

Development of an anhydrotetracycline-inducible gene expression system for solvent-producing *Clostridium acetobutylicum*: A useful tool for strain engineering

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ABSTRACT

Clostridium acetobutylicum is an important solvent (acetone–butanol–ethanol) producing bacterium. However, a stringent, effective, and convenient-to-use inducible gene expression system that can be used for regulating the gene expression strength in *C. acetobutylicum* is currently not available. Here, we report an anhydrotetracycline-inducible gene expression system for solvent-producing bacterium *C. acetobutylicum*. This system consists of a functional chloramphenicol acetyltransferase gene promoter containing *tet* operators (*tetO*), *P*_{thl} promoter (thiolase gene promoter from *C. acetobutylicum*) controlling TetR repressor expression cassette, and the chemical inducer anhydrotetracycline (aTc). The optimized system, designated as pGusA2-2tetO1, allows gene regulation in an inducer aTc concentration-dependent way, with an inducibility of over two orders of magnitude. The stringency of TetR repression supports the introduction of the genes encoding counterselective marker into *C. acetobutylicum*, which can be used to increase the mutant screening efficiency. This aTc-inducible gene expression system will thus increase the genetic manipulation capability for engineering *C. acetobutylicum*.

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1. Introduction

Clostridium acetobutylicum is an important bacterium used in acetone–butanol–ethanol (ABE) fermentation (Jones and Woods, 1986). In the last 20 years, attempts have been made to improve the solvent production efficiency through genetic engineering and metabolic engineering (Lütke-Eversloh and Bahl, 2011; Lee et al., 2008; Papoutsakis, 2008; Sillers et al., 2008; Zhu et al., 2011). Since this Gram-positive, anaerobic and spore-forming bacterium is not easy to work with genetically, a set of genetic manipulation tools are needed to increase the efficiency of strain engineering. For genetic manipulation of *C. acetobutylicum*, gene knockout methods based on homologous recombination using replicative/non-replicative vectors (Green et al., 1996; Harris et al., 2002; Heap and Minton, 2009; Soucaille et al., 2008) or Group II intron retrotransposition (Heap et al., 2007; Shao et al., 2007), and gene knockdown methods based on antisense RNA (asRNA) strategies (Desai and Papoutsakis, 1999; Tummala et al., 2003), have been well established, and widely used for metabolic engineering of *C. acetobutylicum*. However, for gene expression, a stringent, effective, and convenient-to-use inducible gene expression (IGE)

system that can be used for regulating the gene expression strength in *C. acetobutylicum* is currently not available.

Inducible gene expression (IGE) systems refer to the genetic manipulation tools for controlling gene expression using chemical or physical inducers (Terpe, 2006). Conventionally, the IGE systems were used for characterization of gene functions (Bertram and Hillen, 2008), and for production of medically or industrially important proteins/enzymes (Terpe, 2006). Recently, IGE systems were frequently used for expression of genes encoding key enzymes involved in metabolic pathways in strain engineering (Fischer et al., 2008; Lan and Liao, 2011; Martin et al., 2003; Shen et al., 2011), or for construction of biobricks in synthetic biology (Andrianantoandro et al., 2006; Canton et al., 2008). Besides, IGE systems also play important roles in developing bacterial genetic manipulation systems, such as inducible expression of the toxin gene *mazF* as a counter-selectable marker for unmarked chromosomal manipulation in *Bacillus subtilis* (Zhang et al., 2006), and inducible expression of the λ phage *red γ β α* operon for one-step inactivation of chromosomal genes in *Escherichia coli* using PCR products (Datsenko and Wanner, 2000).

With regards to IGE systems in *C. acetobutylicum*, Girbal et al. (2003) briefly described a pXYLgusA vector developed based on the *Staphylococcus xylosus* xylose operon promoter–repressor regulatory system. This system achieved a 17-fold higher glucuronidase activity in *C. acetobutylicum* when xylose was used as the sole carbon source (Girbal et al., 2003). However, the pXYLgusA

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system seems not convenient for use. Since *C. acetobutylicum* strains are usually grown in media containing glucose (such as RCM (Hibsch and Grinsted, 1954), CGM (Roos et al., 1985), or 2YTG (Wiesenborn et al., 1988)), direct addition of xylose will not lead to an induced expression of target gene due to the potential glucose-mediated catabolite repression by CcpA on xylose utilization (Girbal et al., 2003). Thus, moving cells from glucose medium to xylose medium may be needed to achieve the xylose-induced gene expression. In another work, Heap et al. (2007) reported an IPTG-inducible 'fac' promoter derived by inserting the lacO operator of the *E. coli lacZ* gene downstream of the promoter of the *Clostridium pasteurianum* ferredoxin gene. However, using *cat* (encoding chloramphenicol acetyltransferase) as a reporter gene, this systems showed a significant level of basal expression in *C. acetobutylicum* in the absence of IPTG. Because of this, addition of IPTG could only achieve a 10-fold, maximally, increased gene expression. Besides, it was found that the promoter of *recA* in *C. acetobutylicum* could be induced by UV-radiation (Nuyts et al., 2001). However, expression of *lacZ* reporter gene using such promoter only showed 20–30% increase after 2 Gy irradiation (Nuyts et al., 2001), suggesting that *recA* promoter does not fit for the development of IGE system.

An ideal IGE system in *C. acetobutylicum* should fit the commonly used medium and be of low level of basal expression. As illustrated above, the well studied and commonly used sugar-responsive IGE systems (such as lactose, arabinose, xylose, rhamnose) (Terpe, 2006) may not be suitable for *C. acetobutylicum*. Among other IGE systems, the tetracycline (Tc)-responsive IGE system effective in many organisms (Bertram and Hillen, 2008), received our attention. The Tc-responsive IGE system was originally adapted from *E. coli* Tn10 transposon. In Tn10, *tetA* gene encodes a membrane-spanning Tc-exporting protein. The expression of *tetA* is under transcriptional control of the Tc-responsive Tet repressor (TetR) encoded by the divergently oriented *tetR* gene (Chopra and Roberts, 2001). In the promoter of *tetA* gene, there are two tetO operators (tetO1 and tetO2) responsible for TetR binding (Chalmers et al., 2000). In the absence of Tc, TetR tightly binds the tetO in the *tetA* promoter and suppresses the transcription of *tetA*. In the presence of Tc, TetR is bound by Tc, which leads to a conformational change that results in dissociation of TetR from tetO and thus induces expression of *tetA* gene. In practice, Tc is usually replaced by its analog anhydrotetracycline (aTc) as an inducer because of its lower toxicity and higher efficiency of binding TetR (Gossen and Bujard, 1993). Since TetR–tetO is the most

efficiently inducible system of transcriptional regulation known to date, it is commonly used as the IGE systems in prokaryotes and eukaryotes (Orth et al., 2000). Here, we describe the development of a TetR-controlled expression system that allows efficient regulation of gene expression in *C. acetobutylicum*. This IGE system will facilitate future metabolic engineering of *C. acetobutylicum*.

