CRISPR–Cas9D10A nickase-assisted base editing in the solvent producer Clostridium beijerinckii

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Abstract
Clostridium beijerinckii is a potentially important industrial microorganism as it can synthesize valuable chemicals and fuels from various carbon sources. The establishment of convenient to use, effective gene tools with which the organism can be rapidly modified is essential if its full potential is to be realized. Here, we developed a genomic editing tool (pCBEclos) for use in C. beijerinckii based on the fusion of cytidine deaminase (Apobec1), Cas9D10A nickase and uracil DNA glycosylase inhibitor (UGI). Apobec1 and UGI are guided to the target site where they introduce specific base-pair substitutions through the conversion of C·G to T·A. By appropriate choice of target sequence, these nucleotide changes are capable of creating missense mutation or null mutations in a gene. Through optimization of pCBEclos, the system derived, pCBEclos-opt, has been used to rapidly generate four different mutants in C. beijerinckii, in pyrE, xylR, spo0A, and araR. The efficiency of the system was such that they could sometimes be directly obtained following transformation, otherwise only requiring one single restreaking step. Whilst CRISPR–Cas9 nickase systems, such as pNICKclos2.0, have previously been reported in C. beijerinckii, pCBEclos-opt does not rely on homologous recombination, a process that is intrinsically inefficient in clostridia such as C. beijerinckii. As a consequence, bulky editing templates do not need to be included in the knockout plasmids. This both reduces plasmid size and makes their construction simpler, for example, whereas the assembly of pNICKclos2.0 requires six primers for the assembly of a typical knockout plasmid, pCBEclos-opt requires just two primers. The pCBEclos-opt plasmid established here represents a powerful new tool for genome editing in C. beijerinckii, which should be readily applicable to other clostridial species.

KEYWORDS
base editing, Cas9, Clostridium beijerinckii, CRISPR, nickase

1 | INTRODUCTION

Clostridium beijerinckii, a spore-forming, solventogenic, Gram-positive bacterium, is a potentially important industrial strain as it can utilize a variety of carbon-based feedstock to generate valuable chemicals and fuels (Chen & Blaschek, 1999; Dürrre, 1998; Ezeji, Qureshi, & Blaschek, 2007; Y. Gu, Jiang, Yang, & Jiang, 2014; Jiang, Liu, Jiang, Yang, & Yang, 2015; Lee et al., 2008; Thakker, Martinez, Li, San, & Bennett, 2015). The establishment of convenient to use, effective gene tools with which the organism can be rapidly modified is
essential if its full potential is to be realized. Such tools may be used both to provide an in-depth understanding of cell physiology and to enable the robust construction of engineered process organisms. Several genomic editing tools have been developed in C. beijerinckii. Till now, a commonly used procedure is based on gene inactivation by Group II introns, typified by Clostron/Targetron technology (Heap et al., 2010; Heap, Pennington, Cartman, Carter, & Minton, 2007; Shao et al., 2007). Here the presence of intron-encoding protein allows a mobile Group II intron to recognize and insert into a specific site of the genome, resulting in gene disruption. Although Clostron/Targetron technology is effective, it cannot achieve in-frame deletion, large fragment insertion, or base editing. Moreover, in common with any insertional mutagen, it can result in polar effects.

Traditional homologous recombination (HR)-dependent allelic exchange may also be used to edit C. beijerinckii genomes. Its application is reliant on the sequential occurrence of single crossover and double crossover events. These occur naturally but at a very low frequency. According to those cells in which the desired crossovers have taken place need to be detected in the wild-type population through the use of appropriate selective tools. The latter have included the use of counter-selection markers (Al-Hinai, Fast, & Papoutsakis, 2012) or I-SceI endonuclease (N. Zhang et al., 2015). Their use, however, is somewhat laborious, involving numerous restreaking of colonies onto the necessary selective media, and their effectiveness can suffer from a high background of false positives due to spontaneous mutants. A more effective means of selecting the required double crossover mutants is to use Cas9-D10A genome editing strain, which are eliminated on mass leaving only the desired mutant cells. In such a system, typified by the previously described C. beijerinckii CRISPR–Cas9 genome editing tool (Li et al., 2016), all colonies obtained following transformation are in essence mutants. However, whilst the use of CRISPR–Cas9 offers a significant advantage over the use of other counter-selection markers, it remains reliant on HR, which is notoriously inefficient in clostridia and therefore relies on highly efficient DNA transfer. As the frequency of DNA transfer is inversely proportional to plasmid size, the need to incorporate large editing templates in CRISPR–Cas9 vectors for the purposes of HR compromise the system. Moreover, the inclusion of an editing template in the design of the knockout plasmid adds complexity, requiring at least six primers for the assembly of the vector (Li et al., 2016). As the consequence that the availability of an HR-independent C. beijerinckii genomic editing tool that would involve fewer steps for assembly, and use relatively smaller vectors conducive to high transformation frequencies, is highly desirable.

