Engineering *Clostridium acetobutylicum* to permit simultaneous utilization of glucose and xylose: elimination of carbon catabolite repression

by

Mark Raymond Bruder

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Abstract

The industrial gram-positive anaerobe *Clostridium acetobutylicum* is a valued ABE (acetone, butanol, ethanol) solvent producer that is able to utilize a vast array of carbon sources in fermentation. When glucose is present in the growth medium, however, *C. acetobutylicum*, like many gram-positive organisms, exhibits bi-phasic growth characteristics in which glucose is used preferentially over secondary carbon sources, a phenomenon known as carbon catabolite repression (CCR). The secondary carbon source is only utilized when the supply of glucose is exhausted, resulting in inefficient use of complex carbon sources. As biofuel production is sought from cheap feedstock, attention has turned to lignocellulosic biomass. Growth of *C. acetobutylicum* on lignocellulose, however, can be limited by CCR. Here, we present a method to relieve the inhibitory effect of CCR and allow simultaneous utilization of the lignocellulosic sugars glucose and xylose by *C. acetobutylicum*. First, we utilized an *in vivo* gene reporter assay to demonstrate that an identified 14-nucleotide catabolic responsive element (CRE) sequence was sufficient to introduce CCR-mediated transcriptional inhibition, while subsequent mutation of the CRE sequence relieved the inhibitory effect. Next, we demonstrated that *C. acetobutylicum* harboring a CRE-less plasmid-borne xylose and pentose phosphate pathway operon afforded a 7.5-fold increase in xylose utilization in the presence of glucose as compared to a wild-type-CRE plasmid-borne operon, effectively overcoming native CCR effects. The methodology presented here should translate to other members of *Clostridium* that exhibit CCR to enable simultaneous utilization of a vast array of carbon sources.

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Abbreviations:

ABE, acetone-butanol-ethanol

AC, adenylate cyclase protein

ATP, adenosine triphosphate

bp, base pair

CAP, cAMP-associated catabolite gene-activating protein

cAMP, cyclic adenosine monophosphate

CcpA, catabolite control protein A

CCR, carbon catabolite repression

CRE, catabolite responsive element

CRP, cAMP receptor protein

DHAP, Dihydroxyacetone phosphate

EI, enzyme I of PTS

EII, enzyme II of PTS

FBP, Fructose-1, 6-bisphosphate

G3P, Glyceraldehyde-3-phosphate

glcG, glucose-specific PTS enzyme II

HPr, phosphocarrier protein

HPrK, HPr kinase

kbp, kilo base pairs

PEP, phophoenolpyruvate

PTS, PEP-dependent phosphoenolpyruvate-dependent phosphotransferase system

PPP, pentose phosphate pathway

xylA, C. acetobutylicum gene encoding xylose isomerase

xylB, *C.acetobutylicum* gene encoding xylulose kinase

xylT, *C. acetobutylicum* gene encoding xylose-proton symporter

talA, *E. coli* gene encoding transaldolase

tal, *C. acetobutylicum* gene encoding transaldolase

tkt, C. acetobutylicum gene encoding transketolase

Chapter 1- Overview

1.1 Research background

Concerns over energy security, the global supply of fossil fuels, and climate change have led to a realigning of research goals toward renewable, sustainable, and environmentally neutral energy supplies. Presently, biologically produced ethanol and biodiesel represent the two most widely used first-generation biofuels (Peralta-Yahya et al. 2012), however each have substantial limitations that render them unattractive energy sources long term: ethanol has only 70% of the energy density of petroleum derived gasoline and can only be used as an additive to traditional petrol in North America, as opposed to an alternative. Further, ethanol does not fit seamlessly with existing infrastructure, as it has a tendency to absorb water from the air leading to corrosion of pipes and engines, and distillation from fermentation broth is quite labor intensive. Biodiesel, on the other hand, has approximately 91% of the energy density of D2 diesel and can provide an additive or alternative to diesel, however has a propensity for developing wax build-up in the fuel if the temperature is too low, creating potential problems for engines as well as geographic limitations for its production and transport (Peralta-Yahya et al. 2012). Additionally, ethanol and biodiesel are predominantly produced from food crops, creating a link between food prices and fuel prices (Demirbas 2009).

Recently, attention has turned to butanol as an attractive biofuel due to its resemblance to gasoline in terms of physical, chemical, and combustibility properties (Jang et al. 2012; Szwaja and Naber 2010) (Table 1).

Table 1 Comparison of properties of several fuels (Adapted from Szwaja and Naber 2010)

Fuel	Chemical formula	Specific gravity (kg/dm3)	Specific Energy (MJ/kg)	Energy Density (MJ/L)	Octane Number
Methanol	CH_3OH	0.7913	20.08	15.9	99
Ethanol	C_2H_5OH	0.7894	26.83	18.4	100
n-Butanol	C_4H_9OH	0.8097	36	29.2	86
Gasoline	$CH_{1.87}$	0.743	42.9	32	87

Butanol has 84% of the energy density as compared to gasoline, limited miscibility with water and completely miscible in gasoline, and is completely compatible with existing infrastructure, so it is essentially 'ready-made' for integration (Demirbas 2009; Peralta-Yahya et al. 2012).

Butanol produced from microorganisms using cheap, renewable feedstock represents an attractive alternative to petroleum-derived gasoline, and as such, attention has turned to members of the genus *Clostridium* for their ability to produce butanol as a metabolite (Jang et al. 2012). In order to compete economically with gasoline, however, microbial-based production platforms must be engineered for improvements in overall yield and efficiency of conversion (Zheng et al. 2009).

The non-food feedstock lignocellulose is among the most abundant renewable biomass on Earth and is among the most underused resources, consisting of approximately 70% sugars (Peralta-Yahya et al. 2012). Focusing metabolic engineering efforts on utilization of this abundance of stored sugars will serve to make biofuels much more attractive economically as a viable alternative to fossil fuels.

1.2 Research objectives

We hypothesize that relief of CCR of xylose can be achieved by mutating the CRE sequence found in the xylose catabolic operon leading to improved carbon consumption and ABE solvent production.

The overall objectives of this thesis include:

1. Experimentally confirm that a 14-nucleotide putative CRE sequence is sufficient to impart CCR-type inhibition in its non-native context.
2. Provide evidence that mutating this CRE sequence relieves CCR-type inhibition.
3. Investigate the effect of mutating the CRE sequence in its native context to relieve CCR-type inhibition from a secondary carbon source catabolic operon and permit simultaneous co-consumption in the presence of glucose.

1.3 Outline of thesis

Chapter 2 is a review of the relevant literature related to the biotechnological importance of members of the genus *Clostridium,* characterization of the elements of CCR in Gram-positive microorganisms, and a synopsis of specific CCR in *Clostridium* and other Firmicutes. Finally, an overview of genetic and bioprocessing strategies for the circumvention of native CCR effects is presented, with an emphasis on strategies aimed at introducing the ability to co-utilize secondary carbon sources such as xylose in the presence of glucose in *C. acetobutylicum.* In Chapter 3, all materials and methods relevant to this study are described, including strain and plasmid construction, media and cultivation, and analytical analyses. In Chapter 4, the results of this study are stated including, (1) experimental confirmation that a 14-nucleotide CRE sequence is sufficient to impart a phenotype consistent with CCR-type inhibition in a non-native context; (2) mutating the degenerate nucleotides of the CRE consensus sequence does not relieve the observed inhibition, while non-degenerate nucleotides are sensitive; and (3) over-expression of a plasmid-borne, native xylose catabolic operon and pentose phosphate pathway with a mutated CRE imparts a dual substrate co-utilization phenotype on *C. acetobutylicum*. Chapter 5 contains a discussion of the results presented in Chapter 4 and comparison to other relevant studies in the literature. Finally, Chapter 6 states the conclusions of this study and a proposal for future studies.

Chapter 2- Literature Review

2.1 The genus *Clostridium*

Clostridium is among the largest genera of prokaryotes, comprised of more than 150 distinct species that satisfy a traditionally simple set of criteria: Gram-positive rods with the capacity to form endospores, and an obligate anaerobic metabolism. As a result of these simple criteria, *Clostridium* is well known for its extreme phylogenetic heterogeneity. The arrival of 16S rRNA cataloging and accessible genome sequencing technologies, however, has led to a proposed re-organization and re-classification of the taxonomy of the genus, as fewer than half of the historically-classified members of *Clostridium* were found to possess sufficient similarity to the type species, *C. butyricum* (Collins et al. 1994; Gupta and Gao 2009; Lawson et al. 1993; Yutin and Galperin 2013). The heterogeneity of the genus allows members of *Clostridium* to colonize and thrive in incredibly diverse habitats, owing to endospores resistant to oxygen, heat, dessication, and acids (Tracy et al. 2012), and as such, many species of *Clostridium* are recognized for their importance medically, as both causative agents (e.g. *C. botulinum, C. difficile, C. perfringens, C. tetani*, which are implicated in hospital-acquired infections and the source of some of the most potent known biological toxic compounds) (Popoff and Bouvet 2013), and potential therapeutics (e.g. anticancer properties of apathogenic species such as *C. acetobutylicum, C. butyricum,* and *C. novyi*) (Barbé et al. 2005; Bettegowda et al. 2006; Nuyts et al. 2002). Additionally, attention has turned to several apathogenic species of immense industrial biotechnological interest for their diverse substrate utilization and unique metabolic capabilities, including production of alcohols and solvents of industrial importance as bulk chemicals, precursors, and biofuels (Tracy et al. 2012). Of particular interest is production of butanol, which

in recent years has garnered interest for its potential as a biofuel that can substitute for petroleum-derived gasoline in terms of physicochemical and combustibility properties (Jang et al. 2012; Tracy et al. 2012). Butanol production has been investigated in several members of *Clostridium*, including *C. acetobutylicum*, *C. tyrobutyricum*, *C. pasteurianum*, *C. beijerinckii*, and *C. saccharoperbutylacetonicum* (Jiang et al. 2010; Lütke-Eversloh and Bahl 2011; Moon et al. 2011; Noguchi et al. 2013; Xiao et al. 2012), however, fundamental issues regarding process shortcomings including high feedstock cost, poor yield, and product toxicity (Demirbas 2009; Zheng et al. 2009) must be resolved in order to make biobutanol from *Clostridium* an attractive biofuel production platform. To compound this issue, genetic engineering tools are generally lacking in clostridia, as well as seemingly strain-dependent, (Pyne et al. 2014a), resulting in lagging tool advancement and significant difficulty in applying procedures in clostridial species.

2.2 *C. acetobutylicum* and lignocellulosic biomass

To date, the most extensively studied member of *Clostridium is C. acetobutylicum*, and as such, availability of genetic engineering tools is by far the most advanced in this organism (Pyne et al. 2014a). *C. acetobutylicum* is a classical ABE (acetone-butanol-ethanol) fermenter, of which butanol is the primary product. AB fermentation was the dominant production method of acetone and butanol until the introduction of the current petrochemical method in the 1950s, and has since experienced a revival of this process in several countries, including most notably, China (Green 2011; Ni and Sun 2009). While many metabolic engineering strategies have focused on increasing the product yield of butanol from several substrates (Lütke-Eversloh and Bahl 2011; Tracy et al. 2012), including combined gene knock-down and over-expression strategies to

increase solvent formation to 30 g l^{-1} (compared to approximately 20 g l^{-1} for wild-type cultivation) and favor product selectivity toward butanol and ethanol production (as opposed to butanol and acetone for wild-type) (Sillers et al. 2009), relatively few studies have focused on the choice of feedstock itself (Jang et al. 2012; Wang et al. 2014).

While many carbon sources such as glucose are readily converted, they often suffer from high costs that render biofuel production unsustainable economically, or present political and ethical questions of repurposing agricultural land toward biofuel production (Demirbas 2009). Lignocellulosic biomass (plant biomass) is among the most abundant renewable feedstock on Earth, and as such, has garnered attention as a viable substrate for biofuel production (Hill et al. 2006; Jang et al. 2012). Lignocellulose is composed of carbohydrate polymers (cellulose and hemicellulose) of which glucose, xylose, and arabinose are major constituents, and an aromatic polymer (lignin). Conversion of these stored carbohydrates is neither easily nor efficiently attained, requiring pre-treatment to release fermentable sugars and engineering strategies to enhance the metabolic capabilities of microorganisms to utilize the substrate efficiently (Jang et al. 2012; Stephanopoulos 2007).

