ABSTRACT

Title of Document: EVALUATION OF THE TRANSCRIPTION OF SMALL RNA SgrS AND GLUCOSE TRANSPORTER mRNA *ptsG* IN *E. coli* B AND *E. coli* K CULTURES UNDER HIGH GLUCOSE CONDITIONS

Weng Ian Ng, Master of Science, 2009

Directed By: Associate Professor, Nam Sun Wang
Department of Chemical and Biomolecular Engineering

Escherichia coli is commonly used as the production system for recombinant proteins. However, acetate accumulation in fermentation affects cell growth and protein yield. Recent studies have showed that the small RNA SgrS regulates the major glucose transporter mRNA *ptsG* in a post-transcriptional manner when the metabolic intermediate glucose-6-phosphate is accumulated intracellularly in *E. coli* K. Here, comparative analysis of the transcription of SgrS and *ptsG* is performed between *E. coli* B and *E. coli* K cultures in both shake flasks and bioreactor. Both strains expressed SgrS when grown on the non-metabolizable glucose analog α-methyl-glucoside. However, under high glucose conditions, only *E. coli* B showed significant expression of SgrS. This behavior is unaffected by oxygen supply and pH control. *E. coli* B produced less acetate on glucose than *E. coli* K in the bioreactor settings. This provides evidence of a possible connection between SgrS and acetate production in aerobic fermentation of *E. coli*.

EVALUATION OF THE TRANSCRIPTION OF SMALL RNA SgrS AND GLUCOSE TRANSPORTER mRNA *ptsG* IN *E. coli* B and *E. coli* K UNDER HIGH GLUCOSE CONDITIONS

By

Weng Ian Ng

Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Master of Science
2009

Advisory Committee:
Professor Nam Sun Wang, Chair
Dr. Joseph Shiloach, Special Faculty Member
Professor William A. Weigand
Assistant Professor Ganesh Sriram

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Dedication

I would like to dedicate this work to my parents, who love and support me endlessly and bring me to where I am today.

Acknowledgements

I would like to acknowledge the guidance and advices from both of my advisors, Dr. Nam Sun Wang and Dr. Joseph Shiloach. I would particularly like to thank Dr. Shiloach for his support and guidance on the research project, and the opportunities he gave me during the past year and the near future. Additionally, I would like to thank all the members in Dr. Shiloach's lab at National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), National Institutes of Health, especially Dr. Alejandro Negrete, who not only gave me help and advices on research work, but also on developing my career pathway. Last but not least, I would like to thank my committee members, Dr. William A. Weigand and Dr. Ganesh Sriram.

This research was done in the Biotechnology Core Laboratory at NIDDK, National Institutes of Health in Bethesda, Maryland.

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Chapter 1 Introduction and Overview

1.1 Introduction and Significance of the Study

Escherichia coli is the commonly used platform for the commercial production of recombinant proteins for industrial and medical applications. It is always the preferred production system because of its low manufacturing costs and its capability of growing to high cell density, which usually leads to high protein yield. Examples of recombinant proteins produced in *E. coli* include industrial enzymes such as rennin, amylases, proteases and therapeutic proteins such as insulin, growth hormones, and interferons [1].

The common method for recombinant protein production is aerobic high cell density cultures of *E. coli* in a batch or fed-batch process. In these processes, cells are grown to a high density before the culture is induced to produce the desired protein. This is done by growing the cells in a bioreactor with a controlled supply of oxygen and a carbon source, usually glucose. However, the disadvantage of this production system is the accumulation of acetate, which is the by-product of aerobic fermentation in *E. coli*. Acetate accumulation is undesirable because it affects both cell growth and protein production. Thus significant efforts have been put to overcome this problem by improving the fermentation processes and the physiological behavior of *E. coli* by metabolic engineering [1].

Acetate is produced when there is an imbalance between rapid uptake of glucose and its conversion into biomass and products. Glucose is transported into the cells through the phosphoenolpyruvate (PEP): carbohydrate phosphotransferase system (PTS). The regulation of the glucose transporter in the PTS has been characterized at the level of transcription. In recent years, regulation at the post-transcriptional level was discovered. A small non-coding RNA, SgrS, was found to play an important regulatory role in glucose uptake. This small RNA regulates the messenger RNA (mRNA), *ptsG*, encoding the glucose transporter by rapid degradation under glucose phosphate stress and thus preventing further entry of glucose into the cells [2, 3].

These findings in post-transcriptional rapid degradation of the *ptsG* mRNA and the regulatory role of SgrS were observed in *E. coli* K by using α-methyl-glucoside (αMG), a non-metabolizable glucose analog, as the carbon substrate, and in metabolic gene mutants grown in glucose in previous research work [3-5]. However, the role of this small regulatory RNA in glucose uptake has not yet been characterized in *E. coli* B, which is also commonly used for recombinant protein production in the industry. *E. coli* B was found to behave differently in acetate production from *E. coli* K in both batch and fed-batch fermentations [6, 7]. In this study, a comparative analysis of the regulation of glucose uptake by the small RNA SgrS was performed between *E. coli* B and *E. coli* K. This provided a strong evidence for the difference in regulation of glucose uptake between the two strains. Based on this, the possible connection between the small RNA regulation and acetate production will be discovered by further studies. All these work will make a contribution to overcome

acetate production while using *E. coli* as the platform for commercial production of recombinant proteins.

1.2 Overview of Chapters

Chapter 2 provides a brief background divided into four sections: 1) Glucose metabolism; 2) Small RNA regulators; 3) Regulation in response to sugar phosphate stress; and 4) Previous work. In the glucose metabolism section, the PTS as the major route for glucose transport and phosphorylation is described. The metabolic pathways, glycolysis and TCA cycle, followed by the acetate production and consumption are described. The small RNA regulators section contains small regulatory RNAs in *E. coli*. In the regulation in response to sugar phosphate stress section, the cell response to sugar phosphate stress in terms of post-transcriptional regulation is covered. This introduces the rapid degradation of mRNA, the recruiting of the small RNA SgrS, the model of regulatory mechanism, and the bifunctional characteristics of SgrS. The previous work section at the end of this chapter summarizes the previous research work relevant to this study.

Chapter 3 includes the materials and analytical methods employed for physiological studies and data recording.

Chapters 4 presents the results obtained from experiments conducted in shake flasks for three wild type strains (BL21, MG1655, and JM109). The data are analyzed and discussed.

Chapters 5 presents the results obtained from experiments conducted in bioreactor for three wild type strains (BL21, MG1655, and JM109). Real-time PCR results for gene knockout mutants (BL21 *sgrS*$^-$, and MG1655 *sgrS*$^-$) are also showed. The data are analyzed and discussed.

Chapter 6 gives the conclusion of all the work in this study and the future work is proposed to further study the post-transcriptional regulation of glucose uptake in *E. coli*.

Chapter 2 Background

2.1 Glucose Metabolism in Escherichia coli

Bacteria can utilize a wide range of carbon sources, which are translocated across the cytoplasmic membrane catalyzed by a variety of specific transport systems [8]. For growing *Escherichia coli*, glucose is commonly used as the carbon and energy source. The usual route for glucose uptake and phosphorylation is the phosphoenolpyruvate (PEP): phosphotransferase system (PTS). Glucose is transported into the cells by the PTS, and is then metabolized through the Embden-Meyerhof glycolytic pathway (glycolysis) followed by the tricarboxylic acid (TCA) cycle to produce numerous metabolites and energy in the cells. Acetate is produced as a co-product in aerobic fermentation on glucose [8-10].

2.1.1 Phosphoenolpyruvate: Carbohydrate Phosphotransferase System

Apart from being able to utilize a variety of carbon sources, most bacteria can also adapt to the continuously changing environment in order to compete for limiting nutrients. Cells contain sensing systems to detect and respond to changes in the surroundings such as: concentration gradients of nutrients, changes in osmotic strength, oxygen supply, and stress conditions. The phosphoenolpyruvate (PEP): carbohydrate phosphotransferase system (PTS) is both transport and sensing systems

in bacteria. The PTS is involved in both the transport and phosphorylation of a large number of carbohydrates, in the movement of cells towards these carbon sources (chemotaxis), and in the regulation of different metabolic pathways [8, 11].

In *E.coli*, the PTS consists of two general cytoplasmic proteins, enzyme I (EI) and histidine protein (HPr), which participate in the phosphorylation of all PTS sugars. In addition, the PTS contains a number of sugar-specific enzyme II complexes (EIIs). Each EII complex consists of two hydrophilic domains, IIA and IIB, both containing a phosphorylation site, and the membrane-bound hydrophobic domain formed by one or two proteins, IIC and/or IID [8]. The EIIs specific for mannitol (Mtl), glucose (Glc), and mannose (Man) are shown in figure 2.1.

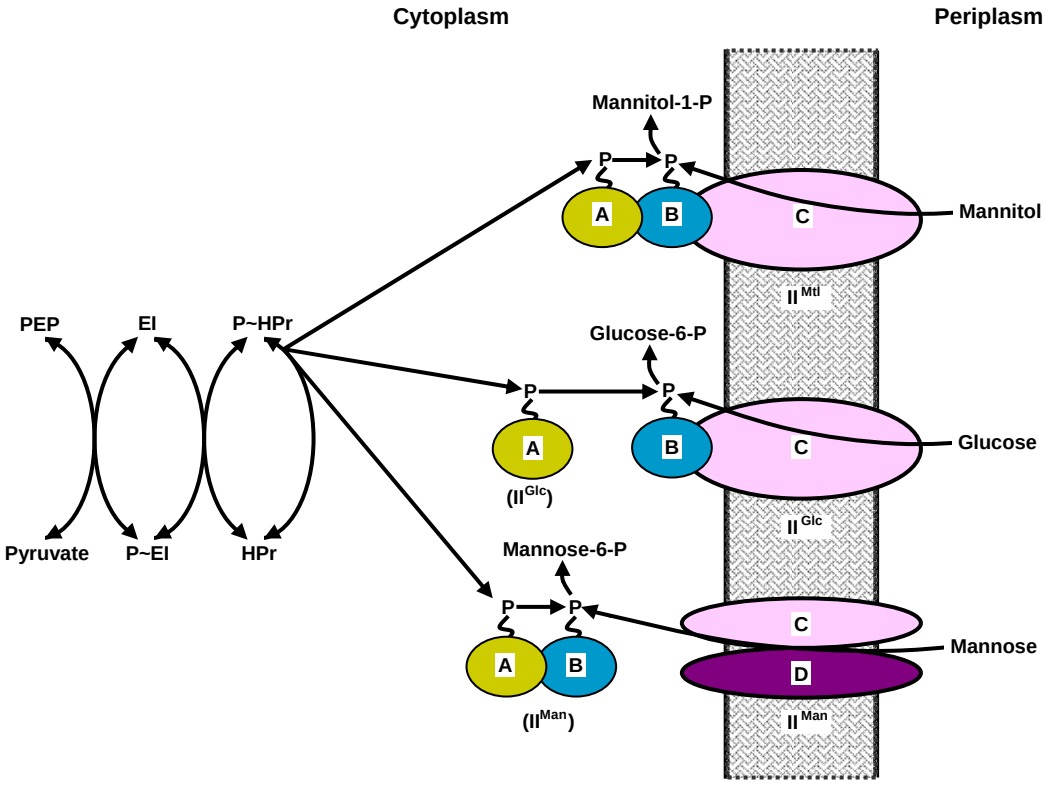


Figure 2.1. The general EI and HPr proteins and the sugar-specific EII complexes in the PTS. Modified from Postma *et al*. 1993 [11].

The EII complex for glucose, or glucose transporter, consists of a cytoplasmic protein IIA^{Glc} and a membrane-bound $IICB^{Glc}$ protein. When glucose is translocated across the membrane, it is concomitantly phosphorylated in the following scheme: the phosphoryl group is transferred from the phosphoenolpyruvate (PEP) to EI, to HPr, to EIIs and finally to the glucose molecule. Other PTS sugars such as mannitol and mannose are transported in similar manner (Figure 2.1).

