



Genome Editing in *Clostridium saccharoperbutylacetonicum* N1-4 with the CRISPR-Cas9 System

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ABSTRACT *Clostridium saccharoperbutylacetonicum* N1-4 is well known as a hyperbutanol-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 clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated 9 (CRISPR-Cas9) system. First, the functionality of the CRISPR-Cas9 system previously customized for *Clostridium beijerinckii* was evaluated in *C. saccharoperbutylacetonicum* by targeting *pta* and *buk*, two essential genes for acetate and butyrate production, respectively. *pta* and *buk* single and 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 guide RNA (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, depending on the fermentation medium. In the *pta buk* double deletion mutant, the butanol production in P2 medium reached 19.0 g/liter, which is one of the highest levels 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 mechanism in this hyperbutanol-producing microorganism and developing strains with desirable butanol-producing features. This tool can be easily adapted for use in closely related 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 areas of metabolic engineering and synthetic biology. The developed mutants can be used as excellent starting strains for development of more robust ones for desirable solvent production.

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

Biofuels produced from renewable biomass provide a feasible solution for addressing problems such as environmental pollution and climate change resulting from fossil fuel consumption. Thus, biofuels are considered appropriate substitutes for fossil

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fuels at their depletion (1). Biobutanol produced through acetone-butanol-ethanol (ABE) fermentation possesses various advantages over ethanol as a fuel source and is considered one of the most promising biofuel candidates for the future (2). Additionally, butanol manifests an 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: *Clostridium acetobutylicum*, *Clostridium beijerinckii*, *Clostridium saccharobutylicum*, and *Clostridium saccharoperbutylacetonicum* (2). Among them, undoubtedly, *C. acetobutylicum* and *C. beijerinckii* have been the most extensively investigated for their ABE fermentation physiology, genomics, genetics, and mutant development (especially in recent years) (4–9). *C. saccharoperbutylacetonicum* N1-4 (ATCC 13564) was isolated in 1959 in Japan and was used for industrial butanol production from then until the late 1980s (10, 11). As a well-known hyper-butanol-producing strain (12), *C. saccharoperbutylacetonicum* N1-4 has been broadly studied for its fermentation characteristics, including 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 of *C. saccharoperbutylacetonicum* has lagged. In addition, there were rare reports concerning attempts at metabolic engineering of 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 an amylase 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 were published about 10 years ago from the same research group. During the preparation of this article, Herman et al. reported the development of a transformation protocol and the metabolic engineering of *C. saccharoperbutylacetonicum* using plasmid-based overexpression and nonreplicative vector-based allelic exchange (21). However, clearly, more efficient and versatile genetic engineering tools for *C. saccharoperbutylacetonicum* are highly desirable 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 phages 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 (23–28). In the CRISPR-Cas9 system, the CRISPR RNA (crRNA) and the *trans*-activating CRISPR RNA (tracrRNA) form a complex 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 double-strand breakage (DSB) (29). Genetic mutations can be introduced through nonhomologous end joining (NHEJ) or homology-directed repair (HDR) by providing a DNA editing template (30). 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 (29). In the past few years, numerous successes in genome editing based on the CRISPR-Cas9 system in various bacteria have been reported, including *Escherichia coli* (31, 32), *S. pneumoniae* (23), *Streptomyces* species (33–35), *Tatumella citrea* (32), *C. pasteurianum* (36), *C. beijerinckii* (8, 26, 34, 37), and *C. acetobutylicum* (38, 39). It was demonstrated that although the CRISPR-Cas9-based genome editing also relies on the targeted DSB from Cas9, the DSB more likely serves as a selection power for the edited mutant cells against the unedited background cells (23, 26).

During the biphasic ABE fermentation, the production of acids is indispensable and plays important roles in the metabolic switch for solvent production (40). In the acidogenic phase, acetate is produced from acetyl coenzyme A (acetyl-CoA) through

the action of phosphotransacetylase (PTA) and acetate kinase (ACK), while butyrate is generated through catalysis by phosphotransbutyrylase (PTB) and butyrate kinase (BUK). Subsequently, during the solventogenic phase, both acids are reassimilated for the production of acetone, butanol, and ethanol (41). Disruption of acid formation pathways has been achieved previously in *C. acetobutylicum* and *C. beijerinckii*; the results demonstrated that the production patterns of both acids and solvents were altered by such manipulation (42–44).

