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Short communication

Markerless genome editing in *Clostridium beijerinckii* using the CRISPR-Cpf1 system

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ABSTRACT

CRISPR-Cpf1 is a type V CRISPR system that has recently been exploited for genome engineering purposes. Compared to the well-known *Streptococcus pyogenes* CRISPR-Cas9 system, the effector protein Cpf1 recognizes T-rich protospacer-adjacent motif (PAM) instead of G-rich PAM (used by CRISPR-Cas9), which could offer a substantial expansion of the existing genetic toolbox for genome editing. In this study, we report the implementation of the *Acidaminococcus* sp. Cpf1 (AsCpf1) for markerless genome engineering in *Clostridium beijerinckii*, a prominent species for biosolvent production through the well-known Acetone-Butanol-Ethanol (ABE) pathway. A lactose inducible promoter was used to control the expression of AsCpf1 to decrease its toxicity, while a constitutive small RNA promoter was employed to drive the expression of pre-crRNA. A One-Step-Assembly (OSA) approach was employed to construct the CRISPR-Cpf1-based vector in one single step, which simplified and streamlined the plasmid construction process. Using the customized CRISPR-Cpf1 system, we successfully deleted *spo0A* and *pta* genes in *C. beijerinckii*, with an editing efficiency of up to 100%. Altogether, our results demonstrated the easy programmability and high efficiency of the CRISPR-Cpf1 system for versatile genome engineering purposes. This study provides valuable guidance and essential references for repurposing the CRISPR-Cpf1 system for genome engineering in other microorganisms.

Biofuels and biochemicals produced from renewable feedstocks offer the potential to address the concerns over the fossil fuel depletion and associated environmental problems. Acetone-butanol-ethanol (ABE) fermentation by solventogenic clostridia represents a promising route for renewable fuel and chemical production (Ren et al., 2016). The most eminent and widely implemented ABE producing strains are generally from four clostridial species, including *Clostridium acetobutylicum*, *C. beijerinckii*, *C. saccharobutylicum*, and *C. saccharoperbutylacetonicum* (Green, 2011). Although extensive research efforts have been invested to improve the ABE production in solventogenic clostridial strains, only very limited achievement has been realized so far. This is mainly because of the susceptibility of the host strain to butanol toxicity and the complicated metabolic regulation of ABE production pathways (Moon et al., 2016). Therefore, versatile genetic engineering tools and techniques are urgently needed to investigate the complex regulation of solventogenesis and construct desirable strains with enhanced capability for solvent production.

From > 20 years ago, tremendous attention has been paid to the development of the genetic engineering tools and techniques for *Clostridium*. In late 1990s, several transposons were employed to generate random mutagenesis in clostridia (Pyne et al., 2014). One of the advantages of this technique is that it is not limited by the unavailability of DNA sequence information; however, the positive mutation rate is generally low and therefore its success is highly dependent on efficient selection methods to screen desired mutants from large populations. ClosTron (or Targetron), which was developed based on the group II intron technology, is a relatively new genetic tool established in 2007 (Heap et al., 2007). Since then, it has been widely employed to various clostridial strains by the *Clostridium* community (Heap et al., 2010; Wang et al., 2013). Unlike the transposon technique, ClosTron can knock out site-specific genes by inserting an intron fragment into the selected chromosomal site. However, the targeting efficiency of ClosTron is generally low, which varies from less than 1% to approximately 60% (Pyne et al., 2014), making the mutant screening a really labor-intensive process in most cases. Furthermore, since the insertion site of ClosTron is predicted by a specific algorithm, ectopic intron

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insertions occasionally occurred for sequences with high similarity (Zhang et al., 2015). In addition, it is hard to find usable insertion sites for a particularly small gene (especially when the interesting gene is shorter than 400 bp). Homologous-recombination-based genome editing tools allow precise chromosomal gene deletion, replacement and insertion. Such tools have been developed for several clostridial strains with the assistance of positive and negative selection markers, such as *mazF*, *codA*, *pyrE*, and *tdk* (Pyne et al., 2014). Nevertheless, most counter-selection markers are limited in their application, as the functionality of these markers is highly dependent on the host strain.

Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) system is an RNA guided immune system in bacteria and archaea (Barrangou and Doudna, 2016). It has been recently exploited as high efficient genome editing tools in various organisms (Barrangou and Doudna, 2016). From 2015, successes have been successively reported for genome engineering in several clostridial strains using the *Streptococcus pyogenes* derived type II CRISPR-Cas9 (spCRISPR-Cas9) system (Wang et al., 2015, 2017). In the spCRISPR-Cas9 system, the maturation of precursor CRISPR RNA (pre-crRNA) is directed by the trans-activating crRNA (tracrRNA) and processed by double-stranded RNA-specific ribonuclease RNase III and Cas9 protein (Deltcheva et al., 2011). The complex of Cas9, crRNA and tracrRNA is then guided by crRNA to the target site containing a signature protospacer-adjacent motif (PAM) “NGG” (N represents any nucleotide) at the 3′-end of the protospacer, and induces a site-specific double-strand breakage. A chimeric guide RNA (gRNA) has been created (combining features of both crRNA and tracrRNA and thus simplifying the overall configuration of spCRISPR-Cas9) and extensively employed to complex with Cas9 for the cleavage of the target DNA (Jinek et al., 2012). Indeed, the spCRISPR-Cas9 system offers a highly specific and relatively easy programmable genetic tool for genome editing in *Clostridium*. Recently, another type V CRISPR-Cpf1 system has also been characterized and engineered for genome editing purposes (Zetsche et al., 2017). Different from the spCRISPR-Cas9 system, the Cpf1 alone is responsible for the maturation of pre-crRNA (Zetsche et al., 2017). Cpf1 can be guided by the single-strand mature crRNA to recognize the sequence "TTTN-N23″ (N represents any nucleotide; TTTN represents the PAM located at 5′-end of the protospacer; N23 represents a 23-bp protospacer) and then cleave the target DNA to a 5-nt staggered cut distal. Therefore, the CRISPR-Cpf1 system has the advantages over spCRISPR-Cas9 system in genome editing for AT-rich microorganisms, such as *Clostridium*, which could offer more candidate sites for recognizing and cleaving. Compared to the spCRISPR-Cas9 system, the CRISPR-Cpf1 system has a compacter configuration with the effector protein (Cpf1) and RNA guide (thus potentially enabling much easier programmability), and has exhibited higher targeting efficiency and capability for genome engineering in specific strains (Jiang et al., 2017; Zetsche et al., 2017). Despite such advantages over CRISPR-Cas9, till now, the application of CRISPR-Cpf1 for genome engineering has only been demonstrated in very limited numbers of bacterial strains (Bayat et al., 2018). Therefore, in this study, we aimed to develop an efficient genome engineering tool for AT-rich *C. beijerinckii* based on the CRISPR-Cpf1 system, providing essential references for the broader application of CRISPR-Cpf1 in AT-rich microorganisms with underdeveloped genetic engineering tools.

Vector pJZ159-AsCpf1 was firstly constructed as the mother vector for generating the customized CRISPR-Cpf1 vector for targeted genome editing (Fig. 1A, Table 1 and Appendix S1). The functional expression of Cpf1 and pre-crRNA is essential for the CRISPR-Cpf1 system to recognize and cleave the specific target site. However, the simultaneous co-expression of Cpf1 and crRNA is usually highly toxic to the host cell and leads to cell death and unsuccessful transformation (Zhang et al., 2018). Thus, a lactose inducible promoter (P*lac*) was selected to drive the expression of AsCpf1 in pJZ159-AsCpf1 to abate the strong toxicity of the nuclease (Wang et al., 2016). Based on pJZ159-AsCpf1, the customized CRISPR-Cpf1-based vectors can be further constructed through a One-Step-Assembly (OSA) approach (Fig. 1B & Appendix S1). Using the OSA approach, the pre-crRNA expression cassette, including small RNA promoter and pre-crRNA (P*sRNA*-pre-crRNA), was clone into pJZ159-AsCpf1 along with two homology arms in one single step. To realize OSA, the homology arms for the target gene deletion were placed immediately downstream of the pre-crRNA expression cassette which potentially led to co-expression of pre-crRNA and homology arms. However, Cpf1 has the capability to process the pre-crRNA (repeat-spacer-repeat) to mature crRNA (repeat-spacer) (Zetsche et al., 2017), and thus the mature crRNA can be split from the whole transcript of pre-crRNA and arms (Fig. 1C). Usually, the construction of the CRISPR-Cas9-based plasmid needs at least two steps to assemble gRNA and two homology arms (Wang et al., 2016, 2017). Therefore, the OSA approach described herein is more convenient and simplifies the vector construction process.

