METABOLIC ENGINEERING OF CENTRAL CARBON METABOLISM IN *ESCHERICHIA COLI*: IMPROVING THE PRODUCTION OF BIOMASS AND METABOLITES

Thesis by

Neilay N. Dedhia

In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1995

(submitted October 24, 1994)

Acknowledgments

I want to express my love and devotion to my parents who have sacrificed so much in terms of personal comfort in raising me and my brothers, in providing me with a fine education and being always there for me.

I want to thank my advisor Prof. James Bailey for his guidance, gentle encouragement, and insightful talks on research philosophy and long-term goals. I have been inspired by his strong character and integrity, cheerful nature, and great communication skills.

Interaction with my friends and colleagues in the Bailey research group gave me a chance of appreciating the role of science in uniting individuals from diverse nations and cultures in a common bond. I thank Dr. Thomas Hottiger and Dr. Juan Diaz Ricci for providing me initial guidance in my research. Dr. Pauli Kallio was of immense help in trouble-shooting molecular biology techniques and I thank Dr. Wolfgang Minas for his help in setting up and repairing equipments. I thank Wilfred Chen and Vassily Hatzimanikatis for their collaborative help in my research. Ruizhen Chen, Wilfred Chen, Phil Tsai and Pablo Umaña were great friends and made the lab a fun place to work in. I thank Pablo Umaña for sharing his tremendous enthusiasm in life. I enjoyed our random musings on science and philosophy. I thank Ruizhen Chen for sharing with me the lonely moments in the third floor Bailey labs after the rest of the group departed for Zürich.

My room-mates for the last four years Ashish Bansal, Mohit Jain and Siddharth Valluri have been my constant companions in fun and play, joy and despair. I thank Ashish

I thank Chesterton for explaining to my logical scientific mind:

"The general fact is simple. Poetry is sane because it floats easily in an infinite sea; reason seeks to cross the infinite sea, and so make it finite. The result is mental exhaustion. To accept everything is an exercise, to understand everything a strain. The poet desires exaltation and expansion, a world to stretch himself in. The poet only asks to get his head into the heavens. It is the logician who tries to get the heavens into his head. And it is his head that splits."

Chesterton in "Orthodoxy"

Abstract

The pathway for central carbon metabolism provides precursors for cell biosynthesis and metabolite synthesis along with ATP and NADH. We investigated the metabolic engineering of one of the branches of the central carbon pathways: the pathway of glycogen synthesis and degradation. We were motivated in selecting the glycogen pathway for genetic manipulation by the literature on acetate production in E. coli. The literature indicates that in aerobic cultures the uptake of nutrients occurred faster than the utilization of the precursors, formed from the nutrients, in making biomass and energy. We decided to sequester the excess carbon in glycogen which is a storage polymer. We also devised vectors to degrade the sequestered glycogen. The effects, possible causes of the effects, and potential applications of the sequestering of carbon in the form of glycogen, sometimes combined with engineered degradation of the sequestered glycogen, have been the subject of this thesis.

This manipulation of the glycogen pathway yielded practically useful results. The metabolic engineering was done in an *Escherichia coli* mutant defective in acetate biosynthesis due to deletion of the *ack* (acetate kinase) and *pta* (phosphotransacetylase) genes. The sequestering of glycogen was achieved by transforming cells with a plasmid containing the glycogen biosynthesis genes *glg*C (encoding ADPG pyrophosphorylase) and *glg*A (encoding glycogen synthase) under the control of the IPTG-inducible *tac* promoter. If glycogen overproduction in the *ack pta* strain grown in complex medium was induced during late log-phase, biomass production increased by 15 - 20% relative to uninduced controls. When glycogen was sequestered and then degraded in *E. coli* cultures grown in minimal medium, by overamplifying the genes for glycogen synthesis and

degradation, then glutamate production was increased almost 3-fold compared to the plasmid-free strain.

When glycogen was sequestered, we observed changes in some of the secreted end-products. We observed that, after overproduction of glycogen, uptake of the previously secreted pyruvate was increased with respect to the control strain, and the CO_2 production rate was also increased. These dual observations suggest an increased activity of the gluconeogenic pathways or the TCA cycle. The increase in glutamate, when glycogen sequestering was combined with degradation, also indicate an increase in TCA flux.

Comparison of cAMP levels with and without glycogen overproduction indicate a higher level in cAMP after glycogen is overproduced. There appears to be a tentative link, though not conclusive, between cAMP synthesis and glycogen synthesis pathway. cAMP is a global regulator of central carbon metabolism including many genes of the TCA cycle enzymes. By affecting the TCA flux, cAMP may be one of the causes behind the pleiotropic effects of glycogen overproduction and degradation.

Table of Contents

Chapter 3   Coordinated Synthesis and Degradation of Glycogen as Tools to Manage Carbon Flow

Chapter 4   Redirection of Carbon Flux to Metabolites: Amplification of Glycogen Synthesis and Degradation Genes in *E. coli* Improves Glutamate Production

Chapter 5   Conclusions and future work

Appendix 1   Modifying a Promoter That is Activated Under Conditions of High Glycolytic Flux to Work in Aerobic Conditions.

x

Chapter 1

Introduction

1.1 Metabolic engineering

Cells of diverse living organisms possess approximately 2000 distinct metabolic reactions which are used to synthesize a large number of chemicals and structural endproducts. Using this vast network of metabolic reactions, a living cell can synthesize a new cell using simple starting chemicals like glucose and some salts. The products of the complex metabolic reactions of the living cell have been used since prehistoric times in the form of fermented dairy products and wine. The discovery of the penicillin compound made by a fungus and it's commercialization heralded the beginning of the modern biologically-based pharmaceutical industry.

The advent of recombinant DNA technology in the early 1970's led to the development of the modern biotechnology industry which enabled novel protein-based therapeutics to be produced by living bacterial cells, yeast cells, and mammalian cells. The availability of genetic engineering techniques also opened up the possibility of rationally altering the metabolic capacities of the living cell at the genetic level. This led to the beginning of the field of metabolic engineering. Several closely-related definitions exist in the literature for this branch of applied science which began to deal with the alteration of the chemical synthesizing capacity of the cell at the molecular genetic level. Bailey (1991) used the term metabolic engineering and defined it as "improvement of cellular activities by manipulation of enzymatic, transport, and regulatory functions of the cell with the use of recombinant DNA technology." Japanese researchers have favored the term "molecular breeding" defined as "breeding of new strains of organisms via direct manipulation of the genes at the molecular level." The pharmaceutical industry has used the term "rational strain development" as a step forward from the traditional term of "strain development"

commonly done through random mutagenesis of the strains and selection of the best mutant strains.

1.2 Examples of metabolic engineering

Metabolic engineering examples have been reviewed by Bailey, 1991 and Cameron and Tong, 1993. Through the purposeful modification of the metabolic capacities of living cells, metabolic engineering can help in:

1) Improving production of metabolites already produced by the living cell.

2) Producing compounds new to the host organisms.

3) Modifying useful overall cell properties and cell physiology.

4) Extending substrate range for growth and product formation by the living cell.

5) Adding new catabolic activities for degradation of toxic chemicals.

Prominent and illustrative examples from each of the above applications of metabolic engineering will be discussed next.

Metabolic engineering can be used to alter organisms so that they will overproduce practically any metabolic intermediate. Numerous metabolites have been overproduced by metabolic engineering including catabolic end-products like CO_2, H_2, acetic acid, ethanol,

butanol, acetone; a large number of antibiotics; various amino acids; polymers like xanthan gum, bacterial cellulose; and lipids. A metabolic intermediate can be overproduced by genetically amplifying an enzyme or a set of enzymes that will increase the flow of precursors into the desired pathway often combined by genetically blocking competing parallel pathways which utilize the same pool of precursors as the desired pathway.

An impressive example of metabolic engineering was the improvement of phenylalanine production (Backman et al. 1990). The researchers overexpressed all phenylalanine genes from *E. coli* , deleted a repressor protein, engineered a new promoter of *pheA*, genetically altered the strain to overcome feedback inhibition of chorismate mutase, used excision vector technology for the tyrosine biosynthetic gene and subjected the strain to selection pressure for resistance to toxic amino acid analogs. The engineered strain produced over 50 g/l of phenylalanine. During the implementation of this strategy, the researchers discovered that the overproduction of some of the enzymes in the phenylalanine pathway is detrimental to product formation. In principle, a theory known as metabolic control theory can be used to study the effects of changes in enzyme activities on changes in fluxes (Kacser and Burns, 1973; Heinrich et al. 1977). Although this theory has been used to model actual metabolic pathways (Galazzo and Bailey, 1990), the theory has not yet been used as a central tool in a rational metabolic engineering strategy. One of the constraints is the accuracy of the prediction depends heavily on accuracy of the kinetic rate expressions and the experimental data.

Amplification of enzymatic activity at a fork in a metabolic pathway has been used not only to augment the flow of precursors to the desired metabolite but also to divert metabolite flow away from undesired metabolites. High amounts of indole glucosinolates in oilseeds is considered commercially undesirable because it lowers the value of the

oilseed meal left after extraction of oil (Chavadez et al. 1994). Canola plants (*Brassica napus*) were transformed with a tryptophan decarboxylase gene from the plant *Catharantus roseus*. This metabolic engineering redirected tryptophan into tryptamine rather than into indole glucosinolates. Indole glusosinolate content of mature seeds from transgenic plants was only 3% of that found in nontransgenic seeds. These results demonstrate how the creation of artificial metabolic sinks could direct metabolite flow and be used to remove the undesirable glucosinolates. In this thesis we created a metabolic sink to direct metabolite flow away from undesired fermentation byproducts as will be explained later.

Metabolic engineering can be used to create chemicals new to the host organism. Examples in this area include the two classical examples of metabolic engineering: production of indigo (Ensley et al. 1983) and the vitamin C precursor, 2-keto-L-gluconic acid (2-KLG) (Anderson et al. 1985). Ensley et al. (1983) discovered that *E. coli* could synthesize indigo. The "indigo" operon containing the naphthalene dioxygenase gene from *Pseudomonas putida* and tryptophanase gene was transformed into *E. coli*. *E. coli* cells were able to convert tryptophan to cis-indole-2,3-dihydrodiol which rearranged to form indoxyl and, in the presence of air, was converted into indigo.

An exciting application of metabolic engineering in creating new chemicals has been the effort to create novel compounds with potential medicinal activity in *Streptomyces*. The "programming" of polyketide synthases to make hybrid complexes capable of catalyzing the synthesis of "hybrid" polyketide-derived carbon chains has enabled the creation of novel compounds (McDaniel et al. 1993; Kao et al. 1994). Polyketide synthases are multifunctional enzymes that catalyze the biosynthesis of a high variety of carbon chains differing in their length and patterns of cyclization. Researchers developed a *Streptomyces* host-vector system for efficient construction and expression of recombinant polyketide

synthases (McDaniel et al. 1993). Using this system several novel compounds were synthesized *in vivo* in significant quantities. Expression of the enzyme 6-deoxyerythronolide B (DEBS) in a heterologous host enabled the synthesis of substantial quantities of two new macrolactones (Kao et al. 1994). The biosynthetic strategy utilized a genetic approach that facilitated rapid structural manipulation of DEBS or other modular polyketide synthases. This approach will allow rational design of biosynthetic products and in the future lead to the generation of diverse polyketide libraries by means of combinatorial cloning of naturally occurring and mutant polyketide synthases.

Cell physiology and overall properties can be modified by metabolic engineering. Growth and product synthesis in many industrial high cell density fermentations are limited by oxygen supply. The aerobic bacterium *Vitreoscilla* lives in poorly aerated environments and synthesizes increased quantities of a hemoglobin molecule in oxygen-limited conditions. Researchers hypothesized that the hemoglobin molecule may play a beneficial role under oxygen-limited conditions in *Vitreoscilla* and decided to investigate whether the hemoglobin molecule may enhance the ability of other industrially useful micro-organisms to adapt to oxygen-limited conditions. Indeed, *Vitreoscilla* hemoglobin gene when expressed in *E. coli* improved the growth rate and product formation under oxygen-limiting conditions (Khosla and Bailey, 1988). This strategy suggests a general design principle for engineering better responses to environmental stresses which limit productivity in bioprocessing: after identifying organisms which have adapted to the stress in nature, the genes that control the stress response may be identified and cloned in the desired organism.

Metabolic engineering can be used to extend the substrate range that a host organism can use for growth and product formation. Efforts have been directed towards

engineering activities to enable the cells to use xylose, the primary pentose sugar in biomass. *Zymomonas mobilis*, a ethanologenic bacterium can use hexoses as substrates for growth but not pentoses like xylose. Metabolic engineering was used to attempt to construct a xylose catabolic pathway in *Zymomonas mobilis* (Feldman et al. 1992). Genes for xylose isomerase and xylulokinase of the xylose catabolic pathway from *Klebsiella pneumoniae* were cloned in *Zymomonas mobilis* but no growth on pentose as sole carbon source occurred. Xylitol phosphate accumulated due to the action of a novel enzyme and an unexpected side-reaction of the introduced xylulokinase enzyme on xylitol. After further iterations of metabolic engineering which included mutagenesis to reduce formation of xylitol phosphate, and amplification of the native transketolase activities by introducing the *tkt* gene from *E. coli*, *Zymomonas mobilis* cultures were able to mediate conversion of small amounts of xylose to CO_2 but no growth on xylose as sole carbon source was detected. Instead sedoheptulose 7-phosphate accumulated intracellularly. A study of the enzyme activities of the pentose phosphate pathway revealed that *Zymomonas mobilis* did not possess the transaldolase activity. This example illustrates the iterative cycle of genetic modification, analysis of the metabolic consequences of this change, and choice of the next genetic modification. In the above strategy, analysis of intracellular metabolite concentrations and better understanding of the biochemistry of the metabolic pathway enabled the choice of the next modification.

Metabolic engineering can be used to create new catabolic activities for biodegradation of toxic chemicals. A strategy which shows the mix-and-match capabilities of metabolic engineering to construct a hybrid organism was developed by Rojo et al. (1987) to enable a *Pseudomonas* strain to simultaneously degrade chloro aromatics and methyl aromatics. Critical enzymes from five different catabolic pathways of three distinct soil bacteria were combined into a functional ortho cleavage route for the degradation of

methyl phenols and methyl benzoates. An ortho cleavage pathway was constructed that consisted of five discrete segments combined from five genes from *Pseudomonas* TOL plasmid, one gene from *Alcaligenes eutrophus* and mutational activation of one chromosomal gene of the host strain. The presence of this pathway in *Pseudomonas* sp. B13 enabled the engineered bacterium to grow well on mixtures of chloro- and methyl-aromatics.

The metabolic reaction network of the cell is highly complex and coupled. There are chances of unanticipated responses to modifications of metabolic engineering. Accounting for such responses will require knowledge of the biochemistry of the involved pathways, the regulation of the pathways at the genetic and enzymatic level and the kinetics of the pathway.

In this thesis, the results represent three applications of metabolic engineering:

Improving the cell densities of cultures of the bacterium *E. coli* by overproduction of glycogen. (discussed in Chapter 2.)

Characterizing control networks to allow controlled synthesis and degradation of glycogen. (discussed in Chapter 3.)

Improving production of glutamate by amplification of the glycogen synthesis and degradation genes. (discussed in Chapter 4.)

1.3 Metabolic engineering to improve culture growth of *E. coli*:growth of *E. coli* and product formation is limited by production of acetate

The bacterium *E. coli* is the most well studied bacterium in the world in terms of it's physiology and genetics. Since the advent of recombinant DNA technology, *E. coli* has been the most popular host for expression of heterologous proteins. The emergence of the biotechnology industry in the early 1980's made *E. coli* an important industrial micro-organism. If a protein does not require post-translational modification like glycosylation, then *E. coli* is the preferred host for producing the protein. As a result, several commercial therapeutic proteins like insulin, human growth hormone, granulocyte macrophage colony stimulating factor are produced in *E. coli*. *E. coli* strains whose metabolic pathways have been altered by metabolic engineering and have been used to produce several metabolites like ethanol (Ingram et al. 1987), phenylalanine (Backman et al. 1990), indigo (Ensley et al. 1983; Mermod et al. 1986), and polyhydroxybutyrate (Peoples and Sinskey 1990). In all such applications of *E. coli* to make heterologous proteins and specialty chemicals, rapid growth of bacteria to the highest possible cell densities is important for maximizing process space-time yields and minimizing costs.

A fundamental problem encountered in growth of *E. coli* cultures is the secretion of acetate during rapid growth. Acetate is a strong inhibitor of *E. coli* growth and, when accumulated beyond a certain concentration, acts as an uncoupler of the membrane potential. Undissociated acetic acid can freely enter the cytoplasm of the cell and after entering, acetic acid can dissociate into acetate and H^+. The usual membrane potential maintains a higher concentration of H^+ outside the cell. As long as metabolic energy is available for the extrusion of protons via energy-dependent transport processes a ΔpH can

be maintained by the cells. The ΔpH collapses as soon as available energy generation by glycolysis drops below the values necessary for sufficient H^+ extrusion. Therefore beyond a certain critical concentration of acetate, the membrane potential across the cell will collapse and further growth will be inhibited. Further the production of acetate can consume a significant fraction of the glucose utilized, detracting from potential yields; it has been estimated that 15.5% of the glucose utilized is lost in acetate (Holms 1986).

The production of acetate by *E. coli* cultures has an effect on the amount of recombinant protein made by the cell. Meyer et al. (1984) have reported that high acetate concentrations, during cultivation of an interferon-producing *E. coli* strain, indicated that the interferon titer was low. The production of interferon was growth-associated and the decrease in interferon titer might be correlated to the reduced growth rate in the presence of acetate. Jensen and Carlsen (1990) showed that the production of recombinant human growth hormone in batch fermentations of *E. coli*, using a medium containing glucose, decreased due to the inhibitory effect of acetic acid. Acetate had an inhibiting effect on the growth of *E. coli* in concentrations greater than 6 g/L, but even at a concentration of 2.4 g/L a significant decrease in the specific production rate of human growth hormone was seen. Sun et al. (1993) showed that acetate was inhibitory to the growth of early induced *E. coli* cells and their expression of recombinant protein, transforming growth factor-alpha-Pseudomonas exotoxin 40 (TGFα-PE40). They showed that the inhibitory level was strain dependent. For *E. coli* JM109 expressing the recombinant protein, 3 g/L of total acetate in the medium decreased TGFalpha-PE40 production by 38.0%. Acetate was less inhibitory to *E. coli* RR1, and RR1 was not affected by adding 2 g/L of acetate. However, 6.7 g/L of total acetate in the medium, decreased TGFα-PE40 production by 21.2%. Thus these results also indicate that higher acetate concentration was associated with inhibition of the recombinant protein TGFα-PE40 expression in *E. coli* JM109. Koh et al. (1992) after

investigating the extent of acetate inhibition on growth rate of different host and recombinant *E. coli* K12 strains showed that acetate inhibition is more significant for recombinant cells than for host cells.

If acetate is harmful for *E. coli* growth, would evolutionary pressures have not eliminated the production of acetate? The sensitivity of *E. coli* is not incompatible with evolutionary selective pressures, since in their natural habitat which is the stomach and intestine of animals and humans, *E. coli* cultures would probably be found with other acetate-consuming organisms. In the natural environment, it is also unlikely that *E. coli* will accumulate in cell densities comparable to the levels achieved in modern bioreactors and thus the acetate concentrations in the vicinity of the cell will be much lower. This observation highlights an important heuristic for strain improvement in metabolic engineering. The natural genotype of a particular organism may not be the most ideal for bioprocess engineering applications where the environment is different and more controlled.

To tackle the problem of acetate production by *E. coli* cultures either by affecting the environment of the cell by using process control strategies in bioreactors or by metabolic engineering of *E. coli*, it is necessary to elucidate the factors which cause acetate to be produced by the cell.

1.4 Factors affecting acetate production

The acetate biosynthesis pathway is shown in Figure 1. The acetate biosynthesis pathway lies at the junction of the glycolytic pathway which converts glucose to acetyl CoA

and the TCA cycle which uses acetyl CoA as a substrate. Two reactions are involved in the synthesis of acetate from acetyl CoA. The two enzymes that catalyze this reaction are phosphotransacetylase, encoded by the *pta* gene, and acetate kinase, encoded by the *ack* gene. It has been speculated that the *E. coli* cultures produce acetate even when grown aerobically because the uptake of nutrients and the generation of biosynthetic precursors occur faster than the utilization of these precursors for the production of biomass and energy (Hollywood et al. 1976; Doelle et al. 1983; Reiling et al. 1985; El-Mansi et al. 1989).

Studies show that specific growth rate and glucose concentrations affect the start-point of acetate production (Hollywood et al. 1976; Zabriskie and Arcuri, 1986). These studies were carried out in defined and complex media in chemostats. Acetate production appeared at specific growth rates which exceeded 0.35 h^{-1} in defined media and 0.2 h^{-1} in complex media. With increasing dilution rates, the specific glucose uptake rate Q_g increased. The increase in Q_g values were associated with increases in glyceraldehyde 3-phosphate dehydrogenase and lactate dehydrogenase. All other enzymes of the glycolytic pathway and the pentose phosphate pathway tested were not affected. Therefore, the increase of these two enzymes may indicate an increasing use of the lower half of the glycolytic pathway or the use of the methyl glyoxal bypass to pyruvate. During the use of the methyl glyoxal bypass, there is no ATP production and it has been speculated that the primary function of the methyl-glyoxal bypass is to effect metabolic uncoupling under conditions in which the energy charge and the glycolytic flux rate is high. Taken as a whole, these observations are in line with the observations that the glucose uptake transport system through the PEP -phosphotransferase system sets the overall pace for glucose utilization and that the level of glycolytic enzymes do not play a role in regulating the glucose uptake (Doelle et al. 1983).

Hollywood *et al.*(1976) varied the glucose concentrations from 0.1 % to 0.5 % at dilution rate ranging from 0.1 hr^{-1} to 0.5 hr^{-1}. The start of acid production occurred at 0.3% glucose input in case of $\mu=0.1\ hr^{-1}$ and 0.2 hr^{-1}, but at 0.1% glucose concentration at $\mu=0.3\ hr^{-1}$ to 0.5 hr^{-1}. This indicates that glucose concentration also causes a switch from aerobic respiration to acid production. The start of repression coincided with the inhibition of the TCA cycle activity due to repression of α ketoglutarate dehydrogenase and isocitrate dehydrogenase. The respiratory enzyme succinate dehydrogenase and NADH dehydrogenase was also repressed while the glycolytic enzymes were not affected.

In *E. coli*, the formation of acid production occurs concomitant with oxidative phosphorylation unlike in yeast where there is a total switch from aerobic respiration to fermentation. The repression of NADH dehydrogenase, succinate dehydrogenase and, in the cases of increased specific growth rate, also of cytochrome d synthesis does not prevent oxidative phosphorylation. The increase in lactate dehydrogenase probably adds an additional entry point in the electron transport chain.

During aerobiosis, the direction of carbon flow through the predominant pentose phosphate shunt does not change, but the increased glyceraldehyde 3-phosphate dehydrogenase suggests an increase flow to pyruvate. This increased flow through the energy-yielding part of the glycolytic pathway may supply additional PEP for the PTS system. The increased accumulation of NADH may restrict carbon flow through the TCA cycle and switche pyruvate flow to acetate through the *pta-ack* pathway.

As a summary, acetate production occurs due to an imbalance between rate of the glucose uptake system and the dissimilatory pathway of the TCA cycle and the respiratory

pathway. At low specific growth and low glucose concentration, there is no flux constraint through the TCA cycle and the respiratory system and hence, no acetate production occurs. At high specific growth rate and also at high glucose concentrations, the flux through the TCA cycle becomes limiting, probably due to low activity of α ketoglutarate dehydrogenase. At still higher growth rates, the flux through the respiratory chain will become limiting.

1.5 Strategies to overcome the growth inhibition by acetate

In cultivation of *E. coli* cultures in commercial bioreactors, usually process control strategies are used to overcome the growth inhibition of acetate. The strategies are based on the principle that lower the growth rate and lower the glucose concentration, the acetate production rate will be lower and involve the use of low dilution rates in a chemostat or slow feeding of nutrients in fed-batch cultures (Meyer et al. 1984; Fiesko and Ritch, 1986; Zabriskie et al. 1986; Jensen and Carlsen, 1990).

The above process control approach may be complemented by metabolic engineering. The first approach involved the use of *E. coli* strains which were blocked in the acetate synthesis pathway. Such mutant strains produced a very low amount of acetate and grew to higher cell densities in fed-batch reactors (Bauer et al. 1990). However, these strains accumulated pyruvate as an unusual metabolic product (Bauer et al. 1990; Diaz-Ricci et al. 1991) which suggested metabolic imbalance. In this thesis, we have studied the metabolic engineering of the glycogen biosynthesis and degradation pathway in *E. coli* strains deficient in the acetate pathway as an approach to improve the cell densities of *E. coli* cultures (described in Chapter 2), to create and characterize a novel regulatory system

(described in Chapter 3), and to improve the production of glutamate metabolite (described in Chapter 4).

1.6 Background on physiology and regulation of glycogen production in *E. coli.*

In *E. coli*, glycogen is mainly produced only under stationary phase conditions. The rate of glycogen synthesis increases 2- to 5- fold on entry to stationary phase when growth ceases because of depletion of an nutrient such as nitrogen, or phosphate, or sulfur, or even because of an unfavorable pH for growth (reviewed by Preiss 1984; Preiss and Romeo, 1989).

This regulation of glycogen production occurs because of both enzymatic regulation and genetic regulation. The pathway from glucose to glycogen is shown in Figure 2. The genes encoding the enzymes for the biosynthesis of glycogen synthesis as well as glycogen degradation are organized in an operon (Preiss 1984). The genes involved are shown in Figure 3.

The regulation of glycogen production occurs at the level of ADPG synthesis, the first step in the pathway, catalyzed by ADPG pyrophosphorylase:

$$ATP + \alpha\text{-glucose-1-P} \rightarrow ADPG + PP_i$$
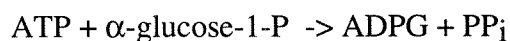
ADPG pyrophosphorylase is regulated by allosteric effectors. Fructose 1,6-biphosphate, a glycolytic cycle intermediate, acts as an activator and AMP acts as an inhibitor. It has been shown that genetic regulation of the glycogen operon plays an important role in the induction of the ADPG pyrophosphorylase and glycogen synthase.

Transcription of glgC and glgA is enhanced 26- and 10-fold respectively by cAMP-CRP complex. ppGpp, the regulatory factor for the global stringent response enhances transcription by 3.6- and 1.8 fold, respectively. The regulatory region has been located in a 250 base-pair region upstream to the glgC gene.

Interestingly, the location of the glycogen phosphorylase gene, which is involved in glycogen degradation is only 18 bp distal to the stop codon of the glgA (glycogen synthase) gene. This suggests that the expression of glgP (glycogen phosphorylase) may be coordinately regulated with that of glgC (ADPG pyrophosphorylase) and glgA (glycogen synthase) as part of a glycogen biosynthesis/catabolic operon. The relative activity of ADPG pyrophosphorylase(allosterically activated by fructose 1,6-biphosphate, inhibited by AMP) versus that of glycogen phosphorylase (inhibited by ADPG and activated by AMP) may thus determine whether glycogen is accumulated, as in early stationary phase, or degraded, as occurs later in stationary phase. However, the maximal rate of glycogen accumulation is several-fold higher than that of degradation and biosynthetic enzymes are 10- to 400-fold in excess of glycogen phosphorylase in glucose grown cells. There is considerable evidence that the regulation of net glycogen synthesis occurs primarily at the biosynthetic level (Romeo et al. 1988).

1.7 Scope of thesis

This thesis investigates the application and effects of metabolic engineering of glycogen synthesis and degradation.

Three applications have been investigated in this thesis. The first application studied the effect of amplification of the glycogen synthesis genes on cell growth. We found that this metabolic engineering strategy increased the cell growth by 20%.

The second application studied the manipulation of the glycogen synthesis and degradation genes in vectors with different control modes of temporal expression of the genes.

The third application studied the effect of metabolic engineering of glycogen synthesis and degradation on metabolite production. We showed that amplification of the glycogen synthesis and degradation genes improved the production of glutamate almost 3-fold as compared to a plasmid-free control strain.

