CRISPR-Cas9^{D10A} nickase-assisted base editing in solvent producer *Clostridium beijerinckii*

Qi Li[1,2], François M. Seys [3], Nigel P. Minton[3], Junjie Yang[2], Yu Jiang[2], Weihong Jiang[2], Sheng Yang[2,4*]

[1]College of Life Sciences, Sichuan Normal University, Longquan, Chengdu 610101, China

[2]Key Laboratory of Synthetic Biology, Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200032, China

[3]Clostridia Research Group, BBSRC/EPSRC Synthetic Biology Research Centre (SBRC), School of Life Sciences, University of Nottingham, Nottingham NG7 2RD, UK

[4] Jiangsu National Synergetic Innovation Center for Advanced Materials, Nanjing 210009, China

AUTHOR INFORMATION

Corresponding author

*E-mail: syang@sibs.ac.cn. Address: 300 Fenglin Road, Shanghai 200032, China.

Author Contributions

Qi Li performed the experiments. Qi Li and Sheng Yang wrote the manuscript. François M. Seys, Nigel P. Minton, Junjie Yang, Weihong Jiang and Yu Jiang designed the experiments and wrote the manuscript.

Conflict of Interest

The authors declare no competing financial interest.

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), $Cas9^{D10A}$ 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 re-streaking 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 knock-out plasmids. This both reduces plasmid size and makes their construction simpler, e.g., whereas the assembly of pNICKclos2.0 requires six primers for the assembly of a typical knock-out 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.

Key words: CRISPR, Cas9, nickase, base editing, *Clostridium beijerinckii*

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 feedstocks to generate valuable chemicals and fuels (Chen & Blaschek, 1999; Dürre, 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

62 procedure is based on gene inactivation by group II introns, typified by
63 Clostron/Targetron technology (Heap et al., 2010; Heap, Pennington, Cartman, Carter,
64 & Minton, 2007; Shao et al., 2007). Here the presence of intron-encoding protein allows
65 a mobile group II intron to recognize and insert into a specific site of the genome,
66 resulting in gene disruption. Although Clostron/Targetron technology is effective, it
67 cannot achieve in-frame deletion, large fragment insertion or base editing. Moreover,
68 in common with any insertional mutagen, it can result in polar effects.

69 Traditional homologous recombination-dependent allelic exchange may also be
70 employed to edit *C. beijerinckii* genomes. Its application is reliant on the sequential
71 occurrence of single crossover and double crossover events. These occur naturally, but
72 at a very low frequency. According those cells in which the desired crossovers have
73 taken place need to be detected in the wild type population through the use of
74 appropriate selective tools. The latter have included the use of counter selection
75 markers (Al-Hinai, Fast, & Papoutsakis, 2012) or I-*Sce*I endonuclease (N. Zhang et al.,
76 2015). Their use, however, is somewhat laborious, involving numerous re-streaking of
77 colonies onto the necessary selective media, and their effectiveness can suffer from a
78 high background of false positives due to spontaneous mutants. A more effective means
79 of selecting the required double crossover mutants is to use CRISPR-Cas9 where the
80 wild type cells are eliminated on mass leaving only the desired mutant cells. In such a
81 system, typified by the previously described *C. beijerinckii* CRISPR-Cas9^{D10A} genome
82 editing tool (Li et al., 2016), all colonies obtained following transformation are in

essence mutants. However, whilst the use of CRISPR-Cas9 offers significant advantage over the use of other counter selection markers, it remains reliant on homologous recombination (HR), which is notoriously inefficient in clostridia and therefore reliant 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 knock-out 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 a 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-Cas 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 (DSB) while the plasmid employed 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. beijerinckkii* NCIMB 8052 by the fusion of $Cas9^{D10A}$ 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 (pCBclos-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 knock-out plasmids. This both reduces plasmid size and makes their construction simpler, e.g., whereas the assembly of pNICKclos2.0 requires six primers for the assembly of a typical knock-out plasmid, pCBEclos-opt requires just two primers.

To our knowledge, this is the first report of the successful application of the $Cas9^{D10A}$ 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 the Supporting information, Table S1. *Escherichia coli* DH5α was used for plasmid construction and maintenance. It was grown in LB medium at 37℃, supplemented where necessary with ampicillin (100 μg/ml). *C. beijerinckii* NCIMB 8052 was used as genome editing strain, it was grown in CGM medium at 37℃ in anaerobic chamber (Thermo Forma, Inc., Waltham, MA, USA). 20 μg/ml 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 PCR, respectively. All restriction enzymes used in this study were purchased from Thermo Fisher Scientific (USA). 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

$Cas9^{D10A}$ nickase and P_{thl} were amplified from the plasmid pNICKclos2.0 (Li et al., 2016) by primers BE-P_{thl}-up/BE-P_{thl}-dn (apo-hm) and Cas9nclos-up/Cas9nclos-dn

144 respectively. The plasmid #73021 purchase from Addgene was used as the template to
145 amplify the Apobec1 and UGI gene by primers Apobec1-hm-up/Apobec1-hm-dn and
146 UGI-hm-up/UGI-hm-dn. The design guideline for sgRNA is as follows: 1. Choose 5'-
147 NGG-3' protospacer-adjacent motif (PAM); 2. The window area (typically from
148 positions 4 to 8 within the N20, counting the end distal PAM to the as position 1) must
149 containing C; 3. Base immediately 5'of the target C should be TC ≥ CC ≥ AC > GC
150 (Komor et al., 2016). The primers *cbei1006*-gRNA1-up1/BE-gRNA-dn were first used
151 to amplify the P_{j23119}-sgRNA-*pyrE* cassette from pNICKclos2.0 which was then used
152 as the template with primers *cbei1006*-gRNA1-up2/BE-gRNA-dn to produce the
153 overlapping extensions at the 5' ends of the P_{j23119}-sgRNA-*pyrE* cassette. Then, P_{j23119}-
154 sgRNA-*pyrE* cassette, P_{thl}, Apobec1, $Cas9^{D10A}$ nickase and UGI were fused with
155 *Bam*HI/*Sma*I linearized pXY1 to generated plasmid pCBEclos-*cbei1006*-g1. Plasmids
156 pCBEclos-*cbei1006*-g2 and pCBEclos-*cbei1006*-g3 were derived from pCBEclos-
157 *cbei1006*-g1 by replacing the 20-bp target sequences. The construction of plasmid
158 pCBEclos-*cbei1006*-g2 has been shown here as an example. Fragment *cbei1006*-
159 gRNA2-A was amplified from plasmid pCBEclos-*cbei1006*-g1 by primers *cbei1006*-
160 gRNA2-up(A-up)/pBEclos-A-dn. Primers pBEclos-B-up/pBEclos-B-dn, pBEclos-C-
161 up/pBEclos-C-dn were used to amplify the fragments BEclos-B, BEclos-C from
162 pCBEclos-*cbei1006*-g1. *cbei1006*-gRNA2-A, BEclos-B and BEclos-C were assembled
163 together to yield plasmid pCBEclos-*cbei1006*-g2. Among them, the fragments BEclos-
164 B and BEclos-C were universal, only the fragment A (e.g. *cbei1006*-gRNA2-A) was

changed for each new plasmid (Fig. S1). For example, during the construction of plasmid pCBEclos-*cbei1006*-g3, only the primers *cbei1006*-gRNA3-up (A-up)/pBEclos-A-dn were used to amplified the fragment *cbei1006*-gRNA3-A, then this fragment was fused with previously amplified fragments BEclos-B and BEclos-C to generate the plasmid pCBEclos-*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. $Cas9^{D10A}$ nickase was amplified from the plasmid pNICKclos2.0 by primers Cas9nclos-up (for opt)/Cas9nclos-dn (for opt). Apobec1, UGI and $Cas9^{D10A}$ nickase amplified here were fused with *Bam*H/*Sma*I linearized pCBEclos-*cbei1006*-g1 to generated plasmid pCBEclos-*cbei1006*-g1-opt.

