Final Report

"Sugar Transport and Metabolism in *Thermotoga*"

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PROJECT GOALS

Members of the genus *Thermotoga* are hyperthermophilic anaerobes that are among the most slowly-evolving members of the phylogenetic domain *Bacteria*. They are the earliest bacterial heterotrophs and utilize the widest spectrum of carbohydrates of any archaeal or bacterial hyperthermophile. They ferment sugars to acetate, carbon dioxide, hydrogen, and minor products *via* the Embden-Meyerhof-Parnas glycolytic pathway. Prior to this investigation, nothing was known about the mechanisms by which sugars are transported into their cells. The publication of the sequence of the genome of *T. maritima* in 1999 provided the means to examine in detail sugar transport and the regulation of the expression of the transporter genes on a global scale. The goal of the two 3-year project periods was to elucidate these mechanisms of sugar transport and begin an exploration of the underlying central catabolic regulatory networks in *Thermotoga* species.

Specific Aims, Period I (9/93-5/97)

Specific Aim #1 – Determination of pathways of carbohydrate catabolism

1. Catabolite repression.

We had observed the phenomenon of catabolite repression while formulating growth medium for *T. neapolitana*. We subsequently found that b-galactosidase activity is inducible and, in general, is repressed in cells grown with glucose present. Both β-galactosidase and β-glucosidase activities are induced by cellobiose and these activities are not repressed when glucose is added in addition. Cyclic AMP is not involved in this regulation.

- Vargas, M. and K. M. Noll. 1995. Catabolite repression in the hyperthermophilic bacterium *Thermotoga neapolitana* is not mediated by cAMP. Abstr. Ann. Meet. Amer. Soc. Microbiol., p. 539.
- Vargas, M. and K. M. Noll. 1996. Catabolite repression in the hyperthermophilic bacterium *Thermotoga neapolitana* is independent of cAMP. Microbiology. 142:139-144.

2. Cloning genes encoding glycolytic enzymes.

In an effort to further explore this regulation and to elucidate the control of carbon flow in *T. neapolitana* cells, we began to identify genes encoding proteins involved in central catabolism and transport. Using a genomic library of *T. neapolitana* DNA in a cosmid vector capable of conjugal transfer to other bacteria, we identified clones expressing glycolytic enzyme activities including phosphoglycerate kinase, triosephosphate isomerase, and enolase through complementation of mutant *E. coli* strains.

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- Yu, J.-S. and K. M. Noll. 2001. The phosphoglycerate kinase-triose phosphate isomerase complex of *Thermotoga neapolitana. In* Adams, M. W. W. and R. M. Kelly., Methods in Enzymology, Hyperthermophilic Enzymes, Part B. Academic Press, Inc., Orlando. pp. 78-89.

Specific Aim #2 – Measurement of carbohydrate transport

1. Glucose transport by *T. neapolitana*

The following conditions were found necessary to measure [^{14}C]-2-deoxy-D-glucose (2-DOG) uptake by resting suspensions of *T. neapolitana* at 77°C: (i) manipulation of the cells under strictly anaerobic conditions including addition of a reducing agent to the cell suspension; (ii)

centrifugation through oil to rapidly separate cells from buffer without lysing them; (iii) addition of an energy source, *e.g.* L-arginine or pyruvate; and (iv) continuous flushing with oxygen-free nitrogen apparently to remove inhibitory hydrogen derived from pyruvate catabolism. We found arginine was catabolized with subsequent ATP synthesis *via* the arginine deiminase pathway. Cells of *T. neapolitana* accumulated 2-DOG to an internal concentration about 25 times greater than the external concentration. This accumulation was dependent upon addition of pyruvate and the accumulated 2-DOG was not phosphorylated following uptake. We found that *T. neapolitana* lacks both glucose-specific and fructose-specific PEP-dependent sugar:phosphotransferase system (PTS) systems. The ability of *T. neapolitana* to concentrate 2-DOG intracellularly is sodium dependent.

- Galperin, M. Y., K. M. Noll, and A. H. Romano. 1996. Active transport of glucose in the hyperthermophilic bacterium *Thermotoga neapolitana*. Archaea: Ecology, Metabolism and Molecular Biology. Gordon Research Conference. Plymouth, NH.
- Galperin, M., K. M. Noll, and A. R. Romano. 1996. The glucose transport system of the hyperthermophilic anaerobic bacterium *Thermotoga neapolitana*. Appl. Environ. Microbiol. 62:2915-2918.

