transport. A polypeptide identified as acetyl-CoA synthetase (ADP-forming) may be involved in ATP synthesis from CoA-thioesters. Components of several putative ion-translocating complexes were detected including an ATP synthase (possibly Na^+-translocating), a Na^+-translocating glutaconyl-CoA decarboxylase, and an ion-translocating Rnf-like complex that may be involved in reverse electron transport. Components of heterodisulfide reductase, a Fe-only hydrogenase, and both periplasmic and cytoplasmic formate dehydrogenases were also detected. The two formate dehydrogenase complexes could be involved in formate cycling to generate an ion gradient across the membrane. This study provides an overview of the cellular machinery involved in energy conservation and electron transport in syntrophic microorganisms that operates close to thermodynamic equilibrium.

Key words: *Syntrophus aciditrophicus*, proteomic, benzoate, crotonate

INTRODUCTION

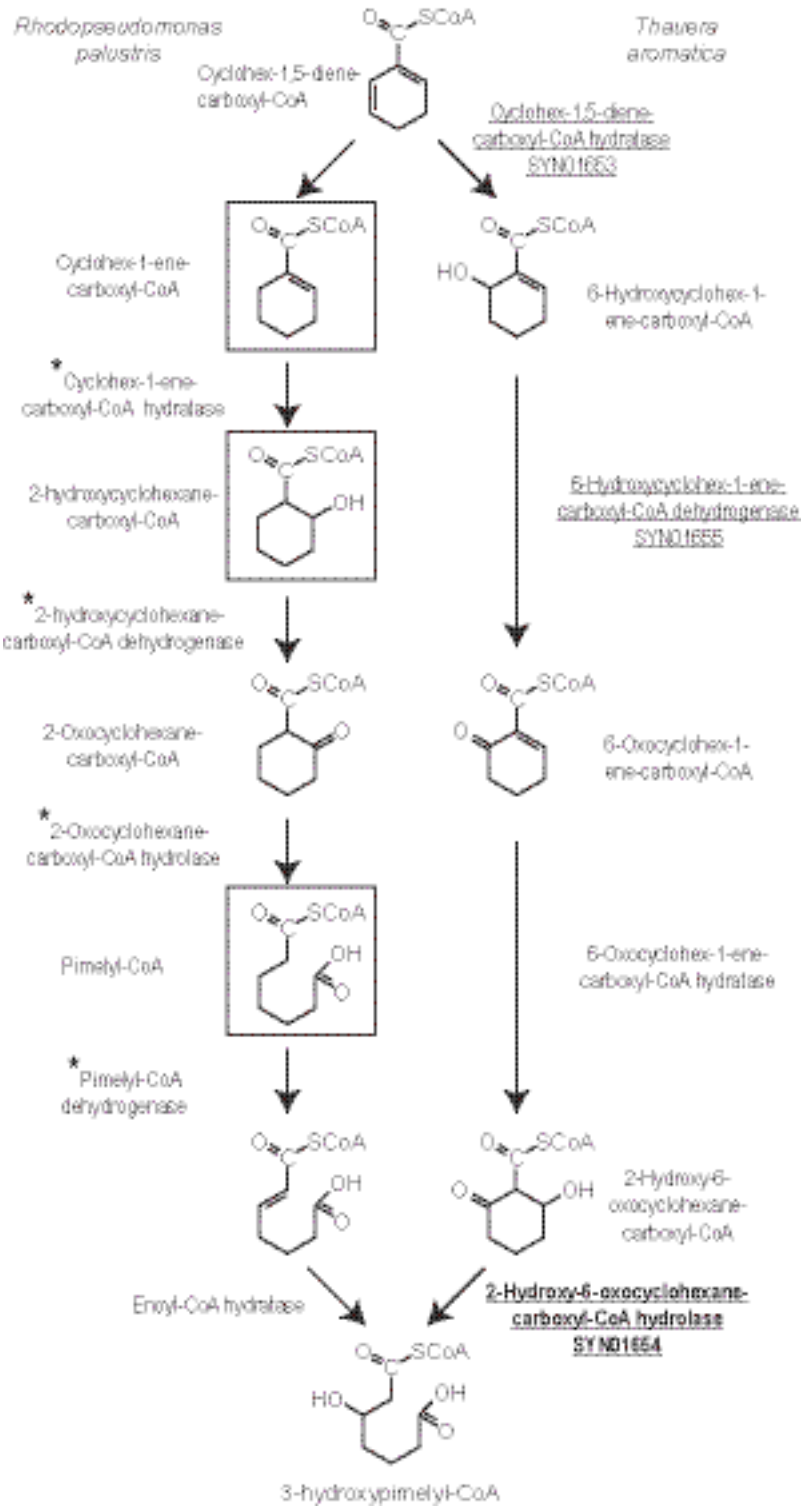
The degradation of natural polymers such as polysaccharides, proteins, nucleic acids, and lipids to CO_2 and CH_4 involves a complex microbial community (50). Fermentative bacteria hydrolyze the polymeric substrates such as polysaccharides, proteins, and lipids and ferment the hydrolysis products to acetate and longer chain fatty acids, CO_2, formate, H_2. Propionate and longer chain fatty acids, alcohols, and some amino acids and aromatic compounds are syntrophically degraded to the methanogenic substrates, H_2, formate, and acetate (41, 50). The syntrophic degradation of fatty acids is often the rate-limiting step so syntrophic metabolism is essential to the efficient operation of many waste treatment facilities. The degradation of syntrophic substrates is thermodynamically unfavorable unless hydrogen and/or formate levels are maintained at low levels by H_2/formate-using microorganisms such as methanogens or sulfate reducers (41). Lastly, two different groups of methanogens, the hydrogenotrophic methanogens and the acetotrophic methanogens, complete the process by converting the acetate, formate and hydrogen made by other microorganisms to methane and carbon dioxide.

In the absence of oxygen, the degradation of many aromatic compounds, such as phenols, halogenated aromatics, and aromatic hydrocarbons, converges to a central intermediate, benzoate and benzoyl-CoA, prior to ring cleavage (21, 27, 29). In methanogenic environments, syntrophic metabolism is required for the conversion of benzoate to methanogenic substrates, acetate, carbon dioxide, hydrogen, and formate

(44). *Syntrophus aciditrophicus* is a Gram-negative bacterium that belongs to the δ Proteobacteria that degrades benzoate and fatty acids in coculture with H_2/formate-using microorganisms (31). It can grow in pure culture with crotonate or benzoate (18). Recent studies have shown that it forms cyclohexane carboxylate and acetate during the fermentation of crotonate (45). The genome of *S. aciditrophicus* has been recently sequenced (43), which provides the first glimpse of the composition and architecture of the enzymatic systems needed to exist on the marginal energy economies of a syntrophic lifestyle.

The reduction of benzoyl-CoA represents a significant energy barrier in anaerobic microorganisms. Benzoyl-CoA reductase has been studied in the denitrifying bacterium, *Thauera aromatica* (6-8, 10-12), and this enzyme hydrolyzes two molecules of ATP to reduce benzoyl-CoA to cyclohex-1-ene carboxyl-CoA (Fig. 4. 1) (6, 7, 20). The enzyme is coded by four genes *bcr*CBAD that are highly identical at the amino acid level to the subunits coded by *bad*DEFG in the phototrophic bacterium *Rhodopseudomonas palustris*. Homologous to these genes could not be detected in genomes of other benzoate-degrading bacteria including *S. aciditrophicus* (45), *Geobacter metallireducens* (56) or *Desulfococcus mulivorans* (48). In recent work, Wischgoll et al. (56) proposed that the reduction of benzoyl-CoA in *G. metallireducens* involves a new type of benzoyl-CoA reductase that may use membrane energy to drive this reaction. Two sets of genes homologous to the putative benzoyl-CoA reductase were detected in the genome of *S. aciditrophicus* (43), but it is not known if these gene systems are expressed.

Figure 4. 1. Anaerobic benzoate degradation pathways similar to *R. palustris* (left) and *T. aromatica* (right). Stars indicate detected enzyme activities; framed molecules indicate detected intermediates when *S. aciditrophicus* was grown with benzoate in coculture with hydrogen-using microorganisms; assignments of gene products to specific steps are underlined; and the genes heterologously expressed and characterized are shown in bold.


Rhodopseudomonas palustris
Thauera aromatica
Cyclohex-1,5-diene-carboxyl-CoA
Cyclohex-1,5-diene-carboxyl-CoA hydratase SYN01653
Cyclohex-1-ene-carboxyl-CoA
6-Hydroxycyclohex-1-ene-carboxyl-CoA
*Cyclohex-1-ene-carboxyl-CoA hydratase
2-hydroxycyclohexane-carboxyl-CoA
6-Hydroxycyclohex-1-ene-carboxyl-CoA dehydrogenase SYN01655
*2-hydroxycyclohexane-carboxyl-CoA dehydrogenase
2-Oxocyclohexane-carboxyl-CoA
6-Oxocyclohex-1-ene-carboxyl-CoA
*2-Oxocyclohexane-carboxyl-CoA hydrolase
Pimelyl-CoA
6-Oxocyclohex-1-ene-carboxyl-CoA hydratase
*Pimelyl-CoA dehydrogenase
2-Hydroxy-6-oxocyclohexane-carboxyl-CoA
Enoyl-CoA hydratase
2-Hydroxy-6-oxocyclohexane-carboxyl-CoA hydrolase SYN01654
3-hydroxypimelyl-CoA

While previous studies detected 2-hydroxycyclohexane carboxylate, cyclohex-1-ene carboxylate and pimelate in culture fluids of *S. aciditrophicus* grown with benzoate and the enzyme activities needed to convert cyclohex-1-ene carboxyl-CoA to pimelyl-CoA in cell-free extracts of *S. aciditrophicus* (Fig. 4. 1), genes homologous to those that encode these proteins in *Rhodopseudomonas palustris* (16, 47) were not detected in the *S. aciditrophicus* genome (43). However, the genome of *S. aciditrophicus* contains three adjacent genes (SYN01655, SYN01654, and SYN01653) (Fig. 4. 1) (43), whose gene products share high homology (36 to 51% identity at the amino acid level) with Had, Oah, and Dch from *T. aromatica*, the 6-hydroxycyclohex-1-ene carboxyl-CoA dehydrogenase, 2-ketocyclohex-1-ene carboxyl-CoA hydrolase, and cyclohex-1,5-diene carboxyl-CoA hydratase, respectively (12). Recently, it was shown that the gene product of SYN01653 has cyclohex-1,5-diene carboxyl-CoA hydratase activity (49), suggesting that a diene intermediate is made during benzoate metabolism in *S. aciditrophicus* as seen in phototrophs and denitrifiers. The presence of the genes for Had, Oah, and Dch in *S. aciditrophicus* suggests that cyclohex-1,5-diene carboxyl-CoA is degraded by the pathway used by denitrifiers (Fig. 4. 1). Genomic analysis suggested that *S. aciditrophicus* synthesizes ATP from acetyl-CoA by an acetyl-CoA synthase (ADP-forming), whose genes appear to be of archaeal origin.

The proteome of *S. aciditrophicus* was investigated to test various hypotheses derived from genomic analysis. Specifically, we wished to determine whether the

proteins involved in benzoate and crotonate metabolism and ATP synthesis once acetyl-CoA is made.

MATERIALS AND METHODS

Strain and growth conditions.

Syntrophus aciditrophicus strain SB (ATCC 700169) was grown in pure culture in the basal medium described by McInerney et al. (42) without rumen fluid (17, 45) and with 20 mM of crotonate or with 20 mM of crotonate and 5 mM of benzoate. Media and stock solutions were prepared according to the anaerobic techniques described by Balch and Wolfe (3). The headspace was pressurized to 172 kPa with N_2/CO_2 (80:20 v/v). Cultivation was conducted at 37°C without shaking. Cells were harvested at mid-log phase of growth by centrifugation (14,300 × *g*, 20min, 4°C) and the cell pellet was washed three times with 50 mM phosphate buffer (pH 7.0). The cell pellets were frozen until utilization.

Protein extraction and sample preparation for 2-D-PAGE.

Cells were washed twice with ice-cold PBS containing protease inhibitors and sonicated for 10 s in ice-cold radioimmune precipitation assay buffer containing 10 mM $NaPO_4$ (pH 7.2), 0.3 M NaCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 2 mM EDTA, protease inhibitor mixture set III (100 mM 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), 80 μM aprotinin, 5 mM bestatin, 1.5 mM E-64, 2 mM leupeptin, 1 mM pepstatin), and phosphatase inhibitor mixture set II (200 mM imidazole, 100 mM sodium fluoride, 115 mM

sodium molybdate, 100 mM sodium orthovanadate, 400 mM sodium tartrate dihydrate) (Calbiochem, La Jolla, CA). Lysates were centrifuged at 1000 × g for 5 min. To remove the salt from the lysates, the supernatant proteins were precipitated with trichloroacetic acid (10% w/v) and 20 mM DTT for 30 min on ice. The precipitate was collected by centrifugation at 20,800 × g for 10 min at 4 °C and washed three times with 10% trichloroacetic acid, 20 mM DTT. Trichloroacetic acid in the precipitate was removed through the extraction with diethyl ether or acetone plus 10 mM DTT. After drying, the pellet was resuspended by sonication in a buffer containing 7 M urea, 2 M thiourea, 4% w/v CHAPS, 100 mM DTT, 0.2% v/v Bio-Lyte pH 3/10:4/6:5/8 (1:0.5:0.5), 5% glycerol, and protease/phosphatase inhibitors (mixture sets II and III). After standing for 1 h at room temperature, the sample was centrifuged at 23,800 × *g* for 10 min at 15 °C, and the supernatants were stored at -80 °C until use for two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). Protein concentration in the samples was estimated by using a commercial Non-interfering Protein Assay (Geno Technology, Inc., St. Louis, MO, USA), and bovine serum albumin as standard.

2-D PAGE.

Approximately 100 μg of whole cell lysate was added to each immobilized pH gradient (IPG) strip, which was rehydrated in 8 M urea, 2% CHAPS, 50 mM DTT, 0.2% Bio- Lyte 3/10 ampholyte, 0.001% bromophenol blue. The pre-isoelectric focusing (IFE) and IEF were performed by using pre-made 17 cm length IPG strips

(pH 3–10 NL) on Multiphor II electrophoresis system (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The pre-IEF was performed linearly from 50 V to 250 V for 2 h, from 250 V to 675 V for 1.5 h, from 675 V to 1250 V for 2 h. IEF was then performed with a linear increase up from 1250 V to 3500 V over 2 h and then held at 3500 V for 19 h to a total of 70 kVh. For the second dimension, the IPG strips were equilibrated in a buffer containing 37.5 mM Tris-HCl, pH 8.8, 20% glycerol, 2% SDS, and 6 M urea with 2% DTT, followed by electrophoresis in a 12% SDS-PAGE on a Protean Plus Dodeca Cell (Bio-Rad). The resulting gel was stained with Sypro-Ruby, visualized under ultraviolet light with a Molecular Imager FX Pro Plus (Bio-Rad).

Protein identification.

Differentially abundance of protein spots were determined based on staining intensity of the Sypro Ruby as determined by the PDQuest software (Bio-Rad). This sensitivity of the software is set to detect a 2-fold increase in staining intensity as a criterion for a significant increase in protein expression. For the purpose of this study, we increased the stringency to 3-fold. The differentially abundant spots and other spots were excised by a spot-excision robot (Proteome Works, Bio-Rad) and deposited into 96-well plates. Gel spots were washed and digested with sequencing-grade trypsin, and the resulting tryptic peptides were extracted using standard protocols.

Peptide sequencing was accomplished with nanoflow high performance liquid chromatography system (LC Packings, Sunnyvale, CA, USA) with a

nanoelectrospray (nano-ESI) interface (Protana, Odense, Denmark) and an Applied Biosystems/Sciex QSTAR® XL (QqTOF) mass spectrometer (Foster City, CA). The samples were first loaded onto a LC Packings PepMap C18 precolumn (150 μm × 3 mm; particle size 5μm) and washed for two minutes with the loading solvent, 0.1% formic acid. The samples were then injected onto a LC Packings PepMap C18 column (75 μm × 150 mm; particle size 5 μm) for nano-LC separation at a flow rate of 220 nL/min. The enzymatic digest samples were dried and redissolved in 0.1% formic acid (FA) solution. For each LC-MS/MS run, typically a 6 μL sample volume was loaded to the precolumn first and washed with the loading solvent of 0.1% FA. The eluents used for the LC were (A) 0.1% formic acid and (B) 95% ACN/5% H_2O/0.1% FA. The following gradient was used for elution: 6% B to 24% B in 18 min, 24% B to 36% B in 6 min, 36% B to 80% B in 2 min and 80% B for 8 min. The column was the finally re-equilibrated with 6% B for 16 min before the next run.

