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Extending CRISPR-Cas9 Technology from Genome Editing to Transcriptional Engineering in the Genus *Clostridium*

Mark R. Bruder,[a] Michael E. Pyne,[a]* Murray Moo-Young,[a] Duane A. Chung,[a,b,c] C. Perry Chou[a]

Department of Chemical Engineering, University of Waterloo, Waterloo, Ontario, Canada[a]; Algaeneers Inc., Hamilton, Ontario, Canada[b]; Neemo Inc., Hamilton, Ontario, Canada[c]

ABSTRACT

The discovery and exploitation of the prokaryotic adaptive immunity system based on clustered regularly interspaced short palindromic repeats (CRISPRs) and CRISPR-associated (Cas) proteins have revolutionized genetic engineering. CRISPR-Cas tools have enabled extensive genome editing as well as efficient modulation of the transcriptional program in a multitude of organisms. Progress in the development of genetic engineering tools for the genus *Clostridium* has lagged behind that of many other prokaryotes, presenting the CRISPR-Cas technology an opportunity to resolve a long-existing issue. Here, we applied the *Streptococcus pyogenes* type II CRISPR-Cas9 (SpCRISPR-Cas9) system for genome editing in *Clostridium acetobutylicum* DSM792. We further explored the utility of the SpCRISPR-Cas9 machinery for gene-specific transcriptional repression. For proof-of-concept demonstration, a plasmid-encoded fluorescent protein gene was used for transcriptional repression in *C. acetobutylicum*. Subsequently, we targeted the carbon catabolite repression (CCR) system of *C. acetobutylicum* through transcriptional repression of the *hprK* gene encoding HPr kinase/phosphorylase, leading to the coutilization of glucose and xylose, which are two abundant carbon sources from lignocellulosic feedstocks. Similar approaches based on SpCRISPR-Cas9 for genome editing and transcriptional repression were also demonstrated in *Clostridium pasteurianum* ATCC 6013. As such, this work lays a foundation for the derivation of clostridial strains for industrial purposes.

IMPORTANCE

After recognizing the industrial potential of *Clostridium* for decades, methods for the genetic manipulation of these anaerobic bacteria are still underdeveloped. This study reports the implementation of CRISPR-Cas technology for genome editing and transcriptional regulation in *Clostridium acetobutylicum*, which is arguably the most common industrial clostridial strain. The developed genetic tools enable simpler, more reliable, and more extensive derivation of *C. acetobutylicum* mutant strains for industrial purposes. Similar approaches were also demonstrated in *Clostridium pasteurianum*, another clostridial strain that is capable of utilizing glycerol as the carbon source for butanol fermentation, and therefore can be arguably applied in other clostridial strains.

Efficient genome and transcriptome modification strategies form the backbone for understanding molecular biology processes to rational design of biological organisms. Within the genus *Clostridium*, the members of which are industrially important production hosts for solvents and biofuels (1), genetic engineering technologies remain immature, owing in part to low transformation efficiencies, inadequate endogenous homologous recombination, and poorly understood physiology and metabolism (2, 3). Although methods for plasmid transfer and gene expression have been established in *Clostridium acetobutylicum*, arguably the most common and well-characterized *Clostridium* species, for more than two decades (4), sophisticated engineering tools for genome editing and transcriptional regulation are lacking (3).

Traditional strategies for genome engineering in *Clostridium* were developed based on the use of nonreplicating and replicating plasmids for chromosomal integration via natural homologous recombination systems of the host organism (3). However, unstable single-crossover homologous recombination events tended to interfere, leading to unwanted insertion of the entire integration vector (5, 6). This drawback necessitated the development of more robust strategies (5, 6). Recently, a gene inactivation approach, in which the mobile group II retrohoming intron of *Lactococcus lactis* (Ll.ltrB) is programmed for site-specific chromosomal insertion, was developed for *Clostridium*. Precise insertion of the intron into the host genomic DNA is facilitated through base pairing between the intron RNA and host DNA and requires essential functions associated with a cognate intron-encoded protein, LtrA (7–10). Although this technology represents a significant advance for targeted gene inactivation in *Clostridium*, it suffers from several drawbacks, including variable frequency of intron insertion, inability to target certain genomic loci, and ectopic intron insertion

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Address correspondence to Duane A. Chung, duane.chung@uwaterloo.ca, or C. Perry Chou, cpchou@uwaterloo.ca.

* Present address: Michael E. Pyne, Department of Biology and Centre for Structural and Functional Genomics, Concordia University, Montréal, Québec, Canada.

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events (7, 8, 11). Further, while a heterologous cargo DNA can be inserted into the Ll.ltrB intron for genomic integration, the targeting efficiency can be severely impacted, limiting the applicability of this technology (12–14). Extensive efforts have been made to improve the frequency and isolation of double-crossover homologous recombination (DCHR) events in *Clostridium* based on the use of genome- and plasmid-borne counterselectable markers (3, 15, 16). Numerous counterselection markers have been derived, including *mazF* and *codA* from *Bacillus subtilis* and *Escherichia coli*, respectively, in addition to several native clostridial genes, such as *pyrE*, *pyrF*, and *galK* (15–18). Although these systems can result in markerless genome editing, they often require specific host genotypes or tedious steps to recycle the counterselection marker to generate markerless mutant strains. Recently, a single-stranded DNA (ssDNA) annealing protein (SSAP) RecT homolog from *Clostridium perfringens* was employed for recombineering in *C. acetobutylicum* (19), suggesting the potential exploitation of bacteriophage-derived SSAPs for homologous recombination.

Industrially useful phenotypes may not be achieved solely through the deletion of endogenous genes or the introduction of heterologous genes due to unpredicted nonlinear and/or interrelated effects (20, 21). Hence, implementing genome engineering with more capacities, such as genome editing and transcriptional regulation, can be useful in developing complex phenotypes (22–24). Similar to the state of genome editing, transcriptional modulation techniques in *Clostridium* are generally lacking despite significant advancement of sophisticated *trans*-acting elements, such as transcription activator-like effectors (TALEs) and zinc finger (ZF) DNA-binding domains fused to various activator and repressor domains in eukaryotes and, more recently, in prokaryotes (3, 22, 25–27). Antisense RNA (asRNA) technology (3, 23), in which an RNA molecule complementary to a target mRNA is transcribed for *in vivo* hybridization with the target mRNA (28), remains the most prevalent transcriptional silencing mechanism employed in *Clostridium*, particularly for metabolic engineering applications (3). However, the gene knockdown efficacy can be difficult to predict, and therefore the technology often requires tedious empirical screening to optimize the target region, sequence length, and secondary structure of the asRNA molecule (28).