1.8 References

Anderson S, Marks CB, Lazarus R, Miller J, Stafford K, Seymour J, Light D, Rastetter W, Estell D (1985) Production of 2-keto-L-gluconate, an intermediate in L-ascorbate synthesis by a genetically modified *Erwinia herbicola*. Science 230:144-149

Backman K, O'Connor MJ, Maruya A, Rudd E, McKay D, Balakrishnan R, Radjai M, DiPasquantinio V, Shoda D, Hatch R, Venkatasubramanian K (1990) Genetic engineering of metabolic pathways applied to the production of phenylalanine. Ann NY Acad of Sci 589:16-24

Bailey JE (1991) Toward a science of metabolic engineering. Science 252:1668-1675

Bauer KA, Benbassat A, Dawson M, Delapuente VT, Neway JO (1990) Improved expression of human interleukin 2 in high cell density fermenter cultures Of *Escherichia Coli* K-12 by a phosphotransacetylase mutant. Appl Environ Microbiol 56:1296-1302

Cameron DC, Tong IT (1993) Cellular and metabolic engineering: An overview. Appl Biochem Biotechnol 38:105-140

Chavadez S, Brisson N, McNeil JN, de Luca V (1994) Redirection of tryptophan leads to production of low indole glucosinolate canola. Proc Natl Acad Sci USA 91: 2166-2170

Diaz-Ricci JC, Regan LC, Bailey JE (1991) Effect of alteration of the acetic-acid synthesis pathway on the fermentation pattern of *Escherichia coli*. Biotechnol Bioeng 38:1318-1324

Doelle HW, Ewings KE, Hollywood NA(1983) Regulation of glucose metabolism in Bacterial Systems. Advances in Biochemical Engineering 23:1-35

El-Mansi EMT, Holms WH (1989) Control of carbon flux to acetate excretion during growth of *Escherichia coli* in batch and continuous cultures. J Gen Microbiol 135:2875-2883

Ensley BD, Ratzkin BJ, Osslund TD, Simon MJ, Wackett LP, Gibson DT (1983) Expression of naphthalene oxidation genes in *Escherichia coli* results in the biosynthesis of indigo. Science 222:167-169

Feldman SD, Sahm H, Sprenger GA (1992) Pentose metabolism in *Zymomonas mobilis* wild-type and recombinant strains. Appl Microbiol Biotechnol 38(3):354-361

Fiesko J, Ritch T (1986) Production of human alpha consensus interferon in recombinant *Escherichia coli*. Chem Eng Comm 45:229-240

Galazzo JL, Bailey JE (1990) Fermentation pathway kinetics and metabolic flux control in suspended and immobilized *Saccharomyces cerevisiae*. Enzyme Microb Technol. 12:162-172

Heinrich R, Rapoport SM, Rapoport TA (1977) Prog Biophys Molec Biol 32:1-82

Hollywood N, Doelle HW (1976) Effect of specific growth rate and glucose concentration on growth and glucose metabolism of *Escherichia Coli* K-12. Microbios 17:23-33

Holms WH (1986) The central metabolic pathways in *Escherichia coli*: Relationship between flux and control at a branch point, efficiency of conversion to biomass and excretion to acetate. Current Topics in Cellular Regulation 28:69-105

Ingram LO, Conway T, Clark DP, Sewell GW, Preston JF (1987) Genetic engineering of ethanol production in *Escherichia coli*. Appl Environ Microbiol 53:2420-2425

Jensen EB, Carlsen S (1990) Production of recombinant human growth hormone in *Escherichia coli* Expression of different precursors and physiological effects of glucose, acetate, and salts. Biotechnol Bioeng 36:1-11

Kacser H, Burns JA (1973) Symp Soc Exp Biol 27:65-104

Kao CM, Katz L, Khosla C (1994) Engineered biosynthesis of a complete macrolactone in a heterologous host. Science 265:509-512

Khosla C, Bailey JE (1988) Heterologous expression of a bacterial hemoglobin improves the growth properties of recombinant *Escherichia coli*. Nature 331:633-635

Koh BT, Nakashimada U, Pfeiffer M, Yap MGS (1992) Comparison of acetate inhibition on growth of host and recombinant *Escherichia coli* K12 strains. Biotechnol Letters 14:1115-1118

Mcdaniel R, Ebert Khosla S, Hopwood DA, Khosla C (1993) Engineered biosynthesis of novel polyketides. Science 262:1546-1550

Mermod N, Harayama S, Timmis KN (1986) New route to bacterial production of indigo. Biotechnology 4:321-324

Meyer HP, Leist C, Fiechter A (1984) Acetate formation in continuous culture of *Escherichia coli* K12-D1 on defined and complex media. Biotechnology 1: 355-358 (1984)

Peoples OP Sinskey AJ (1990) In: Dawes EA (ed) Novel biodegradable microbial polymers, Kluwer Academic Publishers, Netherlands, pp 191-202

Preiss J (1984) Bacterial glycogen synthesis and its regulation. Annu Rev Microbiol 38:419-458

Reiling HE, Laurila H, Fiechter A (1985) Mass culture of *Escherichia coli*: Medium development for low and high density cultivation of *Escherichia coli* B/r in minimal and complex media. J Biotechnol 2:191-206

Romeo T, Kumar A, Preiss J (1988) Analysis of the *Escherichia coli* glycogen gene cluster suggests that catabolic enzymes are encoded among the biosynthetic genes. Gene 70: 363-376

Romeo T, Preiss J (1989) Physiology, biochemistry and genetics of bacterial glycogen synthesis. Adv Microbial Physiol 30:1183-1238

Sun WU, Lee C, George HA, Powell AL, Dahlgren ME, Greasham R, Park CH (1993) Acetate inhibition on growth of recombinant *Escherichia coli* and expresson of fusion protein TGFα-PF40. Biotech Letters 15: 809-814

Zabriskie DW, Arcuri EJ (1986) Factors affecting productivity employing recombinant organisms. Enz Microbiol Technol 8:705-776

1.9 Figure captions

Figure 1: Acetate biosynthesis pathway in *E. coli.*

Figure 2: Glycogen biosynthesis pathway in *E. coli.*

Figure 3: Genetic organization of the glycogen biosyntheis/degradation operon in *E. coli.*

figure 1
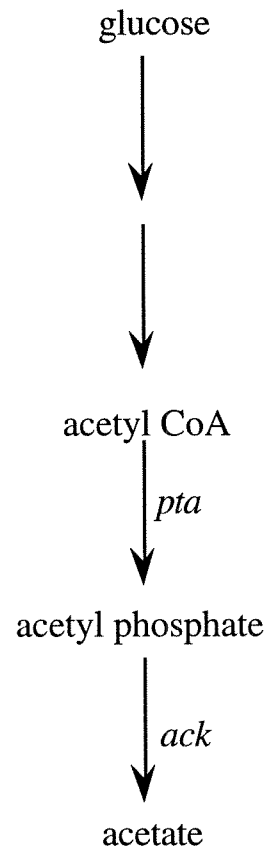
Acetate Synthesis Pathway In *E. coli*
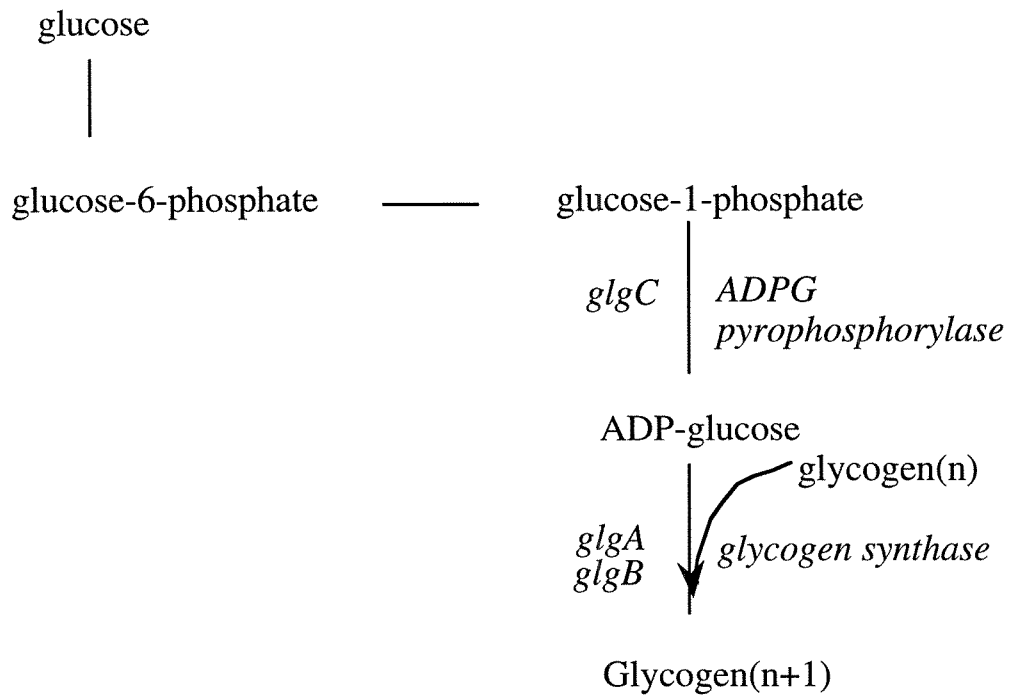
figure 2

Pathway of Glycogen Production in E. coli

figure 3

glg B   glg X   glg C   glg A   glg P   13.6 kb
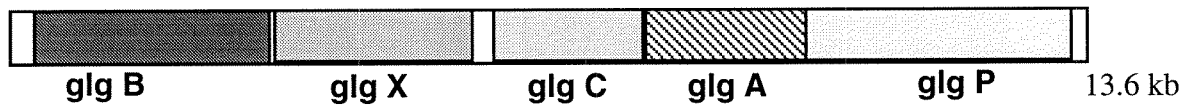
glg B, branching enzyme;
glg X, function unknown;
glg C, ADPG pyrophosphorylase;
glgA, glycogen synthase;
glg P, glycogen phosphorylase

Genetic Organization of the Glycogen Biosynthesis/Degradation Operon

Chapter 2

Overproduction of Glycogen in *Escherichia coli* Blocked in the Acetate Pathway Improves Cell Growth

The initial experiments in this Chapter (described in Figure 2 and Figure 3) were conducted in collaboration with Dr. Thomas Hottiger.

2.1 Abstract

Excessive production of acetate is a problem frequently encountered in aerobic high cell-density fermentations of *Escherichia coli*. Here, we have examined genetic alterations resulting in glycogen overproduction as a possible means to direct the flux of carbon away from the acetate pool. Glycogen overaccumulation was achieved either by using a regulatory *glgQ* mutation or by transforming cells with a plasmid containing the glycogen biosynthesis genes *glg*C (encoding ADPG pyrophosphorylase) and *glg*A (encoding glycogen synthase) under their native promoter. Both strategies resulted in an approximately 5-fold increase in glycogen levels but had no significant effect on acetate excretion. The *glg*C and *glg*A genes were then placed under the control of the IPTG-inducible *tac* promoter, and this construct was used to stimulate glycogen production in a mutant defective in acetate biosynthesis due to deletion of the *ack* (acetate kinase) and *pta* (phosphotransacetylase) genes. If glycogen overproduction in the *ack pta* strain was induced during late log-phase, biomass production increased by 15 - 20% relative to uninduced controls. Glycogen overaccumulation had a significant influence on carbon partitioning: The output of carbon dioxide peaked earlier than in the control strain, and the levels of an unusual fermentation byproduct, pyruvate, were reduced. Exogenous pyruvate was metabolized more rapidly, suggesting higher activity of gluconeogenesis or the TCA cycle as a result of glycogen overproduction. Potential meachnisms of the observed metabolic alterations are discussed. Our results suggest that *ack pta* mutants overproducing glycogen may be a suitable starting point for constructing *Escherichia coli* strains with improved characteristics in high cell densitiy fermentations.

2.2 Introduction

The bacterium *Escherichia coli* is among the most widely used hosts for the expression of heterologous proteins. Its popularity is due to many advantageous characteristics such as the availability of established protocols for genetic manipulation, fast growth on relatively inexpensive media, and well-known behaviour in large-scale fermentations. On the negative side, a fundamental problem encountered in work with this organism is its strong tendency to excrete acetic acid during rapid growth.[2, 9, 14, 17, 23, 31] Acetate is a potent inhibitor of *E. coli* proliferation[17] and, when accumulated beyond a certain concentration, acts as an uncoupler.[4]

Acetate biosynthesis in *E. coli* occurs in two steps.[3, 10, 16] First, acetyl-CoA is converted to acetyl phosphate by phosphotransacetylase, the product of the *pta* gene. Second, acetyl phosphate reacts with ADP to yield acetate and ATP in a process catalyzed by acetate kinase (encoded by the *ack* gene). While acetate biosynthesis provides an important means for ATP synthesis during anaerobic growth, [30] it is not clear why *E. coli* produces acetate even when cultivated aerobically. Apparently in aerobic cultures the uptake of nutrients and the generation of biosynthetic precursors occur faster than the utilization of these precursors for the production of biomass and energy.[1, 6, 7, 12, 18, 23] To reduce acetate excretion, the focus has been on developing optimum feeding strategies. These involve use of low dilution rates in a chemostat or slow feeding of nutrients in fed-batch cultures.[8, 20, 23, 31] Because the rate of biomass production depends directly on the feeding rate, the space-time efficiency of the process is limited by the need to avoid acetate accumulation. This problem might be solved by the use of *E. coli* mutants that do not

produce acetate even during growth in rich medium. The most obvious way to achieve this goal is construction of strains blocked in the acetate biosynthetic pathway, specifically in the acetate kinase (*ack*) and / or phospho-transacetylase (*pta*) genes.[2, 5] Indeed, such strains have been demonstrated to grow to higher cell densities in fed-batch fermentations[2] and, concomitantly, to produce increased amounts of product. However, the maximal growth rate was found to be lower than that of wild-type cells[5] and, in addition, pyruvate accumulated as an unusual by-product,[2, 5] indicating metabolic imbalance.

In cells growing under conditions of carbon input exceeding the capacity of the central pathways, metabolism is balanced in either of the following ways ([7] and references therein):

i) Conversion of metabolic intermediates in excess of requirements to (storage) poymers;

ii) uncoupling of substrate oxidation and energy generation. Excess carbon can then be dissipated as carbon dioxide and excess energy as heat;

iii) excretion of low molecular weight compounds to the medium.

As these metabolic strategies are complementary, it should be possible to eliminate acetate production (an example of strategy iii), for example, by directing carbon flux to a storage polymer (= strategy i). The glycogen pool appeared feasible for sequestering excess carbon, since glycogen is non-toxic and osmotically inactive. Therefore the polymer can accumulate to very high concentrations without adverse effects on cell viability. Glycogen biosynthesis in *E. coli* requires the activities of ADPG pyrophosphorylase, glycogen synthase, and the branching enzyme (encoded by the *glg*C, *glg*A and *glg*B genes, respectively; reviewed in [22]). The *glg*C and *glg*A genes along with the glycogen degradation gene *glg*P are arranged in an operon.[24] In addition to the glycogen

biosynthetic genes, a regulatory gene (*glgQ*) has been isolated[22] that controls the expression levels of the *glg*A, *glg*B and *glg*C gene products.[26]

In this study, we have examined the effects of glycogen overproduction (achieved either by using a *glgQ* mutant or by overexpressing the *glg*A and *glg*C genes from a high copy number plasmid) on acetate excretion and growth characteristics of *E. coli*. Glycogen overaccumulation was induced both in a wild-type background and in a mutant strain blocked in acetate biosynthesis. We show that, while glycogen overproduction in wild-type *E. coli* did not significantly reduce acetate excretion, accumulation of glycogen in an acetate kinase / phospho-transacetylase double mutant virtually eliminated the accumulation of acetate and, at the same time, resulted in higher cell yields and decreased excretion of pyruvate. Therefore, this strategy may potentially be used to create *E. coli* strains with improved performance in high cell-density fermentations.

2.3 Materials and methods

2.3.1 Bacterial strains and plasmids

The following *Escherichia coli* strains were used in this study (the relevant genotypes are given in brackets): *E. coli* B (wild type); AC70RI (*E. coli* B, *glgQ*, see [22]); DH5α (wild type *E. coli* K12); TA3476 (*E. coli* K12 *hisΔ* (*pta-ack-dhuA-hisJ-hisQ-hisP*[16]). Plasmid pPR2[25] was kindly donated to us by Dr. T. Romeo, Texas College of Osteopathic Medicine, Houston, Texas. It is a pUC19 derivative containing the glycogen biosynthesis genes *glg*C and *glg*A under their native promoter. Plasmid pGT100 was constructed by ligating a 2.9 kb *Eco*RI fragment from pPR2 (made blunt-ended by

treatment with Klenow enzyme) into *Sma*I-digested pKVQ4;[28] see reference[27] for detailed protocols). Proper orientation of the insert was checked by digestion with appropriate restriction enzymes.

2.3.2 Media and cultivation

Buffered LB medium was used in all experiments. The medium contained (g / L): Tryptone (10), yeast extract (5), NaCl (5), KH_2PO_4 (3), and K_2HPO_4 (1). Where indicated, glucose was added to a final concentration of 0.2%. Ampicillin (50 mg/l) was supplied during experiments involving plasmid-bearing strains.

Shake flask cultivations were carried out in an orbital shaker at 275 rpm and 37°C. In some experiments, cells were grown in a BioFlo III bench top fermentor (New Brunswick Scientific, Edison, NJ, USA), using 2.5 L of medium and the following cultivation conditions: temperature, 37°C; impeller speed, 400 rpm; air flow rate, 2.0 L/min. The pH was controlled at 7.0 with 2N NaOH / 2N HCl. Foaming was prevented by the addition of Medical Antifoam AF solution (Dow Corning, Midland, MI, USA). Carbon dioxide in the off-gas stream was measured using an infrared analyzer (model 702-075, Infrared Industries, Santa Barbara, CA, USA) connected to an IBM-XT personal computer appropriately equipped for data-logging.

Inoculation of cultures was as follows: A single colony from a freshly streaked out LB-plate was inoculated into 4 ml of buffered LB medium. After ca. 8h, the first stage culture was diluted 200-fold into 50 ml of buffered LB. The second stage culture was allowed to grow overnight and used to inoculate the main culture in fermentor or shake

flasks. The amount of inoculum was adjusted to give a starting OD_{600} (= optical density at 600 nm) of 0.1.

2.3.3 Analytical Methods

Glycogen was assayed essentially as described in reference[11] Pyruvate was determined by HPLC[5], and the results were confirmed by enzymatic analysis, using a SIGMA diagnostic kit. Glucose was measured enzymatically, and acetate was analyzed by GC[13]. The method of Peterson[21] was used for protein assays. SDS polyacrylamide gel electrophoresis was performed as described in Ref. 27.

2.4 Results

In a first experiment, we compared the fermentation characteristics of wild-type *E. coli* B and an *E. coli* B mutant, AC70RI, that harbors a *trans*-acting *glgQ* mutation causing overproduction of glycogen biosynthetic enzymes.[22, 26] Cultures were grown in shake flasks on buffered LB-medium (see Material and Methods) or on the same medium supplemented with 0.2% glucose. As shown in Fig. 2A, the growth curves of *glgQ* cultures were comparable to those of the wild-type, but the mutant cells accumulated 5 - 10 times more glycogen than wild-type controls (Fig. 2B). Both wild-type and mutant had a higher capacity to store glycogen when the medium was supplemented with glucose (Fig. 1B). Despite its higher glycogen content, the *glgQ* mutant did not produce less acetate. However, on LB without glucose, the *glgQ* cells were found to consume the accumulated acetate somewhat more rapidly (Fig. 2C). Both wild-type and *glgQ* mutant accumulated significantly more acetate when growing in the presence of glucose, which fits the idea that acetate production is due to overload of central metabolic pathways.

We hypothesized that our failure to detect any significant effect of glycogen overproduction on acetate excretion might be due to the fact that the *glgQ* cells did not produce enough glycogen to efficiently divert carbon away from glycolysis. Indeed, glycogen concentrations in the experiment shown in Fig. 2 were only ca. 0.1 g / L, whereas roughly tenfold more acetate (ca. 1 g / L) was excreted. We therefore tried to increase glycogen accumulation by transforming the *E. coli* strain DH5α with plasmid pPR2[25] containing the *glgA* and *glgB* glycogen biosynthetic genes under their native promoter. During growth on buffered LB supplemented with 0.2% glucose, DH5α cells

transformed with pPR2 grew at about the same rate as controls but accumulated roughly 4-fold more glycogen (Figs. 3A, B). Peak glycogen concentrations of DH5α/pPR2 were slightly higher than those observed with the *glgQ* strain, AC70RI (Figs. 2, 3). However, acetate levels still were not appreciably reduced relative to the control, although acetate accumulation was delayed (Fig. 3C). If glucose was omitted from the medium, both DH5α/pPR2 and DH5α/pUC19 produced considerably less glycogen. Surprisingly, cells harboring pPR2 grew much more slowly on this medium than controls (Fig. 3A). Most likely, this indicates shortage of some biosynthetic precursor(s). Again, glycogen overproduction did not result in a significant decrease in acetate levels, although the kinetics of acetate excretion differed substantially from those in the control culture (Fig. 3C).

The above data demonstrate that the conversion of metabolic intermediates to the storage polymer, glycogen, is insufficient as a means to reduce acetate excretion. We therefore proceeded to study whether a combination of glycogen overproduction with mutations in the acetate biosynthetic pathway might prove a more appropriate strategy. We hypothesized that the putative metabolic sink created by glycogen overproduction might balance the excess of metabolic intermediates (evidenced by pyruvate excretion[5]) in acetate⁻ mutants. Since plasmid pPR2 contained the glycogen biosynthesis genes under their native promoter and therefore did not allow their regulated expression, we constructed plasmid pGT100 with *glgC* and *glgA* under the control of the inducible *tac* promoter. pGT100 was transformed into strain TA3476 (see [16]) mutated in the *ack* and *pta* genes. In a first experiment, TA3476/pGT100 was grown to late exponential phase on LB medium with 0.2% glucose; then, the *tac* promoter was induced by the addition of various concentrations of IPTG, and the time trajectories of glycogen accumulation were determined. As shown in Fig. 4, glycogen levels were approximately 3-fold higher in the presence of IPTG than in the absence of the inducer. IPTG concentrations as low as 10µM clearly stimulated

glycogen biosynthesis, while 100μM - 1mM IPTG gave maximal levels of the polymer (Fig. 4). In all following experiments, 500μM IPTG was used to ensure full induction of the *tac* promoter. SDS-PAGE analysis indicated that glycogen synthase, the product of the *glg*A gene, appeared within 20 min of IPTG addition (Fig. 5).

We proceded to analyze growth, glycogen production, glucose consumption and byproduct formation of strain TA3476/pGT100 supplemented with either 0 μM or 500 μM IPTG. Since the glycogen levels of TA3476/pGT100 cultures were clearly elevated even in the absence of IPTG, TA3476 cells transformed with pKQ4, the parental vector of pGT100, were used as an additional control. Cultivations were carried out in a bench-top fermentor at constant pH, temperature and impeller speed. Cells were inoculated into buffered LB supplemented with 0.2% glucose, and IPTG (500 μM) was added to induce the *tac* promoter after 3.5h (i.e., in late log-phase). TA3476/pGT100 cells induced with IPTG (and therefore overproducing glycogen, Fig. 6B) grew to a 15 - 20% higher density than both uninduced TA3476/pGT100 cultures and cells harboring the control plasmid pKQV4 (Fig. 6A). Since the growth characteristics of TA3476/pKQV4 were comparable in the presence or absence of IPTG (Fig. 6A), this difference is not due to some non-specific effect of IPTG but reflects the induction of glycogen accumulation. This conclusion is supported by the finding that in cultures treated with various concentrations of IPTG a positive correlation was obtained between the levels of stored glycogen and biomass (protein, see Table 1). Most of the extra-biomass formed as a consequence of glycogen overproduction was synthesized after the exhaustion of glucose from the medium (Figs. 6A, C), suggesting more efficient utilization of non-glucose substrates by TA3476/pGT100 than by controls. Thus, glycogen overaccumulation appeared to enable strain TA3476/pGT100 to maintain a higher metabolic activity during the transition from exponential growth to stationary phase. To further substantiate this hypothesis, we

measured the output of CO_2 during cultivations of TA3476/pGT100 and TA3476/pKQV4, the control with the lowest glycogen content. As shown in Fig. 7B, the total CO_2-output of the two strains did not differ much. However, the specific CO_2 production rate of TA3476/pGT100 increased earlier than that of TA3476/pKQV4 and reached a maximum during the time period when the difference in growth rate relative to the control was most pronounced (Fig. 7A). This supports our interpretation that glycogen overproduction in some way enabled TA3476/pGT100 cells to maintain a higher metabolic shortly before and after the exhaustion of glucose from the medium.

The fermentation by-products other than CO_2 in cultures of IPTG-induced TA3476/pGT100 and controls were analyzed. While none of the strains produced significant amounts of acetate (data not shown; compare reference[5]), considerably lower amounts of pyruvate accumulated in the medium of IPTG-treated TA3476/pGT100 cultures than in that of controls (Fig. 6D). With all strains, pyruvate levels increased only until glucose was exhausted from the medium (Figs. 6C, D), indicating pyruvate production is due to overload of central metabolic pathways. The lower amounts of pyruvate excreted by TA3476/pGT100 cells could be a consequence of either a lower rate of pyruvate synthesis or a higher rate of pyruvate consumption than in the controls. To test whether TA3476/pGT100 was able to metabolize pyruvate more efficiently than the other strains, TA3476/pGT100 cells were cultivated as above (Fig. 6), and a pyruvate pulse was added 15 min after induction of the *tac*-promoter. The pulse dose was chosen such as to give a pyruvate concentration comparable to that maximally reached in the fermentation involving strain TA3476/pKQV4 (excreting the highest amounts of pyruvate). As shown in Fig. 8A, glucose-depleted TA3476/pGT100 cells were found to have a much higher capacity to utilize pyruvate than TA3476/pKQV4 controls. It should be noted that the final density of the TA3476/pGT100 culture in this experiment (as assessed by protein measurements, Fig.

8B) was comparable to that in Fig. 6A. This excludes the possibility that the inferior growth of the control strains was due to toxic effects of the pyruvate accumulation in the experiment shown in Fig. 6.

2.5 Discussion

In this study we have addressed the question whether it was possible by appropriate genetic alterations to design an *E. coli* strain exhibiting favorable growth characteristics yet producing little or no acetate during aerobic cultivation. As an initial step towards this aim, wild-type *E. coli* cells were engineered to overproduce the storage carbohydrate, glycogen. We reasoned that glycogen biosynthesis might reduce carbon flux through the glycolytic pathway, thereby lowering the concentration of metabolic intermediates available for acetate production (see also Introduction). Glycogen overaccumulation was achieved either by using a mutant defective in the regulatory *glgQ* gene,[22] or by transforming wild-type *E. coli* with a high copy plasmid containing the glycogen biosynthesis genes, *glgC* and *glgA* under their native promoter. Although both approaches resulted in cells with significantly elevated glycogen levels, acetate excretion was not appreciably reduced (Figs. 2 and 3). While, in principle, this might be due to the fact that the carbon demand for glycogen synthesis was too low to have significant effects on the intracellular concentration of acetate precursors, this interpretation is difficult to reconcile with our finding that *E. coli* K-12 cells overproducing the *glgA* and *glgC* grew very slowly on LB-medium without glucose (Fig. 3). As this slow growth most likely reflects shortage of some biosynthetic precursor(s) (presumably some metabolite usually provided by glucose catabolism), we assume that glycogen overproduction considerably reduced the levels of at least some metabolic intermediates. Since the strategy nevertheless failed to reduce acetate excretion, it was not investigated any further.

In an alternative approach, we tried combining glycogen overproduction (achieved by overexpression of the *glg*C and *glg*A genes) with mutations in the *ack* and *pta* genes. We hypothesized that the putative excess of biosynthetic precursors resulting from the block in the acetate pathway might be compensated by an increased demand for such precursors in glycogen biosynthesis. Indeed, glycogen hyperaccumulation reduced pyruvate excretion that usually occurs when acetate biosynthesis is inhibited (Fig. 6). However, more importantly, *ack pta* cells expressing the *glg*A and *glg*C genes from a high copy number vector were able to grow to significantly higher cell densities than controls (Fig. 6).

Why did glycogen overproduction increase final cell yields? During early and mid log-phase growth on LB-medium supplemented with glucose, carbon is metabolized primarily by glycolysis, whereas at later stages of growth gluconeogenesis becomes predominant. In our experiments, induction of glycogen accumulation occurred at a time point when cells are supposed to start switching from primarily glycolytic to primarily gluconeogenic metabolism. We speculate that, during gluconeogenic growth, the metabolic sink created as a result of glycogen biosynthesis may promote the assimilaton of non-glucose carbon sources. Thus, induced TA3476/pGT100 cells possibly were able to make use of either a fermentation by-product, or, alternatively, of a non-glucose carbon source in LB that the control cultures were unable to utilize. This interpretation is consistent with the finding that TA3476/pGT100 cultures metabolized at least one such carbon source, namely pyruvate, more rapidly than controls (Figs. 6, 8). Since pyruvate utilization occurred during a phase of active glycogen synthesis, pyruvate probably entered the gluconeogenic pathways. Thus, induction of glycogen overaccumulation in TA3476/pGT100 cells may stimulate gluconeogenesis. This again suggests that the glycogen-overproducing cells were able to assimilate some substrate that did not promote

the growth of the control strains. However, TA3476/pGT100 not only showed faster pyruvate net uptake than controls but also produced more carbon dioxide at about the time when glucose in the medium is exhausted. This possibly points to higher activity of the TCA cycle.

However, we do not so far have any direct evidence in support of either possibility. Therefore, an additional, somewhat more speculative explanation for the unusually high biomass produced by TA3476/pGT100 cultures is offered next. It has long been noted that there are situations in which production of energy in microbial cells is obviously uncoupled from growth (reviewed in Ref 29). While this is most evident for cultures deprived of nutrients other than the energy source, it might also be true during normal growth: According to estimations by Lagunas,[15] only about 40% of the ATP produced by yeast cells is utilized for known metabolic functions, while the rest is spent for unidentified purposes. Similar discrepancies have been reported for *E. coli*.[29] We speculate that glycogen overproduction in an acetate⁻ strain might lead to more tight coupling between growth and energy production. The observation of a more rapid uptake of pyruvate in glycogen overaccumulating cells fits this idea. Pyruvate uptake in *E. coli* occurs by symport with H^+.[19] and more rapid uptake could reflect a higher membrane potential.

While the interpretation of our data is complex, we feel that they may nevertheless be of immediate interest to the practitioner: *E. coli* strains designed in a way similar to TA3476/GT100 could be attractive for industrial high cell-density fermentations, e.g. in the production of heterologous proteins. More work will be required to show whether strains similar to TA3476/pGT100 could be profitably used not only in a batch but also in a fed-batch situation.

2.6 Acknowledgements

Dr. Hottiger's help in analyzing the data and preparing the manuscript is gratefully acknowledged.

2.7 References

1. Andersen, K. B., von Meyenburg, K. 1980. Are growth rates of *Escherichia coli* in batch cultures limited by respiration? J. Bacteriol. **144:** 114-123.

2. Bauer, K. A., Ben Bassat, A., Dawson, M., de la Puente, V., Neway, J. O. 1990. Improved expression of human interleukin-2 in high-cell-densitiy fermentor cultures of *Escherichia coli* K-12 by a phosphotransacteylase mutant. Appl. Environ. Microbiol. **56:** 1296-1302.

3. Brown, T. D. K., Jones-Mortimer, M. C., Kornberg, H. L. 1977. The enzymic interconversions of acetate and acetyl-coenzyme A in *Escherichia coli*. J. Gen. Microbiol. **102:** 327-336.

4. Chen, R., Bailey, J. E. 1993. Observations of aerobic, growing *Escherichia coli* metabolism using an on-line nuclear magnetic resonance spectroscopy system. Biotechnol. Bioeng. **142:** 215-221.

5. Diaz-Ricci, J. C., Regan, L. C., Bailey, J. E. 1991. Effect of alteration of the acetic-acid synthesis pathway on the fermentation pattern of *Escherichia coli*. Biotechnol. Bioeng. **38:** 1318-1324.

6. Doelle, H. W., Ewings, K. R., Hollywood, N. A. 1983. Regulation of glucose metabolism in bacterial systems. Advances in Biochemical Engineering **23:** 1-35.

7.   El-Mansi, E. M. T., Holms, W. H. 1989. Control of carbon flux to acetate excretion during growth of *Escherichia coli* in batch and continuous cultures. J. Gen. Microbiol. **135:** 2875-2883.

8.   Fiesko, J., Ritch, T. 1986. Production of human alpha consensus interferon in recombinant *Escherichia coli.* Chem. Eng. Commun. **45:** 229-240.

9.   Gottschalk, G. 1985. Bacterial metabolism. 2nd edition. Springer, New York.

10.   Guest, J. R. 1979. Anaerobic growth of *Escherichia coli* K12 with fumarate as terminal electron acceptor. Genetic studies with menaquinone and fluoroacetate-resistant mutants. J. Gen. Microbiol. **115:** 259-271.

