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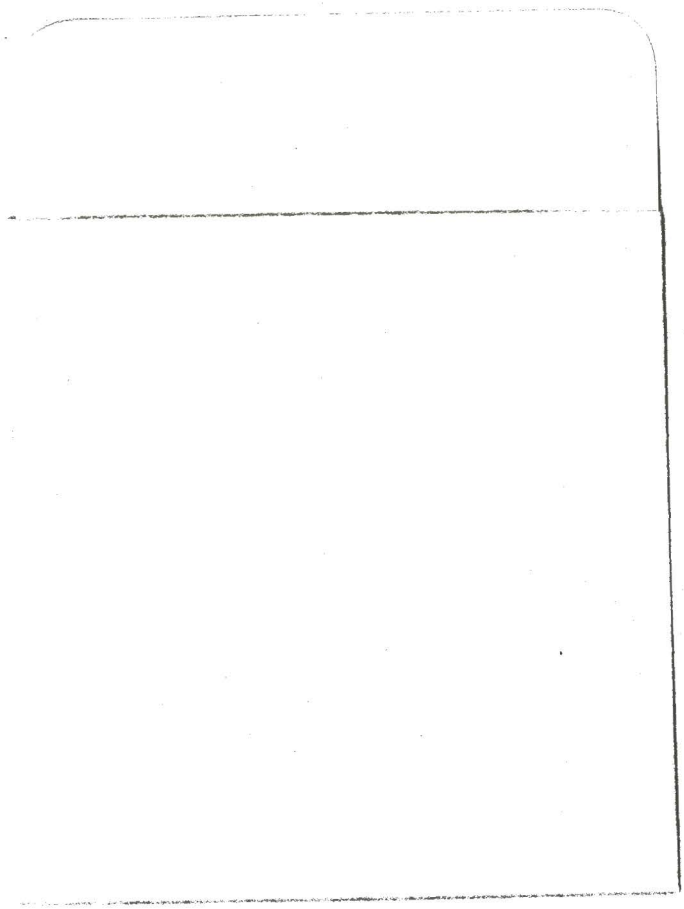

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The *csrA* gene negatively regulates the expression of four genes *glgB*, *glgC*, *glgA* and *glgS* involved in glycogen synthesis. It also negatively regulates *glgY*, which encodes the enzyme glycogen phosphorylase involved in glycogen degradation, but no effect was observed on the glycogen debranching enzyme in this pathway. In addition, *csrA* exhibits a positive effect on the glycolytic enzyme triosephosphate isomerase. No significant effects were observed on the expression of two genes (*zwf* & *gnd*) participating in the pentose phosphate pathway. *In vitro* expression of *glgB*, *glgC* and *glgA* was specifically inhibited by cell extracts containing the *csrA* gene product (CsrA). This study provides evidence that *csrA* encodes an important regulator of intermediary carbon metabolism in *Escherichia coli*.

EFFECTS OF THE PLEIOTROPIC GENE

*csrA* ON GLYCOGEN METABOLISM

IN *ESCHERICHIA COLI*

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# EFFECTS OF THE PLEIOTROPIC GENE *csrA* ON GLYCOGEN METABOLISM IN *ESCHERICHIA COLI*

THESIS

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## LIST OF ILLUSTRATIONS

## LIST OF ABBREVIATIONS

| | |
|---|---|
| BCA | Bicinchoninic acid |
| cAMP | Adenosine 3':5'- cyclic monophosphate |
| CRP | cAMP receptor protein |
| *csrA* | Carbohydrate storage regulator gene |
| CsrA | Carbohydrate storage regulator |
| DHAP | Dihydroxyacetone phosphate |
| DTT | Dithiothreitol |
| EDTA | Ethylenediaminetetraacetate |
| EGTA | Ethylene glycol-bis-(β-aminoethylether)-N,N,N',N'-tetraacetic acid |
| G3P | D-glyceraldehyde-3-phosphate |
| ONPG | o-nitrophenyl β-D-galactopyranoside |
| ORF | Open reading frame |
| PFK | Phosphofructokinase |
| PGI | Phosphoglucoisomerase |
| PYK | Pyruvate kinase |
| ppGpp | Guanosine 5'-diphosphate 3'-diphosphate |
| PTS | Phosphotransferase system |
| TEA | Triethanolamine |
| TR1-5 | *csrA*⁻ mutant |
| *tpi* | Triosephosphate isomerase gene |
| TPI | Triosephosphate isomerase |
| Tris | Tris(hydroxymethyl)aminomethane |
| X-Gal | 5-bromo-4-chloro-3-inodolyl β-D-galactopyranoside |

# INTRODUCTION

Glycogen is a form of stored D-glucose which may serve as a readily metabolized source of energy. It is composed entirely of glucosyl residues, the majority of which are linked together by α-1,4 glucosidic linkages. Branches occur in the glycogen molecule through α-1,6 glucosidic linkages. Glycogen is found in animal tissues and bacteria. In plants, the storage forms of glucose are amylose (exclusive α-1,4 glucosidic linkages) and amylopectin (α-1,4 glucosidic linkages and α-1,6 glucosidic linkages), which constitute starch. In animals, the storage of glycogen is crucial to mammalian homeostasis. Hepatic glycogen in liver and skeletal muscle glycogen serve different roles. Muscle glycogen serves as a fuel reserve for the synthesis of ATP within that tissue, whereas liver glycogen functions as a glucose reserve for the maintenance of blood glucose concentrations (20, 43).

Glycogen is not restricted to any class of bacteria. It accumulates in many bacteria when growth is limited and excess of a carbon source is available (11, 42). Thus glycogen accumulation occurs in stationary phase because of nitrogen, sulfur or phosphate limitation or because of suboptimal pH conditions. While the function of glycogen in bacteria is not rigorously known, it is probably utilized for energy and for carbon needed for the preservation of cell integrity during starvation (42, 43).

The reactions leading to glycogen synthesis in bacteria have been extensively studied since 1964. The synthesis of the α-1,4-glucosidic linkages of bacteria glycogen utilizes the ADP-glucose pathway instead of the UDP-glucose pathway, as in mammalian tissues. The reactions leading to glycogen synthesis require 3 enzymes: ADPglucose pyrophosphorylase (EC 2.7.7.27), glycogen synthase (EC 2.4.1.21) and branching

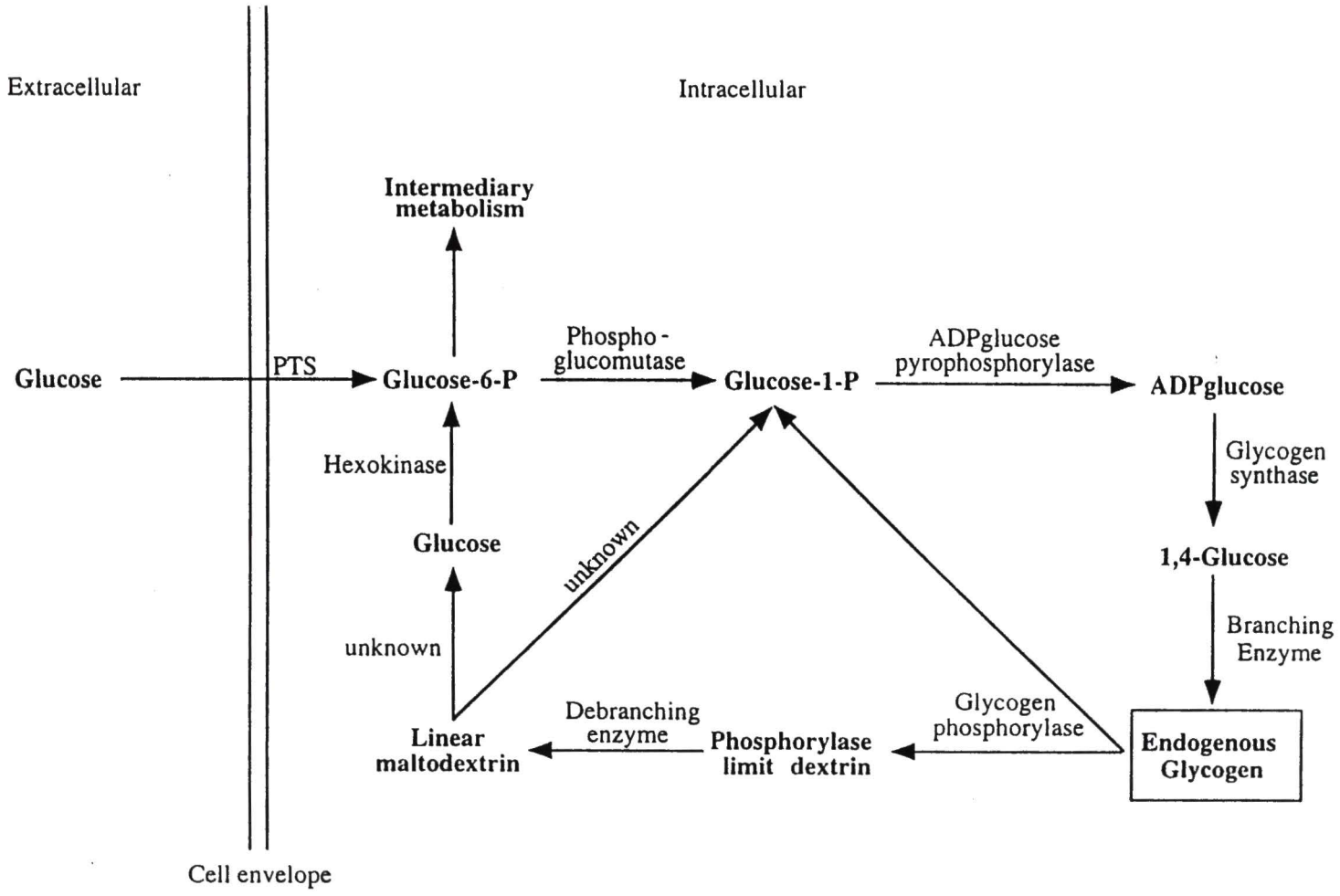
enzyme (EC 2.4.1.18) (38, 39, 41). ADPglucose pyrophosphorylase converts α-glucose-1-phosphate into ADPglucose, the glucosyl moiety of the formed ADPglucose is then transferred by glycogen synthase to either a maltodextrin or glycogen primer to form a new α-1,4-glucosidic bond. Subsequently, branching enzyme catalyzes formation of the α-1,6-glucosidic linkage of glycogen from the growing polyglucose chain (Fig.1).

There is considerable evidence that the glycogen biosynthesis in bacteria is regulated allosterically (43). The allosteric regulation of glycogen synthesis in bacteria is centered exclusively on ADPglucose pyrophosphorylase rather than glycogen synthase, as in mammalian species. In *E. coli*, this enzyme is activated by the glycolytic intermediate fructose-1,6-bisphosphate and inhibited by metabolites associated with energy metabolism, AMP, ADP and inorganic Pi (43). Glycolytic intermediates in the cell may be considered as signals of carbon excess and thus, under conditions of limited growth with excess carbon in the media, the accumulation of glycolytic intermediates may signal the activation of ADPglucose synthesis.

The structural genes for glycogen synthesis are located at approximately 75 min on the *E. coli* chromosome, and the gene order at this location is *glgY-glgA-glgC-glgX-glgB-asd* (11). The nucleotide sequence of the entire *glg* gene cluster has been determined (2, 3, 21, 46, 59). The *glg* genes appear to be transcribed as two tandem-arranged operons, *glgBX* and *glgCAP(Y)*. The *glgB* encodes the glycogen branching enzyme, *glgC* encodes ADP-glucose pyrophosphorylase, and *glgA* encodes glycogen synthase.

When the exogenous carbon source has been either completely utilized or considerably diminished, degradation of the glycogen will occur (43). Interestingly, genes that encode enzymes which are apparently involved in glycogen degradation are also located in the same cluster. The *glgY* gene is located downstream from *glgA*, it encodes glycogen phosphorylase (46) and has been alternatively designated as *glgP* (61). Amino

FIG. 1. **A scheme for metabolism of glycogen in *Escherichia coli*.**


Extracellular
Intracellular
Intermediary metabolism
Glucose
PTS
Glucose-6-P
Phospho - glucomutase
Glucose-1-P
ADPglucose pyrophosphorylase
ADPglucose
Glycogen synthase
1,4-Glucose
Branching Enzyme
Hexokinase
Glucose
unknown
unknown
Linear maltodextrin
Debranching enzyme
Phosphorylase limit dextrin
Glycogen phosphorylase
Endogenous Glycogen
Cell envelope

acid sequence analysis revealed that *glgX* is significantly related to glucan hydrolases and transferases, including α-amylase, pullulanase, the glycogen branching enzyme, and others (46), suggesting that it also may be involved in the catabolism of glycogen.

Glycogen phosphorylase (6, 7) catalyzes phosphorolysis of the α-1,4 glucosidic linkage and converts glycogen to glucose-1-phosphate and phosphorylase limit dextrin. The phosphorylase limit dextrin is further degraded by the glycogen debranching enzyme (17). Debranching enzyme hydrolyzes α-1,6 glucosidic linkages of phosphorylase limit dextrins to generate maltotetraose plus linear maltodextrins, which can be further degraded to glucose-1-phosphate (36, 43, Fig.1). Palmer et al (36), have suggested that maltodextrin phosphorylase and amylomaltase may degrade linear maltodextrin to glucose-1-phosphate and glucose. However, these are enzymes which are induced in the presence of maltose, and another study showed that glycogen degradation in *E. coli* K 12 occurs at the same rate in strains having deletions for the genes encoding amylomaltase and maltodextrin phosphorylase (maltose A region) (9). This strongly indicates that amylomaltase and maltodextrin phosphorylase are not involved in the catabolism of endogenous glycogen. The mode of degradation of endogenous maltotetraose and other low molecular weight linear maltodextrins is as yet unknown *E. coli*.

The expression of the *glg* structural genes in part determines the amount of glycogen that is accumulated by cultures, indicating the glycogen biosynthesis is also under genetic regulation (42). The expression of the *glgCAY* operon is induced in stationary phase and is positively regulated by cAMP-cAMP receptor protein (CRP) and ppGpp, which mediate the catabolite repression and stringent response global regulatory systems, respectively (47, 48). The transcription of *glgCAY* depends upon $\sigma^{70}$ RNA polymerase (42) and is not regulated by the alternative sigma factor $\sigma^{s}$, which is the gene product of *rpoS* or *katF* (2, 13). The expression of the *glgBX* operon is also induced in stationary phase, but is not influenced by cAMP or ppGpp (47, 48).

Glycogen biosynthesis is also potentially controlled *via* the regulated expression of structural genes outside of the *glg* gene cluster. Hengge-Aronis and Fisher (13) have isolated, cloned and sequenced the monocistronic *glgS* gene from *E. coli*, which stimulates glycogen synthesis. It is one member of the class of stationary-phase-induced genes which depend on $\sigma^s$, an alternative sigma factor for expression (22, 31, 53). The expression of *glgS* also requires cAMP (13). A null mutation in *glgS* does not affect the expression of *glgC* or *glgA* gene fusions, and the mechanism by which *glgS* enhances glycogen synthesis is currently unknown.

Studies of glycogen-excess *E. coli* B mutants SG3 and AC70R1, which exhibit enhanced levels of the enzymes in the glycogen synthesis pathway, first suggested that glycogen synthesis is under negative genetic regulation (35, 39, 43). In order to obtain further information about negative regulation of the *glg* genes, Romeo et al (44,45), isolated a collection of transposon mutants that affect glycogen biosynthesis in *E. coli*. Mutations were introduced into a strain that contained a plasmid-encoded *glgC'-'lacZ* fusion (pCZ3-3), and the resulting mutants were stained with iodine vapor to detect intracellular glycogen. The plasmid-encoded β-galactosidase was subsequently determined in glycogen-excess and glycogen-deficient mutants. The glycogen-excess mutant, TR1-5, was found to accumulate approximately 24-fold more glycogen than an isogenic wild-type strain was isolated. The gene affected by the TR1-5 mutation, *csrA* (carbon storage regulator), was subsequently cloned, sequenced, and mapped on the *E. coli* genome and some of its regulatory effects have been studied.

The *csrA* gene is located at 58 min or at position 2830 kb on the physical map of the *E. coli* K-12 genome (18, 44). The *csrA* gene is located between the gene *alaS*, which encodes alanyl-tRNA synthetase (EC 6.1.1.7), and the *serV* operon of tRNA genes, and is transcribed counterclockwise on the chromosome. The *csrA* open reading

frame (ORF) encodes a 61-amino acid polypeptide, which is strongly expressed from a plasmid-encoded clone of *csrA*, pCSR10 (45). This clone complements the TR1-5 (*csrA::kanR*) mutation.

The TR1-5 mutation was also shown to affect glycogen levels by causing elevated expression of genes in both glycogen operons, *glgC* and *glgB*. Levels of ADPglucose pyrophosphorylase expressed from the chromosome were approximately 10-fold higher in the TR1-5 mutant than in an isogenic *csrA*$^+$ strain in the stationary phase. The β-galactosidase activities expressed from the *glgC'-'lacZ* and the *glgB'-'lacZ* translational fusions were seven-fold and three-fold higher in the TR1-5 mutant, respectively, than in an isogenic *csrA*$^+$ strain. It was found that the TR1-5 mutation affects glycogen levels and the expression of the *glgB* and *glgC* genes in both the exponential and stationary phases. The effects of *csrA* on glycogen synthesis were mediated independently of the catabolite repression (cAMP) and stringent response systems (ppGpp).

In addition to its role in the negative regulation of glycogen biosynthesis, the gene *csrA* exhibits pleiotropic effects, suggesting that *csrA* may encode a global regulatory factor (45). First, the expression of a phosphoenolpyruvate carboxykinase (EC 4.1.1.38) operon fusion (*pckA'-lacZ*) was enhanced approximately two-fold throughout both the exponential phase of growth and the stationary phase in the TR1-5 mutant, suggesting that gluconeogenesis may also be under negative control of *csrA* (45). Second, cells containing the TR1-5 mutation were significantly larger than isogenic wild-type strains (maximum four-fold at early stationary stage) under certain growth conditions. It was not determined if this was an effect resulting from the increased storage of glycogen in the mutant strain or was an independent effect of the TR1-5 mutation. Finally, the TR1-5 mutant also exhibited altered surface properties, such as adherence to the glass culture tubes under anaerobic growth on MOPS medium. This adherent phenotype was also complemented by pCSR10.

In a preliminary attempt to determine the mechanism by which *csrA* affects gene expression, analysis of *glgC* transcripts by S1-nuclease protection mapping showed that the steady-state levels of all four *glgC* transcripts (transcript A, B, C, and D) are elevated in the TR1-5 mutant and were severely depressed in a pCSR10-containing strain, indicating that *csrA* affects the transcriptional regulation of *glgC* (26).