2. Materials and methods

2.1. Bacterial strains, plasmids, and primers

Bacterial strains and plasmids used in this work are listed in Table 1, and the primers are listed in Table 2. *E. coli* JM109 was routinely used for vectors construction. All primers were synthesized by Invitrogen (Beijing, China) followed by polyacrylamide gel electrophoresis purification.

2.2. Growth and maintenance conditions

E. coli strains were grown aerobically at 37 °C in Luria-Bertani medium supplemented, when necessary, with ampicillin (100 µg/ml) or chloramphenicol (Cm, 30 µg/ml). All *C. acetobutylicum* strains were grown anaerobically at 37 °C in RCM medium (Hibsch and Grinsted, 1954) supplemented, when necessary, with Cm (30 µg/ml), erythromycin (Em, 50 µg/ml), 5-fluorouracil (5-FU, 50 µg/ml), and anhydrotetracycline (aTc, 100 ng/ml). All *C. acetobutylicum* and *E. coli* strains were maintained frozen in 15% glycerol at –80 °C.

2.3. DNA isolation and manipulation

Total genomic DNA of *C. acetobutylicum* and plasmid DNA of *E. coli* were prepared using an E.Z.N.A Bacterial DNA Isolation Kit and E.Z.N.A Plasmid Extraction Kit (Omega Biotek Inc., Guangzhou, China), respectively. DNA restriction and cloning were performed according to standard procedures (Sambrook and Russell, 2001). Restriction enzymes and T4 DNA ligase were obtained from New England BioLabs (Beijing, China), and used according to the manufacturer's instructions. Electrotransformation of *C. acetobutylicum* was carried out according to the protocol developed by Mermelstein et al. (1992).

Table 1
Bacterial strains and plasmids.

Bacterial strains and plasmids	Characteristics	Sources
Bacterial strains		
<i>E. coli</i> JM109	<i>recA1 mcrB⁺ hsdR17(r_K m_K⁺)</i>	Promega
<i>E. coli</i> XL1-Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB lacI q Z DM15 Tn10 (TetR)]</i>	Stratagene
<i>C. acetobutylicum</i> SMB009	CAC1502::intron	Dong et al. (2010)
<i>C. acetobutylicum</i> SMB011	<i>upp</i> ::intron, derived from SMB009	This work
Plasmids		
pIMP1	MLS ^R Ap ^R , shuttle vector of <i>E. coli</i> - <i>C. acetobutylicum</i>	Mermelstein et al. (1992)
pCm4	pIMP1 containing <i>cat</i> operon (chloramphenicol resistance gene)	This work
pPcm-tetO1	pIMP1 containing Pcm-tetO1- <i>cat</i> cassette	This work
pPcm-tetO2	pIMP1 containing Pcm-tetO2- <i>cat</i> cassette	This work
pPcm-2tetO2	pIMP1 containing Pcm-2tetO2- <i>cat</i> cassette	This work
pPcm-tetO1/2	pIMP1 containing Pcm-tetO1/2- <i>cat</i> cassette	This work
pPcm-tetO1&2	pIMP1 containing Pcm-tetO1&2- <i>cat</i> cassette	This work
pPcm-tetO1-tetR	pPcm-tetO1 containing Pthl- <i>tetR</i> cassette	This work
pGusA2	pPcm-tetO1-tetR containing <i>gusA</i> gene (replacing <i>cat</i>)	This work
pGusA2-2tetO1	pGusA2 containing Pcm-2tetO1 promoter (replacing Pcm-tetO1)	This work
pGusA2-tetO2/1	pGusA2 containing Pcm-tetO2/1 promoter (replacing Pcm-tetO1)	This work
pMazF-2tetO1	pGusA2-2tetO1 containing <i>mazF</i> gene (replacing <i>gusA</i>)	This work
pUPP-2tetO1	pGusA2-2tetO1 containing <i>upp</i> gene (replacing <i>gusA</i>)	This work
pMTL009-upp120/121s	for <i>upp</i> gene disruption by group II intron method	This work

Table 2
Primers used in this study.

Primers	Sequences (5'–3')
295-Pcm-1(Sall)	AGTGTCCACTTGA AAAAATTCACAAAAATGGTATAATATC
296-Pcm-2(BamHI)	CGTGATCCTTA ACTATTTATCAATTCCTGCAATTCGT
297-Pcm-tetO1-1	AGTGTCCACTTGA AACTCTATCATTGATAGAGTATAATATCTTTGTTTCATTAGAGCG
298-Pcm-tetO1/2-1	AGTGTCCACTTGA AACTCTATCATTGATAGAGTATAATACCCTCCTATCAGTG
299-Pcm-tetO1/2-2	GTTATAATACCCTCCTATCAGTGATAGAGAGAATTTGAGAGGGAACCTTAGATGG
300-Pcm-2tetO2-1	AGTGTCCACTCCCTATCAGTGATAGATTGAAATCCCTATCAGTGATAGATATAATATC
301-Pcm-2tetO2-2	ATCCCTATCAGTGATAGATATAATATCTTTGTTTCATTAGAGCGATAAACTTGAATTTG
309-Pthl-tetR-1	ATCGGATCCTATATTGATAAAAAATAATAATAGTGGGTATAAATTAAGTTGTTAGGAGG
310-Pthl-tetR-2	GGTATAATTAAGTTGTTAGGAGGTTAGTTAGAATGTCTAGATTAGATAAAAAGTAAAGTG
311-Pthl-tetR-3	ATCGGAATTCAACTCGACATCTTGGTTACCGTG
312-Pcm-tetO2	AGTGTCCACTTGA AATCCCTATCAGTGATAGATATAATATCTTTGTTTCATTAGAGCG
313-Pcm-tetO1&2-1	AGTGTCCACTTGA AACTCTATCATTGATAGAGTATAATATCTTTGTTTCATTAGAGCTC
314-Pcm-tetO1&2-2	TATAATATCTTTGTTTCATTAGAGCTCCCTATCAGTGATAGAGAGGGAACCTTAGATGG
420-pIMP1	GTGAATCGATTATGCTTTTTCGCGATTCCTC
421-Pcm-tetO1-R1	ATCGTGTACAGGTACCACTAGTGTCTAATGAACAAAGATATTATACT
422-Pcm-tetO1-R2	ATCGTGTACAGGTACCACTAGTACCCTAAGTTCCTCTCAAATTC
509-gusA-1	ATCGACTAGTTTAGGAGGTTAGTTAGAATGTTACGCTCTGTAGAAACC
510-gusA-2	ATCGGATCCACCGTCTCGAGTCATTGTTGCTCCCTCCTGCTCGG
576-Pcm-2tetO1-1	AGTGTCCACTCTATCATTGATAGAGTTGAACTCTATCATTGATAGAGTATAATATC
577-Pcm-tetO2/1-1	AGTGTCCACTCCCTATCAGTGATAGAGATTGAACTCTATCATTGATAGAGTATAATATC
004-upp-1	ATCGACTAGTTTAGGAGGTTAGTTAGAATGAGTAAAGTTACACAAAATATC
005-upp-2	ATCGCTCGAGTTATTTTGTACCGAATAATCTATC
009-MazF-1	ATCGACTAGTTTAGGAGGTTAGTTAGAATGGTAAGCCGATACGTACCCC
010-MazF-2	ATCGCTCGAGTACCAATCAGTACGTTAATTTTGG
243-upp120/121s-IBS	AAAAAAGCTTATAATTATCCTTAGCAATGCTAATGGTGGCCAGATAGGGTG
244-upp120/121s-EBS2	TGAACGCAAGTTTCTAATTTCCGGTTATTGCTCGATAGAGGAAAGTGCT
245-upp120/121s-EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCCATAATGGCTAACTACCTTCTTTGT
10-EBS Universal	CGAAATTAGAACTTGCCTCAGTAAAC
14-007-R1	AGGGTATCCCCAGTTAGTGTAAAGTCTTGG
260-upp120IBS	CCTTAGCAATGCTAATGG
262-upp-1	CAATGGAGGAATGAAATAATGAGTAAAG
263-upp-2	GAGGAAGTTACAGAAGTATTCTACAGG