11.   Gunja-Smith, Z., Patil, N. B., Smith, E. E. 1977. Two pools of glycogen in *Saccharomyces.* J. Bacteriol. **130:** 818-825.

12.   Hollywood, N. W., Doelle, H. W. 1976. Effect of specific growth rate and glucose concentration on growth and glucose metabolism of *Escherichia coli* K12. Microbios **17:** 23-33.

13.   Hottiger, T., Bailey, J. E. 1991. Neither total culture fluorescence nor intracellular fluorescence are indicative of NAP(P)H levels in *Escherichia coli* MG1655. Appl. Microbiol. Biotechnol. **36:** 400-403.

14. Jensen, E. B., Carlsen, S. 1990. Production of recombinant growth hormone in *Escherichia coli*: Expression of different precursors and physiological effects of glucose, acetate, and salts. Biotechnol. Bioeng. **36:** 1-11.

15. Lagunas, R. 1976. Energy metabolism of *Saccharomyces cerevisiae*: Discrepancy between ATP balance and known metabolic functions. Biochim. Biophys. Acta **440:** 661-674.

16. LeVine, S. A., Ardhesir, F., Ames G. F. A. 1980. Isolation and characterization of acetate kinase and phosphotransacetylase mutants of *Escherichia coli* and *Salmonella typhimurium*. J. Bacteriol. **143:** 1081-1085.

17. Luli, G. W., Strohl, W. R. 1990. Comparison of growth, acetate production, and acetate inhibition of *Escherichia coli* strains in batch and fed batch fermentations. Appl. Environ. Microbiol. **56:** 1004-1011.

18. Majewski, R. A., Domach, M. M. 1990. Simple constrained optimization view of acetate overflow in *Escherichia coli*. Biotechnol. Bioeng. **35:** 732-738.

19 Maloney, P. C. 1987. Coupling to an energized membrane: Role of ion-motive gradients in the transduction of metabolic energy. p. 222 - 243. In: Neidhardt, F. C., Ingraham, J. L., Low, K. B., Magasanik, M., Schaechter, M., Umbarger, H. E. (ed.), *Escherichia coli* and *Salmonella typhimurium*. Cellular and molecular biology. American Society for Microbiology, Washington, D.C.

20. Meyer, H.-P., Leist, C., Fiechter, A. 1984. Acetate formation in continuous culture of *Escherichia coli* K12 D1 on defined and complex media. J. Biotechnol. **1:** 355-358.

21. Peterson, G. L. 1977. A simplification of the protein assay method by Lowry et al., which is more generally applicable. Anal. Biochem **83:** 346-356.

22. Preiss, J. 1984. Bacterial glycogen synthesis and its regulation. Annu. Rev. Microbiol. **38:** 419-458.

23. Reiling, H. E., Laurila, H., Fiechter, A. 1985. Mass culture of *Escherichia coli*: Medium development for low and high density cultivation of *Escherichia coli* B/r in minimal and complex media. J. Biotechnol. **2:** 191-206.

24. Romeo, T., Kumar, A., Preiss, J. 1988. Analysis of the *Escherichia coli* glycogen gene cluster suggests that catabolic enzymes are encoded among the biosynthetic genes. Gene **70:** 363-376.

25. Romeo, T., Preiss, J. 1989. Physiology, Biochemistry and Genetics of Bacterial Glycogen Synthesis. Adv. Microbial Physiol. **30:** 1183-1238.

26. Romeo, T., Black, J., Preiss, J. 1990. Genetic regulation of glycogen biosynthesis in *Escherichia coli*: In vivo effects of the catabolite repression and stringent response systems in *glg* gene expression. Curr. Microbiol. **21:** 131-137.

27. Sambrook,J. Fritsch, E. F., Maniatis, T. 1989. Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

28. Strauch, M. A., Spiegelman, G. B., Perego, M., Johnson, W. C., Burbulys, D., Hoch, J. A. 1989. The transiton-state transcription regulator *AbrB* of *Bacillus subtilis* is a DNA-binding protein. EMBO J. **8:** 1615-1623.

29. Tempest D. W., Neijssel, O. M. 1987. Growth yield and energy distribution. p 797 - 806. In: Neidhardt, F. C., Ingraham, J. L., Low, K. B., Magasanik, M., Schaechter, M., Umbarger, H. E. (ed.), *Escherichia coli* and *Salmonella typhimurium.* Cellular and molecular biology. American Society for Microbiology, Washington, D.C.

30. Thauer, R. K., Jungermann, K., Decker, K. 1977. Energy conservation in chemotrophic anaerobic bacteria. Bacteriol. Rev. **41:** 100-180.

31. Zabriskie, D. W., and Arcuri, E. J. 1986. Factors affecting productivity employing recombinant organisms. Enzyme Microbial Technol. **8:** 705-716.

2.8 Tables

Table 1:   Final glycogen and cell protein levels as influenced by the addition of various concentrations of IPTG to cultures of strain TA3476/pGT100. Cultures were grown in shake flasks on buffered LB-medium, and IPTG at the indicated concentrations was supplemented during late exponential phase.

IPTG (μM)	Glycogen (mg / L)	Cell protein (mg / L)
0	126	1431
10	211	1482
100	324	1568
250	328	1558
500	360	1653
1000	363	1689

2.9 Figure captions

Figure 1:   The pathway of glycogen biosynthesis in *Escherichia coli*.

Figure 2:   Growth (cell protein, A), glycogen content (B) and acetate excretion (C) of wild-type *Escherichia coli B* (circles) and *glgQ* mutant AC70RI (squares) during shake flask cultivation on buffered LB-medium (open symbols) or on buffered LB-medium supplemented with 0.2% glucose (closed symbols).

Figure 3:   Growth (cell protein, A), glycogen content (B), and acetate excretion (C) of *Escherichia coli* DH5α transformed with either pUC19 (circles) or the pUC19-derivative pPR2 containing the glycogen biosynthetic genes *glg*A and *glg*C (squares). Cells were cultivated in shake flasks, either on buffered LB-medium (open symbols) or on buffered LB-medium supplemented with 0.2% glucose (squares). Ampicillin was added to all cultures to ensure plasmid maintenance.

Figure 4:   Time trajectories of glycogen accumulation by *E. coli* TA3476 transformed with plasmid pGT100 harboring the glycogen biosynthesis genes *glg*C and *glg*A genes under the control of the IPTG-inducible *tac* promoter. Cells were cultivated in shake flasks on buffered LB-medium supplemented with 0.2% glucose. IPTG at the indicated concentrations was added at 3.5h.

Figure 5:   SDS-polyacrylamide gel electrophoresis of extracts of TA3476/pGT100 and TA3476/pKQ4 cultures. At various time points during shake flask

cultivation on LB-medium with 0.2% glucose, cells were collected by centrifugation and homogenized by sonication. The sonicated extracts were analyzed on 8.5% SDS-polyacrylamide gels. Lanes 1 - 6 correspond to TA3476/pGT100 cultures sampled 3, 4, 5, 6, 7, and 8h post inoculation. Lane 7 corresponds to TA3467/pKQ4 sampled 3h post inoculation. The TA3467/pGT100 culture was supplemented with 500 μM IPTG at 3.5h to induce the *glg*A and *glg*C genes. The positions of the bands coresponding to glycogen synthase, *glg*A (52 kDa) and ADPG pyrophosphorylase, *glg*C (49 kDa) are indicated by arrows. Molecular weight markers (in kDA) are shown on the left.

Figure 6: Growth (cell protein, A), glycogen content (B), glucose consumption (C), and pyruvate excretion (D) of strain TA3476 transformed with plasmids pKQV4 (circles) or pGT100 (squares). Cells were grown in a bioreactor in LB medium supplemented with 0.2% glucose and 50 mg ampicillin / L. 3.5 h after inoculation 500 μM IPTG (closed symbols) or 0 μM IPTG (open symbols) was added to the cultures (arrows). Note the difference in time scale between panels A and B on one hand and panels C and D on the other hand.

Figure 7: Specific CO_2 production rate (A) and cumulative output of CO_2 (B) during cultivations of TA3476/pGT100 and TA3467/pKQV4 under conditions identical to those in Fig. 6. The TA3476/pGT100 culture was supplemented with 500 μM IPTG 3.5h after inoculation.

Figure 8: Pyruvate (A) and growth (B) profiles of strain TA3476/pGT100 as influenced by the addition of a pyruvate pulse (0.4 g/L) during late log phase (arrow). The cultivation parameters were as in Fig. 6. Glycogen accumulation of cells was induced at 3.5h by the addition of 500 μM IPTG. For comparison, the pyruvate and protein profiles

of TA3476/pGT100 (when no exogenous pyruvate was added) and TA3476/pKQV4 are

shown in dotted lines (data from Fig. 6).

Figure 1

glucose

↓

glucose-6-phosphate ⟷ glucose-1-phosphate

$glgC$ ↓ ADP glucose pyrophosphorylase

fructose-6-phosphate ADP-glucose

$glgA$
$glgB$ Glycogen(n)
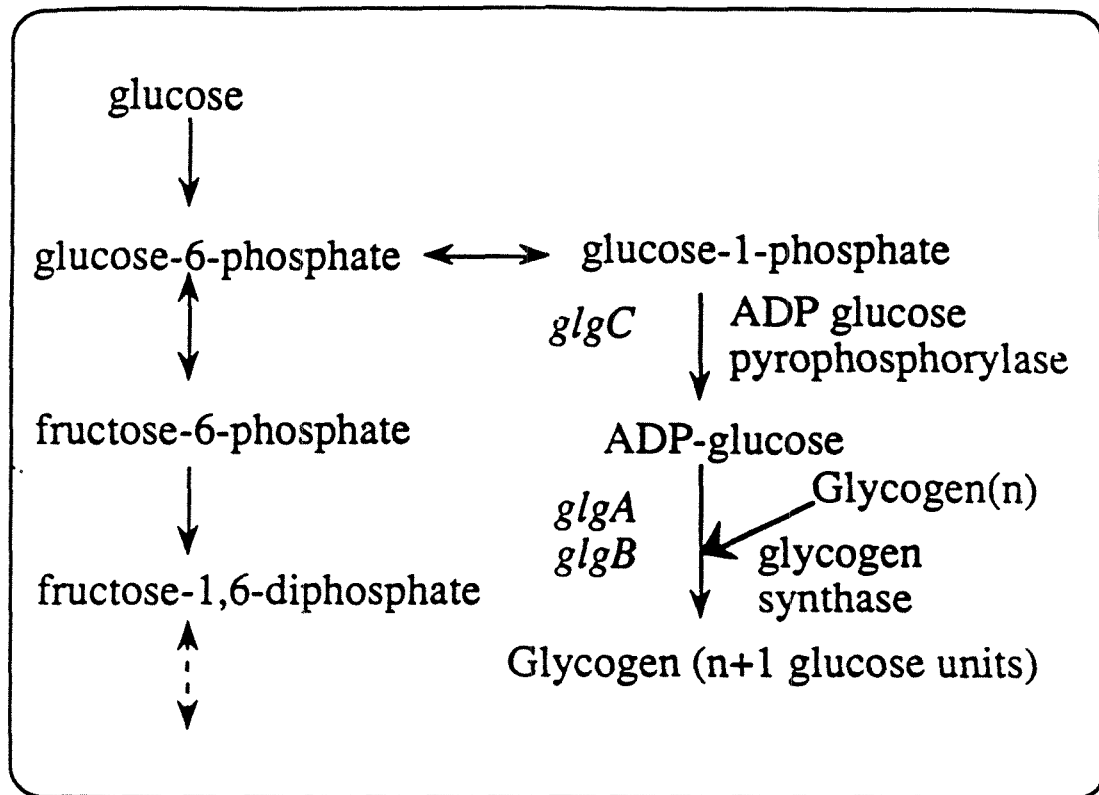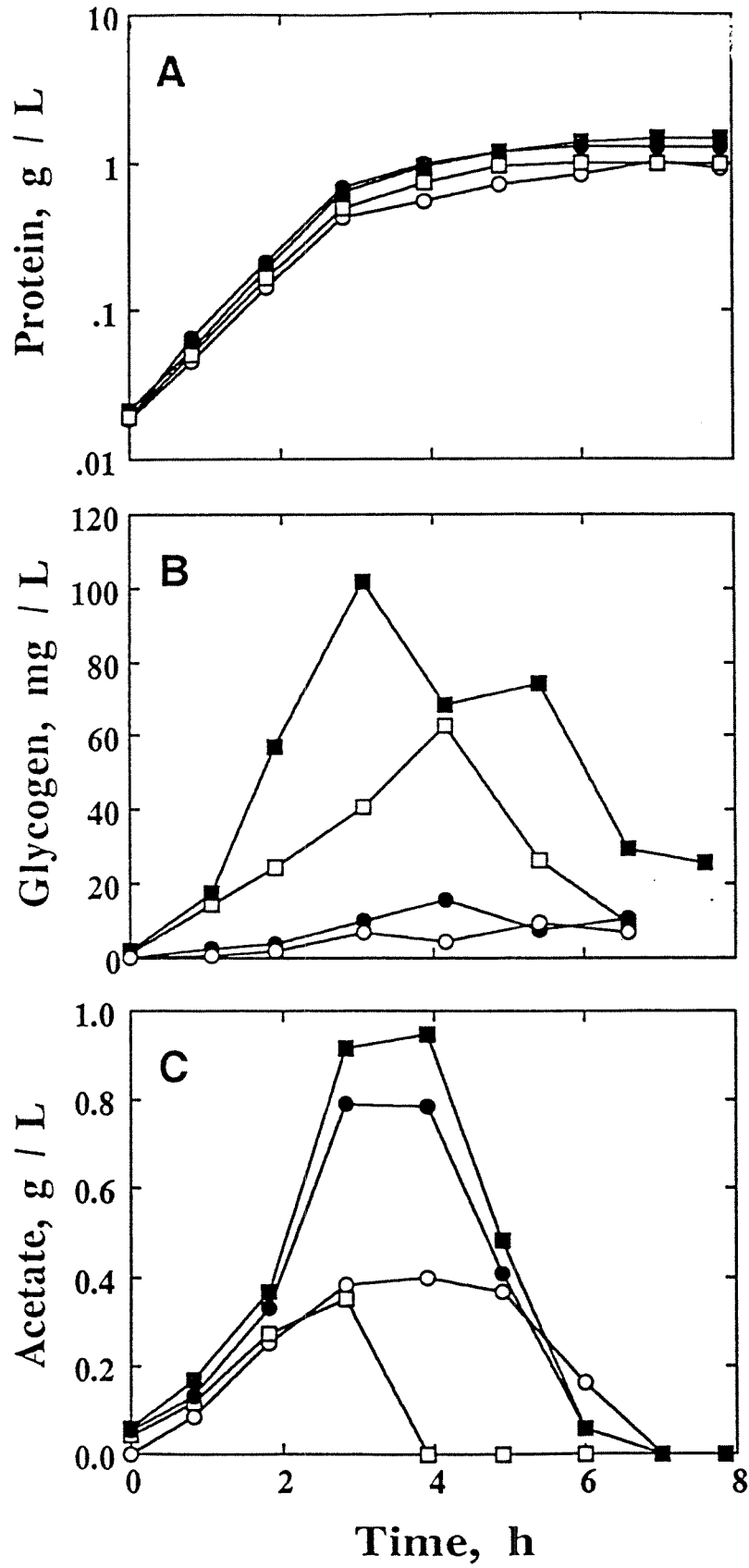
glycogen synthase

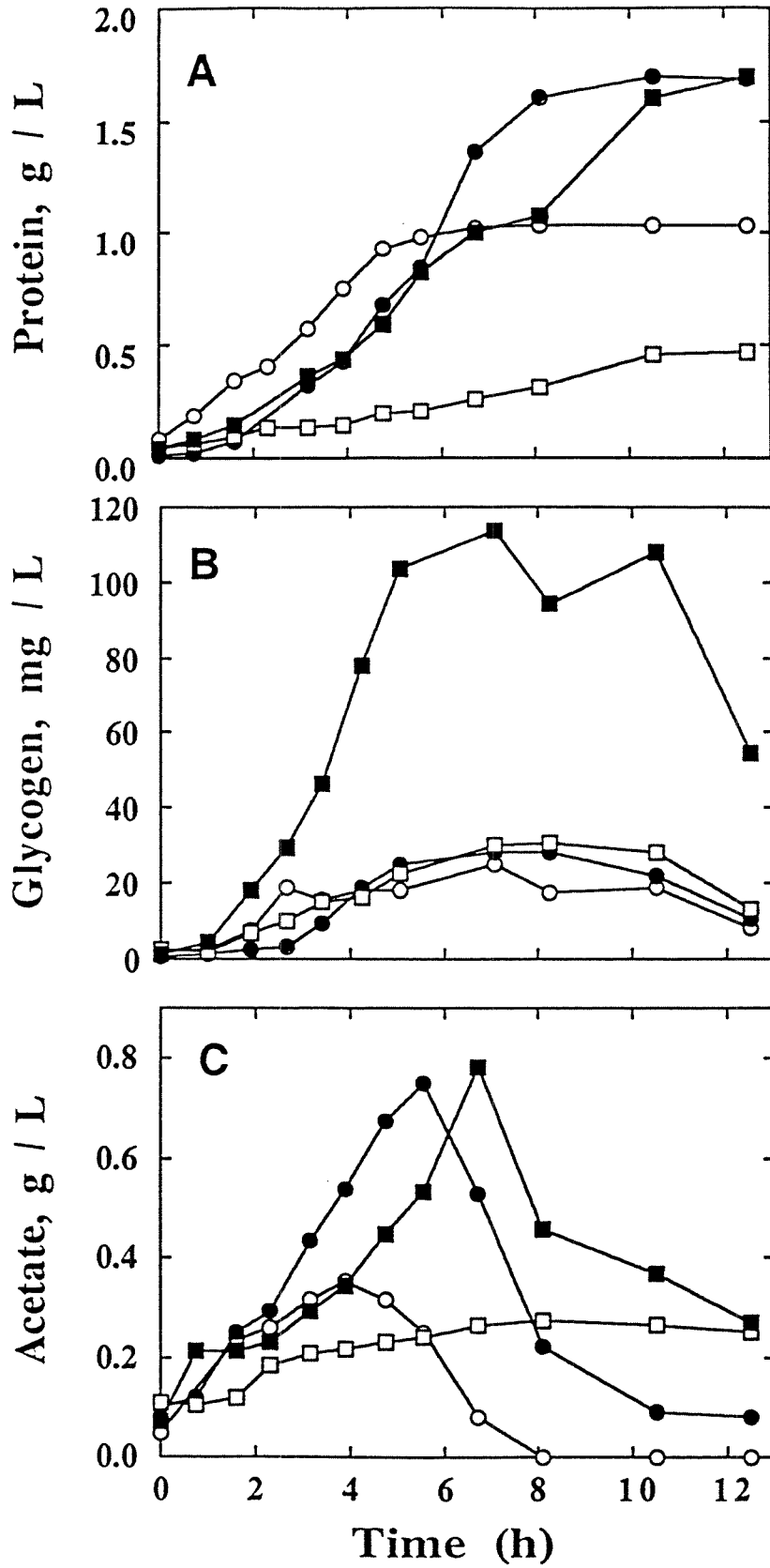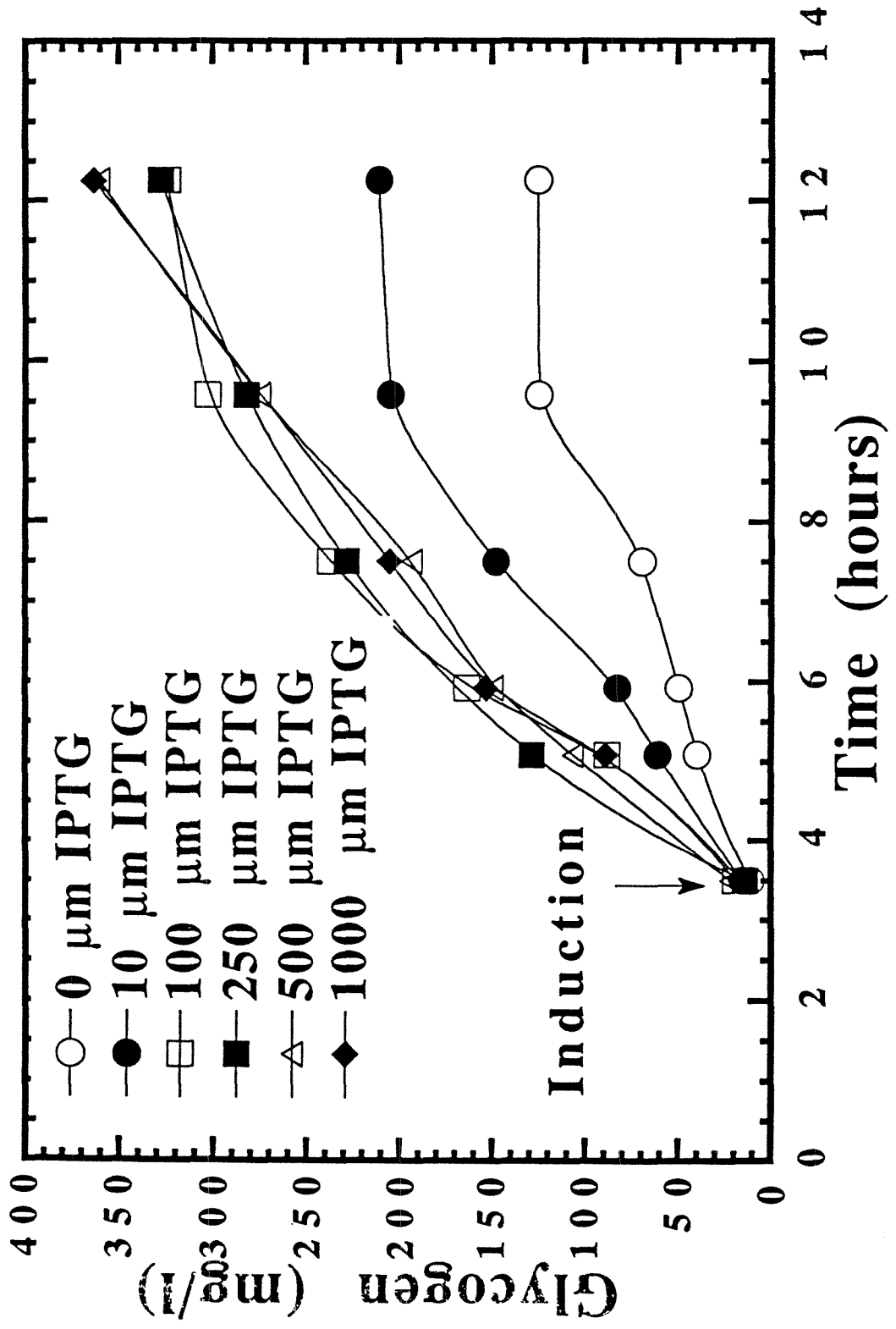fructose-1,6-diphosphate

Glycogen (n+1 glucose units)

Figure 2
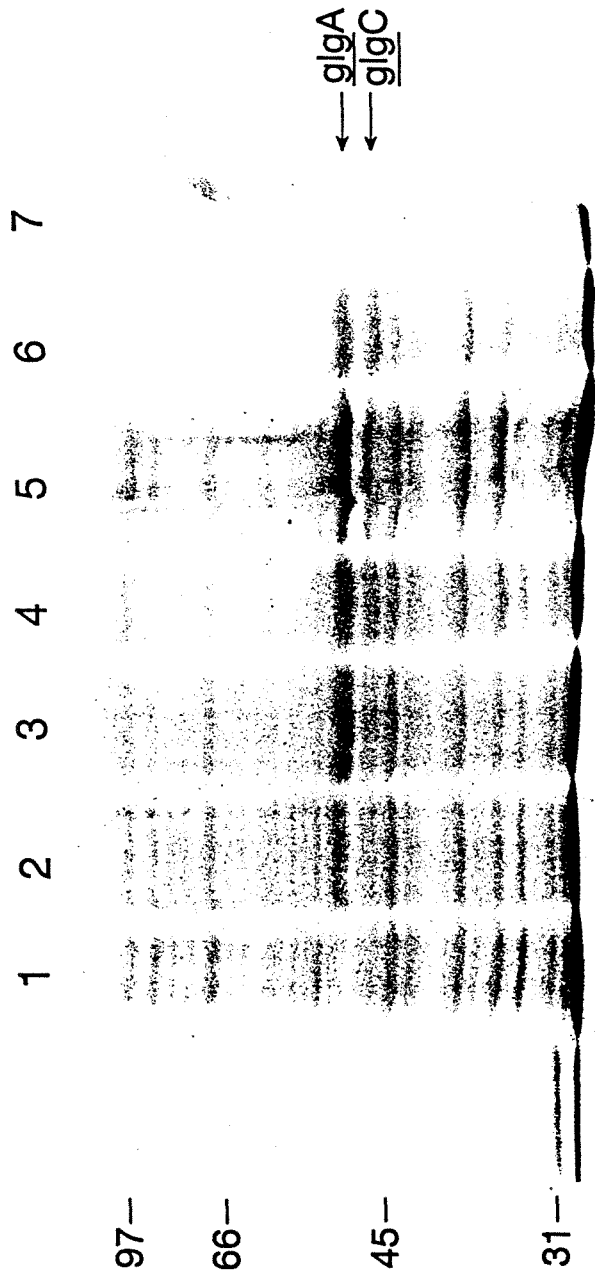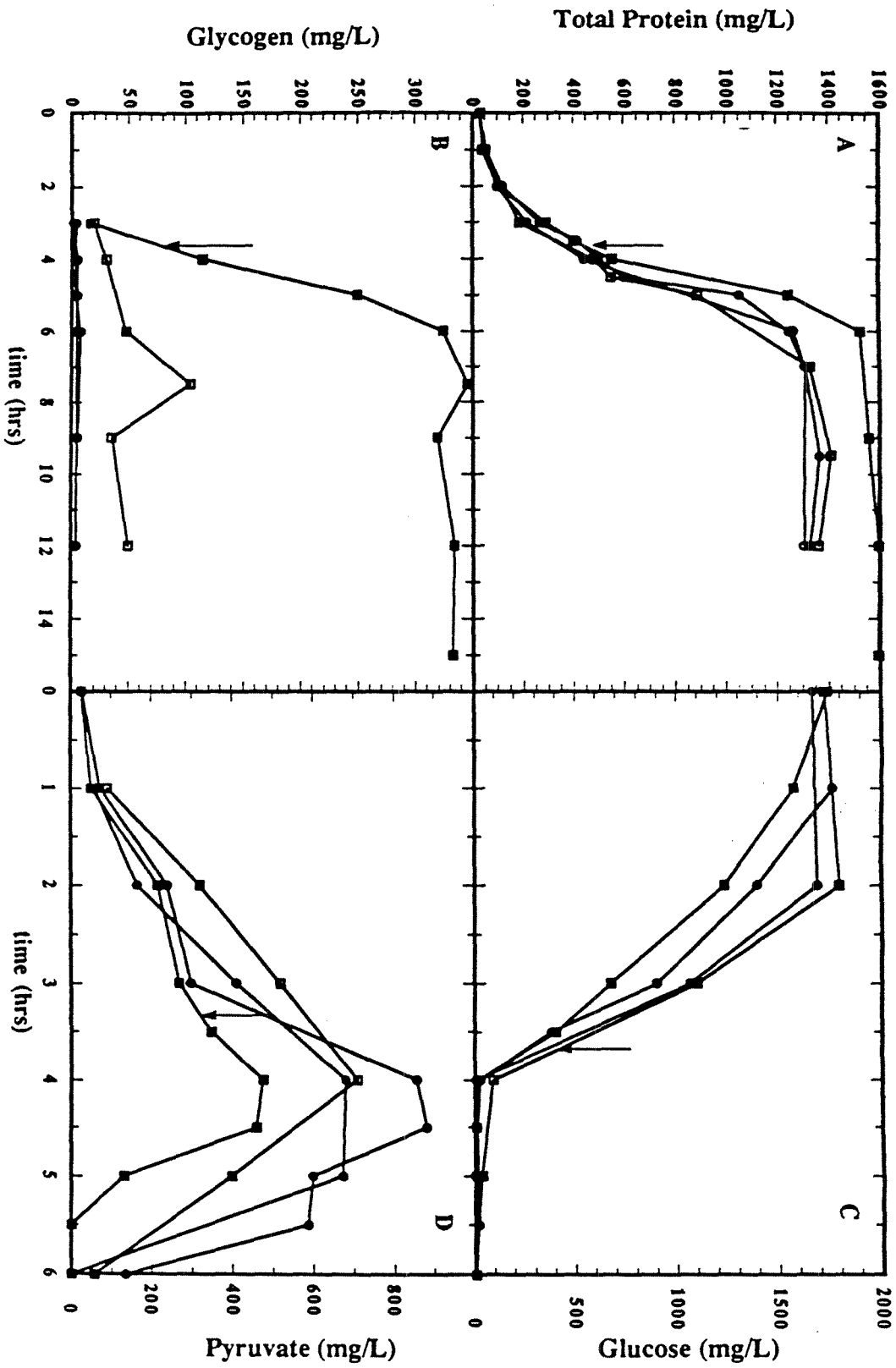
Figure 3

