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Genome editing in *Clostridium saccharoperbutylacetonicum* N1-4 using CRISPR-Cas9 system

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Running Title: *Clostridium saccharoperbutylacetonicum* genome editing

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ABSTRACT

Clostridium saccharoperbutylacetonicum N1-4 is well known as a hyper-butanol-producing strain. However, the lack of genetic engineering tools hinders further elucidation of its solvent production mechanism and development of more robust strains. In this study, we set out to develop an efficient genome engineering system for this microorganism based on the CRISPR-Cas9 system. First, the functionality of the CRISPR-Cas9 system previously customized for *C. beijerinckii* was evaluated in *C. saccharoperbutylacetonicum* by targeting on *pta* and *buk*, two essential genes for acetate and butyrate production, respectively. The *pta*, *buk* single deletion, and the *pta* and *buk* double deletion mutants were successfully obtained based on this system. However, the genome engineering efficiency was rather low (the mutation rate is < 20%). Therefore, the efficiency was further optimized by evaluating various promoters for the gRNA expression. With promoter P_{J23119}, we achieved a mutation rate of 75% for *pta* deletion without serial subculturing as suggested previously for *C. beijerinckii*. Thus, this developed CRISPR-Cas9 system is highly desirable for efficient genome editing in *C. saccharoperbutylacetonicum*. Batch fermentation results revealed that both the acid and solvent production profiles were altered due to the disruption of acid production pathways, however neither acetate nor butyrate production was eliminated with the deletion of the corresponding gene. The butanol production, yield and selectivity were improved in mutants dependent on the fermentation medium. In the *pta-buk* double deletion mutant, the butanol production reached 19.0 g/l in P2 medium, which is one of the highest among the ever reported from batch fermentations.

IMPORTANCE

An efficient CRISPR-Cas9 genome engineering system was developed for *C. saccharoperbutylacetonicum* N1-4. This paves the way for elucidating the solvent production

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mechanism in this hyper-butanol-producing microorganism and developing strains with desirable butanol-producing features. This tool can be easily adapted for use in other close relative microorganisms. As also reported by others, here we demonstrated with solid data that the highly efficient expression of gRNA is the key factor determining the efficiency of CRISPR-Cas9 for genome editing. The protocol developed in this study can provide essential references for other researchers who work in the metabolic engineering and synthetic biology area. The developed mutants can be used as excellent starting strains for development of more robust ones for desirable solvent production.

KEY WORDS

Clostridium saccharoperbutylacetonicum, CRISPR-Cas9, gRNA, genome engineering, *pta*, *buk*, butanol

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INTRODUCTION

Biofuels produced from renewable biomass provide a feasible solution in addressing problems such as environmental pollution and climate change resulted from fossil fuels consumption. Thus, biofuels are considered as appropriate substitutes for fossil fuels at its depletion (1). Biobutanol produced through acetone-butanol-ethanol (ABE) fermentation possesses various superiorities over ethanol as a fuel source and is considered as one of the most promising biofuel candidates for the future (2). Additionally, butanol manifests even higher commodity value when it is considered as the feedstock for numerous industries (3). There are generally four primary solventogenic clostridial species for ABE production: *C. acetobutylicum*, *C. beijerinckii*, *C. saccharobutylicum*, and *C. saccharoperbutylacetonicum* (2). Among them, undoubtedly, *C. acetobutylicum* and *C. beijerinckii* are the most extensively investigated for their ABE fermentation physiology, genomics, genetics and mutant development (especially in recent couple of years) (4-9). *C. saccharoperbutylacetonicum* N1-4 (ATCC 13564) was isolated in 1959 in Japan, and used for industrial butanol production since then until late 1980s (10, 11). As a well-known hyper-butanol-producing strain (12), *C. saccharoperbutylacetonicum* N1-4 has been broadly studied on its fermentation characteristics including the desirable fermentation media and fermentation conditions (13-16). Recently, the genome sequence of *C. saccharoperbutylacetonicum* N1-4 ATCC 27021 (a lysogenic derivative strain of ATCC 13564) has been published (17, 18). However, compared to *C. acetobutylicum* and *C. beijerinckii*, the study of the genomics and genetics on *C. saccharoperbutylacetonicum* was apparently lagging. In addition, there were rare reports concerning the metabolic engineering attempt on this species. So far, to our best knowledge, there are only two reports related to genetic engineering in this strain; one is about the development of a host-vector system and the expression of a amylase

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gene in *C. saccharoperbutylacetonicum* N1-4 (19), and the other is about the downregulation of the hydrogenase gene cluster using the antisense RNA strategy (20). Both reports referred above were published about 10 years ago from the same research group. During the process when we were preparing this manuscript, Herman et al. reported the development of a transformation protocol and the metabolic engineering of *C. saccharoperbutylacetonicum* using plasmid-based overexpression and non-replicative vector based allelic exchange (21). However, clearly, more efficient and versatile genetic engineering tools are highly desirable for *C. saccharoperbutylacetonicum* in order to elucidate its complex fermentation phenotype and regulation mechanism, and develop better strains for butanol production.

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 protect the host from invading foreign DNAs, such as phage or plasmids (22). Recently, the CRISPR system has been engineered as a cutting edge genome engineering tool for both eukaryotic and prokaryotic cells, for which the Type II CRISPR-Cas9 system from *Streptococcus pyogenes* has been mostly employed (23-29). In the CRISPR-Cas9 system, the CRISPR RNA (crRNA) and the trans-activating CRISPR-RNA (tracrRNA) complex together to make a dual tracrRNA:crRNA, directing the Cas9 to the site-specific DNA sequence with the protospacer-adjacent motif (PAM). The endonuclease activity of Cas9 can then lead to the double strand breakage (DSB) (30). Genetic mutations can be introduced through the non-homologous end-joining (NHEJ) or homology directed repair (HDR) by providing a DNA editing template (31). Jinek et al. engineered the dual tracrRNA:crRNA into a single chimeric guide RNA (gRNA) which demonstrated comparable efficiency for the DNA targeting purpose, making the CRISPR-Cas9 system much easier to implement (30). In the past few years,

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113 numerous successes for genome editing based on the CRISPR-Cas9 system have been reported
114 in various bacteria, including *E. coli* (32, 33), *S. pneumonia* (23), *Streptomyces species* (34-36) ,
115 *T. citrea* (33), *C. pasteurianum* (37), *C. beijerinckii* (8, 27, 35, 38) and *C. acetobutylicum* (39,
116 40). It was demonstrated that, although the CRISPR-Cas9 based genome editing also relies on
117 the targeted DSB from Cas9, the DSB more likely serves as a selection power for the edited
118 mutant cells against the unedited background cells (23, 27).

119 During the biphasic ABE fermentation, the production of acids is indispensable and plays
120 important roles in the metabolic switch for solvent production (41). In the acidogenic phase,
121 acetate is produced from acetyl-CoA through the action of phosphotransacetylase (PTA) and
122 acetate kinase (ACK), while butyrate is generated through the catalysis by
123 phosphotransbutyrylase (PTB) and butyrate kinase (BUK). Subsequently during the
124 solventogenic phase, both acids are reassimilated for the production of acetone, butanol and
125 ethanol (42). Disruption of acid formation pathways has been achieved previously in *C.*
126 *acetobutylicum* and *C. beijerinckii*; results demonstrated that the production patterns of both
127 acids and solvents were altered by such manipulation (43-45).

128 Therefore, in this study, we developed an efficient CRISPR-Cas9 based genome editing
129 system for *C. saccharoperbutylacetonicum*. Based on this system, the primary *pta*, *buk* or both
130 *pta* and *buk* genes were deleted, and the fermentation features of the mutants were characterized
131 comparing with the wild type strain. The developed CRISPR-Cas9 system provided a versatile
132 genome engineering tool for *C. saccharoperbutylacetonicum*, making it a reality for easily
133 elucidating the metabolism of this microorganism and developing robust strains for biobutanol
134 production. Additionally, this study provide essential references for relevant researchers in
135 developing customized CRISPR-Cas9 genome engineering tools for other microorganisms.

