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"Control of Sugar Transport and Metabolism in *Zymomonas Mobilis*"

PRINCIPAL INVESTIGATOR: Tyrell Conway

Current Address:

484 West 12th Avenue
Department of Microbiology
The Ohio State University
Columbus, OH 43210

Office: (614) 688-3518
Department: (614) 292-2301
FAX: (614) 688-3519

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FINAL REPORT

A. Specific Objectives of Grant

This research deals with the physiology and genetics of sugar transport and metabolic control in *Zymomonas mobilis*. The specific objectives of the grant are as follows: **First**, the complex interactions of transcriptional, post-transcriptional and translational control mechanisms on regulation of the *glf* operon will be investigated. **Second**, the structure and function of the unique glucose facilitator will be examined by a combination of in vitro and in vivo approaches, making use of the genetically reconstituted system in *E. coli*. **Third**, the possibility that physical association or indirect interactions between the glucose facilitator and glucokinase are involved in metabolic control will be analyzed. **Fourth**, the *Z. mobilis* glucose transport and phosphorylation system will be utilized to metabolically engineer recombinant *E. coli* with altered cell pool metabolite profiles. Work on the third and fourth objectives is complete, work on the first and second objectives is progressing nicely. Publication of this work has been admittedly slow, due primarily to a change in location of the research program from the University of Nebraska to The Ohio State University. However, it should be noted that much of the unpublished data outlined below represent completed studies, and are contained in graduate student theses which are being prepared for submission this summer. Since a full year remains in the current funding period, and the new laboratory is now up and running, we fully expect to make reasonable progress on the remaining objectives and to publish the results in a timely fashion.

B. Summary of Current and Previous Research

The long-term goal of this and related work is to understand the role of gene expression in regulating sugar transport and control of glycolytic metabolism. Prior work on *Zymomonas mobilis* is extensive. The biochemistry and physiology of glycolysis in *Z. mobilis* is now well-understood and the current work on sugar transport provides an important addition to our understanding of the *Z. mobilis* lifestyle.

The genetic mechanisms responsible for regulating balanced expression of the Entner-Doudoroff and glycolytic genes in *Z. mobilis* are now well-understood. Several layers of genetic control, perhaps in a hierarchal arrangement, act in concert to determine the relative abundance of the glycolytic enzymes. These genetic controls involve differential translational efficiency, highly conserved promoter sequences, transcription factors, differential mRNA stabilities, and nucleolytic mRNA processing (Conway, *et al.*, 1991; Barnell, *et al.*, 1992; Liu, *et al.*, 1992; Mejia, *et al.*, 1992).

The importance of gene expression and the consequent balance of glycolytic enzyme synthesis in *Z. mobilis* was investigated by using the glycolytic genes as tools to manipulate specific enzyme levels *in vivo*. The critical genes were subcloned for stable expression in *Z. mobilis*, being placed under control of a regulatable promoter system. Metabolic flux control

analysis revealed that sugar transport and the first two intracellular steps of glucose metabolism exert approximately 60-70% of the total control of flux through the pathway. One very interesting finding of these studies was that overexpression of various combinations of these three rate-controlling enzyme activities resulted in no greater than a 12% increase in pathway flux (Snoep, et al., 1995).

It is clear that glycolytic enzyme synthesis is coordinated to prevent the build-up of toxic metabolic intermediates, the concentrations of which remain low despite very high glucose flux in *Z. mobilis*. The genes specifying the glucose transporter and the first three steps of intracellular glucose metabolism are arranged in an operon, which is carefully regulated at the transcriptional and post-transcriptional levels. The key metabolic intermediate of these early steps, 2-keto-3-deoxy-6-phosphogluconate (KDPG) is toxic (Fuhrman and Conway, M. S. thesis; please see Objective 4 below), but its formation is very carefully controlled biochemically by an optimal *in vivo* balance of these transport and enzyme activities that together exert a very high control of flux from glucose to KDPG (Liu, et al., 1992; Snoep, et al., 1995).