Figure 4

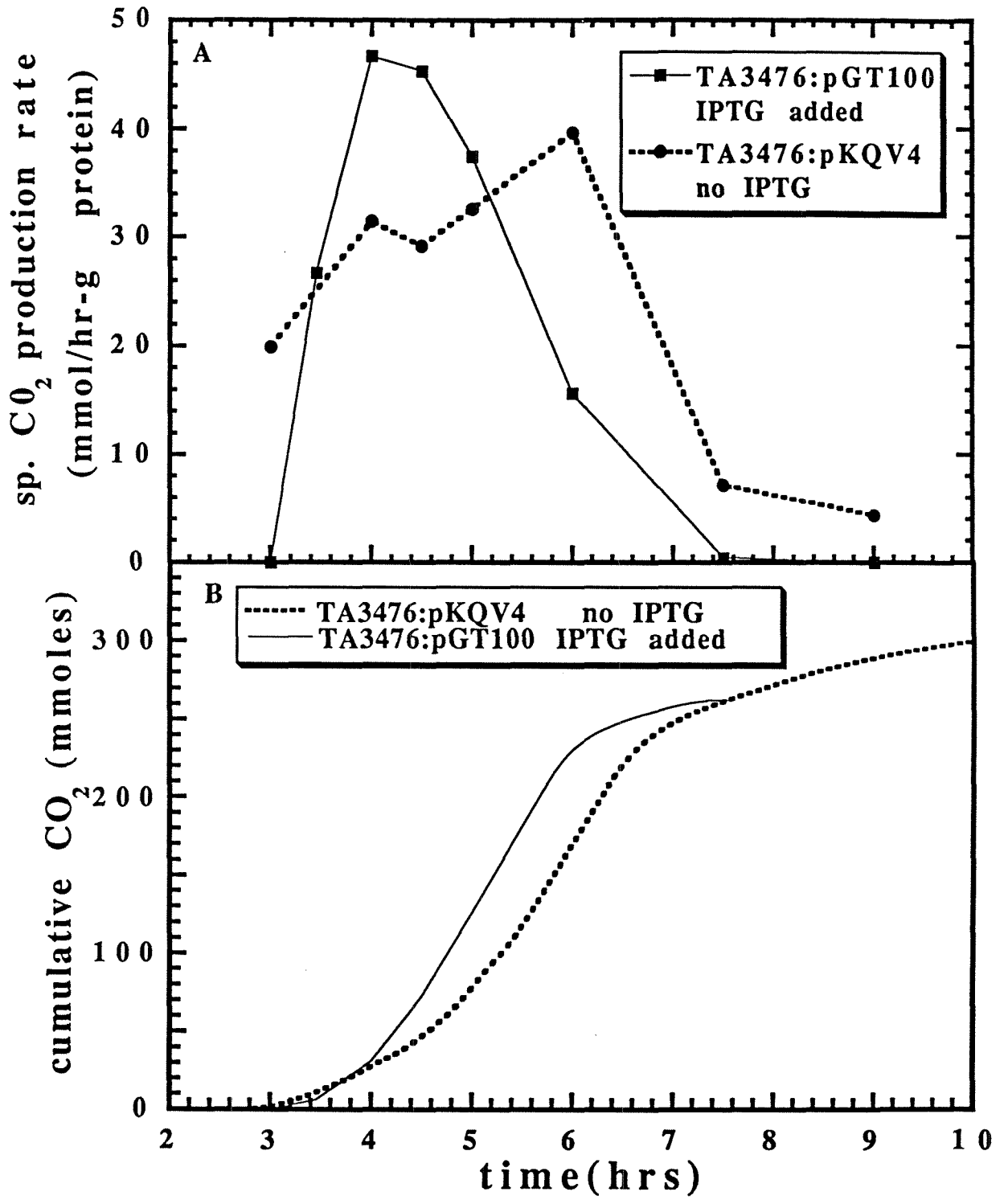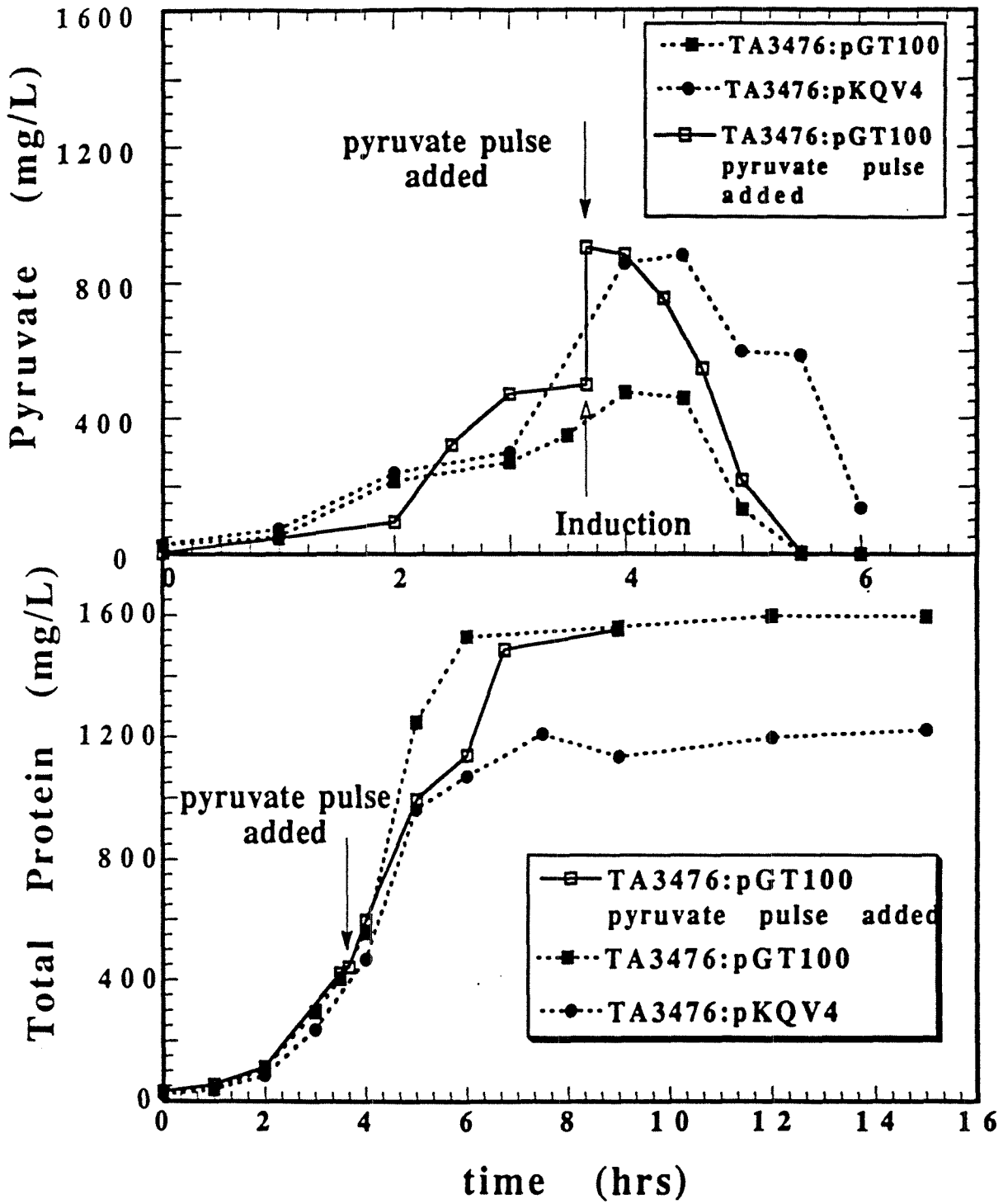# Figure 5

Figure 6

Figure 7

Figure 8

Chapter 3

Coordinated Synthesis and Degradation of Glycogen as Tools to Manage Carbon Flow

In this work, plasmid pMSW2 was constructed by Dr. Wilfred Chen (Section 3.3.3)

3.1 Abstract

The excess flux flowing to inhibitory end-products in *Escherichia coli* could potentially be tapped and redirected to biomass and desired metabolites. As a strategy to study the coordinated synthesis and degradation of glycogen, we have constructed two expression vectors to allow the controlled synthesis and degradation of glycogen. The first vector pMSW2 use the metabolic switch system: the glycogen biosynthesis genes *glgC*, *glgA* along with *lacI* are expressed in an operon constitutively with the help of the λp_L promoter. On adding IPTG the second operon, which includes the glycogen degradation gene *glgP*, is activated and the first operon is repressed. The second expression vector pGTSD100 contains the native *E. coli* operon *glgC-glgA-glgP* under the control of the *tac* promoter. We show that both expression vectors work successfully to control net synthesis and degradation of glycogen. Within 50 minutes of adding IPTG to cultures of strain TA3476 harboring pMSW2, the specific activity of ADPG pyrophosphorylase was reduced by 25% while the specific activity of glycogen phosphorylase increased nearly 2-fold. If IPTG was not added, the activity of *glgC* doubled while the activity of *glgP* stayed nearly constant. Within 30 minutes of adding IPTG to cultures of TA3476:pGTSD100, the specific activity of ADPG pyrophosphorylase increased 40-fold and the specific activity of glycogen phosphorylase also increased 3-fold. Between the time of adding IPTG and the start of net glycogen degradation, there was a delay of nearly 3 hours in cultures of TA3476:pMSW2 and 2.5 hours in cultures of TA3476:pGTSD100. When IPTG is added to TA3476 transformed with pMSW2, the amount of total cell protein at the end of the batch cultivation is about 15% higher compared with the amount of total protein when no IPTG is added. The extra biomass was formed during the glycogen degradation phase. These vectors can be used to study the channeling of carbon to metabolites.

3.2 Introduction

In order to obtain the highest possible yields of recombinant proteins and metabolites in microorganisms and cultured cells, the restructuring of the central pathways of metabolism must be considered. Many microorganisms and cultured cells produce inhibitory catabolic end-products such as acetic acid, lactic acid, and ethanol. For example, *E. coli* has a strong tendency to excrete acetic acid during rapid growth even under aerobic conditions (Reiling et al. 1985; Zabriskie and Acuri, 1986; Bauer at al., 1990; Jensen et al. 1990; Luli and Strohl, 1990). Apparently, the uptake of nutrients and the generation of biosynthetic precursors occur faster than the utilization of these precursors for the production of biomass and energy (Hollywood and Doelle, 1976; Anderson and von Meyenburg, 1980; Doelle et al.., 1983; Reiling et al. 1985; El-Mansi and Holms, 1989; Majewski and Domach, 1990). On a practical basis, the excess flux flowing to products such as acetate could be potentially tapped and redirected to biomass and metabolites.

Controlled synthesis and degradation of glycogen is a potential strategy to manage carbon flow. Glycogen synthesis during exponential phase can store part of the flux which would otherwise flow to unwanted toxic products. Controlled degradation of glycogen during the deceleration growth phase or the stationary phase can be used to channel the flux to biomass or metabolite products. Many important secondary metabolites are produced during stationary phase in certain microorganisms. A large class of important antibiotics are synthesized using hexose phosphate as precursor (Bailey and Ollis, 1986). By coupling the degradation of a storage compound such as glycogen to the antibiotic production pathway, the stored carbon might be routed to secondary metabolite synthesis.

Glycogen biosynthesis in *E. coli* requires the activities of ADPG pyrophosphorylase [EC 2.7.7.27], glycogen synthase [EC 2.4.1.2.1] and the branching enzyme [EC 2.4.1.18] (encoded by the *glgC*, *glgA* and *glgB* genes respectively; reviewed by Preiss, 1984; Preiss and Romeo, 1989). Glycogen degradation requires the activity of glycogen phosphorylase [EC 2.4..1.1] (encoded by the *glgP* gene) (Romeo et al. 1988; Yu et al. 1988; Choi et al. 1989). The *glgC* and *glgA* genes along with the glycogen degradation enzyme *glgP* are apparently cotranscribed in an operon *glgCAP*. Though there are no reports of transcriptional regulation of the *glgP* gene, since the *glgP* gene is located only 18 bp distal to the *glgA* stop codon, it has been suggested that the expression of glycogen phosphorylase may be coordinately regulated with that of ADPG pyrophosphorylase and glycogen synthase as part of a glycogen biosynthetic/catabolic operon (Romeo et al. 1988). Synthesis of glycogen in *E. coli* is controlled in response to a variety of genetic and physiological influences. This is reflected in the array of trans-acting factors that regulate *glgCA* expression. The native promoter of the operon is regulated by several components: it is positively stimulated by cyclic AMP (cAMP), cAMP receptor protein, and guanosine 3'-bisphosphate 5'-bisphosphate (ppGpp) and negatively regulated by *csrA* (Romeo and Preiss, 1989; Romeo et al. 1990; Romeo et al. 1993). Besides the complex transcription pattern observed for *glgCA* , other known regulatory features include the allosteric regulation of ADPG pyrophosphorylase activity (Preiss, 1984; Preiss and Romeo, 1989), and the effects of *katF* and *glgS* on glycogen synthesis via mechanisms that are still unknown (Henge-Aronis et al. 1992; Lange et al. 1991).

Our previous experiments have studied a metabolic engineering strategy which redirects the carbon flux through elimination of wasteful pathways and sequestering of substrate carbon as glycogen (Dedhia et al. 1994). The genes of the glycogen biosynthesis pathway were amplified in *E. coli* TA3476, an *E. coli* mutant lacking the acetate

biosynthesis pathway. This engineered strain overproduced glycogen and grew to higher final cell density compared to *E. coli* TA3476 carrying a control plasmid. There was no appreciable degradation of the overproduced glycogen, suggesting that glycogen degradation is a potential limiting factor.

We have constructed two expression vectors for studying the controlled synthesis and degradation of glycogen. The first expression vector is an application of the "metabolic switch" concept described previously (Chen and Bailey, 1994). This plasmid has two operons. The first operon contains the glycogen synthesis genes, *glgC* and *glgA*, along with *lacI* under the control of the λp_L promoter; the second operon comprises the glycogen degradation gene, *glgP*, along with *cI* under the control of the *tac* promoter. In the absence of IPTG, the first operon is expressed constitutively. When IPTG is added, the λp_L promoter of the first operon is repressed and the *tac* promoter of the second promoter is activated. The repression and activation reinforce each other by cross-regulation. This "metabolic switch" structure enables switching from expression of genes of one metabolic pathway to expression of genes of a second metabolic pathway. This approach is general and can be used for other sets of genes also. The second expression vector contains the native *E. coli glgC-glgA-glgP* operon under the control of the tac promoter. The operon includes 52 bp upstream of the *glgC* start codon. The operon ends 521 bp downstream of the *glgP* gene. Induction of this operon is controlled by the addition of IPTG. We report here the functioning and effectiveness of these vectors in controlled synthesis and degradation of glycogen and the concomitant effects on culture physiology.

3.3 Materials and methods

3.3.1 Strains and plasmids

The genotype of strain TA3476 and characteristics of the plasmids used in this study are shown in Table 1. TA3476 is a mutant defective in acetate biosynthesis due to mutations in the *ack* (acetate kinase) and *pta* (phosphotransacetylase) genes. Plasmid maps are shown in Figure 1. The construction of plasmids is described below.

3.3.2 Polymerase chain reaction (PCR)

The PCR reaction was carried out in a 50 µl final reaction volume containing 2.5 ng of template DNA, 5 µl of 10X reaction buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01%(w/v) gelatin), 8 µl of dNTPs mix (1.25 mM each), 2 mM each of the primers, and 0.5 µl of Taq DNA polymerase (1U/µl) (Perkin Elmer-Cetus). The amplification was carried out for 36 cycles in a DNA thermal cycler (Perkin Elmer -Cetus). The DNA was denatured at 92°C for 1 min, annealed at 42°C for 2 min, and extended at 72°C for 5 min.

3.3.3 Construction of plasmid pMSW2

The polymerase chain reaction was used to synthesize both the *glgC-glgA* and *glgP* structural gene with plasmids pPR2 (Romeo and Preiss, 1989) and pGTC12 (Choi et al. 1989) as templates, respectively. Primers I and II (Figure 2a) were used to amplify the *glgC-glgA* gene. These two primers contained only the Shine-Dalgarno sequence of the

glgC gene but not the transcriptional stop sequence so that the amplified fragment can be used to create a *λpL-glgC-glgA-lacI* operon. Restriction sites *SacI* were created at both ends for subcloning. To synthesize the structural gene of the *glgP* gene, primers III and IV were used (Figure 2b); as in primers I and II, only the Shine-Dalgarno sequence of the *glgP* gene is included. *NsiI* sites were created to facilitate subcloning.

The 2.76 kb *glgC-glgA* fragment generated from PCR amplification was digested with *SacI* overnight. After gel purification, the resulting fragment was subcloned into pUC18 to create pUC*glgC*A. The functionality of the *glgC-glgA* fragment was confirmed by comparing the glycogen content of *E. coli* TA3476 with and without this plasmid (results not shown). This plasmid was then cleaved with *SacI* and the *glgC-glgA* fragment was inserted into the same site of plasmid pλlacI to give pλCAI.

Similarly, the 2.36 *glgP* PCR fragment was cleaved with *NsiI* and subcloned into pSL1180 to yield pSL*glgP*. To construct plasmid pTCIP, the *glgP* fragment was cleaved from pSL*glgP* and inserted into pTCI. Finally, the glycogen metabolic switch construct was obtained by transferring a 5.8 kb *NdeI/BamHI* fragment from pλCAI into the *NdeI/SmaI* sites of pTCIP with blunt end ligation at the *BamHI/SmaI* sites.

3.3.4 Construction of plasmid pGTSD100

Plasmid pGT100 (Dedhia et al. 1994), which carries the *glgC* and *glgA* genes under the control of the *tac* promoter, was digested with *PstI*, treated with Klenow enzyme to create blunt ends and then digested with *NcoI*. This released a 7.6 kb fragment which included the tac promoter, the *glgC* gene and part of the *glgA* gene. Plasmid pGTC12 (Choi et al. 1989) (kindly donated by Dr. Komano, Department of Agricultural University,

Kyoto University, Japan) was digested with *BsaAI* and *NcoI*, which released a 3.9 kb fragment containing the remaining part of the *glgA* gene and the *glgP* gene. The 7.6 kb and 3.9 kb fragment were ligated to create the plasmid pGTSD100. The plasmid pGTSD100 contains the tac promoter followed by the *glgC-glgA-glgP* operon as it exists naturally in the chromosome of *E. coli*.

3.3.5   Medium and culture conditions

The fermentations were carried out in a BioFlo III bench-top fermentor (New Brunswick Scientific, Edison, NJ) containing 2.5 L of medium. The medium used was LB medium buffered with phosphate and supplemented with 0.2% glucose. The medium contained in g/l: bactotryptone, 10; yeast extract, 5; NaCl, 5; KH_2PO_4, 3; K_2HPO_4, 1. The culture conditions were: temperature, 37°C; pH, 7.0; air flow rate, 2.0 liters per minute. pH was controlled with 2N NaOH and 2N HCl. Ampicillin (50 mg/L) was added during fermentations of plasmid-bearing strains. Foam was controlled by the addition of Medical Antifoam AF solution (Dow Corning, Midland, MI.).

Inocula were grown in two stages; this was done to condition the glycogen overproducing strain to growth in liquid medium. The first stage consisted of inoculating a single colony from a freshly streaked plate in 4 ml of medium. After nearly 8 hour, 1:200 dilutions of the first-stage culture were inoculated in 50 ml medium. The second-stage culture was allowed to grow overnight and used to inoculate the main fermentation in fermentor or shake flask. The amount of inoculum added was adjusted to give a starting OD_{600} of 0.1.

3.3.6   Analytical methods

Protein was measured by the method of Peterson (1977). Glycogen was assayed essentially as described by Gunja-Smith et al. (1977).

3.3.7   Assay of glycogen phosphorylase

15 ml of culture from the bioreactor were sampled and centrifuged. The pellets were washed twice with 50 mM potassium phosphate, pH 7.5 and finally suspended in 1 ml of 25 mM potassium phosphate buffer, pH 7.5 containing 0.5% Triton X-100. After sonication for 90s with intermediate cooling, the debris was removed by centrifugation. The cell extracts were assayed for glycogen phosphorylase activity as in Mendicino et al.(1975).

3.3.8   Assay of ADPG pyrophosphorylase.

15 ml of culture from the bioreactor were sampled and centrifuged. The pellets were washed with 25 mM Tris, pH 8.0 and finally the pellets were suspended in 50 mM glycylglycine buffer, pH 7.0 including 5 mM DTT. After sonication for 90 s with intermediate cooling, the cell extracts were assayed for ADPG pyrophosphorylase as in Ozaki and Preiss (1972) using PP^{32}.

3.4 Results

3.4.1 Coordination of glycogen net synthesis and degradation using the metabolic switch plasmid.

According to the design of the plasmid pMSW2, addition of IPTG to cultures carrying pMSW2 will result in transcriptional repression of the glycogen synthesizing genes *glgC* and *glgA* and transcriptional activation of the glycogen degradation gene *glgP*. In our first experiments, we therefore studied the trajectory of enzyme activities of *glgC* and *glgP*, with and without addition of IPTG, in strain TA3476 transformed with plasmid pMSW2. TA3476 is impaired in the acetate synthesis pathway, and we have shown in a previous paper (Dedhia et al. 1994) that TA3476 engineered for glycogen overproduction, with no significant glycogen net degradation, grows to about 15-20% higher cell culture densities than a control strain. Cultivations were carried out in a bench-top bioreactor at constant pH, temperature and impeller speed. Cells were inoculated into buffered LB supplemented with 0.2% glucose, and after 4.66 hour, IPTG (500 µM) was added. Within 50 minutes after addition of IPTG, the specific activity of *glgC* was reduced from ca. 5000 cpm/mg-min to 3700 cpm/mg-min while the specific activity of *glgP* increased from ca. 25 units/mg-min to 40 units/mg-min. If IPTG was not added, the activity of *glgC* in fact increased from 5000 cpm/mg-min to 9000 cpm/mg-min while the activity of *glgP* stayed nearly constant. The wild-type background values of the specific enzyme activities were measured in cultures of TA3476 carrying the control plasmid pKQV4 : the specific activity of *glgC* was 170 cpm/mg-min and the specific activity of *glgP* was 29 units/mg-min.

The trajectory of enzyme activities shows that the "metabolic switch plasmid" pMSW2 is able to shift successfully, when IPTG is added, from the expression of the

enzyme *glgC* to expression of a second enzyme, *glgP*. Within the resolution of our sampling times, the shift appears to take place within 50 minutes of addition of IPTG.

We then proceeded to study the effect of the varying trajectories of the glycogen synthesizing and degradation enzymes on the net rate of glycogen accumulation and degradation in TA3476:pMSW2. As shown in Figure 3, glycogen net synthesis and degradation can be controlled by addition of IPTG in strains carrying pMSW2 or pGTSD100. The glycogen trajectory of strain TA3476:pMSW2 shows three phases with different specific glycogen net synthesis and degradation rates (see Table 2). In the first phase (0 to 4 hour post inoculation), we see the maximum specific net rate of glycogen synthesis. In the second phase (5 to 7.5 hour post inoculation), the net rate of glycogen synthesis is reduced. Finally, in the third phase (7.5 hour to end of cultivation, 15 hour), net glycogen degradation is observed. When no IPTG is added to cultures of TA3476:pMSW2, there is no shift in expression of enzymes: *glgC* continues to be expressed and no induction of *glgP* occurred; there is only one phase in the glycogen trajectory, a phase of net glycogen synthesis (0 to 7.5 hour post inoculation).

3.4.2   Coordination of glycogen net synthesis and degradation using the plasmid pGTSD100.

According to the design of the plasmid pGTSD100, the addition of IPTG to cultures harboring pGTSD100 will induce the tac promoter and the *glgC-glgA-glgP* operon will be transcribed. Cultures of strain TA3476 transformed with plasmid pGTSD100 were grown in a bioreactor under conditions similar to that described for pMSW2, except IPTG was added 3.5 hours after inoculation. Within 30 minutes after addition of IPTG, the

specific activity of *glgC* increased from 1500 cpm/mg-min to ca. 60,000 cpm/mg-min while the specific activity of *glgP* increased from about. 110 units/mg-min to 372 units/mg-min.

When IPTG is added to cultures of pGTSD100 3.5 hours after inoculation, there is a phase of rapid net glycogen synthesis from hours 3.5 to 6, and the glycogen net degradation phase follows during hours 6 to 15 post inoculation (Figure 3 and Table 2).

To compare the glycogen profiles of TA3476:pGTSD100 with a strain overexpressing only the glycogen synthesis genes, we studied the glycogen trajectories of TA3476:pGT100. The strain was grown under conditions identical to that of TA3476:pGTSD100 (IPTG was added 3.5 hours post-inoculation). As shown in Figure 3, the level of glycogen produced in TA3476:pGT100 was nearly 2-fold higher than the maximum level of glycogen produced in TA3476:pGTSD100. No net glycogen degradation was observed in TA3476:pGT100.

3.4.3 Effect of glycogen accumulation and degradation on growth

We studied the effect of net glycogen degradation on growth of *E. coli* TA3476 cultures by following the total protein trajectory of strain TA3476 transformed with pMSW2, with and without addition of IPTG. As shown in Figure 4, when IPTG is added to TA3476 transformed with pMSW2, the amount of total cell protein at the end of the batch cultivation is about 15% higher compared with the amount when no IPTG is added.

The growth trajectories of TA3476:pMSW2 exhibit three phases of growth, while there are two phases of growth when no IPTG is added (see Table 3). Under both conditions, with and without IPTG, the exponential phase, which lasts 5 hours after inoculation, is similar. When IPTG is added, TA3476:pMSW2 exhibits two deceleration growth phases with specific growth rates 0.091 hr^{-1} (from 5 to 7.5 hours) and 0.017 hr^{-1} (from 7.5 to 15 hours). However, when no IPTG is added to cultures of TA3476:pMSW2, there is only one deceleration growth phase with specific growth rate 0. 047 hr^{-1} (from 5 to 7.5 hour); from 7.5 to 15 hours, TA3476:pMSW2 is in stationary phase. The degradation of glycogen possibly contributes to the higher growth rates in the deceleration growth phases in TA3476:pMSW2.

The growth trajectory of TA3476:pGTSD100 is very similar to that of TA3476:pMSW2 (see Table 3) . There are three phases of growth: an exponential phase (from 0 to 5 hours) and two deceleration growth phases with specific growth rates 0.097 hr^{-1} (from 5 to 7.5 hours) and 0.018 hr^{-1} (from 7.5 to 15 hours). Our previous experiments (Dedhia et al. 1994) showed that the growth trajectory of TA3476:pGT100, which overexpresses only the glycogen synthesis genes, shows only two phase of growth: an exponential phase (from 0 to 5 hours) and a deceleration growth phase (from 5 to 7.5 hours); stationary phase spans from hours 7.5 to 15.

The relative amounts of total protein at the end of the batch cultivation for strain TA3476 carrying different plasmids are listed in Table 4.

During the growth of all strains (TA3476:pMSW2 with and without IPTG, TA3476:pGTSD100 and TA3476:pGT100), glucose is exhausted from the medium by 4.0 hours post inoculation (Figure 5). Thus, all the strains accumulate most of the glycogen

after glucose has been exhausted from the medium. Considering the nutrients available in LB medium, it is likely that gluconeogenic pathways contribute to the stored glycogen. Pyruvate is the major organic acid secreted by all strains and the maximum levels are about 0.5 g/L (Figure 5). After the exhaustion of glucose, pyruvate is completely consumed within 5.5 hours post inoculation. Other acids such as acetate, lactate and succinate are not accumulated in significant quantities (< 0.05 g/l) (data not shown). Glycogen levels in all the strains reaches a peak around 6 hours. It is possible that the consumed pyruvate may contribute to glycogen synthesis.

3.5 Discussion

In this study we have constructed vectors which allow coordinated synthesis and degradation of glycogen. We showed that addition of IPTG to cultures of TA3476:pMSW2 resulted in a transition of enzyme activities: the activity of ADPG pyrophosphorylase decreased and that of glycogen phosphorylase increased. On the other hand, when IPTG was added to cultures of TA3476:pGTSD100, the specific activities of both ADPG pyrophosphorylase and glycogen phosphorylase increased.

In the cultivation in which IPTG is added to cultures of TA3476:pMSW2, although the metabolic switch from expression of ADPG pyrophosphorylase to glycogen phosphorylase takes place within 5.5 hours post-inoculation, we observe a net rate of glycogen degradation only 7.5 hours post inoculation. Similarly in the cultivation of TA3476:pGTSD100, ADPG pyrophosphorylase and glycogen phosphorylase are expressed at 4 hours post inoculation while a net rate of glycogen degradation is observed

only 6 hours post inoculation. In cultures of TA3476:pMSW2, the activity of ADPG

pyrophosphorylase is reduced but not eliminated completely after IPTG is added.

Consequently cultures of TA3476:pMSW2 contain both ADPG pyrophosphorylase and

glycogen phosphorylase in phase two of glycogen synthesis (5 to 7.5 hour post

inoculation). By the very nature of the design of plasmid pGTSD100, which is intended to

transcribe the *glgC*, *glgA* and *glgP* genes in an operon, both ADPG pyrophosphorylase

and glycogen phosphorylase are present after the addition of IPTG. Thus due to

coexistence of ADPG pyrophosphorylase and glycogen phosphorylase after addition of

IPTG, the net rate of glycogen synthesis or degradation will be strongly influenced by the

specific activity of ADPG pyrophosphorylase relative to the glycogen phosphorylase

specific activity. It is known that ADPG pyrophosphorylase is allosterically activated by

fructose 1,6 diphosphate and inhibited by 5'-AMP while glycogen phosphorylase is

inhibited by ADPG and activated by AMP (reviewed in Preiss 1984; Preiss and Romeo,

1989). The changing levels of these metabolites will control the specific activities of ADPG

pyrophosphorylase and glycogen phosphorylase. For example, it is probable that levels of

5'-AMP, which are related to the energy charge of the cell, will be lower in phase 2 than

higher in phase 3 because phase 3 has a lower specific growth rate. In wild-type *E. coli* , it

has been suggested that allosteric regulation of ADPG pyrophosphorylase and glycogen

phosphorylase determines whether glycogen is accumulated, as in the early stationary

phase, or degraded, as occurs later in stationary phase (Romeo et al. 1988).

The dynamics of glycogen net synthesis and degradation of TA3476:pGTSD100

differs from that of TA3476:pMSW2: the specific net rate of glycogen synthesis is higher

in TA3476:pGTSD100 and the glycogen net degradation starts earlier. The rate of net

glycogen synthesis is higher in TA3476:pGTSD100 because the activity of the ADPG

pyrophosphorylase is 6-fold higher in TA3476:pGTSD100 compared to that in

TA3476:pMSW2. This larger amount could be due to the difference in the strength of the promoters of *glgC-glgA* and the different copy numbers of pMSW2, whose parent vector is pBR322, and pGTSD100, whose parent vector is pKQV4 (the copy number of pKQV4 is ~5 times that of pBR322). The earlier depolymerization of glycogen in TA3476:pGTSD100 may possibly reflect the effect of the larger amount of glycogen synthesized in TA3476:pGTSD100 on the activity of ADPG pyrophosphorylase or it may reflect a different ratio of degradation enzyme levels to synthesis enzyme levels. The specific rate of net glycogen degradation in TA3476:pGTSD100 is similar to that in TA3476:pMSW2.

The difference in ADPG pyrophosphorylase and glycogen synthase enzyme levels between TA3476:pMSW2 and TA3476:pGTSD100 indicates that, to increase the level of expression of the encoded genes, the next generation of "metabolic switch" plasmid should have a replicon with a higher copy number (the current replicon is from pBR322).