In recent years, the utility of CRISPR–Cas9 in genome editing has been extended through its combination with deaminase enzymes to create a novel strategy for strain engineering which is not reliant on HR. Cytidine deaminase or adenine deaminase is fused to Cas9 effector protein (Cas9 nickase or dCas9) which allows its delivery to the intended DNA target sites by the sgRNA/Cas9 complex. Upon delivery, the deaminase converts nucleotide base pairs C-G to T-A or A-T to G-C. These conversions take place in the absence of Cas9-mediated DNA double-stranded breaks (DSBs) while the plasmid used do not require the relatively large editing templates associated with traditional CRISPR–Cas9 genome editing vectors. To date, the base conversion activity of cytidine deaminase and adenine deaminase has been used in prokaryotes (Banno, Nishida, Arazoe, Mitsunobu, & Kondo, 2018; Gaudelli et al., 2017; T. Gu et al., 2018; Wang, Liu et al., 2018; Wang, Wang et al., 2018; Zheng et al., 2018) and eukaryotes (K. Kim et al., 2017; Y. B. Kim et al., 2017; Komor, Kim, Packer, Zuris, & Liu, 2016; Nishida et al., 2016; Rees et al., 2017; Y. Zhang et al., 2017; Zong et al., 2017), but no deaminase was applied in Clostridium species.

In this study, we established a base editing tool (pCBEclos) in C. beijerinckii NCIMB 8052 by the fusion of Cas9D10A nickase, cytidine deaminase (rat Apobec1) and uracil DNA glycosylase inhibitor (UGI) which was able to efficiently convert specific C-G nucleotide base pairs in the target window sequence to T-A. In its optimized form (pCBEclos-opt) it proved possible to rapidly generate mutants in four different genes, namely pyrE, xylR, spo0A, and araR. The system does not rely on HR, a process that is intrinsically inefficient in clostridia such as C. beijerinckii. As a consequence, bulky editing templates do not need to be included in the knockout plasmids. This both reduces plasmid size and makes their construction simpler, for example, whereas the assembly of pNICKclo2.0 requires six primers for the assembly of a typical knockout plasmid, pCBEclos-opt requires just two primers.

To our knowledge, this is the first report of the successful application of the Cas9D10A nickase and deaminase-mediated base editing in Clostridium. It represents a powerful new tool for genome editing in C. beijerinckii, which should be readily applicable to other clostridial species.

2 | MATERIALS AND METHODS

2.1 | Bacterial strains, media, and reagents

The bacterial strains used in this study are listed in Table S1. Escherichia coli DH5α was used for plasmid construction and maintenance. It was grown in Luria Broth (LB) medium at 37°C, supplemented where necessary with ampicillin (100 µg/ml). C. beijerinckii NCIMB 8052 was used as genome editing strain, it was grown in CGM (Clostridium Growth Medium) medium at 37°C in an anaerobic chamber (Thermo Fisher Scientific, Waltham, MA). Twenty milligrams per liter of erythromycin was supplemented as needed for plasmid selection. For C. beijerinckii NCIMB 8052 pyrE mutant, 20 µg/l uracil was required in CGM medium.

