Received: 3 April 2020 | Revised: 30 April 2020 | Accepted: 19 May 2020

DOI: 10.1002/bit.27435

COMMUNICATION TO THE EDITOR

BIOTECHNOLOGY AND BIOENGINEERING WILEY

Enhancing plasmid transformation efficiency and enabling CRISPR-Cas9/Cpf1-based genome editing in *Clostridium tyrobutyricum*

Jie Zhang[1,2] | Wei Hong[1,3] | Liang Guo[4] | Yifen Wang[1,5] | Yi Wang[1,5]

[1]Department of Biosystems Engineering, Auburn University, Auburn, Alabama

[2]Key Laboratory for Feed Biotechnology of the Ministry of Agriculture and Rural Affairs, Feed Research Institute, Chinese Academy of Agricultural Sciences, Beijing, China

[3]Key Laboratory of Endemic and Ethnic Diseases, Guizhou Medical University, Ministry of Education, Guiyang, China

[4]College of Environmental Science and Engineering, Ocean University of China, Qingdao, China

[5]Center for Bioenergy and Bioproducts, Auburn University, Auburn, Alabama

Correspondence
Yi Wang, Department of Biosystems Engineering, Auburn University, 350 Mell Street, Auburn, AL 36849.
Email: yiwang3@auburn.edu

Funding information
National Institute of Food and Agriculture, Grant/Award Number: 2018-67021-27715; USDA-NIFA, Grant/Award Number: ALA014-1017025; Ocean University of China-Auburn University (OUC-AU) Grants; Auburn University Intramural Grants Program (IGP); Alabama Agricultural Experiment Station

Abstract

Clostridium tyrobutyricum ATCC 25755 is known as a natural hyper-butyrate producer with great potentials as an excellent platform to be engineered for valuable biochemical production from renewable resources. However, limited transformation efficiency and the lack of genetic manipulation tools have hampered the broader applications of this micro-organism. In this study, the effects of Type I restriction-modification system and native plasmid on conjugation efficiency of *C. tyrobutyricum* were investigated through gene deletion. The deletion of Type I restriction endonuclease resulted in a 3.7-fold increase in conjugation efficiency, while the additional elimination of the native plasmid further enhanced conjugation efficiency to $6.05 \pm 0.75 \times 10^3$ CFU/ml-donor, which was 15.3-fold higher than the wild-type strain. Fermentation results indicated that the deletion of those two genetic elements did not significantly influence the end-products production in the resultant mutant Δ*RMI*Δ*NP*. Thanks to the increased conjugation efficiency, the CRISPR-Cas9/Cpf1 systems, which previously could not be implemented in *C. tyrobutyricum*, were successfully employed for genome editing in Δ*RMI*Δ*NP* with an efficiency of 12.5–25%. Altogether, approaches we developed herein offer valuable guidance for establishing efficient DNA transformation methods in nonmodel micro-organisms. The Δ*RMI*Δ*NP* mutant can serve as a great chassis to be engineered for diverse valuable biofuel and biochemical production.

KEYWORDS

Clostridium tyrobutyricum, conjugation, CRISPR-Cas9/Cpf1, genome engineering, restriction-modification system

1 | INTRODUCTION

Clostridium tyrobutyricum is a rod-shaped, Gram-positive, obligate anaerobe that produces butyrate and acetate as its major fermentation products. *C. tyrobutyricum* has received much attention as a potential microbial cell factory for high-efficiency butyrate production using both hexose and pentose sugars as the substrate (Fu et al., 2017). Recently, this organism has been engineered for high-level *n*-butanol (or simply butanol) production by redirection of carbon flow from butyrate to butanol (Zhang, Zong, Hong, Zhang, & Wang, 2018). The replacement of butyrate:acetate CoA transferase gene (*cat1*), the key gene responsible for butyrate production, with an alcohol dehydrogenase gene (*adhE2*) enabled the production of very high titer of 26.2 g/L butanol in the batch

Jie Zhang and Wei Hong contributed equally to this study.

fermentation (Zhang et al., 2018). Although *C. tyrobutyricum* is believed to have great potential for the production of various biochemicals at industrial scales, the genetic tools are still limited for this bacterium and therefore genetic engineering of *C. tyrobutyricum* is time consuming and laborious, mainly due to the low transformation efficiency.

