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Full Length Article

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

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ABSTRACT

Clostridium saccharoperbutylacetonicum N1-4 (HMT) is known as a hyper acetone-butanol-ethanol (ABE) producing strain. Within its genome, there is an endogenous megaplasmid (Csp_135p) of 136 kb, which contains 104 protein-encoding genes and two pseudogenes. Till now, the function of the megaplasmid is unknown; meanwhile the existence of the megaplasmid could cause instability for the strain performance and barrier for efficient genome engineering. In order to investigate the function of the megaplasmid as related to ABE fermentation and other metabolisms, here we successfully eliminated the megaplasmid using the CRISPR-Cas9 system, generating the plasmid-null strain N1-4-C. Results from systematic characterization experiments indicated that the N1-4-C strain exhibited higher plasmid transformation efficiency and better plasmid stability than the N1-4 (HMT) strain. In addition, the N1-4-C strain could produce slightly higher concentration of butanol and ABE, along with more efficient re-assimilation of acids. Since there is a gene encoding a lipase on Csp_135p, we evaluated the capability for ester production of both N1-4-C and N1-4 (HMT) strains. Results demonstrated that the Csp_135p plasmid also contributed to the ester production (such as butyl acetate and butyl butyrate; this is the first report for ester production in *C. saccharoperbutylacetonicum*). This study demonstrated that the CRISPR-Cas9 system can be used as an efficient tool for the curing of endogenous plasmids. The plasmid-null N1-4-C strain can serve as a great platform for the development of more robust strains for biofuel and biochemical production.

1. Introduction

Biobutanol produced through acetone-butanol-ethanol (ABE) fermentation is a valuable biofuel which possesses various advantages over ethanol and a chemical feedstock which has significances to various industries [1]. *Clostridium saccharoperbutylacetonicum* N1-4 (HMT) is well-known as a hyper-butanol-producing strain [2]. Since its isolation, it has been extensively used for ABE production at various scales [3–6]. Recently, the genome sequence of *C. saccharoperbutylacetonicum* N1-4 (HMT) (DSM 14923) has been published [7]. Besides a large genome of 6.666 Mb (the largest genome for all solventogenic clostridial strains the genome of which has been sequenced), a 136 kb megaplasmid (named as Csp_135p) also exists. The megaplasmid contains 104 protein-encoding genes and two pseudogenes. The function of most of these genes are unknown (as hypothetical protein encoding genes) solely based on the bioinformatics prediction. Besides, there are genes annotated as related to potentially important cell metabolism, including the ones encoding various phage associated proteins and a lipase (Table S1). However, to date, there is no experimental evidence to elucidate the exact function of these genes. In many cases, the endogenous plasmid carries very important genetic information that could be essential for the specific cell metabolism. One famous example is the megaplasmid pSOL1 in *C. acetobutylicum* ATCC 824 whose loss leads to the degeneration of the strain (loss of the capacity to produce ABE)

Abbreviations: ABE, acetone-butanol-ethanol; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; CRISPR, clustered regularly interspaced short palindromic repeats; gRNA, guide RNA; DSB, double-strand breakage; cPCR, colony PCR; HPLC, high-performance liquid chromatography; RID, refractive index detector; GC–MS, gas chromatography–mass spectrometry

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because the plasmid carries several key genes responsible for solvent production [8]. Therefore, it is highly desirable to understand the function of the megaplasmid (and the genes) within *C. saccharoperbutylacetonicum* related to ABE production and other metabolisms.

On the other hand, recently, we attempted to introduce some heterologous pathways into *C. saccharoperbutylacetonicum* N1-4 (HMT) to enhance its metabolism for solvent production. However, we noticed that the introduced heterologous plasmids were highly unstable. We suspected that the megaplasmid Csp_135p in *C. saccharoperbutylacetonicum* N1-4 (HMT) might contribute to such instability of the introduced plasmids. This could be due to two reasons: 1) the origin of replication of the heterologous plasmids might be incompatible with that of Csp_135p; 2) some proteins encoded by Csp_135p (such as transposase) might lead to the instability of the introduced plasmids. Therefore, the elimination of Csp_135p could possibly promote the stability of heterologous plasmids, and thus meanwhile improve the plasmid transformation efficiency in *C. saccharoperbutylacetonicum* N1-4 (HMT). This provides us another motivation for carrying out this study.

Various strategies have been employed previously for plasmid curing in microorganisms, most of which involved chemical or physical treatments, such as the treatment with sodium dodecyl sulfate (SDS), acridine orange or heat [9]. However, these methods are not always applicable and can possibly introduce mutations into the host strain. Popping out the plasmid based on the incompatibility of multiple plasmids within the same host is also an efficient method, but generally subculturing of multiple generations is needed to ensure the native plasmid being eliminated by the introduced plasmid. Thus, it usually takes long time to get the plasmid cured [9]. The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated (Cas) system is an RNA-mediated immune system in bacteria and archaea that can efficiently cleave and eliminate the invading foreign DNAs, such as phage or plasmids [10]. It has been widely used for the genome edition as well as plasmid curing [11,12]. In this study, we set out to employ the CRISPR-Cas9 system that we recently developed for genome engineering in *C. saccharoperbutylacetonicum* N1-4 (HMT) [3] to eliminate the endogenous megaplasmid Csp_135p in this strain. Through the characterization of the plasmid-null mutant in comparison to the wild-type strain, we were able to investigate the function of Csp_135p related to ABE production, ester production, plasmid transformation efficiency and stability. This study demonstrated that the CRISPR-Cas9 system can be used as an efficient tool for the curing of endogenous plasmids. Our results provide preliminary insights concerning the function of the endogenous megaplasmid in *C. saccharoperbutylacetonicum*. The plasmid-null strain developed herein can serve as an excellent platform for the development of robust host strains for biofuel and biochemical production.