The major objective of this thesis is to examine the possibility that *csrA* plays a role in the regulation of other genes and enzymes in glycogen metabolism and in other metabolic pathways. First, *csrA* may affect other glycogen biosynthesis genes within the *glg* gene cluster, such as *glgA*, and genes outside of the *glg* gene cluster, such as *glgS*. Second, glycogen catabolism can also affect the intracellular glycogen level in the cell. Glycogen phosphorylase and debranching enzyme are the two major enzymes involved in glycogen catabolism, and *csrA* may also play an important role in regulating these enzymes. Third, metabolic pathways are clearly interconnecting, and glycogen metabolism is closely related to other pathways of carbon metabolism. Perhaps *csrA* also modulates pathways such as glycolysis, gluconeogenesis or the pentose phosphate pathway. Finally, in order to rigorously study the mechanism of genetic regulation by *csrA*, it is important to reconstruct regulation *in vitro*. Therefore, an *in vitro* assay for biologically-active CsrA protein was developed and used to examine the effects of CsrA-containing cell extracts on the expression of glycogen biosynthesis genes. Ultimately, these studies will have significant impact in understanding the physiology of *csrA* and the regulation of bacterial carbohydrate metabolism in general, and will facilitate future efforts to determine the mechanism of *csrA* regulation in glycogen metabolism.

## EXPERIMENTAL PROCEDURES

### Chemicals and reagent

All radiochemicals, [α-$^{35}$S]dATP, translation grade [$^{35}$S] methionine and [$^{14}$C]-glucose-1-phosphate were purchased from Dupont NEN (Wilmington, DE). Pyruvate kinase, phosphoenolpyruvate, α-amylase and amyloglucosidase were from Boehringer Mannheim Biochemicals (Indianapolis, IN). Guanosine 5'-diphosphate 3'-diphosphate (ppGpp) was from Pharmacia Inc. (Piscataway, NJ). Adenosine 3':5'- cyclic monophosphate (cAMP) receptor protein (CRP) was a gift from A. Revzin (Department of Biochemistry, Michigan State University, East Lansing). *Escherichia coli* RNA polymerase, o-nitrophenyl β-D-galactopyranoside (ONPG), micrococcal nuclease, cAMP, AMP, hexokinase, glucose-6-phosphate dehydrogenase, glyceraldehyde-3-phosphate, NADH, α-glycerophosphate dehydrogenase, rabbit muscle phosphorylase b, oyster glycogen, maltodextrin and glucose-1-phosphate were all purchased from Sigma Chemical Co. (St. Louis, MO). The compound 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), Sequenase™ version 2.0 and all DNA sequencing reagents were from U.S. Biochemical Corp. (Cleveland, OH). Restriction enzymes were purchased from either Bethesda Research Laboratories (Gaithersburg, MD) or New England BioLabs (Beverly, MA). Protein and DNA molecular weight standards were from Bethesda Research Laboratories (Gaithersburg, MD). All other biochemical reagents were purchased from commercial sources and were of the highest quality available.

### Bacterial strains, bacteriophages and plasmids

Table I shows the strains, plasmids, and bacteriophages that were used in this study, their sources and relevant genotypes.

Table I

*Bacterial strains, plasmids and phages used in the study.*

| Strain, plasmid and phage | Genotype or description | Source or reference |
|---|---|---|
| *E. coli* strain | | |
| BW3414 | *ΔlacU169* | Barry Wanner |
| TR1-5BW3414[1] | BW3414 *csrA::kanR* | (45) |
| G6 | Hfr *his* $Str^s$ | Maxime Schwartz (15) |
| G6MD3 | Hfr *his thi* $Str^s$ Δ(*malA-asd*) | (54) |
| G6MP11 | Hfr *his thi* $Str^s$ (*malP*) | Maxime Schwartz (15) |
| DH5α | *supE44 ΔlacU169* (Φ80*lacZ*ΔM15)*hsd R17 recA1 endA1 gyrA96 thi-1 relA1* | (1) |
| CAG18642 | *zfh*-3131::Tn*10*; 57.5 min | Carol Gross (56) |
| W3110 | $F^-$ prototroph | Richard Wolf (49) |
| HB354 | W3110 Δ(*argF-lac*)*U169 gnd-128*::ΔMu cts dI ($Ap^r$ Lac)::λp1(209) ($Lac^+$) | Richard Wolf |
| HB582 | W3110 Δ(*argF-lac*) *U169 gnd-217*::ΔMu cts dII ($Ap^r$ Lac)::λp1 (209) ($Lac^+$) (Hyb) | Richard Wolf (49) |
| HB301(λDR52) | ϕ(*zwf'-trpA-lacOZYA*) ($Lac^+$) $bla^+$ | Richard Wolf (49) |
| HB301(λDR104) | ϕ(*zwf'-lacZYA'*) 215 (Hyb)$bla^+$ | Richard Wolf (49) |
| MC4100 | $F^-$, Δ(*arg-lacU169*), *araD139, rpsL150, ptsF25, fibB5301, rbsR, deoC, relA1* | (13) |

| | | |
|---|---|---|
| DW18 | MC4100 ϕ(*glgA::lacZ*) (λ*plac*Mu15) | R. Hengge-Aronis (13) |
| RH105 | MC4100(λRH704)ϕ(*glgS::lacZ*)(hybr) | (13) |
| RH106 | RH105 *rpoS359*::Tn*10* | (13) |
| RH108 | RH105 Δ*cya851* | (13) |
| RH109 | RH105 *rpoS359*::Tn*10*,Δ*cya851* | (13) |
| Plasmids | | |
| pUC19 | Clonig vector, high copy number | (58) |
| pOP12 | Contains *asd* and *glgBXCAY'* genes in pBR322 | (35) |
| pCSR10 | *csrA* gene cloned into pUC19 | (45) |
| pMLB1034 | vector for construction of *'lacZ* translational fusions | (55) |
| pYZ9 | contains *glgCAY'* genes in pMLB1034 | This study |
| phages | | |
| P1*vir* | Strictly lytic P1; forms clear plaques | Carol Gross (56) |

[1] Any strain designation containing the prefix TR1-5 indicates that the wild type (*csrA*$^+$) allele has been replaced by the TR1-5 mutant allele (*csrA::kanR*) by P1*vir* transduction.

Growth conditions and media

Kornberg medium (1.1% $K_2HPO_4$, 0.85% $KH_2PO_4$, 0.6% yeast extract, 0.5% glucose) was used to grow strains for all *glg* gene expression and triose phosphate isomerase studies except where otherwise indicated. In the studies on the expression of glycogen and maltodextrin phosphorylases, cells were grown in Kornberg medium, as above, or in Kornberg medium with 0.5% maltose replacing 0.5% glucose. For growth curve experiments, cultures were inoculated with one volume of an overnight culture per 400 volumes of fresh medium except for the phosphorylase and glycogen assays, which used a 1: 200 inoculum. Solid Kornberg medium containing 1% glucose was routinely used to grow colonies for semi-quantitative staining of glycogen with iodine vapor (45). LB medium (1% tryptone, 1% NaCl, 0.5% yeast extract, 0.2% glucose, pH 7.4, 32) was used for routine laboratory cultures. R-medium plates (1% tryptone, 0.1% yeast extract, 0.8% NaCl, 0.1% glucose, 2 mM $CaCl_2$, 1.2% agar) and R-top agar (R medium containing 0.8% agar) were used for the preparation of P1*vir* lysates (33). Supplements were added as needed at the following concentrations: ampicillin, 100 μg/ml; tetracycline, 10 μg/ml; kanamycin, 100 μg/ml; diaminopimelic acid, 50 μg/ml; X-Gal, 0.04 mg/ml in plates and 0.4 mg/ml in top agar. Liquid cultures were grown at 37°C with gyratory shaking at 250 rpm.

Genetic techniques

Transduction of the TR1-5 mutant allele (*csrA : : kanR*) was conducted by using P1*vir* according to the method of Miller (33). Transductants were isolated either by direct selection for the $Kan^r$ phenotype or by cotransduction of the closely linked $Tet^r$ marker in TR1-5CAG18642 (45).

β-galactosidase assay

Assays were carried out according to Miller (32) with minor modifications (58). An appropriate amount of culture (no more than 150 µl) was added to 0.5 ml of Z buffer (0.1 M sodium phosphate buffer, pH 7.0, containing 0.2 M KCl, 0.002 M $MgSO_4$, 0.1 M 2-mercaptoethanol and 100 µg/ml chloramphenicol). One drop of toluene and 0.25 ml of 300 µg/ml sodium deoxycholate were added to the mixture. The mixture was shaken slowly at 37°C for 30 min to permeablize the cells. After 30 min shaking, the mixture was moved to a 28°C waterbath to cool. Water (for no substrate blanks) or 0.25 ml of 13.3 mM ONPG was added. The reactions were allowed to proceed for 10 min at 28°C and were stopped by the addition of 0.25 ml 1N $K_2CO_3$. The reaction was centrifuged to eliminate the turbidity and absorbance of each reaction was determined at 420 nm, relative to the substrate blank.

Preparation of S-30 and S-200 extracts

The coupled transcription-translation experiments utilized S-30 cell free extracts of *E. coli* strain TR1-5BW3414. The S-30 extract of TR1-5BW3414 and S-200 extracts of TR1-5BW3414 and TR1-5BW3414[pCSR10] were prepared according to Chen and Zubay (8) with modifications (47). The bacterial cells used to prepare S-30 extract were grown to mid-log phase in Kornberg medium with 0.5% glucose at 37°C with a gyratory shaking at 250 rpm . Cells were harvested by centrifugation at 8000 rpm in a JA-14 fixed angle rotor at 4°C and washed twice in 20 ml buffer A (10 mM Tris-Acetate, pH 8.2, 14 mM magnesium acetate, 60 mM potassium acetate). The cell paste was resuspended in buffer A at ratio of 1 g cell per 1.2 ml buffer. The cell suspension was lysed by two passes through a French press at 10,000 psi. Dithiothreitol was added to the lysate immediately after lysis to a final concentration of 1 mM. For the S-30 extract, the cell lysate was centrifuged at 30,000xg in a JA-14 fixed angle rotor at 4°C for 30 min. The supernatant solution was

collected and centrifuged for another 30 min at 4°C. The S-30 extracts were then mixed with translation mix (750 mM Tris-Acetate pH 8.2, 75 mM DTT, 22.5 mM magnesium acetate, 67.5 mM phosphoenolpyruvate, 20 μg/ml pyruvate kinase, 75 μM twenty amino acids, 6 mM ATP) at a ratio of 6.5 : 1. The mixture was incubated at 32°C in a light-protected plastic tube for 80 min and dialyzed overnight against 300 ml buffer A containing 1 mM DTT at 4°C with two changes of buffer. The molecular weight cut off of the dialysis tubing was 12,000-14,000 dalton. Extracts were then treated with micrococcal nuclease (50 U/ml) at 37°C for 20 min in the presence of 1 mM $CaCl_2$ to degrade endogenous nucleic acids. The nuclease was inactivated with 4 mM EGTA pH 8.0 (ethylene glycol-bis-[β-aminoethyl ether]-N,N,N',N'-tetraacetic acid) before the extracts were rapidly frozen in 200 μl aliquots and stored at -80°C. For making S-200 extracts, the cell lysate was centrifuged at 200,000xg in a Beckman TL100.3 rotor for 1 hour at 4°C, the supernatant solution was collected and dialyzed against buffer A containing 1 mM DTT. A membrane with a cut off of 1000 dalton was used for dialysis. After dialysis, the S-200 extracts were treated exactly as the S-30 extract and stored at -80°C.

S-30 coupled transcription-translation assay

Reaction mixtures (35 μl) contained: 0.22 mg TR1-5BW3414 S-30 extract, 11.4 mM magnesium acetate, 20 mM Tris-acetate, pH 8.2, 30 μM $N^{5,10}$-methenyltetra-hydrofolate, 0.452 μg of *E. coli* RNA polymerase, 1 μg pyruvate kinase, 1 μl [$^{35}$S] methionine (1175.0 Ci/mmol, 11.0 mCi/ml), 2 mM dithiothreitol, 30 mM phosphoenolpyruvate pH 6.5, 35 mM ammonium acetate, 2.9 mM ATP, 0.7 mM each CTP, UTP and GTP, 65 mM potassium acetate, 0.8 mM spermidine hydrochloride, 3.6% polyethylene glycol 8000, 10 mM dimethylglutaric acid, pH 6.0, 0.0286 mM methionine, 0.125 mM each of the other 19 amino acids, TR1-5BW3414 or TR1-5BW3414[pCSR10] S-200 extracts (as indicated). The pOP12 plasmid DNA (2 μg) was added to each reaction mixture to start the reaction (47). The reaction mixtures were incubated at 37°C for 1 hour

and terminated by the addition of 35 µl of sodium dodecyl sulfate sample buffer (0.125 M Tris hydrochloride, pH 6.8, 4% sodium dodecyl sulfate, 20% glycerol, 10% 2-mercaptoethanol).

The proteins in the reaction mixture were denatured by boiling at 100°C for 90 seconds. Equal volumes of each reaction were subjected to electrophoresis on 9.5% SDS polyacrylamide slab gels (according to instructions from Hoefer Scientific Instruments, San Francisco, CA). The gels were stained with Coomassie blue R-250 (0.125 g/100 ml in methanol-acetic acid-water [5:1:4]) and destained with acetic acid-methanol-water [1:5:4]. Radiolabeled proteins were detected by fluorography using sodium salicylate (5).

Glycogen Assay

A growth curve was started by inoculating 200 volume of Kornberg medium with 1 volume of overnight BW3414 or TR1-5BW3434 cultures. Glycogen assay was performed according to Preiss et al (40). At each hour, 5 ml of culture was removed and added to 1 ml of 50% trichloroacetic acid and incubated on ice for 10 min. The resulting precipitate was centrifuged for 10 min at 4°C and the pellet was washed twice with 2 ml of 5% trichloroacetic acid. In a microfuge tube, 0.4 ml of 1N NaOH were added to the washed pellets and the suspensions were heated in a boiling water bath for 30 min. Aliquots of the treated suspension were withdrawn for protein and glycogen assay while the tubes were still warm. Glycogen was hydrolyzed and converted to glucose by α-amylase from *Bacillus amyloliquefaciens* (EC 3.2.1.1) and amyloglucosidase from *Aspergillus niger* (EC 3.2.1.3) (4). Cell extracts (0.01 ml) were added to a mixture of 0.29 ml citrate solution (50 mM, pH 4.6), with 0.02 ml amyloglucosidase (144 U/ml) and α-amylase (900 U/ml) and were incubated at 55°C for 15 min. The hydrolysis mix (10 µl) was withdrawn and used to determine the amount of glucose liberated by the hydrolysis of glycogen. Glucose was first converted into glucose-6-phosphate and then 6-

phosphogluconate by the sequential action of hexokinase and glucose-6-phosphate dehydrogenase (Sigma KIT). The reduction of $NAD^+$ was determined by measuring the absorbance at 340 nm.

Triosephosphate Isomerase Assay

*E. coli* cell paste was obtained from cultures grown in Kornberg with 0.5% glucose at 37°C. Cell crude extract was prepared by the method of Pompliano et al (37). The cells were harvested by centrifugation at 5000xg in a JA-14 fixed-angle rotor at 4°C. All subsequent procedures were performed at 4°C. The cell paste was resuspended in 10 mM triethanolamine (TEA) with 1 mM EDTA and 0.1% mercaptoethanol, pH 7.6. Crude lysates were prepared by passing the cell suspension twice through a French press at 10,000 psi. Cell debris was removed by centrifugation (15000xg, 30 min). The activity of TPI was measured spectrophotometrically in the direction of glyceraldehyde 3-phosphate to dihydroxyacetone phosphate by coupling the reaction to α-glycerophosphate dehydrogenase (50). Cell extract (10 µl) was added to an assay mixture (890 µl) containing 50 mM triethanolamine/HCl, pH 7.6, 0.15 mM NADH, 1.5 mM D,L-glyceraldehyde 3-phosphate and 2 units of α-glycerophosphate dehydrogenase. The increase of NADH was determined by measuring the absorbance at 340 nm. One unit of TPI activity is defined as the amount of enzyme required to convert 1µmol of glyceraldehyde 3-phosphate into dihydroxyacetone phosphate per minute.

Glucan phosphorylase assays

Cells were harvested at 4°C by centrifugation and washed twice with ice cold 0.1 M Tris buffer, pH 7.0. The cell paste was resuspended in 0.05 M β-glycerophosphate buffer, pH 6.5, containing 0.03 M 2-mercaptoethanol at a final concentration of 0.25 g wet paste per ml. The cells were lysed by passing twice through a French press at 10,000 psi. Cell debris was removed by centrifugation in a JA-14 rotor at 12,000 rpm for 30 min at

4°C. Glycogen phosphorylase and maltodextrin phosphorylase activities in the supernatant solution was measured by following the rate of incorporation of [$^{14}$C]-glucose from [$^{14}$C]-glucose-1-phosphate into glycogen or maltodextrin as primer (6). Uniformly labeled [$^{14}$C]-glucose-1-phosphate ( 0.001 M, specific activity 3200 cpm / nmol) was added to the mixture of 0.1 M β-glycerophosphate, pH 6.5, containing 0.03 M 2-mercaptoethanol, 0.12 M NaF, 0.4% glycogen or maltodextrin, 0.005 M AMP and crude cell extract (30 μg protein) in a final volume of 0.1 ml (6). The mixture was incubated for 20 min at 37°C. The reaction was terminated by adding 1.4 ml of 75% ethanol containing 1% KCl to the mixture. The reaction was centrifuged in a microcentrifuge (13,000 rpm) for 5 min at room temperature, the precipitate was resuspended in 0.2 ml of $H_2O$ and precipitated again with 1.3 ml of 75% ethanol-1% KCl. The precipitate was separated by centrifugation and resuspended in 0.2 ml of 0.1 M glycine buffer, pH 10, containing 5 mM $ZnCl_2$. Calf intestine alkaline phosphatase (1.5 U) was added to hydrolyze the the residual [$^{14}$C]-glucose-1-phosphate. The mixture was incubated at 37°C for 40 min and precipitated with 1.3 ml of 75% ethanol-1% KCl. The precipitate was obtained from centrifugation again, resuspended in 1 ml of $H_2O$ and radioactivity in the precipitate was quantified by liquid scintillation counting using a Beckman LS6000IC scintillation counter. One unit of enzyme activity is defined as the amount of enzyme which catalyzes the incorporation of 1 nmol [$^{14}$C]-glucose into an ethanol-insoluble form in 20 minutes at 37°C.