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MATERIALS AND METHODS

Bacterial strains, growth conditions, plasmids, and oligonucleotides. All the strains and plasmids used in this study are listed in Table 1, and DNA primers are listed in Table S1 in the supplemental material. *E. coli* strain DH5α was used for DNA cloning. It was grown aerobically at 37ºC in Luria-Bertani (LB) medium supplemented with 100 μg/ml of ampicillin (Amp) as needed. *C. saccharoperbutylacetonicum* N1-4 was grown in an anaerobic chamber (N_2-CO_2-H_2 with volume ratio of 85:10:5) at 35ºC in tryptone-glucose-yeast extract (TGY) medium containing 30 g/l of tryptone, 20 g/l of glucose, 10 g/l of yeast extract, and 1 g/l of L-cysteine (45). TGYL (TGY supplemented with 40 mM of lactose) was used to induce the Cas9 expression. When appropriate, 25 μg/ml of erythromycin (Erm) was added into either TGY or TGYL to make the TGYE or TGYLE medium for *C. saccharoperbutylacetonicum* cultivation.

Plasmid construction. The plasmid pYW34 (27) holding the Cas9 open reading frame (ORF) from *S. pyogenes* under the control of a lactose inducible promoter along with the chimeric gRNA sequence was used as the mother vector to construct plasmids for disrupting *pta* (GenBank: AGF55073.1) and *buk* (GenBank: AGF54071.1) genes. To delete *pta*, the small RNA (sRNA, sCbei_5830) promoter fused with the 20-nt guiding sequence (5'-TCTGGAGCAGTTCATACAAC-3') was amplified from *C. beijerinckii* 8052 genomic DNA using primers YW484 and YW1041, and inserted into the *Btg*ZI site of pYW34 with Gibson Assembly, generating pYW34-*pta*. To achieve the desirable homologous recombination around the *pta* ORF, two homology arms (1076 bp and 1078 bp, respectively) flanking at both sides of the *pta* ORF were amplified from *C. saccharoperbutylacetonicum* genomic DNA with primers YW1035 and YW1036, and YW1037 and YW1038, respectively; then these two fragments were fused together with primers YW1039 and YW1040 through SOE-PCR

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159 (splicing by overlapping extension PCR), and inserted into the *Not*I site of pYW34-*pta*,
160 generating pYW34-deltpta.

161 Similarly, for targeting on *buk*, the fragment containing the sRNA promoter and 20-nt guiding
162 sequence (5'-GGAATGCTTAAGCCAGTAGA-3') was amplified with primers YW484 and
163 YW1053, and then inserted into the *Btg*ZI site of pYW34, generating pYW34-*buk*. One 1 kb
164 homology arm sequence upstream the start codon (ATG) of *buk* was amplified using primers
165 YW949 and YW950, and another 1 kb homology arm sequence downstream the stop codon
166 (TAA) of *buk* was amplified using primers YW951 and YW952. The whole 2 kb homology arm
167 was then obtained through SOE-PCR with primers YW949 and YW952 and inserted into the
168 *Not*I site of pYW34-*buk*, generating the plasmid pYW34-deltbuk.

169 To improve the genomic editing efficiency in *C. saccharoperbutylacetonicum* using the
170 CRISPR-Cas9 system, other strong constitutive promoters were evaluated for the gRNA
171 expression (targeting on the same 20-nt sequence in *pta* as describe above), including the *veg*
172 promoter from *B. subtilis* (P_{vegb}) (46), the hypothetical *veg* (CSPA_RS02900) gene promoter
173 (P_{vegc}) from *C. saccharoperbutylacetonicum*, and the *E. coli* promoter P_{J23119} (47). The P_{vegb}
174 promoter (65 bp) from *B. subtilis* with the 20-nt guiding sequence was amplified using primer
175 pairs YW1340 and YW1342, YW1341 and YW1342 in two steps, and inserted into the *Btg*ZI
176 and *Not*I sites of pYW34, generating pSHW1-*pta*. The P_{vegc} promoter fused with the 20-nt
177 guiding sequence was amplified from *C. saccharoperbutylacetonicum* N1-4 with primers
178 YW1042 and YW1043, and inserted into the *Btg*ZI site of pYW34, generating pSHW2-*pta*.
179 While the P_{J23119} promoter fused with the 20-nt guiding sequence was amplified using primer
180 pairs YW1338 and YW1342, YW1339 and YW1342 in two steps and inserted into the *Btg*ZI and
181 *Not*I sites of pYW34, generating pSHW3-*pta*. Subsequently, the fragment containing the two

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182 homology arm sequences for *pta* deletion was inserted into the *Not*I site of pSHW1-*pta*, pSHW2-
183 *pta* and pSHW3-*pta*, generating pSHW1-deltpta, pSHW2-deltpta and pSHW3-deltpta,
184 respectively.

185 All cloning PCR was carried out using the high fidelity DNA polymerases, either Phusion
186 (New England BioLabs Inc., Ipswich, MA) or PrimeSTAR (Takara Bio USA, Inc., Mountain
187 View, CA). All constructs were verified through Sanger sequencing.

188 **DNA transformation and mutants screening.** Transformation of *C*.
189 *saccharoperbutylacetonicum* N1-4 was carried out with electroporation following the protocol
190 for the transformation of *C*. *beijerinckii* as described by Wang et al. with minor modification
191 (45). *C*. *saccharoperbutylacetonicum* N1-4 was cultivated anaerobically at 35ºC until the optical
192 density of the culture at 600 nm (OD_{600}) reached 0.8-1.0. The cells were harvested through
193 centrifugation at 4,200 g at 4 ºC for 10 min. The cell pellets were washed once with the same
194 volume (as the original cell culture) ice-cold 15% glycerol and then resuspended in 1/20 volume
195 of ice-cold 15% glycerol. Immediately, 1.0 μg of plasmid DNA was added into 400 μl of
196 competent cells and then the mixture was transferred into a precooled 0.2 cm electroporation
197 cuvette. Electroporation was carried out at 1500 V of voltage, 25 μF of capacitance and 300 Ω of
198 resistance using a Gene Pulser Xcell electroporation system (Bio-Rad Laboratories, Hercules,
199 CA) which was connected into the anaerobic chamber. Subsequently, the cells were transferred
200 into 1.6 ml of TGY medium and recovered anaerobically at 35ºC (generally for 10-12 hours until
201 cell growth sign was observed). The recovered cells were spread onto TGYE agar plates and
202 incubated at 35 ºC for about 24 hours under anaerobic conditions until colonies appeared.

203 Following the outgrowth on selective plates, single colonies were picked randomly for colony
204 PCR (cPCR) using primers YW880 and YW881 to confirm the presence of plasmid. The

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transformants were then incubated in TGYE liquid medium and subcultured successively to permit efficient homologous recombinant (38). Starting from the second generation, while the culture was kept being transferred in TGYE medium, it was meanwhile transferred into TGYLE liquid medium for the induction of Cas9 expression and further spread onto TGYLE plates when necessary (that is, when double bands from both the mutant and the wild type were detected through cPCR with TGYLE liquid culture as template). Mutants were screened through cPCR using primers (YW1044 and YW1045 for detecting *pta* deletion; YW953 and YW954 for detecting *buk* deletion) annealing to the chromosomal loci beyond the homologous recombination regions (and thus the primers can only anneal to the chromosome but not the homology arms on the plasmids).

Due to the existence of plasmid harboring homologous sequences within the mutant strain, vector integration event (VIE) could occur during the bacterial genome engineering (27). To detect VIE, pairs of primers (with one anneals to the plasmid and the other anneals to the chromosome) were designed. Specifically, pairs of YW1044/YW200 and YW847/YW1045 were used for detecting VIE of pYW34-deltpta at both the upstream and downstream of the potential VIE, while YW953/YW200 and YW847/YW 954 were used for detecting VIE of pYW34-delbuk in a similar manner (Fig. S2).

Plasmid curing. To cure the donor plasmid pYW34-deltpta or pYW34-deltbuk, mutants that were identified without vector integration were subcultured in TGY medium without antibiotics as described previously (45, 48). After around five cycles of subculturing, the culture was spread onto TGY plates to form colonies. cPCR were performed to confirm the curing of plasmids using primers YW880 and YW881. Colonies lacking PCR products were spread onto TGYE plates to

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227 further confirm the loss of plasmids. Finally, cPCR was conducted to further verify the existence
228 of the desirable mutation.