For genetic engineering of *Clostridium* strains, usually the first and most difficult step is the development of an efficient method to introduce the foreign DNA into host cells. Currently, there are two primary methods that can be used to transform plasmids into *Clostridium*: electrotransformation and conjugation (Wang et al., 2016; Woods et al., 2019). The existence of active restriction-modification (RM) systems in the host which can recognize and eliminate the improperly methylated plasmid is the key factor responsible for impeding the transformation. Such restriction barriers can be overcome by appropriate methylation to protect the transforming plasmid DNA. Methylation can be carried out in vitro using commercially available methyltransferase or in vivo through expressing the corresponding methyltransferase in *Escherichia coli*, for example (Jennert, Tardif, Young, & Young, 2000; Pyne, Moo-Young, Chung, & Chou, 2013). Furthermore, deletion of restriction endonuclease is an alternative option that can construct a mutant accepting unmethylated foreign DNA (Q. Li et al., 2016). Conjugative plasmid transfer, which involves cell-to-cell transferring of plasmid DNA from donor cells to recipient cells, has been proved to be an efficient transformation method for *Clostridium* species, supposedly due to the ability of foreign DNA to escape from host's RM system during conjugation. However, a recent study showed that different methylation types of donor *E. coli* strains also had impact on conjugation efficiency (Woods et al., 2019). Using NEB Express (Dam^+, Dcm^-), instead of the traditionally used CA434 (Dam^+, Dcm^+), as conjugal donor *E. coli* strain, the conjugation efficiency increased greater than 10-fold for *C. autoethanogenum*, *C. sporogenes*, and *C. difficile*. Other than the RM system, to improve the transformation efficiency, there are still many other factors that need to be taken into consideration, such as electrical parameters for electrotransformation and physiological state of donor cells for conjugation (A. Li et al., 2020; Sysoeva, Kim, Rodriguez, Lopatkin, & You, 2020).

Many *Clostridium* strains have been revealed to harbor extrachromosomal elements, including native plasmids. In some instances, the native plasmid which carries essential genetic information could participate in some microbial metabolism. For example, native megaplasmid pSOL1 plays a very important role in solvent production in *C. acetobutylicum* ATCC 824 (Cornillot, Nair, Papoutsakis, & Soucaille, 1997). Elimination of plasmid pSOL1 in *C. acetobutylicum* leads to the loss of capacity to produce acetone-butanol-ethanol. In addition to cell biosynthetic pathways, the native plasmid sometimes has function related to other cell traits. Gu et al. (2019) cured the native megaplasmid Csp_135p in *C. saccharoperbutylacetonicum* N1-4 (HMT) using CRISPR-Cas9. The resultant mutant exhibited better plasmid stability and higher plasmid transformation efficiency, which provides us with another clue that could be applied to increase the transformation efficiency.

Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) system, including Type II CRISPR-Cas9 system and Type V CRISPR-Cpf1 system, have been extensively studied and repurposed as versatile genome editing tools for various organisms (Barrangou & Doudna, 2016). Recently, we have tried to explore CRISPR-Cas9/Cpf1 systems for genome editing in *C. tyrobutyricum*. However, no success was obtained despite multiple attempts, primarily due to the high toxicity of Cas9/Cpf1 nuclease and the low DNA transformation efficiency of *C. tyrobutyricum* (Zhang et al., 2018). Although we have successfully developed the genome editing tool in *C. tyrobutyricum* based on its endogenous Type I-B CRISPR-Cas system, the molecular mechanism of this system is still far from well-known compared to the CRISPR-Cas9/Cpf1 system, limiting its further application (Zhang et al., 2018). Therefore, in this study, we first aimed to boost the conjugation efficiency of *C. tyrobutyricum* by eliminating the RM system and native plasmid. Based on the mutant with enhanced transformation efficiency, we successfully developed the CRISPR-Cas9/Cpf1 systems for genome editing in *C. tyrobutyricum*, which extended the genetic toolbox in this organism.

The complete genome sequence of *C. tyrobutyricum* ATCC 25755, which contains 3,138 protein-coding genes, has been recently reported (Lee, Jang, Han, Kim, & Lee, 2016). By analyzing the genomic sequence in silico using REBASE (Roberts, Vincze, Posfai, & Macelis, 2015), eight putative RM systems were identified, including one putative Type I RM system and seven Type II RM systems (Table 1). The enzymes in *C. tyrobutyricum* RM systems with their hypothetical specificities were then predicted by protein BLAST (http://blast.ncbi.nlm.nih.gov/). However, only limited information can be obtained (Table 1). Generally, the functional subunits (REase, MTases, and S subunit for Type I RM system; REase and MTases for type II RM system) of one RM system are organized in the same operon or adjacent to each other on the chromosome, which could facilitate the coexpression of different subunits and form the functional RM system. Based on such assumption, we predicted that the putative Type I RM system (CTK_C27570, SAM; CTK_C27600, MTase; CTK_C27610, S subunit; CTK_C27620, REase; CTK_C27630, MTase) is the most possible functional RM system in *C. tyrobutyricum*. Thus, this Type I RM system was selected for further testing in this study.