Assay of glycogen debranching enzyme

The substrate for debranching enzyme was phosphorylase limit dextrin. It was prepared by the method of Lee (23) as follows: oyster glycogen (40 mg/ml) was incubated with 5 U/ml of rabbit muscle phosphorylase b (EC 2.4.1.1) in 80 mM sodium phosphate containing 30 mM 2-mercaptoethanol, pH 6.8 at 30°C in a dialysis bag under dialysis against 8 volumes of the same buffer. The buffer was changed several times over a period

of 48 hours. The incubation mixture was then dialyzed against water to remove buffer salts. The incubation mixture was heated at 100°C for 10 min and centrifuged to remove precipitate. The limit dextrin was precipitated by the addition of 3 volumes of ethanol. The precipitate was isolated by centrifugation, washed twice in ethanol and dried in a vacuum oven. The final yield was 50%. For measurement of endogenous glycogen debranching enzyme, cells were harvested by centrifugation, washed with ice cold water twice and disrupted by passing twice through a French press at 10,000 psi in 5 volumes of 0.05 M potassium phosphate buffer, pH 7.0 (17). Cell debris was removed by centrifugation in a JA-14 rotor at 12,000 rpm for 30 min at 4°C. Debranching enzyme activity in cell crude extracts was determined by measuring the increase in reducing power upon incubation of glycogen phosphorylase limit dextrin with cell extract. Enzyme substrate (10 mg) and 100 mM sodium acetate buffer (pH 5.6) were incubated with crude cell extract (50 μg) in a total volume of 0.2 ml at 37°C for 15 min. The mixtures were removed to heat inactivate at 60-70°C. The reducing power was measured in 0.2 ml of the reaction mixture by the method of Nelson (34) using glucose as a standard reducing sugar. A unit of enzyme activity is defined as the amount of enzyme which releases one μmol of reducing end groups per min (17). The visible absorbance spectrum of the iodine-glycogen complex upon treatment with the debranching enzyme was performed as follows (17): 10 mg of oyster glycogen in 0.02 M citrate/phosphate buffer (pH 5.5) was treated with 100 μg of crude cell extract, samples of 0.1 ml were removed at either before incubation or after 24 hrs incubation at 37°C, and were added to 5 ml of iodine reagent (0.02% iodine / 0.2% potassium iodide in 0.05 M HCl). The absorbance of the solutions from 400 nm to 600 nm were scanned in a Hewlett Packard 8452A diode array spectrophotometer.

Molecular biology and nucleotide sequencing

Standard procedures were used for isolation of plasmid DNA and restriction fragments, restriction mapping, transformation and molecular cloning (55). DNA

sequencing was done by the chain termination method of Sanger *et al.* (52) using the Sequenase™ version 2.0 kit under the conditions described by the manufacturer (U. S. Biochemical Corp., Cleveland, OH). For sequencing the junction of the *glgY'-lacZ* fusion , a primer that anneals within *lacZ* was used (GATGTGCTGCAAGGCGATTAAG TTGGGTAACG).

Construction of *lacZ* fusion plasmid

Plasmid pYZ9 contains the entire region of *glgCA* and part of the *glgY* gene fused to the *lacZ* gene. It was constructed as follows: A gel-purified 3.7 kb *Hpa*I restriction fragment of pOP12 was treated with T4 DNA ligase in the presence of pMLB1034, previously linearized with *Sma*I and treated with calf intestinal alkaline phosphatase. The ligation mixture was used to transform the strain DH5α to ampicillin resistance. Blue colonies were isolated on LB medium with ampicillin and X-Gal. The correct construction of the fusion was verified by restriction enzyme mapping and DNA sequencing across the fusion junction.

Protein determination

The estimation of protein concentration was performed by the method of Smith et al. (57) using bovine serum albumin as a standard.

## RESULTS

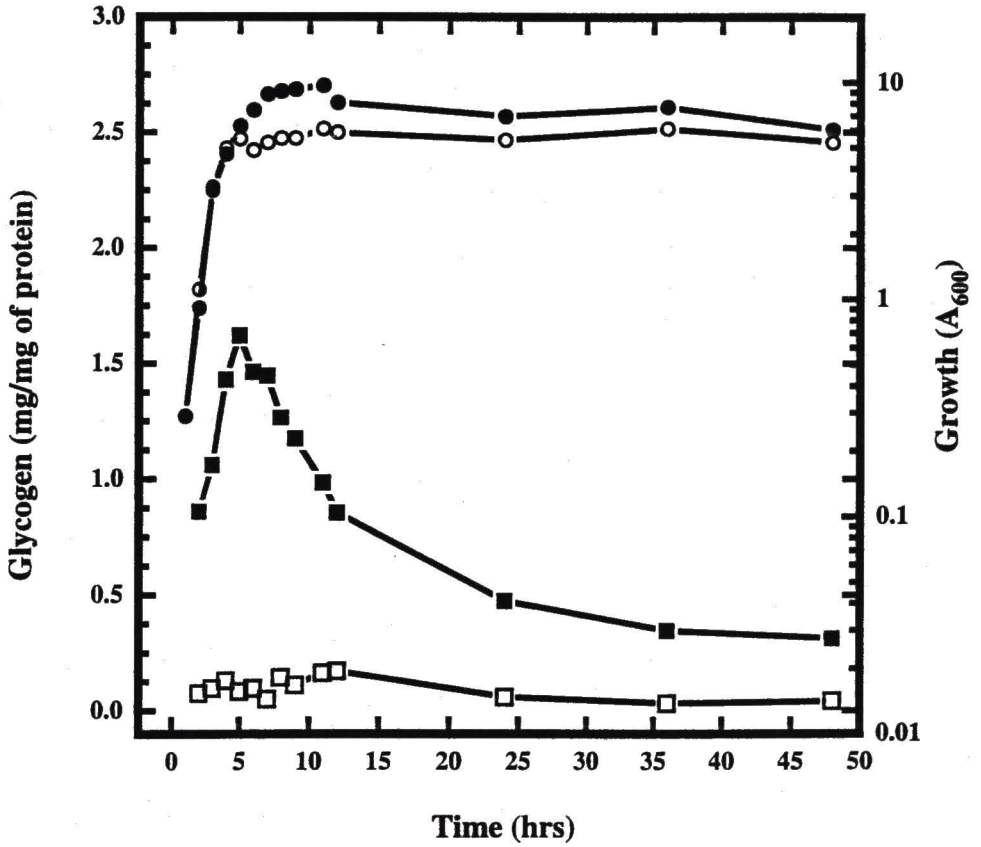
### Effects of the *csrA::kanR* mutation on endogenous glycogen levels

The *csrA::kanR* mutant TR1-5BW3414 was identified originally as a transposition insertion mutant containing high levels of endogenous glycogen (45). The intense staining of colonies of this mutant with iodine vapor suggested that the endogenous glycogen levels in the mutant were much higher than in the parent strain. In order to quantitatively compare the endogenous glycogen levels of BW3414 and TR1-5BW3414 during the growth curve, a quantitative glycogen assay was performed. Glycogen was converted to glucose by hydrolysis with α-amylase and amyloglucosidase and the liberated glucose was quantified using hexokinase and glucose-6-phosphate dehydrogenase coupled enzyme reactions (40). As suggested by iodine staining, the levels of glycogen were significantly higher in the *csrA::kanR* mutant than the isogenic *csrA*$^+$ stain. The maximal difference was approximately 15-fold, and occurred in the early stationary phase (Fig. 2). The maximal amount of glycogen accumulated in the *csrA::kanR* mutant was 1.6 mg glycogen per milligram protein in the cell. Assuming that 55% of the dry weight of *E. coli* is protein, this suggests that approximately 47% {(0.55x1.6)/(1.0+0.55x1.6)x100%} of the dry weight of the mutant is glycogen. After early stationary phase, glycogen levels in the *csrA::kanR* mutant decreased rapidly, at a rate of approximately 0.11 mg glycogen / mg protein · hour. After 24 hours in culture, glycogen levels decreased gradually for the next 24 hours at a rate of 0.007 mg glycogen / mg protein · hour.

### Expression of a *glgA'-'lacZ* translational fusion is enhanced in *csrA::kanR* mutant

Previous studies indicated that the *csrA::kanR* mutant affects glycogen biosynthesis by enhancing the levels of enzymes in the glycogen biosynthesis pathway.

FIG. 2. **Effect of *csrA* on endogenous glycogen levels.** BW3414 (*csrA*$^+$) and TR1-5BW3414 (*csrA::kanR*) were grown in Kornberg medium containing 0.5% glucose. The glycogen levels in BW3414 (open symbols) or TR1-5BW3414 (closed symbols) are shown as squares; growth of the cultures ($A_{600}$) is shown as circles.


3.0
2.5
2.0
1.5
1.0
0.5
0.0
Glycogen (mg/mg of protein)
10
1
0.1
0.01
Growth ($A_{600}$)
0
5
10
15
20
25
30
35
40
45
50
Time (hrs)

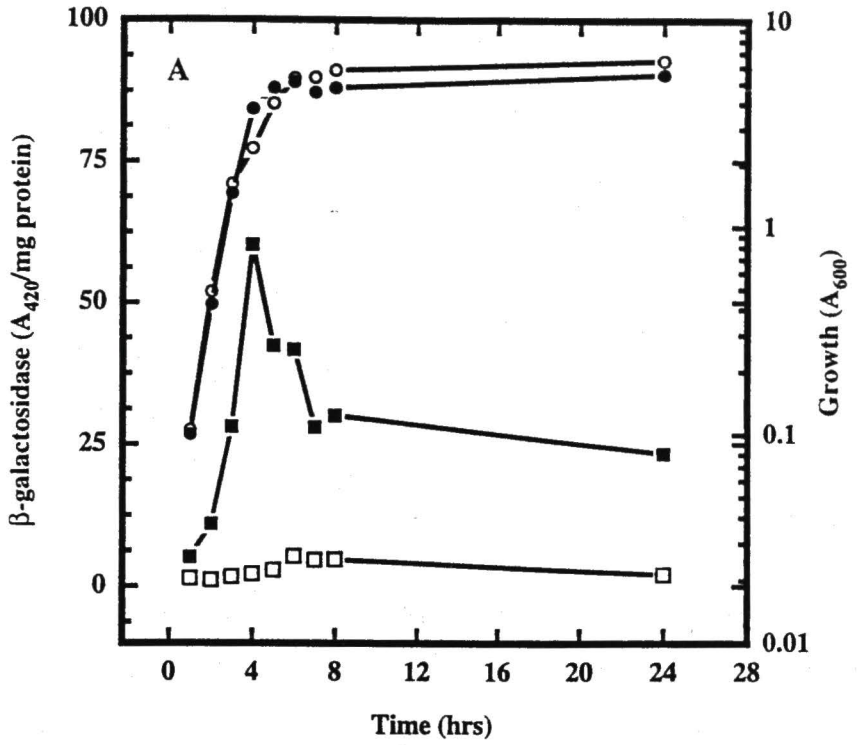
The level of ADP-glucose pyrophosphorylase expressed from the chromosome was approximately ten-fold higher in the *csrA::kanR* mutant than in an isogenic *csrA*+ strain (45). Furthermore, the specific β-galactosidase activities of *glgC'-'lacZ* and *glgB'-'lacZ* were five to seven-fold and three-fold higher, respectively, in the TR1-5 mutant than in the isogenic *csrA*+ strain (45). This indicated that the TR1-5 mutation affects glycogen levels by causing elevated expression of genes in two glycogen operons. The *glgA* gene encodes glycogen synthase and is located immediately downstream from *glgC*. These two genes and *glgP(Y)* have been proposed to occur in an operon in *E. coli* (35). Thus, it was important to determine whether the *csrA::kanR* mutation affects the expression of *glgA* as well as *glgB* and *glgC*.

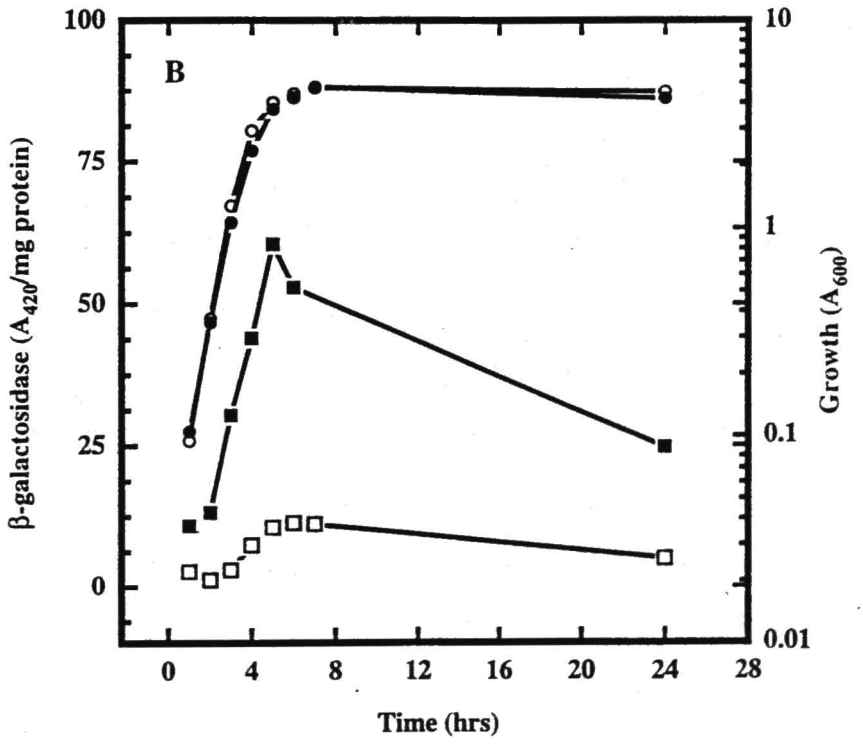
The TR1-5 mutation was transduced into DW18, a strain containing a chromosomally-encoded *glgA'-'lacZ* translational fusion (13). The specific β-galactosidase activity of *glgA'-'lacZ* was higher in the TR1-5 mutant than in the isogenic *csrA*+ strain in both exponential and stationary phases, with a maximal difference of approximately 12-fold in Kornberg medium containing 0.5% glucose and six-fold in LB medium (Fig. 3). In both media, the maximum difference occurred in the early stationary phase. The expression of the *glgA'-'lacZ* fusion was induced when the cell entered the stationary phase, consistent with results previously shown for *glgA* expression (48).

<u>*csrA* negatively affects the expression of *glgS*</u>

Recently, a gene was described which had a strong stimulatory effect on glycogen accumulation in *E. coli*, *glgS*. The monocistronic *glgS* gene maps at 66.6 min on the chromosome, approximately 9 min from the *glg* gene cluster (13). The expression of *glgS* was previously shown to be dependent upon $\sigma^S$ and cAMP (13). To further characterize the effect of *csrA* on glycogen synthesis, the expression of *glgS* gene was examined. The *csrA::kanR* mutation caused an increase in *glgS* expression of about

FIG. 3. **The negative effect of *csrA* on the expression of the *glgA* gene.** Growth-phase-dependent expression of a *glgA'-'lacZ* fusion in DW18 (*csrA*$^+$) and TR1-5DW18 (*csrA::kanR*) was tested on cells grown in (A) Kornberg medium containing 0.5% glucose (B) LB medium. β-galactosidase specific activities expressed from the *glgA'-'lacZ* fusion in DW18 (open symbols) or TR1-5DW18 (closed symbols) are shown as squares; growth of the cultures ($A_{600}$) are shown as circles.


A
β-galactosidase ($A_{420}$/mg protein)
Growth ($A_{600}$)
Time (hrs)
100
75
50
25
0
10
1
0.1
0.01
0
4
8
12
16
20
24
28


B
β-galactosidase ($A_{420}$/mg protein)
Growth ($A_{600}$)
Time (hrs)
100
75
50
25
0
10
1
0.1
0.01
0
4
8
12
16
20
24
28

three-fold in the RH105 (*rpoS* $^{+}$ *cya* $^{+}$) (Fig. 4). As was previously shown, the expression of *glgS* increased dramatically when the culture entered the early stationary phase. Deletion of *cya* in strain RH108 resulted in weaker expression of the *glgS'-'lacZ* fusion, with approximately one third of the β-galactosidase activity being synthesized. TR1-5 mutation caused about 30% increase in *glgS* expression relative to RH108 during stationary phase (Fig. 5). As previously shown, the stationary-phase induction of *glgS:lacZ* was almost entirely dependent upon *rpoS*, and the expression of *glgS* was almost undetectable in strains RH106 and RH109, which lack *rpoS* (Figs. 6, 7). TR1-5 mutation showed no effect on *glgS'-'lacZ* expression in these two strains.

Interestingly, in strains RH105 and RH106, which have a functional *cya* gene, the *csrA:: kanR* mutation did not alter the growth rate in Kornberg containing 0.5% glucose. But when *cya* was deleted from the cell (RH108 and RH109), the growth rate was considerably slower and the culture entered the stationary phase approximately 3-4 hours later than in the isogenic *cya*$^{+}$ strains. When the *csrA:: kanR* mutation was introduced into the *Δcya* strains, it restored the growth rate. Similar effects were observed as either a change in turbidity (Figs. 5, 7) or as an increase in total cellular protein (Fig. 8).

The negative effect of *csrA* on glycogen phosphorylase activity

In order for glycogen to function as an energy storage molecule, it must be degraded or catabolized at the appropriate time. Since *csrA* negatively regulates glycogen biosynthesis, it was appropriate to investigate whether *csrA* also affects glycogen degradation. Glycogen phosphorylase is believed to be a key enzyme involved in the degradation of endogenous glycogen (6); therefore, glycogen phosphorylase activity was determined in BW3414 and its *csrA::kanR* mutant TR1-5BW3414 (Fig. 9). The activity of glycogen phosphorylase was higher in the *csrA::kanR* mutant throughout the growth curve, with a maximal difference of three-fold occurring in the early stationary phase, indicating that *csrA* negatively regulates glycogen phosphorylase (Fig. 9).

FIG. 4. **Expression of *glgS'-'lacZ* translational fusion in RH105 (*csrA*$^+$) and TR1-5RH105 (*csrA::kanR*).** Growth-phase-dependent expression of *glgS'-'lacZ* in RH105 and TR1-5RH105 was tested on cells grown in Kornberg medium containing 0.5% glucose. β-galactosidase specific activities expressed from *glgS'-'lacZ* fusion in RH105 (open symbols) or TR1-5RH105 (closed symbols) are shown as squares; growth of the cultures ($A_{600}$) is shown as circles.