Current studies of sugar transport via the glucose facilitated diffusion transporter, Glf, have brought the physiology of *Z. mobilis* into perspective (Snoep, et al., 1994; Parker, et al., 1995; Parker and Conway, M. S. thesis). *Z. mobilis* can grow on glucose and fructose, but in nature, the primary substrate is sucrose, which is hydrolyzed outside of the cell to release free glucose and fructose. Our data tend to support a model in which a single transporter, Glf, is responsible for transport of both sugars, but with a ten-fold higher apparent affinity for glucose than for fructose. Other experiments have essentially ruled out the possibility that Glf interacts directly with the sugar-specific kinases to regulate transport. Rather, *Z. mobilis* appears to depend upon the very simple mechanism of differential substrate affinity to regulate transport of glucose and fructose. Preferential transport of glucose, leaving a higher concentration of fructose outside of the cell, is important because *Z. mobilis* uses the extracellular fructose as a substrate for reduction to sorbitol, which serves as an osmoprotectant for growth in high sugar environments.

Figure 1. Model relating Glf function with metabolism and physiology.

In summary: Genetic and metabolic studies, together with the sugar transport work that will be described below, have provided a detailed understanding of *Z. mobilis* physiology as it relates to the metabolic lifestyle of the organism in its natural environment (Fig. 1). *Z. mobilis* is found all over the world, primarily in tropical climates, in association with plants that have a very high sugar content in their saps or fruit juices. In most cases the natural substrate for carbon and energy is sucrose, often found at levels exceeding 15%, which is hydrolyzed by

a levan sucrase to release free glucose and fructose. The glucose and fructose appear to compete for transport by a single facilitated diffusion carrier, Glf, which has a higher affinity for glucose, thus leaving a relatively high concentration of fructose outside and available for conversion to sorbitol by glucose-fructose oxidoreductase (GFOR). Sorbitol is then taken up and serves as the primary osmoprotectant in *Z. mobilis*, allowing for growth in very high osmotic environments. Glucose and fructose are metabolized exclusively via the Entner-Doudoroff pathway and fermented to the sole end products ethanol and carbon dioxide. Since respiration is absent, and because ethanol fermentation via this pathway yields only one mole of ATP per mole of glucose, *Z. mobilis* must maintain a very high metabolic flux. This is accomplished by very high cytoplasmic levels of the glycolytic and ethanologenic enzymes, the synthesis of which is balanced primarily at the post-transcriptional level to maintain optimal metabolic regulation. Metabolic flux control studies indicate that approximately two-thirds of the the total flux control resides in sugar transport and the first two intracellular metabolic steps that form the key intermediate of the Entner-Doudoroff pathway, KDPG. This metabolite is known to inhibit growth in several microorganisms under conditions that allow its accumulation. Thus, *Z. mobilis* can be said to have evolved in a highly specialized way for growth in a very narrow ecological niche. Sugar transport rates are balanced to provide osmoprotection and to rapidly feed sugar into metabolism. The biochemistry and genetically determined levels of the glycolytic and ethanologenic enzymes allow the very high carbon flux that *Z. mobilis* requires, but without allowing the build-up of metabolic intermediates that could slow or prevent growth.

C. Specific Objective 1: Genetic Regulation of the *glf* Operon

The *Z. mobilis glf* operon contains four genes (*glf-zwf-edd-glk*) that code for the glucose facilitated diffusion transporter, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydratase, and glucokinase, respectively (Fig. 2). Studies have shown that the *glf* operon is subject to transcriptional regulation, extensive post-transcriptional mRNA processing, and contains complex secondary structures such as a transcriptional terminator in the intergenic region downstream of *glf* and an overlap of the *zwf* and *edd* genes (Barnell, *et al.*, 1992; Liu, *et al.*). Furthermore, the promoters for all of the highly expressed glycolytic genes share a remarkable degree of sequence conservation over a large region, -100 to +15 with respect to the transcriptional initiation site. Most of our work has focused on the structure of the *glf* promoter and transcriptional regulation in response to fructose, leaving the more complex topic of post-

Figure 2. Genetics of the *glf* operon and biochemistry of the Entner-Doudoroff pathway in *Z. mobilis*.

transcriptional regulation for the third year of the grant.

