Energetics and kinetics of anaerobic aromatic and fatty acid degradation

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Michael J. McInerney
Department of Botany and Microbiology
University of Oklahoma
Norman, OK 73019-0245

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I. Summary.

Factors affecting the rate and extent of benzoate degradation by anaerobic syntrophic consortia were studied. Cocultures of a syntrophic benzoate degrader, strain SB, with a hydrogen/formate-using sulfate reducer degraded benzoate to a threshold that depended on the amount of substrate and acetate present. The benzoate threshold was not a function of the inhibition of benzoate degradation capacity by acetate or the toxicity of the undissociated form of acetate. Rather, a critical or minimal Gibb's free energy value may exist where thermodynamic constraints preclude further benzoate degradation. A sensitive assay to detect low formate concentrations was developed to measure the formate levels when the benzoate threshold was reached. We showed that increased acetate concentrations, even when hydrogen and formate levels are low, affects the extent of benzoate degradation, implicating the importance of interspecies acetate transfer.

In addition to benzoate, various saturated and unsaturated fatty acids, 2-methylbutyrate, and methyl esters of fatty acids supported growth in coculture with a hydrogen-using partner. SB is the only syntrophic bacterium known to use both benzoate and fatty acids. Phylogenetic analysis showed that SB clustered with sulfate reducers in the delta subclass of the *Proteobacteria*. SB grew well in coculture with *Desulfoarculus baarsii*, a sulfate reducer that uses formate but not hydrogen. This unequivocally shows that SB can grow by interspecies formate transfer.

Biochemical studies on the fatty acid-degrading, syntrophic bacterium, *Syntrophomonas wolfei*, showed that the D-isomer of 3-hydroxybutyryl-CoA needed for poly-3-hydroxyalkanoate (PHA) synthesis is made by an acetoacetyl-CoA reductase rather than a stereospecific enoyl-CoA hydratase. *S. wolfei* contains a c-type cytochrome that may be involved in electron transport and energy conservation during growth with crotonate. *S. wolfei* also contains low levels formate dehydrogenase activity. Butyrate-grown cells are enzymatically equipped to produce hydrogen rather than formate.

In addition, we have shown that some metal-reducing bacteria can grow by dissimilatory cobalt reduction, and have isolated a phylogenetically distinct dissimilatory iron-reducing bacterium. The physiology and phylogeny of several new halophilic anaerobes was studied. Finally, experiments using glass beads of different sizes showed that pore size restricts that penetration and growth of bacteria, suggesting that pore size is an important factor governing the dispersal and activity of microorganisms in subsurface environments.

II. Kinetics of aromatic acid decomposition.

A. Evidence for thresholds for substrate decay.

The anaerobic transformation of diverse aromatic compounds often results in the formation of benzoic acid (Fuchs et al., 1994). Thus, the factors that affect the rate and extent of benzoate degradation may also influence the anaerobic degradation of other aromatic compounds. In methanogenic environments, benzoate is degraded to acetate, H₂, and formate by syntrophic

bacteria (McInerney, 1991). The degradation of benzoate is thermodynamically unfavorable unless the reaction is coupled to the use of hydrogen and/or formate by organisms such as methanogens. We have isolated a new syntrophic bacterium, strain SB, that degrades benzoate only in coculture with a H₂/formate-using bacterium such as *Desulfovibrio* strain G-11 according to equation a.

We found that cocultures of SB with G-11 degraded benzoate to a threshold concentration below which no further benzoate degradation occurs, even after extended incubations (Hopkins et al., 1995; Warikoo et al., 1996).

Thresholds for the biodegradation of synthetic and natural organic compounds, inorganic nutrients, and anaerobic hydrogen utilization have been reported previously. The existence of thresholds may explain the persistence of trace levels of many organic compounds in natural waters and are important for risk assessment concerning the fate of anthropogenic compounds in the environment. Although the importance of substrate thresholds in microbial ecology is clear, little is known about the cause of thresholds. Most of our work has focused on determining the cause of the benzoate threshold.

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 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 μM . 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 radioactively labeled benzoate to ensure 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 $1.0 \mu\text{M}$ benzoate was observed. A third amendment of benzoate and 6 mM sodium acetate resulted in a threshold of $5.5 \mu\text{M}$ benzoate. The repeated use of benzoate shows that the observed threshold value was not due to a nutritional limitation, the oxidation of the cell suspension, or a 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).

