COMMUNICATION TO THE EDITOR

BIOTECHNOLOGY and BIOENGINEERING

Gene Transcription Repression in *Clostridium beijerinckii* Using CRISPR-dCas9

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ABSTRACT: CRISPR-Cas9 has been explored as a powerful tool for genome engineering for many organisms. Meanwhile, dCas9 which lacks endonuclease activity but can still bind to target loci has been engineered for efficient gene transcription repression. *Clostridium beijerinckii*, an industrially significant species capable of biosolvent production, is generally difficult to metabolically engineer. Recently, we reported our work in developing customized CRISPR-Cas9 system for genome engineering in *C. beijerinckii*. However, in many cases, gene expression repression (rather than actual DNA mutation) is more desirable for various biotechnological applications. Here, we further demonstrated gene transcription repression in *C. beijerinckii* using CRISPR-dCas9. A small RNA promoter was employed to drive the expression of the single chimeric guide RNA targeting on the promoter region of amylase gene, while a constitutive thiolase promoter was used to drive *Streptococcus pyogenes* dCas9 expression. The growth assay on starch agar plates showed qualitatively significant repression of amylase activity in *C. beijerinckii* transformant with CRISPR-dCas9 compared to the control strain. Further amylase activity quantification demonstrated consistent repression (65–97% through the fermentation process) on the activity in the transformant with CRISPR-dCas9 versus in the control. Our results provided essential references for engineering CRISPR-dCas9 as an effective tool for tunable gene transcription repression in diverse microorganisms.

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KEYWORDS: CRISPR-dCas9; CRISPRi; genome engineering; synthetic biology; amylase activity; gene transcription repression

Introduction

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated (Cas) system is an immune system in bacteria and archaea that can efficiently cleave foreign DNA entering the cells (Marraffini and Sontheimer, 2010). Recently, CRISPR-Cas9 has been extensively explored as a revolutionary genome engineering tool for both eukaryotic and prokaryotic organisms. Meanwhile, CRISPR along with the catalytically dead Cas9 (dCas9) which lacks the endonuclease activity but can still bind to the target loci has been engineered for efficient repressive control of gene expression in many hosts. Bikard et al. (2013) reported to achieve programmable transcription repression in *Escherichia coli* using CRISPR-dCas9 by preventing the binding of the RNA polymerase to promoter sequences or as a transcription terminator by blocking the running of the RNA polymerase. Qi et al. (2013) attained sequence-specific gene transcription repression in both *E. coli* and mammalian cells, and proved such CRISPR interference (CRISPRi) system as a simple approach for selectively perturbing gene expression on a genome-wide scale. Smith et al. (2016) systematically analyzed various gRNA designs in order to achieve efficient transcriptional repression in *Saccharomyces cerevisiae* and

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suggested rules for effective library design and genome-wide programmable gene repression purposes. However, generally speaking, until now such attempts for gene transcription repression have rarely been demonstrated in unconventional microorganisms which lack developed genome engineering tools.

Clostridium beijerinckii is considered to have great industrial significance because of its various advantages for biobutanol production from renewable carbonsources. It can naturally produce high butanol concentrations (Chen and Blaschek, 1999); it has the ability to utilize a broader range of substrates, including both 5-carbon and 6-carbon monosaccharides, and disaccharides (Ezeji et al., 2007); it does not contain a megaplasmid (while many other *Clostridium* species do [Cornillot et al., 1997]), rather all genes are located on chromosome. As a result, *C. beijerinckii* is less subject to degeneration during the industrial fermentations (Cornillot et al., 1997). However, as a gram-positive bacterium, *C. beijerinckii* along with other *Clostridium* species is notorious for being difficult to be metabolically engineered due to the lack of metabolic engineering tools. Recently, we reported our work in developing customized CRISPR-Cas9 system for genome engineering in *C. beijerinckii* (Wang et al., 2015, 2016), including gene deletion, gene integration, and single nucleotide modification. However, in many cases, the gene expression repression or knockdown (rather than the actual DNA mutation) is more desirable for various biotechnological applications and synthetic biological manipulations. For example, sometimes the elimination of certain pathways is not always feasible