The genome of *C. acetobutylicum* encodes putative xylose uptake and metabolism genes, including a putative xylose proton-symporter (CAC1345) two putative xylose isomerase genes (CAC1346, CAC2610), and two putative xyulose kinases (CAC1344, CAC2612). Additionally, putative pentose phosphate pathway (PPP) genes have been tentatively identified, including transaldolase and transketolase (CAC1347 and CAC1348, respectively) as well as two possible ribulose epimerases (CAC1730, CAC1349) and ribulose isomerase (CAC2880) (Grimmler et al. 2010; Nölling et al. 2001; Servinsky et al. 2010) (Figure 1). Recently, several studies have produced experimental correlative evidence demonstrating the roles of many of these genes in

xylose metabolism (Grimmler et al. 2010; Jin et al. 2014; Xiao et al. 2011).

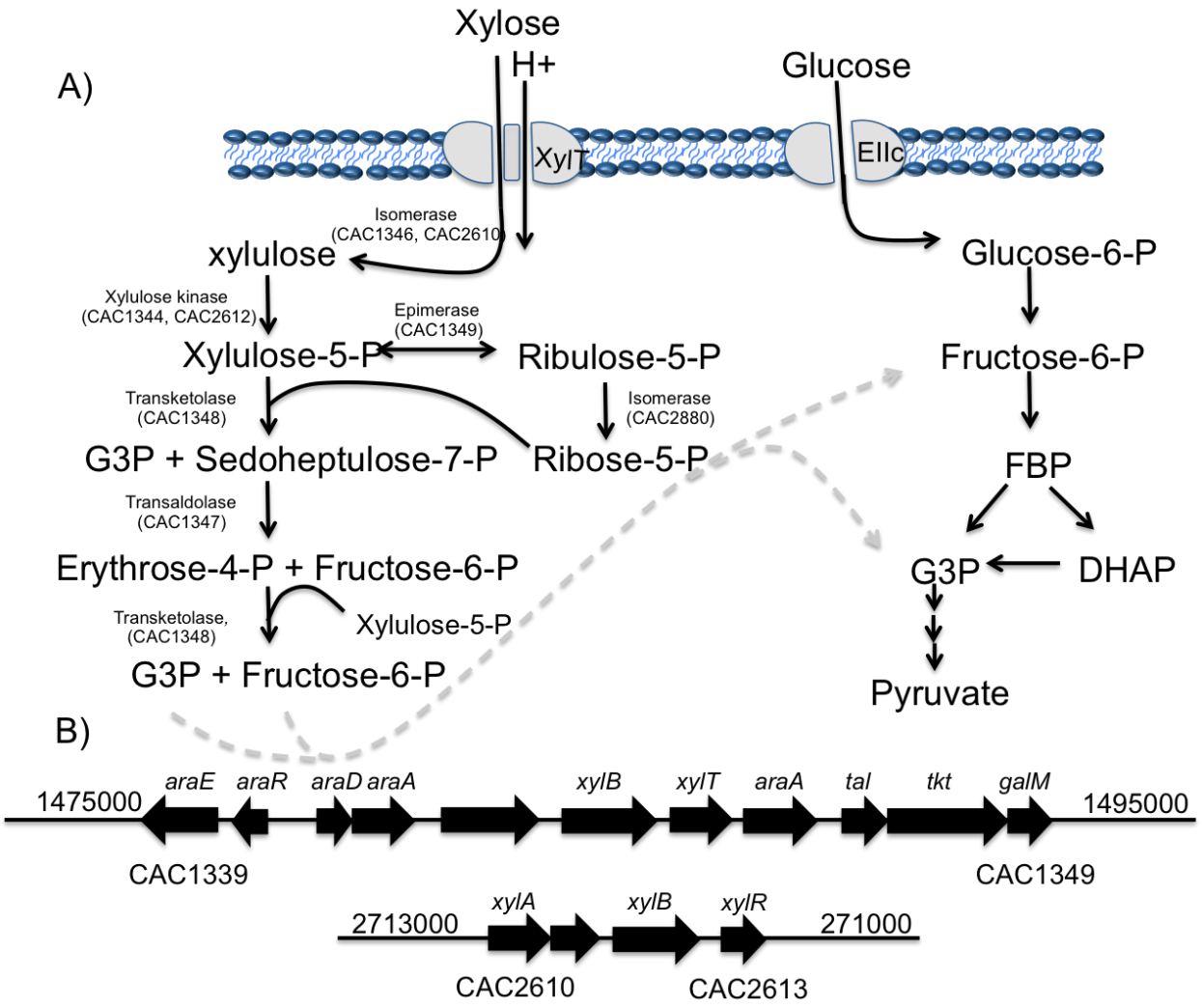

Figure 1 Xylose utilization pathway in *C. acetobutylicum* A) xylose degradation pathway via pentose phosphate pathway and entrance points into glycolysis. B) genomic organization of *C. acetobutylicum* genes involved in xylose degradation.

While *C. acetobutylicum* is able to utilize a remarkable array of carbon sources, including the constituents glucose and xylose of lignocellulosic biomass (Tracy et al. 2012), like many

Gram-positive organisms, including several species of *Clostridium, C. acetobutylicum* is subject to a phenomenon known as carbon catabolite repression (CCR) wherein utilization of secondary carbon sources (i.e. xylose) are restricted when a more preferable, primary carbon source (i.e. glucose) is present (Hueck et al. 1994; Tangney et al. 2003)

2.3 A model of CCR for Firmicutes

CCR has been extensively studied in the ubiquitous Gram-negative model organism *E. coli,* and more recently the molecular mechanism has been elucidated in the model gram-positive Firmicute, *B. subtilis*. In both species, CCR exhibits global transcriptional control over several operon-specific regulatory mechanisms including repression (or lack of activation) of genes encoding secondary-carbon source utilization pathways. Although the CCR mechanism in both organisms is elegantly intertwined with native PTS enzymes that are additionally involved as signaling molecules, the molecular mechanisms of CCR are significantly dissimilar; in *E. coli,* which uses the phosphorylation state of domain A of the glucose-specific enzyme II (EIIA) of the phosphoenolpyruvate-dependent phosphotransferase system (PTS) to direct activity of adenylate cyclase (AC) and consequently the concentration of cyclic AMP (cAMP) inside the cell, cAMP-associated catabolite gene-activator protein (CAP; also called cAMP receptor protein (CRP)) complexes bind and *activate* the promoters of catabolic genes (Gorke and Stulke 2008). In the Gram-positive *B. subtilis*, conversely, CCR is mediated through the *prevention* of transcriptional activation of catabolic gene pathways (Gorke and Stulke 2008). Additionally, CCR is facilitated through an altogether different molecular signaling cascade, although they are mechanically analogous (Gorke and Stulke 2008). Analysis of the *C. acetobutylicum* genome (Nölling et al.

2001; Tangney et al. 2003) has identified all of the requisite proteins involved in the model of CCR in *B. subtilis,* and it is therefore hypothesized that they share similar CCR mechanisms (Tangney et al. 2003), and as such, the model of CCR in *B. subtilis* will now be parlayed to that of *C. acetobutylicum*.

The signaling intermediate utilized in *C. acetobutylicum* is the phospho-carrier protein HPr, which is a component of the PTS, the major carbohydrate transport system in clostridia and other microorganisms (Postma et al. 1993; Tangney and Mitchell 2007) (Figure 2). Briefly, the PTS proteins enzyme I (EI), histone-containing protein (HPr), and enzyme II (EII) form a phosphorylation cascade to link carbohydrate uptake with its simultaneous phosphorylation. In its native PTS context, HPr is phosphorylated at a conserved histidine residue (HPr-His^{16}-P), however in the presence of glycolytic intermediates including fructose-1,6-bisphosphate, the expression of a bi-functional ATP-dependent kinase-phosphorylase, HPr kinase (HPrK), is induced which competitively phosphorylates HPr at a conserved serine residue (HPr-Ser^{46}-P) (Nessler et al. 2003). This serine-phosphorylated HPr forms a complex with catabolite control protein A (CcpA) (HPr-Ser^{46}-P-CcpA), which subsequently forms a dimer by associating with a second HPr-Ser^{46}-P-CcpA, and this complex binds at catabolite responsive element (CRE) sites within the promoter region or coding sequence of transcriptional units to inhibit transcription (Warner and Lolkema 2003). Alternatively, in the presence of inorganic phosphate, HPrK catalyzes the dephosphorylation of serine-phosphorylated HPr, presumably relieving CCR inhibition (Nessler et al. 2003). It is also worth noting that analysis of CCR in *B. subtilis* led to the discovery of a novel HPr-like protein, catabolite repression HPr (Crh), that shares homology with the *B. subtilis* HPr. Crh maintains the conserved Ser46 of HPr, however the HPr His16 is replaced with a glutamine residue in Crh (Galinier et al. 1997). Further, Crh has been

experimentally demonstrated to substitute for HPr in CCR, but not in its PTS function (Galinier et al. 1999; Galinier et al. 1997). A Crh homologue is absent from the *C. acetobutylicum* genome (Nölling et al. 2001).

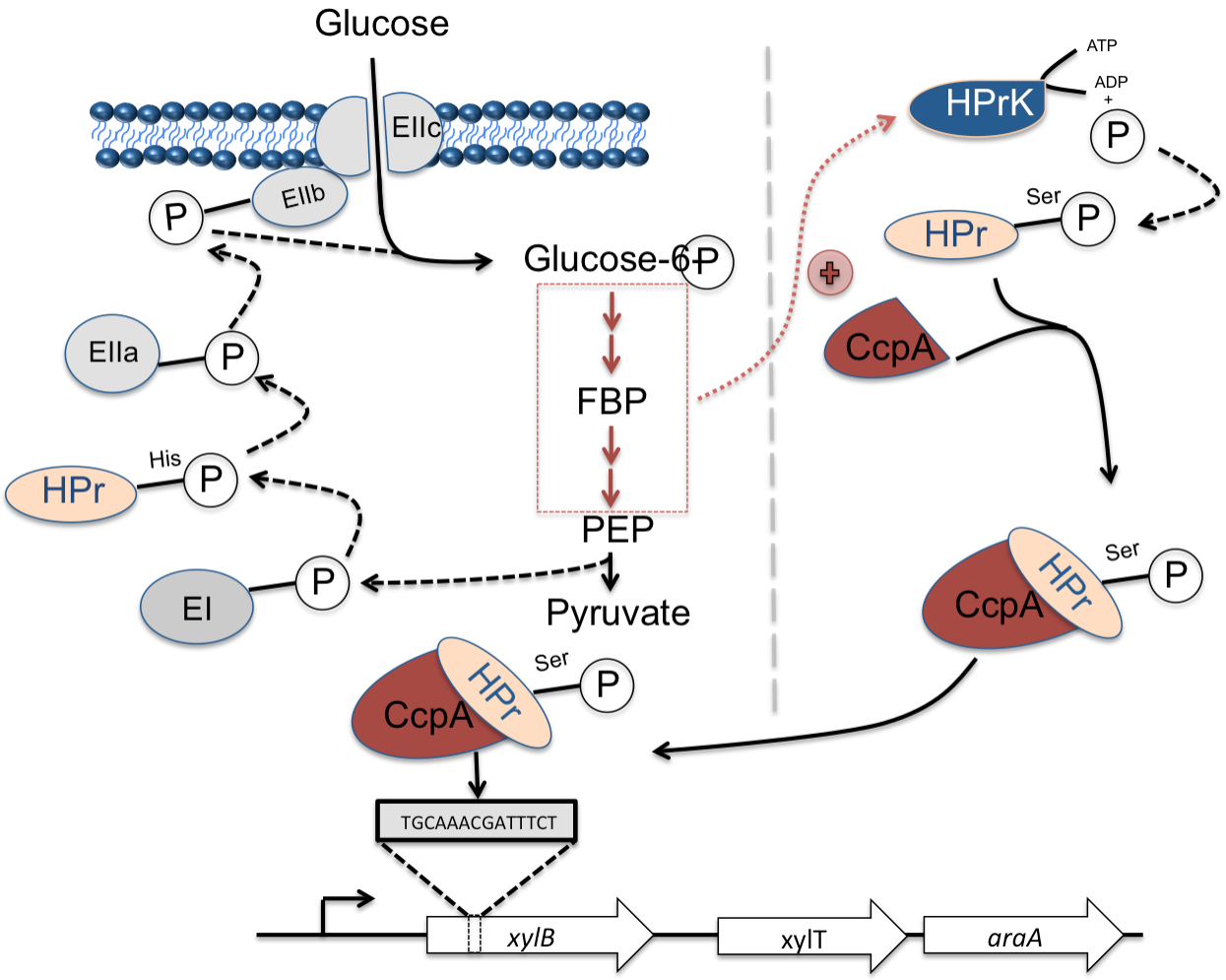

Figure 2 PEP-dependent PTS system and model of CCR-type inhibition. Phospho-relay system to couple glucose transport with its simultaneous phosphorylation. Briefly, phosphate from PEP is transferred in a relay cascade through EI, HPr, and EII to phosphorylate glucose as it is transported into the cell to undergo glycolysis. In the presence of glycolytic intermediates, the expression of HPr Kinase is induced, which competitively phosphorylates HPr at a conserved Ser-46 residue, which forms a complex with CcpA to bind at CRE sites and inhibit transcription.