Benzoate degradation by the SB/G-11 coculture may have been due to the build up of inhibitory levels of hydrogen or formate. The data in Table 1 shows that this possibility can be excluded. Hydrogen and formate levels were low and the free energy change for benzoate degradation when the threshold was reached was favorable. It may be that there is a "critical" or minimal Gibbs free energy value, above which no further benzoate degradation can occur. A value of about -17 to -23 kJ/mol has been proposed as the minimum energy quantum needed to translocate a proton across an energized membrane, equivalent to about $1/4$ to $1/3$ of an ATP (Schink, 1991; Thauer and Morris, 1984). The $\Delta G'$ for benzoate when the threshold is reached is close to this minimum energy value.

Table 1. The effect of acetate on the benzoate threshold value and the energetics of benzoate degradation by cell suspensions of strain SB with *Desulfovibrio* strain G-11. Concentrations were determined when the threshold was reached.

Additions (mM)	Benzoate (μ M)	Acetate (mM)	Hydrogen (Pa)	Formate (μ M)	$\Delta G'^0$ (kJ/mol)
<u>Acetate</u>					
0	<0.2	3.0 (0.6)	0.21 (0.06)	8.9 (0)	-42.2
10	<0.2	13.2 (1.5)	0.08 (0.09)	7.1 (1.6)	-36.4
20	2.3 (0.5)	22.0 (2.5)	0.4 (0.03)	8.1 (0.5)	-30.1
65	29.8 (0.5)	65.7 (0.2)	0.3 (0.1)	7.2 (1.5)	-30.5
<u>NaCl</u>					
65	<0.2	3.1 (0.0)	0.22 (0.0)	9.1 (0.4)	-41.5

Consistent with a thermodynamic explanation for the threshold, the addition of an acetate-using bacterium to cell suspensions with a benzoate threshold resulted in further acetate and benzoate degradation (Warikoo et al., 1996). Secondly, no threshold was observed when a thermodynamically more favorable electron acceptor was used (nitrate versus sulfate) even though the overall kinetics of benzoate degradation were much slower with nitrate than with sulfate as the electron acceptor (Warikoo et al., 1996). This experiment also excluded toxicity of the undissociated form of acetate as the cause of the threshold. A threshold was not observed when nitrate was substituted for sulfate as the electron acceptor, even though both suspensions had the same initial concentration of acetate, and consequently, the same initial concentration of the undissociated form of acetate.

Increasing acetate concentrations inhibited the rate of benzoate degradation (Warikoo et al., 1996). Thus, we needed to determine if the inhibitory activity of acetate result in a benzoate threshold value. The apparent V_{max} and K_m for benzoate degradation decreased with increasing acetate concentration, but the benzoate degradation capacity (V_{max}/k_m) remained comparable. Simulation studies showed that an uncompetitive inhibition model modified to include a term for the threshold best explained the observed inhibition pattern and that the apparent K_i for acetate inhibition of benzoate degradation was 10.0 ± 0.6 mM. Without a threshold term, none of the tested inhibition models predicted a threshold for a benzoate degradation, even when

the initial acetate concentration and the values for $V_{\max}$, k_m , and k_i were varied within reasonable limits. Figure 2 shows an example of these simulation studies with 65 mM acetate. In this figure, simulated benzoate degradation curve was generated using an uncompetitive inhibition model (equation b) and the values for $V_{\max}$, k_m , k_i , and S_0 obtained from experiments with 65 mM acetate.

$$[V_{\max}/(1+I/k_i)] t = S_0 - S + [K_m/(1 + I/k_i)] \ln (S_0/S) \quad (b)$$

Figure 2. Simulation study to determine whether acetate inhibition results in a threshold for benzoate degradation. Symbols: (diamonds) simulation results; (squares) experimental data.