Comparing the glycogen trajectories of TA3476:pGT100 and TA3476:pGTSD100 (which is constructed by the addition of *glgP* gene to pGT100), we can observe the effect of glycogen phosphorylase on the dynamics of glycogen accumulation. The glycogen net synthesis rate in TA3476:pGTSD100 is slightly lower than in TA3476:pGT100, glycogen levels peak about 90 minutes earlier in TA3476:pGTSD100, and the maximum quantity of glycogen accumulated in cultures of TA3476:pGTSD100 is nearly half the maximum quantity observed for TA3476:pGT100. Comparing the glycogen trajectories of TA3476:pMSW2, with and without addition of IPTG, we observe that, after IPTG is added, the glycogen net synthesis rate in TA3476:pMSW2 drops.

All strains which overproduce glycogen (TA3476:pMSW2 induced and uninduced, TA3476:pGTSD100, induced TA3476:pGT100) accumulate more biomass than the control strains TA3476:pKQV4 and uninduced TA3476:pGT100 (Table 4). We do not know definitely how glycogen overaccumulation affects biomass in *E. coli* cells deficient in acetate synthesis , but, for a review of our various hypotheses, the reader may refer to Dedhia et al. (1994).

The difference in biomass between induced and uninduced TA3476:pMSW2 shows that glycogen degradation can contribute to growth. The degraded glycogen can provide energy or biosynthesis precursors or both. When induced, cultures of TA3476:pMSW2 accumulate an extra 170 mg/L protein and degrade about 40 mg/L glycogen. This indicates that inputs other than glycogen have also contributed to the extra biomass.

The vectors pMSW2 and pGTSD100 can be used to study the channeling of stored carbon to metabolites, especially in the stationary phase. One approach would be to add genes coding for enzymes involved in the metabolite's synthesis to the above vectors. Similar vectors can be designed for performing metabolic engineering in microorganisms which produce secondary metabolites. It has been suggested that, recombinant proteins can be made in the stationary phase of *E. coli* cultures by using carbon starvation promoters (Matin 1992). In such cases, the above vectors can be utilized.

3.6 References

Andersen KB, von Meyenburg K (1980) Are growth rates of *Escherichia coli* in batch cultures limited by respiration? J Bacteriol 144: 114 - 123

Bailey JE, Ollis DF (1986) Biochemical Engineering Fundamentals, 2nd ed McGraw-Hill Book Company New York, p 279

Bauer KA, Ben Bassat A, Dawson M, de la Puente V, Neway J0 (1990) Improved expression of human interleukin-2 in high-cell-density fermentor cultures of *Escherichia coli* K- 12 by a phosphotransacteylase mutant. Appl Environ Microbiol 56:1296 -1302

Chen W, Kallio P, Bailey JE (1993) Construction and characterization of a novel cross-regulation system for regulating cloned gene expression in *Escherichia coli*. Gene 130:15-22

Chen W, Bailey JE (1994) Application of the cross-regulation system as a metabolic switch. Biotechnol Bioeng 43:1190-1193

Choi YL, Kawamukai M, Utsumi R, Sakai H, Komano T (1989) Molecular cloning and sequencing of the glycogen phosphorylase gene from *Escherichia coli*. FEBS Letter 243:193-198

Dedhia NN, Hottiger TH, Bailey JE (1994). Overproduction of glycogen in *Escherichia coli* blocked in the acetate pathway improves culture growth. Biotechnol Bioeng *in press*

Doelle HW, Ewings KR, Hollywood NA (1983) Regulation of glucose metabolism in bacterial systems. Advances in Biochemical Engineering 23:1- 35

El-Mansi EMT, Holms WH (1989) Control of carbon flux to acetate excretion during growth of *Escherichia coli* in batch and continuous cultures. J Gen Microbiol 135:2875 - 2883

Gunja-Smith Z, Patil N, Smith EE (1977) Two pools of glycogen in *Saccharomyces*. J Bacteriol 130:818-825

Hengge-Aronis R, Fischer D (1992) Identification and analysis of *glgS*, a novel growth-phase regulated and rpoS-dependent gene involved in glycogen synthesis in *Escherichia coli*. Mol Microbiol 6:1877-1886

Hollywood NW, Doelle HW (1976) Effect of specific growth rate and glucose concentration on growth and glucose metabolism of *Escherichia coli* K12. Microbios 17: 23 - 33

Jensen EB, Carlsen S (1990) Production of recombinant growth hormone in *Escherichia coli*: Expression of different precursors and physiological effects of glucose, acetate, and salts. Biotechnol Bioeng 36: 1 - 11

Lange R, Hengge-Aronis R (1991) Identification of a central regulator of stationary-phase gene expression in *Escherichia coli*. Mol Microbiol 5:49-51

LeVine SA, Ardhesir F, Ames GFA (1980) Isolation and characterization of acetate kinase and phosphotransacetylase mutants of *Escherichia coli* and *Salmonella tvphimurium*. J Bacteriol 143:1081-1085

Luli GW, Strohl WR (1990) Comparison of growth, acetate production, and acetate inhibition of *Escherichia coli* strains in batch and fed batch fermentations. Appl Environ Microbiol 56: 1004 - 1011

Majewski RA, Domach MM (1990). Simple constrained optimization view of acetate overflow in *Escherichia coli*. Biotechnol Bioeng 35:732 - 738

Matin A (1991) The molecular basis of carbon starvation induced general resistance in Escherichia coli. Molecular Microbiol 5:3-10

Matin A (1992) Genetics of bacterial stress response and its applications. Ann NY Acad Sci 665:1-15

Mendicino J, Abou-Issa H, Medicus R, Kratowich N (1975) Fructose-1,6-diphosphate, phosphofructokinase, glycogen synthetase, phosphorylase, and protein kinase from swine kidney. In: Wood (ed) Methods in Enzymology Vol XLII: Complex Carbohydrates Part C. Academic Press, New York, p 262

Ozaki H, Preiss J (1972) ADPG pyrophosphorylase from *Escherichia coli* B. In: Ginsburg V (ed) Methods in Enzymology Vol XXVIII: Complex Carbohydrates Part B. Academic Press, New York, p 389

Peterson GL (1977) A simplification of the protein assay method by Lowry et al. which is more generally applicable. Anal Biochem 83:346 - 356

Preiss J (1984) Bacterial glycogen synthesis and its regulation. Annu Rev Microbiol 38:419-458

Preiss J, Romeo T (1989) Physiology, Biochemistry and Genetics of Bacterial Glycogen Synthesis. Adv Microbial Physiol 30:1183 - 1238

Reiling HE, Laurila H, Fiechter A (1985) Mass culture of *Escherichia coli*: Medium development for low and high density cultivation of *Escherichia coli* B/r in minimal and complex media. J Biotechnol 2:191 - 206

Romeo T, Kumar A, Preiss J (1988) Analysis of the *Escherichia coli* glycogen gene cluster suggests that catabolic enzymes are encoded among the biosynthetic genes. Gene 70: 363 - 376

Romeo T, Preiss J (1989) Genetic regulation of glycogen biosynthesis in *Escherichia coli*: in vitro effects of cyclic AMP and guanosne 5'-diphosphate 3'-diphosphate and analysis of in vivo transcripts. J. Bacteriol: 2773-2782

Romeo T, Black J, Preiss J (1990) Genetic regulation of glycogen biosynthesis in *Escherichia coli*: In vivo effects of the catabolite repression and stringent response systems in *glg* gene expression. Curr Microbiol 21:131-137

Romeo T, Gong M, Liu MY, Brun-Zinkernagel AM (1993) Identification and molecular characterization of *csrA*, a pleiotropic gene from *Escherichia coli* that affects glycogen biosynthesis, gluconeogenesis, cell size and surface properties. J Bacteriol 175:4744-4755

Strauch MA, Spiegelman G, Perego M, Johnson WC, Burbulys D, Hoch JA (1989) The transition-state transcription regulator *AbrB* of *Bacillus subtilis* is a DNA-binding protein. EMBO J 8:1615 - 1623

Yu F, Jen Y, Takeuchi E ,Inouye M ,Nakayama H ,Tagaya M, Fukui T (1988) α-glucan phosphorylase from Escherichia *coli*. Cloning of the gene, and purification and characterization of the protein. J Biol Chem 263:13706-13711

Zabriskie DW, Arcuri EJ (1986) Factors affecting productivity employing recombinant organisms. Enzyme Microbial Technol 8: 705 - 716

3.7 Tables

Table 1. Description of strains and plasmids.

Strain or plasmid	Description	Reference
TA3476	*E. coli* K12 *hisΔ* (*pta-ack-dhΔhisJ-hisQ-hisP*)	LeVine et al. 1980
pGTC12	contains *glgP* gene.	Choi et al. 1989
pPR2	contains *glgC*-glgA gene.	Romeo and Preiss, 1989
pSL1180	commercial cloning vector.	Pharmacia, Piscataway, NJ
pλlacI	contains the lac repressor gene under the control of the lpl promoter (parent vector pPL-Lambda from Pharmacia, NJ)	Chen et al. 1993
pKQV4	cloning vector derived from Pharmacia's cloning vector pKK223-3	Strauch et al. 1989
pGT100	contains *glgC* and *glgA* genes under the control of the tac promoter (parent vector pKQV4).	Dedhia et al. 1994
pGTSD100	contains *glgC*, *glgA* and *glgP* genes under the control of the *tac* promoter (parent vector pKQV4).	this study
pMSW2	contains *glgC*, *glgA*, *lacI* under the *λpL* promoter and *glgP*, *cI* under the *tac* promoter (parent vector pBR322).	this study

Table 2. Specific rates of glycogen synthesis and degradation in different phases of growth.

Construct	Phase	Time post-inoculation, hrs	Synthesis or degradation rate (g glycogen/g protein-hr)
pMSW2 (induced)	Synthesis phase 1	0 to 4 hrs	0.0432
	Synthesis phase 2	5 to 7.5 hrs	0.0163
	Degradation phase	7.5 to 15 hrs	-0.0032
pMSW2 (uninduced)	Synthesis phase	0 to 7.5 hrs	0.0248
pGTSD100	Synthesis phase	3.5 to 5 hrs	0.114
	Degradation phase	5 to 15 hrs	-0.0041

Table 3. Specific growth rates in different phases of growth.

Construct	Phase	Time post-inoculation, hrs	specific growth rate, μ hr^{-1}
pMSW2 (induced)	exponential phase	0 to 5 hrs	0.73
	deceleration growth phase 1	5 to 7.5 hrs	0.091
	deceleration growth phase 2	7.5 to 15 hrs	0.017
pMSW2 (uninduced)	exponential phase	0 to 5 hrs	0.74
	deceleration growth phase 1	5 to 7.5 hrs	0.047
	stationary phase	7.5 to 15 hrs	0.00
pGTSD100	exponential phase	0 to 5 hrs	0.74
	decelaration growth phase 1	5 to 7.5 hrs	0.097
	deceleration growth phase 2	7.5 to 12 hrs	0.018

Table 4. Total protein content at the end of the batch cultivation.

Strain	Final Total Protein (mg/L)
TA3476/pKQV4	1310
TA3476/pGT100	1590
TA3476/pGTSD100	1590
TA3476/pMSW2 (uninduced)	1540
TA3476/pMSW2 (induced)	1710

3.8 Figure captions

Figure 1. Schematic maps of plasmids: (a) pGT100 (b) pGTSD100 (c) pMSW2

Figure 2 (a) Primers I and II used for PCR amplification of *glgC-A* fragment. Sequences homologous to the template pPR2 are underlined. Start codon and stop codon are indicated in italics. (b) Primers III and IV used for PCR amplification of *glgC-A* fragment. Sequences homologous to the template pPR2 are underlined. Start codon and stop codon are indicated in italics

Figure 3. Glycogen trajectories during batch cultivations of different constructs. The cultures were cultivated in a bioreactor in LB supplemented with 0.2% glucose. The arrows indicate the time when IPTG was added to cultures of *E. coli* TA3476:pGT100, TA3476:pMSW2 and TA3476:pGTSD100 at a final concentration of 500 μM.

Figure 4. Total protein trajectories during batch cultivations of different constructs. The growth conditions are described in caption of Figure 3.

Figure 5. Glucose and pyruvate trajectories.

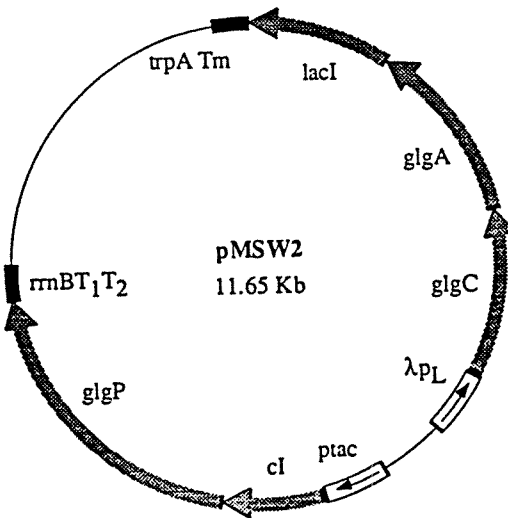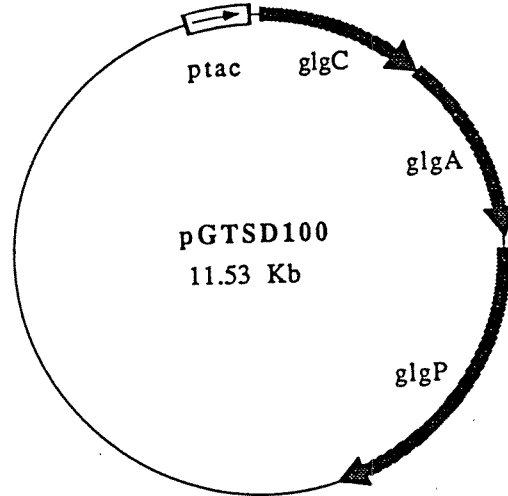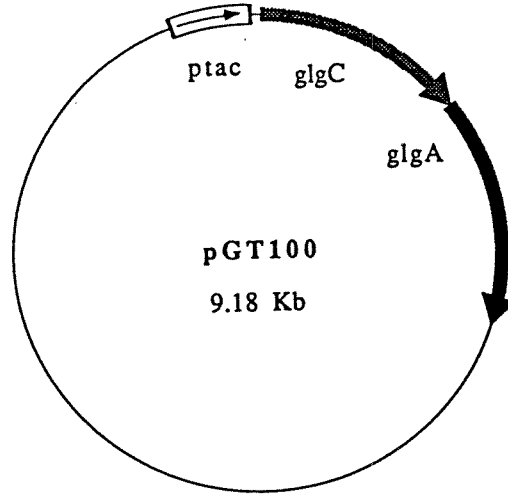## Figure 1

Figure 2

A.

Primer I

5'   3'
GGGAGCTCGG<u>AAGGAAGGAGTTAGTCATGGTTAG</u>

Primer II
5'   3'
CCGAGCTCCCCA<u>CTATTTCGAGCGATAGTAAAGCTC</u>

B.

Primer III

5'   3'
GGATGCATGG<u>CTATCGCTTACAAGCTGATG</u>

Primer IV
5'   3'
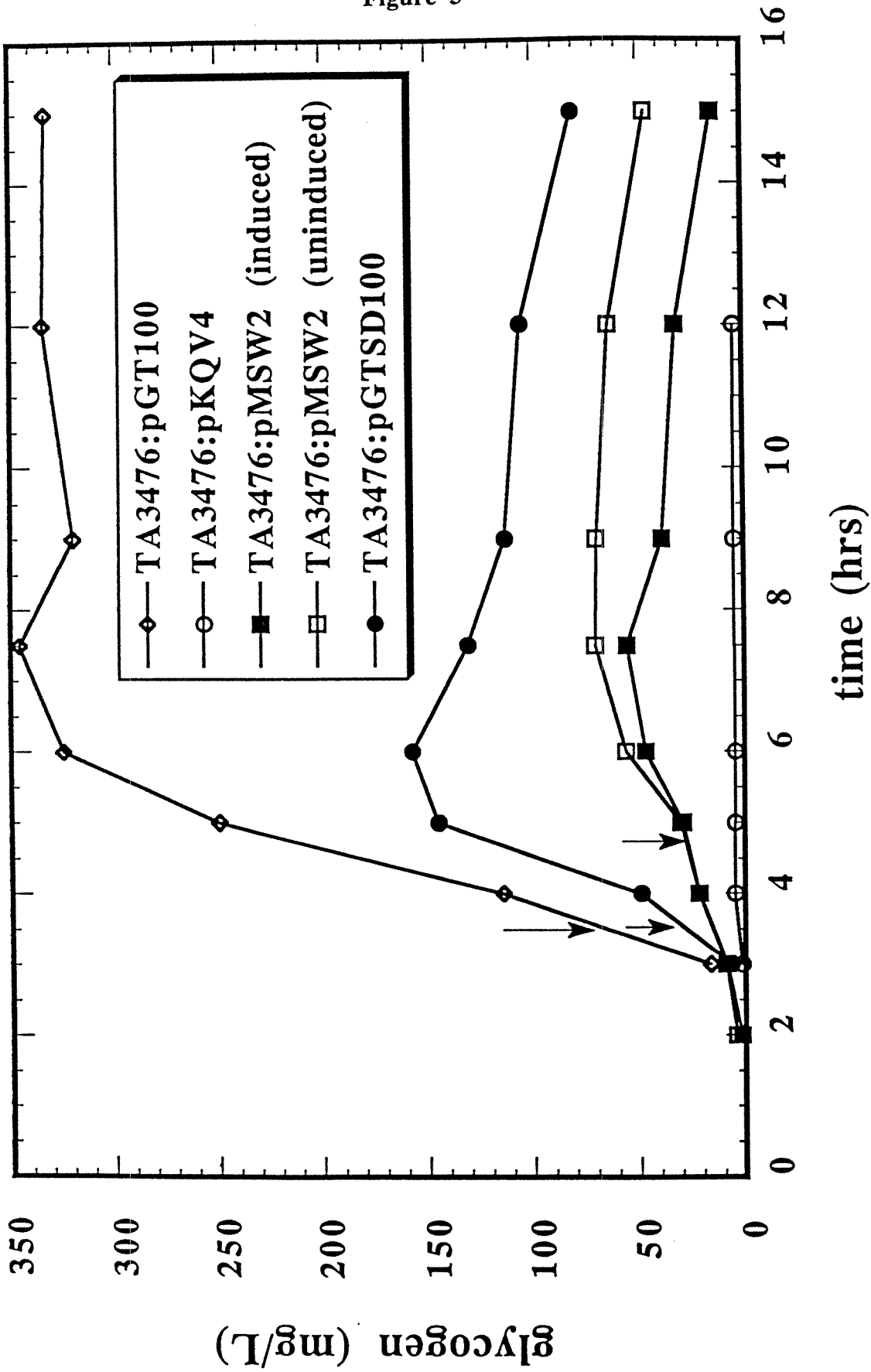CCATGCATCC<u>CTTACAATCTCACCGGATCG</u>

Figure 3

Figure 4

Figure 5
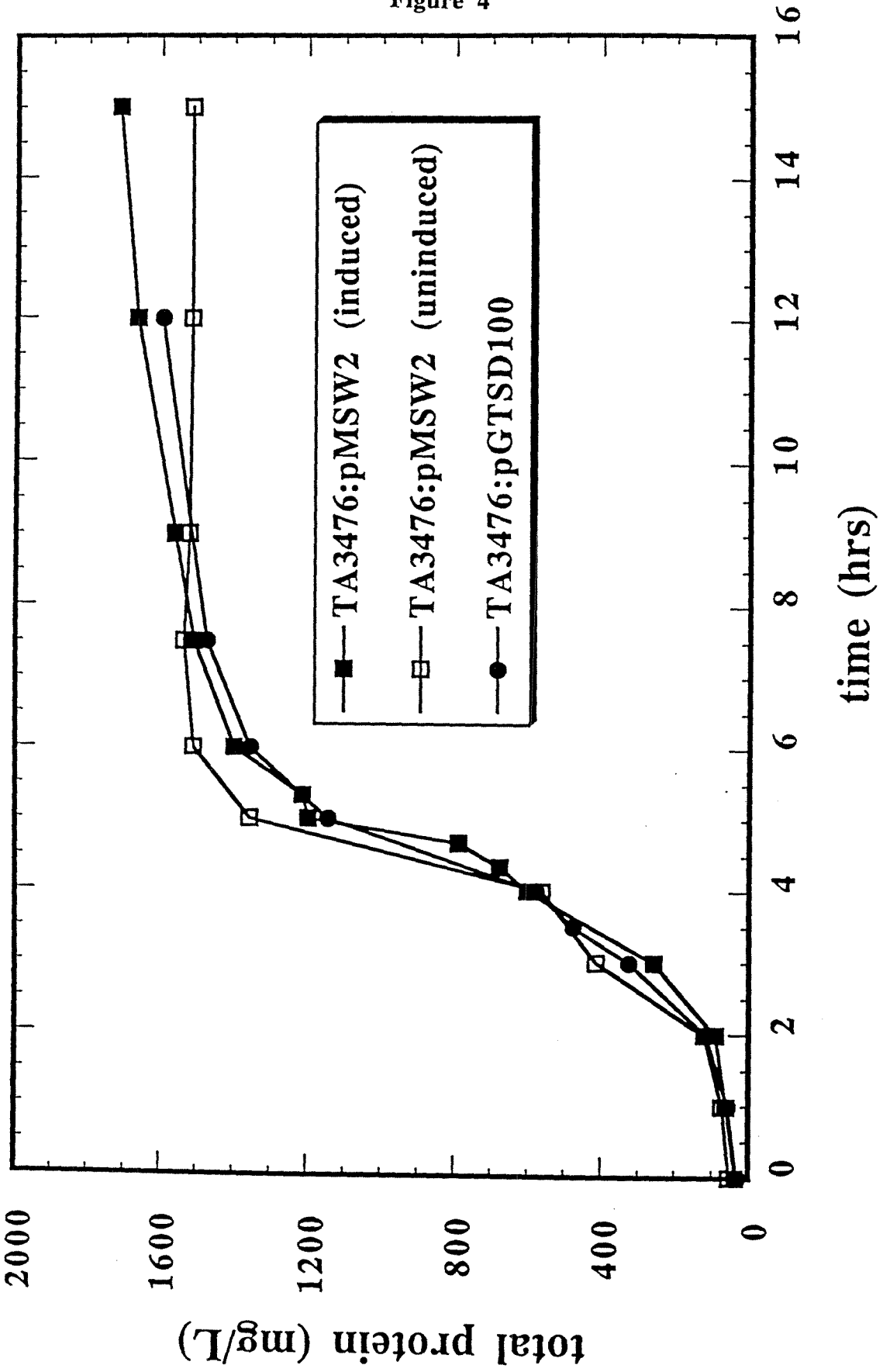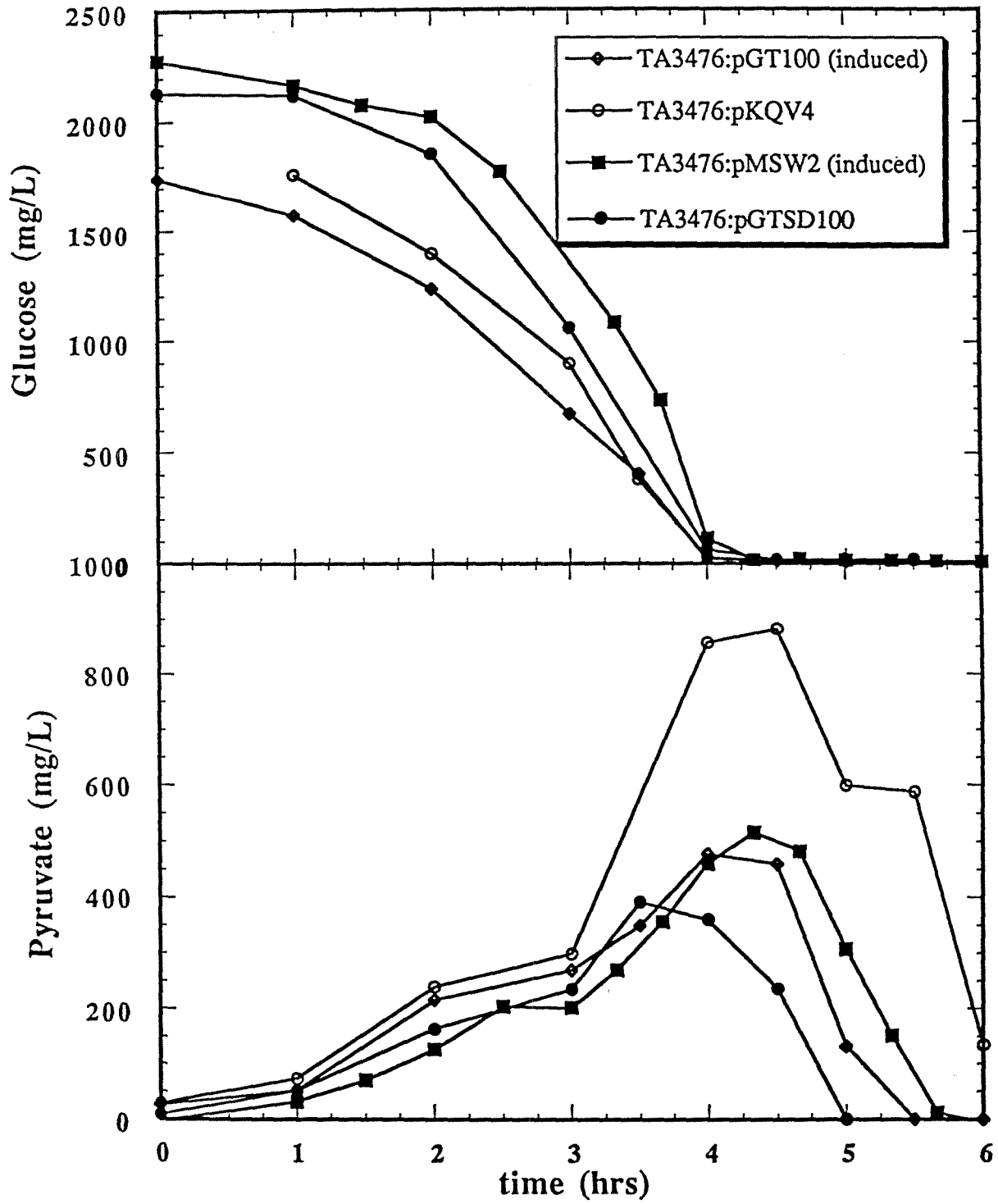
Chapter 4

Redirection of Carbon Flux to Metabolites: Amplification of Glycogen Synthesis and Degradation Genes in *E. coli* Improves Glutamate Production

4.1 Introduction

In this chapter, we have used metabolic engineering to improve the production of a chemical which is already produced by *E. coli*. To improve the production of a particular product of the metabolic reactions of the living cell, the most common approach is to perform metabolic engineering of the specific biosynthetic pathway involved in the synthesis of that metabolite. Thus, metabolic engineering could be used to amplify the enzyme which catalyzes a known rate-limiting reaction, or block competing pathways which utilize the same pool of precursors as the sequence of reactions leading to the desired product. If it is not known *a priori* which reaction in a long series of reaction is rate limiting, then all or part of the enzymes involved in the series of reactions could be amplified. Since the metabolic reactions which are being modified interact in an extremely complex and often unknown manner with the rest of the metabolic network of reactions in the cell, the consequences of a particular metabolic change can be often unexpected. For example, in the classic example of metabolic engineering to improve the production of phenylalanine in *E. coli*, the overproduction of some enzymes in the pathway is detrimental to product formation (Backman et. al., 1990). Therefore, metabolic engineering practitioners need to utilize an iterative cycle of genetic modification, analysis of the metabolic consequences of this change, and choice of the next genetic modification.

Production of many metabolites like ethanol, acetic acid, H_2; antibiotics like tylosin, cephalosporin, doxurubicin; amino acids like L-lysine, L-aspartate, L-arginine, L-threonine; and polymers like xanthan gum and bacterial cellulase have been improved by metabolic engineering of the specific pathways involved in the biosynthesis of these metabolites (reviewed by Bailey et al. 1991, Cameron and Tong, 1993) . For example,

ethanol production in *E. coli* was improved by expressing pyruvate decarboxylase and alcohol dehydrogenase II genes from *Zymomonas mobilis* (Ingram at al., 1987). Xanthan gum production was improved by overexpressing the xanthan synthesis genes in *Xanthomonas campestris* (Pollock and Thorne, 1988). Threonine production in *Brevibacterium lactofermentum* was improved from 17.5 g/l to 33 g/l by amplification of homoserine dehydrogenase and homoserine kinase genes (Nakamori et al., 1987; Morinaga et al. 1987).

The production of metabolites synthesized by the living cell can also be improved by metabolic engineering of reaction pathways other than the specific pathways involved in the biosynthesis of the metabolites. The rationale for this approach to metabolic engineering of metabolite production lays in the fact that all cellular biosynthetic pathways begin with one or another of a small group of twelve elite compounds called precursor metabolites (Neidhardt et al., 1990). These twelve precursor metabolites are glucose-6-phosphate, fructose-6-phosphate, ribose-5-phosphate, erythrose 4-phosphate, triose phosphate, 3-phosphoglycerate, PEP, pyruvate, acetyl CoA, α-ketoglutarate, succinyl CoA, and oxaloacetate. In cells of most living species, these metabolites are produced by reactions of the glycolytic cycle, pentose-phosphate pathway, and TCA cycle. As a result, these pathways are termed the central carbon metabolic pathways. Also the central carbon pathways generate supplies of ATP, and NADPH which are important cosubstrates in many biosynthetic pathways.

Metabolic engineering of the central carbon pathways can be explored to improve the production of metabolites by increasing the availability of the 12 precursor metabolites and biosynthesis cofactors by accelerating some of the key reactions of the central carbon

pathways, changing the regulatory properties of key enzymes or altering the levels of regulatory molecules which control the central carbon metabolic pathways. In *Brevibacterium fermentum*, by cloning of the enzyme PEP carboxylase, which converts PEP to oxaloacetate, the production of threonine was improved by 12% (Sano et al., 1987). Two patents describe the production of 32 g/l glutamate when the enzymes citric acid synthetase from and phosphofructokinase from *E. coli* were cloned in *Corynebacterium* (Katsumata et al., 1988; Katsumata et al., 1988b).

In Chapter 2 and 3, we have shown that overproduction of the glycogen synthesis and degradation enzymes improves biomass production. This manipulation increased production rates of glycogen, CO_2 and uptake rate of pyruvate, suggesting a higher gluconeogenesis flux and TCA cycle flux. In this study, we studied the effect of metabolic engineering of the glycogen synthesis and degradation pathways on metabolite production. Glycogen biosynthesis in *E. coli* requires the activities of ADPG pyrophosphorylase, glycogen synthase and the branching enzyme (encoded by the *glgC*, *glgA* and *glgB* genes respectively; reviewed by Preiss, 1984; Preiss and Romeo, 1989). Glycogen degradation requires the activity of glycogen phosphorylase, encoded by the *glgP* gene (Romeo et al., 1988; Yu et al. 1988; Choi et al. 1989). We report that amplification of the glycogen synthesis genes, *glgC* and *glgA*, and the glycogen degradation gene, *glgP*, in cultures of *E. coli* TA3476 surprisingly improves the production of glutamate almost 3-fold compared to the plasmid-free strain.