2.1.2 Glycolysis and Tricarboxylic Acid Cycle

Glycolysis and the tricarboxylic acid (TCA) cycle are the key biochemical pathways in *E. coli* involved in the aerobic consumption of glucose. *E. coli* cells employ these pathways to produce energy and numerous intermediates required for the biosynthesis of amino acids. After translocation by the PTS, the phosphorylated glucose, glucose-6-phosphate (G6P), is further metabolized down the glycolytic pathway (Figure 2.2). The reaction in which phosphoenolypyruvate (PEP) is converted into pyruvate is coupled with the phosphorylation of EI in the PTS. The pyruvate formed is further converted into acetyl coenzyme A (CoA), which is oxidized through the TCA cycle [9, 10].

2.1.3 Formation and Consumption of Acetate

Acetic acid, which exists as acetate at neutral pH during *E. coli* fermentation, is produced as a co-product when glucose is the carbon source. Acetate accumulates in the culture when carbon flux into the glycolytic cycle exceeds the biosynthesis

demand and the capacity of energy generation. Acetate is thought to transport across the membrane by simple diffusion. Its uncoupling activity interferes the maintenance of a functional pH-gradient across the cell membrane, resulting in inhibition on bacterial growth [12, 13]. It has been reported that acetate concentration as low as 0.5 g/L can inhibit growth and also recombinant protein production. Thus research efforts have been put to reduce acetate accumulation during *E. coli* fermentation on glucose [1].

Two major pathways are involved in acetate production. The first one is the direct conversion of pyruvate into acetate by pyruvate oxidase (*poxB*). Another pathway is the reversible conversion of acetyl-CoA to acetyl phosphate by phophotransacetylase (*pta*) and then to acetate by acetate kinase (*ackA*) [1, 6] (Figure 2.2). On the other hand, cells can utilize acetate by reversing the *pta-ackA* pathway and also by acetyl-CoA synthetase (*acs*). In both cases, acetate is converted back to acetyl-CoA, which is then metabolized through the TCA cycle and the glyoxylate shunt pathway. Cells can also utilize acetate for gluconeogenesis, i.e. the synthesis of glucose [6]. The accumulation of acetate in the culture is thus the net production of acetate minus the consumption.

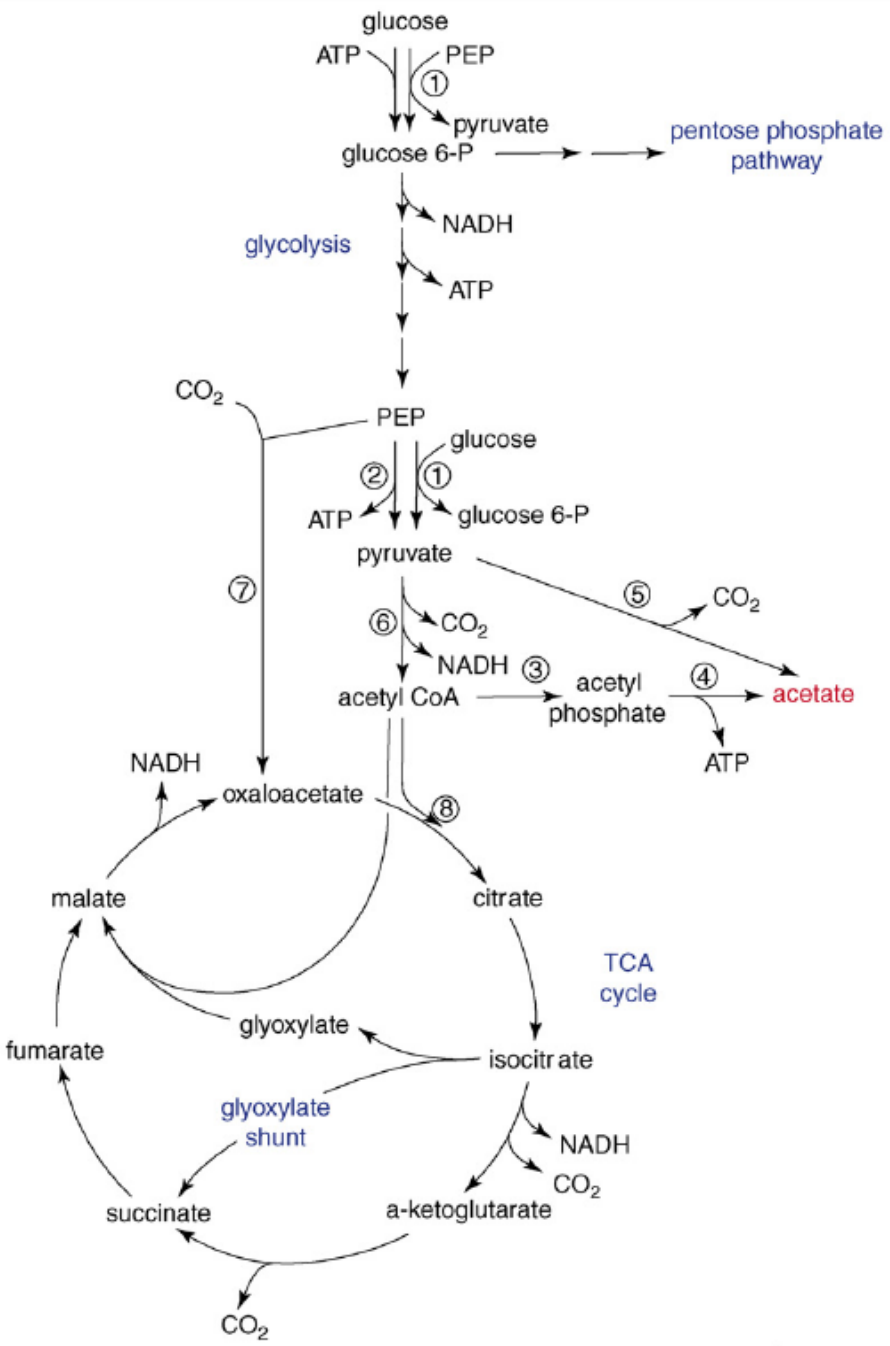


Figure 2.2. Key biochemical pathways in *E. coli* for aerobic consumption of glucose and the synthesis of acetate, carbon dioxide and biomass [1]. 1 – glucose phosphotransferase system (PTS); 2 – pyruvate kinases; 3 – phosphotransacetylase; 4 – acetate kinase; 5 – pyruvate oxidase; 6 – pyruvate dehydrogenase complex; 7 – PEP carboxylase; 8 – citrate synthase.

2.2 Small Regulatory RNAs

Bacteria are capable to grow in a variety of environmental conditions by changing their physiological processes. They have different cellular systems which allow them to sense, respond and recover from environmental stresses and changes in nutrient availability. Regulation at the transcription level was long considered to be the only way to control the diverse cellular activities. However, post-transcriptional control at the mRNA level by small regulatory RNAs (sRNAs) is now considered to play an important role in gene expression as well [14].

Small regulatory RNAs are noncoding RNAs (ncRNAs) widespread in all organisms to control diverse cellular processes. In *E. coli*, more than 100 chromosomally encoded sRNAs have been identified with molecular size ranging from 50 to 250 nucleotides (nt) in length [15]. A large class of these sRNAs regulates gene expression in a post-transcriptional manner through complementary base-pairing to messenger RNAs (mRNAs), while some bind to proteins and modify their activities [14, 16]. These sRNAs are often synthesized and/or activated in response to stress conditions [16]. Parallel to microRNAs and small interfering RNAs (siRNAs) in eukaryotes, sRNAs in *E. coli* act as antisense regulators by pairing with their target mRNAs and affecting their translation and/or stability. The sRNAs may have one or more mRNA targets, and their regulation can be positive or negative. Most of this class of antisense RNAs binds to the RNA chaperone Hfq. The chaperone Hfq is a hexameric protein which increases the stability of the sRNAs and stimulates the base-pairing between sRNAs and the target mRNAs (Table 2.1) [16, 17].

Table 2.1. Examples of Hfq-binding small antisense RNAs [17].

Regulatory protein family	Regulatory protein	Inducing signal	Small RNAs[a]	Targets[b]	References
LysR	OxyR	oxidative stress	OxyS	*fhlA*; *yobF*, *wrbA*, *ybaY*	Altuvia et al. (1997); Argaman and Altuvia (2000); Tjaden et al. (2006)
	GcvA		GcvB	*dpp*, *opp*	Urbanowski et al. (2000); McArthur et al. (2006)
Two-component	OmpR	osmotic shock	OmrA, OmrB	*ompT*, *cirA*, other cell surface genes	Guillier and Gottesman (2006)
	RcsB	cell surface stress	RprA	*rpoS*	Majdalani et al. (2001)
	LuxO	quorum sensing	Qrr1-4 (Vc)	*hapR*	Lenz et al. (2004)
Sigma factor	Sigma E	periplasmic stress	MicA	*ompA*	Udekwu et al. (2005); Johansen et al. (2006)
			RybB	*sigE*	Johansen et al. (2006); K. Thompson and S. Gottesman (in prep.); Vogel and Papenfort (2006)
Fur repressor	Fur	iron limitation	RyhB (Ec) (Vc) PrrF (Pa)	*sodB*, *sdh*, iron-binding proteins	Massé and Gottesman (2002); Wilderman et al. (2004); Davis et al. (2005); Mey et al. (2005)
Mar family	Mar, SoxS, Rob	oxidative stress, antibiotic stress	MicF[c]	*ompF*	Delihas and Forst (2001)
Sugar binding, novel	SgrR	glucose-phosphate accumulation	SgrS	*ptsG*	Vanderpool and Gottesman (2004)
CRP	CRP	glucose limitation	Spot 42	*galK*	Møller et al. (2002a)

[a]Small RNAs were identified in *E. coli* (Ec) unless otherwise indicated. (Vc) *Vibrio cholerae*; (Pa) *Pseudomonas aeruginosa*.
[b]Targets are negatively regulated except for *rpoS*, which is positively regulated.
[c]MicF has also been reported to be regulated by OmpR under some conditions (Ramani et al. 1994).

2.3 Regulation in Response to Sugar Phosphate Stress

2.3.1 Post-transcriptional Regulation of ptsG mRNA

As mentioned in previous section, external glucose is transported and concomitantly phosphorylated by the PTS system in *E. coli*. The PTS membrane-bound enzyme complex $IICB^{Glc}$, a major glucose transporter, is encoded by the gene *ptsG*. The expression of *ptsG* was found to be regulated by two global control

systems at the level of transcription initiation. It is positively regulated by cAMP receptor protein (CRP)-cAMP complex and negatively by the global repressor protein Mlc [18-20]. The intermediate G6P from the PTS is metabolized down the glycolytic pathway. However, its accumulation causes a metabolic stress condition, known as "sugar phosphate stress", in cells and causes toxicity [21]. This stress condition also occurs when there is a block in the early stage of the glycolytic pathway. This can be caused by a mutation in either *pgi* or *pfkA* gene encoding phosphoglucose isomerase or phosphofructokinase, respectively. The mutation in either genes results in an intracellular accumulation of G6P or fructose-6-phosphate (F6P), and a remarkedly reduced level of $IICB^{Glc}$ is observed. This regulation of the glucose transporter is the result of post-transcriptional degradation of *ptsG* mRNA to limit further accumulation of G6P or F6P [4, 5].

When wild-type cells were exposed to α-methyl-glucoside (αMG), a non-metabolizable glucose analog, rapid degradation of *ptsG* mRNA was also observed. This destabilization of mRNA, caused by either a mutation in the glycolytic genes or exposure to αMG, was dependent on RNase E [4]. RNase E is a major endoribonuclease in *E. coli* responsible for the degradation and/or processing of mRNAs and stable RNAs [22].