Recent adaptation of the prokaryotic clustered regularly interspaced short palindromic repeats (CRISPRs) and CRISPR-associated (Cas) proteins has revolutionized genetic engineering (29). CRISPR loci have been identified in approximately 45% of bacteria and in more than 90% of archaea and form the basis of adaptive immunity in prokaryotes (29–31). CRISPR-based immunity generally involves three discrete phases, namely, adaptation, expression, and interference (32). Briefly, adaptation involves the acquisition and incorporation of nucleotide sequence identifiers from invading elements, particularly bacteriophages, into the host chromosome using host Cas proteins. The acquisition, which is conserved among all CRISPR-Cas types, results in the formation of extensive CRISPR arrays as immune "memory" (32). While the expression and interference phases can vary in terms of the specific host Cas proteins that are recruited, the result of an endonuclease-mediated attack onto the invading nucleic acid is identical (33). Among the five CRISPR types (34), the type II system of the Gram-positive human pathogen *Streptococcus pyogenes* represents the most well-characterized one. This *Streptococcus pyogenes* type II CRISPR-Cas9 (SpCRISPR-Cas9) machinery has been successfully implemented for genetic engineering in a multitude of prokaryotic and eukaryotic model organisms, including *E. coli* (35), *B. subtilis* (36), *Saccharomyces cerevisiae* (37), and mouse (38) and human cells (39). In the type II CRISPR system, CRISPR arrays are transcribed into a precursor CRISPR RNA (pre-crRNA), which is cleaved by RNase III to discrete spacer units as mature crRNAs (31). The mature crRNA forms a complex with *trans*-activating crRNA (tracrRNA) and Cas9, and this complex is directed to anneal with the invading DNA at the protospacer to trigger an endonucleolytic attack by Cas9 (31). Recently, through heterologous implementation of SpCRISPR-Cas9 in model organisms for genome editing, single chimeric guide RNA (sgRNA) was used to direct sequence-specific Cas9 binding and cleavage of the target site of the host genome. With simultaneous introduction of editing templates containing designed mutations, mutant cells evading such lethal cleavage can be screened (40).

As the mechanism underlying SpCRISPR-Cas9-based interference is elucidated with the scrutiny of the relevant structural motifs of Cas9 (41), Cas9 mutants can be derived, particularly including a single-stranded DNA nickase Cas9 mutant (Cas9n) (39, 41) and a nuclease-deficient Cas9 (dCas9). dCas9 enables its site-specific DNA binding for transcriptional repression of targeting genes, termed CRISPR interference (CRISPRi) (42) and, therefore, has discerned SpCRISPR-Cas9 as a genetic engineering tool for not only genome editing but also transcriptional regulation (42). As such, SpCRISPR-Cas9 technology potentially offers a simple yet effective solution for overcoming various barriers to genetic engineering of *Clostridium*. To date, SpCRISPR-Cas9 has been heterologously implemented for genome editing in a few clostridial species, including *Clostridium beijerinckii* (43) and *Clostridium cellulolyticum* (44). Prompted by these developments, we exploited SpCRISPR-Cas9 for both genome editing and transcriptional repression in *C. acetobutylicum* DSM792, a common industrial anaerobe. Similar approaches based on SpCRISPR-Cas9 were also demonstrated in *Clostridium pasteurianum* ATCC 6013 and, therefore, can be potentially implemented in other industrially important members of *Clostridium*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and oligonucleotides. Strains and plasmids employed in this study are listed in Table 1. *C. acetobutylicum* (i.e., DSM792) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ; Braunschweig, Germany), and *C. pasteurianum* ATCC 6013 was obtained from the American Type Culture Collection (ATCC; Manassas, VA). *E. coli* strain DH5α was utilized for molecular cloning, and strains ER2275 and ER1821 (New England BioLabs, Ipswich, MA) were used for methylation of shuttle vectors destined for *C. acetobutylicum* and *C. pasteurianum*, respectively. *C. acetobutylicum* and *C. pasteurianum* strains were cultivated anaerobically at 37°C in 2× YTG medium (16 g liter^{-1} tryptone, 10 g liter^{-1} yeast extract, 5 g liter^{-1} NaCl, and 5 g liter^{-1} glucose). If necessary, 10 μg ml^{-1} thiamphenicol or 50 μg ml^{-1} erythromycin was used for the selection of recombinant *C. acetobutylicum* or *C. pasteurianum*. *E. coli* strains were cultivated at 37°C in lysogeny broth (LB) medium (10 g liter^{-1} tryptone, 10 g liter^{-1} NaCl, 5 g liter^{-1} yeast extract), and recombinant *E. coli* cells were selected with 30 μg ml^{-1} kanamycin or 25 μg ml^{-1} chloramphenicol. Desalted oligonucleotides were purchased from Integrated DNA Technologies (IDT; Coralville, IA). The oligonucleotides that were utilized in this study are listed in Table 2.

DNA manipulation and transformation. Vectors destined for *C. acetobutylicum* and *C. pasteurianum* were constructed according to standard procedures (45), methylated in *E. coli* ER2275(pAN3) (16) or ER1821(pFnuDIIMKn) (46), and transformed using electrotransforma-

