Appl Microbiol Biotechnol (2015) 99:7579–7588
DOI 10.1007/s00253-015-6611-4

Elimination of carbon catabolite repression in *Clostridium acetobutylicum*—a journey toward simultaneous use of xylose and glucose

Mark Bruder[1] · **Murray Moo-Young**[1] · **Duane A. Chung**[1,2,3] · **C. Perry Chou**[1]

Received: 26 February 2015 / Revised: 9 April 2015 / Accepted: 12 April 2015 / Published online: 19 May 2015

Abstract The industrial Gram-positive anaerobe *Clostridium acetobutylicum* is a valued acetone, butanol, and ethanol (ABE) 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 biphasic 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 of glucose and xylose by *C. acetobutylicum*. First, we utilized an in vivo gene reporter assay to demonstrate that an identified 14-nucleotide catabolite 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.

Keywords Biofuel · Butanol · *Clostridium acetobutylicum* · Carbon catabolite repression · Catabolic responsive element · Lignocellulose · Xylose

✉ Duane A. Chung
duane.chung@uwaterloo.ca; http://www.neemo.io

✉ C. Perry Chou
cpchou@uwaterloo.ca

Mark Bruder
mark.bruder@uwaterloo.ca

Murray Moo-Young
mooyoung@uwaterloo.ca

[1] Department of Chemical Engineering, University of Waterloo, 200 University Avenue West, Waterloo, Ontario, Canada N2L 3G1

[2] Department of Pathology and Molecular Medicine, McMaster University, Hamilton, Ontario, Canada L8N 3Z5

[3] Neemo Inc., Rm. 5113 Michael G. DeGroote Centre for Learning and Discovery, 1280 Main Street West, Hamilton, Ontario, Canada L8S 4K1

Introduction

The genus *Clostridium* comprised predominantly obligately anaerobic, Gram-positive, endospore-forming, and mesophilic microorganisms (Pyne et al. 2014a). Among them, the solventogenic clostridia can produce acetone, butanol, and ethanol (ABE), of which butanol is a promising biofuel due to its resemblance to gasoline in terms of physicochemical and combustibility properties (Jang et al. 2012). Many clostridia are able to utilize a remarkable range of carbohydrates, including the predominant constituents of lignocellulosic biomass, namely, glucose and xylose (Tracy et al. 2012), and as such, research has aimed to develop genetic engineering tools to rationally enhance the metabolic capabilities of many species of *Clostridium* (Pyne et al. 2014a). However, many Gram-positive organisms, including several species of *Clostridium*, are subject to carbon catabolite repression (CCR) (Hueck et al.

1994; Tangney et al. 2003), in which utilization of carbon sources is abolished in the presence of a preferred carbohydrate such as glucose.

In Gram-positive bacteria, CCR is facilitated through the phospho-carrier protein HPr, which is a component of the phosphoenolpyruvate-dependent phosphotransferase system (PTS), the major carbohydrate transport system in the clostridia (Tangney et al. 2003) (Fig. 1). Briefly, the PTS enzymes I (EI), histone-containing protein (HPr), and enzyme II (EII) form a phosphorylation cascade to link carbohydrate uptake with its simultaneous phosphorylation. In its normal PTS function, HPr is phosphorylated at a conserved histidine residue; however, in the presence of glycolytic intermediates including fructose-1,6-bisphosphate, the expression of an HPr kinase (HPrK) is induced which competitively phosphorylates HPr at a conserved serine residue (HPr-Ser^{46}-P). This serine-phosphorylated HPr forms a complex with catabolite control protein A (CcpA), and the 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). Thus, the presence of glycolytic intermediates triggers alternative phosphorylation within the glucose PTS system and therefore results in the formation of a protein complex that mediates transcriptional inhibition.

