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Energetics and Kinetics of Anaerobic Aromatic and Fatty Acid Degradation

Progress Report

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I. Summary of work accomplished.

Factors influencing the rate and extent of benzoate degradation by the anaerobic syntrophic consortia were studied. Nonlinear regression analysis showed that the cause of the benzoate threshold was not a diminished benzoate degradation capacity. Analysis of cocultures with hydrogen users that differed in their hydrogen utilization capacities showed that the threshold did not depend on the kinetic properties of the syntrophic partner. These data support a thermodynamic explanation for the threshold, and exclude the possibility that a change in the affinity of the enzyme system due to acetate inhibition caused the threshold. Modeling studies showed that the threshold value could be predicted from the concentrations of the end products, assuming a critical Gibb's free energy value. This work shows that interspecies acetate transfer is important in controlling the extent of metabolism by syntrophic organisms.

The characteristics of several anaerobic, halophilic bacteria, phenotypically similar to the recently described *Haloanaerobium salsugo,* were studied. Sequences of the 16sRNA from three of these strains have been completed. Phylogenetic analyses are in progress to delineate the relationships between the newly isolated strains and previously described species. Another new, dissimilatory, metal-reducing bacterium was isolated, and 16sRNA studies indicated that it is in a separate line of descent from *Geobacter* and *Shewanella* species. The electron transport system required for metal reduction in *Shewanella alga* was studied, and the metal reductase activity has been partially purified.

II. Work Accomplished.

A. Kinetics and energetics of benzoate degradation.

Previously, we showed that cocultures of a syntrophic benzoate degrader, SB 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. The V_{max} and K_S estimates of cocultures amended with different sodium acetate concentrations were altered suggesting that the threshold may be explained by diminished benzoate degradation capacity (V_{max}/K_m), or a reduction in the affinity of the enzyme system due to acetate inhibition.

To estimate the kinetic parameters accurately, the type of inhibition and the value of the inhibition constant were determined. The uncompetitive inhibition model adequately described the inhibition of benzoate degradation by acetate, and the inhibition constant (K_i) for acetate was 10.0±0.6 mM. Benzoate threshold values were observed when the initial acetate concentrations were 20 mM or greater. Since a 54% reduction in the apparent V_{max} for benzoate degradation occurred even at the lowest concentration of acetate (10 mM), a decrease in the benzoate degradation capacity could be the cause of the benzoate threshold value. Increasing concentrations of acetate decreased both the V_{max} and the K_m, suggesting that the coculture offset the decrease in V_{max} by increasing the affinity for benzoate. Since the benzoate degradation capacity was similar in cocultures with or without a threshold, this can not be the cause of the threshold.

Experimentally estimated kinetic parameters (V_{max}, K_m, K_i, and S_o) were inserted into the Michaelis-Menton equation modified to include a term for

uncompetitive inhibition to simulate substrate decay curves to determine whether the benzoate threshold could be explained kinetically. The substrate versus time curves generated by this approach did not result in a threshold value for benzoate degradation even when the initial acetate concentration was 100 mM. No benzoate threshold value occurred in simulation when the V_{max}, K_m, and K_i values were increased or decreased by 50%. These studies conclusively show that inhibition of the kinetics of benzoate degradation is not the cause of the threshold.

We found that the rate of benzoate degradation does depend on the kinetics of hydrogen use by the syntrophic partner. The hydrogen-utilization capacity of *Desulfovibrio* strain G-11 is five times greater than that of *Desulfovibrio* strain DG2. When DG2 replaced G-11 as the syntrophic partner, the benzoate degradation capacity of the coculture decreased about five-fold.

The "Gibbs" free energy change for benzoate degradation at the benzoate threshold concentration was about -54 kJ mol^{-1}. These data suggest that there is a critical "Gibbs" free energy value required for the degradation of benzoate. If this is true, it should be possible to estimate the benzoate threshold value from the in situ concentrations of acetate, hydrogen, and formate. We found that there was a close agreement between the experimentally determined benzoate threshold values and those predicted by thermodynamic calculations. This type of approach will be useful in estimating the lowest concentration to which a contaminant will be degradaed in methanogenic environments. A manuscript on this work is currently in preparation.

B. Characterization of a new syntrophic benzoate degrader.

The benzoate-degrading bacterium, strain SB, was isolated in pure culture using crotonate as the energy source. In addition to benzoate, saturated fatty acids including butyrate, hexanoate, heptanoate, octanoate, palmitate, stearate, unsaturated fatty acids including *trans*-2-pentenoate, *trans*-2-hexenoate, *trans*-3-hexenoate, and *trans*-2-octenoate, and methyl esters of butyrate and hexanoate support the growth of SB in coculture with G-11. The DNA base composition of SB is 43 mol% G +C. The phenotypic and genotypic characteristics of strain SB are distinctly different from those of previously described syntrophic bacteria.