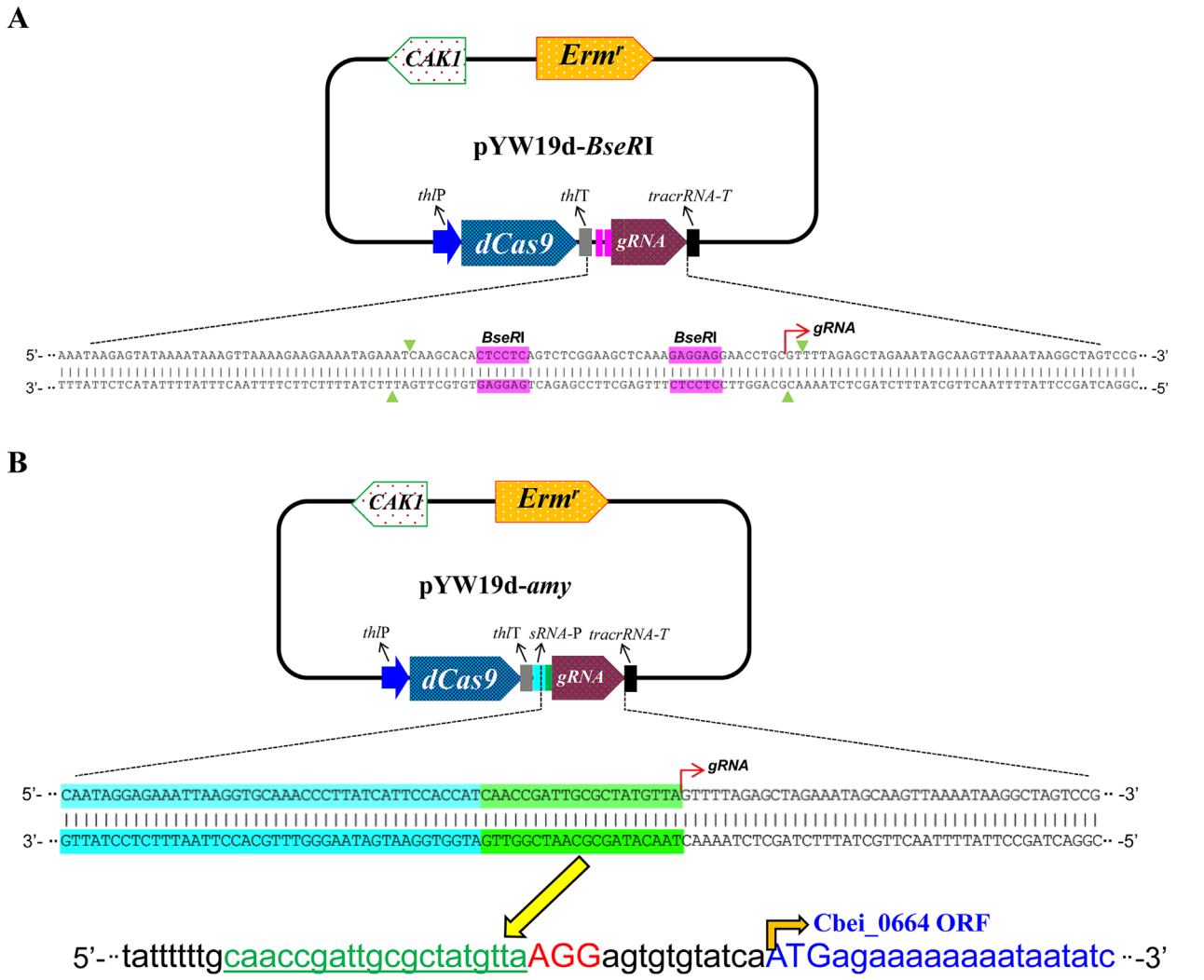

Figure 1. The CRISPR-dCas9 plasmids designed for gene expression repression. **A**: The scheme of pYW19d-*BseR*I, a general vector (and meanwhile as a control vector for transformation) used to construct other vectors (by inserting sCbei_5830 promoter along with 20-nt guiding sequence upstream of gRNA) to target on specific loci using CRISPR-dCas9. Two *BseR*I sites have been included upstream of the gRNA sequence for easy re-targeting purposes. **B**: Scheme of pYW19d-*amy*, with CRISPR-dCas9 targeting on the amylase gene (amy; Cbei_0664) promoter region with 20-nt guiding sequence (5′-CAACCGATTGCGCTATGTTA-3′). The exact target locus on the chromosome was shown underneath the scheme of the pYW19d-amy vector. *thlP*: the promoter of thiolase gene (Cbei_0411); dCas9: *Streptococcus pyogenes* dCas9 ORF amplified from the plasmid pMJ841 (purchased from Addgene) (Jinek et al., 2012); *thlT*: terminator of thiolase gene; sRNA-P: promoter of the small RNA gene (sCbei_5830) (Chen et al., 2011); gRNA: the chimeric single guide RNA (Mali et al., 2013); tracrRNA-T: transcription terminator derived from *S. pyogenes* (Qi et al., 2013); CAK1: *C. beijerinckii* Gram-positive replicon; Ermr: the erythromycin resistant marker.