For online MS and MS/MS analyses, a New Objective (Woburn, MA) PicoTip tip (id. 8μm) was used for spraying with the voltage set at 1750 V. Peptide product ion spectra were automatically recorded during the LC-MS runs by the information-dependent analysis (IDA) on the QSTAR® XL mass spectrometer. Argon was employed as collision gas. Collision energies for maximum fragmentation were automatically calculated using empirical parameters based on the charge and mass-to-charge ratio of the peptide.

Database searching.

Protein identification was accomplished by utilizing the Mascot database search engine (Matrix Science, London, UK). Positive protein identification was based on standard Mascot criteria for statistical analysis of the LC-MS/MS data. The peptide assignments in the database search results were manually inspected for validation. A 10Log (P) score, where P is the probability that the observed match is a random event, of 72 was regarded as significant. All searches were performed with a mass tolerance of 0.005% error (50 ppm).

The completed genome sequence of *S. aciditrophicus* is available in GenBank (accession no. CP000252).

Chemicals.

GTE was obtained from Pharmanex, Inc. (Provo, UT). The purity of the catechins in the GTE was 84%. The Pharmanex GTE is a mixture of many catechin compounds, with EGCG as a major component (43.0% by weight), followed by epicatechin-3-gallate (13.7%), epicatechin (6.0%), gallocatechin gallate (5.6%), epigallocatechin (4.0%), gallocatechin (2.3%), catechin (2.0%), catechin gallate (1.4%). In this study, the concentration was expressed as the amount of GTE per milliliter of media bathing the cells (μg/mL).

Unless stated otherwise, all chemicals were obtained from Sigma (St. Louis, MI, USA). TFA was obtained from Pierce (Rockford, IL, USA). Sequencing-grade trypsin was purchased from Promega (Madison, WI, USA). 17 cm Protean II Ready Gels (12%), 17 cm IPG strips (3/10NL), 10×TGS (Tris-glycine-SDS buffer), DTT,

and Sypro Ruby staining buffer were purchased from Bio-Rad Laboratories, (Hercules, CA, USA).

RT-PCR.

S. aciditrophicus strain SB was grown in pure culture or in coculture with a hydrogen-formate user *Methanospirillum hungatei* strain JF-1. During the exponential phase of growth, cells from each growth condition were harvested and total RNA was obtained by using a RNeasy Mini Kit (Qiagen Inc., Valencia, CA). DNA was removed by using on-column DNA digestion with RNAse-free DNAse Set (Qiagen Inc., Valencia, CA). An additional DNA digestion was performed by using RQ1 RNAse-free DNAse (Promega Corp., Madison, WI). To verify that no DNA remained in the RNA sample, a PCR reaction was carried out with primers that amplify a region of SYN01653, after the on-column treatment and RQ1 DNAse treatment. This region was amplified by using the forward primer 5' GCGAAAGAAGCGGAAGCCATTG 3', and the reverse primer 5' CTGCTCTTGCTGGTGAAATCAG 3', which would amplify a 104 bp region of the SYN01653 gene. The primers were purchased from Invitrogen Corp. (Carlsbad, CA).

One μg of purified RNA was used to synthesize cDNA from each sample by using 200 units of Moloney Murine Virus Reverse Transcriptase (M-MLV RT) from Promega Corp. (Madison, WI), and 1 μg of the reverse primer mentioned above. Gene expression was studied by ampliflying by PCR the 104 bd region of the SYN01653 from undiluted, 10-fold diluted, and 100-fold diluted cDNA obtained. The PCR products were visualized on a 1.5% agarose gel with ethidium bromide.

RESULTS AND DISCUSSION

Proteome of *S. aciditrophicus.*

A combination of two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and nanospray LC/MS/MS analyses identified a total of 356 gene products of *S. aciditrophicus.* These proteins were detected in each grown condition, crotonate plus benzoate and crotonate alone. The proteins belonged to all functional categories with a predominance of the proteins involved in amino acid metabolism, cofactor biosynthesis, and bioenergetics of *S. aciditrophicus* (Table 4. 1). Forty-nine hypothetical proteins or proteins with unknown function were identified, representing 14% of the total polypeptides detected. Among these, 21 were specific to *S. aciditrophicus* with no homologs found in databases (GenBank database), 13 proteins were similar (E-values up to 2E-85) to proteins found in *S. fumaroxidans* based on amino acid sequences, and 11 were similar to other strict anaerobes.

Interestingly, proteomic analysis detected two proteins coded by SYN01186 and SYN01187 that were approximately 37% and 36% identical to the two gene products of DVU2103 and DVU2104, respectively, found in *Desulfovibrio vulgaris* Hildenborough (54) . DVU2103 and DVU2104 are predicted to encode for proteins with iron-sulfur cluster/nucleotide-binding domains. The transcripts of DVU2103 and DVU2104 were shown to be up-regulated three-fold when *D. vulgaris* was switched from a syntrophic to a sulfidogenic lifestyle (54). Homologs to DVU2103 and

Table 4. 1. Functional classification of proteins detected by 2D gel electrophoresis and LC/MS/MS.

Functional categories [a]	Number of proteins identified
Amino acid transport and metabolism	43
Carbohydrate transport and metabolism	18
Cell cycle control, cell division, chromosome partitioning	8
Cell motility	1
Cell wall/membrane/envelope biogenesis	19
Chromatin structure and dynamics	ND
Coenzyme transport and metabolism	24
Defense mechanisms	1
Energy production and conversion	38
Function unknown	18
General function prediction only	22
Inorganic ion transport and metabolism	3
Intracellular trafficking, secretion, and vesicular transport	5
Lipid transport and metabolism	25
Nucleotide transport and metabolism	17
Posttranslational modification, protein turnover, chaperones	25
RNA processing and modification	ND
Replication, recombination and repair	6
Secondary metabolites biosynthesis, transport and catabolism	2
Signal transduction mechanisms	7
Transcription	13
Translation, ribosomal structure and biogenesis	30
Hypothetical proteins	31
Total	356

[a] The protein classification is based on Clusters of Orthogonal Groups (COG's) from http://ncbi.nih.gov

Abbreviation: ND, not detected

DVU2104 have been detected mainly in microorganisms that are known to interact syntrophically with methanogens (54). However, their specific function remains unknown.

Benzoate metabolism.

Differential abundance of proteins between cultures grown with crotonate plus benzoate versus crotonate alone was used to test hypothesis derived from genomic analysis about the metabolism of *S. aciditrophicus*. The determination of spot intensities indicated that there were 25 proteins with differential abundance between the two growth conditions (Table 4. 2). Ten of the differentially abundant proteins are predicted to function in crotonate and/or benzoate metabolism and will be discussed below.

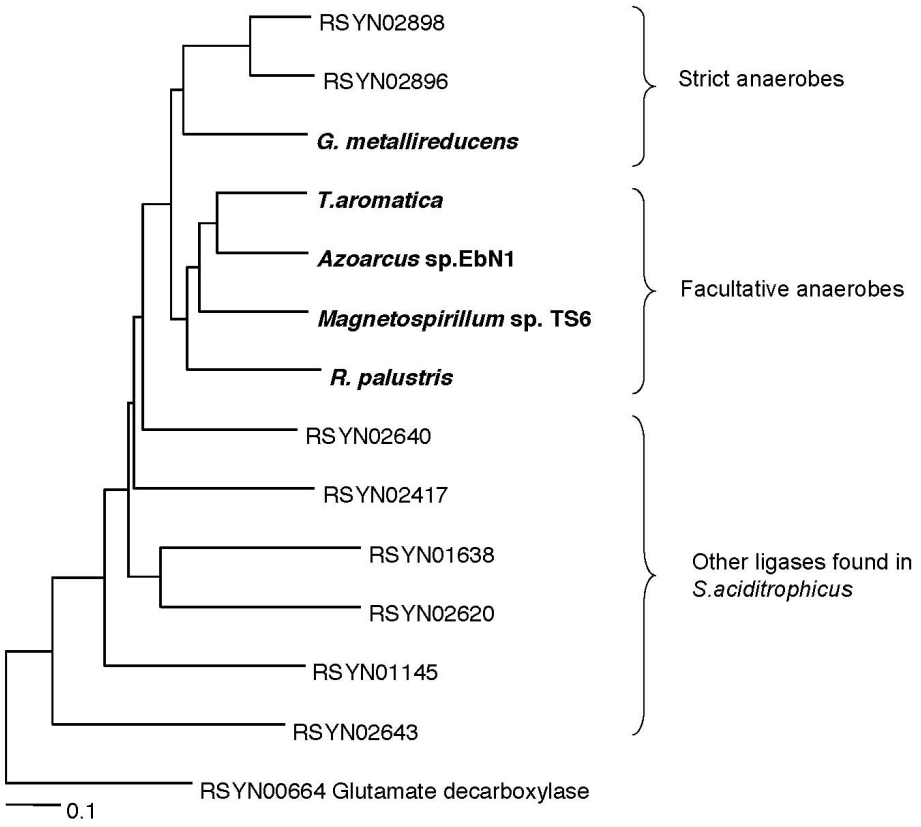
Proteomic data indicated that SYN02898 and SYN02896 gene products were at least three-fold more abundant when *S. aciditrophicus* was grown with crotonate plus benzoate than with crotonate alone, implicating their role in benzoate metabolism. SYN02898 and SYN02896 gene products are both annotated as 4-hydroxybenzoate-CoA ligase (EC 6.2.1.27) / Benzoate-CoA ligase (EC 6.2.1.25). Benzoyl-CoA ligase is used to activated to benzoyl-CoA prior to ring reduction and cleavage (1, 2, 20, 30). Consistent with the annotation, phylogenetic analysis showed that the SYN02898 and SYN02896 gene products were each most closely related to the benzoyl-CoA ligase of *Geobacter metallireducens* (Fig. 4. 2). SYN02898 and SYN02896 gene products share 76% identity with each other and are 52% and 49%

Table 4. 2. Protein differentially abundant on 2D gel electrophoresis detected in *S. aciditrophicus* cell extracts grown with crotonate or with crotonate and benzoate.

Up-regulated with crotonate	
Protein ID	Annotation
SYN01653	Cyclohexa-1,5-diene-1-carboxyl-CoA hydratase
SYN00066	Ribosomal protein L7/L12
SYN01723	Phenylalanyl-tRNA synthetase beta chain
SYN02950	Glucose-6-phoshate isomerase
SYN02536	Isopropylmalate/isocitrate/citramalate synthase
SYN02781	Aspartate kinase, monofunctional class
SYN01378	Pyridoxal phosphate biosynthetic protein PdxJ
SYN00090	2-Isopropylmalate synthase
SYN01297	Cold shock protein
SYN01793	UDP-N-acetylglucosamine-1-carboxyvinyltransferase
SYN02445	Vinylacetyl-CoA delta-isomerase
SYN00070	Transcription antitermination protein
SYN01940	Phospho-2-dehydro-3-deoxyheptanoate aldolase, subtype 2
SYN00479	Acetul/propionyl-CoA carboxylase, alpha subunit
SYN02177	DNA/pantothenate metabolism flavoprotein

Up-regulated with crotonate and benzoate	
Protein ID	Annotation
SYN02898	Benzoate-CoA ligase
SYN03128	Putative fatty acid-CoA ligase
SYN02586	Acyl-CoA dehydrogenase
SYN02896	Benzoate-CoA ligase
SYN00807	Hypothetical cytosolic protein
SYN00257	Heterodisulfide reductase, subunit A
SYN02487	PpiC-type peptidyl-prolyl cis-trans isomerase
SYN00924	Lysyl-tRNA synthetase
SYN02116	Glutaminyl-tRNA synthetase
SYN00481	Glutaconyl-CoA decarboxylase A subunit

Figure 4. 2. Phylogenetic tree for characterized benzoyl-CoA ligases and other ligases found in *S. aciditrophicus*. Functional annotation was based on characterized proteins (bold). Distance and branching order were determined by the neighbor-joining method.


RSYN02898
RSYN02896
G. metallireducens
Strict anaerobes
T.aromatica
Azoarcus sp.EbN1
Magnetospirillum sp. TS6
R. palustris
Facultative anaerobes
RSYN02640
RSYN02417
RSYN01638
RSYN02620
RSYN01145
RSYN02643
Other ligases found in S.aciditrophicus
RSYN00664 Glutamate decarboxylase
0.1

identical, respectively, to the *G. metallireducens* benzoyl-CoA ligase. The benzoyl-CoA ligases of *Azoarcus* sp. EbN1, *Rhodopseudomonas palustris*, *Magnetospirillum* sp. TS6, and *Thauera aromatica* are more distantly related to the above three proteins and form a separate clade (Fig. 4. 2). The other ligases present in the genome of *S. aciditrophicus* are more distantly related to the above gene products. SYN02898 and SYN02896 are in close proximity to each other (440 nucleotides apart) on the *S. aciditrophicus* genome, but are not associated with other genes that could provide further information regarding benzoate metabolism. A 2,3-dihydroxybenzoate-AMP ligase (EC 2.7.7.58) (gene product of SYN01638) was also detected in the proteome. SYN01638 is located in a cluster of genes that may function in benzoyl-CoA reduction (see below).

Proteomic analyses detected the SYN01655, SYN01654, and SYN01653 gene products when *S. aciditrophicus* was grown with either crotonate plus benzoate or crotonate alone (Table 4. 3). These proteins catalyze steps I to III in Figure 4. 1. The enzymatic activity of SYN01653 and SYN01655 genes products has been confirmed by characterizing the respective recombinant protein after cloning and expression in *E. coli* ((49); M. Boll personal communication). The SYN01653 gene product (cyclohex-1,5-diene carboxyl-CoA hydratase) was at least three fold more abundant on 2-D gels of extracts from cells grown with crotonate compared to extracts from cells grown with crotonate and benzoate (Table 4. 2). RT-PCR was used to investigate the expression of SYN01654 under different growth conditions. SYN01654 was expressed under all of the conditions tested, but the data suggest that

Table 4. 3. Proteins identified by 2D gel electrophoresis with predicted role in *Syntrophus aciditrophicus*.

Annotation	Locus
Ligases	
4-Hydroxybenzoate-CoA ligase/Benzoate-CoA ligase	**SYN02898**
4-Hydroxybenzoate-CoA ligase/Benzoate-CoA ligase	**SYN02896**
Long-chain fatty acid-CoA ligase	**SYN03128**
Protein involved in benzoate metabolism	
Heterodisulfide reductase subunit A and related polyferredoxin	**SYN00257,or SYN03215, or SYN01645**[a]
Tungsten-containing aldehyde ferredoxin oxidoreductase	SYN01377,or SYN00261, or SYN03032[b]
Cyclohex-1,5-diene carboxyl-CoA hydratase	**SYN01653**
6-Oxo-cyclohex-1-ene-carbonyl-CoA hydrolase	SYN01654
Putative 6-hydroxycyclohex-1-ene-1-carboxyl-CoA dehydrogenase	SYN01655
Beta oxidation and substrate level phosphorylation	
Enoyl-CoA hydratase	SYN01309
3-Hydroxyacyl-CoAdehydrogenase	SYN01310
Acetyl-CoA:acetyltransferase/thioloase	SYN01681
Acyl-CoA dehydrogenases	SYN02586, SYN02587, and SYN01699
Acetyl-CoA acetyltransferase	SYN02642
Electron transfer flavoprotein α subunit	SYN02637
Electron transfer flavoprotein β subunit	SYN02636
Acetyl-CoA synthetase AMP forming	SYN02635
Acetyl-CoA synthetase ADP forming	SYN00646

Energy production and conversion	
NADH dehydrogenase (ubiquinone), 24 kDa subunit	SYN00153
Pyruvate:ferredoxin oxidoreductase alpha subunit	SYN00154
Biotin carboxyl carrier protein of glutaconyl-CoA decarboxylase	SYN00479
Acyl-CoA dehydrogenase, short-chain specific	SYN00480
Glutaconyl-CoA decarboxylase A subunit	SYN00481
Fe-S oxidoreductase	SYN00259, SYN02421, SYN03213, and SYN01643[b]
Formate dehydrogenase catalytic subunit	SYN00629
Formate dehydrogenase alpha subunit	SYN00630
NADH-quinone oxidoreductase chain F	SYN00631
Formate dehydrogenase iron-sulfur subunit	SYN00633
FdhE	SYN00636
Formylmethanofuran dehydrogenase subunit E	SYN00638
NADH-quinone oxidoreductase chain F	SYN01369
Iron-only hydrogenase	SYN01370
F_0F_1-type ATPase δ subunit	SYN00547
F_0F_1-type ATPase α subunit	SYN00546
F_0F_1-type ATPase γ subunit	SYN00545
F_0F_1-type ATPase β subunit	SYN00544
F_0F_1-type ATPase ε subunit	SYN00543
Fe-S nucleotide-binding protein	SYN01186, SYN01187
Acyl-CoA synthase (ADP-forming)	SYN00646, SYN00647
3-Isopropylmalate dehydrogenase	SYN00088
NADH:ubiquinone oxidoreductase subunit RnfG	SYN01662
NADH:ubiquinone oxidoreductase subunit RnfD	SYN01664

The gene products identified by proteomic analysis that were differentially abundant are.shown in bold. [a] SYN00257 shares from 70% up to 100% identity with SYN02423, SYN01645, and SYN03215. Moreover, the N-terminal sequences of SYN00257, SYN03215, and SYN01645 are highly identical, making it difficult to distinguish which one was encoded.