2. Materials and methods

2.1. Microorganisms and cultivation conditions

All strains and plasmids used in this study are listed in Table 1. *C. saccharoperbutylacetonicum* N1-4 (HMT) (DSM 14923) was requested from DSMZ. *C. saccharoperbutylacetonicum* N1-4 (HMT) was grown in an anaerobic chamber (N_2-CO_2-H_2 with a volume ratio of 85:10:5) at 35 °C in tryptone-glucose-yeast extract (TGY) medium [13]. When needed, clarithromycin (Cla) was added into TGY medium to a final concentration of 30 μg/mL. *Escherichia coli* DH5α was used for routine plasmid propagation and maintenance. It was grown aerobically at 37 °C in Luria-Bertani (LB) medium supplemented with 100 μg/mL of ampicillin (Amp) as needed.

2.2. Plasmid construction

All the primers used in this study are listed in Table S2. The plasmid pYW34, which contains a lactose-inducible promoter driving the expression of the *cas9* gene (from *S. pyogenes*) along with a chimeric gRNA sequence, was used as the mother vector [3,11]. The Phanta Max Super-Fidelity DNA Polymerase (Vazyme Biotech Co., Ltd., Nanjing, China) was used for the PCR to amplify DNA fragments for cloning purposes.

To construct the vector for Csp_135p elimination, the J23119 promoter and the gRNA with 20-nt guide sequence (5′-GAAGAAATTAGC GAGTTATT-3′) targeting on the Csp_135p plasmid were amplified by two rounds of PCR with primers YW1606/YW1342 and YW1339/YW1342 as described previously [3]. The obtained fragment was then inserted into pYW34 (digested with *Btg*ZI and *Not*I) through Gibson Assembly, generating pCP.

To construct the vector for deleting the endogenous CRISPR-Cas system, the J23119 promoter and the gRNA containing the 20-nt guide sequence (5′-CCAAAAGTAGAAGCATATCC-3′) targeting on the *cas21435786* cluster were amplified by two rounds of PCR with primer pairs of YW3478/YW1342 and YW1339/YW1342. The obtained fragment was then inserted into pYW34 (digested with *Btg*ZI and *Not*I) through Gibson Assembly. The two 1-kb homology arms were amplified with primer pairs of YW3474/3475 and YW3476/3477, and then inserted into the above obtained plasmid (digested with *Not*I), generating pYW34-ΔCas.

2.3. Plasmid transformation and mutant strain verification

The competent cell of *C. saccharoperbutylacetonicum* was prepared following the procedure as described in Herman et al. [14] with modifications. Briefly, when the optical density (OD_{600}) of the cell culture reached ~0.8, cells were harvested by centrifugation at 4200*g* and 22 °C for 8 min. Cell pellets were then resuspended with the same volume of electroporation buffer (EPB) (270 mM sucrose, 5 mM NaH_2PO_4 [pH 7.4]). The resuspension was centrifuged again under the same conditions as described above. The supernatant was discarded, and the cell pellets were resuspended in 1/20 vol of SMP buffer (270 mM Sucrose, 1 mM $MgCl_2$ and 7 mM Sodium phosphate). After that, approximately 1.0 μg of plasmid was mixed with 400 μL of competent cells and incubated in a 1.5 mL microcentrifuge tube on ice for 30 min. The mixture was then transferred into a 2-mm electroporation cuvette and electroporation was applied right way with a voltage of 1000 V, capacitance of 25 μF and resistance of 300 Ω using a Gene Pulser Xcell electroporation system (Bio-Rad Laboratories, Hercules, CA). Subsequently, the culture was transferred into 2 mL pre-warmed TGY medium and incubated at 35 °C. Upon recovery (about 3 h after electroporation), the culture was spread onto TGY agar plates containing 30 μg/mL clarithromycin (TGYC plates).

After plates were incubated at 35 °C in the anaerobic chamber for about 24 h, single colonies were picked and inoculated into TGYC liquid medium. After cell growth sign was observed, the culture was spread onto TGYLC plates (TGYC supplemented with 40 mM lactose). Colony PCR (cPCR) was then performed with primer pairs of YW1607/YW1608 and YW1609/YW1610, to verify the elimination of Csp_135p. The verified Csp_135p-null mutant was subcultured in TGY liquid medium for 3–5 generations to cure the pCP plasmid [3]. The finally obtained plasmid-free strain was designated as *C. saccharoperbutylacetonicum* N1-4-C.

Similar procedure was employed to confirm the deletion of the endogenous CRISPR-Cas system, except that the pair of primers YW3472/3473 were used.

