Understanding and elimination of carbon catabolite repression in *Escherichia coli*

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Abstract

Microorganisms often encounter a mixture of different carbon sources and therefore have control systems to selectively take up and metabolize those substrates that promise the best success in competition with other species through rapid growth. The aim of this thesis is to understand and eliminate carbon catabolite repression (CCR) in *Escherichia coli* for efficient utilization of multiple energy and carbon sources simultaneously. We studied a new CCR hierarchy that causes the preferential utilization of sugars (arabinose, galactose, glucose, mannose, and xylose) over a short-chain fatty acid (propionate). Meanwhile, the native promoters of xylose catabolic genes and xylose transporter genes were replaced with synthetic constitutive promoters to construct an *E. coli* strain capable of co-metabolizing glucose and xylose by eliminating the CCR of xylose metabolism by glucose. We showed that such an approach can provide a potential to eliminate CCR. This knowledge will be valuable to help strain improvement strategies for the simultaneous consumption of sugar mixtures, leading to shorter fermentation time and higher substrate range and productivity.

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Figure 11. Comparative mRNA expression of both *xylA* and *xylF* in respective wild type and XYL1 grown in minimal media supplemented with glucose and D-xylose. Both *xylA* and *xylF* genes were continuously transcribed in XYL1 regardless of the presence of glucose, whereas wild type didn't express them at all in the presence of glucose.

Figure 12. Comparison of glucose and xylose utilization in (A) Wild type, (B) XYL1 and (C) XYL2. Blue line represents the concentration of glucose while red line represents the concentration of xylose.

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Nomenclature

CCR: Carbon catabolite repression

E.coli: *Escherichia coli*

PTS: Phosphotransferase system

HPr: Histidine protein

PEP: Phosphoenolpyruvate

cAMP: Cyclic adenosine monophosphate

CRP: cAMP receptor protein

LacI: *lac* repressor

Acetyl-CoA: Acetyl-coenzyme A

IHF: Integration host factor

PPP: Pentose phosphate pathway

IPTG: Isopropyl β-D-1-thiogalactopyranoside

Chapter 1

General introduction

Global warming and oil crisis cause the changes from existing petrochemical industry to biochemical industry. Lignocellulose is suitable raw material to such current of the world. It provides renewable sugar sources and is less expensive. Lignocellulosic hydrolysates contain mixtures of glucose, xylose, arabinose and small amount of galactose and mannose[1,2]. However, when they are used as carbon sources, *Escherichia coli* cannot utilize all sugars simultaneously but the strain preferentially uses glucose even though *E. coli* has metabolic pathways corresponding to lignocellulosic sugars. In other words, glucose represses the metabolism of other sugars in the cell. Such phenomenon is carbon catabolite repression (CCR) [3]. It is a main factor causing inefficient production of bio-fuels or bio-chemicals as it increases the fermentation time and decrease the productivity [4]. Therefore, it is one of the most important things to develop the strategy to eliminate CCR in *E. coli* for future industries.

The CCR in *E. coli* is mediated by a combination of inducer exclusion, global regulatory proteins, and cAMP-CRP complex [3]. The major sugar transport system for hexose sugars, the phosphotransferase system (PTS), is a potential candidate responsible for CCR (Fig. 1). It consists of a series of five conserved functional domains, namely, enzyme (E) I, histidine protein (HPr), EIIA, and EIIc, which, together, form a cascade of phosphorylated intermediates that transfer phosphate from phosphoenolpyruvate (PEP) to the incoming sugar [5]. Thus, PTS-mediated CCR mechanism responds to the phosphorylation level of a PTS protein which is an indicator of the metabolic state in the cell.

Glucose-specific $EIIA^{Glc}$ is a key component in CCR in *E. coli*. In the presence of glucose (Fig. 1A), dephosphorylated $EIIA^{Glc}$ of the PTS inactivates several non-PTS sugar transport systems such as lactose, maltose, and melibiose [6]. Such phenomenon is called inducer exclusion because the transported non-PTS sugar acts as an inducer of the corresponding catabolic operon. Meanwhile, $EIIA^{Glc}$ is phosphorylated in the absence of glucose. Both forms of dephosphorylated and phosphorylated $EIIA^{Glc}$ can bind to adenylate cyclase but only phosphorylated form is able to activate adenylate cyclase leading to the synthesis of cAMP. Increased concentration of cAMP forms a complex with cAMP receptor protein (CRP) and thus this complex activates the promoters of catabolic genes. Thus the cAMP-CRP complex is a global regulator of the sugar operons [7].

Consequently, such cAMP-CRP complex mediated regulation of catabolic gene expression causes the duration of lag phase during diauxic growth while inducer exclusion is mainly involved in repression

of catabolic genes [3]. In addition to such global regulatory system mediated CCR, operon specific regulatory system also participates in CCR. For instance, in the case of *lac* operon, the operon is expressed constitutively even in the presence of glucose when *lac* repressor gene (LacI) is inactivated by mutation or addition of IPTG and hence diauxic growth was removed [8]. Therefore, for *lac* operon, inducer exclusion is a major factor for CCR while cAMP-CRP has an indirect regulatory role [8]. Thus, relative roles of operon specific regulatory system and cAMP-CRP for CCR are different from operon to operon. For that reason, in order to understand CCR, studying each catabolic system is required.

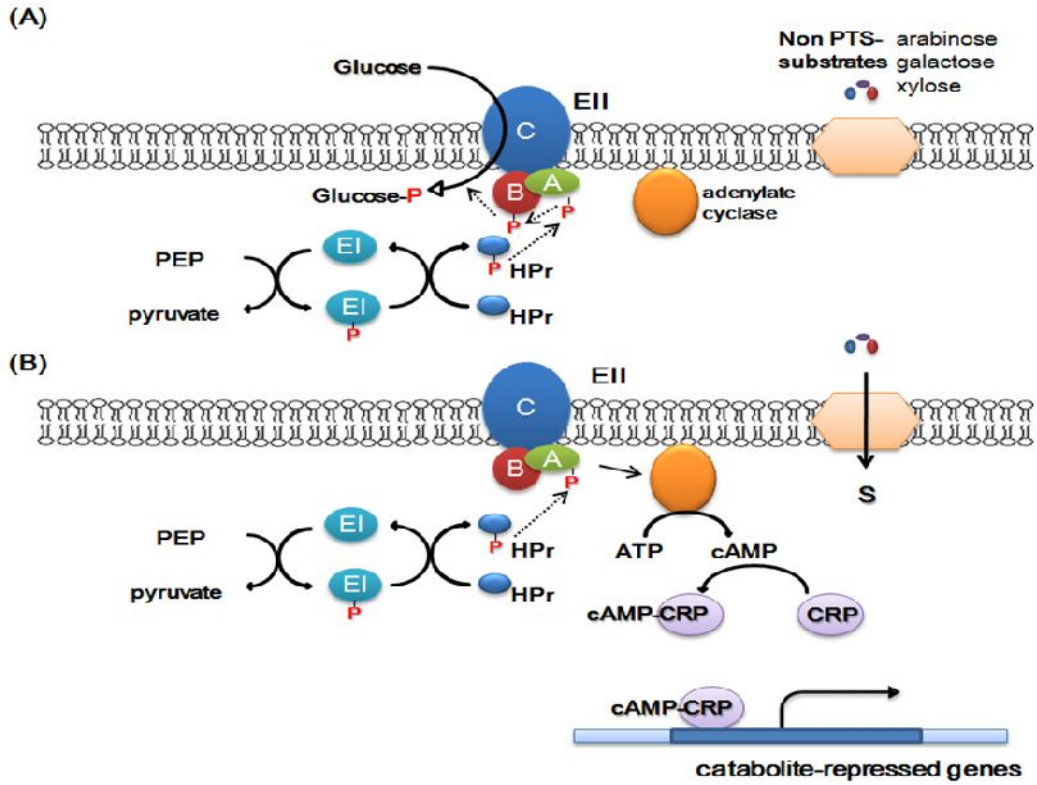


Figure 1. Phosphotransferase system (PTS) causing carbon catabolite repression in *E. coli*.

PTS consists of five conserved functional domains, Enzyme I (EI), Histidine protein (HPr), Enzyme IIA (IIA), Enzyme IIB (IIB) and Enzyme IIC (IIC). These domains transfer the phosphate group from phospho-enol-pyruvate (PEP) to the incoming PTS sugars. (A) In the presence of glucose, dephosphorylated IIA in the PTS limits the transportation of non PTS substrate and the expression of carbon repressed gene. (B) In the absence of glucose, phosphorylated IIA in the PTS induce the transportation of non PTS substrates and the expression of the gene involving in catabolite metabolism that can utilize non PTS sugars [4].

cAMP (cyclic adenosine monophosphate), ATP (adenosine triphosphate), CRP (cAMP receptor protein), S (non-PTS substrate), A (Enzyme IIA), B(Enzyme IIB), C(Enzyme IIC), P (phosphate group).

1.1 Propionate catabolism in *E. coli*

Most fatty acids are catabolized to acetyl-coenzyme A (CoA) via β-oxidation. However, propionate, which is short fatty acid, is metabolized to pyruvate through another route. 2-Methylcitrate cycle is such route for the breakdown of propionate in *E. coli* [9]. The genes required for a 2-methylcitrate pathway, constitute a locus composed of two divergently transcribed units. One is the single gene *prpR*, which encodes the activator protein and the other contains the *prpBCDE* operon encoding the enzymes for 2-methylcitrate pathway (Fig. 2). The *prpBCDE* operon is positively regulated by PrpR together with the integration of the host factor (IHF) [10]. Both promoters of *prpR* and *prpBCDE* are activated by the cAMP-CRP complex. Therefore, the *prpBCDE* operon is directly or indirectly regulated by the cAMP-CRP complex through PrpR [11]. Thus, the propionate metabolism is subject to catabolite repression through CRP, global regulatory protein.