Therefore, in this study, we developed an efficient CRISPR-Cas9-based genome editing system for *C. saccharoperbutylacetonicum*. Based on this system, the primary *pta* gene, *buk* gene, or both were deleted, and the fermentation features of the mutants compared with those of the wild-type strain were characterized. The developed CRISPR-Cas9 system provided a versatile genome engineering tool for *C. saccharoperbutylacetonicum*, allowing easy elucidation of the metabolism of this microorganism and development of robust strains for biobutanol production. Additionally, this study provides essential references for researchers developing customized CRISPR-Cas9 genome engineering tools for other microorganisms.

RESULTS

***pta* deletion in *C. saccharoperbutylacetonicum* N1-4.** In previous work, Wang et al. developed a customized CRISPR-Cas9 system for efficient genome editing in *C. beijerinckii* (26). Here, we first evaluated whether we could apply this tool directly for genome engineering in *C. saccharoperbutylacetonicum*. We selected *pta* as our first target gene because it was demonstrated that the deletion of this gene is achievable in other solventogenic clostridial strains and also because the deletion of this gene can potentially lead to enhanced butanol production (40, 41). The plasmid pYW34-deltpta was constructed using the “general” CRISPR-Cas9 vector pYW34 for *C. beijerinckii* as the mother vector (26). It was transformed into *C. saccharoperbutylacetonicum* through electroporation, with a transformation efficiency of 1.6×10^4 CFU/ μ g DNA. After 24 h of cultivation, single colonies were picked and subcultured in tryptone-glucose-yeast extract (TGY) liquid medium plus erythromycin (TGYE). In order to evaluate the genome engineering efficiency in *C. saccharoperbutylacetonicum* of the CRISPR-Cas9 that was customized for *C. beijerinckii*, we started to induce the expression of the Cas9 from the second generation of the transferring culture. That is, starting from the second generation, while the culture continued to be transferred in TGYE medium, it was also transferred into TGYE liquid medium supplemented with lactose (TGYLE). Colony PCR (cPCR) was performed with primers YW1044 and YW1045 using the liquid culture in TGYLE as the template to test whether the PCR band from the *pta* deletion mutant could be detected. In this case, the *pta* deletion mutant would generate a band of 2,278 bp, compared to a band of 3,280 bp that would be generated from the wild type. If the mutant band was visible, then the culture from TGYLE liquid medium was spread onto TGYLE agar plates and the same cPCR was performed to identify the mutant from the colonies. Interestingly, from the second generation of the subculture, we started to detect the mutant band (beside the wild-type band) from the liquid culture (see Fig. S3 in the supplemental material). However, no mutant was able to be identified from the colony grown on the plates from either the second or the third generation of culture (for each generation, at least 48 colonies were randomly screened), while for the fourth generation, three colonies out of 16 were detected with the mutant band (see Fig. S4 in the supplemental material).

As described by Wang et al., vector integration events (VIE) could occur during the genome editing of the bacterial chromosome using the CRISPR-Cas9 system (26). Here, we further detected VIE using primer pairs YW1044/YW200 and YW847/YW1045, and the expected PCR bands for VIE were detected for both ends in all the three mutant colonies (see Fig. S5 in the supplemental material). One of the three colonies was picked and respread onto new TGYLE agar plates. Further cPCR for detecting VIE was performed, and two out of 36 screened colonies showed no PCR bands for VIE. These two mutant colonies were then inoculated into TGY liquid medium and subcultured in