Type I system is the most complex RM system, consisting of three subunits and forming pentameric protein (2REases + 2MTases + S) (Loenen, Dryden, Raleigh, & Wilson, 2014). The 2MTases + S module acts as a methyltransferase, while the REase subunit is essential for the restriction endonuclease activity. Although the recognition sequence of Type I RM system in *C. tyrobutyricum* was unknown based on the protein sequence information, it can be reasonably assumed that deletion of REase subunit (CTK_C27620) could eliminate the restriction endonuclease activity and therefore increase the transformation efficiency. Usually, the deletion of restriction endonuclease could increase the electrotransformation efficiency (Cui et al., 2012); however, the effect of RM system deletion on conjugation efficiency has been rarely reported. CTK_C27620 was knocked out using the endogenous CRISPR-Cas

TABLE 1 Restriction-modification systems in *Clostridium tyrobutyricum*

No.	Coordinates	Gene tag	Type	Function	Specificity (% sequence identity)
1	2751136-2752260	CTK_C27570	I	SAM	(*Clostridium kluyveri*; 91%)
	2753098-2754687	CTK_C27600		MTase	Unknown (*Clostridium carboxidivorans*; 93%)
	2754899-2756116	CTK_C27610		S subunit	Unknown (*Clostridium magnum*; 48%)
	2756189-2759398	CTK_C27620		REase	Unknown (*Clostridium liquoris*; 93%)
	2759395-2759970	CTK_C27630		MTase	Unknown (*Clostridium luticellarii*; 94%)
2	910111-911817	CTK_C08830	II	MTase	Unknown (*C. luticellarii*; 55%)
3	1038023-1038778	CTK_C10400	II	MTase	Unknown (*C. luticellarii*; 90%)
4	1090314-1091069	CTK_C11240	II	MTase	Unknown (*C. luticellarii*; 90%)
5	2264587-2265141	CTK_C22880	II	MTase	MT-A70: GATC (*Clostridium symbiosum*; 75%)
6	2300988-2302232	CTK_C23300	II	MTase	Unknown (*Clostridium ljungdahlii*; 95%)
7	2320058-2320915	CTK_C23510	II	MTase	Unknown (*Haloimpatiens lingqiaonensis*; 94%)
	2320905-2321993	CTK_C23520		MTase	Unknown (*H. lingqiaonensis*; 92%)
8	2359550-2361205	CTK_C23910	II	MTase	Unknown (*C. kluyveri*; 54%)

Abbreviations: MTase, methyltransferase; REase, restriction endonuclease; SAM, s-adenosyl methionine protein.

system (Zhang et al., 2018; Figure S1a), resulting in mutant Δ*RMI* (RMI, Type I RM system). Conjugation efficiency of mutant Δ*RMI* was then tested using plasmid pMTL82151. The results showed that the conjugation efficiency of Δ*RMI* reached $1.45 \pm 0.15 \times 10^3$ CFU/ml-donor, which increased by 3.7-fold compared with that of the wild-type strain ($3.95 \pm 0.35 \times 10^2$ CFU/ml-donor; Table 2). These results suggested that the ability of *C. tyrobutyricum* to accept foreign DNA via conjugation was improved by deletion of the REase subunit.

Strain *C. tyrobutyricum* ATCC 25755 contains a 63-kbp native plasmid named pCTK01 (Lee et al., 2016). Seventy-eight genes are identified on pCTK01; however, few of them have clearly known functions which include those encoding putative conjugal plasmid transfer proteins (CTK_P00290-410; Table 3). With those proteins, native plasmid pCTK01 might have the capability of transferring itself to other micro-organisms. However, bacterial cells containing the conjugal plasmid transfer system could create a remarkable barrier, such as surface exclusion, against possible damaging conjugative transfer from other donor cells (Thomas & Nielsen, 2005). On the other hand, the large native plasmid is actually a big metabolic burden for the host cell. Therefore, deletion of this native plasmid may facilitate the growth of *C. tyrobutyricum* and further enhance the conjugation efficiency.

To delete pCTK01, vector pJZ154-P*lac*-34*NP* (NP, native plasmid) was constructed based on the endogenous CRISPR-Cas system which carried a synthetic CRISPR array targeting on pCTK01 (specifically on the gene encoding prevent-host-death family protein, CTK_RS15030) and contained no homology arms (Table S1). This vector was successfully transformed into mutant Δ*RMI*. Different from the gene deletion on the chromosome, homologous recombination was not involved when the native plasmid pCTK01 was targeted, leading to the breakage and loss of the plasmid (Figure S1b). The resultant mutant was designated as Δ*RMI*Δ*NP*. Conjugation test with plasmid pMTL82151 was then carried out. Results demonstrated that the conjugation efficiency of Δ*RMI*Δ*NP* reached $6.05 \pm 0.75 \times 10^3$ CFU/ml-donor, which increased by 4.2 and 15.3-folds compared with Δ*RMI* and the wild-type strain, respectively (Table 2), suggesting the elimination of potential barrier for conjugal plasmid transfer posed by the native plasmid. Furthermore, the growth of Δ*RMI*Δ*NP* was also improved compared with Δ*RMI* and the wild-type strain (Figure 1b), indicating the alleviation of metabolic burden for the host cell by deleting the large native plasmid.