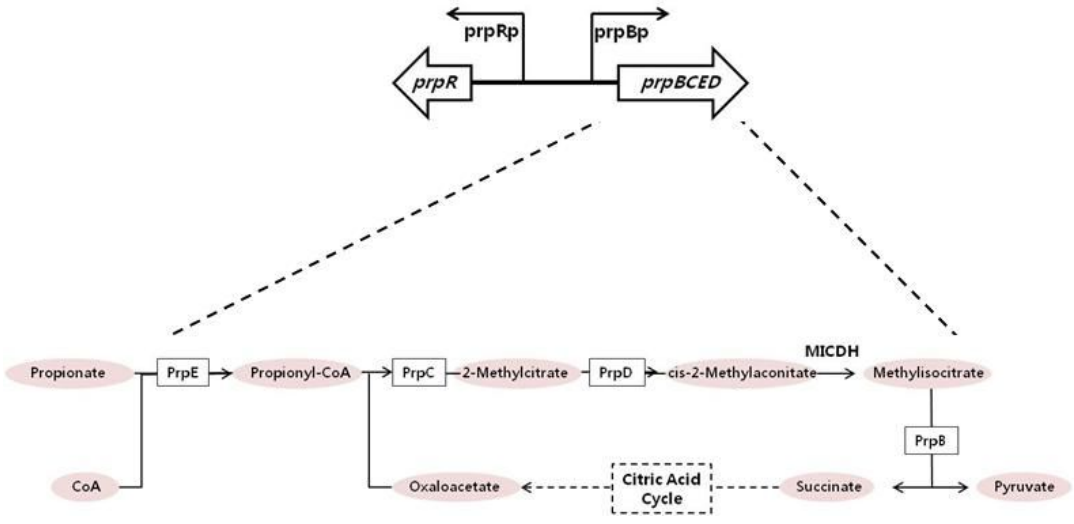


Figure 2. Propionate catabolism via 2-methylcitrate cycle.
PrpE (propionate-CoA ligase), PrpC (2-methylcitrate synthase), PrpD (2-methylcitrate dehydratase), MICDH (2-methylisocitrate dehydratase), PrpB (methylisocitrate lyase)

1.2 Xylose catabolism in *E. coli*

In *E. coli*, D-xylose is dissimilated through the pentose phosphate pathway (PPP) [12]. Before entering the PPP, xylose is isomerized into xylulose and then phosphorylated into xylulose-5-phosphate [13]. An operon which is composed of two catabolic genes, *xylA* (xylose isomerase) and *xylB* (xylulokinase) is involved in such xylose metabolism (Fig. 3). Xylose uptake occurs through two different transporters which are a high-affinity ABC transporter (XylFGH) and a low-affinity proton symporter (XylE) [14].

XylFGH is the dominant xylose uptake system while XylE has little activity even under high-xylose concentrations (50 mM)[14]. Therefore, *xyl* genes are organized into two major transcriptional units, *xylAB* and *xylFGH*. The *xylA* and *xylF* are bidirectionally transcribed by the promoters, P_{xylA} and P_{xylF}, respectively. These operons are positively regulated by XylR which exists in the downstream of *xylFGH* [15] . In addition to such operon specific regulation, *xyl* genes are also up-regulated by the cAMP-CRP complex, which is global regulation indicating that the regulation is modulated by catabolite repression [16].

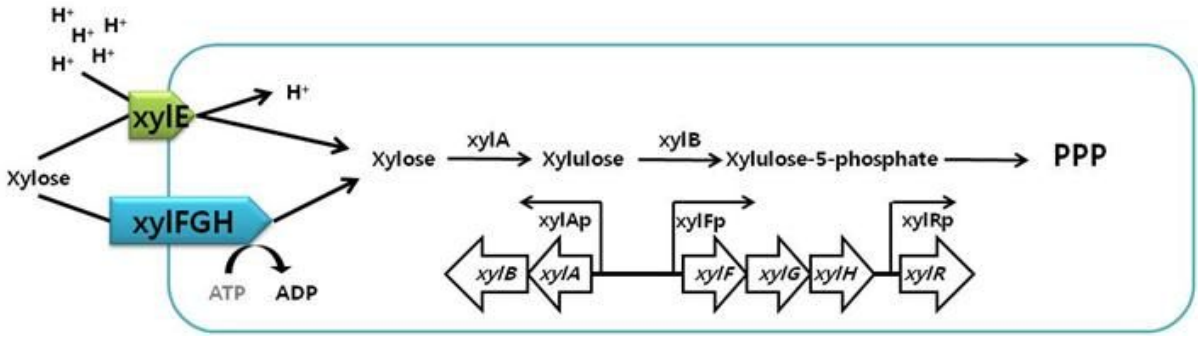


Figure 3. A schematic of xylose uptake and metabolism in *E. coli*
xylA (xylose isomerase), *xylB* (xylulokinase), *xylF* (xylose-binding protein), *xylG* (ATP-binding protein), *xylH* (membrane transporter), PPP (pentose phosphate pathway)

1.3 CRP (cyclic AMP receptor protein)

CRP can function as a transcription factor for a number of sugar catabolic genes and transporter genes only when CRP binds to cAMP [17]. The binding of cAMP to CRP leads to allosteric transition in CRP, causing increased affinity for the CRP recognition sequence located near promoter regions. CRP is composed of two subunits, larger amino terminal domain responsible for cAMP binding, and the carboxy domain responsible for DNA binding[17] (Fig. 4). It has been proposed that bound cAMP changes the relative orientation between these two domains, and alters the relative orientation of the two subunits within a CRP dimer. Therefore, native CRP absolutely needs cAMP to be its active form[18]. However, several mutations in CRP such as CRP* showed apparent reduced dependence on cAMP. Such *crp** was assumed to have same conformational change evoked by cAMP. Several types of *crp** provided information on a class of mutation in *crp* that allow them to function in the absence of cAMP. It has been reported that α D- helix of the DNA's binding domain is an important determinant for the CRP* phenotype[19]. Even though the region is not located in areas of the protein which are directly in contact with DNA or cAMP, the mutation in α D- helix defines conformational change presented during the allosteric transition. On the other hand, it has been reported that serine at position 83, threonine at position 127 and serine at position 128 which lie in the cyclic nucleotide binding pocket, responsible for the contact between cAMP and CRP, are not important determinants of cAMP-binding affinity for CRP* phenotype[20].

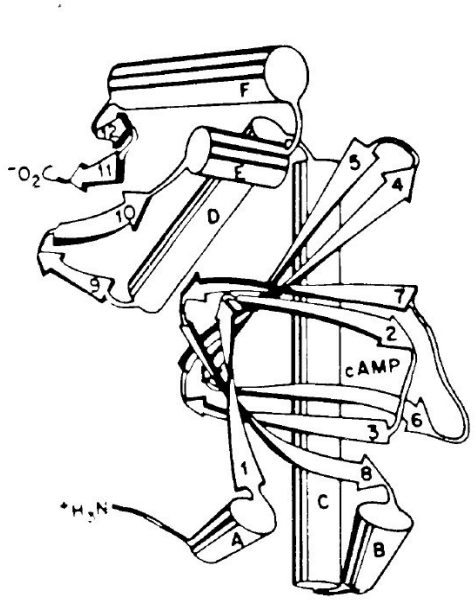


Figure 4. A schematic drawing of a CRP monomer[17]. The regions that are α-helices are represented as cylinders lettered A through F. The regions in β-conformation are represented as arrows 1 through 12. The larger amino-terminal domain consists of α helices A through C and β-sheets 1 through 8. The smaller carboxy-terminal domain consists of the D through F α helices and β-sheets 9 though 12. The two domains are connected covalently by a tetrapeptide segment between the C and D α helices[17].

Chapter 2

The mechanism of sugar-mediated catabolite repression of the propionate catabolic genes in *Escherichia coli*

2.1 Summary

Carbon catabolite repression (CCR) is a well-known phenomenon that involves the preferential utilization of glucose as a carbon source. Cyclic adenosine monophosphate (cAMP) and the cAMP receptor protein (CRP) mediate CCR. Recently, a second CCR hierarchy that leads to the preferential consumption of arabinose over xylose, mediated by arabinose-bound AraC, has been identified. In this study, we report yet another CCR hierarchy that causes the preferential utilization of sugars (arabinose, galactose, glucose, mannose, and xylose) over a short-chain fatty acid (propionate).

Expression of the propionate catabolic (*prpBCDE)* genes is down-regulated in the presence of these sugars. Sugar-mediated repression of the propionate catabolic genes is independent of sugar-specific regulators such as AraC and dependent on global regulators of sugar transport such as the cAMP-CRP complex and the Phosphotransferase System (PTS). Inhibition of the *prpBCDE* promoter is encountered during rapid sugar uptake and metabolism. This unique regulatory crosstalk between sugar metabolism and fatty acid metabolism may help provide new insights into CRP-dependent catabolite repression acting in conjunction with non-carbohydrate metabolism.

2.2 Introduction

Carbon catabolite repression (CCR) is an important global regulatory system in various bacteria that allows them to preferentially utilize the most energy-efficient carbon source in a mixture [21]. To date, CCR in *Escherichia coli* is believed to be mediated by a cyclic adenosine mono-phosphate (cAMP)–cAMP receptor protein (CRP) complex [22]. Preferential utilization of glucose over other sugars is a central phenomenon of CCR in *E. coli*. When glucose is the predominant sugar in the medium, cAMP

synthesis is inhibited. Because other sugar metabolizing operons are positively regulated by the cAMP-CRP complex, their catabolic genes are expressed only after the depletion of glucose [22].
Although catabolite repression by glucose has been studied extensively, CCR between sugars other than glucose is still incompletes. For example, the order of preference among pentose sugars has been established: arabinose followed by xylose followed by ribose. Complex transcriptional regulation by proteins like AraC and XylR ensure the preferential utilization of arabinose and xylose over ribose [23].

Recently, the regulatory mechanisms leading to the preferential use of arabinose over xylose have been identified. Arabinose interacts with AraC and represses the xylose promoter causing cells to utilize arabinose first and then xylose. Xylose, in turn, inhibits the expression of genes that regulate D-ribose metabolism through the transcriptional repressor, XylR [23]. These results demonstrate that the expression of genes regulating the metabolism of one carbon source is significantly altered by the presence of other carbon sources in the culture medium. Therefore, there exists a hierarchy for carbon source utilization that is not only limited to glucose but also exists among other sugar sources.