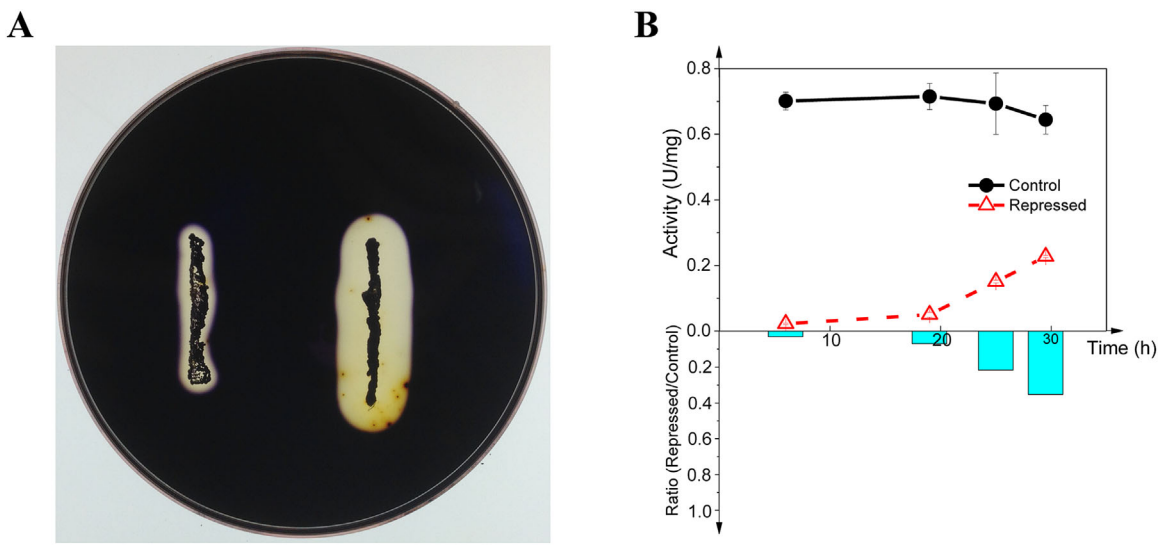

Figure 2. The CRISPR-dCas9 mediated gene transcription regulation led to efficient repression on amylase gene in *C. beijerinckii*. **A**: The amylolytic activity assay with starch agar plate (strained with iodine vapor after the culture was incubated for 48h). Left: the dCas9 repressed culture (Repressed): containing pYW19d-*amy* (dCas9 vector targeting on the promoter region of Cbei_0664, amylase gene). Right: the control culture (Control): containing pYW19d-*Bse*RI (control vector). **B**: The amylolytic activity in both Repressed and Control cultures was quantified as previously described (Annous and Blaschek, 1990). The top profiles represent the amylolytic activity (U/mg) in the two cultures through the fermentation process, while the bottom bar plots represent the ratio of amylolytic activity in Repressed culture versus in Control culture at each fermentation time point.

or easily achieved, as they could be essential for redox balances or the deletion/mutation might be lethal to the cells (Cooksley et al., 2012). Therefore, here, we further attempted to demonstrate the gene transcription repression in *C. beijerinckii* using the CRISPR-dCas9 system.

