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STUDYING AND IMPROVING XYLOSE UPTAKE AND UTILIZATION IN *ESCHERICHIA COLI*

A Dissertation in

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by

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ABSTRACT

Efficient microbial conversion of biomass into renewable fuels and value-added chemicals remains an important goal in biotechnology. Substrate cost is still a major factor in bioproduction of fuel and chemicals. Utilization of lower-valued substrates such as lignocellulose can reduce the cost of bioprocesses thereby enabling them to compete with traditional chemical synthesis. Xylose, which is the second most abundant sugar in nature and a major constituent of hemicellulose in lignocellulosic biomass and wastes, can play an important role in this contest. *Escherichia coli* is capable of utilizing a wide range of sugars as carbon and energy sources and producing native metabolites and non-native compounds. In *E. coli*, xylose uptake occurs primarily through a high-affinity, ATP-binding cassette transporter (XylFGH), although a second, low-affinity proton symporter (XylE) is also present. The efficiency of xylose utilization in this organism is therefore suboptimal due to energetic requirements for xylose uptake.

Escherichia coli W3110 was previously engineered to co-utilize glucose and xylose by replacing the wild-type *crp* gene with a *crp** mutant encoding a cAMP-independent CRP variant. Subsequent deletion of the *xylB* gene (encoding xylulokinase) and expression of xylose reductase from *Candida boidinii* (CbXR) resulted in a strain which produces xylitol from glucose-xylose mixtures. Our results show that although xylose is negligibly metabolized by wild-type *E. coli* W3110 in the presence of glucose (classic diauxic growth), xylose transport and xylitol production do occur in the presence of glucose in wild-type *E. coli* expressing XR, and xylitol production is significantly improved in a mutant strain expressing cAMP-independent CRP (CRP*). These results

indicate that either the native xylose transporters are not tightly controlled by CRP or additional transport mechanisms exist. Finally, growth on xylose by *E. coli* W3110 strains with deletions in both native xylose transporters is recovered to nearly that of wild-type using high concentrations of xylose (~100 mM), demonstrating that xylose is transported by at least one other, low-affinity system.

Accurate modeling of xylose metabolism in *E. coli* in the presence of high concentrations of xylose requires a more thorough understanding of xylose uptake mechanisms and their energy requirements. Conversion of xylose to xylitol (which is secreted) provides a measure of xylose transport without requiring xylose metabolism. This system allows studies of how different native and heterologous transporters (e.g., deleted or overexpressed) influence xylose uptake under various conditions (e.g. in the presence of glucose) and not coupled to growth or expression of genes specific to xylose metabolism.

We have examined the contributions of the native *E. coli* xylose transporters (the D-xylose/proton symporter XylE and the D-xylose ABC transporter XylFGH) and CRP* to xylitol production in the presence of glucose and xylose. The final batch xylitol titer with strain PC09 (Δ*xylB* and *crp**) is reduced by 40% upon deletion of *xylG* and by 60% upon deletion of both *xyl* transporters. This shows that, up to 40% of xylose transport occurs via secondary transport. This uptake is apparently not affected by individual deletions in other transporters known to be promiscuous. Xylitol production by the wild-type strain (W3110) expressing CbXR is not reduced when *xylE* and *xylG* are deleted, demonstrating tight regulation of the xylose transporters by CRP and revealing significant secondary

xylose transport. Finally, plasmid expression of XylE or XylFGH with CbXR in PC07 (Δ*xylB* and wild-type *crp*) growing on glucose results in xylitol titers similar to that achieved with PC09 and provides an alternative strategy to the use of CRP*. Studies that correlate anaerobic growth of engineered strains on xylose to energy yield indicate that secondary xylose transport is largely energy dependent (not diffusion).

E. coli K-12 strains W3110 and MG1655 and wild-type *E. coli* B were compared as platforms for xylitol production from glucose-xylose mixtures using either plasmid-based expression of a xylose transporter (XylE or XylFGH) or replacing the native *crp* gene with a mutant (*crp**). The engineered strains were compared in fed-batch fermentations and as non-growing resting cells. Expression of CRP* in the *E. coli* B strains tested was unable to enhance xylose uptake in the presence of glucose. Xylitol production was similar for the (*crp**, Δ*xylB*)-derivatives of W3110 and MG1655 expressing CbXR (average specific productivities of ~0.43 g xylitol g cdw^{-1} h^{-1} in fed-batch fermentation). In contrast, results varied substantially between different Δ*xylB*-derivative strains coexpressing either XylE or XylFGH. Thus, the differences in genetic background between these strains can profoundly influence metabolic engineering strategies.

While previous studies have examined the effects of expressing CRP* mutants on the expression of specific catabolic genes, little is known about the global transcriptional effects of CRP* expression. We also compare the transcriptome of *E. coli* W3110 (expressing wild-type CRP) to that of mutant strain PC05 (expressing CRP*) in the presence and absence of glucose.

While the simplest model of CRP*-mediated gene expression assumes insensitivity to glucose (or cAMP), our results show that gene expression in the context of CRP* is very different from that of wild-type in the absence of glucose, and is influenced by the presence of glucose. 238 genes were found to respond differently to glucose in PC05 compared to W3110. Genes whose expression is significantly altered by glucose in strain W3110 are generally not altered to the same extent in strain PCO5 and the glucose effect is significantly suppressed in this strain. We present a detailed transcription analysis and relate these results to phenotypic differences between strains expressing wild-type CRP and CRP*. Notably, CRP* expression in the presence of glucose, results in an elevated intracellular NADPH concentration and reduced NADH concentration relative to wild-type CRP. Meanwhile, a more drastic decrease in the NADPH/NADP$^+$ ratio is observed for the case of CRP* expression in strains engineered to reduce xylose to xylitol via NADPH-dependent xylose reductase during glucose metabolism.

TABLE OF CONTENTS

LIST OF FIGURES x

LIST OF TABLES xiii

ACKNOWLEDGEMENTS xv

Chapter 1 Introduction 1

1.1 Hemicellulose and xylose 1
1.2 Biotechnology 3
1.3 Xylitol 4
1.4 Xylose uptake in *E. coli* 6
1.5 Energetics of xylose uptake and xylitol production 9
1.6 References 10

Chapter 2 Bioproduction of sugar alcohols (xylitol and sorbitol) 13

2.1 Summary 13
2.2 Introduction 13
2.3 Xylitol 15
2.4 Sorbitol 21
2.5 Conclusions 23
2.6 References 25

Chapter 3 Role of xylose transporters in xylitol production from engineered *Escherichia coli* 28

3.1 Abstract 28
3.2 Introduction 29
3.3 Materials and methods 33
3.3.1 General 33
3.3.2 Genetic methods 34
3.3.3 Shake-flask cultures 38
3.3.4 Bioreactor cultivations 38
3.4 Results 39
3.4.1 Influence of *xyl* transporter deletions on xylitol production 39
3.4.2 Overexpression of Xyl transporters 43
3.5 Discussion 48
3.6 Reference 52

Chapter 4 Comparison between *Escherichia coli* K-12 strains W3110 and MG1655 and wild-type *E. coli* B as platforms for xylitol production 56

4.1 Abstract....56
4.2 Introduction....57
4.3 Materials and methods....59
4.3.1 General....59
4.3.2 Genetic methods....60
4.3.3 Shake-flask cultures....62
4.3.4 Fermentations....63
4.3.5 Resting cells....64
4.4 Results and discussion....65
4.4.1 Expression of CRP*....65
4.4.2 Expression of xylose transporters....71
4.4.3 Resting cell comparison....73
4.5 Conclusions....74
4.6 References....75

Chapter 5 Transcriptional effects of CRP* expression in *Escherichia coli*....78

5.1 Abstract....78
5.2 Introduction....79
5.3 Materials and methods....82
5.3.1 General....82
5.3.2 Growth conditions....83
5.3.3 Cell harvesting and preparation of RNA....84
5.3.4 Labeling, hybridization and scanning....84
5.3.5 Microarray data analysis....85
5.3.6 Real-time, Reverse Transcription PCR....86
5.3.7 Cofactor measurements....87
5.4 Results....89
5.4.1 Genome-wide transcriptional effects of glucose and CRP*....90
5.4.2 Real-Time Reverse Transcription PCR....100
5.5 Discussion....101
5.5.1 Cofactor analysis....103
5.5.2 Expression of “unnecessary” genes....106
5.5.3 Catabolite repression and inducer exclusion....107
5.6 Conclusions....108
5.7 References....108

Appendix A Strains, plasmids and primers....113

A.1 Strains....113
A.2 Plasmids....117
A.3 Primers....118

Appendix B Methods and protocols....120

B.1 Preparation of P1 lysate 120
B.2 P1 Transduction 121
B.3 Spot titer the lysate 122
B.4 Screening for constitutive sugar utilization using triphenyl tetrazolium chloride (TPTC) 122

Appendix C Microarray statistical analysis 124

C.1 Written code in R for pair-wise comparisons: 124

LIST OF FIGURES

Figure **1-1**: Xylose transport systems in *E. coli.* XylE is a D-xylose proton symporter which uses the proton gradient as a source of energy. XylFGH belongs to the ATP binding cassette ("ABC") family of transporters and requires one ATP per D-xylose transported. 8

Figure **1-2**: Overview of xylose uptake and xylitol production in *E. coli* (Cirino et al., 2006) 10

Figure **2-1**: Pathway for xylose metabolism in yeast. XR, xylose reductase; xDH, xylitol dehydrogenase; XK, xylulokinase; X-5-P, xylulose-5-phosphate; PPP, pentose phosphate pathway. 15

Figure **2-2**: Pathways for mannitol production in heterofermentative and homofermentative LAB, and for sorbitol production in *L. casei* and *L. plantarum*. **Enzymes:** 1. Glucose permease; 2. Fructose permease; 3. Glucokinase; 4. Fructokinase; 5. Phosphoglucose isomerase; 6. Mannitol-1-phosphate dehydrogenase; 7. PEP-dependent PTS; 8. Mannitol dehydrogenase; 9. Mannitol-1-phosphatase*; 10. Unspecified hexitol transport*; 11. Sorbitol-6-phosphate dehydrogenase; 12. Mechanism unspecified*; 13. Lactate dehydrogenase. **Abbreviations:** PTS, phosphotransferase system; 6PG, 6-phosphogluconate; EMP, Embden-Meyerhof-Parnas. *Reactions 9 + 10 and reaction 12 may occur via PTS (Ferain et al., 1996)... 22

Figure **3-1**: Comparison between strains with or without deletions in xylose transporters (*xylG* and/or *xylE*), and expressing wild-type *crp* vs. *crp**. All strains also express CbXR from pLOI3815. Cultures were grown in shake-flasks containing LB medium supplemented with approximately 100 mM glucose, 300 mM xylose and 50 mM MOPS buffer. (a) Time-courses of xylitol production. (b) Time-courses of glucose concentration. Data represent the average of at least two values, with standard deviations shown as error bars.. 41

Figure **3-2**: Bioreactor cultivation profiles comparing induced expression of XylE or XylFGH in PC07 (wt *crp*) to strain PC09 (*crp**). All three strains also express CbXR. Cultures initially contained approximately 100 mM glucose and 300 mM xylose in minimal medium. Glucose was added in 50 mM doses upon depletion and xylose was maintained above 100 mM. DO was not allowed to drop below 5% and pH was maintained at 7.0. (a) Time-courses of xylitol production. (b) Time-courses of glucose consumption. Acetate concentration at 66 h is given in parentheses (the average value from at least two cultivations, with standard deviation reported). 46

Figure **3-3**: Extended fed-batch cultivation of PC07 + pPCC207 growing in minimal medium. Conditions are as described for Figure **3-2**. 47

Figure **4-1**: Fermentation profiles: xylitol production (a-c), glucose consumption (d-f), and growth (d-f). Strains PC09 and FL05 carry plasmid pLOI3815 and express CbXR (a, d). Strains PC07, FL03 and FL07 either carry plasmid pPCC107 and co-express XylE with CbXR (b, e) or carry plasmid pPCC207 and co-express XylFGH with CbXR (c, f). Fed-batch fermentations (30°C, pH=7, DO>20%) contained minimal medium initially supplemented with 5 g yeast extract l^{-1}, 300 mM xylose and 100 mM glucose. Sugars were then fed manually: glucose was fed in 50 mM doses upon each glucose depletion and xylose was added to maintain concentrations above 100 mM. IPTG was added to the vessels prior to inoculation (300 μM). 69

Figure **4-2**: Post-induction profiles of xylitol production and glucose consumption for PC09 and FL05 carrying pLOI3815 (expressing CbXR) in high cell density fermentations. Cells were first grown in the bioreactors in minimal medium under glucose-limited conditions to a density of ~5 g cdw l^{-1}. During the growth phase, glucose (500 g l^{-1}) supplemented with ammonium sulfate (7.5 g l^{-1}) was fed at rate of 0.6 ml h^{-1}. Xylitol production was initiated by inducing with IPTG (300 μM), and adding glucose (50 mM), and xylose (200) mM. Glucose was then fed in 50 mM doses upon each glucose depletion and xylose was added to maintain concentrations above 100 mM during the production phase. Yeast extract was not used. 71

Figure **5-1**: Genome-wide transcriptional effects of glucose in strain W3110 expressing wild-type CRP, presented as expression ratios for individual genes showing significant differential expression in the presence and absence of glucose (WT G – WT). **1a)** 629 genes show significant changes in expression level in response to the presence of glucose in strain W3110. **1b)** The changes in expression levels of the same genes shown in **1a** in CRP* strain PC05, in response to glucose. Gene names and expression levels are given in supplementary Table Sup3. 92

Figure **5-2**: Genome-wide transcriptional effects of glucose in CRP* strain PC05, presented as expression ratios for individual genes showing significant differential expression in the presence and absence of glucose (CRP* G – CRP*). **2a)** 80 genes show significant changes in expression level for strain PC05. Only 29 are upregulated. **2b)** The changes in expression levels of the same genes shown in **2a** for strain W3110, in response to glucose. Gene names and expression levels are given in supplementary Table Sup4 94

Figure **5-3**: Genes that respond differently to the presence of glucose in W3110 compared to PC05, (CRP* G – CRP*) – (WT G – WT). **3a)** Ratio of

expression levels of these 238 genes in strain W3110 in the presence and absence of glucose (WT G / WT). **3b)** Ratio of expression levels of the same genes shown in **3a** in strain PC05, in the presence and absence of glucose (CRP* G / CRP*). Gene names and expression levels are given in supplementary Table Sup5. 98

Figure **5-4**: Significant genome-wide transcription effects of expressing CRP* (strain PC05) instead of wild-type CRP (W3110) in the presence of glucose, presented as individual gene expression ratios (CRP* G – WT G). 349 genes show significant changes in their expression levels between CRP* G and WT G conditions. Gene names and expression levels are given in supplementary Table Sup6. 100

LIST OF TABLES

Table **1-1**: Approximate composition of hardwood and softwood (Jeffries, 1983).... 2

Table **2-1**: Summary of metabolic engineering strategies discussed in this chapter. .. 24

Table **3-2**: Strains and plasmids used in this study.[a] 36

Table **3-3**: Summary of the 72-h time-point results for cultures depicted in Figure **3-1**.. 42

Table **3-4**: Comparison of results from cultures of strain PC07 harboring various plasmids with or without xylose transporters. 44

Table **3-5**: Specific growth rates of W3110 and *xyl* transporter deletion strains growing in xylose minimal medium (h^{-1} ± standard deviation). 50

Table **4-1**: Strains and plasmids used in this study 61

Table **4-2**: Sugar consumption by W3110, MG1655, *E. coli* B, and their respective *crp** derivatives. 66

Table **4-3**: Xylitol production and glucose consumption in W3110, *E. coli* B, and their derivatives. 67

Table **4-4**: Summary of the 63-hour time point results for fermentations depicted in Figure **4-1**. 70

Table **4-5**: Results from resting cells after 48 hours of biotransformation in minimal medium. 74

Table **5-1**: Primers and probes sequences used for RT-PCR 87

Table **5-2**: Expression levels for the genes discussed in this paper. Supplementary Table Sup2, a complete version of Table **5-2** presenting all signal values for the *E. coli* K-12 genome, can be found in supplementary material. Four different conditions were tested in this study: W3110 in LB medium (WT), W3110 in LB+glucose medium (WT G), PC05 in LB medium (CRP*), and PC05 in LB+glucose medium (CRP* G). 90

Table **5-3**: Summary of genome-wide effects of CRP* expression. 91

Table **5-4**: Comparison between expression levels (signal values) of the wild-type strain genes in response to the presence of glucose in two different studies. This comparison focuses on genes involved in central metabolism. 97

Table **5-5**: Comparison between microarray and real-time reverse transcription PCR results. Data are presented as fold changes (signal ratio) for all conditions tested. 101

Table **5-6**: Intracellular cofactor concentrations (μM (g cdw)$^{-1}$) and ratios for strains engineered to produce xylitol. W3110Δ*xylB* harboring plasmid pPCC207 expresses CbXR and XylFGH, while PC05Δ*xylB* harboring plasmid pPLOI3815 expresses CbXR. Strains were grown in the presence of glucose only (G) or in glucose plus xylose (GX), enabling xylose reduction during glucose metabolism. Standard deviations were less than 15% unless noted. 105

Table **A-1**: Strains 113

Table **A-2**: Plasmids 117

Table **A-3**: Primers 118

Table **B-1**: Tubes contents 121

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Chapter 1

Introduction

1.1 Hemicellulose and xylose

Hemicelluloses are cell wall polysaccharides that can be found in large quantities in most trees and vegetable fibers. They are the world's second most abundant family of polymers after cellulose and thus represent an enormous renewable resource for industry. Annually, 60 billion tons of hemicelluloses are produced on the earth and remain almost completely unused (Xu et al., 2006b). These branched polymers are composed of several monosaccharides with a weak structure compared to crystalline cellulose, which is formed linearly through ß1→4 glycosidic bonds. Hemicelluloses from woody plants are mostly composed of D-xylose, D-mannose, D-galactose, D-glucose, L-arabinose, 4-O-methylglucuronic acid, D-galacturonic acid and glucuronic acid. Hemicelluloses from grasses and cereal straws have a smaller variety of sugar residues including D-xylose, L-arabinose, D-glucose, and D-galactose (Xu et al., 2006a). Hemicelluloses are major components of lignocellulosic materials. In addition to neutral sugars, uronic acids and acetal groups are their other major structural components (present as their respective anhydrides). Hemicellulosic sugar anhydrides constitute 26% of the dry weight of hardwoods, 22% of softwoods and an average of 25% of other major agricultural residues. Table **1-1** shows the approximate composition of hardwood and softwood (Jeffries, 1983).

Table **1-1**: Approximate composition of hardwood and softwood (% of total dry weight) (Jeffries, 1983)

	Hemicellulosic sugars[a]	Hemicellulose[b]	Cellulose[c]	Lignin[d]
Hardwoods	26.2	34	45	21
Softwoods	22.3	28	43	29

[a] Reported as anhydrides
[b] Includes acetyl and uronic acid residues
[c] Residual glucan following acid prehydrolysis
[d] Analyzed as Klason lignin (acid-insoluble)

Hemicellulosic sugars, especially D-xylose, are as shown, relatively abundant in agricultural residues and plants wastes. Moreover, due to the random, amorphous structure with little strength of hemicelluloses their recovery by acid hydrolysis is easier and more efficient than the recovery of D-glucose from cellulose which has a crystalline, strong structure that, is resistant to hydrolysis. These advantages make hemicellulosic sugars a favorable feed stock in the biotechnology industry, especially for production of ethanol and other biochemicals (Jeffries, 1983).

After glucose, xylose is the second most abundant sugar in nature. While xylose can be used as substrate for fuel ethanol and value added chemicals like xylitol, it must be fermented simultaneously with glucose to make the process economical. Even though fermentable starch and sucrose are obtained from agricultural by-products, wheat or corn straw and cane bagasse, substrate cost still remains the major factor in fermentative production of fuel ethanol. Reducing production cost is the only way to make ethanol a competitive fuel. This requires expansion of the fermentation process towards utilization of lower-value substrates, such as lignocellulose. Lignocellulosic biomass which is

composed of a mixture of carbohydrate polymers (cellulose and hemicellulose) the most abundant raw material in nature that comes from hardwood, softwood, grasses, and agricultural residues (Lee, 1997). Although the glucose, the main constituent of cellulose hydrolysate, can be fermented to ethanol easily and efficiently, bioconversion of xylose to ethanol and other chemicals is still a biochemical challenge. Especially if xylose is present along with glucose, it needs to be used to make the ethanol production process economical (Aristidou and Penttila, 2000).

1.2 Biotechnology

The microbial world contains organisms that can breakdown a wide range of organic compounds and produce native metabolites as well as non-native materials. In contrast to chemical syntheses which usually need high quality and pure substrates, biocatalitic processes can use complex raw materials. This reduces the process costs due to removing the substrate purification step. This is not the only advantage of using bioprocess. Greener chemistry, high product specificity, low temperature and low pressure production and low energy consumption are the other aspects that make this approach favorable compared to traditional chemical synthesis. Consequently, there has been a shift toward using biotechnology and biological raw materials in production technology. Fermentation and other bioprocesses are competing with traditional production processes and becoming increasingly important in the production of chemicals. Using novel biotechnology techniques, biomaterial waste which should be disposed in negative cost can be used as renewable resources and converted into useful

materials, such as renewable fuel and additives, value added chemicals, intermediate fermentation compounds and a wide variety of organic chemicals like amino acids, enzymes and antibiotics (Thomsen, 2005, Kamm and Kamm, 2004).

Specifications of yield, productivity, wide-substrate and product range, ethanol tolerance, process and biomass disposal cost can not all be obtained with a wild type organism (Aristidou and Penttila, 2000), so genetic manipulation and metabolic engineering seem to be the most efficient way to achieve the desired properties from microorganisms. Metabolic engineering plays an important role in biotechnology increasing growth rate and remains as a powerful tool to rearrange the new fuel and chemical industries. Application of metabolic engineering and genetic manipulation in a wide range of biotechnology processes has made far goals reachable and has received considerable success and appreciation (Kawaguchi et al., 2006, Kim et al., 2006, Lara et al., 2006, Lee and Cho, 2006, Sanchez et al., 2006, Trinh et al., 2006, Watts et al., 2006, Lin et al., 2005, Causey et al., 2003, Aristidou and Penttila, 2000). Engineering of biocatalysts and bioprocesses can be performed at the enzyme level, the host cell level or the whole process level for the best performance (Schmid et al., 2001).

1.3 Xylitol

Xylitol is a relatively value added chemical with a growing market. It is a polyol with a wide range of applications in food and health industries. Xylitol is a natural sweetener, with a sweetening power equal to sucrose, which is not fermentable by the caries-inducive oral microflora, promoting oral health (Peldyak and Makinen, 2002).

Because it has a negative heat of solution xylitol gives an excellent taste and a refreshing-cooling sensation in the mouth. Also its inability to undergo a Maillard reaction, the reaction responsible for darkening and reduction in the nutritional value of proteins, inhibits change in the taste and color of prepared food during storage. Xylitol metabolism in human body is not insulin mediated making it recommended for diabetic patients (Parajo et al., 1998, Emodi, 1978). Xylitol production by engineered *E. coli* strains can be a measure of xylose uptake in study of influence of different xylose transporters, without requiring further xylose metabolism.

Since the 1970s, xylitol has been produced by catalytic hydrogenation of purified xylose through an expensive, high temperature and pressure process(Silva et al., 2006). The bioproduction advantages of xylitol have made it favorable for industry to switch from traditional chemical synthesis to novel bio based processes. Although most efforts toward the biosynthesis of xylitol have focused on natural and genetically engineered yeast strains, bacteria, especially *E. coli,* could be a good choice as an alternative. *E. coli* is a fast growing bacterium that uses inexpensive growth medium and has a well-characterized metabolism. It is also easy to manipulate, has the capability to produce a wide range of products, and can utilize both hexose and pentose based sugars. Drawing upon these advantages, Cirino and coworkers have engineered *E. coli* to both simultaneously uptake xylose and glucose, and partition xylose for xylitol production (Cirino et al., 2006).

1.4 Xylose uptake in *E. coli*

A xylose transport system for *E. coli* was first reported by David and Wiesmeyer in 1970 (David and Wiesmeyer, 1970). They showed that the xylose permease system is inducible and specific for D-xylose. The system was able to transport xylose against a concentration gradient by expending energy. It took almost one decade before Shamanna and Sanderson proved that there were two transport systems for xylose in *E. coli* (Shamanna and Sanderson, 1979). Based on kinetic studies, they measured apparent K_m values of 110 and 24 μM for the two transport systems. In 1980, Lam and coworkers discovered the proton linked D-xylose transport system which showed an alkaline pH change while taking up xylose. They tested different mechanisms to find if the transporter was energized by a proton-motive force, directly by ATP or acetyl phosphate, or phosphorilated by phosphoenolpyruvate. In addition to alkaline pH behavior, the energized xylose transport system was found to be inhibited by uncoupling agents and insensitive to arsenate or fluoride. Based on the observations, they concluded transport system is energized by a proton-motive force (Lam et al., 1980). They reported an apparent K_m value of 23.9±2.4 μM for the transport system which was consistent with previous reported values (Shamanna and Sanderson, 1979). It was postulated that the lack of other transport activity might be due to repression effects of other sugars used in the experiments, especially glucose and glycerol.

Later on, Ahlem and coworkers purified periplasmic D-xylose-binding protein from Escherichia coli K-12 (Ahlem et al., 1982). This osmotic shock-releasable binding protein was thought to be part of the high affinity xylose transport system that was

reported earlier (Shamanna and Sanderson, 1979). They also found that the binding protein was produced while growing in media containing both xylose and glycerol. This result was consistent with L-arabinose and D-ribose transport systems which include both low affinity and high affinity mechanisms with a similar binding protein serving as part of high affinity one (Parsons and Hogg, 1974, Willis and Furlong, 1974). In 1984 Davis and coworkers did a series of experiments that confirmed the presence of at least two xylose transport systems in *E. coli*, one proton-linked and the other associated with a binding protein (Davis et al., 1984). In order to be consistent with the names applied for the two arabinose transport system genes *araE* and *araF* the gene for xylose-proton symport was designated *xylE* and gene for the binding protein system *xylF* (Kolodrubetz and Schleif, 1981). It was found that *xylE* is located at 91.4 min between *pgi* and *malB* genes in the *E. coli* genome.