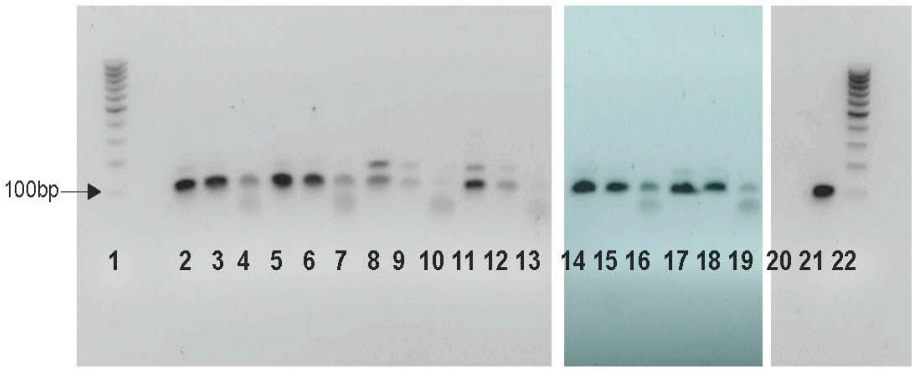
[b] SYN01377 shares over 60% identity with RSYN00261, RSYN03032, and RSYN01640 and they all have highly identical N-terminal sequence, therefore it is difficult to know which one of the genes were actually expressed.

SYN01653 was up-regulated when *S. aciditrophicus* was grown with crotonate in pure culture or in coculture with *Methanospirillum hungatei* (Fig. 4. 3, lines 2 to 7) compared to growth with benzoate in pure culture or in coculture (Fig. 4. 3, lines 8 to 13). Thus, *S. aciditrophicus* makes the enzymes that convert benzoyl-CoA to 3-hydroxylpimelyl-CoA under diverse growth conditions, suggesting that the degradation pathway used for benzoate degradation is similar to that found in denitrifiers (Fig. 4. 1).

The expression of SYN01654 and the abundance of the SYN01653 gene product were somewhat contradictory to what one would expect for genes involved in benzoate metabolism in that this set of genes was not induced with benzoate. Mouttaki et al. (45) showed that *S. aciditrophicus* ferments crotonate to acetate, cyclohexane carboxylate and some benzoate. The gene products of SYN01653-1655 may be involved in the formation of benzoate under these growth conditions. SYN01653 gene product (cyclohex-1,5-diene carboxyl-CoA hydratase) has already been shown to function in both directions, the hydration of cyclohex-1,5-diene carboxyl-CoA and the dehydration of 6-hydroxy- cyclohex-1,5-diene-carboxyl-CoA (49). The lower levels of expression of the above genes when benzoate is present is consistent with the fact that almost all of the benzoate is reduced to cyclohexane carboxylate rather than being degraded to acetate and carbon dioxide when *S. aciditrophicus* is grown with crotonate and benzoate (Chapter 2).

The ring reduction of benzoyl-CoA to cyclohex-1,5-diene-carboxyl-CoA represents a considerable energy barrier. In the denitrifying bacterium *T. aromatica*,

Figure 4. 3. Reverse transcriptase PCR studies of SYN01653 coding for cyclohex-1,5-diene-1-carboxyl-CoA hydratase. The cDNA was obtained by reverse transcription of RNA purified from *Syntrophus aciditrophicus* grown on different substrates using a specific primer. The cells were grown on crotonate pure culture (lanes 2-4), crotonate in coculture with *Methanospirillum hungatei* strain JF-1 (lanes 5-7), benzoate pure culture (lanes 8-10), benzoate in coculture with JF-1 (lanes 11-13), crotonate + benzoate pure culture (lanes 14-16), and cyclohexane carboxylate in coculture with JF-1 (lanes 17-19). A 1 Kb ladder was used (lanes 1 and 22). The genomic DNA of *S. aciditrophicus* was used as a positive control (lane 21). A PCR reaction without template was used as a negative control (lane 20). The cDNA used as templates were undiluted (lanes 2, 5, 8, 11, 14, and 17), 10-fold diluted (lanes 3, 6, 9, 12, 15, and 18), and 100-fold diluted (4, 7, 10, 13, 16, and 19).


100bp
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22

this energy barrier is overcome by coupling the reaction to the hydrolysis of two molecules of ATP (55). The benzoyl-CoA reductase in *T. aromatica* is a 170 KDa protein consisting of four subunits coded by *bcrABCD* (12). Homologous proteins with high degree of identity are present in *A. evansii* and *R. palustris* (4, 15, 16). The subunits BcrA and BcrD of *T. aromatica* each contain one ATP binding site and one [4Fe-4S] cluster (9) and these subunits have high similarity to HgdC, the activator of 2-hydroxyglutaryl-CoA dehydrogenase (9, 26), involved in glutamate metabolism in *Acidaminococcus fermentans* (46). Hydroxyglutaryl-CoA dehydratase catalyses the chemically difficult *syn*-elimination of water from *(R)*-2-hydroxyglutaryl-CoA to form *(E)*-glutaconyl-CoA (24-26). An amino acid alignment of HgdC subunits from *Clostridium symbiosum*, BcrD from *T. aromatica*, BadG from *R. palustris,* and the predicted gene product of SYN00371 from *S. aciditrophicus* show several conserved regions (Fig. 4. 4). Each protein has an ATP-binding domain each containing two phosphate-binding motifs (DXG) and an adenosine-binding motif (GG) (Fig. 4. 4) (25, 35). However, *S. aciditrophicus* genome does not contain genes whose gene products have similarity to BcrBC (BLAST e < -05), which are the substrate reduction modules of benzoyl-CoA reductase in *T. aromatica* (55). Also, no homologs corresponding to HgdA or HgdB of *A. fermentans* (BLAST e < -05) that form component D of hydroxyglutaryl-CoA dehydratase, which has the hydratase function, were found in *S. aciditrophicus* genome. The lack of homologs to BcrBC in the genomes of *S. aciditrophicus* suggests that another enzyme system must be used for benzoyl-CoA reduction. Consistent with the genomic information from *S.*

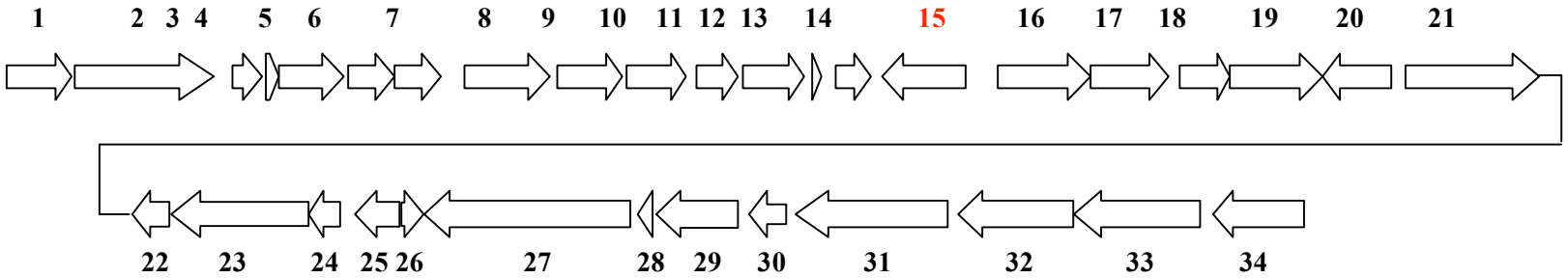
Figure 4. 4. Clustal alignment of amino acid sequences of HgdC, the activator of hydroxyglytaryl-CoA dehydratase from *Clostridium symbiosum* and the BcrD and BadG from *Thauera aromatica* and *Rhodopseudomonas palustris* respectively, one of the subunits of benzoyl-CoA reductase. The ATP-binding motif and conserved cysteines are shown in bold.

```
BcrD    T. aromatica          MTITAGIDIGTGAVKTVLFRVEGDKTEWLAKRNDRIRQRDPFKLAEEAYNGLLEEAGL
BadG    R. palustris          MTITSGIDVGTGAIKVVVFEVDGDQERCLGKRVERVRQRDPLKLAGDIYDDLLRETGL
HgdC    C. symbiosum         MSGIYTLGIDVGSTASKCIVLKDG-----KEIVAKSLIDVGAGTSGPQRAIEAVLNEAGM
SYN0371 S. aciditrophicus      MRIAGIDIGSRTVKLAILEEG-----KLVLSRKTLTSYNPLETAQELIGDTVFDA--
                              ***.*. . *  .                .              . ..
BcrD    T. aromatica         KASDVDYVATTGEG---ESLAFHTGHFYSMTTHARGAVYLNPEARAVLDIGALHGRAIRN
BadG    R. palustris         ARADVAYCATTGEG---ESLTFHTGHFYSMTTHARGAIYLNPGSRAVLDTGALHGRAIRM
HgdC    C. symbiosum         KKEDMAYTLATGYGRTSLMDGIADKQMSELSCHAKGATFLFPNVHTVIDIGGQDVKVLHI
SYN0371 S. aciditrophicus    -------LTATGYGRHLIKGHLDCPVISEIKAFAVGSRFFSQDCSSILDIGGQDTKAISL
                                .** *              . . * *. .      ...* *    . .

BcrD    T. aromatica         DERGKVETYKMTSQCASGSGQFLENIARYLGIAQDEIGSLSTQADNPEVVSSICAVLAET
BadG    R. palustris         DERGKVLAYKMTSQCASGSGQFLENIARYLGIAQDEIGSLSQRADNPEKVSGICAVLAET
HgdC    C. symbiosum         DN-GAMTNFQMNDKCAAGTGRFLDVMARVLEVKVEDLGRLGAMSRKKVGISSTCTVFAES
SYN0371 S. aciditrophicus    DSDGNMRKFEMNDKCAAGTGRFLEVMATALGFSLEEFAQAALSAEKAVKINSTCTVFAES
                             *  * .  . *. .**.*.*.**. .*  *    ..      .    .   *.* **.

BcrD    T. aromatica         DVINMVSRGISAPNILKGIHISMAGRLAKLLKSVGARDGVVLCTGGLALDEGLLKTLNES
BadG    R. palustris         DVINMVSRGISSPNILRGIHESMADRLAKLLKTLGSLDGTVQMTGGLALDAGLVEAMKDA
HgdC    C. symbiosum         EVISQLAMGTDKCDIIDGIHRSVAHRVTGLAHRIGVVP-DVVMTGGVAQNEGVVKALQDE
SYN0371 S. aciditrophicus    EVVSLTAKGVPRNEVALGIHKAIVSRSVGLLKKVPVPG-KIFFAGGVALNECVRVLLEEE
                             .*.   . *     .  *** ..  *   * . .      .  .**.*    .   . .

BcrD    T. aromatica         IQEQKMAVVAYNHPDSPYAGAIGAALWGAFRHEKLARLGQQQVAEAA
BadG    R. palustris         VVKAKVDVAIESHPDAIYAGAIGAALWGAFRHKRLADMAHAA-----
HgdC    C. symbiosum         LGCP-----INTSPLTQYNGALGAALLAWQAASRRQSNS--------
SYN0371 S. aciditrophicus    MASTVFVP-----SDPQIVGAVGAALSAL------------------
                                        **.****
```

aciditrophicus is the a fact that an ATP-dependent, benzoyl-CoA reductase activity has not detected in a number of the benzoate-degrading anaerobes including *Geobacter metallireducens* (56), *Desulfococcus multivorans* (48), and *Syntrophus gentianae* (53).

Wischgoll et al. (56) found a benzoate-induced gene cluster in *G. metallireducens* that contained a gene whose protein product (BamB, annotated as an aldehyde dehydrogenase) may function as the benzoyl-CoA reductase. Adjacent to *bamB* on the genome of *G. metallireducens* are genes encoding for a heterodisulfide reductase subunits, other iron-sulfur proteins, and subunits of the NADH:ubiquinone oxidoreductase (Table 4.4). The presence of the latter genes suggested that membrane energy may be involved in benzoyl-CoA reduction (56). Proteomic analyses of *S. aciditrophicus* provides evidence that such a system functions in *S. aciditrophicus* (Tables 4. 2 and 4. 3). Heterodisulfide reductase subunit A was induced when *S. aciditrophicus* was grown with crotonate and benzoate compared to crotonate alone (Table 4. 2), implicating it in benzoate degradation. The *S*. *aciditrophicus* genome contains five gene clusters that contain homologs of heterodisulfide reductase subunit A, two of which have genes with high sequence similarity and synteny as the benzoate-induced genes in *G. metallireducens* (43). Due to high similarity in the amino acid sequences of the SYN00257, SYN02423, SYN03215, and SYN01645 gene products, especially at the N-terminal region where the peptide sequence information was obtained, one cannot determine which gene encoded for heterodisulfide reductase A subunit. However, the gene product of SYN01638 was

Table 4. 4. Gene cluster of *S. aciditrophicus* genome containing genes involved in crotonate and benzoate metabolism.



Gene #	Gene ID	Annotation
1	RSYN01673	Hypothetical protein
2	RSYN01672	Molybdopterin dependent oxidoreductase
3	RSYN01671	Hypothetical membrane spanning protein
4	RSYN01669	Hypothetical protein
5	RSYN01668	Iron-sulfur cluster assembly/repair protein ApbE (signal peptide)
6	RSYN01666	Antibiotic export ATP-binding protein (ABC transporter)
7	RSYN01665	Antibiotic export permease protein (ABC transporter)
8	**RSYN01664**	**Predicted NADH:ubiquinone oxidoreductase, subunit rnfC**
9	RSYN01663	Predicted NADH:ubiquinone oxidoreductase, subunit RnfD
10	**RSYN01662**	**Predicted NADH:ubiquinone oxidoreductase, subunit RnfG (signal peptide)**
11	RSYN01661	Predicted NADH:ubiquinone oxidoreductase, subunit Rnf E
12	RSYN01659	Predicted NADH:ubiquinone oxidoreductase, subunit RnfA
13	RSYN03073	Hypothetical exported protein (signal peptide)
14	RSYN01658	Predicted NADH:ubiquinone oxidoreductase, subunit RnfB
15	RSYN03074	Transposase
16	**RSYN01655**	**6-Hydroxycyclohex-1-ene-1-carboxyl-CoA dehydrogenase**
17	**RSYN01654**	**Putative ring hydrolyzing enzyme/Enoyl-CoA hydratase/isomerase family**
18	**RSYN01653**	**Cyclohex-1,5-diene-1-carboxyl-CoA hydratase**
19	RSYN01652	Xaa-Pro aminopeptidase
20	RSYN01651	Radical SAM superfamily protein (Predicted Fe-S oxidoreductase)
21	**RSYN01650**	**Putative tRNA (5-carboxymethylaminomethyl-2-thiouridylate) synthase subunit GidA**

22	RSYN01649	Uncharacterized anaerobic dehydrogenase (2Fe-2S iron-sulfur cluster binding domain, and 4Fe-4S binding domain)
23	RSYN01648	NADH:ubiquinone oxidoreductase, NADH-binding (51 kD) subunit
24	RSYN01647	NADH:ubiquinone oxidoreductase 24 kD subunit
25	RSYN01646	F_{420}-non-reducing hydrogenase vhc iron-sulfur subunit D
26	RSYN03075	Hypothetical membrane spanning protein
27	**RSYN01645**	**Heterodisulfide reductase subunit A related polyferredoxin**
28	RSYN01644	Heterodisulfide reductase, subunit A and related polyferredoxins
29	RSYN01643	CoB—CoM heterodisulfide reductase, subunit hdrD
30	RSYN01642	Fe-S-cluster-containing hydrogenase/Ferredoxin
31	**RSYN01640**	**Tungsten-containing aldehyde ferredoxin oxidoreductase**
32	RSYN01639	Di- and tricarboxylate transporters (Sodium:sulfate symporter transmembrane region)
33	RSYN01638	2,3-Dihydroxybenzoate-AMP ligase
34	RSYN01637	Transposase

The gene product identified by proteomic analysis are shown in bold.

detected showing that one of the two gene clusters that contains genes with high similarity and synteny as the benzoate-induced gene system in *G. metallireducens* (56) is expressed in *S. aciditrophicus*. A Fe-S oxidoreductase encoded by one of the genes clustered with genes for heterodisulfide reductase A was detected. Again, due to high amino acid sequence similarity, it is not possible to determine which gene encoded this proteins, SYN00259, SYN02421, or SYN03213. Thus, it is likely that enzymatic machinery used to reduce benzoyl-CoA in *S. aciditrophicus* is similar to that used in *G. metallireducens* (56).