The CcpA protein belongs to the LacI/GalR family of allosteric transcriptional regulators, which acts in a so-called roadblock mechanism through binding onto an operator sequence to create a physical barrier to transcription mediated by the host RNA polymerase (Swint-Kruse and Matthews 2009). Structural

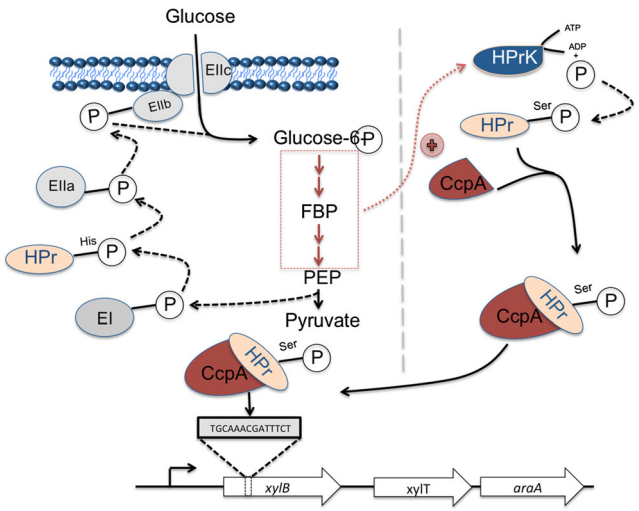

Fig. 1 PEP-dependent PTS system and model of CCR-type inhibition: phospho-relay system to couple glucose transport with its simultaneous phosphorylation. Briefly, phosphate from PEP is transferred in a relay cascade through EI, HPr, and EII domains A and B to phosphorylate glucose as it is transported 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

characterization of several members of this family has been conducted, including LacI and PurR regulators from *Escherichia coli* (Choi and Zalkin 1992; Lewis et al. 1996; Schumacher et al. 1994) and CcpA regulators from *Bacillus subtilis* (Schumacher et al. 2011), *Bacillus megaterium* (Schumacher et al. 2004), and *Lactococcus lactis* (Loll et al. 2007). These studies have shed light on the general architecture and molecular mechanism of these regulators (Schumacher et al. 2004; Schumacher et al. 2006; Schumacher et al. 2007).

Although CCR has been extensively studied in the model Gram-positive Firmicute *B. subtilis*, relatively little investigation has been directed toward CCR in the clostridia (Gorke and Stulke 2008). Aside from observations of phenotypes consistent with CCR-type inhibition in members of *Clostridium* (Gorke and Stulke 2008), experimental analysis of CCR has been limited to *Clostridium acetobutylicum*, including identification and some experimental confirmation of the requisite elements of CCR in the genome (Tangney et al. 2003), transcriptional profiling of *C. acetobutylicum* grown on mixtures of glucose and xylose, and putative CRE site identification using the consensus CRE sequence of *B. subtilis* as the query sequence (Grimmler et al. 2010). Recently, efforts have been made to attenuate the effect of CCR on the xylose catabolic operon of *C. acetobutylicum*, including intron-mediated knockout 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). While putative CRE sequences have been identified in the *C. acetobutylicum* genome (Grimmler et al. 2010; Ren et al. 2012; Rodionov et al. 2001), the requirement of the CRE sequence for CcpA-mediated CCR in *C. acetobutylicum* has not been determined. Elimination of CcpA binding sites could provide an effective and easily implemented strategy for eliminating CCR without the aforementioned drawbacks.

Here, we show that a *cis*-active CRE element is independently sufficient to both impart and, when mutated, remove CCR-mediated inhibition of gene expression in *C. acetobutylicum* through in vivo reporter gene assays and phenotypic derivation. The CRE site sequence chosen for study is from the *C. acetobutylicum* xylose catabolic operon, whose expression is subject to CCR. The proposed genetic strategy by disrupting such catabolic inhibition via mutation

of endogenous CRE sequences should be applicable to any microorganisms with the glucose-induced CCR systems.

Materials and methods

Bacterial strains, plasmids, and oligonucleotides

Bacterial strains and plasmids employed in this work are listed in Table 1, and oligonucleotides in Table 2. *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.

Growth and maintenance conditions

E. coli strains were cultivated aerobically at 37 °C in lysogeny broth (LB), 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.

DNA isolation, manipulation, and electrotransformation

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 electrotransformed to *C. acetobutylicum* as described previously (Mermelstein and Papoutsakis 1993). Restriction enzymes, standard *Taq* DNA Polymerase,

Table 1 Strains and plasmids employed in this study

Strains or plasmids	Relevant characteristics	Source or reference
Strains		
E. coli DH5α	*hsdR recA1 endA1*	Lab stock
E. coli ER2275	*hsdR mcrA recA1 endA1*	NEB
C. acetobutylicum DSM 792	Wild-type	DSMZ
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 14nt 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

NEB New England Biolabs, *DSMZ* Deutsche Sammlung von Mikroorganismen Und Zellkuturen GmbH

Table 2 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

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.