Finally Davis and Henderson cloned and sequenced the *xylE* gene (Davis and Henderson, 1987). *xylE* contains 1473 base pairs encoding for a 491 amino acid protein which is likely the only protein responsible for xylose-H^+ symport. Like other sugar-proton symport proteins, it is highly hydrophobic and there is a high degree of homology with AraE in its structure. While analyzing the *E. coli* genome in 1994 Sofia and coworkers identified three genes downstream of *xylF*. These genes encoded proteins which were similar to the components of ABC-type transport systems and the *araC* regulator. As with *xylF* they proposed the ATP-binding protein gene be called *xylG* and membrane compound of transporter be called *xylH* due to homology to the arabinose transport system. They also named the regulatory protein gene *xylR* (Sofia et al., 1994).

Song and Park characterized the functions and regulation of *xyl* operon. They concluded that transcription regulation of the XylA (xylose isomerase), XylB (xylulokinase) and XylFGH transporter is mediated by XylR, and the cylic-AMP receptor protein CRP (Song and Park, 1997).

In conclusion, xylose uptake in *E. coli* occurs through two transport systems (Figure **1-1**). XylE is a D-xylose proton symporter which uses the proton gradient as a source of energy. It possesses a relatively low affinity with high K_m values between 63 and 169 μM. XylFGH, the other D-xylose transport system, belongs to the ATP binding cassette ("ABC") family of transporters and requires one ATP per D-xylose transported. XylFGH exhibits a high affinity with an apparent K_m between 0.2 and 4 μM (Sumiya et al., 1995).

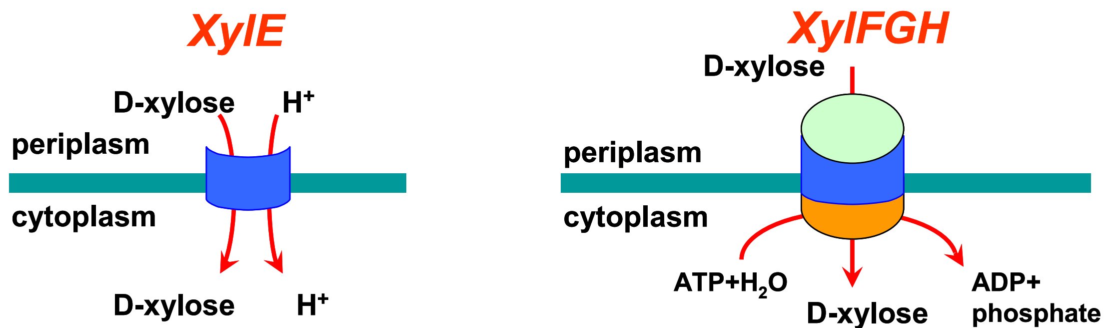

Figure **1-1**: Xylose transport systems in *E. coli.* XylE is a D-xylose proton symporter which uses the proton gradient as a source of energy. XylFGH belongs to the ATP binding cassette ("ABC") family of transporters and requires one ATP per D-xylose transported.

1.5 Energetics of xylose uptake and xylitol production

E. coli has been engineered to produce xylitol from xylose while using glucose as the cofactor regeneration system substrate through central metabolism required for cofactor-dependent, heterologous xylose reduction reaction (Cirino et al., 2006). Due to energy requirements for cofactor regeneration, xylose utilization (especially in anaerobic culture) and subsequently xylitol production can not be optimized when the energy source is shared with other energy sinks. The ATP requirement for xylose transport is a key limitation to xylose utilization and xylitol production when XylFGH plays the main role in xylose uptake. Therefore, the efficiency of xylose utilization in this organism is not optimized due to energetic requirements for nutrient uptake. Engineering a less energy-intensive uptake mechanism is, therefore, a prerequisite for further metabolic optimization and is the main goal in this work. Figure **1-2** shows the overview of xylose uptake and xylitol production in *E. coli*.

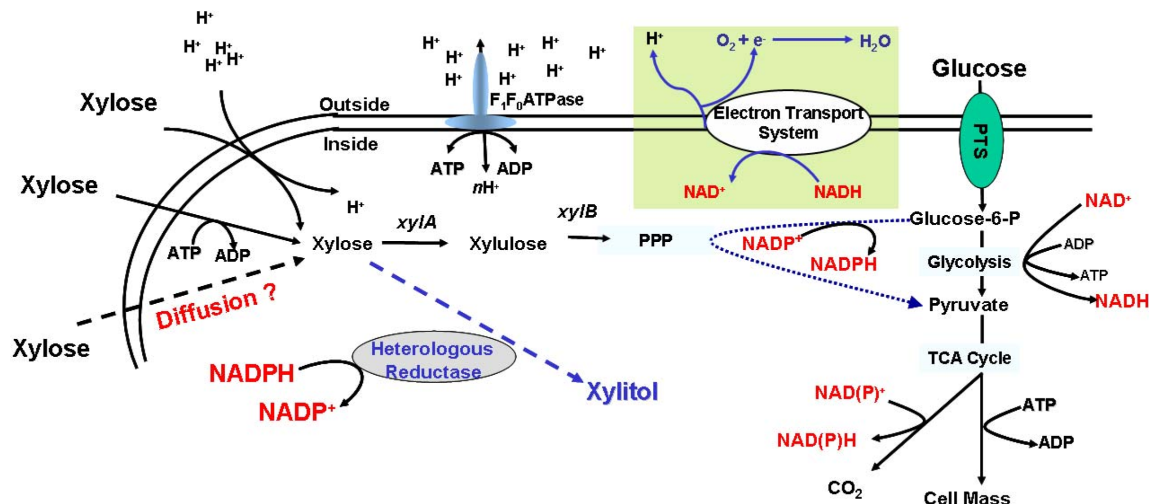

Figure **1-2**: Overview of xylose uptake and xylitol production in *E. coli* (Cirino et al., 2006)

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Chapter 2

Bioproduction of sugar alcohols (xylitol and sorbitol)

2.1 Summary

Sugar alcohols find applications in pharmaceuticals, oral and personal care products, and as intermediates in chemical synthesis. While industrial-scale production of these compounds has generally involved catalytic hydrogenation of sugars, microbial-based processes receive increasing attention. The past few years have seen a variety of interesting metabolic engineering efforts to improve the capabilities of bacteria and yeasts to overproduce xylitol, mannitol and sorbitol. Examples include heterologous expression of yeast xylose reductase in *Escherichia coli* for production of xylitol, co-expression of formate dehydrogenase, mannitol dehydrogenase and a glucose facilitator protein in *Corynebacterium glutamicum* for mannitol production from fructose and formate, and overexpression of sorbitol-6-phosphate dehydrogenase in lactate dehydrogenase-deficient *Lactobacillus plantarum* to achieve nearly maximum theoretical yields of sorbitol from glucose.

2.2 Introduction

Sugar alcohols are a class of polyols in which a sugar's carbonyl (aldehyde or ketone) is reduced to the corresponding primary or secondary hydroxyl group. They have similar characteristics as sugar and are used to improve the nutritional profile of food

products due to health-promoting properties such as lower caloric content, noncariogenicity, and low glycemic index and insulin response (Granstrom et al., 2007a, Schiweck et al., 2003). Other auspicious qualities as food additives include high enthalpies of solution and lack of reactive carbonyls. Sugar alcohols additionally find many applications in pharmaceuticals, chemicals production, oral and personal care and animal nutrition (Silveira and Jonas, 2002). They are found naturally in fruits and vegetables and are produced by microorganisms, serving as carbohydrate reserves, storage of reducing power, translocatory compounds and osmoprotectants.

Traditional industrial production of most sugar alcohols is by hydrogenation of sugars over nickel catalysts under high temperature and pressure conditions (Schiweck et al., 2003). Biosynthetic routes offer the potential for safer, environmentally friendly production with enhanced product specificity. Enzyme-based processes for production of sugar alcohols via sugar reduction have been investigated but are not within the scope of this review. However, costs associated with enzyme preparations and cofactor regeneration for *in vitro* synthesis of sugar alcohols contribute to the general perception that the use of whole cells presents a more attractive biological approach to produce these compounds from crude sugar feedstocks. Here I review recent metabolic engineering efforts to improve microbial production of the common sugar alcohols xylitol and sorbitol.

2.3 Xylitol

Xylitol is a five-carbon sugar alcohol obtained from xylose reduction. The annual xylitol market is estimated to be \$340 million, priced at \$4-5 kg^{-1} (Kadam et al., 2008). Xylitol has received the most recent attention of all the sugar alcohols, particularly as it pertains to microbial production and metabolic engineering. As depicted in Figure **2-1**, yeasts naturally produce xylitol as an intermediate during D-xylose metabolism. Xylose reductase (XR) is typically an NADPH-dependent enzyme, while xylitol dehydrogenase (XDH) requires NAD^+ (Granstrom et al., 2007a). Cofactor imbalance results in secretion of xylitol as a xylose fermentation by-product.

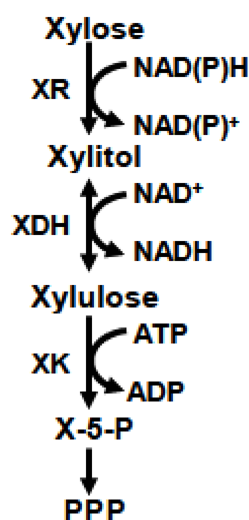

Figure **2-1**: Pathway for xylose metabolism in yeast. XR, xylose reductase; xDH, xylitol dehydrogenase; XK, xylulokinase; X-5-P, xylulose-5-phosphate; PPP, pentose phosphate pathway.

Among the yeast strains that naturally produce xylitol, *Candida sp* have received the most recent attention for developing microbial-based xylitol processes (Ko et al., 2008, Granstrom et al., 2007b). Using a fed-batch submerged membrane bioreactor with cell recycle, *Candida tropicalis* produced 12 g l^{-1} h^{-1} xylitol from xylose, with glucose as co-substrate (Kwon et al., 2006b). This is among the highest productivities reported. Ko and coworkers produced xylitol aerobically from xylose with glycerol as a co-substrate, achieving a volumetric productivity of 3.2 g l^{-1} h^{-1} and a yield of 0.98 mol xylitol (mol xylose)$^{-1}$ in a XDH gene (*XYL2*)-disrupted mutant of *C. tropicalis* (Ko et al., 2006). While *Pichia stipitis* does not naturally overproduce xylitol, mutant strains with disruptions in alcohol dehydrogenase, xylitol dehydrogenase, or D-xylulokinase have been shown to produce xylitol (Jin et al., 2005).

Saccharomyces cerevisiae lacks native xylose-specific transporters and is not an efficient xylose-utilizing organism (Jeffries, 2006). The popularity of *S. cerevisiae* has led to research to improve xylose fermentation by this organism, yielding recombinant strains capable of high levels of xylitol production (Jeppsson et al., 2006). Efforts to increase xylose transport and utilization have included expression of homologous and heterologous transporters (Hahn-Hagerdal et al., 2007, Saloheimo et al., 2007). While *S. cerevisiae* does possess at least one native aldose reductase capable of reducing xylose (NADPH-dependent GRE3) (Ford and Ellis, 2001), endogenous expression or activity of this enzyme is apparently insufficient for appreciable xylitol production. Overexpression of endogenous glucose-6-phosphate dehydrogenase (G6PDH) encoded by *ZWF1* to increase NADPH availability in a recombinant strain of *S. cerevisiae* harboring *P. stipitis* XR gene (*XYL1*) (Chung et al., 2002) resulted in 6-fold increased G6PDH activity and

increased volumetric xylitol productivity (from 1.6 to 2.0 g xylitol l^{-1} h^{-1}) in glucose-limited, fed-batch cultivations containing xylose (Kwon et al., 2006a). To further increase pentose phosphate pathway (PPP) flux, phosphoglucose isomerase (PGI) activity encoded by *PGI1* was reduced by replacing the *PGI1* promoter with the *ADHI* (encoding alcohol dehydrogenase I) promoter (Oh et al., 2007). Simultaneous overexpression of G6PDH and attenuation of PGI in a recombinant *S. cerevisiae* strain expressing *P. stipitis XYL1* resulted in a specific productivity of 0.34 g xylitol (g cdw h)$^{-1}$ in glucose-limited, fed-batch cultivations, which is 90% higher than the specific productivity of the parent strain expressing only *XYL1* (Oh et al., 2007). Glucose served as the primary source of reducing equivalents for xylose reduction, with 4.2 mole xylitol produced per mole glucose consumed in rich medium.

Xylitol production from glucose was also demonstrated using engineered *S. cerevisiae* (Toivari et al., 2007). The strain design included deletion of transketolase (resulting in accumulation of D-xylulose-5-phosphate and secretion of ribitol and pentose sugars) followed by expression of XDH from *P. stipitis* (*XYL2)* and overexpression of *DOG1* encoding sugar phosphate phosphatase. Ribitol was a co-product and the total sugar alcohol yield was at most 0.042 mol (mol glucose)$^{-1}$ (Toivari et al., 2007).

Bacteria offer several potential advantages over yeasts for the production of xylitol. Cirino and coworkers recently described approaches to produce xylitol from engineered *Escherichia coli* (Khankal et al., 2008a, Cirino et al., 2006). The objective was to use glucose metabolism as source of reducing equivalents to drive either direct xylose reduction (via expression of an XR) or conversion of xylose to xylulose (via the native xylose isomerase) followed by reduction to xylitol (via expression of a XDH). A

xylulokinase (*xylB*) deletion prevented xylose metabolism. To overcome glucose repression of xylose transporter gene expression, the native *crp* gene was replaced with a catabolite repression mutant ("*crp**") (Cirino et al., 2006). Several XRs and XDHs were screened in *E. coli*, and the NADPH-dependent XR from *Candida boidinii* (CbXR) allowed for the highest levels of xylitol production in batch cultures (38 g l^{-1} in 46 hours using minimal medium) (Cirino et al., 2006). Use of resting cells allowed for a higher yield on reducing equivalents delivered to the reductase reaction: 1.7 moles xylitol were produced per mole glucose consumed in batch culture, compared to 4.7 moles xylitol produced per mole glucose consumed by resting cells in minimal medium (theoretically, ~7-10 moles xylitol can be produced from xylose per mole glucose consumed, depending on the mechanism of xylose transport (Khankal et al., 2008a)). The contribution of key enzymes in *E. coli* central metabolism toward NADPH supply for xylitol production was also examined (Chin et al., 2009). NADPH availability limited xylitol production in resting cells, and was increased by increasing flux through PPP during glucose metabolism. Excess reducing equivalents in the form of NADH (resulting from glucose oxidation) did not translate into available NADPH for xylitol production (Chin et al., 2009).

An alternative to the use of *crp** in *E. coli* is plasmid-based overexpression of xylose transporters (Khankal et al., 2008a). Overexpressing the ATP-dependent XylFGH xylose transport system from *E. coli* resulted in an average specific xylitol productivity of 0.33 g (g cdw h)$^{-1}$ in a fed-batch fermentation using mineral salts medium (Khankal et al., 2008a). Alternate approaches to xylitol production from glucose-xylose mixtures (expression of *crp**, *xylE* or *xylFGH*) were compared in three common *E. coli* host strains

(K-12 strains W3110 and MG1655, and wild-type *E. coli* B), and differences in host strain genetic background was found to significantly impact metabolic engineering strategies (Khankal et al., 2008b).

In a similar approach as described above, a xylitol-producing *E. coli* strain was constructed by chromosomal insertion of NADPH-dependent XR from *Kluyveromyces lactis* (*XYL1*) and the *E. coli* D-xylose permease (*xylE*) under control of an IPTG-inducible promoter (Hibi et al., 2007). Transcriptome analysis of the strain under conditions where xylitol is produced versus not produced revealed that xylitol production down-regulated 56 genes, which were considered factors related to reduced NADPH supply. The 56 individual gene deletions were studied and a *yhbC*-deficient strain showed the highest improvements in xylitol production (increasing xylitol productivity from 0.68 to 0.81 g l^{-1} h^{-1}). YhbC is uncharacterized, but is potentially a regulatory factor.

E. coli XylB was shown to phosphorylate xylitol to xylitol-phosphate, resulting in inhibited growth on xylose and poor xylitol production (Akinterinwa and Cirino, 2009). In contrast, xylitol is a poor substrate for the xylulokinase Xyl3 from *P. stipitis*. To construct an *E. coli* strain capable of producing xylitol while also metabolizing xylose as a source of carbon and energy, *xylB* was deleted and Xyl3 was expressed instead.

XRs naturally show relaxed sugar specificity and are able to reduce L-arabinose. This is problematic when xylitol is the desired product from plant hemicellulose raw materials containing both D-xylose and L-arabinose. Zhao and coworkers characterized an XR from *Neurospora crassa* (NcXR), and showed that it naturally had higher selectivity for D-xylose over L-arabinose (~2.4), compared to several other XRs (Woodyer et al., 2005). Using both error-prone PCR and iterative targeted site-saturation

mutagenesis (TSSM), combined with high-throughput screening and a clever genetic selection for specificity toward D-xylose reduction, they were able to enhance the substrate specificity of NcXR to ~16.5, with a moderate loss in catalytic efficiency (Nair and Zhao, 2008). The mutant NcXR also increased selectivity toward xylitol production in recombinant *E. coli* resting cells. Xylitol production by engineered *E. coli* using hemicellulose feedstocks has potential to become a commercially viable process (zuChem; URL: http://www.zuchem.com/).

Using glucose as the energy source, xylitol was produced from xylose in a recombinant *Lactococcus lactis* strain by expressing XR from *P. stipitis* (*XYL1*) (Nyyssola et al., 2005). A glucose-limited, fed-batch fermentation produced 2.5 mol xylitol (mol glucose)$^{-1}$ and 1 mol xylitol (mol xylose)$^{-1}$ (xylose was not metabolized) at the rate of 2.7 g xylitol l^{-1} h^{-1} over 20 hours. Co-expression of a xylose transporter with XR did not improve xylitol production.

Finally, Povelainen and Miasnikov reported xylitol (and ribitol) production from glucose in engineered strains of *Bacillus subtilis* (Povelainen and Miasnikov, 2007). Expression of D-xylitol phosphate dehydrogenase (XPDH) from *Lactobacillus rhamnosus* or *Clostridium difficile* in a pentulose-producing mutant of *B. subtilis* (GX7) (Povelainen and Miasnikov, 2006) resulted in xylitol production with a yield of 0.26-0.27 mol xylitol (mol glucose)$^{-1}$ in rich medium containing 10% glucose. Dephosphorylation of pentitol-phosphates was presumably due to an intracellular or membrane-associated sugar phosphate phosphatase (Povelainen and Miasnikov, 2007).

2.4 Sorbitol

D-Sorbitol (D-glucitol) is a popular six-carbon sugar alcohol (a stereoisomer of mannitol) with an estimated annual production of over 500,000 tons and finding applications in the food industry and as a building block for pharmaceutical products (Silveira and Jonas, 2002). Early studies for biotechnological production of sorbitol primarily focused on the bacterium *Z. mobilis,* which naturally can convert fructose and glucose to sorbitol (via glucose-fructose oxidoreductase) for osmoprotection (Silveira and Jonas, 2002, Loos et al., 1994).

The ability of the fermentative food-grade LAB *L. plantarum* and *Lactobacillus casei* to use different electron acceptors for NAD^+ regeneration via dehydrogenases makes them valuable platforms for polyols production through metabolic engineering (Wisselink et al., 2002, Ferain et al., 1996). Ladero and coworkers produced sorbitol from F6P in an engineered strain of *L. plantarum* by reversing the sorbitol catabolic pathway (Ladero et al., 2007). Figure **2-2** depicts the reactions involved in sorbitol production in this organism. Two native sorbitol-6-phosphate dehydrogenase (Stl6PDH) genes were identified and overexpressed in a mutant strain of *L. plantarum* lacking LDH. Using resting cells with glucose as substrate, the mutant strain produced sorbitol with a yield up to 0.65 mol (mol glucose)$^{-1}$, which is near the maximum theoretical value of 0.67 mol (mol glucose)$^{-1}$ (Ladero et al., 2007). Sorbitol was not detected when Stl6PDH was not overexpressed. Mannitol was also produced due to natively expressed Mtl1PDH activity competing for the common substrate F6P.

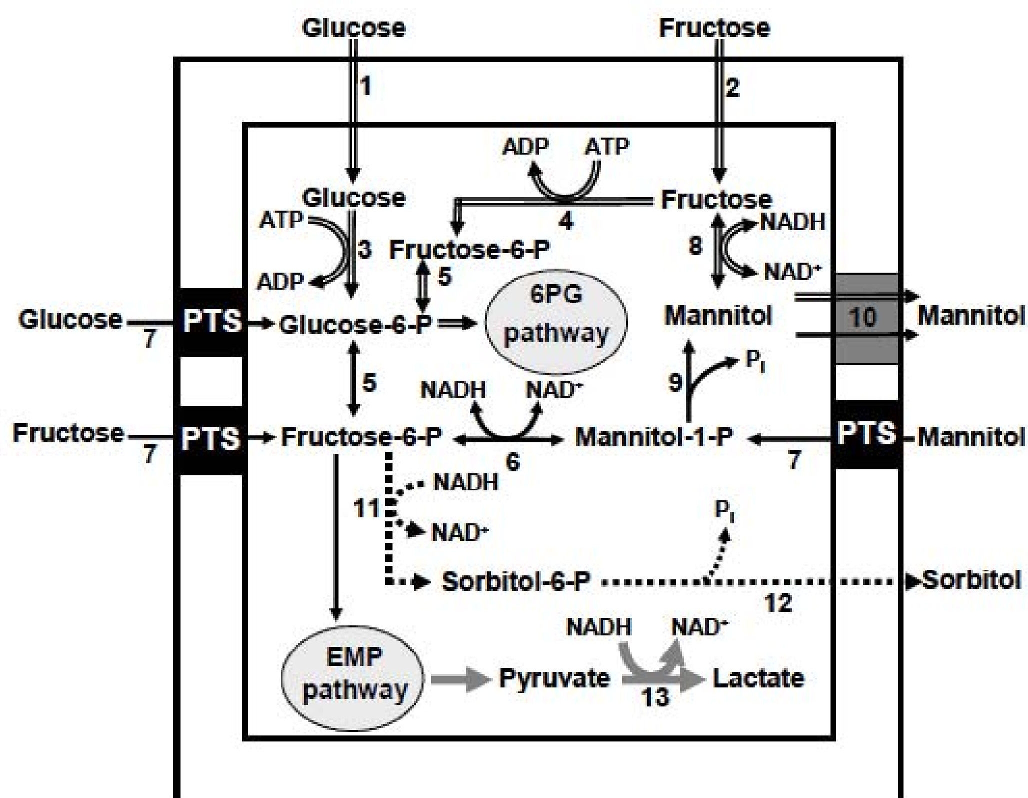

Figure **2-2**: Pathways for mannitol production in heterofermentative and homofermentative LAB, and for sorbitol production in *L. casei* and *L. plantarum*. **Enzymes:** 1. Glucose permease; 2. Fructose permease; 3. Glucokinase; 4. Fructokinase; 5. Phosphoglucose isomerase; 6. Mannitol-1-phosphate dehydrogenase; 7. PEP-dependent PTS; 8. Mannitol dehydrogenase; 9. Mannitol-1-phosphatase*; 10. Unspecified hexitol transport*; 11. Sorbitol-6-phosphate dehydrogenase; 12. Mechanism unspecified*; 13. Lactate dehydrogenase. **Abbreviations:** PTS, phosphotransferase system; 6PG, 6-phosphogluconate; EMP, Embden-Meyerhof-Parnas. *Reactions 9 + 10 and reaction 12 may occur via PTS (Ferain et al., 1996).

In a similar study, a recombinant strain of sorbitol-producing *L. casei* was constructed by integrating a Stl6PDH-encoding gene (*gutF*) (Yebra and Perez-Martinez, 2002) into the chromosomal lactose operon (Nissen et al., 2005). While the parent strain

produced negligible amounts of sorbitol, the engineered strain produced 0.024 mol sorbitol (mol glucose)$^{-1}$ from glucose in resting cells pre-grown on lactose. Deletion of lactate dehydrogenase (*ldhL*) increased sorbitol production to 0.043 mol (mol glucose)$^{-1}$, presumably due to elevated NADH/NAD^{+} ratios. The mechanism of sorbitol-6-phosphate dephosphorylation is unknown, but may occur via a hexitol phosphate-specific EII of the PTS.

2.5 Conclusions

Microbial production of xylitol and sorbitol has received considerable attention in recent years, and the metabolic engineering strategies addressed in this chapter are summarized in Table **2-1**. While many organisms naturally produce these compounds, genetic modification strategies have allowed for their enhanced production. LAB have been particularly exploited in that regard. Alternately, organisms which do not naturally produce a sugar alcohol have been provided the facilities to do so through metabolic engineering. *E. coli* has proven an effective host organism in that category. As biorefining technology improves and renewable, agricultural-based chemicals continue to gain importance, so will the demand and applications of sugar alcohols grow. Biological production routes are increasingly attractive, and some microbial-based processes for sugar alcohols are already commercially viable.

Table **2-1**: Summary of metabolic engineering strategies discussed in this chapter.

