Final Report:

Ethanologenic Enzymes of Zymomonas mobilis

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Zymomonas mobilis is a unique microorganism in being both obligately fermentative and utilizing a Entner-Doudoroff pathway for glycolysis. Glycolytic flux in this organism is readily measured as evolved carbon dioxide, ethanol, or glucose consumed and exceeds 1 μ mole glucose/min per mg cell protein. To support this rapid glycolysis, approximately 50% of cytoplasmic protein is devoted to the 13 glycolytic and fermentative enzymes which constitute this central catabolic pathway. Only 1 ATP (net) is produced from each glucose metabolized. During the past grant period, we have completed the characterization of 11 of the 13 glycolytic genes from *Z. mobilis* together with complementary but separate DOE-funded research by a former post-doc and collaborator, Dr. Tyrrell Conway.

Research funded in my lab by DOE, Division of Energy Biosciences can be divided into three sections: A, Fundamental studies; B. Applied studies and utility; and C. Miscellaneous investigations.

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A. FUNDAMENTAL STUDIES

1) High level expression of glycolytic enzymes results from unusually stable messages. The most distinctive features of these glycolytic genes is their unusually stable messages, 10-18 min half-life. It is our hypothesis that this message stability represents the primary determinant of high level expression in *Z. mobilis*. Other supporting characteristics include the presence or tandem or multiple transcriptional initiation sites, canonical ribosomal-binding sites, biased codon usage, and little turnover by proteolysis. These promoters, terminators, and RBS serve as genetic elements which can be used to facilitate expression of homologous or heterologous genes in *Z. mobilis*.

2) The relative abundance of glycolytic enzymes among operons is determined primarily by differences in mRNA stability. Two-dimensional polyacrylamide gel electrophoresis methods were developed which allowed the unambiguous identification and separation of all 13 glycolytic and fermentative enzymes, facilitating the quantitation individual enzymes (uniformly labelled) and functional message levels (pulse-labelled). These results were compared to estimates of message stability. The abundance of individual glycolytic enzymes was directly related to the abundance and half-life of individual each respective message. Message stability appears to be the fundamental feature separating biosynthetic genes needed at low abundance from highly expressed glycolytic genes.

3) The relative expression of the *gap* and *pgk* genes within the *gappgk* operon is also determined by message stability. The *gap* gene product is 2X to 4X more abundant than the *pgk* gene product. The full length message is less stable than an upstream fragment containing the *gap* gene. Destruction of the full length message is initiated by cleavage within the coding region of the *pgk* message, eliminating further translation. The resulting upstream fragment is rapidly degraded by 3' exonucleases to yield a stable fragment containing a complete *gap* coding region. This stable *gap* fragment is bordered on both the 5' and 3' ends by stem loop structures which are essential for stability. Mutational analysis indicated that the 3' stem encompassing the transcriptional terminator downstream from *pgk* is required to prevent immediate degradation of the full-length

gappgk message. The intercistronic stem loop region bounding *gap* was essential to facilitate intercistronic processing within *pgk*. The 5' stems upstream from *gap* were also essential for message stability. As Dr. Conway has shown, a complex scheme of message processing also appears to regulate expression of 4 glycolytic genes in the *glf* operon.

4) Control of glycolytic flux. Shuttle vectors with containing *lacI^q* and a *tac* promoter were used to express glycolytic genes individually and in combination in *Z. mobilis*. Partial control was achieved. Overexpression of most glycolytic enzyme resulted in negligible change in flux or a negative effect of flux. This negative effect of flux can be readily explained by protein burden for highly expressed genes. The extent of this burden has been predicted from a theoretical basis and confirmed by direct measurement. Expression of only two glycolytic genes resulted in a significant increase in flux, *glk* encoding glucokinase and *zwf* encoding glucose 6-phosphate dehydrogenase. These data can be used to infer flux control of as high as 70% for the combination of both genes. In the presence of 4% ethanol, *lacI^q* control was much tighter for unknown reasons. Flux measurements with 4% ethanol exhibited an excellent dose-dependent relationship with *zwf* expression (series of IPTG concentrations) indicating near complete control by this single enzyme. These results suggest that increased production of *zwf* may improve the rate of ethanol production by *Z. mobilis* and reduce the progressive slowing of glycolysis which normally occurs during the fermentative accumulation of ethanol.