Promoters within the operon. A search for promoters by guanylyl transferase capping of 5' mRNA ends failed to reveal any true transcription start sites within the operon, other than the *glf* promoter itself. In addition, a computer search based upon the consensus sequence derived from several highly expressed *Z. mobilis* genes did not show any significant matches within the *glf* operon. An alternative approach for identifying internal promoters, involving *lacZ* fusion analysis, will be conducted during the coming year.

Fructose signaling and transcriptional regulation. Previous work indicated that a three-fold induction of *glf* transcription occurs in cells grown on fructose, compared to glucose (Barnell, et al., 1992). The same was found to be true for fructokinase and phosphoglucose isomerase, two key enzymes that are involved in fructose, but not glucose, metabolism (Zembruski, et al., 1992; Hesman, et al., 1991). The experiments described below address our working hypothesis that Glf has a higher affinity for glucose than for fructose and that *glf* expression is elevated for growth on fructose in order to allow the required flux of fructose into the cell. Current work has focused on the structure of the *glf* promoter and potential regulatory domains that might be responsible for the response to fructose. Two new vectors, pTC305 and pTC306, are being used for construction of *lacZ* protein and operon fusions, respectively (Fig. 3). A PCR approach was used to construct a series of deletions of the *glf* promoter as *lacZ* protein and operon fusions. PCR primers with *Xba*I and *Kpn*I restriction sites, respectively, were designed to cover the desired 5' and 3' endpoints. The PCR-amplified promoter fragments were cloned into the fusion vectors with special care to ensure that the 3' ends generated in-frame fusions when ligated into pTC305. These constructions were expressed in recombinant *Z. mobilis* and characterized with respect to relative transcriptional activity on different carbon sources and under various growth conditions. β -galactosidase activities were normalized with respect to plasmid copy number and relative mRNA concentrations.

The results of some of these experiments are shown in Table 1. Constructions which delete the promoter to -40 at the 5' end or to -20 from the 3' end, with respect to the transcriptional start site, completely eliminate promoter activity in protein fusions and greatly diminish activity in operon fusions. Operon fusions with 5' endpoints at -228 having 3' endpoints within the *glf* gene (+230 and +136), at the *glf* start codon (+120), or at the transcription initiation site (+1) all show normally regulated activity (data not shown). A domain within the

Figure 3. Broad host shuttle vector for construction of *lac* protein and operon fusions.

promoter region, located between -80 and -60, is responsible for the increased rate of transcription observed when cells are grown on fructose; deletions that eliminate this region show constitutive expression levels regardless of the carbon source. Expression of the full length promoter is also elevated for growth on sucrose, but to a lesser extent than for fructose alone (data not shown). It can be concluded that the fully functional *glf* promoter covers a large region from -80 to +1.

Table1. Relative activity of *glf* promoter fragments as *lacZ* fusions expressed in recombinant *Z. mobilis*.

<i>lacZ</i> Fusion Construct		-Galactosidase Activity (mMol/min/mg) ³	
Promoter Frag ¹	Vector ²	10% Glucose	10% Fructose
-228--+136	pTC305	0.42	1.62
-100--+136	pTC305	0.29	1.40
-60--+136	pTC305	0.30	0.31
-40--+136	pTC305	0.01	0.01
-228--+136	pTC306	2.80	4.79
-100--+136	pTC306	1.82	4.55
-80--+136	pTC306	1.25	4.04
-60--+136	pTC306	1.19	1.29
-40--+136	pTC306	0.60	0.64
-20--+136	pTC306	0.41	0.38
-228---20	pTC306	0.28	0.06

¹5' and 3' endpoints of PCR generated promoter fragments cloned as *Xba*I to *Kpn*I inserts.

²pTC305, *lacZ* protein fusions; pTC306, *lacZ* operon fusions.

³b-Galactosidase measured during mid-log phase and normalized for plasmid copy number.