The DNA polymerase KOD plus Neo and KOD FX (Toyobo, Osaka, Japan) were used for high fidelity DNA amplification and colony polymerase chain reaction (PCR), respectively. All restriction enzymes used in this study were purchased from Thermo Fisher Scientific. The plasmids used in this study were assembled by ClonExpress One Step Cloning Kit (Vazyme Biotech Co. Ltd, Nanjing, China). DNA purification and plasmids extracting were performed by kits purchased from Axygen (Hangzhou, China).

2.2 | Plasmid construction

Cas9D10A nickase and P<sub>inv</sub> were amplified from the plasmid pNICKclo2.0 (Li et al., 2016) by primers BE-P<sub>inv</sub>up/BE-P<sub>inv</sub>dn
(apo-hm) and Cas9nclos-up/Cas9nclos-dn, respectively. The plasmid #73021 purchase from Addgene was used as the template to amplify the Apobec1 and UGI gene by primers Apobec1-hm-up/ Apobec1-hm-dn and UGI-hm-up/UGI-hm-dn. The design guideline for sgRNA is as follows: (a) Choose 5'-NGG-3' protospacer adjacent motif (PAM); (b) The window area (typically from positions 4–8 within the N20, counting the end distal PAM to the as position 1) must containing C; (c) Base immediately 5' of the target C should be TC > CC > AC > GC (Komor et al., 2016). The primers cbei1006-gRNA1-up/BE-gRNA-dn were first used to amplify the P223119-sgRNA-pyrE cassette from pNICKclos2.0 which was then used as the template with primers cbei1006-gRNA1-up/BE-gRNA-dn to produce the overlapping extensions at the 5’ ends of the P223119-sgRNA-pyrE cassette. Then, P223119-sgRNA-pyrE cassette, Pbnl, Apobec1, Cas9D10A nickase, and UGI were fused with BamHI/Smal linearized pXY1 to generated plasmid pCEClos-cbei1006-g1. Plasmids pCEClos-cbei1006-g2 and pCEClos-cbei1006-g3 were derived from pCEClos-cbei1006-g1 by replacing the 20-bp target sequences. The construction of plasmid pCEClos-cbei1006-g2 has been shown here as an example. Fragment cbei1006-gRNA2-A was amplified from plasmid pCEClos-cbei1006-g1 by primers cbei1006-gRNA2-up(A-up)/pBEclos-A-dn. Primers pBEclos-B-up/pBEclos-B-dn, pBEclos-C-up/pBEclos-C-dn were used to amplify the fragments BEclos-B, BEclos-C from pCEClos-cbei1006-g1. cbei1006-gRNA2-A, BEclos-B and BEclos-C were assembled together to yield plasmid pCEClos-cbei1006-g2.

Among them, the fragments BEclos-B and BEclos-C were universal, only the fragment A (e.g., cbei1006-gRNA2-A) was changed for each new plasmid (Figure S1). For example, during the construction of plasmid pCEClos-cbei1006-g3, only the primers cbei1006-gRNA3-up(A-up)/pBEclos-A-dn were used to amplify the fragment cbei1006-gRNA3-A, then this fragment was fused with previously amplified fragments BEclos-B and BEclos-C to generate the plasmid pCEClos-cbei1006-g3.

Codon optimization of genes Apobec1 and UGI were performed by GenScript Biotech Corp in Nanjing. Primers Apobec1-opt-up/Apobec1-opt-dn, UGI-opt-up/UGI-opt-dn were adopted to amplify the optimized Apobec1 and UGI genes, respectively. Cas9D10A nickase was amplified from the plasmid pNICKclos2.0 by primers Cas9nclos-up (for opt)/Cas9nclos-dn (for opt). Apobec1, UGI, and Cas9D10A nickase amplified here were fused with BamHI/Smal linearized pCEClos-cbei1006-g1 to generated plasmid pCEClos-cbei1006-g1-opt.

