Energetics and Kinetics of Anaerobic Aromatic and Fatty Acid Degradation

Progress Report

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I. Summary of work accomplished.

The kinetics of benzoate degradation by the anaerobic syntrophic bacterium, *Syntrophus buswellii*, in coculture with different sulfate reducers was studied with sulfate or nitrate as the electron acceptor. A threshold value for benzoate degradation dependent on the acetate concentration was observed with sulfate, but not nitrate, as the electron acceptor. No threshold was observed in tricultures containing an acetate-using sulfate reducer. The addition of the acetate-using sulfate reducer to cocultures that had degraded benzoate to its threshold value resulted in further degradation of benzoate to levels below the analytical detection limit (ca. 200 nM). These data are consistent with a thermodynamic explanation for the threshold, and exclude the possibility that the threshold was the result of the inhibitory action of the undissociated form of acetate.

The apparent K_m for benzoate degradation was lower in cell suspensions that received higher acetate concentrations than in cocultures with lower acetate concentrations (<10 mM). An 88% reduction in the V_{max} for benzoate degradation by cell suspensions was observed when 65 mM acetate was added. The Gibbs free energy change for benzoate degradation when the benzoate threshold was reached was about -30 kJ per mole; the Gibbs free energy change for benzoate degradation in cell suspensions without a threshold was <-40 kJ per mole. This suggests that there is a critical Gibbs free energy value required for benzoate degradation.

A new species of halophilic anaerobic bacteria has been described, *Haloanaerobium salsugo*. Several new metal-reducing bacteria have been isolated, one of which is a new species in the genus *Geobacter*, *G. acetooxidans*. Studies on *G. acetooxidans* show that the organisms can grow by dissimilatory sulfur reduction as well as dissimilatory metal reduction. This and the close phylogenetic relatedness to sulfate-reducing bacteria suggest that metal-reducing bacteria are important in the cycling of both iron and sulfur.

II. Work Accomplished.

A. Energetics of benzoate degradation.

Syntrophus buswellii in coculture with *Desulfovibrio* strain G11 degraded benzoate to a threshold value below which no further degradation occurred. The value of threshold benzoate concentration depended on the amount of acetate added, ranging from 2.4 μ M with 20 mM sodium acetate to 30.0 μ M with 65 mM sodium acetate. The V_{max} and K_s estimates for benzoate degradation in cocultures amended with different sodium acetate concentrations were calculated by fitting the data to the integrated form of the Michaelis-Menten equation, modified include a term for the benzoate threshold concentration where required. Increasing concentrations of acetate inhibited the rate of benzoate degradation. An 88% reduction in the V_{max} for benzoate degradation was observed when the coculture was amended with 65 mM

sodium acetate ($11.52 \mu\text{mol h}^{-1} \text{mg}^{-1}$ of protein), compared to unamended controls ($96.71 \mu\text{mol h}^{-1} \text{mg}^{-1}$ of protein). The K_s decreased from $20.48 \mu\text{M}$ with 10 mM acetate to $2.66 \mu\text{M}$ with 20 mM acetate amended. The addition of equimolar concentrations of sodium chloride additions did not result in a threshold for benzoate, and did not affect the rate of benzoate degradation. The apparent K_i for acetate inhibition was 7.0 mM .

The "Gibbs" free energy for benzoate degradation at the benzoate threshold concentration was -31.77 to $-29.74 \text{ kJ mol}^{-1}$. In cell suspensions where a threshold was not observed, the "Gibbs" free energy change associated with the benzoate degradation was -40.42 to $-44.74 \text{ kJ mol}^{-1}$. These data suggest that there is a critical "Gibbs" free energy value required for the degradation of benzoate.