2.3.2 Regulation of Small RNA SgrS

SgrS (sugar transport-related sRNA) is a small RNA of 227 nt in length. It was first identified in a global screen for RNAs that bind to the RNA chaperon Hfq [23]. Overexpression of *sgrS* in cells caused a growth defect when glucose was the

only carbon source. When cells lacking SgrS were cultured with αMG, growth inhibition was observed. It was found that SgrS was induced when there was an intracellular accumulation of G6P or αMG6P. The synthesis of SgrS in response to glucose phosphate stress is activated by SgrR, a transcription factor divergently transcribed from the *sgrS* gene. SgrR is a 551 aa protein with a N-terminal DNA-binding domain and a C-terminal solute binding domain [3]. By binding to the *sgrR-sgrS* intergenic region, SgrR activates the transcription of *sgrS* and also autoregulates its own transcription negatively [24].

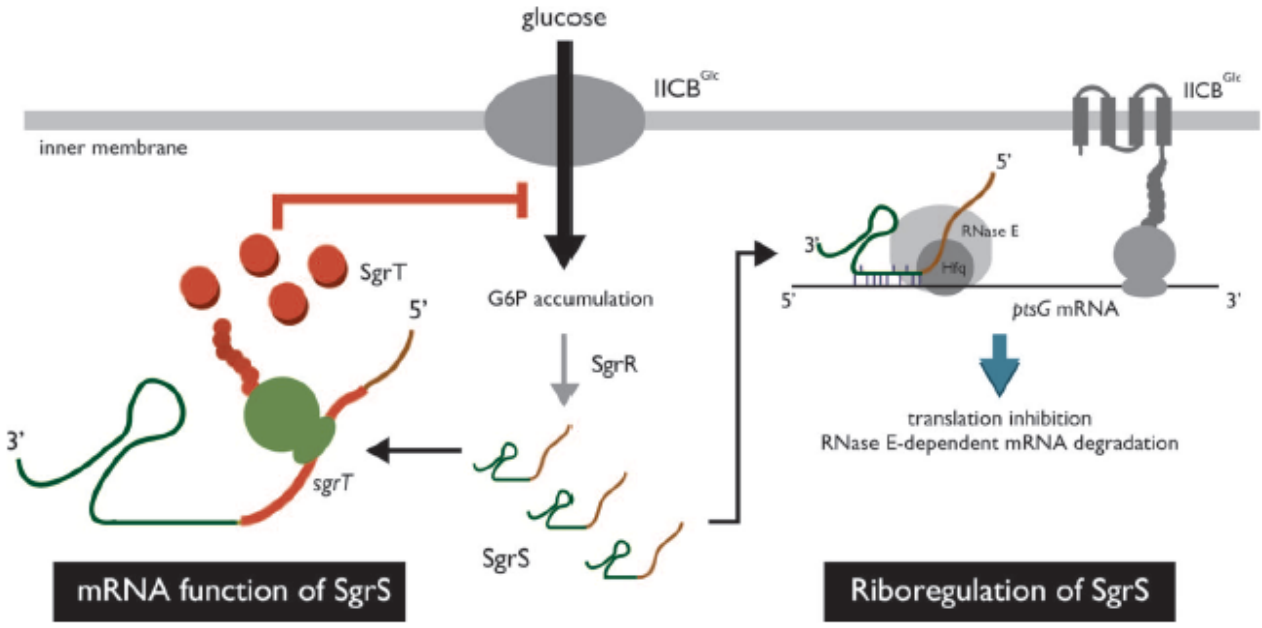


Figure 2.3. Dual function of SgrS under glucose phosphate stress [25].

The expression of SgrS has two effects on its target *ptsG* mRNA: translational repression and destabilization of the message [3, 26]. Translational

repression is primarily responsible for *ptsG* gene silencing under glucose phosphate stress [26]. This translational silencing of the message is caused by the base-pairing between SgrS and *ptsG* exclusively [27]. Also, SgrS causes rapid degradation of *ptsG* mRNA by antisense base-pairing through the RNA chaperone Hfq and affecting its stability [3] (Figure 2.3).

The region where SgrS-*ptsG* base-pairing occurs possibly lies near the Shine-Dalgarno (SD) sequence and AUG start codon on the *ptsG* mRNA. This region shows a 32-nt-long partial complementary between SgrS and *ptsG* [3]. Within this region, six base pairs around the SD sequence of *ptsG* mRNA are crucial for SgrS action (Figure 2.4) [28].

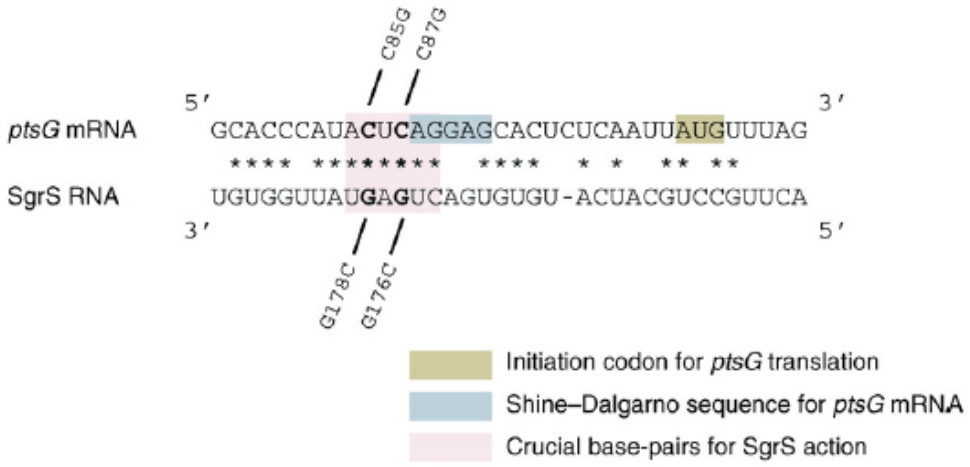


Figure 2.4. Crucial base pairs for SgrS action on *ptsG* mRNA. The predicted base pairs are marked by asterisks [29].

2.3.3 Mechanism of mRNA Destabilization

The Hfq-binding small RNA SgrS mediates the destabilization of *ptsG* mRNA in an RNase-E dependent manner. RNase E consists of three domains: an N-terminal ribonucleolytic region, a central RNA-binding domain, and a C-terminal scaffold region. This endonuclease forms a multiprotein complex, called the RNA degradosome, with 3' exoribonuclease (polynucleotide phosphorylase, PNPase), a DEAD-box RNA helicase (RNA helicase B, RhlB), and a glycolytic enzyme (enolase) [22]. The RNA chaperone Hfq associates with RNase E through its C-terminal scaffold region when this region is not occupied by other degradosome components [30].

When there is an accumulation of G6P in the cells, the small RNA SgrS is highly expressed and associates with RNase E through Hfq to form a ribonucleoprotein complexes (RNP) (Figure 2.5). This RNP becomes a specialized RNA decay machine, which targets the *ptsG* mRNA by the small RNA SgrS [30]. The role of Hfq in this model is to facilitate the SgrS-*ptsG* binding and to associate RNase E with SgrS [28, 30]. After initial cleavage by this RNP, further degradation of the mRNA may involve the RNA degradosome components and other ribonucleases [30].

Apart from recruiting RNase E and Hfq, the membrane localization and insertion properties of $IICB^{Glc}$ protein are also required for the rapid degradation of *ptsG* mRNA [31]. The major glucose transporter $IICB^{Glc}$ consists of an N-terminal domain containing eight transmembrane segments and a C-terminal cytoplasmic domain [32]. The model proposed is that the nascent peptide of $IICB^{Glc}$ is

cotranslationally inserted, bringing the *ptsG* mRNA close to the membrane. This membrane localization of the mRNA may reduce the efficiency of ribosome binding, thus allowing SgrS along with Hfq to base-pair with *ptsG* and resulting in RNase E-dependent degradation of the message [31].

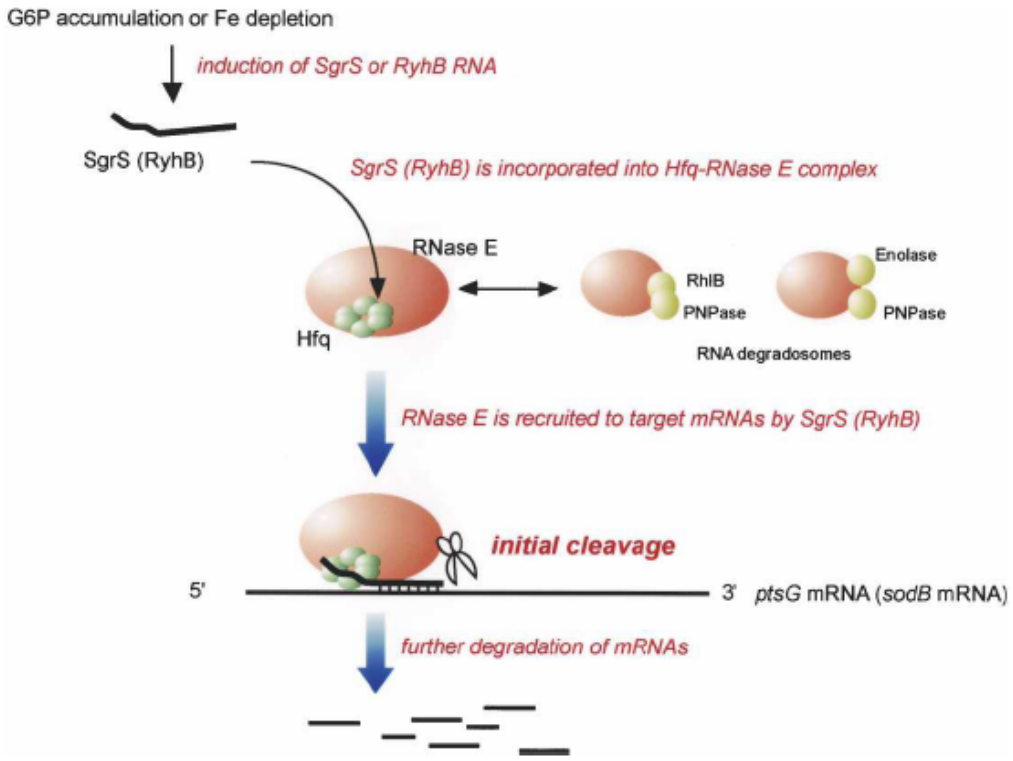


Figure 2.5. Model of small RNA/Hfq/RNase E formation and subsequent action on target mRNA [30].

2.3.4 Bifunctional Characteristics of SgrS

Besides acting as an antisense regulator, the small RNA SgrS acts as a coding mRNA. Upstream of the 3' region where it binds *ptsG*, a conserved ORF (open reading frame) in the 5' region was identified as SgrT, a 43 aa polypeptide. The polypeptide SgrT rescues cells growing on αMG and completely inhibits growth on glucose even without base-pairing degradation. This implies a physiological redundancy in response to phosphosugar stress. SgrT has no effect on *ptsG* mRNA or $IICB^{Glc}$ protein levels. It was proposed that this polypeptide has an effect upon activity of $IICB^{Glc}$ by either "plugging" the transporter channel or inhibiting $IICB^{Glc}$ phosphorylation through protein-protein interactions. The discovery of SgrT suggested that the riboregulation of SgrS is to stop new synthesis of the glucose transporter, while the mRNA function of SgrS is to inhibit the activity of the preexisting transporter proteins [33] (Figure 2.3).