Plasmid pCEClos-cbei1006-g2-opt, pCEClos-cbei1006-g3-opt, pCEClos-cbei14456-opt, pCEClos-cbei2385-g1-opt, pCEClos-cbei2385-g2-opt (Addgene deposits no. 118215), and pCEClos-cbei1712-opt were derived from pCEClos-cbei1006-g1-opt by replacing the 20-bp target sequences. The construction process of these plasmids was similar to the unoptimized pCEClos series of plasmids. Here, only the construction of plasmid pCEClos-cbei1712-opt has been shown as an example. Fragment cbei1712-gRNA-A was amplified from plasmid pCEClos-cbei1006-g1-opt by primers cbei1712-gRNA-up(A-up)/pBEclos-A-dn. Primers pBEclos-B-up/pBEclos-B-dn and pBEclos-C-up/pBEclos-C-dn were used to amplify the fragments BEclos-B-opt, BEclos-C-opt from pCEClos-cbei1006-g1-opt. cbei1712-gRNA-A, BEclos-B-opt, and BEclos-C-opt were assembled together to yield plasmid pCEClos-cbei1712-opt. Similarly, the fragments BEclos-B-opt and BEclos-C-opt were universal for constructing the optimized pCEClos series of plasmids (Figure S1).

2.3 | Electroporation and screening of mutant strains

Plasmids were transformed into C. beijerinckii NCIMB 8052 using a previously reported electroporation protocol (Mermelstein, Welker, Bennett, & Papoutsakis, 1992). The recovered cells were spread on CGM agar supplemented with an appropriate amount of erythromycin and incubated at 37°C for approximately 2 days. The primers listed in the Table S2 were used for colony PCR, which was undertaken when the transformants were visible on the CGM agar plates. Then, the PCR products were extracted and sequenced to confirm the desired mutation events. For screening of the pyrE mutants, the CGM medium was supplemented with 400 μg/L 5-fluoroorotic acid (5-FOA). Colony PCR was undertaken on a selection of random colonies growing on the CGM agar containing 5-FOA, to confirm the expected mutation.

2.4 | Plasmid curing

To eliminate the plasmids used in this study, mutants were first cultivated in 5 ml of CGM medium without any antibiotic (T1). After growing for 12 hr, 50 μl of the T1 broth was used to inoculate 5 ml of fresh CGM medium and grown for 12 hr until the OD600 reached 0.8. The culture was diluted appropriately and aliquots of cells spread on a nonselective CGM agar plate. The individual colonies were patch plated onto CGM agar with and without erythromycin (20 μg/ml). The cells that grew on nonselective medium, but were unable to grow on erythromycin CGM agar, were deemed to have been cured of their plasmids.

2.5 | Fermentation and data analysis

The fermentation of strains 8052WT, 8052xylR(TargeTron; Xiao et al., 2012) and 8052xylR(BE; xylR was disrupted by pCEClos-opt) were performed anaerobically in XHP2 medium (Xiao et al., 2012) at 37°C with xylose(60 g/L) as the carbon source for 72 hr. Five milliliters of liquid CGM was inoculated with the single colony at 37°C for about 12 hr, then approximately 5% (v/v) of the inoculum was transferred into XHP2 medium for fermentation when the optical density at 600 nm (OD600) of the cells reached 0.8–1.0. The concentrations of xylose were determined with high-performance liquid chromatography (1200 series; Agilent, Santa Clara), as described previously (Ren et al., 2010). Cell density (OD600) was measured using a DU730 spectrophotometer (Beckman Coulter, Brea).
3 | RESULTS

3.1 Establishment of CRISPR–Cas9<sup>D10A</sup> nickase-mediated base editing system pCBEclos-opt in *C. beijerinckii* NCIMB 8052

To employ the deaminase-mediated base editing in *C. beijerinckii* NCIMB 8052, we combined all functional components of the desired system into a single plasmid, pCBEclos (Figure 1a). Transcription of the sgRNA was placed under the control of the P<sub>j23119</sub> promoter, and expression of the fusion protein of deaminase (rat Apobec1), Cas9<sup>D10A</sup> nickase and UGI under the control of the P<sub>thl</sub> promoter. Cas9<sup>D10A</sup> nickase targets the nonedited strand and generates a nick, which promotes the use of the edited strand as a template for the repair of the nicked strand (Komor et al., 2016; Komor et al., 2017). UGI suppresses excision of the uracil base generated by the cytosine deaminase and accelerates mutagenesis (Banno et al., 2018; Komor et al., 2017) (Figure 1a). To verify the desired mutation events generated via plasmid pCBEclos, DNA fragments amplified by colony PCR of cells growing on counter-selective media were subject to Sanger sequencing for verifying the counter-selective genes; and colony PCR and sequencing were directly performed from the transformants for nonselectable genes (Figure 1b).