C. tyrobutyricum is a well-known hyper-butyrate producer and recently has been engineered for efficient butanol production

TABLE 2 Summary of conjugation efficiency and genome editing efficiency for different plasmids used in *C. tyrobutyricum* and its derivatives

Plasmid	Strain	Conjugation efficiency (CFU/ml-donor)	Genome editing efficiency	Reference
pMTL82151	Wild-type	$3.95 \pm 0.35 \times 10^2$	–	This study
	Δ*RMI*	$1.45 \pm 0.15 \times 10^3$	–	This study
	Δ*RMI*Δ*NP*	$6.05 \pm 0.75 \times 10^3$	–	This study
pJZ23-Cas9-spo0A	Wild-type	0	–	Zhang et al. (2018)
	Δ*RMI*Δ*NP*	1.5 ± 0.5	25% (2/8)	This study
pJZ60-AsCpf1-spo0A	Wild-type	0	–	Zhang et al. (2018)
	Δ*RMI*Δ*NP*	40 ± 8	12.5% (1/8)	This study

TABLE 3 Putative genes on pCTK01 for conjugal plasmid transfer

No.	Coordinates	Gene tag	Function
1	22926-24950	CTK_P00290	MobA/MobL family protein (*Lactobacillus ultunensis*, 33%)
2	24986-25177	CTK_P00300	Replication protein (*Staphylococcus gallinarum*, 32%)
3	25339-26409	CTK_P00310	hypothetical protein
4	26411-26581	CTK_P00320	Ribbon-helix-helix domain-containing (DNA-binding) protein (*Clostridium kluyveri*, 75%)
5	26721-27059	CTK_P00330	Cag pathogenicity island, type IV secretory system (conjugal transfer protein; *Pontibacillus litoralis*, 51%)
6	27074-27478	CTK_P00340	FtsK/SpoIIIE family protein (conjugal transfer protein) (*Lactobacillus parabuchneri*, 39%)
7	27478-28086	CTK_P00350	Type IV secretory pathway, VirB4 component (*Bacillus camelliae*, 48%)
8	28097-30109	CTK_P00360	Type IV secretory pathway, VirB4 component (*Bacillus simplex*, 57%)
9	30097-31419	CTK_P00370	Conjugation protein *TraF* (*Staphylococcus aureus*, 30%)
10	31416-32372	CTK_P00380	Cell wall-associated hydrolase, invasion-associated protein (*Clostridium pasteurianum*, 55%)
11	32388-32969	CTK_P00390	hypothetical protein
12	32972-33436	CTK_P00400	hypothetical protein
13	33429-35060	CTK_P00410	Type IV secretion-system coupling protein virD4 (*Bacillus* sp. OV166, 67%)

(Zhang et al., 2018), suggesting that this strain is a robust workhorse for valuable biochemical production. There were possibilities that the capacity of Δ*RMI*Δ*NP* for biochemical production were impaired due to the deletion of the Type I restriction endonuclease and native plasmid. Batch fermentations were thus carried out with wild-type strain, Δ*RMI*, and Δ*RMI*Δ*NP* to testify whether this was the case. As seen in Figure 1c,d, the deletion of Type I restriction endonuclease did not influence the acetate and butyrate production. Interestingly,