Beta-oxidation and substrate level phosphorylation.

Proteomic analysis detected all of the proteins needed to convert acyl-CoA substrates to acetyl-CoA in cultures *S. aciditrophicus* grown with crotonate alone and with crotonate plus benzoate (Table 4. 3). The detected proteins include with the corresponding gene designation in parentheses: an enoyl-CoA hydratase (SYN01309), a 3-hydroxyacyl-CoAdehydrogenase (SYN01310), two acetyl-CoA acetyltransferases/ thiolases (SYN001681, SYN02642), electron transfer flavoprotein α and β subunits (SYN02637 and SYN02636, respectively), and four acyl-CoA dehydrogenases (SYN00480, SYN02586, SYN02587, SYN01699). Gene products of SYN03128 (long-chain, fatty acid-CoA ligase (EC 6.2.1.3)) and of SYN02586 and SYN02587 (acyl-CoA dehydrogenases, medium-chain specific (EC 1.3.99.3) and short-chain specific (EC 1.3.99.2), respectively) were induced during growth with crotonate plus benzoate (Table 4. 2). Small amounts of glutarate and pimelate were

detected when *S. aciditrophicus* was grown in pure culture with benzoate or crotonate or in coculture with benzoate (17, 18, 45), which explains the need for multiple acyl-CoA dehydrogenases and acyl-CoA ligases. (45) showed that *S. aciditrophicus* synthesizes cyclohexane carboxylate by the condensation of two-carbon intermediates. Thus, at least two acetyl-CoA acetyltransferases would be needed for growth with crotonate, one to degrade intermediates of fatty and aromatic acid metabolism to acetyl-CoA, and the other to synthesize 3-ketoacyl-CoA intermediates needed to form cyclohexane carboxylate from crotonate. Moreover, the role of the SYN02635 gene product (annotated as an acetyl-CoA synthetase (AMP-forming)) may be to reutilize excreted acetate to form acetyl-CoA for cyclohexane carboxylate formation (38, 45).

Genes SYN02635 to SYN02640 are located with in the same region of the *S. aciditrophicus* genome, suggestive of an operon. In addition to the acetyl-CoA synthetase (AMP-forming)) (SYN02635) and electron transfer flavoprotein α and β subunits (SYN02637 and SYN02636, respectively), whose gene products were detected in the proteome, genes for a membrane-bound, Fe-S oxidoreductase (SYN02638), a fatty acid transporter (SYN03639), and a long-chain fatty acid ligase (SYN02640) are present. The linkage SYN2638 with the genes for the electron transfer flavoprotein is consistent with the SYN2638 gene product functioning to couple in electron flow during the β–oxidation of acyl-CoA intermediates to membrane redox processes.

The two soluble components of the ion-translocating, glutaconyl-CoA decarboxylase, the biotin-carrier protein (SYN00479) and subunit A (SYN00481) along with an acyl-CoA dehydrogenase whose genes (SYN00480) are located with the genes for the above proteins, were detected in the proteome of *S. aciditrophicus*. This enzyme system functions to generate a sodium gradient by the decarboxylation of glutaconyl-CoA derived during benzoate degradation (13, 53). It could also function to synthesize glutaconyl-CoA for the synthesis of cyclohexane carboxylate during the fermentation of crotonate (45). ^{13}C-labeling studies showed that carboxylation occurred after the synthesis of the four-carbon intermediate during cyclohexane carboxylate formation.

Many fermentative bacteria use acetate kinase to synthesize ATP (22). The genome of *S. aciditrophicus* lacks a homolog for acetate kinase, but has two gene clusters, each with a butyrate kinase gene and two phosphate acetyl/butyryl transferase genes (45), which could function to make ATP during fatty and aromatic acid metabolism in *S. aciditrophicus*. Cell-free extracts of benzoate-grown cocultures and crotonate-grown pure cultures had low acetate kinase activity and phosphotransacetylase activity was not detected in cell-free extracts of crotonate-grown, pure cultures (17). The inability to detect or low activity could be due to non-optimal assay conditions. Proteomic analysis, however, did not detect any of the gene products from the above gene systems (Supplemental Table), which strongly argues against a phosphotransferase-kinase system for ATP synthesis in *S. aciditrophicus*. McInerney et al. (43) hypothesized that ATP is synthesized from acetyl-CoA by an acetyl-CoA synthase (ADP-forming) of archaeal origin because the genome of *S. aciditrophicus* contains nine homologs for this enzyme. Consistent with this hypothesis, proteomic

analysis detected the SYN00646 gene product, the β subunit of an acetyl-CoA synthase (ADP-forming).

Bioenergetics.

The key proteins involved in energy production and conversion that were identified by proteomic analysis in *S. aciditrophicus* are summarized in Table 4. 3. Five subunits of a gene cluster coding for a periplasmic and a cytoplasmic formate dehydrogenase complex (SYN00625-SYN00638) were detected. The periplasmic formate dehydrogenase is coded by four genes, SYN00632 to SYN00635. SYN00635 and SYN00634 code for a protein similar to the larger subunit of *E.coli* FdnG and is the active site of the enzyme (5). SYN00633 codes for the iron-sulfur component and its gene product was detected. SYN00632 codes for cytochrome B556, which forms the membrane anchor of the protein. The cytoplasmic formate dehydrogenase is coded by two genes SYN00629 and SYN00630, for the catalytic and iron-sulfur components, respectively. Both gene products were detected. The same gene cluster contains genes coding for a ferredoxin, an NADH:quinone oxidoreductase, a putative heterodisulfide reductase subunit A, F_{420} non-reducing hydrogenase, and maturation proteins. Their protein products are predicted to be cytoplasmic enzymes, which might function in electron transport with as yet undetermined membrane protein. The NADH:quinone oxidoreductase and one of the maturation proteins was detected in the proteome. The presence of cytoplasmic and periplasmic formate dehydrogenases in the proteome is consistent with energy conservation by a formate cycle where proton

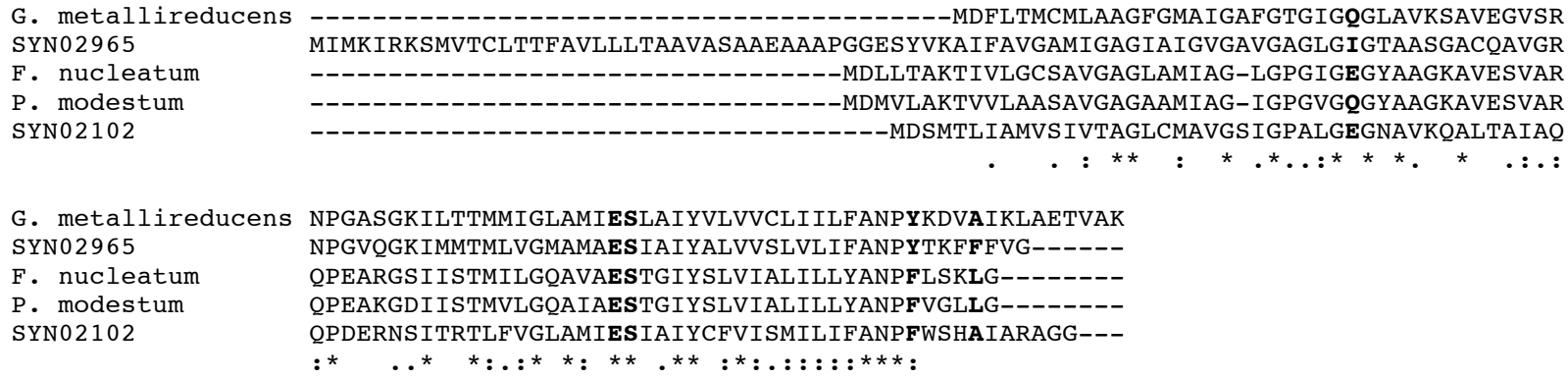
consumption by the cytosolic formate synthesis is coupled to proton production in the periplasm by formate oxidation (28, 43).

A cytoplasmic Fe-only hydrogenase was detected by proteomics (Table 4. 3) that provides a mechanism for hydrogen formation by *S. aciditrophicus*. The presence of hydrogenases and two formate dehydrogenases indicates that *S. aciditrophicus* uses both hydrogen or formate to reoxidize its reduced cofactors.

Major components of F_1F_0 ATP synthase were identified (Table 4. 3). The ATP synthase needs all three subunits of the F_0 module a, b, and c to form a functional ion-translocating particle. (52). Biochemical labeling studies in *Propionigenium modestum* have demonstrated that the Na^+-binding site is located at the conserved acidic residue Glu65 within the membrane of the c subunit (36). Amino acid alignment of the c subunit of *P. modestum* and *S. aciditrophicus* SYN00588 revealed the presence of the conserved amino acid Glu suggesting that the F_1F_0 ATP synthase uses Na^+ rather than H^+ as a coupling ion (Fig. 4. 5). The a subunit was shown as well to be responsible for the coupling ion selectivity (34). The amino acid sequence of SYN00588 annotated as ATP synthase a subunit did not show all the conserved amino acids of the Na^+-translocating of *P. modestum*, therefore it is not certain whether this subunit uses Na^+ or H^+ as a coupling ion. Further studies should be carried out to determine the coupling ion used for ATP synthesis in *S. aciditrophicus*.

Two subunits of the Rnf complex were detected by proteomics (Table 4. 3). The Rnf complex was studied in *R. capsulatus* and was shown to be a new family of

Figure 4. 5. Alignment of deduced amino acid of two ATPase c subunit from *Syntrophus aciditrophicus* (SYN #) and *Geobacter metallireducens*, and amino acid sequences of Na^+-specific F_1F_0-ATPase of *Propionigenium modestum* and *Fusobacterium nucleatum*. The amino acids demonstrated to be Na^+-speacific are shown in bold.


G. metallireducens ----------MDFLTMCMLAAGFGMAIGAFGTGIGQGLAVKSAVEGVSR
SYN02965 MIMKIRKSMVTCLTTFAVLLLTAAVASAAEAAAPGGESYVKAIFAVGAMIGAGIAIGVGAVGAGLGIGTAASGACQAVGR
F. nucleatum ----------MDLLTAKTIVLGCSAVGAGLAMIAG-LGPGIGEGYAAGKAVESVAR
P. modestum ----------MDMVLAKTVVLAASAVGAGAAMIAG-IGPGVGQGYAAGKAVESVAR
SYN02102 ----------MDSMTLIAMVSIVTAGLCMAVGSIGPALGEGNAVKQALTAIAQ
NPGASGKILTTMMIGLAMIESLAIYVLVVCLIILFANPYKDVAIKLAETVAK
NPGVQGKIMMTMLVGMAMAESIAIYALVVSLVLIFANPYTKFFFVG------
QPEARGSIISTMILGQAVAESTGIYSLVIALILLYANPFLSKLG--------
QPEAKGDIISTMVLGQAIAESTGIYSLVIALILLYANPFVGLLG--------
QPDERNSITRTLFVGLAMIESIAIYCFVISMILIFANPFWSHAIARAGG---

energy coupling oxidoreductase that functions in making reduced ferredoxin needed nitrogen fixation (51). However, this complex is found in *S. aciditrophicus* which does not fix nitrogen. The function of the Rnf complex in *S. aciditrophicus* may be to achieve the energetically unfavorable redox reduction of ferredoxin from NADH (37). Pyruvate is hypothesized to be made in *S. aciditrophicus* (43) by the carboxylation of acetyl-CoA, with reduced ferredoxin as the electron donor. The alpha subunit of the pyruvate:ferredoxin oxidoreductase was also detected.

Stress response.

Aerobic bacteria deal with hyperoxic stress by using two main enzymes superoxide dismutase (SOD) and catalase the disproportionation of superoxide and hydrogen peroxide, respectively. It was initially thought that strict anaerobic bacteria did not possess either of the two enzymes (40). However, in *D. gigas*, the activity of both enzymes was demonstrated (14). *S. aciditrophicus* genome contains a gene coding for iron-containing SOD (SYN03202), and its expression was confirmed by 2D gel electrophoresis when *S. aciditrophicus* was grown anaerobically with crotonate. An alternative oxidative stress response exists in anaerobic bacteria and archaea where reduction rather than disproportionation of superoxide or hydrogen peroxide is used (39). Some strict anaerobes such as *D. vulgaris* Hildenborough possess a cytoplasmic rubredoxin oxidoreductase (or desulfoferredoxin) and a non-haem iron protein, rubrerythrin and nigerythrin that reduce hydrogen peroxide (19). *S. aciditrophicus* genome does not contain a rubredoxin oxidoreductase but proteomic

analysis showed the expression of three rubrerythrin proteins (SYN00469, SYN02523, and SYN02559), which might be an alternative to the catalase activity. Although the genome has a gene coding for catalase, its protein product was not detected.

Other proteins involved in stress resistance were detected by proteomic analysis, including two universal stress proteins (SYN01539, and SYN02544), whose expression is enhanced by a variety of stress conditions, two cold shock proteins (SYN00019 and SYN01437) which have been implicated in various cellular processes including adaptation to low temperatures, cellular growth, nutrient stress, and stationary phase regulation (23), and 4 small heat shock proteins (Hsp20) (SYN00029, SYN00030, SYN00031, and SYN01907), whose role is mainly to act as chaperones to protect other proteins against heat-induced denaturation and aggregation (33).

CONCLUDING REMARKS

In this study, we provided an overview of *S. aciditrophicus* proteome data when grown with an unsaturated fatty acid or an aromatic acid compound. The results suggested that, as described for *G. metallireducens* (56) *, S. aciditrophicus* may possess a novel mechanism for benzoate reduction involving a tungsten/molybdenum-containing aldehyde ferredoxin oxidoreductase and heterodisulfide reductase components where the energy needed for ring reduction is probably driven by a membrane potential (56). Although previous studies have shown the detection of intermediates and enzyme activities similar to *R. palustris* during benzoate metabolism, the proteomic data revealed proteins that were analogous to *T. aromatica*. This supports the hypothesize that two possible routes for benzoate metabolism exist in *S. aciditrophicus*, benzoate degradation to acetate and carbon dioxide and benzoate reduction to cyclohexane carboxylate as proposed previously (17, 18)

The syntrophic lifestyle of such microorganisms occurs in an environment where free energy supplies are very limited. Therefore, microorganisms capable of syntrophic metabolism must have developed efficient energy conservation systems (32, 50). This idea gains support from the detection of an ion-translocating Rnf complex, which that may be involved in reverse electron transport, a sodium-translocating glutaconyl-CoA decarboxylase, and cytoplasmic and periplasmic

formate dehydrogenases that would separate formate synthesis and hydrolysis across the cell membrane to form a proton/sodium gradient.

The detection of the same intermediates involved in cyclohexane carboxylate degradation during cyclohexane carboxylate formation during crotonate metabolism (45) suggests that at least part of the cyclohexane carboxylate degradation pathway is reversible. A key step would be that performed by the glutaconyl-CoA decarboxylase, which could reversibly carboxylate or decaboxylate crotonyl-CoA and glutaconyl-CoA, respectively, depending on whether cyclohexane carboxylate is being formed or degraded. Two subunits of this enzyme complex were detected under both conditions as were a number of β–oxidation enzymes, which is consistent with the hypothesis that the same pathway is used to degrade and form cyclohexane carboxylate.