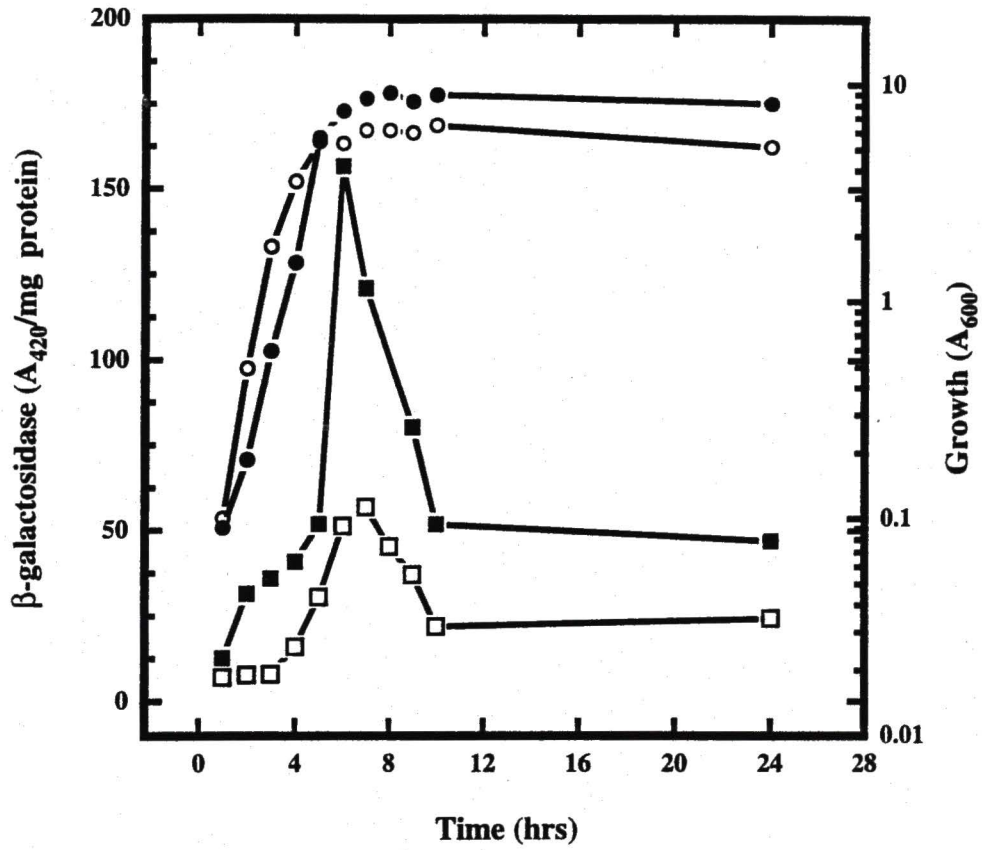

200
150
100
50
0
β-galactosidase (A420/mg protein)
10
1
0.1
0.01
Growth (A600)
0
4
8
12
16
20
24
28
Time (hrs)

FIG. 5. **Expression of *glgS'-'lacZ* translational fusion in RH108 (*rpoS*$^+$ *Δcya csrA*$^+$) and TR1-5RH108 (*rpoS*$^+$*ΔcyacsrA::kanR* ).** Growth-phase-dependent expression of *glgS'-'lacZ* in RH108 and TR1-5RH108 was tested on cells grown in Kornberg medium containing 0.5% glucose. β-galactosidase specific activities expressed from *glgS'-'lacZ* fusion in RH108 (open symbols) or TR1-5RH108 (closed symbols) are shown as squares; growth of the cultures ($A_{600}$) is shown as circles.

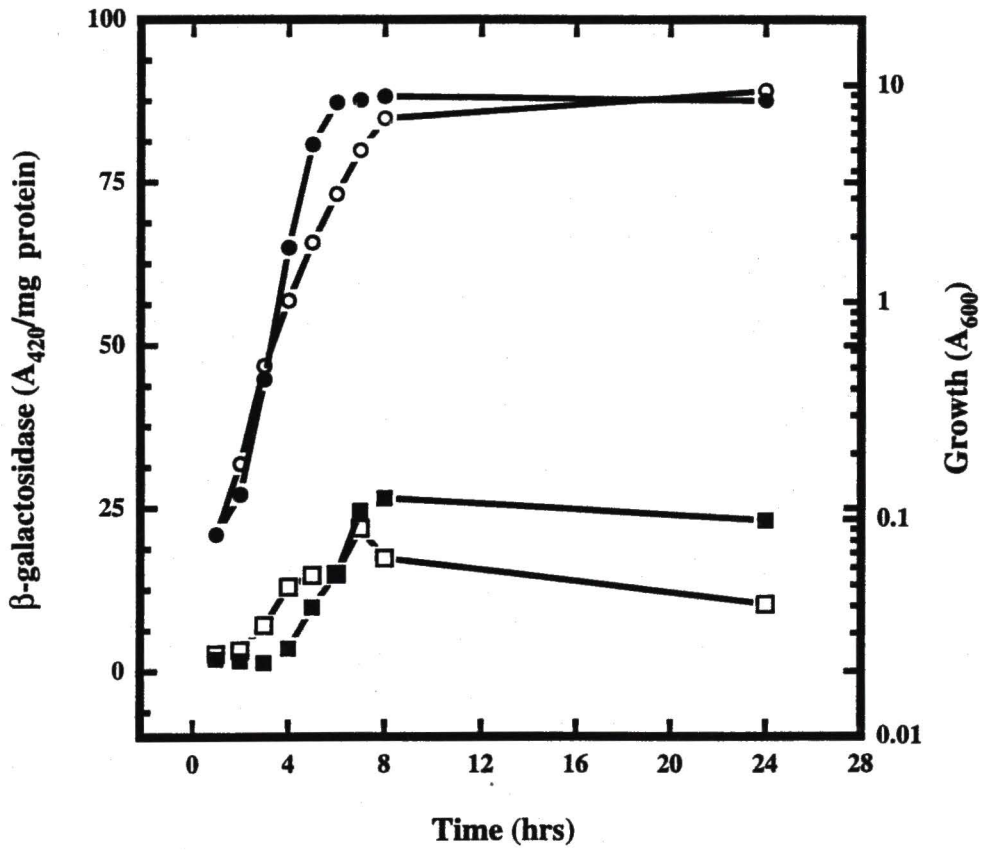

β-galactosidase ($A_{420}$/mg protein)
Growth ($A_{600}$)
Time (hrs)
100
75
50
25
0
10
1
0.1
0.01
0
4
8
12
16
20
24
28

FIG. 6. **Expression of *glgS'-'lacZ* translational fusion in RH106 (*rpoS::*Tn*10 cya*$^+$ *csrA*$^+$) and TR1-5RH106 (*rpoS::*Tn*10 cya*$^+$ *csrA::kanR*).** Growth-phase-dependent expression of *glgS'-'lacZ* in RH106 and TR1-5RH106 was tested on cells grown in Kornberg medium containing 0.5% glucose. β-galactosidase specific activities expressed from *glgS'-'lacZ* fusion in RH106 (open symbols) or TR1-5RH106 (closed symbols) are shown as squares; growth of the cultures ($A_{600}$) is shown as circles.

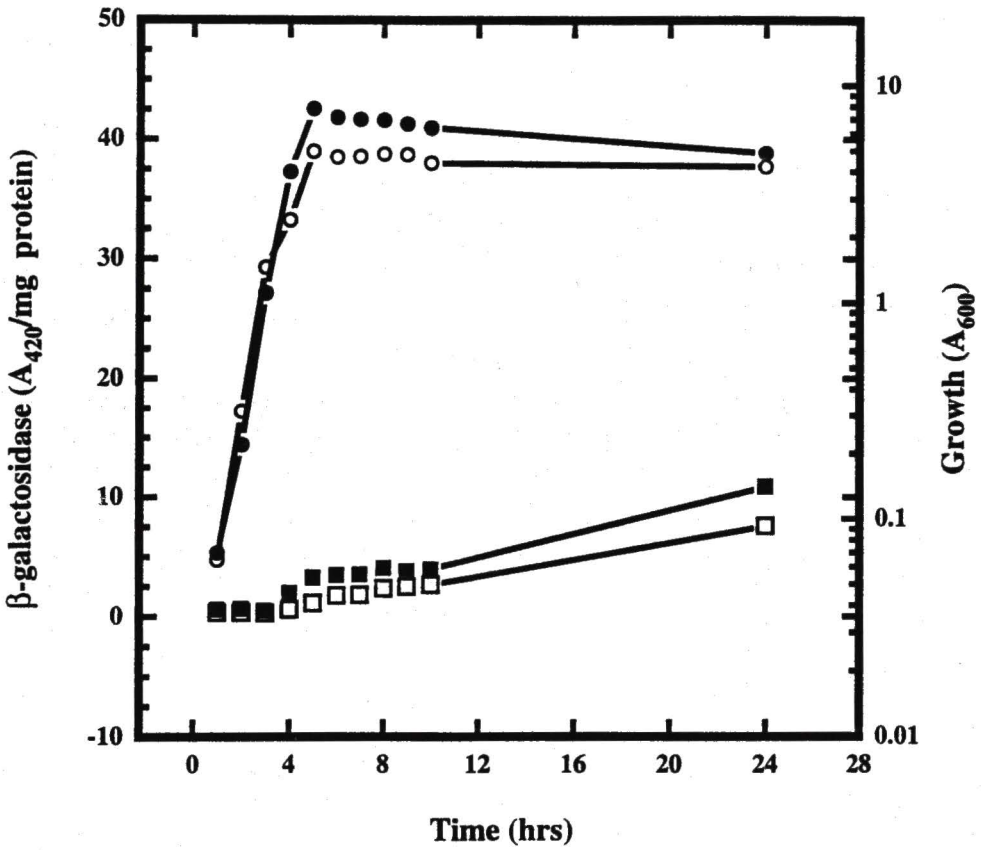

50
40
30
20
10
0
-10
0
4
8
12
16
20
24
28
10
1
0.1
0.01
β-galactosidase ($A_{420}$/mg protein)
Growth ($A_{600}$)
Time (hrs)

FIG. 7. **Expression of *glgS'-'lacZ* translational fusion in RH109 (*rpoS::*Tn*10 Δcya csrA*$^+$) and TR1-5RH109 (*rpoS::*Tn*10 Δcya csrA::kanR*).** Growth-phase-dependent expression of *glgS'-'lacZ* in RH105 and TR1-5RH105 was tested on cells grown in Kornberg medium containing 0.5% glucose. β-galactosidase specific activities expressed from *glgS'-'lacZ* fusion in RH109 (open symbols) or TR1-5RH109 (closed symbols) are shown as squares; growth of the cultures ($A_{600}$) is shown as circles.

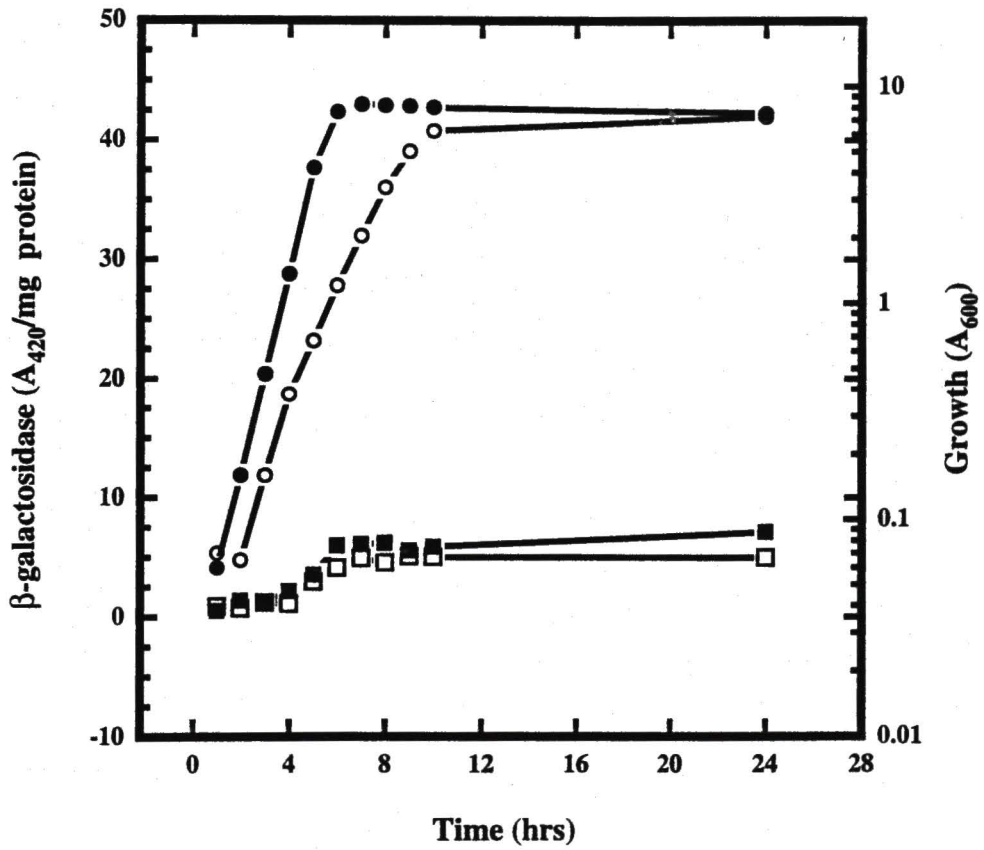
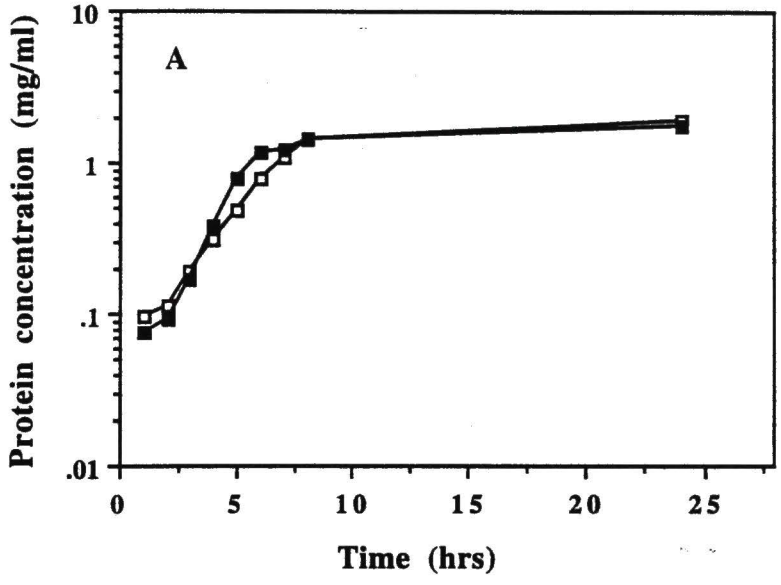

β-galactosidase ($A_{420}$/mg protein)
Growth ($A_{600}$)
Time (hrs)
50
40
30
20
10
0
-10
10
1
0.1
0.01
0
4
8
12
16
20
24
28

FIG. 8. **Effects of *csrA* on growth rate of *Δcya* strains.** Total cellular protein was measured throughout the growth curve in *cya* deleted strains (open squares) and their *csrA::kanR* mutants (closed squares). (A) RH108 (*rpoS*$^+$ *Δcya csrA*$^+$) and TR1-5RH108 (*rpoS*$^+$ *Δcya csrA::kanR*) (B) RH109 (*rpoS::*Tn*10 Δcya csrA*$^+$) and TR1-5RH109 (*rpoS::*Tn*10 Δcya csrA::kanR*).


A
Protein concentration (mg/ml)
10
1
.1
.01
0
5
10
15
20
25
Time (hrs)

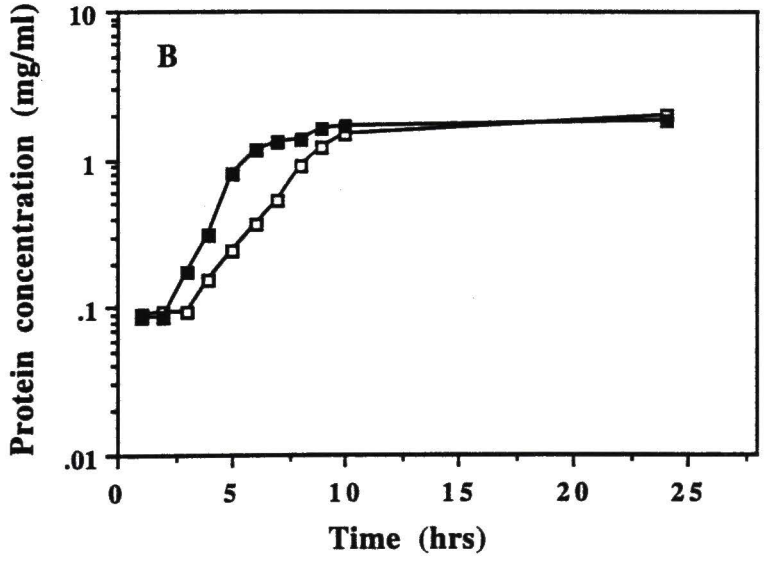
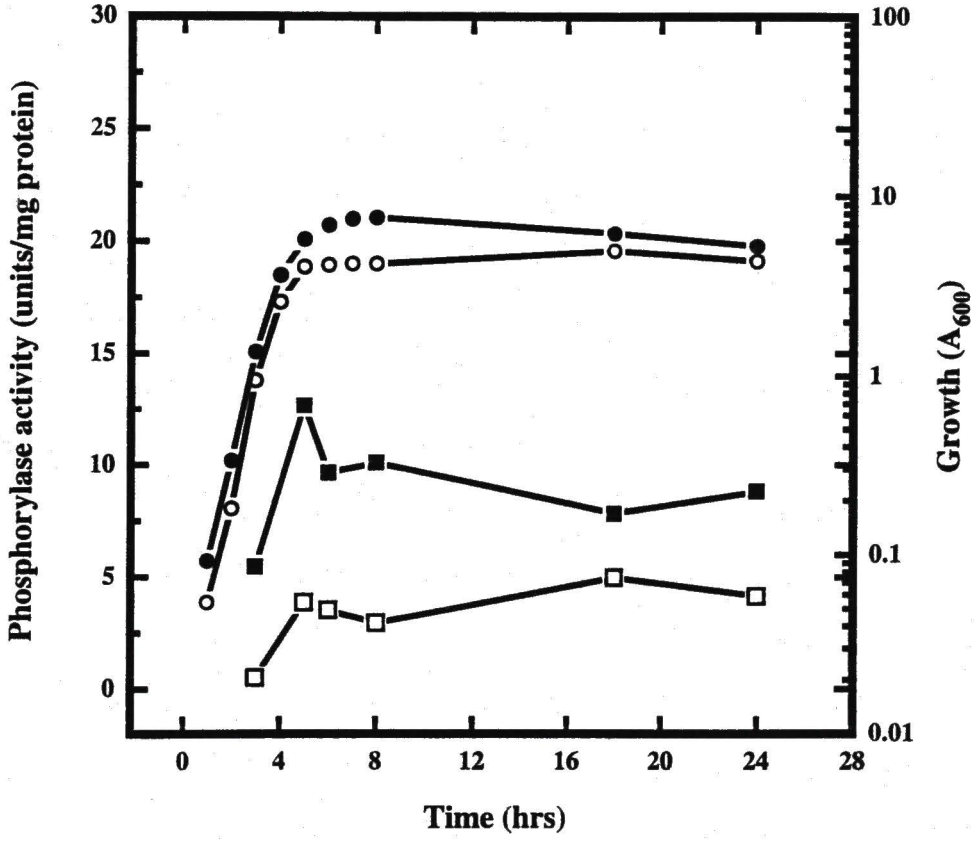

B
Protein concentration (mg/ml)
10
1
.1
.01
0
5
10
15
20
25
Time (hrs)

FIG. 9. **Glycogen phosphorylase specific activities in BW3414 (*csrA*$^+$) and TR1-5BW3414 (*csrA::kanR*).** Cultures were grown in Kornberg medium containing 0.5% glucose. Glycogen phosphorylase specific activities were determined in BW3414 (open symbols) or in TR1-5BW3414 (closed symbols) and are shown as squares; growth of the cultures ($A_{600}$) is shown as circles.