2.4. Vector constructions

The fragment of CAT expression cassette (containing the promoter Pcm and the *cat* gene encoding chloramphenicol acetyltransferase) was cloned from pMTL007 vector (Heap et al., 2007) employed in *C. acetobutylicum* by PCR using primers 295-Pcm-1(Sall) and 296-Pcm-2(BamHI), and was ligated into the Sall and BamHI sites of the *E. coli*-*C. acetobutylicum* shuttle vector pIMP1 (Mermelstein et al., 1992) to generate the vector pCm4. Based on pCm4, five combinations of primers, i.e., 297-Pcm-tetO1-1 & 296-Pcm-2(BamHI), 298-Pcm-tetO1/2-1 & 299-Pcm-tetO1/2-2 & 296-Pcm-2(BamHI), 300-Pcm-2tetO2-1 & 301-Pcm-2tetO2-2 & 296-Pcm-2(BamHI), 312-Pcm-tetO2-1 & 296-Pcm-2(BamHI), and 313-Pcm-tetO1&2-1 & 314-Pcm-tetO1&2-2 & 296-Pcm-2(BamHI), were used for PCR, to modify the Pcm promoter in pCm4 to be Pcm-tetO, resulting in five vectors pPcm-tetO1, pPcm-tetO2, pPcm-2tetO2, pPcm-tetO1/2, and pPcm-tetO1&2, respectively. It should be noticed that in above manipulation, we used two forward primers to achieve the modification for some configurations (i.e., Pcm-2tetO2, Pcm-tetO1/2, and Pcm-tetO1&2), because of the length limit of synthesized primer.

For introduction of *tetR* expression cassette into pPcm-tetO1, firstly, two forward primers 309-Pthl-tetR-1 and 310-Pthl-tetR-2 and a reverse primer 311-Pthl-tetR-3 were designed to introduce the miniPthl (a derivative of thiolase gene promoter from *C. acetobutylicum*) in front of *tetR* by PCR, using the genomic DNA of *E. coli* XL1-Blue as template, then the resulted PCR product miniPthl-*tetR* was ligated into pPcm-tetO1 by BamHI and EcoRI sites to generate pPcm-tetO1-tetR.

To construct a *gusA* expression vector based on pPcm-tetO1-tetR, we firstly used primers 420-pIMP1 (ClaI) and 422-Pcm-tetO1-R2 (SpeI) to get the Pcm-tetO1 promoter fragment containing the cloning sites in the brackets from pPcm-tetO1-tetR. We then amplified the *gusA* gene from the genomic DNA of *E. coli*

JM109 strain using primers 509-gusA-1 (with SpeI and the RBS sequence of thiolase gene from *C. acetobutylicum*) and 510-gusA-2 (XhoI/Agel/BamHI). Finally the two fragments were cloned into pPcm-tetO1-tetR using ClaI, SpeI, and BamHI sites, to replace the Pcm-tetO1-*cat* cassette. The resulted vector was designated pGusA2. To improve the expression stringency of *gusA* in pGusA2, we further modified the Pcm-tetO1 promoter by introducing tetO1 or tetO2 in front of the –35 region, using primers 576-Pcm-2tetO1-1 and 577-Pcm-tetO2/1-1, generating pGusA2-2tetO1 and pGusA2-tetO2/1. Based on pGusA2-2tetO1, two *upp* and *mazF* expression vectors were constructed. The *upp* and *mazF* genes were amplified from the genomic DNA of *C. acetobutylicum* SMB009 or *E. coli* JM109 using primer pairs 004-upp-1 & 005-upp-2 or 009-MazF-1 & 010-MazF-2, respectively. The resulted PCR products were cloned into pGusA-2tetO1 to replace the *gusA* gene, generating pUpp-2tetO1 and pMazF-2tetO1 vectors.

2.5. Construction of *upp* (CAC2879) disrupted mutant *C. acetobutylicum* SMB011

The Restriction–Modification deficient mutant *C. acetobutylicum* SMB009 capable of accepting unmethylated DNA (Dong et al., 2010), was used as the parental strain for disruption of *upp* (CAC2879) gene encoding uracil phosphoribosyltransferase (Nolling et al., 2001) by group II intron method. Firstly, intron insertion site was selected at the position of 120/121 (numbered from the initiator codon of the ORF) on sense strand (marked as upp120/121s), and the intron re-targeting PCR primers (i.e., 243-upp120/121s-IBS, 244-upp120/121s-EBS2, and 245-upp120/121s-EBS1d), were designed according to a published computer algorithm (Perutka et al., 2004). One-tube SOEing PCR were used to amplify and assemble the 353 bp PCR product which contains the modified IBS, EBS1d, and EBS2 sequences that are responsible for intron targeting. PCR was performed

according to the Targetron Gene Knockout System kit Protocol (<http://www.sigmaaldrich.com/life-science/functional-genomics-and-rnai/targetron.html>). The targeting region was cloned into pMTL009 (Dong et al., 2010) to replace the original intron fragment. The resulted construct, designated as pMTL009-upp120/121s, was confirmed by DNA sequencing using primer 14-007-R1 (Heap et al., 2007). Unmethylated pMTL009-upp120/121s was then electrotransformed into the strain SMB009 according to the protocol developed by Mermelstein et al. (1992), resulting in strain SMB009(pMTL009-upp120/121s). Because it is known that the disruption of *upp* could lead to 5-FU (5-fluorouracil) resistance (Soucaille et al., 2008), SMB009(pMTL009-upp120/121s) was directly spread on RCM agar containing 5-FU of 50 µg/ml. Then the culture was spread on the RCM solid plate without antibiotics and incubated for 24 h in an anaerobic chamber. The colonies formed were restreaked on RCM solid plate in around 1 cm² square, and their genomic DNA was isolated and screened by PCR using primers 262-upp-1 & 245-upp120/121s-EBS1d, located on the chromosome and intron, respectively. For further research, one of the positive colonies was selected to cure the plasmid, generating the mutant strain designated as SMB011. To further verify the disruption of CAC2634, PCR using primers 262-upp-1, and 263-upp-2 flanking the insertion site was performed and the resulted PCR product was sequenced to confirm the insertion event. The 5-FU resistance of SMB011 was confirmed on agar plate containing 5-FU of 50 µg/ml, and its parent strain SMB009 was used as the negative control.