2.4. Evaluation of the plasmid transformation efficiency and stability

N1-4 (HMT) and N1-4-C strains were grown anaerobically in TGY medium until OD_{600} reached ~0.8. Competent cells were prepared as described above. One μg of each plasmid (pMTL82151, pMTL83151, pMTL85151, pMTL82151-Hox, pMTL82151-GLE and pMTL82151-icmAB) was used to transform N1-4 (HMT) and N1-4-C, respectively.

Table 1
Strains and plasmids used in this study.

Strains and plasmids	Relevant genotype and characteristics	Source
Strains		
C. saccharoperbutylacetonicum N1-4 (HMT)	DSM 14923 (= ATCC 27021), wild-type strain	DSMZ
C. saccharoperbutylacetonicum N1-4-C	N1-4 (HMT), ΔCsp_135p	This study
C. saccharoperbutylacetonicum N1-4-C ΔCas	N1-4-C, Δ*cas21435786*	This study
E. coli DH5α	F^-, φ80d*lacZ*ΔM1, Δ(*lacZYA-argF*)U169, *deoR*, *recA*1, *endA*1, *hsdR*17(r_k^-, m_k^+), *phoA*, *supE*44, λ^- *thi*-1, *gyrA*96, *relA*1	NEB
Plasmids		
pYW34	CAK *ori*, Amp^r, Erm^r, Plac::Cas9, gRNA	[11]
pCP	pYW34 derivative, P_{sRNA}::20-nt gRNA targeting Csp_135p	This study
pYW34-ΔCas	pYW34 derivative, P_{sRNA}::20-nt gRNA targeting *cas21435786* and homology arms	This study
pMTL82151	pBP1 *ori*, *catP*, ColE1, *tra*	[28]
pMTL83151	pCB102 *ori*, *catP*, ColE1, *tra*	[28]
pMTL85151	pIM13 *ori*, *catP*, ColE1, *tra*	[28]
pMTL82151-icmAB	pMTL82151 derivation with the *icmAB* expression cassette	This study
pMTL82151-Hox	pMTL82151-derivation with *HoxFUYHWI* from *Ralstonia eutropha*	This study
pMTL82151-GLE	pMTL82151-derivation with *glmSLE* from *C. cochlearium*	This study

Colonies (once appeared) were enumerated to determine the plasmid transformation efficiency.

To determine the plasmid stability, the N1-4 (pMTL82151-icmAB) and N1-4-C (pMTL82151-icmAB) strains were subcultured for six times, respectively. PCR (using primers YW1175/YW1176 to amplify the *icmAB* fragment) was performed after each generation of subculturing to verify the existence of the gene fragment.

2.5. Inspection of the existence of phage

The N1-4 (HMT) and N1-4-C strains were cultured in TGY medium until OD_{600} reached ~0.2. Mitomycin C (2 μg/mL) was then applied to induce the release of possibly existent temperate phage [15–17]. After 20 min of incubation at 35 °C, the supernatant containing mitomycin C was removed by centrifugation at 4200*g* for 20 min and the harvested cells were resuspended at the same volume of fresh TGY medium. The OD_{600} was measured in the following several hours until the end of cell lysis. After that, cells were filtered using 0.2 μm filter and purified through $CsCl_2$ density gradient centrifugation and then stored at 4 °C in SM buffer (0.58% NaCl, 0.2% $MgSO_4{\cdot}7H_2O$, 1 mol/L pH 7.5 Tris–HCl) [18]. The obtained N1-4-C sample was used to infect N1-4 (HMT) strain through the double agar overlay plaque assay. For the assay, 100 μL of each serially diluted sample as obtained above was mixed with 100 μL of N1-4 (HMT) culture (OD_{600} ~ 0.8) along with 15 mL of TGY medium (containing 0.8% agar). The mixtures were cultivated at 35 °C for 24 h to observe the appearance of bacteriophage plaques on the plate.

2.6. Fermentation

For ABE fermentation, *C. saccharoperbutylacetonicum* strains were cultivated in TGY medium in the anaerobic chamber until OD_{600} reached 0.8. Then the seed culture was inoculated at an inoculation ratio of 5% into 100 mL P2 medium (containing 80 g/L glucose, 6 g/L tryptone, and 2 g/L yeast extract) in a 250-mL bottle [13]. The fermentation was performed at 30 °C with an agitation of 150 rpm. Samples were taken for analysis at 88 h. For ester production, the fermentation was carried out under the same conditions except that 1/5 vol (of the volume of the fermentation broth) of *n*-hexadecane was added into the fermentation for ester extraction and samples were taken for analysis at 120 h.

2.7. Analytical procedures

Concentrations of acetone, ethanol, butanol, acetic acid, butyric acid and glucose were measured by a high-performance liquid chromatography (HPLC, Model 1260 Infinity series, Agilent Technologies, Santa Clara, CA) with a refractive index Detector (RID), equipped with an Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA). The column was eluted with 5 mM H_2SO_4 at a flow rate of 0.6 mL/min at 25 °C. The concentration of the ester in the n-hexadecane phase was determined by a gas chromatography-mass spectrometry (GC–MS, Model 6890N, Agilent Technologies, Santa Clara, CA) equipped with an HP-5 column (60 m × 0.25 mm, 0.25 mm film thickness). Helium was used as the carrier gas. The initial temperature of the oven was set at 30 °C for 2 min, followed by a ramp of 10 °C/min to reach 300 °C, and a ramp of 2 °C/min to reach the final temperature of 320 °C, and then held for 2 min. The detector was kept at 225 °C [19]. The reported ester concentration is the determined value in the extractant phase.