Phosphorylase activity (units/mg protein)
Growth ($A_{600}$)
Time (hrs)
30
25
20
15
10
5
0
100
10
1
0.1
0.01
0
4
8
12
16
20
24
28

In addition to glycogen phosphorylase, *E. coli* also synthesizes a maltodextrin phosphorylase, which is induced by growth on maltose and is specific for low molecular weight α-1,4-glucans (54). Although these properties distinguish the two phosphorylases, it was at least possible that our assays may have also detected maltodextrin phosphorylase. Thus, phosphorylase activity was assayed in a *malP* mutant, strain G6MP11 and its *csrA::kanR* mutant. Strain G6MP11 has been shown not to be able to synthesize maltodextrin phosphorylase due to a mutation in the *malP* structural gene (15). The presence of the *csrA::kanR* mutation in G6MP11 also resulted in elevated activity of glycogen phosphorylase (3~4-fold) when glucose-grown cells were assayed on glycogen as an acceptor (Table II). This clearly demonstrated that *csrA* negatively regulates glycogen phosphorylase.

Table II also shows the incorporation of [$^{14}$C]-glucose from [$^{14}$C]-glucose-1-P into two different polyglucose acceptors in various strains. Glucose-grown cells consistently showed high GLY/DEX ratios (about 4 : 1); maltose-grown cells showed low GLY/DEX ratios except *malP* mutant G6MP11. Maltose induced a 50-fold increase in the activity of the extract toward dextrin, but only ten-fold increase when glycogen was used as a substrate in both BW3414 and G6. Thus, using glycogen as an acceptor in glucose-grown cells, the glucan phosphorylase activity was predominantly glycogen phosphorylase; using dextrin as an acceptor in maltose-grown cells, the glucan phosphorylase activity was mainly maltodextrin phosphorylase. When the *csrA::kanR* mutation was introduced into strains, glycogen phosphorylase activity increased 2-fold in strains G6 and BW3414, which have both *glgY* and *malP*. On the other hand, the *csrA::kanR* mutation slightly decreased (by about 20%) the maltodextrin phosphorylase activity in strains G6 and BW3414. Finally, G6MD3 is a strain which lacks both *glgY* and *malP*. As expected, the glycogen phosphorylase and maltodextrin phosphorylase activities in this strain and its *csrA::kanR* mutant were undetectable (Table II).

Table II

*Effects of the csrA::kanR mutation on glucan phosphorylase activities.* [1]

| Strain (relevant genotype) | Phosphorylase activity (units/mg protein)[2] | |
|---|---|---|
| | Glycogen as acceptor | Dextrin as acceptor |
| Glucose grown | | |
| BW3414 | 4.5 ± 0.6 | 1.1 ± 0.2 |
| TR1-5BW3414 (*csrA::kanR*) | 6.9 ± 0.7 | 1.3 ± 0.2 |
| G6 | 4.4 ± 0.4 | 2.2 ± 0.1 |
| TR1-5G6 (*csrA::kanR*) | 9.1 ± 0.5 | 4.4 ± 0.5 |
| G6MP11 (*malP*) | 1.7 ± 0.4 | 0.4 ± 0.2 |
| TR1-5G6MP11 (*csrA::kanR,malP*) | 5.7 ± 0.3 | 1.3 ± 0.1 |
| G6MD3 (*ΔmalP, ΔglgBXCAY*) | < 0.2 | < 0.2 |
| TR1-5G6MD3 (*csrA::kanR ΔmalP, ΔglgBXCAY*) | < 0.2 | < 0.2 |
| Maltose grown | | |
| BW3414 | 43.5 ± 2.7 | 49.0 ± 2.2 |
| TR1-5BW3414 | 29.0 ± 2.4 | 36.9 ± 1.7 |
| G6 | 61.8 ± 5.4 | 100.4 ± 7.2 |
| TR1-5G6 | 42.3 ± 3.4 | 76.2 ± 6.3 |
| G6MP11 | 3.4 ± 0.3 | 1.7 ± 0.3 |
| TR1-5G6MP11 | 5.2 ± 0.5 | 2.2 ± 0.2 |
| G6MD3 | < 0.2 | < 0.2 |
| TR1-5G6MD3 | < 0.2 | < 0.2 |

[1]Cultures were grown at 37°C with gyratory shaking in Kornberg medium with either 0.5% glucose or 0.5% maltose. They were harvested in late stationary phase (18 hrs).

[2]One unit of activity is defined as that amount of enzyme which catalyzes the incorporation of 1 nmol [$^{14}$C]-glucose into a glucose acceptor in 20 min at 37°C.

In order to test whether the effect of *csrA* on glycogen phosphorylase was mediated at the level of *glgY* expression, a *glgY'-'lacZ* translational fusion was constructed. The expression of this gene fusion was determined in both *csrA*$^+$ and *csrA::kanR* strains. As shown in Fig. 10, the specific β-galactosidase activity expressed from *glgY'-'lacZ* fusion was three-fold higher in TR1-5BW3414 than BW3414 in stationary phase. During this experiment, the culture was monitored for the stability of the *glgY'-'lacZ* fusion by plating on X-Gal. After 24 hours growth, the TR1-5BW3414[pYZ9] strain contained about 50% white revertants while the *csrA*$^+$ strain showed no revertants of the *lac*$^+$ phenotype. This indicated that the three-fold difference of β-galactosidase between *csrA*$^+$ and *csrA::kanR* is probably an underestimate of the effect of *csrA* on expression. The effect of *csrA* on the *glgY* gene further support the evidence that *csrA* negatively regulates glycogen phosphorylase (Table II).

Effects of *csrA* on glycogen debranching enzyme

Glycogen phosphorylase can not bypass the α-1,6 glucosidic linkage. Therefore, the complete degradation of glycogen also requires the involvement of a glycogen debranching enzyme that is able to hydrolyze this linkage (17). In order to test the effects of *csrA* on the debranching enzyme, the debranching enzyme activity was determined in BW3414 and its *csrA::kanR* mutant TR1-5BW3414. Table III shows that the levels of glycogen debranching enzyme in BW3414 and TR1-5BW3414 were much lower than the levels of the other enzymes involved in glycogen metabolism, and that debranching enzyme activity was approximately the same in BW3414 and TR1-5BW3414 at late stationary phase of growth (18 hrs). This indicates that *csrA* may not regulate the debranching enzyme. Semiquantitative analysis of the hydrolysis of the α-1,6 branches of glycogen was obtained by the examination of the visible absorbance spectrum of the iodine-glycogen complex. When oyster glycogen was incubated with the cell crude extracts for 24 hrs

FIG. 10. **Expression of *glgY'-'lacZ* translational fusion in BW3414 (*csrA*$^+$) and TR1-5BW3414 (*csrA::kanR*).** Cultures containing plasmid pYZ9 were grown in Kornberg medium containing 0.5% glucose. β-galactosidase specific activities expressed from *glgY'-'lacZ* fusion in BW3414 (open symbols) or TR1-5BW3414 (closed symbols) are shown as squares; growth of the cultures ($A_{600}$) is shown as circles.

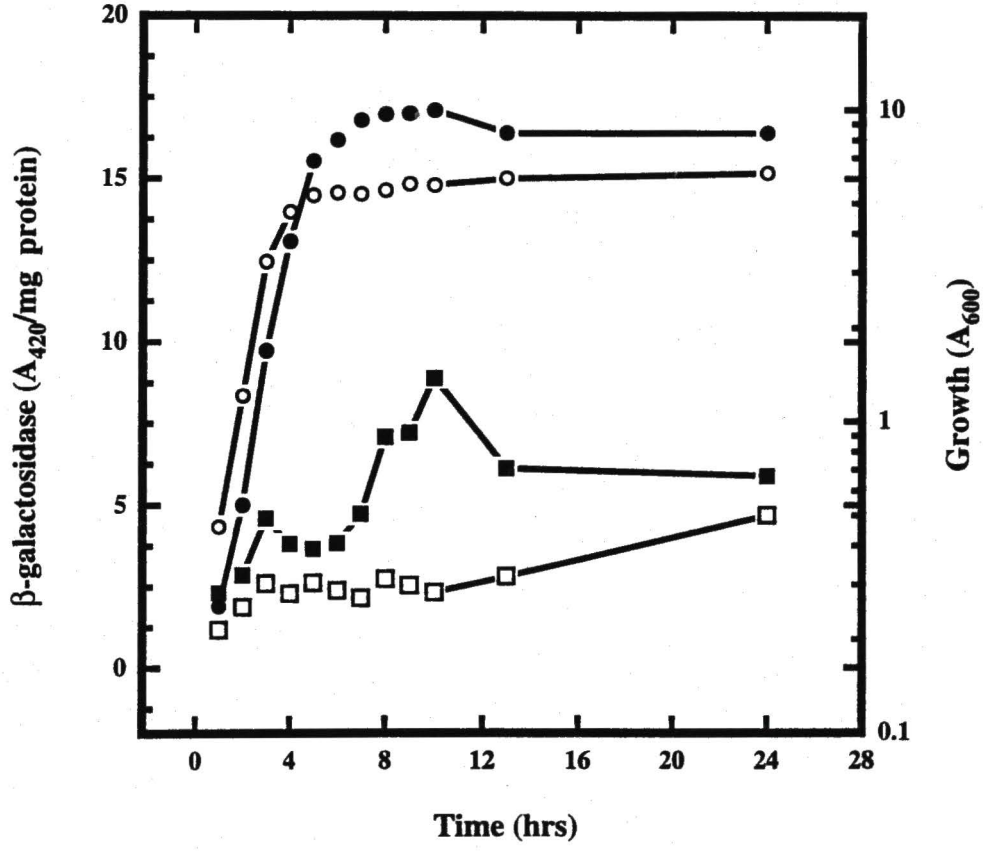

β-galactosidase ($A_{420}$/mg protein)
Growth ($A_{600}$)
Time (hrs)
20
15
10
5
0
10
1
0.1
0
4
8
12
16
20
24
28

Table III

*Levels of glycogen debranching enzyme activity in different E. coli strains and their TR1-5 mutants.* [1]

| Strain | Debranching enzyme activity (units/mg protein)[2] |
|---|---|
| BW3414 | 0.012 ± 0.0012 |
| TR1-5BW3414 | 0.010 ± 0.0005 |
| G6MD3 | < 0.0015 |
| TR1-5G6MD3 | < 0.0015 |

[1]Cultures were grown at 37°C with gyratory shaking in Kornberg medium containing 0.5% glucose. They were harvested in late stationary phase (18 hrs).
[2]One unit of enzyme activity is defined as the amount of enzyme which releases one μmol of reducing end groups per min at 37°C.

under previously established conditions (17), the absorbance maximum was shifted from 430 nm to 450 nm by the BW3414 extract, and from 430 nm to 443 nm by the TR1-5BW3414 extract.

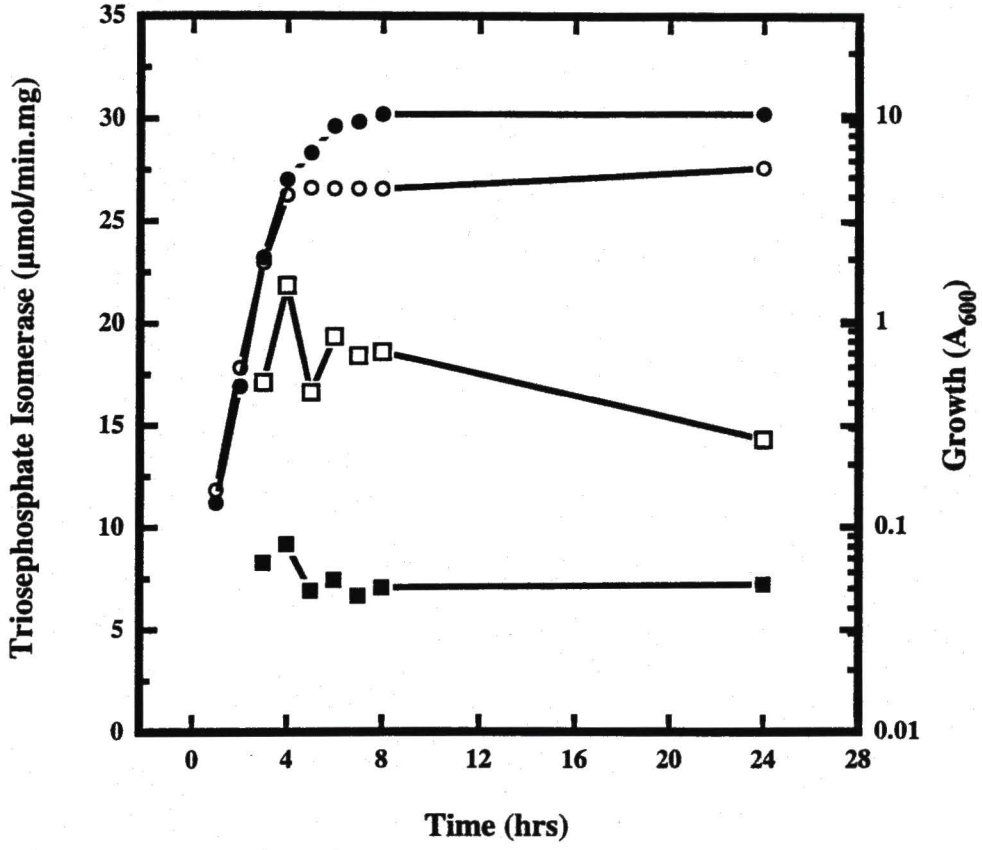
The glycogen debranching enzyme has very low activity toward native high molecular weight glycogen (17). However, it is highly active on glycogen which has been partially degraded with glycogen phosphorylase. In order to ascertain whether the higher cellular glycogen level in TR1-5BW3414 interferes with the debranching assay, crude extract of BW3414 and TR1-5BW3414 were assayed with or without the addition of heat inactivated TR1-5BW3414 crude extract. Similar activities were obtained with both BW3414 and TR1-5 BW3414 in the presence or absence of heat-treated extract (data not shown). Therefore, endogenous glycogen did not affect the debranching enzyme assay.

Similar experiments were carried out with a mutant strain, G6MD3, which lacks the entire *glg* gene cluster. The debranching enzyme activity in this strain and its *csrA::kanR* mutant were undetectable (Table III), suggesting that the gene encoding the debranching enzyme may have been deleted in G6MD3. Alternatively, the expression of this gene may depend upon the accumulation of intracellular glycogen.

Specific activity of triosephosphate isomerase is decreased by the *csrA::kanR* mutation

Previous studies indicated that both glycogen synthesis and gluconeogenesis were negatively regulated by *csrA* (45). To test whether *csrA* may affect the expression of genes in other metabolic pathways, triosephosphate isomerase, a bidirectional enzyme in Embden-Meyerhof pathway was examined in both *csrA*$^+$ and *csrA::kanR* strains. It is established that the levels of Embden-Meyerhof enzymes generally maintained at significantly higher levels to meet the glycolytic needs of *E. coli* relative to the gluconeogenic requirements. Therefore, regulation of TPI will primarily determine carbon flux in the glycolytic direction. TPI converts dihydroxyacetone phosphate to glyceraldehyde-3-phosphate in glycolysis. According to Fig.11, the *csrA*$^+$ strain has a

FIG. 11. **Triosephosphate isomerase specific activities in BW3414 (*csrA*$^+$) and TR1-5BW3414 (*csrA::kanR*).** Cultures were grown in Kornberg medium containing 0.5% glucose. Triose phosphate isomerase specific activities were determined in BW3414 (open symbols) or in TR1-5BW3414 (closed symbols) and are shown as squares; growth of the cultures ($A_{600}$) is shown as circles.


Triosephosphate Isomerase (µmol/min.mg)
35
30
25
20
15
10
5
0
0
4
8
12
16
20
24
28
Time (hrs)
10
1
0.1
0.01
Growth ($A_{600}$)

two to three-fold higher specific TPI activity than the isogenic *csrA::kanR* strain. This difference in TPI activity may be either due to the direct effect of *csrA* on *tpi* or an indirect effect which *csrA* exerts on carbon flux resulting from its effects on glycogen synthesis in the two different strains. These possibilities were tested by an experiment utilizing a strain incapable of glycogen synthesis, G6MD3. G6MD3 has a deletion of the entire *glg* gene cluster, thus it is not able to synthesize glycogen. The specific activity of TPI was found to be two-fold higher in G6MD3 than its isogenic *csrA::kanR* strain (Fig. 12). Therefore, *csrA* positively affects the glycolysis pathway enzyme TPI, independent of the ability of the strain to synthesize glycogen.

To determine whether the difference in TPI activity in the *csrA*$^+$ and *csrA::kanR* strains was due to the inactivation of the *csrA* gene, rather than possible polarity of the kan$^R$ insertion mutation, a multicopy plasmid containing a functional *csrA* gene, pCSR10, was tested for its ability to complement the *csrA::kanR* mutation. TPI activity was compared in 3 isogenic strains: BW3414[pUC19], TR1-5BW3414[pUC19] and TR1-5BW3414[pCSR10]. As shown in Fig. 13, TPI activity was two-fold higher in BW3414[pUC19] than in the *csrA::kanR* strain, TR1-5BW3414[pUC19]. When pCSR10 was introduced into TR1-5BW3414, it complemented the negative effects of *csrA::kanR* mutation on TPI. TPI activity in the mutant was elevated to the level of BW3414, further indicating that the difference in TPI activity was due to the *csrA* gene. Therefore, *csrA* positively regulates TPI in glycolysis pathway. Moreover, when *csrA* gene was overexpressed from the plasmid pCSR10, the negatively regulated genes were consistently found to be expressed at levels lower than in the *csrA*$^+$ strain. TPI activity, which is subject to positive regulation by *csrA*, was nevertheless not expressed at higher levels in the pCSR10-containing strain relative to the *csrA*$^+$ strain. This indicates that the positive effect of *csrA* on TPI activity is saturated with only one functional copy of *csrA* gene.

FIG. 12. **Triosephosphate isomerase specific activities in glycogen-deficient strains G6MD3 (*csrA*$^+$) and TR1-5G6MD3 (*csrA::kanR*).** Cultures were grown in Kornberg medium containing 0.5% glucose. Triose phosphate isomerase specific activities were determined in G6MD3 (open symbols) or in TR1-5G6MD3 (closed symbols) and are shown as squares; growth of the cultures ($A_{600}$) is shown as circles.