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APPENDIX 1

Microbial Oil Recovery from sand-packs using a biosurfactant synthesized by *Bacillus* strain JF-2

ABSTRACT

The effectiveness of *Bacillus* strain JF-2, and its biosurfactant in recovering residual oil was tested in sand packed columns. Oil was recovered from columns during the injection of the supernatant of JF-2 culture. This shows that the biosurfactant present in the supernatant can enhance oil recovery. The addition of cells and nutrients to columns lead to sporadic oil recovery due to sporadic growth of strain JF-2 in the columns. Future experiments will focus on strategies to enhance the in-situ growth and metabolism by altering the nutrient mixture, inoculum size and incubation time.

Key words: MEOR, *Bacillus* strain JF-2, biosurfactant.

INTRODUCTION

Microbially enhanced oil recovery (MEOR) is an important tertiary recovery technology utilizing microorganisms and/or their metabolic products for the recovery of residual oil. EOR technology, by using a chemical surfactant, can recover around 30% of the oil remaining in the reservoir (2). However, the expense of using chemical surfactants has limited the application of chemical EOR. In addition, chemical surfactants may be difficult to degrade which presents an environmental risk if a spill occurs. Biosurfactants are biodegradable, which makes them environmentally friendly.

Biosurfactant are amphiphilic molecules that lower the interfacial tension between two different phases. The reduction in interfacial tension reduces the high capillary forces that entrap oil in small pores in the reservoir rock. Biosurfactants also act as emulsifiers to increase the apparent water solubility of oil and to reduce its viscosity. Both of these mechanisms make oil more mobile (4).

Bacillus strain JF-2 synthesizes a biosurfactant, a lipoprotein, able to decrease the surface tension from 72 to 27 mN/m under anaerobic conditions (5)**.** This microorganism can grow under both aerobic and anaerobic conditions in the presence of high salinity and elevated temperature (up to 50°C). The biosurfactant is stable at pH values between 5.5 and 12, which is the typical condition of the most oil reservoirs. These characteristics make *Bacillus* strain JF-2 a good candidate for in-situ MEOR (7).

The purpose of this work is to test the effectiveness of using the biosurfactant synthesized by *Bacillus* strain JF-2 to recover residual oil. Our first goal was to determine if oil can be recovered by adding the biosurfactant alone or if the in situ biosurfactant production is necessary to improve oil recovery. Indeed, a localized production might be needed to generate sufficiently high biosurfactant concentration and perhaps additional compounds synthesized by the microorganism that can act as co-surfactant. To test this hypothesis, the experiment was conducted using sand-packs columns incubated under anaerobic conditions at elevated temperatures.

MATERIALS AND METHODS

Microorganisms and growth conditions

Bacillus strain JF-2 (ATCC # 39307) was grown anaerobically in medium E. Medium E contained (all components per liter of medium): 2.7g KH_2PO_4, 13.9 K_2HPO_4, 50g NaCl, 10g glucose, 1g yeast extract, 1g $NaNO_3$, 1g $(NH_4)_2SO_4$, 30g Proteose Peptone #2, and 10 ml of a metal solution. The metal solution (a modified Wolin's trace metal solution) contained: 1g EDTA, 3g $MnSO_4 \cdot H_2O$, 0.1g $FeSO_4 \cdot 7H_2O$, 0.1g $CaCl_2 \cdot 2H_2O$, 0.1g $CoCl_2 \cdot 2H_2O$, 0.1g $ZnSO_4 \cdot 2H_2O$, 0.01g $CuSO_4 \cdot 5H_2O$, 0.01g $AlK(SO_4)_2$, 0.01g H_3BO_3, 0.01g $Na_2MoO_4.7H_2O$, 25g $MgSO_4$.

The bacterial enumeration was done by plating onto Plate Count Agar (Difco) which contained 5 g Bacto-tryptone (a pancreatic digest of casein), 2.55 g Bacto-yeast extract, 15 g Bacto-dextrose, 1g glucose, 15 g Bacto-agar, or by using a selective medium which is medium E added with 15 g of agar. The serial dilution of the samples was done by using a solution of 2.7g KH_2PO_4, 13.9g K_2HPO_4 and 50g NaCl (pH of 7). Direct microscopic counting was done using a Petroff-Hausser counter (1/400 square mm, 1/50 mm deep) (3).

Sand-packed preparation

Plexiglass columns were filled with sand (quartz sand with a mesh size of 100). The sand was packed inside the columns with continuous vibration to ensure a homogenous packing density to avoid formation of layers. The columns were weighed before and after packing to determine the weight of the sand by difference.

Brine saturation

The packs were placed in the horizontal position and the brine (degassed nanopure water with 5% NaCl) was injected into one end of the column while applying a vacuum to the other end. Three pore volumes of brine were flooded through each column to saturate the sand and to remove any residual air that was entrapped in the column. Each column was weighted before and after brine saturation and from the brine density (ρ_b=1.0519), the pore volume was calculated (Table A1. 1). Between each step, the tubes joining the reservoir flasks and the columns were disconnected, and the columns were sealed with caps.

Oil drainage

Next, each column was saturated with crude oil by using a peristaltic pump. The packs were placed in the vertical position and the inlet and the outlet ends were connected to the oil container and the collecting flasks, respectively. The oil (ρ_o=0.8108) was injected from the top to ensure a maximum coverage of the column and to utilize gravity to displace the brine from the column. Three pore volumes of oil were used to saturate each column to obtain irreducible water saturation. The difference between the initial oil volume and the total oil collected was the volume of oil in each column.

Recovery of oil by brine flood

Each column was placed in the vertical position connected to the peristaltic pump and brine was injected into the bottom of the column. Since the displacing fluid (brine) is denser than the displaced fluid (oil), the displacement efficiency is

Table A1. 1. Petrophysical data of the sand-packs.

Columns #	Volume of the empty packs (ml)	Volume of the sand added (ml)	Liquid pore volume (ml)	Amount of oil after drainage (ml)	Amount of oil remaining after brine flood (ml)	Residual oil saturation (% of pore volume)	Oil recovery following the water flood (% of total oil)
1	280.1	208.6	73	60	24	33	60
2	286.3	209.3	73	62	25	34	59
3	270.6	195.7	72	58	21	29	63
4	284.5	206.6	70	58	24	34	58
5	283.2	204.4	74	64	30	40	53

improved if the displacing fluid is injected from the bottom. Three pore volumes of brine were injected into each column and this was sufficient to reach residual oil saturation in each column.

Microbial treatments.

Inoculation. JF-2 was grown in flasks containing 500ml of medium E under anaerobic conditions at 37^{o}C. After 48 hours, the absorbance of the culture at 600nm reached a value of 0.58 and the surface tension was 38 mN/m. The culture was centrifuged (11,300 x g; 15min). The pellet was resuspended in sterile medium E to give a final concentration of $8.1*10^{6}$ cells/ml. Two columns (columns 1 and 2) each received 1 pore volume of the JF-2 suspension in medium E. Two others columns (columns 3 and 4) each received one pore volume of the supernatant of the JF-2 culture. A fifth column (column 5) served as a control and was treated with 1 pore volume of brine. All fluid injections were made by using a peristaltic pump and the fluids were injected into the bottom of each column.

First nutrient treatment. Columns 1 and 2 were each flooded with 2 pore volumes of medium E with $8.1*10^{6}$ cells/ml of JF-2. Columns 3 and 4 each received 2 pore volumes of the supernatant of a JF-2 culture with a surface tension of 38 mN/m, prepared as described above. Column 5 received 2 pore volumes of brine. The columns were sealed and incubated for 60 hours at 40^{o}C.

After the incubation, the column were flooded with 2 pore volumes of brine and the effluent of each column was collected in sealed, 60 ml, plastic syringes, which were positioned vertically to allow the separation of oil, gas and liquid phases.

The amount of gas, oil and liquid from each column was measured volumetrically in the syringes.

The gas that was collected was injected into sealed serum bottles containing a solution of 5% of NaCl at pH 2 to maintain any gas that was produced in the gas phase. The liquid phase of the effluent was collected and frozen until subsequent analysis.

Second nutrient treatment. A second nutrient treatment was done by flooding each column (columns 1 to 5) with 1 pore volume of sterile medium E, no cells were added, using the procedure described above. The columns were incubated for 9 days at 40°C. After the incubation time, each column was flooded with 2 pore volumes of brine following the same procedure than the one described above.

Analytical techniques

The surface tension was measured by using a Fisher Tensiometer model 215. The tensiometer was calibrated using a 1g weight. The surface tension of nanopure water was measured as a standard (72.5 mM/m). All the samples were measured at room temperature.

CO_2 concentration was quantified by using a gas chromatograph equipped with a thermal conductivity detector (TCD). Helium was the carrier gas at a flow rate of 16 ml/min. The column was an 80/100 Poropak Q 6'*1/8''. The injector and detector temperatures were 105°C, the column temperature was 60°C, and the TCD filament was at 160°C. CO_2 standards were made in helium-containing bottles with various CO_2 concentrations (2% to 8%).

Glucose concentration was measured by using the sulfuric acid/phenol reaction (3). The absorbance was read at 488 nm against the blank prepared without glucose. The concentrations of sugars are then determined in the samples from a standard curve prepared by plotting the absorbance of standards versus the concentration of glucose.

RESULTS

The surfactant production is an important mechanism for the microbially enhanced oil recovery process. A biosurfactant produced by *Bacillus* strain JF-2 has been shown to significantly reduce the interfacial tension between oil and water (6, 8).

The first experiment was conducted to determine if this biosurfactant recover oil recovery from sand-packs on its own or if the introduction of JF-2 is needed for the localized production of the biosurfactant or other products. Previous work done by Bryant et al. (1), suggested that the production of gas, organic acids and solvents by the microorganism might be involved in MEOR.

Our work was conducted in sand-packs columns, and primary treatment with brine recovered between 53 and 63.8% of the oil (Table A1. 1).

First nutrient treatment.

Two columns (columns 1 and 2) were treated with cells of JF-2 at a final cell concentration of $8.1*10^6$ cells/ml and medium E and the two other columns were treated with a supernatant of JF-2 culture with a surface tension of 38 mM/m, due to the biosurfactant synthesized by *Bacillus* strain JF-2. Immediately after injecting the supernatant of the JF-2 culture, 1.5 ml of oil was recovered from the column 3, and 0.5 ml from the column 4 probably due to the biosurfactant contained in the supernatant. No oil was recovered from the other columns during the injection phase.

The columns were incubated for 60 hours to allow for in-situ growth and metabolism, and then the columns were flooded with brine. For the two columns that received cells and nutrients, oil was recovered from only one column after the brine flood (Table A1. 2). The effluent of both of these columns had surface tension of 41 to 43 mN/m (Table A1. 2), which indicates that the amount of biosurfactant produced did not exceed the critical micelle concentration. Little glucose and nitrate were consumed in column 1 indicating that little in-situ metabolism occurred. Significant depletion of glucose and nitrate occurred in the column 2, suggesting that in-situ metabolism did occur.

Oil was produced from columns that received the supernatant of a JF-2 culture and allowed to incubate as described above (Table A1. 2). In column 3, where 2 ml of oil was recovered by the brine flood, the surface tension was 28 mN/m, indicating that the critical micelle concentration was reached. In column 4, little oil was recovered, the surface tension was in excess of 40 mN/m, indicating that the biosurfactant concentration did not exceed the critical micelle concentration. Effluent analysis showed that significant amounts of glucose and nitrate were present (Table A1. 3). These nutrients could have supported the in-situ growth and metabolism of the small number of JF-2 cells that were present in the supernatant solution.

Gas was produced from the columns that received nutrients or the supernatant of a JF-2 culture. No gas or oil was recovered from the control that received only brine.

Table A1 .2. Experimental results obtained after the first and the second nutrient treatment.

	Column #	Treatment no.	Volume of oil recovered (ml)	Gas produced (ml)	Residual oil recovery (%)	Surface tension (mN/m)
Inoculated columns	1	1	-	4.5	-	43
		2	High turbidity of the effluent	4	ND	36
	2	1	1	4	4	41
		2	Turbid effluent	3.5	ND	43
Cell-free columns	3	1	2	4.5	10	28
		2	Turbid effluent	3.5	ND	41
	4	1	<0.5	5	<2%	36
		2	Turbid	4	ND	39
Untreated column	5	1	-	-	-	73
		2	-	-	-	50

Table A1. 3. Columns effluent analysis after the first and the second nutrient treatment.

Column no.	Treatment no.	Glucose concentration (g/l)	Nitrate concentration (mM)	CO_2 Concentration (μM)
1	1	9.2	11.7	2
	2	3.5	3.82	3
2	1	8.3	10.6	2
	2	9	12.9	1
3	1	6.1	2.71	4
	2	9.2	13.0	1
4	1	6.1	2.58	3
	2	9.1	13.1	2
Initial concentration in columns 1 and 2		9.4	14.8	-
Initial concentration in columns 3 and 4		9.2	12.4	-

Second nutrient treatment.

Since little growth was evident after the first treatment, one pore volume of medium E was added to each column and the incubation time was extended to 9 days to stimulate in-situ growth. The control column (#5) also received nutrients. No oil was observed after the injection of the medium into the columns. Thus, the medium itself is not sufficient to recover oil.

After incubation, the columns were flooded with brine and turbidity was apparent in the effluent of all the columns except the column 5 (Table A1. 2). Microscopic analysis showed that the turbidity was mainly due to the presence of very small oil particles. The average diameter of the oil drops were 1.6, 2, 5.8, 5, 6 μm for columns 1, 2, 3, 4, respectively. This emulsion was very stable and the turbidity remained for an extended period of time. This made quantification of the oil recovered very difficult.

Gas was collected with the effluent of column1 through 4 but not from column 5. Surface tensions of all the effluent samples were above 40 mN/m. Thus, the biosurfactant concentration was less than the critical micelle concentration. Significant loss of glucose and nitrate was observed only in column 1 (Table A1. 3).

Effluent samples were placed onto plates of medium E and plate count agar. Only 5 colonies were detected in plates of medium E inoculated and undiluted effluent from the column 1. No bacteria growth was observed on plate count agar. Thus little or no JF-2 cells were recovered in the effluent (< 30 per ml).

DISCUSSION

The addition of the supernatant of a culture of JF-2 to oil-containing porous material did result in oil recovery. This shows that the biosurfactant is effective in recovering oil when the concentration is near the critical micelle concentration.

At present, we have insufficient data to conclude whether the in-situ production of the biosurfactant is more effective than the injection of biosurfactant solutions. We do know that in the one core where the surface tension of the effluent was at or below the critical micelle concentration, a significant amount of oil (2 ml) was produced (Table A1. 2).

It is apparent that significant growth of JF-2 did not occur when the columns were flooded with medium E. This is surprising since this medium supports excellent anaerobic growth of JF-2 in liquid culture. Experiments will continue to determine whether longer incubation times, larger inoculum sizes and addition medium components will be needed to obtain significant growth of JF-2 in porous materials.

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APPENDIX 2

Bacillus strain JF-2 growth and biosurfactant synthesis under anaerobic conditions in sand-pack columns.

ABSTRACT

Bacillus strain JF-2 (JF-2) grew and synthesized biosurfactant under anaerobic conditions in sand-pack columns. Biosurfactant production was confirmed using surface tension measurements. The surface tension of the column effluent was 38 mN/m when the columns were injected with an inoculum size of 10^8 cells/ml. A slightly higher surface tension (44 mN/m) was obtained when the inoculum size was 10^4 cells/ml. However, JF-2 was not able to grow in the presence of the particular crude oil used in this experiment.

Key words: MEOR, *Bacillus* strain JF-2, biosurfactant.

INTRODUCTION

Oil is an essential energy source and continued economic growth increases the demand for oil. Conventional production technologies are able to recover approximately 30 to 50% of oil originally in place (2). The target of enhanced oil recovery (EOR) is to increase oil reserves by improving oil recovery. However the large capital or high chemical/energy cost of current EOR technologies limits their application (1). An alternative technology to improve oil recovery is to use microorganisms, called microbially enhanced oil recovery (MEOR).

It has been shown that MEOR methods hold promise as an economical alternative to conventional EOR process. MEOR capitalizes on naturally occurring substances and processes; and, since environmental compatibility is becoming an increasingly important factor in the selection of industrial chemicals, MEOR processes could result in both economical and environmentally friendly methods. With further research, it may be possible to produce large amounts of useful products such as biosurfactants from inexpensive and renewable resources.