229 **Fermentation.** Bach fermentation was performed in BioFlo 115 benchtop bioreactors (New
230 Brunswick Scientific Co., Enfield, CT) with a working volume of 1.5 liters. P2 medium or the
231 modified P2 (MP2) medium (45) were used as the fermentation medium, and 80 g/l glucose was
232 used as the sole carbon source. To generate an anaerobic condition, oxygen-free nitrogen was
233 sparged through the fermentation broth starting several hours before the inoculation until the cell
234 culture initiates its own gas production.

235 Mutant strains as well as the wild type (stored as glycerol stocks) were propagated in TGY
236 medium in anaerobic chamber until the OD_{600} reached 0.8. Then the culture was inoculated into
237 the reactor at a 5% (vol/vol) inoculum ratio. Fermentation was performed at 30 ºC with 50 rpm
238 agitation. The pH was controlled above 5.0 throughout the fermentation by adding 1 M NaOH.
239 All fermentations were performed in duplicate.

240 **Analytical methods.** Cell density (OD_{600}) was measured with an Ultrospec 10 cell density
241 meter (Amersham Biosciences Corp., Piscataway, NJ). The NBS BioCommand software (New
242 Brunswick Scientific Co, Inc., Edison, NJ) was used to record pH profiles in real time
243 throughout the fermentation. Concentrations of ABE (acetone, butanol and ethanol), acids
244 (acetate, butyrate and lactate) as well as glucose were determined using a high-performance
245 liquid chromatography (Agilent Technologies 1260 Infinity series, CA) with a refractive index
246 Detector (RID) and a diode array ultraviolet detector (DAD), equipped with a Aminex HPX-
247 87H column (Bio-Rad Laboratories, Hercules, CA). The column was eluted with 0.005 N H_2SO_4
248 with a flow rate of 0.6 ml/min at 25 ºC.

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250 **RESULTS**

251 ***pta* deletion in *C. saccharoperbutylacetonicum* N1-4.** In the previous work, Wang et al.
252 developed the customized CRISPR-Cas9 system for efficient genome editing in *C. beijerinckii*
253 (27). Here first we evaluated whether we could apply this tool directly for genome engineering in
254 *C. saccharoperbutylacetonicum*. We selected *pta* as our first target gene because it was
255 demonstrated that the deletion of this gene is achievable in other solventogenic clostridial strains,
256 and also the deletion of this gene can potentially lead to enhanced butanol production (41, 42).
257 The plasmid pYW34-deltpta was constructed using the 'general' CRISPR-Cas9 vector pYW34
258 for *C. beijerinckii* as mother vector (27). It was transformed into *C. saccharoperbutylacetonicum*
259 through electroporation, with a transformation efficiency of 1.6×10^4 cfu/μg DNA. After 24 h of
260 cultivation, single colonies were picked and subcultured in TGYE liquid medium. In order to
261 evaluate the genome engineering efficiency in *C. saccharoperbutylacetonicum* of the CRISPR-
262 Cas9 that was customized for *C. beijerinckii*, we started to induce the expression of the Cas9
263 from the second generation of the transferring culture. That is, starting from the second
264 generation, while the culture was kept being transferred in TGYE medium, it was meanwhile
265 transferred into TGYLE liquid medium. cPCR was performed with primers YW1044 and
266 YW1045 using the liquid culture in TGYLE as template to test whether the PCR band from the
267 *pta* deletion mutant can be detected. In this case, the *pta* deletion mutant would generate a band
268 of 2,278 bp compared to a band of 3,280 bp that would be generated from the wild type. If the
269 mutant band was visible, then the culture from TYGLE liquid medium would be spread onto
270 TGYLE agar plates, and same cPCR would be performed to identify the mutant from the
271 colonies. Interestingly, from the second generation of the subculture, we started to detect the
272 mutant band (beside the wild type band) from the liquid culture (Fig. S3). However, no mutant

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273 was able to be identified from the colony grown on the plates from either the second or the third
274 generation of culture (for each generation, at least 48 colonies were randomly screened). While
275 for the fourth generation, three colonies out of 16 were detected with the mutant band (Fig. S4).
276 As described by Wang et al., VIE could occur during the genome editing of bacterial
277 chromosome using CRISPR-Cas9 system (27). Here, we further detected VIE using primers
278 pairs of YW1044/YW200 and YW847/YW1045, and the expected PCR bands for VIE were
279 detected for both ends in all the three mutant colonies (Fig. S5). Then one of the three colonies
280 was picked and re-spread onto new TGYLE agar plates. Further cPCR for detecting VIE was
281 performed and two out of 36 screened colonies showed no PCR bands for VIE. Then, these two
282 mutant colonies were inoculated into TGY liquid medium and subcultured in the same medium
283 for 7 generations for plasmid curing purpose. The culture was then spread onto TGY plates and
284 cPCR with YW880 and YW881 were conducted to detect the existence of the plasmid. It was
285 shown that none of the tested colonies showed visible PCR band and thus confirmed the curing
286 of the plasmid. Selected colonies (tested as mutants without plasmid) were spread onto the
287 TGYE plates and no colonies were grown after 48 hour of cultivation, further verifying the loss
288 of the plasmid. Finally, the mutation in the strain was further verified through cPCR using
289 primers YW1044 and YW1045 (Fig. 1A). The clean mutant strain with the whole *pta* ORF
290 deleted was named as *C*. *saccharoperbutylacetonicum* deltpta.
291 ***buk* deletion in *C. saccharoperbutylacetonicum* N1-4.** In a similar manner, we attempted to
292 delete *buk* in *C*. *saccharoperbutylacetonicum* N1-4. The plasmid pYW34-deltbuk was
293 transformed into *C*. *saccharoperbutylacetonicum* N1-4 wild-type, and the transformed cells were
294 plated onto TGYE plates. A transformation efficiency of 1.5×10^4 cfu/μg DNA was obtained.
295 Single colonies were picked and cultivated successively in TGYE liquid medium. From the

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second generation, cultures were also transferred into TGYLE for induction of Cas9 expression followed by plating on TGYLE plates. cPCR was performed with primers YW953 and YW954 to detect the mutation in the TGYLE liquid culture or on the plates. No mutant band was observed from the PCR using the liquid culture as template until the seventh generation. For the cPCR with the colonies from the seventh generation, two colonies out of 32 tested demonstrated double bands (2,061 bp for mutant and 3,119 bp for wild type) (data not shown). The colony with relative brighter mutant band was picked and replated onto a new TGYLE plate. Further screening of colonies from the replating indicated that 11 out of 16 colonies demonstrated double bands and 1 out of 16 colonies showed the pure mutant band (Fig. S6). VIE was also detected in this mutant using primers YW953/YW200 and YW847/YW 954 (date not shown). Through further replating and screening, the colonies without VIE were identified. Plasmid curing was then carried out and finally clean *buk* deletion mutant was obtained (Lane 3 in Fig. 1B). The mutant was designated as *C. saccharoperbutylacetonicum* deltbuk.

***buk* deletion in *C. saccharoperbutylacetonicum* deltpta.** In a further step, we tried to achieve a double knockouts, that is, knocking out *buk* using *C. saccharoperbutylacetonicum* deltpta as the starting strain. The plasmid pYW34-deltbuk was transformed into *C. saccharoperbutylacetonicum* deltpta. Through the same procedure as discussed above, the cPCR did not show visible mutant bands using the 2^{nd}-6^{th} generation of the TGYLE liquid culture as template. In the liquid culture of the seventh generation, both the mutant and the wild type bands were observed. For the cPCR with the colonies plated from the seven generation culture, four out of 32 tested colonies exhibited double bands (2,061 bp for the mutant and 3,119 bp for the wild type) (data not shown). Further screening of colonies from the re-plating indicated that two out of 16 colonies showed the pure mutant band (Fig. S7). VIE was also detected in both colonies.

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319 Through further replating, screening, and subculturing (for plasmid curing), the clean *pta* and
320 *buk* double knockout mutant was obtained (Lane 5 in Fig. 1C). The mutant was designated as *C*.
321 *saccharoperbutylacetonicum* deltptabuk.