Plasmid pCBEclos-*cbei1006*-g2-opt, pCBEclos-*cbei1006*-g3-opt, pCBEclos-*cbei4456*-opt, pCBEclos-*cbei2385*-g1-opt, pCBEclos-*cbei2385*-g2-opt (Addgene deposits No. 118215) and pCBEclos-*cbei1712*-opt were derived from pCBEclos-*cbei1006*-g1-opt by replacing the 20-bp target sequences. The construction process of these plasmids was similar to the unoptimized pCBEclos series of plasmids. Here, only the construction of plasmid pCBEclos-*cbei1712*-opt has been shown as an example. Fragment *cbei1712*-gRNA-A was amplified from plasmid pCBEclos-*cbei1006*-g1-opt by primers *cbei1712*-gRNA-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

186 BEclos-B-opt, BEclos-C-opt from pCBEclos-*cbei1006*-g1-opt. *cbei1712*-gRNA-A,
187 BEclos-B-opt and BEclos-C-opt were assembled together to yield plasmid pCBEclos-
188 *cbei1712*-opt. Similarly, the fragments BEclos-B-opt and BEclos-C-opt were universal
189 for constructing the optimized pCBEclos series of plasmids (Fig. S1).

190 **2.3 Electroporation and screening of mutant strains**

191 Plasmids were transformed into *C. beijerinckii* NCIMB 8052 using a previously
192 reported electroporation protocol (Mermelstein, Welker, Bennett, & Papoutsakis, 1992).
193 The recovered cells were spread on CGM agar supplemented with an appropriate
194 amount of erythromycin and incubated at 37℃ for approximately 2 days. The primers
195 listed in the Supplementary Table S2 were used for colony PCR, which was undertaken
196 when the transformants were visible on the CGM agar plates. Then, the PCR products
197 were extracted and sequenced to confirm the desired mutation events. For screening of
198 the *pyrE* mutants, the CGM medium was supplemented with 400 μg/l 5-fluroorotic acid
199 (5-FOA). Colony PCR was undertaken on a selection of random colonies growing on
200 the CGM agar containing 5-FOA, to confirm the expected mutation.

201 **2.4 Plasmid curing**

202 To eliminate the plasmids used in this study, mutants were first cultivated in 5 ml of
203 CGM medium without any antibiotic (T1). After growing for 12 h, 50 μl of the T1 broth
204 was used to inoculate 5 ml of fresh CGM medium and grown for 12 h until the OD_{600}
205 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, 8052*xylR*(TargeTron) (Xiao et al., 2012) and 8052*xylR*(BE)(*xylR* was disrupted by pCBEclos-opt) were performed anaerobically in XHP_2 medium (Xiao et al., 2012) at 37℃ with xylose(60 g/l) as the carbon source for 72 h. 5 ml of liquid CGM was inoculated with single colony at 37 °C for about 12h, then ~5% (v/v) of the inoculum was transferred into XHP2 medium for fermentation when the optical density at 600 nm (OD_{600}) of the cells reached 0.8–1.0. The concentrations of xylose were determined with high-performance liquid chromatography (1200 series; Agilent), as described previously (Ren et al., 2010). Cell density (OD_{600}) was measured using a DU730 spectrophotometer (Beckman Colter).

3 Results

3.1 Establishment of CRISPR-Cas9^{D10A} 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_{j23119} promoter, and expression of the fusion protein of deaminase (rat Apobec1), $Cas9^{D10A}$ nickase and UGI under the control of the P_{thl} promoter. $Cas9^{D10A}$ nickase targets the non-edited strand and generates a nick, which promotes the use of the edited strand as 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 non-selectable 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

246 randomly selected colonies were subject to colony PCR and the amplified DNA
247 fragment subject to Sanger sequencing. All of the sequence reads obtained were wild
248 type. To ascertain whether mutant cells were present within the population, all of the
249 55 primary transformants were patch plated onto CGM agar media supplemented with
250 5-FOA. Of these, 22 were found to be resistant to 5-FOA. However, even after an
251 extended period of time, these colonies grew poorly (Figure 2A). Further screening of
252 a randomly selected 9 representatives of these 22 clones by Sanger sequencing of the
253 DNA fragment amplified by colony PCR indicate that all 9 contained the expected
254 mutational change (Figure 2B).

255 Our hypothesis to explain this observation is that the initial transformant colonies
256 are composed of a mixture of wild type and mutant cells in which the former vastly
257 predominate. The ratio of mutant to wild type is such that the mutant sequence cannot
258 be detected as it is swamped by the wild type. As an additional consequence, the
259 transformants grow poorly when initially plated on agar media containing 5-FOA as
260 they are predominately wild type cells, which are sensitive to this uracil analog.
261 Additionally, vector maintenance and constitutive expression of the codon un-
262 optimized fusion protein might also be responsible for reduced growth. Thus, our initial
263 base editing system (designated pCBEclos) although functional in *C. beijerinckii*
264 NCIMB 8052, was deemed relatively inefficient.

265 We also selected two other target sites (Fig. S2A) within the *pyrE* gene to test
266 pCBEclos. Similar to pCBEclos-*cbei1006*-g1, 20 out of 22 transformants obtained with

plasmid pCBEclos-*cbei1006*-g2 were found to grow on CGM agar containing 5-FOA, albeit weakly. In contrast, no cells resistant to 5-FOA were obtained with plasmid pCBEclos-*cbei1006*-g3 (Fig. S2B). 9 out of those 20 clones obtained from pCBEclos-*cbei1006*-g2 which grew on 5-FOA medium were all shown by Sanger sequencing of amplified PCR products, to contained the desired mutations (Fig. S2C). The results indicated that the selection of different target sites on the same gene was not a fruitful way to improve the efficiency of the initially established base editing plasmid pCBEclos.

As the Addgene-derived Apobec1 and UGI genes used in the pCBEclos plasmid system were optimized for expression in human cells, they may not be well expressed in *Clostridium*. This could explain the poor efficiency of pCBEclos. Accordingly, we elected to optimize the Apobec1 and UGI codons used based on *C. beijerinckii* NCIMB 8052 genome codon usage. The humanized components on plasmid pCBEclos-*cbei1006*-g1 were thereafter replaced with the *Clostridium* optimized Apobec1 and UGI genes to generate plasmid pCBEclos-*cbei1006*-g1-opt. Following the procedure showed in Figure 1B, plasmid pCBEclos-*cbei1006*-g1-opt was electroporated into *C. beijerinckii* NCIMB 8052 and transformed cells plated onto CGM agar supplemented with erythromycin. The transformation frequencies obtained equated to 18.2 CFU/μg DNA. Sanger sequencing of the amplified DNA 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 re-streaked 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 un-optimized 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 about 2-folds via pCBEclos-opt system, compared to the previous pCBEclos system (Figure 3D).

Successive rounds of base editing require that the initially used editing plasmid is cured from the cell if an additional mutation is required. In order 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 (Fig. S3). These data indicated that curing of plasmid pCBEclos-*cbei1006*-g1 from the cells took place with 100% efficiency after only two subcultures.

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 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 3 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 re-streaking of one of the transformants (Figure 4B).

329 The plasmid pCBEclos-*cbei2385*-g1-opt targeting *xylR* was transformed in *C.*
330 *beijerinckii* NCIMB 8052 and yielded 3 transformants that were screened by colony
331 PCR and Sanger sequencing. The sequencing results showed that 2 transformants were
332 mixtures (Figure 4C), while the last colony was wild-type. A pure mutant could be
333 obtained by single-round re-streaking one of the mixed colonies on the CGM agar
334 (Figure 4D).

335 In the case of the *xylR* gene, further improvements in mutagenesis efficiency were
336 sought by changing the target sequence of pCBEclos-opt. Accordingly, the 20-bp spacer
337 on plasmid pCBEclos-*cbei2385*-g1-opt was replaced to yield pCBEclos-*cbei2385*-g2-
338 opt. The latter was found to be electroporated into 8052 with efficiency of 29.8 CFU/μg
339 DNA. Sanger sequencing of the colony PCR product of five randomly selected
340 transformants showed that one of them was a pure mutant (Figure 5A), one was a
341 mixture and the other three were wild type. As previously, re-streaking of a mixed clone
342 onto CGM agar and subsequent testing of individual single colonies easily allowed the
343 isolation of a pure mutant (Fig. S4A). Thus, in contrast to the previous plasmid targeting
344 *xylR*, pCBEclos-*cbei2385*-g1-opt, changing the target site to that present in pCBEclos-
345 *cbei2385*-g2-opt allowed the direct isolation of a pure mutant. As *xylR* was inactivated
346 via TargeTron previously (Xiao et al., 2012) and it was related to xylose consumption.
347 In order to test the fermentation phenotype of *xylR* mutant we obtained by pCBEclos-
348 opt, we fist cleared the plasmid pCBEclos-*cbei2385*-g2-opt with efficiency of 34/39 to
349 obtain the strain 8052*xylR*(BE) (Fig. S5A). Then, 8052WT, 8052*xylR*(BE) as well as

8052*xylR*(TargeTron) (Xiao et al., 2012) were cultured in XHP2 medium containing 60 g/l xylose for 72h. The results showed that the fermentation phenotype of 8052(BE) was close to the 8052*xylR*(TargeTron), both mutants consumed 10% more xylose than strain 8052WT (Fig. S5B).