4.2 Materials and methods

4.2.1 Strains and plasmids

The *Escherichia coli* strain used in this study was TA3476, an *E. coli K12* mutant lacking the acetate biosynthesis pathway. The genotype of TA3476 is : *E. coli* K12 *hisΔ* (*pta-ackA-dhuA-hisJ-hisQ-hisP*).

Using the set of plasmids pGT100, pGTSD100 described in Chapters 2-3, and adding the *alaB* gene to each of these plasmids, we constructed the plasmids pGTALA100, pGTSDALA100 (see Figure 1). The constructions of these plasmids are described below.

4.2.2 Construction of plasmid pGTALA100

Plasmid pJAL198, containing the *alaB* gene, was digested with SmaI/HincII which released a 1.7 kb fragment containing the *alaB* gene. pGT100, containing the glycogen synthesis genes *glgC* and *glgA* was linearized with AflII, and treated with Klenow enzyme to create blunt ends. The AflII fragment of pGT100 was ligated with the 1.7 kb fragment from pJAL198. to create the plasmid pGTALA100. The orientation of the resulting plasmids was checked with restriction digests and used to isolate the plasmid pGTALA100 which contained the *alaB* gene downstream of the glycogen synthesis genes and in the same orientation as the glycogen synthesis genes.

4.2.3 Construction of plasmid pGTSDALA100

Plasmid pJAL198, containing the *alaB* gene, was digested with SmaI/HincII which released a 1.7 kb fragment. This 1.7 kb fragment was ligated with BsaAI-digested pGTC12. The orientation of the resulting plasmids was checked with restriction digests and used to isolate the plasmid pDALA100. Plasmid pDALA100 contained part of the *glgA* gene, the entire *glgP* gene and the *alaB* gene. pDALA100 was digested with SphI, treated with Klenow enzyme to create blunt ends and then partially digested with NcoI. The 5.6 kb fragment was purified. Plasmid pGT100 was digested with PstI, treated with T4 DNA polymerase to blunt the 3' overhanging ends and then digested with NcoI. This released a 7.7 kb fragment. The 7.7 kb fragment and the 5.6 kb were ligated to create the plasmid pGTSDALA100. Plasmid pGTSDALA100 contains the tac promoter followed by the *glgC-glgA-glgP* operon. The *alaB* gene fragment is inserted downstream of the *glgC-glgA-glgP* operon in the same orientation as the operon.

4.2.4   Medium and cultivation conditions

Clark-Maaløe minimal medium (Clark and Maaløe, 1967) was used in this study because the experiments required medium free of precipitated salts. Clark- Maaløe medium contains 136 mM Na^+, 30 mM NH_4^+, 22 mM K^+, 2.1 mM Mg^{2+}, 90 µM Ca^{2+}, 1.7 µM Fe^{3+}, 15 mM SO_4^{2-}, 42 mM $H_2PO_4^-$, 22 mM HPO_4^{2-}, and 56 mM Cl^-. The resulting pH of the medium is 7.0. 2.0 g $(NH_4)_2SO_4$, 6.0 g Na_2HPO_4, 3.0 g KH_2PO_4, 3.0 g NaCl, 0.011 g Na_2SO_4 was dissolved in 200 ml water and called solution A. Solution B contained 0.2 g $MgCl_2$, 0.01 g $CaCl_2$, and 0.0005 g $FeCl_3$ dissolved in 800 ml water. Solution B contains the metals in dilute solution while solution A is a concentrated solution of the phosphates and other readily water-soluble salts. A and B are mixed after autoclaving by pouring A into B. No precipitate is formed.

For reasons explained in the Results section, 5 g/l glucose and 2 g/l fructose were used as carbon sources. 70 mg/l carnosine was also added. The shake-flask experiments were carried out in 250 ml medium contained in 1 L flasks. Innova 4000 shaker (New Brunswick Scientific, Inc, New Jersey) was used with temperature set at 37.0 °C and the shaker speed set at 275 rpm.

4.2.5   Assay of glutamate

1.0 ml of culture from the bioreactor were sampled and centrifuged. Ammonium ions were removed by adjusting 200 µl of the supernatant to pH 9.0 and lyophilizing the solution in a speed-vac. After resuspending the lyophilized sample in 200 µl water, glutamate was assayed enzymatically with glutamate dehydrogenase and NAD^+ according to the protocol described in (Bernt and Bergmeyer, 1974). The concentration of the assay mixture components were 300 mM glycine, 250 mM hydrazine, 1.0 mM ADP and 1.6 mM β-NAD^+. Glutamate dehydrogenase (L-Glutamate:NAD oxidoreductase, deaminating; EC 1.4.1.3) was added to catalyze the following reaction:

$$\text{L-glutamate} + NAD^+ + H_2O \leftrightarrow \alpha\text{-ketoglutarate} + NADH + NH_4^+$$
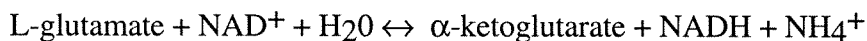
The increase in NADH, as measured by absorbance at 340 nm, is proportional to the amount of glutamate. A calibration curve was constructed using glutamate standards and is shown in Figure 2.

4.2.6   Assay of intracellular cAMP

10 ml of culture from bioreactor were sampled and filtered rapidly through a 0.45 micron filter (type HA, Millipore Corp.) by vacuum filtration. The filters were immediately

suspended in 2.0 ml of 0.1 N HCl. The time delay between withdrawing sample from the bioreactor and immersing the filter in HCl was 30 to 45 seconds. The tube containing the filter and HCl were immediately incubated for 10 min in boiling water. After incubation, 1.2 ml of the liquid was withdrawn and lyophilized in a speed-vac. The lyophilized extract was suspended in 200 μl of 50 mM acetate buffer at pH 4.0. Samples of the extracts were assayed for their concentration of cAMP by using a protocol kindly provided by Amersham Corp., Arlington Heights, IL which is similar to the protocol used in their commercial cyclic AMP[^{3}H] assay kit (catalog number TRK.432). The assay involves competitive binding of cAMP and [^{3}H] cyclic AMP to cyclic AMP dependent kinase. The binding assay was done in a total volume of 200 μl and the final concentrations of the constituents of the assay mixture were: 50 mM Tris pH 7.5, 4 mM EDTA, [^{3}H] cAMP (stock concentration as obtained from New England Nuclear Corp., Boston, MA is 30-50 Ci/mmol)5 μl, 3':5' cyclic AMP dependent protein kinase (Sigma catalog number P5511) 5 μg, and assay sample 50 μl. For calibration, instead of the assay sample a known amount of standard cAMP (sodium salt) was added. The mixture was incubated at 4° C for 2 hours. After incubation, 100 μl of charcoal suspension (3.5% acid washed charcoal in 50 mM Tris pH 7.5, 4 mM EDTA, 2% BSA) was added. The tubes were centrifuged for 1 minute, 200 μl of supernatant was withdrawn and placed in scintillation vial for counting ^{3}H. The ratio of ^{3}H counts in the tube in which no standard cAMP is added to counts in the tube to which standard cAMP is added is linearly proportional to the amount of cAMP in the assay tube.

4.3 Results

The aim of this study was to study the effect of glycogen overproduction and degradation on production of amino acids. To assay for amino acids, bacterial cultures have to be grown in minimal medium. In minimal medium containing glucose as the sole carbon source, TA3476 did not grow well after the addition of IPTG. A series of dual carbon sources were investigated: glucose-fructose, glucose-lactate, glucose-succinate, and glucose-glycerol. Cultures of TA3476:pGTALA100 were grown in Clark-Maaløe medium containing the above combination of carbon sources. IPTG was added to induce the plasmid genes. The final cell densities reached are shown in Table 1. Cultures of TA3476:pGTALA100 grew best in minimal medium containing glucose-glycerol and glucose-fructose. The combination of glucose-fructose was chosen for future experiments. 70 mg/l Carnosine (β-Ala-His) was also added to the medium. Carnosine serves as the histidine source because in TA3476 the histidine biosynthesis and transport genes are deleted. Carnosine is hydrolyzed within the cell to β-alanine and L-histidine. The concentration of alanine produced by hydrolysis of 70 mg/l carnosine is 27 mg/l which is too low to affect or contribute significantly to the concentration of the predominant amino acid, glutamate, produced in this study.

The original aim of this study was to study the effect on alanine production of metabolic engineering of the glycogen biosynthesis and degradation. Alanine is synthesized from pyruvate by the action of the enzyme pyruvate-alanine amino-transferase encoded by the *alaB* gene (Wang et al., 1987). The *alaB* gene was used to construct the plasmids pGTALA100 and pGTSDALA100. These plasmids contained the *alaB* gene along with the glycogen synthesis genes and glycogen synthesis plus degradation genes respectively.

This study was started by analyzing all the amino acids produced by cultures of TA3476:pKQV4 and TA3476:pGTSDALA100. Cultures of TA3476:pKQV4, TA3476:pGTSDAlA100 were grown in shake flasks in Clark-Maaløe minimal medium supplemented with 5 g/l glucose, 2 g/l fructose and 70 mg/l carnosine. IPTG, to a final concentration of 500 μM, was added to both cultures at 6.66 hours. At the end of the batch cultivation (38 hours post inoculation), a complete amino-acid analysis of the fermentation medium was done by chromatography. Representative chromatograms of amino acids found in the culture medium of TA3476:pKQV4 and TA3476:pGTSDALA100 is shown in Figure 3 and Figure 4 respectively. The calculated concentrations of all the amino acids is shown in Table 2. This analysis revealed surprisingly the presence of an unusually large amount of glutamate in TA3476:pGTSDALA100. Other amino acids, including alanine, were not present in significant quantities.

To investigate the surprising increase in glutamate production, we studied the effect of different combinations of genes on glutamate production. In one set of cultivations we studied the glutamate production trajectories in TA3476, TA3476:pKQV4, TA3476:pGT100, TA3476:pGTSD100. In another set of experiments, we studied the glutamate trajectories in TA3476:pGTALA100 and TA3476:pGTSDALA100. pGTALA100 and pGTSDALA100 were created by adding the *alaB* gene to the plasmids pGT100 and pGTSD100, respectively. Cultures of TA3476, TA3476:pKQV4, TA3476:pGT100, TA3476:pGTSD100, TA3476:pALA100, TA3476:pGTALA100, TA3476:pGTSDALA100 were grown in shake flasks in Clark-Maaløe minimal medium supplemented with 5 g/l glucose, 2 g/l fructose and 70 mg/l carnosine. IPTG, to a final concentration of 500 μM, was added to all cultures at 6.66 hours.

We then analyzed enzymatically the glutamate trajectories in all the strains (Figures 5, 6). The maximum level of glutamate produced by cultures of TA3476 is 390 mg/l, TA3476:pKQV4 180 mg/l , TA3476:pGT100 650 mg/l and TA3476:pGTSD100 1110 mg/l, TA3476:pGTALA100 315 mg/l, TA3476:pGTSDALA100 1450 mg/l. Several runs of these shake-flask experiments were repeated. In further duplicate or triplicate runs the maximum glutamate concentrations varied as follows: TA3476: 390 mg/l, 316 mg/l; TA3476:pKQV4: 100, 100 mg/l; TA3476:pGT100: 370 mg/l, 340 mg/l; TA3476:pGTSD100: 940 mg/l; TA3476:pGTSDALA100: 870 mg/l, 880 mg/l.

The glucose consumption trajectories of TA3476:pGT100 and TA3476:pGTSD100 cultures are shown in Figure 7. TA3476 and TA3476:pKQV4 produced no glycogen. The maximum amount of glycogen in TA3476:pGT100 and TA3476:pGTSD100 is about 70 mg/l. This glycogen amount is low compared to the increase of glutamate by 700 mg/l in TA3476:pGTSD100 compared to the plasmid-free strain.

As further controls, cultures of TA3476:pGTSD100 and TA3476:pGTSDALA100 were grown without addition of IPTG. Medium and growth conditions were similar to that described above. The glutamate trajectories are shown in Figure 8. Without induction of the plasmid genes by IPTG, the maximum level of glutamate produced by cultures of TA3476:pGTSD100 is 125 mg/l and by TA3476:pGTSDALA100, 220 mg/l. This proves that induction of the glycogen synthesis and degradation genes in TA3476:pGTSD100 cultures is responsible for the increase in glutamate levels.

We had noted earlier (in chapter 2) that glycogen overproduction may cause an increase in the activity of the gluconeogenic cycle and the TCA cycle. We hypothesized that cAMP, a global regulator of central carbon metabolism, may be involved in causing some

of these changes. To test the hypothesis of correlation between cAMP levels and glycogen overproduction, we conducted measurements of intracellular cAMP concentrations in cultures of TA3476:pGT100 and TA3476:pKQV4 grown in a bioreactor. The cultures were grown in buffered LB medium supplemented with 0.2% glucose. The operating conditions of the bioreactor were the same as the conditions described for bioreactor operation in Chapter 2. Cultures of TA3476:pGT100 were induced by addition of IPTG (final concentration 500 µM) at 3.5 hours and to discount the effect of IPTG on cAMP levels, we added IPTG to cultures of the control strain TA3476:pKQV4 too. Intracellular concentrations of cAMP were measured at various time intervals. The cAMP trajectories are shown in Figure 9. For each time point, the radioactive assay measurements were done on duplicate samples. The errors in the measurement are about ±10%. Within 45 minutes of the addition of IPTG, the cAMP levels in cultures which overproduced glycogen increased while the cAMP levels in the control cultures did not increase. This experiment was repeated twice. An higher peak in cAMP levels, 50 minutes after addition of IPTG was again observed.

The above experiment suggested that there may exist an indirect link between the activity of the glycogen pathway and cAMP levels. cAMP levels increased when glycogen was overproduced. If cAMP levels increase, the flux through the TCA cycle can increase and glutamate production can also increase. Since the previously discussed cAMP assay was carried in LB medium, the results cannot be directly extrapolated to the earlier experiments in this study which were carried out in minimal medium. If cAMP is involved in the increase of glutamate production, then adding exogenous cAMP to the plasmid-free strain TA3476 should result in increase in glutamate. We tested this hypothesis by growing cultures of TA3476 in medium and conditions described earlier. 500 µM cAMP was added

to the cultures at 9.25 hours post-inoculation. The glutamate trajectories produced by cultures of TA3476 when exogenous cAMP is added are shown in Figure 10.

4.4 Discussion

Previous work on metabolic engineering to improve the production of metabolites has mostly focused on the specific biosynthesis pathways involved in the synthesis of the desired metabolite. A few studies have shown that metabolic engineering of the central carbon pathways can also improve the production of metabolites. This study provides an additional example to show that genetic engineering of pathways far removed from a particular metabolite pathway can improve the production of a metabolite. We showed that genetic engineering of the glycogen biosynthesis and degradation pathway surprisingly improved the production of the amino-acid glutamate.

Our initial assumption in constructing a system which allowed controlled synthesis and degradation of glycogen was to trap the unwanted carbon flux in glycogen and then reutilize the stored carbon in the glycogen in the synthesis of the desired metabolite. In the present study, glycogen was produced in low quantities (ca. 70 mg/l) compared to the amount of increase in glutamate in TA3476:pGTSD100 (700 mg/l). Therefore direct channeling of the carbon in the stored glycogen to glutamate through the glycolytic pathway and the TCA cycle is not the dominant cause in the increase of glutamate levels.

When cultures of TA3476 carried the control plasmid pKQV4, the production of glutamate decreased to about half the levels produced by cultures of the plasmid-free strain. When only the glycogen synthesis genes were amplified in cultures of TA3476:pGT100, in

two experimental runs the glutamate levels increased from the levels produced by TA3476:pKQV4 cultures and reached levels similar to that produced by the plasmid-free strain; in one experimental run, the production of glutamate increased a further 50% over the plasmid-free strain levels.

When the degradation gene was also amplified, the 3-fold increase in glutamate compared to the plasmid-free strain (and 6-fold compared to TA3476:pKQV4) suggests that the addition of the degradation enzyme activity plays a role in the increase of the glutamate. We showed in Chapter 3 that plasmid pGTSD100, after addition of IPTG, coexpresses the glycogen synthesis enzyme, ADPG pyrophosphorylase, and the degradation enzyme, glycogen phosphorylase. The coexpression of the two enzyme activities led to a growth phase in which glycogen synthesis and degradation existed simultaneously. The data from chapter 3 (Figure 3), shows a decrease in glycogen synthesis rate in TA3476:pGTSD100 after induction compared to TA3476:pGT100. This suggests the existence of a futile cycle between glycogen synthesis and degradation. Glycogen synthesis requires the use of ATP, and thus the operation of a futile cycle will waste cellular ATP supply. The higher utilization of ATP may force the cell to maintain a higher catabolic turnover rate. Glutamate is synthesized from α-ketoglutarate, an intermediate of the TCA cycle. A higher catabolic turnover rate could possibly lead to an increased flux through the TCA cycle and an increased rate of glutamate production. In a previously reported metabolic engineering strategy (Rogers and Hiller, 1990), a futile cycle with phosphofructokinase was created in baker's yeast by expressing cloned fructose 1.6-bisphosphatase from a yeast glycerophosphate dehydrogenase promoter that is induced by glucose. This strain consumed more ATP and produced 20 to 25% more CO_2 than the wild-type.

The results do not provide any conclusive evidence that the addition of the alanine-glutamate amino-transferase increases the production of glutamate over the levels produced by amplifying the glycogen synthesis and degradation genes. Although one run with cultures of TA3476:pGTSDALA100 did show a 30% increase in the glutamate levels, two other runs produced glutamate concentrations similar to the concentrations in TA3476:pGTSD100.

Although Figure 10 shows a increase in glutamate levels by 115 mg/l within 30 minutes of adding cAMP, the experimental errors inherent in a single data point (glutamate concentrations determined at 9.25 hours or 9.75 hours) does not allow us to firmly conclude that cAMP played a role in increasing the glutamate levels in TA3476 cultures. But our previous observations on changes in end-products when glycogen is overproduced in TA3476:pGT100 [the increase in pyruvate uptake (Chapter 2, Figure 8a), specific CO_2 production rate (Chapter 2, Figure 7a).,and biomass (Chapter 2, Figure 6A)] suggests that the involvement of cAMP in affecting cell physiology in cultures of TA3476:pGT100 and TA3476:pGTSD100 remains a viable hypothesis to partially account for the observation in this study of increase in glutamate levels in TA3476:pGT100 and TA3476:pGTSD100 cultures compared to TA3476:pKQV4 cultures. cAMP can play a role in all of these pleiotropic effects of glycogen overproduction and degradation by affecting the TCA flux.

This example in metabolic engineering demonstrates that genetic alteration of the central carbon metabolic pathways can alter the pattern of carbon fluxes. In the present study the improvement of metabolite production appears to have been caused mainly by the existence of a futile cycle between glycogen synthesis and degradation. This example also highlights the complexity of metabolic changes which may occur from genetic engineering of the central carbon pathways, and the occurrence of physiological responses that cannot

be anticipated. *A priori*, we could not have anticipated that changes in the glycogen synthesis and degradation would result in improvement of glutamate production.

4.5 References

Backman K, O'Connor MJ, Maruya A, Rudd E, McKay D, Balakrishnan R, Radjai M, DiPasquantinio V, Shoda D, Hatch R, Venkatasubramanian K (1990) Genetic engineering of metabolic pathways applied to the production of phenylalanine. Ann NY Acad of Sci 589:16-24

Bailey JE (1991) Toward a science of metabolic engineering. Science 252:1668-1675

Bernt E, Bergmeyer HU (1974) L-Glutamate:UV assay with glutamate dehydrogenase and NAD. In: Bergmeyer (ed) Methods of enzymatic analysis, Academic Press, New York, pp 1704-1707

Cameron DC, Tong IT (1993) Cellular and metabolic engineering: An overview. Appl Biochem Biotechnol 38:105-140

Choi YL, Kawamukai M, Utsumi R, Sakai H, Komano T (1989) Molecular cloning and sequencing of the glycogen phosphorylase gene from *Escherichia coli*. FEBS Letter 243:193-198

Clark DJ, Maaløe O.(1967) DNA replication and the division cycle in *Escherichia coli*. J Mol Biol 23:99-112

Ingram LO, Conway T, Clark DP, Sewell GW, Preston JF (1987) Genetic engineering of ethanol production in *Escherichia coli*. Appl Environ Microbiol 53:2420-2425

Katsumata R, Yokoi H, Kino K (1988) L-Glutamic acid and L-proline, and it's manufacture with recombinant Corynebacterium and Brevibacterium. Jpn. Kokai Tokkyo Koho JP 63/102692, A2; Chem Abstr 1988 109:188807q

Katsumata R, Yokoi H, Kino K (1988) L-Glutamic acid and L-proline, their recombinant manufacture with Corynebacterium and Brevibacterium. Jpn. Kokai Tokkyo Koho JP 63/119688, A2; Chem Abstr 1989 110:113201z

Morinaga Y, Takagi H, Ishida M, Miwa K, Sato T, Nakamori S, Sano K (1987) Threonine production by co-existence of cloned genes coding homoserine dehydrogenase and homoserine kinase in *Brevibacterium lactofermentum*. Agric Biol Chem 51:93-100

Nakamori S, Ishida M, Takagi H, Ito K, Miwa K, Sano K (1987) Improved l-threonine production by the amplification of the gene encoding homoserine dehydrogenase in *Brevibacterium lactofermentum*. Agric Biol Chem 51:87-91

Neidhardt FC, Ingraham JL, Schaecter M (1990) Physiology of the bacterial cell, Sinauer Associates Inc, Sunderland pp 133.

Pollock TJ, Thorne L (1988) Enhanced manufacturing of santhan gum with genetically modified Xanthomonas. Eur Pat Appl EP 287363; Chem Abstr 1989 105:93587z

Preiss J (1984) Bacterial glycogen synthesis and its regulation. Annu Rev Microbiol 38:419-458

Preiss J, Romeo T (1989) Physiology, Biochemistry and Genetics of Bacterial Glycogen Synthesis. Adv Microbial Physiol 30:1183 - 1238

Rogers DT, Hiller E (1990) In: Heslot H (ed) Proceedings of the sixth international symposium on genetics of industrial microorganisms, vol 1, Societé française de microbiologie, Strasbourg, pp 533-544

Romeo T, Kumar A, Preiss J (1988) Analysis of the *Escherichia coli* glycogen gene cluster suggests that catabolic enzymes are encoded among the biosynthetic genes. Gene 70: 363 - 376

Sano K, Ito K, Miwa K, Nakamori S (1987) Amplification of the phosphoenolpyruvate carboxylase gene of *Brevibacterium lactofermentum* to improve amino-acid production. Agric Biol Chem 51: 597-599

Wang MD, Buckley L, Berg CM (1987) Cloning of genes that suppress an *Escherichia coli* K-12 alanine auxotroph when present in multicopy plasmids. J Bacteriol 169:5610-5614

Yu F, Jen Y, Takeuchi E ,Inouye M ,Nakayama H ,Tagaya M, Fukui T (1988) α-glucan phosphorylase from *Escherichia coli*. Cloning of the gene, and purification and characterization of the protein. J Biol Chem 263:13706-13711

4.6 Tables

Table 1

Cultures of TA3476:pGTALA100 were grown in a shaker in 5 ml Clark-Maaløe minimal medium along with dual-carbon sources showed in column 1. The starting glucose concentration was 5 g/l and the concentration of the second carbon source was 2 g/l. 70 mg/l L-Carnosine and 50 mg/l ampicillin were also added to the medium. The cell densities at the end of the cultivation (24 hrs.. post-inoculation) are shown in column 2.

Carbon source	Final O.D.$_{600}$
Glucose-Glycerol	1.29
Glucose-Fructose	1.18
Glucose-Succinate	0.84
Glucose-Lactate	0.502

Table 2

Amino Acid	Retention time, (min)	Glutamate (g/l) TA3476:pGTALA100	Glutamate (g/l) TA3476:pKQV4
Aspartic acid	2.03	0.003	0.000
Glutamic acid	2.47	0.156	0.869
Serine	3.15	0.003	0.000
Glycine	3.57	0.003	0.000
Histidine	3.77	0.004	0.000
Arginine	4.37	0.004	0.000
Threonine	4.82	0.008	0.007
Alanine	5.2	0.007	0.003
Tyrosine	7.83	0.005	0.000
Valine	8.78	0.003	0.000
Methionine	9.05	0.003	0.000
Cysteine	9.88	0.003	0.000
Isoleucine	10.9	0.004	0.000
Leucine		0.003	0.000
Phenylalanine		0.004	0.000
Lysine		0.001	0.013

4.7 Figure captions

Figure 1: Plasmid maps of pGTALA100 and pGTSDALA100.

Figure 2: Calibration curve for assay for glutamate

Figure 3: Chromatogram of amino acids found in the culture medium of TA3476:pKQV4. Cultures of TA3476:pKQV4 were grown in a 1L shake flask containing 250 ml of Clark-Maaløe medium supplemented with 5g/L glucose, 2 g/L fructose, 70 mg/L carnosine, and 50 mg/L ampicillin. The temperature of the cultivation was set at 37.0 ° C. The sample for the analysis was withdrawn at 34 hours post-inoculation and the chromatographic run was conducted by the Microchemical facility at Caltech.

Figure 4: Chromatogram of amino acids found in the culture medium of TA3476:pGTSDALA100. The growth conditions of the cultures were similar to that described for Figure 3. IPTG to a final concentration of 500 μM was added at 10.66 hours post-inoculation. The sample for the chromatographic analysis was withdrawn at 38 hours post-inoculation.

Figure 5: Trajectories of optical density (at 600 nm) and glutamate concentration in cultures of TA3476, TA3476:pGT100 and TA3476:pGTSD100. Growth condition were as described in Figure 3. Plasmid-bearing strains were induced at 10.66 hours.

Figure 6: Trajectories of optical density (at 600 nm) and glutamate concentration in cultures of TA3476:pGTALA100 and TA3476:pGTSDALA100. Growth condition were as described in Figure 3. Plasmid-bearing strains were induced at 10.66 hours.

Figure 7: Glucose consumption profiles in cultures of TA3476:pGT100 and TA3476:pGTSD100.

Figure 8: Glutamate profiles in control cultures of TA3476:pGTSD100 and TA3476:pGTSDALA100 when no IPTG was added. Growth condition were as described in Figure 3. Plasmid-bearing strains were induced at 10.66 hours.

Figure 9: Intracellular concentration of cAMP in cultures of TA3476:pKQV4 and TA3476:pGT100. The cultures were grown in 2500 ml of phosphate-buffered LB in a BioFlo III bioreactor. The operating conditions of the bioreactor were set at: temperature, 37° C; air flowrate 2 L/min, agitation, 400 rpm, pH 7.0. IPTG was added to both the cultures at 3.5 hours as indicated by the arrow.