Many of the experiments using the full *glf* operon did not express individual components as expected in *E. coli* or in *Z. mobilis*. Our results suggest that multiple promoters may exist within the *glf* operon which also contribute to the differential expression of component genes.

5) Despite the low ATP yield per glucose in *Z. mobilis*, rapid glycolysis in this organism produces ATP at roughly twice the rate which is needed to support the maximum rate of growth. After dilution from stationary phase, the maximum rate of growth is achieved when flux reaches 50% of maximal specific activity. The protein burden created by overexpression of individual glycolytic enzymes can be used to reduce the rate of glycolytic flux. Doubling time is not appreciably

affected until flux declines to a level equivalent to 50% of the maximum specific activity.

Inhibition of growth with chloramphenicol leads to a 50% reduction in glycolytic flux. These results are consistent with a spillover metabolism as described by Dr. Russell for the disposal of excess ATP and regeneration of ADP, an essential feature for continued glycolysis.

Inhibition of membrane ATPase with DCCD results in an initial 20% inhibition of flux followed by recovery to the full flux rate during a 15 min period. DCCD-sensitive ATP hydrolyzing activity in French press extracts is half of total ATP hydrolyzing activity. The unusual *Z. mobilis* alkaline phosphatase is the second most abundant ATP hydrolyzing activity in these extracts. This enzyme does not seem to be a scavenger enzyme since it is not phosphate repressible and it is most active on nucleotides such as ATP with little activity for sugar phosphates.

We are pursuing the physiological role of this enzyme in *Z. mobilis*. Thus far we have described the cloning and sequencing. Suicide vectors are being constructed to reverse engineer knockout mutations by homologous recombination. Controlled expression of this gene in *Z. mobilis* may also test the hypothesis that ATP turnover/ADP regeneration is limiting during periods of maximum flux. Such a finding would provide an excellent basis for rational improvements the rate of ethanol production.

6) ADHII (adhB), a new family of alcohol dehydrogenase. The adhB gene from *Z. mobilis* represents the first member of a new family of alcohol dehydrogenase which have subsequently been found to be widely distributed in bacteria with homologues in yeast and mammalian systems. The unusual ADHB enzyme in *Z. mobilis* requires iron for activity, although homologues vary in their metal requirements.

7) AdhB was discovered to be stress protein in *Z. mobilis* which is induced by heat shock and by ethanol shock. This is the first time that a fermentative enzyme has been identified as such a prominent stress responsive gene in a microorganism although several glycolytic genes have been reported to exhibit a weak heat shock response in yeasts. However, *pdc* and *adh* are stress responsive genes in plants which are induced in root tissue in response to water-logged conditions

(anoxia).

8) Identification of two of the abundant cytoplasmic proteins in *Z. mobilis* as *groES* and *groEL*, cloning and characterization of these genes. The *groESL* products are very abundant in *Z. mobilis* even prior to significant accumulation of ethanol. These increase with ethanol in the beer. Both genes share high homology with genes from organisms which do not produce ethanol as major fermentation products. DnaJ and DnaK proteins were also tentatively identified in 2-D gels.

9) Cloning, sequencing and characterization of the principal alkaline phosphatase gene (*phoD*) in *Z. mobilis*. This gene was truly unusual and delineates a new family of phosphatases. It exhibited no appreciable homology to other phosphatases. However, segments exhibited partial homology to pyruvate kinase and to mammalian nucleotide phosphodiesterase (membrane-bound). We feel that this gene may have an important physiological role in ATP turnover. Since publication of the sequence in GenBank, we have been contacted by two groups which have identified homologues with unknown function from other bacteria. In two cases, these gene were in the regions encoding flagellar apparatus. It is tempting to speculate such energy consuming flagellar processes could be involved in the dissipation of excess ATP by *Z. mobilis*. Although many strains do not appear to be motile in directed sense, flagellar apparatus coupled with F1F0 ATPase could provide a futile cycle whose sole function is energy dissipation.