Organism	Genetic Modifications	Substrate	Yield and/or Productivity	References
Xylitol				
C. tropicalis	Disrupted *XYL2*	Xylose + Glycerol	0.96 mol (mol xyl)$^{-1}$; 3.2 g l^{-1} h^{-1}	(Ko et al., 2006)
S. cerevisiae	Overexpressed G6PDH, expressed Xyl1, attenuated PGI activity	Xylose + Glucose	0.34 g (g cdw h)$^{-1}$	(Oh et al., 2007)
E. coli	Expressed *C. boidinii* XR, replaced *crp* with *crp**, deleted *xylB*	Xylose + Glucose	4.7 mol (mol gluc)$^{-1}$	(Cirino et al., 2006)
E. coli	Expressed *C. boidinii* XR, deleted *xylB*, overexpressed XylE or XylFGH	Xylose + Glucose	0.33 g (g cdw h)$^{-1}$	(Khankal et al., 2008a)
E. coli	Expressed XylE and *K. lactis* XR, deleted *xylA*, *yhbC*-deficient	Xylose + Glucose	0.81 g l^{-1} h^{-1}	(Hibi et al., 2007)
L. lactis	Expressed *P. stipitis* XR	Xylose + Glucose	2.5 mol (mol gluc)$^{-1}$; 2.7 g l-1 h-1	(Nyyssola et al., 2005)
B. subtilis	Expressed XPDH, deleted *rpi*, transketolase-deficient	Glucose	0.27 mol (mol gluc)$^{-1}$	(Povelainen and Miasnikov, 2007)
Sorbitol				
L. plantarum	Overexpressed Stl6PDH, LDH-deficient	Glucose	0.65 mol (mol gluc)$^{-1}$	(Ladero et al., 2007)
L. casei	Expressed Stl6PDH, deleted *ldhL*	Glucose	0.04 mol (mol gluc)$^{-1}$	(Nissen et al., 2005)

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Chapter 3

Role of xylose transporters in xylitol production from engineered *Escherichia coli*

3.1 Abstract

Escherichia coli W3110 was previously engineered to co-utilize glucose and xylose by replacing the wild-type *crp* gene with a *crp** mutant encoding a cAMP-independent CRP variant (Cirino et al., 2006). Subsequent deletion of the *xylB* gene (encoding xylulokinase) and expression of xylose reductase from *Candida boidinii* (CbXR) resulted in a strain which produces xylitol from glucose–xylose mixtures. In this study we examine the contributions of the native *E. coli* xylose transporters (the D-xylose/proton symporter XylE and the D-xylose ABC transporter XylFGH) and CRP* to xylitol production in the presence of glucose and xylose. The final batch xylitol titer with strain PC09 (Δ*xylB* and *crp**) is reduced by 40% upon deletion of *xylG* and by 60% upon deletion of both *xyl* transporters. Xylitol production by the wild-type strain (W3110) expressing CbXR is not reduced when *xylE* and *xylG* are deleted, demonstrating tight regulation of the xylose transporters by CRP and revealing significant secondary xylose transport. Finally, plasmid expression of XylE or XylFGH with CbXR in PC07 (Δ*xylB* and wild-type *crp*) growing on glucose results in xylitol titers similar to that achieved with PC09 and provides an alternative strategy to the use of CRP*.

3.2 Introduction

Escherichia coli is an important platform organism for biological conversion of renewable biomass feedstocks into fuels and chemicals (Wendisch et al., 2006, Dien et al., 2003, Aristidou and Penttila, 2000). It is fast growing, its metabolism is well understood, and it can produce a wide range of products. One attractive characteristic of *E. coli* is its ability to metabolize both hexose and pentose sugars. This makes possible the use of hexose and pentose sugar mixtures as substrate, which significantly reduces fermentation process costs relative to pure sugar feeds (Aristidou and Penttila, 2000, Lee, 1997). Xylose is the second most abundant sugar in nature (after glucose), found most commonly in the form of xylan in hemicelluloses (Jeffries, 1983), and xylose recovery by acid hydrolysis is easier and more efficient than recovery of glucose from cellulose (Xu et al., 2006).

Two different D-xylose-specific transport systems have been identified from *E. coli* and characterized. XylE is a member of the major facilitator superfamily (MFS) of transporters (Griffith et al., 1992) and acts as a relatively low-affinity D-xylose/proton symporter (Km for xylose between 63 and 169 μM (Sumiya et al., 1995)). The proteins XylF, XylG, and XylH comprise a high-affinity D-xylose transporter belonging to the ATP binding cassette (ABC) family of transporters (apparent Km between 0.2 and 4 μM (Sumiya et al., 1995)). Sequence homology analysis suggests that XylF is the periplasmic D-xylose-binding protein (Ahlem et al., 1982), XylG is the ATP-binding protein, and XylH is the membrane component of the ABC transporter (Sofia et al., 1994). *E. coli* K-12 strains growing in a mixture of glucose and xylose exhibit typical diauxie, whereby glucose is preferentially utilized (Cirino et al., 2006, Hasona et al., 2004, Hernandez-

Montalvo et al., 2001, Stulke and Hillen, 1999). The xylose transporter genes *xylFGH*, along with catabolic genes *xylA* and *xylB* are co-regulated by CRP and the XylR transcriptional activator (Song and Park, 1997). While less is known about regulation of *xylE*, it is putatively under CRP control (Robison et al., 1998), and was shown to be induced in cells grown on xylose relative to glucose (Gonzalez et al., 2002). There is also indirect evidence of XylR-mediated control of *xylE* (Song and Park, 1997).

Hasona coworkers compared the growth of *E. coli* strains with deletions in either *xylE* or *xylG* (but not both) (Hasona et al., 2004). Deleting *xylG* reduced aerobic growth on 20 mM xylose by 47%, while anaerobic growth decreased by 84% in 60 mM xylose. Under the same conditions, a *xylE* deletion only reduced growth by 9% and 21%, respectively. They concluded that XylE is not a primary source of xylose transport and the XylFGH system is dominant even under conditions in which both transporters are saturated.

We recently engineered *E. coli* W3110 to co-utilize glucose and xylose by replacing the native *crp* gene with *crp**, encoding a cAMP-independent CRP mutant (Cirino et al., 2006). We speculated that CRP* would increase expression of *xyl* genes and potentially other (promiscuous) transporters in the presence of glucose. Xylose reduction to xylitol in the presence of glucose-xylose mixtures was then possible by expressing an NADPH-dependent xylose reductase from *Candida boidinii* (CbXR) from plasmid pLOI3815. Xylitol is a natural, nutritive sweetener with applications in food preparation (Emodi, 1978), oral health products (Peldyak and Makinen, 2002), diabetes and obesity diets (Pepper and Olinger, 1988), and as a value-added building block

chemical from biomass refining (Granstrom et al., 2007, Werpy T, 2004, Parajo et al., 1998).

Cultures of wild-type strain W3110 and strain PC07 (carrying a deletion in *xylB*, encoding xylulokinase) expressing CbXR and initially containing 300 mM xylose and 100 mM glucose each produced less than 60 mM xylitol and secreted more than 60 mM acetate, while strain PC09 (Δ*xylB* and *crp**) expressing CbXR produced 275 mM xylitol (Cirino et al., 2006) and very little acetate. In all cultures tested (including with different reductases) there was an inverse correlation between xylitol production and acetate production: ability to reduce xylose enabled more complete oxidation of glucose carbon. We were therefore curious whether *crp** played an important role in this apparent delayed acetate overflow metabolism (Wolfe, 2005), beyond increasing xylose transport (e.g., by increasing TCA cycle activity (Veit et al., 2007, Vemuri et al., 2006).

Another parameter of interest was the xylitol yield ("Y_{RPG}"), defined as moles of xylose reduced (or xylitol produced) per mole of glucose consumed, which for (non-growing) resting cells of strain PC09 was 4-5 mol mol^{-1}. As expected, the theoretical maximum xylitol yield, calculated using a stoichiometric network model of *E. coli* metabolism (SimPheny^{TM} software, Genomatica Inc.; model adapted from (Reed et al., 2003)) is influenced by the mode of xylose transport. The *in silico* strain is modeled as PC09 (Δ*xylB*, able to uptake glucose and xylose) expressing NADPH-dependent xylose reductase, with energy-free xylitol secretion. As shown in Table **3-1** for a fixed growth rate of 0.01 h^{-1} (essentially non-growing), increasing energy requirements for xylose transport significantly reduces the theoretical maximum Y_{RPG}. As a reference, we include the yield for the hypothetical case of xylose uptake via facilitated diffusion.

Table **3-1**: Maximum theoretical xylitol yields as a function of xylose transport mechanism.

Transport mechanism	Xylose transporter	Max Y_{RPG}[a]
Xylose/H^+ symport	XylE	9.2
ATP binding cassette	XylFGH	6.9
Facilitated diffusion	-------	10.3

[a] Y_{RPG}: Xylitol produced per glucose consumed (mol mol^{-1}). Xylose is not consumed as a carbon or energy source. The specific growth rate was fixed at a low value of 0.01 h^{-1} and glucose consumption was set to 10 mmol h^{-1} g^{-1} cell dry weight.

While this modeled strain may not accurately reflect the actual metabolism of PC09 resting cells, it is clear from these simulations that an understanding of the mode of xylose uptake in *E. coli* is important for accurately modeling xylose metabolism or xylitol production. In general, the energetic and kinetic properties of transporters play an important role in our pursuit of bio-based renewable fuels and chemicals from biomass (Stephanopoulos, 2007). The present study is aimed at understanding what roles XylE, XylFGH and CRP* play in *E. coli* strains engineered for xylitol production. We find that deleting both Xyl transporters from strain PC09 does not eliminate xylitol production, although it is reduced by 60%. Secondary xylose transport, presumably via diffusion and/or promiscuous transporter activity, is therefore significant under the high xylose concentrations used in this study. Inducible plasmid expression of XylE or XylFGH in strain PC07 (wild-type *crp*) expressing CbXR and growing on glucose results in xylitol production levels similar to those achieved with strain PC09 (with *crp**) and provides insights into the influence of *crp** expression on xylitol and acetate production.

3.3 Materials and methods

3.3.1 General

All strains were maintained on plates containing either Luria–Bertani (LB) medium (10 g tryptone, 5 g yeast extract, 5 g NaCl, and 15 g agar per liter) or minimalmedium containing glucose (2%), mineral salts (3.4 g KH_2PO_4, 5.2 g K_2HPO_4, 3.3 g $(NH_4)_2HPO_4$, 0.25 g $MgSO_4.7H_2O$, 15 mg $CaCl_2.2H_2O$, and 0.5 mg thiamine per liter), and agar (15 g l^{-1}). Liquid LB and minimal media contained glucose and xylose concentrations as indicated in the text. Liquid minimal medium additionally contained trace metals from a stock solution (1 ml l^{-1}) (Causey et al., 2003). 4-Morpholino-propane-sulfonic acid (MOPS) was added to all shake-flask cultures for pH control (50mM, pH 7.4). Isopropyl-β-D-thiogalactopyranoside (IPTG) was used to induce plasmid genes under control of a *tac* promoter. Our previous study (Cirino et al., 2006) and preliminary results during this study showed that increasing the concentration of IPTG beyond 100 μM had no effect on xylitol production in batch cultures. A higher concentration of IPTG (300 μM) was used in fed-batch cultivations to ensure sufficient availability of IPTG in spite of higher cell densities, culture dilution during sugar feeding, and possible degradation of IPTG. Plasmids were maintained by inclusion of kanamycin monosulfate (50 μg ml^{-1}). Cell culture optical density was measured at 600 nm (OD_{600}) using a SPECTRAmax PLUS^{384} spectrophotometer (Molecular Devices). Cell dry weight (cdw) was calculated to be 0.3 g l^{-1} for OD_{600} at 1.0.

Aerobic batch culture specific growth rates (μ) in xylose minimal medium were determined as follows. Strains were conditioned on minimal medium plates containing

glucose (xylose was not used in plates, to prevent occurrence of mutations which might confer increased xylose uptake in transporter deletion strains). Colonies from these plates were used to inoculate overnight cultures in glucose minimal medium. These cultures were used to inoculate 100 mM xylose minimal medium cultures, which were shaken aerobically at 37 °C until cultures entered logarithmic growth phase (OD~0.7). The cells were then harvested by centrifugation, washed in minimal medium lacking xylose, resuspended in minimal medium to an OD of 0.15 and aliquotted into microtiter plates. Xylose was added to various final concentrations, and the cultures were shaken at 37 °C in a plate reader, with cell density monitored over time (absorbance at 600 nm). Specific growth rates were calculated based on the exponential increases in cell density, before appreciable depletion of xylose (exponential growth curve fits generally had R^2 values of ~0.99). Data reported represent the average of four independent cultures.

Xylitol, xylose, xylulose, glucose, and organic acid concentrations were determined as described (Cirino et al., 2006) using a Shimadzu LC-10AD HPLC equipped with a UV-monitor (210 nm) and refractive index detector (RID). Products were separated using an Aminex HPX-87H column (Bio-Rad Laboratories) with 4 mM H_2SO_4 as the mobile phase (0.5 ml min^{-1}, 45°C).

3.3.2 Genetic methods

Standard methods were used for plasmid construction, phage P1 transduction, electroporation, PCR and cloning (Sambrook and Russell, 2001, Miller, 1992). Strains used in this study are listed in Table **3-2**. *E. coli* K-12 W3110 (ATCC 27325) was used as the wild-type strain. Methods for construction of strains PC07 carrying *xylB* deletion

(W3110 and Δ*xylB*) and PC09 (W3110, Δ*xylB* and *crp**) were described previously (Cirino et al., 2006). Strains AH257 (W3110, Δ*xylE*::FRT-Km-FRT) and AH280 (W3110, Δ*xylG*::FRT-Km-FRT) were obtained from Shanmugam (Hasona et al., 2004). These two strains were used for introducing the *xylE* and *xylG* deletions into other strains via P1 phage transduction. FRT-flanked antibiotic resistance was deleted as described (Datsenko and Wanner, 2000). Following phage transduction and elimination of kanamycin resistance, the region of chromosome flanking each deleted *xyl* gene was amplified using PCR, and the PCR products were imaged on gels and sequenced to verify gene deletion. Inability to amplify *xylE* or *xylG* from their respective deletion strains further confirmed that these genes were eliminated.

Table **3-2**: Strains and plasmids used in this study.[a]

Strains/Plasmids	Relevant Characteristics	Reference
<u>Strains</u>		
W3110	Wild-type	ATCC 27325
AH257	W3110, Δ*xylE*::*FRT-Km-FRT* (Km^R)	(Hasona et al., 2004)
AH280	W3110, Δ*xylG*::*FRT-Km-FRT* (Km^R)	(Hasona et al., 2004)
PC05	W3110, *crp**::Tn*10* (Tet^R)	(Cirino et al., 2006)
PC07	W3110, Δ*xylB*::*FRT*	(Cirino et al., 2006)
PC08	PC05, Δ*xylB*::*FRT-aac-FRT* (Tet^R, Apr^R)	(Cirino et al., 2006)
PC09	PC05, Δ*xylB*::*FRT* (Tet^R)	(Cirino et al., 2006)
RK14	W3110, Δ*xylG*::*FRT*	This study
RK15	RK14, Δ*xylE*::*FRT-Km-FRT* (Km^R)	This study
RK16	RK14, Δ*xylE*::*FRT*	This study
RK27	PC08, Δ*xylG*::*FRT-Km-FRT* (Tet^R, Apr^R, Km^R)	This study
RK28	PC09, Δ*xylG*::*FRT* (Tet^R)	This study
RK33	RK28, Δ*xylE*::*FRT-Km-FRT* (Tet^R, Km^R)	This study
RK37	RK28, Δ*xylE*::*FRT* (Tet^R)	This study
<u>Plasmids</u>		
pLOI3809	*Km,* pBR322-origin vector for expression of XR or XDH, under control of *tac* promoter	(Cirino et al., 2006)
pLOI3815	pLOI3809 carrying CbXR gene	(Cirino et al., 2006)
pPCC203	pLOI3809 carrying *xylE* gene	This study
pPCC205	pLOI3809 carrying *xylFGH* genes	This study
pPCC107	pLOI3809 carrying CbXR-*xylE* operon	This study
pPCC207	pLOI3809 carrying CbXR-*xylFGH* operon	This study

[a] Tet = tetracycline, Apr = apramycin, Km = kanamycin

Plasmids used in this study are listed in Table **3-2**. Plasmids pLOI3809 and pLOI3815 were described previously (Cirino et al., 2006). Briefly, pLOI3809 is a medium copy vector containing a kanamycin resistance marker. Insertion of the xylose reductase gene from *C. boidinii* (CbXR, GenBank accession no. AF451326) downstream of a *tac* promoter and upstream of a transcription termination sequence in plasmid pLOI3809 resulted in plasmid pLOI3815. Plasmid pPCC201 was constructed as follows: the *xylFGH* operon was amplified from *E. coli* W3110 genomic DNA using iProof™ high fidelity polymerase (Bio-Rad Laboratories) with forward primer *xylFfor* (5'-CAGAAGGCCCTACACCATGAA-3') and reverse primer *xylHrev* (5'-AACTCAAAACCGGTAATACGTAACC-3'). The PCR product was TOPO-cloned into the pCR 2.1-TOPO cloning vector (Invitrogen) resulting in plasmid pPCC201 (verified by digestion analysis and DNA sequencing). The *xylFGH* fragment was then isolated by digestion with EcoRI. The 3.9 kb fragment was ligated into the EcoRI site of pLOI3809 and pLOI3815, resulting in expression plasmids pPCC205 and pPCC207, respectively. Plasmid pPCC207 contains the tetracistronic *CbXR-xylFGH* operon under control of a single *tac* promoter. Plasmid pPCC202 (*xylE* TOPO-cloned) was constructed in a similar manner as pPCC201 using primers *xylEfor* (5'-AGGAGGATACATATGAATACCCA GTATAATTCCAGTT-3') and *xylErev* (5'-GTGCTGGACAGGAAGATTAC-3'). Plasmid pPCC203 (*xylE* cloned into pLOI3809) was constructed in a similar manner as plasmid pPCC205 (ligation at the EcoRI site). To construct plasmid pPCC107, a 1.5 kb *xylE* gene fragment from pPCC203was isolated by digestion with HindIII followed by Klenow treatment and digestion with BglII. The *xylE* fragment was ligated downstream of the CbXR gene in pLOI3815 which was digested sequentially with SmaI and BglII.

3.3.3 Shake-flask cultures

All shake-flask culture experiments were performed as described previously (Cirino et al., 2006) in duplicate or triplicate, and all data points reported are the average of at least two experiments. Shake-flask cultures for xylitol production contained 50 ml medium in 250 ml baffled flasks (approximate initial concentrations: 300 mM xylose, 100 mM glucose) and were grown at 30°C and 250 rpm. Seed cultures were prepared by inoculating 3 ml of medium (13 mm x 100 mm tube) with a few colonies from a fresh plate (LB plates for LB cultures, minimal medium plates containing 2% glucose for minimal medium cultures). Seeds were grown to an OD_{600} of 2.0-4.0, and shake-flask cultures were inoculated directly from the seed cultures by dilution to a final OD_{600} of 0.1. Enzyme expression was induced with 100 μM IPTG in shake-flasks at the time of inoculation.

3.3.4 Bioreactor cultivations

Experiments were performed in a SixFors, parallel fermentation system (ATR). Each bioreactor had a working volume of 500 ml with the temperature (30 ^{o}C), pH (7.0), agitation, and dissolved oxygen (DO) controlled using the IRIS software supplied by the manufacturer. pH was controlled by adding 1M KOH. DO concentration was initially 100% (air saturation) and was allowed to drop to no less than 5% saturation during each cultivation by oxygen enrichment and by varying the agitation rate (between 400 and 750 rpm).A sterile air/O_2 mixture was continuously fed at a rate of 500 ml min^{-1} and the air/O_2 ratio was controlled using an automatic three-way valve.

Fed-batch cultivations contained minimal medium as described above (without MOPS), initially containing approximately 300 mM xylose and 100 mM glucose. Culture supernatants were analyzed by HPLC throughout the cultivations, and sugars were added manually: glucose was fed in 50 mM doses upon each glucose depletion and xylose was added to maintain concentrations above 100 mM. The cultivation seed culture was prepared as follows: colonies growing on fresh minimal plates (2% glucose) were used to inoculate 3 ml pre-seed cultures containing 100 mM glucose and 50 mM MOPS. Pre-seeds were grown at 37°C to OD_{600} of ~ 2.5, and were used to inoculate (by dilution) 50 ml seed cultures in 250 ml flasks grown at 30°C and 250 rpm. When the OD_{600} was between 1 and 2 (log phase), seeds were harvested by centrifugation (3750 rpm, room temperature) and cells were resuspended in 5 ml fresh medium to provide an inoculum of 30 mgcdwl^{-1} in the 500 ml working volume (OD_{600} ~ 0.1). IPTG was added to the vessels prior to inoculation. Controlled cultivations were performed in triplicate and showed reproducible growth and metabolite profiles. Average values are difficult to calculate due to differences in sampling and sugar feeding times between replicated runs, so data in Figure **3-2** and Figure **3-3** are for a single, representative cultivation.

3.4 Results

3.4.1 Influence of *xyl* transporter deletions on xylitol production

Strains and plasmids used in this study are listed in Table **3-2**. We first sought to determine to what extent the ATP-dependent transporter XylFGH contributes to xylitol production by strain PC09 in the presence of glucose and xylose. Figure **3-1a** depicts the

time-courses of xylitol production from shake-flask batch cultures of strains expressing CbXR from plasmid pLOI3815, and Figure **3-1b** depicts the glucose concentration profiles of these cultures. Table **3-3** lists pertinent culture data after 72 h. Cultures initially contained approximately 100 mM glucose and 300 mM xylose. Xylitol produced is essentially equal to xylose consumed in all cases with minor differences between these values resulting from evaporation (xylitol concentrations are slightly elevated). Strains PC09, RK28 and RK37 (*crp** strains) show similar growth profiles. PC09 produces 290 mMxylitol in 72 h, converting essentially all xylose in the medium to xylitol. Deletion of the *xylG* gene (strain RK28) results in a 40% decrease in the final xylitol titer (174 mM). Subsequent deletion of *xylE* (RK37) further reduces xylitol production to a final titer of 113 mM, although xylitol production is notably not eliminated. In addition to the xylose transporters, xylose clearly enters the cells through as yet un-characterized mechanisms. Based on the low affinity of this "secondary" transport (see Section **3.5**), we attribute this to non-specific uptake. Assuming this non-specific uptake is not increased or up-regulated as a result of *xyl* transporter deletions, up to 40% of xylitol produced by strain PC09 may be attributed to secondary transport when xylose concentrations are high (e.g., >100 mM).

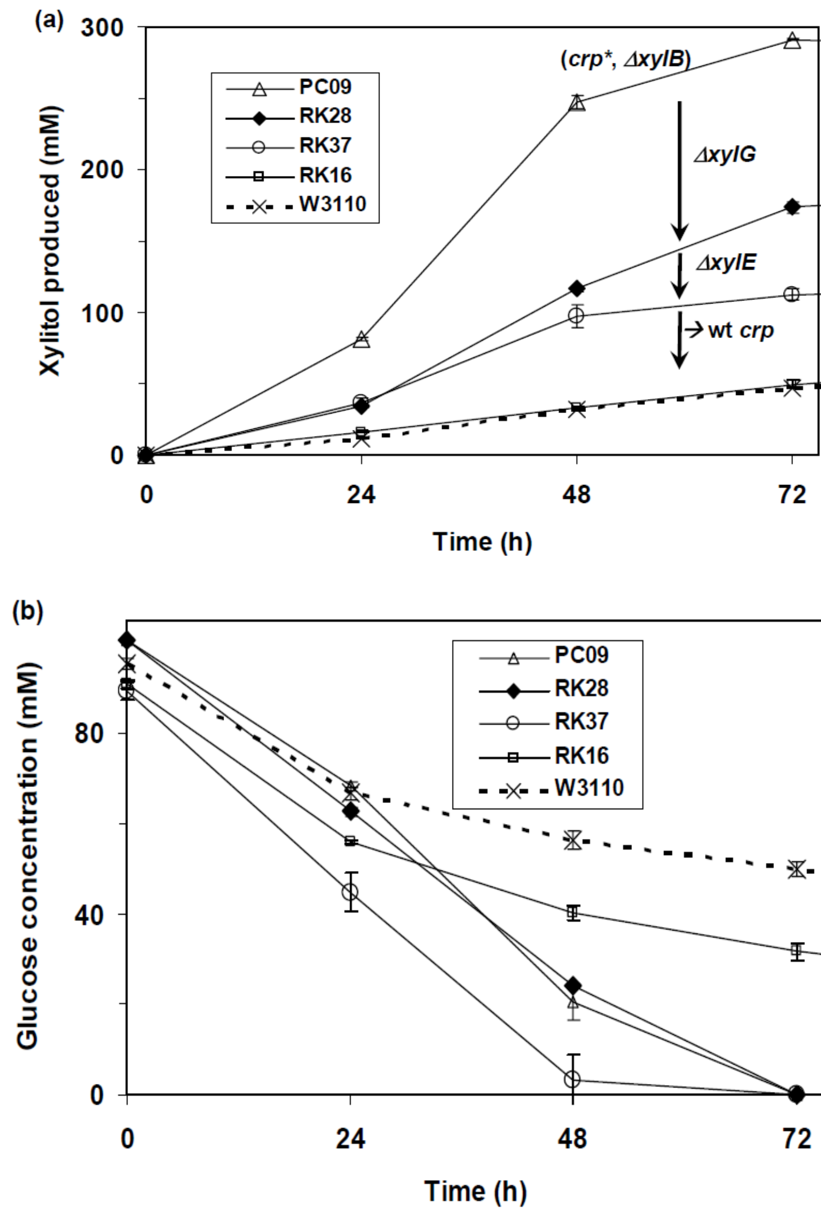

Figure **3-1**: Comparison between strains with or without deletions in xylose transporters (*xylG* and/or *xylE*), and expressing wild-type *crp* vs. *crp**. All strains also express CbXR from pLOI3815. Cultures were grown in shake-flasks containing LB medium supplemented with approximately 100 mM glucose, 300 mM xylose and 50 mM MOPS buffer. (a) Time-courses of xylitol production. (b) Time-courses of glucose concentration. Data represent the average of at least two values, with standard deviations shown as error bars.

Table **3-3**: Summary of the 72-h time-point results for cultures depicted in Figure **3-1**.

	Glucose consumed (mM)	Xylose consumed (mM)	Xylitol produced (mM)	Acetate produced (mM)	Cell OD_{600}	PH
PC09	101	289	291	0.4	7.9	7.6
RK28	101	167	174	0.4	8.9	7.6
RK37	89	106	113	0.4	7.4	7.5
RK16	59	41	50	48	5.9±0.7	4.6
W3110	46	35	47	64	5.8	4.6

Standard deviations were less than 10% of the average except where indicated.

Xylitol production by wild-type strain W3110 expressing CbXR is nearly identical to xylitol production by RK16 carrying deletions in both *xyl* transporters (~50 mM), and this xylitol titer is 56% lower than that produced by strain RK37 (carrying *crp** but otherwise isogenic). This strongly suggests that the XylE and XylFGH transporters are not responsible for xylose uptake in the presence of glucose with wild-type CRP (they are tightly regulated by CRP), but rather secondary uptake is solely responsible for xylitol production in this case. W3110 and PC07 (carrying the *xylB* deletion) behave identically in the glucose-xylose mixture, which is expected if *xylA* and/or *xylB* are also tightly regulated by CRP and xylose is therefore not metabolized (xylose consumed is equal to xylitol produced).