The pyrE gene (cbei1006) encoding orotate phosphoribosyltransferase was selected as the first target gene in *C. beijerinckii* NCIMB 8052. Inactivation of the pyrE gene leads to uracil auxotrophy and to resistance to the uracil analog 5-FOA (Ehsaan et al., 2016; Tripathi et al., 2010), making such mutants readily distinguishable from wild-type cells. Accordingly, the plasmid pCBEclos-cbei1006-g1 carrying the spacer that targets the pyrE gene was electroporated into *C. beijerinckii* NCIMB 8052 and a total of 55 transformants from those obtained on CGM media supplemented with erythromycin. To establish if any of these transformants were mutants, a total of 20 randomly selected colonies were subject to colony PCR and the amplified DNA fragment subject to Sanger sequencing. All of the sequences reads obtained were wild type. To ascertain whether mutant cells were present within the population, all of the 55 primary transformants were patch plated onto CGM agar media supplemented with 5-FOA. Of these, 22 were found to be resistant to 5-FOA. However, even after an extended period of time, these colonies grew poorly (Figure 2a). Further screening of a randomly selected 9 representatives of these 22 clones by Sanger sequencing of the DNA fragment amplified by colony PCR indicate that all 9 contained the expected mutational change (Figure 2b).

Our hypothesis to explain this observation is that the initial transformant colonies are composed of a mixture of wild-type and mutant cells in which the former vastly predominate. The ratio of...
In parallel to the above, 49 primary transformants obtained by colony PCR of six randomly selected transformants revealed that three of them contained the desired mutational changes. However, the reads obtained comprised a mixture of wild type and mutant reads in the target region (Figure 3a). These cells were therefore restreaked once onto fresh CGM agar plates and two of single colonies tested again by Sanger sequencing of the PCR amplified product. All of the purified colonies appeared to be clean mutants with no detectable wild-type sequence (Figure 3c). In parallel to the above, 49 primary pCBEclos-cbei1006-g1-opt transformants were patch plated onto CGM agar containing 5-FOA. On the basis of their growth, 46 out of the 49 colonies were found to be resistant to 5-FOA. Moreover, in this case, the growth observed was vigorous, in contrast to the poor growth previously obtained when using the unoptimized pCBEclos system (Figure 3b). The new base editing tool was designation the pCBEclos-opt system. In contrast to pCBEclos, clones containing the desired C-G to T-A mutations obtained simply by plating cells electroporated with the pCBEclos-opt system onto CGM media containing erythromycin. The detection of the desired mutants using the pCBEclos system requires subsequent screening of primary transformants on selective media (Figure 3d). Moreover, the ratio of positive 5-FOA resistant colonies was improved by approximately two-fold via pCBEclos-opt system, compared with the previous pCBEclos system (Figure 3d).

Successive rounds of base editing require that the initially used editing plasmid is cured of the cell if an additional mutation is required. To test the efficiency of plasmid curing, the edited C. beijerinckii NCIMB 8052 containing the expected pyrE mutation was cultured in nonselective liquid CGM that was supplemented with exogenous uracil (20 μg/L). After two subcultures, clonal populations were isolated by plating to single colonies on nonselective CGM plates and these single colonies were patch plated onto CGM agar with and without erythromycin supplementation. The result showed that all 56 colonies could grow on the nonselective CGM medium, but they were sensitive to erythromycin (Figure S3). These data indicated that curing of plasmid pCBEclos-cbei1006-g1 from the cells took place with 100% efficiency after only two subcultures.

