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

Progess Report

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Michael J. McInerney

Department of Botany and Microbiology

University of Oklahoma

770 Van Vleet Oval

Norman, Oklahoma 73019

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I. Summary.

The kinetics of benzoate degradation by *Syntrophus buswellii* grown in coculture with *Desulfovibrio* strain G11 was determined. Benzoate was degradaded to a threshold value where no futher benzoate degradation was detected even after long incubation times. The threshold was detected when benzoate was measured spetrophotometrically or radioisotopically indicating that it was not the result of a detection problem. The addition of sodium acetate, but not sodium chloride, was found to affect the threshold value with higher threshold values observed at higher acetate concentrations. Thermodynamic considerations suggest that threshold was the result of the reaction approaching thermodynamic equilibrium. These studies show that the existence of a threshold must be considered when using kinetic models to predict the fate of a chemical in an anaerobic environment.

Syntrophus buswellii was isolated in pure culture with crotonate as the substrate. Also, an anaerobic, fermentative bacterium that metabolizes 3-chlorophenoxyacetate and phenoxyacetate to the corresponding phenol was isolated in pure culture. These data show that the bacterium catalyzes a novel aryl ether cleavage reaction under anaerobic conditions.

The synthesis of poly-3-hydroxyalkanoate (PHA) in *Syntrophomonas wolfei* was studied. The bacterium synthesized PHA directly from the ß-oxidation intermediate during the initial stages of growth. Later, PHA was made by the condensation of two acetyl-CoA molecules. *S. wolfei* contains an acetoacetyl-CoA reductase activity that synthesizes D-3-hydroxybutyryl-CoA from acetoacetyl-CoA using either NADH or NADPH suggesting that acetoacetyl-CoA rather than crotonyl-CoA is the ß-oxidation intermediate used for PHA synthesis. The genes for PHA synthesis in *S. wolfei* have been cloned into *Escherichia coli*.

II. Work Accomplished.

A. Kinetics of benzoate degradation.

The kinetics of benzoate degradation by an anaerobic, syntrophic, benzoate-degrading coculture of *Syntrophus buswellii* and *Desulfovibrio vulgaris* strain G11 was studied using progress curve analysis. The rate of benzoate consumption by washed cell suspensions of the coculture exhibited zero-order kinetics initially and then mixed-order kinetics below 40 to 50 µM benzoate. Continued first-order decay following the mixed-order region was not observed. The coculture appeared to metabolize benzoate to a threshold value where no further change in benzoate concentration occurred even after extended incubation periods. Typically, a threshold value of 1 to 3 µM benzoate was observed in the cell suspensions following overnight consumption of the initial 1 mM benzoate and the subsequent use of an additional 200 µM benzoate.

Experiments using radioactive benzoate showed that the threshold value was not the result of a detection problem. The washed cell suspension degraded 1 mM labeled benzoate until a threshold value of 214 nM was reached. The subsequent

addition of labeled benzoate with 10 mM sodium acetate resulted in a threshold value of 7.4 μM.

The addition of sodium acetate, but not sodium chloride was found to affect the threshold value. To investigate the influence of acetate, progress curves were conducted with cell suspensions which were serially amended with sodium benzoate and sodium acetate. The cell suspension degraded the initial 500 μM benzoate to below the detection limit of the high-pressure liquid chromatograph (HPLC) (<500 nM). The second addition of 500 μM benzoate with 6 mM sodium acetate resulted in a threshold of 1.0 μM benzoate. The third addition of 500 μM benzoate plus 6 mM 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 thresholds was probably not due to nutritional limitations, oxidation of the cell suspension, or the build-up of toxic by-products. A series of control experiments where NaCl was added in equimillimolar amounts instead of sodium acetate was conducted. After each amendment, benzoate was degraded to a level below the detection of the HPLC, indicating that the threshold was not the result of increasing ionic strength of inhibition by sodium cations.