10) Considerable effort was expended to investigate the possible existence of glycolytic complexes in *Z. mobilis* with little conclusive results. All glycolytic enzyme were either purified in my lab or obtained from Dr. R.K. Scopes, a collaborator. Polyclonal antibodies were prepared for each enzyme. Electron microscopy gold-labelled antibodies suggested associations between alcohol dehydrogenase I and other glycolytic enzymes. Attempts to further substantiate this with gel filtration methods were unsuccessful; glycolytic enzyme were either bound or completely retarded by large pore Biorad HPLC columns. These columns are quite expensive. However, it is possible that an alternative matrix would have provided resolution. Other attempts to demonstrate association relied on immunobeads containing secondary antibodies. Indeed, antibodies to

individual glycolytic enzymes contained significant levels other enzymes when precipitated by gentle binding to immunobeads. These experiments are still in progress and are supportive of complexes.

11) Cloning and sequencing of the *Z. mobilis* DNA methylase. This methylase serves as tool for the construction of a variety of new vectors, greatly improving our ability to genetically manipulate *Z. mobilis*.

B. APPLIED STUDIES AND UTILITY - metabolic engineering, source of genes for others

Our *Z. mobilis* genes encoding the ethanol pathway (*adhB* and *pdc*) have been used to engineer novel biocatalysts which are capable of converting all of the sugar constituents found in lignocellulose into ethanol with greater than 90% of the theoretical yield. Prior to this, no organisms in nature could efficiently convert the pentoses of hemicellulose into any single product of value. Intensive investigations since the oil crisis of the 1960's had failed to find such organisms from nature or to successfully construct such organisms. Our work has been regarded as an important step toward the commercialization of woody waste to fuel ethanol, a replacement for part of the imported petroleum.

This work demonstrated that fermentation pathways could be exchanged among organisms using the tools of genetics, and that central metabolism could be redirected in this manner. The success of this approach has served as an impetus for research by others and to some extent as a justification for funding in this area with goals ranging from reducing cavities to "direct" conversion of sunlight to ethanol.

The PET operon which we developed has now been used to engineer Gram negative bacteria with considerable success. We have integrated these gene into the chromosome to produce stable organisms which express 5%-8% of their cellular protein as the *Z. mobilis* PDC and ADHII. Progress has been made in engineering Gram positive bacteria for ethanol production (*B. subtilis*,

Lactobacillus, *Strep. mutans* for replacement therapy to reduce carries, etc.), blue greens, yeasts and higher plants. Glycolytic genes isolated during the past DOE award have been used as probes by many investigators to isolate genes in other organisms.

New biocatalysts have been engineered by our lab for both hemicellulose and cellulose-based fermentations. These have been licensed and are nearing commercial demonstration. These have been shown to effectively ferment industrial hemicellulose hydrolysates as effectively as laboratory sugars. Increased ethanol tolerance, the basis for the current submission, is a priority need to improve the utility of these biocatalysts and to decrease the costs of fuel ethanol production.

C. MISCELLANEOUS INVESTIGATIONS

- 1) Replacement of *E. coli* PTS glucose pathway by *Z. mobilis* glucose facilitator and glucokinase.
- 2) Direct recovery of functional genes for hydrolases such as cellulase using DNA isolated from microbial consortia (anaerobic digester) - genes from uncultured, perhaps unculturable organisms. This work was done in collaboration with Dr. K.T. Shanmugam in our department.
- 3) Several collaborative investigations with Dr. Jensen have been fruitful. We assisted in the work with the cyclohexadienyl dehydrogenase gene and have recently provided his group with a sequenced aminotransferase gene.
- 4) A putative lactate dehydrogenase gene was found downstream from *pgm*, now being studied by a collaborator.
- 5) We have cloned and sequenced the PTS *cel* genes from *B. stearothermophilus*, the first cellobiose transport genes ever characterized in a Gram positive organism. We have also characterized the *ptsHI* operon from this organisms and discovered that this operon contains a third small gene which may serve some regulatory function.