TABLE 1 Strains and plasmids employed in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strain		
Escherichia coli		
DH5α	F$^-$ *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG* ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 hsdR17*(r_K^- m_K^+), λ$^-$	Lab stock
ER2275	*trp-31 his-1 tonA2 rpsL104 supE44 xyl-7 mtl-2 metB1 e14*$^+$ Δ(*lac*)*U169 endA1 recA1 R (zgb-210*::Tn*10)* Tet^s Δ(*mcr-hsd-mrr*)*114*::*1510* [F¢ *proAB lacI*^{q}*Z*Δ*M15*] *zzf*::mini-Tn*10*	New England BioLabs
ER1821	F$^-$ *glnV44 e14*$^-$ (McrA$^-$) *rfbD1? relA1? endA1 spoT1? thi-1* Δ(*mcrC-mrr*)*114*::IS*10*	New England BioLabs
Clostridium acetobutylicum		
DSM792 (ATCC 824)	Wild type	DSMZ
792Δ*cac1502*	DSM 792; Δ*cac1502*	This study
792Δ*cac1502*::*afp*	DSM 792; Δ*cac1502*::*afp*	This study
Clostridium pasteurianum		
ATCC 6013	Wild type	ATCC
6013Δ*cpaAIR*::*afp*	ATCC 6013; Δ*cpaAIR*::*afp*	This study
Plasmid		
p85Cas9	Derived from pMTL85141 by inserting *cas9* with the native promoter; Tm^r/Cm^r; ColE1; pIM13	(48)
p85delCas9	Derived from p85cas9 by deleting the native *cas9* promoter; Tm^r/Cm^r; ColE1; pIM13	(48)
pAN3	A plasmid containing the gene encoding Φ3TI methyltransferase for methylation of *E. coli-C. acetobutylicum* shuttle vectors; Km^r; p15A	(16)
pCas9gRNA-AFP	Derived from pCas9gRNA-cpaAIR by replacing the original sgRNA with the one targeting the *afp* gene; *cas9*; Tm^r/Cm^r; ColE1; pIM13	This study
pCas9gRNA-cac824I	Derived from pCas9gRNA-cpaAIR by replacing the original sgRNA with the one targeting the *C. acetobutylicum cac1502* gene; *cas9*; Tm^r/Cm^r; ColE1; pIM13	This study
pCas9gRNA-cpaAIR	Derived from pMTL85141 by inserting *cas9* whose expression is regulated by the *C. pasteurianum* thiolase promoter and sgRNA whose expression is regulated by the *C. beijerinckii* sCbei5830 small RNA promoter targeting the *C. pasteurianum cpaAIR* gene; Tm^r/Cm^r; ColE1; pIM13	(48)
pCas9gRNA-delcpAFP	Derived from pCas9gRNA-cpAIR by inserting the *cpaAIR* replacement editing cassette with homologous region flanking P_{thl}::*afp* from pGlow-CK^{XN}-Pp1; *cas9*; sgRNA targeting the *cpaAIR* gene; Tm^r/Cm^r; ColE1; pIM13	This study
pCas9gRNA-delcac824I-500	Derived from pCas9gRNA-cac824I by inserting the *cac1502* deletion editing cassette with 500 bp of homologous region; *cas9*; sgRNA targeting the *cac1502* gene; Tm^r/Cm^r; ColE1; pIM13	This study
pCas9gRNA-delcac824I-1000	Derived from pCas9gRNA-cac824I by inserting the *cac1502* deletion editing cassette with 1 kbp of homologous region; *cas9*; sgRNA targeting the *cac1502* gene; Tm^r/Cm^r; ColE1; pIM13	This study
pCas9gRNA-delcac824I::AFP	Derived from pCas9gRNA-cac824I by inserting the *cac1502* replacement editing cassette with homologous region flanking P_{thl}::*afp* from pGlow-CK^{XN}-Pp1; *cas9*; sgRNA targeting the *cac1502* gene; Tm^r/Cm^r; ColE1; pIM13	This study
pCRISPRi	Derived from pCas9gRNA-AFP by replacing *cas9* with its nuclease-deficient variant *dcas9*; sgRNA targeting the *afp* gene; Tm^r/Cm^r; ColE1; pIM13	This study
pCRISPRi-AFP	Derived from pCas9gRNA-AFP by replacing *cas9* with its nuclease-deficient variant *dcas9* and inserting the P_{thl}::*afp* cassette from pGlow-CK^{XN}-Pp1; sgRNA targeting the non-template strand of the *afp* gene; Tm^r/Cm^r; ColE1; pIM13	This study
pCRISPRi-AFPT	Derived from pCRISPRi-AFP by replacing the original sgRNA with the one targeting the template strand of the *afp* gene; *dcas9*; P_{thl}::*afp*; Tm^r/Cm^r; ColE1; pIM13	This study
pCRISPRi-Cont	Derived from pCas9gRNA-cpaAIR by replacing *cas9* with its nuclease-deficient variant *dcas9* and inserting the P_{thl}::*afp* cassette from pGlow-CK^{XN}-Pp1; sgRNA targeting the *C. pasteurianum cpaAIR* gene; Tm^r/Cm^r; ColE1; pIM13	This study
pCRISPRi-GlpX	Derived from pCRISPRi by replacing the original sgRNA with the one targeting the nontemplate strand of the *C. acetobutylicum glpX* gene; *dcas9*; Tm^r/Cm^r; ColE1; pIM13	This study
pCRISPRi-HPrK	Derived from pCRISPRi by replacing the original sgRNA with the one targeting the nontemplate strand of the *C. acetobutylicum hprK* gene; *dcas9*; Tm^r/Cm^r; ColE1; pIM13	This study
pGlow-CK^{XN}-Pp1	A plasmid containing the *afp* gene whose expression is regulated by the *C. acetobutylicum* thiolase promoter (P_{thl}::*afp*); Ap^r; Ert^r; ColE1; pIM13	Evocatal GmbH
pMTL85141	An *E. coli-Clostridium* shuttle vector; Tm^r/Cm^r; ColE1; pIM13	(47)

TABLE 2 Oligonucleotides employed in this study

Oligonucleotide	Sequence (5′ to 3′)[a]
Cas9.invpcr.cac824I.f	TCTTTATTATCTTTAAATAGA ATGGTGGAATGATAAGGGTTTG
Cas9.invpcr.r	GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAG
Cas9.invpcr.Evo.f	GCATTTGAAAGATTAACAGGAT ATGGTGGAATGATAAGGGTTTG
CAC824I.SOE1.f	CGATCGGACCATAGAGGAAACTTGTTTATAAGC
CAC824I.SOE1.r	TGCCATAGTAACCACCTCTAAAC TGCCATAGTAACCACCTCTAAAC
CAC824I.SOE2.f	GTTTAGAGGTGGTTACTATGGCAATTCCGGAACAACTTTAGAGACTG
CAC824I.SOE2.r	CGATCGCCTCCATTTTAAGCCTGTCATG
CAC824I-500.f	CGATCG GAGTAATGTATGTCCAACGTTAACG
CAC824I-500.r	CGATCG GGTCTAAAATATGGCCTCTGTATTC
CAC824I.SOE1.Evo.r	GCCATGCTCTGACG TGCCATAGTAACCACCTCTAAAC
CAC824I.SOE2.Evo.f	TAGAGCTGAAGTTATATATGATTATCATTCCGGAACAACTTTAGAGACTG
Evoglow.SOE.f	CGTCAGAGCATGGCTTTATG
Evoglow.SOE.r	ATAATCATATATAACTTCAGCTCTAGGC
PvuI.f	CGATCG CCCTTCCCAACAGTTGC
PvuI.r	CGATCG GTGCGGGCCTCTTCGCTATTA
D10A.f	CTCAATAGGCTTA**GCT**ATCGGCACAAATAGCGTC
D10A.r	ATTTGTGCCGAT**AGC**CTAAGCCTATTGAGTATTTCTTATCCA
H840A.f	**GCA**ATTGTTCCACAAAGTTTCCTTAAAG
H840A.r	GTGGAACAAT**TGC**ATCGACATCATAATCACTTAAACG
Evoglow.f	GAGGCCCGCACCGATCGCGTCAGAGCATGGCTTTATG
Evoglow.r	TTGGGAAGGGCGATCGGATAATCATATATAACTTCAGCTCTAGGC
Cas9.Evo.T.f	TAAGCTGAAGAAGTTTTGCATTTATG ATGGTGGAATGATAAGGGTTTG
Cas9.invpcr.HPrK.f	AAGAGTACAATAGGTGGCTTTTAA ATGGTGGAATGATAAGGGTTTG
Cas9.invpcr.GlpX.f	GGAGCAAAAGGTGCTATAGATATAAA ATGGTGGAATGATAAGGGTTTG
Cac1502colpcr.f	GGTTCTGATAGAAAAATGTGAGGAAG
Cac1502colpcr.r	TCCATTGGCTTTAGCTAGATGTT
AFPcolpcr.f	TGCGCAGATGATATACTTTATCAAGATGCTAGA
delcpaAIR.PvuI.S	CGATCGGTCCTAAAAGCAGGGTATGAAGTCCATTAG
delcpaAIR.AFP.SOE.AS	GCCATGCTCTGACGGAATAGAATGTTGTTCGATAGGCATCC
delcpaAIR.AFP.SOE.S	TAGAGCTGAAGTTATATATGATTATCCAGATAGAAGTCCTAGACCTCAAG
delcpaAIR.PvuI.AS	CGATCGGCTTAGCTGGTAAGAAGCAAGGTCTT
cpaAIR.AS	ATAGGTGGATTCCCTTGTCAAGATTTTAGC

[a] Underlined text indicates restriction sites and bold text indicates nucleotide substitutions for *dcas9*.

tion (4, 46). After transformation, colonies were allowed to sporulate for 5 to 7 days before experiments were carried out. Commercial kits for DNA purification and agarose gel extraction were purchased from Bio Basic Inc. (Markham, ON, Canada). A NEBuilder HiFi DNA assembly cloning kit was purchased from New England BioLabs (Whitby, ON). All commercial kits and enzymes were used according to the manufacturers' recommendations.