Thermodynamic calculations using the actual concentrations of hydrogen, acetate and benzoate determined at the end of the progress curve showed that the change in free energy approached zero when the threshold value was reached. This suggests that as end products increase and the benzoate concentrations decrease, the reaction approaches thermodynamic equilibrium and further degradation of benzoate becomes energetically unfavorable.

The existence of a threshold for metabolism will be an important consideration when modeling substrate turnover in anaerobic environments. Kinetics models that assume continued first-order decay of the substrate will eventually fail to predict accurately the fate of the substrate at low in situ concentrations where a threshold for metabolism exists. If the threshold is the result of the reaction approaching thermodynamic equilibrium, then the fate of the substrate may be predicted from a simple thermodynamic calculation using the in situ concentrations of substrate and products.

We have cocultures growing with nitrate and sulfite as electron acceptors. By using different electron acceptors, the thermodynamics of the reaction can be changed which will allow us to determine if the threshold value is controlled by the thermodynamics of the reaction.

B. Isolation of aromatic degraders.

The syntrophic benzoate-degrading bacterium, *Syntrophus buswellii*, has been isolated in pure culture using crotonate as the energy source. In pure culture, benzoate degradation was not observed. However, when *S. buswellii* was recombined with the hydrogen-using sulfate reducer, benzoate degradation was observed. Recent phylogenetic studies show that the fatty acid-degrading syntrophic bacteria form a separate and distinct group of in the gram-positive line of decent. Phylogenetic studies of *S. buswellii* will show whether this is a property of other

syntrophic bacteria.

The characterization of the anaerobic bacterium that catalyzes an aryl ether cleavage reaction, strain SG-1, continued. Strain SG-1 is an anaerobic, spore-forming, gram-negative rod. In pure culture, SG-1 metabolized 3-chlorophenoxyacetate to 3-chlorophenol. The nature of the product from the side chain is under investigation. Phenoxyacetate was degraded with the appearance of p-hydroxyphenol, phenol, and benzoate as products. The organism also used crotonate and several sugars for growth. Crotonate was fermented to acetate, butyrate, ethanol, and formate. The latter two compounds are not produced by other known crotonate degraders. These products were produced from glucose except that lactate rather than butyrate was made.

C. Physiological studies on *Syntrophomonas wolfei*.

1. Formate metabolism by *S. wolfei*.

An important question regarding the metabolism of syntrophic bacteria is whether H_2 of formate is the extracellular intermediate involved in interspecies hydrogen transfer. Last year, we showed that *S. wolfei* catalyzes a formate-bicarbonate exchange reaction, but the rate of formate degradation was very slow. The specific activities of hydrogenase in the pure culture and percoll separated cells of *S. wolfei* grown in coculture with *Methanospirillum hungatei* were 700 to 900 times higher than formate dehydrogenase specific activities. Also, levels of hydrogenase in *M. hungatei* from the coculture were much higher than formate dehydrogenase activities. These data suggest that both organisms when grown under conditions that require interspecies hydrogen transfer preferentially synthesize hydrogenase rather than formate dehydrogenase.

We have now shown that butyrate-degrading cocultures of *S. wolfei* with a methanogen that uses only hydrogen for methanogenesis grow at the same rate as cocultures containing a methanogen that can use either hydrogen or formate. This shows that hydrogen is produced by *S. wolfei* and that butyrate degradation can occur in the absence of formate use by a methanogen. We developed a spectrofluorescent method to detect very low levels of formate (50 to 1000 nM). This method was used to determine if formate is produced by *S. wolfei*. We found that the both cocultures produced small amounts of formate during growth with butyrate or crotonate. However, the levels are much smaller than that required by mass transfer considerations to support the observed rate of methanogenesis. Thus, at least for *S. wolfei*, formate production and use is not required for butyrate metabolism.