PUBLICATIONS, PRESENTATIONS AND AWARDS RESULTING FROM DOE-SPONSORED RESEARCH

PUBLICATIONS

Ingram, L.O., J.B. Doran, D.S. Beall, T.A. Brooks, B.E. Wood, X. Lai, and L. Yomano. 1995. Genetic engineering of bacteria for the conversion of cellulosic biomass to ethanol. ACS Symposium. Submitted for publication.

Asghari, A., R.J. Bothast, J.B. Doran, and L.O. Ingram. 1995. Ethanol production from hemicellulose hydrolysates of agricultural residues using genetically engineered *Escherichia coli* strain KO11. ACS Symposium. Submitted for publication.

Snoep, J.L., N. Arfman, L.P. Yomano, H.V. Westerhoff, T. Conway, and L.O. Ingram. Control of glycolytic flux in *Zymomonas mobilis* by gene products from the *glf-zwf-edd-glk* operon. Submitted for publication.

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Parker, C., W.O. Barnell, J.L. Snoep, L.O. Ingram and T. Conway. 1995. Characterization of the *Zymomonas mobilis* glucose facilitator gene product (*glf*) in recombinant *Escherichia coli*: examination of transport mechanism, kinetics and the role of glucokinase in glucose transport. Mol. Microbiol. 15: IN PRESS.

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- Barbosa, M. de F.S., L.P. Yomano, and L.O. Ingram. 1994. Cloning, sequencing, and expression of stress genes from the ethanol-producing bacterium *Zymomonas mobilis*: The *groESL* operon. Gene 148:51-57.
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genes from *Clostridium thermocellum*. Appl. Environ. Microbiol. 58: 2103-2110.

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Barbosa, M. de F.S., M.J. Beck, J.E. Fein, D. Potts, and L.O. Ingram. 1992. Efficient fermentation of *Pinus* sp. acid hydrolysates by an ethanologenic strain of *Escherichia coli*. Appl. Environ. Microbiol. 58:1382-1384.

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Utt, E.A., C.K. Eddy, K.F. Keshav, and L.O. Ingram. 1991. Sequencing and expression of the *Butyrivibrio fibrisolvens* *xylB* gene encoding a novel bifunctional protein with β -D-xylopyranosidase and α -L-arabinofuranosidase activities. Appl. Env. Microbiol. 57:1227-1234.

Ohta, K., D.S. Beall, K.T. Shanmugam, and L.O. Ingram. 1991. Genetic improvement of

Escherichia coli for ethanol production: chromosomal integration of *Zymomonas mobilis* genes encoding pyruvate decarboxylase and alcohol dehydrogenase II. Appl. Environ. Microbiol. 57:893-900.

SYMPOSIA PRESENTATIONS

Joint USDA & DOE Ethanol Biofuel Conference, Chicago, 1992

American Chemical Society, San Francisco, 1992

Agriculture and Ecology Conference, Univ. of Viscosa, Brazil, 1992

Genecor/Iowa Electric Biotechnology Conference, Iowa City, 1992

Gordon Conference on BioCatalysis, New Hampshire, 1992

9th International Biotechnology Symposium, Washington D.C., 1992

American Chemical Society, Denver, 1993

Dutch Microbial Physiology Platform, Delft, Netherlands, 1993

International Energy Agency, Helsinki, Finland, 1993

International Congress on Chemicals from Biotechnology, Hannover, Germany, 1993

American Society for Microbiology, Washington D.C. (1995)

American Chemical Society, 2 symposia, Anaheim (1995)

HONORS AND AWARDS

Commendation from the Florida Senate and Florida House, U.S. House of Representatives, 1991

University of Florida Research Achievement Award, 1991

U.S. Department of Commerce, Landmark Patent No. 5,000,000, 1991

University of Florida Research Achievement Award, 1992

U.S. Department of Agriculture, Distinguished Service Award, 1993

Distinguished Inventor Award, Florida Small Business Development Agency, 1994