![Image](136x604 to 460x733)

**FIGURE 2** Mutagenesis of pyrE gene in C. beijerinckii NCIMB 8052 via pCBEclos system. (a) C. beijerinckii were spread on CGM plates containing 5-FOA after transformation with plasmid pCBEclos-cbei1006-g1. "-" represents the negative control; (b) Sequence alignment of the pyrE mutants edited by pCBEclos system after selection on 5-FOA plates. The bolded and underlined sequence is the targeted N20 site, the red underlined is the PAM sequence, and the mutated nucleotides are highlighted in green. 5-FOA: 5-fluoroorotic acid; C. beijerinckii: *Clostridium beijerinckii*; CGM: clostridium growth medium; PAM: protospacer adjacent motif [Color figure can be viewed at wileyonlinelibrary.com]
3.2 Expansion of the pCBEclos-opt system to further genes in *C. beijerinckii* NCIMB 8052

After demonstrating the functionality of cytidine deaminase-based gene editing on the *pyrE* gene, we further expanded the pCBEclos-opt system to other genes in *C. beijerinckii* NCIMB 8052 (namely, *araR* or *cbei4456*, encoding a GntR family transcriptional regulator; *xylR* or *cbei2385*, encoding the transcriptional regulator of xylose metabolism; and *spo0A* or *cbei1712*, encoding a response regulator receiver protein).

Accordingly, *C. beijerinckii* NCIMB 8052 was transformed with plasmid pCBEclos-cbei4456-opt encoding a sgRNA that targets *araR*. In this case, all three transformants obtained harbored the desired C-G to T-A mutation. However, as with *pyrE*, all three represented a mixed population composed of the wild-type and desired mutant (Figure 4a). One pure colony harboring the desired mutation could be isolated after single-round restreaking of one of the transformants (Figure 4b).

The plasmid pCBEclos-cbei2385-g1-opt targeting *xylR* was transformed in *C. beijerinckii* NCIMB 8052 and yielded three transformants that were screened by colony PCR and Sanger sequencing. The sequencing results showed that two transformants were mixtures (Figure 4c), while the last colony was wild type. A pure mutant could be obtained by single-round restreaking one of the mixed colonies on the CGM agar (Figure 4d).

In the case of the *xylR* gene, further improvements in mutagenesis efficiency were sought by changing the target sequence of pCBEclos-opt. Accordingly, the 20-bp spacer on plasmid pCBEclos-cbei2385-g1-opt was replaced to yield pCBEclos-cbei2385-g2-opt. The latter was found to be electroporated into 8052 with efficiency of 29.8 CFU/μg DNA. Sanger sequencing of the colony PCR product of five randomly selected transformants showed that one of them was a pure mutant (Figure 5a), one was a mixture and the other three were wild type. As previously, restreaking of a mixed clone onto CGM agar and subsequent testing of individual single colonies easily allowed the isolation of a pure mutant (Figure S4A). Thus, in contrast to the previous plasmid targeting *xylR*, pCBEclos-cbei2385-g1-opt, changing the target site to that present in pCBEclos-cbei2385-g2-opt allowed the direct isolation of a pure mutant. As *xylR* was inactivated via TargeTron previously (Xiao et al., 2012) and it was related to xylose consumption, its ability to ferment xylose was tested. We first cured the plasmid pCBEclos-cbei2385-g2-opt with the efficiency of 34/39 to obtain the strain 8052*xyR*(BE) (Figure S5A). Then, 8052WT, 8052*xyR*(BE) as well as 8052*xyR*(TargeTron) (Xiao et al., 2012) were cultured in XHP2 medium containing 60 g/L xylose for 72 hr.

The results showed that the fermentation phenotype of 8052(BE) was close to the 8052WT, 8052*xyR*(BE) (Figure S5A). Then, 8052WT, 8052*xyR*(BE) and 8052*xyR*(TargeTron) were cultured in XHP2 medium containing 60 g/L xylose for 72 hr. The results showed that the fermentation phenotype of 8052(BE) was close to the 8052*xyR*(TargeTron), both mutants consumed 10% more xylose than strain 8052WT (Figure S5B).