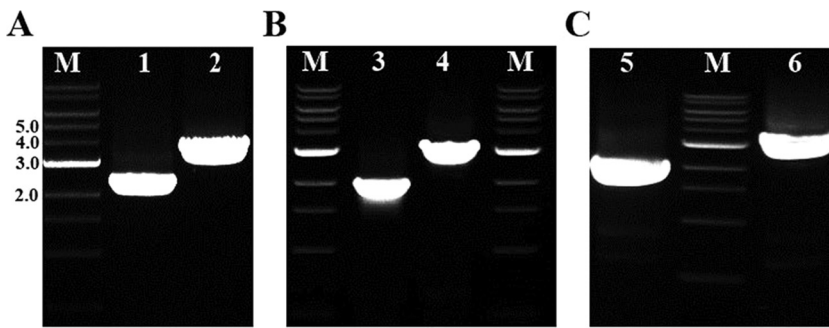


FIG 1 Colony PCR (cPCR) results confirm the deletion of the *pta* and *buk* genes. (A) cPCR results using primers YW1044 and YW1045 flanking the upstream and downstream sequences of the homologous recombination region of the *pta* gene in *C. saccharoperbutylacetonicum* deltpa (lane 1, 2,278 bp) and the wild type (lane 2, 3,280 bp). (B) cPCR results using primers YW953 and YW954 flanking the upstream and downstream sequences of the homologous recombination region of the *buk* gene in *C. saccharoperbutylacetonicum* deltbuk (lane 3, 2,061 bp) and the wild type (lane 4, 3,119 bp). (C) cPCR results using primers YW953 and YW954 flanking the upstream and downstream sequences of the homologous recombination region of the *buk* gene in *C. saccharoperbutylacetonicum* deltpabuk (lane 5, 2,061 bp) and the *C. saccharoperbutylacetonicum* deltpa strain (lane 6, 3,119 bp). The NEB 1-kb DNA ladder was used as the marker (lane M), with numbers on the left representing the band length in kb.

the same medium for 7 generations for plasmid curing. The culture was then spread onto TGY plates, and cPCRs with YW880 and YW881 were conducted to detect the existence of the plasmid. It was shown that none of the tested colonies showed a visible PCR band, thus confirming the curing of the plasmid. Selected colonies (tested as mutants without a plasmid) were spread onto the TGYE plates, and no colonies were grown after 48 h of cultivation, further verifying the loss of the plasmid. Finally, the mutation in the strain was further verified through cPCR using primers YW1044 and YW1045 (Fig. 1A). The clean mutant strain with the whole *pta* open reading frame (ORF) deleted was named *C. saccharoperbutylacetonicum* deltpa.

***buk* deletion in *C. saccharoperbutylacetonicum* N1-4.** In a similar manner, we attempted to delete *buk* in *C. saccharoperbutylacetonicum* N1-4. The plasmid pYW34-deltbuk was transformed into wild-type *C. saccharoperbutylacetonicum* N1-4, and the transformed cells were plated onto TGYE plates. A transformation efficiency of 1.5×10^4 CFU/ μ g DNA was obtained. Single colonies were picked and cultivated successively in TGYE liquid medium. From the second generation, cultures were also transferred into TGYE for induction of Cas9 expression, followed by plating on TGYE plates. cPCR was performed with primers YW953 and YW954 to detect the mutation in the TGYE liquid culture or on the plates. No mutant band was observed from the PCR using the liquid culture as the 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 the mutant and 3,119 bp for the wild type) (data not shown). The colony with the brighter mutant band was picked and replated onto a new TGYE 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 (see Fig. S6 in the supplemental material). VIE were also detected in this mutant using primers YW953/YW200 and YW847/YW954 (data not shown). Through further replating and screening, the colonies without VIE were identified. Plasmid curing was then carried out, and finally a clean *buk* deletion mutant was obtained (Fig. 1B, lane 3). The mutant was designated *C. saccharoperbutylacetonicum* deltbuk.

***buk* deletion in *C. saccharoperbutylacetonicum* deltpa.** In a further step, we tried to achieve a double knockout, that is, knocking out *buk* using *C. saccharoperbutylacetonicum* deltpa as the starting strain. The plasmid pYW34-deltbuk was transformed into *C. saccharoperbutylacetonicum* deltpa. Through the same procedure as discussed above, the cPCR did not show visible mutant bands using the second to sixth generations of the TGYE liquid culture as the template. In the liquid culture of the seventh

TABLE 1 Comparison of genome editing efficiencies using CRISPR-Cas9 with different promoters for gRNA expression