2. The effects of non-metabolizable glucose analogs on cell growth.

Growth of *T. neapolitana* cells was only partly inhibited by methyl-a-D-glucopyranoside (1-100 mM), whereas 2-deoxy-D-glucose (2-DOG) (10 mM) completely suppressed cell growth. The effects of these analogs were partly sensitive to the presence of glucose in the growth media. It was concluded that a-methylglucoside (αMG) and 2-DOG can be used as non-metabolizable substrates of the glucose transport system of *T. neapolitana*. In addition, the growth inhibition by 2-DOG can be exploited for isolation of analog-resistant mutants. Analyses of these mutants may provide further insights into transport mechanisms.

- Noll, K. M. and M. Vargas. 1997. Recent advances in genetic analyses of hyperthermophilic archaea and bacteria. Arch. Microbiol. 168:73-80.

Specific Aims, Period II (6/97-5/02)

Specific Aim #1 – Membrane energetics of glucose transport

Using methods we developed to examine both bioenergetics and DNA transformation, we attempted to prepare membrane vesicles of *T. neapolitana* to study membrane energetics and transport under more controlled conditions. This work began during a stay in the laboratory of Dr. Gerhard Gottschalk at the Universität Göttingen, Germany. These vesicles are phase light and exhibit a number of interesting enzymatic activities including hydrogenase, an NADH:methyl viologen oxidoreductase, and, importantly for these studies, ATPase. Preliminary experiments were performed in the laboratory of Dr. Wil Konings to determine the integrity of the vesicles prepared by these methods. These vesicles could not maintain a membrane potential as measured by accumulation of the fluorescent dye DiSC3.

To examine the energetic needs for sugar transport in whole cells, we are measured growth yields as a function of different growth substrates. For this work we developed a simple chemostat to grow *T. maritima*. We measured growth constants and yields for cells grown on glucose, maltose and lactose to calculate the energy required for transport of each sugar. Although informative, the data lack the precision necessary to definitively identify the transport mechanisms involved.

- Noll, K. M. 1997 "Sulfur reduction by the hyperthermophilic bacterium *Thermotoga neapolitana*." Presented at the Fakultät für Biologie, Universität Konstanz, Germany.
- Noll, K. M. 1997 "Carbohydrate transport and membrane energetics in *Thermotoga*." Presented at the Department of Microbiology, University of Groningen, Haren, The Netherlands.

- Noll, K. M. 1997 "Bioenergetics in the hyperthermophilic bacterium *Thermotoga.*" Presented at the Institut für Mikrobiologie, Universität Göttingen, Göttingen, Germany
- Käslin, S. A., S. E. Childers, and K. M. Noll. 1998. Membrane-associated redox activities in *Thermotoga neapolitana.* Arch. Microbiol. Arch. Microbiol. 170:297-303.
- Yu, J.-S., M. Vargas, and K. M. Noll. 1999. A PCR-based method used to detect liposome-mediated transformation of the hyperthermophile *Thermotoga.* Abstr. ASM Conference on Biodiversity, Chicago, IL, p. 67.
- Brancieri, M. A. 1999. Determination of substrate transport characteristics in *Thermotoga maritima* during carbon-limited, continuous culture. MS Thesis, University of Connecticut.
- Noll, K. M. and S. E. Childers. 2000. Sulfur metabolism among hyperthermophiles. *In* J. Seckbach (ed.), Journey to Diverse Microbial Worlds, Kluwer Academic Publishers, the Netherlands. pp. 93-105.
- Yu, J.-S., M. Vargas, C. Mityas, and K. M. Noll. 2001. Liposome-mediated DNA uptake and transient expression in *Thermotoga.* Extremophiles 5:53-60.