There are a number of ways in which microorganisms may affect the mobilization of oil within reservoirs (10). The accumulation of biomass and polymers synthesized by the microorganisms themselves can plug the most permeable regions redirecting the recovery fluid to previously bypassed zones. The production of polymers can increase the viscosity of the injected fluids and thus positively affect the mobility rates. The capillary number may be increased by a reduction in interfacial

tension due to surfactant production. The gas produced by the microorganisms can cause a local repressurization within the reservoir. The production of acids will dissolve the limestone matrices and improve oil recovery by changing the porosity. JF-2 is able to synthesize a biosurfactant significantly decreases both the surface tension and the interfacial tension between oil and water and it is potentially useful in enhanced oil recovery (4, 5, 9).

In previous experiments, metabolic indicators (such as the loss of glucose, and/or the production of metabolic byproducts) were not detected in the column effluent of sand-pack columns saturated with oil and inoculated with JF-2 (see appendix 1). While some glucose loss was detected, it was not possible to associate this loss with microbial growth. No viable bacteria were detected in the effluent. In an effort to clarify the reasons for this, several hypothesis were tested: i) JF-2 is not able to grow in a sand environment due to a contact inhibition with the sand; ii) the sand contains some competing microorganisms which prevent JF-2 from growing; and iii) JF-2 growth is inhibited by the presence of particular crude oil that was used.

The purpose of the following studies was to test these hypotheses to determine what factor was responsible for the inhibition of JF-2 growth. First we determined if JF-2 was able to grow in the sand-pack environment under anaerobic conditions by measuring metabolic by-products synthesized from glucose and by plating the effluent onto medium to see if JF-2 was present. Secondly, we determined if the presence of this particular crude oil was inhibitory to JF-2.

MATERIALS AND METHODS

Microorganisms and growth conditions.

Bacillus strain JF-2 (ATCC # 39307) was grown anaerobically in medium E. Medium E contained (per liter): 2.7g KH_2PO_4, 13.9 K_2HPO_4, 50g NaCl, 10g glucose, 1 g yeast extract, 1 g $NaNO_3$, 1 g $(NH_4)_2SO_4$, 30 g Proteose Peptone #2, and 10 ml of a metal solution. The metal solution (a modified Wolin's trace metal solution) contained: 1 g EDTA, 3 g $MnSO_4 \cdot H_2O$, 0.1 g $FeSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.1 g $CoCl_2 \cdot 2H_2O$, 0.1 g $ZnSO_4 \cdot 2H_2O$, 0.01 g $CuSO_4 \cdot 5H_2O$, 0.01 g AlK $(SO_4)_2$, 0.01 g H_3BO_3, 0.01 g $Na_2MoO_4.7H_2O$, 25 g $MgSO_4$. Bacterial enumeration was performed by plating onto medium E containing 1.5 g/l of agar. The serial dilution was performed using a sterilized solution containing 2.7 g KH_2PO_4, 13.9 g K_2HPO_4, and 50 g NaCl (pH of 6.8).

Sand-pack preparation.

Plexiglass columns were filled with sand (quartz with a density of 2.65 kg/l) and packed with continuous vibrations to ensure a homogenous packing density and avoid the formation of layers. The columns were sealed with plastic stoppers and a butyl rubber septum was used to allow aseptic and anaerobic additions to the columns and to maintain anaerobic conditions. The ends were capped by using polypropylene filters with a pore size of 20 μm.

Each column was weighed before and after packing with sand. It will give the sand weight. Knowing the density of sand, the pore volume was determined by

calculated the difference between the column volume and the sand volume. The pore volume was also determined by weighing the columns before and after the brine flooding. From the brine density (ρ_b= 1.05196), and the difference in weight before and after the flooding, the liquid pore volume was calculated. These two techniques gave similar estimates for the pore volume that differed by only 1-1.4% (Table A2. 1). To ensure anaerobic conditions, the columns were flushed 3 times with nitrogen gas and then placed in an anaerobic chamber for 24 hours.

Brine saturation.

The columns were injected with brine (degassed nanopure water with 5% NaCl using positive pressure of the fluid reservoir to push fluid into the columns. Two pore volumes were injected through the columns to saturate them and remove the gas trapped inside.

Columns treatment.

A 200 ml solution of medium E inoculated with JF-2 was incubated under anaerobic conditions at 40°C for 20 hours. The surface tension was 30 mN/m. The cell concentration was determined using a Petroff Hausser counting chamber (1/400 square mm, 1/50 mm deep) (3). The cells were pelleted by centrifugation and resuspended in sterile medium E to give a cell concentration of 10^4 or 10^8 cells/ml.

Two columns (1 and 2) were treated with one pore volume of medium E which contained a cell concentration of 10^4 cells/ml. A third column (3) was treated with one pore volume of medium E containing a cell concentration of 10^8 cells/ml. A fourth column (4) was treated with sterile medium E and served as the control. Each

column was flooded by using a positive pressure of nitrogen gas to push he liquid into the column. The sand-packs were incubated at 40°C.

Pressure measurements.

The pressure inside the columns was measured by using a Cole-Parmer digital gauge. JF-2 produces CO_2 and N_2 as end product of its metabolism, so gas production was used as an indicator of in situ growth (Table A2. 2). When the rate of increase in the gas pressure stopped, the columns were flooded with two pore volumes of brine, and samples were collected into 30 ml syringes. The effluent was collected in 20 ml samples. The liquid effluent was frozen until subsequent analysis.

JF-2 growth in the presence of crude oil.

Serum bottles were filled with 50 g of sand and flushed with nitrogen for 5 minutes to ensure anaerobic conditions. Each bottle received 30 ml of medium E and 1 ml of oil. The bottles were inoculated with JF-2 and incubated for 20 hours at 40°C. The positive controls consisted of inoculating medium E with JF-2 in the absence of sand and oil. The negative control consisted of an uninoculated medium with sand and oil. The sand was previously sterilized at 120°C during 20 minutes. The experiment was done in triplicate

Balch tubes containing 20 ml of medium E supplemented with 1 ml of crude oil were inoculated with JF-2. The oil was previously sterilized at 120°C during 20 minutes. The positive control consisted of tubes without oil. The tubes were all kept under anaerobic conditions. This was performed in triplicate.

Table A2. 1. Petrophysical data of the sand-packs.

Column no.	Weight of sand (g)	Volume of sand (g)	Pore volume (cm^3)	Weight of brine (g)	Pore volume (cm^3)
1	357.02	134.7245	67.0808	71.1	67.5881
2	358.98	135.4642	66.3412	70.5	67.0177
3	354.93	133.9358	67.8695	72.2	68.6338
4	353.29	133.4260	68.3793	72.9	69.3182

Table A2. 2. Change in pressure in sand-pack columns after inoculation (psi).

Column no.	Time				
	20 hours	48 hours	4 days	5 days	11 days
1	3.6	7.1	6.4	6.4	6.2
2	3.5	9.6	8.5	8.0	-
3	8.7	11.7	11.3	10.6	-
4	1.7	7.4	5.2	5.2	-

DNA Blot.

Colony transfer: Cells were transferred to a nylon membrane from a petri dish by placing the nylon membrane on the surface of the petri dish. The cells were then lysed by placing the membrane in a solution of 0.5 N of NaOH, and allowed to stand for 10 minutes at room temperature. The membrane was transferred first to a filter paper soaked with 0.5 M Tris HCl (pH 7.5) for 5 minutes, then to a filter paper soaked with a solution containing 0.5 M Tris HCl (pH 7.5) and 1.5 M NaCl for 5 minutes, and finally transferred to a filter paper soaked with 2x SSC solution (20x SSC solution is 173.3 g of NaCl and 88.2 g of sodium citrate per liter at a pH 7.8). To immobilize the DNA onto the membrane, the membrane was baked under vacuum for 2 hours at 80°C.

DNA Blotting. The membrane was placed in a hybridization glass tube (Fisher Biotech) containing 20 ml prehybridization solution (DIG Eazy Hyb, Boehringer Mannheim) per 100 cm^2 of membrane surface area, and incubated in Hybridization Incubator (Fisher Biotech) at 37-42°C for 2 hours. The prehybridization solution was discarded and the prehybridization solution containing the labeled probe srfA1 (5-25 ng/ml) was added, and incubated in Hybridization Incubator at 37-42°C overnight. At the end of the hybridization, the hybridization solution was poured into a tube. The membrane was washed in 2x washing solution (300 mM NaCl, 30 mM sodium citrate, 0.1% SDS, pH 7.0) at room temperature for 10 min. The membrane was washed again in 0.5x washing solution (75 mM NaCl, 7.5 mM sodium citrate, 0.1% SDS, pH 7.0) at 68°C for 30 min.

Chemiluminescent Detection. After hybridization and post-hybridization washes, the membrane was equilibrated in washing buffer (100 mM maleic acid, 150 mM NaCl; pH 7.5; 0.3% (v/v) Tween®20) for 1 minute. The membrane was blocked by gently agitating it in blocking solution (100 mM maleic acid, 150 mM NaCl; pH 7.5; 1% (w/v) Blocking reagent [Boehringer Mannheim]) for 30-60 minutes. The blocking solution was removed and the membrane was incubated in the antibody solution (the Anti-Digoxigenin-AP in blocking solution [1:100000 v/v], Boehringer Mannheim) for 30 minutes. After the antibody solution was discarded, the membrane was washed in washing buffer for 30 minutes. The washing buffer was removed and the membrane was equilibrated in detection buffer (100 mM Tris-HCl, 100 mM NaCl; pH 9.5) for two minutes. The membrane was placed between two sheets of acetate (PhotoGene Development Folders, Gibco BRL) and 0.5 ml (per 100 cm^2) of the Chemiluminescent substrate (CSPD® 1:100 in detection buffer, Boehringer Mannheim) was then added on top of the membrane, scattering the drops over the surface of the membrane. With a damp tissue, the top sheet of plastic was wiped gently to remove any bubbles present under the sheet and to create a liquid seal around the membrane. The filter was incubated for 5 minutes. The semi-dry membranes were sealed in acetate sheets. The membrane was incubated at 37°C for 15 minutes. For detection of the Chemiluminescent signal, the membrane was exposed to Lumi-Film (Boehringer Mannheim) for 15-20 minutes.

Analytical techniques.

The surface tension was measured using a Fisher Tensiometer model 215. The surface tension of nanopure water was measured as a standard (73 mN/m). All the samples were measured at room temperature.

The concentration of glucose was measured by phenol sulfuric method (3). The absorbance was read at 488 nm against the blank prepared without glucose. The concentrations of glucose were determined in the samples from a standard curve prepared by plotting the absorbance of standards versus the concentration of glucose.

Nitrate concentrations were determined by using a Dionex Ion Chromatography system with an AS4A-SC 4-mm particle-size column, a model CD 20 conductivity detector, and a mobile phase of 1.8 mM sodium carbonate and 1.7 mM sodium bicarbonate delivered at 2ml/min.

Acetate and butanediol concentrations were determined using a gas chromatography equipped with a flame ionization detector and a glass column. The samples as well as the standards were diluted in a solution of 30 mM of oxalic acid.

The concentration of biosurfactant in the samples was quantified by HPLC analysis. A C18 column was used with a mobile phase of 70% methanol and 30% of 10 mM phosphate buffer. The HPLC was run at a flow rate of 1 ml/min and the injection volume was 20 µl. A UV detector was used with a wavelength set at 210 nm. Sample preparation is described in the previous chapter of this report.

RESULTS AND DISCUSSION

Sand-pack experiment.

The first experiment was conducted to determine if JF-2 was able to grow and produce biosurfactant in a sand environment under anaerobic conditions. It was also important to determine if inoculum size influenced biosurfactant production. The columns were inoculated with two different cell concentrations of JF-2: two columns (1 and 2) were treated with a cell concentration of 10^4 cells/ml and a third column (3) was treated with a cell concentration of 10^8 cells/ml. The fourth column was treated with uninoculated medium E and served as a control. After 5 days of incubation, the columns were flooded with brine. The first column was allowed to incubate for a longer period of time (11 days) in order to determine if a longer incubation time was necessary for biosurfactant production.

The surface tension measured in the two first columns was reduced to 44 mN/m, which indicated the production of biosurfactant. The surface tension of the third column where the inoculum size was higher, 10^8 cells/ml reached a value of 38 mN/m. HPLC analysis was not able to detect the presence of biosurfactant. Significant depletion of glucose and nitrate was observed in all the columns indicating metabolic activity. This suggested that JF-2 grew and metabolized its substrates inside the 2, 3 sand-pack columns. The end -product analysis revealed the production of acetate and butanediol in all the columns, even in the uninoculated

column (Table A2. 3), potentially due to contamination. The metabolic activity in column 4 was probably due to organisms present in the sand grains.

The effluent from each column was plated onto medium E to determine the viable cell concentration in the effluent of each column. The effluent cell concentration was $11.5x10^4$, $8x10^4$, $15x10^5$ and $16.4x10^3$ cells/ml for columns 1, 2, 3 and 4, respectively. The cells were transferred onto a membrane and lysed to expose the DNA and to hybridize it with a probe corresponding to one of the genes involved in the biosynthesis of biosurfactant to determine if JF-2 was growing inside the columns. This method showed 85 spots at a dilution of 10^{-2} for the column 1, 5 spots at a dilution of 10^{-3} for the column 2, 8 spots at a dilution of 10^{-4} for column 3 and 4 spots at a dilution of 10^{-2} for column 4. This showed that JF-2 represented about 20 to 75% of the viable cell population and the presence of this other bacteria did not prevent JF-2 from growing and synthesizing its biosurfactant.

The surface tension in the fourth column did not decrease and the number of JF-2 cells observed was very low. These data suggest that the cell concentration is important for biosurfactant synthesis and that biosurfactant production depends on the presence of JF-2.

Previous work (under aerobic conditions) showed that biosurfactant synthesis in JF-2 occurs during the exponential phase of growth and when cells reach the stationary phase the biosurfactant concentration starts to decrease until it completely disappears from the culture broth (5, 7, 8). However as shown with the first column, which was incubated for 11 days, the surface tension was the same as that found in

Table A2. 3. Data analysis from JF-2 growing in sand-packs columns.

Column no.	Effluent collected	Surface tension (mN/m)		Glucose concentration (mM)		Nitrate concentration (mM)		Acetate concentration (mM)	Butanediol concentration (mM)
		Initial	Final	Initial	Final	Initial	Final	Initial	Final
1	1/3 from the top	58	44	63.9	2.9	15.0	0	17.4	7.57
	1/3 from the middle		47		2.5		0	12.8	6.09
2	1/3 from the top	60	44	58.9	4.8	15.9	0	18.4	5.94
	1/3 from the middle		48		3.4		0	16.5	5.48
3	1/3 from the top	52	38	59.4	3.3	16.1	0	17.6	9.04
	1/3 from the middle		41		3.3		0	18.8	8.41
4	1/3 from the top	59	60	57.8	4.2	15.8	0	16.9	4.89
	1/3 from the middle		58		2.8		0	13.3	3.05

the column incubated for only 5 days of incubation. The anaerobic conditions may prevent the internalization of the biosurfactant molecules by the microorganisms.

The spatial distribution of metabolic end-products such as glucose, nitrate, acetate and butanediol was examined to determine if these products are uniformly present or present along a gradient within the column. The columns have a total liquid volume of about 67-68 ml. During the brine flood, one pore volume of the effluent was collected in 3 separate syringes (20 ml each time) corresponding to a third of the liquid volume of the column, the first third from the distal, the second third from the center and the last corresponding to the proximal end of the column relative to the inoculation site. The analyses showed that the distribution of products along the column decreased slightly (Table A2. 3). For example, in column 1 the first third had a glucose concentration of 2.9 mM and the second third had a glucose concentration of 2.5 mM. Similarly, the acetate concentration at the first third was 17.4 mM and 12.8 mM in the second sample. The butanediol concentration was 7.6 mM and 6.1 mM respectively. These data suggest uniform growth of JF-2 throughout the column.

JF-2 growth in the presence of crude oil.

From the data given above, it was determined that the presence of sand or competing microorganisms did not inhibit JF-2 growth. So, the second experiment was conducted to determine if the particular oil used in the experiment was actually responsible for inhibiting growth of JF-2. As JF-2 was isolated from oil-field injection brine (5,7) it was evident that JF-2 was able to grow in the presence of crude

oil. However, in this case, the oil in use did appear to inhibit growth of JF-2. Each time oil was added to the medium inoculated with cells, no bacterial growth occurred and no metabolic activity was detected since neither glucose nor nitrate were depleted (Table A2. 4). Since no growth was detected in bottles using sterilized sand and oil, the inhibition was not due to competition from other microorganisms. This data confirmed that that particular crude oil used in the sand packs inhibited JF-2 growth.