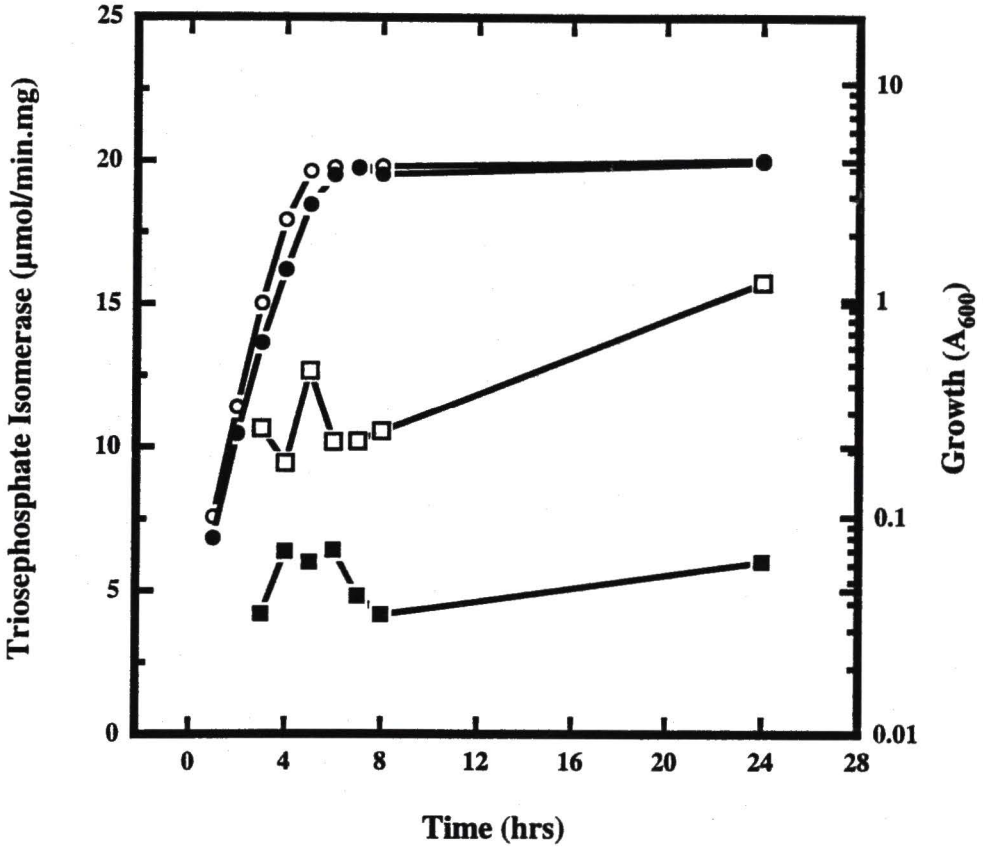
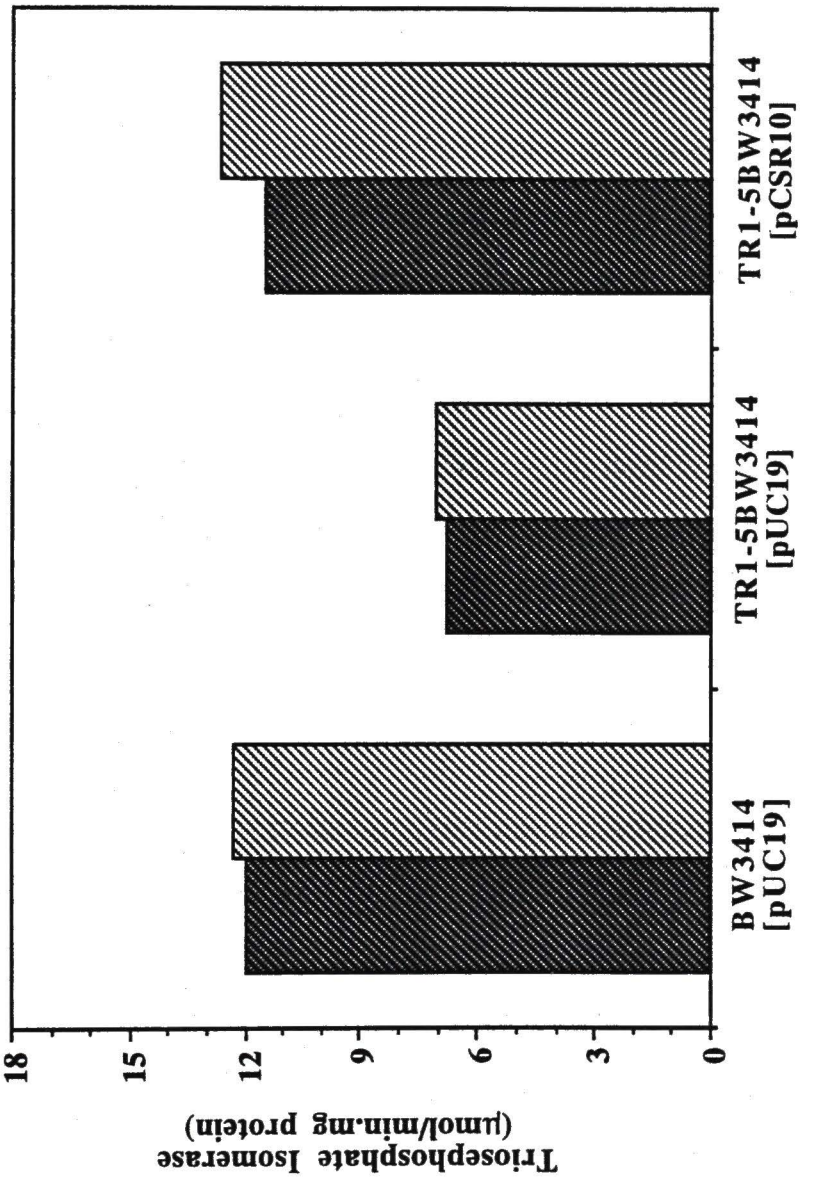

Triosephosphate Isomerase (µmol/min.mg)
25
20
15
10
5
0
0
4
8
12
16
20
24
28
Time (hrs)
10
1
0.1
0.01
Growth ($A_{600}$)

FIG. 13. **Complementation of the negative effect of the *csrA::kanR* mutation on TPI activity by pCSR10, a multicopy plasmid which encodes *csrA*.** Triosephosphate isomerase specific activities were measured in BW3414[pUC19], TR1-5BW3414[pUC19] and TR1-5BW3414[pCSR10]. Cells were grown in Kornberg medium containing 0.5% glucose. Specific activities of triose phosphate isomerase were determined at mid-log phase (5hr) (dark shading) and late stationary phase (24hr) (light shading).


Triosephosphate Isomerase
(μmol/min.mg protein)
0
3
6
9
12
15
18
BW3414
[pUC19]
TR1-5BW3414
[pUC19]
TR1-5BW3414
[pCSR10]

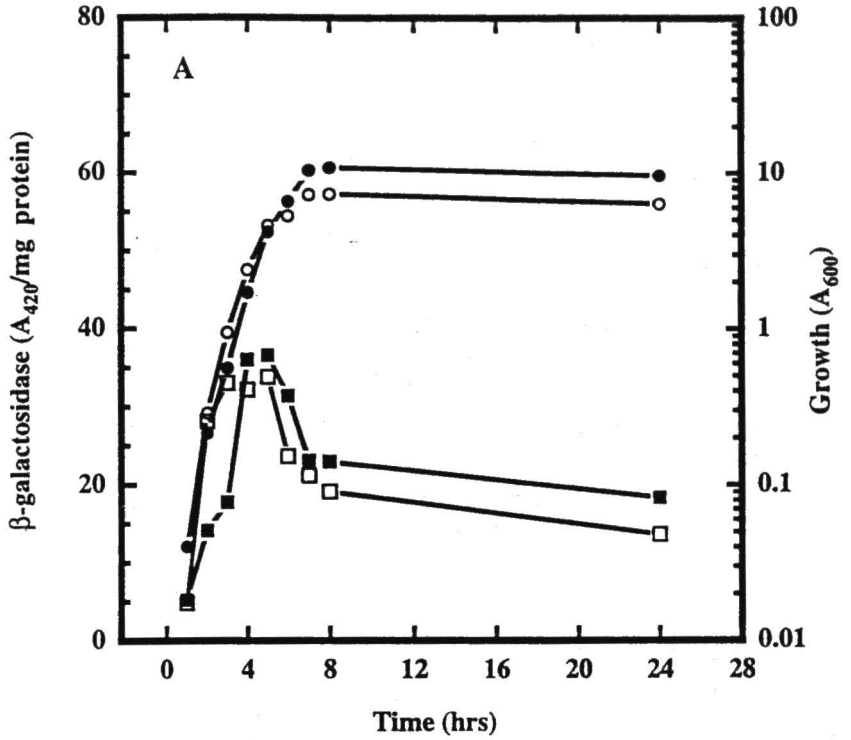
The *csrA* gene does not significantly affect the expression of genes in the pentose phosphate pathway

In aerobically growing *Escherichia coli*, the predominant pathway for glucose catabolism is through the glycolytic pathway to yield pyruvate, which is oxidized to $CO_2$ in the citric acid cycle (30). An alternative pathway is the pentose phosphate pathway, which is used to provide NADPH for reductive biosynthesis and to provide ribose-5-phosphate for nucleotide and nucleic acid biosynthesis (30). Since *csrA* positively regulates TPI activity in the glycolysis pathway, it was of interest to determine whether it regulates the enzymes in pentose phosphate pathway as well. The expression of two genes, *gnd* and *zwf*, in pentose phosphate pathway were examined. The gene *zwf* encodes glucose-6-phosphate dehydrogenase, which converts glucose-6-phosphate to 6-phosphogluconolactone; *gnd* encodes 6-phosphogluconate dehydrogenase, which converts 6-phosphogluconate to ribulose-5-phosphate and $CO_2$. Figures 14 and 15 show that *csrA* has little or no effect on the expression of either *gnd* and *zwf* transcriptional and translational *lacZ* fusions, suggesting that *csrA* probably plays no role in regulating the pentose phosphate pathway.

CsrA-containing S-200 extracts inhibit the in vitro transcription-translation of *glg* genes and alter the relative expression of genes within the *glgCAY* operon

The *csrA* gene was observed to negatively affect the expression of *glgC*, *glgB* and *glgA in vivo* (45). In order to rigorously study the mechanism of genetic regulation by *csrA*, it was important to reconstruct regulation *in vitro*. The S-30 transcriptional and translational assay was chosen for initial studies, which should allow the effects of *csrA* to be detected regardless of which step of transcription or translation that it may regulate. S-30 extracts which contained the cellular factors needed for transcription-translation of plasmid-encoded genes were prepared from the *csrA*$^-$ strain TR1-5BW3414. S-200

FIG. 14. **Expression of *gnd::lacZ* fusions in *csrA*$^+$ and *csrA::kanR* strains.** (A) Expression of *gnd-'lacZ* transcriptional fusions in HB354 and TR1-5HB354; (B) Expression of *gnd'-'lacZ* translational fusions in HB582 and TR1-5HB582. β-galactosidase specific activities expressed from *gnd::lacZ* fusions in *csrA*$^+$ strains (open symbols) or *csrA::kanR* strains (closed symbols) and are shown as squares; growth of the cultures ($A_{600}$) are shown as circles.


A
β-galactosidase ($A_{420}$/mg protein)
Growth ($A_{600}$)
Time (hrs)
0
20
40
60
80
0.01
0.1
1
10
100
0
4
8
12
16
20
24
28

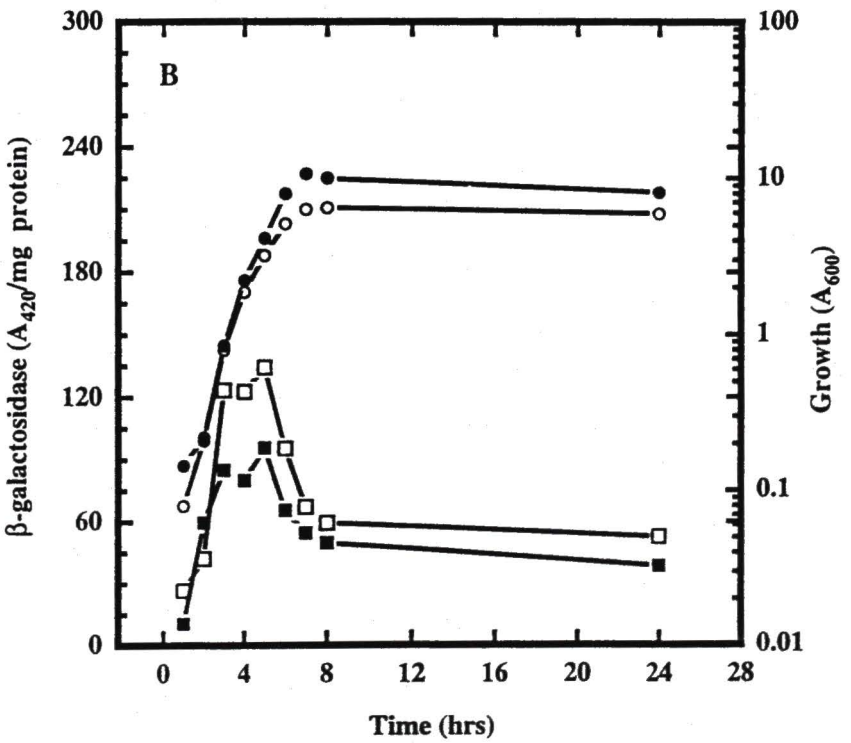
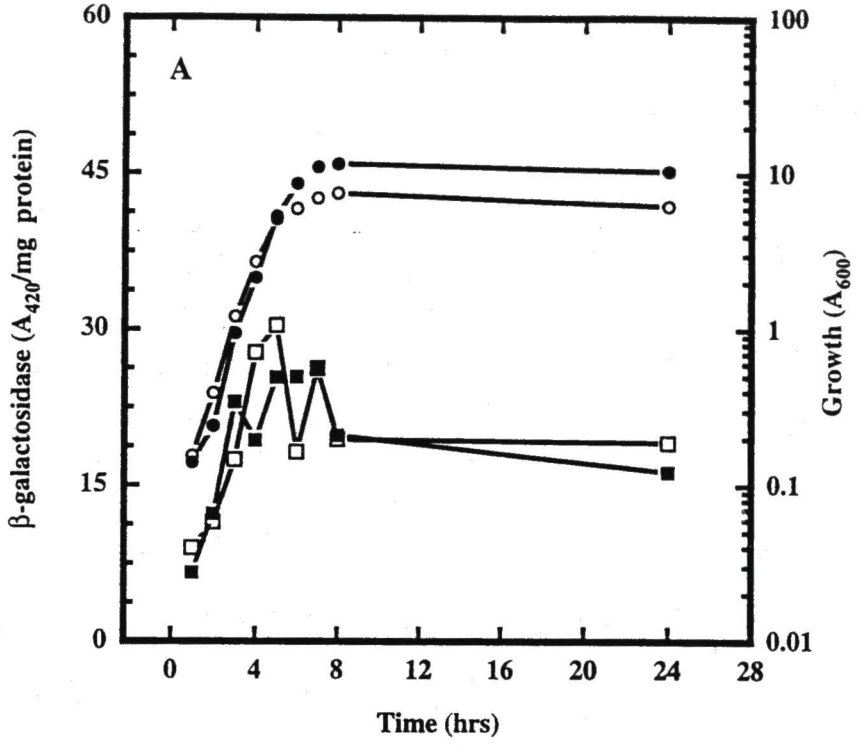
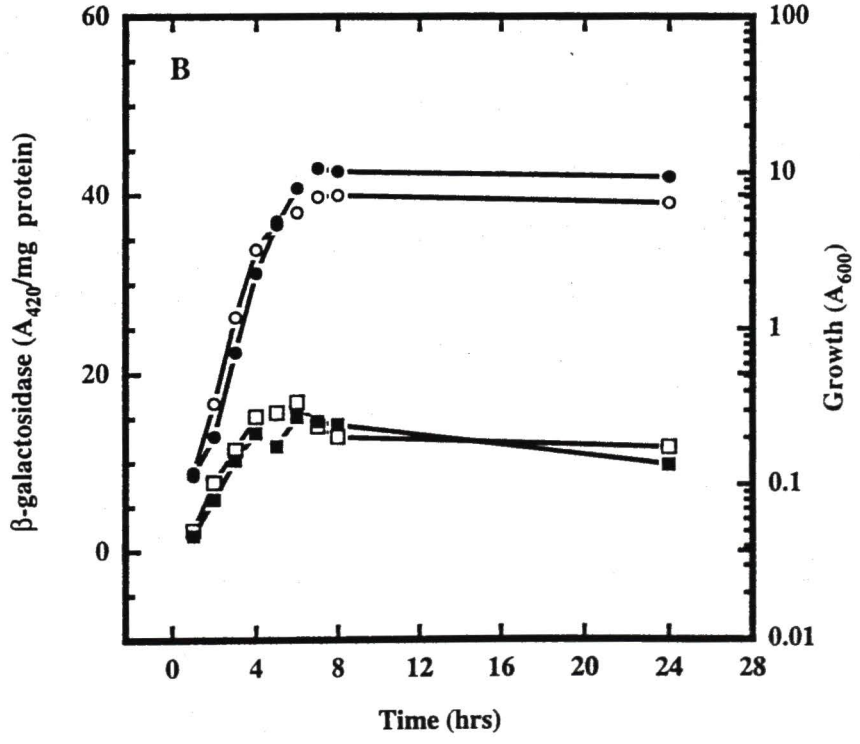

B
β-galactosidase ($A_{420}$/mg protein)
Growth ($A_{600}$)
Time (hrs)
0
60
120
180
240
300
0.01
0.1
1
10
100
0
4
8
12
16
20
24
28

FIG. 15. **Expression of *zwf::lacZ* fusions in *csrA*$^+$ and *csrA::kanR* strains.** (A) Expression of *zwf-'lacZ* transcriptional fusions in HB301(λDR52) and TR1-5HB301(λDR52); (B) Expression of *zwf'-'lacZ* translational fusions in HB301(λDR104) and TR1-5HB301(λDR104). β-galactosidase specific activities expressed from *zwf::lacZ* fusions in *csrA*$^+$ strains (open symbols) or *csrA::kanR* strains (closed symbols) and are shown as squares; growth of the cultures ($A_{600}$) are shown as circles.