The preferential utilization of the best available nutrition may be an adaptation that allows bacteria to survive a competitive environment. However, the elimination of catabolite repression in industrial hosts is a potentially important mechanism to increase yields particularly when lignocellulosic biomass is used as a substrate [24]. Lignocellulosic biomass is composed of a mixture of sugars including arabinose, galactose, glucose, mannose, and xylose [25,26]. CCR among these sugar sources hampers the efficient production of bio-products. Because catabolite repression might lead to long fermentation times and impede downstream processes in industrial settings, substantial efforts have been recently devoted to eliminate CCR in engineered *E. coli* strains. Therefore, a thorough understanding of all aspects of CCR is a necessary prerequisite to engineering *E. coli* that can ferment many potential carbon sources. In particular, very little is known about the CCR that occurs between carbohydrate and non-carbohydrate substrates.

This study investigated a novel CCR hierarchy that exists between carbohydrate (sugars) and non-carbohydrate (propionate) based carbon sources. The mechanism of sugar-mediated CCR of fatty acid catabolic genes was investigated using arabinose (sugar) and propionate (short chain fatty acid) as a proof-of-concept. We propose a possible mechanism of CCR of the propionate catabolic genes *prpBCDE* in the presence of various mono-sugars in *E. coli*.

2.3 Materials and methods

2.3.1 Bacterial strains and media

The strains used are listed in Table 1. *E. coli* MG1655 was used as the parental strain in this study. Cells were grown at 37°C in Luria-Bertani broth (LB) supplemented with suitable antibiotics (kanamycin at 50 µg/mL or ampicillin at 100 µg/mL). The *prpBCDE* promoter (P_{prpB}) was induced using 20 mM sodium propionate (pH 8.0). For promoter activity assays, the LB medium was supplemented with 100 mM sodium phosphate buffer (pH 7.0) to avoid any pH-related changes in cellular metabolism. Cell growth was monitored by measuring optical density at 600 nm (OD_{600}) using a Biochrom Libra S22 spectrophotometer (Biochrom, Cambridge, England).

Table 1. *E. coli* strains and plasmids used in this study

Strains/plasmids	Description/genotype	Reference/source
Strains		
E. coli MG1655	Wild-type	[27]
CP12CHB CRP*	MG1655 with P_{CP12}-*chb*, *crp*::*crp** (T127I)	This study
JARA1	MG1655 with P_{CP25}-*araB*, P_{CP6}-*araF*, Δ*araC*::FRT	In this study
JARA2	MG1655 with Δ*araA*::FRT	In this study
JARA35	JAR1 adapted for 35 days	In this study
Plasmids		
pSIM5	λ-Red recombinase expression plasmid and temperature sensitive replication	[28]
pCP20	Yeast FLP recombinase gene controlled by *c*I repressor and temperature sensitive replication.	[29]
pKD13	Template plasmid for gene disruption. The resistance gene is flanked by FRT sites. *oriR6K*-gamma origin requiring the *pir* + *E. coli*.	[28]

2.3.2 Strain construction

Promoter replacement, gene knockout, and gene replacement were performed in *E. coli* MG1655 using the lambda Red recombination system as described previously [28,30]. Two synthetic constitutive promoters (CP6 and CP25) with β-galactosidase activities of 280 and 528 Miller units, respectively, in *E. coli* were used for promoter replacement (Table 2) [31].

Strain JARA1 was constructed by replacing the arabinose-inducible promoters of *araBAD* and *araF* with the synthetic constitutive promoters CP25 and CP6, respectively, and by deleting *araC*. The engineered strain was adapted in arabinose minimal medium for 35 days by sub-culturing cells in fresh medium every time the culture reached an OD_{600} of 1.0. The resulting strain, JARA35, was used to analyze the effect of AraC-independent arabinose metabolism on CCR by observing *prpBCDE* expression.

To construct strains carrying a cAMP-insensitive CRP (encoded by *crp**), the native *crp* gene was first deleted and was then replaced with the *crp** gene (W3110 CRP with T127I) constructed by splice overlap extension (SOE)-PCR [30]. Strains carrying the mutant *crp* were easily identified by their faster growth rate when compared to the *crp*-knockout strains. The recombinant mutants were verified by PCR-amplification of genomic DNA isolated from the transformants and sequence analysis. The primers used for strain construction are listed in Table 2.

Table 2. Primers used in the study

Strain construction	Primer sequence
SOEing CP6 promoter to Kanamycin cassette	
	5′-CAT AGC TGT TTC CTG TGT GAA CAG TAC TCA GTT ATT ATA TCA TCC GG-3′
	5′-TCA GTT ATT ATA TCA TCC GGA AAT ATC TGT GTC AAG AAT AAA CTC C-3′
	5′-CTG TGT CAA GAA TAA ACT CCC ACA TGA TTC CGG GGA TCC GTC GAC C-3′
SOEing CP25 promoter to Kanamycin cassette	
	5′-CAT AGC TGT TTC CTG TGT GAA CAG TAC TAT GTG ATT ATA CCA GCC CCC-3′
	5′-ATG TGA TTA TAC CAG CCC CCT CAC TAC ATG TCA AGA ATA AAC TGC-3′
	5′-ACA TGT CAA GAA TAA ACT GCC AAA GAT TCC GGG GAT CCG TCG ACC-3′
Promoter exchange	
P_{CP6}-*araF*	5′-GGT AAT GCG GCC TAT TGA CTG GTT AAA AAG AAG ACA TCC CGC ATG GGT AGT GTA GGC TGG AGC TGC TTC G-3′
	5′-GAC ATA ACG GCT GCC AGA CCA ATG GCT GCC AGG GCT TTA GTA AAT TTG TGC ATA GCT GTT TCC TGT GTG AAC AGT ACT-3′
P_{CP25}-*araB*	5′-CCC TAT GCT ACT CCG TCA AGC CGT CAA TTG TCT GAT TCG TTA CCA AGT GTA GGC TGG AGC TGC TTC G-3′
	5′-TCG CAC AGA ATC ACT GCC AAA ATC GAG GCC AAT TGC AAT CGC CAT AGC TGT TTC CTG TGT GAA C-3′
Gene deletion/insertion	
crp deletion	5′-CTA CCA GGT AAC GCG CCA CTC CGA CGG GAT TAA CGA GTG CCG TAA ACG ACG TGT AGG CTG GAG CTG CTT CG-3′
	5′-GGC GTT ATC TGG CTC TGG AGA AAG CTT ATA ACA GAG GAT AAC CGC GCA TGA TTC CGG GGA TCC GTC GAC C-3′
*crp** insertion	5′-GGC GTT ATC TGG CTC TGG AGA AAG CTT ATA ACA GAG GAT AAC CGC GCA TGG TGC TTG GCA AAC CGC AAA CAG ACC CG-3′
	5′-ACT TGC AGA CGA CGC GCC ATC TGT GCA GAC-3′
	5′-CTT CTG ATG CGT TTG TCT GCA CAG ATG GCG CGT CGT CTG CAA GTC ATT TCA GAG AAA GTG GGC AAC CTG GCG TTC CTC GAC GTG ACG GGC CGC ATT ACA-3′
	5′-ATT CCG GGG ATC CGT CGA CCT TAA CGA GTG CCG TAA ACG ACG ATG GTT TTA CCG TGT GCG GAG-3′
araA deletion	5′-GAC TCT ATA AGG ACA CGA TAA TGA CGA TTT TTG ATA ATT ATG AAG TGT GGT TTG TCA TTG TGT AGG CTG GAG CTG CTT C-3′
	5′-TAC ATA CCG GAT GCG GCT ACT TAG CGA CGA AAC CCG TAA TAC ACT TCG TTC CAG CGC AGC ATT CCG GGG ATC CGT CGA CC-3′
araC deletion	5′-TGG CCC CGG TGC ATT TTT TAA ATA CTC GCG AGA AAT AGA GGT GTA GGC TGG AGC TGC TTC G-3′
	5′-ATA AGC GGG GTT ACC GGT TGG GTT AGC GAG AAG AGC CAG TAT TCC GGG GAT CCG TCG ACC -3′

2.3.3 Assay of in vivo promoter activities

The *gfpuv* gene expressed under the control of the *prpBCDE* promoter was used as a reporter (pPro7(E)-*gfp*) to assess promoter activity [32]. Strains harboring the reporter plasmid, pPro7(E)-*gfp,* were cultured overnight in LB medium supplemented with ampicillin. Cells grown overnight were sub-cultured (1:100 dilution) into 5 mL of fresh LB medium supplemented with ampicillin and sodium phosphate buffer. When the cell density reached an OD_{600} of 0.5, 20 mM propionate and 2 g/L of sugar were added to induce the culture, which was then transferred to 96-well plates. Cell growth and GFP expression were monitored every 10 minutes by measuring OD_{600} and fluorescence emission at 535 nm (excitation at 475 nm), respectively, using the Tecan SpectraFluor Plus plate reader (Tecan-US, Durham, NC).

2.3.4 HPLC analysis

The amount of residual arabinose in the medium was measured with a Shimadzu HPLC station equipped with a SupelCogel Pb column and a refractive index detector (Shimadzu, Japan). 500 μL of overnight culture was used to inoculate 50 mL of fresh LB medium supplemented with 2 g/L arabinose in a 250 mL shake flask and grown at 37°C in a shaking incubator. One milliliter of sample was collected every 1 hour, centrifuged to remove cells, and then boiled and filtered to remove protein precipitates. Twenty microliters of the processed sample was injected into the column maintained at 80°C. HPLC grade water was used as a mobile phase at a flow rate of 0.5 mL/min.