322 **Optimization of CRISPR-Cas9 efficiency by evaluating various promoters for gRNA**
323 **expression.** With the customized CRISPR-Cas9 system, nearly 100% genome engineering
324 efficiency could be achieved in *C*. *beijerinckii* (27). Here, when we applied this system directly
325 to *C*. *saccharoperbutylacetonicum*, desirable genome engineering purposes were achieved, but
326 the efficiency was rather low (the mutation rate was less than 20% following the induction of
327 Cas9 expression). We hypothesized that this might be because the selection power (against the
328 unedited background cells) from the CRISPR-Cas9 was not very effective in *C*.
329 *saccharoperbutylacetonicum*. For the functionality of the CRISPR-Cas9 system, the high level of
330 gRNA expression is essential and thus could be the limiting factor in many cases, as indicated by
331 many previous studies (49, 50). Therefore, we decided to enhance the CRISPR-Cas9 efficiency
332 in *C*. *saccharoperbutylacetonicum* by identifying a promoter for more efficient gRNA expression.
333 For this purpose, the *veg* promoter from *B*. *subtilis* (46), the hypothetical *veg* promoter from *C*.
334 *saccharoperbutylacetonicum*, and the *E*. *coli* promoter J23119 (47) were selected and evaluated.
335 In addition, for an easy comparison, we still targeted on *pta* deletion, and used exactly the same
336 20-nt guiding sequence and the same homology arms. The corresponding vectors pSHW1-*pta*,
337 pSHW2-*pta*, pSHW3-*pta* were constructed and transformed into *C*. *saccharoperbutylacetonicum*
338 N1-4. Following the same procedure, the *pta* deletion mutant was generated and the genome
339 editing efficiency was evaluated.

340 As illustrated in Table 2, for the genome engineering with CRISPR-Cas9 using four different
341 promoters for gRNA expression, mutant bands were all started to be detected from the second

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342 generation of the TGYLE liquid culture. However, for the colonies grown from the replating of
343 the second generation of liquid culture, mutants were only identified (with cPCR screening for at
344 least 48 colonies for each, unless mutants were detected with screening on fewer colonies) from
345 the transformation with pSHW1-*pta*, pSHW3-*pta*, with a mutation rate of 12.5% and 75%,
346 respectively. While for the transformation with pSHW2-*pta*, no mutant was identified until the
347 7^{th} generation, in which one mutant was obtained out of 16 screened colonies (representing a
348 mutation rate of 6.25%). These results demonstrated that the various promoters for gRNA
349 expression in CRISPR-Cas9 led to the genome engineering efficiency in *C.*
350 *saccharoperbutylacetonicum* in the following order: $P_{J23119} > P_{vegb} > P_{sRNA} > P_{vegc}$. Therefore, the
351 CRISPR-Cas9 system with J23119 promoter for gRNA expression (and the lactose inducible
352 promoter for Cas9 expression) constructed here could be used as an efficient genome editing tool
353 in *C. saccharoperbutylacetonicum*. Mutants can be easily obtained after one generation of
354 cultivation in TGYE and one additional generation of incubation in TGYLE for the induction of
355 Cas9 expression. Surely, the efficiency could be well dependent on the chromosomal target and
356 the exact genome engineering purpose, which warrants further investigation in the future.
357 As a summary, the general strategy for gene deletion and mutant screening was demonstrated
358 in Figure S1. After transformation of the plasmid DNA containing the desirable construct for
359 deleting the targeted gene (*pta* or *buk* in this case), recombinant strains were subcultured
360 successively to permit efficient homologous recombination. During this process, both double
361 crossover (A1 in Fig. S1) and single crossover (B1 and C1 in Fig. S1) could possibly occur,
362 generating the chromosome with the clean gene deletion (A2 in Fig. S1) and the chromosome
363 with vector integrated (B2 and C2 in Fig. S1). Moreover, some recombinant strains might still be
364 the wild type containing the transformed plasmid (D1 in Fig. S1). Then lactose-containing

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365 medium was used to induce the expression of Cas9 which would lead to the DSB on the
366 chromosome wherever the target site (the 20 nt guiding sequence in gRNA) still existed (B2, C2
367 and D2 in Fig. S1). Finally, only the mutant with the clean deletion of *pta* or *buk* survived from
368 the selection and could be screened through cPCR.

369 **Fermentation of the mutants in P2 medium.** The kinetics of batch fermentation in P2 medium
370 with mutants as well as the wild type were demonstrated in Figure 2. Though slower growth rate
371 was observed when the *pta* or *buk* gene was deleted (Table 3), both *C*.
372 *saccharoperbutylacetonicum* deltpta and *C*. *saccharoperbutylacetonicum* deltbuk displayed
373 similar growth kinetics with the wild type strain (Fig. 2A). The slowest growth was observed in
374 the mutant with the deletion of both *pta* and *buk*. It showed a much longer lag phase and reached
375 a maximum OD_{600} of about 5.5%-14.4% lower than the other three strains. As indicated in
376 Figure 2B, *C*. *saccharoperbutylacetonicum* deltptabuk also showed the slowest glucose
377 consumption, with 73.1 g/l was consumed in 60 hours. While, 70.2, 69.5 and 71.5 g/l glucose
378 was consumed within 48 hours in *C*. *saccharoperbutylacetonicum* deltpta, deltbuk and the wild-
379 type strain, respectively. The pH profiles were illustrated in Figure 2C. In all strains, with the
380 production of acids from the beginning of the fermentation, the pH dropped quickly to 5.0 (and
381 was then controlled at ≥ 5.0); corresponding to the initiation of solventogenesis, the pH increased
382 to a peak followed by another slight decrease and finally rose back to a high plateau between 5.7
383 and 6.0. Comparatively, mutant with single gene deletion (*pta* or *buk*) dropped to pH 5.0 at about
384 8 h and 9 h, respectively, similar to the wild type (8 h) but stayed at 5.0 for a longer time (5
385 hours for deltpta and 6 hours for deltbuk) than the wild type (3 hours) before increase to the
386 higher level. For the double knockout mutant, deltptabuk, corresponding to the slowest growth, it

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387 took longer time (about 12 h) for the pH to decrease to pH 5.0, and stay at this point for more
388 than 10 hours before increase to pH 5.9, generating the highest peak as seen in Figure 2C.

389 As illustrated in Figures 2D and E, deletion the whole ORFs of the *pta* or *buk* gene did not
390 completely eliminate the formation of acetate or butyrate, indicating that additional acetate- and
391 butyrate-forming pathways were probably present in *C*. *saccharoperbutylacetonicum*. Compared
392 to the wild type, *pta* deletion decreased the acetate production slightly by 0.2 g/l and 0.1 g/l in *C*.
393 *saccharoperbutylacetonicum* deltpta and *C*. *saccharoperbutylacetonicum* deltptabuk,
394 respectively (Fig. 2D, Table 3). To make up energy (ATP) lose caused by *pta* deletion, *C*.
395 *saccharoperbutylacetonicum* deltpta and *C*. *saccharoperbutylacetonicum* deltptabuk increased
396 the production of butyrate by 0.4 g/l (14.8%) and 0.2 g/l (7.4%) respectively than that obtained in
397 wild type (Fig. 2E, Table 3). Similarly, in the mutant deltbuk with *buk* deletion, butyrate
398 production was decreased by 7.4%, while acetate production was increased significantly by 0.3
399 g/l (a 14.3% increase comparing to the wild type). In addition to acetate and butyrate, in a similar
400 manner, lactate was also produced (the maximum is ≥ 1.6 g/l) and reassimilated in all the strains
401 (Fig. 2F). Deletion of *pta* or *buk* led to the increase of lactate in the mutants, especially in *C*.
402 *saccharoperbutylacetonicum* deltptabuk in which lactate reached to a peak level of 1.9 g/l,
403 representing an 18.8% improvement compared to the wild type.

404 ABE started to be produced in the early exponential phase in all mutants and wild type (Figs.
405 2G, H and I). ABE production from the mutants and the wild type was also compared in Table 3.
406 Compared to the wild type, *C*. *saccharoperbutylacetonicum* deltpta, except for a later initiation
407 for solvent production, demonstrated very similar acetone, butanol and ethanol production
408 profiles, and also similar butanol and ABE yields as well as butanol selectivity (butanol/ABE
409 ratio). *C*. *saccharoperbutylacetonicum* deltbuk generated the comparable amount of butanol than

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410 wild type, but decreased production of acetone (by 11.7%) and ethanol (16.7%), resulting in a
411 slight increase in the selectivity (71.3% vs 68.2%). When compared to wild type, *C*.
412 *saccharoperbutylacetonicum* deltptabuk produced 15.6% less acetone, but 20.8% higher ethanol.
413 This strain also produced much higher butanol (1.3 g/l more than the wild type, reaching 19.0
414 g/L) with slightly higher butanol yield. The butanol selectivity was also higher (72.4% vs 68.2%).