Plasmid construction. All CRISPR expression vectors described in this section have a pMTL85141 backbone and consist of the *S. pyogenes cas9* or *dcas9* gene, whose expression was regulated by the constitutive *C. pasteurianum* thiolase promoter, and the chimeric sgRNA, whose expression was regulated by the *C. beijerinckii* sCbei5830 small RNA promoter, as described previously (43, 47, 48). Plasmid pCas9gRNA-cpaAIR (48) was used as the template in an inverse PCR with primers Cas9.invpcr.cac824I.f and Cas9.invpcr.r to generate pCas9gRNA-cac824I. Similarly, plasmid pCas9gRNA-cpaAIR was used as the template in an inverse PCR with primers Cas9.invpcr.Evo.f and Cas9.invpcr.r to generate pCas9gRNA-AFP. In these constructs, the sgRNA was designed to target the *C. acetobutylicum cac1502* gene (pCas9gRNA-cac824I) or the anaerobic fluorescent protein (*afp*) gene (49) (pCas9gRNA-AFP) with spacer sequences TCTATTTAAAGATA ATAAAGA and ATCCTGTTAATCTTTCAAATGC, respectively. For genome editing, pCas9gRNA-cac824I was modified by inserting the editing template. To do this, splicing by overlap extension PCR (SOE PCR) was used to fuse the 5′ and 3′ homologous regions, which are 1,015 bp and 961 bp, respectively, to the *cac1502* gene using primer set CAC824I.SOE1.f and CAC824I.SOE1.r and set CAC824I.SOE2.f and CAC824I.SOE2.r. The resulting PCR product was digested with PvuI and ligated to the similarly digested pCas9gRNA-cac824I, generating pCas9gRNA-delcac824I-1000. To generate pCas9gRNA-delcac824I-500, in which the ~1-kbp homologous regions were replaced with 500-bp regions, the editing template of ~1 kbp was PCR amplified from pCas9gRNA-delcac824I-1000 using primer pair CAC824I-500.f and CAC824I-500.r, digested with PvuI, and ligated to the PvuI-digested pCas9gRNA-cac824I. Finally, to construct pCas9gRNA-delcac824I::AFP, SOE PCR was used to fuse 1,015 bp and 961 bp of the homologous regions to the *cac1502* gene with a 781-bp PCR segment comprised of the *afp* gene and *C. acetobutylicum* thiolase promoter (P_{thl}) from pGlow-CK^{XN}-Pp1 using primer sets CAC824I.SOE1.f and CAC824I.SOE1.Evo.r, Evoglow.SOE.f and Evoglow.SOE.r, and CAC824I.SOE2.Evo.f and CAC824I.SOE2.r. The resulting PCR product was digested with PvuI and ligated to the similarly digested pCas9gRNA-cac824I to generate pCas9gRNA-delcac824I::AFP.

Plasmid pCas9gRNA-delcpAFP, consisting of *cas9*, the sgRNA targeting the *C. pasteurianum cpaAIR* gene, and the *afp* integration cassette, was constructed as follows: PCR products of 1,028 bp of 5′ homologous region sequences and 1,057 bp of 3′ homologous region sequences to the *cpaAIR* gene were generated using primer set delcpaAIR.PvuI.S and delcpaAIR.AFP.SOE.AS and primer set delcpaAIR.AFP.SOE.S and delcpaAIR.PvuI.AS, respectively. These PCR products were spliced in a three-way SOE PCR with the 781-bp *afp*-containing PCR product that was generated using the primer set Evoglow.SOE.f and Evoglow.SOE.r. The resulting PCR product was digested with PvuI and ligated with the similarly digested p85Cas9gRNA-delcpaAIR to generate pCas9gRNA-delcpAFP.

To generate pCRISPRi-AFP and pCRISPRi-Cont, Gibson assembly

(50) was used to assemble PCR products with overlapping regions as in the following: using either pCas9gRNA-AFP (as the template for the construction of pCRISPRi and pCRISPRi-AFP) or pCas9gRNA-cpaAIR (for the construction of pCRISPRi-Cont), an ~3.3-kbp PCR product was generated from both templates using primers PvuI.f and D10A.r. These PCR products differed only in the targeting sequence of the sgRNA. Additionally, ~2.5-kbp and ~1.8-kbp PCR products were generated using primers D10A.f and H840.r and primers H840A.f and PvuI.r, respectively, using pCas9gRNA-AFP as the template. The primers D10A.f/r and H840A.f/r were designed to incorporate the nucleotide substitutions required to mutate *cas9* to the endonuclease-deficient variant *dcas9*. An ~800-bp PCR product containing the P_{thl}::*afp* sequence was also amplified from pGlow-CK^{XN}-Pp1 using primers Evoglow.f and Evoglow.r. Finally, the four PCR products (i.e., the ~3.3 kbp containing the targeted sgRNA to the *afp* gene or the *C. pasteurianum cpaAIR* gene, and the ~2.5-kbp, ~1.8-kbp, and ~800-bp fragments) were used in Gibson assembly reactions in a 50°C thermocycler for 1 h to generate pCRISPRi-AFP and pCRISPRi-Cont. An additional control vector targeted to the *afp* gene (pCRISPRi), in which the P_{thl}::*afp*-containing segment was omitted (i.e., ~800-bp fragment), was also constructed using Gibson assembly. Finally, pCRISPRi-AFPT was derived using inverse PCR to replace the spacer region of the pCRISPRi-AFP with the sequence CATAAATGCAAAACTTCTTCAG CTTA using primers Cas9.Evo.T.f and Cas9.invpcr.r. Plasmids pCRISPRi-HPrK and pCRISPRi-GlpX were constructed using inverse PCR with pCRISPRi as the template and primer sets Cas9.invpcr.HPrK.f and Cas9.invpcr.r, with the spacer sequence TTAAAAGCCACCTA TTGTACTCTT for pCRISPRi-HPrK, and Cas9.invpcr.GlpX.f and Cas9.invpcr.r, with the spacer sequence TTTATATCTATAGCACCTTTT GCTCC for pCRISPRi-GlpX, respectively.