Acetate was the only secreted metabolite (other than xylitol) that accumulated to any appreciable extent in these cultures (e.g., formate, lactate and pyruvate never exceeded 10 mM). Strains W3110 and RK16 secrete acetate continuously throughout the culture, causing a drop in culture pH, reduced glucose consumption, and lower final OD's (Table **3-3**). In contrast, cultures of *crp** strains do not accumulate significant amounts of acetate and all glucose is consumed.

3.4.2 Overexpression of Xyl transporters

We next tested whether induced expression of plasmid-borne XylE or XylFGH under control of a glucose-insensitive promoter (i.e., *tac*) could sufficiently enhance xylose uptake and availability to facilitate high levels of xylitol production in a wild-type *crp* strain growing in glucose. Plasmid pPCC107 contains the *xylE* gene placed downstream of CbXR (under p*tac* control). Similarly, pPCC207 contains the *xylFGH* sequence downstream of CbXR. Strains of PC07 (W3110,Δ*xylB*) harboring either pPCC107, pPCC207, or control plasmids pLOI3815 (expressing only CbXR) or pLOI3809 (no gene) were compared in batch, shake-flask, minimal medium cultures initially containing 100 mM glucose and 300 mM xylose. Results from analysis of these cultures at 72 h are presented in Table **3-4**. Co-expression of either transporter with CbXR drastically enhances xylose transport beyond that of non-specific uptake (i.e., with plasmid pLOI3815), resulting in significant improvements in xylitol production (132 mM vs. 25 mM). Among these strains, ability to uptake xylose and produce xylitol does not correlate with reduced acetate secretion, but rather reduced cell yield. In fact, PC07 + pPCC207 expressing XylFGH consumed the most glucose and produced the most acetate.

Table **3-4**: Comparison of results from cultures of strain PC07 harboring various plasmids with or without xylose transporters.

Plasmid	Xylitol Produced (mM)	Glucose consumed (mM)	Acetate produced (mM)	Final pH	OD
pLOI3809	0 ± 0.7	89	34 ± 19	4.7	6.8
pLOI3815	25 ± 4	65 ± 7.4	30 ± 3	4.9	6.1 ± 1.3
pPCC107	132	62	32	5.4	3.4
pPCC207	132	98	47	4.7	4.6

Cultures were grown in minimal medium initially containing 100 mM glucose and 300. mM xylose. Time-points at 72 h are shown. Standard deviations were less than 10% of the average except where indicated.

These shake-flask experiments provided incentive to pursue higher xylitol productivity using these plasmids in fed-batch, controlled cultivations. We first compared strains PC07 + pPCC107 and PC07 + pPCC207 to our traditional xylitol-producing *crp** strain PC09 + pLOI3815. The bioreactors initially contained 500 ml minimal medium (no MOPS buffer) with approximately 100 mM glucose plus 300 mM xylose. pH was maintained at 7.0 and dissolved oxygen (DO) started at 100% (relative to air saturation) and was not allowed to drop below 5%. Xylose concentrations were manually maintained above 100 mM by xylose addition in 100 mM doses, and glucose was fed in 50 mM doses upon each glucose depletion. More information regarding the seed and cultivation protocols can be found in section **3.3.4**. Figure **3-2a** shows the time-courses of xylitol production for these strains, and Figure **3-2b** depicts their glucose consumption profiles, with the acetate concentration at 66 h given in parentheses. Co-expression of either transporter with CbXR in PC07 results in xylitol production similar to that achieved from strain PC09 expressing only CbXR. Xylose is not metabolized by the cells due to the *xylB* deletion. However, as previously reported (Cirino et al., 2006), strain PC09 does secrete

up to 25 mM xylulose in minimal medium containing xylose, due to expression of xylose isomerase (XylA) (not shown). This does not occur in PC07 because *xylA* is tightly regulated by wild-type CRP, so xylose consumption is essentially equal to xylitol production. Whereas in the previous shake-flask cultures PC07 + pPCC207 (expressing XylFGH) produced more acetate than PC07 + pPCC107 (expressing XylE), under these controlled bioreactor conditions the strain expressing XylFGH produced low amounts of acetate and behaves similar to PC09 + pLOI3815. This reduced acetate is likely a result of maintaining higher DO levels as cell density increased (Phue and Shiloach, 2005) and/or controlling constant pH (both significantly affect culture performance).

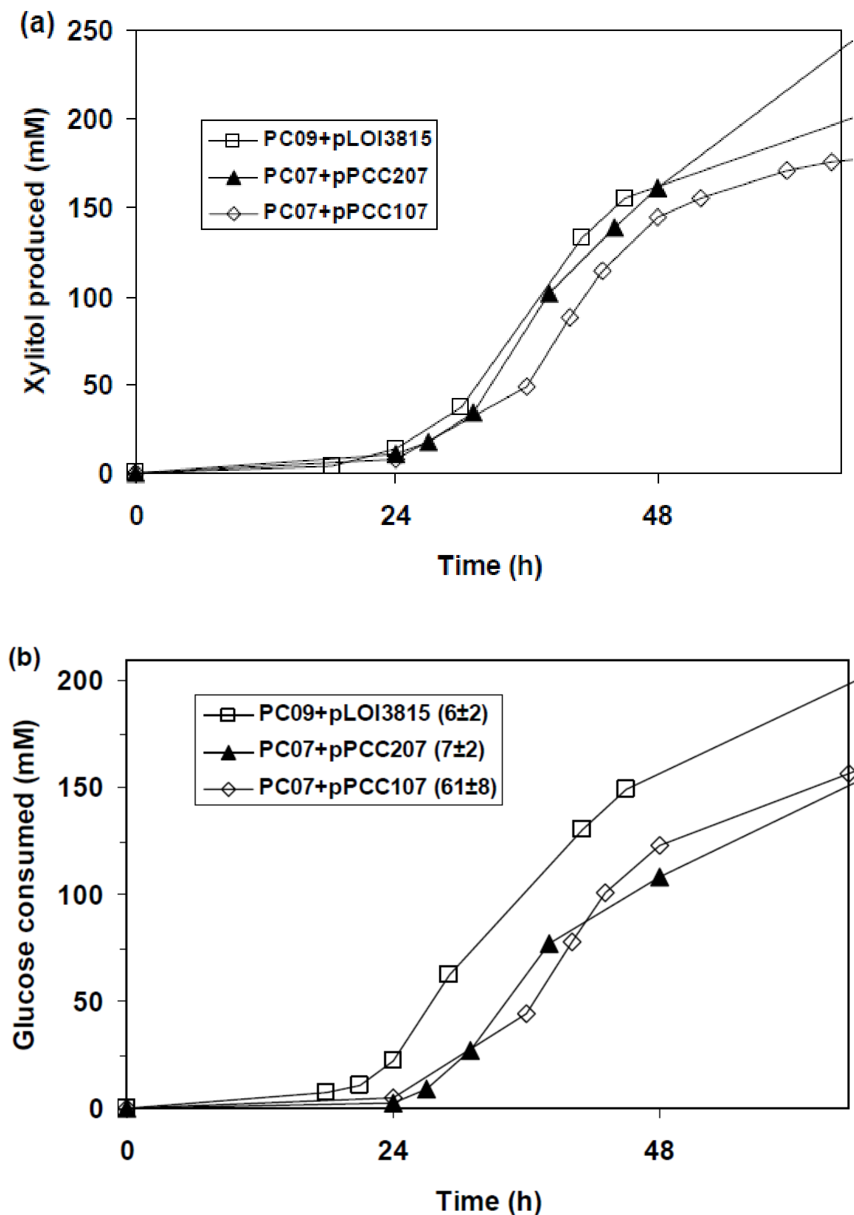

Figure **3-2**: Bioreactor cultivation profiles comparing induced expression of XylE or XylFGH in PC07 (wt *crp*) to strain PC09 (*crp**). All three strains also express CbXR. Cultures initially contained approximately 100 mM glucose and 300 mM xylose in minimal medium. Glucose was added in 50 mM doses upon depletion and xylose was maintained above 100 mM. DO was not allowed to drop below 5% and pH was maintained at 7.0. (a) Time-courses of xylitol production. (b) Time-courses of glucose consumption. Acetate concentration at 66 h is given in parentheses (the average value from at least two cultivations, with standard deviation reported).

Finally, extended fed-batch bioreactor cultivation was performed with strain PC07 + pPCC207 in glucose-xylose minimal medium, and these results are presented in Figure **3-3**. This strain produced ~225 mM xylitol (34 g l^{-1}) in 48 h and ~370 mM (56 g l^{-1}) in 96 h. Overall yields were ~20 xylitol per dry biomass (g g^{-1}) and 1.7 xylitol per glucose consumed (mol mol^{-1}), and the average specific xylitol productivity from 24 to 96 h was ~0.33 g (g cdw^{-1}) h^{-1}. These results are similar to what was previously reported for strain PC09 in a 10 l, fed-batch cultivation (Cirino et al., 2006). Notably, essentially all glucose and xylose are consumed, with no xylulose and little acetate production. The similarity in titer and yield achieved with PC09 + pLOI3815 and PC07 + pPCC207 suggests that in these strains xylitol production is not limited by transport of xylose.

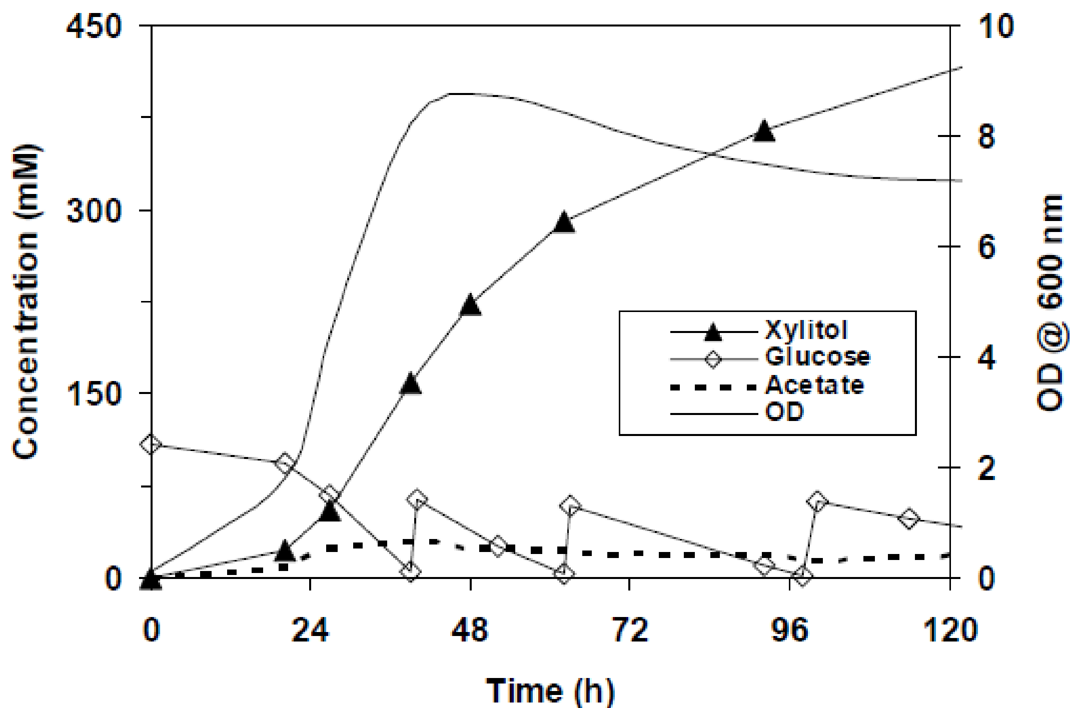

Figure **3-3**: Extended fed-batch cultivation of PC07 + pPCC207 growing in minimal medium. Conditions are as described for Figure **3-2**.

3.5 Discussion

Microbial carbohydrate transporter proteins are generally considered promiscuous; many studies have demonstrated relaxed specificity for a variety of uptake systems (Hamacher et al., 2002, Chaillou et al., 1999, Snoep et al., 1994, Dimarco and Romano, 1985, Iida et al., 1984, Heller et al., 1980). As a result, multiple transporters may be acting on a substrate, provided they are expressed. It is therefore important to characterize a strain's specific and non-specific substrate transport mechanisms when engineering and modeling metabolic pathways (Stephanopoulos, 2007).

We previously used a *crp* E. coli* mutant to transport xylose and enable xylitol production while metabolizing glucose. In this context, xylitol production serves as a platform for studying NADPH trafficking in whole-cells catalyzing a heterologous, NADPH-dependent transformation, where the substrate to the transformation is readily transported into the cell (thus avoiding problems associated with limited substrate availability (Chen, 2007)). Here we investigated the contributions of the xylose transporters XylE and XylFGH in xylitol production. Deletion of *xylG* significantly lowers xylitol production, in agreement with the finding that XylFGH is the primary xylose transporter even in high xylose concentrations (Hasona et al., 2004). Xylitol production by strains with deletions in both *xylE* and *xylG* suggests that secondary xylose uptake is considerable (up to 40% for the *crp** strain) when xylose concentrations are high.

Measuring xylitol production allowed us to study xylose uptake in growing cultures without requiring xylose metabolism, so the effects of glucose and CRP on xylose transport could be investigated. Our results demonstrate tight regulation of *xylE*

and *xylFGH* by the glucose/cAMP/CRP system (even in the presence of xylose), or at least these genes are not active enough in the presence of glucose (with wild-type *crp*) to measure a contribution to xylitol production. Considering the known promiscuity of many transporters, it was not surprising to find that xylitol production was not abolished in RK16 and RK37. However, the extent to which secondary uptake contributes to xylitol production was unexpected and led us to question whether this activity was in fact "nonspecific". The growth rates on xylose-limited minimal medium should provide estimates of the relative *in vivo* affinities of cellular transport activities (i.e., in the context of metabolism and growth). Table **3-5** lists specific growth rates from batch cultures with different initial xylose concentrations, for the wild-type and *xyl* deletion strains. Strains were not previously conditioned to growth on xylose plates in these experiments. Growth rates were calculated at the onset of log-phase growth, as described in Section **3.3.1**. Strain RK16 grows much slower than W3110 at low xylose concentrations (e.g., 80-fold slower than W3110 in 0.5 mM xylose), but growth is recovered to near that of wild type with xylose concentrations in the 100 mM range. The *xylE* and *xylG* deletion strains (AH257 and AH280, respectively) show intermediate growth rates in low xylose concentrations, with Δ*xylG* impairing growth much more significantly than Δ*xylE* (as previously reported (Hasona et al., 2004)). Similar trends in growth rates were observed on minimal xylose plates.

Table **3-5**: Specific growth rates of W3110 and *xyl* transporter deletion strains growing in xylose minimal medium (h^{-1} ± standard deviation).

Xylose concentration	W3110	AH257	AH280	RK16
0.5 mM	0.16 ± 0.02	0.06 ± 0.01	0.03 ± 0.02	0.002 ± 0.002
10 mM	0.18 ± 0.02	0.15 ± 0.01	0.10 ± 0.01	0.09 ± 0.01
20 mM	0.19 ± 0.03	0.18 ± 0.01	0.11 ± 0.01	0.11 ± 0.07
100 mM	0.25 ± 0.03	0.24 ± 0.04	0.20 ± 0.04	0.22 ± 0.04

Given the low apparent affinity of RK16 for xylose (half-maximal growth rate at ~20 mM), it is unlikely that the secondary uptake is the result of a xylose-specific transporter. Likely promiscuous culprits include transporters of arabinose (AraE) (Hasona et al., 2004), galactose (GalP) (Hernandez-Montalvo et al., 2003), glycerol (GlpF) (Heller et al., 1980), lactose (LacY) (Kaback, 2005, Heller and Wilson, 1979), and pts systems (Kornberg et al., 2000, Kornberg and Riordan, 1976). Importantly, many of these genes were reported to be significantly up-regulated when growing on xylose versus glucose (in the absence of the native inducers for these genes) (Gonzalez et al., 2002). The inclusion of IPTG for xylitol production favors the possibility that LacY mediates xylose uptake in *crp** strains (perhaps explaining the difference in xylitol production between RK16 and RK37). The possibility that diffusion of xylose through the cell membrane contributes to secondary transport also should not be ruled out (Sacerdote and Szostak, 2005).

A common challenge during the development of microbial based processes is to reduce acetate accumulation in aerobic fermentations (Veit et al., 2007, Wolfe, 2005, Lee, 1996). It is not clear if the reduced acetate overflow metabolism observed during xylitol production with strain PC09 (Cirino et al., 2006) is simply due to increased

availability of xylose in the *crp** strain (increasing internalized xylose increases NADPH oxidation via CbXR, promoting acetyl-CoA oxidation and reduced acetate secretion (Vemuri et al., 2006, Wolfe, 2005)), or whether *crp** plays additional, pleiotropic roles in this phenotype. For example, CRP* may contribute to increased expression of TCA cycle genes under conditions of excess glucose (Phue and Shiloach, 2005, Gosset et al., 2004, Neidhardt and Curtiss, 1996). In combination with a heterologous cofactor sink, this could suppress the onset of overflow metabolism, as was recently demonstrated by expression of NADH oxidase in *E. coli* (Vemuri et al., 2006). Note that *crp** in the absence of xylitol production is not sufficient to reduce acetate overflow: strain PC09 + pLOI3815 growing on glucose (no xylose) and strain PC09 + pLOI3809 (no CbXR) growing in glucose + xylose both secrete high levels of acetate, similar to that observed with wild-type *crp* (data not shown). Increasing internal xylose availability by overexpressing xylose transporters in PC07 drastically increases xylitol production, but this is not sufficient to alleviate acetate production in shake-flasks (Table **3-4**). The observed differences in acetate secretion between RK16 and RK37 (Table **3-3**), and between PC09 and PC07 (Table **3-4** and Figure **3-2b**) implicate additional *crp** effects (beyond xylose uptake). While warranting further investigation, these results support the hypothesis that *crp** promotes reduced acetate overflow.

Kwon and coworkers reported 12 g l^{-1} h^{-1} xylitol produced during a fed-batch, cell-recycle fermentation of *C. tropicalis* in a submerged membrane bioreactor, with final cell density of 76 g l^{-1} (Kwon et al., 2006). With little optimization, PC07 + pPCC207 (expressing XylFGH) produced 56 g l^{-1} xylitol in a 96 h fed-batch cultivation using strictly defined mineral salts medium, and with a final cell density of ~2.4 g l^{-1} (notably

low). While this level of xylitol production is not competitive with the best yeast processes, this strain presents an alternative to PC09 as a starting point for improving xylitol production by *E. coli* through metabolic engineering and process optimization. Importantly, average-specific productivity (~0.33 g (g cdw^{-1}) h^{-1}) is considerably higher than that reported for *C. tropicalis* (~0.22 g (g cdw^{-1}) h^{-1}), and significant improvements are expected from high-density resting cells with optimized oxygen control.

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Chapter 4

Comparison between *Escherichia coli* K-12 strains W3110 and MG1655 and wild-type *E. coli* B as platforms for xylitol production

4.1 Abstract

Escherichia coli W3110 was previously engineered to produce xylitol from a mixture of glucose plus xylose by expressing xylose reductase (CbXR) and deleting xylulokinase (Δ*xylB*), combined with either plasmid-based expression of a xylose transporter (XylE or XylFGH) (Khankal et al., 2008) or replacing the native *crp* gene with a mutant (*crp**) that alleviates glucose repression of xylose transport (Cirino et al., 2006). In this study, *E. coli* K-12 strains W3110 and MG1655 and wild-type *E. coli* B were compared as platforms for xylitol production from glucose-xylose mixtures using these same strategies. The engineered strains were compared in fed-batch fermentations and as non-growing resting cells. Expression of CRP* in the *E. coli* B strains tested was unable to enhance xylose uptake in the presence of glucose. Xylitol production was similar for the (*crp**, Δ*xylB*)-derivatives of W3110 and MG1655 expressing CbXR (average specific productivities of ~0.43 g xylitol g cdw^{-1} h^{-1} in fed-batch fermentation). In contrast, results varied substantially between different Δ*xylB*-derivative strains coexpressing either XylE or XylFGH. Thus, the differences in genetic background between these strains can profoundly influence metabolic engineering strategies.

4.2 Introduction

Escherichia coli strains are an established platform organism for bacterial physiology studies, metabolic engineering and overproduction of native and non-native small molecules. Of the many laboratory strains available, the two closely related non-pathogenic *E. coli* K-12 sub-strains MG1655 and W3110 are the most widely used and well-characterized, including the availability of complete genome sequences (Hayashi et al., 2006, Blattner et al., 1997), and comparative "omic" analyses (Herring and Palsson, 2007, Vijayendran et al., 2007, Hayashi et al., 2006). Although these two strains have nearly identical genome structures and show very similar growth behavior in controlled cultivation, significant differences in gene expression between W3110 and MG1655 were observed by Vijayendran and co-workers (Vijayendran et al., 2007). One of the most notable differences between these strains is in the sequence and function of the RNA polymerase sigma factor 38 RpoS (σ^s), involved in general stress response and stationary phase gene expression (Hengge-Aronis, 2002). The *rpoS* sequence in MG1655 encodes a 330 amino acid protein. Different lab stocks of W3110 have been shown to carry different alleles of *rpoS* (Subbarayan and Sarkar, 2004, Jishage and Ishihama, 1997). The W3110 strain used in our study carries a common allele that differs from that in MG1655 by a C→T transition at nucleotide position 97, resulting in an amber mutation at codon 33 (CAG→TAG). Many of the differences in gene expression between MG1655 and W3110 (having the same *rpoS* sequence as ours) identified by Vijayendran were in genes related to stress response and attributed to different roles of RpoS (Vijayendran et al., 2007).

E. coli B-type strains have several potential biotechnological advantages over *E. coli* K12 strains, including reduced acetate production and higher growth rates and yields on recombinant proteins (Shiloach et al., 1996, Luli and Strohl, 1990). Transcription and metabolic flux analyses comparing B-type strain BL21 and K-12 strain JM109 suggest that increased flux through anaplerotic pathways and the glyoxylate shunt pathway are largely re*sponsi*ble for reduced acetate overflow metabolism and elevated biomass yields with *E. coli* B (Phue et al., 2007, Phue and Shiloach, 2004, Noronha et al., 2000). Wild-type *E. coli* B obtained from the Coli Genetic Stock Center (strain CGSC 5365) expresses a 330-amino acid allele of RpoS in which codon 33 is GAG, as is common for B-type *E. col*i strains (Atlung et al., 2002).

We previously described engineering *E. coli* W3110 to produce xylitol during growth on a mixture of glucose and xylose (Khankal et al., 2008, Cirino et al., 2006). In one approach to overcome catabolite repression, xylose uptake and xylitol production in the presence of glucose was enabled by the plasmid-based co-expression of CbXR and a xylose transport system (either XylE or XylFGH) (Khankal et al., 2008). In a second approach, the gene coding for the wild-type cAMP receptor protein (CRP) was replaced with a *crp* variant (*crp**) that enabled xylose uptake and metabolism in the presence of glucose. Subsequent deletion of *xylB* (encoding xylulokinase) prevented xylose metabolism, while expression of NADPH-dependent xylose reductase from *Candida boidinii* (CbXR) resulted in xylose reduction to xylitol during glucose metabolism (Cirino et al., 2006).

The noteworthy differences in metabolism between K-12 type strains W3110 and MG1655 and B-type *E. coli* stress the importance of selecting an appropriate ‘‘platform’’

E. coli strain for testing metabolic engineering strategies. In this study, we compare our two approaches for xylitol production in all three of these common lab strains. The engineered strains are compared in controlled, fed-batch fermentations and as non-growing resting cells. We find that CRP* relieves glucose repression of xylose transport in MG1655 but not *E. coli* B. In fed-batch fermentation xylitol production was similar for the (*crp**, Δ*xylB*)-derivatives of W3110 and MG1655 expressing CbXR. Resting cell performance was also similar for these strains, although the yield of xylitol produced per glucose consumed was higher for the W3110 derivative (4.1 compared to 3.2). In contrast, different Δ*xylB* -derivative strains co-expressing either XylE or XylFGH with CbXR yielded significantly different results that do not obviously reflect the documented differences in genetic and growth properties of the wild-type strains.

4.3 Materials and methods

4.3.1 General

All strains were maintained on plates containing either Luria-Bertani (LB) medium or minimal medium containing mineral salts (per liter: 3.4 g KH_2PO_4, 5.2 g K_2HPO_4, 3.3 g $(NH_4)_2HPO_4$, 0.25 g $MgSO_4.7H_2O$, 15 mg $CaCl_2.2H_2O$, 0.5 mg thiamine), glucose (20 g l^{-1}), and agar (15 g l^{-1}). Liquid LB and minimal media contained glucose and xylose concentrations as indicated. Liquid minimal medium additionally contained trace metals from a stock solution (1 ml l^{-1}) (Causey et al., 2003) and where indicated was supplemented with 5 g yeast extract l^{-1}. 4-Morpholino-propane-sulfonic acid (MOPS) was added to all shake-flask cultures for pH control (50 or 100 mM, pH 7.4). Isopropyl-

β-D-thiogalactopyranoside (IPTG) was used to induce plasmid genes under control of a *tac* promoter. Plasmids were maintained by inclusion of kanamycin monosulfate (50 μg ml^{-1}). Cell dry weight (cdw) was calculated to be 0.33 g l^{-1} for OD_{600} at 1.0. Xylitol, xylose, xylulose, glucose, and organic acid concentrations were determined as described (Cirino et al., 2006).

4.3.2 Genetic methods

Standard methods were used for plasmid construction, phage P1 transduction, electroporation, PCR and cloning. Strains used in this study and their sources are listed in Table **4-1**. Two different wild-type *E. coli* B strains were tested: American Type Culture Collection (ATCC) strain 11303 and *E. coli* Genetic Stock Center (CGSC) strain 5365. Construction of strains PC05, PC07, and PC09 was described previously (Cirino et al., 2006). P1 phage lysates of strain ET25 (*crp**::Tn10) (Eppler and Boos, 1999) and PC06 (W3110, *ΔxylB*::FRT-*aac*-FRT) (Cirino et al., 2006) were respectively used to initiate propagation of *crp** and the *xylB* deletion to other strains. Transductants were selected based on resistance to apramycin (for *ΔxylB*) or tetracycline (for *crp**). Due to the different restriction systems between *E. coli* K-12 strains and B-type strains, B strain WA837 (r_B^-, m_B^+) was used as an intermediate host for transduction of *crp** and the *xylB* deletion into *E. coli* B (Dien et al., 2001).