Attempting to edit *spo0A*, plasmid pCBEclos-*cbei1712*-opt was introduced into 8052, and transformants were obtained with transformation of 110.6 CFU/μg DNA. 2 out of 6 random picked colonies were pure mutated colonies, 3 were mixed colonies, and 1 was pure wild type (Figure 5B). Pure mutated strains could be isolated from all 3 mixed colonies (Fig. S4B).

4 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 homologous recombination mechanisms. Based on this principle, CRISPR-Cas9 mediated genome editing has been widely used in bacteria. However, some bacteria have inefficient HR system and lack a functional non-homologous end joining (NHEJ) 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. bejerinckii* NCIMB 8052 is one of these bacteria

370 lacking an effective DSB repair pathway. One such HR independent tool available in
371 *C. bejerinckii* NCIMB 8052 is the group II intron-based gene inactivation, but it is as
372 precise as Cas9-mediated genome editing and it has polar effects.
373 In this study, we first established a CRISPR-mediated base editing tool pCBEclos
374 in *Clostridium* by the fusion of Apobec1, $Cas9^{D10A}$ nickase and UGI. The conversion
375 of C·G to T·A at the target sites were realized via pCBEclos in *C. bejerinckii* NCIMB
376 8052. We initially established the pCBEclos system by directly applying Apobec1 and
377 UGI obtained from Addgene. However, the pCBEclos plasmid was inefficient and it
378 required selective medium to screen the edited strains, such as culturing the *pyrE*
379 mutants on 5-FOA plates. This pCBEclos system with poor efficiency is not suitable
380 for genes that do not exhibit a selectable phenotype. Fortunately, the base editing
381 efficiency was greatly improved after the optimization of Apobec1 and UGI, and the
382 desired mutants of *pyrE*、*xylR*、*spo0A* or *araR* could be directly detected in the
383 transformants of *C. bejerinckii* NCIMB 8052 via this optimized pCBEclos-opt system.
384 Furthermore, the loss of plasmid pCBEclos-*cbei1006*-g1-opt after gene editing was
385 achieved with efficiency of 100% after only two subcultures, allowing for successive
386 rounds of base editing. When mixed colonies of wild-type cells and mutants were
387 obtained, pure colonies harboring the desired mutation could be isolated by subsequent
388 re-streaking of the mixed colonies. Targeting a different locus within the *xylR* allowed
389 to isolate pure colonies of the desired genotype without the need of a re-streak. This
390 improvement in mutagenesis efficiency might reinforce the hypothesis of Komor et al

391 (2016), that the base immediately 5’ and 3’ of the target C may result in the different
392 editing efficiency.

393 The whole process of Cas9^{D10A} nickase-mediated base editing, including
394 electroporation, editing, identification and plasmid curing, only took five days. Unlike
395 the pNICKclos2.0 system we established previously, pCBEclos-opt does not rely on
396 homologous recombination, and as such DNA repair templates are not required when
397 using this system to edit gene. Therefore, the assembly of pCBEclos-opt is easier than
398 pNICKclos2.0, requiring only two primers instead of six. PCR amplification is
399 performed to obtain the part A that contains the new 20-bp target sequence, then this
400 part A is fused with the universal part B and C to generate the new plasmid (Fig. S1)
401 using ClonExpress One Step Cloning Kit. Its high genome editing efficiency of and the
402 simplicity of its assembly make pCBEclos-opt a useful genome editing tool in
403 *Clostridium*. If mutagenesis efficiency can be improved, a plasmid library of
404 pCBEclos-opt containing sgRNAs targeting each gene in *C. bejerinckii* NCIMB 8052
405 could be used to produce a mutant library that could be selected against a desired
406 phenotype.

407 In summary, this study is the first report that successfully applied Cas9^{D10A}
408 nickase-mediated base editing tool in *Clostridium*. A similar strategy would likely be
409 effective in other *Clostridium* strains. The base editing plasmid pCBEclos-opt we
410 established here will accelerate the metabolic engineering of *Clostridium* for the
411 optimization of chemicals and solvents in the future.

412

413 Acknowledgements

414 This work is supported by the grant from the National Natural Science Foundation of
415 China (31500068, 31870019) and the Natural Science Foundation of Shanghai
416 (18ZR1446800).

417

418 Supporting Information

419 Table S1: Plasmids and strains used in this study.

420 Table S2: Oligonucleotides used in this study.

421 Figure S1: Schematic for construction of pCBEclos series plasmids.

422 Figure S2: Target sites on *pyrE* gene were changed to test the pCBEclos system.

423 Figure S3: Clearance of plasmid pCBEclos-*cbei1006*-g1-opt.

424 Figure S4: Purification of the mixed *xylR* and *spo0A* mutants on plates.

425 Figure S5: Clearance of plasmid pCBEclos-*cbei2385*-g2-opt and the xylose
426 consumption of 8052WT, 8052xylR(TargeTron) and 8052xylR(BE).

427

428 References

429 Al-Hinai, M. A., Fast, A. G., & Papoutsakis, E. T. (2012). Novel system for efficient

isolation of *Clostridium* double-crossover allelic exchange mutants enabling markerless chromosomal gene deletions and DNA integration. *Appl Environ Microbiol, 78*(22), 8112-8121. doi:10.1128/AEM.02214-12

Banno, S., Nishida, K., Arazoe, T., Mitsunobu, H., & Kondo, A. (2018). Deaminase-mediated multiplex genome editing in *Escherichia coli*. *Nat Microbiol* doi:10.1038/s41564-017-0102-6

Chen, C. K., & Blaschek, H. P. (1999). Acetate enhances solvent production and prevents degeneration in *Clostridium beijerinckii* BA101. *Appl Microbiol Biotechnol, 52*(2), 170-173. doi:10.1007/s002530051504

Dürre, P. (1998). New insights and novel developments in clostridial acetone/butanol/isopropanol fermentation. *Appl Microbiol Biotechnol, 49*(6), 639-648.

Ehsaan, M., Kuit, W., Zhang, Y., Cartman, S. T., Heap, J. T., Winzer, K., & Minton, N. P. (2016). Mutant generation by allelic exchange and genome resequencing of the biobutanol organism *Clostridium acetobutylicum* ATCC 824. *Biotechnol Biofuels, 9*, 4. doi:10.1186/s13068-015-0410-0

Ezeji, T., Qureshi, N., & Blaschek, H. P. (2007). Butanol production from agricultural residues: Impact of degradation products on *Clostridium beijerinckii* growth and butanol fermentation. *Biotechnol Bioeng, 97*(6), 1460-1469. doi:10.1002/bit.21373

Gaudelli, N. M., Komor, A. C., Rees, H. A., Packer, M. S., Badran, A. H., Bryson, D.

I., & Liu, D. R. (2017). Programmable base editing of A*T to G*C in genomic DNA without DNA cleavage. *Nature, 551*(7681), 464-471. doi:10.1038/nature24644

Gu, T., Zhao, S., Pi, Y., Chen, W., Chen, C., Liu, Q., . . . Ji, Q. (2018). Highly efficient base editing in *Staphylococcus aureus* using an engineered CRISPR RNA-guided cytidine deaminase. *Chem Sci, 9*(12), 3248-3253. doi:10.1039/c8sc00637g

Gu, Y., Jiang, Y., Yang, S., & Jiang, W. (2014). Utilization of economical substrate-derived carbohydrates by solventogenic clostridia: pathway dissection, regulation and engineering. *Curr Opin Biotechnol, 29*, 124-131.