2.4 Previous Work

The transcription of SgrS and its effect on *ptsG* have been studied in *E. coli* K strains (MG1655, DJ480, W3110, PP6, and IT1568) exclusively. The methodology used to elucidate the effect of accumulation of G6P or αMG6P on the transcriptions was growing the cells in LB media up to an OD_{600} value of 0.3-0.6. Either a final concentration of 0.4% or 1% glucose or αMG was added to the media. Cells were incubated for 10-20 min with the selected carbon substrates before harvesting for

RNA extraction [3-5, 26, 31, 34]. Vanderpool and Gottesman, 2004 performed a time-course study on the αMG sensitivity for 30 min. SgrS and *ptsG* transcription was analyzed with and without exposure to αMG at different time intervals [3]. All those experiments were carried out in shake flasks at 37ºC. No correlation between growth parameters (OD_{600}, pH, acetate concentration, glucose concentration) and transcriptions analyzed by Northern blots has been studied (Table 2.2).

Table 2.2. Differences and similarities between previous studies and this study.

	Previous studies	This study
Bacterial strains	*E. coli* K (MG1655, DJ480, W3110, PP6, IT1568)	*E. coli* K (MG1655, JM109) *E. coli* B (BL21)
Scale	Shake flasks	Shake flasks Bioreactor
Media	LB with 0.4% or 1% glucose or αMG	LB with 2% glucose or 1% αMG Modified LB with 4% glucose
Sampling	One-point measurement after incubation for 10-20 min	Time course at 1-hour intervals until stationary phase was reached
Analysis	Cell growth, or Northern blots	Cell culture parameters (OD_{600}, pH, glucose, acetate) Northern blots with internal controls Real-time PCR

Chapter 3 Materials and Methods

3.1 Materials

3.1.1 Bacterial Strains and Sources

The *Escherichia coli* strains studied are described in Table 3.1.

Table 3.1. Bacterial strains used in this study.

Strain	Description	Source
MG1655	Wild-type *E. coli* K-12 Genotype: F^-, λ-, *ilv*G-, *rfb*-50, *rph*-1	Biotech Unit / NIDDK
BL21	Wild-type *E. coli* B Genotype: F^-, *omp*T, *hsd*S_B(r_B^-, m_B^-), *gal*, *dcm*	Novagen EMD Biosciences
JM109	Wild-type *E. coli* K-12 Genotype: *end*A1, *gyr*A96, *hsd*R17(r_k^-, m_k^+), *mcr*B^+, *rec*A1, *rel*A1, *sup*E44, *thi*-1, Δ(*lac-pro*AB), F'[*tra*D36, *pro*AB, *lac*I^qZΔM15]	Promega
BL21(DE3)	Genotype: F^-, *omp*T, *hsd*S_B(r_B^-, m_B^-), *gal*, *dcm* (DE3)	VWR International
JM109(DE3)	Genotype: *end*A1, *rec*A1, *gyr*A96, *thi*, *hsd*R17(r_k^-, m_k^+), *rel*A1, *sup*E44, λ-, Δ(*lac-pro*AB), [F', *tra*D36, *pro*AB, *lac*I^qZΔM15], IDE3	Promega
BL21 *sgrS* $^-$	BL21 Δ*sgrS*	Dr. Lee, S.J., NCI
JM109 *sgrS* $^-$	JM109 Δ*sgrS*	Dr. Lee, S.J., NCI

3.1.2 Growth Conditions and Sample Preparation

Shake Flask

Cells were grown in Luria-Bertani (LB) medium (Lennox) which contained 10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl. Overnight cultures were used to inoculate 300 mL fresh media in vented baffled shake flasks to reach an initial OD_{600} of ~0.03, which incubated at 37 ºC with agitation at 200 rpm. When the cultures reached an OD_{600} of ~0.3, glucose was added to the media to a final concentration of 20 g/L, the final concentration of α-methyl-glucoside was 1% (w/v). The stock solutions used were 50% (w/v) glucose and 50% (w/v) α-methyl-glucoside. Samples were collected in 1.5 mL fractions in eppendorf tubes and centrifuged at 15,700×g for 5 min. The cell pellets and the supernatant were separated and stored immediately at -80 ºC for RNA extraction and acetate quantification, respectively. Cell growth was monitored by OD_{600} absorbance until stationary phase was reached.

Bioreactor

Studies were performed in a B. Braun fermentor equipped with Rushton impellers, data acquisition and an adaptive control system. The pH sensor was calibrated with standard solutions pH 7.0 and pH 4.0, and the O_2 sensor was calibrated to 0% using nitrogen and to 100% air saturation. Modified LB medium containing 10 g/L tryptone, 5g/L yeast extract (15g/L for MG1655 and JM109), 5g/L NaCl, and 5g/L K_2HPO_4 was used. After sterilization in autoclave, media was supplemented with 10 mM $MgSO_4$, 1 mL/L trace metal solution, and 40 g/L glucose. Overnight cultures were used to inoculate 3.0 L of LB medium to reach an initial OD-

$_{600}$ of ~0.3. Parameters monitored and controlled included temperature at 37 ºC, pH 7.0 by addition of 15% (w/v) NH_4OH, and dissolved oxygen at 30% of air saturation by agitation and air/oxygen flow. Samples were collected and prepared in the same way as those in the shake flask experiments mentioned above.

3.2 Analytical Methods

3.2.1 Cell Density

Cell density was determined by the measurement of optical density at a wavelength of 600 nm (OD_{600}). 1:10 serial dilutions of samples were performed accordingly to obtain an $OD_{600} < 1.0$ with a Pharmacia Biotech Ultrospec 3000 UV/Visible spectrophotometer.

3.2.2 pH

The offline pH measurement of the collected samples from shake flasks was measured by Radiometer Analytical PHM220 Lab pH Meter.

3.2.3 Glucose Measurement

The concentration of glucose (g/L) in the culture medium was determined by YSI 2700 SELECT Biochemistry Analyzer. The YSI 2700 is a biosensor containing

the enzyme, glucose oxidase, which is specific for the substrate glucose, immobilized between two membrane layers. Glucose is oxidized as it enters the enzyme layer and produces hydrogen peroxide, which then passes through the second membrane layer and is oxidized at a platinum electrode. The current generated is proportional to the glucose concentration of the culture medium. Dilutions of the collected samples were performed accordingly until the concentration was less than 10 g/L.

3.2.4 Acetate Measurement

Frozen aliquots of supernatant were thawed and centrifuged at 15,700×g for 5 min to remove any trace of cells or solids. The concentration of acetate was determined by high-pressure liquid chromatography (HPLC) analysis. The HP 1100 Series HPLC Systems (HewlettPackard) equipped with a UV diodearray detector and an Aminex resin-based HPX-87H column (Bio-Rad) was used. The mobile phase was 4 mM H_2SO_4 at a flow rate of 0.6 mL/min, running at 35˚C.

Three standards (0.1 g/L, 1.0 g/L, and 10 g/L) were analyzed along with the samples to generate a calibration curve. The peaks corresponding to the acetate were selected according to the retention time of the standards (14-15 min). Areas of the selected peaks were used to calculate the acetate concentration (g/L) from the calibration curve.

3.2.5 RNA Extraction

Cells were lysed by resuspending the collected cell pellets in a solution consisting of 0.5% SDS (sodium dodecyl sulfate), 20 mM NaAc (sodium acetate), and 10 mM EDTA (ethylenediaminetetraacetic acid). Contaminants were removed by three subsequent extractions by hot acid phenol:chloroform, pre-heated at 65 ºC. Samples were then centrifuged at 14,800×g for 10 min. The RNA-containing supernatant was further purified by phenol:chlorform isoamyl alcohol to remove any protein contamination. RNA was then precipitated out by 99% ethanol and the samples were placed at -80°C for at least 15 min. The RNA pellets were obtained by centrifugation at 20,200×g for 15 min. They were then washed with 70% ethanol, air-dried, and resuspended in DEPC-treated water. The concentration (ng/μL) and purity of RNA were determined by Thermo Scientific NanoDrop 1000 UV-Vis Spectrophotometer, which measured the absorbance ratios of A_{260}/A_{280} (~2.0 for pure RNA) and A_{260}/A_{230} (1.8-2.2 for pure nucleic acid).

3.2.6 Northern Blot

Resolution of ptsG and ompA

The total RNA extracted and purified by the hot phenol method was analyzed by Northern Blot. Samples of 5 μg total RNA were resolved by 1.2% agarose gel at 90 V for 3 h in 1X MOPS buffer to analyze *ptsG* and *ompA*. The RNA was then blotted onto a Zeta-Probe GT membrane (Bio-Rad) by the downward capillary transfer method (TurboBlotter™, Whatman) using 20X SSC as the transfer buffer following the manufacturer's protocol.

Resolution of SgrS and SsrA

For SgrS and SsrA analysis, 5 μg of total RNA was resolved by 10% polyacrylamide gel (Bio-Rad Criterion TBE-Urea Precast Gel) at 75 V for 2.5 h using 1X TBE buffer. Electrophoretic transfer running at 80 V for 1 h or 15 V overnight (depending on size of the transfer cell and cooling system according to the manufacturer's protocol) at 4 °C was used to blot RNA onto a Zeta-Probe GT membrane.

Detection

The blotted membranes were fixed by UV-induced crosslinking (UV Stratalinker® 1800) by using the auto-crosslink mode at 120,000 μJoules. Prehybridization for 30 min, followed by hybridization with the specific biotinylated DNA probes (Table 3.2) overnight at a concentration of 100 ng/μL were both performed in 10 mL ULTRAhyb buffer (Ambion) in rolling bottles at 42 °C. After hybridization, the membranes were subsequently washed and conjugated with streptavidin-alkaline phosphatase following the BrightStar Biodetect Kit (Ambion). Chemiluminscent signals induced upon addition of the substrate, CDP-Star, were detected by the Fujifilm LAS-4000 imaging system. Serial images at a set time interval were collected and the image before saturation point denoted by red color in the system was recorded for RNA expression analysis.

Stripping for Re-probing

For detection of the internal controls (*ompA* and SsrA), the membranes were stripped by putting the membranes into boiling 0.5% SDS for 10 min. The transcriptions of the two housekeeping genes were then detected by the same procedure starting from prehybridization step.

3.2.7 Real-time PCR

Isolated RNA samples were first treated with DNase I, Amp Grade (Invitrogen) to remove any contaminant genomic DNA according to the manufacturer's protocol. Real-time PCR was then performed using Power SYBR Green RNA-to-C_T^{TM} 1-Step Kit (Applied Biosystems) with the corresponding forward and reverse primers (Table 2). 10 ng RNA samples were used to synthesize the corresponding cDNA, which were amplified in the standard thermal cycling conditions: reverse transcription at 48 °C for 30 min, activation of DNA polymerase at 95 °C for 10 min, and 40 cycles of denaturation at 95 °C for 15 s and annealing/extending at 60 °C for 1 min.

Cycle threshold (C_T) values were averaged from replicates of both targets (*ptsG* and SgrS) and endogenous controls (*ompA* and SsrA). Measurements of the targets were normalized using the endogenous controls, and the relative quantities of targets were analyzed by the comparative C_T ($\Delta\Delta C_T$) method. This gave the fold changes between normalized target quantities.

Table 3.2. Oligonucleotides used in this study.

Oligonucleotide	5'-3' sequence
ptsG probe[1]	Biosg/CAG CCA GCT GAA ATT CGC GGA ACC GAC GCC CAG CAG
SgrS probe[1]	Biosg/GCA ACC AGC ACA ACT TCG CTG TCG CGG TAA AAT AGT G
ompA probe[2]	Biosg/CCA TTG TTG TTG ATG AAA CCA GTG TCA TGG TAC TGG GAC CAG C
SsrA probe[2]	Biosg/CGC CAC TAA CAA ACT AGC CTG ATT AAG TTT TAA CGC TTC A
ptsG-F	CTG CCC GCC GTT GTA TCG CA
ptsG-R	CAG CGC GGA TAC GCC ATC GT
SgrS-F	GGG TGC CCC ATG CGT CAG TT
SgrS-R	GCA CAA CTT CGC TGT CGC GG
ompA-F	CGG TCT TCG CTG GCG GTG TT
ompA-R	TCC GGA CGA GTG CCG ATG GT
SsrA-F	CCC TGC CTG GGG TTG AAG CG
SsrA-R	GGG AGT TGA ACC CGC GTC CG

[1] Reference [3]; [2] Reference [35].