415 **Fermentation of the mutants in MP2 medium.** To exclude the interference of acetate pre-
416 added in P2 medium for the fermentation kinetics, MP2 medium was used to carry out further
417 fermentation with the mutants as well as the wild type (Fig. 3). Compared to the fermentation
418 with P2 medium, a longer lag phase of around 12 h in MP2 was observed for the wild type and *C*.
419 *saccharoperbutylacetonicum* deltbuk, and prolonged lag phage of around 24 h was observed for
420 both *C*. *saccharoperbutylacetonicum* deltpta and deltptabuk (Fig. 3A). When entered into the
421 exponential phase, all the mutants grew more slowly and reached much lower maximal OD than
422 the wild type (Table 3). Corresponding to the slow growth, all mutants consumed glucose more
423 slowly especially when both the *pta* and *buk* genes were deleted (Fig. 3B). Within 60 h, 77.8 g/l,
424 64.6 g/l and 69.9 g/l glucose was consumed by the wild type, *C*. *saccharoperbutylacetonicum*
425 deltpta and *C*. *saccharoperbutylacetonicum* deltbuk, respectively; while in *C*.
426 *saccharoperbutylacetonicum* deltptabuk, 84 hours were taken to consume 68.3 g/l glucose.

427 As illustrated in Figure 3C, corresponding well with the long lag phase in cell growth, it took a
428 long time for the pH to drop to pH 5.0 (about 20 h, 28 h, 24 h and 28 h for the wild type, deltpta,
429 deltbuk and deltptabuk strain, respectively). Similarly, the pH was held at pH 5.0 for a much
430 longer time (10-20 hours) than that in P2 medium before it increased to high levels. Production
431 profiles for the acetate and the butyrate were demonstrated in Figures 3D and E. Without pre-
432 supplemented acetate in MP2, the deletion of the major acetate-producing gene *pta* led to an

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433 obvious decrease of acetate production by 14.5% and a significant increase of butyrate
434 production by 52.0% in *C*. *saccharoperbutylacetonicum* deltpta when compared to the wild type.
435 While when the *buk* gene responsible for butyrate production was knocked out, a 20.0% decrease
436 of butyrate production and a 175.4% increase of acetate production were observed in *C*.
437 *saccharoperbutylacetonicum* deltbuk compared to the wild type. In *C*.
438 *saccharoperbutylacetonicum* deltptabuk, the peak acetate production was comparable to that in
439 the wild type, but the peak butyrate production was 44.0% more than that in the wild type (Table
440 3). Besides lactate and butyrate, high level lactate was also detected in all the strains with MP2
441 (Fig. 3F). Compared with the lactate production in wild type, an improvement of 25.0% and 8.3%
442 was observed in *C*. *saccharoperbutylacetonicum* deltbuk and deltptabuk, respectively. While *C*.
443 *saccharoperbutylacetonicum* deltpta demonstrated the highest lactate production with an 83.3%
444 increase compared to the wild type (Table 3).

445 The ABE production profiles were shown in Figures 3G-3I, and also summarized in Table 3.
446 Compared to the wild type (18.4 g/l), the butanol production was slightly improved to 18.7 g/l
447 and 18.8 g/l, respectively in *C*. *saccharoperbutylacetonicum* deltpta and deltptabuk. But the
448 butanol yield was improved remarkably by 13.0% in these two mutants, due to their declined
449 consumption of sugars. The butanol production in *C*. *saccharoperbutylacetonicum* deltbuk
450 decreased by 0.7 g/l compared with the wild type; however, the similar butanol yield was still
451 obtained in *C*. *saccharoperbutylacetonicum* deltbuk as in the wild type, also due to the declined
452 consumption of sugar. Compared to the wild type, lower levels of acetone production were
453 observed in all three mutants, with a 42.9%, 11.7% and 39.0% decrease in *C*.
454 *saccharoperbutylacetonicum* deltpta, deltbuk and deltptabuk, respectively. Except for *C*.
455 *saccharoperbutylacetonicum* deltbuk which generated the same ethanol production with the wild

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type, the ethanol production was reduced by 15.2% and 38.9% in *C. saccharoperbutylacetonicum* deltpta and deltptabuk, respectively. The butanol selectivity was improved by ~10% in both *C. saccharoperbutylacetonicum* deltpta and deltptabuk, while that in deltbuk increase slightly.

DISCUSSION

C. saccharoperbutylacetonicum N1-4 is well known as a hyper-butanol-producing microorganism and has been used for commercial butanol production in Japan since 1960s until the shutdown of the butanol production facilities (11, 12, 51). As the type strain of the species of *C. saccharoperbutylacetonicum*, N1-4 strain is very different from other extensively studied solventogenic clostridial strains, such as *C. acetobutylicum* (ATCC 824) and *C. beijerinckii* (NCIMB 8052), in terms of both genotype and phenotype (52, 53). *C. saccharoperbutylacetonicum* (N1-4) has the largest genome of 6.7 Mb among all the solventogenic clostridia characterized so far (51). It also has a 136 kb megaplasmid which contains no genes apparently related to solvent formation. This is very different from *C. beijerinckii* who does not have a plasmid, and from *C. acetobutylicum* who contains a 192 kb megaplasmid the loss of which leads to the strain degeneration (54). The DNA transformation in *C. saccharoperbutylacetonicum* is difficult, demonstrating possibly a very different methylome comparing to the previously reported (55-57). Actually, an amenable transformation protocol has just been reported very recently (21). On the other hand, *C. saccharoperbutylacetonicum* demonstrates very robust cell growth (reaching up to OD_{600} of 16.0 as was overserved in this study, which has never been seen in the batch fermentation with either *C. beijerinckii* or *C. acetobutylicum*), very high butanol production capability, high butanol tolerance and selectivity.

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479 It also has a comparatively low sporulation frequency and can perform ABE fermentation using a
480 wide range of carbohydrates (12, 53, 58). However, compared with other ABE-producing strains
481 such as *C. acetobutylicum* and *C. beijerinckii*, the study of the genomics and genetics on *C.*
482 *saccharoperbutylacetonicum* is apparently lagging. To elucidate the complex fermentation
483 metabolism in *C. saccharoperbutylacetonicum* and further develop improved strains for solvent
484 production, the development of a versatile genetic engineering system is highly desirable. In this
485 study, an efficient genome editing toolkit was developed based on the CRISPR-Cas9 system,
486 paving the way for efficient genomic engineering in *C. saccharoperbutylacetonicum*. Based on
487 this system, single and double knockout mutants were generated related to the acid production
488 pathways.

489 First, we evaluated the CRISPR-Cas9 system that was customized for *C. beijerinckii* for
490 genome engineering in *C. saccharoperbutylacetonicum* (38). The *pta* deletion mutant was
491 obtained in the fourth generation of subculture with a mutation rate of 18.75%, and the *buk*
492 deletion mutant was obtained in the seventh generation of subculture with a mutation rate of 6.25%
493 (12.5% for *buk* deletion in generating the *pta-buk* double deletion mutant). The varied efficiency
494 in deleting different genes indicated that the CRISPR-Cas9 genome engineering efficiency could
495 be affected by different target sites and homology arm sequences as previously reported (50).
496 Notwithstanding, the efficiency was much lower than that was observed when the same system
497 was used for genome engineering in *C. beijerinckii* (in that case, generally nearly 100% mutation
498 rate was observed for various genome engineering purposes) (27). This result was not surprising
499 considering the differences between these two strains. Obviously, this customized high efficient
500 genome engineering system for *C. beijerinckii* does not implement well in *C.*
501 *saccharoperbutylacetonicum*.