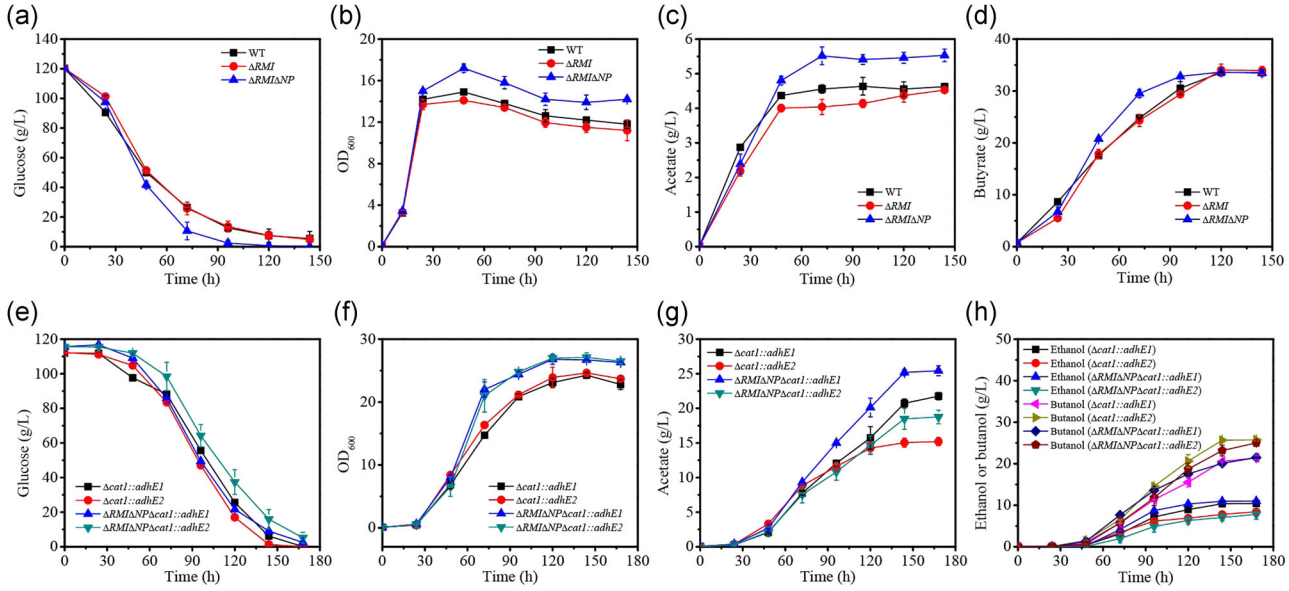

FIGURE 1 Batch fermentation profiles of *Clostridium tyrobutyricum* wild-type strain, Δ*RMI*, and Δ*RMI*Δ*NP* at 37°C (a–d) and Δ*cat1*::*adhE1*, Δ*cat1*::*adhE2*, Δ*RMI*Δ*NP*Δ*cat1*::*adhE1* and Δ*RMI*Δ*NP*Δ*cat1*::*adhE2* at 20°C (e–h). (a, e) Glucose consumption; (b, f) cell growth; (c, g) acetate production; (d) butyrate production; (h) ethanol and butanol production. Reported values are based on three independent replicates [Color figure can be viewed at wileyonlinelibrary.com]

the deletion of native plasmid slightly increased the acetate production, while did not significantly influence the butyrate production. Moreover, to verify whether the genetic manipulation would influence the production of other biochemicals (particularly butanol), mutant Δ*RMI*Δ*NP* was engineered to produce butanol by replacing the butyrate biosynthesis pathway with the butanol producing pathway according to our previous study (Zhang et al., 2018), generating mutant Δ*RMI*Δ*NP*Δ*cat1*::*adhE1* and Δ*RMI*Δ*NP*Δ*cat1*::*adhE2*. Since lower temperature could reduce butanol toxicity and enhance butanol production (Zhang et al., 2018), batch fermentations were then carried out at 20°C with Δ*RMI*Δ*NP*Δ*cat1*::*adhE1* and Δ*RMI*Δ*NP*Δ*cat1*::*adhE2* to test their ethanol and butanol production, using Δ*cat1*::*adhE1* and Δ*cat1*::*adhE2* as the control, respectively. Results showed that ethanol and butanol production were at the similar levels before and after the deletion of Type I restriction endonuclease and native plasmid (Figure 1h). However, the cell growth and acetate production were slightly enhanced after the deletion of the two genetic elements, which were coincident with the result for acid production (Figure 1). Collectively, the deletion of RM system and native plasmid did not significantly influence the end-products production capacity of *C. tyrobutyricum*, suggesting that the mutant Δ*RMI*Δ*NP* with higher conjugation efficiency can serve as a better chassis strain (than the wild-type strain) to be engineered for biofuel and biochemical production.

Recently, the Type II CRISPR-Cas9 and Type V CRISPR-Cpf1 systems have been employed as highly efficient genome editing tools in several Gram-positive bacteria (Hong, Zhang, Cui, Wang, & Wang, 2018; Jiang et al., 2017; Wang et al., 2015). However, previously the exploration of these systems for genome editing in *C. tyrobutyricum* was failed, due to the low DNA transformation efficiency (Zhang et al., 2018). The obtaining of mutant Δ*RMI*Δ*NP* offered us an opportunity to establish these systems in *C. tyrobutyricum*. Therefore, Cas9-based plasmid pJZ23-Cas9-*spo0A* and Cpf1-based plasmid pJZ60-AsCpf1-*spo0A* (Zhang et al., 2018), which were used for *spo0A* (the master regulator for sporulation) deletion and previously could not be introduced into *C. tyrobutyricum* wild-type strain, were selected and tried to transform into Δ*RMI*Δ*NP*. As expected, transformants were obtained with pJZ23-Cas9-*spo0A* and pJZ60-AsCpf1-*spo0A*, with overall conjugation efficiencies of 1.5 ± 0.5 CFU/ml-donor and 40.0 ± 8.0 CFU/ml-donor (Table 2), respectively. This further confirmed the enhanced conjugation efficiency of Δ*RMI*Δ*NP* compared to the wild-type strain. The resultant transformants (with either pJZ23-Cas9-*spo0A* or pJZ60-AsCpf1-*spo0A*) were then plated onto TGYLT plates to induce the expression of Cas9/Cpf1 nuclease. Eight colonies appeared on the selection plates were randomly picked to screen the *spo0A* deletion mutants through colony polymerase chain reaction (cPCR). Results showed that two out of eight of the tested clones were Δ*RMI*Δ*NP*Δ-*spo0A* mutants from the transformants harboring pJZ23-Cas9-*spo0A*, representing an editing efficiency of 25% (Table 2 and Figure S2a). For transformants harboring pJZ60-AsCpf1-*spo0A*, out of eight randomly picked colonies, the PCR of one colony (12.5%) demonstrated pure mutant band of *spo0A* deletion, while the PCR of another demonstrated mixed bands containing both mutant band and wild-type band (Table 2 and Figure S2b). These results demonstrated that genome editing can be achieved in Δ*RMI*Δ*NP* based on the CRISPR-Cas9/Cpf1 systems.