Experiments designed to look at the time course of expression of a full-length *lacZ* protein fusion (-228--+136::pTC305) during batch growth indicate that the *glf* promoter is regulated in a complicated fashion (Fig. 4). These cultures were inoculated from starter cultures that were pregrown to early stationary phase on glucose. For fructose-grown cultures, expression increases during log growth, the overall extent of expression is more than two-fold higher than on glucose, and expression remains high well into stationary phase. A similar result was obtained for sucrose, but with highest expression occurring earlier in log phase and falling to a lower level by mid log phase. This is in stark contrast to a culture growing on 10% glucose which shows a burst

of expression during entry into log phase, then returns to a lower level by the middle of log phase

Figure 4. Growth and B-Galactosidase activity of *Z. mobilis* (-228--+136::pTC305). A, 10% glucose; B, 10% fructose; C, 10% sucrose.

and drops nearly to zero in stationary phase. Interestingly, cultures grown on low concentrations of glucose (0.1%) show relatively high expression of the full-length protein fusion, without the modulation of activity seen on high glucose.

Cultures grown on low concentrations of fructose showed essentially no difference from cultures grown on high fructose. Increased *glf* expression at the onset of log phase might be a secondary effect created by a need to synthesize Glk in order to initiate growth, since Glk enzyme levels are low in stationary phase cells. While this line of experiments is beginning to reveal some of the details of promoter structure and regulation in *Z. mobilis*, much remains to be done during the next funding period.

D. Specific Objective 2: Structure and Function of Glf

The structure and function of Glf are of particular interest because Glf is the only prokaryotic sugar facilitator that has been cloned and characterized at the molecular level. It is felt that our most important contribution will be to improve understanding of *Z. mobilis* physiology by studying the gene expression and biochemistry of sugar transport. While the structure of Glf with respect to substrate recognition and the non-energy-dependent transport process is important, we have focused on a functional characterization of Glf with respect to transport kinetics and substrate specificity, making a considerable effort to understand these biochemical properties in terms of the physiology of *Z. mobilis* growth and metabolism.

Glf transport kinetics in recombinant E. coli. The *Z. mobilis glf* and *glk* genes are able to complement glucose transport and phosphorylation in an *E. coli* mutant (Snoep, *et al.*, 1994). This was significant, at least in part because no other glucose facilitator had ever been expressed in a foreign host. Experiments with the genetically reconstituted transport system in recombinant *E. coli* allowed us to isolate Glf function, important in order to avoid the potential complications

of multiple transport systems or interaction of Glf with cytoplasmic enzymes in the native host. The kinetics of sugar transport have been measured in recombinant *E. coli* expressing the *Z. mobilis glf* gene and the results published (Parker, *et al.*, 1995). The rates of glucose transport and growth of the recombinant *E. coli* were shown to be proportional to the level of *glf* expression. The recombinant *E. coli* was shown to be capable of influx counterflow when preloaded with glucose and had an apparent K_m for glucose of approximately 2.5 mM, consistent with the function of Glf as a low affinity glucose facilitator. One question that had never been properly addressed was whether *Z. mobilis* possesses a single transporter for both glucose and fructose or has multiple transporters. While transport of fructose by the recombinant *E. coli* expressing *glf* was clearly shown to be Glf-dependent, we were not able to measure a reasonable K_m value for fructose (> 500 mM). Several glucose analogues were able to compete with glucose for transport by recombinant *E. coli* expressing *glf*, but there was only a modest competition by fructose, indicating that Glf is fairly specific for glucose. While the results of fructose transport assays by recombinant *E. coli* did not completely rule out the possibility of very low affinity for fructose, the apparent specificity of Glf for glucose led us to conclude that it is possible that *Z. mobilis* utilizes a different transporter(s) for fructose.

Sugar transport kinetics in Z. mobilis. Subsequently, we turned our attention to kinetic analysis of sugar transport by *Z. mobilis*. The data shown in Table 2, indicate that the apparent K_m for glucose is approximately 13 mM, while the apparent K_m for fructose is approximately 185 mM (Parker and Conway, M. S. thesis). There is an obvious 2 to 3-fold increase in the V_{max} for

Table 2. Kinetic values for sugar transport by *Z. mobilis* CP4.