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As indicated by previous reports (27, 59), the CRISPR-Cas9 based genome engineering in bacterial strains very likely relies on the selection power of the Cas9 endonuclease against the background cells induced by the DSB. The gRNA expression is especially essential for the functionality of the whole system (49, 50). Therefore, we further set out to identify a more powerful promoter for gRNA expression. The *veg* promoter of the Gram-positive *B. subtilis* (P_{vegb}) has been employed for efficiency gRNA expression in *B. subtilis* (46). Therefore, we decided to evaluate its efficiency for gRNA expression in *C. saccharoperbutylacetonicum*; meanwhile, the endogenous hypothetical *veg* promoter in *C. saccharoperbutylacetonicum* (P_{vegc}) was also selected. Additionally, the *E. coli* promoter P_{J23119} has been proved to be effective for gRNA expression in *E. coli* and recently in solventogenic *C. acetobutylicum* and *C. beijerinckii* (34, 39, 47, 60). So, this promoter was also selected to drive the gRNA expression in this study. As shown in Table 2, by following the same procedure to obtain *pta* deletion mutant, it was determined that the order of the promoters for efficient gRNA expression (and thus the genome editing efficiency) is: $P_{J23119} > P_{vegb} > P_{sRNA} > P_{vegc}$. With P_{J23119}, a high mutation rate of 75% was obtained through screening the colonies from the second generation of subculture. This represents a very desirable genome engineering efficiency. Though the mutation rate was not as high as that obtained in *C. beijerinckii* (100%), mutants can be easily obtained through screening after one generation of subculturing in TGYE followed by one generation of incubation in TGYLE without serial transfers as done in *C. beijerinckii* (27). Comparatively, lower efficiency was obtained with P_{vegc} for gRNA expression. It is worthwhile to point out that, the total 325 bp including the whole P_{vegc} region was used in this test because the exact transcription start site of the *veg* gene was unknown. It is possible that an improved efficiency can be achieved if we did a careful selection of the P_{vegc} sequence to be used for gRNA expression (especially, the RBS was

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525 likely redundant and might have a negative impact since gRNA is a non-coding RNA). It has
526 been reported that the strong expression of gRNA is essential for the functionality of the
527 CRISPR-Cas9 system (49, 50). Along this line, in this study, we provided solid data to verify
528 that the achievement of potent gRNA expression is the critical step to develop a high efficient
529 CRISPR-Cas9 system for genome engineering.

530 As reported in other *Clostridium* strains (45, 61), the inactivation of the *pta* gene did not
531 completely eliminate the acetate formation, indicating that additional acetate-forming pathways
532 might exist in *C. saccharoperbutylacetonicum*. It needs to point out that the acid production
533 pathways in many of the previous characterized mutants were disrupted with TargeTron based
534 intron-insertion approaches (44, 45). It is suspected that residual activities of the enzymes
535 encoded by disrupted genes can still exist in the mutant. In this study, the clean and complete
536 deletion of the primary acid production genes was obtained using the CRISPR-Cas9 system; the
537 corresponding acid production in the mutant has not been eliminated either. In the fermentation
538 with P2 medium, the deletion of *pta* resulted in a slight decrease of acetate production in *C.*
539 *saccharoperbutylacetonicum* deltpta and deltptabuk (Fig. 2D and Table 3). Since ATP is the
540 fundamental demand of cell growth and other metabolisms (62), and more ATP per glucose can
541 be generated through the acetate formation pathway (than the butyrate formation pathway), the
542 deletion of *pta* imposed a severe metabolic burden on cells (63). To compensate the energy loss
543 and avoid significant decline in overall cell growth, more carbon flow were directed to the
544 butyrate formation, leading to a > 7% increase of butyrate production in both *C.*
545 *saccharoperbutylacetonicum* deltpta and deltptabuk. Similarly, the deletion of *buk* gene led to a
546 14.3% increase of acetate production in *C. saccharoperbutylacetonicum* deltbuk, and a slight
547 decrease of butyrate production. Production of butyrate was still observed in *C.*

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548 *saccharoperbutylacetonicum* deltbuk and deltptabuk, suggesting the existence of other active
549 butyrate formation pathway genes. Besides *buk* (GenBank: AGF54071.1) that was deleted in this
550 study, there are another two butyrate kinase encoding genes in the *C.*
551 *saccharoperbutylacetonicum* N1-4 genome, which are denoted as butyrate kinase 1
552 (WP_015392233.1) and butyrate kinase 2 (WP_015395384.1). Further study is in progress in our
553 lab to evaluate the contributions of these three butyrate kinases on butyrate production in this
554 microorganism.

555 In MP2 medium without pre-added acetate, long lag phases were observed in all the mutants
556 as well as in the wild type (Fig. 3A), indicating a positive effect of acetate on cell growth as also
557 reported previously (64, 65). The mutants showed slower growth rate than the wild type (Table
558 3); the disruption of acid production pathways might lead to the declined energy generation and
559 thus the metabolic burden on cell growth (61). In MP2 medium with the elimination of the
560 interference from the pre-added acetate, *C. saccharoperbutylacetonicum* deltpta clearly
561 demonstrated reduced acetate production and meanwhile enhanced butyrate production to
562 compensate the ATP generation. Similarly, the *buk* deletion in *C. saccharoperbutylacetonicum*
563 deltbuk led to apparent decrease of butyrate production and a significant increase of acetate
564 production to balance the ATP generation. These results confirmed that the deleted *pta*
565 (GenBank: AGF55073.1) and *buk* (GenBank: AGF54071.1) are the primary acid formation
566 pathway genes for acetate and butyrate production, respectively, although there are other
567 annotated homologous genes exist. A comprehensive transcriptomic analysis is desirable to
568 further enhance the genome annotation and assess the function of each specific gene (66, 67).

569 As reported previously, lactate dehydrogenase in solventogenic clostridia only shows
570 remarkable activities under certain growth conditions such as iron limitation (68). However, we

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571 observed that the wild type *C*. *saccharoperbutylacetonicum* N1-4 produced 1.6 g/l lactate (at the
572 peak level) in P2 medium and 1.2 g/l in the MP2 medium, which was even more than the
573 production of acetate. While under the similar condition, less than 0.2 g/l lactate was produced
574 by *C*. *beijerinckii* 8052 (45) and less than 0.1 g/l lactate was observed in *C*. *acetobutylicum*
575 ATCC 824 (68). Therefore, the lactate formation pathway may act more actively in *C*.
576 *saccharoperbutylacetonicum* N1-4. Moreover, there are six genes annotated as lactate
577 dehydrogenase in *C*. *saccharoperbutylacetonicum*; the action of these genes and the mechanism
578 for lactate production and re-utilization in *C*. *saccharoperbutylacetonicum* need to be further
579 elucidated. Similar with the results in *C*. *beijerinckii* (45), deletion of *pta* or *buk* led to the
580 improvement of lactate production; especially in the *pta* deletion mutant, the lactate production
581 was increased by 83.3% in MP2 medium when compared to the wild type. It was reported that
582 the lactate formation pathway can serve as a less efficient method for energy generation and
583 NADH oxidation when the pathway for the disposal of protons and electrons by the generation
584 of molecular hydrogen is inhibited (45). The increase of lactate production may help mitigate for
585 the decreased ATP production in the mutants.

586 In P2 medium, the deletion of *buk* gene in *C*. *saccharoperbutylacetonicum* deltbuk slightly
587 increased the butanol production and butanol yield while significantly decreased the production
588 of acetone (by 11.7%) and ethanol (by 16.7%), resulting in a higher butanol selection. In *C*.
589 *saccharoperbutylacetonicum* deltptabuk, the acetone production was decreased by 15.6%, and
590 the butanol production was improved by 1.3 g/l, reaching 19.0 g/l. Therefore, a much higher
591 butanol selectivity (72.4%, comparing 68.2% in the wild type) was observed. The butanol
592 production reported here represents one of the highest among the ever reported from batch
593 fermentations for biobutanol production (44). However, a significant improvement in solvent

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production in the deltptabuk strain comparing to the wild type was not observed as previously reported in the mutant of *C. acetobutylicum* with the same homologous genes disrupted (44) . This from another angle indicated that *C. saccharoperbutylacetonicum* is different from the extensively studied canonical *C. acetobutylicum* in terms of the regulation of solvent production metabolism. Butanol producing strains can rarely tolerate > 2% butanol, and thus the butanol production titer in the regular batch fermentation can hardly exceed 20.0 g/L (69). Butanol tolerance is considered as one of the major bottlenecks to improve the butanol production in the host workhorse. Therefore, the further enhancement of butanol tolerance through systematic metabolic engineering might be a rational strategy in order to further improve the final butanol production in *C. saccharoperbutylacetonicum*. With MP2 medium, the butanol yield was improved by 13.0% in both *C. saccharoperbutylacetonicum* deltpta and deltptabuk than that from the wild type. Furthermore, the butanol selectivity was also improved by ~10% in *C. saccharoperbutylacetonicum* deltpta and deltptabuk when compared to the wild type in the fermentation with MP2 medium. With these desirable butanol-producing features, the mutants constructed in this study can be used as excellent starting strains to construct more robust ones for butanol production through metabolic engineering. Considering that the ABE fermentation is medium dependent (as shown with P2 and MP2 media) (45), fermentation conditions can be further optimized for the mutants to achieve more desirable solvent production.