Heap, J. T., Kuehne, S. A., Ehsaan, M., Cartman, S. T., Cooksley, C. M., Scott, J. C., & Minton, N. P. (2010). The ClosTron: Mutagenesis in Clostridium refined and streamlined. *J Microbiol Methods, 80*(1), 49-55. doi:10.1016/j.mimet.2009.10.018

Heap, J. T., Pennington, O. J., Cartman, S. T., Carter, G. P., & Minton, N. P. (2007). The ClosTron: a universal gene knock-out system for the genus *Clostridium*. *J Microbiol Methods, 70*(3), 452-464. doi:10.1016/j.mimet.2007.05.021

Jiang, Y., Liu, J., Jiang, W., Yang, Y., & Yang, S. (2015). Current status and prospects of industrial bio-production of n-butanol in China. *Biotechnol Adv, 33*(7), 1493-1501. doi:10.1016/j.biotechadv.2014.10.007

Kim, K., Ryu, S.-M., Kim, S.-T., Baek, G., Kim, D., Lim, K., . . . Kim, J.-S. (2017).

Highly efficient RNA-guided base editing in mouse embryos. *Nat Biotechnol, 35*(5), 435-+. doi:10.1038/nbt.3816

Kim, Y. B., Komor, A. C., Levy, J. M., Packer, M. S., Zhao, K. T., & Liu, D. R. (2017). Increasing the genome-targeting scope and precision of base editing with engineered Cas9-cytidine deaminase fusions. *Nat Biotechnol, 35*(4), 371-+. doi:10.1038/nbt.3803

Komor, A. C., Kim, Y. B., Packer, M. S., Zuris, J. A., & Liu, D. R. (2016). Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. *Nature, 533*(7603), 420-+. doi:10.1038/nature17946

Komor, A. C., Zhao, K. T., Packer, M. S., Gaudelli, N. M., Waterbury, A. L., Koblan, L. W., . . . Liu, D. R. (2017). Improved base excision repair inhibition and bacteriophage Mu Gam protein yields C:G-to-T: A base editors with higher efficiency and product purity. *Sci. Adv, 3*(8). doi:10.1126/sciadv.aao4774

Lee, S. Y., Park, J. H., Jang, S. H., Nielsen, L. K., Kim, J., & Jung, K. S. (2008). Fermentative butanol production by Clostridia. *Biotechnol Bioeng, 101*(2), 209-228. doi:10.1002/bit.22003

Li, Q., Chen, J., Minton, N. P., Zhang, Y., Wen, Z., Liu, J., . . . Yang, S. (2016). CRISPR-based genome editing and expression control systems in *Clostridium acetobutylicum* and *Clostridium beijerinckii*. *Biotechnol J, 11*(7), 961-972. doi:10.1002/biot.201600053

Mermelstein, L. D., Welker, N. E., Bennett, G. N., & Papoutsakis, E. T. (1992).

493 Expression of cloned homologous fermentative genes in *Clostridium*
494 *acetobutylicum* ATCC 824. *Nat Biotechnol, 10*(2), 190-195.
495 Nishida, K., Arazoe, T., Yachie, N., Banno, S., Kakimoto, M., Tabata, M., . . . Kondo,
496 A. (2016). Targeted nucleotide editing using hybrid prokaryotic and vertebrate
497 adaptive immune systems. *Science, 353*(6305). doi:10.1126/science.aaf8729
498 Rees, H. A., Komor, A. C., Yeh, W.-H., Caetano-Lopes, J., Warman, M., Edge, A. S. B.,
499 & Liu, D. R. (2017). Improving the DNA specificity and applicability of base
500 editing through protein engineering and protein delivery. *Nat commun, 8*,
501 15790-15790. doi:10.1038/ncomms15790
502 Ren, C., Gu, Y., Hu, S., Wu, Y., Wang, P., Yang, Y., . . . Jiang, W. (2010). Identification
503 and inactivation of pleiotropic regulator CcpA to eliminate glucose repression
504 of xylose utilization in Clostridium acetobutylicum. *Metab Eng*, 12(5), 446-454.
505 doi: 10.1016/j.ymben.2010.05.002
506 Shao, L., Hu, S., Yang, Y., Gu, Y., Chen, J., Yang, Y., . . . Yang, S. (2007). Targeted
507 gene disruption by use of a group II intron (targetron) vector in *Clostridium*
508 *acetobutylicum*. *Cell Res, 17*(11), 963-965. doi:10.1038/cr.2007.91
509 Thakker, C., Martinez, I., Li, W., San, K. Y., & Bennett, G. N. (2015). Metabolic
510 engineering of carbon and redox flow in the production of small organic acids.
511 *J Ind Microbiol Biotechnol, 42*(3), 403-422. doi:10.1007/s10295-014-1560-y
512 Tripathi, S. A., Olson, D. G., Argyros, D. A., Miller, B. B., Barrett, T. F., Murphy, D.
513 M., . . . Caiazza, N. C. (2010). Development of *pyrF*-based genetic system for

514 targeted gene deletion in *Clostridium thermocellum* and creation of a pta mutant.
515 *Appl Environ Microbiol*, *76*(19), 6591-6599. doi:10.1128/AEM.01484-10
516 Wang, Y., Liu, Y., Liu, J., Guo, Y., Fan, L., Ni, X., . . . Ma, Y. (2018). MACBETH:
517 Multiplex automated *Corynebacterium glutamicum* base editing method. *Metab*
518 *Eng*, *47*, 200-210. doi:10.1016/j.ymben.2018.02.016
519 Wang, Y., Wang, S., Chen, W., Song, L., Zhang, Y., Shen, Z., . . . Ji, Q. (2018). Precise
520 and efficient genome editing in *Klebsiella pneumoniae* using CRISPR-Cas9 and
521 CRISPR-assisted cytidine deaminase. *Appl Environ Microbiol*.
522 doi:10.1128/aem.01834-18
523 Xiao, H., Li, Z., Jiang, Y., Yang, Y., Jiang, W., Gu, Y., & Yang, S. (2012). Metabolic
524 engineering of D-xylose pathway in Clostridium beijerinckii to optimize solvent
525 production from xylose mother liquid. *Metab Eng*, 14(5), 569-578. doi:
526 10.1016/j.ymben.2012.05.003
527 Zhang, N., Shao, L., Jiang, Y., Gu, Y., Li, Q., Liu, J., . . . Yang, S. (2015). I-SceI-
528 mediated scarless gene modification via allelic exchange in *Clostridium*. *J*
529 *Microbiol Methods*, *108*, 49-60. doi:10.1016/j.mimet.2014.11.004
530 Zhang, Y., Qin, W., Lu, X., Xu, J., Huang, H., Bai, H., . . . Lin, S. (2017). Programmable
531 base editing of zebrafish genome using a modified CRISPR-Cas9 system. *Nat*
532 *commun*, *8*. doi:10.1038/s41467-017-00175-6
533 Zheng, K., Wang, Y., Li, N., Jiang, F.-F., Wu, C.-X., Liu, F., . . . Liu, Z.-F. (2018).
534 Highly efficient base editing in bacteria using a Cas9-cytidine deaminase fusion.

Communications biology, 1, 32-32. doi:10.1038/s42003-018-0035-5

Zong, Y., Wang, Y., Li, C., Zhang, R., Chen, K., Ran, Y., . . . Gao, C. (2017). Precise base editing in rice, wheat and maize with a Cas9-cytidine deaminase fusion. *Nat Biotechnol, 35*(5), 438-+. doi:10.1038/nbt.3811

Figure legends

Figure 1: Base editing in *C. beijerinckii* NCIMB 8052. (A) Design and strategy for CRISPR-Cas9^{D10A} nickase-mediated C·G to T·A conversion in *C. beijerinckii*. Guide RNA, Apobec1, Cas9^{D10A} nickase and UGI are all expressed on one plasmid pCBEclos. (B) The procedure for confirming the mutant strain edited by pCBEclos in *C. beijerinckii*. After assembly of pBEclos, it is electroporated into *C. beijerinckii* NCIMB 8052 and cells are plated on CGM plates with erythromycin to select for pBEclos. In the case of *pyrE* mutagenesis, an additional selection step is carried out by patching single colonies on CGM plates with erythromycin and 5-FOA. Colonies from both the transformation plate and the mutant selection plate are finally screened by PCR amplification and subsequent Sanger sequencing. When revealed by the sequencing

results, mixed colonies are re-streaked on CGM plates with appropriate antibiotic for isolation of pure colonies and re-sequencing.

