**Effect of Community Structure on the Kinetics of Anaerobic Degradation of Aromatic
Compounds**

Progress Report

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I. Summary of work accomplished.

The physiology of fatty acid metabolism and the kinetics of benzoate degradation by anaerobic syntrophic bacteria were studied during the course of this project. We have shown that: a threshold for benzoate degradation by a syntrophic coculture of *Syntrophus buswellii* and *Desulfovibrio* strain G11 exists and the value of the threshold depends on the amount of benzoate and acetate suggesting a thermodynamic limitation. The existence of a threshold for substrate degradation is an important factor for reactions that operate near thermodynamic equilibrium is an important factor and in predicting the fate of substrates in methanogenic environments.

Syntrophomonas wolfei has the enzymatic ability to produce formate and that low levels of formate are made during growth in pure culture with crotonate or in coculture with butyrate. However, the high specific activities of hydrogenase compared to formate dehydrogenase indicate that hydrogen rather than formate is the intermediate involved in the interspecies transfer of reducing equivalents. However, the possible production of formate must be considered in assessing the energetics of syntrophic bacteria.

We have isolated *Syntrophus buswellii* and a novel anaerobic bacteria that catalyzes an aryl-ether cleavage reaction using crotonate as the energy source. Several novel obligately halophilic anaerobes from hypersaline oil reservoir brines were isolated and characterized. Two of these degraded pyrogallate with the production of acetate.

We have shown that *S. wolfei* synthesizes poly- β -hydroxyalkanoate (PHA) by two routes, directly from a β -oxidation intermediate without cleaving a C-C bond and by the condensation of two acetyl-CoA molecules. The formation of D-3-hydroxyacyl-CoA needed for PHA synthesis occurs by the activity of a acetoacetyl-CoA reductase rather than a enoyl-CoA hydratase. The genes for PHA synthesis in *S. wolfei* have been cloned into *Escherichia coli*. We have developed methods to purify the enzymes involved in β -oxidation in *S. wolfei* and have purified and characterized the 3-ketoacyl-CoA thiolase. The nutritional requirements and the use of various fatty acids by *S. wolfei* were studied. The enzymes involved in crotonate metabolism and the role of cytochrome c were also studied.

II. Work accomplished

A. Kinetics of benzoate degradation

The kinetics of benzoate degradation by a washed cell suspension of *Syntrophus buswellii* in coculture with *Desulfovibrio* strain G11 was studied using progress curve analysis. Cell suspensions were incubated overnight in the presence of 1 mM sodium benzoate unless otherwise indicated to insure that the cell suspension was metabolically active. The rate of benzoate degradation exhibited zero-order kinetics then mixed-order kinetics as the concentration of benzoate decreased below 40 to 50 μ M. Continued first-order consumption of benzoate by the coculture as predicted by Michaelis-Menton kinetics was not observed. The degradation of benzoate approached a

threshold concentration below which no further degradation occurred, even after extended incubations. This pattern was consistently observed in repeated experiments with different benzoate concentrations and cell densities. A typical threshold of 1 to 3 μM benzoate was observed in cell suspensions following overnight consumption of the initial 1 mM benzoate and the subsequent use of an additional 200 μM benzoate.

Experiments were repeated using radioactive benzoate to insure that the threshold was not the result of a detection problem. In this experiment, benzoate degradation was followed immediately after resuspension of the cell pellet. The cell suspension immediately degraded the labeled benzoate until an apparent threshold of 214 nM was reached. The suspension was amended again with labeled benzoate and 10 mM sodium acetate; the cell suspension degraded the benzoate until a new threshold of 7.5 μM benzoate was reached.

The threshold value was dependent on how much benzoate the cell suspension received since cell suspensions that received higher levels of benzoate had higher threshold values. This suggested that the amount of acetate produced from the added benzoate influenced the threshold value. To test this hypothesis, the rate of benzoate degradation was followed in cell suspensions that had been serially amended with sodium benzoate and 6 mM sodium acetate (Fig. 1).

Figure 1. Effect of acetate on benzoate degradation by the *S. buswellii* coculture. The arrow indicates the addition of 6 mM sodium acetate and the indicated sodium benzoate concentration.