Plasmid used for transformation	Promoter ^a	Generation for:		No. of mutants/ no. of colonies screened (mutation rate, %)
		Mutant band starting to be detected from TGYLE liquid culture using cPCR	Obtainment of mutant strain	
pYW34-deltpta	P _{sRNA}	Second	Fourth	3/16 (18.75)
pSHW1-deltpta	P _{veg_b}	Second	Second	2/16 (12.5)
pSHW2-deltpta	P _{veg_c}	Second	Seventh	1/16 (6.25)
pSHW3-deltpta	P _{J23119}	Second	Second	12/16 (75)

^aP_{sRNA}, small RNA promoter from *C. beijerinckii*; P_{veg_b}, veg promoter from *B. subtilis*; P_{veg_c}, hypothetical veg gene promoter from *C. saccharoperbutylacetonicum*; P_{J23119}, J23119 promoter from *E. coli*.

generation, both the mutant and the wild-type bands were observed. For the cPCR with the colonies plated from the seventh-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 replating indicated that two out of 16 colonies showed the pure mutant band (see Fig. S7 in the supplemental material). VIE were also detected in both colonies. Through further replating, screening, and subculturing (for plasmid curing), the clean *pta buk* double knockout mutant was obtained (Fig. 1C, lane 5). The mutant was designated *C. saccharoperbutylacetonicum* deltp_{tabuk}.

Optimization of CRISPR-Cas9 efficiency by evaluating various promoters for gRNA expression. With the customized CRISPR-Cas9 system, nearly 100% genome engineering efficiency could be achieved in *C. beijerinckii* (26). Here, when we applied this system directly to *C. saccharoperbutylacetonicum*, desirable results for genome engineering purposes were achieved, but the efficiency was rather low (the mutation rate was less than 20% following the induction of Cas9 expression). We hypothesized that this might be because the selection power (against the unedited background cells) from the CRISPR-Cas9 was not very effective in *C. saccharoperbutylacetonicum*. For the functionality of the CRISPR-Cas9 system, a high level of gRNA expression is essential and thus this could be the limiting factor in many cases, as indicated by many previous studies (45, 46). Therefore, we decided to enhance the CRISPR-Cas9 efficiency in *C. saccharoperbutylacetonicum* by identifying a promoter for more efficient gRNA expression. For this purpose, the veg promoter from *Bacillus subtilis* (P_{veg_b}) (47), the hypothetical veg promoter from *C. saccharoperbutylacetonicum* (P_{veg_c}), and the *E. coli* promoter J23119 (48) were selected and evaluated. In addition, for easy comparison, we still targeted *pta* deletion and used exactly the same 20-nucleotide (nt) guiding sequence fused to the small RNA (sRNA) promoter (P_{sRNA}) and the same homology arms. The corresponding vectors pSHW1-*pta*, pSHW2-*pta*, and pSHW3-*pta* were constructed and transformed into *C. saccharoperbutylacetonicum* N1-4. Following the same procedure, the *pta* deletion mutant was generated and the genome editing efficiency was evaluated.

As illustrated in Table 1, for the genome engineering with CRISPR-Cas9 using four different promoters for gRNA expression, mutant bands all started to be detected from the second generation of the TGYLE liquid culture. However, for the colonies grown from the replating of the second generation of liquid culture, mutants were identified (with cPCR screening for at least 48 colonies for each, unless mutants were detected with screening on fewer colonies) only from the transformations with pSHW1-*pta* and pSHW3-*pta*, with mutation rates of 12.5% and 75%, respectively, while for the transformation with pSHW2-*pta*, no mutant was identified until the seventh generation, where one mutant was obtained out of 16 screened colonies (representing a mutation rate of 6.25%). These results demonstrated that the various promoters for gRNA expression in CRISPR-Cas9 led to genome engineering efficiency in *C. saccharoperbutylacetonicum* in the order P_{J23119} > P_{veg_b} > P_{sRNA} > P_{veg_c}. Therefore, the CRISPR-Cas9 system with the J23119 promoter for gRNA expression (and the lactose-inducible promoter for Cas9 expression) constructed here could be used as an efficient genome

editing tool in *C. saccharoperbutylacetonicum*. Mutants can be easily obtained after one generation of cultivation in TGYE and one additional generation of incubation in TGYE for the induction of Cas9 expression. Of course, the efficiency could well be dependent on the chromosomal target and the exact genome engineering purpose, which warrants further investigation in the future.