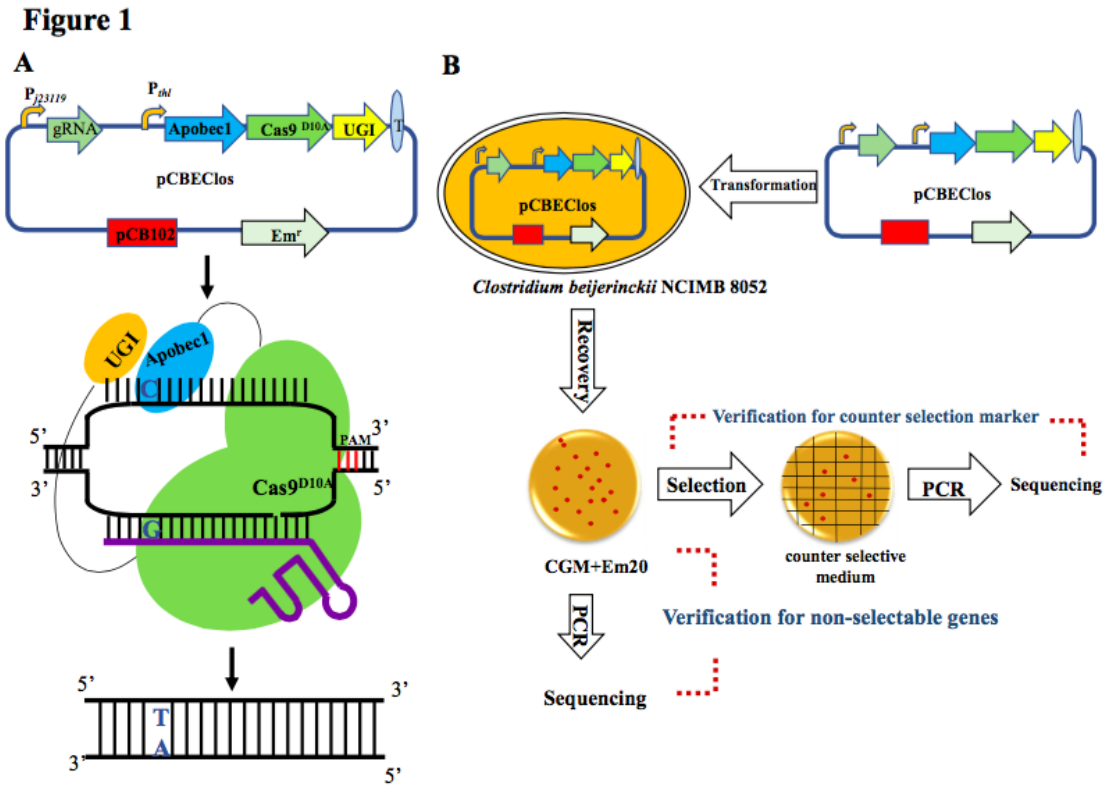
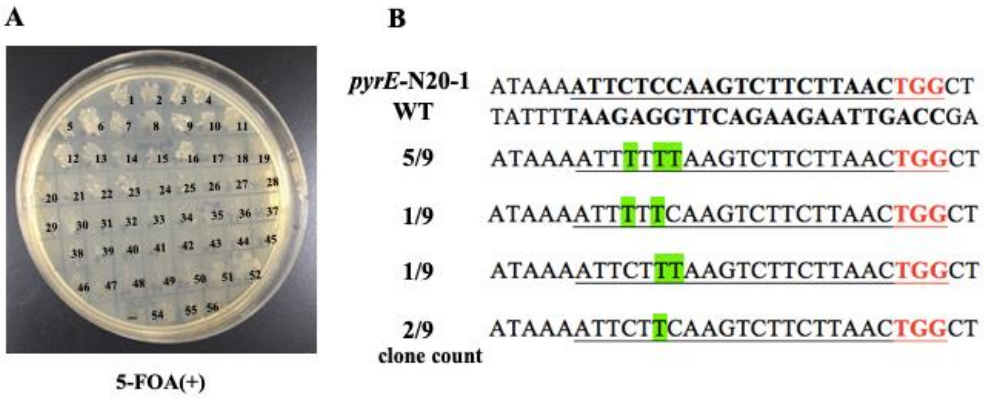


Figure 2: Mutagenesis of *pyrE* gene in *C. beijerinckii* NCIMB 8052 via pCBEclos system. (A) *C. beijerinckii* were spread on CGM plates containing 5-FOA after transformation with plasmid pCBEclos-*cbei1006*-g1, "-" represents the negative control; (B) Sequence alignment of the *pyrE* mutants edited 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.

Figure 2



565

566 **Figure 3: Base editing efficiency was improved by pCBEclos-opt carrying the**
567 **codon optimized Apobec1 and UGI.** (A) Sequence alignment of the *pyrE* mutants
568 obtained by transformation with pCBEclos-*cbei1006*-g1-opt; (B) *C. beijerinckii*
569 carrying the plasmid pCBEclos-*cbei1006*-g1-opt were patched on plates containing 5-
570 FOA, "-" represents the negative control; (C) Sequence alignment of the *pyrE* mutants
571 isolated after re-streaking mixed colonies on CGM+ erythromycin plate; (D) The ratio
572 of positive transformants and 5-FOA resistant strains was compared between the
573 pCBEclos and pCBEclos-opt. The bolded and underlined sequence is the targeted N20
574 site, the red underlined is the PAM sequence, and the mutated nucleotides are
575 highlighted in green.

576

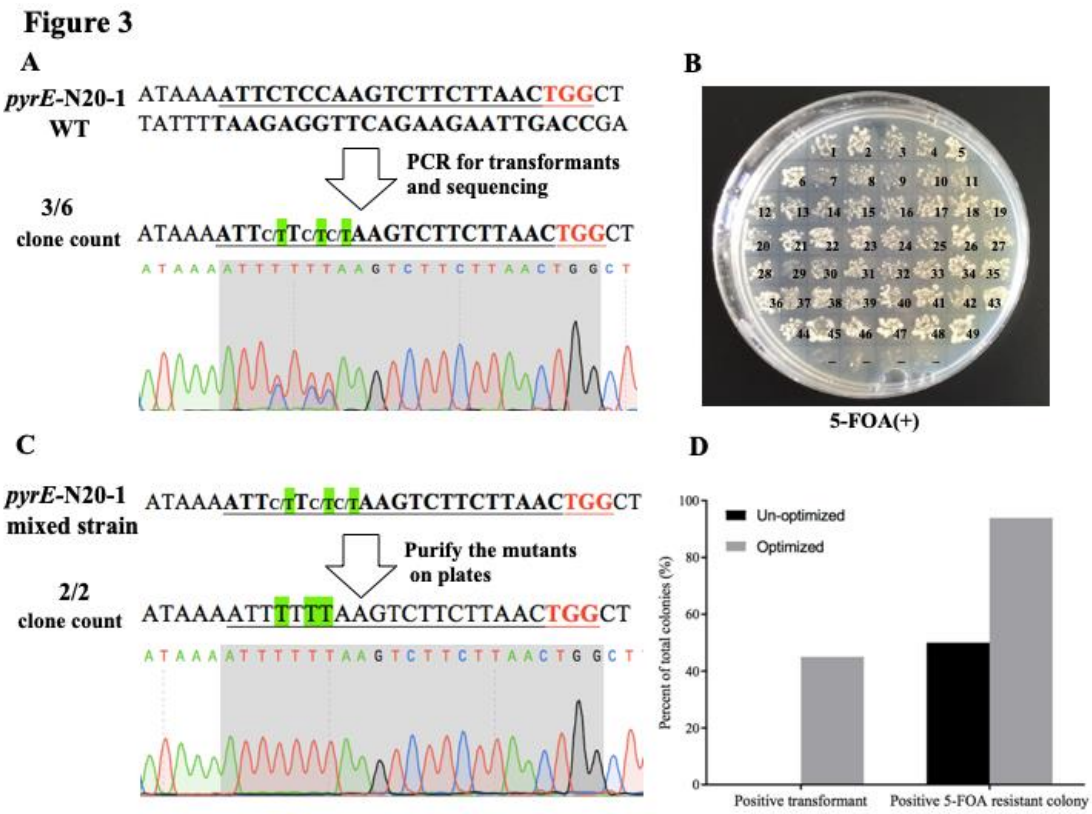
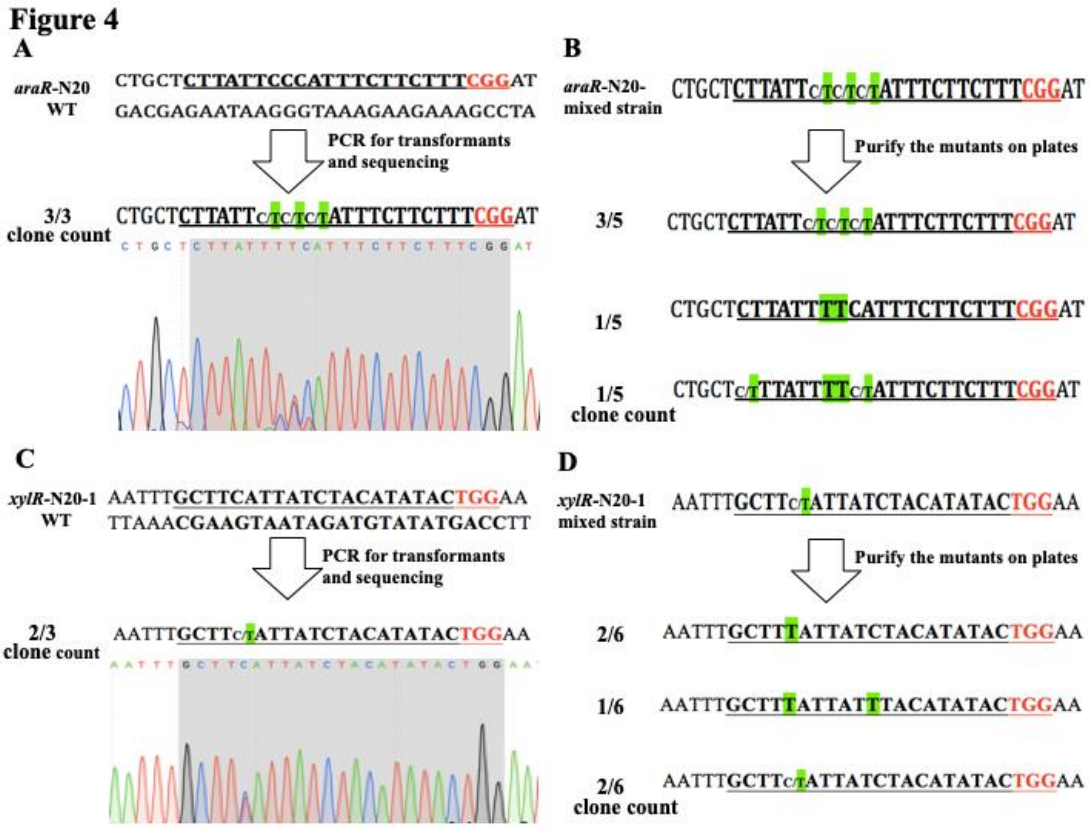