Table A2. 4. Effect of sand, oil and competing organisms on the metabolic activity of JF-2.

Treatment	Glucose concentration (mM)		Nitrate concentration (mM)	
	Initial	Final	Initial	Final
Medium E + JF-2	53.7	20	13.8	0.45
Medium E + JF-2 + oil	53.5	51	14.7	13.8
Medium E + JF-2 + sand	53.6	18	14.5	1.3
Medium E + JF-2 + sand + oil	52	53	14.7	13.8
Medium E + JF-2 + sterilized sand	51.3	26	14.9	2.83
Medium E + JF-2 + sterilized sand oil	51	54	13	15
Medium E + sand + oil	55	48	16	15.7

CONCLUSION

JF-2 is able to grow under anaerobic conditions in a sand environment and produce its biosurfactant. These qualities make it suitable for understanding the MEOR processes in laboratory sand-pack columns. It is able to synthesize its biosurfactant at a detectable level if the inoculum size is above 10^8 cells/ml. However the experiment revealed that the particular crude oil used in our experiment inhibited JF-2 from growing, and this phenomenon was not due to a competing microorganism. However, other crude oils can be used which are not inhibitory.

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APPENDIX 3

Competition of *Bacillus* strain JF-2 with natural microbial population.

ABSTRACT

The addition of Proteose peptone to groundwater microcosms inoculated with *Bacillus* strain JF-2 resulted in the production of 2,3-butanediol, a fermentation end product characteristic of *Bacillus* species. This metabolite was also detected in microcosms that did not receive an inoculum of *Bacillus* strain JF-2 as long as Proteose peptone was present. In groundwater microcosms amended with glucose and 27 mM nitrate or Proteose peptone and 27 mM nitrate that were inoculated with *Bacillus* strain JF-2, up to 90% of the viable microbial population contained genes for the production of the lipopeptide biosurfactant. Further analyses of the microbial cells that contained the biosurfactant genes showed that they grew anaerobically at high salt concentrations and some made biosurfactants in liquid culture. All of these are characteristics of *Bacillus* strain JF-2. These studies showed that it is possible to enhance the growth of bacteria with the potential to produce lipopeptide biosurfactants by selective nutrient additions. In fact, nearly all of the cells in the population had the genetic potential to make lipopeptide biosurfactants with certain

nutrient amendments. It is possible to control the dynamics of natural microbial populations during microbial oil recovery processes by nutrient manipulations.

Key words: MEOR, *Bacillus* strain JF-2, biosurfactant.

INTRODUCTION

It is becoming increasingly important to develop enhanced oil recovery (EOR) techniques to recover additional oil from existing wells (15). The microbial EOR process has some promise. The biosurfactants produced by microorganisms are naturally occurring substances and thus should not persist for long period of time in the environment. Because microbial growth occurs at exponential rates, it should be possible to produce large amounts of useful products rapidly from inexpensive and renewable resources. Lastly, some microbial biosurfactants are known to significantly decrease the interfacial tension between oil and water. The lipopeptide biosurfactant produced by *Bacillus* strain JF-2 reduces the surface tension from 72 to 27 mN m -1 and the interfacial tension to between oil and water to less than 10^{-3} mN/m (6, 11, 12). This organism can grow and produce the lipopeptide biosurfactant under anaerobic conditions in the presence of high salt concentrations and at elevated temperatures. These are the environmental conditions found in many oil reservoirs.

There are several different strategies for employing biosurfactants in MEOR. One approach might be the production of biosurfactant in bioreactors ex-situ and subsequent injection of the biosurfactant into the reservoir. This approach would require the development of cultures that make very large concentrations of biosurfactants in order for product recovery to be economically feasible. The injection of a biosurfactant-producing microorganism into a reservoir and the subsequent stimulation of its growth and metabolism in the reservoir is another approach. This

approach has been shown to be successful in a previous MEOR field trial (2). Localized production of biosurfactant might be an efficient mechanism to recover residual oil. However, this claim has never been tested. A third approach would be to selectively stimulate biosurfactant-producing bacteria that occur naturally in oil reservoirs. However, it is not know whether organisms that make biosurfactants are commonly found in oil reservoirs. These last two approaches both require mechanisms to stimulate selectively a) the growth of biosurfactant-producing organisms and b) the production of their biosurfactant. Previous studies have shown that the injection of nutrients in oil reservoirs will stimulate microbial metabolism (2, 7, 10). The presence of gases, solvents, acids, and surface-active agents have been detected in field studies. However, most of these products are a consequence of the central energy metabolism of the microorganism. Biosurfactants are secondary metabolites that may not be needed for the growth of cells. Thus, it is not yet clear whether it is possible to stimulate the production of such molecules selectively.

The objective of this work to determine if the nutrient formulation previously developped to allow the anaerobic growth of *Bacillus* strain JF-2 will allow this organism to establish itself in a mixed microbial community. The experiment was designed to simulate the conditions that might occur during the application of a MEOR process. A readily available source of subsurface microorganisms was groundwater obtained from a local anaerobic aquifer whose microbiology has been studies in detail (4).

MATERIALS AND METHODS

Source of groundwater.

The groundwater was collected in May 2001 from the aquifer underlying the landfill in Norman, OK. The groundwater samples were extracted from the well #40 at 3.75 m depth by using a peristaltic pump (4). The groundwater was flushed with 100% nitrogen gas and then stored in the anaerobic chamber at room temperature until used.

Microorganisms and growth conditions.

Bacillus strain JF-2 (ATCC # 39307) was grown aerobically in medium E at 37°C during 24 hours. Medium E contained (per liter): 2.7g KH_2PO_4, 13.9 K_2HPO_4, 50g NaCl, 10g glucose, 1 g yeast extract, 1 g $NaNO_3$, 1 g $(NH_4)_2SO_4$, 30 g Proteose Peptone #2, and 10 ml of a metal solution. The metal solution (a modified Wolin's trace metal solution) contained: 1 g EDTA, 3 g $MnSO_4 \cdot H_2O$, 0.1 g $FeSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.1 g $CoCl_2 \cdot 2H_2O$, 0.1 g $ZnSO_4 \cdot 2H_2O$, 0.01 g $CuSO_4 \cdot 5H_2O$, 0.01 g $AlK(SO_4)_2$, 0.01 g H_3BO_3, 0.01 g $Na_2MoO_4.7H_2O$, 25 g $MgSO_4$.

Flasks with 200 ml of medium E were inoculated with *Bacillus* strain JF-2 and then grown aerobically at 37°C for 20 hours. After growth, the cells were harvested by centrifugation (11,300 x g; 15min; at 10°C) and the cell pellet was resuspended in a sterile 50 mM of TES buffer (pH 7.0). One ml of this cell suspension was used to inoculate each microcosm.

Microcosm preparation.

Each microcosm was prepared by adding 50 ml of groundwater to sterile serum bottles inside of the anaerobic chamber (1). The serum bottles were capped with rubber stoppers and sealed with aluminum caps and taken out of the anaerobic chamber. The gas phase of each serum bottle was exchanged with 100% nitrogen by evacuation under vacuum and respressurization with the above gas phase (1). Nutrients were added to the serum bottles in different combinations by adding 0.5 ml of a sterile, anaerobically prepared stock solution using a sterile syringe and needle flushed with nitrogen gas in order to maintain anaerobic conditions (1). The stock solutions used were: 2.7 M nitrate, 0.12 M nitrate, 30% Proteose peptone #2, 57.4 mM K_2HPO_4 and 0.55 M glucose. The final concentrations of the respective nutrient in the microcosm were: 27 mM or 1.2 mM of nitrate, 0.3% of Proteose peptone, 0.574 mM of K_2HPO_4 and 5.5 mM of glucose. The stock solutions were sterilized by autoclaving at 121°C for 20 minutes, except for glucose, which was sterilized by passage through a 0.22μm filter. After nutrients were added to the microcosms, 1 ml of the JF-2 cell suspension was added to each serum bottle. Each nutrient treatment was done in triplicate. The serum bottles were incubated at 23°C inside the anaerobic chamber. Samples were taken by using syringes with needles previously flushed with nitrogen gas after 1, 10 and 30 days of incubation. A second set of microcosms was prepared as above but it did not receive an inoculum of JF-2 and only 20 ml rather than 50 ml of groundwater was added to each serum bottle. These microcosms were sampled after immediately after preparation and after 10 days of incubation.

Analytical techniques.

Bacterial enumeration was performed by plating onto 1/10X Plate Count Agar containing (per liter): 5 g of tryptone, pancreatic digest of casein USP, 2.5 g of yeast extract, 1 g of dextrose-glucose, 15 g of agar. The medium was diluted 10-fold and 13.5 g of agar was added prior to preparation of the plates. A 1:10 serial dilution until $1:10^7$ dilution was performed with samples from the microcosm using sterile, Nanopure water. Each agar plate received 0.1ml of the diluted cell suspension. The plates were incubated at 23°C inside the anaerobic chamber.

The surface tension of microcosms and cultures was measured using a Fisher Tensiometer model 215. Nanopure water was used as a standard (73 mN/m). All the samples were measured at room temperature.

The concentration of glucose was measured by the phenol sulfuric method (5). The absorbance was read at 488 nm against the blank prepared without glucose. The concentration of glucose was determined from a standard curve prepared by plotting the absorbance versus the concentration of glucose of standards.

Nitrate concentration was determined by using a Dionex Ion Chromatography system with an AS4A-SC 4-mm particle-size column, a model CD 20 conductivity detector, and a mobile phase of 1.8 mM sodium carbonate and 1.7 mM sodium bicarbonate delivered at 2ml/min.

Acetate and butanediol concentrations were determined using a gas chromatograph (GC) equipped with a flame ionization detector (FID) and a 6’ glass column packed with Carbopack TM B-DA 80/120 4% Carbowax 20M resin. The GC

was set to a flow rate of 24 ml/min of helium and operated with an injector temperature of 200°C and a detector temperature of 200°C. A thermal gradient from 155°C to 185°C with temperature increasing at 3°C per min was used. Samples as well as standards were diluted in a solution of 30 mM of oxalic acid prior to injection.

The concentration of biosurfactant in the samples was quantified by high pressure liquid chromatography analysis. A C18 column was used with a mobile phase of 70% methanol and 30% of 10 mM phosphate buffer (pH 6.8). The HPLC was run at a flow rate of 1 ml/min and the injection volume was 20 µl. A UV detector was used with a wavelength set at 210 nm (9).

Ammonium concentration was measured by using the indophenol blue reaction (5). The ammonium standard was prepared by dissolving 381.9 mg of anhydrous NH_4Cl in 1 liter of Nanopure water. One ml of this solution contained 122 µg of NH_3. The samples were centrifuged (11,300 x g; 15min; at 10°C) to remove cells and debris. The supernatant was removed and diluted in nanopure water to a final volume of 5 ml. To the diluted samples, the following reagents were added: 25µl of 0.003 M MnSO4, 0.25 ml of hypochlorite reagent (10 ml of 5% sodium hypochlorite solution in 40 ml of Nanopure water), and 0.30ml of phenate reagent (2.5 g of NaOH and 10 g of phenol in 100 ml solution). The absorbance was read at 630 nm against the blank prepared with the above reagents. The concentration of ammonium was determined from a standard curve prepared by plotting the absorbance of standards versus their concentration.

DNA Blot analysis.

Cells were transferred to a nylon membrane from agar plates by placing the nylon membrane on the surface of the agar plates. The cells were then lysed by placing the membrane in a solution of 0.5 N of NaOH for 10 minutes at room temperature. The membrane was transferred first to a filter paper soaked with 0.5 M Tris HCl (pH 7.5) for 5 minutes, second to a filter paper soaked with a solution containing 0.5 M Tris HCl (pH 7.5) and 1.5 M NaCl for 5 minutes, and finally transferred to a filter paper soaked with 2x SSC solution (2x SSC solution contained 17.33 g of NaCl and 8.82 g of sodium citrate per liter, pH 7.8). The membrane was baked under vacuum for 2 hours at 80^{o}C to immobilize the DNA onto the membrane.

The membrane with immobilized DNA was placed in a hybridization glass tube (Fisher Biotech) containing 20 ml prehybridization solution (DIG Eazy Hyb, Boehringer Mannheim) per 100 cm^2 of membrane surface area, and incubated in hybridization incubator (Fisher Biotech) at 37-42°C for 2 hours. The prehybridization solution was discarded and the prehybridization solution containing the labeled probe srfA1 (5-25 ng/ml) was added. The membrane was then incubated in hybridization incubator at 37-42°C overnight. The hybridization solution was discarded into a tube. The membrane was washed in 2x washing solution (300 mM NaCl, 30 mM sodium citrate, 0.1% SDS, pH 7.0) at room temperature for 10 min. The membrane was washed again in 0.5x washing solution (75 mM NaCl, 7.5 mM sodium citrate, 0.1% SDS, pH 7.0) at 68°C for 30 min.

Chemiluminescent Detection.

After hybridization and post-hybridization washes, the membrane was equilibrated in washing buffer (100 mM maleic acid, 150 mM NaCl; pH 7.5; 0.3% (v/v) Tween®20) for 1 minute. The membrane was blocked by gently agitating it in blocking solution (100 mM maleic acid, 150 mM NaCl; pH 7.5; 1% (w/v) Blocking reagent (Boehringer Mannheim) for 30-60 minutes. The blocking solution was removed and the membrane was incubated in the antibody solution (the Anti-Digoxigenin-AP in blocking solution [1:100000 v/v], Boehringer Mannheim) for 30 minutes. After the antibody solution was discarded, the membrane was washed in washing buffer for 30 minutes. The washing buffer was removed and the membrane was equilibrated in detection buffer (100 mM Tris-HCl, 100 mM NaCl; pH 9.5) for two minutes. The membrane was placed between two sheets of acetate (PhotoGene Development Folders, Gibco BRL) and 0.5 ml (per 100 cm^2) of the Chemiluminescent substrate (CSPD® 1:100 in detection buffer, Boehringer Mannheim) was then added on top of the membrane by scattering the drops over the surface of the membrane. With a damp tissue, the top sheet of plastic was wiped gently to remove any bubbles present under the sheet and to create a liquid seal around the membrane. The filter was incubated for 5 minutes. The semi-dry membranes were sealed in acetate sheets. The membrane was incubated at 37°C for 15 minutes. For detection of the chemiluminescent signal, the membrane was exposed to Lumi-Film (Boehringer Mannheim) for 15-20 minutes.

RESULTS

Anaerobic metabolism in groundwater microcosms inoculated with *Bacillus* strain JF-2.

The ability of *Bacillus* strain JF-2 to establish itself in the presence of natural microbial population was evaluated by using groundwater as a model subsurface microbial population. Groundwater amended with nutrients known to support the growth of strain JF-2 was utilized. The depletion of nutrients, the production of characteristic products and the presence of the biosurfactant genes in bacteria isolated from the groundwater incubations were used as indicators that JF-2 or physiologically similar bacteria established themselves in the mixed microbial community. Previous study has shown that the addition of Proteose Peptone to Medium E stimulated anaerobic growth of JF-2. We also found that 2,3- butanediol was produced by strain JF-2 under these conditions. Many species of the genus *Bacillus* species produce 2,3-butanediol as a product of carbohydrate fermentation as well as use nitrate as electron acceptor in the absence of oxygen (14). We used two nitrate concentrations (1.2 and 27 mM) since indications in the literature suggest that high concentrations of nitrate selectively enrich for *Bacillus licheniformis* and related species, such as *B. mojavensis*.

The initial glucose concentration in the microcosms was approximately 5 mM. Glucose depletion was evident in many of the microcosms that contained Proteose peptone and were inoculated with strain JF-2 (Table A3. 1).