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819 **TABLE 1** Strains and plasmids used in this study

Name	Characteristics*	Sources or references
Strains		
E. coli DH5α	F⁻ *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20* φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 hsdR17($r_K^-m_K^+$) λ⁻	NEB
C. beijerinckii NCIMB 8052	ATCC 51743	ATCC
C. saccharoperbutylacetonicum N1-4	ATCC 13564, wild type strain	ATCC
deltpta	N1-4 Δ*pta*	This work
deltbuk	N1-4 Δ*buk*	This work
deltptabuk	N1-4 Δ*pta* Δ*buk*	This work
Plasmids		
pYW34	CAK1 ori Amp^r Erm^r Plac::Cas9 gRNA	(27)
pYW34-*pta*	pYW34 P_{sRNA}::20nt-gRNA targeting on *pta*	This work
pYW34-deltpta	pYW34-pta::homology arm sequences	This work
pYW34-*buk*	pYW34 P_{sRNA}::20nt-gRNA targeting on *buk*	This work
pYW34-deltbuk	pYW34-*buk*::homology arm sequences	This work
pSHW1-*pta*	pYW34 P_{vegb}::20 nt-gRNA targeting on *pta*	This work
pSHW2-*pta*	pYW34 P_{vegc}::20 nt-gRNA targeting on *pta*	This work
pSHW3-*pta*	pYW34 P_{J23119} ::20 nt-gRNA targeting on *pta*	This work
pSHW1-deltpta	pSHW1-pta::homologous arm sequences	This work
pSHW2-deltpta	pSHW2-*pta*::homologous arm sequences	This work
pSHW3-deltpta	pSHW3-*pta*::homologous arm sequences	This work

820 * $P_{sRNA,}$ the small RNA promoter from *C. beijerinckii*; $P_{vegb,}$ the *veg* promoter from *B. subtilis*; P_{vegc}, the hypothetical

821 *veg* gene promoter from *C. saccharoperbutylacetonicum*; $P_{J23119,}$ the J23119 promoter from *E. coli*.

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829 **TABLE 2** Comparison of genome editing efficiency using CRISPR-Cas9 with different

830 promoters for gRNA expression

Plasmids used for transformation	Promoter*	Mutant band started to be detected from TGYLE liquid culture using cPCR	Obtainment of mutant strain	Mutation rate
pYW34-deltpta	P_{sRNA}	Second generation	fourth generation	3/16 (18.75%)
pSHW1-deltpta	P_{vegb}	Second generation	Second generation	2/16 (12.5%)
pSHW2-deltpta	P_{vegc}	Second generation	seventh generation	1/16 (6.25%)
pSHW3-deltpta	P_{J23119}	Second generation	Second generation	12/16 (75%)

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832 * $P_{sRNA,}$ the small RNA promoter from *C*. *beijerinckii*; $P_{vegb,}$ the *veg* promoter from *B*. *subtilis*; P_{vegc}, the hypothetical

833 *veg* gene promoter from *C*. *saccharoperbutylacetonicum*; $P_{J23119,}$ the J23119 promoter from *E*. *coli*.

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849 **TABLE 3** Summary of the fermentation results for the *C. saccharoperbutylacetonicum* mutants

850 comparing to the wild type strain[a].

	Fermentation results with mutants and wild type in two media							
	P2 medium				MP2 medium			
Characteristics	N1-4	deltpta	deltbuk	deltptabuk	N1-4	deltpta	deltbuk	deltptabuk
Growth rate (/h)	0.42±0.01	0.38±0.02	0.36±0.03	0.29±0.01	0.66±0.01	0.41±0.01	0.56±0.03	0.35±0.02
Maximum OD_{600}	14.6±0.2	13.2±0.2	13.8±0.5	12.5±0.3	16.0±0.2	14.7±0.15	13.8±0.4	11.8±0.2
Peak acetate (g/l)[b]	2.1±0.03	1.9±0.04	2.4±0.01	2.0±0.01	0.69±0.01	0.59±0.02	1.9±0.01	0.67±0.02
Peak butyrate (g/l)	2.7±0.02	3.1±0.04	2.5±0.02	2.9±0.01	2.5±0.05	3.8±0.05	2.0±0.03	3.6±0.05
Peak lactate (g/l)	1.6±0.05	1.7±0.03	1.7±0.01	1.9±0.01	1.2±0.05	2.2±0.15	1.5±0.05	1.3±0.1
Butanol (g/l)[c]	17.7±0.1	17.7±0.2	17.9±0.2	19±0.1	18.4±0.02	18.7±0.05	17.7±0.1	18.8±0.15
Butanol yield	0.24±0.01	0.24±0.01	0.25±0.01	0.25±0.01	0.23±0.01	0.26±0.02	0.24±0.02	0.26±0.01
Acetone (g/l)	7.7±0.05	7.5±0.1	6.8±0.05	6.5±0.05	7.7±0.02	4.4±0.05	6.8±0.05	4.7±0.1
Ethanol (g/l)	0.72±0.02	0.78±0.01	0.6±0.04	0.87±0.02	0.72±0.01	0.61±0.01	0.73±0.02	0.44±0.01
Total ABE (g/l)	25.9±0.16	25.8±0.06	25.2±0.08	26.3±0.03	26.8±0.02	23.6±0.11	25.2±0.17	23.8±0.22
Butanol selectivity[d] (g/g)	68.2±0.05%	68.3±0.05%	71.3±0.01%	72.4±0.25%	68.5±0.1%	79±0.2%	70.3±0.1%	78.7±0.1%

851 [a] N1-4: *C. saccharoperbutylacetonicum* N1-4 wild type; deltpta: *C. saccharoperbutylacetonicum* deltpta;

852 deltbuk: *C. saccharoperbutylacetonicum* deltbuk; deltptabuk: *C. saccharoperbutylacetonicum* deltptabuk.

853 [b] There was approximately 1.7 g/l of acetate pre-added in the P2 medium.

854 [c] The reported titers were the maximum values after the solvent production reached plateaus.

855 [d] Butnaol selectivity: the ratio of Butanol/Total ABE.

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858 **FIGURE LEGENDS**

859 **Figure 1.** Colony PCR (cPCR) results confirmed the deletion of *pta* and *buk* genes. (A) cPCR
860 results using primers YW1044 and YW1045 flanking the upstream and downstream of the
861 homologous recombination region of *pta* gene in *C*. *saccharoperbutylacetonicum* deltpta (Lane 1,
862 2,278 bp) and the wild type (Lane 2, 3,280 bp); (B) cPCR results using primers YW953 and
863 YW954 flanking the upstream and downstream of the homologous recombination region of *buk*
864 gene in the *C*. *saccharoperbutylacetonicum* deltbuk (Lane 3, 2,061 bp) and the wild type (Lane 4,
865 3,119 bp); (C) cPCR results using primers YW953 and YW954 flanking the upstream and
866 downstream of the homologous recombination region of *buk* gene in *C*.
867 *saccharoperbutylacetonicum* deltptabuk (Lane 5, 2,061 bp) and the *C*.
868 *saccharoperbutylacetonicum* deltpta strain (Lane 6, 3,119 bp). The NEB 1-kb DNA ladder was
869 used as the marker (Lane M) with numbers on the left representing the band length in kb.