Growth Conditions	^{14}C -Substrate	K_m (mM)	V_{max} (nmol/min/mg)
10% glucose	glucose	13.4	210
10% fructose	glucose	14.6	376
10% glucose	fructose	583	3295
10% glucose	fructose w/ FCCP	201	1253
10% fructose	fructose	167	3000

transport of both sugars when the cells are grown on fructose by comparison to glucose, consistent with the 3-fold higher *glf* mRNA levels in cells grown on fructose (Barnell, *et al.*, 1992). It is worth noting that the apparent K_m of Glf for glucose in recombinant *E. coli* is significantly lower (5-fold) than the K_m for glucose measured in wild type *Z. mobilis*, perhaps due to differences in membrane lipid composition, and that it was not possible to measure a K_m value for fructose in the recombinant *E. coli* strains that we used. In fact, initial attempts to measure fructose transport by *Z. mobilis* cells grown on glucose, but not fructose, gave similarly strange high apparent K_m values for fructose. During the course of these experiments it was recalled that

Z. mobilis has the periplasmic enzyme glucose fructose oxidoreductase (GFOR) which reduces fructose to the osmoprotectant molecule sorbitol by oxidizing glucose to gluconate. Therefore, the fructose kinetic experiment was repeated in the presence of the ATPase inhibitor, FCCP, which should theoretically eliminate energy-dependent secondary transport systems. Glucose-grown cells treated with FCCP had more reasonable kinetic values -- an apparent K_m for fructose of 201 mM and a V_{max} of 1253 nmol/min/mg, consistent with our hypothesis that cells grown on glucose were able to convert some of the radioactive fructose to sorbitol which served as a solute for an energy-dependent secondary transport system. There was no effect of FCCP treatment on fructose-grown cells since glucose was not available as a substrate of GFOR. These experiments clearly demonstrate that *Z. mobilis* transports glucose with an apparent affinity that is an order of magnitude greater than for fructose, but the V_{max} for fructose is about 6 to 8-fold higher for fructose than for glucose. Unfortunately, nothing was concluded with regard to the presence or absence of additional transporters for fructose.

Physiology of sugar transport by Z. mobilis. There are two observations which suggest that *Z. mobilis* will preferentially metabolize glucose when presented with a mixture of glucose and fructose (or sucrose). First, there is the higher apparent affinity of *Z. mobilis* for glucose. Second, kinetic analysis of the fructose-specific kinase revealed that fructose phosphorylation is inhibited by free glucose (at least *in vitro*; Table 3), while the glucose-specific kinase is unaffected by fructose or fructose-6-phosphate (data not shown). In order to evaluate whether or not *Z. mobilis* can co-metabolize glucose and fructose, the effect of adding glucose to a culture growing on fructose was tested (Fig. 5). The results indicated that metabolism of fructose can occur at a significant rate with glucose in the growth medium, albeit with a temporary lowering of the rate of incorporation of radioactive fructose due to co-metabolism of glucose. The results of this experiment led to the simple conclusion that glucose and fructose are transported and metabolized simultaneously, and that inhibition of fructokinase by free glucose did not appear to occur *in vivo*.

Table 3. Inhibition of *Z. mobilis* fructokinase activity by free glucose.

Growth Conditions ¹ (% Fruct/% Gluc in growth medium)	Fructokinase Specific Activity ² (concentrations of Fructose and Glucose in assay mixture)				
	10 mM Fruc; 0 Gluc	10 mM Fruc; 1.25 mM Gluc	10 mM Fruc; 5 mM Gluc	10 mM Fruc; 10 mM Gluc	1.25 mM Fruc; 10 mM Gluc
1% F only	1.6	1.5	0.67	0.36	0.10
1% F/0.2% G	1.3	1.0	0.50	0.29	0.06

¹ *Z. mobilis* CP4-M2, a glucokinase negative mutant grown on YEP medium with given sugar concentrations.

² Fructokinase activity measured in crude extracts with given substrate concentrations (mM/min/mg).

In a clear case of running one experiment too many, we decided to repeat the fructose incorporation experiment with a mutant strain, *Z. mobilis* CP4-M2, that is defective in

Figure 5. Growth (top) and incorporation (bottom) of radioactive fructose by *Z. mobilis* CP4 in the presence (squares) or absence (circles) of added glucose.

Figure 6. Growth (top) and incorporation (bottom) of radioactive fructose by *glk*⁻ *Z. mobilis* CP4-M2 in the presence (squares) or absence (circles) of added glucose.