2.4 Results

2.4.1 Catabolite repression of propionate catabolic genes in the presence of sugars

The change in expression levels of propionate catabolic (*prpBCDE*) genes of *E. coli* MG1655 in the presence of various sugars (arabinose, galactose, glucose, IPTG, lactose, mannose, and xylose) was

analyzed by following GFP expression from the *prpBCDE* promoter (*PprpB*). All metabolizable sugars at concentrations above 2 g/L downregulated *PprpB* promoter activity to background levels (Fig. 5). By contrast, *prpBCDE* promoter activity was unaffected by the non-metabolizable lactose analogue IPTG (Fig. 5); *prpB* promoter activity was also not repressed at low concentrations of the metabolizable sugars (less than 1 g/L). Hence, we hypothesized that the repression of *PprpB* promoter activity is mediated by a complex interplay of regulatory proteins and intermediate metabolites produced during sugar metabolism.

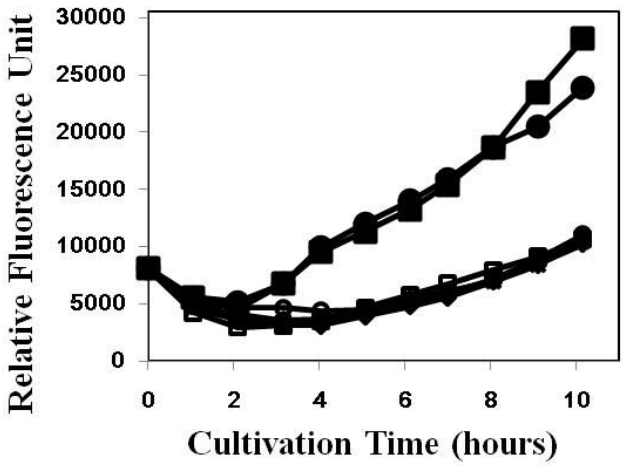


Figure 5: Comparison of *prpB* promoter activity in the absence of sugar (closed squares) or in the presence of different sugars such as arabinose (asterisks), galactose (open diamonds), glucose (open square), IPTG (closed circles), mannose (closed diamonds), and xylose (open circles) in MG1655.

2.4.2 *prpBCDE* promoter activity in catabolite de-repressed strains of *E. coli*

To determine if transcription from the *prpBCDE* promoter is subject to catabolite repression in a manner similar to that of other sugar operons, we analyzed the effect of sugar on the *prpB* promoter activity in catabolite de-repressed strains such as a strain carrying the *crp** (T127I) gene or PTS gene-knockout strain [33]. The native *crp* gene of *E. coli* MG1655 was replaced by a mutant (referred to as *crp**) that can function independent of cAMP. A single amino acid substitution (T127I) in CRP alters the cAMP binding domain without disturbing the DNA binding domain and the activation domain.

CRP* could not restore *prpB* promoter activity in the presence of glucose or arabinose. There was no significant difference in growth between strains carrying *crp* and *crp** in minimal medium supplemented with those two sugars. By contrast, CRP* helped in relief mannose-mediated CCR of propionate catabolic genes when induced during the mid log phase (Fig. 6A). CRP* also helped in the partial relief galactose- and xylose-mediated CCR of propionate catabolic genes (Fig. 6A). However, we observed impaired growth of strains carrying *crp** in minimal medium supplemented with mannose, galactose, or xylose as the sole carbon source. Mannose, galactose, and xylose also repressed *prpB* promoter activity in *crp**-containing strains when sugar and propionate were added to the cells in the early log phase.

P_{prpB} promoter activity was analyzed in a PTS-knockout strain. Deletion of the PTS system renders cells incapable of growth on mannose minimal medium and causes an impairment of growth in glucose minimal medium. As observed in the CRP* strain, ΔPTS strains with impaired growth in glucose and mannose showed less catabolite repression of propionate catabolic genes in the presence of these sugars than wild-type strains (Fig. 6B). These results indicate that the repression of *prpB* promoter activity is directly correlated with rapid sugar transport and indirectly correlated with cAMP levels.

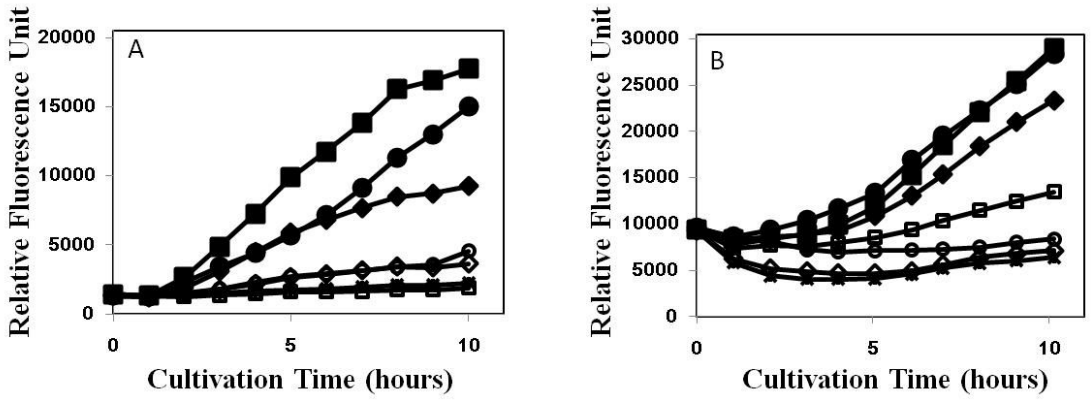


Figure 6. Comparison of *prpB* promoter activity in the absence of sugar (closed squares) or in the presence of different sugars such as arabinose (asterisks), galactose (open diamonds), glucose (open square), IPTG (closed circles), mannose (closed diamonds), and xylose (open circles) in the CRP* (A) and ΔPTS (B) strains.

2.4.3 Effect of the sugar-specific regulator AraC on the expression of propionate catabolic genes

We next asked whether sugar specific regulators such as AraC, Mlc, XylR, or GalS regulate the *prpB* promoter. Because all sugars tested in this study influenced the *prpB* promoter in a similar manner, we used arabinose as a proof-of-concept to determine the effect of different sugars on the propionate catabolic (*prp*) operon. It has also been reported recently that AraC is a key player involved in the repression of xylose metabolism [34]. AraC is a positive regulator of all three arabinose operons (*araBAD*, *araE*, and *araFGH*) in *E. coli*. Therefore, deletion of *araC* hampers their ability to transport and metabolize arabinose.

In order to investigate the effect of AraC on the regulation of propionate catabolism without disturbing arabinose metabolism, we constructed an *araC*-knockout mutant capable of metabolizing arabinose by deleting the *araC* gene and replacing the native AraC-regulated promoters of 2 different arabinose operons with synthetic constitutive promoters (CP25 for *araBAD* and CP6 for *araFGH*). The resulting strain, JARA1, was able to utilize arabinose albeit slowly in minimal media containing arabinose as a sole carbon source.

The engineered strain showed no repression of *prpB* promoter activity in the presence of arabinose (Fig. 9). Relief of catabolite repression of the propionate catabolic genes in the presence of arabinose in the JARA1 strain might be due to slow arabinose uptake, and hence, the JARA1 strain was adapted in minimal medium containing arabinose as the sole carbon source. Efficient AraC independent arabinose metabolism was achieved after 35 days of adaptation on arabinose as a sole carbon source. The adapted strain, was designated JARA35, was used for further study. Efficient utilization of arabinose by strain JARA35 was confirmed by measuring residual arabinose concentrations in the culture media (data not shown).

We tested the effect of AraC-independent arabinose metabolism on propionate catabolism using JARA35. Activity of the *prpB* promoter was reduced to background levels in the presence of arabinose even in strain JARA35 (Fig. 7). This result indicates that AraC may not be directly involved in arabinose-mediated catabolite repression of propionate catabolic genes.

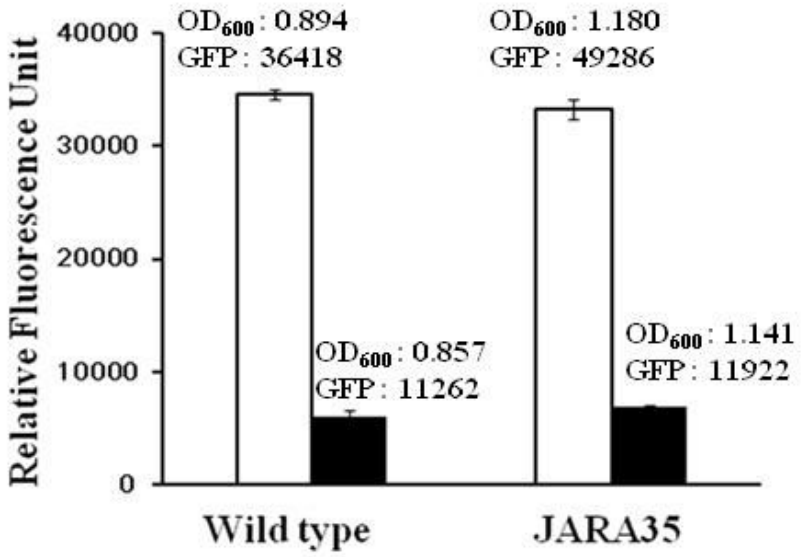


Figure 7. A comparison of *prpB* promoter activity in wild-type and JARA35 strains grown in the absence or the presence of arabinose (white and black, respectively). When the cell density reached an OD_{600} of 0.5, the culture was induced with 20 mM propionate, and 2 g/L of arabinose was then added. Promoter activity was measured after 10 h of growth in 96-well microplates. Error bars indicate the standard deviation of experiments performed in triplicate.

2.4.4 Effect of sugar metabolites on propionate metabolism

The effect of arabinose intermediates on propionate catabolism was tested using an *araA* null mutant. The *araA* null mutant JARA2 is capable of transporting arabinose but cannot metabolize the transported arabinose as it lacks L-arabinose isomerase, an enzyme that catalyzes the first step of arabinose metabolism.