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.

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 h and resuspended 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 min. The crude extract was harvested by centrifugation, and the β-galactosidase assay was performed as described previously

(Tummala et al. 1999) and presented as specific activity in Miller units, which is the standard unit for estimating β-galactosidase concentration in the cell by measuring its enzymatic activity (Garcia et al. 2011). All β-galactosidase activity assays were carried out in triplicate.

Anaerobic static-flask fermentations were carried out in 250-ml flasks 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 80 °C water bath and 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-h intervals for A_{600} measurements as well as HPLC analysis. All fermentations were carried out in duplicate.

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).

Results

Introduction of a 14-nucleotide 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 putative xylulose kinase-encoding *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 (Fig. 2). Upon electrotransformation 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 Fig. 2, *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 messenger RNA 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 (i.e., the sequence did not resemble the structure of the CRE consensus sequence, TGNAANCGNNNNCN). The plasmid pHT3catP-Pthl-14nt was introduced to *C. acetobutylicum*, and the resulting recombinant strain was assayed for β-galactosidase activity. As shown in Fig. 2, 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.

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., TGNAANCGNN NNCN) 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, but the activity was significantly lower than that of the positive

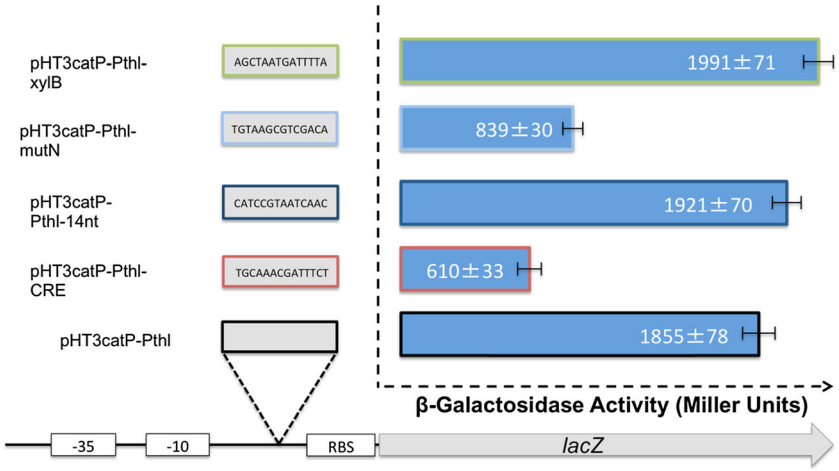

Fig. 2 β-galactosidase activities and relevant plasmid structure for *C. acetobutylicum* harboring plasmids, pHT3catP-Pthl, pHT3catP-Pthl-CRE (sequence 5′ TGCAAACGATTTCT 3′), pHT3catP-Pthl-14nt (5′ CATCCGTAATCAAC 3′), pHT3catP-Pthl-mutN (5′ TGTAAGCGTC GACA 3′), and pHT3catP-Pthl-xylBmut (5′ AGCTAATGATTTTA 3′). Strains were grown in CGM medium to mid-exponential phase and 16 OD units were harvested and assayed for β-galactosidase activity. β-galactosidase assays were carried out in triplicate

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, some mutations may provide slight modulation of the inhibitory 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 (Fig. 2). 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.

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 overexpression 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 (Fig. 3), 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 Fig. 3, after 84 h of cultivation, the strain overexpressing 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 h, 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 (Fig. 3, 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 allows *C. acetobutylicum* to utilize glucose and xylose concurrently.