Table A3. 1. Glucose consumption and acetate and 2,3-butanediol production in microcosms inoculated with *Bacillus* JF-2. [1]

Treatment	Glucose (mM)		Acetate (mM)		Butanediol (mM)	
	Day 1	Day 10	Day 1	Day 10	Day 1	Day 10
Glucose	5.0±1.95	0.2±0.05	0.4±0.4	18±5.3	ND	ND
Glucose + high NO_3^-	5.2±0.04	0.2±0.05	0.4±0.07	0.7±0.4	ND	ND
Glucose + low NO_3^-	5.3±0.24	0.2±0.3	0.3±0.3	18±0.4	ND	ND
Glucose + high NO_3^- + PP[2]	1.8±0.70	0.4±0.04	12±5.2	4.9±3.7	0.34±0.20	0.33±0.1
Glucose + low NO_3^- + PP	2.3±2.6	0.3±0.03	13±1.1	3.32±0.2	0.46±0.2	0.69±0.4
Glucose + high NO_3^- + PP+ PO_4^-	1.5±0.50	0.3±0.03	13±2.1	11±6.6	0.44±0.1	0.76±0.4
Glucose + low NO_3^- + PP+ PO_4^-	1.6±0.10	0.3±0.06	14±2.5	33±1.6	0.69±0.4	0.65±0.03
PP + high NO_3^-	0.5±0.06	0.3±0.03	1.6±0.2	6.0±1.1	0.43±0.6	0.22±0.02
PP + low NO_3^-	0.6±0.02	0.2±0.01	2.9±1.6	12±5.3	0.1±0.03	0.41±0.2
PP	0.5±0.06	0.3±0.04	3.6±0.4	19±3.2	0.2±0.16	0.71±0.04
Unamended	0.0±0.00	0.0±0.00	0.1±0.2	1.7±0.4	0.0±0.00	0.0±0.00

[1] The data are means ± standard deviations of triplicate incubations. Microcosms were incubated anaerobically at 23°C.

[2] Abbreviations: PP, Proteose peptone; ND, not detected.

After 10 days of incubation, glucose consumption in excess of 90% of the initial amount added was evident in all of the microcosms. Acetate was produced after 10 days of incubation. Glucose-amended microcosms without Proteose peptone and without nitrate or with low nitrate concentrations (1.2 mM) produced about 18 mM of acetate. About 11 mM acetate would be expected from the anaerobic degradation of 5.5 mM glucose. Production of acetate at concentrations above that expected from the amount of glucose added suggested that additional carbon sources were present in the groundwater to support anaerobic metabolism. Large amounts of acetate were produced (in excess of 30 mM) when Proteose peptone was added to glucose-amended microcosms with low nitrate concentrations. The increase in acetate production may have been due to the metabolism of Proteose peptone itself since microcosms amended with only Proteose peptone had an acetate concentration of about 19 mM after 10 days of incubation (Table A3. 1). In microcosms with high nitrate (27 mM), acetate concentrations were 0.7 mM in glucose-amended microcosms, 4.9 in microcosms with glucose and Proteose peptone, 11 mM in microcosms with glucose, Proteose peptone and phosphate, and 6.0 mM in microcosms with Proteose peptone (Table A3. 1). The lower acetate concentrations in microcosms amended with high nitrate compared to those with low nitrate suggests that nitrate respiration accounted for more of the reducing equivalents when nitrate levels were high.

Interestingly, 2,3-butanediol, a fermentation product known to be synthesized by *Bacillus* species, was produced in the enriched groundwater only when Proteose peptone was added to the solutions.

The nitrate was added to the groundwater as an electron acceptor in the absence of oxygen. It can be also used as a nitrogen source for some microorganisms. Previous work done by Cozzarelli et al. (4) showed that the nitrate is reduced to ammonium in groundwater from the landfill aquifer. Analysis of the microcosms for these two compounds showed depletion in the nitrate and an increase in ammonium concentration (Table A3. 2). It was expected that one mole of nitrate would be reduced to one mole of ammonium. The fact that the amount of ammonium produced was less than stoichiometrically predicted from nitrate consumption suggests that the nitrate may have been transformed to another nitrogen compound, either nitrite or nitrogen gas. These latter two compounds were not quantified in our experiment. However, the fact that nitrate was consumed and ammonium was produced showed that anaerobic nitrate metabolism did occur in our microcosms.

The surface tension was measured in samples collected from each microcosm. However no significant decrease of surface tension (<55 Nm/cm) was detected (data not shown).

Table A3. 2. Nitrate utilization and ammonium production in microcosms inoculated with *Bacillus* strain JF-2. [1]

Treatment	Nitrate (mM)		Ammonium (mM)	
	Day 1	Day 10	Day 1	Day 10
Glucose	0.52±0.01	0.52±0.00	2.35±1.7	2.99±0.38
Glucose + high NO_3^-	27.4±2.6	4.17±1.48	2.28±0.59	2.40±2.1
Glucose + low NO_3^-	1.39±0.13	0.52±0.00	2.35±0.93	3.82±0.52
Glucose + high NO_3^- + PP[2]	8.17±5.0	0.51±0.01	3.57±1.6	11.0±1.0
Glucose + low NO_3^- + PP	0.45±0.01	0.52±0.00	4.90±1.4	10.9±2.6
Glucose + high NO_3^- + PP + PO_4	15.6±7.89	0.49±0.04	3.70±2.4	9.18±2.1
Glucose + low NO_3^- + PP + PO_4	0.45±0.011	0.52±0.00	5.51±1.3	9.41±1.3
PP + high NO_3^-	12.5±3.83	0.52±0.00	2.42±2.1	10.0±3.4
PP + low NO_3^-	0.45±0.004	0.52±0.00	4.29±1.0	12.3±2.9
PP	0.45±0.003	0.53±0.00	4.56±0.37	8.61±2.1
Unamended	0.47±0.043	0.53±0.00	1.36±1.2	2.41±2.1

[1] The data are means ± standard deviations of triplicate incubations. Microcosms were incubated anaerobically at 23°C.

[2] Abbreviations: PP, Proteose peptone.

Anaerobic metabolism in uninoculated groundwater microcosms.

A control was carried out by amending nutrients to groundwater without inoculating it with JF-2. After 10 days of incubation, the glucose concentrations in glucose-amended microcosms ranged from 2.8 to 3.6 mM, regardless of the nitrate concentration (Table A3. 3). When microcosms were inoculated with JF-2, glucose concentrations were all below 1 mM (Table A1. 1). Acetate and 2,3-butanediol concentrations were lower in microcosms that were not inoculated with JF-2 (Table A3. 3) compared to the respective microcosms that were inoculated with JF-2 (Table A3. 1).

The presence of Proteose peptone enhanced the metabolism of microorganisms naturally present in the groundwater. The concentration of acetate and 2,3-butanediol was higher in microcosms that received Proteose peptone compared to the respective microcosm that did not receive Proteose peptone (Table A3. 3). Up to 0.76 mM 2,3-butanediol was detected, which is similar to levels found in microcosms that were inoculated with JF-2 (Table A3. 1). Evidently, the microorganisms naturally present in groundwater have the ability to produce 2,3-butanediol. Large amounts of nitrate were consumed in microcosms that were not inoculate with JF-2 (Table A3. 4). Ammonium production was limited, precluding major conclusions concerning the mechanism of metabolism of nitrate by the indigenous microorganisms.

Table A3. 3. Acetate and 2,3-butanediol production and glucose consumption in groundwater that was not inoculated with *Bacillus* strain JF-2.

Treatment	Glucose (mM)		Acetate (mM)		Butanediol (mM)	
	Initial	Day 10	Initial	Day 10	Initial	Day 10
Glucose	3.35±0.68	2.79±1.36	0.020±0.03	4.5±0.03	ND	0.07±0.09
Glucose + high NO_3^-	3.86±0.04	3.70±0.22	0.229±0.32	ND[1]	ND	ND
Glucose + low NO_3^-	3.51±0.49	3.67±0.18	0.086±0.01	0.7±0.05	ND	0.01±0.02
PP[1]	0.34±0.03	0.14±0.01	0.126±0.05	22±1.13	ND	0.77±0.08
PP + high NO_3^-	0.72±0.07	0.22±0.01	0.065±0.03	8.5±1.23	ND	0.07±0.08
PP + low NO_3^-	0.46±0.02	0.19±0.01	0.113±0.03	19±0.60	ND	0.45±0.20
Unamended	0.08±0.00	0.08±0.01	0.154±0.01	ND	ND	ND

[1]Abbreviations: PP, Proteose peptone; ND, not detected.

[2] The data are means ± standard deviations of triplicate incubations. Microcosms were incubated anaerobically at 23°C.

Table A3. 4. Effect of nutrient additions on nitrate and ammonium concentrations in groundwater that was not inoculated with *Bacillus* strain JF-2.

Treatment	Nitrate (mM)		Ammonium (mM)	
	Initial	Day 10	Initial	Day 10
Glucose	0.00±0.00	0.137±0.01	5.33±0.18	5.70±0.35
Glucose + high NO_3^-	35.6±3.98	0.109±0.02	5.55±0.62	5.61±0.64
Glucose + low NO_3^-	0.10±0.02	0.055±0.05	4.68±0.48	6.16±1.08
PP	0.12±0.00	0.007±0.00	3.33±2.12	4.71±4.25
PP + high NO_3^-	36.2±3.47	0.003±0.00	3.91±4.26	6.10±2.70
PP + low NO_3^-	0.10±0.02	0.007±0.00	4.58±3.64	4.79±0.95
Unamended	0.12±0.01	0.004±0.00	5.21±0.71	6.03±0.45

[1] Abbreviations: PP, Proteose peptone.

Presence of microorganisms with biosurfactant genes.

Molecular analysis was used to determine which nutrient amendment allowed the establishment of JF-2 or a similar microorganism that contained biosurfactant genes. The gene probe designed in McInerney et al. (13) was used to determine the percentage of the total viable cell population that contained genes for biosurfactant production.

A sample of amended groundwater from each bottle was plated on diluted PCA medium to allow the growth of the most numerous microorganisms in the microcosms. A dot blot was done on these plated to determine which of the colonies had cells that contained the biosurfactant genes. The probe used to hybridize to the DNA extracted from the colonies corresponded to a region of one of the genes involved in the synthesis of *Bacillus subtilis* biosurfactant, a lipopeptide almost identical to the biosurfactant synthesized by *Bacillus* strain JF-2 (8). Mcinerney et al. (13) showed that the probe would be specific to *Bacillus* strain JF-2 biosurfactant genes. The dot blot analysis revealed that, in microcosms amended with glucose and 27 mM of nitrate and microcosms amended with the Proteose peptone and 27 mM of nitrate, 90% of the total viable microbial population contained microorganisms that had surfactin synthetase-like genes (Table A3. 5).

The colonies that hybridized with the biosurfactant gene probe and colonies that did not hybridize with the gene probe were further analyzed to determine if they had physiologies similar to *Bacillus* strain JF-2 (Table A3. 6). A total of 15 positive

colonies that contained the biosurfactant gene as revealed by the dot blot analysis and 15 colonies which did not hybridize with the probe were inoculated into Medium E and Nutrient Broth. These cultures were incubated under aerobic and anaerobic conditions at two temperatures, 23°C and 37°C. Medium E contains high salt concentrations which is selective for *Bacillus* strain JF-2-like organisms. Also, *Bacillus* strain JF-2 and related organisms are known to grown anaerobically in this medium. Only colonies that contained the biosurfactant genes as revealed by dot blot analysis grew in Medium E or Nutrient Broth (Table A3. 6). Colonies that did not hybridize with the probe did not grow in either of these two media at either temperature, but they did grow on dilute PCA agar. In addition, the surface tension was measured from cultures that grew in these media. Those cells that were obtained from microcosms with Proteose peptone or glucose and 27 mM of nitrate lowered the surface tension of the medium to 49 Nm/m. Thus, microbial cells that grew in the microcosms amended with glucose and 27 mM nitrate or with Proteose peptone and 27 mM nitrate were predominately cells that contained biosurfactant genes similar to those needed to produce a lipopeptide biosurfactant. These cells also grew anaerobically in high salt medium and at elevated temperatures (37°C), both characteristics of *Bacillus* strain JF-2 and related organisms.

Table A3. 5. Viable cell concentration and number of organisms that contained biosurfactant genes in groundwater inoculated with *Bacillus* strain JF-2.

Treatment	Cell concentration (viable cells per ml)		Percentage of cells with biosurfactant genes [1]
	Day 1	Day 10	
Glucose	1.20E+08	TNTC[2]	-[3]
Glucose + high NO_3^-	8.40E+08	1.48E+09	90
Glucose + low NO_3^-	2.75E+08	8.12E+07	ND[2]
Glucose + high NO_3^- + PP[2]	TNTC	1.32E+09	-
Glucose + low NO_3^- + PP	1.38E+07	1.27E+06	4.3
Glucose + high NO_3^- + PP + PO_4^-	3.37E+08	8.50E+07	-
Glucose + lowNO_3^- + PP + PO_4^-	3.35E+06	ND	ND
PP + high NO_3^-	1.62E+09	2.87E+07	90
PP + low NO_3^-	2.61E+08	1.11E+07	ND
PP	1.19E+08	5.83E+07	15
Unamended	9.15E+06	ND	-

[1] Cells of colonies from plate count agar were transferred to a membrane and then hybridized with a probe for the biosurfactant gene (srfA). The percentage corresponds to the number of total culturable cells that contained the biosurfactant gene.
[2] Abbreviations: PP, proteose peptone; TNTC, too numerous to count; ND, not detected.
[3] -, not determined.

Table A3. 6. Confirmation that colonies that tested positive with the biosurfactant gene probe were physiologically similar to *Bacillus* strain JF-2.

Colony Type	Medium E				Nutrient Broth			
	Aerobic		Anaerobic		Aerobic		Anaerobic	
	23°C	37°C	23°C	37°C	23°C	37°C	23°C	37°C
Positive colonies[1]	10/15	10/15	10/15	8/15	11/15	12/15	3/15	1/15
Negative colonies	0/15	0/15	0/15	0/15	0/15	0/15	0/15	0/15

[1]The positive colonies were those that hybridized with the probe srfA corresponding to the biosurfactant genes. The negative colonies were those that did not hybridize to the above probe, indicating that they did not carry the biosurfactant genes.

DISCUSSION

The depletion of nitrate and glucose and the production of acetate, 2,3-butanediol and ammonium showed that the nutrient amendments stimulated bacterial metabolism in microcosms inoculated with JF-2 and in those that did not receive JF-2. The butanediol, a product known to be synthesized by members of the genus *Bacillus* (14) was detected in the groundwater supplemented by Proteose peptone. Butanediol was detected when only glucose was added. These data show the importance of Proteose peptone and nitrate concentration in enhancing the production of a specific microbial product, 2,3- butanediol. Thus, nutrient additions can lead to the selective stimulation of particular microbial metabolism. The analysis of the microbial fermentation products in the microcosms showed that acetate and butanediol are the major compounds synthesized with glucose as carbon source. The amount of acetate and butanediol produced in the groundwater supplemented by Proteose peptone alone showed that the addition of this nutrient is sufficient to support bacterial growth and bacterial metabolism. Butanediol is of particular importance for MEOR, because it can serve as a co-surfactant, a solvent, enhancing the activity of biosurfactants.

Molecular analysis showed the importance of either Proteose peptone or a high level of nitrate (27 mM) in enhancing the growth of microorganisms that contain genes for the synthesis of lipopeptide biosurfactants even in a mixed microbial population where many different kinds of microorganisms are present. It is

remarkable that this approach resulted microbial population where almost all of the viable microbial cell (90%) have the potential to make lipopeptide biosurfactants. Further investigations confirmed that the cells that tested positive for the biosurfactant genes were physiologically similar to *Bacillus* strain JF-2. Several of these strains did produce a biosurfactant when grown in liquid culture. Our work does show that we can manipulate mixed microbial communities to selectively enhance for physiologies that are needed for enhanced oil recovery. However, further investigations should be done to determine minimal concentrations of each nutrient needed to select for biosurfactant-producing *bacilli*.

A puzzling result is that a decrease of surface tension and the lipopeptide biosurfactant were not detected in any of the microcosms, even those where 90% of the cultivable cells contained the biosurfactant genes. Previous publications noticed the loss of the biosurfactant during aging of the culture (7, 10, 11). Due to sample size limitations, we were only able to take one sample for surface tension measurements from each microcosm. This was done after microbial metabolism and growth ceased (30 days). In many cases, viable cell numbers had already begun to decline by 30 days. It is entirely possible that the biosurfactant was synthesized and disappeared from the culture by the time we sampled. However, we do know that the most numerous organisms isolated from these microcosms did produce a biosurfactant when grown in liquid culture.

This work confirms our studies with sand-packed columns that showed the growth of JF-2 and the production of 2.3-butanediol and biosurfactant after inoculation and addition of glucose, nitrate and Proteose peptone. These sand-packs were not sterilized before inoculation, but this shows that our nutrient regime will allow strain JF-2 to establish itself in a mixed microbial community under the salinities seen in many oil reservoirs. This is an important point since many oil reservoirs may not contain an indigenous population of microorganisms that make biosurfactants. Our work shows that, in model systems, we can inoculate with *Bacillus* strain JF-2 and that it can maintain itself and synthesize its biosurfactant when specific nutrients: glucose, Proteose peptone and nitrate are added.

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