2.6. *GusA* activity assay

GusA activity was measured by monitoring cleavage of the glucuronidase substrate 4-methylumbelliferyl- β -glucuronide (4-MUG) (Gallagher, 1992). The assay was adapted to be performed in a 96-well plate format. 10 ml of cells were harvested by centrifugation at 5000 \times g at 4 °C for 10 min and washed once with 10 ml of ice-cold TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The resulted *C. acetobutylicum* cell pellets were resuspended in 1 ml of *GusA* buffer (50 mM Sodium Phosphate pH 7.0, 1 mM EDTA). The cells were sonicated on ice for 10 min using a Sonifier JY92-IIID (Nanjing XinCHEN Biotech Company, China) with the following conditions: 3 s of sonication with a 3 s interval, 200 W. The resulted lysate was collected and centrifuged at 7000g at 4 °C for 10 min. Total protein concentrations in the supernatant were determined by the Bradford method (Bradford, 1976) with bovine serum albumin as a standard, analyzed on a Spectra MAX190 plate reader (Molecular Devices Corporation, USA) set to measure absorbance at 595 nm. For 4-MUG assay, the assay reaction consisted of 50 µl sample extract and prewarmed 450 µl Assay Buffer (*GusA* Buffer containing 2 mM 4-MUG). The reaction solution was transferred to an opaque 96-well plate (200 µl/hole, duplicate), and incubated at 37 °C. Fluorescence kinetic curves were recorded on a TECAN infinite M200 plate reader (Tecan, USA) every 1 min for 30 min, at 455 nm (emission) when excited at 365 nm, with a gain value of 50. In this work, the slope value (F455/min) of fluorescence kinetic curve was recognized as the *GusA* activity units (U) of a sample, and then standardized by protein concentration (mg) to accommodate differences in samples. Final *GusA* activity values were recorded as U/mg.

3. Results

3.1. Construction of the model vector pCm4 with *CAT* expression cassette

To construct an inducible promoter in *C. acetobutylicum*, a functional parental promoter and an indicator gene for evaluating

the promoter activity, are needed. It is known that the *CAT* expression cassette (containing the promoter Pcm and the *cat* gene encoding chloramphenicol acetyltransferase) is functional in *C. acetobutylicum* and can confer the host chloramphenicol resistance (Heap et al., 2007). We therefore selected the *CAT* expression cassette from the vector pMTL007 previously used in *C. acetobutylicum* (Heap et al., 2007) as an indicator to construct the model vector pCm4 (Fig. 1A). The promoter region (Pcm) was its shortest sequence (76 bp) starting from the –35 region so as to be easily modified via synthesis of primers. The pCm4 was transformed into the Restriction-Modification system deficient strain *C. acetobutylicum* SMB009 (Dong et al., 2010) that we constructed previously, and showed chloramphenicol resistance as expected (Fig. 1C), demonstrating the *CAT* expression cassette is functional in this new construct.

3.2. Designing of the *Pcm-tetO* hybrid promoters

Previous work has shown that the configurations of *tetO* operators may differ from different promoters and hosts (Bertram and Hillen, 2008), suggesting that the configurations of *tetO* operators need to be screened to fit for a given host strain. The determining factors for an effective configuration of *tetO* include (1) which *tetO* operators to be used: *tetO1*, *tetO2*, or both and (2) which location in the promoter: between –35 and –10 region, following –10 region, or in front of –35 region. Inspired by the reported successful aTc-IGE systems (Bertram and Hillen, 2008), we designed five different configurations (Fig. 1B). Pcm-*tetO1* and Pcm-*tetO2* containing a *tetO1* or *tetO2* between –35 and –10 region were the two basal promoters. Based on Pcm-*tetO1*, a copy of *tetO2* was introduced downstream of –10 region, generating Pcm-*tetO1/2* (*tetO2* overlaps transcription start site) and Pcm-*tetO1&2* (*tetO2* follows transcription start site). Based on Pcm-*tetO2*, another copy of *tetO2* was introduced upstream of –35 region to generate Pcm-2*tetO2*. The resulted five vectors (pPcm-*tetO1*, pPcm-*tetO2*, pPcm-2*tetO2*, pPcm-*tetO1/2*, and pPcm-*tetO1&2*) were also tested for chloramphenicol resistance. Only pPcm-*tetO1* showed chloramphenicol resistance, suggesting that the configuration of (–35 region)–(*tetO1*)–(–10 region) did not destroy the function of Pcm promoter (Fig. 1C). Therefore, the pPcm-*tetO1* was selected for further work.

3.3. *Pcm-tetO1* promoter could be repressed by *TetR*

Using the pPcm-*tetO1* as a starting vector, an expression cassette of *TetR* repressor was introduced downstream of Pcm-*tetO1-cat*. The commonly used and native constitutive promoter of thiolase gene (*Pthl*) (Stim-Herndon et al., 1995) was selected to express *tetR* in *C. acetobutylicum*. To facilitate the ligation of the promoter *Pthl* with *tetR* gene via PCR method, we used a minimal *Pthl* promoter (*miniPthl*) in which the 84 bp fragment between its transcription start site and ribosome binding site (RBS) was deleted (Fig. 2A). The resulted vector pPcm-*tetO1-tetR* was then electrotransformed into *C. acetobutylicum* SMB009 to detect chloramphenicol resistance. Using its parental vector pPcm-*tetO1* as control, pPcm-*tetO1-tetR* showed chloramphenicol sensitivity (Fig. 2B). The results indicated that introducing a functional *TetR* led to the repression of *cat* gene expression controlled by Pcm-*tetO1* promoter.