Benzoate metabolism was studied in cell-free extracts of strain SB. Benzoyl-coenzyme A (CoA) synthetase, glutaryl-CoA dehydrogenase, enoyl-CoA hydratase, L-3- hydroxyacyl-CoA dehydrogenase, 3-ketothiolase, phosphotransacetylase, and acetate kinase activities were detected in both benzoate and crotonate grown cells. Cell-free extracts of SB were able to activate 3-hydroxybenzoate, 2-fluorobenzoate, 3-flurobenzoate, and 4-flurobenzoate. The pathway for benzoate degradation appears to be similar to that observed for the metabolism of trihydroxybenzenes. This work will be presented at the next ASM meeting; an abstract of the work is enclosed.

C. Characterization of novel halophiles and metal-reducing bacteria.

Physiological characterization of three halophilic anaerobic fermentative bacteria isolated from oil field brines has been completed. The 16S RNA from these strains has been sequenced. Sequence analysis indicates that two of the strains are closely related, and the third is not. Further analyses are in progress to determine the phylogenetic relationships between these organisms and described taxa. DNA-DNA hybridization studies will be required to define the taxonomic status of these strains.

We have characterized another new metal-reducing strain. Ribosomal RNA sequencing indicates that this organism is in a separate line of decent from other metal-reducing bacteria.

We have studied that electron transfer components required for the reduction of Fe(III), CO(III), and Cr(IV) by strain BrY. Hydrogen-dependent metal-reducing activities were found in both the soluble and membrane fractions. The soluble fraction required the addition of menadione for metal reduction. Fe(III), Co(III), and Cr(IV) reductase activities had similar Km values, and had similar responses to changes in temperature and ionic strength. Difference spectra showed the presence of a cytochrome *c*. Hydrogen reduced cytochrome *c* was reoxidized by the addition of CO, Fe(III), Co(III), and Cr(IV). This reoxidation was not inhibited by cyanide or azide. Dicumarol and HOQNO inhibited the reduction of cytochrome *c* , and metal reduction with hydrogen as the electron donor. These results suggest that electron transport to Fe(III), Co(III), and Cr(IV) proceeds through a menaquinone and a *c* -type cytochrome, and that the *c* -type cytochrome may be the metal reductase.

We have also found that several thermophilic and extremely thermophilc produce cell-associated emulsifiers. The emulsifiers are stable at high temperatures and salt concentrations. These emulsifiers may be useful for applications involving enhanced oil recovery. A preprint describing this work is enclosed.

III. Publications and Presentations (items from this year are indicated in bold)

A. Manuscripts published (reprints of new publication 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. *wolfei.* Arch. Microbiol. 159:16-20.

4. V. K. Bhupathiraju, A. Oren, P. K. Sharma, R. S. Tanner, C. R. Woese, and M. J. McInerney. 1994. *Haloanaerobium salsugo* sp. nov., a moderately halophilic, anaerobic bacterium from a subterranean brine. will be submitted to Int. J. System. Bacteriol. 44:3752-3759.

5. P. K. Sharma and M. J. McInerney. 1994. Effect of grain size on bacterial penetration, reproduction, and metabolic activity in subsurface materials. submitted to Appl. Environ. Microbiol. 60:1481-1486.

6. Caccavo, Jr., F., D. J. Lonergan, D. R. Lovley, J. F. Stolz, and M. J. McInerney. 1994. *Geobacter sulfurreducens* sp. nov., a hydrogen- and acetate-oxidizing dissimilatory metal-reducing microorganism. Appl. Environ. Microbiol 60:3752-3759.

B. Manuscripts submitted (preprint enclosed).

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

2. Trebbau-Acevedo, G., and M. J. McInerney. Emulsifying activity in thermophilic and extremely thermophilic microorganisms. Submitted to J. Indust. Microbiol.

C. Manuscripts in preparation.

1. V. Warikoo and M. J. McInerney. Kinetics and thermodynamics of benzoate degradation by a syntrophic coculture: importance of interspecies acetate transfer. in preparation for Appl. Environ. Microbiol.

2. V. Warikoo and M. J. McInerney. Kinetics of benzoate degradation by sulfate-reducing bacteria. In preparation for Appl. Environ. Microbiol.

3. V. Warikoo and M. J. McInerney. Role of formate in interspecies electron transfer in a syntrophic benzoate-degradaing coculture. 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 wolfei*. in preparation for J. Bacteriol.

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

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

8. Bhupathiraju, V. K., and M. J. McInerney. Characterization of a novel syntrophic bacterium that metaboliszes both benzoate and fatty acids. 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.

92rd 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 92rd annual meeting of the American Society for Microbiology, Atlanta, GA, May 16-20, Abstract Q-130, p. 369.

4. V. K. Bhupathiraju, R. S. Tanner, and M. J. McInerney. Characterization of fermentative halophilic anaerobes from subterranean brines. Presented at the 93rd General Meeting of the American Society for Microbiology, Las Vegas, May 23-27.

5. F. Caccavo, Jr., and M. J. McInerney. Electron transport in the dissimilatory metal-reducing microorganism, strain BrY. Presented at the 93rd General Meeting of the American Society for Microbiology, Las Vegas, May 23-27.

6. V. Warikoo, and M. J. McInerney. Energetics and kinetics of anaerobic benzoate degradation. Presented at the 93rd General Meeting of the American Society for Microbiology, Las Vegas, May 23-27.

Preprints, reprints and conference abstracts removed for separate processing