2.4 Structural characterization of CcpA and its cognate DNA binding site CRE

The CcpA protein belongs to the LacI/GalR family of allosteric transcriptional regulators, which act in a so-called "roadblock" mechanism in which the regulator binds at an operator sequence to provide a physical barrier to transcript elongation by the host RNA Polymerase (Swint-Kruse and Matthews 2009). CcpA from *C. acetobutylicum* shares 63% similarity at the amino acid level with CcpA from *B. subtilis* and *B. megaterium*, and 48% similarity with the more distantly related family-member LacI from *E. coli*. Although the structure of CcpA from *C. acetobutylicum* has not been solved, structural characterization of several members of this family is available, including *apo*- and *holoenzyme* structures of the archetypal LacI and PurR regulators from *E. coli* (Choi and Zalkin 1992; Lewis et al. 1996; Schumacher et al. 1994), as well as several CcpA regulators, including those from *B. subtilis* (Schumacher et al. 2011), *B. megaterium* (Schumacher et al. 2004), and *L. lactis* (Loll et al. 2007). These studies have formed the basis for detailed structural analysis of the general architecture of these regulators, as well as meticulous exploration of the specific amino acid residues that are involved in the various functions of the proteins, including dimerization, effector binding, and DNA binding (Schumacher et al. 2004; Schumacher et al. 2006; Schumacher et al. 2007).

Analysis of CRE sequences in *B. subtilis* has led to the derivation of a 14-nucleotide consensus sequence with bi-fold symmetry and considerable degeneracy (Miwa et al. 2000). This has led to the investigation of how CcpA tolerates considerable degeneracy in the CRE sites to bind with high-affinity at such diverse sequences. To address this question, crystal structures of the CcpA-(HPr-Ser^{46}-P) dimer complex bound to various CRE sites have been solved in *B. subtilis* and *B. megaterium* (Schumacher et al. 2004; Schumacher et al. 2011) (Figure 3). The most striking observations from these studies revealed that the CcpA-bound operators display

differing bend angles, or kinking, which are accommodated by flexibility of CcpA. It is this plasticity that permits CcpA binding at diverse operators. CcpA is composed of a DNA-binding domain at its N-terminal, which is comprised of two distinct DNA-binding elements: a three-helix bundle of which helices 1 and 2 form the DNA major-groove-binding helix-turn-helix motif (HTH) connected via a flexible linkage to a hinge-helix motif, which inserts into the minor-groove of the central CpG step of the CRE sequence and contributes to the kinking of the DNA (Schumacher et al. 2004). Furthermore, these crystal structures exposed the specific amino acid residues responsible for binding CRE site DNA and shed light on the significant degeneracy tolerated in the CRE site as well as the critical, specified, and most conserved nucleotides in the CRE sequence (i.e T**G**NAAN**CG**NWNN**C**W, where the most conserved bases are denoted in bold font; N, any base; W, A or T) (Schumacher et al. 2011). Interestingly, it appears as though there are only two, conserved, side chain – base interactions that are specific; the major groove contacts from residue arginine-22 (Arg22) of the HTH motif to guanine-2 (Gua2) of CRE, and the minor-groove associating hinge-helix residue Leucine-56 (Leu56) to the central CpG step of the CRE (Schumacher et al. 2011). It appears as though it is both the DNA-kinking minor-groove interactions of the dimer-related "leucine levers" of the hinge-helix (Leu56 residues from each CcpA monomer of the dimer complex) that insert into the minor groove of the central CpG step and allows the formation of operator-specific, HTH-major groove contacts between Arg22 and Gua2 (Schumacher et al. 2011) that are indispensible to CRE site binding. Indeed, mutation of only the central CG to TA abolished CCR-type inhibition in *B. megaterium* (Gosseringer et al. 1997), and mutating or deleting any of the most conserved bases significantly reduced transcriptional repression of amylase activity in *B. subtilis* (Weickert and Chambliss 1990). Other CcpA-CRE site interactions are formed through weak but specific hydrogen bonding

between the hinge-helix backbone atoms of alanine-52 (Ala52) and Ala57 with the nucleotides of the central CpG step, and van der Waals' interactions between aspargine-29 (Asn29) and thymine-1 (Thy1) and an additional stacking interaction between Arg22 and Thy1, termed a 5'-pyrimidine-guanine-3' interaction (Schumacher et al. 2011). Additionally, there are numerous non-specific side chain-phosphate backbone interactions and other van der Waals' interactions that contribute to binding affinity, but not specificity (Schumacher et al. 2011). In CcpA of *C. acetobutylicum*, each of these amino acid residues is conserved, except for Ala57, which is a lysine residue. Of the N-terminal 59 amino acids comprising the DNA-binding domain and linker motif, 32 amino acid residues are identical, and an additional 12 have similar side-chains (Figure 3). Given this similarity, it is reasonable to suggest a similar DNA-binding structure mechanism in *C. acetobutylicum* as in *B. subtilis* and *B. megaterium.*

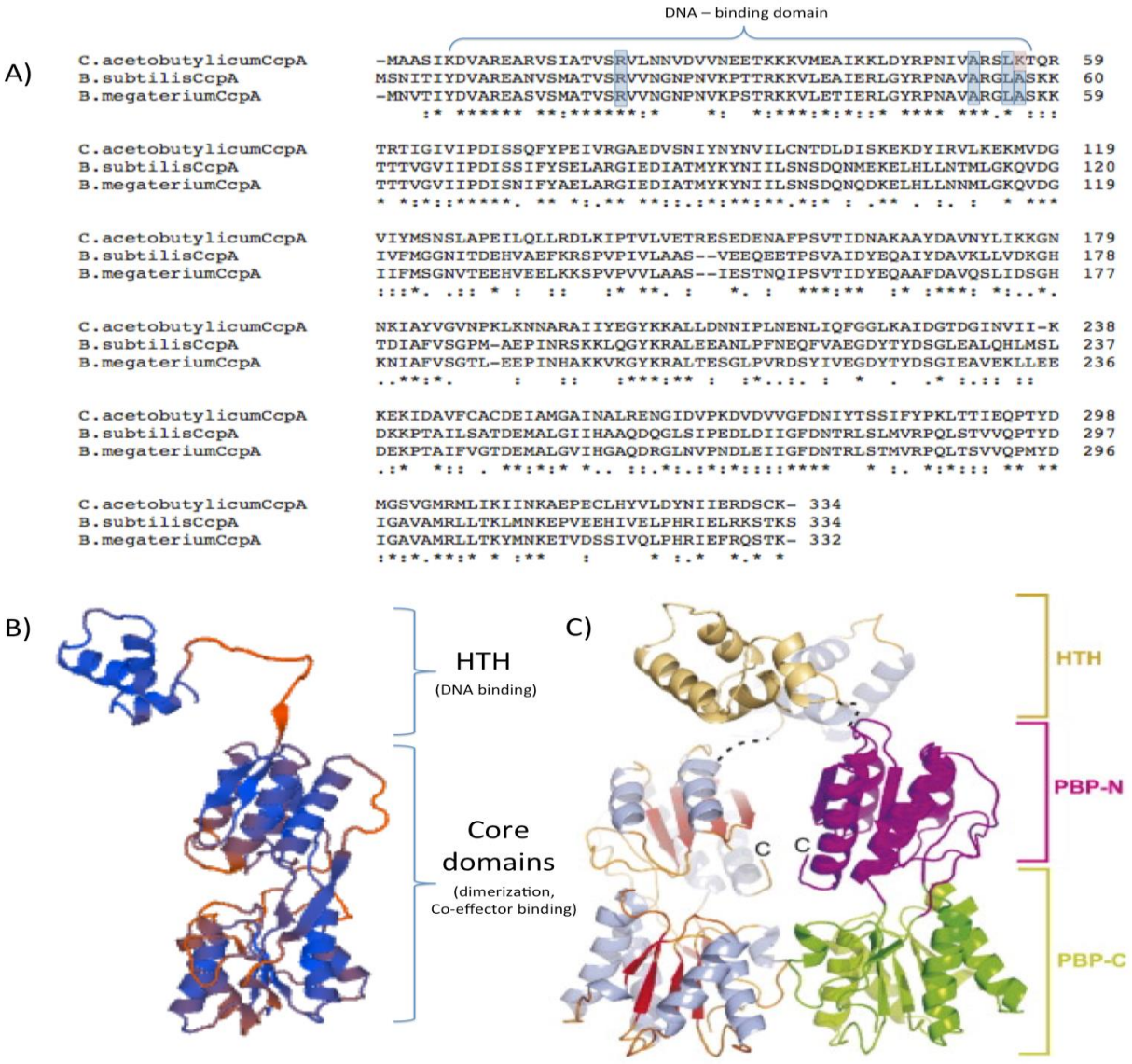

Figure 3 CcpA of *C. acetobutylicum* A) Multiple sequence alignment of the amino acid sequences of CcpA from *C. acetobutylicum*, *B. subtilis*, and *B. megaterium* showing the DNA binding region and relevant amino acid residues necessary for CRE binding (shaded blue). (* indicates conserved residue; : denotes similar residue) B) Ribbon representation of *C. acetobutylicum* CcpA monomer, using solved *B. megaterium* CcpA (*apo*-form) structure as template. C) Ribbon representation of dimeric CcpA (*apo*-form) from *B. megaterium* (image taken from Loll et al. 2007)

2.5 Elements of CCR in *Clostridium* and other Firmicutes

With the increasing availability of genome sequences for members of *Clostridium,* including the *C. acetobutylicum* annotated genome, analysis of the elements of CCR has progressed. Though most effort is directed in *C. acetobutylicum,* evidence of CCR has been found in other members of *Clostridium,* including: observations of diauxic growth when glucose is present in *C. tyrobutyricum* and *C. saccharoperbutylacetonicum;* regulation of expression of cellulase components during growth on cellobiose in both *C. cellulolyticum* and *C. thermocellum;* and identification of potential CREs as well as components necessary for CCR including putative CcpA, HPrK and Crh orthologs in *C. cellulolyticum,* and putative CcpA, HPr, and HPrK in *C. thermocellum* (Abdou et al. 2008; Gorke and Stulke 2008; Jiang et al. 2010; Noguchi et al. 2013; Zhang and Lynd 2005). Interestingly, although a putative Crh containing the conserved Ser46 but not His15 was found in *C. cellulolyticum*, no HPr homolog was found, consistent with the absence of a putative PTS enzyme II-encoding gene in the *C. cellulolyticum* genome (Abdou et al. 2008). *C. beijerinckii,* a close relative of *C. acetobutylicum* and native AB fermenter capable of utilizing several hexose and pentose carbon sources, on the other hand, appears not to be subject to CCR-type regulation, as it has been shown to utilize both glucose and xylose concurrently in batch fermentation (Xiao et al. 2012).