3. Results and discussion

3.1. Endogenous plasmid elimination

Many strategies have been previously employed for plasmid curing in microorganisms [9]. However, these methods are often laborious and sometimes introduce mutations in the host strain. The CRISPR-Cas9 system can target on the endogenous plasmid specifically to make double-strand breakage (DSB), thus eliminating the plasmid efficiently [20].

The schematic of this work is illustrated in Fig. 1. Recently, in our group, we have developed the customized CRISPR-Cas9 system for genome engineering in *C. saccharoperbutylacetonicum* N1-4 (HMT) [3]. Here, we attempted to employ the CRISPR-Cas9 system to eliminate the Csp_135p plasmid within the N1-4 (HMT) strain. The pCP plasmid carrying Cas9 targeting on the Csp_135p plasmid was constructed and transformed into the N1-4 (HMT) strain. After the induction of Cas9 expression, cPCR results showed that more than 83% of the colonies were mutants with Csp_135p plasmid eliminated (Fig. 2). Then, the pCP plasmid within the mutant was cured. The obtained clean mutant strain was designated as N1-4-C. These results demonstrated that the CRISPR-Cas9 system is a powerful tool to eliminate the endogenous plasmid in the bacterial host.

3.2. Effects of Csp_135p elimination on plasmid transformation efficiency and stability

The existence of the megaplasmid in N1-4 (HMT) could have negative impact on the plasmid transformation efficiency. Thus, the elimination of Csp_135p within N1-4-C would lead to the improved plasmid transformation efficiency. To test this hypothesis, we compared the transformation efficiency of plasmids pMTL82151, pMTL83151, pMTL85151, pMTL82151-Hox, pMTL82151-GLE and pMTL82151-