Table **4-1**: Strains and plasmids used in this study

	Relevant Characteristics	Reference
Strains		
W3110	Wild-type	ATCC 27325
MG1655	Wild-type	ATCC 47076
E. coli B	Wild-type	CGSC 5365
E. coli B	Wild-type	ATCC 11303
PC05	W3110, *crp**::Tn*10*	(Cirino et al., 2006)
PC07	W3110, Δ*xylB*	(Cirino et al., 2006)
PC09	PC05, Δ*xylB*	(Cirino et al., 2006)
FL01	MG1655, *crp**::Tn*10*	This study
FL03	MG1655, Δ*xylB*	This study
FL05	FL01, Δ*xylB*	This study
FL06	*E. coli* B, *crp**::Tn*10*	This study
FL07	*E. coli* B, Δ*xylB*	This study
FL08	FL06, Δ*xylB*	This study
Plasmids		
pLOI3815	pBR322-origin vector for expression of CbXR, under control of *tac* promoter	(Cirino et al., 2006)
pPCC107	pLOI3815 carrying *xylE*	(Khankal et al., 2008)
pPCC207	pLOI3815 carrying *xylFGH*	(Khankal et al., 2008)

Plasmids used in this study are listed in Table **4-1**. Plasmid pLOI3815 was described previously (Cirino et al., 2006). Briefly, it is a medium copy pBR322-origin vector carrying a kanamycin resistance marker and the xylose reductase gene from *Candida boidinii*, which is located downstream of *tac* promoter and upstream of a

transcription termination sequence. Plasmids pPCC107 and pPCC207 were described previously (Khankal et al., 2008). Briefly, xylose transporter genes *xylE* (xylose-proton symporter) and *xylFGH* (ATP-dependent xylose transporter) were cloned downstream of *CbXR* in pLOI3815 to make pPCC107 and pPCC207 plasmids, respectively. The *CbXR-xylE* operon in pPCC107 and *CbXR-xylFGH* operon in pPCC207 are each under control of a single *tac* promoter upstream.

4.3.3 Shake-flask cultures

All shake-flask culture experiments were performed in baffled flasks as described previously (Cirino et al., 2006) in duplicate or triplicate, and all data points reported are the average of at least two experiments. Cultures expressing CbXR were grown at 30°C; all others were grown at 37°C. For LB medium experiments, seed cultures were prepared by inoculating 10 ml of LB medium with a few colonies from a fresh LB plate. Seed cultures for minimal medium shake-flask experiments (with or without 5 g yeast extract l^{-1}) were prepared as follows: 10 ml LB pre-seed cultures were first grown overnight by inoculation with colonies from LB plates, and a few drops of those overnight cultures were used to inoculate 10 ml minimal medium seed cultures (with or without yeast extract) containing 5 g glucose l^{-1}, in 125 ml flasks. Seed cultures were grown to a density of 0.6-1.2 g cdw l^{-1}, and shake-flask cultures were inoculated directly from the seed cultures by dilution to 0.033 g cdw l^{-1}. Enzyme expression was induced with 100 μM IPTG in shake-flasks at the time of inoculation.

4.3.4 Fermentations

Experiments were performed in a SixFors parallel fermentation system (ATR). Each bioreactor had a working volume of 500 ml with the temperature (30°C), pH (7.0), agitation, and dissolved oxygen (DO) controlled. pH was controlled by adding 1 M KOH and antifoam was added manually if required. DO concentration was initially 100% (air saturation) and was allowed to drop to no less than 20% saturation during each fermentation by oxygen enrichment and by varying the agitation rate (between 400 and 800 rpm). A sterile air/O_2 mixture was continuously fed at a rate of 500 ml min^{-1} and the air/O_2 ratio was controlled using an automatic three-way valve.

For fermentations containing yeast extract, seed cultures were prepared as follows: colonies growing on fresh LB plates were used to inoculate 10 ml pre-seed cultures in minimal media containing 5 g yeast extract l^{-1}, 5 g glucose l^{-1}, and 50 mM MOPS. Pre-seeds were grown at 37°C overnight and were used to inoculate (by dilution) 50 ml seed cultures in 250 ml flasks grown at 30°C and 250 rpm. When the density was between 0.33 and 0.66 g cdw l^{-1}, seeds were harvested by centrifugation and cells were resuspended in 5 ml spent medium to provide an inoculum of 0.033 g cdw l^{-1} in the 500 ml working volume.

For high cell density fermentations performed in minimal medium without yeast extract, seed cultures were prepared as follows: colonies growing on fresh minimal plates were used to inoculate 3 ml pre-seed cultures (minimal medium) containing 100 mM glucose and 50 mM MOPS. Pre-seeds were grown at 37°C to ~0.8 g cdw l^{-1}, and were used to inoculate 50 ml minimal medium seed cultures in 250 ml flasks grown at 30°C and 250 rpm. When the density was between 0.33 and 0.66 g cdw l^{-1}, seeds were

harvested by centrifugation and cells were resuspended in 5 ml spent medium to provide an inoculum of 0.033 g cdw l^{-1} in the 500 ml working volume.

4.3.5 Resting cells

Resting cells were prepared using a protocol described previously (Cirino et al., 2006) and adapted as follows. Colonies growing on fresh LB plates were used to inoculate 10 ml overnight LB cultures. These cultures were then used to inoculate pre-seed cultures in minimal medium containing 5 g glucose l^{-1}. Pre-seed cultures were grown at 37°C to a density of 0.6-1.2 g cdw l^{-1}, and were used to inoculate seed cultures (25 ml minimal medium supplemented with 50 mM glucose, 50 mM xylose, and 50 mM MOPS) to a density of 0.033 g cdw l^{-1} (in 250 ml baffled flasks). After 9-10 hours of growth (density between 0.33 and 0.5 g cdw l^{-1}), the cultures were used to inoculate 200 ml of minimal medium supplemented with 100 mM glucose, 50 mM xylose, 50 mM MOPS, and 100 μM IPTG (in 1 l flasks) and were grown at 30°C and 250 rpm. When the cells reached a density between 0.6 and 1.2 g cdw l^{-1}, chloramphenicol (50 μg ml^{-1}) was added and the cells were harvested by centrifugation, washed twice in minimal medium lacking a carbon and nitrogen source, and resuspended to a ‘‘working density’’ of 0.66 g cdw l^{-1} in 30 ml minimal medium containing 50 mM glucose and 300 mM xylose, but lacking a nitrogen source. Cell densities did not change significantly after resuspension for approximately two days. The reported data was collected after 48 hours of biotransformation. Background levels of xylitol production were noted in control experiments in which glucose was not added to the resting cells. When calculating the molar yield of xylitol per glucose consumed (Y_{RPG}) and productivity, the background

level of xylitol produced in the absence of glucose was subtracted from the amount of xylitol produced in the presence of glucose.

4.4 Results and discussion

4.4.1 Expression of CRP*

The CRP* mutant used in this study confers a maltose$^+$, lactose$^+$ phenotype in an *E. coli* K-12 *cya* mutant (Sabourin and Beckwith, 1975) . The sequence of wild-type CRP is identical in strains MG1655 and *E. coli* B, while W3110 contains a single base substitution in the *crp* gene corresponding to a T28K mutation (Hayashi et al., 2006). Relative to the MG1655 CRP sequence, the CRP* used carries three amino acid substitutions: I112L, T127I, and A144T (and therefore an additional T28K substitution relative to that of W3110). Previous studies on a variety of CRP* mutants described in the literature demonstrate the complex regulatory properties of these proteins: all remain at least partly responsive to cyclic nucleotides, and the extent of "relief of catabolite repression" varies significantly depending on the CRP* mutant and gene under investigation (Karimova et al., 2004, Belduz et al., 1993, Aiba et al., 1985). Sugar consumption by W3110, MG1655, *E. coli* B, and their respective *crp** derivatives (PC05, FL01 and FL06) growing in LB medium supplemented with glucose and xylose is summarized in Table **4-2** . None of the wild-type strains consumed a significant amount of xylose in the presence of glucose. We previously described the ability of W3110-derivative strain PC05 to co-utilize glucose and xylose as a result of expressing CRP* in place of CRP (Cirino et al., 2006). Strain FL01 showed a sugar consumption pattern

similar to PC05. In contrast, replacing *crp* with *crp** in *E. coli* B (strain FL06) did not show the desired co-consumption phenotype (note this strain does consume xylose in the absence of glucose). This inability of CRP* to enable xylose consumption in the presence of glucose was also observed for ATCC 11303 wild-type *E. coli* B-strain (not shown).

Table **4-2**: Sugar consumption by W3110, MG1655, *E. coli* B, and their respective *crp** derivatives.

Strain	Glucose consumed (mM)	Xylose consumed (mM)
W3110	38	2
PC05	9	29
MG1655	40 ± 4	0
FL01	9	26
E. coli B	44	0
FL06	47	0

Results are given for 8-hour time point from shake-flask cultures grown at 37°C and containing LB medium supplemented with 100 mM each of glucose, xylose and MOPS buffer. Standard deviations were less than 10% unless noted.

While FL06 could not metabolize xylose in the presence of glucose, it was still possible that xylose transport (but not metabolism) was up-regulated in the context of *crp**. We therefore tested the ability of FL08 (*crp* ΔxylB*) expressing CbXR to produce xylitol while growing in a glucose-xylose mixture, compared to wild-type *E. coli* B, FL07, and the analogous W3110-derivative strains. As shown in Table **4-3**, similar low levels of xylitol are produced by W3110 and *E. coli* B and their *ΔxylB*-derivatives (neither XylA nor XylB play a role since neither is expressed in the presence of glucose). Whereas strain PC09 (expressing *crp**) produces approximately 270 mM xylitol, strain FL08 produced a level of xylitol similar to FL07 and wild-type *E. coli* B.

Table **4-3**: Xylitol production and glucose consumption in W3110, *E. coli* B, and their derivatives.

Strain	Glucose consumed (mM)	Xylitol produced mM)
W3110	50	58
PC07	50	58
PC09	94	269
B5365	117	78
FL07	89	61
FL08	95	69

Strains harboring plasmid pLOI3815 were grown in shake-flasks containing LB medium supplemented with xylose (300 mM), glucose (100 mM), and MOPS (50 mM). Results represent the 96-hour time point. No significant amounts of lactate, pyruvate, formate, or ethanol were produced by any of the cultures.

There are several possible explanations for why this *crp** mutation in *E. coli* B does not alleviate catabolite repression, as it does in the K-12 strains. Even though the wild-type *crp* sequence is essentially the same for all strains, the regulatory regions where CRP operates may be different in the B strain, such that the mutations in *crp** cause unfavorable interactions between CRP* and operator DNA or RNA polymerase, at the site for *xyl* regulation. It is also possible that intracellular cyclic nucleotides play a role in the diauxic relief observed for the K-12 strains, while those nucleotide levels may be significantly different in *E. coli* B. Another possibility is that the co-regulator of *xyl* genes (XylR), which has been characterized in a K-12 strain (Song and Park, 1997), may function differently in *E. coli* B or not interact with CRP* in the same manner that it does in the K-12 strains.

In addition to CRP-mediated control over catabolic genes, catabolite repression also results from “inducer exclusion”, in which enzyme IIA^{Glc} of the phosphotransferase system (PTS) inhibits the transport of molecules that serve to induce their respective catabolic operons (Hogema et al., 1998, Saier and Crasnier, 1996). It is possible that in

W3110 and MG1655 xylose transport is not inhibited by the PTS (or at least is not sufficiently inhibited to prevent the CRP* effect), while xylose exclusion is more significant in the B strain. One strong argument against this xylose exclusion explanation is the observation that while xylose metabolism is not active in the presence of glucose, xylose transport is also not eliminated. Table **4-3** shows that expressing CbXR in all strains tested results in low levels of xylitol production from glucose-xylose mixture. Thus, FL08 is capable of low levels of xylose transport in the presence of glucose, similar to wild-type *E. coli* B and FL07. If CRP* functioned as a transcriptional activator, even in the presence of strong inducer exclusion, expression of *xylA* and *xylB* would be expected in FL06, and would result in xylose metabolism by this strain.

We next compared the performance of strains PC09 and FL05 harboring plasmid pLOI3815 in controlled, fed-batch fermentations. Figure **4-1a, d** depicts the profiles of xylitol production, glucose consumption, and growth for these fermentations, and the average results from three independent runs are summarized in Table **4-4**. In all cases, xylose consumption corresponds to xylitol production (that is, no xylose was metabolized due to the *xylB* deletion, and xylulose secretion was minimal). In contrast to the considerable differences in the performance of PC07 and FL03 reported below (using plasmid pPCC107 or pPCC207), the *crp** analogue strains perform very similarly when expressing CbXR. Although PC09 consumed approximately 20% more glucose and secreted significantly more acetate (46 mM compared to 12 mM), both strains had an average specific productivity of ~0.43 g xylitol g cdw^{-1} h^{-1} which is the highest xylitol productivity reported by our lab or others (Kwon et al., 2006).

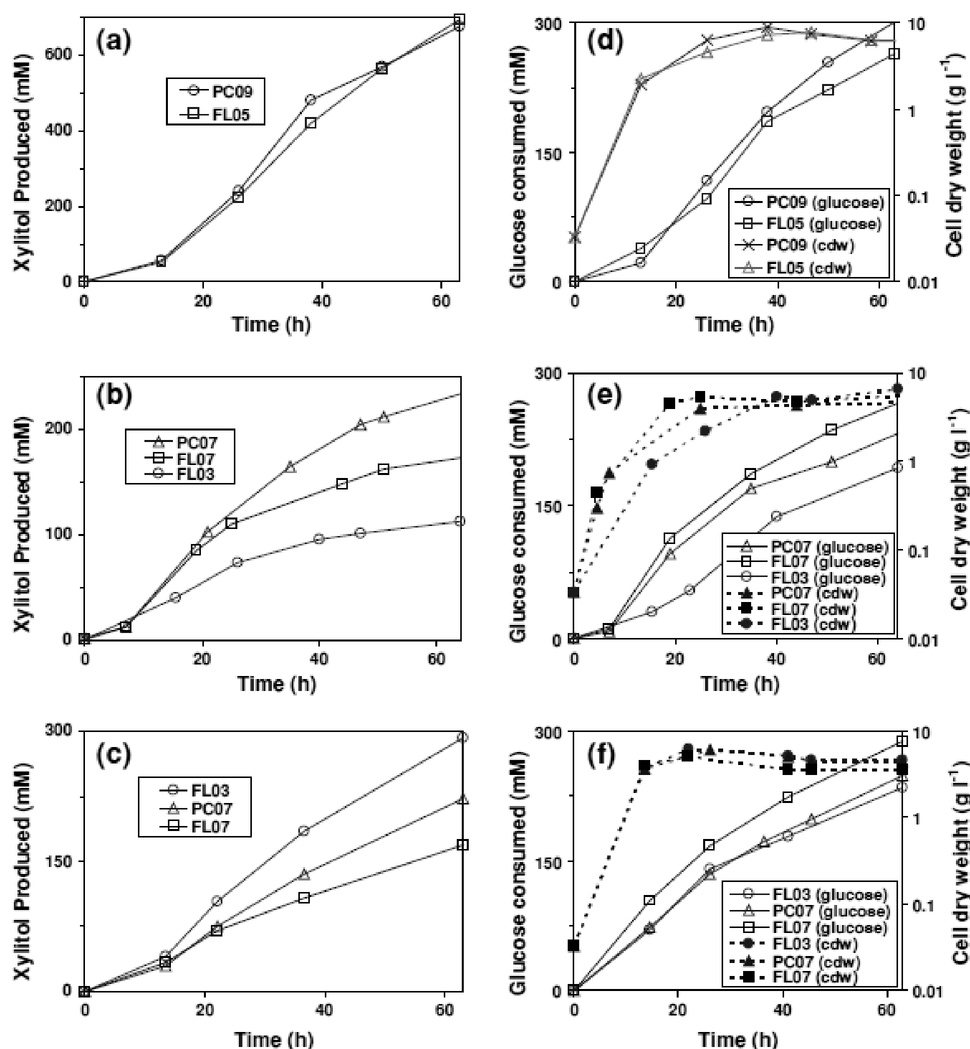

Figure **4-1**: Fermentation profiles: xylitol production (a-c), glucose consumption (d-f), and growth (d-f). Strains PC09 and FL05 carry plasmid pLOI3815 and express CbXR (a, d). Strains PC07, FL03 and FL07 either carry plasmid pPCC107 and co-express XylE with CbXR (b, e) or carry plasmid pPCC207 and co-express XylFGH with CbXR (c, f). Fed-batch fermentations (30°C, pH=7, DO>20%) contained minimal medium initially supplemented with 5 g yeast extract l^{-1}, 300 mM xylose and 100 mM glucose. Sugars were then fed manually: glucose was fed in 50 mM doses upon each glucose depletion and xylose was added to maintain concentrations above 100 mM. IPTG was added to the vessels prior to inoculation (300 μM).

Table **4-4**: Summary of the 63-hour time point results for fermentations depicted in Figure **4-1**.

Strain	Glucose consumed (mM)	Xylitol produced (mM)	Acetate produced (mM)	Cell dry weight (g l^{-1})
PC09 + pLOI3815	301	680	46 ± 5	6.5
FL05 + pLOI3815	252	657	12 ± 6	6.1
PC07 + pPCC107	226	233	8	4.5
FL03 + pPCC107	189	112	18	6.4
FL07 + pPCC107	263	172	3	5.4
PC07 + pPCC207	248	223	6	4.5
FL03 + pPCC207	234	293	29	4.6
FL07 + pPCC207	288	169	6	3.6

In all cases, xylose consumption corresponds to xylitol production (no xylose was metabolized and xylulose secretion was minimal). Standard deviations were less than 10% of the average except where indicated.

We then compared these two strains in higher density fermentations using minimal medium without the addition of yeast extract. In this high cell density approach the cultures were first grown under glucose-limited (glucose feeding) conditions without the addition of IPTG or xylose, until cell densities reached approximately to 5 g cdw l^{-1}, at which time IPTG and xylose were included. Figure **4-2** depicts the time-courses of these fermentations, starting from the onset of induction. Following induction, strain PC09 + pLOI3815 grew to a final cell density of 8.3 g cdw l^{-1}, while FL05 + pLOI3815 grew to 7.4 g cdw l^{-1}. Again, both strains performed similarly, although PC09 ultimately “out-performs” FL05 with regard to higher xylitol production, lower glucose consumption and lower acetate secretion. Both strains produced ~400 mM xylitol in 24 hours and showed overall specific productivities of 0.27 (PC09) and 0.24 (FL05) g xylitol g cdw^{-1} h^{-1} from the time of induction.

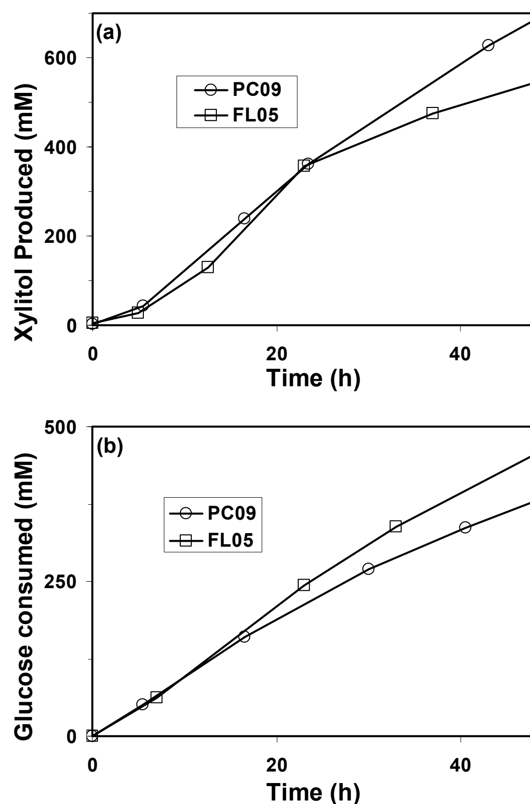

Figure **4-2**: Post-induction profiles of xylitol production and glucose consumption for PC09 and FL05 carrying pLOI3815 (expressing CbXR) in high cell density fermentations. Cells were first grown in the bioreactors in minimal medium under glucose-limited conditions to a density of ~5 g cdw l^{-1}. During the growth phase, glucose (500 g l^{-1}) supplemented with ammonium sulfate (7.5 g l^{-1}) was fed at rate of 0.6 ml h^{-1}. Xylitol production was initiated by inducing with IPTG (300 μM), and adding glucose (50 mM), and xylose (200) mM. Glucose was then fed in 50 mM doses upon each glucose depletion and xylose was added to maintain concentrations above 100 mM during the production phase. Yeast extract was not used.

4.4.2 Expression of xylose transporters

We previously reported xylitol production by PC07 harboring plasmids pLOI3815, pPCC107 or pPCC207 in minimal medium shake-flaks cultures containing glucose and xylose. Whereas PC07+pLOI3815 produced only 25 mM xylitol in 72 hours, PC07+pPCC107 and PC07+pPCC207 each produced ~132 mM xylitol in the same

amount of time (Khankal et al., 2008). Similar increases in xylitol production due to co-expression of XylE or XylFGH (i.e., using pPCC107 pr pPCC207 compared to pLOI3815) were observed for both FL03 and FL07 in minimal medium shake-flask cultures (not shown). During the course of this study we discovered that for the K-12 strains, the relative improvement in xylitol production when XylE is co-expressed is less significant in rich medium (LB) than in minimal medium or minimal medium supplemented with 5 g yeast extract l^{-1} (not shown). As an example, the increase in shake-flask xylitol production by PC07 harboring pPCC107 instead of pLOI3815 is ~400% when growing in minimal medium (with glucose and xylose) but no significant increase was observed when growing in LB supplemented with glucose and xylose. In contrast, the relative benefit of expressing XylE was similar for B-type strain FL07 in both rich and minimal medium cultures. While it is difficult to interpret these findings, one possible explanation is that for the K-12 strains, one or more ingredients in the tryptone indirectly inhibit XylE (e.g., by inducing expression of an inhibitor).

We next compared co-expression of CbXR with XylE or XylFGH in strains PC07, FL03 and FL07 in controlled fed-batch fermentations containing mineral salts medium and initially containing 300 mM xylose, 100 mM glucose, and 5 g yeast extract l^{-1}. These fermentation profiles are depicted in Figure **4-1b, c, e, f**, and the results after 63 h are summarized in Table **4-4**. While PC09 and FL05 behaved very similarly in the fermentations described above (with plasmid pLOI3815), their analogues PC07 and FL03 are considerably more different from one another, depending on which plasmid is used. W3110-derivative PC07 is the best performing strain for xylitol production with plasmid pPCC107, giving the highest xylitol/glucose ratio and with little net acetate production.

MG1655-derivative FL03 produced the least xylitol (112 mM at 63 h) and secreted the most acetate. In contrast, the MG1655-derivative FL03 out-performs the other two strains with plasmid pPCC207, although acetate secretion is notably higher than for PC07 or FL07. In this case, the B-type derivative FL07 produced the least amount of xylitol. The observed differences between these strain/plasmid combinations are significant and hardly predictable. While the physiological impacts of expressing XylE differ from those of expressing XylFGH (in terms of functional expression levels, membrane association and transporter energy requirements), the differences in strain performance with pPCC107 compared to pPCC207 were not consistent between the three strains. The drastic difference in the performance of strain FL03 expressing XylFGH (producing 293 mM xylitol) relative to XylE (producing 112 mM xylitol) is noteworthy, particularly considering that PC07 and FL07 each show similar fermentation profiles with both plasmids.

4.4.3 Resting cell comparison

Finally, we compared the resting cell performance of strains PC09 and FL05 harboring pLOI3815 and strains PC07, FL03 and FL07 harboring pPCC107, all in minimal medium containing glucose and xylose. Here, the primary performance "determinants" are the molar yield of xylitol produced per glucose consumed (Y_{RPG}) and the total xylitol productivity. Results from these cultures are summarized in Table **4-5**. Note that in calculating Y_{RPG} , xylitol production is corrected for the "background" amount of xylitol produced in cultures not containing glucose. Similar to what was observed in fermentations, strains with *crp** (PC09 and FL05) had higher xylitol

productivities, yields and titers compared to those co-expressing XylE from pPCC107. The lower yield with FL05 compared to PC09 is reflected in a higher rate of glucose consumption. The productivities of the *crp** strains were similar to those reported above for the high cell density fermentations (also in minimal medium). FL03 + pPCC107 produced the least amount of xylitol in resting cells, as was also the case in fermentations. In general this strain was not well-behaved and gave the least reproducible data.

Table **4-5**: Results from resting cells after 48 hours of biotransformation in minimal medium.

Strain	Glucose consumed (mM)	Total xylitol produced (mM)	Acetate produced (mM)	Yield xylitol / glucose	Overall Productivity (g xylitol g cdw^{-1} h^{-1})
PC09 + pLOI3815	14	71	2.6 ± 0.6	4.1	0.34
FL05 + pLOI3815	20	75	1.4	3.2	0.36
PC07 + pPCC107	20	60	7.4	2.8	0.29
FL03 + pPCC107	12 ± 6	29 ± 3	4.8 ± 1.6	2.5 ± 1.1	0.14
FL07 + pPCC107	16	46 ± 5	6.4 ± 1.1	2.7	0.22

The cell density was 0.66 g cdw l^{-1}, and aeration was achieved by shaking at 250 rpm in baffled flasks. Standard deviations were less than 10% unless noted.

4.5 Conclusions

We have compared two different metabolic engineering approaches for xylitol production from three common lab strains of *E. coli* growing in a glucose-xylose mixture. The differences and similarities in performance between the different engineered strains depended entirely on the nature of the genetic modifications being employed. The use of CRP*/pLOI3815 in general gave better results than overexpressing a xylose transporter, although CRP* did not alleviate repression of xylose uptake in *E. coli* B (and PC09 had

slight advantages over FL05). Importantly, no single strain out-performed the others in all scenarios tested, and the relative performances of the engineered strains are not likely to be rationalized based on the documented differences in genetic background, growth characteristics or gene expression patterns of the wild-type strains.