Figure 7. Growth of *Z. mobilis* CP4 on fructose in the presence (squares) or absence (circles) of added 3-O-methyl glucose (top) or xylose (bottom).

glucokinase and can only grow on fructose. The result we obtained was a complete surprise, eventually leading us to a new level of understanding of *Z. mobilis* sugar transport physiology. The experiment in Fig. 6 shows that growth and incorporation of radioactive fructose are severely inhibited by addition of glucose to the growth medium, in stark contrast to the analogous result with the wildtype (Fig. 5). The only obvious difference between the *glk*⁻ and wildtype strains was the inability of the mutant to metabolize glucose and the inhibitory effect of glucose could clearly be attributed to the glucokinase mutation in *Z. mobilis* CP4-M2, since genetic complementation of the mutant with the *glk* gene restored its ability to co-metabolize glucose and fructose. Therefore, the experiment was repeated for the wildtype, but this time with the non-metabolizable glucose analogues, 3-O-methyl glucose and xylose (Fig. 7). An equimolar concentration of 3-O-methyl glucose, which was known to severely inhibit Gtf-mediated glucose transport (Parker, *et al.*, 1995), resulted in complete growth inhibition. Likewise, a 100-fold excess of xylose,

Figure 9. Short-term uptake of 30 mM ¹⁴C-fructose by *Z. mobilis* CP4 grown on glucose (top) or on fructose (bottom) in the presence (filled boxes) or absence (open boxes) of 30 mM glucose.

which was known to be a modest inhibitor of Glf-mediated glucose transport, resulted in complete growth inhibition.

Figure 8. Short-term uptake of 30 mM ^{14}C -fructose by *Z. mobilis* CP4-M2 grown on fructose in the presence (filled boxes) or absence (open boxes) of 30 mM glucose.

Fructose uptakes were conducted in order to test whether the inhibitory effect of glucose on fructose metabolism by the *glk*⁻ mutant was the result of transport inhibition. The experiment shown in Fig. 8 indicates that severe inhibition of fructose uptake occurs in the presence of equimolar glucose. The analogous experiment with the wildtype, shown in Fig. 9, indicates that in this strain there is also a significant, but in this

case incomplete, inhibition of fructose uptake in the presence of equimolar glucose. The wildtype grown on fructose has a five-fold higher initial velocity of fructose transport as compared to cells grown on glucose, and the inhibition of fructose transport by glucose occurs to the same extent whether the cells were grown on glucose or on fructose. These results can be most easily explained by a model in which there is a single transporter, Glf, responsible for transport of both glucose and fructose, but with an order of magnitude lower affinity for fructose. While the decreased rate of fructose transport can explain why the wildtype incorporates fructose more slowly when growing on mixtures of glucose and fructose (Fig. 5), it does not explain why the *glk*⁻ mutant was completely unable to grow on the mixture (Fig. 6), or why growth was inhibited by the presence of non-metabolizable glucose analogues (Fig. 7).

In order to understand how a non-metabolizable substrate could inhibit growth on fructose we had to consider the properties of a facilitated diffusion transporter. These transporters have the capacity for influx, efflux, and exchange, the latter referring to a swap of solute at either face of the membrane. Normally, metabolism is very rapid, ensuring that the concentration of sugar inside the cell is lower than the concentration outside of the cell, and consequently the net flow of solute is into the cell with minimal efflux or exchange. This all changes when the solute cannot be metabolized: the solute equilibrates across the membrane, net influx and efflux are equal, and the exchange reaction predominates. Quite simply, the recognition sites of the transport protein are continuously occupied by a high affinity substrate due to the exchange reaction. In the presence of a non-metabolizable solute for which Glf has higher affinity (ie. glucose), fructose is no longer transported because it cannot displace the high-affinity substrate from the transport protein.