In summary, we successfully disrupted the RM system and native plasmid in *C. tyrobutyricum*. Results demonstrated that the deletion of RM system and native plasmid is beneficial to remove the barriers created by these two genetic elements in the conjugation process. Finally, the CRISPR-Cas9/Cpf1 systems were successfully implemented in the mutant with high conjugation efficiency for genome editing, expanding the genetic toolbox for unlocking the potential of this organism for valuable biofuel and biochemical production. Our results offer essential references and valuable guidance for boosting the conjugative plasmid transformation efficiency and developing CRISPR-Cas9/Cpf1-based genome engineering tools for microorganisms with underdeveloped genetic manipulation toolkits.

2 | MATERIALS AND METHODS

2.1 | Bacterial strains and cultivation

All the bacterial strains used in this study are listed in Table S1. The *E. coli* strain NEB Express (New England BioLabs Inc., Ipswich, MA) used for general propagation of recombinant plasmids and *E. coli* CA434 strain used as the donor strain for conjugation were cultivated in liquid Luria–Bertani (LB) broth or on solid LB agar plates supplemented with 30 μg/ml chloramphenicol (Cm) or 50 μg/ml kanamycin (Kan) as needed. *C. tyrobutyricum* ATCC 25755 (KCTC 5387) was routinely propagated at 37°C in tryptone-glucose-yeast extract (TGY) medium under strict anaerobic condition (Wang et al., 2016). To select and maintain *C. tyrobutyricum* transformants and mutants, 40 mM lactose, 15 μg/ml thiamphenicol (Tm), or 250 μg/ml D-cycloserine was added into the medium when needed.

2.2 | Plasmid construction

All the plasmids used in this study are listed in Table S1. The primers are listed in Table S2. The Phanta Max Super-Fidelity DNA Polymerase (Vazyme Biotech Co., Ltd., Nanjing, China) was used for PCR to amplify DNA fragments for cloning purposes. The Type I-B CRISPR genome editing plasmids were constructed through Gibson Assembly following the procedure as described previously (Zhang et al., 2018). In brief, plasmid pHW12-P*lac*-34*RMI* was constructed by cloning a synthetic CRISPR expression cassette (targeting on Type I restriction endonuclease subunit R) and two homology arms (for the deletion of Type I restriction endonuclease subunit R through homologous recombination) into pMTL82151 between *EcoR*I and *Kpn*I sites, and between *Kpn*I and *BamH*I sites, respectively. The synthetic CRISPR expression cassette contained a lactose inducible promoter, a 34-nt spacer sequence (5′-TCCAATAATGATAGTATGCTTGCTACCA ATCAAG-3′) flanked by two 30-nt direct repeat sequences (5′-GTTGAACCTTAACATGAGATGTATTTAAAT-3′) and the terminator sequence of the endogenous CRISPR array of *C. tyrobutyricum*.

The lactose inducible promoter was amplified from vector pYW34-*BtgZI* (Wang et al., 2016). The terminator sequence and two homology arms (~500 bp each) were amplified from the genomic DNA of *C. tyrobutyricum* (Table S2). The 34-nt spacer and two 30-nt direct repeat sequences were included in the reverse primer for amplifying the lactose inducible promoter and the forward primer for amplifying the terminator. To construct plasmid pJZ154-P*lac*-34*NP*, the synthetic CRISPR expression cassette, containing a 34-nt spacer sequence (5′-AATTGATCGTATGATAGGATTACTGTATCAATAT-3′) targeting on the native plasmid of *C. tyrobutyricum*, was cloned into the *EcoR*I and *Kpn*I sites of pMTL82151.