A CRISPR-dCas9 vector pYW19d-*amy* was designed to target on the promoter region of the amylase gene (Cbei_0664) (Fig. 1B). Both transformations with pYW19d-*amy* and the control vector (pYW19d-*Bse*RI) achieved similar transformation efficiencies (4.17×10^3 and 3.20×10^3 cfu/μg-DNA, respectively). Amylolytic activity plate assay was conducted with the transformants. Starch plates were prepared containing 30 g/L of tryptone, 15 g/L of soluble starch, 5 g/L of glucose, 10 g/L of yeast extract, and 1 g/L of L-cysteine, supplemented with 25 μg/mL of Erm. The glycerol frozen stocks of pYW19d-*amy* and pYW19d-*Bse*RI transformants were dipped with sterilized pipette tips and stripped in parallel onto the surface of the starch plate. After incubating for 48 h at 35°C under anaerobic conditions, the plate was stained with iodine vapor, which was generated by heating iodine crystals in a water bath at 80°C (Annous and Blaschek, 1991). After iodine staining, the clear zone for the dCas9 repressed culture was much narrower than that for the control strain, indicating that the amylolytic activity was significantly repressed in the strain hosting pYW19d-*amy* (Fig. 2A). In addition, amylolytic activity was quantified as previously described (Annous and Blaschek, 1990). Both amylase repressed culture and control culture were grown in P2 medium containing 20 g/L starch and 1 g/L yeast extract supplemented with 25 μg/mL Erm. Cells were collected at various time points for amylolytic enzyme activity quantification. Results illustrated that the amylolytic activity in the dCas9 repressed culture was significantly inhibited, representing only 3–35% of the activity as in the control culture (Fig. 2B).

Here, we selected amylase gene (*amy*) as the target because amylase activity is easy to detect and quantify as demonstrated in our previous study (Annous and Blaschek, 1991). Based on our RNA-Seq data (Wang et al., 2011, 2012), out of the five annotated amylase genes, Cbei_0664 which demonstrated the highest expression was selected as the target (Fig. S1). Results demonstrated that amylolytic activity of the culture that had been transformed with *amy*-targeting dCas9 vector was severely repressed. However, it is interesting that, although amylolytic activity in the repressed culture was much lower than in the control culture, the activity increased (and thus the difference from the control decreased) over the time course of the fermentation (Fig. 2B). This may be because the strength of the thiolase gene promoter (*thl*P) decreased over the time course as indicated by our RNA-Seq data (Fig. S2) (Wang et al., 2012). Therefore, specific gene repression purposes may be achieved through careful selection of promoters for dCas9 expression (along with the selection of dCas9 target sites on the chromosome). High-throughput RNA-Seq analyses would definitely provide essential reference for this purpose.

In conclusion, we developed an efficient gene transcription repression system by taking advantage of the CRISPR-dCas9 system. By targeting on the promoter region of the amylase gene in *C. beijerinckii*, the overall amylase activity over the fermentation process has been severely repressed (up to 97% compared to the control). Gene transcription repression with CRISPR-dCas9 is efficient, tunable, reversible, and technically repression of multiple genes can be achieved simultaneously. Therefore, such a tool can bring about tremendous flexibility for various bioengineering applications. The CRISPR-dCas9 (or the so-called CRISPRi) system represents another elegant tool for the metabolic engineers and

Table I. Strains and plasmids used in this study.

Strains/plasmids	Description and relevant characteristics	Source/reference
Strains		
C. beijerinckii		
NCIMB 8052	Wild-type	Lab stock
E. coli		
DH5α	Electrocompetent cell	NEB[a]
ER2925	Electrocompetent cell	NEB[a]
Plasmids		
pTJ1	Ap[r], Erm[r], *E. coli-C. beijerinckii* shuttle vector[b]	Wang et al. (2013)
pMJ841	pET-derived His_6-MBP expression vector with *S. pyogenes* Cas9 (D10A/H840A double mutant)	Jinek et al. (2012)
pYW19d-*BseR*I	General dCas9 vector, without protospacer sequence, with two *BseR*I sites for easy re-targeting purposes	This study
pYW19d-*amy*	dCas9 vector targeting on amylase gene (Cbei_0664) promoter region	This study

[a]New England Biolabs Inc., Ipswich, MA.
[b]Ap, ampicillin; Erm, erythromycin.

synthetic biologists to carry out versatile biological manipulations in diverse microorganisms.

Materials and Methods

Strains, Culture Conditions, Plasmids, and Oligonucleotides

The bacterial strains and plasmids used in this study are listed in Table I. The DNA oligonucleotide sequences are listed in Table II. *E. coli* transformants were grown aerobically at 37°C in LB medium supplemented with 100 μg/mL of ampicillin. *C. beijerinckii* 8052 wild-type strain and transformants were grown anaerobically at 35°C in tryptone-glucose-yeast extract (TGY) medium (unless otherwise indicated) containing 30 g/L of tryptone, 20 g/L of glucose, 10 g/L of yeast extract, and 1 g/L of L-cysteine, supplemented with 25 μg/mL of erythromycin (Erm) as needed (Wang et al., 2013). Clostridial transformation was conducted through electroporation as described previously (Wang et al., 2016).