Search for additional sugar transporters. Several clones were isolated from the *Z. mobilis* gene library based on their ability to restore glucose or fructose metabolism in the *E. coli*

carbohydrate transport mutant JM1100 and another strain, ZSC113, as indicated by red colony formation on MacConkey plates and/or restored ability to grow on minimal glucose plates. Transport activity was confirmed in several of the clones by assay of radioactive glucose uptake. Two of these plasmids were characterized by restriction mapping to ensure that they were different from *glf*, subcloned to localize the structural genes, and subjected to sequence analysis. With 10 kb of very good sequence data in hand from the two clones, sequence analysis failed to reveal any open reading frames that could possibly encode a transport protein, or for that matter, any open reading frames that could encode very hydrophobic peptides that even resemble membrane proteins. Even more surprising was our inability to identify *any* open reading frames within the subcloned regions that specified transport activity. The search for additional sugar transporters in *Z. mobilis* has not been abandoned. We are currently using another strain for selection of transport genes by genetic complementation, but probably will not attempt more time-intensive approaches, such as PCR or use of heterologous gene probes, unless we obtain good physiological/biochemical evidence for the presence of additional transporters.

E. Specific Objective 3: Interactions of Glf and Glucokinase

Experiments designed to investigate the effect of Glk activity on Glf-dependent glucose transport indicated that the maximal velocity of glucose transport in a recombinant *E. coli* strain was not affected by altering the level of Glk activity *in vivo* (Parker, *et al.*, 1995). Also, the results of kinetic experiments indicated that Glk did not enhance the apparent K_m of Glf for glucose when Glf and Glk were co-expressed in a recombinant *E. coli*. The results of these experiments strongly suggest that Glf and Glk do not interact at the cytoplasmic face of the membrane to control transport rate or metabolic flux. Furthermore, the physiological studies of *Z. mobilis* growth and sugar transport described above suggested that glucose phosphorylation is simply regulated by the concentration of free glucose in the cytoplasm. While it remains a formal possibility that Glf and Glk are loosely associated in some kind of metabolic complex to channel glucose to Glk, it was felt that this type of association would be very difficult to study and therefore has not been pursued further.

F. Specific Objective 4: Metabolic Engineering and Metabolite Pools in *E. coli*

PEP pools in recombinant E. coli. During the course of this work, recombinant *E. coli* strains were constructed which utilize a pathway for glucose transport and phosphorylation that is unique to *E. coli*. The recombinant *E. coli* transports glucose via facilitated diffusion and phosphorylates it at the expense of ATP, a considerably different process from PTS transport and phosphorylation at the expense of PEP (phosphoenol pyruvate). Two immediate and obvious differences from the wildtype *E. coli* are the potential increase in PEP pool size and the availability of free glucose inside the cell. The consequences of these alterations on metabolism were examined by HPLC-based measurements of metabolite concentrations in the cell pool. We were quite surprised to find that there are absolutely no differences in the pool sizes of PEP or ATP in the recombinant *E. coli* expressing the *Z. mobilis glf* and *glk* genes when compared to wildtype *E. coli*. Moreover, there were no significant differences in the pool sizes of other

phosphorylated intermediates of glycolysis. (These data were removed from the paper by Parker, *et al.*, 1995 on request of the editor.) These experiments indicate that there is considerable "slack" in *E. coli* metabolism. In other words, the metabolic perturbation created by changing the phosphoryl donor that initiates glucose metabolism was absorbed through adjustments in flux control by other enzymes of the pathway.

KDPG accumulation and growth stasis in E. coli. We have ascribed the need for *Z. mobilis* to evolve extensive genetic regulation and precise control of carbon flux to toxicity of KDPG, the key intermediate of the Entner-Doudoroff pathway. In fact, the accumulation of phosphorylated metabolites has long been known to inhibit cell growth, and KDPG is one such

Figure 10. Addition of gluconate (arrow) to an *E. coli* mutant (*edd⁺, edd⁺*) growing on minimal glucose medium. KDPG concentrations are indicated in brackets (mM).