The cell suspension degraded benzoate to an amount below the detection limit of the high pressure liquid chromatograph (HPLC) system ($<0.5 \mu\text{M}$). Following a second amendment of benzoate with the addition of 6 mM sodium acetate, a threshold of 1.0 μM benzoate was observed. A third amendment of benzoate and 6 mM sodium acetate resulted in a threshold of 5.5 μM benzoate. The initial rate of benzoate degradation was similar after each amendment, indicating that the observed increase in the threshold value was not due to nutritional

limitations, oxidation of the cell suspension, or build up of toxic by-products. A series of control experiments were conducted using cell suspensions that had been amended with benzoate and equimillimolar amounts of sodium chloride instead of sodium acetate. With each amendment of benzoate and NaCl, benzoate degradation continued to a level below the detection limit of the HPLC (data not shown).

The partial pressure of hydrogen of 1×10^{-4} atm was measured at the end of a progress curve when the threshold value for benzoate was $5.5 \mu\text{M}$ and the final acetate concentration was 17.9 mM . This gives a Gibbs free energy value of about -8.3 kJ/mole of benzoate which suggests that the Gibbs free energy value is close to thermodynamic equilibrium when the threshold is reached. Experiments are in progress to further investigate the effect of acetate on the threshold value for benzoate degradation.

The observation of a threshold for benzoate degradation may have some interesting implications concerning the degradation of other aromatic compounds. Many of the Environmental Protection Agency's priority pollutants contain aromatic moieties. Benzoate is a key intermediate in the degradation of many of these compounds. Thus, the efficient degradation of benzoate may be crucial in insuring optimum carbon flow and the degradation of important pollutants. Measuring in situ acetate and hydrogen levels could allow one to estimate based on thermodynamic considerations, the minimum level to which benzoate could be metabolized in a given methanogenic environment. Such thermodynamic considerations may be applicable to other biological reactions that operate near thermodynamic equilibrium. Substrate disappearance models based on the use of first-order or second-order kinetics assume first-order decay below the apparent k_m of the reaction. The existence of a threshold for substrate degradation by syntrophic associations that operate near thermodynamic equilibrium is an important factor affecting the application of such models in predicting substrate decay. Modeling attempts may have to include an estimate of the threshold as a parameter in order to predict accurately the environmental persistence of pollutants.

B. Role of formate in the metabolism of butyrate by *Syntrophomonas wolfei*.

Syntrophomonas wolfei is an anaerobic, syntrophic bacterium that degrades butyrate and other short-chain saturated fatty acids to acetate, propionate, and hydrogen only when grown in association with hydrogen-using bacteria (McInerney et al., 1979, 1981). Because *S. wolfei* could be grown in coculture with either *Methanobacterium bryantii* or *Methanobrevibacter arbophilicum*, two methanogenic bacteria that use hydrogen but not formate, it was concluded that the degradation of butyrate by these cocultures involved the interspecies transfer of hydrogen. However, these results did not exclude the possibility that formate could also serve as an intermediate (Thiele and Zeikus, 1988; Boone et al., 1989).

Recent work shows that it is possible to separate cells of *S. wolfei* from those of the hydrogen user by centrifugation in Percoll gradients and also to grow *S. wolfei* in pure culture with crotonate. Using these methods, we have shown that *S. wolfei* has the enzymatic capability to metabolize formate and that

low levels of formate are produced when *S. wolfei* is grown in pure culture or in coculture.

When whole cell suspensions of *S. wolfei* grown in pure culture were incubated with various concentrations of ^{14}C -labeled formate (1 to 20 μM), the radioactivity in formate decreased with a concomitant increase in radioactivity in bicarbonate. These data show that whole cell suspensions of *S. wolfei* catalyzed a formate-bicarbonate exchange reaction which indicates that *S. wolfei* can metabolize very low concentrations of ^{14}C -labeled formate. No transformation of the label was observed when boiled cells suspensions were used.

Similar experiments were conducted using cells of *S. wolfei* and *M. hungatei* separated from each other after growth in coculture with butyrate. Here, both the change in formate concentration and radioactivity was followed. The specific rates of formate metabolism for cells of *S. wolfei* and *M. hungatei* were 0.032 and 0.4 $\mu\text{mol}/\text{min}/\text{mg}$ of protein, respectively. The decrease in radioactivity in formate was much faster than the rate of formate metabolism.