3.4. Repression of *TetR* against *Pcm-tetO1* promoter can be released by aTc

To test if addition of aTc can release the repression of *TetR* against Pcm-*tetO1* promoter, strain SMB009(pPcm-*tetO1-tetR*) was incubated in RCM medium with/without aTc (Fig. 3A) to detect the chloramphenicol resistance. The results showed that

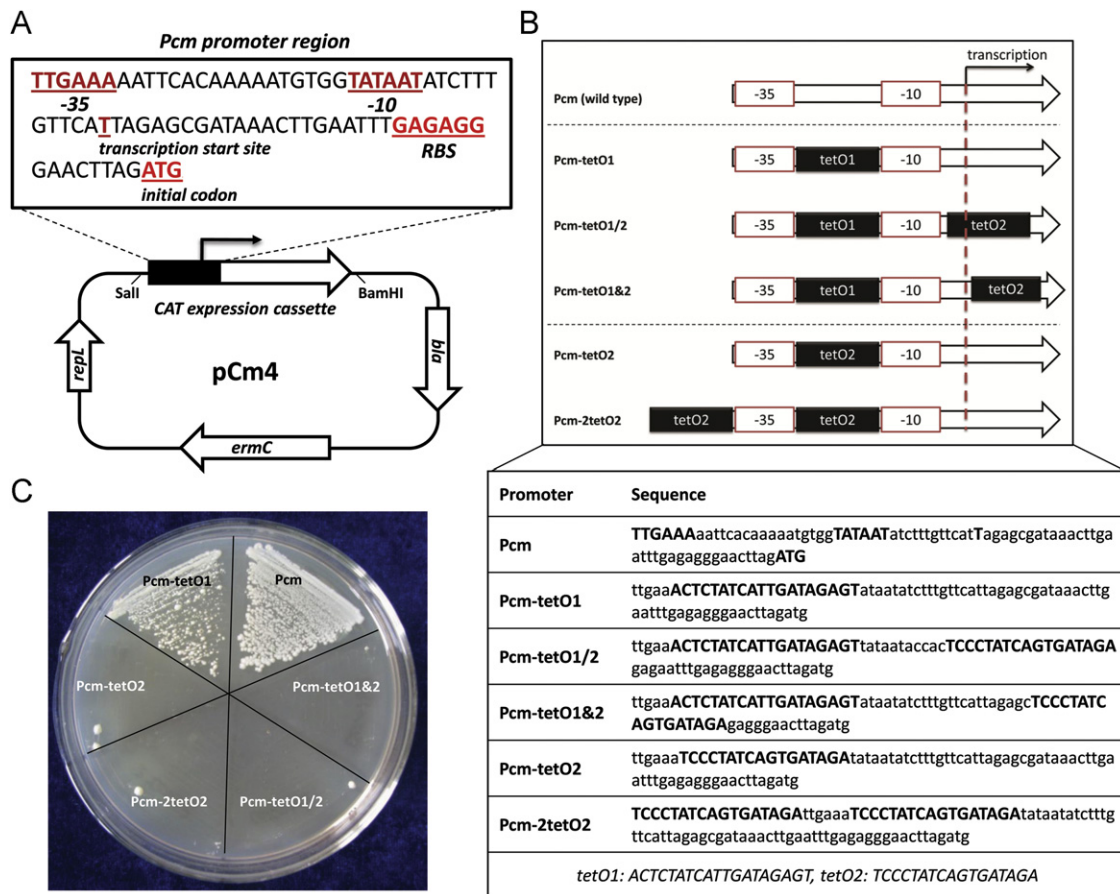


Fig. 1. Construction of hybridized promoters Pcm-tetO in *C. acetobutylicum*. (A) The model vector pCm4 with chloramphenicol acetyltransferase (CAT) expression cassette was constructed for inducible promoter screening. CAT expression cassette was directly cloned from pMTL007 vector and ligated into the Sall and BamHI cloning sites of pIMP1 to generate pCm4. The Pcm promoter region was annotated according to the sequence of GenBank No. M74769.1. (B) The schematic show of hybridized promoters Pcm-tetO and their sequences. The bold letters stand for the functional regions. (C) Functional analysis of the hybridized promoters in *C. acetobutylicum* SMB009 on RCM agar plate containing chloramphenicol of 30 µg/ml.

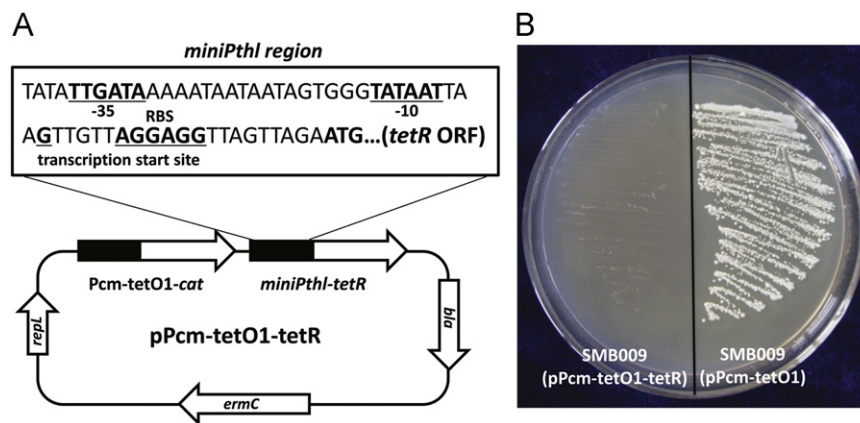


Fig. 2. Inhibition of TetR against the function of Pcm-tetO1 promoter. (A) Expression of *tetR* gene using miniPthl promoter in pPcm-tetO1 vector. The miniPthl region was annotated according to Stim-Herndon et al. (1995). (B) Analysis of the inhibition of TetR against the function of Pcm-tetO1 promoter on RCM agar plate containing chloramphenicol of 30 µg/ml.

aTc-induced culture grew on plate containing both aTc and Cm (Fig. 3B), but not on Cm only, implying that the Cm resistance need to be continuously induced by aTc during growth. The uninduced cell could not grow at either conditions (Fig. 3C and D), indicating that Cm resistance needs to be pre-induced by aTc before spreading. Through the Cm resistance experiments, we conclude that the reconstructed promoter Pcm-tetO1, repressor

TetR, and inducer aTc, constitute a primary IGE system in *C. acetobutylicum*.