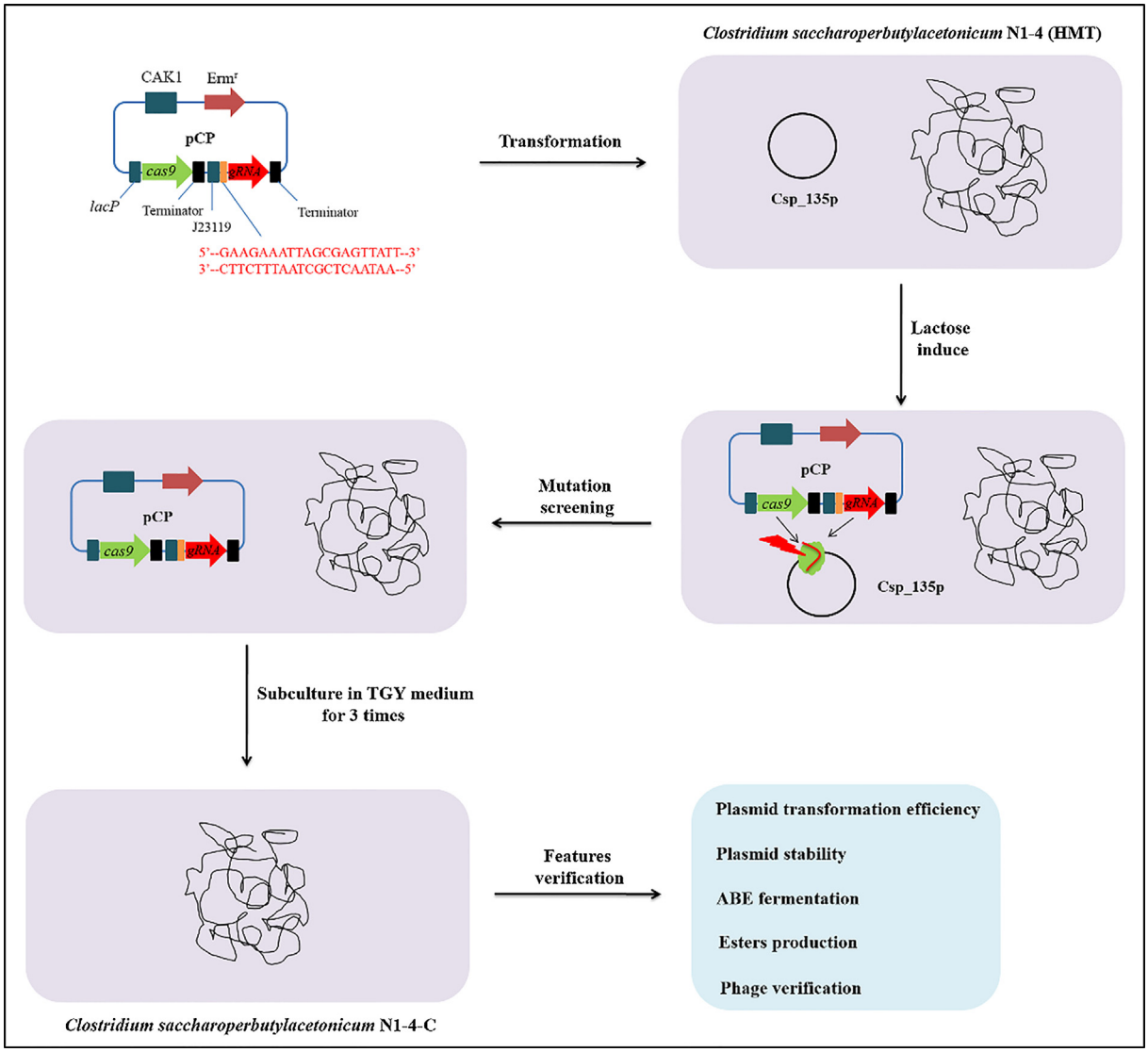

Fig. 1. The elimination of the endogenous Csp_135p plasmid from *Clostridium saccharoperbutylacetonicum* N1-4 (HMT) using the CRISPR-Cas9 system. Cas9: *Streptococcus pyogenes cas9* ORF; gRNA: the chimeric single guide RNA; *lacP*: lactose inducible promoter; J23119: a synthetic constitutive promoter; CAK1: *Clostridium* Gram-positive replicon; Erm^r: erythromycin resistant marker.

icmAB in N1-4 (HMT) and N1-4-C, respectively (Table 2). Transformation efficiencies of empty plasmids pMTL82151, pMTL83151 and pMTL85151 were all much higher ($> 1 \times 10^3$) than those carrying heterogeneous pathways. It is also obvious that the transformation efficiencies of all the plasmids in N1-4-C were higher than in N1-4 (HMT) by 1.8–7.8 folds. This suggested that the elimination of Csp_135p significantly improved the plasmid transformation efficiency. This provided valuable references for ones who work with microbial strains that are difficult to transform; the elimination of the endogenous plasmid (if exists) could be an effective way to boost the transformation efficiency. However, this is only applicable when the elimination of the endogenous plasmid won't result in remarkable negative impacts on the microbial metabolism.

Based on our previous experiences, we observed that the introduced heterogeneous plasmid within N1-4 (HMT) was not stable; the heterogeneous plasmid can be lost during subculturing or the long-term fermentation process (data not shown). We reasoned that the curing of Csp_135p could alleviate or address this problem. To test this hypothesis, the pMTL82151-icmAB plasmid was transformed into N1-4 (HMT) and N1-4-C, respectively, and the obtained N1-4 (pMTL82151-icmAB) and N1-4-C (pMTL82151-icmAB) strains were subcultured for six times in TGY medium. PCR was performed for each generation of subcultured cells to verify the stability of the pMTL82151-icmAB plasmid. As shown in Fig. S1, the plasmid was stable after one generation of subculturing and a clean specific PCR band could be obtained in both strains (2nd generation). Staring from the 3rd generation, an unspecific shorter band was started to be observed; however, comparatively, in N1-4-C (pMTL82151-icmAB), the unspecific band was much weaker than the specific one, while in N1-4 (pMTL82151-icmAB), the unspecific band was slightly stronger than the specific one. With further subculturing, in the 4th generation, in N1-4-C (pMTL82151-icmAB), the unspecific band was still much weaker than the specific one, while in N1-4 (pMTL82151-icmAB), the unspecific band became very strong and the specific one became very weak. In the 5th generation, the specific band in N1-4-C (pMTL82151-icmAB) became weak and meanwhile the unspecific one could not be visible. While in N1-4 (pMTL82151-icmAB), the unspecific band became even stronger and the specific one kept weak. For the 6th generation, the specific band in N1-4-C (pMTL82151-icmAB) was still weak and meanwhile there were several other unspecific bands were observed (but they were all much weaker than the specific band). In N1-4 (pMTL82151-icmAB), the unspecific band turned very strong and the specific band is hardly visible. These results

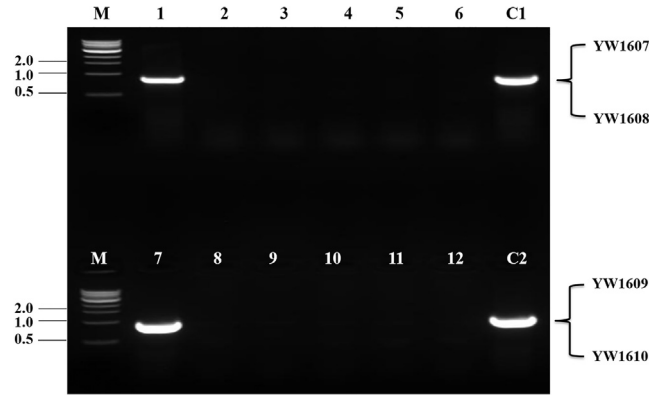

Fig. 2. Confirmation of the Csp_135p plasmid elimination by PCR. Lane M: The NEB 1-kb DNA ladder, with numbers on the left representing the band length in kb; Lanes 1–6: PCR results of six randomly picked colonies with primers YW1607/YW1608. Lane C1: the PCR result of the control N1-4 (HMT) strain with primers YW1607/YW1608. Lanes 7–12: PCR results of six randomly picked colonies (the same colonies as in Lanes 1–6; the colony used in Lane 7 is the same as in Lane 1, the colony used in Lane 8 is the same as in Lane 2, and so on) with primers YW1609/YW1610. Lane C2: the PCR result of the control N1-4 (HMT) strain with primers YW1609/YW1610.