Interestingly, *prpB* promoter activity was partially relieved from arabinose-mediated repression in JARA2 (Fig. 8). This result suggests that the arabinose transport process or the transported arabinose itself might be partly involved in arabinose-mediated catabolite repression of propionate catabolic genes. Rapid arabinose (sugar) metabolism causes a decrease in the phosphoenol pyruvate: pyruvate ratio bringing about dephosphorylation of $EIIA^{glu}$, which represses adenylate cyclase activity leading to decreased cAMP synthesis [35]. This is consistent with increased cAMP levels in a pyruvate kinase (*pykF*)-knockout mutant [36]. Therefore, the partial relief of *prp* operon repression in the presence of arabinose in JARA2 may be a result of limited cAMP availability. The impaired growth shown in JARA2 might be caused by transported arabinose that is toxic to the cell.

We hypothesize that sugar metabolites, as well as sugar transport, responsible for decreasing intracellular cAMP level might be key factors in the repression of propionate catabolic genes. To test this hypothesis, we followed *prpB* promoter activity in JARA1 that is capable of either transporting or metabolizing arabinose very slowly, JARA2 that is only capable of transporting arabinose, and JARA35 that is capable of both transporting and metabolizing arabinose (Fig. 9). The JARA1 strain showed no repression, the JARA2 strain exhibited partial repression, and the JARA35 strain showed strong repression of *prpB* promoter activity. Therefore, we conclude that the *prp* promoter is repressed by sugar transport that is indirectly related to intracellular cAMP levels.

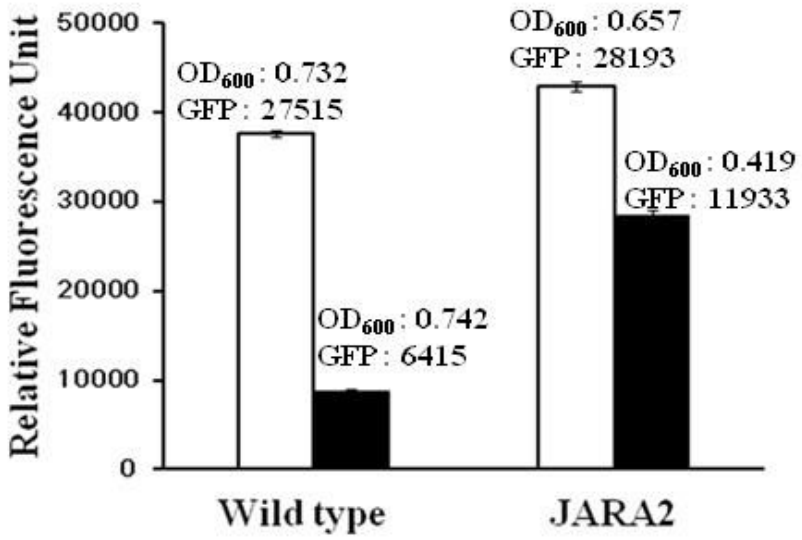
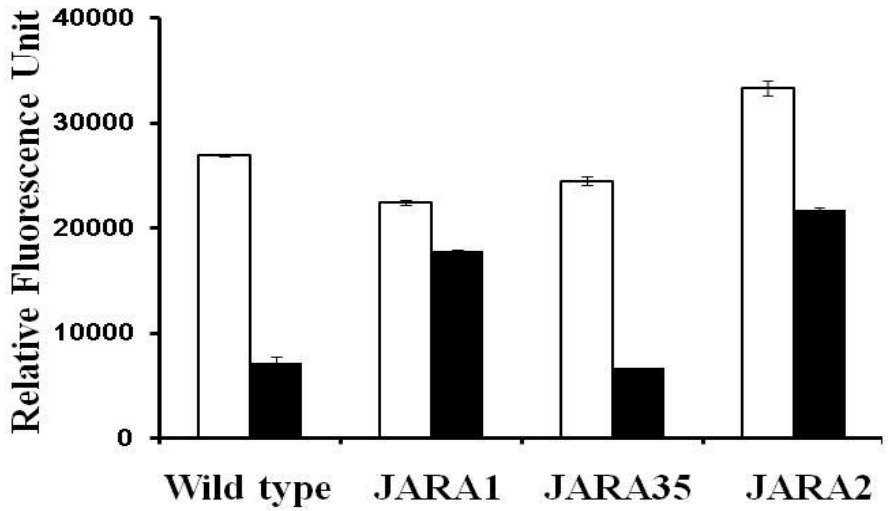


Figure 8. Comparison of *prpB* promoter activity in wild-type and JARA2 strains grown in the absence or the presence of arabinose (white and black, respectively). This experiment was performed using the same culture method as described in Fig. 7. Error bars indicate the standard deviation of experiments performed in triplicate.



	Wildtype		JARA1		JARA35		JARA2	
	Ara^-	Ara^+	Ara^-	Ara^+	Ara^-	Ara^+	Ara^-	Ara^+
OD_{600}	1.056	1.2	1.02	0.7	0.917	1.232	0.803	0.577
GFP	28401	8640	23040	13282	22478	8210	26755	12548

Figure 9. Comparison of the *prpB* promoter activity in the absence (white) or the presence (black) of arabinose in wild-type, JARA1, JARA2, and JARA35 strains. This experiment was performed using the same culture method as described in Fig. 7. Promoter activity was measured after 6 h of growth in 96-well microplates. Error bars indicate the standard deviation of experiments performed in triplicate.

2.5 Discussion

2.5.1 A third hierarchy of CCR

While a model for glucose effects via the cAMP-CRP complex is well established, the glucose transporter model provides an alternate explanation for CCR [37]. The regulatory mechanisms underlying CCR are more complex than previously expected. Repression of the maltose operon by glycerol-3-phosphate, an alcohol intermediate, has also been reported [38]. Methylglyoxal is believed to be a key factor responsible for CCR under high sugar concentrations [39,40,41]. More recently, arabinose-bound AraC has been proposed to regulate xylose metabolism in the presence of arabinose [34]. While all these models involve the transcriptional regulation of sugar operons, evidence exists for antitermination-mediated regulation of CCR as in the *bgl* operon of *E. coli* [42].

2.5.2 Role of CRP in propionate promoter activity

The *prpB* promoter was predicted to have a consensus CRP binding site [11]. A previous report indicates that the deletion of *crp* or *cya* results in a reduction of *prpB* promoter activity, implying a direct role of CRP in activating the *prp* operon [11,16]. Therefore, we hypothesized that reduced levels of the cAMP-CRP complex during sugar uptake may play a key role in the down-regulation of the *prpB* promoter. cAMP levels are reduced during sugar uptake and tend to increase only after sugar depletion [43]. Higher concentrations of sugar greatly reduce intracellular cAMP levels [44] and, hence, might be responsible for the repression of the *prp* operon. By contrast, when sugar concentrations are very low, cAMP levels may not be significantly affected and hence the *prp* operon is not repressed. This finding is consistent with previous results that implicate the cAMP-CRP complex, in addition to the *prp* operon regulatory protein PrpR, as a positive regulator of the *prpB* promoter [11,16,45,46].

2.5.3 Correlation between sugar uptake rate and cAMP concentration

Impairment of sugar uptake does not affect *prp* operon activity in the presence of sugar. For example, the *prp* operon is unaffected in the presence of arabinose in Δ*araC* strain that is incapable of arabinose uptake. The *prp* operon is also not affected in the presence of arabinose in JARA1 and 2 strains that exhibit impaired growth in arabinose minimal medium. Further, *prpB* promoter activity is not reduced in the *crp** strain in the presence of mannose, galactose, or xylose, because the CRP* strain exhibits impaired growth in minimal medium containing these sugars. The *prpB* promoter activity is not affected by mannose in *Δ*PTS strains that are incapable of growing on mannose minimal medium.

These lines of evidence are consistent with our hypothesis that repression of the *prp* operon is determined by the ability of the cell to uptake and metabolize a particular sugar. It is a well-known fact that the level of cAMP is greatly reduced during rapid sugar uptake and metabolism [16]. Impairment in sugar uptake directly correlates with high levels of cAMP that may be made available for the activation of the *prpB* promoter. Concentration of cAMP does not increase significantly even after complete depletion of a particular sugar [37].

However, the inability of CRP* (that is believed to work independent of cAMP) to completely relieve sugar-mediated catabolite repression of propionate catabolic genes suggests that a complex interplay of regulatory events control propionate catabolism. Consistent with this idea, *prpB* promoter activity is reduced in the *crp**-containing strain when compared to its activity in the wild-type strain. A CRP* mutant more specifically targeting the propionate promoter might help elucidate the direct involvement of cAMP in the repression of the propionate promoter.

2.5.4 A need for regulation of propionate catabolism

2-Methyl citrate, a breakdown product of propionate, has been previously characterized to be toxic to *Salmonella typhimurium* [47]. The toxicity of 2-methyl citrate may necessitate the tight regulation of the *prp* operon. Cells might also tightly regulate the *prp* operon in order to avoid the production of propionic/pyruvic or succinic acid from propionate. As a cell can derive all the basic components for growth from sugar, propionate, a secondary carbon source, might be subject to anaplerotic reactions leading to acid production that is detrimental to cell growth, providing a compelling reason for *E. coli*

to regulate the *prp* operon [48]. Although the exact molecular mechanisms underlying the inhibitor effect of sugars on propionate metabolism could not be established, we postulate that cAMP might be a major player in this complex regulation. However, we cannot ignore the fact that cAMP is not the sole player in this repression.

In summary, *prpB* promoter activity is completely downregulated to basal levels in the presence of about 2 g/L of different sugars such as arabinose, galactose, glucose, lactose, mannose, and xylose. We propose a third hierarchy of CCR in *E. coli* between sugar and fatty acid metabolism. However, the mechanistic underpinnings of CCR appear more complex than expected. Rapid transport and metabolism of sugars is necessary for this repression. We assume that a change in cAMP levels during the transport and metabolism of sugar might be a major determinant of the repression of the *prpB* operon.