In order to demonstrate the functionality of CRISPR-Cpf1 in *C. beijerinckii*, the *spo0A* gene which is the master regulator for sporulation was selected as the first target gene to delete. Plasmid pJZ160 used for *spo0A* deletion was constructed via OSA and transformed into *C. beijerinckii* NCIMB 8052 under the selection of erythromycin (Erm). Both transformations with pJZ160 and the mother vector (pJZ159-AsCpf1) achieved similar transformation efficiencies ($1.3 \pm 0.3 \times 10^2$ and $1.4 \pm 0.1 \times 10^2$ CFU/μg-plasmid, respectively). The obtained transformants were cultivated in Tryptone-Glucose-Yeast extract (TGY) liquid medium containing 30 μg/mL Erm (TGYE), and then spread onto TGY plates supplemented with 40 mM lactose and 30 μg/mL Erm (TGYLE) to induce the expression of AsCpf1 nuclease (Fig. 1D). Twenty-four resultant colonies were randomly picked and screened by colony PCR to detect the *spo0A* deletion. Results showed that all tested colonies were Δ*spo0A* mutants, representing an editing efficiency of 100% (Fig. 1E & Table 2). In addition to *spo0A*, the *pta* gene (encoding phosphotransacetylase) involved in acetic acid production was selected as another target gene to delete. Plasmid pJZ161 designed for *pta* deletion was successfully transformed into *C. beijerinckii* NCIMB 8052 with transformation efficiency of $1.1 \pm 0.4 \times 10^2$ CFU/μg-plasmid, and the resultant transformants were then spread onto TGYLE plates. Subsequently, mutant screening was performed with colony PCR. Similar as for *spo0A* deletion, 100% editing efficiency was observed for *pta* deletion (Fig. 1E & Table 2). The mutations in both Δ*spo0A* and Δ*pta* mutants were further verified by Sanger sequencing (data not shown). These results demonstrated that efficient genome editing can be achieved in *C. beijerinckii* using the inducible CRISPR-Cpf1 system.

The *spo0A* gene and *pta* gene in *C. beijerinckii* have been knocked out previously using various genetic tools, including ClosTron and CRISPR-Cas9 (Table 2) (Heap et al., 2010; Wang et al., 2013, 2015, 2016). For the approach with ClosTron, the vector construction could be completed in one step which was considerably faster than the approach with CRISPR-Cas9. However, the editing efficiency of ClosTron was relatively low. For *spo0A* and *pta* deletion respectively, editing efficiencies of 25% and 28.6% were observed (Heap et al., 2010; Wang et al., 2013). In addition, the gene knockout with ClosTron is essentially a disruption of the gene function through insertion; polar effects can potentially observed (Heap et al., 2007). In contrast, up to 100% efficiencies were obtained for the clean deletion of *spo0A* or *pta* when CRISPR-Cas9 or CRISPR-Cpf1 was employed. Taken together, these data demonstrated that the CRISPR-Cpf1 system developed in this study is a powerful genome engineering tool with easy programmability and high efficiency.

In summary, we successfully exploited the CRISPR-Cpf1 system for genome engineering in *C. beijerinckii*. By using the OSA approach, the construction of the CRISPR-Cpf1 plasmid was simplified and streamlined. With a lactose inducible promoter for AsCpf1 expression, markerless genome editing was achieved with efficiency up to 100%. Overall, the CRISPR-Cpf1 system developed in this study extends the existing genetic toolbox for genome engineering in *C. beijerinckii*. The CRISPR-Cpf1 platform developed herein can be easily adapted to other

