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**One Carbon Metabolism in Anaerobic Bacteria:
Regulation of Carbon and Electron Flow
During Organic Acid Production**

Grant No. DE-FB02-87ER13719

PROGRESS REPORT

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SUMMARY OF PROGRESS ON EXPERIMENTAL OBJECTIVES

- A. *B. methylotrophicum* - a model to understand control of carbon and electron flow in relation to inter- and intra-molecular H₂ transfer.

Objective 1: To elucidate the metabolic pathway of solvent production in *B. methylotrophicum*.

We have shown that *B. methylotrophicum* can ferment CO into ethanol and butanol at low pH values. We have completed studies which demonstrate that the organism produces an NAD-linked butaldehyde and acetaldehyde reductase and an NAD-linked alcohol dehydrogenase. A manuscript on these studies is being prepared for submission and publication in *Appl. Environ. Microbiol.*

Objective 2: To characterize and purify hydrogenase in relation to establishment of physiological function.

The effect of pH on glucose fermentation and hydrogenase activity and pyridine nucleotide ferredoxin oxidoreductase of *Butyribacterium methylotrophicum* was investigated with an anaerobic continuous culture system. The amount of acetate and butyrate formed and the *in vivo* levels of hydrogenase and *in vitro* levels of pyridine nucleotide ferredoxin oxidoreductase were studied under different pH values for steady-state growth. The molar ratio of butyrate/acetate was shifted from 0.35 at pH 7.0 to 1.21 at pH 5.5. Uptake hydrogenase activity and ferredoxin-NAD oxidoreductase increased while production hydrogenase and NADH-ferredoxin oxidoreductase decreased in cells grown at pH 5.5 versus pH 7.0. The addition of exogenous hydrogen did not increase the butyrate/acetate ratio at pH 7.0 or pH 5.5. The results indicate that growth pH values regulate both hydrogenase activity and pyridine nucleotide ferredoxin oxidoreductase levels such that at acid pH values more intermediary electron flow is directed towards butyrate production than H₂ production. A manuscript on these studies is being prepared for submission to publication in *Appl. Environ. Microbiol.*

We have begun studies on cloning the genes for and purification of soluble versus membrane-bound hydrogenase.

- B. *A. succiniciproducens* a model to understand CO₂ fixation in relation to control of carbon and electron flow.

Objective 3: Elucidate the biochemical mechanism for metabolic regulation of the succinate fermentation.

This objective has been completed and published (see Samuelov et al., 1991, *Appl. Environ. Microbiol.* 57:3013-3019).

CO₂ serves as an electron acceptor and a positive regulator of PEP: carboxykinase and a negative regulator of lactate hydrogenase and alcohol dehydrogenase. The PEP carboxykinase functions in catabolic CO₂ fixation and CO₂ switches glucose metabolism from a fermentative into a respiratory process.

Phosphoenolpyruvate carboxykinase from the obligate anaerobe *Anaerobiospirillum succiniciproducens* was purified 18-fold. The enzyme was monomeric with an M_r of 57 000 ± 2 000. The enzyme was oxygen stable and had a pH optimum of 6.5-7.1 and was stable from pH 5.0 to 9.0. The N-terminal amino acid sequence of the enzyme was compared with the N-terminal sequences from six other phosphoenolpyruvate carboxykinases from different organisms and showed the most similarity to the enzyme from *Escherichia coli*. The enzyme displayed Michaelis-Menten kinetics for the substrate PEP and the co-substrate bicarbonate with a K_m of 0.54 mM and 17 mM, respectively. The enzyme required Mn⁺² or CO⁺² in addition to Mg⁺² to exhibit maximum activity. pCMB inhibited activity and PEP protected the enzyme against inactivation suggesting that an essential cysteine may be in the active site. A manuscript on these studies was forwarded to *Biochem. J.* for publication consideration.

We have begun cloning the PEPCK gene using probes made toward the N-terminal amino acid sequence.

Objective 4: Characterize the electron flow pathway key oxidoreductases and provide evidence for ETP during CO₂ reduction to succinate.

In light of our exciting findings above, we have changed this objective to cloning the gene for and characterization of the structure and function of PEP carboxykinase, a key catabolic CO₂ fixing enzyme, in anaerobes.

- C. Heteroacidogens - models to understand the physiobiochemical function of formate metabolism in anaerobes.

Objective 5: Characterization of CO₂ reductase a key catabolic CO₂ fixing enzyme in anaerobes.

Prior to our studies, CO₂ reductase activity was only reported in *C. pasteurianum* and functions in anabolism not catabolism. We have shown that *Sarcina ventriculi* produces CO₂ reductase but lacks formate dehydrogenase and that the enzyme is used catabolically when cells make high formate levels (i.e., > 30 M) (see Lowe and Zeikus, 1991, Arch. Microbiol. 155:325-329). Notably, we have detected CO reductase in methanogens which lack formate dehydrogenase. A manuscript on these studies is prepared for submission to publish in *Appl. Environ. Microbiol.*

Our attempts to purify the enzyme in cell extracts has failed to date but we continue to attempt this in *S. ventriculi*, *B. methylophilum*, *M. barkeri*, and *A.*

succiniciproducens. Because of the lack of progress on this objective of the grant, we have added the new objectives below.

- D. *S. ventriculi*: a model for understanding the influence of low pH on one carbon metabolism and C & E flow.