The specific activities of hydrogenase and formate dehydrogenase in cell-free extracts of *S. wolfei* grown in pure culture or of cells of *S. wolfei* and of *M. hungatei* separated from a butyrate-degrading coculture were determined by measuring the rate of substrate-dependent reduction of benzyl viologen (Table 1). Formate dehydrogenase activity was detected in cells-free extracts of *S. wolfei* grown in pure culture and in coculture. Although this activity was measured as formate utilization, these data suggest that *S. wolfei* has the enzymatic ability to produce formate if the measured activity is reversible. However, the specific activity of hydrogenase was 700 to 900 times higher than that of formate dehydrogenase in *S. wolfei*. The specific activities of these two enzymes did not change dramatically when *S. wolfei* was grown in pure culture or in coculture. Cell-free extracts of *M. hungatei* obtained from the coculture had much higher specific activities of hydrogenase compared to formate dehydrogenase.

Table 1. Formate dehydrogenase and hydrogenase activities in cell-free extracts of *S. wolfei* grown in pure culture and of separated cells of *S. wolfei* and *M. hungatei* obtained from a butyrate-degrading coculture.^a

Culture	Specific activity ($\mu\text{mol}/\text{min}/\text{mg}$)	
	Formate dehydrogenase	Hydrogenase
<i>S. wolfei</i> (pure culture)	0.0075	5.3
<i>S. wolfei</i> (coculture)	0.0035	3.2
<i>M. hungatei</i> (coculture)	0.15	58.9

These results showed that *S. wolfei* catalyzes a rapid formate-bicarbonate exchange reaction and slowly metabolizes formate. These data suggest that *S. wolfei* does have the required enzymatic activities to produce

formate. Cell-free extracts of *S. wolfei* have much higher specific activities for hydrogenase compared to formate dehydrogenase and the specific activities of these two enzymes do not markedly change when *S. wolfei* is grown in pure culture or in coculture. Cell-free extracts of *M. hungatei* obtained from a butyrate-degrading coculture had much higher hydrogenase activity compared to formate dehydrogenase activity. The large capacity of *S. wolfei* and of *M. hungatei* to metabolize hydrogen is consistent with hydrogen being the intermediate for interspecies transfer of reducing equivalents during butyrate degradation by the coculture. However, the fact that *S. wolfei* has the ability to metabolize formate suggests that formate may play a role in syntrophic metabolism.

In other studies, we have shown that the growth rates and final absorbances obtained by butyrate-degrading cocultures of *S. wolfei* with *M. hungatei* and *M. bryantii* were similar. This shows that the absence of formate utilization does not affect the growth or metabolism of *S. wolfei*. We have also developed a sensitive method to detect low concentrations of formate (minimum detection limit of about 30 nM) in bacterial culture fluids. In pure culture with crotonate, a maximum formate concentration of 0.55 μM was detected. The maximum formate concentrations in *M. hungatei* and *M. bryantii* cocultures were 0.49 and 0.33, respectively. Boone et al. (1989) predicted that a concentration of 16 μM was required so that the diffusion of formate would be fast enough to account for the observed rates of methanogenesis in a butyrate-degrading coculture. Thus, formate concentrations are 30-fold lower than that needed (Boone et al., 1989). These data again are consistent with hydrogen rather than formate being the intermediate used for the interspecies transfer of reducing equivalents.

C. An anaerobic bacterium that metabolizes phenoxyacetates.

An anaerobic bacterium that catalyzes the cleavage of an aryl-ether linkage was isolated in pure culture from an 2,4,5-trichlorophenoxyacetate-degrading enrichment of aseptically obtained aquifer material. The bacterium, strain SG-1, is an anaerobic, Gram-negative, spore-forming rod. It was isolated in pure culture by serial dilution and inoculation of crotonate agar roll tubes. Crotonate and several mono- and disaccharides were the only compounds that supported the growth of SG-1 in a mineral salts medium containing B-vitamins. SG-1 fermented crotonate (100 mM) to 112 mM acetate, 37.4 mM butyrate, 3.4 mM ethanol, and 1.0 mM formate. The latter two compounds are not produced by other crotonate-fermenting anaerobes. These products were produced from glucose except that lactate rather than butyrate was made. Crotonate-grown, but not glucose-grown, cultures degraded 50 μM of phenoxyacetate or 2-chlorophenoxyacetate to trace amounts within 72 h. The phenoxyacetates alone did not support the growth of SG-1. Phenol and 2-chlorophenol were detected as intermediates of phenoxyacetate and 2-chlorophenoxyacetate degradation, respectively. Phenol was further metabolized to compound with a retention time identical to that of benzoate and another as yet unidentified metabolite. 2-chlorophenol was not further metabolized. Higher concentrations of the phenoxyacetates were inhibitory to growth with crotonate. The fact that the corresponding phenol was produced from the respective phenoxyacetate