In *C. acetobutylicum*, all of the components required in the *B. subtilis* model of CCR are present in the genome sequence (Tangney et al. 2003), and transcriptional profiling of *C. acetobutylicum* grown on different carbon sources has been conducted (Grimmler et al. 2010; Rodionov et al. 2001; Servinsky et al. 2010). Perhaps most notably, several transcriptional units were repressed when glucose was included in growth media, including operons encoding putative xylose and arabinose utilization genes as well as genes encoding putative pentose

phosphate pathway (PPP). Additionally, a genome-wide search for putative CREs was conducted, revealing 27 potential CRE sites within either the promoter region or coding sequence at a maximum distance of 200 bp from the start codon, including CREs upstream of the start codon of a putative arabinose utilization gene, and in the coding sequence of the first gene in the putative xylose utilization operon (Grimmler et al. 2010) (Table 2). This analysis agrees with previous searches for CRE sequences in *B. subtilis*, which suggested CCR predominance over genes involved in catabolism of secondary carbon sources, nitrogen sources, and other PTS components (Grimmler et al. 2010; Marciniak et al. 2012; Miwa et al. 2000).

Aside from being extensively studied in *B. subtilis* and *B. megaterium* (Fujita 2009), evidence of CCR and its molecular basis has been found in several other Firmicutes, including other members of *Bacillus, Lactobacillus, Lactococcus,* and *Staphylococcus* (Egeter and Brückner 1996; Gorke and Stulke 2008; Mahr et al. 2000), and CcpA has been implicated in expression of virulence genes in *S. pyogenes* and *S. pneumonia*e*, L. monocytogenes*, *S. aureus*, and members of the pathogenic clostridia*, C. perfringens* and *C. difficile* (Antunes et al. 2011; Gorke and Stulke 2008; Seidl et al. 2006; Varga et al. 2004). Further, in *B. subtilis* and more recently in *C. acetobutylicum*, the pleiotropic nature of CcpA has also been demonstrated experimentally: the *B. subtilis* genes *ackA*, *pta*, and the *ilv*-leu operon are activated by CcpA (Fujita 2009), and CcpA is required for full activation of the *sol* locus of *C. acetobutylicum* (Wu et al. 2015).

Table 2 CRE sites identified in *C. acetobutylicum* (Adapted from Grimmler et all. 2010)

Gene ID	Gene Function	Transcriptional profile		CcpA binding site	
		Glucose	Xylose	Sequence	Position[a]
CA_P0066	*ptn*A, mannose-specific PTS component IIAB	*	*	TGCAATCGGGTGCG	+49
CA_P0098	*amy*A, alpha-amylase			TGAAAACGATTACT	+87
CA_P0120	Xylan degradation protein	*	*	TGCAAACGTTAACA	+25
CA_C0154	*mtl*A, PTS system, mannitose-specifc IIBC component	*	*	TGAAAACGATCGCA	-64
CA_C0164	ATP transporter			TGAAAACGCTATCA	-24
CA_C0531	RpiR family transcriptional regulator			TGAAAACGATCTCC	+44
CA_C0672	Fision threonyl-tRNA synthetase (N-terminal) and uridine kinase	*	*	TGGAAGCGTAAACG	+116
CA_C0674	L-Serine dehydratase, alpha chain			TGAAATCGGCTGCT	+148
CA_C1339	*ara*E, arabinose sugar-proton symporter		|	TGAAAGCGATTACC	-136
CA_C1344	*xylB*, sugar kinase, xylulose kinase	*	|	TGCAAACGATTTCT	+95
CA_C1353	PTS system IIC component	|		TGTAAACGGTATCT	+174
CA_C1407	PTS system, beta-glucosides-specific IIABC component	|	*	TGTAACCGTTATCA	+152
CA_C1551	Nitroreductase family protein			TGCAAGCGCCATCA	+91
CA_C2166	Nucleoside-diphosphate-sugar epimerase	|		TGTAACCGTATTCC	+70
CA_C2252	Alpha-glucosidase			TGCAATCGATTTCA	-70
CA_C2807	Endo-1,3(4)-beta-glucanase family protein 16		|	TGCAAACGTATTCA	+79
CA_C2891	Bifunctional alpha-glucosidase/glycosidase		|	TGCAATCGTTTTCC	-59

* signal intensity intensity after subtracting background and background standard deviation ≤100, | ≥2 fold up-regulation comparing exponential growth on glucose and xylose

[a] distance to start codon

2.6 Genetic and non-genetic strategies for circumvention of CCR

Despite a sound understanding of the elements of CCR, few studies have circumvented CCR through endogenous genetic engineering strategies in either *Clostridium* or other Gram-positive organisms such as *B. subtilis*. Rather through media tailoring efforts, co-fermentation strategies, or engineering xylose utilization capabilities through over-expression of heterologous genes. As recently as 2012, a recombinant *B. subtilis* strain with an introduced xylose-utilization operon from *E. coli* was able to utilize glucose and xylose simultaneously to produce 2,3-butanediol (Liu et al. 2012). In other members of *Clostridium*, CCR was circumvented in *C. saccharoperbutylacetonicum* by fermentation with xylose and cellobiose, a disaccharide comprised of two monomers of glucose covalently bonded by a β(1,4)-glycosidic linkage, and transported across the membrane as a disaccharide before being hydrolyzed to two glucose monosaccharides inside the cell (Noguchi et al. 2013).

The insights derived from structural characterization of CcpA from *B. subtilis* and *B. megaterium* has led to protein engineering and investigation of mutants of the CcpA protein and characterization of the phenotypic response these mutations have on CCR-type inhibition, including mutations in the co-effector binding and DNA binding regions of CcpA from *L. casei* and *B. megaterium* (Esteban et al. 2004; Kraus et al. 1998; Küster et al. 1999).

Recently, efforts have been made to attenuate the effect of CCR on the xylose catabolic operon of *C. acetobutylicum*, including intron-mediated knock-out of the genes *ccpA* (Ren et al. 2010) and *glcG*, which encodes EII of the PTS (Xiao et al. 2011), and the generation of a *ccpA* mutant deficient in co-effector HPr-Ser^{46}-P binding (Wu et al. 2015). Though these strategies proved to be successful in relieving carbon-inhibition, they suffer from potential drawbacks;

ccpA knockout mutants have shown impaired growth rates, failure to activate overflow metabolism and other biosynthetic pathways (Egeter and Brückner 1996; Grundy et al. 1993; Hueck and Hillen 1995; Ren et al. 2010; Tobisch et al. 1999; Tojo et al. 2005), and efficient sporulation (Varga et al. 2004). On the other hand, *glcG* knockout mutants and strains with mutated *ccpA* have shown severely impaired growth on glucose (Eiteman et al. 2008; Paulsen et al. 1998; Wu et al. 2015). Additionally, knockouts of PTS enzyme II may not be possible, as is the case for *C. cellulolyticum*, which appears to not possess this gene in its genome (Abdou et al. 2008).

Chapter 3- Materials and Methods

3.1 Bacterial strains and plasmids

Bacterial strains and plasmids employed in this work are listed in Table 3, and oligonucleotides in Table 4. *E. coli* DH5α was utilized for vector construction and routine plasmid maintenance, and ER2275 (pAN3) (Al-Hinai et al. 2012) for methylation of *E. coli-C. acetobutylicum* shuttle vectors destined for electrotransformation to *C. acetobutylicum*. Vectors pHT3 and pHT5 (Tummala et al. 1999) were kindly provided by Professor Terry Papoutsakis (University of Delaware; Newark, DE). Oligonucleotides were synthesized by Integrated DNA Technologies (IDT; Coralville, IA) at the 25 nM scale using standard desalting.

Table 3. Strains and plasmids used in this study

Strains or plasmids	**Relevant characteristics[a]**	**Source or reference[a]**
Strains		
E. coli		
DH5α	*hsdR recA1 endA1*	Lab stock
ER2275	*hsdR mcrA recA1 endA1*	NEB
C. acetobutylicum DSM 792	Wild-type	DSMZ
792-Pthl	792/pHT3catP-Pthl	This study
792 -CRE	792/pHT3catP-Pthl-CRE	This study
792-14nt	792/pHT3catP-Pthl-14nt	This study
792 -mutN	792/pHT3catP-Pthl-mutN	This study
792-xylB	792/pHT3catP-Pthl-xylB	This study
792-xyl-wt	792/pMTLxyloperon-wt	This study

792-CRE-free	792/pMTLxyloperon	This study
Plasmids		
pAN3	Φ3TI; Km^R; p15A	(Al-Hinai et al. 2012)
pHT3	Ap^R; Em^R; ColE1; repL; *LacZ*	(Tummala et al. 1999)
pHT3catP	Cm^R/Tm^R ; ColE1; repL; *LacZ*	Unpublished data
pHT5	Ap^R; Em^R; ColE1; repL; *thl* promoter; *LacZ*	(Tummala et al. 1999)
pMTL007C-E6	Cm^R/Tm^R ; ColE1; repL	(Pyne et al. 2014b)
pHT3catP-Pthl	Cm^R/Tm^R ; ColE1; repL; P*thl* upstream of LacZ	This study
pHT3catP-Pthl-CRE	Cm^R/Tm^R ; ColE1; repL; *Pthl* with CRE sequence inserted upstream of *LacZ*	This study
pHT3catP-Pthl-14nt	Cm^R/Tm^R ; ColE1; repL; *Pthl* with 14 nt replacing CRE sequence; *LacZ*	This study

pHT3catP-Pthl-mutN	Cm^R/Tm^R ; ColE1; repL; *Pthl* with mutated degenerate nucleotides of CRE sequence; *LacZ*	This study
pHT3catP-Pthl-xylB	Cm^R/Tm^R ; ColE1; repL; *Pthl* with mutated CRE sequence; *LacZ*	This study
pMTLxyloperon-wt	Cm^R/Tm^R ; ColE1; repL; *cac1344-1349* with ~300 bp of homology upstream and downstream	This study
pMTLxyloperon	Cm^R/Tm^R ; ColE1; repL; *cac1344-1349* with ~300 bp of homology upstream and downstream; mutated CRE	This study

Table 4 Oligonucleotides employed in this study

Oligonucleotide	Sequence (5' – 3')
Pthl.S	GCACCATATGCTCGTAGAGCACACGGTTTAACG
Pthl.AS	GGATCCCTACGGGGTAACAGATAAACCATTTCAATC
Pthl.CRE.AS	GGATCCAGAAATCGTTTGCACTACGGGGTAACAGATAAACCATTTCAATC
Pthl.14nt.AS	GGATCCNNNNNNNNNNNNNCTACGGGGTAACAGATAAACCATTTCAATC
Pthl.mutN.AS	GGATCCTGTCGACGCTTACACTACGGGGTAACAGATAAACCATTTCAATC
Pthl.xylB.AS	GGATCCTAAAATCATTAGCTCTACGGGGTAACAGATAAACCATTTCAATC
xylB.S	ACATTCGTGAGGTTGGAGAAG
xylB.AS	CTTTTAGATTAAATGCTCTACAGCTGC
xylB.mut.S	AGCTAATGATTTTAGTATATTAGCTAGCGGAAGCTTTGAATGG
xylB.mut.AS	ATAAGGACAGCTTTAATTCGAGTAGAACCA
1/2xyloperon.S1	GTAGAGCATTTAATCTAAAAGTAAAATTTAGAAGATTATCAG
1/2xyloperon.AS1	GATATCTGTTAAAGGATGTTTTATTAATTG
1/2xyloperon.S2	CAATTAATAAAACATCCTTTAACAGATATCGGAATAG
1/2xyloperon.AS2	CTGAGAATATTGTAGGAGATCTTCTAGAAAGATTGCCATCCTTTATCCT
repLcatPcolE1pMTL.S	ATCGATCGCCGCATTCACTTCTTTTC
repLcatPcolE1pMTL.AS	GCGGCCGCATCCACAGAATCAGGGGATAACG

3.2 Cultivation and maintenance conditions

E. coli strains were cultivated aerobically at 37 °C in lysogeny broth (LB) (10 g l^{-1} tryptone, 5 g l^{-1} yeast extract, 10 g l^{-1} NaCl) and recombinant derivatives were selected, when necessary, with chloramphenicol (25 µg ml^{-1}), or kanamycin (30 µg ml^{-1}). *C. acetobutylicum* DSM 792 was cultivated anaerobically at 37 °C in 2×YTG (pH 5.2) medium (16 g l^{-1} tryptone, 10 g l^{-1} yeast extract, 5 g l^{-1} glucose, 4 g l^{-1} NaCl), *Clostridium* Growth Medium (CGM) (Abdou et al. 2008), or P2 minimal medium (Baer et al. 1987), within an anaerobic containment chamber (Plas-Labs; Lansing, MI) containing an atmosphere of 5% CO_2, 10% H_2, and 85% N_2. Strict anaerobic conditions were maintained and monitored through the use of a palladium catalyst fixed to the heater of the chamber and addition of resazurin (1 mg l^{-1}) to all solid and liquid media preparations. Recombinant *C. acetobutylicum* strains were selected, where necessary, with thiamphenicol (10 µg ml^{-1}). Recombinant *E. coli* and *C. acetobutylicum* were stored frozen in 15% glycerol at -80 °C or as sporulated colonies on solidified 2×YTG (pH 5.8) agar plates.