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

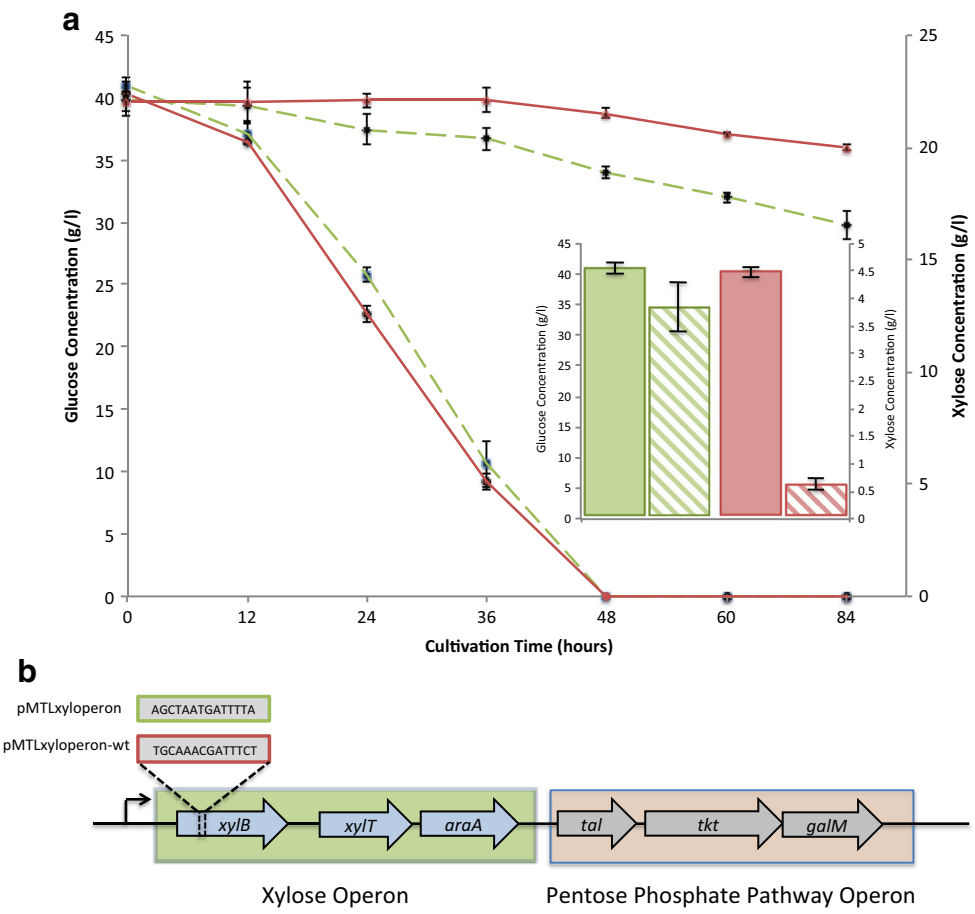

Fig. 3 **a** Residual glucose and xylose concentration time-course and consumption at 48 h (*inset*; *solid bar* denotes glucose consumption and *striped lines* denote xylose) for wild-type *C. acetobutylicum* (*red solid lines*) and *C. acetobutylicum* harboring a plasmid-borne CRE-free xylose operon (*green dashed lines*) 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 h 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′)

overexpression and knockout (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 bifold 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 italics) (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 accept that they share a similar DNA-binding structural mechanism (unpublished data; Wu et al. 2015). By studying the specific amino acid–nucleotide interactions between CcpA and CRE, respectively, and using the CRE consensus sequence of *B. subtilis* and the one proposed for *C. acetobutylicum* (Miwa et al. 2000; Ren et al. 2012), as well as mutational studies performed in other organisms (Kim and Chambliss 1997; Marasco et al. 2002; Weickert and Chambliss 1990), we have used the reported putative CRE sequences in addition to our own search (Grimmler et al. 2010; Ren et al. 2012; unpublished data) to analyze and identify potentially active CRE sites in the *C. acetobutylicum* genome, specifically associated with genes involved in xylose utilization (data not shown).

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 that 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 bifold 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).

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 (data not shown). 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 expression of the *E. coli talA* gene encoding transaldolase; overexpression of native xylose-dissimilation genes *xylT*, *xylA*, and *xylB*; and overexpression 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 *Clostridium 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 *Clostridium cellulolyticum*, *Clostridium thermocellum*, *Clostridium tyrobutyricum*, and *Clostridium 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 downregulated 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).