2.3 | Conjugative plasmid transformation of *C. tyrobutyricum*

All plasmids used in this study were transformed into *C. tyrobutyricum* by conjugation as described previously with modifications (Zhang et al., 2018). First, recombinant plasmids were transformed into donor strain *E. coli* CA434 by electroporation. Then, the resulting transformants were cultivated in LB medium containing 30 μg/ml Cm and 50 μg/ml Kan. When the optical density at 600 nm (OD_{600}) reached 1.5–2.0, about 1 ml *E. coli* CA434 cells were collected by centrifugation, washed once with equivalent volume of fresh LB medium, and mixed with 400 μl of *C. tyrobutyricum* culture (which had an OD_{600} of 2.0–3.0). The cell mixture was then spotted onto TGY agar plate and incubated at 37°C in the anaerobic chamber. After mating for 24 hr, the cell mixture was collected with 1 ml TGY medium and replated onto TGY plates containing 15 μg/ml Tm and 250 μg/ml D-cycloserine. Plates were incubated at 37°C for 48–96 hr until colonies appeared.

2.4 | Mutant screening and plasmid curing

To screen the desirable mutants, transformant colonies of *C. tyrobutyricum* were first inoculated into TGY liquid medium containing 15 μg/ml Tm (TGYT) and further spread onto TGY plates with addition of 40 mM lactose and 15 μg/ml Tm (TGYLT) for the induction of synthetic CRISPR array or Cas9/Cpf1 expression. The plates were incubated at 37°C for 48–96 hr until colonies were observed. The desirable mutants were then screened through cPCR.

To cure the plasmid in the mutant, the newly obtained mutant was serially transferred (with a 1% v/v inoculum) in Tm-free TGY liquid medium. After about 10 rounds of successive transferring, the cells were spread onto Tm-free TGY plates for colony development. Plasmid-cured mutants were screened by both cPCR (using primers to amplify a plasmid specific region) and Tm selection.

2.5 | Batch fermentation

The fermentation medium used in this study contains the following ingredients (in g/L): yeast extract, 5; tryptone, 5; $(NH_4)_2SO_4$, 3; K_2HPO_4, 1.5; $MgSO_4{\cdot}7H_2O$, 0.6; $FeSO_4{\cdot}7H_2O$, 0.03; L-cysteine, 1; glucose, 120. Oxygen-free nitrogen was used to flush through the fermentation medium for 1–2 hr before inoculation to generate anaerobic conditions. The *C. tyrobutyricum* strain was first precultured in TGY medium at 37°C until OD_{600} reached 1.5 and then 12.5 ml of the actively growing seed culture was inoculated into a 500-ml bioreactor (GS-MFC; Shanghai Gu Xin Biological Technology Co., Shanghai, China) containing 250 ml fermentation medium. During the fermentation, temperature and pH were maintained at 37°C and >6.0, respectively. All fermentations were performed in triplicate.

2.6 | Analytical methods

The cell density was monitored by measuring OD_{600} using a cell density meter (Ultrospec 10; Biochrom Ltd., Cambridge, England). The concentration of glucose, acetate, ethanol, butyrate, and butanol was analyzed using a high-performance liquid chromatography system (Agilent 1260 series; Agilent Technologies, Santa Clara, CA) equipped with an Aminex HPX-87H column (Bio-Rad, Hercules, CA) and a refractive index detector. A total of 5 mM sulfuric acid was used as the mobile phase with a flow rate of 0.6 ml/min at 25°C.

ACKNOWLEDGMENTS

This study was supported by the Agriculture and Food Research Initiative Competitive Grant no. 2018-67021-27715 from the USDA National Institute of Food and Agriculture (NIFA), the Ocean University of China-Auburn University (OUC-AU) Grants program, Auburn University Intramural Grants Program (IGP), the USDA-NIFA Hatch project (ALA014-1017025), and the Alabama Agricultural Experiment Station. The authors thank Dr. Hans Blaschek (University of Illinois at Urbana-Champaign) for providing pYW34-*BtgZI*, Dr. Mike Young (Aberystwyth University, UK) for providing *E. coli* CA434, and Dr. Nigel Minton (University of Nottingham, UK) for providing pMTL82151. Auburn University has filed a patent application covering the work described in this article (Provisional Application No.: 63/004,657). The application names Y. W. and J. Z. as inventors.

ORCID

Yi Wang http://orcid.org/0000-0002-0192-3195

REFERENCES

Barrangou, R., & Doudna, J. A. (2016). Applications of CRISPR technologies in research and beyond. *Nature Biotechnology*, *34*, 933–941.

Cornillot, E., Nair, R. V., Papoutsakis, E. T., & Soucaille, P. (1997). The genes for butanol and acetone formation in *Clostridium acetobutylicum* ATCC 824 reside on a large plasmid whose loss leads to degeneration of the strain. *Journal of Bacteriology*, *179*(17), 5442–5447.

Cui, G.-Z., Hong, W., Zhang, J., Li, W.-L, Feng, Y., Liu, Y.-J., & Cui, Q. (2012). Targeted gene engineering in *Clostridium cellulolyticum* H10 without methylation. *Journal of Microbiological Methods*, *89*(3), 201–208.

Fu, H., Yu, L., Lin, M., Wang, J., Xiu, Z., & Yang, S.-T. (2017). Metabolic engineering of *Clostridium tyrobutyricum* for enhanced butyric acid production from glucose and xylose. *Metabolic Engineering*, *40*, 50–58.