3.3 DNA isolation, manipulation, and electrotransformation

Plasmid DNA was extracted from *E. coli* and purified using an EZ-10 Spin Column Plasmid DNA Miniprep Kit (Bio Basic, Inc.; Markham, ON). Linear DNA restriction fragments and PCR products were purified either from agarose gels or directly using an EZ-10 Spin Column DNA Gel Kit (Bio Basic, Inc.; Markham, ON). Vectors destined for *C. acetobutylicum* were constructed in *E. coli* according to standard procedures (Sambrook et al. 1989), methylated in *E. coli* ER2275 (pAN3) (Al-Hinai et al. 2012), and electro-transformed to *C. acetobutylicum* as described previously (Mermelstein and Papoutsakis 1993).

Restriction enzymes, Standard *Taq* DNA Polymerase, Phusion High-Fidelity DNA Polymerase, and Quick Ligation Kit were purchased from New England Biolabs (Whitby, ON). In-Fusion HD Cloning Kit was purchased from Clontech Laboratories (Mountain View, CA), and CloneJET PCR Cloning Kit was purchased from Thermo Scientific (Ottawa, ON). All commercial enzymes and kits were used according to the manufacturer's instructions.

3.4 Vector construction

Plasmid pHT3catP is a derivative of pHT3 wherein the erythromycin selectable marker is replaced by the chloramphenicol/thiamphenicol *catP* selectable marker by sub-cloning a 1.3 kbp *Cla*I + *Sac*I restriction fragment of pSY6catP and ligating to the similarly digested pHT3 (Pyne et al. 2013).

To construct plasmids pHT3catP-Pthl, pHT3catP-Pthl-CRE, pHT3catP-Pthl-14nt, pHT3catP-Pthl-mutN, and pHT3catP-Pthl-xylB, a 301 bp region corresponding to the *thiolase (thl)* promoter region of plasmid pHT5 was amplified by PCR, (primers Pthl.S + Pthl.AS, Pthl.S + Pthl.CRE.AS, Pthl.S +Pthl.14nt.AS, Pthl.S + Pthl.mutN.AS, and Pthl.S + Pthl.xylB.mut.AS, respectively), digested with *Bst*API + *Bam*HI, and ligated to the *Bst*API + *Bam*HI restriction sites of pHT3catP.

For construction of pMTLxyloperon and pMTLxyloperon-wt, first a 1.8 kbp region corresponding to the *xylB* gene (CAC1344) and 232 bp of upstream DNA were PCR amplified from *C. acetobutylicum* DSM 792 genomic DNA using primers xylB.PCR.S + xylB.PCR.AS, and ligated to pJET1.2 blunt cloning vector, yielding pJET-xylB. The putative CRE sequence was then mutated in the resulting vector using inverse-PCR (primers xylB.mut.S + xylB.mut.AS) to yield pJET-xylB-mut. Next, in two successive In-Fusion

cloning steps, 3.9 kbp (primers 1/2xyloperon.S1 + 1/2xyloperon.AS1) and 3.3 kbp (primers 1/2xyloperon.S2 + 1/2xyloperon.AS2) PCR fragments making up the rest of the xylose and pentose phosphate pathway operon as well as 169 bp of downstream sequence were amplified from genomic DNA and fused to the vector backbones of pJET-xylB-mut and pJET-xylB, creating pJET1.2xyloperon, and pJET1.2xyloperon-wt, respectively. Finally, a 2.5 kbp PCR fragment comprising *repL* clostridial ORI, *catP* for dual selection of chloramphenicol and thiamphenicol for *E. coli* and *C. acetobutylicum*, respectively, and the *E. coli* ORI *colE1* was amplified from vector pMTL007-E6, digested with *Cla*I + *Not*I, and ligated to the similarly digested pJET1.2xyloperon and pJET1.2xyloperon-wt to generate pMTLxyloperon and pMTLxyloperon-wt using primers repLcatPcolEIpMTL.S and repLcatPcolEIpMTL.AS.

3.5 Enzyme assays and fermentation

Cells were collected from 50-ml cultures grown in CGM to mid-exponential phase (A_{600} ~ 1.2-1.5) with glucose as the sole carbon source, pelleted by centrifugation, and the pellet was immediately frozen at -80 °C. Frozen cell pellets were thawed on ice for 1 hour and re-suspended to an A_{600} of ~16 OD units in Z buffer. Lysozyme (1 mg ml^{-1}) was added and the cells were incubated in a 37 °C water bath for 45 minutes. The crude extract was harvested by centrifugation and the β-galactosidase assay was performed as described previously (Tummala et al. 1999). All β-galactosidase activity assays were carried out in triplicate.

Anaerobic static-flask fermentations were carried out with 50 ml working volume in P2 medium with glucose (40 g l^{-1}) and xylose (20 g l^{-1}) as carbon sources. Recombinant *C.*

acetobutylicum harboring either the wild-type xylose operon and pentose phosphate pathway genes or the mutated CRE version were heat shocked in a 80°C water bath, cooled on ice, and serial dilutions were prepared. The cultures were incubated overnight, and the fermentation flasks were inoculated with 1% of the highest dilution-factor culture that was turbid and growing exponentially. Samples were taken at 12-hour intervals for A_{600} measurements as well as HPLC analysis. All fermentations were carried out in duplicate.

3.6 Analytical Analyses

Cell density measurements were conducted by measuring the A_{600} using a spectrophotometer (DU520, Beckman Coulter, Fullerton, CA). Dilutions were made using saline as appropriate to ensure the accuracy of OD_{600} measurements. For measurement of glucose, xylose, and ABE solvents concentration, HPLC (LC-10AT, Shimadzu, Kyoto, Japan) with a refractive index detector (RID-10A, Shimadzu, Kyoto, Japan) and a chromatographic column (Aminex HPX-87H, Bio-Rad Laboratories, CA, USA) was employed. The column temperature was maintained at 65 °C and the mobile phase was 5 mM H_2SO_4 (pH 2.0) running at 0.6 mL/min. Data acquisition and analysis were performed using the Clarity Lite Chromatographic Station (Clarity Lite, DataApex, Prague, The Czech Republic).

3.7 CRE search

Analogously to previous studies in *B. subtilis* and recently in *C. acetobutylicum*, we performed our own search for CRE sequences in the *C. acetobutylicum* ATCC 824 genome (chromosome and megaplasmid) using an almost completely degenerate query sequence TGNNNNCGNNNNCN (N, any base) using the PRODORIC database and the virtual footprint version 3.0 (Grote et al. 2009).

Chapter 4- Results

4.1 Introduction of a 14-nucleotide catabolite responsive element (CRE) is sufficient to result in CCR-type inhibition

We first aimed to evaluate the sufficiency of a putative 14-nucleotide CRE sequence to confer CCR-type inhibition by constructing a derivative of the *lacZ* gene reporter vector pHT3catP (Pyne et al. 2013) in which the sequence of the putative CcpA binding site in the *xylB* gene of *C. acetobutylicum* (Grimmler et al. 2010) is inserted between the *thl* promoter and the ribosome binding site (RBS) of the *lacZ* coding sequence (Figure 4). Upon electro-transformation of the promoter-less pHT3catP and pHT3catP-Pthl controls, and pHT3catP-Pthl-CRE, the resulting recombinant *C. acetobutylicum* strains were assessed for β-galactosidase activity in CGM using glucose as the carbon source for cultivation. As shown in Figure 4, *C. acetobutylicum* harboring the promoter-less vector yielded negligible β-galactosidase activity (data not shown), while the pHT3catP-Pthl harboring strain, in which the endogenous *thl* promoter is driving expression of the *lacZ* reporter gene, exhibited a high β-galactosidase activity of approximately 2000 Miller Units (MU). Conversely, the β-galactosidase activity of *C. acetobutylicum* harboring pHT3catP-Pthl-CRE was approximately 600 MU, a reduction of approximately 70% as compared to *C. acetobutylicum* harboring the CRE-less plasmid construct. Since the only difference between the vectors pHT3catP-Pthl-CRE and pHT3catP-Pthl is the introduced putative 14-nucleotide CRE sequence from the *xylB* gene, the observed decrease in β -galactosidase activity suggests that the presence of the CRE sequence is responsible for the CCR-inhibition of the transcription

of the *lacZ* gene.

To ensure that the inhibition of *lacZ* expression was not associated with the increased length of the mRNA transcript or some other promoter perturbation issue, we constructed another derivative of pHT3catP, i.e. pHT3catP-Pthl-14nt, to include 14 additional random nucleotides in the same placement and orientation as the introduced CRE sequence. The sequence of the random 14 nucleotides was examined to ensure its inability to act as a putative CRE element. The plasmid pHT3catP-Pthl-14nt was introduced to *C. acetobutylicum* and the resulting recombinant strain was assayed for β-galactosidase activity. As shown in Figure 4, the β-galactosidase activity of this recombinant strain was similar to that of the positive control of *C. acetobutylicum* harboring pHT3catP-Pthl, approximately 2000 MU. Taken together, this data suggests that the presence of the specific 14-nucleotide consensus sequence can potentially activate CCR-mediated inhibition of the *lacZ* gene expression.

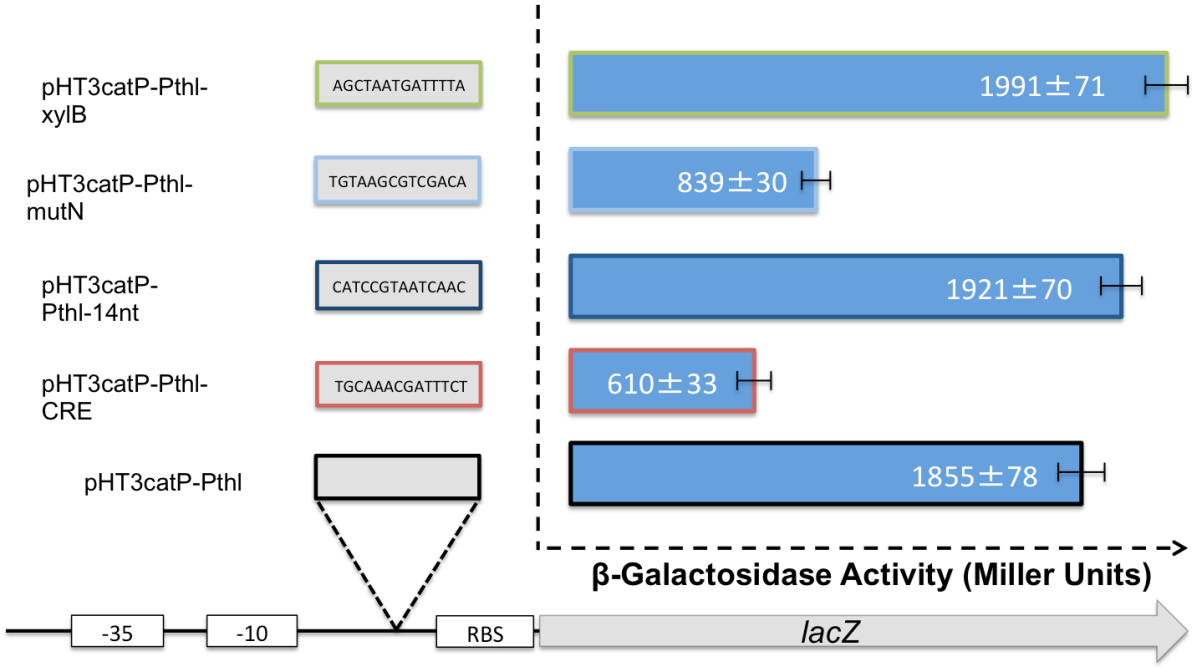

Figure 4 β-galactosidase activities and relevant plasmid structure for *C. acetobutylicum* harboring plasmids pHT3catP-Pthl, pHT3catP-Pthl-CRE (sequence 5' TGCAAACGATTTCT 3'), pHT3catP-Pthl-14nt (5' CATCCGTAATCAAC 3'), pHT3catP-Pthl-mutN (5' TGTAAGCGTCGACA 3'), and pHT3catP-Pthl-xylBmut (5' AGCTAATGATTTTA 3'). Strains were grown in CGM medium to mid-exponential phase and 16OD units were harvested and assayed for β-galactosidase activity. β-galactosidase assays were carried out in triplicate.