Cultivation. For a proof-of-concept demonstration of CRISPRi, anaerobic static flask cultivations were carried out in 250-ml flasks using 2× YTG medium with glucose (20 g $liter^{-1}$) as the carbon source. Sporulated cells of recombinant *C. acetobutylicum* (i.e., DSM792) or *C. pasteurianum* (i.e., ATCC 6013) strains harboring pGlow-CK^{XN}-Pp1, pMTL85141, pCRISPRi-AFP, pCRISPRi-Cont, or pCRISPRi-AFPT (for DSM792 only) were heat shocked in an 80°C water bath and cooled on ice. The seed cultures were incubated overnight, and flasks containing the fresh medium were inoculated with 1% volume of the seed culture containing exponentially growing cells. Thiamphenicol was added to ensure plasmid maintenance. Upon reaching a cell density of ~1 to 1.5 optical density at 600 nm (OD_{600}), the cultivated cells at 1 OD unit were harvested, pelleted by centrifugation, and resuspended in anaerobic phosphate-buffered saline (PBS). This procedure was repeated three times. The harvested cells were finally resuspended in PBS containing 10% formalin and incubated for 15 min for cell fixation. The fixed cells were serially diluted in PBS to a dilution factor of 10^{-6} and pipetted to a black-bottomed 96-well plate. The 96-well plate was wrapped tightly with a Parafilm tape, and samples were analyzed in the plate reader and flow cytometer immediately. All experiments were carried out in triplicate.

For characterization of the relief of carbon catabolite repression (CCR) using CRISPRi, anaerobic static flask cultivations were carried out in 250-ml flasks using 2× YTGX medium with glucose (20 g $liter^{-1}$) and xylose (10 g $liter^{-1}$) as the carbon sources. Sporulated recombinant *C. acetobutylicum* strains, i.e., DSM792 harboring pCRISPRi-HPrK, pCRISPRi-GlpX, or pCRISPRi, were heat shocked in an 80°C water bath and cooled on ice. The seed cultures were incubated overnight, and flasks containing the fresh medium and thiamphenicol were inoculated with 1% of the seed culture containing exponentially growing cells. Samples were taken at 12-h intervals for cell density measurement as well as metabolite analysis of cell-free medium. All cultivations were carried out in triplicate.

Analyses. Cell density in OD_{600} was measured using a spectrophotometer (DU520; Beckman Coulter, Fullerton, CA). For measurement of glucose, xylose, and metabolite concentrations, the high-performance liquid chromatography (HPLC) system (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 65°C, and the mobile phase was 5 mM H_2SO_4 (pH 2.0) running at 0.6 ml/min. HPLC data acquisition and analysis were performed using the Clarity Lite chromatographic station (DataApex, Prague, The Czech Republic).

A Synergy 4 hybrid microplate reader (BioTek, Winooski, VT) and ImageStream X Mark II imaging flow cytometer (Amnis, Seattle, WA) were employed for AFP fluorescence intensity analysis. Plate reader samples were analyzed at an excitation of 450 nm and an emission at 495 nm. Flow cytometry experiments were conducted with AFP being excited using a 488-nm solid-state laser and detected in the range of 480 to 560 nm. Ten thousand events were collected while thresholding on area versus aspect ratio to remove noise. The flow cytometry data were analyzed with Ideas software (Amnis, Seattle WA).

Tests for the statistical significance of differences in fluorescence intensity between strains were performed using the Student's *t* test with a significance level value of 5%.

RESULTS

Implementation of SpCRISPR-Cas9 for markerless gene deletion and gene replacement in *C. acetobutylicum*. We implemented SpCRISPR-Cas9 for genome editing in *C. acetobutylicum* by first targeting the *cac1502* gene (encoding the restriction endonuclease Cac824I), which was previously disrupted using group II intron technology (51). The protospacer adjacent motif (PAM) sequence associated with Cas9 binding (i.e.,5′-NGG-3′) (52) was present adjacent to the targeting protospacer within the *cac1502* gene (Fig. 1). Transformation (at three attempts) of *C. acetobutylicum* DSM792 with pCas9gRNA-cac824I (expressing *cas9* and sgRNA targeting the *cac1502* gene) produced no recombinant colonies. As the toxicity associated with *cas9* expression was reported previously (53–55), we constructed two additional plasmids, i.e., p85Cas9 and p85delCas9, which harbor *cas9* with the native gene promoter (p85Cas9) and with the promoter being deleted (p85delCas9). Additionally, both plasmids lack the targeting spacer and guide RNA sequences for generating a double-stranded DNA break on the host chromosome. Transfer of p85Cas9 and p85delCas9 to DSM792 was successful, although the transformation efficiency was rather low for p85Cas9 (1.8 ± 0.2 CFU μg^{-1} DNA) compared with its promoterless variant p85delCas9 (150 ± 8 CFU μg^{-1} DNA), ostensibly due to Cas9 toxicity. Taken together, these data suggest that the lethal effect upon transferring pCas9gRNA-cac824I may be associated with the Cas9-mediated double-stranded DNA break in the presence of Cas9 toxicity.

For genome editing via double-crossover homologous recombination (DCHR) in *C. acetobutylicum*, we constructed two additional vectors that harbor an editing template in addition to the *cas9* gene and sgRNA coding sequence. The editing template contains a truncated version of the *cac1502* gene (lacking the protospacer and PAM sequences) flanked by either an ~500-bp or an ~1-kbp homologous region, yielding pCas9gRNA-delcac824I-500 and pCas9gRNA-delcac824I-1000, respectively (Fig. 1). Upon transferring pCas9gRNA-delcac824I-500 or pCas9gRNA-delcac824I-1000 to DSM792, the truncated *cac1502* editing cassette on the plasmid was expected to replace the wild-type *cac1502* locus on the *C. acetobutylicum* genome through DCHR so that mutant cells could avoid the lethal double-stranded DNA cleavage by Cas9 (Fig. 1 and 2A). Despite the drastically reduced efficiency, we were able to establish the transformation of DSM792 with

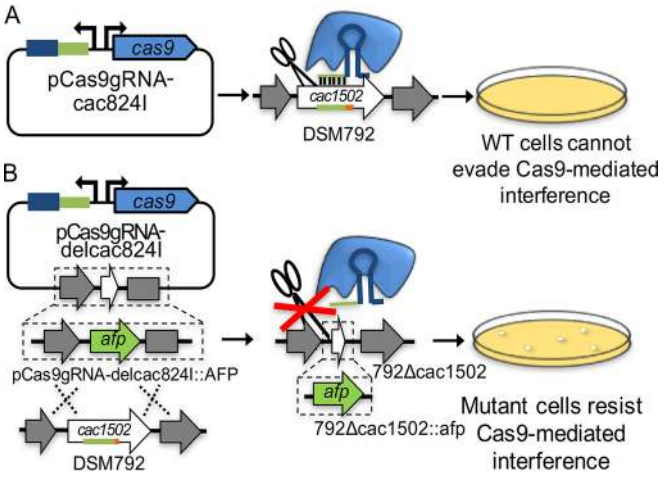

FIG 1 Genome editing in *C. acetobutylicum* using the type II CRISPR-Cas9 system of *S. pyogenes*. Expression of a plasmid-encoded Cas9 with a programmable sgRNA results in Cas9-mediated interference of the *C. acetobutylicum* chromosome and cell death. (A) Introduction of a double-stranded DNA break at the *cac1502* locus was achieved by targeting a sgRNA, consisting of the Cas9 binding handle sequence (blue) and a spacer sequence complementary to the protospacer sequence present on the *C. acetobutylicum* chromosome (green). Cas9 is guided to the target locus by the sgRNA through base pairing of the spacer sequence to the chromosomal protospacer, and recognition of the *S. pyogenes* PAM element (5′-NGG-3′, orange) by Cas9 initiates strand separation of the host chromosome and endonucleolytic activity of Cas9 resulting in the introduction of a double-stranded DNA break. (B) Introduction of a *cac1502* editing cassette to the CRISPR expression plasmid allows DCHR between the plasmid and the host chromosome. Two alternative DCHR events are shown, one introducing a deletion (pCas9gRNA-delcac824I) and one introducing a gene replacement with *afp* (pCas9gRNA-delcac8241-afp). Each of these events is expected to result in the deletion of the *cac1502* coding sequence as well as the protospacer and PAM element required for recognition and cleavage by Cas9 and the establishment of transformation.