Only when the above model was modified to include a term for a substrate threshold (equation c) did the simulation predict a threshold for benzoate degradation (data not shown). Thus, the existence of a benzoate degradation threshold can not be solely explained as a result of acetate acting as an uncompetitive inhibitor.

$$[V_{\max}/(1+I/k_i)] t = S_0 - S + [(K_m + S_c)/(1 + I/k_i)] \ln [(S_c - S_0)/(S_c - S)] \quad (c)$$

$V_{\max}$ is the maximal velocity, K_m is the half-saturation Michealis-Menten constant, K_i is the inhibition constant, I is the inhibitor concentration, S is the substrate concentration, S_0 is the initial substrate concentration, S_c is the substrate threshold concentration, and t is time.

An important implication of this work is that the extent of benzoate degradation can be estimated from thermodynamic considerations. If a critical $\Delta G'$ for benzoate degradation exists, then the extent of benzoate degradation can be estimated from the concentrations of acetate, hydrogen and formate at the end of the experiment. Warikoo et al. (1996) showed that there was fairly good agreement between the experimentally obtained benzoate threshold values and those predicted using $\Delta G'_{\text{crit}}$. Our work shows that both the rate and extent of substrate degradation by syntrophic consortia are controlled by the efficient removal of end products. In particular, we highlight the need for interspecies acetate transfer.

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 ensuring 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 can be metabolized in a given 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. Kinetics of benzoate degradation by sulfate-reducing bacteria.

We have characterized the kinetics of benzoate degradation by pure cultures of sulfate-reducing bacteria (V. Warikoo and M. J. McInerney, unpublished observations). The $V_{\max}$ and K_m for benzoate degradation by *Desulfotomaculum sapomandans* were 8.4 ± 0.7 nmol/min/mg and 8.6 ± 0.1 μ M, respectively, and that for *Desulfovibrio multivorans* were 38.9 ± 4.6 nmol/min/mg and 6.4 ± 1.0 μ M, respectively. The $V_{\max}$ and k_m for benzoate degradation by SB in coculture with *Desulfovibrio* strain G-11 were 110.9 ± 5.3

nmol/min/mg and 41.7 ± 3.9 μ M, respectively. The capacities for benzoate degradation ($V_{\max}/K_m$) by the sulfate reducers and SB were comparable, 2.6 per min for SB cocultures and 1.0 to 6.0 per min for the sulfate reducers. Thus, syntrophic associations should be able to compete for benzoate with sulfate-reducing bacteria when sulfate concentrations are high. However, it appears that the degradation of fatty and aromatic acids in sulfate-reducing sediments does not involve syntrophic interactions (Banat and Nedwell, 1983). Why syntrophic associations are not involved in the degradation of these compounds under sulfate-reducing conditions needs further study.

III. Characterization of new syntrophic bacterium, strain SB.

Strain SB degrades benzoate and certain fatty acids in coculture with hydrogen/formate-using bacteria. Strain SB is a strictly anaerobic, gram-negative, rod-shaped bacterium that occurs singly or in pairs. In coculture with a hydrogen/formate-using sulfate reducer, SB produced about three moles of acetate per mole of benzoate, with the remaining benzoate carbon presumably converted to carbon dioxide or formate. In addition to benzoate, saturated fatty acids such as butyrate, hexanoate, heptanoate, octanoate, palmitate, stearate, unsaturated fatty acids including trans-2-pentenoate, trans-2-hexenoate, trans-3-hexenoate, trans-2-octenoate and methyl esters of butyrate and hexanoate also served as energy sources for SB in coculture with a sulfate reducer. These substrates did not support the growth of SB in pure culture. Strain SB could be grown in pure culture, independent of the sulfate reducer, with crotonate as the substrate. The major end-products of crotonate metabolism by SB were acetate and butyrate.

Phylogenetic analysis of the 16S rRNA sequences placed strain SB in the delta subclass of the *Proteobacteria*, with sulfate-reducing bacteria. Strain SB was most closely related to *Syntrophus* species. However, phenotypic and genotypic characteristics of strain SB are considerably different from those of the two described *Syntrophus* strains. Thus, a new species, *Syntrophus acidotrophicus*, is proposed for this organism. This work shows that several phylogenetic groupings of syntrophic bacteria exist. Similar conclusions have been reached by others (Harmsen et al., 1993; Wallrabenstein et al., 1995).