4.2 Mutating key nucleotides of the CRE sequence can potentially affect CCR-type inhibition

The putative consensus CRE sequence established in previous CCR studies in *B. subtilis* (Miwa et al. 2000) was used as a query sequence to search the *C. acetobutylicum* genome for putative CRE elements (Grimmler et al. 2010). However, the sequence has several degenerate nucleotides (i.e. N; any base). Indeed, 7 of the 14 nucleotides (i.e. TGNAANCGNNNNCN) used in the search query are undefined. We mutated the degenerate nucleotides within our introduced CRE sequence to determine their effect on mediating CCR. Interestingly, mutating the degenerate nucleotides in the consensus sequence (i.e. pHT3catP-Pthl-mutN) resulted in approximately 137% of the β-galactosidase activity as compared to the CRE vector construct pHT3catP-Pthl-CRE (Figure 4), but the activity was significantly lower than that of the positive control of pHT3catP-Pthl, implying that the CCR-type inhibition was still present. The results suggest that the degenerate nucleotides are largely insensitive to CCR-type inhibition, however mutations may have a modest effect.

We next sought to investigate the effect of mutating the non-degenerate nucleotides by constructing pHT3catP-Pthl-xylB, in which the endogenous CRE sequence found within the *xylB* coding sequence was replaced with a mutated version. We constrained our mutations to "silent" ones without altering the encoding amino acids in the event that we would explore similar mutations in the native *xylB* gene. Indeed, cultivation of *C. acetobutylicum* harboring pHT3catP-Pthl-xylB exhibited a β-galactosidase activity similar to that of the positive control of approximately 2000 MU (Figure 4). The results suggest that these non-degenerate nucleotides of the CRE consensus sequence are critical for CCR-type inhibition and therefore

can be proper target nucleotides for mutation in order to relieve the CCR associated with the expression of the xylose operon.

4.3 Expression of a CRE-sequence-free xylose operon relieves CCR inhibition and enables simultaneous glucose and xylose dissimilation in C. acetobutylicum

Our gene reporter assay data suggested a feasible strategy to abolish CCR-mediated inhibition by introducing silent mutations within the CRE sequence in the *C. acetobutylicum* xylose operon. To demonstrate this, we investigated the effect of over-expression of a plasmid-borne xylose operon with a compromised CRE sequence on CCR of xylose in the presence of glucose. We constructed two expression vectors, containing the endogenous xylose operon with either the native CRE sequence (pMTLxyloperon-wt) or a mutated CRE sequence (pMTLxyloperon containing the same silent mutations as those in pHT3catP-Pthl-xylB). Additionally, note that the two vectors also include the pentose phosphate pathway (PPP) genes (Figure 5), to avoid potential limitation for xylose utilization, as it is unclear whether the native PPP genes (i.e. CAC1347-CAC1349) in *C. acetobutylicum* are transcribed as a separate operon or as part of the CCR-controlled operon encoding xylose dissimilation genes (i.e. CAC1344-CAC1346) (Grimmler et al. 2010; Paredes et al. 2004). The resulting recombinant *C. acetobutylicum* strains were cultivated for simultaneous co-dissimilation of glucose and xylose in P2 medium containing 40 g l^{-1} glucose and 20 g l^{-1} xylose as carbon sources. As shown in Figure 5, after 84 hours of cultivation, the strain over-expressing the wild-type xylose operon consumed approximately 2 g l^{-1} of xylose, or approximately 10% of the available xylose, while the strain harboring the CRE-free xylose operon utilized approximately 6 g l^{-1}, or 30%. More importantly, during the first 48 hours, in which glucose

was present in the media, the *C. acetobutylicum* strain with the CRE-free operon consumed 7.5-fold more xylose than that with the wild-type operon (Figure 5 inset). This result suggests that mutating the non-degenerate nucleotides of the CRE consensus sequence is sufficient to relieve the CCR-mediated repression associated with the expression of the xylose and PPP operons and therefore allow *C. acetobutylicum* to utilize glucose and xylose concurrently.

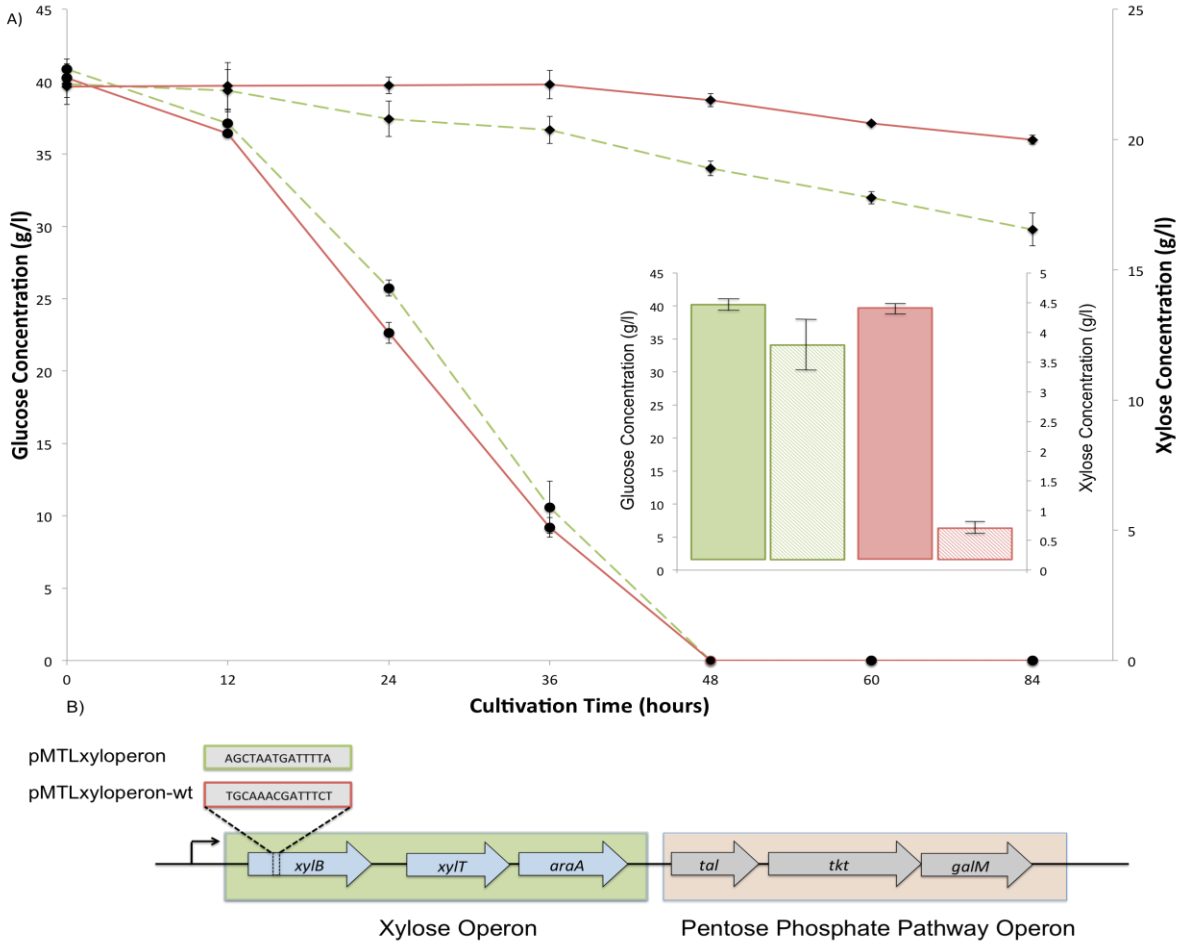

Figure 5 Time-course cultivation profile for *C. acetobutylicum* A) Residual glucose (circles) and xylose (diamonds) concentration time-course and consumption at 48 hours (inset; solid bar denotes glucose consumption and striped lines denotes xylose) for wild-type *C. acetobutylicum* (red, solid) and *C. acetobutylicum* harboring a plasmid-borne CRE-free xylose operon (green, dashed) in static-flask fermentation. Cells were grown in P2 minimal medium grown on a mixture of glucose (40 g/l) and xylose (20 g/l). Samples were taken every 12 hours for analysis. Fermentations were performed in duplicate. B) Schematic drawing representing the relevant structure of pMTLxyloperon (top; sequence 5' AGCTAATGATTTTA 3') and pMTLxyloperon-wt (bottom; 5' TGCAAACGATTTCT 3').

Chapter 5- Discussion

Genetic manipulation techniques have enabled rational metabolic engineering approaches among the clostridia (Pyne et al. 2014a). A great deal of attention has been directed toward increasing the production of target metabolites through gene over-expression and knock-out (Lütke-Eversloh and Bahl 2011; Ren et al. 2010; Xiao et al. 2011), as well as enhancing a microorganism's ability to assimilate carbon sources (Gu et al. 2009; Jin et al. 2014; Ren et al. 2010). CCR is a common transcriptional inhibitory mechanism limiting carbon assimilation in the clostridia. Although recent studies have circumvented CCR through media-tailoring strategies (Noguchi et al. 2013), applicable genetic-based approaches to relieve CCR is needed for *Clostridium*.

Analysis of CRE sequences in *B. subtilis* has led to the identification of a 14-nucleotide consensus sequence with bi-fold symmetry and considerable degeneracy (Miwa et al. 2000). This degeneracy of CRE operator sequences suggests high tolerance associated with the catabolite control protein, CcpA, upon its binding onto the CRE sites with such diverse sequences (i.e. T**G**NAAN**CG**NWNN**C**W, N, any base; W, A or T; most conserved bases are denoted in bold) (Schumacher et al. 2004; Schumacher et al. 2011). Major molecular interactions between key amino acids of *B. subtilis* CcpA and CRE nucleotides were identified for better understanding of the CcpA-CRE binding nature (Schumacher et al. 2011). Because of the high similarity in CcpA between *C. acetobutylicum* and *B. subtilis*, it is reasonable to assume that they share a similar DNA-binding structural mechanism.

Our search for CRE sequences in the *C. acetobutylicum* chromosome and megaplasmid sequences identified 782 matches in 268 gene-coding sequences and inter-

genic regions (Supplementary Figure 1), and 29 matches in 6 gene-coding sequences and inter-genic regions (Supplementary Figure 2), respectively, including each of the CRE sites reported previously (Grimmler et al. 2010). Importantly, no novel CRE sites were identified in either genomic loci encompassing the xylose degradation pathways (i.e. CAC1344-1349 or CAC2610-2613) other than those previously reported. No further analysis was conducted with respect to this data, as the almost complete degeneracy of the query sequence is very likely to match several instances of completely coincidental sequence having no CCR functionality and even those instances where the match shows higher stringency, some may not be functional due to sequence context, as suggested for *B. subtilis* (Miwa et al. 2000; Turinsky et al. 1998).