3.5. aTc-inducible expression of *gusA* in *C. acetobutylicum*

In order to quantify the inducibility of Pcm-tetO1-tetR, we used the *gusA* gene (Gallagher, 1992) encoding β-glucuronidase

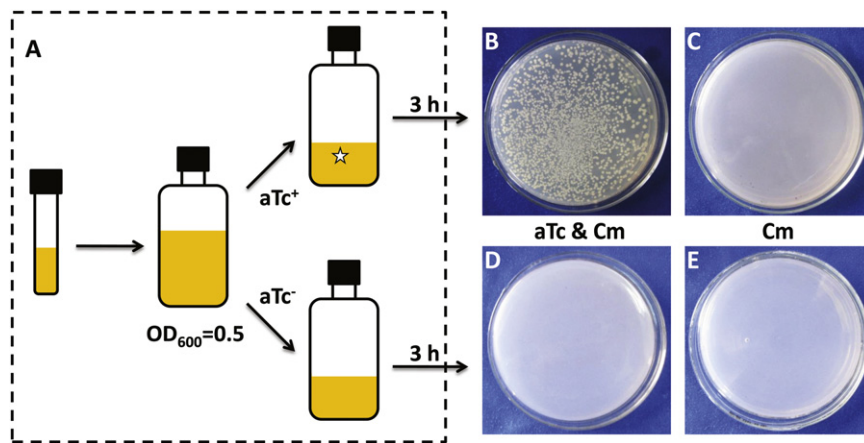


Fig. 3. Release of the inhibition of TetR against Pcm-tetO1 promoter by aTc. (A) Incubation of strain SMB009(pPcm-tetO1-tetR) with/without aTc. The strain SMB009(pPcm-tetO1-tetR) was firstly cultured to an OD₆₀₀ of 0.5; then the culture was divided into two aliquots, and aTc was added to reach 100 ng/ml for one aliquot; after 3 h of incubation at 37 °C, proper amounts of cells were spread onto RCM agar plates containing chloramphenicol of 30 µg/ml with/without 100 ng/ml aTc, for detection of chloramphenicol resistance. (B–E) The chloramphenicol resistance test on RCM agar plates containing chloramphenicol with/without aTc. Cm: chloramphenicol of 30 µg/ml. aTc: anhydrotetracycline of 100 ng/ml.

(GusA) from *E. coli* JM109 as a reporter gene. The *cat* gene in pPcm-tetO1 was replaced by *gusA* gene, generating pGusA2 (Fig. 4A). The vector pGusA2 was then electrotransformed into *C. acetobutylicum* SMB009. The resulted strain SMB009(pGusA2) was cultivated and induced with aTc as described in the legend of Fig. 3A, followed by measuring the activity of GusA. Fig. 4B shows that GusA activities in strain SMB009(pGusA2) were 7.6 ± 3.5 U/mg under non-inducing conditions, and 315.3 ± 37.5 U/mg under inducing conditions. Thus, addition of 100 ng/ml aTc could lead to a 41-fold higher GusA expression.

3.6. Improvement of Pcm-tetO1 expression stringency

To improve the expression stringency of *gusA* under the control of Pcm-tetO1 promoter in pGusA2, we further modified the Pcm-tetO1 promoter by introduction of a copy of tetO1 or tetO2 in front of –35 region (Fig. 5A) to strengthen the capability of binding TetR. The resulted vectors pGusA2-2tetO1 and pGusA2-tetO2/1 were electrotransformed into strain SMB009 for GusA activity analysis (Fig. 5B). The GusA activity of strain SMB009(pGusA2-2tetO1) and SMB009(pGusA2-tetO2/1) under non-inducing conditions were 2.4 ± 0.1 U/mg and 0.7 ± 0.5 U/mg, respectively, suggesting an improved stringency. Although the basal expression level of strain SMB009(pGusA2-tetO2/1) is very low, the GusA activity (109.3 ± 60.1 U/mg) is also much lower than that of strain SMB009(pGusA2-2tetO1) (286.6 ± 68.3 U/mg) upon aTc induction, suggesting the promoter activity of pGusA2-tetO2/1 was impaired. The GusA activity was also histochemically tested using X-Gluc (5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid) as substrate (Jefferson et al., 1986), which is qualitatively consistent with the quantitative analysis on GusA activities (Fig. 5B). To maintain promoter activity, pGusA2-2tetO1 was selected for further study.

In order to test if the expression of GusA is aTc concentration-dependent, SMB009(pGusA2-2tetO1) was cultivated in the presence of different concentrations of aTc. Quantification of GusA activities upon induction for 3 h demonstrated that the expression strength is significantly dependent on the concentrations of aTc (Fig. 5C). The maximal induced GusA activity was 313-fold that of the non-induced condition when 200 ng/ml aTc was added. However, as the concentrations of aTc increases, the strain showed significant growth inhibition, especially when aTc concentration exceeded 200 ng/ml (Fig. 5D). Considering the induction strength of aTc and the effect on growth, 100 ng/ml aTc was selected as a general working concentration.

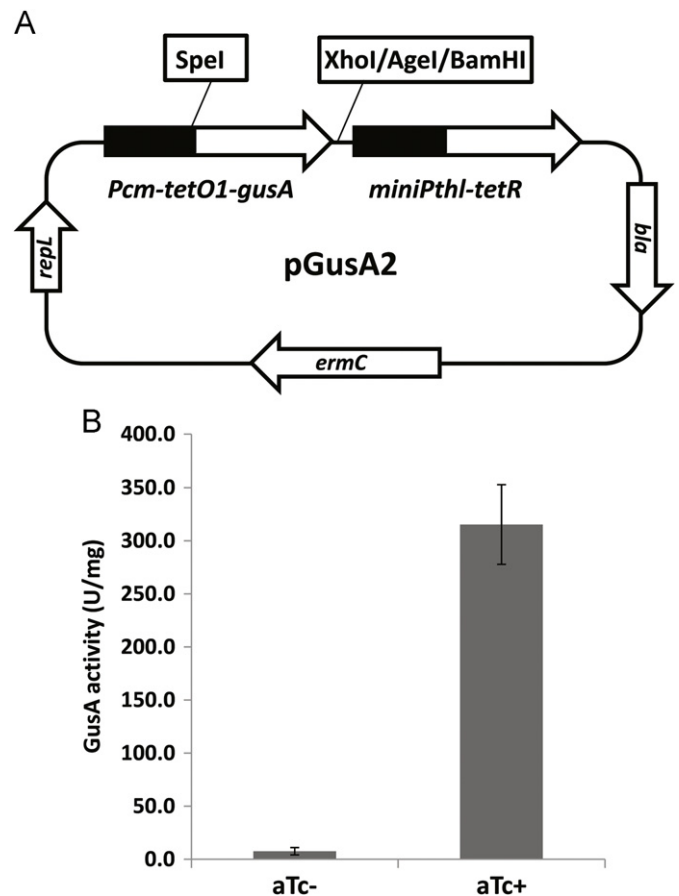


Fig. 4. Inducible expression of β -glucuronidase gene (*gusA*) of *E. coli* in *C. acetobutylicum* SMB009. (A) The vector pGusA2 for inducible expression of *gusA*. (B) Analysis of GusA activity of SMB009(pGusA2) after induction by 100 ng/ml aTc for 3 h.