Figure 10: Glutamate profile in cultures of TA3476. Exogenous cAMP to a final concentration of 500 μm was added to the cultures at 9.25 hours post-inoculation. The growth conditions were as described in figure 3 except no ampicillin was added to the medium.
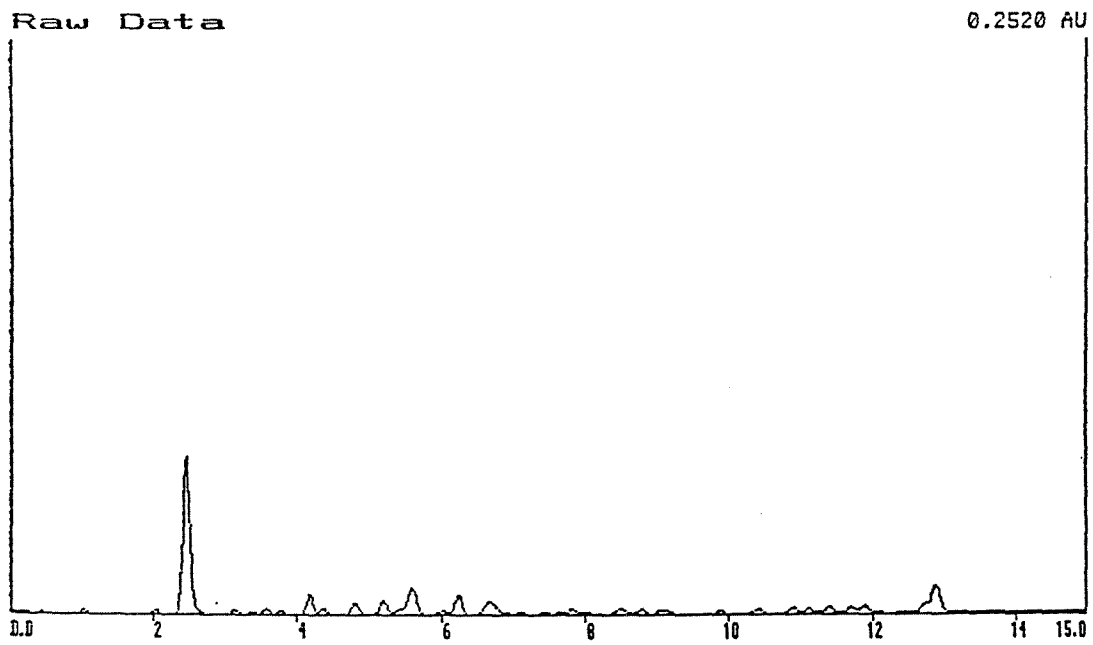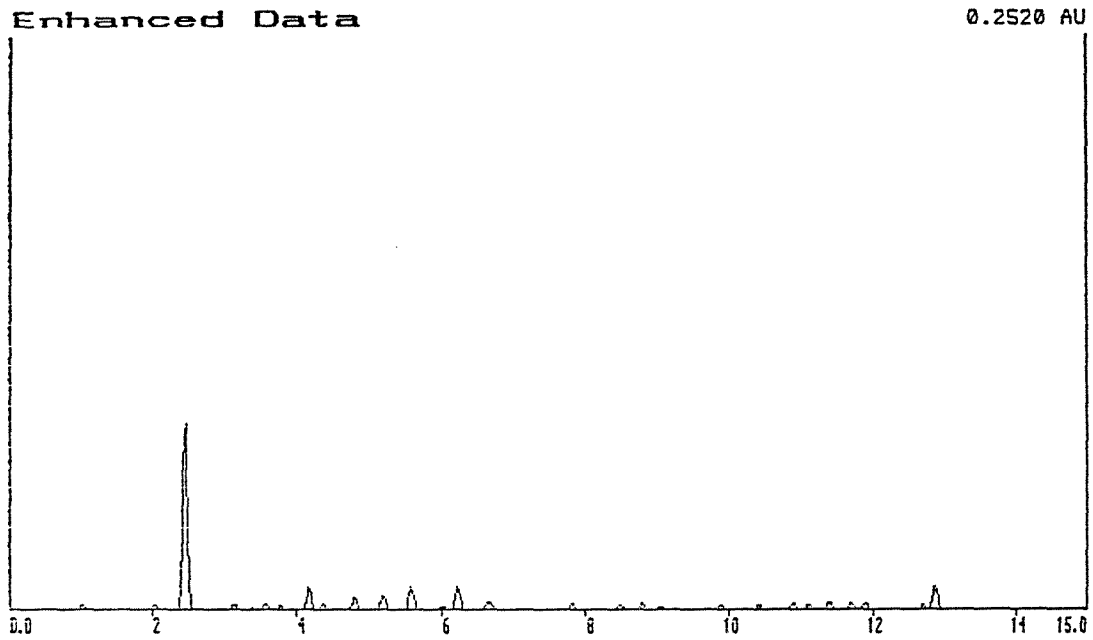
Figure 1

Figure 2

glutamate conct (g/l) = -0.0012 + 0.029805*ΔA_{340}

R= 0.99879

ΔA_{340}

glutamate (g/l)
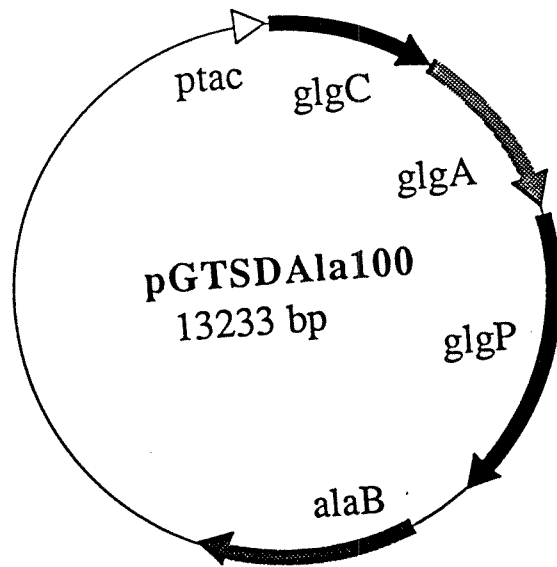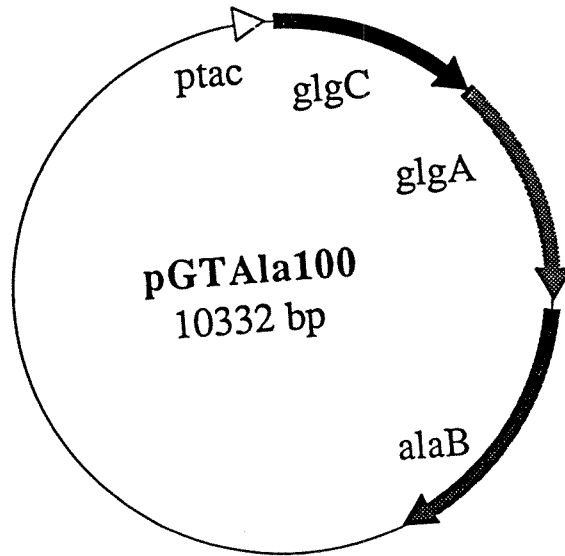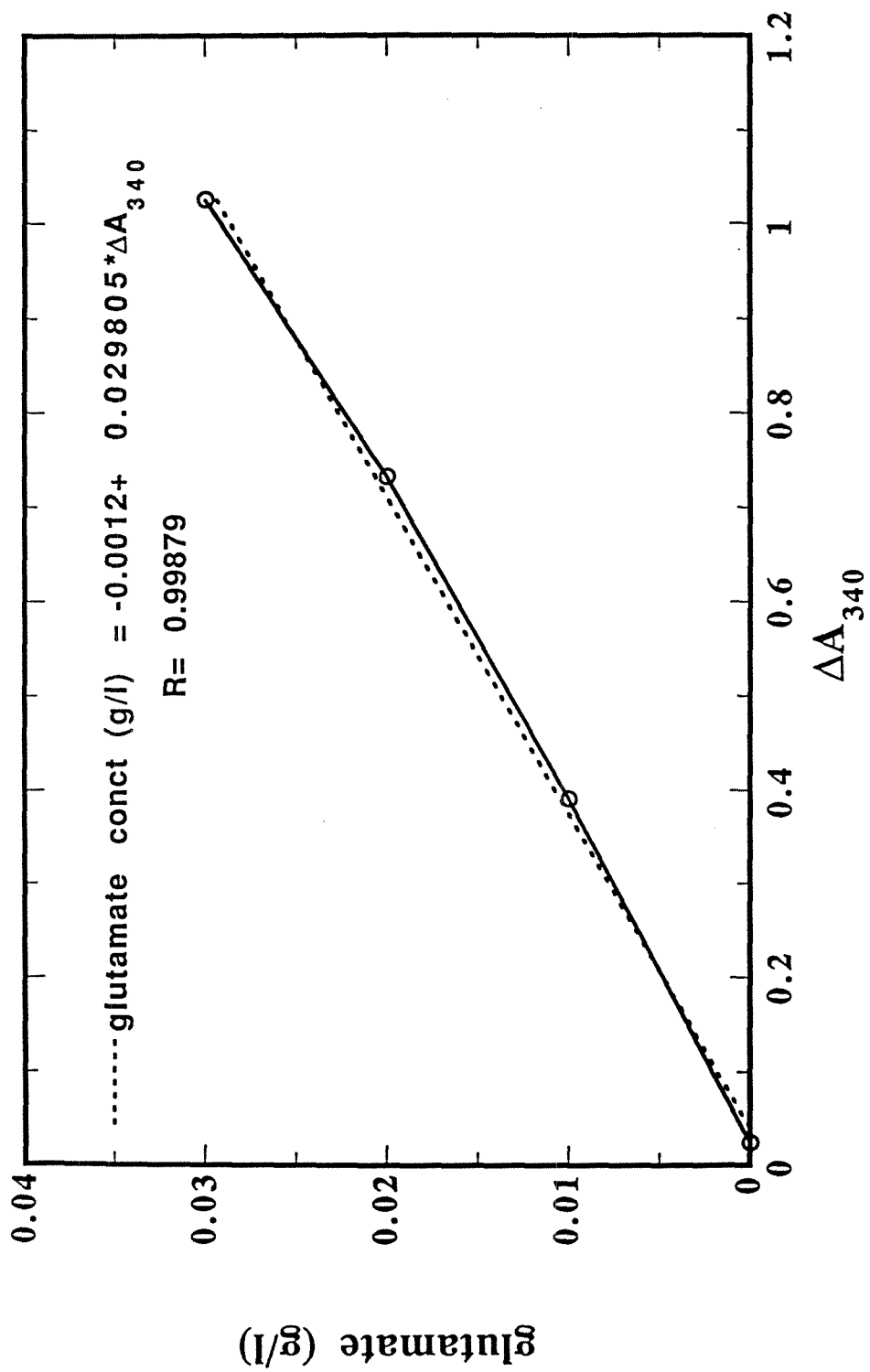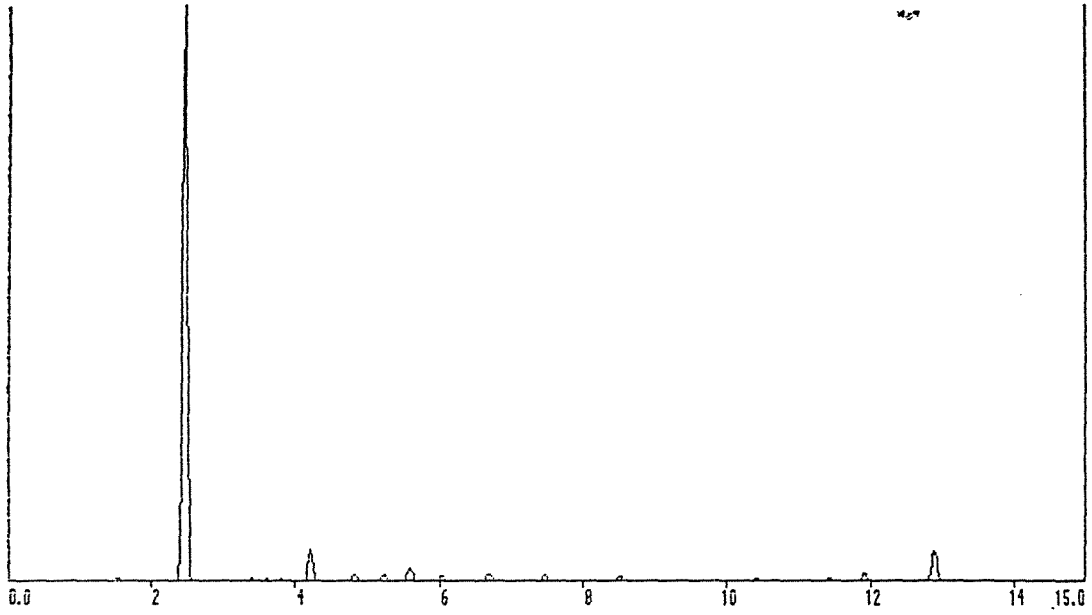
Figure 3

Chromatogram Report

Sample ID: 16701004   (initiated 6/16/93 5:40pm)   BASELINE CORRECTED

Enhanced Data   0.2520 AU

Raw Data   0.2520 AU

Figure 4

Chromatogram Report

Sample ID: 16701007   (initiated   6/16/93 8:08pm)   BASELINE CORRECTED

Enhanced Data   0.2520 AU
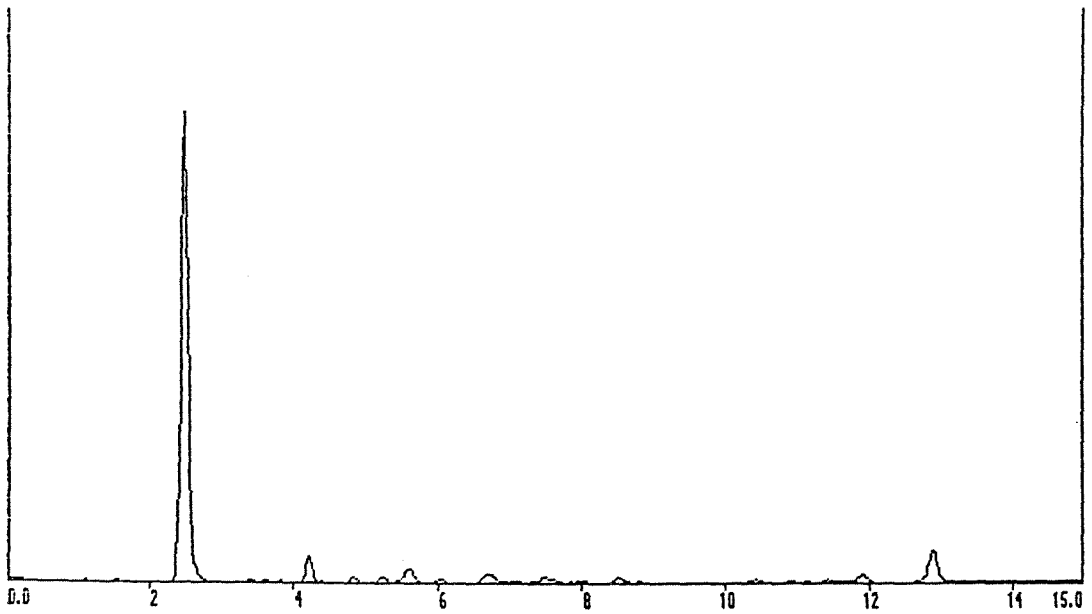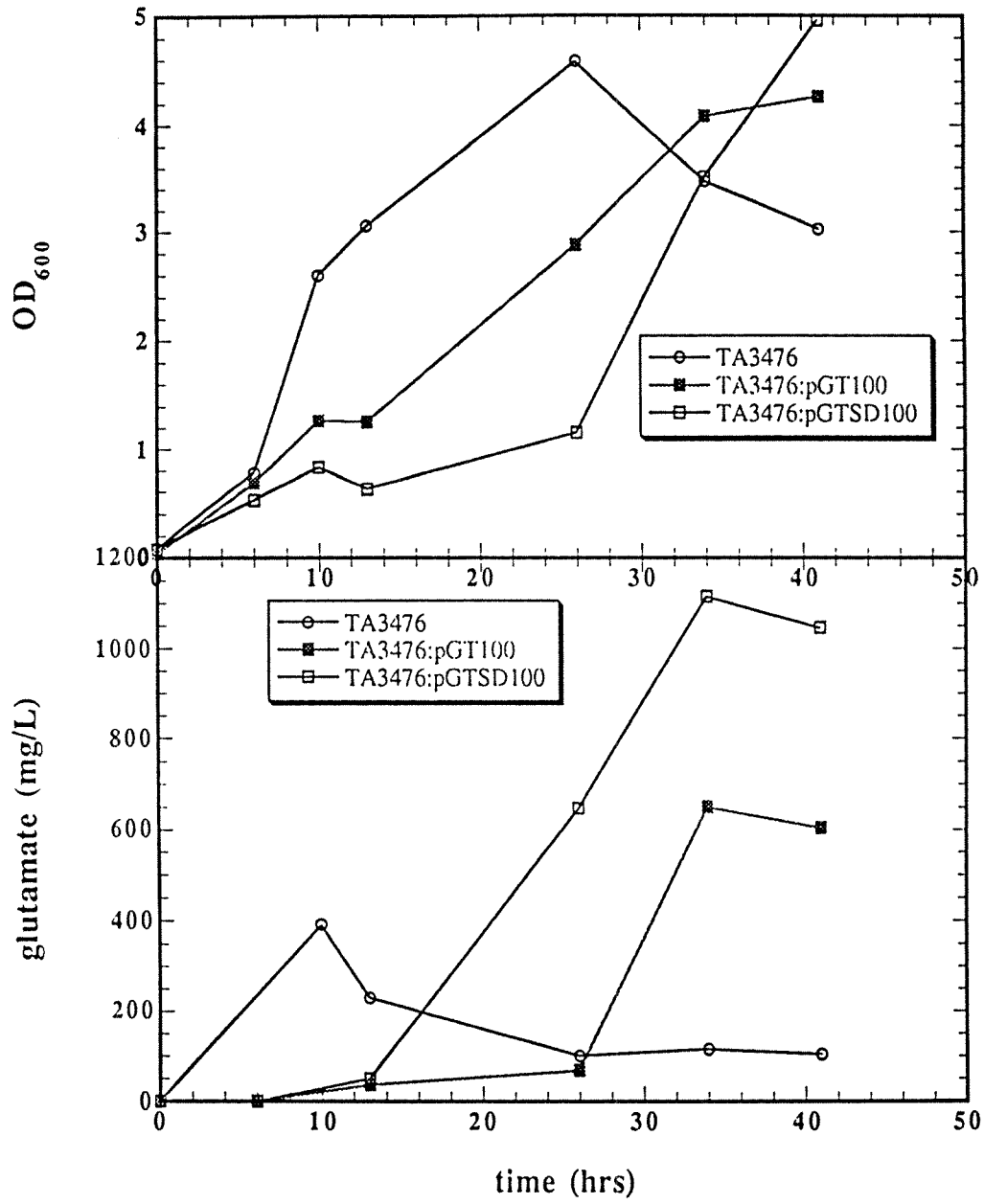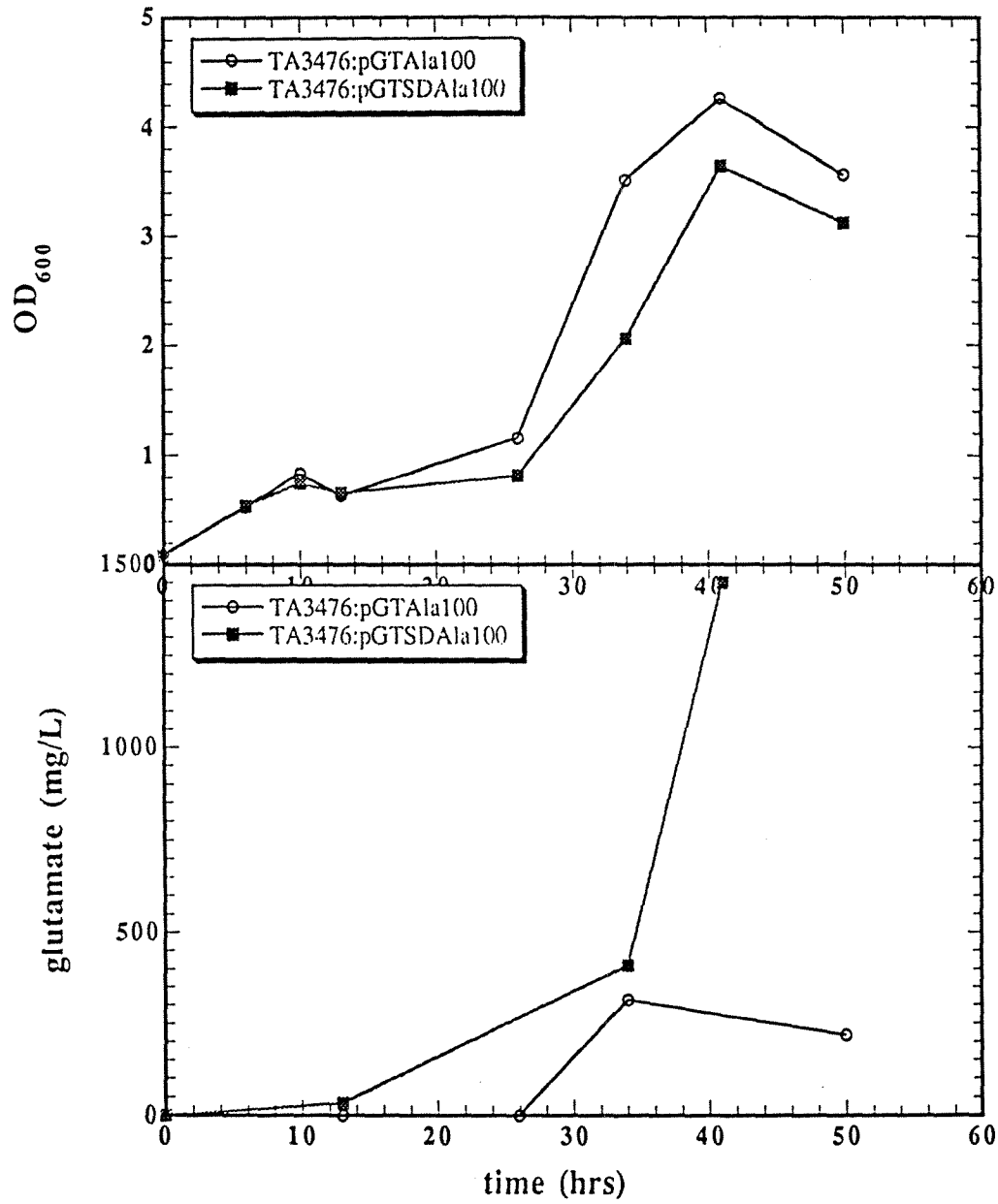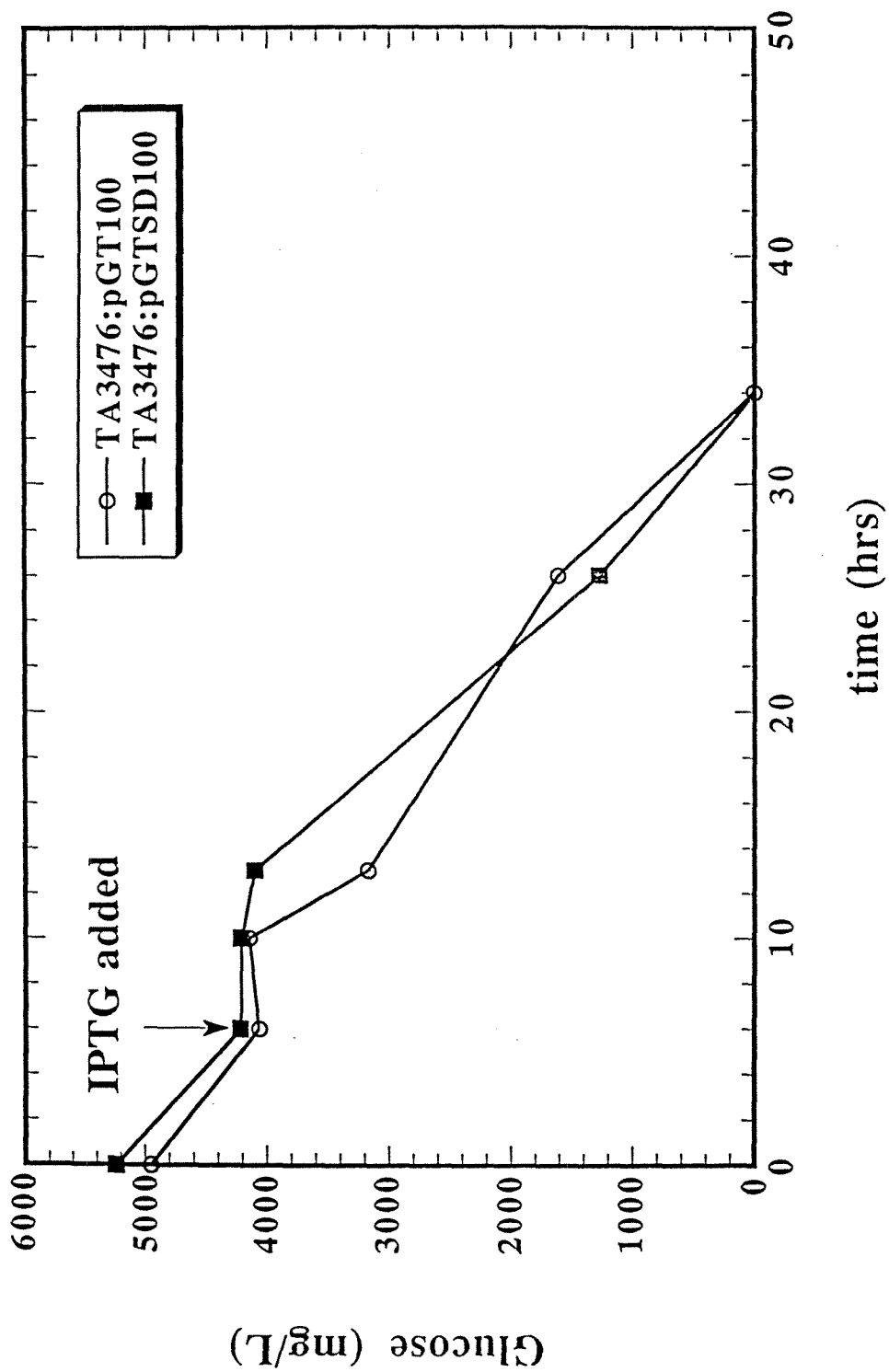

Raw Data   0.2520 AU

Figure 5

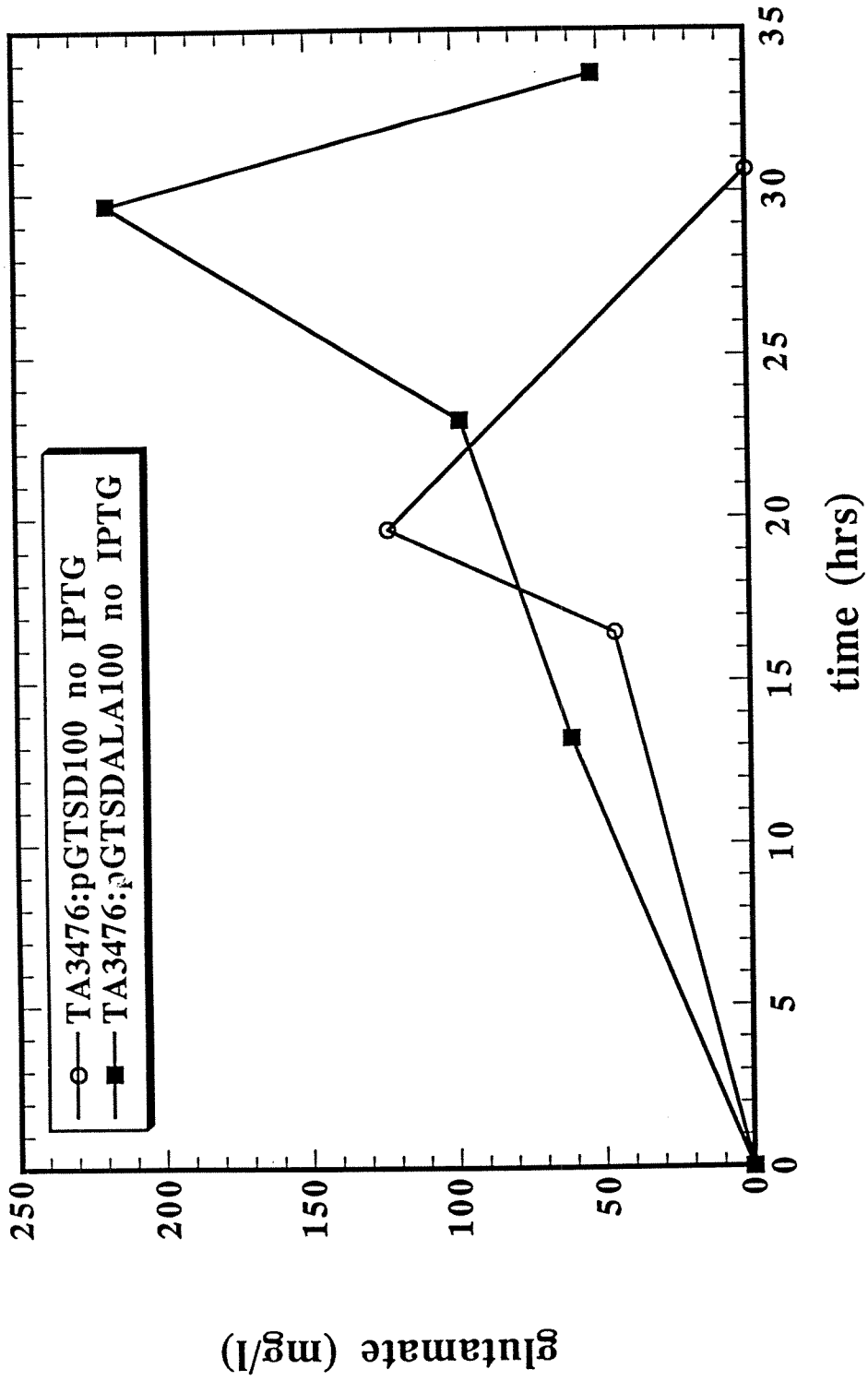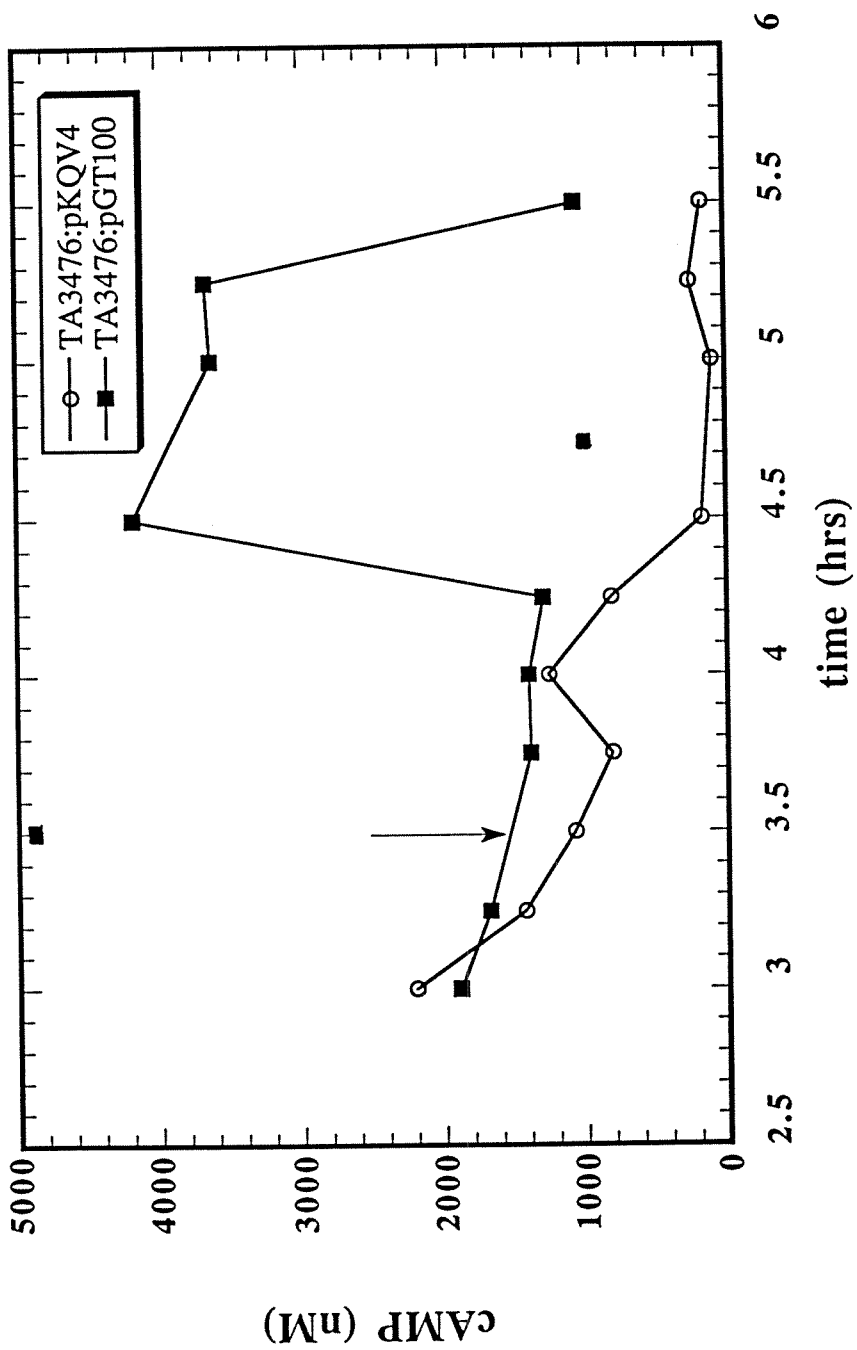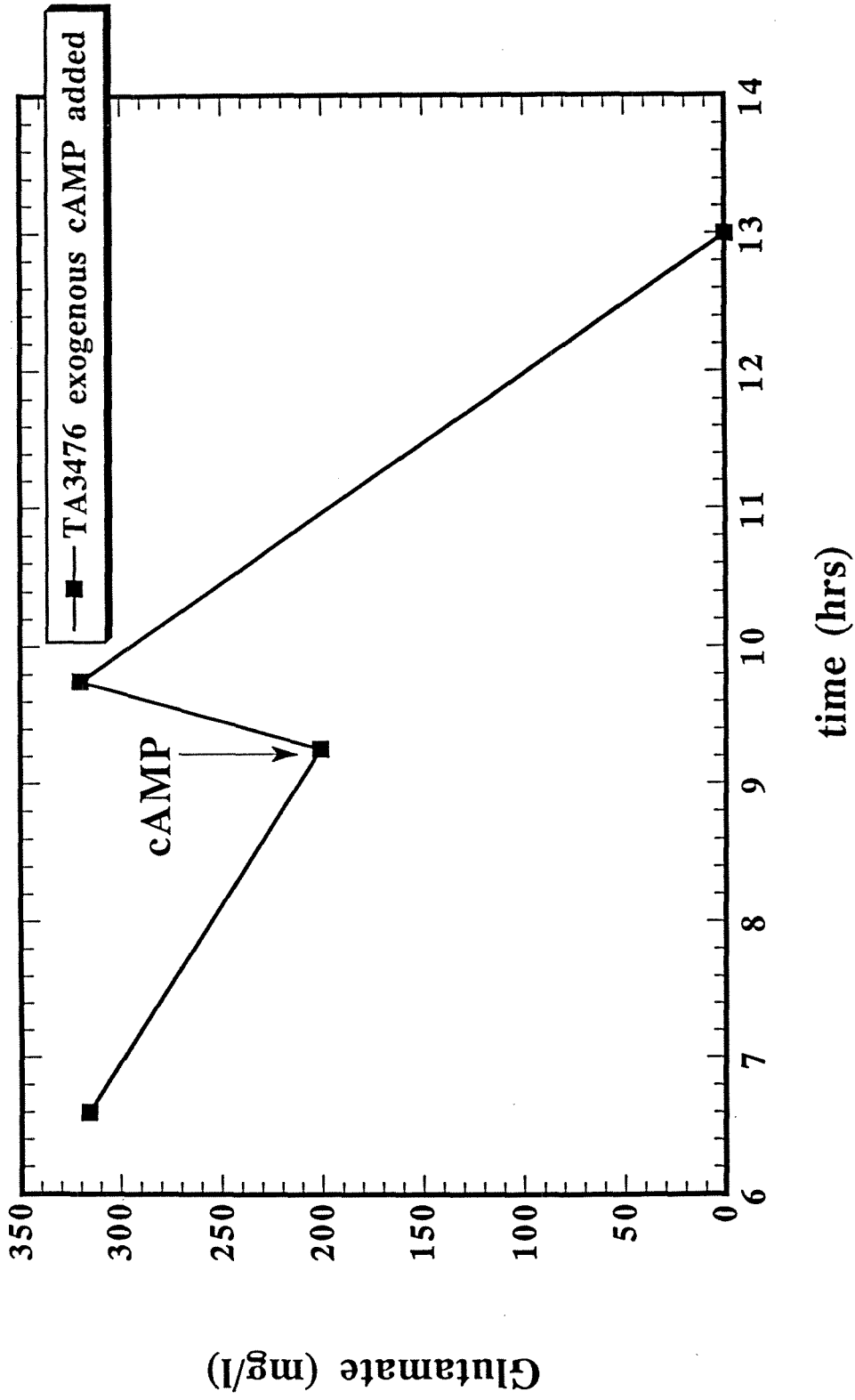# Figure 6

Figure 7

Figure 8

Figure 9

Figure 10

Chapter 5

Conclusions and future work

5.1 Conclusions

The commercially useful products made by *Escherichia coli* and other micro-organisms can be classified broadly into proteins and small molecules. Genetic engineering offers powerful tools to improve the production of these products. In this thesis, we created and studied metabolic engineering strategies in *E. coli* with potential to improve the production of both proteins and small molecules.