2. Production of poly-ß-hydroxyalkanoate (PHA).

S. wolfei has two route for the synthesis of PHA. The first route involves the direct incorporation of the ß-oxidation intermediate without cleavage of the C-C bond. The second route involves the condensation and subsequent reduction of two acetyl-

CoA molecules. The composition of PHA during growth of *S. wolfei* with trans-2-pentenoate or one of several hexenoates as the substrate was followed to determine the relative contribution of each route for PHA synthesis during growth. The predominant route for the synthesis of PHA was by the condensation of and subsequent reduction of two acetyl-CoA molecules. The ratio of the C-5 and C-4 monomer in PHA was followed during growth. Immediately after inoculation, this ratio increased indicating that PHA was synthesized by the direct incorporation route. However, the ratio rapidly decreased with time as the amount of C-4 monomer in PHA increased suggesting that the shortly after growth begins the predominant pathway for PHA synthesis is from the condensation route.

We have partially purified an acetoacetyl-CoA reductase activity that synthesizes D-3-hydroxybutyryl-CoA from acetoacetyl-CoA. This enzyme in unusual in that it can use either NADH or NADPH. In other bacteria, the synthesis of PHA usually requires NADPH. This would present a problem in *S. wolfei* since ß-oxidation produces NADH and a transhydrogenase activity would be required for the interconversion.

I have recently completed a sabbatical at the University of Oklahoma Health Sciences Center where I was able to construct a recombinant DNA library of the *S. wolfei* genome in *Escherichia coli*. Some of the recombinants were shown to synthesize large amounts of PHA showing that the genes for PHA synthesis in *S. wolfei* have been cloned into *E. coli*. Work is underway to determine which enzymes have been cloned. Hopefully, this will be the start of a molecular system to study the physiology of syntrophic bacteria.

3. Purification of the ß-oxidation enzymes.

Purification and characterization of the enzymes involved in ß-oxidation has been difficult since most fractionation procedures result in the significant loss of activity. Becaus of this, it has been difficult to determine whether any purification has been obtained since specific activities do not increase and at times they decrease. We are performing reconstitution experiments to determine whether a factor needed for activity has been lost during the purification protocol. Analysis of the most purified fractions indicate that contaminating proteins have been removed and that there are about 8 to 10 subunits that copurify with the three ß-oxidation enzyme activities. Thus, were are not that far away from achieving a pure protein.

4. Electron transport chain in *S. wolfei*.

S. wolfei contains a c-type cytochrome that was localized in the membrane fraction of the cell. Over 95% of the total activity of the four ß-oxidation enzymes was found in the soluble fraction. The c-type cytochrome was reduced by dithionite and hydrogen, but not by NAD(P)H, $FADH_2$, crotonyl-CoA, butyryl-CoA, or 3-hydroxybutyryl-CoA. These data suggest that the c-type cytochrome is involved in hydrogen production during the oxidation of the CoA substrates.

D. Publications, Presentations, and Theses.

1. Publications during the last year.

Amos, D. A., and M. J. McInerney. 1990. Growth of *Syntrophomonas wolfei* on unsaturated short chain fatty acids. Arch. Microbiol. 154: 31-36.

Beaty, P. S., and M. J. McInerney. 1990. Nutritional features of *Syntrophomonas wolfei.* Appl. Environ. Microbiol. 56: 3223-3224.

Amos, D. A., and M. J. McInerney. 1991. Composition of poly-ß-hydroxyalkanoate from *Syntrophomonas wolfei* grown on unsaturated fatty acid substrates. Arch. Microbiol. in press.

2. Presentations.

Warikoo, V., M. J. McInerney, and S. Gibson. 1990. Metabolism of phenoxyacetates by an anaerobic fermentative bacterium. Presented at the annual meeting of the American Society for Microbiology, Anaheim CA, May 13 to 17, 1990.

3. Theses and Dissertations.

Amos, D. A. 1989. Poly-ß-hydroxyalkanoate production and other physiological traits of *Syntrophomonas wolfei* subsp. *wolfei*. Ph. D. Dissertation, University of Oklahoma, Norman, OK.

Hopkins, B. T. 1990. Benzoate degradation by an anaerobic syntrophic coculture: evidence for a threshold. M. S. Thesis, University of Oklahoma, Norman, OK.

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