Figure 4: Mutagenesis of gene *araR* and *xylR* in *C. beijerinckii* NCIMB 8052 via pCBEclos-opt system. (A) Sequence alignment of the *araR* mutants obtained by transformation with pCBEclos-*cbei3835*-g1-opt; (B) The pure *araR* mutant was obtained after single-round streaking the mixed colony on plate. (C) Sequence alignment of the *xylR* mutants obtained by transformation with pCBEclos-*cbei2385*-g1-opt. (D) The pure *xylR* mutant was isolated by single-round streaking a mixed colony on plate. 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.



586

587 **Figure 5: Pure mutants were obtained directly from the transformants of *C.***

588 ***beijerinckii* NCIMB 8052 via plasmid pCBEclos-opt.** (A) Sequence alignment of the

589 *xylR* pure mutant obtained by transformation with pCBEclos-*cbei2385*-g2-opt; (B)

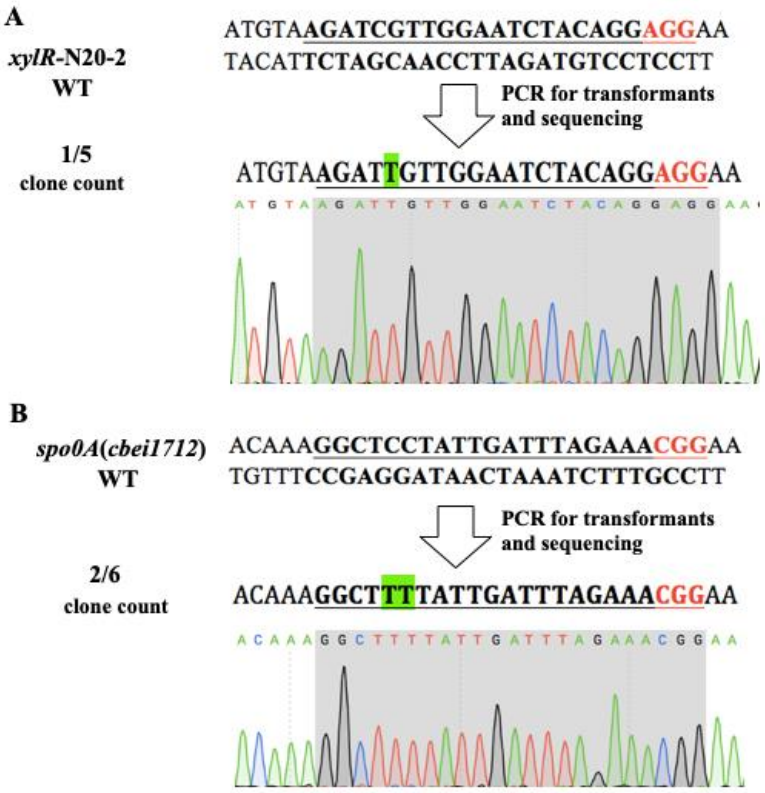
590 Sequence alignment of the *spo0A* pure mutant obtained by transformation with

591 pCBEclos-*spo0A*-g2-opt. The bolded and underlined sequence is the targeted N20 site,

592 the red underlined is the PAM sequence, and the mutated nucleotides are highlighted in

593 green.

Figure 5


A
xylR-N20-2
WT
ATGTAAGATCGTTGGAATCTACAGGAGGAA
TACATTCTAGCAACCTTAGATGTCCTCCTT
PCR for transformants
and sequencing
1/5
clone count
ATGTAAGATTGTTGGAATCTACAGGAGGAA
B
spo0A(cbei1712)
WT
ACAAAGGCTCCTATTGATTTAGAAACGGAA
TGTTTCCGAGGATAACTAAATCTTTGCCTT
PCR for transformants
and sequencing
2/6
clone count
ACAAAGGCTTTTATTGATTTAGAAACGGAA

606 **Supporting information, Table S1: Plasmids and strains used in this study.**

Strains or plasmids	Description	Source or reference
Strains		
C. beijerinckii NCIMB 8052	Wild type	NCIMB
E. coli DH5α	Commercial transformation host	GIBCO BRL, Life Technologies
Plasmids		
pXY1	pCB102, MLS^R, P_{thl} promotor,ColE1 origin , Amp^R, *E.coli-Clostridium* shuttle vector	This study
pCBEclos-*cbei1006*-g1	Derived from pXY1-Cas9n, pJ23119-sgRNA1-*cbei1006*, P_{thl}-rAPOBEC1-XTEN Cas9n-UGI	This study
pCBEclos-*cbei1006*-g2	Derived from pCBEclos-*cbei1006*-g1, pJ23119-sgRNA2-*cbei1006*	This study
pCBEclos-*cbei1006*-g3	Derived from pCBEclos-*cbei1006*-g1, pJ23119-sgRNA3-*cbei1006*	This study
pCBEclos-*cbei1006*-g1-opt	Derived from pCBEclos-*cbei1006*-g1, P_{thl}-rAPOBEC1(optimized)-XTEN Cas9n-UGI(optimized)	This study
pCBEclos-*cbei4456*-g1-opt	Derived from pCBEclos-*cbei1006*-g1-opt, P_{thl}-rAPOBEC1(optimized)-XTEN Cas9n-UGI(optimized)	This study
pCBEclos-*cbei2385*-g1-opt	Derived from pCBEclos-*cbei1006*-g1-opt, P_{thl}-rAPOBEC1(optimized)-XTEN Cas9n-UGI(optimized)	This study
pCBEclos-*cbei2385*-g2-opt	Derived from pCBEclos-*cbei1006*-g1, P_{thl}-rAPOBEC1(optimized)-XTEN Cas9n-UGI(optimized)	This study
pCBEclos-*cbei1712*-opt	Derived from pCBEclos-*cbei1006*-g1-opt, pJ23119-sgRNA-*cbei1712*	This study