Ideally, the proposed genetic strategy for CRE mutation should be targeted on the host chromosome. However, genome modification and editing tools remain somewhat elusive in the clostridia (Pyne et al. 2014a). As these technologies are continually developed and improved for the clostridia, manipulating the CRE sequence of catabolically repressed operons to relieve CCR is expected to enhance carbon utilization for more effective and economic bio-manufacturing.

Acknowledgments This work was supported in part by the Natural Sciences and Engineering Research Council of Canada (NSERC; grant number STPGP 430106–12) and the Canada Research Chairs (CRC; grant number 950–211471) programs.

Conflict of interest DAC is a founder and employee of Neemo Inc., at which MB has also been employed. Neemo Inc. has a financial interest in the production of biofuels using clostridial microorganisms. The remaining authors declare no competing interests.

Authors' contributions MB helped conceive the study, participated in its design and coordination, carried out the experiments, and drafted the manuscript. MMY participated in the study design and coordination. DAC and CPC helped conceive the study, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

References

Abdou L, Boileau C, de Philip P, Pagès S, Fiérobe H-P, Tardif C (2008) Transcriptional regulation of the *Clostridium cellulolyticum cip-cel* operon: a complex mechanism involving a catabolite-responsive element. J Bacteriol 190:1499–1506. doi:10.1128/jb.01160-07

Al-Hinai MA, Fast AG, Papoutsakis ET (2012) Novel system for efficient isolation of *Clostridium* double-crossover allelic exchange mutants enabling markerless chromosomal gene deletions and DNA integration. Appl Environ Microbiol 78:8112–8121. doi:10.1128/AEM.02214-12

Baer SH, Blaschek HP, Smith TL (1987) Effect of butanol challenge and temperature on lipid composition and membrane fluidity of butanol-tolerant *Clostridium acetobutylicum*. Appl Environ Microbiol 53:2854–2861

Bowles LK, Ellefson WL (1985) Effects of butanol on *Clostridium acetobutylicum*. Appl Environ Microbiol 50:1165–1170

Choi KY, Zalkin H (1992) Structural characterization and corepressor binding of the *Escherichia coli* purine repressor. J Bacteriol 174:6207–6214

Egeter O, Brückner R (1996) Catabolite repression mediated by the catabolite control protein CcpA in *Staphylococcus xylosus*. Mol Microbiol 21:739–749. doi:10.1046/j.1365-2958.1996.301398.x

Eiteman MA, Lee SA, Altman E (2008) A co-fermentation strategy to consume sugar mixtures effectively. J Biol Eng 2:3–3. doi:10.1186/1754-1611-2-3

Garcia Hernan G, Lee Heun J, Boedicker James Q, Phillips R (2011) Comparison and calibration of different reporters for quantitative analysis of gene expression. Biophys J 101:535–544. doi:10.1016/j.bpj.2011.06.026

Gorke B, Stulke J (2008) Carbon catabolite repression in bacteria: many ways to make the most out of nutrients. Nat Rev Microbiol 6:613–624. doi:10.1038/nrmicro1932

Grimmler C, Held C, Liebl W, Ehrenreich A (2010) Transcriptional analysis of catabolite repression in *Clostridium acetobutylicum* growing on mixtures of d-glucose and d-xylose. J Biotechnol 150:315–323. doi:10.1016/j.jbiotec.2010.09.938

Grundy FJ, Waters DA, Allen SH, Henkin TM (1993) Regulation of the *Bacillus subtilis* acetate kinase gene by CcpA. J Bacteriol 175:7348–7355

Gu Y, Li J, Zhang L, Chen J, Niu L, Yang Y, Yang S, Jiang W (2009) Improvement of xylose utilization in *Clostridium acetobutylicum* via expression of the *talA* gene encoding transaldolase from *Escherichia coli*. J Biotechnol 143:284–287. doi:10.1016/j.jbiotec.2009.08.009

Hu S, Zheng H, Gu Y, Zhao J, Zhang W, Yang Y, Wang S, Zhao G, Yang S, Jiang W (2011) Comparative genomic and transcriptomic analysis revealed genetic characteristics related to solvent formation and xylose utilization in *Clostridium acetobutylicum* EA 2018. BMC Genomics 12 doi:10.1186/1471-2164-12-93