In order to determine whether the observed threshold was due to thermodynamic constraints caused by high acetate concentrations or was the result of the inhibitory action of undissociated form of acetate, cell suspensions were amended with 20 mM sodium acetate and either nitrate or sulfate as the terminal electron acceptor. The rates of benzoate degradation with nitrate ($15.26 \mu\text{mol h}^{-1} \text{mg}^{-1}$ of protein) and sulfate ($15.03 \mu\text{mol h}^{-1} \text{mg}^{-1}$ of protein) were similar. In the suspensions with nitrate as the electron acceptor, benzoate was degraded to below the HPLC detection limit (200 nM). However, in the cell suspensions with sulfate as the terminal electron acceptor, a threshold for benzoate was observed. Since both the cell suspensions had the same initial concentration of acetate, and nitrate reduction is a thermodynamically more favorable than sulfate reduction, these results suggests that the threshold was the result of thermodynamic limitations rather than the inhibition by the undissociated form of acetate. Consistent with this conclusion is the observation that the addition of *D. baarsii*, an acetate-using sulfate reducer, to cell suspensions where a threshold concentration had been reached, resulted in further degradation of benzoate to levels below the analytical detection limit.

The benzoate-degrading syntrophic bacterium grows in coculture with *Desulfovibrio baarsii*, which uses formate, but not H_2 . This shows that *S. buswellii* produces formate during benzoate degradation, and that formate can be the sole intermediate involved in interspecies electron transfer. Studies are underway to compare the effects of inhibitors of hydrogenase and formate dehydrogenase on the growth and metabolism of *S. buswellii* in coculture with *D. baarsii* or strain G11.

Studies on the enzymology of benzoate degradation show that *S. buswellii* activates benzoate using a Coenzyme A ligase reaction rather than a Coenzyme A transferase reaction. The ligase reaction requires energy in the form of ATP, which suggests that the threshold for benzoate may result from the inability of the organisms to activate the substrate.

Several lines of evidence support the conclusion that the enzymes involved in β -oxidation and poly- β -hydroxyalkanoate synthesis in *Syntrophomonas wolfei* form a loosely associated complex. An acetoacetyl-CoA thiolase was purified to homogeneity as determined by native gel electrophoresis. The purified acetoacetyl-CoA thiolase

fractions contained low levels of crotonase activity, but a separate crotonase enzyme was not discernible by native gel electrophoresis. The L-3-hydroxyacyl-Coenzyme A dehydrogenase was purified to near homogeneity. Only one protein fraction was detected after gel filtration chromatography. In addition to high levels of L-3-hydroxyacyl-Coenzyme A dehydrogenase activity, crotonase and β -ketothiolase activities were also detected.

Phylogenetic and physiological characterization of one of the halophilic anaerobic fermentative bacteria isolated from oil field brines has shown it to be a new species of *Haloanaerobium*, *H. salsugo*. A manuscript that describes this organism is enclosed.

Two new dissimilatory metal-reducing bacteria have been isolated. One isolate, strain PCA, is an obligately anaerobic, non-motile, gram-negative rod that uses acetate or hydrogen as the electron donor. A variety of iron minerals (ferric phosphate, ferric oxyhydroxide, or ferric citrate), and cobalt serve as the electron acceptor. The organism can also grow with sulfur as the electron acceptor. This is the first report of an metal-reducing bacterium that uses both hydrogen and acetate for growth. Physiologically, it resembles both the metal-reducing bacterium, *Geobacter metaloreducans*, and the dissimilatory sulfur-reducing species in the genus *Desulfuromonas*. This suggests that organisms involved in sulfur transformations may also be those responsible for metal cycling in nature. Phylogenetic analysis shows that strain PCA is a new species in the genus, *Geobacter*. We have recently isolated a metal-reducing bacterium that oxidizes butyrate or palmitate. This is the first organism isolated that can degrade fatty acids linked to metal reduction, and provides the missing link in the trophic structure of iron-reducing environments.