The pathway for benzoate metabolism by strain SB was studied by demonstrating the presence of key enzymatic activities (Bhupathiraju and McNerney, 1995). Cell extracts of benzoate-grown cocultures of *S. acidotrophicus* contained a benzoyl-CoA ligase activity. In addition to benzoate, cell extracts also contained acyl-CoA ligase activities for 2-fluorobenzoate, 3-fluorobenzoate, 4-fluorobenzoate and 3-hydroxybenzoate. These activities were not detected in cell extracts of the sulfate reducer grown in pure culture or in crotonate-grown pure cultures of *S. acidotrophicus*. Cell extracts also contained cyclohex-1-ene-1-carboxyl-CoA hydratase, 2-hydroxycyclohexene-CoA dehydrogenase, and 2-ketocyclohexane carboxyl-CoA hydrolase activities. These activities were not detected in the pure culture of the sulfate reducer. The specific activities of these enzymes were 8 to 25-fold higher in benzoate-grown cocultures than in crotonate-grown pure cultures of *S. acidotrophicus*. Cell extracts of benzoate-grown cocultures and crotonate-

grown pure cultures of *S. acidotrophicus* contained high levels pimelyl-CoA and glutaryl-CoA dehydrogenase activities. These activities were not detected in the pure culture of the sulfate reducer. The specific activities of these enzymes were 2-fold higher in benzoate-grown cocultures than in crotonate-grown pure cultures of *S. acidotrophicus*. *S. acidotrophicus* contained all of the enzyme activities required for the conversion of crotonyl-CoA to acetate, including enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, 3-keto-CoA thiolase, phosphotransacetylase and acetate kinase. The presence of the above enzyme activities are consistent with a pathway for benzoate metabolism where benzoate is activated to benzoyl-CoA by a ligase activity and transformed in a series of reactions via cyclo-1-ene-1-carboxyl-CoA, pimelyl-CoA, and glutaryl-CoA to three molecules of acetate and one molecule of carbon dioxide or formate.

IV. Role of formate in benzoate metabolism.

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, 1991). 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).

Whether hydrogen, formate or both serves as the interspecies electron carrier in syntrophic associations has been a matter of considerable interest in the last several years. We have found that cells of each member of the butyrate-degrading syntrophic coculture, *Syntrophomonas wolfei* and *Methanospirillum hungatei*, are enzymatically equipped for hydrogen rather than formate use (N. Q. Wofford and M. J. McInerney, unpublished data). 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 2). 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, assuming that 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 2).

Table 2. 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

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.

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).

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.

V. 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; McInerney et al., 1992). 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 enzymes involved in crotonate metabolism in *S. wolfei* were studied (McInerney and Wofford, 1992). 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. Formate dehydrogenase activity was detected in pure cultures of *S. wolfei*, but the activity was low, suggesting a biosynthetic rather than a catabolic role for this enzyme. 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 molar growth yields of *S. wolfei* with crotonate are much higher than those of other crotonate-using anaerobes, indicating that *S. wolfei* may gain energy by electron transport. 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.

The synthesis of PHA in *S. wolfei* has been studied (Amos and McInerney, 1989, 1991 and 1993). 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 pentenoate and hexenoate 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.

S. wolfei synthesizes poly-3-hydroxyalkanoates using an acetoacetyl-CoA reductase reaction (Amos and McInerney, 1989, 1993). This enzyme was separated from the 3-hydroxyacyl-CoA dehydrogenase involved in β -oxidation by ion exchange chromatography. This enzyme was partially purified and the most pure fractions used either NADH or NADPH as the electron donor. 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 showed that *S. wolfei* makes D-(-)-3-hydroxybutyryl-CoA by an acetoacetyl-CoA reductase activity rather than by a stereospecific enoyl-CoA hydratase and that the reducing equivalents required for PHA synthesis from acetoacetyl-CoA can be supplied from NADH made in β -oxidation.

The 3-ketoacyl-CoA thiolase from *S. wolfei* is the key enzyme in that controls the synthesis of PHA from acetyl-CoA. This enzyme was purified to near homogeneity from *S. wolfei* (K. Kealy, M. S. Thesis, University of Oklahoma, 1992). The enzyme 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 homotetrameric 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. It uses acetoacetyl-CoA but not 3-ketooctanoyl-CoA, indicating that it is specific for short-chain, 3-ketoacyl-CoA substrates. The condensation reaction is strongly inhibited by CoA (apparent k_i of 3.6 μ M) while the thiolysis reaction is competitively inhibited

by acetyl-CoA (apparent k_i of 500 μ M). This indicates that the synthesis of PHA from acetyl-CoA is favored when the concentration of free CoA is low, and the acetyl-CoA concentration is high. These conditions presumably occur during the latter stages of growth.