Hueck CJ, Hillen W (1995) Catabolite repression in *Bacillus subtilis*: a global regulatory mechanism for the gram-positive bacteria? Mol Microbiol 15:395–401. doi:10.1111/j.1365-2958.1995.tb02252.x

Hueck CJ, Hillen W, Saier MH Jr (1994) Analysis of a cis-active sequence mediating catabolite repression in gram-positive bacteria. Res Microbiol 145:503–518. doi:10.1016/0923-2508(94)90028-

Jang Y-S, Malaviya A, Cho C, Lee J, Lee SY (2012) Butanol production from renewable biomass by clostridia. Bioresour Technol 123:653–663. doi:10.1016/j.biortech.2012.07.104

Jiang L, Cai J, Wang J, Liang S, Xu Z, Yang S-T (2010) Phosphoenolpyruvate-dependent phosphorylation of sucrose by *Clostridium tyrobutyricum* ZJU 8235: evidence for the phosphotransferase transport system. Bioresour Technol 101:304–309. doi:10.1016/j.biortech.2009.08.024

Jin L, Zhang H, Chen L, Yang C, Yang S, Jiang W, Gu Y (2014) Combined overexpression of genes involved in pentose phosphate pathway enables enhanced d-xylose utilization by *Clostridium acetobutylicum*. J Biotechnol 173:7–9. doi:10.1016/j.jbiotec.2014.01.002

Kim J-H, Chambliss GH (1997) Contacts between *Bacillus subtilis* catabolite regulatory protein CcpA and amyO target site. Nucleic Acids Res 25:3490–3496. doi:10.1093/nar/25.17.3490

Lewis M, Chang G, Horton NC, Kercher MA, Pace HC, Schumacher MA, Brennan RG, Lu P (1996) Crystal structure of the lactose operon repressor and its complexes with DNA and inducer. Science 271:1247–1254. doi:10.1126/science.271.5253.1247

Li Z, Xiao H, Jiang W, Jiang Y, Yang S (2013) Improvement of solvent production from xylose mother liquor by engineering the xylose metabolic pathway in *Clostridium acetobutylicum* EA 2018. Appl Biochem Biotechnol 171:555–568. doi:10.1007/s12010-013-0414-9

Loll B, Kowalczyk M, Alings C, Chieduch A, Bardowski J, Saenger W, Biesiadka J (2007) Structure of the transcription regulator CcpA from *Lactococcus lactis*. Acta Crystallogr Sect D: Biol Crystallogr 63:431–436. doi:10.1107/S0907444907000546

Lütke-Eversloh T, Bahl H (2011) Metabolic engineering of *Clostridium acetobutylicum*: recent advances to improve butanol production. Curr Opin Biotechnol 22:634–647. doi:10.1016/j.copbio.2011.01.011

Marasco R, Muscariello L, Rigano M, Sacco M (2002) Mutational analysis of the *bglH* catabolite-responsive element (cre) in *Lactobacillus plantarum*. FEMS Microbiol Lett 208:143–146. doi:10.1111/j.1574-6968.2002.tb11074.x

Marciniak B, Pabijaniak M, de Jong A, Duhring R, Seidel G, Hillen W, Kuipers O (2012) High- and low-affinity cre boxes for CcpA binding in *Bacillus subtilis* revealed by genome-wide analysis. BMC Genomics 13:401. doi:10.1186/1471-2164-13-401

Mermelstein L, Papoutsakis E (1993) In vivo methylation in *Escherichia coli* by the *Bacillus subtilis* phage phi 3T I methyltransferase to protect plasmids from restriction upon transformation of *Clostridium acetobutylicum* ATCC 824. Appl Environ Microbiol 59:1077–1081

Miwa Y, Nakata A, Ogiwara A, Yamamoto M, Fujita Y (2000) Evaluation and characterization of catabolite-responsive elements (cre) of *Bacillus subtilis*. Nucleic Acids Res 28:1206–1210. doi:10.1093/nar/28.5.1206

Noguchi T, Tashiro Y, Yoshida T, Zheng J, Sakai K, Sonomoto K (2013) Efficient butanol production without carbon catabolite repression from mixed sugars with *Clostridium saccharoperbutylacetonicum* N1-4. J Biosci Bioeng 116:716–721. doi:10.1016/j.jbiosc.2013.05.030