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Chapter 5

Transcriptional effects of CRP* expression in *Escherichia coli*

5.1 Abstract

Escherichia coli exhibits diauxic growth in sugar mixtures due to CRP-mediated catabolite repression and inducer exclusion related to phosphotransferase system enzyme activity. Replacement of the native *crp* gene with a catabolite repression mutant (referred to as *crp**) alleviates diauxic effects in *E. coli* and enables co-utilization of glucose and other sugars. While previous studies have examined the effects of expressing CRP* mutants on the expression of specific catabolic genes, little is known about the global transcriptional effects of CRP* expression. In this study, we compare the transcriptome of *E. coli* W3110 (expressing wild-type CRP) to that of mutant strain PC05 (expressing CRP*) in the presence and absence of glucose.

While the simplest model of CRP*-mediated gene expression assumes insensitivity to glucose (or cAMP), our results show that gene expression in the context of CRP* is very different from that of wild-type in the absence of glucose, and is influenced by the presence of glucose. 238 genes were found to respond differently to glucose in PC05 compared to W3110. Genes whose expression is significantly altered by glucose in strain W3110 are generally not altered to the same extent in strain PCO5 and the glucose effect is significantly suppressed in this strain. We present a detailed transcription analysis and relate these results to phenotypic differences between strains

expressing wild-type CRP and CRP*. Notably, CRP* expression in the presence of glucose results in an elevated intracellular NADPH concentration and reduced NADH concentration relative to wild-type CRP. Meanwhile, a more drastic decrease in the NADPH/NADP$^+$ ratio is observed for the case of CRP* expression in strains engineered to reduce xylose to xylitol via NADPH-dependent xylose reductase during glucose metabolism.

5.2 Introduction

E. coli growing in a mixture of sugars exhibits diauxic growth characteristics, whereby glucose is preferentially assimilated before other sugars. This is due to CRP-mediated catabolite repression and inducer exclusion related to phosphotransferase system enzyme activity. It is well established that cyclic AMP (cAMP) and its receptor protein (CRP) are involved in transcriptional activation of catabolic genes (Sabourin and Beckwith, 1975, Emmer et al., 1970), but the details of catabolite repression and inducer exclusion mechanisms and their relation to the levels of cAMP and CRP (also known as CAP) are not clear and have motivated many studies (Nanchen et al., 2008, Bettenbrock et al., 2007, Karimova et al., 2004, Tagami and Aiba, 1995, Saier and Reizer, 1994).

Inducer exclusion is a result of dephosphorylation of enzyme IIA^{Glc} of PTS (Eppler and Boos, 1999, Hogema et al., 1998) and catabolite repression is associated with altered levels of cAMP (Kolb et al., 1993, Botsford and Harman, 1992, Ullmann and Danchin, 1983) and CRP (Eppler and Boos, 1999, Inada et al., 1996). Enzyme IIA^{Glc}, when unphosphorylated, inhibits activity of other transport systems (non-PTS transporter)

(Saier, 1989, Nelson et al., 1983, Osumi and Saier, 1982) and adenylate cyclase (encoded by *cya*). In its phosphorylated form, Enzyme IIA^{Glc} stimulates adenylate cyclase activity, resulting in higher intracellular levels of cAMP (Amin and Peterkofsky, 1995, Yang and Epstein, 1983) and the cAMP-CRP complex (global transcription activator).

Efforts to study or alleviate catabolite repression mediated by CRP have resulted in a series of CRP mutants isolated from strains lacking adenylate cyclase and having an apparent reduced dependence on cAMP for activating catabolic genes (called CRP*, CRP-in or CAP^{c}) (Guidirontani et al., 1981, Botsford and Drexler, 1978, Sabourin and Beckwith, 1975). Genetically different *crp** strains reported are also phenotypically different, showing different sensitivities to cyclic nucleotides and relieving catabolic repression of select genes examined to different extents (Harman et al., 1986). For example, six different *crp** mutants isolated after UV treatment and selection for a $lactose^{+}$ phenotype in an adenylate cyclase-deficient *E. coli* strain showed a variety of utilization patterns for different sugars (lactose, maltose, arabinose, xylose, ribose, mannose, mannitol) as well as different levels of activation of the *lac* operon by cAMP or cGMP (Aiba et al., 1985). Similar examples have been reported by others (Karimova et al., 2004, Harman et al., 1986).

Ability to co-utilize sugars via relief of catabolite repression during microbial production of value-added chemicals has potential to improve bioproduction process economics (Aristidou and Penttila, 2000). We previously engineered *E. coli* to produce xylitol from xylose while metabolizing glucose as a source of carbon and energy (xylose metabolism is disabled) (Khankal et al., 2008a, Cirino et al., 2006). Expression of CRP* was an effective approach to promote expression of xylose transporters and enhance

xylitol production in the presence of glucose. Although plasmid-based, CRP-independent expression of xylose transporters in wild-type *crp* strains also enhances xylose uptake and xylitol production in the presence of glucose (Khankal et al., 2008a), the favorable effects of CRP* expression were found to go beyond improving xylose transport and to include other beneficial phenotypes such as reduced acetate production and higher yields on xylose reduced per mole of glucose consumed (Khankal et al., 2008b).

While CRP*s have been studied at the molecular level and the effects of expressing CRP* mutants on the expression of specific catabolic genes have been reported, the global transcriptional effects and regulatory consequences of CRP* expression is not known. Here, we report the results of comparisons between the transcriptome of *E. coli* W3110 (expressing wild-type CRP) and that of mutant strain PC05 (expressing CRP*) in the presence and absence of glucose through microarray analysis. Our results show that gene expression in PC05 is drastically different from that of W3110 in both the presence and absence of glucose, and that while expression of the CRP* allele used in this study has the general effect of suppressing transcriptional changes due to glucose, a significant response to glucose nonetheless remains. Results are analyzed in light of the observed differences between wild-type and CRP* strains during xylitol production. We identify many genes showing differential expression that are consistent with the observed elevated levels of glucose oxidation and NADPH-dependent xylose reduction for PC05 compared to W3110. A subsequent intracellular cofactor analysis reveals CRP*-correlated effects on cofactor levels that are consistent with the observed expression changes.

5.3 Materials and methods

5.3.1 General

E. coli K-12 strain W3110 (ATCC 27325) and its derivatives were maintained on plates containing Luria-Bertani (LB) medium (10 g tryptone, 5 g yeast extract, 5 g NaCl, and 15 g agar per liter). Methods for construction of strains PC05 (W3110 and *crp**), PC07 (W3110Δ*xylB*), and PC09 (PC05Δ*xylB*) were described previously (Cirino et al., 2006). Briefly, the *crp** gene and *xylB* deletion were introduced into W3110 via P1 phage transduction using a lysate from strain ET25 (*crp**::Tn10) (Eppler and Boos, 1999) and PC06 (W3110, Δ*xylB*::FRT-*aac*-FRT) (Cirino et al., 2006) followed by selections on tetracycline (for crp*) or apramycin (for Δ*xylB*) plates. Plasmid pLOI3815 is a medium copy, pBR322-origin vector carrying a kanamycin resistance marker and the xylose reductase gene from *Candida boidinii*, which is located downstream of *tac* promoter and upstream of a transcription termination sequence (Cirino et al., 2006). Xylose transporter genes *xylFGH* (ATP-dependent xylose transporter system) were cloned downstream of CbXR in pLOI3815 to make plasmid pPCC207.

Amino acid substitutions in the CRP* were confirmed by sequencing. The *crp** phenotype was verified in two ways. First, several Tet^R transductants were grown in LB medium containing glucose (1%) and xylose (1%). Cells were harvested at mid logarithmic growth phase and washed twice in phosphate buffer containing kanamycin (50 mg/mL). After allowing time for residual sugars to be cleared, the cells were resuspended a final time in buffer containing xylose (1%), kanamycin, and 1%

triphenyltetrazolium chloride (TTC). Reduction of TTC results in red color formation and indicates constitutive xylose utilization. The *crp** phenotype was additionally confirmed using HPLC to verify simultaneous glucose and xylose consumption in batch cultures (Cirino et al., 2006).

5.3.2 Growth conditions

Four different conditions were tested in this study: W3110 in LB medium (WT), W3110 in LB+glucose medium (WT G), PC05 in LB medium (CRP*), and PC05 in LB+glucose medium (CRP* G). All experiments were performed at least in triplicate and all data reported are the average of at least three experiments. Cell culture optical density was measured at 600 nm (OD_{600}) using a SPECTRAMax PLUS^{384} spectrophotometer (Molecular Devices). Cells grown for harvesting were prepared briefly as follows. Overnight pre-seed cultures were prepared by inoculating 3 ml of LB medium (in 13 x 100 mm tube) with a few colonies from a fresh LB plate. The overnight cultures were used to inoculate, to an OD_{600} of 0.1, 50 ml LB media (with or without 0.4% glucose supplementation) seed cultures in a 250 ml shake-flask. The seed culture were grown at 37°C to an OD_{600} of ~2 and then were used to directly inoculate, to an OD_{600} of 0.02, 100 ml LB media (with or without 0.4% glucose supplementation) cultures in a 500 ml flask. These cultures were grown at 37°C and 250 rpm to an OD_{600} of 0.5.

5.3.3 Cell harvesting and preparation of RNA

Cells from the 100 ml culture were harvested at an OD_{600} of 0.5 (early logarithmic growth phase) by immediately placing on ice, transferring to 50 ml falcon tubes and centrifuging at 4°C for 5 minutes before treating with lysozyme. Promega PureYield™ RNA Midiprep System kit was used for RNA extraction. As a preliminary check, RNA yield and quality were determined by spectrophotometry according to the manufacturer's protocol and the integrity of the purified RNA was determined by formaldehyde agarose gel electrophoresis.

5.3.4 Labeling, hybridization and scanning

Total RNA concentration and purity were determined using a NanoDrop spectrophotometer and total RNA integrity was examined using an Agilent Bioanalyzer. Total RNA of sufficient concentration, purity, and integrity was labeled and subsequently hybridized to Affymetrix GeneChip microarrays by the Penn State DNA Microarray Facility according to the manufacturer's instructions (Affymetrix Inc, Santa Clara, CA). Briefly, 10 μg of total RNA was converted to cDNA using random primed reverse transcription. cDNA was purified by removing the RNA via hydrolysis with NaOH and then neutralizing the solution. Purified cDNA was fragmented and subsequently end-labeled with biotin. Fragmented, end-labeled cDNA was dissolved in hybridization cocktail and hybridized to Affymetrix GeneChip *E. coli* Genome 2.0 Arrays (approximately 10000 probe set) for 16 hours at 45°C. The details of GeneChip *E. coli* Genome 2.0 Arrays are described by Affymetrix (http://www.affymetrix.com).

After hybridization, the hybridization cocktail was removed and the arrays were washed to remove unbound and non-specifically bound cDNA. Hybridization was detected by staining the arrays with streptavidin phycoerythrin. All washing and staining was performed using the Affymetrix GeneChip Fluidics Station 450 according to the manufacturer's instructions (Affymetrix Inc, Santa Clara, CA). Stained arrays were scanned using the Affymetrix GCS3000 7G scanner.

5.3.5 Microarray data analysis

A minimum of three data sets was generated for each of the four different conditions tested (based on the combination of the strains W3110 and PC05 in LB and LB+glucose media). Affymetrix Expression Console™ software (Version 1.1) was used for background adjustment, normalization and summarization of chip level data in the form of feature intensity (CEL) files in order to generate probe set summarization (CHP) files, using the probe logarithmic intensity error (PLIER) method. Data from CHP files were then exported to a Microsoft Excel spreadsheet for further analysis. Signal values for 10208 probsets from GeneChip *E. coli* Genome 2.0 Arrays were filtered to extract probe set data for only the *E. coli* K-12 strain. All calculations and analyses were performed on the 4070 genes remaining after filtration.

Signal values were transformed to the log base for the pair-wise comparisons. A linear model was fitted to each gene using the Bioconductor software package LIMMA (Smyth, 2005, Smyth, 2004) in the R environment (http://www.r-project.org). The linear model coefficients were used to calculate significant differences in expression levels for

all pair-wise comparisons. The P-values were adjusted by the Benjamini-Hochberg method (Benjamini and Hochberg, 1995) and genes with a P-value of <0.05 were considered as those with significantly different expression levels under different conditions tested. Data are reported as expression levels (signal values) or ratios of expression levels. Written code in R (http://www.r-project.org) can be found in Appendix C. Gene Annotations were transformed from AFFY probe set ID's to Entrez gene IDs using NETAFFX on the Affymetrix website (http://www.affymetrix.com). The online database for annotation, visualization and integrated discovery (DAVID) (Huang da et al., 2009, Dennis et al., 2003) and Kyoto Encyclopedia of Genes and Genome (KEGG) (Kanehisa et al., 2004) were used for pathway visualization and gene ontology (GO) classification.

5.3.6 Real-time, Reverse Transcription PCR

Total RNA samples were isolated the same way as for microarray studies. *ppsA* (phosphoenolpyruvate synthase), *pntA* (membrane-bound proton-translocating pyridine nucleotide transhydrogenase), and *rpsQ* (30S ribosomal subunit protein S17) were selected for confirmation by real-time reverse transcription PCR with *rrsH* (encoding 16S ribosomal RNA) as a control. Primer and probe sequences used for RT-PCR are listed in Table **5-1** (supplementary Table Sup1a (http://www.che.psu.edu/Faculty/Cirino))and were designed by Deborah S. Grove of the Penn State Nucleic Acid Facility using Primer Express v2.0 (Applied Biosystems, Foster City, CA). Probes were synthesized by Biosearch (Novato, CA). The Applied Biosystems High Capacity cDNA Reverse

Transcription Kit (part number 4368813) was used for reverse transcription according to the manufacturer's instructions for cDNA production. cDNA was amplified in an ABI 7300 real-time machine using TaqMan® Universal PCR Master Mix, No AmpErase® UNG (part number 4324018). Output was analyzed using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001).

Table **5-1**: Primers and probes sequences used for RT-PCR

Primers	Sequence	Probe sequence
pntARB	TAATTAGCTTCGGCGCTGGTT	CACCACCGCGCTTATTCCAGGC
pntAF	GGCAAAAGAGGTCGATATCATTG	
rpsQFB	TGTTGCTATCGAACGTTTTGTGA	CACCCGATCTACGGTAAATTCATCAAGCG
rpsQRB	CGTCATGTACGTGCAGTTTGG	
ppsF	TGTTTCCGTTCCGAATGGTT	ACCGCCGACGCGTTTAACCAGTTTC
ppsR	TGGTTTACGCCGCTTTGG	
rrsHF	CTACGGGAGGCAGCAGTG	TGSACAATGGGCGMAAGCCTG
rrsHR	TGCCAGCAGCCGCGGTAATAC	

5.3.7 Cofactor measurements

The cofactor analysis used in this study is based on the methods developed by Bernofsky and Swan (Bernofsk.C and Swan, 1973), and modified by Gibon and Larher (Gibon and Larher, 1997), and Walton and Stewart (Walton and Stewart, 2004). To investigate the effect of xylitol production on intracellular NADP(H) and NAD(H) levels, cofactor concentrations and ratios were measured and compared in PC05Δ*xylB* strain harboring pLOI3815 and W3110Δ*xylB* harboring pPCC207. Seed cultures were grown at 30°C to an OD_{600} of ~2 and then were used to directly inoculate, to an OD_{600} of 0.02, 100 ml LB medium supplemented with 100 mM glucose, 100 mM xylose (or 200 mM

glucose for non-xylitol producing conditions), 50 mM MOPS, Kanamycin monosulfate (50 μM) and Isopropyl-B-D-thiogalactopyranoside (IPTG, 50 μM) in a 500 ml flask. These cultures were grown at 37°C and 250 rpm to an OD_{600} of 0.5. Cells were harvested by pelleting (4°C, 15 min, 3750 rpm) to achieve a final OD_{600} of 30 in 1 ml. To isolate the oxidized forms, the pellet was resuspended in 0.5 ml of 0.3 M HCl, 50 mM Tricine-NaOH (pH 8.0). To isolate the reduced forms, the pellet was resuspended in 0.5 ml of 0.3 M NaOH. All samples were then heated to 60°C for 7 minutes followed by a neutralization step (0.5 ml 0.3 M NaOH for oxidized forms, 0.3 ml 0.3 M HCl, 0.2 ml 1.0 M Tricine-NaOH (pH 8.0) for reduced forms). The neutralized solutions were then centrifuged (4°C, 60 min, 13000 rpm) and the supernatants were transferred to a new microcentrifuge tube.

Cofactor levels were measured in a 96-well microtiter plate. Either 40 μl of oxidized sample and 40 μl 0.1 M NaCl, or 80 μl of reduced sample was aliquoted to a single well. The 2X stock solution of the reaction mixture consisted of equal volumes of 1.0 M Tricine-NaOH (pH 8.0), 4.2 mM MTT, 40 mM EDTA, 1.67 mM PES, and substrate (either 5 M ethanol or 25 mM glucose-6-phosphate). After addition of the appropriate reaction mixture (ethanol for NAD(H), glucose-6-phosphate for NADP(H)), the plate was incubated at 37°C for 5 minutes. To start the reaction, either 10 units/ml alcohol dehydrogenase (from 100 units/ml stock) or 0.27 units/ml glucose-6-phosphate dehydrogenase (from 2.7 units/ml stock) was added. The formation of reduced MTT was monitored using a SpectraMax[384] plate reader, taking readings every 15 seconds for 10 minutes using a wavelength of 570 nm while being incubated at 37°C. The cofactor concentration of the samples was interpolated by comparing the rate of reaction to that

observed in a concentration curve run on the same plate, and subtracting the rate from the background of the sample (reaction without enzyme).

5.4 Results

The *E. coli* W3110-derivative CRP* strains used in our studies are derived from *E. coli* donor strain ET25 (Eppler and Boos, 1999), which expresses a CRP* mutant with three amino acid substitutions (I112L, T127I, and A144T) identical to those found in an earlier characterized CRP* strain CA8404 (Sabourin and Beckwith, 1975). Amino acid position 127 lies in the cAMP binding pocket, and T127I or T127L mutations occur frequently in CRP* alleles (Gorshkova et al., 1995, Harman et al., 1986), presumably serving to reduce the cAMP requirement to form an activating CRP complex (Gorshkova et al., 1995). Mutation A144T is also frequently found in different CRP* alleles (Karimova et al., 2004, Belduz et al., 1993, Harman et al., 1986) and can exhibit the CRP* phenotype to some extent even as the only mutation in the protein (Garges and Adhya, 1985). This position lies in the DNA binding domain of CRP and is suggested to improve affinity of the protein for CRP binding sites (Garges and Adhya, 1985). A fourth base substitution in the *crp** sequence results in a T28K mutation, which is the result of native differences in the *crp* sequence between W3110 and the donor strain.

5.4.1 Genome-wide transcriptional effects of glucose and CRP*

Average signal values for the genes that are discussed in this chapter are presented in Table **5-2**. Supplementary Table Sup2 contains signal values for the complete probe set data for the *E. coli* K-12 genome. The coefficients of the linear model which was fitted to signal values for each gene were used to calculate significant differences in expression levels for all pair-wise comparisons. P-values of <0.05 were considered significantly different expression levels in pair-wise comparisons. Table **5-3** summarizes the genome-wide effects of CRP* expression under the conditions tested.

Table **5-2**: Expression levels for the genes discussed in this paper. Supplementary Table Sup2, a complete version of Table **5-2** presenting all signal values for the *E. coli* K-12 genome, can be found in supplementary material. Four different conditions were tested in this study: W3110 in LB medium (WT), W3110 in LB+glucose medium (WT G), PC05 in LB medium (CRP*), and PC05 in LB+glucose medium (CRP* G).

gene	WT G	WT	CRP* G	CRP*	WT G / WT	CRP* G / CRP*	CRP* G / WT G
acs	56	223	203	386	0.25	0.52	3.63
asnA	594	1741	1409	1880	0.34	0.75	2.37
fumA	956	3284	4013	3738	0.29	1.07	4.20
glyA	1613	3148	3353	3448	0.51	0.97	2.08
maeB	579	1420	1509	1839	0.41	0.82	2.61
mdh	5878	9397	12603	15821	0.62	0.80	2.14
pntA	3946	6915	7400	7086	0.57	1.04	1.88
pntB	1736	2546	3057	3083	0.68	0.99	1.76
ppsA	247	6026	1325	3494	0.04	0.38	5.36
sdhA	1033	7257	3292	3154	0.14	1.04	3.19
sdhB	895	5160	2407	2521	0.17	0.95	2.69
sdhD	556	5466	2568	1980	0.10	0.77	4.62
sthA	3236	3764	1936	2741	0.86	0.71	0.60
tdcB	147	4614	7708	13552	0.03	0.57	52.44
thrA	811	2686	1997	1279	0.30	1.56	2.46
thrB	849	2857	2165	1353	0.30	1.60	2.55
thrC	783	1722	1594	1378	0.45	1.16	2.04
tnaA	688	16366	10805	23343	0.04	0.46	15.70

Table **5-3**: Summary of genome-wide effects of CRP* expression.

Conditions compared	Number of genes showing significant differential expression	Up-regulated	Down-regulated	Genes in common with WT G - WT comparison
WT G - WT	629	375	254	629
CRP* G - CRP*	80	29	51	43
(CRP* G - CRP*) - (WT G - WT)	238	-----	-----	198
CRP* G – WT G	349	232	117	163
CRP* - WT	553	392	161	205
CRP* G - WT	481	330	151	218

Transcriptome analysis of strain W3110 reveals that 629 genes show significant changes in expression level in response to the presence of glucose (comparison between WT G and WT in Figure **5-1**a). 375 of these genes are upregulated by glucose, as depicted in Figure **5-1**a. Catabolic genes, membrane-related components, and sugar transporters (especially non-glucose PTS related enzymes) represent a large portion of genes repressed by glucose.

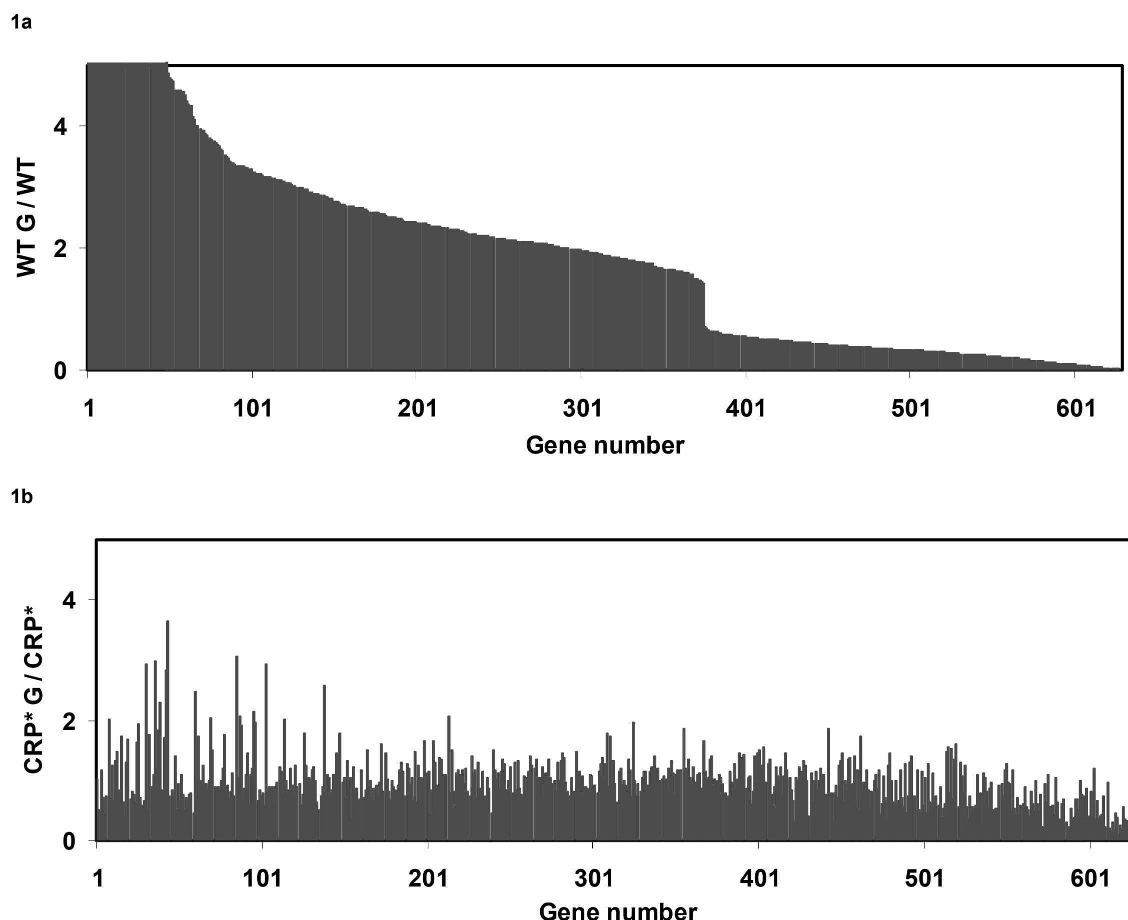

Figure **5-1**: Genome-wide transcriptional effects of glucose in strain W3110 expressing wild-type CRP, presented as expression ratios for individual genes showing significant differential expression in the presence and absence of glucose (WT G – WT). **1a)** 629 genes show significant changes in expression level in response to the presence of glucose in strain W3110. **1b)** The changes in expression levels of the same genes shown in **1a** in CRP* strain PC05, in response to glucose. Gene names and expression levels are given in supplementary Table Sup3.

Figure **5-1**b depicts the changes in expression levels of the same genes shown in Figure **5-1**a for CRP mutant strain PC05 in response to glucose. The average WT G / WT ratio for the genes which are upregulated in W3110 in the presence of glucose is 4.06 while the average CRP* G / CRP* ratio for the same genes is 1.07. For downregulated

genes in W3110 by glucose, WT G / WT and CRP* G / CRP* ratios are 0.32 and 0.81 respectively. These results show that genes whose expression is significantly altered by glucose in strain W3110 are generally not altered to the same extent in strain PCO5 and that CRP* suppresses this effect of glucose.