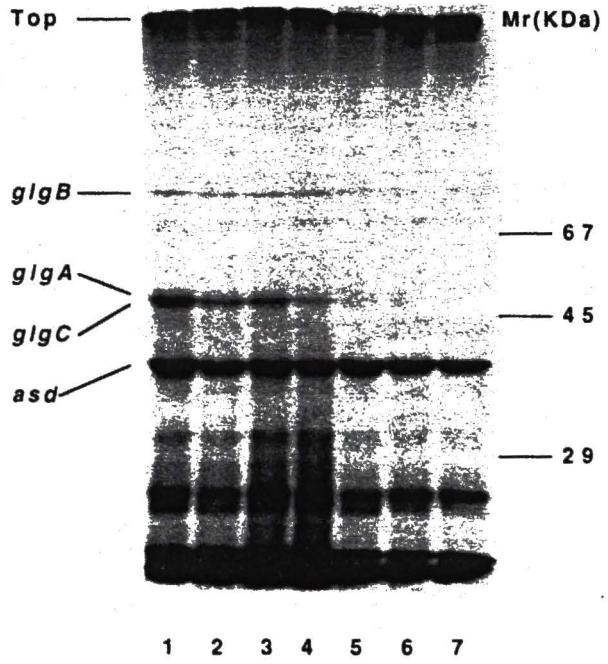

A
β-galactosidase ($A_{420}$/mg protein)
Growth ($A_{600}$)
Time (hrs)
60
45
30
15
0
100
10
1
0.1
0.01
0
4
8
12
16
20
24
28


B
β-galactosidase ($A_{420}$/mg protein)
Growth ($A_{600}$)
Time (hrs)
60
40
20
0
100
10
1
0.1
0.01
0
4
8
12
16
20
24
28

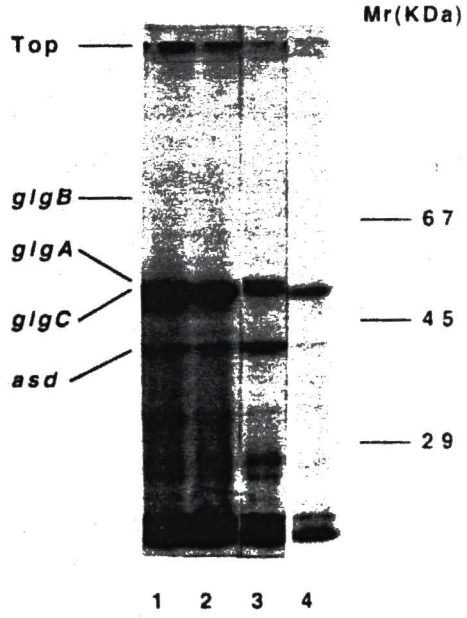
extracts, which had been depleted of macromolecular complexes (e.g. ribosomes), were also prepared from this strain and from another strain that overexpressed the *csrA* gene, TR1-5BW3414[pCSR10]. Both kinds of extracts were treated with micrococcal nuclease to degrade endogenous nucleic acids. Extracts from the latter strain contained elevated levels of CsrA protein, as determined by Western blot analysis (25). As previously shown, the *in vitro* synthesis of the products of four genes: *glgB*, *glgA*, *glgC* and *asd* (aspartate semialdehyde dehydrogenase) absolutely depended upon the addition of pOP12 plasmid to the reactions (47; Fig. 16). As observed previously, the addition of the activators: cAMP, ppGpp and CRP increased the pOP12-directed synthesis of ADP glucose pyrophosphorylase (*glgC*) and glycogen synthase (*glgA*), but did not alter *glgB* expression. The addition of the CsrA-containing S-200 extract strongly inhibited *glgC* expression in both the basal reactions and in reactions which were activated via cAMP, CRP and ppGpp (Fig.16). CsrA-containing extracts caused little inhibition of *glgA* expression. This result contrasts with the observation made on the experiment of *glgA'-'lacZ* fusion (Fig. 3). Therefore, the expression ratio of *glgA/glgC* was much greater in the presence of CsrA in both the activated and inactivated reactions. The expression of *asd* showed little or no effect of the CsrA-containing extract in the absence of activators cAMP-CRP and ppGpp, and was enhanced by the addition of the CsrA-containing extract in the presence of these activators.

FIG. 16. **Effects of S-200 extracts from *csrA*-deficient or *csrA*-overexpressing strains on the expression of pOP12-encoded genes.** Transcription-translation reaction (35µl) were conducted using an S-30 extract prepared from TR1-5BW3414 (*csrA::kanR*) and were analyzed as described in Materials and Methods. The positions of unlabeled standards of glycogen branching enzyme (*glgB*), ADPglucose pyrophosphorylase (*glgC*), and glycogen synthase (*glgA*), bovine serum albumen (67), ovalbumen (45), and carbonic anhydrase (29) were determined by Coomassie blue staining. The quantity of S-200 protein (µg) from strain TR1-5BW3414 (TR) or from TR1-5BW3414[pCSR10] that was added to each reaction mixture prior to starting the reactions with pOP12 plasmid DNA (2 µg) is indicated. The reactions shown in the right panel contained 2 µg CRP, 100 µM cAMP, and 250 µM ppGpp; reactions in the left panel lacked these factors. Lane 4 of the right panel shows a lighter exposure of lane 2 to allow a better assessment of the effect of the CsrA-containing extract on the relative expression *glgC* and *glgA*.

| TR S-200 (μg) | 0 | 5 | 20 | 50 | 0 | 0 | 0 |
|---|---|---|---|---|---|---|---|
| pCSR S-200(μg) | 0 | 0 | 0 | 0 | 5 | 20 | 50 |




| TR S-200 (μg) | 0 | 5 | 0 | 5 |
|---|---|---|---|---|
| pCSR S-200 (μg) | 0 | 0 | 5 | 0 |

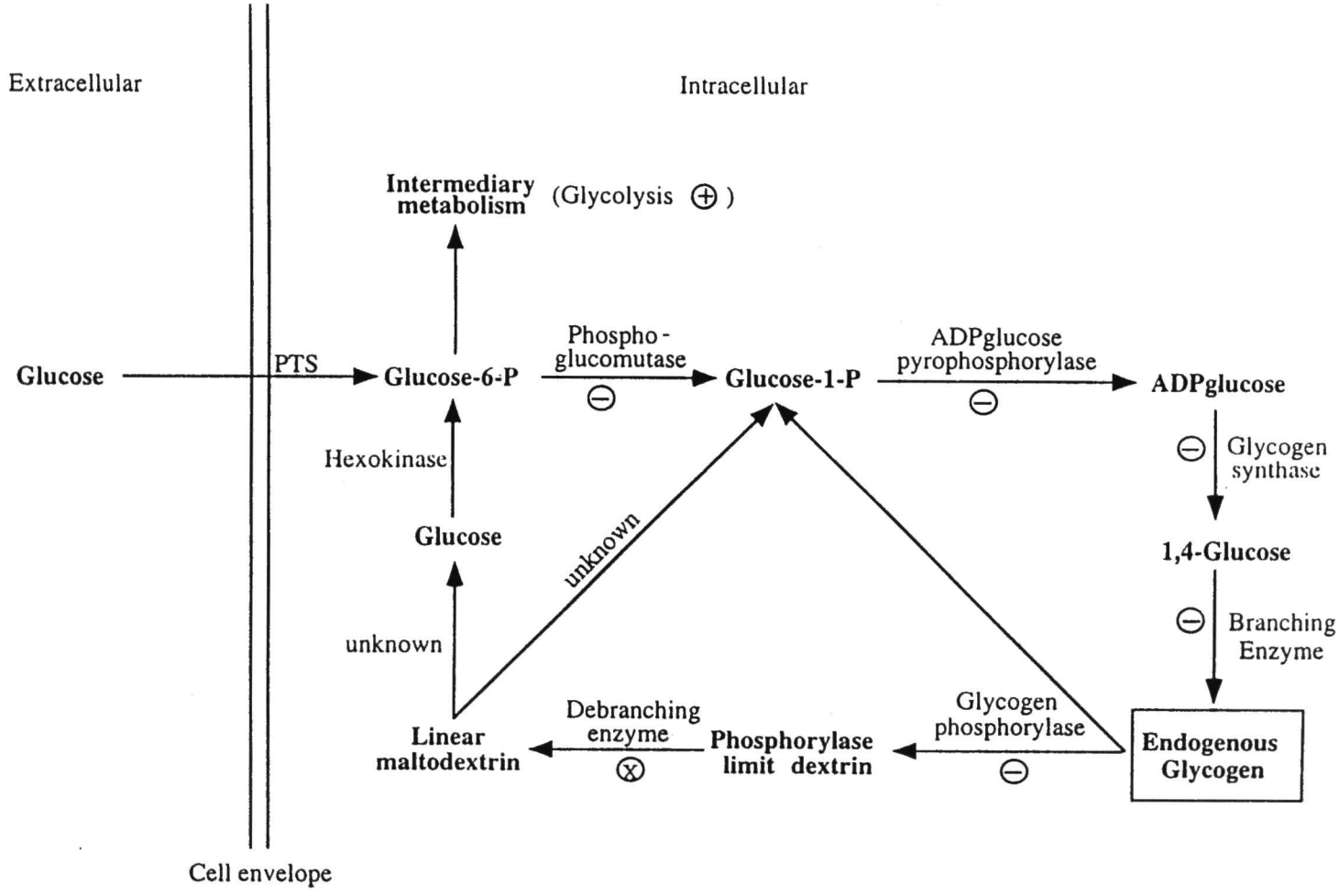


# DISCUSSION

*E. coli* is able to rapidly grow in the presence of abundant nutrients in either the natural or laboratory environment, but inevitably the batch culture will exhaust available nutrients. In order to prepare for and survive conditions of nutrient exhaustion, *E. coli* redirects its metabolism from one that sustains growth to one that maintains viability during starvation conditions. Glycogen is a readily metabolized endogenous source of energy for *E. coli* to utilize for surviving during starvation. Although much has been learned about the biochemistry of glycogen metabolism in *E. coli* cells, the investigation of the genetic details of its regulation is still in the early stage. One important regulator in this process, *csrA*, was previously found to negatively regulate glycogen biosynthesis (45). Until the investigation in this thesis, little was known about the role of *csrA* in glycogen degradation or in other metabolic pathways. Evidence from this thesis indicates that (1) *csrA* negatively regulates both the synthesis and the degradation of glycogen (summarized in Fig. 17); (2) *csrA* positively regulates TPI enzymatic activity, suggesting a role in the regulation of glycolysis; (3) effects of *csrA* on the growth rate of *Δcya* strains suggests an even wider physiological role than regulation of carbon flux; (4) negative regulation of *glgS* by *csrA* is *rpoS* dependent, allowing for a potential role of *csrA* in the regulation of *rpoS*, which itself is a central regulatory gene for early stationary phase gene expression (13); and (5) *glgX* may encode a glycogen debranching enzyme.

The enhancement of endogenous glycogen levels in a *csrA::kanR* mutant (TR1-5BW3414) has been clearly demonstrated by iodine vapor staining (45). However, the quantitative measurement of the glycogen levels throughout the growth curve in *csrA*$^+$ and *csrA::kanR* strains shown in this thesis provided a fundamental insight to the effects of

FIG. 17. **The regulation of glycogen metabolism in *E. coli* by *csrA*.** Negative regulation (-), positive regulation (+), no regulation (x).


Extracellular
Intracellular
Intermediary metabolism
(Glycolysis ⊕)
Glucose
PTS
Glucose-6-P
Phospho - glucomutase
Glucose-1-P
ADPglucose pyrophosphorylase
ADPglucose
Glycogen synthase
1,4-Glucose
Branching Enzyme
Hexokinase
Glucose
unknown
unknown
Linear maltodextrin
Debranching enzyme
Phosphorylase limit dextrin
Glycogen phosphorylase
Endogenous Glycogen
Cell envelope

*csrA* on intracellular glycogen synthesis and degradation. The glycogen curve of TR1-5BW3414 exhibited up to 15-fold enhancement of endogenous glycogen levels caused by the *csrA::kanR* mutation in the early stationary phase. The rapid increase (0.25 mg glycogen / mg protein · hour) and decline (0.11 mg glycogen / mg protein · hour) observed in the TR1-5BW3414 glycogen levels suggests that *csrA* negatively regulates glycogen degradation as well as synthesis (Fig. 2).

The inhibition of glycogen biosynthesis by *csrA* can be attributed to its negative effects on the expression of at least four genes involved in glycogen synthesis, *glgC* (ADP-glucose pyrophosphorylase), *glgA* (glycogen synthase), *glgB* (glycogen branching enzyme), and *glgS* (a novel gene involved in glycogen synthesis), which are constituted in three different operons. The regulation of glycogen synthesis involves a complex assemblage of factors which adjust the rate of synthesis according to the physiological status of the cell. These factors have been rigorously shown to affect glycogen synthesis on at least two levels; allosteric regulation of ADP-glucose pyrophosphorylase and genetic regulation of the expression of the structural genes *glgC*, *glgA*, and *glgB*. When cells grow in the presence of excess glucose, glucose can either be catabolized *via* glycolysis to provide energy or be stored in the form of glycogen. During exponential growth, nucleic acid and protein synthesis in the cell consume large amounts of ATP, and the ATP level is relatively low (about 70%) compared to the stationary phase (51). In order to produce ATP to meet these requirements, cells need to metabolize available carbohydrate through glycolysis rather than to store it through glycogen synthesis. Therefore, *E. coli* may need a negative regulatory system to control glycogen synthesis. The *csrA* gene may facilitate this task in two aspects. (1) stimulating the glycolytic pathway by its positive regulation on TPI and other enzymes in the glycolytic pathway (51). (2) blocking the flux of carbon into glycogen by negatively regulating the expression of glycogen biosynthetic genes. It is well established that the glycolytic intermediate fructose-1,6-bisphosphate is a potent activator of

ADP-glucose pyrophosphorylase (43). When the cell predominantly utilizes the glycolytic pathway, a considerable amount of fructose-1,6-bisphosphate accumulates in the cell, and should stimulate glycogen biosynthesis (28). Therefore, it is imperative that the cell has a negative regulator of glycogen synthesis in order to impede the positive allosteric effect of fructose-1,6-bisphosphate on ADP-glucose pyrophosphorylase. This will assure that the bulk of carbon flux goes through the energy producing pathways rather than into the storage pathway during exponential phase. However, the disruption of *csrA* does not alter the pattern of induction of *glg* genes in the stationary phase of growth, suggesting that *csrA* regulation is not responsible for growth phase control. Therefore, the physiological parameters to which *csrA* responds still need to be clarified.

*E. coli* accumulates glycogen in the early stationary phase and degrades glycogen to meet its energy need when exogenous carbon sources become limiting. Since the synthetic genes (*glgC* and *glgA*) and the degradative gene (*glgY*) are clustered in the same operon (2, 22, 49, 61), the cell may synthesize ADP-glucose pyrophosphorylase, glycogen synthase and glycogen phosphorylase at the same time, thereby allowing their activities to be controlled through allosteric regulation. During glycogen synthesis, ADP-glucose inhibits glycogen phosphorylase whereas, in the degradation of glycogen, AMP activates glycogen phosphorylase (6) and inhibits ADP-glucose pyrophosphorylase (43). Our studies show that *csrA* also negatively regulates glycogen degradation *via* its effect on glycogen phosphorylase. The physiological significance of why the cell needs such a negative system to control the degradation of glycogen is still unclear. Studies suggest that the intracellular glycogen provides energy for the cell, and therefore, can retard the degradation of RNA and protein, and prolongs the survival time during starvation (29). Thus, the cell presumably degrades glycogen at slow rate only to meet its survival energy requirements. The negative regulation of *csrA* on glycogen phosphorylase levels

ultimately reduces the rate of glycogen degradation and thereby may extend the bacterial survival time during starvation.

In addition to glycogen phosphorylase, glycogen debranching enzyme is another enzyme participating in glycogen degradation. It hydrolyzes α-1,6-glucosidic linkages, to allow further degradation of glycogen from phosphorylase limit dextrin to linear maltodextrins. Studies showed that *csrA* has no effect on this enzyme (Table III). This observation may be considered in light of two other observations. First, *csrA* exhibits stronger negative effects on the *glgA* and *glgC* genes which encode enzymes catalyzing the reactions needed for the synthesis of the linear chains (α-1.4 linkages), relative to the *glgB* gene which encodes the branching enzyme (α-1,6 linkages). The β-galactosidase activity expressed from the *glgC'-'lacZ* and *glgA'-'lacZ* translational fusions was approximately seven-fold and twelve-fold higher, respectively, in TR1-5 mutant than in an isogenic *csrA*$^+$ strain, but only 2~3-fold higher for *glgB'-'lacZ* fusion in TR1-5 mutant. The glycogen curve of TR1-5BW3414 indicates that *csrA* also negatively regulates glycogen degradation. According to the relative strength of the effects that *csrA* exhibited on the enzymes needed to form the α-1,4 polymers and the α-1,6 linkages, we may predict that *csrA* should exhibit greater regulation of the enzyme which breaks down the α-1,4 linkage (glycogen phosphorylase) relative to the enzyme which hydrolyzes the α-1,6 linkage (debranching enzyme). Second, *csrA* has weaker effects on the glycogen degradation enzyme, glycogen phosphorylase, compared to its effects on the glycogen synthetic genes. Enzymes for catabolism of glycogen are found at extremely low levels (20, 43) and the debranching enzyme activity is even lower than that of glycogen phosphorylase (17). The *csrA::kanR* mutation elevates the glycogen phosphorylase activity approximately three-fold. The relative level of *csrA* effect on glycogen phosphorylase is approximately one-third to one-fourth of its effects on the *glgC* and

*glgA* genes. Base upon these comparisons, it is not surprising that *csrA* exerts no regulation on the debranching enzyme.

In G6MD3, a strain which has the entire *glg* gene cluster deleted, the activity of the glycogen debranching enzyme was undetectable, suggesting that the gene which encodes the debranching enzyme may be located within *glg* gene cluster. Based upon sequence homology, previous studies have suggested that *glgX* most likely encodes a glucosyl-transferase or glucan hydrolase (46). The amino acid sequence of *glgX* was found to have significant similarity to mammalian and bacterial α-amylases, bacterial pullulanase and glucosyltransferase (46). The *E. coli* glycogen branching enzyme also shows extensive sequence similarity with *glgX* (46) and with the rabbit muscle and human muscle debranching enzymes (24). Therefore, *glgX* may encode an *E. coli* glycogen debranching enzyme. However, purification of the *glgX* gene product is necessary to provide a definitive proof of this hypothesis.

Carbon flux through the Embden-Meyerhof pathway must be regulated if a glycolytic/gluconeogenic futile cycle is to be avoided. This thesis shows that TPI which converts dihydroxyacetone phosphate to glyceraldehyde-3-phosphate in the glycolytic pathway is positively regulated by *csrA*. Sabnis et al, have shown that in addition to TPI, phosphoglucoisomerase (PGI), enolase, phosphofructokinase I (PFK I) and pyruvate kinase F (PYK F) are also under the positive control of *csrA* (51). This positive effect of *csrA* on PGI and PFK I will cause the accumulation of larger amounts of DHAP and G3P in the *csrA*$^+$ cell compared to the *csrA::kanR* mutant. Since *csrA* also positively regulates enolase and PYK F, a greater amount of the G3P will ultimately be metabolized to pyruvate *via* the glycolytic pathway. The increased TPI activity induced by *csrA* responds to this need for increased G3P substrate. Another explanation that might be considered to account for the positive effect of *csrA* on TPI activity is to avoid the

conversion of DHAP by methylglyoxal synthase to methylglyoxal, which is extremely toxic to bacterial cells. Although glyoxylase could convert methylglyoxal to D-lactate, studies have shown that this enzyme is of low capacity and insufficient to prevent methylglyoxal toxicity in the *tpi* mutant (12). *csrA* may increase TPI conversion of DHAP to G3P to avoid accumulation of toxic methylglyoxal levels under conditions of increased DHAP synthesis.