suggests that the aryl ether linkage was cleaved. This is the first report of a pure culture of an anaerobic bacterium that can cleave an aryl ether linkage. Studies are in progress to determine the mechanism of this novel reaction.

D. Poly- β -hydroxyalkanoate (PHA) synthesis in *Syntrophomonas wolfei*.

The synthesis of PHA in *S. wolfei* has been studied (Amos and McInerney, 1989 and 1991; Amos and McInerney, submitted for publication, see preprint). These studies show that PHA is synthesized in *S. wolfei* by two routes, the direct incorporation of a β -oxidation intermediate without cleavage of a C-C bond and by the condensation and subsequent reduction of two acetyl-CoA molecules. PHA was produced during exponential growth in pure culture with crotonate as the energy source or in coculture with butyrate as the energy source. After growth stopped, PHA was metabolized, consistent with its role as a carbon storage material. PHA in cultures grown with pentanoate and hexanoate substrates was a copolyester containing mostly a 4-carbon monomer and small amounts of a monomer with the same chain length as the substrate. Labeling studies and analysis of the monomeric composition showed that early in growth PHA is made by the incorporation of an intermediate without cleaving a C-C bond. Later, PHA is made by a pathway in equilibrium with the acetate pool. Altering the C to N ratio of the medium did not affect the amount of PHA (per mg of protein) or its monomeric composition. These data plus the fact that PHA was made during the exponential phase of growth suggest that its synthesis is regulated in a manner different from that found in other bacteria.

A pyridine nucleotide-dependent acetoacetyl-CoA reductase which formed D-(-)-3-hydroxybutyryl-CoA was partially purified from *S. wolfei*. This enzyme was separated from the 3-hydroxyacyl-CoA dehydrogenase involved in β -oxidation by ion exchange chromatography. The acetoacetyl-CoA reductase used both NADH and NADPH. Thus, the NADH formed during β -oxidation can directly supply the reducing equivalents needed for PHA synthesis without the need for a transhydrogenase activity or some other mechanism to interconvert NADH and NADPH. These studies indicate that *S. wolfei* synthesizes PHA either from the 3-ketoacyl-CoA intermediate generated in β -oxidation or from the condensation of two acetyl-CoA molecules rather than from an enoyl-CoA intermediate.

Recently, the genes for PHA synthesis in *S. wolfei* have been cloned into *Escherichia coli* (M. J. McInerney, J. Palmer, and J. R. Sokatch, unpublished data). These genes are expressed and *E. coli* recombinants carrying *S. wolfei* DNA synthesize from 10 to 33 μ g PHA per mg of protein. These data suggest that the genes needed for the synthesis of PHA in *S. wolfei* are tightly clustered together and may form an operon. These data were obtained during my sabbatical leave in Dr. Sokatch's laboratory last spring. This summer I will continue these studies in his laboratory. I hope to determine which genes are involved and their sequence. This will give me further training in molecular biology and I hope to be able to incorporate molecular techniques in the study of *S. wolfei*.

E. Physiology of *S. wolfei*

Work on the nutrition, metabolism of unsaturated compounds, and the effects of organic acid anions on the growth of *S. wolfei* were completed during the course of this project (Beaty and McInerney, 1989 and 1990; Amos and McInerney, 1990). The addition of five B-vitamins, iron and cobalt were required for good growth of *S. wolfei* in a defined medium with crotonate as the energy source. *S. wolfei* used a variety of mono- and diunsaturated acids 5 to 6 carbons in length for growth in pure culture. Analysis of the fermentation products showed that part of the substrate was β -oxidized and the remainder served as an electron acceptor. Propionate was made from a hexadienoate suggesting that this compound was degraded by another pathway in addition to β -oxidation. Molar growth yields ranged from 8.4 to 17.5 mg (dry wt) per mmol of substrate degraded and suggested that energy was conserved by substrate-level phosphorylation and from the reduction of the unsaturated substrate.