Chapter 3

Engineered *Escherichia coli* capable of co-utilization of glucose and xylose

3.1 Summary

The presence of glucose inhibits xylose utilization in *E. coli* and decreases yield and productivity of the major sugar fermentation due to sequential utilization of xylose after glucose. As an approach to overcome this drawback, *E. coli* MG1655 was engineered for co-utilization of glucose and xylose by replacing the native promoters of xylose catabolic genes (*xylAB*) and xylose transporter genes (*xylFGH*) with synthetic constitutive promoters, CP25 and CP6, respectively. The engineered strain (XYL) grew very slow in xylose-containing minimal medium. Evolutionary adaptation of the strain by repeated subculture in xylose-containing minimal medium led to an increase in the rate of cell growth on xylose. The adapted *E. coli* (XYL1) continuously expressed xylose metabolic genes even in the presence of glucose and/or arabinose. However, glucose still inhibited xylose utilization in the engineered strain, indicating that the engineered xylose metabolic pathway is subject to CCR by the glucose.

3.2 Introduction

Even though xylose is the most abundant pentose sugar in lignocellulosic hydrolysates, it cannot be used efficiently for the production of value-added building block chemicals or bio-fuels from biomass refining because of the carbon catabolite repression (CCR). To avoid this problem, there were many studies to co-metabolize glucose and xylose which are main composition of lignocellulose through relief of CCR in *E. coli*. Many researches related with both PTS system and CRP protein which are considered as a main factor of CCR have been carried out [26,33,49,50]. In relation with the studies about the PTS system, it was verified that *E. coli* IT1168 (W3110, *ptsG*::Tn5) [26] and FBR14 (W3110, *ldhA*::Kn, Δ*pfl*::Cm, *zce726*::Tn10 *ptsG*$^{+}$21 and *pdc*$^{+}$/*adhB*$^{+}$[pLOI297]) [26] can partially co-metabolize arabinose and xylose with glucose, indicating that inactivation of *ptsG* helps overcome CCR. Meanwhile, *E. coli* carrying a deletion of *ptsH, ptsI* and *crr* genes exhibited a pleoitropic effect due to inadequate PTS and could not grow on both PTS and non-PTS sugars [33,49]. However, this phenotype

(PTS$^-$ Glucose$^-$) was restored depending on the activation of GalP, which can transport glucose nonspecifically, and the over-expression of glucokinase gene, which can phosphorylate glucose transported in the cell [33,49]. Such PTS$^-$ Glucose$^+$ mutants utilized arabinose and glucose simultaneously and showed partial repression of xylose utilization when grown with glucose and xylose [33]. Recently, the *E. coli* W3110 PTS$^-$ Glucose$^-$ strain, VH30 (W3110 Δ*ptsH*, *ptsI*,*crr*::*kan*^{R}, Glucose$^-$), was evolved in minimal media with glucose as the sole carbon source under anaerobic conditions to obtain glucose$^+$ phenotype [50]. The resulting strains, designated as VH30N2 (PTS$^-$Glucose$^+$ derivative of VH30 selected by an anaerobic continuous culture method) and VH30N4 (PTS$^-$Glucose$^+$ derivative of VH30 selected by an anaerobic continuous culture method) consumed xylose and glucose in a simultaneous manner when cultured in glucose and xylose mixture, indicating that catabolite repression is relieved in these strains [50]. Such results indicate that the inactivation of the PTS system helps release CCR.

CRP protein can stimulate transcription of sugar catabolic genes and transporter genes only with cAMP. Therefore, a series of CRP mutants (*crp**) were isolated from strains lacking adenylate cyclase in order to allow *E. coli* to have an apparent reduced dependence on cAMP for activating catabolic genes [37,51,52,53]. The *crp** mutant strain PC05 (W3110, *crp**::Tn*10* (Tet^R) showed increased xylose uptake in the presence of glucose [14]. Hankal et al. found that in this PC05 strain, the expression of genes involved in sugar transport and catabolism was increased [52]. However, despite the increase in the expression of catabolic genes, they still exhibited glucose effect. The *crp** mutant also gives us hope that it is potentially possible to release CCR with further metabolic engineering strategies.

Previous studies have focused on the manipulation of these global regulatory systems. However, such approaches cause another problem such as inhibition of glucose utilization caused by inactivation of *ptsG* and thus could not allow *E. coli* to co-utilize xylose and glucose efficiently [54]. Further, recently, it was verified that arabinose represses xylose metabolism through AraC which is arabinose operon specific regulator protein [34]. To date, there were a number of strategies to remove the glucose-mediated repression on xylose metabolism while arabinose-mediated repression of that was ignored. In this study, we focused on the continuous gene expression of both xylose catabolic genes and transporter genes without any regulatory interference by glucose and arabinose by replacing the native promoters of xylose catabolic genes (*xylAB*) and xylose transporter genes (*xylFGH*) with synthetic constitutive promoters, CP25 and CP6, respectively.

3.3 Materials and Methods

3.3.1 Bacterial strains and media

The strains used are listed in Table 3. *E. coli* MG1655 was used as a parental strain in this study. Strain construction were made using Luria broth containing antibiotics as appropriate (kanamycin at 50 μg/mL or ampicillin at 100 μg/mL or chloramphenicol at 30 μg/mL). Temperature-conditional plasmids were grown at 30°C. All others were grown at 37°C. Except during constructions, engineered strains were cultured in minimal medium supplemented with suitable sugars.

Table 3. *E. coli* strains and plasmids used in this work

Strains/plasmids	Description/genotype	Reference/source
Strains		
E. coli MG1655	Wild-type	[27]
XYL	MG1655 with P_{CP25}-*xylA,* P_{CP6}-*xylF*	This study
XYL1	XYL adapted for 20 days	This study
XYL2	XYL1 with *crp*::*crp** (I121L,T127L)	This study
Plasmids		
pSIM5	λ-Red recombinase expression plasmid and temperature sensitive replication	[28]
pCP20	Yeast FLP recombinase gene controlled by *c*I repressor and temperature sensitive replication.	[29]
pKD13	Template plasmid for gene disruption. The resistance gene is flanked by FRT sites. *oriR6K*-gamma origin requiring the *pir* + *E. coli*.	[28]

3.3.2 Strain construction

Promoter replacements and gene deletions were performed using the λ red system [26, 27]. For promoter replacement, two synthetic constitutive promoters (CP6 and CP25) with a ß-galactosidase activities of 280, and 528 Miller units in *E. coli*, respectively, were used (Table 4) [28].
XYL was constructed by replacing the native promoter of *xylAB* and *xylFGH* with synthetic constitutive promoters, CP25 and CP6, respectively. The engineered strain was adapted in xylose minimal media for 20 days by sub-culturing cells to fresh medium every time the culture reached an OD_{600} of 1.0. The resulting strain was designated as XYL1.

To construct XYL2 carrying a cAMP-independent CRP (encoded by *crp**), the native *crp* gene was first deleted and was then replaced with the *crp** gene (CRP with I112L, T127I) constructed by splice overlap extension (SOE)-PCR [29]. Strains carrying the mutant *crp* were easily screened based on their faster growth rate compared with the *crp* knock-out strains. All engineered strains were verified by the phenotype and sequence analysis. The primers used for strain construction are listed in Table 4.

Table 4. Primers used in this study

Strain construction	Primer sequence
SOEing CP6 promoter to Kanamycin cassette	
	5′-CAT AGC TGT TTC CTG TGT GAA CAG TAC TCA GTT ATT ATA TCA TCC GG-3′
	5′-TCA GTT ATT ATA TCA TCC GGA AAT ATC TGT GTC AAG AAT AAA CTC C-3′
	5′-CTG TGT CAA GAA TAA ACT CCC ACA TGA TTC CGG GGA TCC GTC GAC C-3′
SOEing CP25 promoter to Kanamycin cassette	
	5′-CAT AGC TGT TTC CTG TGT GAA CAG TAC TAT GTG ATT ATA CCA GCC CCC-3′
	5′-ATG TGA TTA TAC CAG CCC CCT CAC TAC ATG TCA AGA ATA AAC TGC-3′
	5′-ACA TGT CAA GAA TAA ACT GCC AAA GAT TCC GGG GAT CCG TCG ACC-3′
Promoter exchange	
P_{CP25}-*xylA*	5'-TGA GCC TTC ATA ACG AAC GCG ATC GAG CTG GTC AAA ATA GGC TTG CAT AGC TGT TTC CTG TGT GAA-3'
	5'-TTG TTG CGC AAT TGT ACT TAT TGC ATT TTT CTC TTC GAG GAA TTA CCC AGT TTC ATC AAT TCC GGG GAT CCG TCG ACC-3'
P_{CP6}-*xylF*	5'-TGA TGA AAC TGG GTA ATT CCT CGA AGA GAA AAA TGC AAT AAG TAC AAT TGC GCA ACA AGT GTA GGC TGG AGC TGC TTC G-3'
	5'-AAG CAG GAG TGA GGT GCA AAG GGT GAG TAG AAT GTT CTT TAT TTT CAT AGC TGT TTC CTG TGT GAA C-3'
Gene deletion/insertion	
crp deletion	5′-CTA CCA GGT AAC GCG CCA CTC CGA CGG GAT TAA CGA GTG CCG TAA ACG ACG TGT AGG CTG GAG CTG CTT CG-3′
	5′-GGC GTT ATC TGG CTC TGG AGA AAG CTT ATA ACA GAG GAT AAC CGC GCA TGA TTC CGG GGA TCC GTC GAC C-3′
*crp** insertion	5′-GGC GTT ATC TGG CTC TGG AGA AAG CTT ATA ACA GAG GAT AAC CGC GCA TGG TGC TTG GCA AAC CGC AAA CAG ACC CG-3′
	5′-ACT TGC AGA CGA CGC GCC ATC TGT GCA GAC-3′
	5′-CTT CTG ATG CGT TTG TCT GCA CAG ATG GCG CGT CGT CTG CAA GTC ATT TCA GAG AAA GTG GGC AAC CTG GCG TTC CTC GAC GTG ACG GGC CGC ATT ACA-3′
	5′-ATT CCG GGG ATC CGT CGA CCT TAA CGA GTG CCG TAA ACG ACG ATG GTT TTA CCG TGT GCG GAG-3′
RT-PCR	
xylA-FP	5′- ATG GCG GTG GCT ATT TCA TG -3′
xylA-RP	5′-TTA TTT GTC GAA CAG ATA ATG G-3′
xylF-FP	5′- GCT TAC GAC CGT ATG ATT AAC G-3′
xylF-RP	5′- ATT TCT GCG GCA GTA TTT GC-3′

3.3.3 Batch culture

500 µL overnight grown cells in LB were inoculated (1:100) into 50 mL of M9 media supplemented with 2 mM $MgSO_4$, 0.1 mM $CaCl_2$ and suitable concentration of test sugars (xylose, glucose, arabinose) in 250-mL flask and grown at 37°C in a shaking incubator. Cell density was obtained by measuring the OD_{600} using a Biochrom Libra S22 spectrophotometer. Residual sugar was analyzed using high-pressure liquid chromatography (HPLC).