Attempting to edit *spo0A*, plasmid pCBEclos-cbei1712-opt was introduced into 8052, and transformants were obtained at a frequency of 110.6 CFU/μg DNA. Two out of six randomly picked colonies were pure mutants, three were mixed colonies, and one was pure wild type (Figure 5b). Pure mutated strains could be isolated from all three mixed colonies (Figure S4B).
DISCUSSION

Genome editing tools based on CRISPR–Cas9 systems traditionally introduce a DSB at a specific locus under the guidance of a sgRNA. During the repair of the DSB, precise genome editing can be achieved in the presence of a donor DNA template by exploiting the host’s HR mechanisms. On the basis of this principle, CRISPR–Cas9-mediated genome editing has been widely used in bacteria. However, some bacteria have inefficient HR system and lack a functional nonhomologous end-joining repair pathway, which prevents the repair of Cas9-mediated DSBs and results in cell death. Therefore, it is necessary to establish HR-independent genome editing tools in such bacteria.

C. beijerinckii NCIMB 8052 is one of these bacteria lacking an effective DSB repair pathway. One such HR-independent tool available in C. beijerinckii NCIMB 8052 is the Group II intron-based gene inactivation, but it is not as precise as Cas9-mediated genome editing and it has polar effects.

In this study, we first established a CRISPR-mediated base editing tool pCBEclos in C. beijerinckii by the fusion of Apobec1, Cas9<sup>D10A</sup> nickase and UGI. The conversion of C·G to T·A at the target sites were realized via pCBEclos in C. beijerinckii NCIMB 8052. We initially established the pCBEclos system by directly applying Apobec1 and UGI obtained from the Addgene. However, the pCBEclos plasmid was inefficient and it required selective medium to screen the edited strains, such as culturing the pyrE mutants on 5-FOA plates. This pCBEclos system with poor efficiency is not suitable for genes that do not exhibit a selectable phenotype. Fortunately, the base editing
efficiency was greatly improved after the optimization of Apobec1 and UGI, and the desired mutants of pyrE, xylR, spo0A, or araR could be directly detected in the transformants of C. beijerinckii NCIMB 8052 via this optimized pCBEclos-opt system. Furthermore, the loss of plasmid pCBEclos-chel1006-g1-opt after gene editing was achieved with an efficiency of 100% after only two subcultures, allowing for successive rounds of base editing. When mixed colonies of wild-type cells and mutants were obtained, pure colonies harboring the desired mutation could be isolated by subsequent restreaking of the mixed colonies. Targeting a different locus within the xylR allowed the isolation of pure colonies of the desired genotype without the need of a restreak. This improvement in mutagenesis efficiency might reinforce the hypothesis of Komor et al. (2016), that the base immediately 5′ and 3′ of the target C may result in the different editing efficiency.

The whole process of Cas9D10A nickase-mediated base editing, including electroporation, editing, identification, and plasmid curing, only took five days. Unlike the pNICKclos2.0 system we established previously, pCBEclos-opt does not rely on HR, and as such DNA repair templates are not required when using this system to edit gene. Therefore, the assembly of pCBEclos-opt is easier than pNICKclos2.0, requiring only two primers instead of six. PCR amplification is performed to obtain the part A that contains the new 20-bp target sequence, then this part A is fused with the universal part B and C to generate the new plasmid (Figure S1) using ClonExpress One Step Cloning Kit. Its high genome editing efficiency and of the simplicity of its assembly make pCBEclos-opt a useful genome editing tool in Clostridium. If mutagenesis efficiency can be improved, a plasmid library of pCBEclos-opt containing sgRNAs targeting each gene in C. beijerinckii NCIMB 8052 could be used to produce a mutant library that could be selected against the desired phenotype.

In summary, this study is the first report that successfully applied Cas9D10A nickase-mediated base editing tool in Clostridium. A similar strategy would likely be effective in other Clostridium strains. The base editing plasmid pCBEclos-opt we established here will accelerate the metabolic engineering of Clostridium for the optimization of chemicals and solvents in the future.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

AUTHOR CONTRIBUTIONS

Q.L. performed the experiments. Q.L. and S.Y. wrote the manuscript. F.M.S., N.P.M., J.Y., W.J., and Y.J. designed the experiments and wrote the manuscript.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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