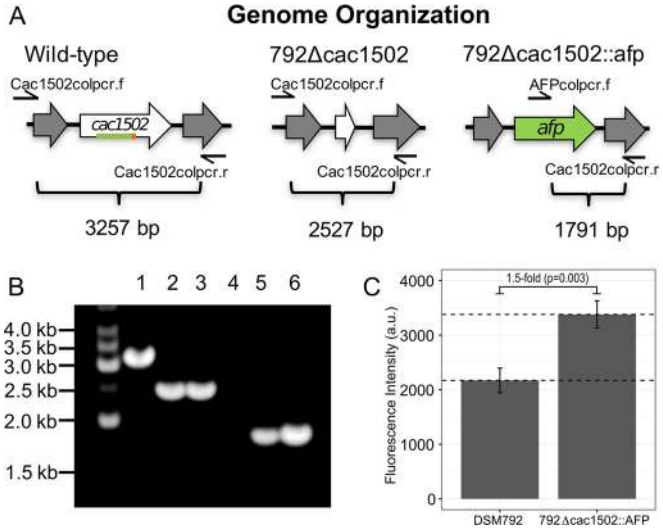

FIG 2 Confirmation of gene deletion and replacement using SpCRISPR-Cas in *C. acetobutylicum*. (A) Schematic depicting the genomic structures of the *C. acetobutylicum* wild type (DSM792), markerless deletion of *cac1502* (792Δ*cac1502*), and markerless gene replacement of *cac1502* with P_{thl}::*afp* (792Δ*cac1502*::*afp*) and the primers used for colony PCR verification of genome editing. (B) Colony PCR verification of gene editing (lane 1, unedited *C. acetobutylicum* genomic DNA; lanes 2 and 3, verification of genome editing using ~500 bp and ~1 kbp of the homologous region, respectively; lane 4, negative control for primers AFPcolpcr.f and cac1502colPCR.r using unedited *C. acetobutylicum* genomic DNA as the template; lanes 5 and 6, verification of P_{thl}::*afp* integration at the *cac1502* locus). (C) Fluorescence intensity of the *C. acetobutylicum* wild-type and AFP-producing mutant via fluorescent plate reader.

pCas9gRNA-delcac824I-500 (0.2 ± 0.0 CFU μg^{-1} DNA) and pCas9gRNA-delcac824I-1000 (0.38 ± 0.05 CFU μg^{-1} DNA), and all resulting mutants were verified for deletion of *cac1502* by colony PCR (Fig. 2B). The results support the hypothesis that Cas9-mediated DNA cleavage occurred site specifically within *cac1502* on the *C. acetobutylicum* genome. The increased length of the homologous region in the editing template appears to improve DCHR efficiency, generating more mutant cells.

To extend the utility of Cas9-mediated genome editing in *Clostridium*, we also explored targeted gene replacement by replacing the wild-type *cac1502* gene on the *C. acetobutylicum* genome with a cargo DNA fusion of the *C. acetobutylicum* thiolase gene promoter (P_{thl}) and the *afp* gene (P_{thl}::*afp*) (49). Similar to the aforementioned editing template, this cargo DNA of P_{thl}::*afp* (~800 bp) was flanked by ~1 kbp of homologous sequences adjacent to both sides of *cac1502*. The fused DNA cassette was inserted into pCas9gRNA-cac824I as an editing template, yielding pCas9gRNA-delcac824I::AFP. The resulting plasmid was used to transform DSM792, yielding an efficiency of 0.4 ± 0.1 CFU μg^{-1} DNA. Two colonies were confirmed for the genomic presence of the fused DNA cassette via colony PCR (Fig. 2B). After curing the pCas9gRNA-delcac824I::AFP plasmid (9), the chromosomally expressed AFP was assessed for the resulting mutant 792Δ*cac1502*::*afp* using a fluorescent plate reader. Green fluorescence intensity in the mutant was 56% higher than it was in the wild-type control, indicating successful genomic integration and functional expression of *afp* (Fig. 2C).

Repurposing SpCRISPR-Cas9 for transcriptional repression in *Clostridium*. For extensive application of SpCRISPR-Cas9 in *Clostridium*, we repurposed Cas9 for transcriptional repression by introducing two amino acid residue substitutions (i.e., D10A and H840A) that inactivate its endonucleolytic activity (42). The strategy of using a nuclease-deficient Cas9 (dCas9), which can nonetheless be guided to target a gene locus for interfering with various proteins involved in transcription, such as RNA polymerase and transcription factors, is known as CRISPR interference (CRISPRi) (42). To conduct CRISPRi in *C. acetobutylicum*, we selected a plasmid-encoded AFP as the reporter protein. In addition to a fluorescent plate reader for measuring the overall culture fluorescence, we applied flow cytometry to discern the fluorescence among individual cells.

C. acetobutylicum cells, i.e., DSM792 harboring either pGLOW-CK^{XN}-Pp1 or the control vector pMTL85141, were cultivated and analyzed. A strong fluorescent signal was detected for DSM792 harboring pGLOW-CK^{XN}-Pp1, and its fluorescence intensity was approximately 650% higher than that of the control, implying that AFP was functionally expressed (Fig. 3A). For evaluation of transcriptional repression, DSM792 harboring pCRISPRi-AFP and pCRISPRi-Cont were cultivated and analyzed. While DSM792 harboring pCRISPRi-Cont had a fluorescence intensity similar to that of DSM792 harboring pGLOW-CK^{XN}-Pp1, the fluorescence intensity for DSM792 harboring pCRISPRi-AFP was only ~10% that of DSM792 harboring pCRISPRi-Cont (Fig. 3A). These results suggest that significant