Through analysis of the purported CRE sequence with gene reporter assays, we identified a partially conserved CRE sequence associated with CCR in *C. acetobutylicum*. Interestingly, a recent genome-wide study of *B. subtilis* revealed CRE boxes of differing affinity for CcpA (Marciniak et al. 2012). Comparing the CRE sequence found in the *xylB* gene of *C. acetobutylicum* with those identified in *B. subtilis* revealed that the *xylB* CRE sequence would be classified as a low-affinity CRE binding site. Similar to the CRE sequence of *B. subtilis* (Marciniak et al. 2012; Weickert and Chambliss 1990), mutating the degenerate nucleotides of the consensus sequence of the *xylB* CRE retained significant CCR-type inhibition, suggesting the relatively insignificant role these nucleotides play in this transcriptional repression mechanism. However, further examination of the mutated sequence (i.e. TGTAAGCGTCGACA) revealed divergence from the optimal bi-fold symmetry observed for many operator sequences (Sadler et al. 1983). This divergence

ostensibly reduced the binding affinity of CcpA to the CRE site and thereby the CCR-mediated inhibitory effect, as reflected by a marked 37% increase in β-galactosidase activity compared to the original CRE sequence (i.e.TGCAAACGATTTCT). As such, it appears as though mutating the degenerate nucleotides of CRE may have modest, potentially sequence-specific modulation on the effects of CCR by altering the chemical and spatial environment of the CcpA-DNA binding interface.

While recombinant *C. acetobutylicum* containing the CRE-free xylose operon was able to assimilate xylose in the presence of glucose more effectively based on its relief from CCR, such concurrent utilization of xylose and glucose imparted only a modest effect on growth and solvent titer advantage (Supplementary Table 1). This may be associated with the fact that xylose, even acting as the sole substrate, is a relatively poor carbon source for *C. acetobutylicum* potentially due to poor affinity of the transporter (encoded by *xylT)* for xylose substrate or weak enzymatic activity of xylose-dissimilation enzymes (i.e. xylose isomerase and xylulose kinase encoded *xylA* and *xylB*, respectively) (Jin et al. 2014; Xiao et al. 2011). Considerable effort has been directed toward enhancing xylose utilization in *C. acetobutylicum*, including over-expression of the *E. coli talA* gene encoding transaldolase, over-expression of native xylose-dissimilation genes *xylT, xylA,* and *xylB*, and over-expression of the genes associated with the native pentose phosphate pathway (Gu et al. 2009; Jin et al. 2014; Xiao et al. 2011). Additionally, recent studies have shown improved xylose utilization in *C. beijerinckii* (Xiao et al. 2012) and *C. acetobutylicum* strains ATCC 824 and EA 2018 (Hu et al. 2011; Li et al. 2013), in which the *xylR* gene encoding a putative D-xylose repressor was inactivated, suggesting a possible secondary repression mechanism

that is independent of CCR. While all these genetic strategies improved the overall rate of xylose utilization, it appeared that the engineered strains often consumed minimal xylose during the initial cultivation stage due to underlying deficiencies in the biochemical properties of the xylose-utilization enzymes. As such, heterologous expression of the xylose-utilization genes from more efficient xylose-utilizing microorganisms, such as *Klebsiella oxytoca* (Ohta et al. 1991) or *Klebsiella pneumoniae* (Yu and Saddler 1983*)*, could provide a promising solution. On the other hand, it has also been proposed that butanol inhibition would be more drastic for *C. acetobutylicum* grown on xylose (Ounine et al. 1985) due to effects on cell membrane functionality (Bowles and Ellefson 1985), suggesting that effort to mitigate butanol inhibition can be critical in advancing *C. acetobutylicum* as a production host for lignocellulosic butanol.

With the identification of CRE sequences, our proposed methodology for relieving CCR can be applied to other CCR-inhibited catabolic operons in *C. acetobutylicum*, such as the arabinose, sucrose, lactose, and maltose operons (Tangney et al. 2003; Xiao et al. 2011; Yu et al. 2007), or even to other species of *Clostridium*, such as *C. cellulolyticum, C. thermocellum*, *C. tyrobutyricum,* and *C. saccharoperbutylacetonicum,* for which evidence of CCR has been identified (Abdou et al. 2008; Gorke and Stulke 2008; Jiang et al. 2010; Noguchi et al. 2013; Zhang and Lynd 2005). While the improvement in xylose utilization based on relieving CCR may be limited in *C. acetobutylicum* due to the aforementioned drawbacks, this approach can be advantageous to other species of *Clostridium*, in particular *C. cellulolyticum* and *C. thermocellum,* both of which possess native cellulosomes for degradation of cellulosic materials. It has been suggested that the expression of cellulosomic

components is subject to CCR-type regulation and the expression of these components may be down regulated as glucose is liberated from cellulose into the medium (Abdou et al. 2008; Zhang and Lynd 2005). Furthermore, targeting CRE sites for mutation presents an alternative method of CCR-relief to disruption of *glcG*, the gene encoding the sugar-specific Enzyme II (EII) of the PEP-dependent PTS for glucose transport and phosphorylation, in *C. acetobutylicum* strains ATCC 824 and EA 2018 (Li et al. 2013; Xiao et al. 2011) since impaired cell growth on glucose was observed for similar mutants of other microorganisms (Eiteman et al. 2008; Paulsen et al. 1998). Also, this gene disruption may not be feasible in microorganisms that do not possess a native glucose PTS system, such as *C. cellulolyticum* (Abdou et al. 2008).

Chapter 6- Conclusions and Recommendations

In this study, we presented a method for relieving CCR of xylose through mutation of a CRE sequence within an operon involved in xylose metabolism, successfully permitting xylose utilization in the presence of glucose. We believe that this method presents a viable alternative to those recently published for which the potential drawbacks have been discussed. Further, relief of CCR on a local scale (i.e. for an individual operon or carbon source) as opposed to globally (i.e. recombinant strains such as *glcG* knockout and CcpA mutants) may be beneficial in that the pleiotropic effectiveness of the global transcriptional regulator CcpA are retained, thereby maintaining regulation of gene expression similar to that of the wild-type strain. In this way, this method may reduce the overall metabolic burden of diverting cellular resources to producing mRNA transcripts and protein products of genes that are not required given the growth conditions, and instead focus host gene expression machinery and other cellular resources to only those gene pathways for which expression is required or desired.

Ideally, the proposed genetic strategy for CRE mutation should be targeted on the host chromosome. Plasmid-based relief of CCR for concurrent xylose and glucose utilization was used as a target for this proof of concept study due to technical restrictions related to the genomic organization of *C. acetobutylicum* as well as the lack of available genetic engineering tools that permit efficient chromosomal modification. As such, the utility of this genetic strategy would be affirmed by systematically manipulating the CRE sequence of catabolically repressed operons on the host chromosome to relieve CCR, leading to the derivation of *C. acetobutylicum* strains specialized for utilization of desired feedstock while

simultaneously minimizing the increased metabolic burden of ungoverned transcription of unneeded genes, as well as that of maintaining an extra-chromosomal plasmid for gene over-expression. Further, this strategy should be transferrable to other members of *Clostridium* and potentially members of Firmicutes for which CCR restricts secondary carbon source utilization, such as those discussed previously.

As evidenced in this study and in agreement with previous reports, relief from the effects of CCR afforded minimal benefit on ABE production. Two potential reasons for this discussed in this study and in the literature are due to *C. acetobutylicum* being an innately poor xylose utilizer, and butanol toxicity. Therefore, these two issues warrant further study. Using a chromosomally engineered *C. acetobutylicum* strain capable of simultaneous glucose and xylose co-consumption as a host strain, metabolic engineering strategies aimed at improving the utilization of xylose should be pursued. Identification of potential bottle necks in the xylose utilization pathway and pentose phosphate pathway could be analyzed through various biochemical assays to evaluate enzyme kinetics, and through analysis of the transcriptome of the recombinant strain to observe changes in transcript levels compared to the wild-type. In conjunction with the mutated CRE sequence, the promoter driving expression of the xylose utilization pathway could be engineered for higher transcription, potentially leading to greater xylose utilization efficiency. Additionally, protein engineering studies could be undertaken to identify mutants of *xylA, xylB, xylT,* and proteins of the pentose phosphate pathway that show increased specificity and affinity for xylose as substrate. To address the issue of butanol toxicity, it has been suggested that butanol affects the lipid bi-layer membrane structure, as well as inhibits carbon transport across the

membrane, highlighting the necessity of detailed analyses and characterization of these effects to understand the role butanol toxicity plays as well as how to direct efforts to overcome this obstacle. Detailed transcriptome studies may aid in shedding light on any changes in gene expression or endogenous SOS response of *C. acetobutylicum* under butanol challenge and suggest the most prominent course of action to improve ABE production from xylose. Alternatively, engineering the constituent profile of the lipid bi-layer may offer improvements in reducing butanol toxicity.

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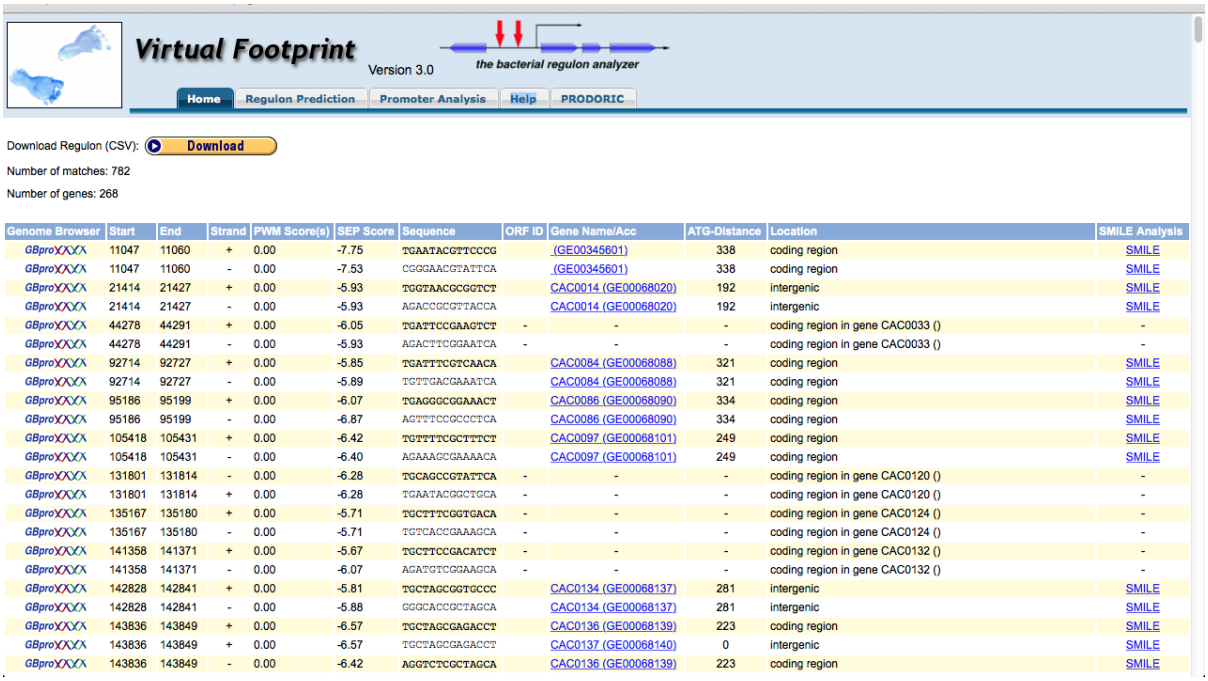
Appendix A