Table II. DNA oligonucleotides used in this study.

Name	Sequence (5′–3′)
IDT gBlock	
gBLK01	GTCAGCTAGGAGGTGACTGATATAAATTAAGATTTAAA AAGGTTACTATGATAATTCTCATGGTAACCTTTTTT TATTAAATAAGAGTATAAAATAAAGTTAAAAGAAGA AAATAGAAATCAAGCACACTCCTCAGTCTCGGAAGC TCAAAGAGGAGGAACCTGCGTTTTAGAGCTAGAAA TAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTT GAAAAAGTGGCACCGAGTCGGTGCTTTTTTTCTAT GGAGAAATCTAGATCAGCATGATGTCTGACTAGTAC AGATACATTATTATGTATCAAAATATAAAATACTTGC TCAAAGGTTACTTAAGTATTTGTTCTGTTCAATTTT GAAAGACTAAGTCTTTCAATGTTCTTTGAAAATTGC ACATAGATTTAATGTATATAAAATACAACAAAGCCAA GAATAAATATTCTTTGTGATATGACTAATAATTAGCG GCCGCCAGTGTGATGGTTACTAGT
Primers	
P01	AAACTGAATTGATTGGGCCCGAAGAATAGCAGATGCTA TATTAAAATATTTT
P02	GCCTATTGAGTATTTCTTATCCATGTTTGACCTCCTAAA ATTTTATAGATTATTT
P03	AAAATTTTAGGAGGTCAAACATGGATAAGAAATACTCA ATAGGCTT
P04	GTAACCTTTTTAAATCTTAATTTATATCAGTCACCTCCT AGCTGAC
P05	AAAGTTAAAAGAAGAAAATAGAAATATAATCTTTAATT TGAAAAGATTTAAG
P06	TTGCTATTTCTAGCTCTAAAACTAACATAGCGCAATCGG TTGATGGTGGAATGATAAGGG

Plasmid Construction

The control vector pYW19d-*BseR*I was constructed using Gibson Assembly as follows. The strong constitutive *thl*P was selected for dCas9 expression, and was amplified using primers P01 and P02 from *C. beijerinckii* genomic DNA (gDNA). The dCas9 open reading frame (ORF) was amplified from the plasmid pMJ841 (purchased from Addgene) (Jinek et al., 2012) using primers P03 and P04. A 495 bp gBlock (gBLK01 in Table II, including *thl*T, a 45 bp random sequence containing two *BseR*I sites fused with the gRNA sequence, and a transcription terminator derived from *S. pyogenes*) was synthesized by IDT. The three fragments describe above (*thl*P, dCas9 ORF, and the gBLK01) were finally assembled and inserted into pTJ1 between the *Apa*I and *Not*I restriction enzyme sites, and the resultant vector was named as pYW19d-*BseR*I.

Vector pYW19d-*amy* was constructed based on pYW19d-*BseR*I. First, the insert PCR fragment was amplified using primers P05 and P06 (Table II) with *C. beijerinckii* gDNA as template (in order to amplify the sRNA sCbei_5830 promoter [Wang et al., 2015, 2016] along with 20-nt guiding sequence upstream of gRNA). Then pYW19d-*BseR*I was digested with *BseR*I and the obtain PCR fragment was inserted into the vector through Gibson Assembly.

Amylolytic Activity Quantification

Both amylase repressed culture and control culture were grown in P2 medium containing 20 g/L starch and 1 g/L yeast extract supplemented with 25 μg/mL Erm. Cells were collected over the fermentation process for amylolytic activity quantification. Further supplementation of 25 μg/mL Erm to the fermentation was done after the third sample was taken (19 h) (Fig. 2B). Total amylolytic activity was determined as described previously (Annous and

Blaschek, 1990), and the total protein concentration was determined using the Pierce bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's protocol. One unit of amylolytic enzyme activity is defined as the amount of enzyme producing 1 μmol of reducing sugar per min under the reaction conditions specified. Finally, enzyme activity was reported in units per milligram of total protein (U/mg) (Annous and Blaschek, 1990).

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Authors' Contributions

YW, TL, YSJ, and HPB conceived the idea and planned the experiments. YW, ZTZ, SOS, and PL performed the experiment. YW, SOS, TL, YSJ, and HPB wrote the manuscript.

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Supporting Information

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