3.3.4 HPLC analysis

The amount of residual sugars in the medium was measured with a Shimadzu HPLC station equipped with a refractive index detector (Shimadzu) and a SIL-20A autosampler (Shimadzu). A Bio-Rad Anemix 300 mm x 7.8 mm HPX-87P column was used to separate the sugars, as per manufacturer's recommendations. The column was run at 0.6 mL/min at 80°C with water as the mobile phase. For sampling, 500 µL of overnight grown cells was inoculated into 50 mL of fresh LB medium supplemented with appropriate concentrations of test sugars in 250-mL flask and grown at 37°C in a shaking incubator. 1 ml of grown cultures was collected every 2 hours, centrifuged to remove cells and then boiled and filtered to remove protein precipitate particles. The samples were transferred in auto-injector vials and were loaded in to auto sampler. Samples were injected in a volume of 50 µL using a SIL-20A autosampler (Shimadzu).

3.3.5 RNA isolation

Wild type *E. coli* MG1655 and XYL1 overnight grown in LB were inoculated into 5 mL of M9 media with a ratio of 1:100. All media were supplemented with 2 g/L glucose and 2 g/L D-xylose. 500 µL cells were harvested from the cultures reaching mid-logarithmic growth after 5 hours and the total RNA was extracted by using a commercial Qiagene RNeasy minikit according to the manufacturer's protocol.

3.3.6 Reverse transcription polymerase chain reaction (RT-PCR)

Before performing RT-PCR assay, precipitated RNA was treated with DNase I at 37°C for 1hour, in order to remove DNA contamination. The DNase I was inactivated by adding EDTA (final 5 mM) and incubation at 65°C for 10min. This RNA preparation was used as a template for RT-PCR. cDNA synthesis and amplification was performed at once by using Bioneer AccuPower RT/PCR Premix kit. Primers were designed to give 500bp PCR products from *xylA* or/and *xylF* genes, respectively, as listed in Table4.

3.4 Results

3.4.1 Strain construction and characterization

Glucose inhibits xylose metabolism by reducing intracellular cAMP-CRP complex availability required for the transcription of xylose catabolic operon (*xylAB*) and transporter (*xylFGH*)[15,55] , while arabinose represses the transcription of xylose catabolic operon (*xylAB*) through AraC, which is an arabinose operon specific regulatory protein [56].

Therefore, for continuous xylose metabolism, which is repressed by AraC and cAMP-CRP at the transcriptional level in *E. coli*, we replaced the native promoters of two different xylose operons, which are *xylAB* for xylose metabolism and *xylFGH* for xylose transport, with synthetic constitutive promoters, CP25 and CP6, respectively.

However, the resulting strain, XYL showed severely slow growth rate in minimal media containing xylose as a sole carbon (Fig. 10). Therefore, in order to restore the growth on xylose media, the engineered strain was evolved in minimal media containing 2g/L D-xylose as the sole carbon source. After 20 days of adaptation the adapted strain, designated as XYL1, showed efficient xylose metabolism much similar to that of the wild type (Fig. 10).

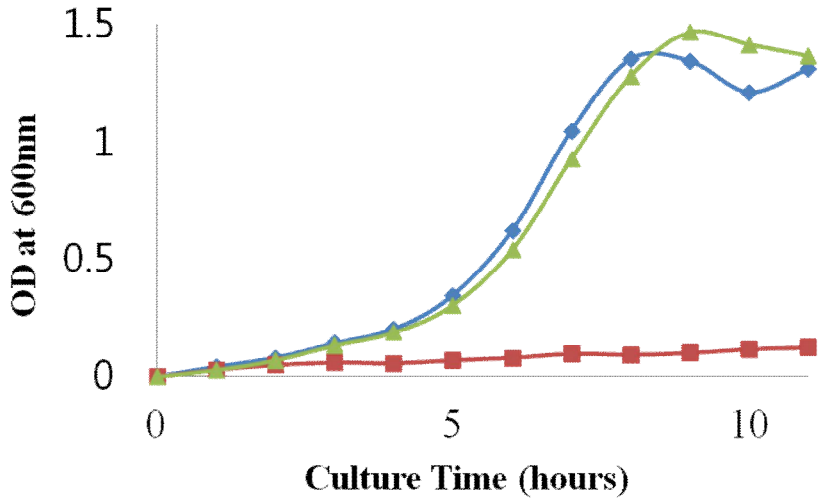


Figure 10. Comparative growth of wild type, XYL and XYL1 in xylose (2g/L) containing minimal media at 37°C. XYL showed much lower growth rate than wild type. After adaptation in minimal media supplemented with 2g/L D-xylose as a carbon source for 20 days, the resulting strain, XYL1 restored its growth rate in minimal media with 2g/L D-xylose as much as wild type. Blue line (wild type), Red line (XYL), Green line (XYL1)

3.4.2 The effect of *xylA* and *xylF* promoter replacement with the synthetic constitutive promoters on the expression of the xylose metabolic genes in the presence of glucose

Using RT-PCR, we tested whether xylose operons in XYL1 are expressed constitutively even in the presence of glucose. As a result, XYL1 exhibited continuous transcription of both genes of *xylA* and *xylF* even in the presence of glucose whereas wild type didn't transcribe *xylA* and *xylF* at all in the same culture condition (Fig. 11). Therefore, XYL1 was used for further study.

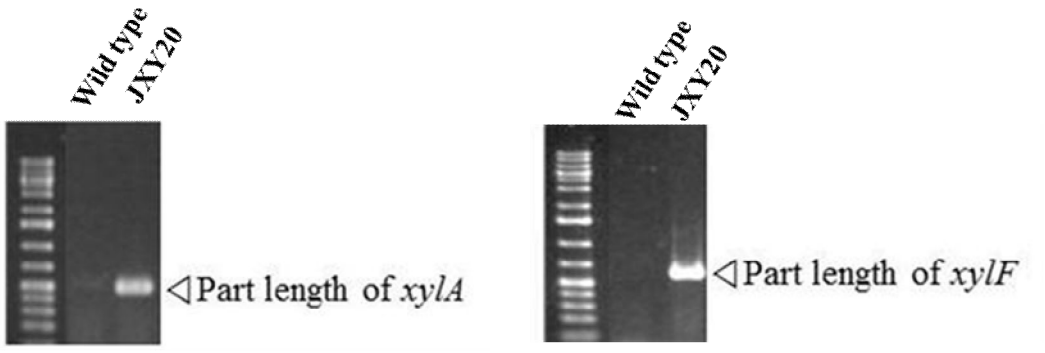


Figure 11. Comparative mRNA expression of both *xylA* and *xylF* in respective wild type and XYL1 grown in minimal media supplemented with glucose and D-xylose. Both *xylA* and *xylF* genes were continuously transcribed in XYL1 regardless of the presence of glucose, whereas wild type didn't express them at all in the presence of glucose.

3.4.3 Xylose utilization in the presence of glucose in XYL1

Next we tested whether the strain XYL1 obtain the capacity to co-metabolize glucose and xylose using HPLC. XYL1 and the wild type strain MG1655, were grown in minimal medium supplemented with 2g/L each of glucose and D-xylose. Supernatants were analyzed at various time points to ascertain their sugar utilization. From this experiment, we found that XYL1 still showed the repression of xylose metabolism in the presence of glucose even though two different xylose operons, *xylAB* and *xylFGH,* in the XYL1 are under the control of constitutive promoters, respectively (Fig. 12A, 12B). XYL1 presented faster xylose consumption rate after deletion of glucose as compared with wild type, indicating that glucose effect was slightly released.

In order to increase the xylose uptake rate through non-specific xylose transporters, we replaced the *crp* gene with *crp** gene (I121L, T127L) encoding cAMP independent CRP* protein in XYL1. It has been reported that *crp** increase the xylose uptake rate by activating an unknown non-specific xylose transporter in addition to the xylose specific transporters which are *xylE*, and *xylFGH* [57]. Since xylose uptake through the non-specific xylose transporter is considerable (up to 40%) when xylose concentration is high [57], we supposed that the expression CRP* protein in XYL1 might help relief of CCR on xylose metabolism by glucose.

Against our expectations, however, the CPR* phenotype didn't have any effect on relief of CCR on xylose metabolism by glucose (Fig. 12C). Based on this result, we found that the constitutive transcription of xylose catabolic genes and transporter genes helps release glucose effect slightly by increasing the rate of xylose consumption after deletion of glucose, but still exhibits strong catabolite repression. Therefore, we concluded that there is another factor causing the repression of xylose metabolism by glucose beyond transcriptional level in addition to the transcriptional level as assumed previously[58].