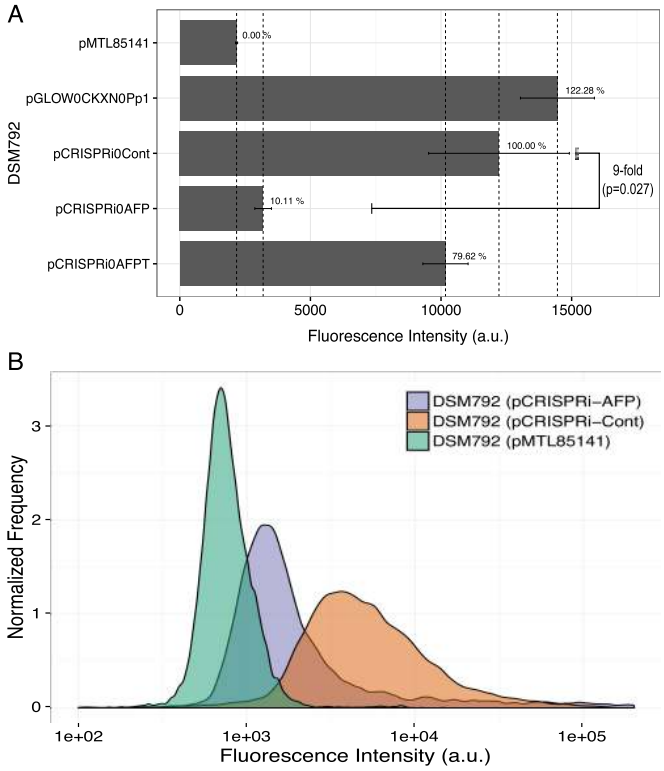

FIG 3 CRISPRi-mediated transcriptional repression of *afp* in *Clostridium*. (A) Mean fluorescence intensity for DSM792 harboring plasmids pMTL85141, pGLOW-CK^{XN}-Pp1, pCRISPRi-Cont, pCRISPRi-AFP, and pCRISPRi-AFPT analyzed by fluorescent plate reader. (B) Histogram of fluorescence intensity of DSM792 harboring pCRISPRi-Cont, empty control vector pMTL85141, or pCRISPRi-AFP as analyzed by flow cytometry.

transcriptional repression is associated with the expression of *dcas9* and sgRNA targeting the nontemplate strand of the *afp* gene. Flow cytometry further revealed a substantial reduction in fluorescence intensity for individual cells of DSM792 harboring pCRISPRi-AFP compared with that for individual cells of DSM792 harboring pCRISPRi-Cont (Fig. 3B). To evaluate the DNA strand-binding bias of dCas9 that was observed previously (42), we derived pCRISPRi-AFPT, in which the sgRNA was modified to target the template strand of *afp*. DSM792 harboring pCRISPRi-AFPT did not show significant transcriptional repression, which is in agreement with the previous report (Fig. 3A) (42).

Employing CRISPRi for relief of carbon catabolite repression in *C. acetobutylicum*. In light of the effective transcriptional repression of episomal *afp*, we conducted CRISPRi by targeting an endogenous gene on the *C. acetobutylicum* genome. Based on the mechanistic model of CCR for Gram-positive *Firmicutes* (56–58), we selected the *hprK* gene (*cac1089*), encoding HPr phosphorylase/kinase (HPrK), for repression. HPrK has been reported to competitively modulate phosphorylation of glucose phosphotransferase HPr (58). The *hprK* gene resides on the *C. acetobutylicum* chromosome as the second gene of a two-gene operon, with the *glpX* gene (*cac1088* encoding a putative fructose 1,6-bisphosphatase) located immediately upstream (59). We therefore derived pCRISPRi-GlpX and pCRISPRi-HprK, respectively, targeting *glpX* and *hprK* for transcriptional repression. Upon respectively transferring pCRISPRi-HprK, pCRISPRi-GlpX and the control plasmid pCRISPRi to DSM792, these recombinant *C. acetobutylicum* cells were cultivated in a medium containing glucose and xylose. While xylose was hardly metabolized by the control strain of DSM792 harboring pCRISPRi (less than 2%) even when glucose was depleted, the simultaneous utilization of glucose and xylose was observed in both strains of DSM792 harboring pCRISPRi-HprK and DSM792 harboring pCRISPRi-GlpX, indicating their metabolic relief from CCR (Fig. 4). Note that xylose utilization was slightly better for DSM792 harboring pCRISPRi-GlpX (~30% of xylose was consumed in 72 h) than for DSM792 harboring pCRISPRi-HPrK (~21% of xylose was consumed in 72 h).

General implementation of the SpCRISPR-Cas9 technology in *Clostridium*. Given the successful implementation of SpCRISPR-Cas9 for genome editing and transcriptional repression in *C. acetobutylicum*, we also explored similar genetic strategies in other members of *Clostridium*. Using the industrially important 1,3-propanediol- and butanol-producing *C. pasteurianum*, both Cas9-mediated genome editing (see Fig. S1 in the supplemental material and published elsewhere [48]) and transcriptional repression (see Fig. S2 in the supplemental material) were successfully demonstrated. The results suggest that similar approaches may be applicable to most, if not all, members of *Clostridium*.

DISCUSSION

Several genome editing strategies have been recently implemented in *Clostridium*, with donor editing cassettes in replicative plasmids being integrated into the host genome (15, 16, 43, 44). Mediated by DCHR based on a homologous region as short as 300 bp, genomic integration of cargo DNA up to ~6.5 kbp in a single step and ~40 kbp through multiple sequential integration events in *C. acetobutylicum* was demonstrated (15, 16). Despite the success of these systems, poor endogenous homologous recombination and low transformation efficiency in *Clostridium* present a great challenge (3). In this report, we explored site-specific genome editing in *C. acetobutylicum* through DCHR. Because of infrequent homologous recombination in *C. acetobutylicum*, the implementation of an effective screening tool, such as CRISPR, for the selection of edited cells can enhance screening of mutant cells. CRISPR systems have been identified in ~70% of members of *Clostridium*, including the solventogenic clostridia (30, 48, 60). While a native CRISPR system has been recently unveiled in *C. acetobutylicum* strain GXAS18-1 (61), no such homologs are detected in *C. acetobutylicum* strains DSM792, DSM1731, or EA2018 (60). Using the editing template of a double-stranded DNA in a plasmid containing the SpCRISPR-Cas9 machinery to replace *cac1502* via DCHR, site-specific genome editing was successfully demonstrated. Although the efficiency of genome editing was 100%, the overall number of edited cells was low. This may be a combinational effect of notoriously poor transformation and the low frequency of homologous recombination in *C. acetobutylicum*, as well as Cas9-induced toxicity. In addition, the large size of the constructed plasmids for genome editing may exacerbate this limitation. While using longer homologous regions in the editing template appeared to improve the yield of edited cells (0.38 ± 0.05 CFU μg^{-1} DNA for pCas9gRNA-delcac824I-1000 versus 0.2 ± 0.0 CFU μg^{-1} DNA for pCas9gRNA-delcac824I-500), the transformation efficiency remained low.