We have shown that low pH stimulates synthesis of pyruvate decarboxylase in *S. ventriculi*. This causes a shift from formic and acetic production to ethanol production. Pyruvate decarboxylase was the key branch point enzyme regulating the alteration of C & E flow (see Lowe and Zeikus, Arch. Microbiol. 155:325-329).

Objective 6: Cloning the gene for and characterization of pyruvate decarboxylase structure-function.

The pyruvate decarboxylase from *S. ventriculi* was purified and biochemically characterized (see Lowe and Zeikus, 1992, J. Gen. Microbiol., in press). The enzyme shows closer homology to pyruvate decarboxylase of yeast than *Zymomonas*.

Nucleotide probes have been made based on the pyruvate decarboxylase N-terminal sequence and DNA fragments which hybridize to the probes have been isolated.

Objective 7: Characterization of membrane structure function properties in relation to growth at pH 7.0 versus 3.0.

Changes in the composition of membrane lipids in a strictly anaerobic, facultative acidophilic eubacterium, *Sarcina ventriculi* were studied in response to various forms of environmental stress. Changes in lipid composition and structure occurred in response to environmental pH. At neutral pH, the predominant membrane fatty acids ranged in chain length from C14 to C18. However, when cells were grown at pH 3.0, a family of unique very long chain α,ω -dicarboxylic fatty acids containing 32 to 36 carbon atoms were synthesized and accounted for 50% of the total membrane fatty acids. It is proposed that these unusual fatty acids negate the damage to membrane stability caused by deleterious fermentation products (acetic acid, formic acid, and ethanol) formed during growth. Addition of alcohols, either metabolic (0.25 M ethanol) and non-metabolic (0.05 M butanol) to cells grown at pH 7.0, or thermal stress (growth temperature at pH 7.0 was raised from 37°C to 45°C or 55°C) also resulted in the synthesis of these very long chain fatty acids. Thus, synthesis of these unusual membrane lipids appears to be in response to diverse environmental parameters. Synthesis of these very long chain α,ω -dicarboxylic acids was reversed by returning the temperature back to 37°C. These more hydrophobic fatty acids may serve to enhance the stability of the membrane by decreasing the degrees of freedom of molecular motions among the membrane components hence

maintaining the optimal fluidity. A manuscript on these studies has been submitted for consideration and publication in *Journal of Bacteriology*.

Studies underway will determine if ATPase activity and organic acid uptake or differences in cells are regulated as a function of pH extremes for growth.

PUBLICATIONS WITH DOE GRANT SUPPORT

1. Lowe, S., and J. G. Zeikus. 1991. Metabolic regulation of carbon and electron flow as a function of pH during growth of *Sarcina ventriculi*. *Arch. Microbiol.* 155:325-329.
2. Samuelov, N.S., R. Lamed, S. Lowe and J. G. Zeikus. 1991. Influence of CO₂-HCO₃⁻ levels and pH on growth succinate production and enzyme activities of *Anaerobiospirillum succiniciproducens*. *Appl. Environ. Microbiol.* 57:3013-3019.
3. Lowe, S., and J. G. Zeikus. 1992. Purification and characterization of pyruvate decarboxylase from *Sarcina ventriculi*. *J. Gen. Microbiol.* (in press).

ABSTRACTS

1. Kemner, J.M., and J. G. Zeikus. 1990. Evidence for Cytochrome *b* Function in Acetate Catabolism by *Methanosarcina barkeri* MS. Annual Meeting of the American Society for Microbiology.
2. Wu, W.-M., R. F. Hickey, and J. G. Zeikus. 1991. Formate Synthesis from Hydrogen by Hydrogen Utilizing Methanogens. Annual Meeting of the American Society for Microbiology.
3. Podkovyrov, S., and J. G. Zeikus. 1992. Purification and Characterization of Phosphoenol-pyruvate Carboxykinase from *Anaerobiospirillum succiniciproducens*. Annual Meeting of the American Society for Microbiology.

PRESENTATIONS

August, 1990. "Regulation of Solventogenesis in CAB by bioelectrochemical controls of H₂ and Organic Acid Metabolism" International Workshop on the Regulation of Metabolism, Genetics, and Development of the Solvent-Forming Clostridia, Salisbury, England.

September, 1990. "Physiological Control of Oxychemical Fermentations: Enhanced Production of Alcohols and Methane by Anaerobic Bacteria." Biomass and Production Session, IUMS Congress: Bacteriology & Mycology, Osaka, Japan.

October, 1990. "Emerging Microbial Enzyme Technologies for Utilization of Renewable Resources", Texas Biotechnology Symposium, University of Texas, Austin, Texas.

October, 1991. "Emerging Microbial and Enzyme Technologies for Renewable Resource Utilization", University of Nebraska, Lincoln, Nebraska.

October, 1991. "Emerging Microbial and Enzyme Technology for Renewable Resource Utilization", invited presentation, Symposium on Biotechnology and the Bacterial Cell, Michigan State University, East Lansing, Michigan.

November, 1991. "Emerging Microbial and Enzyme Technologies for Renewable Resource Utilization", American Society for Microbiology Southeast Branch Meeting, Atlanta, Georgia.

STUDENTS TRAINED

1. Dr. Jer-Song Shieh, Completed postdoctorate
2. Dr. Wei-Min Wu, Completed doctorate
3. Dr. Susan Lowe, Current postdoctorate
4. Dr. Sergey Podkovyrov, Current postdoctorate
5. Dr. Bassam Annous, Current postdoctorate

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