Specific Aim #2 – Maltose transport

An initial series of experiments showed that radiolabel from ^{14}C-α-methylglucoside (a possible maltose analog) accumulates in cells. Subsequently we detected high affinity maltose and glucose binding activities in cell-free extracts of *Thermotoga neapolitana* and *Thermotoga maritima.* These binding activities are distinct and specific. That is, ^{14}C-maltose binding was blocked much more strongly by non-radioactive maltose than by non-radioactive glucose, and ^{14}C-glucose binding was blocked much more strongly by non-radioactive glucose than by non-radioactive maltose. Binding of both glucose and maltose was optimum at 50°C, but strong binding (within 80% of maximum) took place at 77°C, the optimum growth temperature of these *Thermotoga* species. Binding constants (K_d) were measured for each binding activity, and found to be of the same order of magnitude: 2 mM for glucose and 3 mM for maltose. All these characteristics indicate strongly the presence of binding proteins of the type found in a number of bacteria that participate in high-affinity active transport systems that are energized by phosphate bond energy (as opposed to a protonmotive force). In our experiments with *T. neapolitana* and *T. maritima*, we found all maltose and glucose-binding activity in the 100,000 x g supernatant of a cell-free extract (prepared by French Press) and none in an octyl glucoside extract of the membrane fraction.

We began an effort to relate sugar binding activities to specific gene products by expressing recombinant sugar binding protein-encoding genes in *E. coli* and then measuring their abilities to bind various sugars using fluorescence emission spectroscopy. We have found that the two ORFs assigned as periplasmic maltose binding proteins, TM1204 and TM1839, do bind maltose and with slightly different affinities (K_d 5.2 μM and 7.4 μM, respectively). However, TM1839, but not TM1204, binds trehalose (K_d 12.4 μM). We found that neither of these proteins binds glucose, lactose, cellobiose or galactose.

- Nanavati, D., A. H. Romano, and K. M. Noll. 2000. Periplasmic sugar binding protein activities in *Thermotoga maritima.* Abstr. Extremophiles 2000, Hamburg, Germany. p. 57.
- Nanavati, D., K. M. Noll, and A. H. Romano. 2002. Periplasmic maltose and glucose binding protein activities from *Thermotoga maritima* cells. Annual Meeting of the Connecticut Valley chapter of the American Society for Microbiology, Storrs, CT.
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Specific Aim #3 – Mechanisms of catabolite repression

T. neapolitana prioritizes its use of carbohydrates and that this process does not involve cyclic AMP. We also found that lactose transporter and β-galactosidase activities are coordinately regulated. It is of great interest to identify which genes are regulated by catabolite repression to understand global regulation of carbon catabolism in *Thermotoga.* We initially attempted to use random, arbitrarily-primed PCR (RAP-PCR) to non-specifically amplify transcripts from the

cDNAs from lactose- and glucose-grown cells. It became clear that this approach cannot provide reproducible patterns of differentially expressed genes nor provide numerous potential candidate genes for further study. This work also pointed out that batch-grown cells are responding not only to the available carbon source, but also to their growth phase. Consequently we developed a continuous culture method to obtain cells for examinations of mRNA content using DNA microarray technology in collaboration with the Institute for Genomic Research (TIGR).

We conducted replicate continuous cultures on each of glucose, maltose or lactose and examined gene expression using a full genome, 1879-ORF DNA microarray. Our preliminary data show genes relevant to maltose catabolism appear to be expressed at levels similar to those in cells grown on glucose with no transporter genes showing higher expression in maltose-grown cells as compared with glucose-grown cells. Our microarray data are supported by dot blot hybridizations using exponential growth phase, batch-grown cells. The dot blot data showed a putative periplasmic maltose binding protein-encoding gene, TM1839, is expressed at just over twice the level in maltose-grown cells than it is in glucose-grown cells. In contrast, the both known maltose binding protein genes (TM1839 and TM1204) are expressed at much higher levels in lactose-grown cells relative to glucose-grown cells as evidenced both in our microarray and dot-blot data. Using data such as these in combination with our fluorescence-derived binding data, we hope to begin to define the regulatory networks responsible for catabolite-mediated control of gene expression.

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- Noll, K. M. 1998. "*Thermotoga*: Promises, promises." Presented at The Institute for Genomic Research, Gaithersburg, MD.
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- Nguyen, T. N., K. M. Borges, A. H. Romano, and K. M. Noll. 1999. Transcriptional regulation of carbohydrase gene expression in *Thermotoga neapolitana.* Abstr. ASM Conference on Biodiversity, Chicago, IL, p. 67.
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- Nguyen, T. N., M. A. Brancieri, Ejaz, A. D., A. M Mikula, K. E. Nelson, A. H. Romano, S. R. Gill, and K. M. Noll. 2002. A simple chemostat system used to examine differential gene expression in the hyperthermophile *Thermotoga maritima.* Abstr. Ann. Meet. Amer. Soc. Microbiol., Salt Lake City, UT.
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