Chapter 4 Studies in Shake Flasks

4.1 Introduction

Glucose phosphate stress occurs in cells when there is an intracellular accumulation of G6P or αMG6P. The response is the rapid degradation of *ptsG* mRNA by the RNase E-dependent base-pairing with the small RNA SgrS, preventing further accumulation of G6P or αMG6P [2]. It was shown that when cells were exposed to αMG, there was no detection of *ptsG* expression in Northern blots. However, when cells were grown in glucose, there was no degradation of the mRNA [3, 4, 34]. All these studies were done in *E. coli* K-12 wild type or derived strains as mentioned in the previous work section in chapter 2. Until now, there are no studies done in *E. coli* B, which is a popular strain used for production of recombinant proteins.

In previous studies, analysis was done with a one-time-point measurement upon exposure of cells to the carbon substrates, αMG and glucose, for 10-20 min. In the study presented in this chapter, a comparative analysis of the transcription of SgrS and *ptsG* between *E. coli* B (BL21) and *E. coli* K (MG1655 and JM109) was performed using the same carbon substrates. Cultures were grown in shake flasks at 37 °C, and a time-course study was performed across the bacterial cell growth profile. Cell growth parameters OD_{600} and pH were recorded along with measurements of glucose and acetate concentrations. Internal controls, SsrA (small stable RNA) and

ompA (outer membrane protein A) were also analyzed along with SgrS and *ptsG* by Northern blots.

4.2 Results

4.2.1 Growth Parameters

When cells were grown in 1% αMG, BL21 reached stationary phase with a final OD_{600} of ~4.0 after exposure to αMG for 3 hours (Figure 4.1 a). The pH started to go up from the first hour and reached pH 8.0. For MG1655, it took 5 hours after αMG introduction to reach a similar OD of ~4.0, while the pH began to increase after 2 hours (Figure 4.1 b). The OD_{600} of JM109 culture was the lowest among the three strains, approximately 2.5 OD units (Figure 4.1 c). The pH started to go up in a similar behavior as MG1655. For each culture, there was a negligible amount of glucose detected. The highest acetate concentration reached in the three strains was approximately 0.4-0.6 g/L.

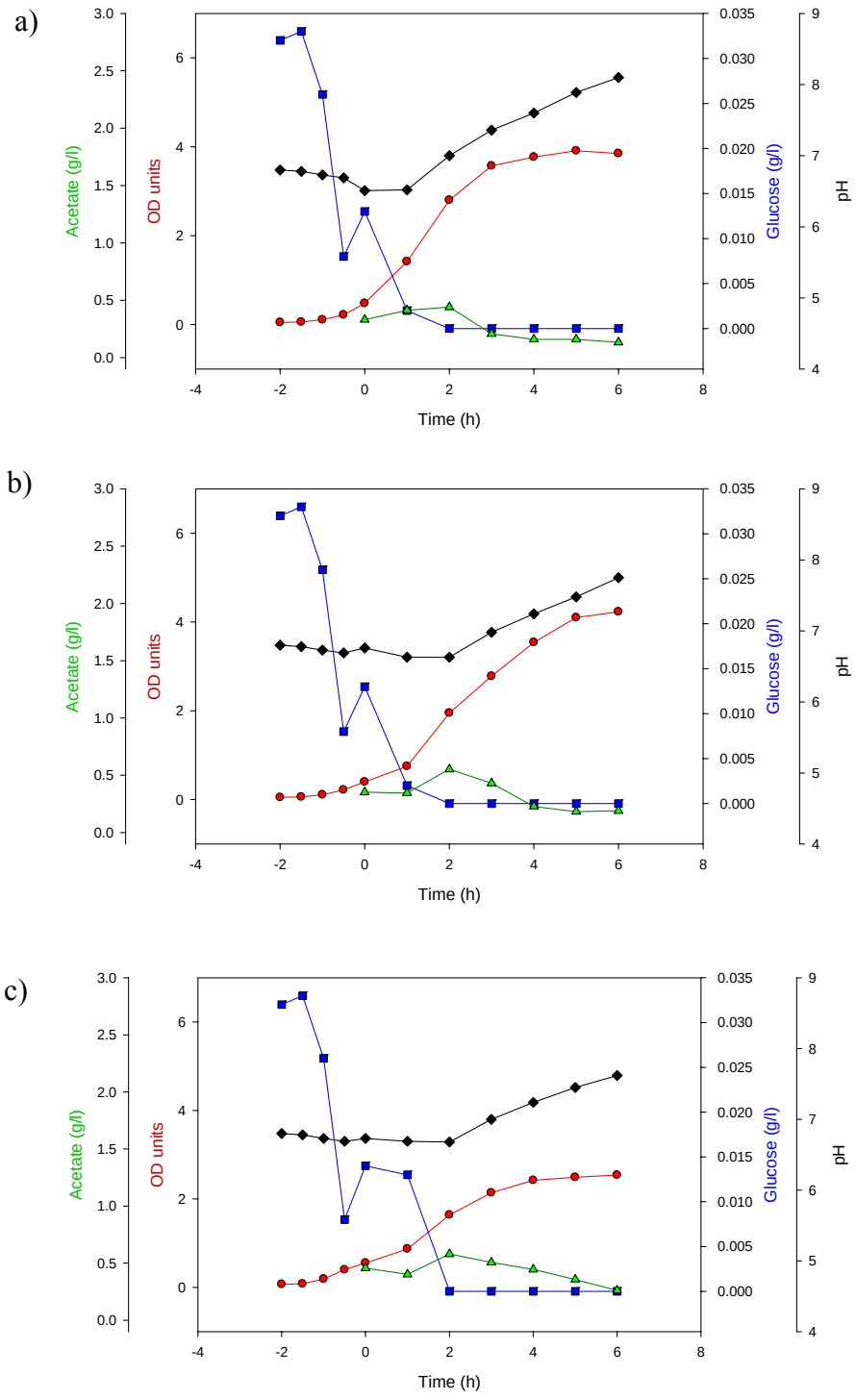


Figure 4.1. Growth parameters of wild type *E. coli* a) BL21, b) MG1655, c) JM109, growing in LB with addition of 1% αMG at time 0. ● - OD_{600}; ■ - glucose (g/L); ▲ - acetate (g/L); and ♦ - pH.

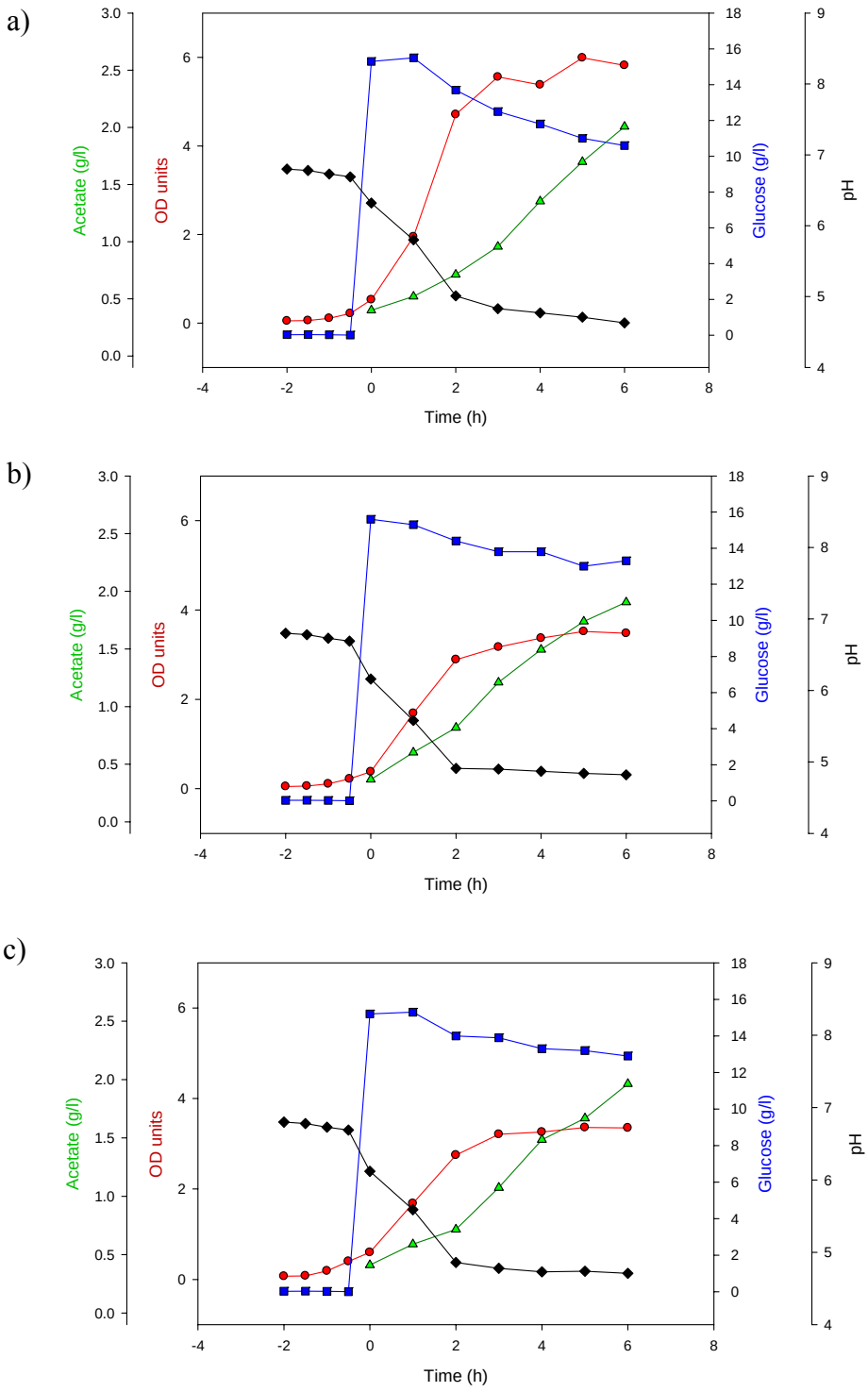


Figure 4.2. Growth parameters of wild type *E. coli* a) BL21, b) MG1655, c) JM109, growing in LB with addition of 20 g/L glucose at time 0. ● - OD_{600}; ■ - glucose (g/L); ▲ - acetate (g/L); and ♦ - pH.

When the substrate was glucose, the three strains behaved differently. BL21 grew to a higher OD_{600} (~6.0) compared to its growth in αMG (Figure 4.2 a). MG1655 grew to a similar OD_{600} as in αMG (OD_{600} 3.5-4.0), although in glucose condition, the cells reached the stationary phase at about hour 4, which was two hours earlier than the growth in αMG condition (Figure 4.2 b). The growth rate was much faster in the exponential phase, while the growth in αMG was comparatively more linear. For JM109, cell density reached an OD_{600} of ~3.5, which is 1 OD unit higher than that in αMG condition (Figure 4.2 c). In all three cultures, the initial concentration of glucose was approximately 16.0 g/L. The BL21 culture showed the greatest drop in glucose concentration, from 16.0 g/L to 10.5 g/L. The final concentrations of glucose for both MG1655 and JM109 were similar, ~13.0 g/L. The pH in the three cultures with glucose decreased significantly from the addition of glucose to a final pH ~4.7 while the acetate accumulated to a concentration of ~2.0 g/L at the end of the growth.