Figure 11. HPLC chromatograms showing KDPG accumulation in the culture shown in Fig. 10. Cell pools were extracted at the indicated times (min.).

metabolite for which circumstantial evidence of toxicity is available in *E. coli*, *Pseudomonas aeruginosa*, and a few other Gram negatives. However, a carefully measured correlation between KDPG accumulation and growth inhibition was never made, nor had the mechanism of KDPG toxicity been addressed. We chose to study this problem in *E. coli* because of the availability of strains that allowed us to specifically induce KDPG accumulation by manipulating the culture medium. The experiment in Figs. 10 and 11 shows the result of adding gluconate to an *E. coli eda* mutant growing in log phase on a permissive substrate (Fuhrman and Conway, M. S. thesis). The intracellular KDPG pool increased to 3.9 mM within 15 sec and reached as high as 15.5 mM after 20 min. This experiment demonstrates quite convincingly that the immediate inhibition of growth caused by blocked carbon flow through the Entner-Doudoroff pathway is correlated with accumulation of KDPG. Plating experiments showed that the effect of KDPG accumulation is bacteriostatic, not bactericidal. One possible mechanism of the growth inhibition resulting from KDPG accumulation could be competitive inhibition of 6-phosphogluconate

dehydrogenase, the key enzyme for entry of carbon into the pentose phosphate pathway which is important for generation of several precursor metabolites, including ribose. In support of this hypothesis, addition of the biosynthetic end products generated from the precursor metabolites of the pentose phosphate pathway to the growth medium completely prevented the growth inhibition that normally results from accumulation of KDPG (data not shown).

G. Literature Cited

- Barnell, W. O., J. Liu, T. L. Hesman, M. C. O'Neill, and T. Conway. 1992. The *Zymomonas mobilis* *glf*, *zwf*, *edd*, and *glk* genes form an operon: Localization of the promoter and identification of a conserved sequence in the regulatory region. *J. Bacteriol.* **174**: 2816-2823.
- Bhattacharya, M., L. Fuhrman, A. Ingram, K. W. Nickerson, and T. Conway. 1995. Single-run separation and detection of multiple metabolic intermediates by anion exchange-HPLC and application to cell pool extracts prepared from *Escherichia coli*. (submitted)
- Conway, T., R. Fliege, D. Jones-Kilpatrick, J. Liu, W.O. Barnell, and S. E. Egan. 1991. Cloning, characterization, and expression of the *Zymomonas mobilis* *eda* gene that encodes 2- keto-3-deoxy-6-phosphogluconate aldolase of the Entner-Doudoroff pathway. *Molec. Microbiol.* **5**: 2901-2911.
- Hesman, T.L., W.O. Barnell and T. Conway. 1991. Cloning, characterization and sequence analysis of a phosphoglucose isomerase gene (*pgi*) from *Zymomonas mobilis* that is subject to carbon source-dependent regulation. *J. Bacteriol.* **173**: 3215-3223.
- Liu, J., W. O. Barnell, and T. Conway. 1992. The polycistronic mRNA of the *Zymomonas mobilis* *glf-zwf-edd-gl*k operon is subject to complex transcript processing. *J. Bacteriol.* **174**: 2824-2833.
- Mejia, J.P., M.E. Burnett, H. An, W.O. Barnell, K.F. Keshav, T. Conway and L.O. Ingram. 1992. Coordination of *Zymomonas mobilis* glycolytic and fermentative enzymes: A simple hypothesis based on mRNA stability. *J. Bacteriol.* **174**: 6438-6443.
- Parker, C. T., 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. *Molecular Microbiol.* **15**: 795-802.
- Snoep, J. L., N. Arfmann, L. P. Yomano, H. V. Westerhoff, T. Conway, and L. O. Ingram. 1994. Control of glycolytic flux in *Zymomonas mobilis* by gene products from the *glf-zwf-edd-gl*k operon. *Molecular Microbiol.* (submitted)
- Snoep, J. L., N. Arfman, L. P. Yomano, R. K. Fliege, T. Conway, and L. O. Ingram. 1994. Reconstitution of glucose uptake and phosphorylation in a glucose negative mutant of *Escherichia coli* by using the *Zymomonas mobilis* genes encoding the glucose facilitator protein and glucokinase. *J. Bacteriol.* **176**: 2133-2135.
- Zembrzuski, B., P. Chilco, S.-L. Liu, J. Liu, T. Conway, and R. K. Scopes. 1992. The fructokinase gene from *Zymomonas mobilis*: Cloning, sequencing, expression and structural comparison with other hexose kinases. *J. Bacteriol.* **174**: 3455-3460.