VI. Other projects.

Students that enter my laboratory are given a project involving the isolation an interesting type of microorganism or a project concerning subsurface microbiology to gain experience in anaerobic cultivation methods and expertise in microbial diversity and physiology. Several of these projects have lead to publications that were partially supported by this grant.

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 μ M 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.

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 proteinacious material for growth and acetate production.

Two new species in the genus, *Haloanaerobium*, have been described (Bhupathiraju et al., 1994). Both strains are strictly anaerobic, moderately halophilic, gram-negative bacteria that were isolated from highly saline oil field brine. Strain VS-752 produced acetate, ethanol, carbon dioxide, and hydrogen from glucose. Although the glucose fermentation products of strain VS-752

suggested that it was a *Halobacteroides* species, 16S rRNA sequencing showed that it was phylogenetically most closely related to *Haloanaerobium* species. We have recently found that the type strain of *Halobacteroides acetothylicus* should be placed within the genus *Haloanaerobium*. A new species of *Haloanaerobium*, *Haloanaerobium salsugo*, is proposed for strain VS-752.

Three fermentative halophilic anaerobic bacteria, strains VS-732, VS-751, and VS-511, were found to members of the genus, *Haloanaerobium*. However, phenotypic and genotypic characterization showed that they differed from other described species in this genus. Each of these strains had very high DNA-DNA homology with each other, but not with any of the other described *Haloanaerobium* species. These three strains are placed in a new species, *Halobacterium kushnerii* (V. K. Bhupathiraju, Ph. D. Dissertation, University of Oklahoma, 1996)

A new metal-reducing bacterium that uses both acetate and hydrogen as electron donors, and that can grow by dissimilatory cobalt reduction was isolated and described (Caccavo, Jr. et al., 1993). This organism, called *Geobacter sulfurreducens*, is the first metal reducer described that can use both hydrogen and acetate. The ability of *G. sulfurreducens* to use elemental sulfur as an electron acceptor provides further evidence of the interrelationship between the iron and sulfur cycles.

A phylogenetically distinct metal-reducing bacterium is described. Along with *Flexistipes sinusarabici*, it forms a separate line of descent within the eubacteria (Caccavo, Jr. et al., 1996). Recent work in collaboration with J. D. Coates shows that *G. ferrireducens* uses proline and propionate as electron donors for dissimilatory iron reduction. This is the first report of a metal reducer that uses fatty acids other than formate or acetate.

A Venezuelan student had a hypothesis that the unusual lipids found in the *Archaea* could be effective as emulsifiers or biosurfactants in enhanced oil recovery (Trebbaud de Acevedo and McInerney, 1996). She set up several enrichments and isolated thermophilic eubacteria and archaebacteria that emulsified oil. Further characterization of a methanogenic strain showed that the emulsifier was a cell-wall associated protein. This work may provide an explanation why the stimulation of microbial growth in the reservoir results in increased oil production. The stimulation of microbial growth would increase the amount of bioemulsifiers present which would prevent the formation of paraffin deposits that may prevent oil from flowing into the well.

The mechanisms of migration of bacteria through subsurface materials in the absence of fluid flow was studied (Sharma et al., 1993; Sharma and McInerney, 1994). We found that bacteria can penetrate porous materials at rates that approximate those found in many aquifers. Motility was an important factor that controls the rate of penetration; however, nonmotile mutants were still able to penetrate porous material. Chemotaxis was not required for penetration. However, chemotaxis did affect the mode of penetration. Nonchemotactic mutants penetrated porous materials in a diffuse manner, while the chemotactic parental strain penetrated the porous materials in an ordered manner. For the latter strain, the migration pattern appeared band-like with high concentrations of cells detected at the front. In another paper, we show that growth and metabolism of bacteria in small pores is restricted. Thus, pore size may be an

important factor governing the penetration and dispersal of microorganisms in subsurface reservoirs and, consequently, the biodegradation of organic compounds in these reservoirs.

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VIII. Publications and Theses supported by this project.

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