Gu, Y., Feng, J., Zhang, Z.-T., Wang, S., Guo, L., Wang, Y., & Wang, Y. (2019). Curing the endogenous megaplasmid in *Clostridium saccharoperbutylacetonicum* N1-4 (HMT) using CRISPR-Cas9 and preliminary investigation of the role of the plasmid for the strain metabolism. *Fuel*, *236*, 1559–1566.

Hong, W., Zhang, J., Cui, G., Wang, L., & Wang, Y. (2018). Multiplexed CRISPR-Cpf1-mediated genome editing in *Clostridium difficile* toward the understanding of pathogenesis of *C. difficile* infection. *ACS Synthetic Biology*, *7*(6), 1588–1600.

Jennert, K. C. B., Tardif, C., Young, D. I., & Young, M. (2000). Gene transfer to *Clostridium cellulolyticum* ATCC 35319. *Microbiology*, *146*, 3071–3080.

Jiang, Y., Qian, F., Yang, J., Liu, Y., Dong, F., Xu, C., … Li, Y. (2017). CRISPR-Cpf1 assisted genome editing of *Corynebacterium glutamicum*. *Nature Communications*, *8*, 15179.

Lee, J., Jang, Y. S., Han, M. J., Kim, J. Y., & Lee, S. Y. (2016). Deciphering *Clostridium tyrobutyricum* metabolism based on the whole-genome sequence and proteome analyses. *mBio*, *7*(3), e00743-16.

Li, A., Wen, Z., Fang, D., Lu, M., Ma, Y., Xie, Q., & Jin, M. (2020). Developing *Clostridium diolis* as a biorefinery chassis by genetic manipulation. *Bioresource Technology*, *305*, 123066.

Li, Q., Chen, J., Minton, N. P., Zhang, Y., Wen, Z., Liu, J., … Yang, J. (2016). CRISPR-based genome editing and expression control systems in *Clostridium acetobutylicum* and *Clostridium beijerinckii*. *Biotechnology Journal*, *11*(7), 961–972.

Loenen, W. A. M., Dryden, D. T. F., Raleigh, E. A., & Wilson, G. G. (2014). Type I restriction enzymes and their relatives. *Nucleic Acids Research*, *42*(1), 20–44.

Pyne, M. E., Moo-Young, M., Chung, D. A., & Chou, C. P. (2013). Development of an electrotransformation protocol for genetic manipulation of *Clostridium pasteurianum*. *Biotechnology for Biofuels*, *6*(1), 50.

Roberts, R. J., Vincze, T., Posfai, J., & Macelis, D. (2015). REBASE—a database for DNA restriction and modification: Enzymes, genes and genomes. *Nucleic Acids Research*, *43*(Database issue), D298–D299.

Sysoeva, T. A., Kim, Y., Rodriguez, J., Lopatkin, A. J., & You, L. (2020). Growth-stage-dependent regulation of conjugation. *AIChE Journal*, *66*(3), e16848.

Thomas, C. M., & Nielsen, K. M. (2005). Mechanisms of, and barriers to, horizontal gene transfer between bacteria. *Nature Reviews Microbiology*, *3*, 711–721.

Wang, Y., Zhang, Z. T., Seo, S. O., Choi, K., Lu, T., Jin, Y. S., & Blaschek, H. P. (2015). Markerless chromosomal gene deletion in *Clostridium beijerinckii* using CRISPR/Cas9 system. *Journal of Biotechnology*, *200*, 1–5.

Wang, Y., Zhang, Z. T., Seo, S. O., Lynn, P., Lu, T., Jin, Y. S., & Blaschek, H. P. (2016). Bacterial genome editing with CRISPR-Cas9: Deletion, integration, single nucleotide modification, and desirable "clean" mutant selection in *Clostridium beijerinckii* as an example. *ACS Synthetic Biology*, *5*(7), 721–732.

Woods, C., Humphreys, C. M., Rodrigues, R. M., Ingle, P., Rowe, P., Henstra, A. M., … Minton, N. P. (2019). A novel conjugal donor strain for improved DNA transfer into *Clostridium spp*. *Anaerobe*, *59*, 184–191.

Zhang, J., Zong, W., Hong, W., Zhang, Z. T., & Wang, Y. (2018). Exploiting endogenous CRISPR-Cas system for multiplex genome editing in *Clostridium tyrobutyricum* and engineer the strain for high-level butanol production. *Metabolic Engineering*, *47*, 49–59.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Zhang J, Hong W, Guo L, Wang Y, Wang Y. Enhancing plasmid transformation efficiency and enabling CRISPR-Cas9/Cpf1-based genome editing in *Clostridium tyrobutyricum*. *Biotechnology and Bioengineering*. 2020;117:2911–2917. https://doi.org/10.1002/bit.27435