4.2.2 Northern Blots

When exposed to αMG, all three *E. coli* strains expressed SgrS, of which the abundance increased slightly with time (Figure 4.3 a). The blot for *ptsG* mRNA shows faint signals of expression for all the three strains, with the expression in BL21 being the lowest. When cells were grown in glucose, only BL21 expressed SgrS, while MG1655 and JM109 did not (Figure 4.3 b). The *ptsG* mRNA expression for

BL21 was correspondingly lower than the expression of the other two strains, of which the intensity of bands was strong and clear.

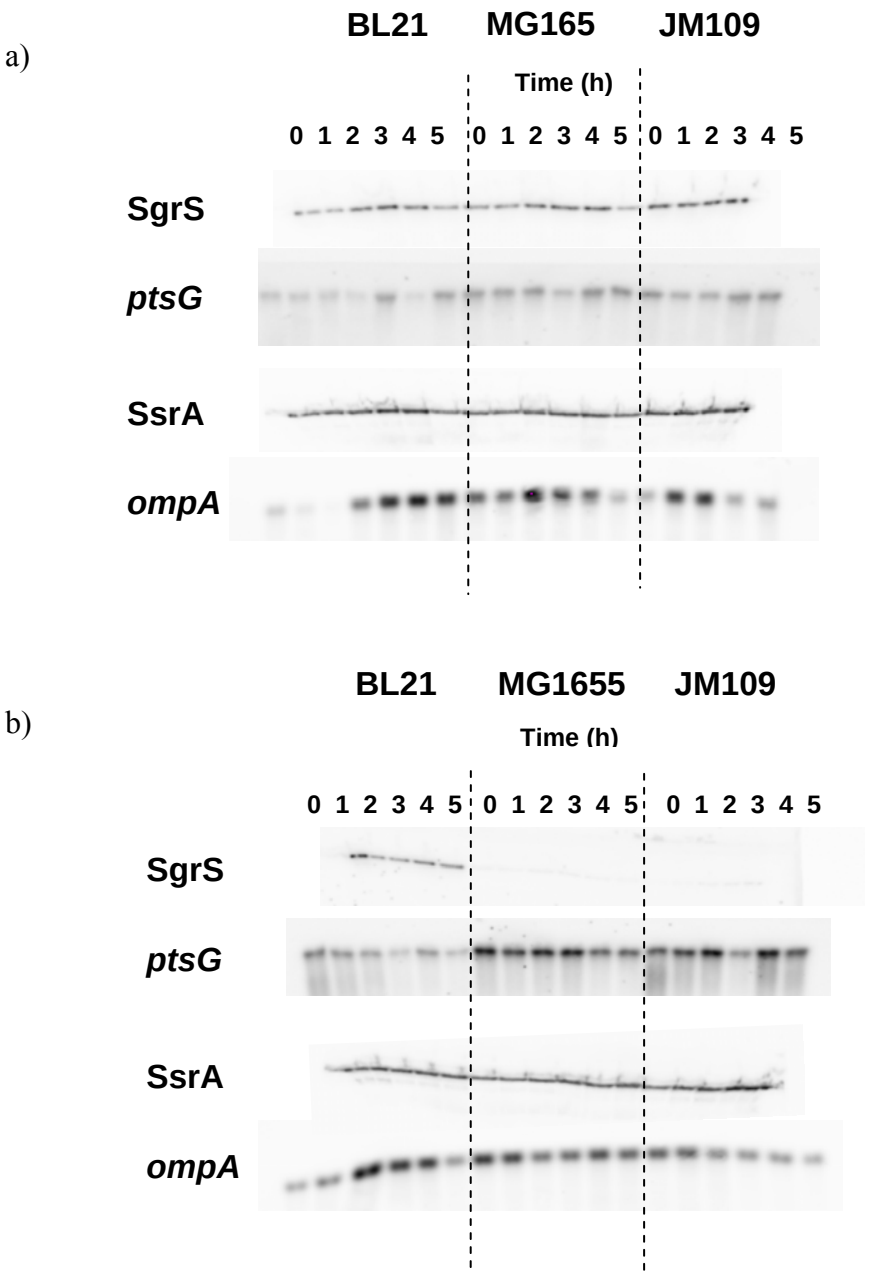


Figure 4.3. Expression of SgrS and *ptsG* with SsrA and *ompA* as the internal controls, respectively, in wild type *E. coli* BL21, MG1655, and JM109 growing in LB with addition of a) 1% αMG, b) 20 g/L glucose, at time 0.

In this study, it was noticed that the exposure time for detecting chemiluminescent signals from separate membranes would affect the accuracy of comparing the transcription levels among different strains. To avoid this problem, all the samples were loaded on the same membrane and signals were detected for the same exposure time for comparative analysis. This eliminated the problem in different time exposures among membranes and allowed accurate comparative analysis of the transcription levels.

4.3 Discussion

Previous studies have shown that the small non-conding RNA SgrS mediates rapid degradation of its target glucose transporter mRNA *ptsG* when there is an intracellular accumulation of G6P or αMG6P [3, 4]. When cells were grown on glucose, SgrS was not expressed and thus *ptsG* mRNA was not destabilized [4, 34]. All these phenomena and studies about SgrS-*ptsG* regulation were observed and performed in *E. coli* K strains.

In the present work, the question whether *E. coli* B uses the same SgrS-*ptsG* regulatory mechanism as *E. coli* K was addressed. To do this, three strains BL21 (*E. coli* B), MG1655 and JM109 (*E. coli* K) cells were grown in LB media supplemented with 1% αMG. The results of Northern blots showed that all three strains expressed SgrS to destabilize *ptsG* to prevent further accumulation of αMG6P. This means that

E. coli B also recruits the SgrS-*ptsG* regulation when exposed to αMG, the same physiological response as observed in *E. coli* K.

From the growth profiles of the three strains in αMG and glucose, lower final cell densities were observed in all three strains grown in αMG. BL21 had a decrease of 2 OD units at the final cell densities in αMG. MG1655 had similar final cell densities in both carbon substrates, but the exponential growth phase was slower in αMG. *E. coli* JM109 also had a 1 OD unit lower final cell density in αMG compared to that in glucose. These corresponded to the same phenomenon observed by Vanderpool and Gottesman, 2004, which showed that DJ480 wild type cells (derivative of MG1655) slowed down on growing temporarily when 1% αMG was added to the medium at the mid-log phase [3]. In the present study, although SgrS was expressed to prevent further accumulation of the non-metabolizable phosphosugar, cell growth was slowed down or limited when the media was supplemented with αMG. This is probably due to blocking of the glycolytic pathway by the accumulating αMG6P and thus the cells cannot generate enough energy and intermediary metabolites necessary for growth.

When cells were grown in glucose, there was a significant difference between *E. coli* B and *E. coli* K in the regulation of glucose uptake by the SgrS-*ptsG* mechanism. Only BL21 expressed SgrS, which in turn caused destabilization of *ptsG* mRNA. However, there was still a detectable expression of *ptsG* as shown on the Northern blot, meaning that the mRNA was not fully degraded by the small RNA. On the other hand, MG1655 and JM109 had strong expressions of *ptsG* mRNA and did not activate the transcription of SgrS. This correlates with previous studies, which

showed that *ptsG* was not degraded in glucose [4, 34]. This physiological difference between *E. coli* B and *E. coli* K and the incomplete degradation of *ptsG* in *E. coli* B can be explained by several possibilities. First, *E. coli* K does not accumulate G6P when grown in high glucose condition and thus did not express SgrS, while there can be some accumulation of G6P or other possible intermediates in *E. coli* B that signals the cells to activate the regulation by SgrS. This means only regulation at the transcription level is used to control glucose uptake *in E. coli* K, but *E. coli* B use both the transcriptional and post-transcriptional regulations for glucose uptake. The possible accumulation of G6P that triggers SgrS activation can be due to how the cells direct carbon flux through the metabolic pathway and the metabolic rate. Second, SgrS expression in *E. coli* B does not fully degrade all *ptsG*, meaning that there is still *ptsG* mRNA present in the cells. The SgrS-*ptsG* regulatory mechanism does not destabilize all the mRNA, but it may help to slow down the synthesis of new glucose transporters. Also, there may be a role that SgrS plays besides base-pairing with *ptsG* to perform the regulation, for instance, encoding the polypeptide SgrT. This polypeptide was found to be able to rescue cells from glucose phosphate stress [33]. Other roles that might involve in this regulation have not yet been discovered.

Chapter 5 Studies in Bioreactors

5.1 Introduction

In the previous chapter, studies in shake flasks were presented. The results showed that only *E. coli* B (BL21) expressed SgrS when the cells were grown in high glucose condition. The other two strains of *E. coli* K (MG1655 and JM109) in glucose did not show SgrS expression, which agreed with the previously published results [4, 34]. The previously published work was done in shake flasks at 37 °C, without oxygen and pH control. In this condition, cells cannot grow to high density. Cells grown in a bioreactor can reach high cell density as a result of an adequate supply of oxygen to the fast-growing cells and pH control at the optimum growing condition (pH 7.0) throughout the whole fermentation process. The availability of glucose depends on the process mode, i.e. batch or fed-batch processes. As high cell densities usually yield high protein concentrations, industries employ fermentation in bioreactors for the commercial production of recombinant proteins.

As *E. coli* B and *E. coli* K respond differently to high glucose condition in shake flasks, it is interesting to further study this difference in the well-controlled bioreactor settings. In this study, a 3-liter batch fermentation process was used, in which all nutrients (10 mM $MgSO_4$, and 1 mL/L trace metal solution) and 40g/L glucose were added before inoculation. Growth parameters including OD_{600}, pH, and

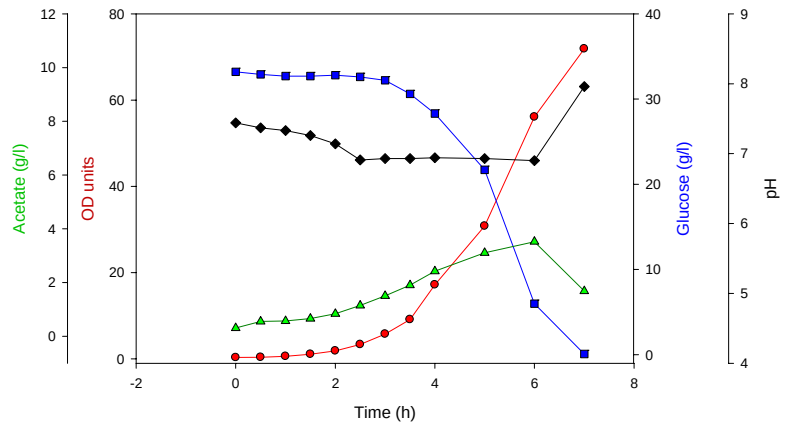
dissolved oxygen (DO) were monitored and samples were collected until glucose was depleted and the pH increased. Time-course analysis of SgrS and *ptsG* expression was performed. Real-time PCR was used to quantify SgrS and *ptsG*, which confirmed the results obtained from the Northern blot analysis. The *sgrS* gene-knockout mutants of BL21 and MG1655 were created and the transcriptions of SgrS and *ptsG* were analyzed by real-time PCR.