Methods have been developed to purify the enzymes involved in β -oxidation in *S. wolfei*. Greater than 95% of the activities for acyl-CoA dehydrogenase, enoyl-CoA hydratase, 3-hydroxybutyryl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase were found in the soluble fraction after ultracentrifugation. The acyl-CoA dehydrogenase is acidic and elutes from a DEAE-biogel column with 500 mM KCl. The acyl-CoA dehydrogenase activity coelutes with a flavin absorbance suggesting that the enzyme is a flavoprotein. Ion exchange and hydroxylapatite chromatography separated the 3-ketoacyl-CoA thiolase from the other two β -oxidation enzymes and affinity chromatography brought 3-ketoacyl-CoA thiolase to near homogeneity. The 3-hydroxyacyl-CoA dehydrogenase was also purified to near homogeneity using ion exchange, hydroxylapatite, and affinity chromatography. These studies show that the β -oxidation enzymes can be disassociated from each other and are not subunits of a multienzyme complex as found in *E. coli*. However, combining fractions containing the 3-hydroxyacyl-CoA dehydrogenase and the enoyl-CoA hydratase resulted in a dramatic increase in activity of each enzyme suggesting that they may form a complex that is easily disassociated.

The 3-ketoacyl-CoA thiolase from *S. wolfei* appears to be composed of a single polypeptide with a molecular weight of about 41,000. Gel filtration chromatography indicates that the native molecular weight is about 160,000 indicative of a tetrameric subunit organization. The K_m and V_{max} for the thiolytic cleavage of acetoacetyl-CoA are 62 μ M and 128 μ moles/min/mg, respectively.

The enzymes involved in crotonate metabolism in *S. wolfei* were studied. Cell-free extracts of *S. wolfei* grown with crotonate in pure culture or in coculture with *M. hungatei* contained a crotonyl-CoA: acetate CoA transferase activity that was not found in butyrate-grown coculture. This suggests that the long lag times observed before *S. wolfei* grew with crotonate were initially due to the inability to activate crotonate. CO dehydrogenase was not detected. *S. wolfei* has the enzyme activities to form butyrate using a CoA transferase activity, but not by a phosphotransbutylase activity or enoate reductase activity. A c-type cytochrome and a menaquinone were detected in *S. wolfei* suggesting the presence of an electron transport system. The c-type cytochrome was found mainly in the membrane fraction and was reduced by hydrogen, but not by NADH or 3-hydroxybutyryl-CoA. *S. wolfei* contains a pyridine nucleotide-linked hydrogenase system that stoichiometrically produces hydrogen from NADH.

This system was assayed by quantitating the amount of methane produced when cell-free extracts of *S. wolfei* were incubated in the presence of *M. bryantii*. The fact hydrogen is produced from NADH and that the c-type cytochrome is reduced by hydrogen and not NADH suggests that *S. wolfei* has a hydrogen recycling system when it grows with crotonate as the energy source.

F. Other work

We have isolated *S. buswellii* in pure culture with crotonate as the energy source. This is a significant breakthrough since it will facilitate the study of the physiology of this important bacterium. Currently, we are growing cells for Dr. Tanner so that the 16S RNA sequence can be done. This will provide additional information on the phylogeny of syntrophic bacteria and help determine whether this group of organisms form a cluster distinct from other known bacteria.

We have completed a study of the ecology of a hypersaline oil-producing reservoir. We have found that the addition of 15% NaCl to the medium greatly increased the concentrations of bacteria which we were able to detect. Anaerobic heterotrophic populations were higher than that of aerobic heterotrophic populations. We have isolated a great number of heterotrophic anaerobes from these brines. All of these organisms are obligately halophilic and grow optimally in medium with 12 % NaCl. These bacteria use a wide range of sugars and proteinaceous material for growth and acetate production. Two of these halophiles used pyrogallol (a trihydroxybenzene) for growth and acetate production. This is the first report of an extreme halophile that can grow with this compound.

We also have isolated three new types of obligately halophilic sulfate reducers. We also have enrichments for halophilic methanogens that use acetate or hydrogen and carbon dioxide.

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IV. Publications and Theses supported by this project.

A. Published papers.

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B. Papers submitted for publication.

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C. Manuscripts in preparation.

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D. Thesis and Dissertations

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