Table 2
Plasmid transformation efficiencies in *Clostridium saccharoperbutylacetonicum* N1-4 (HMT) and N1-4-C strains.

Plasmids	N1-4 (HMT) (cfu/μg plasmid DNA)	N1-4-C (cfu/μg plasmid DNA)
pMTL82151	$(4.66 \pm 0.66) \times 10^3$	$(9.00 \pm 0.48) \times 10^3$
pMTL83151	$(6.71 \pm 0.31) \times 10^3$	$(1.18 \pm 0.12) \times 10^4$
pMTL85151	$(2.84 \pm 0.24) \times 10^3$	$(9.45 \pm 0.65) \times 10^3$
pMTL82151-icmAB	$(9.32 \pm 0.56) \times 10^2$	$(2.07 \pm 0.07) \times 10^3$
pMTL82151-Hox	$(3.68 \pm 0.13) \times 10^2$	$(2.88 \pm 0.37) \times 10^3$
pMTL82151-GLE	$(5.70 \pm 1.70) \times 10^1$	$(2.92 \pm 0.23) \times 10^2$

indicated that the Csp_135p megaplasmid within N1-4 (HMT) might contribute to the instability of the introduced heterogeneous plasmid. It is inferred that the transposase encoded by the gene on Csp_135p might be partly responsible for this. The elimination of Csp_135p could mitigate but could not completely solve this problem. There might have been some other factors in the chromosome leading to the instability of the heterologous plasmid in N1-4 (HMT). From the genome sequence of the N1-4 (HMT) strain, it is shown that a Type-I CRISPR-Cas system exists in the N1-4 (HMT) chromosome. The endogenous CRISPR-Cas system provides adaptive immunity in bacteria ad archaea, which can act against invading genetic elements such as phages and plasmids [21]. Therefore, we further deleted the endogenous CRISPR-Cas system and the obtained strain was named as N1-4-C ΔCas. We investigated the stability of the transformed plasmid in the N1-4-C ΔCas strain. Unfortunately, similar instability of the plasmid was observed as in N1-4-C (data not shown). Thus, we excluded the endogenous CRISPR-Cas system as a possible mechanism that led to the plasmid instability. As we shown above, although the curing of megaplasmid can help improve the stability of the transformed plasmid, there are still likely other unknown factors that are responsible for the instability of the heterogeneous plasmid. This warrants further investigation in the future.

3.3. Effects of Csp_135p elimination on ABE production

C. saccharoperbutylacetonicum N1-4 (HMT) is known as a robust strain for ABE production [22]. Here, we further investigated the effect of Csp_135p elimination on ABE production in the host strain. As shown in Fig. 3, the cell growth of N1-4-C was slower than N1-4 (HMT). The OD_{600} of N1-4 (HMT) reached the maximum at around 52 h, and then declined; while the OD_{600} of N1-4-C reached the maximum (about the same level) at 76 h, after which the cell density decreased (Fig. 3a). The consumption of glucose in N1-4-C was also slightly slower than N1-4 (HMT) before 52 h. Afterwards, N1-4 (HMT) stopped consuming glucose (with a total of 75.2 g/L glucose consumed), while N1-4-C stopped consuming glucose after 64 h, with a total of 78.1 g/L glucose been consumed (Fig. 3b). The fact that N1-4-C grew slower than the wild-type strain is unexpected because we originally anticipated that the curing of the megaplasmid would help relieve the metabolic burden in the host and thus elevate the cell growth. Interestingly, similar results have been reported in *C. butyricum* type E strain [23]. With the elimination of the two megaplasmids from the wild-type host, the mutant Δmp strain grew slower than the wild-type under either the optimal conditions or limiting conditions of pH and salt concentrations [23]. We inferred that the elimination of the megaplasmid might have broken the original intracellular metabolic balance in the host, thereby led to delayed or decreased cell growth.

Corresponding to the cell growth and glucose consumption kinetics, the production of acetone, butanol and ethanol in N1-4-C was all slightly slower than that in N1-4 (HMT), but all reached slightly higher concentrations by the end of the fermentation (Fig. 3c–e). This led to a slight increase of total ABE production (26.2 g/L) in N1-4-C than that in N1-4 (HMT) (24.8 g/L) (Fig. 3f). ABE fermentation is a biphasic process in which acetic acid and butyric acid are first produced at the acidogenesis phase and then re-assimilated for ABE production in the solventogenic phase [24]. As shown in Fig. 3g–h, compared to N1-4 (HMT), the production of fatty acids in N1-4-C was also slightly delayed, but reached higher maximum levels (2.80 g/L vs. 2.60 g/L for acetic acid, and 1.26 g/L vs. 0.79 g/L for butyric acid). Then the acids in both strains were re-assimilated in the late stage of the fermentation, with N1-4-C had slightly better re-assimilation of acids (especially for butyric acid). The Csp_135p plasmid, with a size of 136,188 bp, carries 106 annotated genes many of which encode phage related proteins. The replication and maintenance of this endogenous megaplasmid including the expression of the phage related genes might shunt the metabolic flux that would otherwise be used for solvent production. Therefore, the curing of Csp_135p could help save the energy and substrate, and thus increase acid production and further ABE production (although such increment is minor) in the N1-4-C strain.