Figure **5-2** depicts that fewer genes show significant changes in expression level for strain PC05 (80 genes) compared to W3110 (629 genes) when grown in the presence versus absence of glucose. 29 of these genes are upregulated in the presence of glucose. This confirms the expected role of CRP* in the alleviation of glucose repression. Only 43 genes are common between those of Figure **5-1** and Figure **5-2**. In contrast to W3110, the number of genes that are repressed in the presence of glucose in PC05 is greater than the number of genes that are upregulated. Only 3% of genes that are upregulated in W3110 in the presence of glucose are also upregulated in PC05 in the same condition, while 12% of glucose-repressed genes in W3110 are also repressed by glucose in PC05.

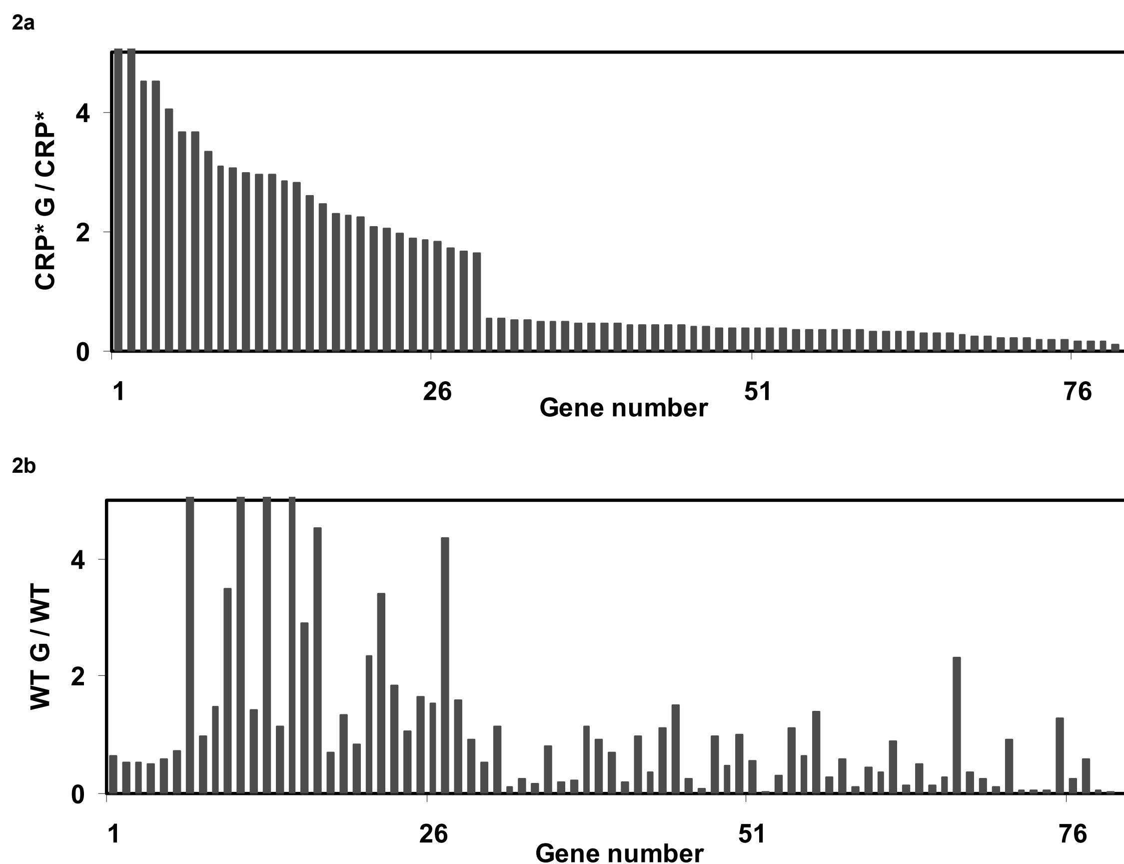

Figure **5-2**: Genome-wide transcriptional effects of glucose in CRP* strain PC05, presented as expression ratios for individual genes showing significant differential expression in the presence and absence of glucose (CRP* G – CRP*). **2a)** 80 genes show significant changes in expression level for strain PC05. Only 29 are upregulated. **2b)** The changes in expression levels of the same genes shown in **2a** for strain W3110, in response to glucose. Gene names and expression levels are given in supplementary Table Sup4

418 genes in the *E. coli* genome are suggested to be regulated in part by CRP, as reported by the most current EcoCyc database (Keseler et al., 2009). While the modes of regulation of many of these genes are complicated and not well understood (often involving multiple transcription factor binding sites), the CRP-cAMP complex is assigned to be a transcriptional activator for approximately 321 genes (implying

upregulation in the absence of glucose) and a repressor for approximately 46 genes (implying downregulation in the absence of glucose) (in some cases the role of CRP is dual or unclear). While 629 genes show significant changes in their expression levels in response to the presence of glucose in W3110 (Figure **5-1**), only 19% of them (118 out of 629) have a CRP binding site close to their start codon (these genes are highlighted in green in supplementary Table Sup3). This result is perhaps not unexpected when considering that most genes under CRP control are also regulated by other transcription factors (Keseler et al., 2009). Of these 118 genes, 25 are upregulated while 93 show reduced expression in the presence of glucose. 77 out of the 93 genes downregulated in glucose are described in Ecocyc as being activated by CRP-cAMP, showing good agreement with the expected inverse relationship between glucose presence and CRP-cAMP activity. Meanwhile only 23 of the 80 genes showing significant differential expression in the presence of glucose for strain PC05 are present among the list of 418 genes believed to be directly regulated by CRP (highlighted in green in supplementary Table Sup4; 7 are upregulated in glucose, and 13 out of the remaining 16 downregulated genes are reported to be activated by CRP-cAMP and therefore expected to show lower expression in the presence of glucose), again demonstrating significant alleviation of catabolite repression. Thus, the majority of expression changes resulting from the presence of glucose or the CRP* mutations are not directly related to altered regulation by CRP at CRP binding sites, but rather due to secondary effects resulting from a smaller number of direct, CRP-mediated expression differences.

In a study of CRP-dependent gene expression, Gosset and coworkers reported transcriptome analysis of CRP-dependent genes in another *E. coli* K-12 strain BW25113

(Gosset et al., 2004). In Table **5-4** we compare their result to results from our study for common conditions tested (i.e., WT G - WT). While their study did not examine CRP*, this comparison provides an indication of the consistency of glucose-responsive gene expression among different but similar strains. Our comparison focuses on genes involved in central metabolism and shows that the genes which are subject to glucose repression in BW25113 (such as *aceA* (isocitrate lyase monomer), *aldA* (aldehyde dehydrogenase A), *sdhA* (succinate dehydrogenase) and *sucA* (oxoglutarate dehydrogenase)) are also downregulated in the presence of glucose for W3110. However, not all the genes which are upregulated in the presence of glucose in BW25113 are upregulated in W3110 under the same conditions (examples are *aceE* (pyruvate dehydrogenase E1 component), *guaB* (IMP dehydrogenase), *rpsQ* (30S ribosomal subunit protein S17)). This is likely to be due to differences between these two strains (Khankal et al., 2008b, Vijayendran et al., 2007, Baba et al., 2006, Hayashi et al., 2006) as well as the differences in experimental methods.

Table **5-4**: Comparison between expression levels (signal values) of the wild-type strain genes in response to the presence of glucose in two different studies. This comparison focuses on genes involved in central metabolism.

Gene symbol	This study		(Gosset et al., 2004)	
	WT G	WT G / WT	WT G	WT G / WT
Upregulated genes in the study by Gosset and coworkers (Gosset et al., 2004)				
aceE	7646	0.58	8177	4.4
fis	3058	0.99	5726	6.9
guaB	4670	0.61	2749	4.2
ptsG	13731	3.50	2387	3.2
rpls	8353	0.84	5704	2.7
rpmE	13204	1.18	8773	3.9
rpsQ	6405	0.51	3311	2.7
rpsT	10344	1.8	24476	2.6
spf	11308	3.40	26801	11.2
Downregulated genes in the study by Gosset and coworkers (Gosset et al., 2004)				
aceA	1806	0.57	498	0.2
aceB	1466	0.65	296	0.2
aldA	370	0.15	305	0.1
fumA	956	0.29	819	0.3
gltA	126	0.27	452	0.1
mdh	5878	0.62	996	0.2
pckA	6733	0.56	765	0.3
sdhA	1033	0.14	614	0.2
sdhB	556	0.10	401	0.2
sucA	3988	0.47	822	0.2
sucB	6087	0.60	802	0.1
sucC	5447	0.59	1250	0.2
sucD	3439	0.58	526	0.1

To investigate which genes respond differently to glucose in PC05 compared to W3110, an interaction term ((CRP* G - CRP*) - (WT G - WT)) was examined with the same criteria as pair-wise comparisons. This comparison reports the difference of differences and reveals that 238 genes respond differently to glucose presence in W3110 compared to PC05, as illustrated in Figure **5-3**a and Figure **5-3**b (listed in supplementary Table Sup5).

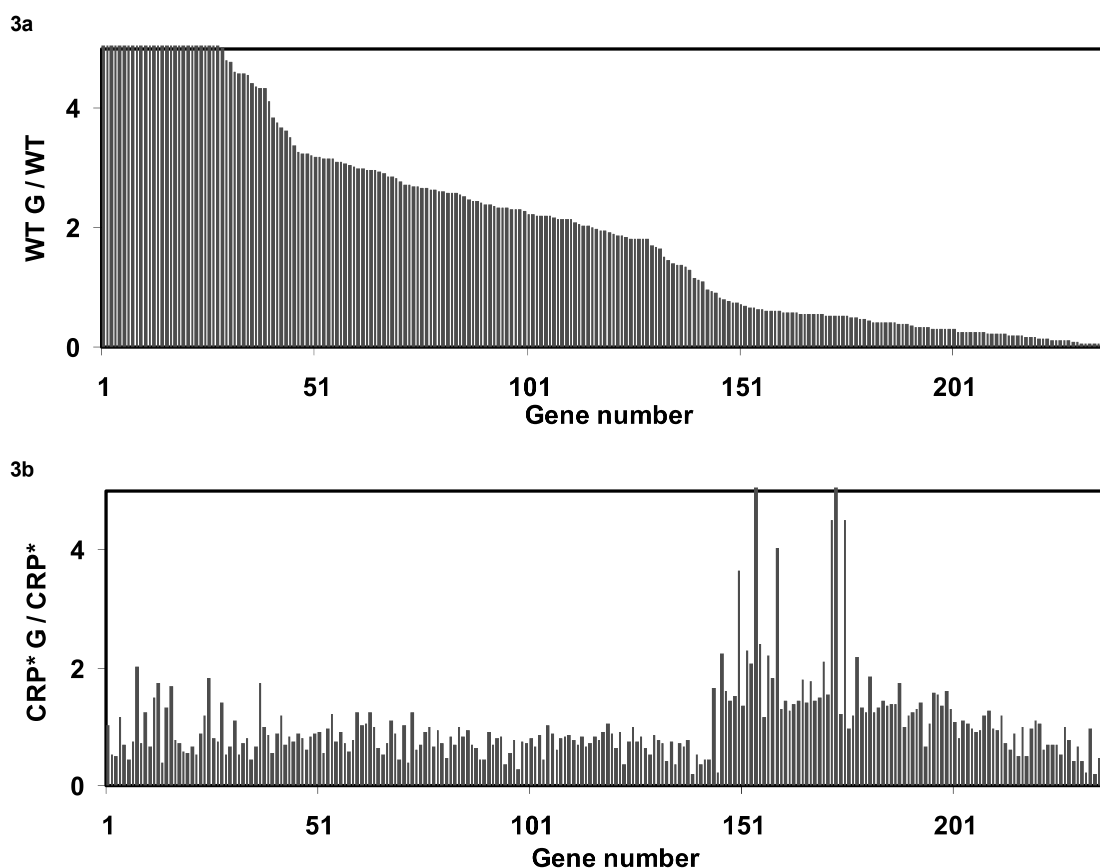

Figure **5-3**: Genes that respond differently to the presence of glucose in W3110 compared to PC05, (CRP* G – CRP*) – (WT G – WT). **3a)** Ratio of expression levels of these 238 genes in strain W3110 in the presence and absence of glucose (WT G / WT). **3b)** Ratio of expression levels of the same genes shown in **3a** in strain PC05, in the presence and absence of glucose (CRP* G / CRP*). Gene names and expression levels are given in supplementary Table Sup5.

Pair-wise comparison between the CRP* G and WT G conditions identifies the changes in transcriptional levels of genes affected by CRP* in the presence of glucose. In this category, 349 genes show significant changes in their expression levels (Table **5-3**), as depicted in Figure **5-4** and as listed in supplementary Table Sup6. Pair-wise comparison of gene expression in PC05 and W3110 grown on LB (without glucose) reveals changes in gene transcription levels as a result of the CRP* mutation. This comparison shows that 553 genes are expressed differently (392 of them are upregulated) between these two strains in the absence of glucose. These results are summarized in Table **5-3** and the specific genes are listed in supplementary Table Sup7. Finally, to examine the extent to which CRP* reduces the glucose effect, we performed a pair-wise test between two conditions: PC05 in the presence of glucose (CRP* G) and W3110 in the absence of glucose (WT). Our results show that the transcriptional levels of 481 genes are significantly different between these two conditions. These results are summarized in Table **5-3** and the specific genes and expression values are listed in supplementary Table Sup8.

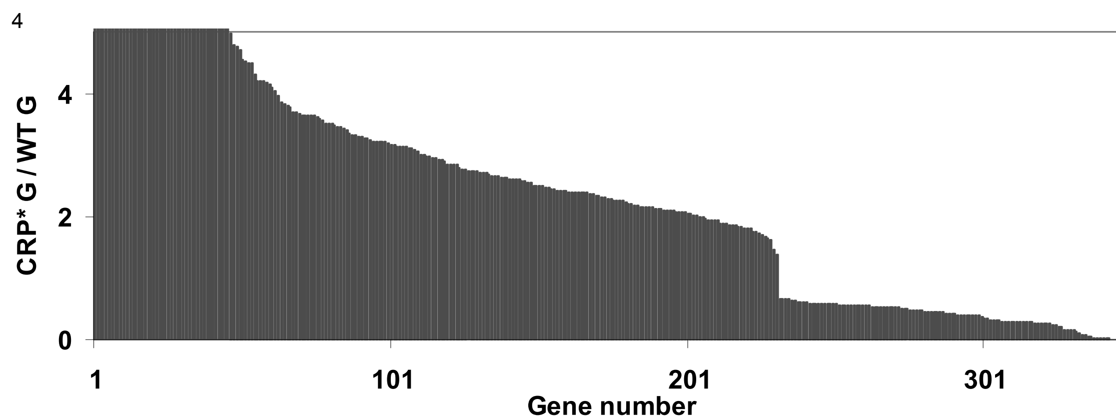

Figure **5-4**: Significant genome-wide transcription effects of expressing CRP* (strain PC05) instead of wild-type CRP (W3110) in the presence of glucose, presented as individual gene expression ratios (CRP* G – WT G). 349 genes show significant changes in their expression levels between CRP* G and WT G conditions. Gene names and expression levels are given in supplementary Table Sup6.

5.4.2 Real-Time Reverse Transcription PCR

To confirm the microarray results, the transcript levels of *ppsA* and *pntA,* both of which showed significant changes in their transcriptional levels under the various conditions tested, were compared by real-time reverse transcription PCR. The transcript of *rpsQ* was also analyzed, as this gene was noted to respond quite differently to the presence of glucose with wild-type CRP in our study as compared to the study by Gosset (WT G / WT value of 0.51 compared to 2.7) (Gosset et al., 2004). Data are presented in Table **5-5** (supplementary Table Sup1b) as fold-changes (signal ratios) for all conditions tested, and show a good agreement between microarray and real-time RT-PCR results.

Table **5-5**: Comparison between microarray and real-time reverse transcription PCR

results. Data are presented as fold changes (signal ratio) for all conditions tested.

	ppsA		*pntA*		*rpsQ*	
Ratio	Microarray	RT-PCR	Microarray	RT-PCR	Microarray	RT-PCR
WT G / WT	0.04	0.03	0.57	0.41	0.51	0.27
CRP* G / CRP*	0.38	0.26	1.04	1.08	1.22	1.97
CRP* G / WT G	5.36	4.00	1.87	1.51	1.47	1.10

5.5 Discussion

We previously used a CRP* strain to promote expression of xylose transporters in the presence of glucose to produce xylitol from a glucose+xylose mixture, with xylose metabolism disabled (Cirino et al., 2006). Plasmid-based, CRP-independent expression of xylose transporters in wild-type *crp* strains was an alternative strategy we explored to enhance xylose uptake and xylitol production in the presence of glucose (Khankal et al., 2008a). However the favorable effects of CRP* expression were found to go beyond improving xylose transport and to include other beneficial phenotypes such as reduced acetate production and higher yields on xylose reduced per mole of glucose consumed (Khankal et al., 2008b).

In *E. coli*, complete oxidation of glucose during aerobic growth requires that respiration and anabolic metabolism consume reducing equivalents as they are generated (Fuhrer and Sauer, 2009). Elevated glucose flux beyond the capacity of respiration and growth results in incomplete oxidation and acid secretion. Heterologous, NADPH-dependent xylitol production can act as an added electron sink in strains producing xylitol from a mixture of glucose and xylose, and an increased ability to produce xylitol during aerobic growth is expected to increase glucose oxidation and tricarboxylic acid (TCA)

cycle flux, provided reducing equivalents from NADH can be converted to NADPH. Alternately, increased expression of genes involved in glucose oxidation would allow for increased xylitol production, provided reducing equivalents can be delivered as NADPH. Our transcription analysis sheds light on the observed ability of CRP* strains expressing xylose reductase to produce more xylitol and secrete less acetate than similar wild-type CRP strains constitutively expressing a xylose transporter (as reported previously (Khankal et al., 2008a, Cirino et al., 2006)).

TCA cycle genes involved in reactions between succinate and oxaloacetate are upregulated in PC05 compared to W3110 in the presence of glucose, as listed in Table **5-2**. These include *sdhA*, *sdhB*, *sdhD* (encoding succinate dehydrogenase), *fumA* (encoding fumarate hydratase class I), and *mdh* (encoding malate dehydrogenase). All of these genes are known to have CRP binding sites in their promoter regions, so increased expression in PC05 is likely due to direct regulatory effects of CRP*. A strain with a more active TCA cycle potentially increases glucose oxidation, produces more NADPH and produces less acetate (Wolfe, 2005, Phue and Shiloach, 2004). Upregulation of *acs* (acetyl-CoA synthetase) in CRP* G compared to WT G (3.63-fold) is difficult to interpret but may promote acetate assimilation instead of accumulation. Also shown in Table **5-2**, *sthA* encoding soluble pyridine nucleotide transhydrogenase is differentially expressed in CRP* G compared to WT G (CRP * G / WT G ratio of 0.6). SthA is believed to primarily oxidize NADPH to regenerate NADH (Fuhrer and Sauer, 2009) and an increase in NADPH demand corresponds to reduced *sthA* expression (Sauer et al., 2004, Canonaco et al., 2001). As shown in Table **5-2** , *sthA* expression is lower with CRP* G compared to WT G. Transcriptional control of *sthA* is not well understood, but the lack of an apparent

CRP binding site suggests that reduced *sthA* expression may be a result of increased NADPH demand rather than a direct result of CRP*-mediated control. Consistent with this apparent elevated demand for NADPH is the fact that the genes encoding both subunits of the membrane-bound, proton-translocating pyridine nucleotide transhydrogenase (*pntA* and *pntB*) are upregulated in CRP* G compared to WT G(1.88 and 1.76-fold respectively). PntAB has been reported to produce 35-45% of the NADPH required for *E. coli* biosynthesis during aerobic growth (Sauer et al., 2004). Interestingly, the *maeB* gene encoding NADP-linked malic enzyme can serve as another source of NADPH regeneration in *E. coli* (Mahajan et al., 1990) and also shows a higher level of transcription in PC05 compared to W3110 in the presence of glucose (CRP* G / WT G ratio of 2.6). Changes in transcriptional patterns of the above mentioned genes can be in response to increased demands for NADPH in CRP* strains, perhaps as this relates to apparently increased anabolic demands (described below).

5.5.1 Cofactor analysis

In order to better understand how CRP* expression may influence NADPH availability for xylose reduction, intracellular cofactor concentrations were quantified for wild-type and CRP* strains engineered to produce xylitol. To prevent xylose metabolism in these strains, *xylB* encoding xylulokinase was deleted (Khankal et al., 2008a, Cirino et al., 2006). The wild-type CRP strain (W3110*ΔxylB*) was transformed with plasmid pPCC207 for inducible co-expression of an NADPH-dependent xylose reductase (CbXR) and the *E. coli* ATP-dependent xylose transporter system (XylFGH) (Khankal et al.,

2008a), while the CRP* strain (PC05*ΔxylB*) was transformed with plasmid pLOI3815 for CbXR expression (Cirino et al., 2006). The strains were then compared after growth on glucose (no xylitol production) versus growth on glucose plus xylose (resulting in xylose reduction).

Results from the intracellular cofactor concentration measurements are summarized in Table **5-6**. In the absence of xylose, the NADPH concentration is significantly higher in the CRP* strain (0.8 versus 0.5 μM (g cdw)$^{-1}$). Meanwhile, given the ability to reduce xylose to xylitol, the NADPH concentration falls to a much lower level in the CRP* strain (from 0.8 to 0.1 μM (g cdw)$^{-1}$) compared to wild-type CRP (from 0.5 to 0.3 μM (g cdw)$^{-1}$). While the NADPH/NADP$^+$ ratios are nearly identical for both strains in the absence of glucose, the significant consumption of NADPH during xylose reduction coincides with a significantly larger drop in the NADPH/NADP$^+$ ratio for CRP* (from 0.09 to 0.01) compared to wild-type CRP (from 0.10 to 0.05). Also note that the oxidized NADP$^+$ concentrations are significantly elevated in the CRP* strain, while both NADH and NAD$^+$ concentrations are much lower. The net effects are higher total NADP(H) (i.e. NADPH plus NADP$^+$) concentrations and lower NAD(H) concentrations in the CRP* strains compared to wild-type. It is also noteworthy that the NADH/NAD$^+$ ratio is significantly lower in the CRP* strain under both conditions tested.

Table **5-6**: Intracellular cofactor concentrations (μM (g cdw)$^{-1}$) and ratios for strains engineered to produce xylitol. W3110Δ*xylB* harboring plasmid pPCC207 expresses CbXR and XylFGH, while PC05Δ*xylB* harboring plasmid pPLOI3815 expresses CbXR. Strains were grown in the presence of glucose only (G) or in glucose plus xylose (GX), enabling xylose reduction during glucose metabolism. Standard deviations were less than 15% unless noted.

	NADH	NAD^+	NADPH	$NADP^+$	NADH /NAD+	NADPH /NADP+	NAD(H)	NADP(H)
W3110Δ*xylB* + pPCC207 GX	10.3	47.9	0.3±0.05	5.8	0.22	0.05	58.2	6.1
W3110Δ*xylB* + pPCC207 G	10±1.8	47.9	0.5	5.1±1.0	0.21	0.10	57.9	5.6
PC05Δ*xylB* + pLOI3815 GX	1.8±0.7	33.1	0.1±0.04	9.9±2.3	0.05	0.01	34.9	10.0
PC05Δ*xylB* + pLOI3815 G	3.8±0.7	39.2	0.8	8.7±2.6	0.10	0.09	43.1	9.6

While the mechanisms by which cells maintain redox balance are not well understood, it is clear that both the concentrations and ratios of reduced and oxidized cofactors play a critical role in the coupling between anabolic and catabolic metabolism. The known regulatory influences of NADH levels and/or the $NADH/NAD^+$ ratio on a number of enzymes involved in central metabolism and glucose oxidation (e.g. pyruvate dehydrogenase (Kim et al., 2008, Shen and Atkinson, 1970), citrate synthase (Maurus et al., 2003, Nguyen et al., 2001) and α-ketoglutarate dehydrogenase (Smith and Neidhardt, 1983)) suggest that the reduced NADH levels indirectly related to CRP* expression are likely to promote conditions favorable for NADPH-dependent xylitol production during glucose metabolism (e.g. elevated TCA cycle flux and more complete glucose oxidation). To ensure continued growth, *E. coli* balances intracellular concentrations and ratios of the reduced and oxidized cofactors through a complex interplay between catabolic

metabolism, anabolic metabolism, redox-sensitive regulation (both genetic and allosteric) and transhydrogenase activities (Durnin et al., 2009, Fuhrer and Sauer, 2009, Bizouarn et al., 2002, Arner and Holmgren, 2000, Boonstra et al., 1999). While the mechanisms of maintaining redox balance are not well understood, the expression level of enzymes and regulators involved in redox metabolism clearly plays a critical role. It is thus perhaps not surprising that altering the activity of a global regulator has a significant impact on cofactor concentrations and the range of attainable redox states. CRP* expression causes increased production of NADPH relative to NADH, along with (or perhaps due to) increased tolerance to a range of NADPH levels and NADPH/NADP$^+$ ratios, leading to elevated xylitol production in our engineered strains.

5.5.2 Expression of "unnecessary" genes

Most of the genes that are differentially expressed between PC05 and W3110 in the presence of glucose (231 out of 349) are upregulated (Figure **5-4**), supporting the generally assumed behavior of CRP* in alleviating glucose-dependent catabolite repression. The otherwise unnecessary upregulation of these genes likely causes a significant increase in demand for carbon and energy, helping to explain the slower growth rate observed for PC05 compared to W3110 (Cirino et al., 2006). Notable genes that fall into this upregulated category include many involved in amino acids metabolism, such as *tnaA* (encoding tryptophanase), *thrA* (aspartokinase I and homoserine dehydrogenase I), *glyA* (serine hydroxymethyl transferase), *tdcB* (threonine dehydratase), *thrC* (threonine synthase), *thrB* (homoserine kinase), and *asnA* (asparagine synthetase A)

(expression data are given in Table **5-2**). Upregulation of amino acid metabolism pathways may be in response to increased protein synthesis demands caused by upregulation of other genes.