607

608

609

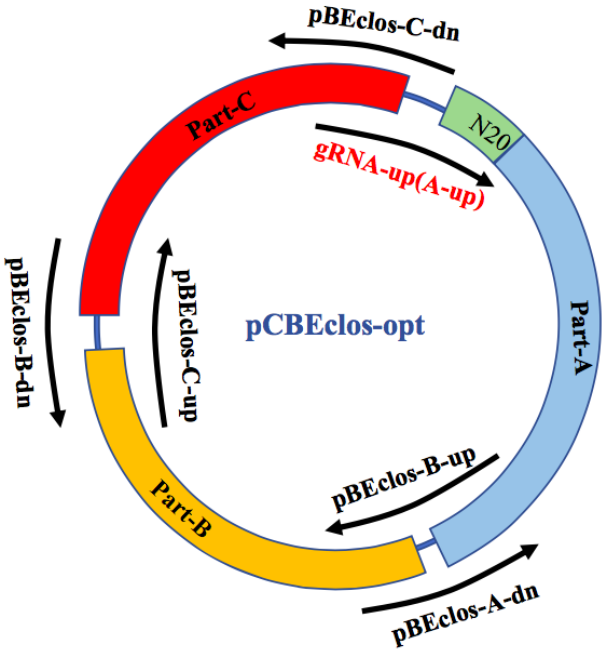
610 **Supporting information, Table S2: Oligonucleotides used in this study.**

Oligos	Sequence (5'→3')
cbei1006-gRNA1-up1	aggtataatactagt**attctccaagtcttcttaac**gttttagagctagaaatagcaagt
cbei1006-gRNA1-up2	aatgtgctgcattgacagctagctcagtcctaggtataatactagtattctccaagtct
BE-gRNA-dn	ctattattatttttatcaatatattttgttaaaaaggtaccaaaaaagcaccgactcgg
BE-P_{thl}-up	agtcggtgctttttttggtacctttttaacaaaatatattgataaaaataataatagtgg
BE-P_{thl}-dn(apo-hm)	gccactgggccagtctctgagctcatggatcctctaactaacctcctaaattttgatac
Apobec1-hm-up	gttaccccgtatcaaaatttaggaggttagttagaggatccatgagctcagagactggc
Apobec1-hm-dn	cgctatttgtgccgatagctaagcctattgagtatttcttatcactttcgggtgtggcg
Cas9nclos-up	cccgggacctcagagtccgccacacccgaaagtgataagaaatactcaataggcttagc
Cas9nclos-dn	tccttttcaataatatctgacagattagtagaaccaccagagtcacctcctagctgact
UGI-hm-up	tgaaacacgcattgatttgagtcagctaggaggtgactctggtggttctactaatctgt
UGI-hm-dn	gtcacgacgttgtaaaacgacggccagtgaattcccgggttaagaaccaccagagagca

cbei1006-gRNA2-up(A-up)	attgacagctagctcagtcctaggtataatactagt**aacttccgccattgtaacta**gttttagagctagaaatagcaag
cbei1006-gRNA3-up(A-up)	attgacagctagctcagtcctaggtataatactagt**ttgtgccatagttacaatgg**gttttagagctagaaatagcaag
pBEclos-A-dn	ttgactacttcttcacttgga
pBEclos-B-up	gttctgataaaaatcgtggtaaa
pBEclos-B-dn	atcctttgatcttttctacgg
pBEclos-C-up	taacgtgagttttcgttcca
pBEclos-C-dn	actagtattatacctaggactgag
Apobec1-opt-up	ttaccccgtatcaaaatttaggaggttagttagaggatccatgtcaagtgaaacaggac
Apobec1-opt-dn	tatttgtgccgatagctaagcctattgagtatttcttatcagattcaggagttgcagat
Cas9nclos-up(for opt)	ccaggaacatcagaatctgcaactcctgaatctgataagaaatactcaataggcttagc
Cas9nclos-dn(for opt)	tctttttctattatatctgaaagatttgttgatcctccactgtcacctcctagctgact
UGI-opt-up	tttatgaaacacgcattgatttgagtcagctaggaggtgacagtggaggatcaacaaat
UGI-opt-dn	cgacgttgtaaaacgacggccagtgaattcccgggttatgatcctccagataacatttt
cbe44565-gRNA1-up(A-up)	attgacagctagctcagtcctaggtataatactagt**cttattcccatttcttctttg**ttttagagctagaaatagcaag
cbei2385-gRNA1-up(A-up)	attgacagctagctcagtcctaggtataatactagt**gcttcattatctacatatac**gttttagagctagaaatagcaag
cbei2385-gRNA2-up(A-up)	attgacagctagctcagtcctaggtataatactagt**agatcgttggaatctacagg**gttttagagctagaaatagcaag
cbei2385-verf-up	ttgatagaagtaaatcacagtaaaataaaag
cbei2385-verf-dn	gaagcatacacatctatgaattctc
cbei1712-gRNA-up(A-up)	attgacagctagctcagtcctaggtataatactagt**ggctcctattgatttagaaa**gttttagagctagaaatagcaag
cbei4456-verf-up	gggttacataaaggccct
cbei4456-verf-dn	ttaaactctagaacaagaatctctaaca
cbei1006-verf-up	acgagattataggaataatataaattgatc
cbei1006-verf-dn	tcacagtcctgagaaacatatat
cbei1712-verf-up	atacaatgcaattggaaaaggt
cbei1712-verf-dn	atttgttggcttacctttatcat

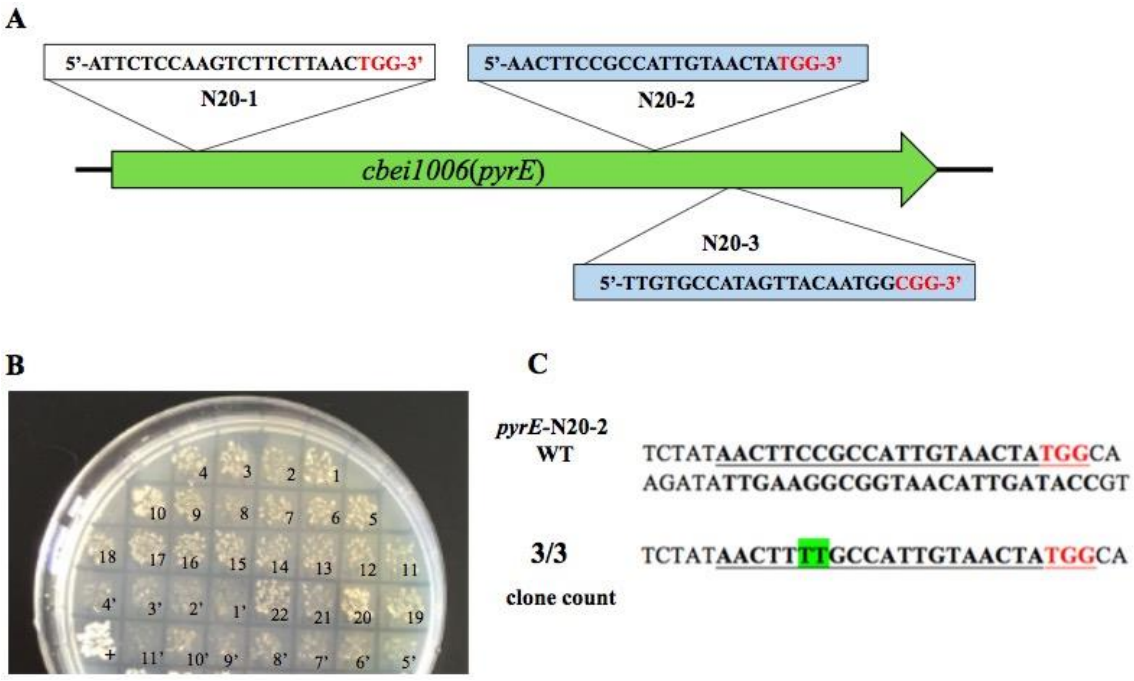
The bolded and underlined sequence represent the target sites used in base editing.