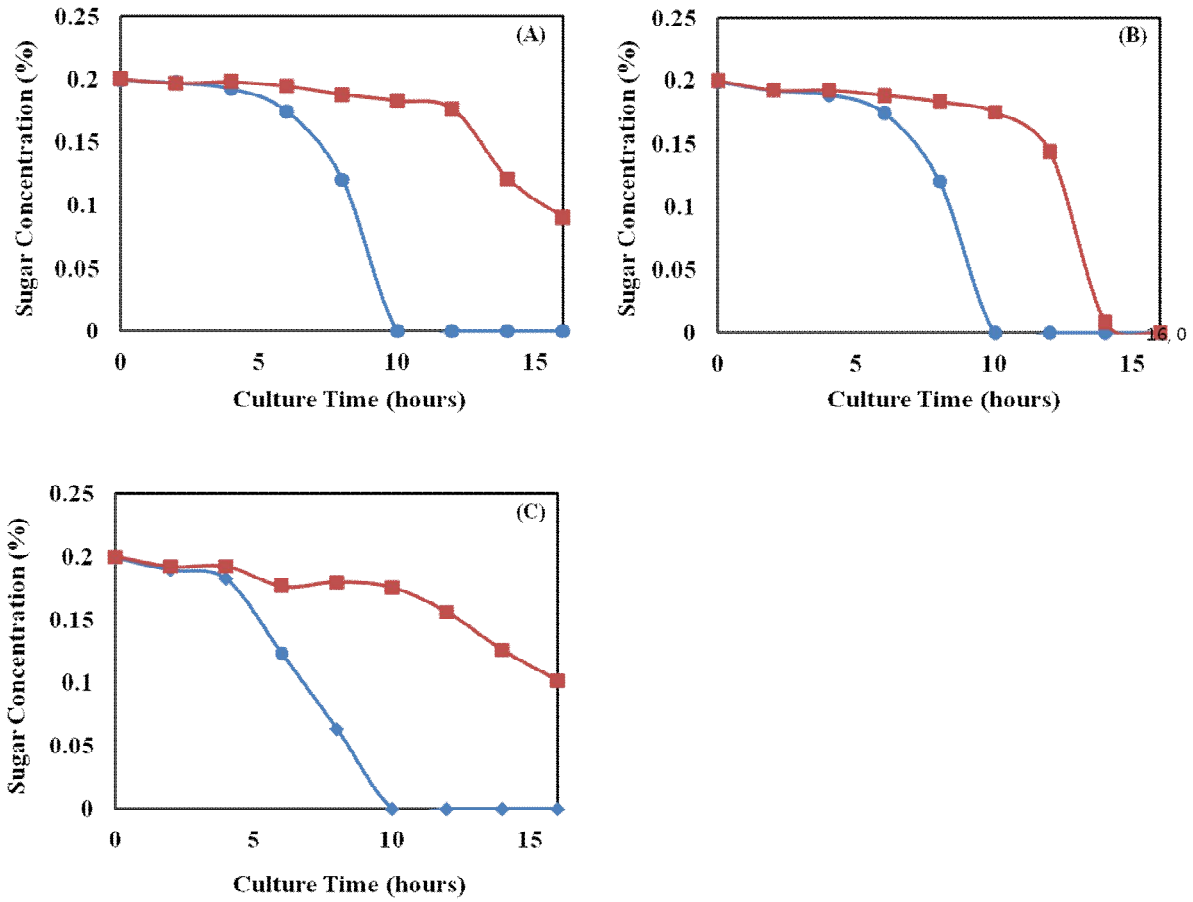


Figure 12. Comparison of glucose and xylose utilization in (A) Wild type, (B) XYL1 and (C) XYL2. Blue line represents the concentration of glucose while red line represents the concentration of xylose.

3.4.4 Xylose utilization in the presence of both glucose and arabinose in XYL1

We measured the xylose utilization pattern in XYL1 in the presence of both arabinose and glucose since lignocellulose contains both sugars. In the same manner with previous method, sugar utilization was analyzed in minimal media supplemented with 2 g/L each of D-xylose, L-arabinose and glucose in wild type and XYL1. Wild type was shown to utilize glucose, arabinose and xylose sequentially as already known (Fig. 13A). Interestingly, XYL1consumed the sugars inside the media in order of glucose, xylose and arabinose (Fig. 13B).

Taken together, we found that the glucose effect on xylose metabolism in XYL1was slightly released and the inhibition of xylose metabolism by arabinose was also reduced in the presence of both arabinose and glucose. This result also supported that catabolite repression might be controlled at the post-transcriptional level as shown in Figure 12. Therefore, to eliminate catabolite repression, further studies on post-transcriptional regulation on xylose metabolism by glucose should be needed.

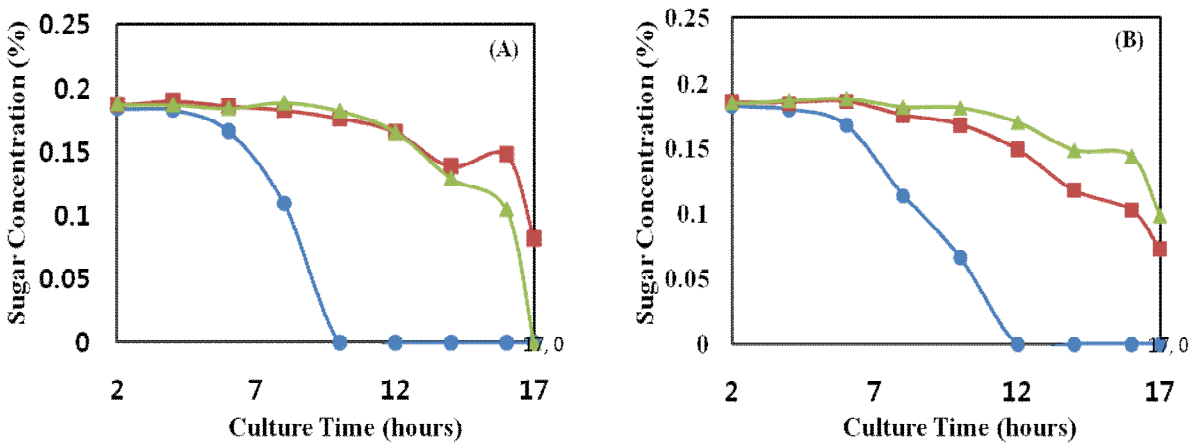


Figure 13. Sugar utilization pattern in wild type (A), XYL1 (B) in minimal media supplemented with 2g/L L-arabinose, 2g/L D-xylose and glucose. Blue line (glucose concentration), Red line (xylose concentration), Green line (arabinose concentration)

3.5 Discussion

The aim of the current work was to construct a CCR de-repressed strain capable of co-metabolizing glucose and xylose through continuous expression of the genes for xylose catabolism regardless of the presence of both glucose and arabinose in order to increase the yield of the bio-fuels or bio-chemicals. Previous studies have focused on the elimination of factors causing CCR such as *crp*, *mlc*, *ptsG*, *pgi*, and *mgsA* gene which are involved in the transcriptional catabolite regulation mechanism [54]. However, even though these corresponding gene knockout mutants showed release of CCR, the glucose uptake rate became much lower as compared to the uptake rate of the wild type due to the inactivation of *ptsG* in all the mutants considered[54]. Further, previous studies ignored arabinose mediated CCR on xylose metabolism. Therefore, continuous gene expression involved in xylose catabolism might be useful for efficient co-metabolism of xylose and glucose without any inhibition of glucose metabolism and arabinose mediated regulatory interference.

To do this, we replaced the native promoter of two different xylose operons, which are *xylAB* for xylose catabolism and *xylFGH* for xylose transporter. However, the resulting strain XYL showed impaired growth on xylose media. For efficient xylose utilization, XYL was adapted for 20days in xylose containing minimal media using the sub-culturing method. As a result the adapted strain, XYL1, obtained the efficient xylose utilization similar to the wild type. Such long-term evolution experiments change their gene expression to allow *E. coli* to adjust the new conditions [59]. In other words, it makes rigorous connections between genetic changes and phenotypic outcomes in bacterial cells [60]. Therefore, it is a most useful tool for gene engineering due to short time to experiment as well as simple experimental procedures. However, the evolutionary dynamics of regulatory systems are largely unexplored.

Meanwhile, XYL1 obtained from such evolution experiment still showed strong glucose effect even though XYL1 showed continuous expression of xylose operons. In addition, XYL2(CRP with I121Land T127I) didn't show the relief of CCR even though a previous study showed the CRP* (I121L, T127I and A144T) increase the xylose uptake rate through xylose non-specific transporters [57]. It indicates that genetically different *crp** exhibited different relieving catabolic repression of select genes examined to different extent [18,52]. Therefore, the construction of *crp** sensitive to xylose metabolism will be the subject of a future study. Strong glucose effect investigated in XYL1 demonstrated that the xylose metabolic gene expressions are repressed beyond transcription level in the presence of glucose. Our results clearly demonstrate that CCR is not just at the transcriptional

level as previously assumed, but that different sugar transport and catabolism genes show complex transcriptional responses to glucose and their respective carbon sources [58].

It has been already known that the expression of *ptsG* enconding $EIIBC^{Glc}$, the membrane-specific component of the glucose Phosphotransferase system (PTS), is also controlled post-transcriptionally in response to phosphor-sugar stress[61]. More recently, it has been reported that base-pairing RNA Spot42 plays a broad role in catabolite repression in *E. coli* [62]. Spot42 sRNA is abundant in the presence of glucose and hence it represses numerous transport and catabolism genes at the post-transcriptional level in the presence of glucose.

Further, CRP represses Spot42 and thus these regulators accelerate gene repression when the preferred carbon source appears, and delay gene activation when the preferred carbon source disappears [62]. It was also identified that one of the genes regulated by Spot42 is *xylF*. This might be one of the reasons why XYL1 still showed the strong glucose effect even continuous transcription of xylose operons. Therefore, manipulation of the *spf* gene encoding Spot42 might help continuous gene expression for xylose metabolism. Thus, study at the diverse expression level is required for the continuous xylose metabolism.

In summary, we proposed a new approach to release CCR which focused on the continuous gene expression of xylose specific operons without any other regulatory interference in lignocellulose sugar mixtures whereas previous study focused on the manipulation of the regulatory proteins causing CCR. We allowed *E. coli* to express the genes involved in xylose metabolism continuously by replacing the native promoters of *xylAB* and *xylFGH* with synthetic constitutive promoters. The XYL1 strain slightly released glucose effect and was free of the repression by arabinose in the mixture of arabinose, xylose and glucose. This engineered strain is still far away from industrial biofuel production. However, to do this, xylose consumption rate in XYL1should be increased accompanied by the diverse studies about the regulation of gene expression.

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