5.5.3 Catabolite repression and inducer exclusion

A relationship between the phosphorylation state of enzyme IIA^{Glc} and the intracellular "phosphoenolpyruvate (PEP)/pyruvate" ratio has been suggested (Hogema et al., 1998). Decreased levels of phosphorylated enzyme IIA^{Glc} is usually accompanied by decreased PEP/pyruvate ratios. The crucial role of the unphosphorylated form of enzyme IIA^{Glc} in catabolite repression and inducer exclusion is well documented (Eppler and Boos, 1999, Hogema et al., 1998, Amin and Peterkofsky, 1995, Saier, 1989, Nelson et al., 1983, Yang and Epstein, 1983, Osumi and Saier, 1982). Enzyme IIA^{Glc}, when unphosphorylated, inhibits activity of other transport systems and also inhibits activity of adenylate cyclase. As shown in Table **5-2**, phosphoenolpyruvate synthase (encoded by *ppsA*) is expressed to a higher level in PC05 compared to W3110 in the presence of glucose (CRP* G / WT G ratio of 5.35). Upregulation of this enzyme which mediates conversion of pyruvate to PEP may increase the intracellular PEP/pyruvate ratio, resulting in an increase in the phosphorylated form of enzyme IIA^{Glc}. This in turn may increase adenylate cyclase activity (Amin and Peterkofsky, 1995, Yang and Epstein, 1983) and further help to alleviate catabolite repression and inducer exclusion in PC05.

5.6 Conclusions

We have used microarray analysis to compare the transcriptomes of *E. coli* W3110 expressing wild-type CRP and mutant strain PC05 expressing CRP* in the presence and absence of glucose. Table **5-3** summarizes the genome-wide effects of CRP* expression under the conditions tested. Gene expression in the context of CRP* in the presence of glucose is very different from that of wild-type in the absence of glucose. Although fewer genes show expression sensitivity to glucose in PC05 compared to W3110, CRP* does not completely eliminate glucose effects. Many genes showing significant differential expression in CRP* versus wild-type CRP help to explain the observed differences in cofactor levels and metabolic behavior of CRP* strains.

5.7 References

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Appendix A

Strains, plasmids and primers

A.1 Strains

Table **A-1**: Strains

	Strain	Genotype	Description	Date
1	RK01	BW27752 ΔKan	Used pFTA to Kick out Kan	Apr-05
2	RK02	PC05Φ(ΔxylG FRT-kan-FRT)	Used AH280 phage library	May-05
3	RK03	PC05Φ(ΔxylE FRT-kan-FRT)	Used AH257 phage library	May-05
4	RK04	PC07Φ(ΔxylG FRT-kan-FRT)	Used AH280 phage library	May-05
5	RK05	PC07Φ(ΔxylE FRT-kan-FRT)	Used AH257 phage library	May-05
6	RK06	PC05Φ(ΔxylB FRT-app-FRT)	Used PC06 phage library	Jun-05
7	AHO7	AH01ΔKan	Used pFTA to Kick out Kan	Jun-05
8	RK07	RK06Φ(ΔxylG FRT-kan-FRT)	Used AH280 phage library	Jun-05
9	RK08	RK06Φ(ΔxylE FRT-kan-FRT)	Used AH257 phage library	Jun-05
10	RK09	RK02 ΔKan	Used pFTA to Kick out Kan	Jun-05
11	RK10	RK03 ΔKan	Used pFTA to Kick out Kan	Jun-05
12	RK11	RK04 ΔKan	Used pFTA to Kick out Kan	Jun-05
13	RK12	RK05 ΔKan	Used pFTA to Kick out Kan	Jun-05
14	RK13	RK06 Δapp	Used pFTA to Kick out app	Jun-05
15	RK14	AH280 ΔKan	Used pFTA to Kick out Kan	Jun-05
16	RK15	Rk14Φ(ΔxylE FRT-kan-FRT)	Used AH257 phage library	Jun-05
17	RK16	Rk15 ΔKan	Used pFTA to Kick out Kan	Jul-05
18	Rk17	AH257 ΔKan	Used pFTA to Kick out Kan	Jul-05
19	PP03	PP01 ΔApp	Used pFTA to Kick out App	Jul-05
20	PP04	PP02 ΔApp	Used pFTA to Kick out App	Jul-05
21	RK18	W3110Φ(ΔxylG FRT-kan-FRT)	Used AH280 phage library	Jul-05
22	RK19	W3110Φ(ΔxylE FRT-kan-FRT)	Used AH257 phage library	Jul-05
23	RK20	W3110Φ(CRP *TET)	Used ET23 phage library	Aug-05
24	RK21	RK20Φ(ΔxylB FRT-app-FRT)	Used PC06 phage library	Aug-05
25	RK22	W3110Φ(araEp Pcp13 -araE)	Used BW27752 phage library	Aug-05

Table **A-1**, continue

	Strain	Genotype	Description	Date
26	RK23	RK21 Δapp	Used pFTA to Kick out App	Aug-05
27	RK24	RK18 ΔKan	Used pFTA to Kick out Kan	Aug-05
28	RK25	RK19 ΔKan	Used pFTA to Kick out Kan	Aug-05
29	RK26	RK22 ΔKan	Used pFTA to Kick out Kan	Aug-05
30	RK27	RK21Φ(ΔxylG FRT-Kan-FRT)	Used AH280 phage library	Aug-05
31	RK28	RK27 Δ Kan, ΔApp	Used pFTA to Kick out Kan and App	Sep-05
32	RK29	W3110Φ(Δcrp FRT-kan-FRT)	Used JWK5702_2 phage library	Nov-05
33	RK30	RK16Φ(Δcrp FRT-kan-FRT)	Used JWK5702_2 phage library	Nov-05
34	RK31	RK29 ΔKan	Used pFTA to Kick out Kan	Dec-05
35	RK32	RK30 ΔKan	Used pFTA to Kick out Kan	Dec-05
36	RK33	RK28Φ(ΔxylE FRT-Kan-FRT)	Used AH257 phage library	Dec-05
37	RK34	JC17Φ(ΔxylG FRT-Kan-FRT)	Used AH280 phage library	Dec-05
38	RK35	W3110Φ(ΔglpF FRT-Kan-FRT)	Used JWK3898-1 phage library	Dec-05
39	RK36	JC29Φ(ΔxylG FRT-Kan-FRT)	Used AH280 phage library	Dec-05
40	RK37	RK33 ΔKan	Used pFTA to Kick out Kan	Jan-06
41	RK38	RK34 ΔKan	Used pFTA to Kick out Kan	Jan-06
42	RK39	RK35 ΔKan	Used pFTA to Kick out Kan	Jan-06
43	RK40	RK36 ΔKan	Used pFTA to Kick out Kan	Jan-06
44	RK41	RK16Φ(ΔglpF FRT-Kan-FRT)	Used JWK3898-1 phage library	Jan-06
45	RK42	W3110Φ(ΔptsI FRT-Kan-FRT)	Used JWK2409-1 phage library	Jan-06
46	RK43	RK16Φ(ΔptsI FRT-Kan-FRT)	Used JWK2409-1 phage library	Jan-06
47	RK44	RK41 ΔKan	Used pFTA to Kick out Kan	Jan-06
48	RK45	RK42 ΔKan	Used pFTA to Kick out Kan	Jan-06
49	RK46	RK43 ΔKan	Used pFTA to Kick out Kan	Jan-06
50	RK47	W3110Φ(ΔgalP FRT-Kan-FRT)	Used JWK2910-2 phage library	Feb-06
51	RK48	RK16Φ(ΔgalP FRT-Kan-FRT)	Used JWK2910-2 phage library	Feb-06
52	RK49	W3110Φ(ΔptsH FRT-Kan-FRT)	Used JWK2408-3 phage library	Feb-06
53	RK50	RK16Φ(ΔptsH FRT-Kan-FRT)	Used JWK2408-3 phage library	Feb-06
54	RK51	RK47 ΔKan	Used pFTA to Kick out Kan	Feb-06
55	RK52	RK48 ΔKan	Used pFTA to Kick out Kan	Feb-06
56	RK53	RK49 ΔKan	Used pFTA to Kick out Kan	Feb-06

Table **A-1**, continue

	Strain	Genotype	Description	Date
57	RK54	RK50 ΔKan	Used pFTA to Kick out Kan	Feb-06
58	RK55	W3110Φ(ΔaraF FRT-Kan-FRT)	Used JWK1889-1 phage library	Apr-06
59	RK56	RK16Φ(ΔaraF FRT-Kan-FRT)	Used JWK1889-1 phage library	Apr-06
60	RK57	W3110Φ(ΔxylF FRT-Kan-FRT)	Used JWK3538-1 phage library	Apr-06
61	RK58	RK16Φ(ΔxylF FRT-Kan-FRT)	Used JWK3538-1 phage library	Apr-06
62	RK59	W3110Φ(ΔaraE FRT-Kan-FRT)	Used JWK2809-1 phage library	Apr-06
63	RK60	RK16Φ(ΔaraE FRT-Kan-FRT)	Used JWK2809-1 phage library	Apr-06
64	RK61	RK55 ΔKan	Used pFTA to Kick out Kan	Apr-06
65	RK62	RK56 ΔKan	Used pFTA to Kick out Kan	Apr-06
66	RK63	RK57 ΔKan	Used pFTA to Kick out Kan	Apr-06
67	RK64	RK58 ΔKan	Used pFTA to Kick out Kan	Apr-06
68	RK65	RK59 ΔKan	Used pFTA to Kick out Kan	Apr-06
69	RK66	RK60 ΔKan	Used pFTA to Kick out Kan	Apr-06
70	RK67	W3110Φ(ΔxylR FRT-Kan-FRT)	Used JWK3541-2 phage library	Jun-06
71	RK68	RK16Φ(ΔxylR FRT-Kan-FRT)	Used JWK3541-2 phage library	Jun-06
72	RK69	RK67 ΔKan	Used pFTA to Kick out Kan	Jun-06
73	RK70	RK68 ΔKan	Used pFTA to Kick out Kan	Jun-06
74	RK71	AH280(ΔxylB FRT-App-FRT)	Used PC06 phage library	Jul-06
75	RK72	PC06(ΔxylG FRT-Kan-FRT)	Used AH280 phage library	Jul-06
76	RK73	RK71 ΔKan	Used pFTA to Kick out Kan	Sep-06
77	RK74	RK72 ΔKan	Used pFTA to Kick out Kan	Sep-06
78	RK75	SZ31 ΔKan	Used pFTA to Kick out Kan	Sep-06
79	RK76	SE2358 ΔKan	Used pFTA to Kick out Kan	Sep-06
80	RK77	RK75(ΔxylG FRT-Kan-FRT)	Used AH280 phage library	Sep-06
81	RK78	RK76(ΔxylG FRT-Kan-FRT)	Used AH280 phage library	Sep-06
82	RK79	RK77 ΔKan	Used pFTA to Kick out Kan	Oct-06
83	RK80	RK78 ΔKan	Used pFTA to Kick out Kan	Oct-06
84	RK81	RK23Φ(ΔglpF FRT-Kan-FRT)	Used JWK3898-1 phage library	May-07
85	RK82	RK81 ΔKan	Used pFTA to Kick out Kan	Jun-07
86	RK83	W3110Φ(ΔlacZ-A FRT-cmr-FRT)	Used BW26438 phage library	Aug-07
87	RK84	RK16Φ(ΔlacZ-A FRT-cmr-FRT)	Used BW26438 phage library	Aug-07

Table **A-1**, continue

	Strain	Genotype	Description	Date
88	RK85	RK37Φ(ΔlacZ-A FRT-cmr-FRT)	Used BW26438 phage library	Aug-07
89	RK86	Rk21Φ(ΔxylE FRT-kan-FRT)	Used AH257 phage library	Aug-07
90	RK87	RK83 ΔCmr	Used pFTA to Kick out Cmr	Aug-07
91	RK88	RK84 ΔCmr	Used pFTA to Kick out Cmr	Aug-07
92	RK89	RK85 ΔCmr	Used pFTA to Kick out Cmr	Aug-07
93	RK90	RK86 ΔKan	Used pFTA to Kick out Kan	Aug-07
94	RK91	Rk23Φ(ΔxylE FRT-kan-FRT)	Used AH257 phage library	Dec-07
95	RK92	RK91 ΔKan	Used pFTA to Kick out Kan	Dec-07
96	RK93	Rk23Φ(ΔxylE FRT-kan-FRT)	Used JW3991-1 phage library	Jan-08
97	RK94	RK23Φ(ΔlacZ-A FRT-cmr-FRT)	Used BW26438 phage library	Jan-08
98	RK95	RK37Φ(ΔlacZ-A FRT-cmr-FRT)	Used BW26438 phage library	Jan-08
99	RK96	Rk92Φ(ΔxylG FRT-kan-FRT)	Used AH280 phage library	Jan-08
100	RK97	RK93 ΔKan	Used pFTA to Kick out Kan	Jan-08
101	RK98	RK94 ΔCmr	Used pFTA to Kick out Cmr	Jan-08
102	RK99	RK95 ΔCmr	Used pFTA to Kick out Cmr	Jan-08
103	RK100			
104	RK101	RK96 ΔKan	Used pFTA to Kick out Kan	Jan-08
105	RK102	W3110Φ(ΔxylE FRT-kan-FRT)	Used JW3991-1 phage library	Dec-08
106	RK103	RK14Φ(ΔxylE FRT-kan-FRT)	Used JW3991-1 phage library	Dec-08
107	RK104	RK102 ΔKan	Used pCP20 to kick out kan	Feb-09
108	RK105	RK103 ΔKan	Used pFTA to Kick out Kan	Jan-09
109	RK106	RK24Φ(ΔxylE FRT-kan-FRT)	Used JW3991-1 phage library	Mar-09
110	RK107	RK106 ΔKan	Used pFTA to Kick out Kan	Mar-09
111	RK108	RK107Φ(ΔaraE FRT-Kan-FRT)	Used JWK2809-1 phage library	Mar-09
112	RK109	RK107Φ(ΔaraF FRT-Kan-FRT)	Used JWK1889-1 phage library	Mar-09
113	RK110	RK107Φ(ΔlacZ-A FRT-cmr-FRT)	Used BW26438 phage library	Mar-09
114	RK111	RK108 ΔKan	Used pFTA to Kick out Kan	Apr-09
115	RK112	RK109 ΔKan	Used pFTA to Kick out Kan	Apr-09
116	RK113	RK110 ΔCmr	Used pFTA to Kick out Cmr	Apr-09
117	RK114	RK111Φ(ΔaraF FRT-Kan-FRT)	Used JWK1889-1 phage library	Apr-09
118	RK115	RK111Φ(ΔlacZ-A FRT-cmr-FRT)	Used BW26438 phage library	Apr-09
119	RK116	RK114 ΔKan	Used pFTA to Kick out Kan	Apr-09
120	RK117	RK115 ΔCmr	Used pFTA to Kick out Cmr	Apr-09
121	RK118	RK116Φ(ΔlacZ-A FRT-cmr-FRT)	Used BW26438 phage library	Apr-09
122	RK119	RK118 ΔCmr	Used pFTA to Kick out Cmr	May-09

A.2 Plasmids

Table **A-2**: Plasmids

	Plasmid	Vector	Insert
1	pPCC201	pCR 2.1-TOPO	*xylFGH*
2	pPCC202	pCR 2.1-TOPO	*xylE*
3	pPCC203	pLOI3809	*xylE*
4	pPCC204	pCR 2.1-TOPO	*araE*
5	pPCC205	pLOI3809	*xylFGH*
6	pPCC206	pLOI3809	*araE*
7	pPCC207	pLOI3815	*xylFGH*
8	pPCC208	pLOI3809	*pncB*
9	pPCC209	pLOI3815	*pncB*
10	pPCC210	pLOI3809	*glf* (*Z. mobilis*)
11	pPCC211	pLOI3815	*glf* (*Z. mobilis*)
12	pPCC212	pLOI3809	*nadK*
13	pPCC213	pLOI3815	*nadK*
14	pPCC107*	pLOI3815	*xylE*

*Made by Jonathan Chin

A.3 Primers

Table **A-3**: Primers

	Primer	Sequence (5'-3')	Date
1	4765 XYLF For	CAGAAGGCCCTACACCATGAA	04/2005
2	4059 XylHfor1	CGCCGCCGATGCCGAAGTGG	01/2005
3	4060 XylHrev1	GGCCATCTCGCGCTCCCTGG	01/2005
4	4766 XYLH Rev	AACTCAAAACCGGTAATACGTAACC	04/2005
5	4763 XYLE For	TGGTCTAAGGCAGGTCTG	04/2005
6	4789 XYLE For Rbs	AGGAGATATCATATGAATACCCAGTATAATTCC	04/2005
7	4922 XYLE New for	AGGAGGATACATATGAATACCCAGTATAATTCCAGTT	04/2005
8	XYLE NEW FOR (invitro.)	AGGAGGATACATATGAATACCCAGTATAATTCCAGTT	06/2005
9	XylE3 for	TCTGCATTACCGATCACCATCGT	06/2005
10	XYLE NEW UP	AAGGCAGGTCTGAATGAATACC	04/2005
11	4764 XYLE Rev	GTGCTGGACAGGAAGATTAC	04/2005
12	4797 XYLE REV TE	AAAGTCAGCACGCCGAAATG	04/2005
13	XYLE REV TE (invitro.)	AAAGTCAGCACGCCGAAATG	06/2005
14	xylE rev far (invitro.)	AGGAAGCTCGCCACTGTGAA	07/2005
15	BMEG XYLT FOR (invitro.)	CGTGAATTCAGGAGATATTATGAAACAAAATCGAA ACTCATTATATAT	06/2005
16	BMEG XYLT REV (invitro.)	CTAGGTACCTTATATGGCCTTCTTACTCTTTGAA	06/2005
17	TH XYLE FOR (invitro.)	CGTGAATTCAGGAGATATTATGAATAATAAAGGATT AAATCGCTATG	06/2005
18	TH XYLE REV (invitro.)	CTAGGTACCTTATAACCAAGTATTTTCTAATTGTTC AAG	06/2005
19	4773 ARAE For	GAGCATATGGTTACTATCAATACGGAATC	04/2005
20	4770 ARAE Rev	CACATCCGGCCCGTGAAATC	04/2005
21	4796 ARAE REV TE	AACGGCCAAGTGCCCAATCT	04/2005
22	4798 GLF FOR	AGGAGATATCATATGAGTTCTGAAAGTAGTCAGGG	04/2005
23	4799 GLF REV	CAGCCAAAGCAAGTTTAACTA	04/2005
24	5188 CRPF	TTCGGCAATCCAGAGACAGC	05/2005
25	5189 CRPR	TACCAGGTAACGCGCCACTC	05/2005
26	5190 XYLF F2	AACGCGCTAACCGCCAATAA	05/2005
27	5191 XYLH R2	AGCCTAACCAGACGTCAC	05/2005
28	201 xylR for	TGATGGATGTACCGACCTTC	01/2006
29	202 xylR rev	AGCCTTATCCGACTTGTCAG	01/2006
30	203 glpF for	CGGCACGCCTTGCAGATTAC	01/2006
31	204 glpF rev	GCGCTGCGACACGCTAATGA	01/2006

Table A-3, continue

	Primer	Sequence (5'-3')	Date
32	205 ptsI for	GTTACCATTACCGCTCCGAA	01/2006
33	206 ptsI rev	ATACCGAAGTGGACGAACAG	01/2006
34	207 ptsH for	CAGCCTGTCGGAACTGGTAT	01/2006
35	208 ptsH rev	CTGCTCCAGCTCCTCATCTT	01/2006
36	209 galP for	GTGCCTTCTGAACAACTGAC	01/2006
37	210 galP rev	GTGCGGTACCACTTCTTCAA	01/2006
38	211 araF For	AGGTAATGCGGCCTATTGAC	02/2006
39	212 araF rev	CGAGATGCAGTTCCTGGTAA	02/2006
40	213 araE for	GGACTGCCGAGTGACCTGAT	03/2006
41	214 xylFGH seq1	CGTACCGGCAGCAATACGTT	06/2006
42	215 xylFGH seq2	CTGCTTGCGGTTGAATGCTG	06/2006
43	216 xylFGH seq3	ACCGCCATTACCGGAACGAT	06/2006
44	217 xylFGH seq4	GACCACCGATGGTGCCTACT	06/2006
45	218 xylR 270	AGCGTTGCCGGAGCAGTAAT	06/2006
46	219 xylR 1902	AACGGTTAATCGCGGCGCAT	06/2006
47	220 lacA rev	GGCATGATGCGACGCTTGTT	06/2007
48	221 lacZ for	GCGCGTTGGCCGATTCATTA	06/2007
49	222 cat C1	TTATACGCAAGGCGACAAGG	06/2007
50	223 cat C2	GATCTTCCGTCACAGGTAGG	06/2007
51	224 pncB for	CGCGAGAATTCAGGAGGACAGCTATGACACAATTCGC TTCTCC	03/2009
52	225 pncB rev	CCGCGGGTACCTTAACTGGCTTTTTTAATATGCGGAAGGTC G	03/2009
53	226 zmglf for	CGCGAGAATTCAGGAGGACAGCTATGAGTTCTGAAAG	03/2009
54	227 zmglf rev	TCGAAGGTACCCTACTTCTGGGAGCGCCACATCTCCT	03/2009
55	228 nadK for	CGCGAGAATTCAGGAGGACAGCTATGAATAATCATTT CAAGTG	03/2009
56	229 nadK rev	CCGCGGGTACCTTAGAATAATTTTTTTGACCAGCCGAGCTT GGTGC	03/2009

Appendix B

Methods and protocols

Methods and protocols used in the experiments are explained in detail in "Materials and methods" section of each chapter. Here, protocols which are considered too detailed to be mentioned in the body of the chapters or are not directly related to the main subject are presented. These protocols are modified for using in our lab considering our needs and available equipment.

B.1 Preparation of P1 lysate

1. Dilute a fresh overnight of donor strain 1:100 in LB (no selection). Inoculate two cultures (15 ml): first one to be infected by P1 phage, the second one to be used as lysis control.
2. Grow the cultures to an OD_{600} of 0.2.
3. Adjust $CaCl_2$ in the media to 10 mM final (add ~1.6 ml of 0.1 M $CaCl_2$ to 15 ml culture). Inoculate one of the cultures with 150 μl of P1 virus lysate.
4. Continue to grow cells until lysis (4-6 hours) at 37 ^oC. You may see small clumps of cell debris and the lysed culture will appear clearer than the uninfected control culture.

5. Add ~0.5 ml $CHCl_3$ to the culture and transfer it to a 15 ml conical tube. Mix or vortex lightly. Use glass pipette whenever you touch chloroform. Keep polypropylene 15 ml conical tubes on ice.
6. Spin lysed culture (3750 RPM, 10 min, 4^oC) to pellet cell debris. Transfer the supernatant to a clean glass tube containing 0.5 ml $CHCl_3$. $CHCl_3$ helps to avoid getting background in subsequent transductions.

B.2 P1 Transduction

1. Grow an overnight culture of the recipient strain.
2. Spin down 1 ml of the culture in the microcentrifuge tube (5000 RPM, 5 Minutes).
3. Remove 0.5 ml of supernatant and resuspend the cell pellet in the rest and make it to the final concentrations of 10 mM $MgSO_4$ and 5 mM $CaCl_2$ (add 5 µl of 1.0 M $MgSO_4$ and 25 µl of 0.1 M $CaCl_2$ to the 0.5 ml of culture).
4. Prepare 5 tubes as follows (Table **B-1)**

Table **B-1**: Tubes contents

Tube	Cell (ml)	P1 lysate (µl)
1	0.1	------
2	0.1	10
3	0.1	50
4	0.1	100
5	-------	100

5. Incubate at 37^oC for 30 minutes, no shaking.
6. Add 0.1 ml 1 M NaCitrate to each tube and mix.
7. Add 1 ml LB to each tube. Incubate 1 – 1.5 hours at 37^oC (no shaking).

8. Spin down the cells. Dump supernatant leaving 100 - 150 µl and plate on the appropriate antibiotic media (use beads).

B.3 Spot titer the lysate

1. Spin down an overnight culture and resuspend the pellet in ½ volume of 10 mM Mg SO_4 and 5 mM $CaCl_2$ (5000 RPM for 5 minutes).
2. Add 100 µl of the cell suspension to 3 ml of top agar.
3. Pour on a plate and allow solidifying.
4. Make serial dilutions of the lysate, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} (make dilutions in LB, mix and change tips between solutions)
5. Spot 10 µl on to the plate of each solution.
6. Incubate face up at 37°C.

B.4 Screening for constitutive sugar utilization using triphenyl tetrazolium chloride (TPTC)

1. Grow 3 ml overnight culture in LB + glucose (1%) + xylose (1%) + MOPS (50 mM) to the OD_{600} of 1-2.
2. Spin down and resuspend in 1.5 ml buffer (50 mM KPO_4, pH 7.4 + 100 mg/ml Kanamycin. Let sit for 30 minutes.
3. Repeat pellet and resuspension in 1.5 ml three more times.
4. On last wash, divide sample into 3 X 500 µl in separate tubes before centrifuging.
5. Resuspend each pellet in one of these three solutions:

A: PO_4 + Kan + 1% TPTC

B: PO_4 + Kan + 0.5% glucose + 1% TPTC

C: PO_4 + Kan + 0.5% xylose + 1% TPTC

6. Incubate at 37°C in dark and observe over time.
7. Tubes with utilizing sugar strain will turn pink. Take a picture of tubes.

Appendix C

Microarray statistical analysis

C.1 Written code in R for pair-wise comparisons:

```
setwd("C:/Documents and Settings/khankal/My
Documents/project/rwork/Ecolik12")
crp <- read.csv("input.csv",header=T)
dim(crp)
crp[1,]
library(limma)
y <- as.matrix(crp[,3:16])
dim(y)
y10<-log(y,base=10)
targets <- c(rep('A',5),rep('B',3),'C','D','D','C','D','C')
f<-factor(targets,levels=c('A','B','C','D'))
design<-model.matrix(~0+f)
fit <- lmFit(y10,design)
contrast.matrix<-makeContrasts((fA-fC),levels=design)
fit3 <- contrasts.fit(fit,contrast.matrix)
fit4 <- eBayes(fit3)
p.values <- fit4$p.value
```

```
ids <- as.character(crp[,2])

p.val<-p.adjust(p.values[,1],method="BH")

ord<-order(p.val)

ord.sign<-subset(ord,p.val[ord]<0.05)

ids.sign<-ids[ord.sign]

write.table(ids.sign,file="studyset.txt",sep="

",col.names=F,row.names=F,quote=F)

y3<-y[ord.sign, ]

aa<-cbind(ids.sign,y3)

write.table(aa,file="aa.txt",sep=" ",col.names=F,row.names=F,quote=F)

results<-decideTests(fit4)

vennDiagram(results)
```

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