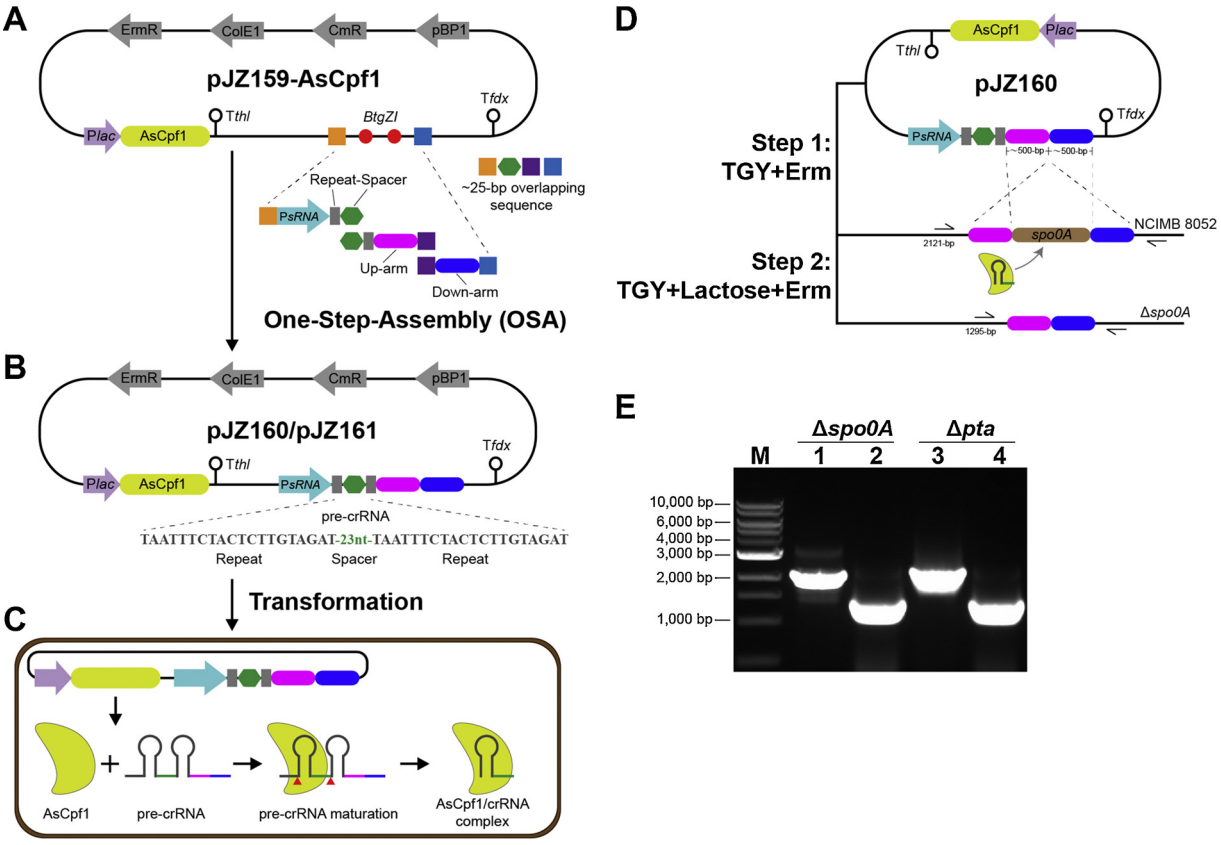

Fig. 1. The CRISPR-Cpf1 mediated genome editing in *C. beijerinckii*. (A) Construction of the mother vector pJZ159-AsCpf1. The lactose inducible promoter (P*lac*) was used to drive the expression of AsCpf1. Two *BtgZI* sites were inserted to facilitate the construction of the customized CRISPR-Cpf1 plasmids for targeted genome editing. (B) Construction of plasmids pJZ160/pJZ161 using the One-Step-Assembly (OSA) approach. The pre-crRNA expression cassette (P*sRNA*-pre-crRNA), upstream homology arm (Up-arm) and downstream homology arm (Down-arm) were cloned into *BtgZI* sites of pJZ159-AsCpf1 in one step using Gibson Assembly. (C) The maturation of pre-crRNA. Although the two homology arms are co-expressed with pre-crRNA, the Cpf1 nuclease has the capability of processing pre-crRNA (repeat-spacer-repeat) to mature crRNA (repeat-spacer). (D) Schematic diagram illustrating the workflow of *spo0A* deletion using the CRISPR-Cpf1 system. The ~500-bp Up-arm and ~500-bp Down-arm were used for the deletion of *spo0A* through homologous recombination. The Δ*spo0A* mutant was obtained after two screening steps. In Step 1, plasmid pJZ160 was transformed into *C. beijerinckii* under the selection of Erm. In Step 2, AsCpf1 was induced to express with lactose; the AsCpf1/crRNA complex was formed, which could recognize the site-specific *spo0A* gene target and induce a double-strand breakage. So the wild type background cells were eliminated, leaving only the Δ*spo0A* mutant. Pairs of half arrows and the numbers in the figure indicate the colony PCR flanking regions and the size of corresponding amplicons, respectively. (E) Diagnostic PCR verification of Δ*spo0A* and Δ*pta* mutants. M, NEB 1-kb DNA marker (with numbers on the left representing the corresponding band size); Lane 1, unedited *C. beijerinckii* (wild type, 2,121-bp); Lane 2, mutant with *spo0A* deletion (Δ*spo0A*, 1,295-bp); Lane 3, unedited *C. beijerinckii* (wild type, 2,261-bp); Lane 4, mutant with *pta* deletion (Δ*pta*, 1,240-bp).

Table 1
Bacterial strains and plasmids used in this study.