Last year, my graduate student showed that several iron-reducing bacteria can grow by dissimilatory reduction of cobalt. This was an important discovery since it shows that cobalt can be involved in important biological oxidation/reduction reactions. We are currently purifying the enzyme(s) involved in metal reduction. Both iron and cobalt reductase activities are localized in the cytoplasmic membrane. The activity can be solubilized with Triton X-100, but activity is lost upon further purification. We are currently determining whether the loss of activity is due to inactivation of the protein or the loss of a needed component.

III. Publications and Presentations

A. Manuscripts published (reprints attached).

1. M. J. McInerney and N. Q. Wofford. 1992. Enzymes involved in crotonate metabolism in *Syntrophomonas wolfei*. Arch. Microbiol. 158: 344-349.
2. M. J. McInerney, D. A. Amos, K. S. Kealy, and J. A. Palmer. 1992. Synthesis and function of polyhydroxyalkanoates in anaerobic syntrophic bacteria. FEMS Microbiol. Rev. 103:195-206.
3. D. A. Amos and M. J. McInerney. 1993. Formation of D-3-hydroxybutyryl-

coenzyme A by an acetoacetyl-coenzyme A reductase in *Syntrophomonas wolfeii* subsp. *wolfeii*. Arch. Microbiol. 159:16-20.

B. Manuscripts submitted (preprints enclosed).

1. V. K. Bhupathiraju, A. Oren, P. K. Sharma, R. S. Tanner, C. R. Woese, and M. J. McInerney. *Haloanaerobium salsugo* sp. nov., a moderately halophilic, anaerobic bacterium from a subterranean brine. will be submitted to Int. J. System. Bacteriol. by the end of the year.

2. P. K. Sharma and M. J. McInerney. Effect of grain size on bacterial penetration, reproduction, and metabolic activity in subsurface materials. submitted to Appl. Environ. Microbiol.

C. Manuscripts in preparation.

1. B. S. Hopkins and M. J. McInerney. Benzoate degradation by a syntrophic coculture: evidence for a threshold for benzoate degradation, and growth of the benzoate degrader in pure culture. in preparation for Appl. Environ. Microbiol.

2. V. Warikoo and M. J. McInerney. Energetics and kinetics of benzoate degradation by a syntrophic coculture. in preparation for Appl. Environ. Microbiol.

3. V. Warikoo and M. J. McInerney. Thermodynamic basis for a threshold for benzoate degradation in *Syntrophus buswellii*. In preparation for Appl. Environ. Microbiol.

4. K. Kealy and M. J. McInerney. Purification and properties of an acetoacetyl-coenzyme A thiolase involved in polyhydroxyalkanoate synthesis in *Syntrophomonas wolfeii*. in preparation for J. Bacteriol.

5. K. Kealy and M. J. McInerney. Evidence that the β -oxidation enzymes form a multienzyme complex in *Syntrophomonas wolfeii*. in preparation for J. Bacteriol.

6. M. J. McInerney and N. Q. Wofford. H_2 production from NADH by cell-free extracts of *Syntrophomonas wolfeii*. in preparation for Appl. Environ. Microbiol.

7. P. S. Beaty, P. J. Reynolds, N. Q. Wofford, and M. J. McInerney. Metabolism of formate in *Syntrophomonas wolfeii*. in preparation for Arch. Microbiol.

C. Presentations.

1. M. J. McInerney, K. S. Kealy, and N. Q. Wofford. 1993. Biochemistry of syntrophic fatty acid oxidation. Conference on Multicellular and Interactive Behavior of

Bacteria. Woods Hole, MA, March 28-April 1. Abstract #29, p. 15.

2. M. J. McInerney. 1993. Specific physiological interactions within biofilms. 93rd annual meeting of the American Society for Microbiology, Atlanta, GA, May 16-20, Session 46.

3. F. Caccavo, Jr. and M. J. McInerney. 1993. Isolation and characterization of an acetate-oxidizing, Fe(III)-reducing microorganism from Norman, Oklahoma. Presented at the 93rd annual meeting of the American Society for Microbiology, Atlanta, GA, May 16-20, Abstract Q-130, p. 369.

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