No evidence has been found to determine why the effects of *csrA* on TPI activity seems to be maximal with only a single chromosomal copy of *csrA* gene. However, two hypotheses may be proposed: (1) The *csrA* gene product may directly bind to *tpi* transcript to either stabilize the transcript or permit its translation. The *tpi* transcript binding site for CsrA could be saturated by the levels of the CsrA protein which are expressed from a single *csrA*$^+$ allele. (2) Alternatively, *csrA* may positively regulate the expression of a gene which has a positive effect on *tpi*. This putative gene may achieve maximal effect on *tpi* at a low level of expression. When the purified CsrA protein is obtained, its effects on glycolytic genes in an S-30 transcription and translation assay could allow theses hypotheses to be tested. This would aid in determining whether the effect is direct or indirect, and would help to establish the saturating concentration of CsrA protein.

Studies in this thesis show that *csrA* has a strong negative effect on *glgS* expression when *rpoS* is present in the cell, but when *rpoS* is mutated in the cell, *csrA* no longer regulates the *glgS* gene. It is known that *glgS* is regulated by cAMP and *rpoS* gene product ($\sigma^s$), although *glgS* expression depends entirely on $\sigma^s$ under conditions of maximal glycogen synthesis, i.e. excess glucose and limited nitrogen (13). The stationary phase-specific sigma factor $\sigma^s$ is a regulatory molecule controlling the transcription of a number of genes during stationary phase (16, 27). It is required for development of resistance against pH fluctuations, oxidative stress and heat shock in starved cells. It is also involved in cell morphology and cell division (16, 27). The *rpoS* gene not only

controls other growth phase-regulated genes, but is itself a stationary phase induced gene (16, 27). The observation that *csrA* gene did not regulate *glgS* in the *rpoS* mutant may have three possible explanations. First, the *glgS* gene expression is extremely low in the *rpoS* mutant (about one tenth of the *glgS* expression in the *rpoS*$^+$ strain) when cells were grown in Kornberg medium containing 0.5% glucose. Based upon this level of the expression, it is possible that the regulation could not be observed. A second hypothesis is that the regulation of *glgS* by *csrA* is dependent on *rpoS*. Evidences from previous studies disprove the possibility that *rpoS* gene regulates *csrA*. It has been established that both *glgA* and *glgC* are not under *rpoS* control (13), but are negatively regulated by *csrA*. If *csrA* was under *rpoS* regulation, then both *glgA* and *glgC* gene should be regulated by *rpoS* as well. Therefore, *rpoS* does not regulate *csrA*. It is possible however, that *csrA* negatively regulates *rpoS*. Since the *glgS* expression is dependent on *rpoS*, the *csrA::kanR* mutation may elevate the expression of *glgS* through *rpoS*. When *rpoS* was mutated in the cell, *csrA* lost its regulation target and its indirect effect on *glgS* gene was, therefore, abolished. The third possibility is that the CsrA protein may bind to only one of the two *glgS* transcripts. The *glgS* gene has two transcripts, *glgS*p1 and *glgS*p2. The *glgS*p1 transcript was absent in a *cya* mutant, whereas an *rpoS* mutant did not synthesize the *glgS*p2 transcript (13). The CsrA protein may only interact with the *glgS*p2 transcript, thus the regulation of *glgS* by *csrA* could not be achieved in an *rpoS* mutant due to the absence of the *glgS*p2 transcript.

The expression of *glgS* also relies on cAMP. When *cya* is deleted from the cell, it not only decreases the expression of *glgS*, it also greatly decreases the growth rate of the culture. The *csrA::kanR* mutation was found to restore the growth rate in *Δcya* strains. cAMP-CRP is a well studied system that regulates gene expression (19). Previous studies on *glgA* and *glgC* genes have established that *csrA* negatively regulates some genes which are stimulated by cAMP. Therefore, it is possible that some genes in *E. coli* that are

important in determining the rapid growth of the cell are also under positive regulation of cAMP and negative regulation of *csrA*. When cAMP is not present in the cell, the expression of these genes would be elevated by the *csrA::kanR* mutation.

The gene *csrA* did not influence the expression of pentose phosphate genes *gnd* and *zwf*. The major role of the pentose phosphate pathway is to provide ribose-5-phosphate for nucleotide and nucleic acid biosynthesis, and to provide NADPH for reductive biosynthesis (30). Our current information indicates that the regulation of gene expression by *csrA* seems to focus on the control of carbon metabolism in response to cellular energy needs rather than the control of nucleotide and nucleic acid synthesis. Moreover, since *csrA* positively regulates the glycolytic pathway, the amount of NADH which is generated from conversion of G3P to 1,3-bisphosphoglycerate is also increased by *csrA*. Therefore, it may not be necessary to control the level of the reducing compound NADPH generated from the pentose phosphate pathway.

The observation that the *csrA* gene product inhibits the *in vitro* expression of the the *glgC*, *glgB* and *glgA* genes is similar to the results for the expression of *lacZ* fusions of these genes. Inhibition by the CsrA-containing extract *in vitro* is also independent from the effects of cAMP-CRP and ppGpp. The effects of *csrA* on the expression of glycogen synthesis genes *in vivo* and *in vitro* further demonstrates the regulatory role of *csrA* in glycogen synthesis pathway. Although CsrA-containing extracts exhibited weak effects on the *in vitro* expression of *glgA*, the *in vivo* expression of a chromosomal *glgA'-'lacZ* fusion was strongly regulated *via csrA*. This apparent discrepancy may be due to the fact that the pOP12 plasmid, which was used as the *in vitro* template for *glg* expression, does not contain the entire *glgY* gene, and the *glgCAY'* transcript that it encodes lacks half of the *glgY* coding region and a putative stem and loop structure following *glgY* (46), which may protect the native *glgA* transcript against 3' to 5' degradation *in vivo* (14). Studies have been conducted to explore the mechanism of

*csrA* regulation on glycogen metabolism, and have indicated that *csrA* destabilizes the *glgC* mRNA (26). The major transcript of *glgC* (transcript A and B) was elevated more than ten-fold in the *csrA::kanR* strain (26). The major route of *glgCAY* transcript decay *in vivo* appears to be *csrA*-mediated 5' to 3' degradation, indicating that the chromosomally-encoded transcript is very stable toward 3' to 5' degradation (14). However, degradation of the *glgCAY'* transcript from the 3' to 5' direction in the absence of *csrA* would favor the expression of *glgC* relative to that of *glgA*. In the S-30 transcription translation experiments, the addition of the CsrA-containing extract may have caused *glgCAY'* to be rapidly degraded in the 5' to 3' direction, shifting the relative expression in favor of *glgA* (Fig. 16).

The variety of mechanisms which participate in the regulation of glycogen metabolism in *E. coli* attests to the importance of this process to the cell. The complexity of *csrA* regulatory patterns has raised many more questions than have been answered. In order to address such questions and to identify the precise role of *csrA* during prolonged starvation, a variety of studies could be conducted. Viability tests on both *csrA*$^+$ and *csrA::kanR* strains could provide strong evidence as to whether *csrA* affects cell survival during starvation. Further studies of the relationship of *csrA* and *rpoS* could be performed through S-30 transcriptional and translational assay, revealing an even broader view of *csrA* regulation. The effects of the CsrA protein on the expression of glycolytic genes could be tested, in order to determine whether the CsrA protein can directly stimulate gene expression. Lastly, the effects of *csrA* on enzymes and genes in other carbon metabolic pathways should be explored.

## BIBLIOGRAPHY


1. Ausubel, F.M., R. Brent, R. E. Kingston, D. D. Moore, J. Seidman, J. A. Smith, and K. Struhl. (ed.) 1989. Current Protocols in Molecular Biology. John Wiley & Sons, Inc. New York.
2. Baecker, P. A., C. E. Furlong, and J. Preiss. 1983. Biosynthesis of bacterial glycogen. Primary structure of *Escherichia coli* ADPglucose synthetase as deduced from the nucleotide sequence of the *glgC* gene. J. Biol. Chem. 258:5084-5088.
3. Baecker, P. A., E. Greenberg, and J. Preiss. 1986. Biosynthesis of bacterial glycogen. Primary structure of *Escherichia coli* 1,4-α-D-glucan: 1,4-α-D-glucan 6-α-D-(1,4-α-D-glucano)-transferase as deduced from the nucleotide sequence of the *glgB* gene. J. Biol. Chem. 261:8738-8743.
4. Beutler, H. O. Metabolites 1, Carbohydrates, Starch: 1-10. *In* H. U. Bergmeyer (ed.) Methods of Enzymatic Analysis, Third Edition, Volume VI. Academic Press, New York and London.
5. Chamberlain, J. P. 1979. Fluorographic detection of radioactivity in polyacrylamide gels with the water-soluble flour, sodium salicylate. Anal. Biochem. 98, 132-135.
6. Chen, G. S., and I. H. Segel. 1968. *Escherichia coli* polyglucose phosphorylases. Arch. Biochem. Biophys. 127:164-174.
7. Chen, G. S., and I. H. Segel. 1968. Purification and properties of glycogen phosphorylase from *Escherichia coli.* Arch. Biochem. Biophys. 127:175-186.
8. Chen, H. Z., and G. Zubay. 1983. Prokaryotic coupled transcription-translation. p. 674-690. *In* R. Wu, L. Grossman, and K. Moldave (ed.) Methods in Enzymology. Vol. 101. Academic Press, London.

9. Creuzat-Sigal, N., C. Frixon. 1977. Catabolism of glycogen in *Escherichia coli.* FEMS Microbiol, Lett. 1:235-238.

10. Damotte, M., J. Cattaneo, N. Sigal and J. Puig. 1968. Mutants of *Escherichia coli* K 12 altered in their ability to store glycogen. BBRC 32:916-920.

11. Dawes, E. A., and P. J. Senior. 1973. The role and regulation of energy reserve polymers in micro-organisms. Adv. Microbiol. Physiol. 10:135-267.

12. Fraenkel, D. G. Glycolysis, pentose phosphate pathway, and Entner-Doudoroff pathway. p. 142-150. *In* F. C. Neidhardt (ed), *Escherichia coli* and *Salmonella Typhimurium* Cellular and Molecular Biology. American Society for Microbiology, Washington, D. C.

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

14. Higgins, C. F., H. C. Causton, G. S. C. Dance, and E. A. Mudd. The role of the 3' end in mRNA stability and decay. 1993. p. 13-27. *In* J. G. Belasco and G. Brawerman (eds.), Control of messenger RNA stability. Academic Press, San Diego, CA.

15. Hofnung, M., M. Schwartz, and D. Hatfield. 1971. Complementation studies in maltose-A region of the *Escherichia coli* K12 genetic map. J. Mol. Biol. 61:681-694.

16. Huisman, G., and R. Kolter. Regulation of gene expression at the onset of stationary phase in *Escherichia coli.* 1994. p. 21-40. *In* P. Piggot (ed.), Regulation of bacterial differentiation. American Society for Microbiology, Washington, D. C.

17. Jeanningros, R., N. Creuzet-Sigal, C. Frixon and J. Cattaneo. 1975. Purification and properties of a debranching enzyme from *Escherichia coli*. Biochimica et Biophysica Acta 438:186-199.

18. Kohara, Y., K. Akiyama, and K. Isono. 1987. The physical map of the whole *E. coli* chromosome: Application of a new strategy for rapid analysis and sorting of a large genomic library. Cell 50:495-508.

19. Kolb, A., S. Busby, H. Buc, S. Garges, and S. Adhya. 1993. Transcriptional regulation by cAMP and its receptor protein. Annu. Rev. Biochem. 62:749-795.

20. Krebs, E. G., and J. Preiss. 1975. Regulatory mechanisms in glycogen metabolism. MTP Int. Rev. Sci. Carbohydr. Biochem. Ser. I 5:337-389.

21. Kumar, A., C. E. Larsen, and J. Preiss. 1986. Biosynthesis of bacterial glycogen. Primary structure of *Escherichia coli* ADPglucose: α-1,4-glucan, 4-glucosyl transferase as deduced from the nucleotide sequence of the *glgA* gene. J. Biol. Chem. 261:16256-16259.

22. Lange, R., and R. Hengge-Aronis. 1991. Identification of a central regulator of stationary-phase gene expression in *Escherichia coli*. Mol. Microbiol. 5:49-59.

23. Lee, E. Y. C. 1971. The action of sweet potato β-amylase on glycogen and amylopectin: Formation of a novel limit dextrin. Arch. Biochem. Biophys. 146:488-492.

24. Liu, W., M. L. Castro, J. Takrama, P. T. Bilous, T. Vinayagamoorthy, N. B. Madsen, and R. C. Bleackley. 1993. Molecular cloning, sequencing, and analysis of cDNA for rabbit muscle glycogen debranching enzyme. Arch. Biochem. Biophys. 306::232-239.

25. Liu, M. Y., and T. Romeo, unpublished data.

26. Liu, M. Y., H. Yang, and T. Romeo. The product of the pleiotropic *Escherichia coli* gene *csrA* modulates glycogen biosynthesis via effects on messenger RNA stability. J. Bacteriol. (accepted).

27. Loewen, P. C., and R. Hengge-Aronis. 1994. The role of the sigma factor $\sigma^S$ (KatF) in bacterial global regulation. Annu. Rev. Microbiol. 48:53-80.

28. Lowry, O. H., J. Carter, J. B. Ward, and L. Glaser. 1971 The effect of carbon and nitrogen sources on the level of metabolic intermediates in *Escherichia coli*. J. Biol. Chem. 21:6511-6521.

29. Mason, C. A., and T. Egli. 1993. Dynamics of microbial growth in the decelerating and stationary phase of batch culture. p. 81-102. *In* S. Kjelleberg (ed.), Starvation in Bacteria. Plenum Press, New York.

30. Mathews, C. K., and K. E. van Holde. 1990. Biochemistry. The Benjamin/ Cummings Publishing Company, Inc.

31. McCann, M. P., J. P. Kidwell, and A. Martin. 1991. The putative $\sigma$ factor *katF* has a central role in development of starvation-mediated general resistance in *Escherichia coli*. J. Bacteriol. 173:4188-4194.

32. Miller, J. H. 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

33. Miller, J. H. 1992. A Short Course in Bacterial Genetics. A Laboratory Manual and Handbook for *Escherichia coli* and Related Bacteria. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

34. Nelson, N. 1944. A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. 153:375-380.

35. Okita, T. W., R. L. Rodriguez, and J. Preiss. 1981. Biosynthesis of bacterial glycogen. Cloning of the glycogen biosynthetic enzyme structural genes of *Escherichia coli*. J. Biol. Chem. 256: 6944-6952.

36. Palmer, T. N., G. Wober, and W. J. Whelan. 1973. The pathway of exogenous and endogenous carbohydrate utilization in *Escherichia coli*: A dual function for the enzymes of the maltose operon. Eur. J. Biochem. 39: 601-612.

37. Pompliano, D. L., A. Peyman, and J. R. Knowles. 1990. Stabilization of a reaction intermediate as a catalytic device: Definition of the functional role of the flexible loop in triosephosphate isomerase. Biochemistry 29:3186-3194.

38. Preiss, J. 1969. The regulation of the biosynthesis of α-1,4 glucans in bacteria and plants. p. 125-160. *In* B. L. Horecker and E. R. Stadtman (ed.) Curr. Top. Cell. Regul. 1. Academic press, London.

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

40. Preiss, J., C. Lammel, and E. Greenberg. 1976. Biosynthesis of bacterial glycogen. Kinetic studies of a glucose-1-P adenylyl-transferase (EC 2.7.7.27) from glycogen excess mutant of *Escherichia coli* B. Arch. Biochem. Biophy. 174:105-119.

41. Preiss, J. and T. Romeo. 1989. Physiology, biochemistry and genetics of bacterial glycogen synthesis. p. 183-233. *In* A. H. Rose and D. Tempest (ed.) Advances in Microbial Physiology Vol. 30. Academic Press, London.

42. Preiss, J. and T. Romeo. 1994. Molecular biology and regulatory aspects of glycogen biosynthesis in bacteria. p. 301-327. *In* W. E. Cohn and K. Moldave (ed.) Progress in Nucleic Acid Research and Molecular Biology Vol. 47. Academic Press, San Diego, CA.

43. Preiss, J., and D. A. Walsh. 1981. The comparative biochemistry of glycogen and starch. p. 200- 314 . *In* V. Ginsburg (ed.) Biology of Carbohydrates Vol. 1. Wiley, New York.

44. Romeo, T., and M. Gong. 1993. Genetic and physical mapping of the regulatory gene *csrA* on the *Escherichia coli* K-12 chromosome. J. Bacteriol.175:5740-5741.

45. Romeo,T., M. Gong, M.Y. Liu and A.-M. Brun-Zinkernagel. 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.

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

47. Romeo, T., and J. Preiss. 1989. Genetic regulation of glycogen biosynthesis in *Escherichia coli*: *In vitro* effects of cyclic AMP and guanosine 5'-diphosphate 3'-diphosphate and analysis of *in vivo* transcripts. J. Bacteriol. 171:2773-2782.

48. Romeo, T., and J. Preiss. 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.

49. Rowley, D. L., A. J. Pease, and R. E. Wolf. 1991. Genetic and physical analyses of the growth rate-dependent regulation of *Escherichia coli zwf* expression. J. Bacteriol. 173:4660-4667.

50. Rozacky, E. E., T. H. Sawyer, R. A. Barton, and R. W. Gracy. 1971. Studies on human triosephosphate isomerase. Arch. Biochem. Biophys. 146:312-320.

51. Sabnis, N. A., H. Yang and T. Romeo, unpublished data.

52. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463-5467.

53. Seigele, D. A., and R. Kolter. 1992. Life after log. J. Bacteriol. 174:345-348.

54. Schwartz, M. 1966. Location of the maltose A and B loci on the genetic map of *Escherichia coli*. J. Bacteriol. 92:1083-1089.

55. Silhavy, T. J., M. L. Berman, and L. W. Enquist. 1984. Experiments with Gene Fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

56. Singer, M., T. A. Baker, G. Schnitzler, S. M. Deischel, M. Goel, W. Dove, K. J. Jaacks, A. D. Grossman, J. W. Erickson, and C. A. Gross. 1989. A collection of genetically linked alternating antibiotic resistance elements for genetic mapping of *Escherichia coli*. Microbiol. Rev. 53:1-24.

57. Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk. 1985. Measurement of protein using bicinchoninic acid. Anal Biochem. 150:76-85.

58. Yanish-Perron, C., J. Vieria, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33:103-109.

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