3.4. Effects of Csp_135p elimination on ester production

Within Csp_135p, there is a gene (*CSPA_RS28700*) encoding a lipase protein. Lipase generally catalyzes the hydrolysis of lipid, however it can also be used for the production of ester based on its reversible catalysis activity [19]. The N1-4 (HMT) strain can produce acids including acetic acid and butyric acid, and alcohols including ethanol and butanol. Thus, these acids and alcohols could be possibly catalyzed by the endogenous lipase to produce esters. As expected (Fig. 4), the N1-4 (HMT) strain produced small amount of butyl acetate and butyl butyrate, which was 147.0 mg/L and 20.5 mg/L, respectively. The N-4-C strain also produced these two esters, however with much lower concentrations (60.3 mg/L butyl acetate and 11.5 mg/L butyl butyrate) than those in the N1-4 (HMT) strain. These results suggested that the elimination of Csp_135p in N1-4-C indeed diminished the capability of the host strain for ester synthesis. However, little amount of esters can still be produced in N1-4-C, indicating that there might be other lipase genes active for the ester production. In the chromosome of *C. saccharoperbutylacetonicum*, the gene *CSPA_C45280* was also annotated as a lipase encoding gene. The ester production in N1-4-C was about half of that in the wild-type N1-4 (HMT) strain; thus we conclude that both of these two lipase genes (Cspa_c45280 and Capa_RS28700) contribute (about equally) to the ester production. We further compared these two enzymes with the two well-studied lipases, CALB from *Candida antarctica* [25] and TLL from *Thermomyces lanuginosus* [26]. The amino