870 **Figure 2.** Batch fermentation profiles of the *C*. *saccharoperbutylacetonicum* mutants compared
871 to the wild type in P2 medium. (A) Cell growth; (B) Glucose consumption; (C) pH profiles; (D)
872 Acetate production; (E) Butyrate production; (F) Lactate production; (G) Acetone production; (H)
873 Ethanol production; (I) Butanol production. N1-4: *C*. *saccharoperbutylacetonicum* N1-4; deltpta:
874 *C*. *saccharoperbutylacetonicum* deltpta; deltbuk: *C*. *saccharoperbutylacetonicum* deltbuk;
875 deltptabuk: *C*. *saccharoperbutylacetonicum* deltptabuk. Fermentation was carried out in
876 replicates, and one batch was reported here as representative.

877 **Figure 3.** Batch fermentation profiles of the *C*. *saccharoperbutylacetonicum* mutants compared
878 to the wild type in MP2 medium. (A) Cell growth; (B) Glucose consumption; (C) pH profiles; (D)
879 Acetate production; (E) Butyrate production; (F) Lactate production; (G) Acetone production; (H)
880 Ethanol production; (I) Butanol production. N1-4: *C*. *saccharoperbutylacetonicum* N1-4; deltpta:

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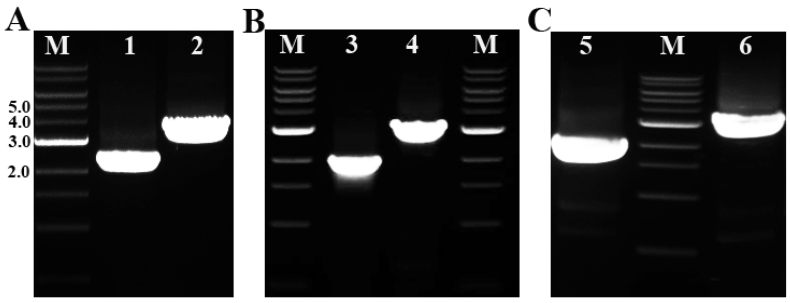
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881 *C*. *saccharoperbutylacetonicum* deltpta; deltbuk: *C*. *saccharoperbutylacetonicum* deltbuk;
882 deltptabuk: *C*. *saccharoperbutylacetonicum* deltptabuk. Fermentation were carried out in
883 replicates, and one batch was reported here as representative.

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887 **FIG 1**
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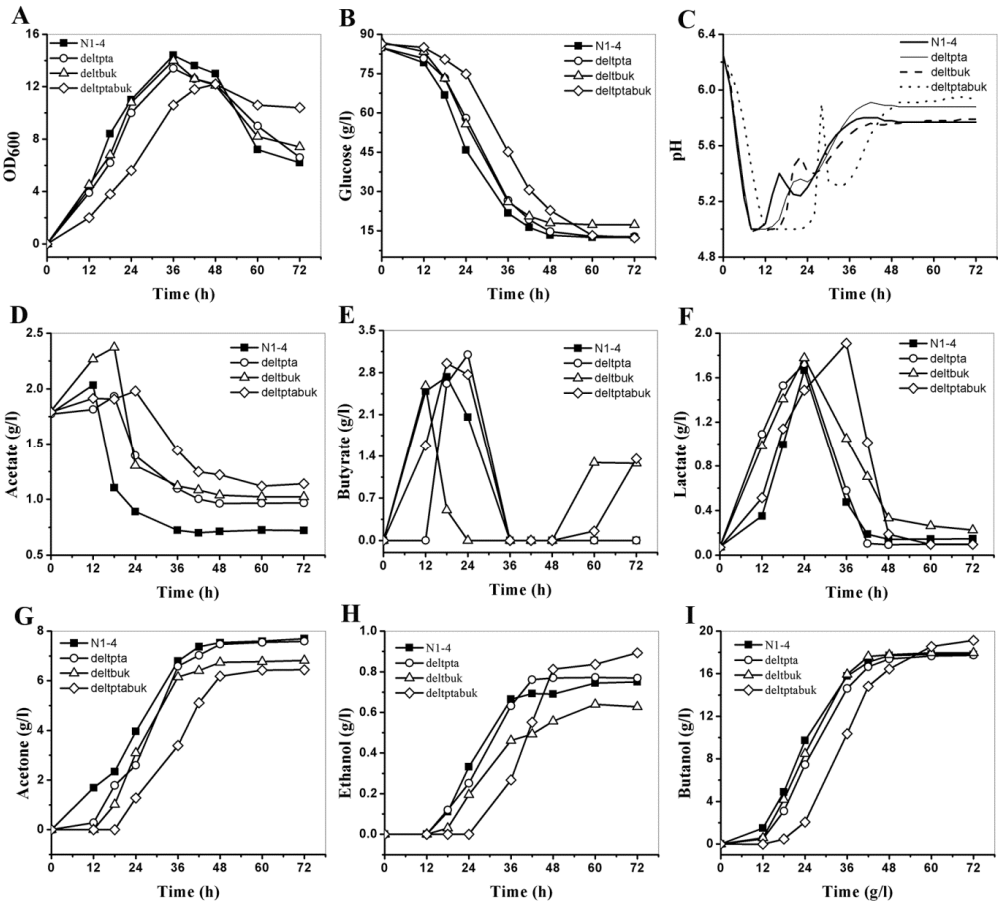


FIG 2

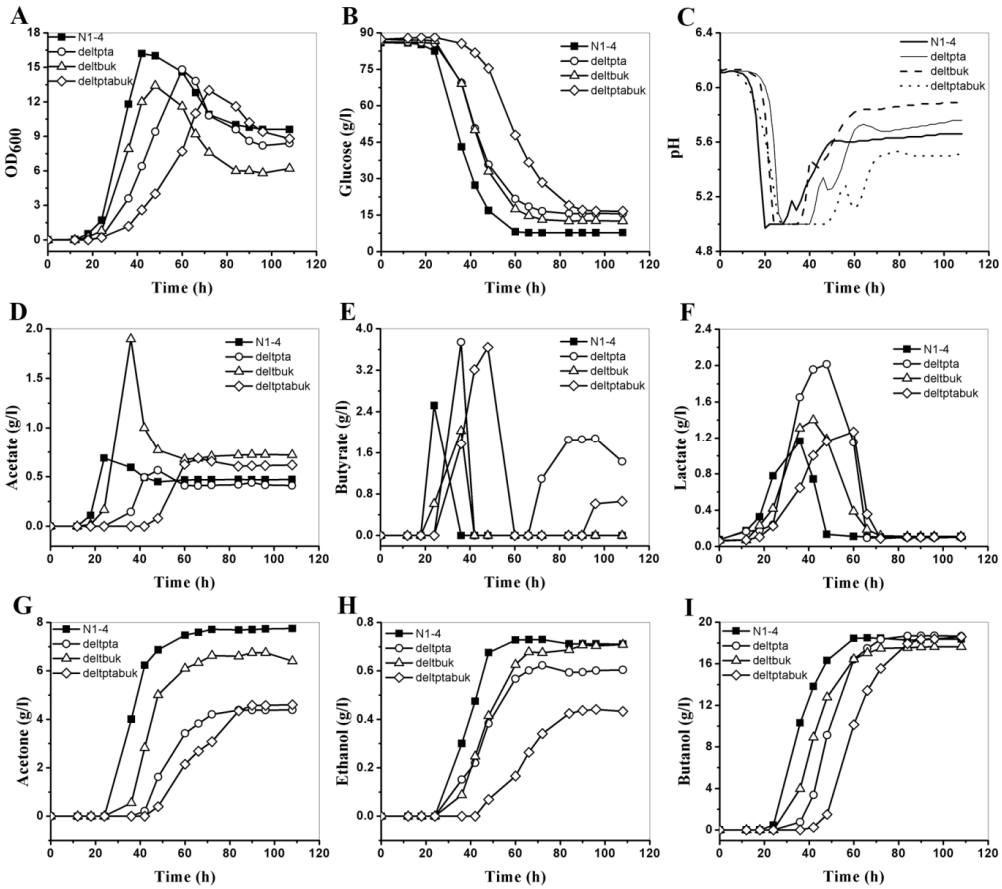
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902 **FIG 3**

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