5.2 Results

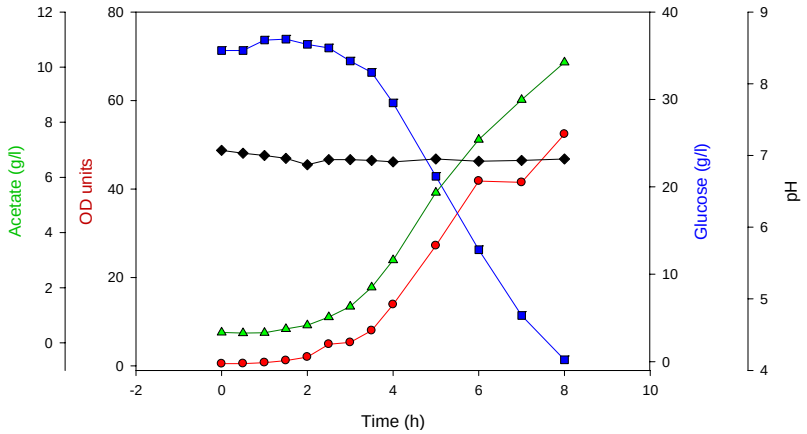
5.2.1 Growth Parameters

Of the three *E. coli* wild type cultures, BL21 reached the highest cell density OD_{600} ~70 in 7 hours of fermentation (Figure 5.1 a). Acetate production reached 3.5 g/L after 6 hours, and then dropped to ~1.5 g/L. The pH increased as glucose was depleted in the medium after 7 hours. MG1655 had a final cell density OD_{600} ~50 in an 8-hour fermentation, with an acetate production of 10 g/L at the end of the run (Figure 5.1 b). JM109 grew to OD_{600} 30 after 8.5 hours of fermentation (Figure 5.1 c). Acetate production reached approximately 10 g/L, which was similar to MG1655.

a)



b)



c)

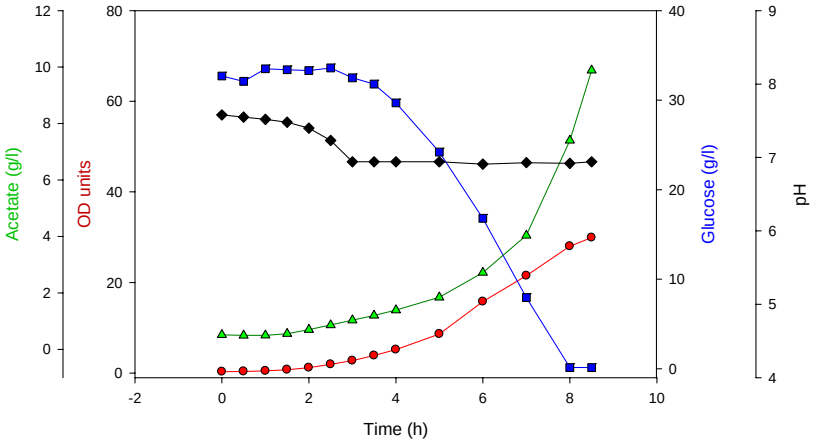
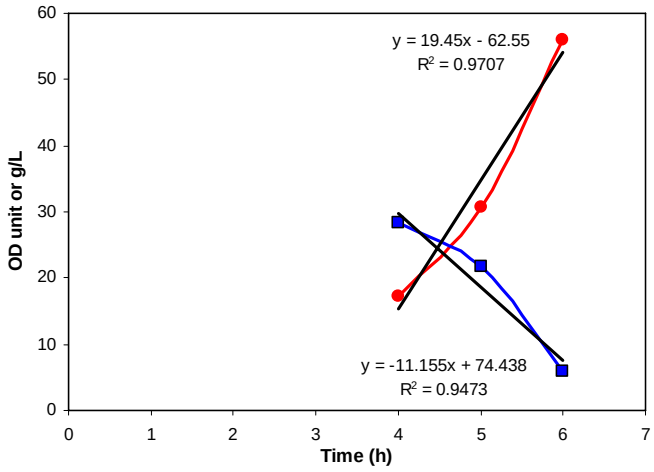
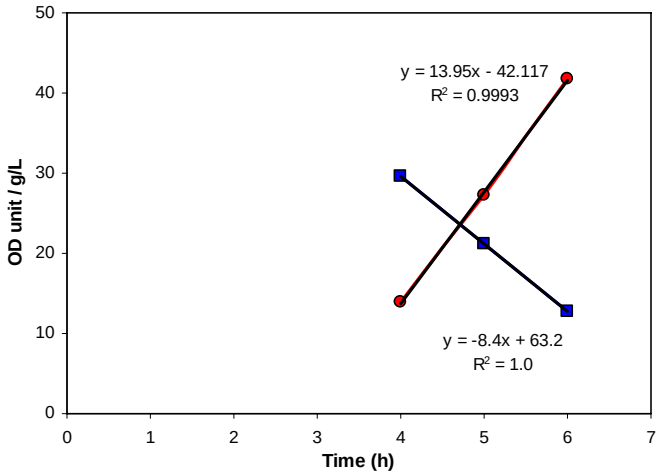


Figure 5.1. Growth parameters of wild type *E. coli* a) BL21, b) MG1655, c) JM109, growing in modified LB with 40 g/L glucose in a 3L bioreactor. ● - OD_{600}; ■ - glucose (g/L); ▲ - acetate (g/L); and ♦ - pH.

a)



b)



c)

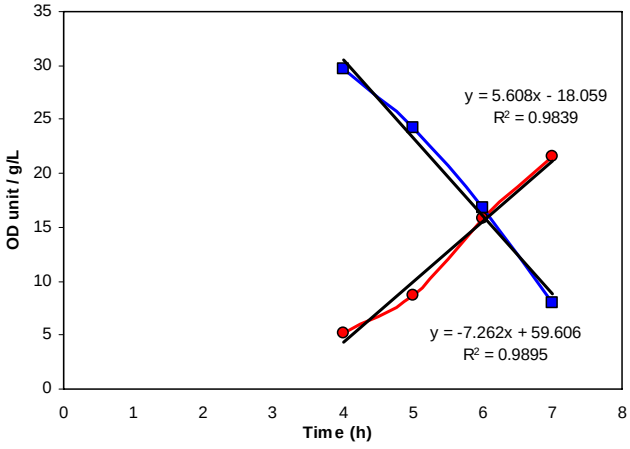


Figure 5.2. Calculation of the rate of glucose consumption of wild type *E. coli* a) BL21, b) MG1655, c) JM109, growing in modified LB with 40 g/L glucose in a 3L bioreactor during the mid-log phase. ● - OD_{600}; ■ - glucose.

The rates of glucose consumption of the three *E. coli* strains during the mid log growth phase were calculated based on the graphs presented in figure 5.2. The glucose consumption rate of BL21 and MG1655 were similar, ~0.6 g per OD unit, between 4-6 hours of fermentation. JM109 consumed glucose at a faster rate, 1.29 g per OD unit, which was doubled than those of the other two strains. The rate of glucose consumption for JM109 was analyzed between 4-7 hours of the run.

5.2.2 Northern Blots

Northern blot analysis was done to compare the SgrS and *ptsG* expressions in the three *E. coli* strains, BL21, MG1655, and JM109. Similar to the results obtained in shake flasks, BL21 showed more intense signals of SgrS compared to the two *E. coli* K strains MG1655 and JM109 (Figure 5.3). The *ptsG* expression in BL21 was lower than the other two strains, of which there were high expressions of the mRNA especially in the time 0. This may be due to the adjusting of the inoculums from the overnight cultures to high glucose condition.

5.2.3 Real-time PCR

The relative quantities of SgrS and *ptsG* expressions were analyzed by the comparative C_T (threshold cycle) method with real-time PCR. The expressions of each gene denoted by the C_T values were averaged from three technical replicates. Expressions of the target genes (SgrS and *ptsG*) were first normalized by the corresponding endogenous controls (SsrA and *ompA*) for each sample taken at hour 2

from fermentation cultures in bioreactor. The normalized quantities were then calculated by the $2^{-\Delta\Delta CT}$ method to give the fold changes compared to JM109 wild type [36]. The results obtained corresponded to the results from Northern blots. SgrS expression was the highest in BL21 wild type, a 4.7-fold more than in JM109 (Figure 5.4 a). MG1655 had a one-fold higher expression of SgrS compared with JM109. The *ptsG* expression in BL21 wild type was the lowest, 0.7 fold less than JM109 (Figure 5.4 b). The PCR results also confirmed the knock out of *sgrS* gene.

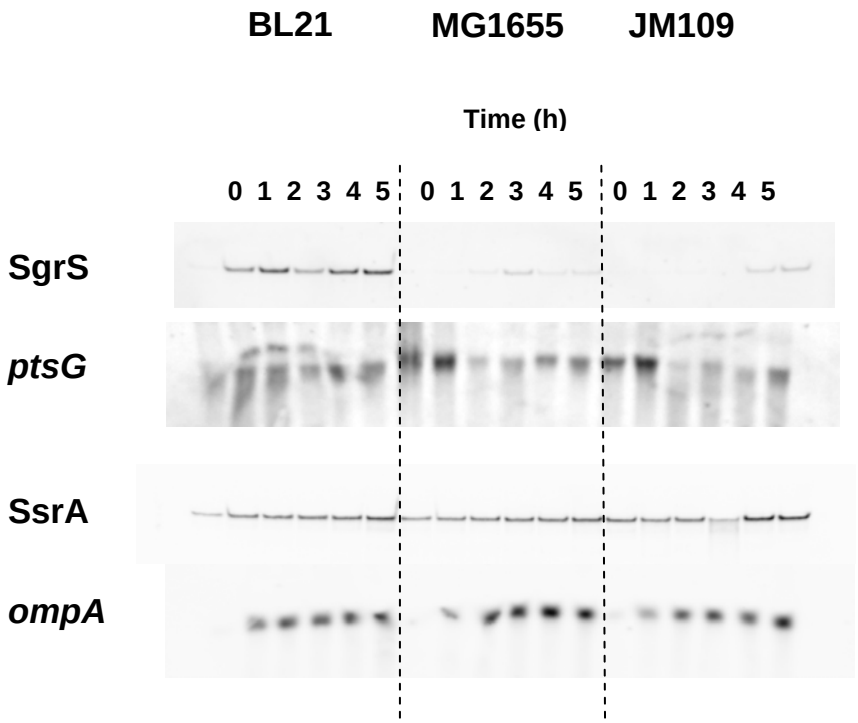


Figure 5.3. Expression of SgrS and *ptsG* with SsrA and *ompA* as the internal controls, respectively, in wild type *E. coli* BL21, MG1655, and JM109 growing in modified LB with 40 g/L glucose in a 3L bioreactor.