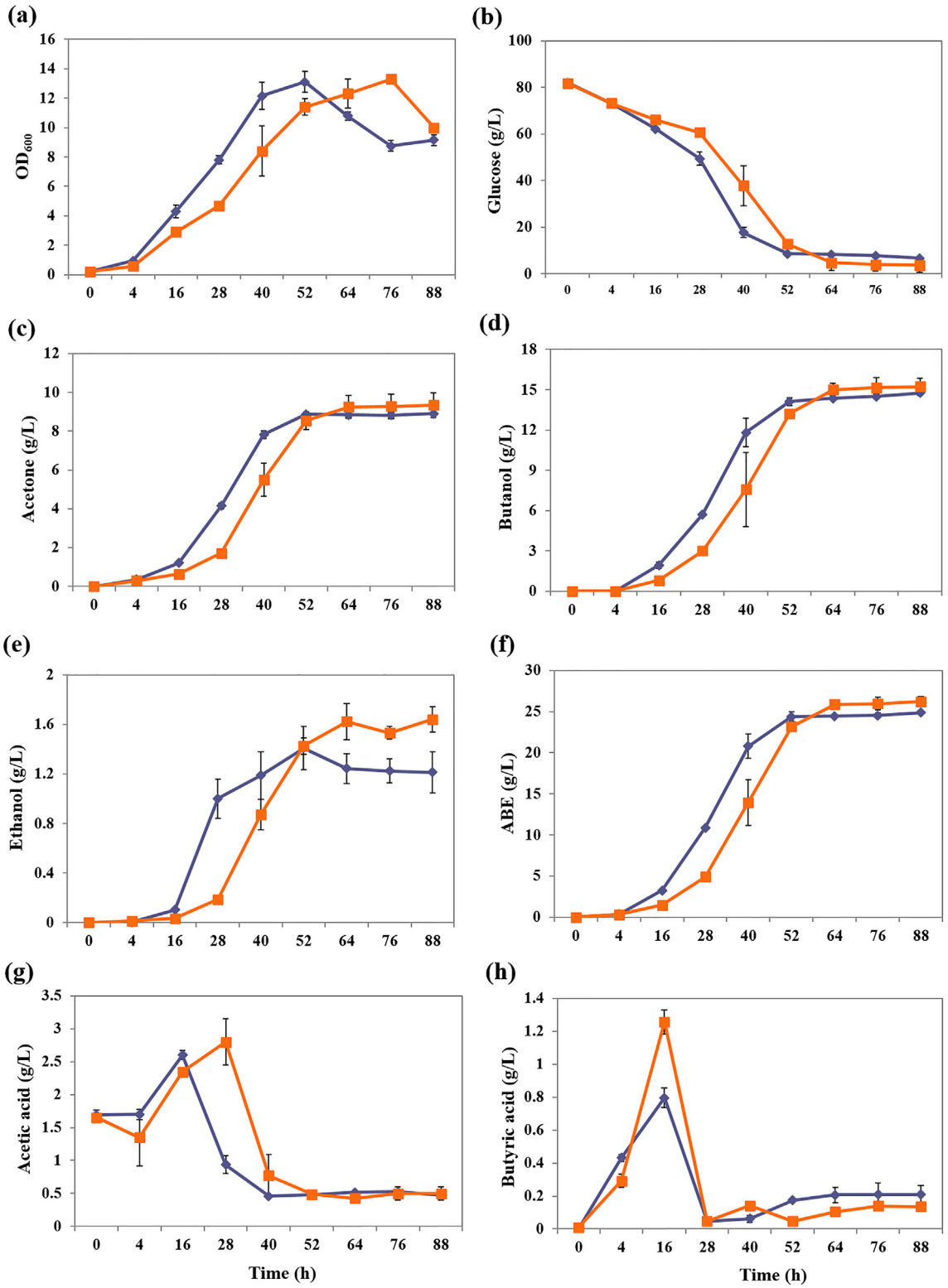

Fig. 3. ABE fermentation results using *Clostridium saccharoperbutylacetonicum* N1-4 (HMT) and N1-4-C. (a) Cell growth profiles; (b) Glucose consumption; (c) Acetone; (d) Butanol; (e) Ethanol; (f) Total ABE; (g) Acetic acid; (h) Butyric acid.

acid sequences of Cspa_c45280 and Capa_RS28700 share a high identity of 52.1%, but they share low similarities (< 11%) with eihter CALB or TLL (Fig. S2).

3.5. Verification of the existence of temperate bacteriophages on Csp_135p

Csp_135p has a size of 136,188 bp carrying 106 annotated genes (104 protein encoding genes and two pseudogenes). As shown in Table S1, besides the hypothetical proteins, majority of the annotated genes encode phage related proteins such as virion structure proteins,

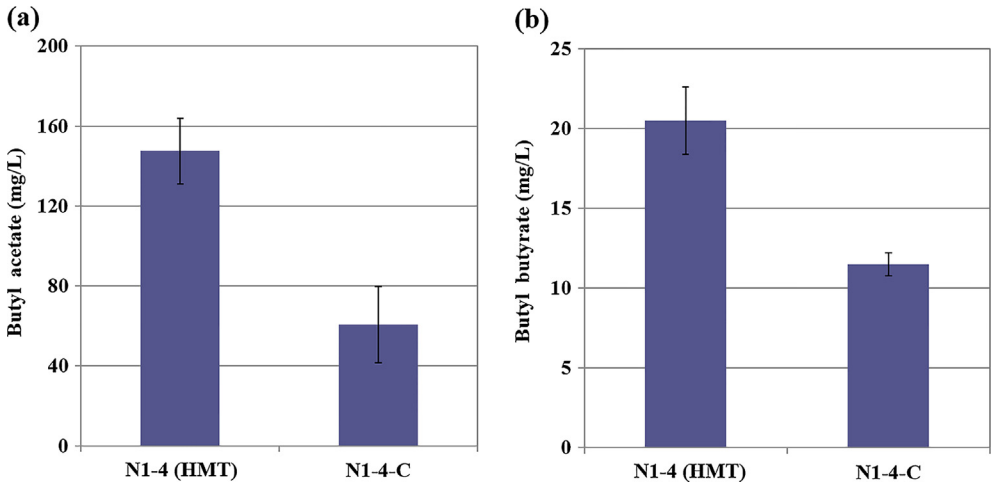

Fig. 4. The production of esters in *Clostridium saccharoperbutylacetonicum* N1-4 (HMT) and N1-4-C. (a) Butyl acetate; (b) Butyl butyrate.

baseplate proteins, tail sheath subunits and holing-like toxins. Thus, we speculated that Csp_135p could be a temperate bacteriophage, which exists as a plasmid in the N1-4 (HMT) strain. To test this hypothesis, we applied mitomycin C into the N1-4 (HMT) culture to induce the possible release of phage. As showed in Fig. S3, both N1-4 (HMT) and N1-4-C strains were subjected to severe cell lysis after the induction with mitomycin C. Additionally, double agar overlay plaque assays were performed with N1-4 (HMT) infected with the N1-4-C cell lysate, and plaques were observed on the double agar plate (data not shown). With these results, it is hard to draw the conclusion that Csp_135 is a temperate bacteriophage, because the cell lysis and appearance of plaques could also be caused by the existence of HMT phage and bacteriocins on the chromosome [17,27]. To exclude such possibilities and further elucidate the megaplasmid function as related to bacteriophage metabolism, the HMT phage genome and the bacteriocins synthetic pathways on the chromosome need to be deleted. Such attempts are currently underway in our lab.

4. Conclusions

In this study, the megaplasmid Csp_135p in *C. saccharoperbutylacetonicum* N1-4 (HMT) was eliminated using the CRISPR-Cas9 system. The mutant N1-4-C strain was characterized in comparison with the wild-type N1-4 (HMT) strain, in order to elucidate the function of the Csp_135p plasmid. Results demonstrated that the elimination of Csp_135p could improve the plasmid transformation efficiency and stability; it could also increase the ABE production in the plasmid-null mutant. The *CSPA_RS28700* gene on the plasmid was confirmed to contribute to the ester biosynthesis. Meanwhile, experiments were carried out to determine whether Csp_135p is a temperate bacteriophage. However, firm conclusions are hard to reach at this point and further investigation is warranted on this matter. This study demonstrated that the CRISPR-Cas9 system is a convenient and efficient tool for curing endogenous plasmid from the microbial host. In addition, the plasmid-free strain can serve as a great platform for the development of robust strains for the production of various biofuels and biochemicals.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fuel.2018.09.030.

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