3.7. Applications of pGusA2-2tetO1 IGE system

To examine the applicability of the newly developed pGusA2-2tetO1 IGE system, two genes that are usually used in counter-selection genetic manipulation systems were selected to test for inducible expression in *C. acetobutylicum*. One is the *upp* gene encoding uracil phosphoribosyltransferase (UPP) whose function

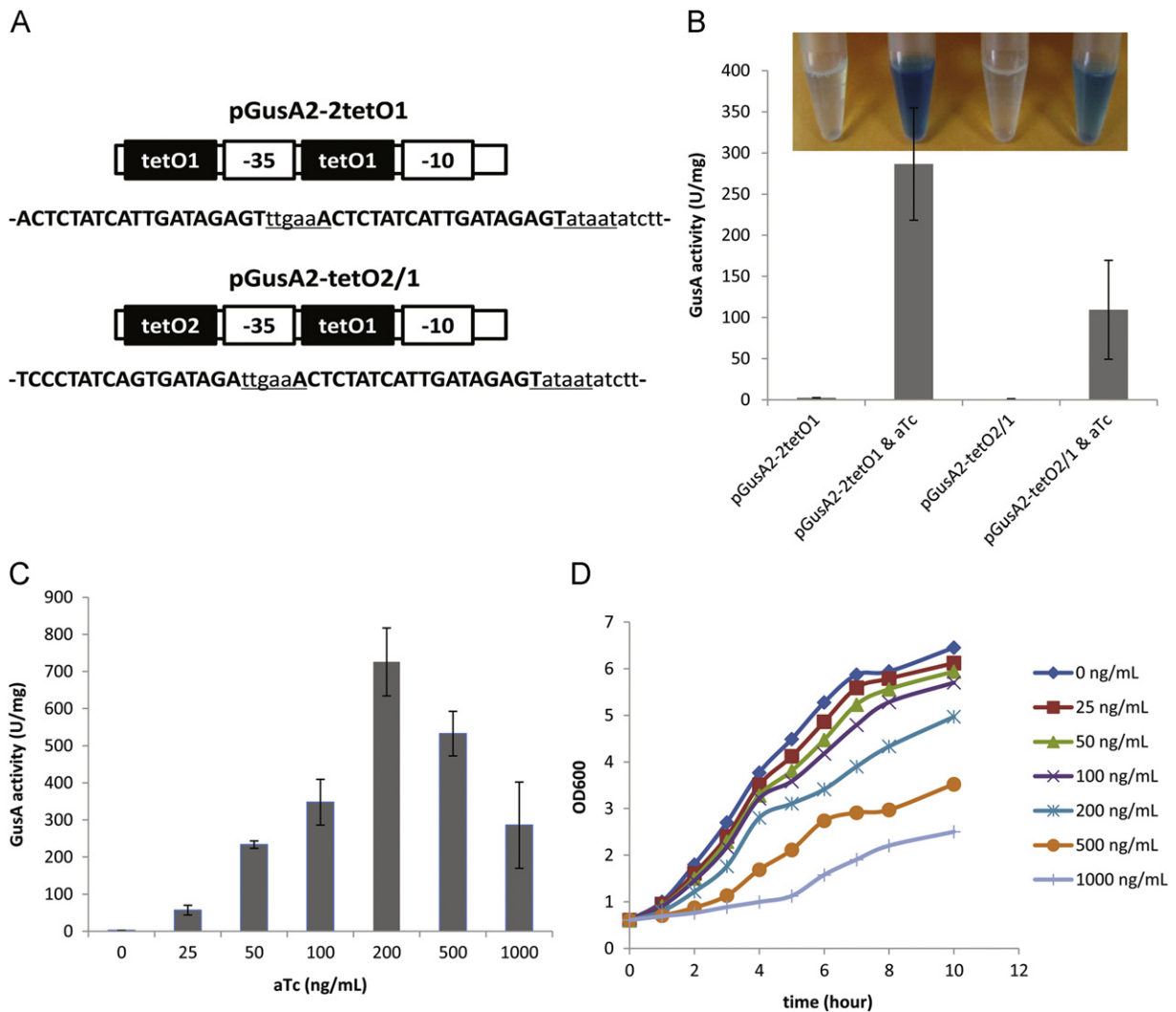


Fig. 5. Analysis of the inducibility for pGusA2-2tetO1 and pGusA2-tetO2/1. (A) The structure of inducible promoter in pGusA2-2tetO1 and pGusA2-tetO2/1. The sequence underlined is -35 region or -10 region of a promoter. The capitalized sequence is tetO1 or tetO2 operator. (B) Analysis of the GusA activity of pGusA2-2tetO1 and pGusA2-tetO2/1 in presence/absence of 100 ng/ml aTc using *C. acetobutylicum* SMB009 as the host. The samples were obtained after 3 h induction. The blue colors of solutions in the tubes are results of histochemical test of GusA activity using X-Gluc as substrate. The tubes correspond to the samples indicated in the downstream figure vertically. (C) aTc concentration-responsive induction of GusA. Strain SMB009(pGusA2-2tetO1) was grown into log phase ($OD_{600}=0.5$) and increasing amounts of aTc were added. Three hours later, GusA activities were determined. (D) Growth curves in the presence of aTc. Strain SMB009(pGusA2-2tetO1) was grown with increasing concentrations of aTc and OD_{600} values were recorded every 1 h. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

is to convert 5-fluorouracile (5-FU) to a toxic product. Cells having UPP activity cannot grow on a 5-FU medium (Soucaille et al., 2008). The other is *mazF* encoding a toxin protein MazF that cleaves cellular mRNAs specifically at ACA site to block protein synthesis leading to cell death (Zhang et al., 2003). The *upp* and *mazF* genes were amplified from *C. acetobutylicum* SMB009 and *E. coli* JM109, respectively, and were cloned into the pGusA-2tetO1 by replacing the *gusA* gene, generating pUPP-2tetO1 and pMazF-2tetO1 vectors. pUPP-2tetO1 was electrotransformed into 5-FU resistant mutant *C. acetobutylicum* SMB011 (see Section 2.5 for detail) for *upp* expression, and pMazF-2tetO1 was electrotransformed into *C. acetobutylicum* SMB009 for *mazF* expression. As shown in Fig. 6A, strain SMB011(pUPP-2tetO1) showed regular growth in RCM agar medium containing 5-FU, but no growth in the presence of aTc, indicating that the *upp* gene was inducibly expressed. Using strain SMB009(pGusA2-2tetO1) as control, strain SMB009(pMazF-2tetO1) grew in the absence of aTc, but not in the presence of aTc, conceiving the toxicity of the inducibly produced MazF (Fig. 6B).