Strains/ Plasmids	Relevant characteristic	Sources
Strains		
E. coli		
NEB Express	*fhuA2 [lon] ompT gal sulA11 R(mcr-73::miniTn10–Tet^S)2 [dcm] R(zgb-210::Tn10–Tet^S) endA1 Δ(mcrC-mrr)114::IS10*	New England BioLabs
C. beijerinckii		
NCIMB 8052	ATCC 51743, wild type stain	NCIMB
Δ*spo0A*	Derived from NCIMB 8052, with *spo0A* gene deleted	This work
Δ*pta*	Derived from NCIMB 8052, with *pta* gene deleted	This work
Plasmids		
pMTL82151	*E. coli-Clostridium* shuttle vector, pBP1 ori, Cm^R, ColE1 ori, TraJ	Heap et al. (2009)
pYW34-*BtgZI*	CAK1 ori, ColE1 ori, Amp^R, Erm^R, P*lac*-Cas9, gRNA	Wang et al. (2016)
pDEST-hisMBP-AsCpf1-EC	M13 ori, Amp^R, AsCpf1 (*E.coli* codon optimized)	Hur et al. (2016)
pWH34-AsCpf1	pMTL82151 derivative; P*lac*-AsCpf1-T*thl*	This work
pJZ159-AsCpf1	pWH34-AsCpf1 derivative; contain additional Erm marker	This work
pJZ160	pJZ159-AsCpf1 derivative; 23nt-spacer targeting on *spo0A* gene of NCIMB 8052; two homology arms for gene editing	This work
pJZ161	pJZ159-AsCpf1 derivative; 23nt-spacer targeting on *pta* gene of NCIMB 8052; two homology arms for gene editing	This work

Table 2
Comparison of the mechanism, plasmid construction process, and genome editing efficiencies of various genetic tools developed for *C. beijerinckii*.

Genetic tool	Mechanism	Target gene	Plasmid construction	Editing efficiency	Reference
ClosTron/TargeTron	Insertion disruption	*spo0A*	One step	25% (1/4)	Heap et al. (2010)
		pta	One step	28.6% (2/7)	Wang et al. (2013)
CRISPR-Cas9	Clean deletion	*spo0A*	Two steps	100% (5/5)	Wang et al. (2015)
		pta	Two steps	100% (8/8)	Wang et al. (2016)
CRISPR-Cpf1	Clean deletion	*spo0A*	One step	100% (24/24)	This work
		pta	One step	100% (24/24)	This work

microorganisms.

Conflict of interest

The authors declare no financial or commercial conflict of interest.

Author contribution statement

Y.W., J.Z., and W.H. devised the research; J.Z., W.H., W.Z., and P.W. performed the experiments; Y.W., W.H. and J.Z. wrote the pape.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jbiotec.2018.07.040.

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