The pathway for central carbon metabolism provides precursors for cell biosynthesis and metabolite synthesis along with ATP and NADH and also NADPH if the pentose phosphate pathway is included. We investigated the metabolic engineering of one of the branches of the central carbon pathways: the pathway of glycogen synthesis and degradation. We were motivated in selecting the glycogen pathway for genetic manipulation by the literature on acetate production in *E. coli*. The literature reviewed in Chapter 1 indicates that in aerobic cultures the uptake of nutrients occurred faster than the utilization of the precursors, formed from the nutrients, in making biomass and energy. We decided to sequester the excess carbon in glycogen which is a storage polymer. We also devised vectors to degrade the sequestered glycogen. The effects, possible causes of the effects, and potential applications of the sequestering of carbon in the form of glycogen, sometimes combined with engineered degradation of the sequestered glycogen, have been the subject of this thesis.

This manipulation of the glycogen pathway yielded practically useful results. The sequestering of glycogen was achieved by overamplifying the genes of the glycogen biosynthetic pathway. This sequestering of glycogen improved culture densities by 20%.

When glycogen was sequestered and then degraded, by overamplifying the genes for glycogen synthesis and degradation, then glutamate production was increased almost 3-fold compared to the plasmid-free strain.

The original reasoning behind using glycogen as a buffer to store excess carbon was that it would reduce the conversion of glycolytic precursors to unwanted acid products. Later degrading the sequestered glycogen when cells become carbon-limited ought to redirect the stored carbon to biomass and metabolites.

The reasons behind the effects of our metabolic engineering cannot be explained totally by the above hypothesis. The first surprise was that sequestering of glycogen without degradation of glycogen increased cell growth. The carbon stored in glycogen was not degraded significantly by the chromosomally encoded glycogen phosphorylase. Although acid by-products such as pyruvate did decrease, the pyruvate levels with and without glycogen overproduction were not high enough to cause toxic effects. The second surprise was that the co-amplification of the glycogen synthesis and degradation genes increased glutamate production. The amount of increase in glutamate (nearly 1000 mg/L) was far greater than the observed maximum level of glycogen (10 mg/L). The storage of carbon in glycogen followed by redirection of degraded glycogen precursors to glutamate cannot be the dominant cause.

When glycogen was sequestered, we observed changes in some of the secreted end-products. We observed that, after overproduction of glycogen, uptake of the previously secreted pyruvate was increased with respect to the control strain, and the CO_2 production rate was also increased. These dual observations suggest an increased activity of the gluconeogenic pathways or the TCA cycle. The increase in glutamate, when

glycogen sequestering was combined with degradation, also indicate an increase in TCA flux.

Comparison of cAMP levels with and without glycogen overproduction indicate a higher level in cAMP after glycogen is overproduced. There appears to be a tentative link, though not conclusive, between cAMP synthesis and glycogen synthesis pathway. cAMP is a global regulator of central carbon metabolism including many genes of the TCA cycle enzymes. By affecting the TCA flux, cAMP may be one of the causes behind the pleiotropic effects of glycogen overproduction and degradation. We do not know how the glycogen pathway influences the pathway producing cAMP excepting the observation that these two pathways utilize a common intermediate, glucose-6-phosphate.

In future work, the link between cAMP and glycogen pathway can be investigated in more depth. Vectors with cAMP-responsive promoters and a reporter gene can be co-amplified with the glycogen synthesis genes. The observation of a correlation between the amounts of reporter protein and glycogen will provide further direct evidence of the link between cAMP and the glycogen pathway. In principle, the addition of exogenous cAMP to cultures of glycogen overproducing strains and controls can also help determine whether cAMP levels influence cell physiology and yields. Cell yield comparisons are best done in the controlled environmental conditions of a bioreactor but the high cost of adding expensive cAMP to bioreactor cultures is a potential constraint.

5.2 Future work in metabolic engineering of central carbon metabolism

Another strategy for overcoming the inhibition of *E. coli* growth by acetate is to isolate mutants under selection conditions that favor acetic acid-tolerant cells. In principle, a number of mutations leading to acetic acid tolerance can be envisioned (Baronofsky et al. 1984). For example, although mutant cells with membrane lipids sufficiently altered to be significantly more impermeable to acetic acid might be obtainable, acetic acid that is generated inside the cell must exit and this makes such strains unlikely. A proton-driven active transport system for acetic acid efflux, like the lactate exporter in streptococci (Otto et al. 1980), could function to alkanize the cell's interior. It is unlikely that such systems can be produced by mutations. Such transport systems will have to be installed in *E. coli* by metabolic engineering using heterologous genes.

Central metabolic pathways are complex and interact with many pathways. The possible increase in TCA cycle activity due to glycogen overproduction suggests that though global control and branch point control imposes constraint on flux changes, there does exist some potential for increasing flux through the important TCA cycle. In future work, the challenge of improving TCA cycle activity can be explored. During exponential growth under glucose repressive conditions, the levels of the TCA cycle enzymes are low (Gray 1966; Hollywood 1976). It has been observed that the levels of α-ketoglutarate dehydrogenase and succinate dehydrogenase are especially low during glucose repressive conditions (Hollywood 1976).

Genetic analysis of the citric acid cycle has shown that the genes for citrate synthase, succinate dehydrogenase, a-ketoglutarate dehydrogenase and succinyl-CoA

synthetase are organized in a cluster (Miles and Guest, 1987). cAMP receptor protein (CRP) binding sites have been identified in some of the promoters of these genes. Catabolite repression is probably responsible for the low levels of the enzymes in this cluster during repressive conditions. Removing the CRP binding sites from the promoter is one potential means of increasing TCA cycle activity during early phase of aerobic growth. For bioreactor fermentations, an ideal *E. coli* industrial strain would use the TCA cycle obligately instead of the mix of fermentation pathways and TCA cycle that *E. coli* currently uses.

Other challenges in central carbon metabolism include the development of *E. coli* strains that can use electron acceptors other than O_2 for respiration. After the O_2/H_2O redox couple, the NO_3^-/NO_2^- is next best in terms of efficiency as measured by ΔG. *E. coli* does use NO_3^- as a electron acceptor during anaerobic respiration but the reduced product NO_2^- is toxic at high concentrations. There exist micro-organisms such as *Paracoccus denitrificans* and *Pseudomonas aeruginosa* which can reduce NO_2^- to N_2. All the genes and proteins involved in this pathway are yet to be cloned and characterized. After they are cloned, the entire respiration pathway which reduces NO_3^- to N_2 can be potentially reconstituted in *E. coli.*

The power requirements of aeration constitute a major cost of large bioreactor operations. Besides, O_2 limitation is a barrier to higher cell yields in high cell density fermentations. Since the NO_3^- species is more diffusible than O_2, *E. coli* using nitrate respiration may potentially offer higher cell yields. Alternatively, O_2 can be used to grow the cultures to high cell densities and then a switch can be made to anaerobic respiration in which the cultures use NO_3^- as a electron acceptor. This switch can be accomplished by using native regulatory promoters or vectors similar to the kind characterized in this thesis.

The advances in *E. coli* genome mapping, the discovery of more and more genes from diverse organisms, development of complex regulatory networks, availability of efficient genetic engineering techniques will allow increasingly complex metabolic engineering strategies to be implemented in the future.

5.3 References

Baronofsky JJ, Schreurs WJA, Kashket ER (1984) Uncoupling by acetic acid limits growth of and acetogenesis by *Clostridium thermoaceticum*. Appl Environ Microbiol 48: 1134-1139

Gray GT, Wimpenny JWT, Mossman MR (1966) Regulation of metabolism in facultative bacteria II: Effects of aerobiosis, anaerobiosis and nutrition on the formation of the Kreb cycle enzymes in *Escherichia coli*. Biochim Biophys Acta 117:33-41

Hollywood N, Doelle HW (1976) Effect of specific growth rate and glucose concentration on growth and glucose metabolism of *Escherichia Coli* K-12. Microbios 17:23-33

Miles JS, Guest. JR (1987) Molecular genetic aspects of the citric acid cycle of *Escherichia coli*. Biochem Soc Symp 54:45-65

Otto R, Sonnenberg ASM, Veldkamp H, Konings WN (1980) Generation of an electrochemical proton gradient in *Streptococcus cremoris* by lactate efflux. Proc Natl Acad Sci USA 77:5502-5506

Appendix 1

Modifying a Promoter That is Activated Under Conditions of High Glycolytic Flux to Work in Aerobic Conditions.

A1.1   Introduction

E. coli produces acetate under aerobic conditions or pyruvate if the acetate pathway is blocked genetically. The reason for the acid byproduct production is an imbalance between the glucose uptake rate and the catabolic rate of the TCA cycle and the respiratory system. Therefore acid production is a response to excess flux through the glycolytic pathway. A promoter which responds to excess flux through the glycolytic pathway will be useful in channeling the excess carbon through any artificially engineered operon leading to the synthesis of the desired product.

We have identified one candidate promoter which can be useful in sensing excess flux through the glycolytic pathway. This is the promoter of the pyruvate formate lyase (*pfl*) gene. Pyruvate formate lyase enzyme catalyzes the cleavage of pyruvate into acetyl-CoA and formate and is a crucial enzyme for anaerobic fermentation in *E. coli* (Knappe and Sawers, 1990). This enzyme is produced only under anaerobic conditions.

The genetic regulation of the *pfl* promoter is complex (Sawers and Bock, 1988; Sawers and Bock, 1989). S1 nuclease protection experiments and primer extensions have identified that transcription is initiated from at least six promoters which spanned 1.2 kb (figure 1). The *pfl* promoter is under the control of *fnr* protein and is induced 10-fold further under anaerobic conditions. There are two sequences within the regulatory region that show strong homology with the consensus recognition sequences proposed for the *fnr* protein.

The promoter is further regulated by pyruvate. Exogenously added pyruvate causes a slight induction (1.5 times) over the anaerobic levels. When a *pfl* mutant strain is grown anaerobically with glucose as the carbon source, there is a strong induction of the *pfl* promoter compared to a wild-type strain. A *pfl* mutant strain will be blocked in further processing of pyruvate under anaerobic conditions. This shows that either pyruvate or a glycolytic intermediate between glucose and pyruvate functions as an inducer. These features of the *pfl* promoter suggest that it can be used as a signal for excess carbon flux.

The *pfl* promoter is expressed only under anaerobic conditions whereas the regime of acetate production in which we are interested is the aerobic regime. Sawers et al. showed that addition of exogenous pyruvate to aerobically growing cells could not bring about the induction of the *pfl* expression. However when a *pfl* mutant strain is grown aerobically with glucose as the carbon source, the level of expression of the *pfl* promoter (fused to a reporter gene) was significantly higher than in the wild type. Aerobically grown cells contain substantial amounts of the *pfl* enzyme (in an inactive form) and anaerobiosis causes a 10-fold increase. After this increase, *pfl* protein amounts reach 2.7% of the cytoplasmic protein. Thus we should expect that there will be sufficient level of expression from the *pfl* promoter under aerobic conditions and that there will be some further induction under conditions of high glycolytic flux as suggested by the expression level of the *pfl* promoter in the *pfl* mutant strain under aerobic conditions.

In this work, we have modified the *pfl* promoter so that it may work better under aerobic conditions. We have created deletions of the two *fnr* 'boxes'. Near one of the *fnr* boxes, there is the presumptive -35 region of one of the six promoters. This sequence, TTCTCA, differs from the consensus -35 sequence, TTGACA, by 2 bases. By using a

primer which is degenerate at two bases , ~TT(C/G)(T/A)A~, we have created a library of clones that contain four different promoters.

A1.2   Materials and methods

Plasmids and primers

Plasmid pRM23 (Sawers and Bock, 1989) containing 1.5 kb of the *pfl* promoter was kindly provided by Dr. Sawers from University of Munich, Lehrstuhl Mikrobiologie, Germany. Primers were synthesized by the Microchemical Facility at Caltech.

Modification of the *pfl* promoter

The modifications in the promoter were done by PCR using the splicing-by-overlap-extension method (Hemsley et al. 1989; Ho et al. 1989; Horton et al. 1990). In this technique, four primers are used (figure 2). Primer 1 and primer 2 are used to generate product AB and primer 3 and primer 4 are used to generate product CD. Primer 2 and primer 3 are designed such that they share a region of homology. When these two fragments are mixed, denatured and reannealed, the 3 '- end of the top strand of product AB anneals onto the 3'-end of the bottom strand of fragment CD, and this overlap can be extended to form the recombinant product. Modifications like insertions, deletions, point changes can be incorporated into primers 2 and 3.

The 4 primers used in the modification of the *pfl* promoter are shown below:

Primer 1: 5' cgggatcccgcctacgcaatgtaggcttaa **3'**

Primer 2: 3' at<u>gc ccg gat att cgg tcc g</u> **5'**

Primer 3: 5' <u>cg ggc cta taa gcc agg c</u>
tatataaattt(c/g)(t/a)**catctataatgctt 3'**

Primer 4: 3' gtgtttcacgtggtatgaaa **5'**

The underlined regions represent the complementary regions of primer 2 and primer 3. The bold letters represent the modifications incorporated. The modifications in primer 3 are deletion of a 11 base pair sequence, TATTTGGATAA, which represents one half-site of the *fnr* consensus sequence region. This deletion is just after the complementary underlined region and indicated by |. The other modifications include replacement of c by (g/c) and t by (t/a) in primer 3 and the addition of the *BamHI* linker sequence cgggatcccg in primer 1. The second *fnr* box is upstream of primer 1. The whole sequence upstream of primer 1 is not represented in the final modified promoter.

PCR was done using primer 1 and primer 2 to generate 325 bp product I and using primers 3 and 4 to generate 567 bp product II. The template used was plasmid pRM23 (Sawers and Bock, 1989) which has a 1.9 kb insert of the *pfl* promoter. 500 µg of the plasmid was used as template. The other components of the buffer were according to the specifications of the manufacturer (Perkin-Elmer-Cetus). PCR was conducted in a Precision Scientific Genetic Thermal Cycler. The conditions during the PCR were 1 min at 94 °C, 2 min at 42 °C and 3 min at 74 °C. The magnesium concentration of the buffer had to be optimized to observe specific amplification. For primer pair 1 and 3, the optimum concentration was 4 mM and for primer pair 3 and 4 the optimum concentration was 1.5 mM. Half of the amplified product I (25 µl) and half of the amplified product II was used

as templates for the final round of PCR using primers 1 and 2. The concentration of magnesium in the buffer was 4 mM.

This generated the final product, the 857 bp modified *pfl* promoter. This DNA mixture contains a mixture of potentially 4 different clones due to the presence of two degenerate bases. This product was digested by *BamHI* and *SfiI*. This 857 bp fragment can be used to replace the corresponding position of the native *pfl* promoter in pRM23.

A1.3   Results

The modifications done in the *pfl* promoter are shown in figure 3. Two sequences which resembled the *fnr* consensus sequences were deleted. At the same time 2 bases in the presumptive -35 region of promoter number 6 were replaced by degenerate bases. This was done because the sequence of the presumptive -35 region of one of the six promoters, TTCTCA, differs from the consensus -35 sequence, TTGACA, by 2 bases. The third base C was replaced by G/C and the fourth base replaced by A/T. This was done because the *pfl* promoter, with the *fnr* deletion will have much weaker activity. If we have a promoter that is closer to the consensus, we may compensate for the loss of that activity. We did not replace it by the consensus sequence because, if the promoter became too strong, then pyruvate will not be able to act as a transcriptional activator.

Initial efforts to use the PCR fragment containing 857 base pairs of the modified *pfl* promoter to replace the corresponding stretch of base pairs in the native *pfl* promoter were unsuccessful. After the promoter has been reconstituted in plasmid pRM23, the promoter can be tested for activity under aerobic conditions and conditions of high aerobic flux.

References

Hemsley A, Arnheim N, Toney MD, Cortopassi G, Galas DJ (1989) A simple method for site directed mutagenesis using the polymerase chain reaction. Nucl Acid Res, 17:6545-6551

Ho SN, Hunt HD, Horton RM, Pullen JK, Pease LR (1989) Site directed mutagenesis by overlap extension using the polymerase chain reaction. Gene 77:51-59

Hollywood N, Doelle HW (1976) Effect of specific growth rate and glucose concentration on growth and glucose metabolism of *Escherichia Coli* K-12. Microbios 17:23-33

Horton RM, Cai ZL, Ho SN, Pease LR (1990) Gene splicing by overlap extension: tailor made genes using the polymerase chain reaction. Biotechniques 8(5): 528

Knappe J, Sawers G (1990) A radical chemical route to Acetyl CoA : the anaerobically induced pyruvate formate lyase system of *Escherichia coli*. Fems Microbiol Rev 7: 383-398

Sawers G, Bock A(1989) Novel transcriptional control of the pyruvate formate lyase gene-upstream regulatory sequences and multiple promoters regulate anaerobic expression. J Bacteriol 171:2485-2498

Sawers G, Wagner AFV., Bock A (1989) Transcription initiation at multiple promoters of the *pfl* gene by E sigma 70 dependent transcription *in vitro* and heterologous expression in *Pseudomonas putida in vivo*. J Bacteriol 171: 4930-4937

A1.4   Figure captions

Figure 1: Summary of the transcriptional organization of the *pfl* operon. Tr₁ to Tr₇ represent 5' ends of the various transcripts. The small solid boxes indicate sequences which are inferred to be involved in *fnr* binding, as deduced from sequence similarities with regulatory regions of other *fnr*-dependent genes.
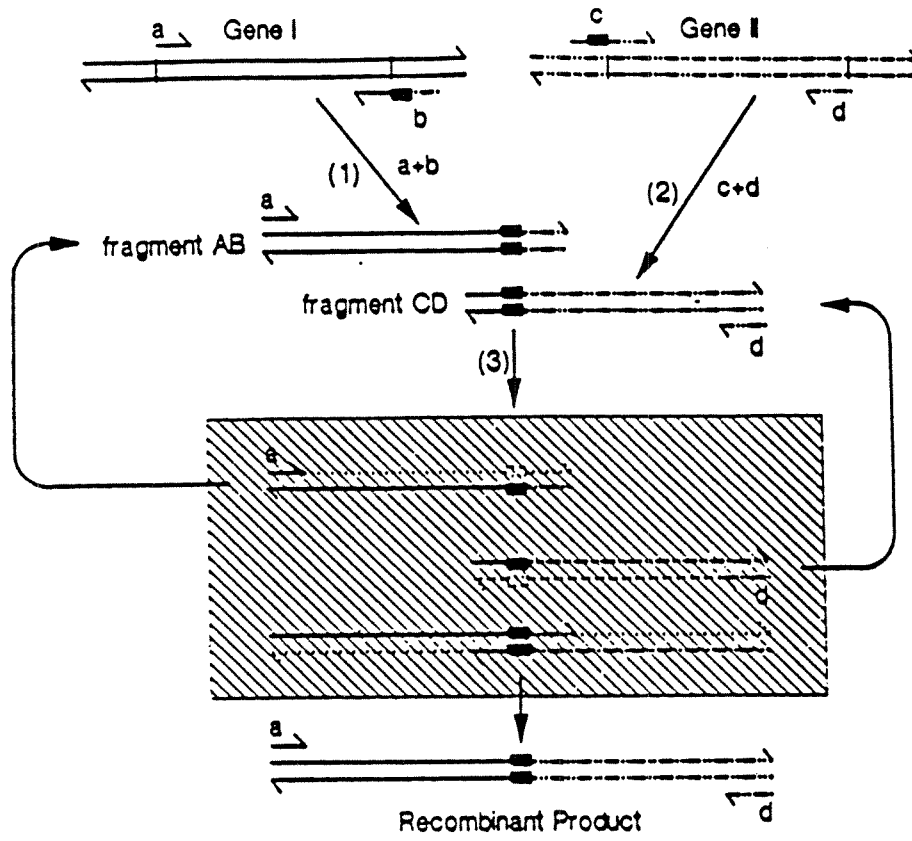
Figure 2: Principle of gene splicing by overlap extension (SOE)

Figure 3: Modifications done in the *pfl* promoter. The two *fnr* sites are shown boxed, the positions of the primers are shown, the -35, -10 and the transcript start point of the sixth transcript is shown.
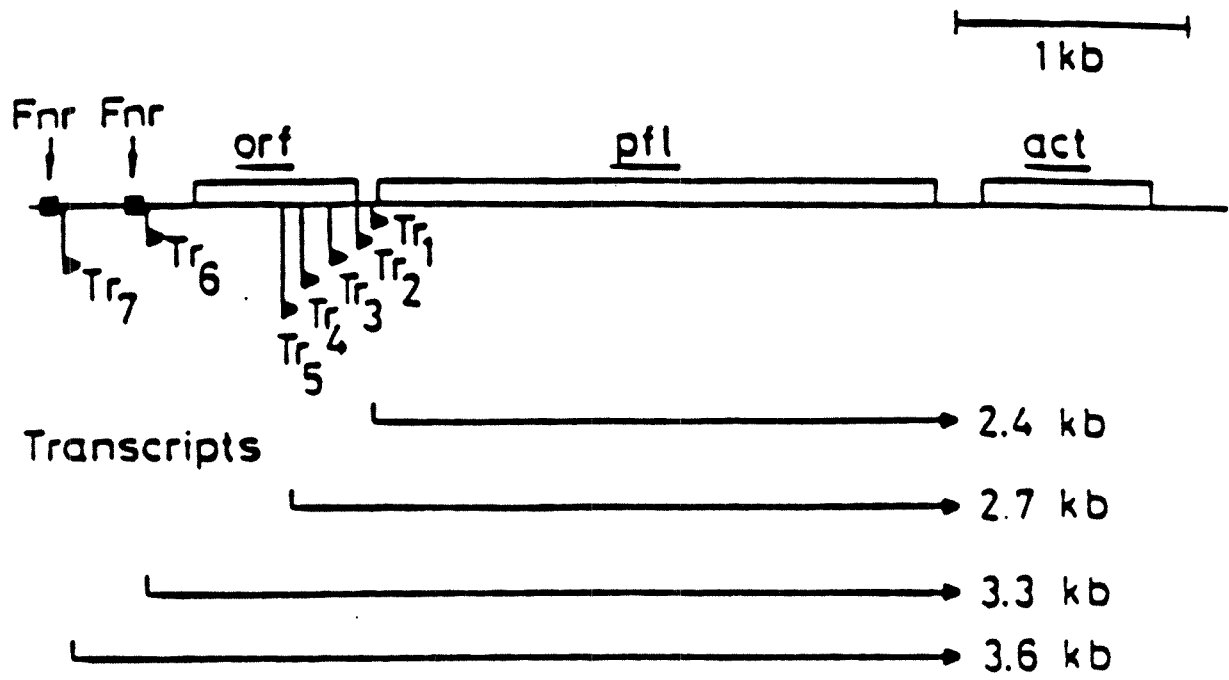
Figure 1

Figure 2

Figure 3

```
  1 TTTGCTGCAC ATCAGTCGTT GTTGAAGGCC  TACGAAAAGC TGCAGCGCGC CAAAGCAGCA 60

 61 TTCTGGGCAA AATAAAATCA AATAGCTAC   GCAATGTAGG CTTAATGATT AGTCTGAGTT 120
```

_/ 1_

```
121 ATATTACGGG GCTTTTTTT AATGCCCCGC  TTTACATATA TTTGCATTAA TAAAATAATT 180
```
→ Transcript 7

```
181 GTAATTATAA GGTTAAATAT CGGTAATTTG 'TATTTAATAA ATACGATCGA TATTGTTACT 240

241 TTATTCGCCT GATGCTCCCT TTTAATTAAC  TGTTTTAGCG GAGGATGCGG AAAAAATTCA 300

301 ACTCATTTGT TAATTTTTAA AATTTATTTT  TATTTGGATA ATCAAATATT TACTCCGTAT 360
```
_P3_
```
361 TTGCATAAAA ACCATGCGAG TTACGGGCCT  ATAAGCCAGG CGAGATATGA TCTATATCAA 420
```
_P2_
```
421 TTTCTCA TCT ATAATGCTTT GTTAGTATCT  CGTCCCGAC TTAATAAAGA GAGAGTTAGT 480
```
-35   -10
→ Transcript 6

```
481 GTGAAAGCTG ACAACCCTTT TGATCTTTTA  CTTCCTGCTG CAATGGCCAA AGTGGCCGAA 540

541 GAGGCGGGTG TCTATAAAGC AACGAAACAT  CCGCTTAAGA CTTTCTATCT GGCGATTACC 600

601 GCCGGTGTTT TCATCTCAAT CGCATTCGTC  TTCTATATCA CAGCAACCAC TGGCACAGGC 660

661 ACAATGCCCT TCGGCATGGC AAAACTGGTT  GGCGGCATTT GCTTCTCTCT GGGGCTGATT 720

721 CTTTGTGTTG TCTGCGGAGC CGATCTCTTT  ACTTCCACCG TGTTGATTGT TGTTGCTAAG 780

781 GCGAGTGGGC GCATCACCTG GGGTCAGTTG  GCGAAAAACT GGCTAAATGT CTATTTTGGC 840

841 AACCTGGTCG GCGCACTGCT GTTTGTACTT  TTAATGTGGC TTTCCGGCGA GTATATGACC 900

901 GCAAATGGTC AATGGGGACT AAACGTCCTA  CAAACCGCCG ACCACAAAGT GCACCATACT 960
```
_P4_
```
961 TTTATTGAGG CCGTCTGTCT TGGTATCCTG  GCAAACCTGA TGGTATGTCT GGCAGTATGG 1020

1021 ATGAGTTATT CTGGCCGCAG CCTGATGGAC  AAAGCGTTCA TTATGGTGCT GCCGGTCGCG 1080

1081 ATGTTTGTTG CCAGCGGTTT TGAGCACAGT  ATCGCAAACA TGTTTATGAT CCCGATGGGT 1140

1141 ATTGTAATCC GCGACTTCGC ATCCCCGGAA  TTTTGGACCG CAGTCGGTTC TGCACCGGAA 1200

1201 AATTTTTCTC ACCTGACCGT GATGAATTTC  ATCACTGATA ACCTGATTCC GGTTACGATC 1260

1261 GGCAACATTA TCGGTGGTGG TTTGTTGGTT  GGGTTGACAT ACTGGGTCAT TTACCTGCGT 1320

1321 GAAAACGACC ACCATTAATG GTTGTCGAAG  TACGCAGTAA ATAAAAATC CACTTAAGAA 1380
```
→ Transcript 1
```
1381 GGTAGGTGTT ACATGTCCGA GCTTAATGAA  AAGTTAGCCA CAGCCTGGGA A        1431
```
+1
ribosome
binding
site