Virtual Footprint Version 3.0 — the bacterial regulon analyzer

Home | Regulon Prediction | Promoter Analysis | Help | PRODORIC

Download Regulon (CSV): Download

Number of matches: 782

Number of genes: 268

Genome Browser	Start	End	Strand	PWM Score(s)	SEP Score	Sequence	ORF ID	Gene Name/Acc	ATG-Distance	Location	SMILE Analysis
GBproXXXX	11047	11060	+	0.00	-7.75	TGAATACGTTCCCG		(GE00345601)	338	coding region	SMILE
GBproXXXX	11047	11060	-	0.00	-7.53	CGGGAACGTATTCA		(GE00345601)	338	coding region	SMILE
GBproXXXX	21414	21427	+	0.00	-5.93	TGGTAACGCGGTCT		CAC0014 (GE00068020)	192	intergenic	SMILE
GBproXXXX	21414	21427	-	0.00	-5.93	AGACCGCGTTACCA		CAC0014 (GE00068020)	192	intergenic	SMILE
GBproXXXX	44278	44291	+	0.00	-6.05	TGATTCCGAAGTCT	-	-	-	coding region in gene CAC0033 ()	-
GBproXXXX	44278	44291	-	0.00	-5.93	AGACTTCGGAATCA	-	-	-	coding region in gene CAC0033 ()	-
GBproXXXX	92714	92727	+	0.00	-5.85	TGATTTCGTCAACA		CAC0084 (GE00068088)	321	coding region	SMILE
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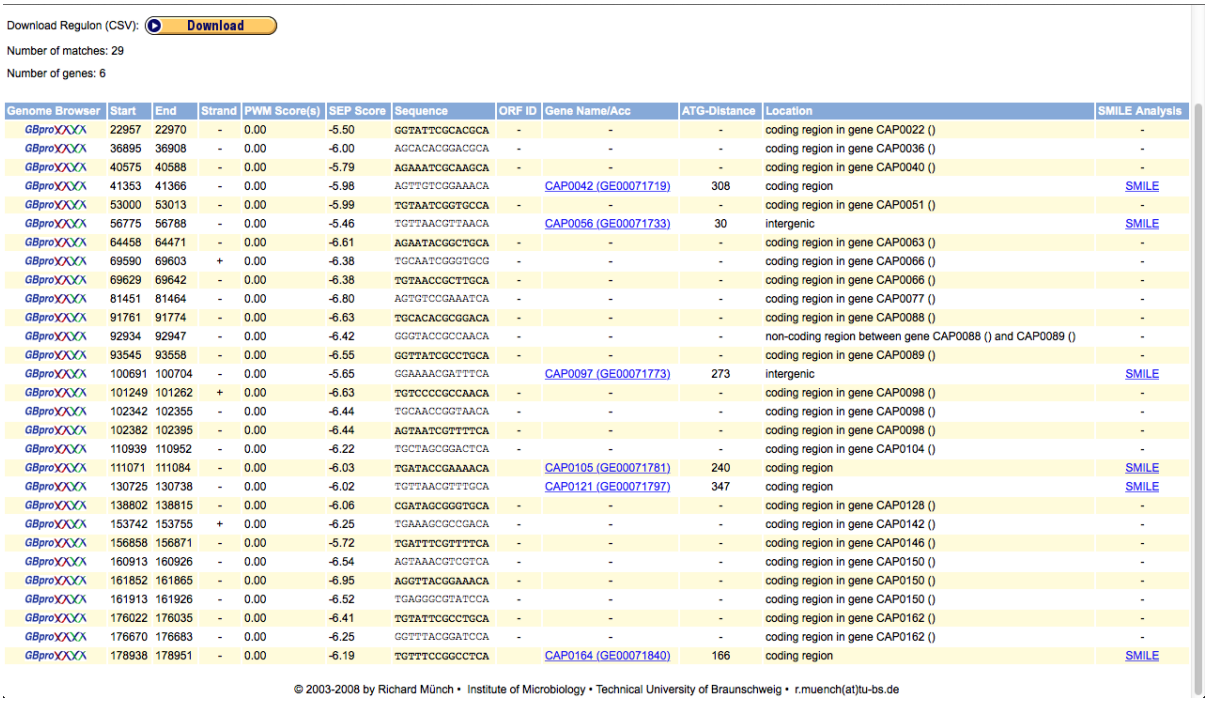
GBproXXXX	95186	95199	+	0.00	-6.07	TGAGGGCGGAAACT		CAC0086 (GE00068090)	334	coding region	SMILE
GBproXXXX	95186	95199	-	0.00	-6.87	AGTTTCCGCCCTCA		CAC0086 (GE00068090)	334	coding region	SMILE
GBproXXXX	105418	105431	+	0.00	-6.42	TGTTTTCGCTTTCT		CAC0097 (GE00068101)	249	coding region	SMILE
GBproXXXX	105418	105431	-	0.00	-6.40	AGAAAGCGAAAACA		CAC0097 (GE00068101)	249	coding region	SMILE
GBproXXXX	131801	131814	-	0.00	-6.28	TGCAGCCGTATTCA	-	-	-	coding region in gene CAC0120 ()	-
GBproXXXX	131801	131814	+	0.00	-6.28	TGAATACGGCTGCA	-	-	-	coding region in gene CAC0120 ()	-
GBproXXXX	135167	135180	+	0.00	-5.71	TGCTTTCGGTGACA	-	-	-	coding region in gene CAC0124 ()	-
GBproXXXX	135167	135180	-	0.00	-5.71	TGTCACCGAAAGCA	-	-	-	coding region in gene CAC0124 ()	-
GBproXXXX	141358	141371	+	0.00	-5.67	TGCTTCCGACATCT	-	-	-	coding region in gene CAC0132 ()	-
GBproXXXX	141358	141371	-	0.00	-6.07	AGATGTCGGAAGCA	-	-	-	coding region in gene CAC0132 ()	-
GBproXXXX	142828	142841	+	0.00	-5.81	TGCTAGCGGTGCCC		CAC0134 (GE00068137)	281	intergenic	SMILE
GBproXXXX	142828	142841	-	0.00	-5.88	GGGCACCGCTAGCA		CAC0134 (GE00068137)	281	intergenic	SMILE
GBproXXXX	143836	143849	+	0.00	-6.57	TGCTAGCGAGACCT		CAC0136 (GE00068139)	223	coding region	SMILE
GBproXXXX	143836	143849	+	0.00	-6.57	TGCTAGCGAGACCT		CAC0137 (GE00068140)	0	intergenic	SMILE
GBproXXXX	143836	143849	-	0.00	-6.42	AGGTCTCGCTAGCA		CAC0136 (GE00068139)	223	coding region	SMILE

Supplementary Figure 1 Screenshot of partial CRE search of *C. acetobutylicum* chromosome.

Appendix B



Download Regulon (CSV): Download

Number of matches: 29

Number of genes: 6

Genome Browser	Start	End	Strand	PWM Score(s)	SEP Score	Sequence	ORF ID	Gene Name/Acc	ATG-Distance	Location	SMILE Analysis
GBproXXXX	22957	22970	-	0.00	-5.50	GGTATTCGCACGCA	-	-	-	coding region in gene CAP0022 ()	-
GBproXXXX	36895	36908	-	0.00	-6.00	AGCACACGGACGCA	-	-	-	coding region in gene CAP0036 ()	-
GBproXXXX	40575	40588	-	0.00	-5.79	AGAAATCGCAAGCA	-	-	-	coding region in gene CAP0040 ()	-
GBproXXXX	41353	41366	-	0.00	-5.98	AGTTGTCGGAAACA		CAP0042 (GE00071719)	308	coding region	SMILE
GBproXXXX	53000	53013	-	0.00	-5.99	TGTAATCGGTGCCA	-	-	-	coding region in gene CAP0051 ()	-
GBproXXXX	56775	56788	-	0.00	-5.46	TGTTAACGTTAACA		CAP0056 (GE00071733)	30	intergenic	SMILE
GBproXXXX	64458	64471	-	0.00	-6.61	AGAATACGGCTGCA	-	-	-	coding region in gene CAP0063 ()	-
GBproXXXX	69590	69603	+	0.00	-6.38	TGCAATCGGGTGCG	-	-	-	coding region in gene CAP0066 ()	-
GBproXXXX	69629	69642	-	0.00	-6.38	TGTAACCGCTTGCA	-	-	-	coding region in gene CAP0066 ()	-
GBproXXXX	81451	81464	-	0.00	-6.80	AGTGTCCGAAATCA	-	-	-	coding region in gene CAP0077 ()	-
GBproXXXX	91761	91774	-	0.00	-6.63	TGCACACGCGGACA	-	-	-	coding region in gene CAP0088 ()	-
GBproXXXX	92934	92947	-	0.00	-6.42	GGGTACCGCCAACA	-	-	-	non-coding region between gene CAP0088 () and CAP0089 ()	-
GBproXXXX	93545	93558	-	0.00	-6.55	GGTTATCGCCTGCA	-	-	-	coding region in gene CAP0089 ()	-
GBproXXXX	100691	100704	-	0.00	-5.65	GGAAAACGATTTCA		CAP0097 (GE00071773)	273	intergenic	SMILE
GBproXXXX	101249	101262	+	0.00	-6.63	TGTCCCCGCCAACA	-	-	-	coding region in gene CAP0098 ()	-
GBproXXXX	102342	102355	-	0.00	-6.44	TGCAACCGGTAACA	-	-	-	coding region in gene CAP0098 ()	-
GBproXXXX	102382	102395	-	0.00	-6.44	AGTAATCGTTTTCA	-	-	-	coding region in gene CAP0098 ()	-
GBproXXXX	110939	110952	-	0.00	-6.22	TGCTAGCGGACTCA	-	-	-	coding region in gene CAP0104 ()	-
GBproXXXX	111071	111084	-	0.00	-6.03	TGATACCGAAAACA		CAP0105 (GE00071781)	240	coding region	SMILE
GBproXXXX	130725	130738	-	0.00	-6.02	TGTTAACGTTTGCA		CAP0121 (GE00071797)	347	coding region	SMILE
GBproXXXX	138802	138815	-	0.00	-6.06	CGATAGCGGGTGCA	-	-	-	coding region in gene CAP0128 ()	-
GBproXXXX	153742	153755	+	0.00	-6.25	TGAAAGCGCCGACA	-	-	-	coding region in gene CAP0142 ()	-
GBproXXXX	156858	156871	-	0.00	-5.72	TGATTTCGTTTTCA	-	-	-	coding region in gene CAP0146 ()	-
GBproXXXX	160913	160926	-	0.00	-6.54	AGTAAACGTCGTCA	-	-	-	coding region in gene CAP0150 ()	-
GBproXXXX	161852	161865	-	0.00	-6.95	AGGTTACGGAAACA	-	-	-	coding region in gene CAP0150 ()	-
GBproXXXX	161913	161926	-	0.00	-6.52	TGAGGGCGTATCCA	-	-	-	coding region in gene CAP0150 ()	-
GBproXXXX	176022	176035	-	0.00	-6.41	TGTATTCGCCTGCA	-	-	-	coding region in gene CAP0162 ()	-
GBproXXXX	176670	176683	-	0.00	-6.25	GGTTTACGGATCCA	-	-	-	coding region in gene CAP0162 ()	-
GBproXXXX	178938	178951	-	0.00	-6.19	TGTTTCCGGCCTCA		CAP0164 (GE00071840)	166	coding region	SMILE

Supplementary Figure 2 Screenshot of CRE search of *C. acetobutylicum* megaplasmid

Appendix C

Supplementary Table 1 Cultivation parameters for strains harboring plasmids pMTLxyloperon-wt (wildtype) and pMTLxyloperon (CRE-free) after 60 hours of batch cultivation

	Initial Carbon ($g\ l^{-1}$)		Residual Carbon ($g\ l^{-1}$)		Products ($g\ l^{-1}$)		Max OD_{600}[a]	μ_{net} (h^{-1})[b]	Productivity ($g\ l^{-1}\ h^{-1}$)	ABE yield (g/g)	Butanol yield (g/g)
	Glucose	**Xylose**	**Glucose**	**Xylose**	**Butanol**	**ABE**					
Wild-type	40.7	22.3	0	20.6	8.1	12.8	4.3	0.2	0.21	0.3	0.19
CRE-free	40.6	22.7	0	17.9	8.3	14.6	4.7	0.23	0.24	0.32	0.18

[a] Maximum OD_{600} was observed after 36 hours of cultivation

[b] based on observed log phase between 12 and 24 hours of cultivation