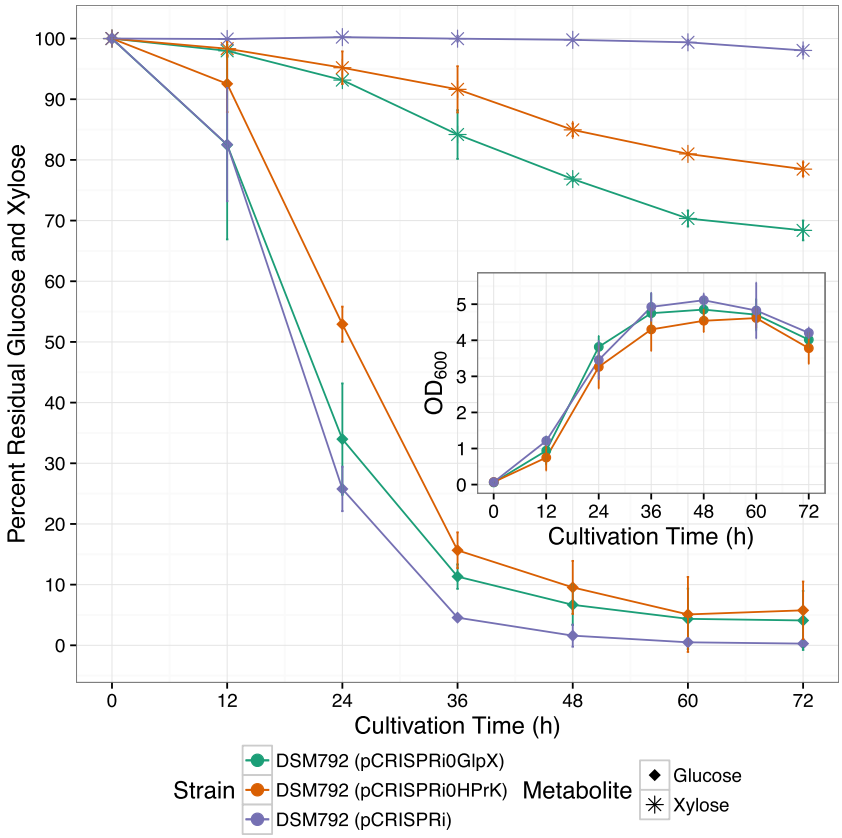

FIG 4 CRISPRi-mediated repression of HPrK leading to the coconsumption of glucose and xylose. Percent residual glucose and xylose and growth curve (inset) for DSM792 harboring plasmids pCRISPRi, pCRISPRi-GlpX, and pCRISPRi-HPrk.

Toxicity associated with Cas9 has been implicated in several previous reports (53–55), and this can significantly reduce the transformation efficiency of *C. acetobutylicum*. Additionally, numerous attempts to construct *E. coli-Clostridium* vectors with *cas9* and sgRNA expression that was regulated by strong, constitutive, and endogenous clostridial promoters, such as thiolase or ferredoxin promoters, yielded only *E. coli* transformants with deletions/mutations in the resulting plasmids (data not shown). Only plasmids with sgRNA expression that was regulated by the *C. beijerinckii* small RNA promoter sCbei5830 were successfully derived without any mutations. As the thiolase and ferredoxin promoters have both been shown to be active in *E. coli* (62, 63), we speculate that the mutation effects may be associated with nonspecific lethal targeting of the expressed sgRNA in *E. coli* and the sCbei5830 promoter may not be recognized by *E. coli*. These results highlight the importance of promoter selection for functional expression of sgRNA.

Given the significant advances in genome editing over the past decades, modulating the transcriptome of an organism to derive complex and multigenic phenotypes is still considered challenging (64, 65). Various strategies have been envisioned, particularly engineering components of the transcriptional machinery by fusing synthetic ZF or TALE DNA-binding domains with transcriptional activator and repressor domains to elicit novel phenotypes (21, 25, 66). In *Clostridium*, transcriptional engineering has been limited to asRNA-mediated repression of endogenous genes, which was implemented in several members of *Clostridium* to various degrees of success (3), ostensibly due to difficulties in identifying sequences with desirable features for targeting as well as the effect of the secondary structure of the asRNA molecule on targeting efficacy (23, 28, 67). A simple and effective method for modifying the transcriptional program in *Clostridium* is critical for further development of industrial strains. Here, transcriptional repression mediated by CRISPRi in *C. acetobutylicum* was explored by expressing *dcas9* along with a targeting sgRNA. With the nontemplate strand of an episomal *afp* gene being targeted in DSM792, the repression efficiency achieved 90% (Fig. 3A). In agreement with the previous study (42), employing an sgRNA targeting the template strand resulted in insignificant repression, as it was reported that the Cas9-sgRNA heteroduplex recognizes the PAM on the nontemplate DNA strand (40, 52). These results corroborate well with previously reported CRISPRi-mediated transcriptional repression in *E. coli* (42) and *Mycobacterium tuberculosis* (68), where significant levels of transcriptional repression were observed.

Note that flow cytometry analysis of *C. acetobutylicum* cultures not only showed a general trend of CRISPRi-associated repression at the single-cell level but also culture heterogeneity, leading to a reduction in the overall *afp* expression level (Fig. 3C). The stochastic nature of gene expression arising from randomness in transcription and translation has been well documented in both prokaryotic and eukaryotic organisms, leading to cell-to-cell variation in the mRNA and protein levels for a genetically homogeneous population of cells (69, 70). Additionally, the asynchronous replication of cells within the population results in heterogeneity in physiological and metabolic state, which can affect the regula-

tion of gene expression (70, 71). The effectiveness of CRISPRi at a single-cell resolution is evidenced by the left-shifted mode and narrowed histogram distribution for DSM792 harboring pCRISPRi-AFP, compared to the right-shifted mode, broad, and longer tailed distribution for DSM792, which harbors the control plasmid pCRISPRi-Cont (Fig. 3C).

CRISPRi was applied for transcriptional repression of endogenous *hprK* and *glpX* in *C. acetobutylicum*, leading to the relief of CCR. In Gram-positive *Firmicutes*, the bifunctional protein HPr kinase/phosphorylase (HPrK) regulates the phosphorylation state of glucose phosphotransferase, i.e., histone-containing protein (HPr), which complexes with the pleiotropic transcriptional regulatory protein carbon control protein A (CcpA) for its DNA binding at the catabolite responsive elements (CREs) to inhibit transcription of carbon catabolic operons (56, 72). Importantly, phosphorylation of HPr at Ser46 by HPrK was reported to result in an ~1,000-fold greater affinity for complexing with CcpA than HPr that is not phosphorylated (58). The *hprK* (*cac1089*) gene exists as the second gene in a two-gene operon, immediately downstream of *glpX* (*cac1088*) encoding a putative fructose 1,6-bisphosphatase, which modulates the concentrations of the glycolytic regulatory hexoses of fructose 1,6-bisphosphate and fructose 6-phosphate and therefore strengthens CcpA-dependent transcriptional regulation (57, 59, 73, 74). Our results show that downregulation of GlpX and/or HPrK via CRISPRi may potentially relieve CCR since both DSM792 harboring pCRISPRi-GlpX and DSM792 harboring pCRISPRi-HPrK consumed glucose and xylose simultaneously (Fig. 4). The difference in the level of CCR relief between the two gene targets may be associated with either the biochemical mechanism of CCR or the targeting efficiency of dCas9, which is knowingly affected by the sgRNA secondary structure and the location of the sgRNA target sequence within the gene (42, 68). Our results suggest the potential applicability of CRISPRi in manipulating or even optimizing complex and multigenic phenotypes in *C. acetobutylicum* for industrial purposes.

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M.R.B. helped conceive of the study, participated in its design and coordination, carried out the experiments, and drafted the manuscript. M.E.P. participated in coordination of the study and assisted with experimental procedures. M.M.Y. participated in the study design and coordination. D.A.C. and C.P.C. helped conceive of the study, participated in its design and coordination, and helped draft the manuscript. All authors read and approved the final manuscript.

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