Ohta K, Beall DS, Mejia JP, Shanmugam KT, Ingram LO (1991) Metabolic engineering of *Klebsiella oxytoca* M5A1 for ethanol production from xylose and glucose. Appl Environ Microbiol 57:2810–2815

Ounine K, Petitdemange H, Raval G, Gay R (1985) Regulation and butanol inhibition of d-xylose and d-glucose uptake in *Clostridium acetobutylicum*. Appl Environ Microbiol 49:874–878

Paredes CJ, Rigoutsos I, Papoutsakis ET (2004) Transcriptional organization of the *Clostridium acetobutylicum* genome. Nucleic Acids Res 32:1973–1981. doi:10.1093/nar/gkh509

Paulsen IT, Chauvaux S, Choi P, Saier MH (1998) Characterization of glucose-specific catabolite repression-resistant mutants of *Bacillus subtilis*: identification of a novel hexose:H+ symporter. J Bacteriol 180:498–504

Pyne ME, Bruder M, Moo-Young M, Chung DA, Chou CP (2014a) Technical guide for genetic advancement of underdeveloped and intractable *Clostridium*. Biotechnol Adv 32:623–641. doi:10.1016/j.biotechadv.2014.04.003

Pyne ME, Moo-Young M, Chung DA, Chou CP (2013) Development of an electrotransformation protocol for genetic manipulation of *Clostridium pasteurianum*. Biotechnol Biofuels 6:50

Pyne ME, Moo-Young M, Chung DA, Chou CP (2014b) Expansion of the genetic toolkit for metabolic engineering of *Clostridium pasteurianum*: chromosomal gene disruption of the endogenous CpaAI restriction enzyme. Biotechnol Biofuels 7:163

Ren C, Gu Y, Hu S, Wu Y, Wang P, Yang Y, Yang C, Yang S, Jiang W (2010) Identification and inactivation of pleiotropic regulator CcpA to eliminate glucose repression of xylose utilization in *Clostridium acetobutylicum*. Metab Eng 12:446–454. doi:10.1016/j.ymben.2010.05.002

Ren C, Gu Y, Wu Y, Zhang W, Yang C, Yang S, Jiang W (2012) Pleiotropic functions of catabolite control protein CcpA in butanol-producing *Clostridium acetobutylicum*. BMC Genomics 13:349. doi:10.1186/1471-2164-13-349

Rodionov DA, Mironov AA, Gelfand MS (2001) Transcriptional regulation of pentose utilisation systems in the *Bacillus/Clostridium* group of bacteria. FEMS Microbiol Lett 205:305–314. doi:10.1111/j.1574-6968.2001.tb10965.x

Sadler JR, Sasmor H, Betz JL (1983) A perfectly symmetric lac operator binds the lac repressor very tightly. Proc Natl Acad Sci U S A 80:6785–6789

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning, vol 2. Cold spring harbor laboratory press, New York

Schumacher M, Allen G, Diel M, Seidel G, Hillen W, Brennan R (2004) Structural basis for allosteric control of the transcription regulator CcpA by the phosphoprotein HPr-Ser46-P. Cell 118:731–741

Schumacher MA, Choi KY, Zalkin H, Brennan RG (1994) Crystal structure of LacI member, PurR, bound to DNA: minor groove binding by alpha helices. Science 266:763–770. doi:10.1126/science.7973627

Schumacher MA, Seidel G, Hillen W, Brennan RG (2006) Phosphoprotein Crh-Ser46-P displays altered binding to CcpA to effect carbon catabolite regulation. J Biol Chem 281:6793–6800. doi:10.1074/jbc.M509977200

Schumacher MA, Seidel G, Hillen W, Brennan RG (2007) Structural mechanism for the fine-tuning of CcpA function by the small molecule effectors glucose 6-phosphate and fructose 1,6-bisphosphate. J Mol Biol 368:1042–1050. doi:10.1016/j.jmb.2007.02.054

Schumacher MA, Sprehe M, Bartholomae M, Hillen W, Brennan RG (2011) Structures of carbon catabolite protein A-(HPr-Ser46-P) bound to diverse catabolite response element sites reveal the basis for high-affinity binding to degenerate DNA operators. Nucleic Acids Res 39:2931–2942. doi:10.1093/nar/gkq1177