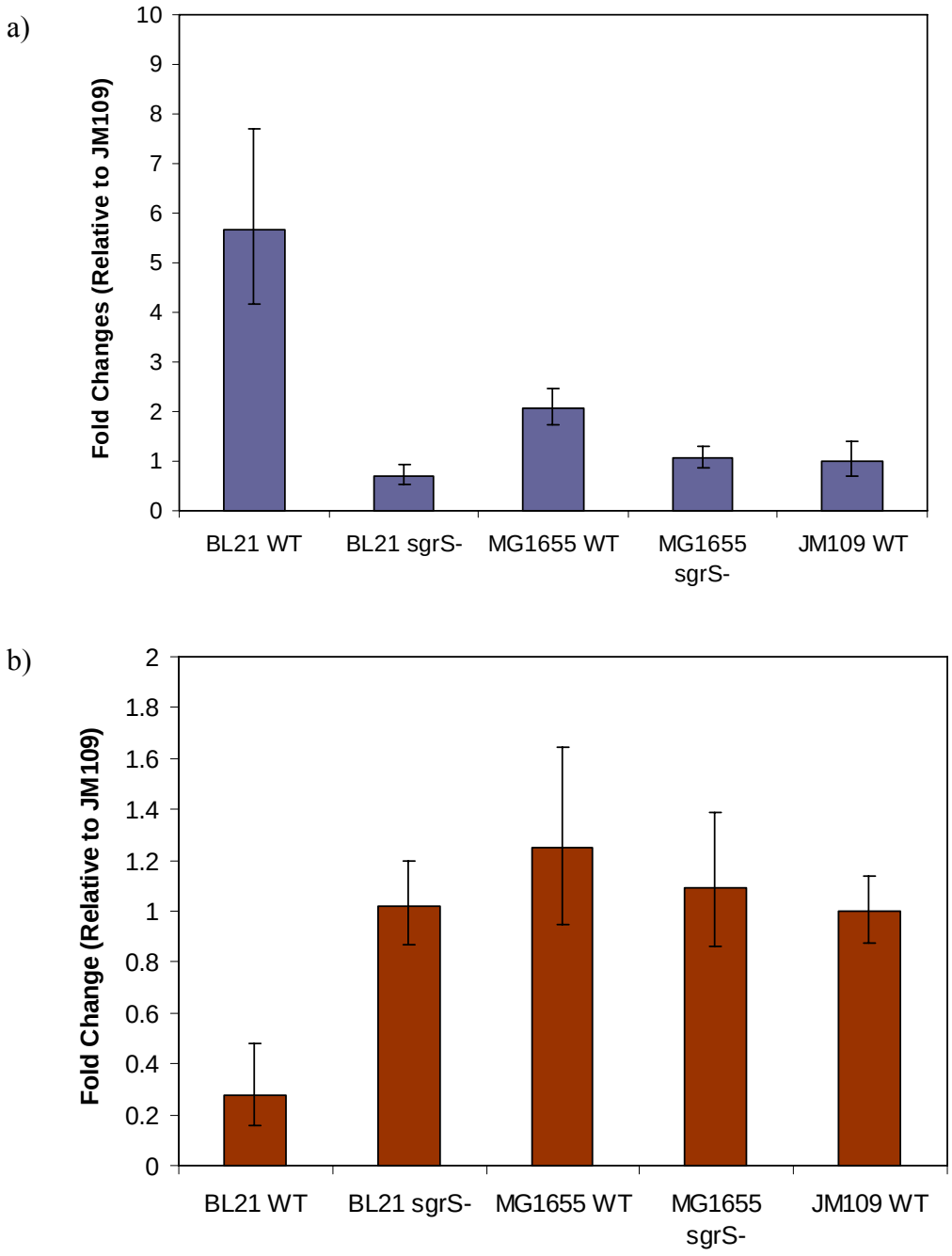


Figure 5.4. Relative quantities of expression of a) SgrS, normalized by SsrA, and b) *ptsG*, normalized by *ompA*, in *E. coli* BL21, BL21 *sgrS* ¯, MG1655, and MG1655 *sgrS* ¯, to expressions in JM109 at hour 2 of cultures growing in modified LB with 40 g/L glucose in a 3L bioreactor by real-time PCR.

5.3 Discussion

The difference in regulation of glucose uptake was studied between *E. coli* B and *E. coli* K grown in vented shake flasks at 37 °C without pH control. The results presented in the previous chapter show that only *E. coli* B expresses SgrS when grown in LB containing glucose, while *E. coli* K does not express SgrS in the same growth condition. This brought up the question whether this difference will also appear when cells are grown in bioreactor with well-controlled pH and oxygen supply.

Northern blot analysis showed that the three strains grown in bioreactor had similar patterns in SgrS expression as found in the analysis of the shake flask experiments. Only *E. coli* B, expressed significant amount of SgrS in glucose, while the target mRNA *ptsG* was not completely degraded. This means both pH control and oxygen supply do not affect the expression of SgrS and its regulation of *ptsG* in BL21. For the growth profiles, BL21 had the highest final cell density but the lowest acetate production. MG1655 and JM109 had lower cell densities, but both strains produced high concentration of acetate by the end of the run. The cell densities and acetate production results obtained agreed with published work [6, 7].

During the mid-log phase of cell growth, the rate of glucose consumption in JM109 was two-folded of those in BL21 and MG1655. This implied that the high acetate production in JM109 was caused by the high uptake rate of glucose and high activation of the metabolic pathways directing the carbon flux to acetate formation, as proposed by Phue *et al*., 2005. [6]. Although MG1655 had the same glucose uptake

rate as BL21, it produced as high acetate concentration as JM109. This was possibly because MG1655 also directed most of its carbon flux towards the acetate synthetic routes. Both MG1655 and JM109 used only transcriptional regulation of glucose uptake, though they had different glucose uptake rate during the mid-log phase. On the other hand, as mentioned previously, BL21 used both transcriptional and post-transcriptional level of glucose uptake regulation. Its lower rate of glucose consumption, together with the high activation of TCA cycle and gluconeogenesis proposed in previous studies, explained its low acetate production (Figure 5.5) [6].

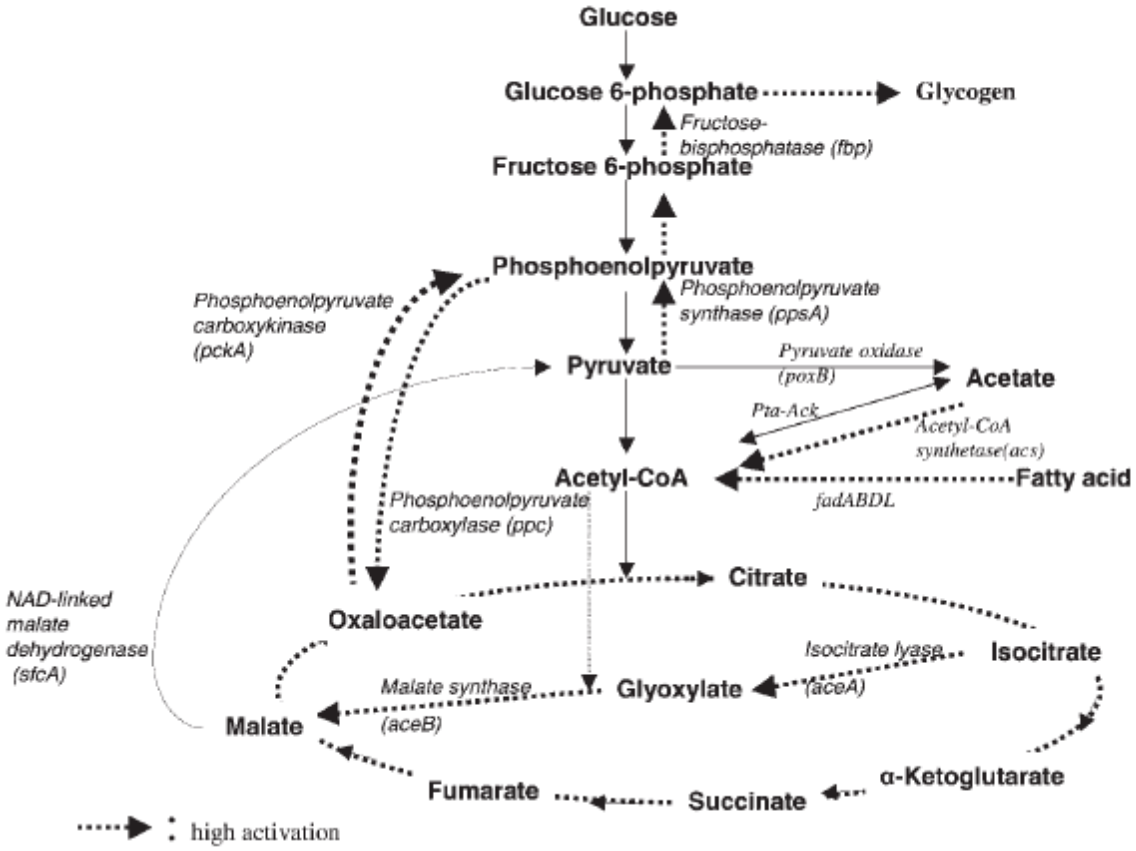


Figure 5.5. Proposed pathways for acetate accumulation during high glucose batch fermentation of E. coli BL21 [6].

This shows that in BL21, SgrS plays a role in controlling its glucose uptake. The *ptsG* was not completely degraded upon expression of SgrS. It is possible that SgrS is expressed at a level just to lower the synthetic rate of glucose transporter by destabilizing part of the pre-existing *ptsG* mRNA. The SgrS transcripts may also involve in encoding the polypeptide SgrT, which in turn regulates the glucose uptake by acting on the transporter proteins directly. Furthermore, the activation of SgrS gives a hint that some signaling molecules, such as G6P, may accumulate and trigger the small RNA expression. From the proposed acetate accumulation pathways (Figure 5.5), other possible signaling molecules include phosphoenolpyruvate (PEP), as the phosphoenolpyruvate carboxykinase pathway is highly activated under high glucose batch fermentation [6]. PEP molecules may accumulate when the rate of gluconeogenesis is not fast enough to convert all PEP into F6P.

The connection between SgrS and acetate production in *E. coli* B is not clear, therefore, studying how SgrS causes variations in acetate concentration will be useful for genetic modifications of *E. coli* to overcome the acetate production.

Chapter 6 Conclusions and Future Work

6.1 Conclusions

The present work provides evidence about the difference between *E. coli* B and *E. coli* K in the transcription of the small RNA SgrS in response to glucose in the growth media. Both strains expressed SgrS for post-transcriptional regulation of the glucose transporter mRNA *ptsG* when they were exposed to the non-metabolizable glucose analog αMG. However, only *E. coli* B expressed significant amount of SgrS when cells were grown in high glucose conditions. The patterns of SgrS expression in *E. coli* B were the same for cultures in both shake flasks and bioreactors. This means transcription of SgrS and *ptsG* is not affected by pH control or dissolved oxygen in the media. In both growing conditions, *ptsG* was not fully degraded upon expression of SgrS in *E. coli* B. This implied that the level of SgrS expressed might just be enough to destabilize part of the pre-existing *ptsG* mRNA. It is also possible that, in addition to the role in post-transcriptional regulation of the mRNA, SgrS may also involve in other regulatory roles such as encoding the polypeptide SgrT.

As there are significant differences in SgrS expression and acetate production between *E. coli* B and *E. coli* K, it is possible that there is a connection between the regulation of SgrS and acetate production in *E. coli* cultures. If this is the case, this will provide another way to perform genetic modifications in cells for overcoming the

problem of acetate accumulation in high cell density cultures used for recombinant protein production.

In addition, real-time PCR was developed for comparative quantification of the transcription of small RNA and mRNA. Until now, the analytical work on small RNA has only been done by Northern blots. The present study is the first one to use real-time PCR to quantify small RNA, and the results correlate to those obtained by Northern blots. Thus real-time PCR provides faster way to analyze and quantify the small RNA expressed in cells.

6.2 Future Work

The present work is the beginning of the investigations on the role of the small RNA SgrS in *E. coli* B. As there is a physiological difference between *E. coli* B and *E. coli* K in glucose regulation, the next step will be to understand how this difference contributes to the distinct characteristics of the two strains. First, it will be interesting to learn how the *sgrS* knockout and overexpressing mutants of *E. coli* B and *E. coli* K behave in response to high glucose concentrations in the expression of *ptsG* mRNA, cell growth and acetate production. The roles of SgrS can be studied by analyzing the transcriptions of SgrR and SgrT, and the corelation of SgrS to the amount of intermediary metabolites in the metabolic pathways. From this, it may be possible to identify the molecule which activates the transcription of SgrS, and to know how the carbon flux changes according to the level of SgrS expression. The regulation of

glucose uptake in both *E. coli* B and *E. coli* K can be investigated by elucidating both the transcriptional and post-transcriptional level of regulation, i.e. by measuring the level of CRP-cAMP, Mlc, SgrR, SgrS, SgrT, and *ptsG* in different wild type and mutant strains. Most importantly, the acetate production in different strains can be quantified and we can see how the expression levels of different genes, especially SgrS, cause the differences in acetate production. This provides useful information for the genetic modifications of *E. coli* strains to reduce formation of the by-product acetate, which is a long-term research goal for the industrial production of recombinant proteins.

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