Supporting information, Figure. S1: Schematic for construction of pCBEclos series plasmids.



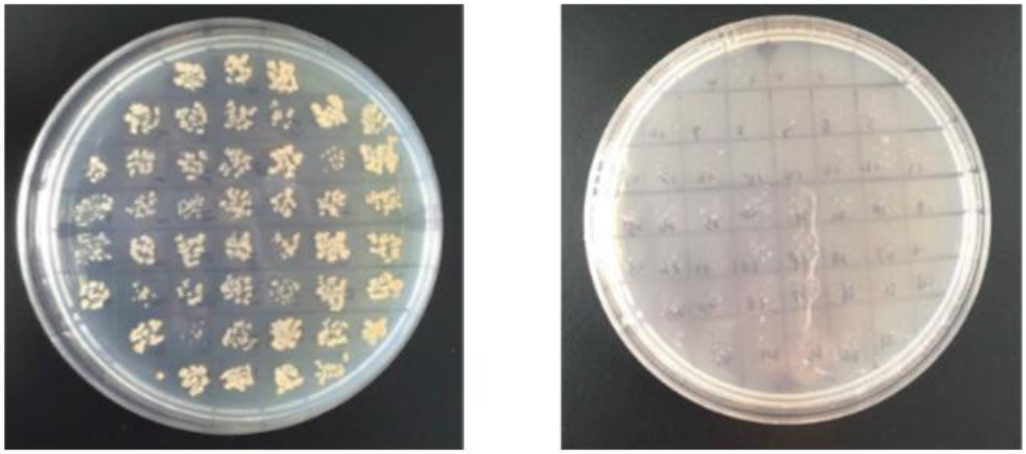
Primers pBEclos-B-up/pBEclos-B-dn, pBEclos-C-up/pBEclos-C-dn are used to amplify the universal fragments BEclos-B-opt(part B), BEclos-C-opt(part C). Only the part A is changed for the construction of new plasmid by the primer gRNA-up(A-up) and the universal primer pBEclos-A-dn. Then, part A, part B and part C are assembled to generated the new base editing plasmid.

Supporting information, Figure. S2: Target sites on *pyrE* gene were changed to test the pCBEclos system.



(A) The sequence of target sites in the *pyrE* gene; (B) *C. beijerinckii* were spread on plates containing 5-FOA after transformation with plasmid pCBEclos-*cbei1006*-g2 and pCBEclos-*cbei1006*-g3; Strains 1-22 are the transformants of plasmid pCBEclos-*cbei1006*-g2, while strains 1'-11' are the transformants of plasmid pCBEclos-*cbei1006*-g3; "+" represents the positive control; (C) 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.

Supporting information, Figure. S3: Clearance of plasmid pCBEclos-*cbei1006*-g1-opt.



638

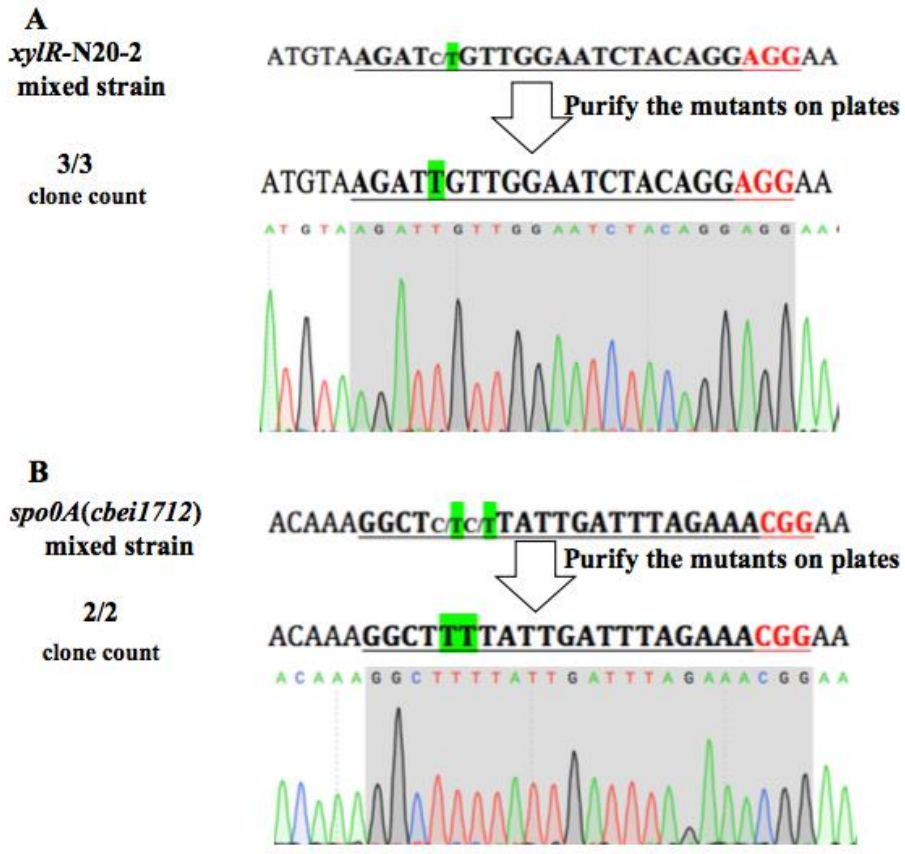
639

640

641

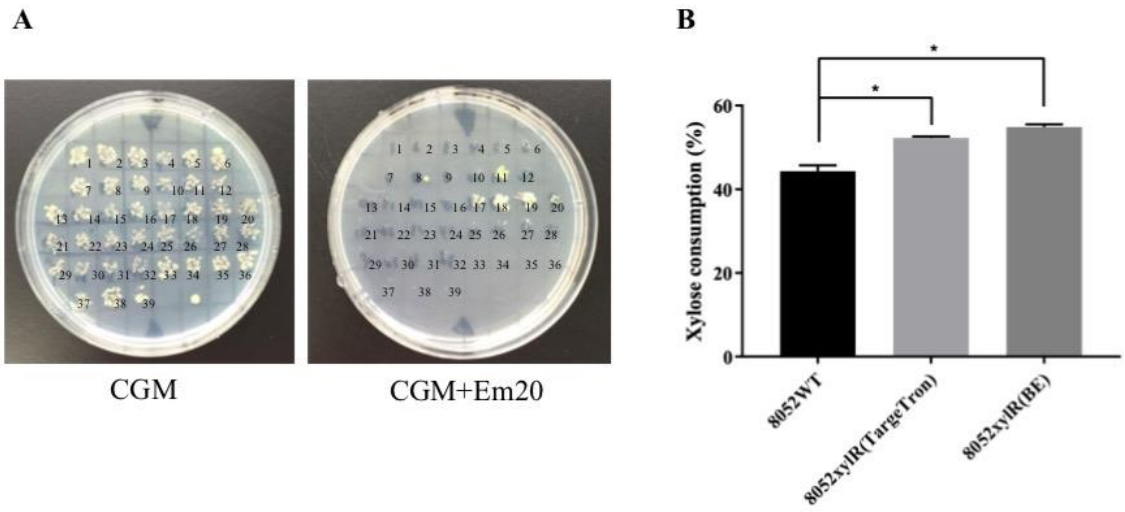
642 **Supporting information, Figure. S4: Purification of the mixed *xylR* and *spo0A***

643 **mutants on plates.**



(A) Sequence alignment of the pure *xylR* mutants after streaking the mixed strain on plate. (B) Sequence alignment of the pure *spo0A* mutants after streaking the mixed strain on plate.

Supporting information, Figure. S5: Clearance of plasmid pCBEclos-*cbei2385*-g2-opt and the xylose consumption of 8052WT, 8052*xylR*(TargeTron) and 8052*xylR*(BE).



653

654 (A) Clearance of plasmid pCBEclos-*cbei2385*-g2-opt. (B) Xylose consumption of

655 strains 8052WT, 8052*xylR*(TargeTron) (*xylR* was disrupted by TargeTron technology)

656 and 8052*xylR*(BE) (*xylR* was disrupted by pCBE-opt) in XHP2 medium containing 60

657 g/l D-xylose. Samples were taken after 72 h of fermentation. Fermentations were

658 performed in triplicate.

659

660

661

662

663

664

665