Swint-Kruse L, Matthews KS (2009) Allostery in the LacI/GalR family: variations on a theme. Curr Opin Microbiol 12:129–137. doi:10.1016/j.mib.2009.01.009

Tangney M, Galinier A, Deutscher J, Mitchell WJ (2003) Analysis of the elements of catabolite repression in *Clostridium acetobutylicum* ATCC 824. J Mol Microbiol Biotechnol 6:6–11. doi:10.1159/000073403

Tobisch S, Zühlke D, Bernhardt J, Stülke J, Hecker M (1999) Role of CcpA in regulation of the central pathways of carbon catabolism in *Bacillus subtilis*. J Bacteriol 181:6996–7004

Tojo S, Satomura T, Morisaki K, Deutscher J, Hirooka K, Fujita Y (2005) Elaborate transcription regulation of the *Bacillus subtilis ilv*-leu operon involved in the biosynthesis of branched-chain amino acids through global regulators of CcpA, CodY and TnrA. Mol Microbiol 56:1560–1573

Tracy BP, Jones SW, Fast AG, Indurthi DC, Papoutsakis ET (2012) Clostridia: the importance of their exceptional substrate and metabolite diversity for biofuel and biorefinery applications. Curr Opin Biotechnol 23:364–381. doi:10.1016/j.copbio.2011.10.008

Tummala SB, Welker NE, Papoutsakis ET (1999) Development and characterization of a gene expression reporter system for *Clostridium acetobutylicum* ATCC 824. Appl Environ Microbiol 65:3793–3799

Varga J, Stirewalt VL, Melville SB (2004) The CcpA protein is necessary for efficient sporulation and enterotoxin gene (cpe) regulation in *Clostridium perfringens*. J Bacteriol 186:5221–5229. doi:10.1128/jb.186.16.5221-5229.2004

Warner JB, Lolkema JS (2003) CcpA-dependent carbon catabolite repression in bacteria. Microbiol Mol Biol Rev 67:475–490. doi:10.1128/mmbr.67.4.475-490.2003

Weickert MJ, Chambliss GH (1990) Site-directed mutagenesis of a catabolite repression operator sequence in *Bacillus subtilis*. Proc Natl Acad Sci U S A 87:6238–6242. doi:10.1073/pnas.87.16.6238

Wu Y, Yang Y, Ren C, Yang C, Yang S, Gu Y, Jiang W (2015) Molecular modulation of pleiotropic regulator CcpA for glucose and xylose coutilization by solvent-producing *Clostridium acetobutylicum*. Metab Eng 28:169–179. doi:10.1016/j.ymben.2015.01.006

Xiao H, Gu Y, Ning Y, Yang Y, Mitchell WJ, Jiang W, Yang S (2011) Confirmation and elimination of xylose metabolism bottlenecks in glucose phosphoenolpyruvate-dependent phosphotransferase system-deficient *Clostridium acetobutylicum* for simultaneous utilization of glucose, xylose, and arabinose. Appl Environ Microbiol 77:7886–7895. doi:10.1128/aem.00644-11

Xiao H, Li Z, Jiang Y, Yang Y, Jiang W, Gu Y, Yang S (2012) Metabolic engineering of d-xylose pathway in *Clostridium beijerinckii* to optimize solvent production from xylose mother liquid. Metab Eng 14:569–578. doi:10.1016/j.ymben.2012.05.003

Yu EK, Saddler JN (1983) Fed-batch approach to production of 2,3-butanediol by *Klebsiella pneumoniae* grown on high substrate concentrations. Appl Environ Microbiol 46:630–635

Yu Y, Tangney M, Aass HC, Mitchell WJ (2007) Analysis of the mechanism and regulation of lactose transport and metabolism in *Clostridium acetobutylicum* ATCC 824. Appl Environ Microbiol 73:1842–1850. doi:10.1128/aem.02082-06

Zhang Y-HP, Lynd LR (2005) Regulation of cellulase synthesis in batch and continuous cultures of *Clostridium thermocellum*. J Bacteriol 187:99–106. doi:10.1128/jb.187.1.99-106.2005