4. Discussion

A stringent and effective IGE system should be a useful tool for study of gene function, and also for genetically manipulation of important bacteria. However, the IGE systems for the genus *Clostridium* are not very well developed in spite of the medical and industrial importance of this genus. To our knowledge, except for *C. acetobutylicum*, there were two reports on developing clostridial IGE systems. One is the IPTG-inducible 'fac' promoter used in *Clostridium sporogenes*, *Clostridium botulinum*, and *Clostridium difficile* (Heap et al., 2007), and the other is based on a lactose-inducible promoter P_{bgaL} for controlled gene expression in pathogenic *Clostridium perfringens* (Hartman et al., 2011). In an attempt to develop an effective IGE system for solvent-producing *C. acetobutylicum*, we made use of the *E. coli* Tn10 encoded tet (tetracycline) regulatory system, and the P_{cm} promoter of chloramphenicol acetyltransferase gene which is active in *C. acetobutylicum*, generating one plasmid-based IGE system pGusA2-2tetO1. Three characteristics of the tet regulatory system may

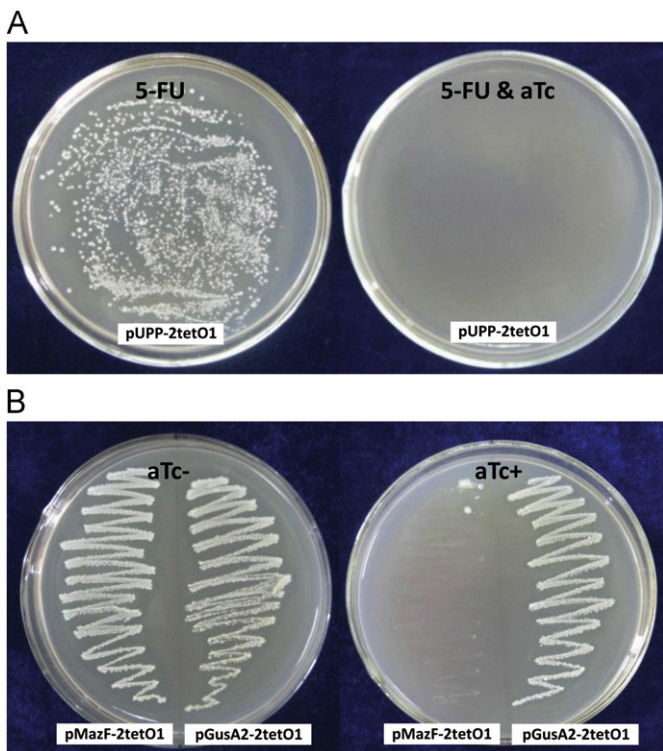


Fig. 6. Inducible expression of *upp* and *mazF* gene in *C. acetobutylicum*. (A) The *upp*-disrupted strain *C. acetobutylicum* SMB011 was used as the host for inducible expression of its native *upp* gene by the vector pUpp-2tetO1. The two RCM agar plates contain 5-FU (50 $\mu\text{g/ml}$), or 5-FU (50 $\mu\text{g/ml}$) with aTc (100 ng/ml) as indicated in the figure. (B) The strain *C. acetobutylicum* SMB009 was used for the inducible expression of *mazF* gene from *E. coli* JM109 by the vector pMazF-2tetO1. One of two RCM agar plates contains aTc of 100 ng/ml (aTc+), the other not (aTc-). All of the agar plates in (A) and (B) contain erythromycin of 50 $\mu\text{g/ml}$ for vector maintenance.

lead to the success of this work. Firstly, a high binding constant ($K_a \sim 10^{11}/\text{M}$) of TetR to tetO implies a high occupancy of tetO and, thus, contributes to efficient repression (Rodríguez-García et al., 2005). Secondly, binding of Tc to TetR reduces the affinity of TetR for tetO by nine orders of magnitude, which guarantees that the silent and the active states of tetO are efficiently discriminated (Rodríguez-García et al., 2005). Finally, Tc/aTc inducer can cross biological membranes by diffusion, enabling these inducers to penetrate most bacterial and eukaryotic cells (Ehrt et al., 2005). Therefore, this work may provide a valuable reference to develop a tet regulatory system based IGE system in other anaerobic and low GC content bacteria, especially for *Clostridium* species.

In the construction process of aTc-responsive IGE system in *C. acetobutylicum*, a functional promoter Pcm-tetO1 was screened out from five different configurations of hybridizing tet operators (tetO1 and tetO2) and Pcm promoter. The interesting thing is that, the replacement of tetO1 in Pcm-tetO1 with tetO2 (Pcm-tetO2) led to the promoter inactivation. This may explain why Pcm-2tetO2, a Pcm-tetO2 derivative with an additional tetO2 copy in front of -35 region of Pcm-tetO2, also inactivated the promoter activity. In addition, it may also explain why the other two tetO1 derivatives, Pcm-tetO1/2 and Pcm-tetO1&2, which both had a tetO2 copy following -10 region, lost the promoter function. It seems that the tetO2 in Pcm might be incompatible with RNA polymerase in *C. acetobutylicum*, or be bound by an unknown protein from the host, leading to transcription inhibition. In the functional Pcm-tetO1, we also introduced a tetO2 or tetO1 in front of -35 region, to generate a Pcm-tetO2/1 or Pcm-2tetO1. Although the promoter Pcm-tetO2/1 did not lose its promoter

activity, the efficiency for expression showed two-fold decrease compared with Pcm-2tetO1, under inducing conditions. From the above results, it is suggested that tetO2 has a negative effect on Pcm promoter activity. However, for other reported tetO2-containing promoters, such as P_{myc1tetO} in *Mycobacterium tuberculosis* (Ehrt et al., 2005) and $P_{\text{LtetO-1}}$ in *E. coli* (Lutz and Bujard, 1997), the tetO2 did not show the phenomenon as described above, indicating this effect may be promoter or host specific.

The Pcm-tetO2/1 promoter could control a more stringent expression than Pcm-2tetO1 did in *C. acetobutylicum*. However, the relatively low inducibility of Pcm-tetO2/1 may limit its application. The Pcm-2tetO1 has shown a strong inducer concentration-dependant gene expression. And the experiments of turn-on/off the expression of *mazF* gene and *upp* gene using pGusA2-2tetO1 vector, showed that the stringency of Pcm-2tetO1 could support the introduction of toxic genes into *C. acetobutylicum*. Hence, pGusA2-2tetO1 was selected as the aTc-IGE system for *C. acetobutylicum* in our further research. In addition, *mazF* gene, which has been applied in some microbes for genetic manipulations (Yang et al., 2009; Zhang et al., 2006), was confirmed for the first time to be a feasible counter-selectable marker in *C. acetobutylicum* in this work, thus may contribute to the development of genetic manipulation tools in *C. acetobutylicum*. The inducible expression of *mazF* could be used for convenient plasmid curing in Clostron system (Heap et al., 2007) for gene inactivation, or for fast screening of double-crossover mutants in homologous recombination based gene knockout method (Green et al., 1996; Harris et al., 2002; Heap and Minton, 2009; Soucaille et al., 2008). Moreover, genes most likely require to be expressed to different levels to balance its function and effect on cellular physiology. The inducer dose-responsive characteristic of pGusA2-2tetO1 may provide a useful tool for fine-tuning gene expression (Kashiwagi et al., 2009) in *C. acetobutylicum*.

5. Conclusions

An efficient and stringent IGE system designated as pGusA2-2tetO1, was successfully developed for *C. acetobutylicum*. This system, which allows turning genes on/off and regulating gene expression strength, will be an important genetic manipulation tool for metabolic engineering of the solvent-producing *C. acetobutylicum*.

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