As a summary, the general strategy for gene deletion and mutant screening is shown in Fig. S1 in the supplemental material. After transformation of the plasmid DNA containing the desirable construct for deleting the targeted gene (*pta* or *buk* in this case), recombinant strains were subcultured successively to permit efficient homologous recombination. During this process, both double crossovers (Fig. S1, A1) and single crossovers (Fig. S1, B1 and C1) could possibly occur, generating a chromosome with a clean gene deletion (Fig. S1, A2) and a chromosome with vector integrated (Fig. S1, B2 and C2). Moreover, some recombinant strains might still be the wild type containing the transformed plasmid (Fig. S1, D1). Lactose-containing medium was then used to induce the expression of Cas9, which would lead to the DSB on the chromosome wherever the target site (the 20-nt guiding sequence in gRNA) still existed (Fig. S1, B2, C2, and D2). Finally, only the mutant with the clean deletion of *pta* or *buk* survived from the selection and could be screened through cPCR.

Fermentation of the mutants in P2 medium. The kinetics of batch fermentation in P2 medium with mutants as well as the wild type are demonstrated in Fig. 2. Though a lower growth rate was observed when the *pta* or *buk* gene was deleted (Table 2), both *C. saccharoperbutylacetonicum* Δ ptat and *C. saccharoperbutylacetonicum* Δ buk displayed growth kinetics similar to those of the wild-type strain (Fig. 2A). The slowest growth was observed in the mutant with the deletion of both *pta* and *buk*. It showed a much longer lag phase and reached a maximum optical density at 600 nm (OD_{600}) of about 5.5% to 14.4% lower than those of the other three strains. As indicated in Fig. 2B, *C. saccharoperbutylacetonicum* Δ ptabuk also showed the slowest glucose consumption, with 73.1 g/liter consumed in 60 h, while 70.2, 69.5, and 71.5 g/liter glucose were consumed within 48 h in *C. saccharoperbutylacetonicum* Δ ptat and Δ buk and the wild-type strain, respectively. The pH profiles are illustrated in Fig. 2C. In all strains, with the production of acids from the beginning of the fermentation, the pH dropped quickly to 5.0 (and was then controlled at ≥ 5.0); corresponding to the initiation of solventogenesis, the pH increased to a peak, which was followed by another slight decrease, and finally rose back to a high plateau between 5.7 and 6.0. In comparison, mutants with single gene deletion (*pta* or *buk*) dropped to pH 5.0 at about 8 h and 9 h, respectively, similar to the wild type (8 h), but stayed at 5.0 for a longer time (5 h for Δ ptat and 6 h for Δ buk) than the wild type (3 h) before increasing to the higher level. For the double knockout mutant, Δ ptabuk, corresponding to the slowest growth, it took longer (about 12 h) for the pH to decrease to pH 5.0, and it stayed at this point for more than 10 h before increasing to pH 5.9, generating the highest peak (Fig. 2C).

As illustrated in Fig. 2D and E, deletion of the whole ORF of the *pta* or *buk* gene did not completely eliminate the formation of acetate or butyrate, indicating that additional acetate- and butyrate-forming pathways were probably present in *C. saccharoperbutylacetonicum*. Compared to that in the wild type, *pta* deletion decreased the acetate production slightly, by 0.2 g/liter and 0.1 g/liter in *C. saccharoperbutylacetonicum* Δ ptat and *C. saccharoperbutylacetonicum* Δ ptabuk, respectively (Fig. 2D; Table 2). To make up energy (ATP) loss caused by *pta* deletion, *C. saccharoperbutylacetonicum* Δ ptat and *C. saccharoperbutylacetonicum* Δ ptabuk increased the production of butyrate by 0.4 g/liter (14.8%) and 0.2 g/liter (7.4%), respectively, compared to that in the wild type (Fig. 2E; Table 2). Similarly, in the mutant Δ buk with a *buk* deletion, butyrate production was decreased by 7.4%, while acetate production was increased significantly by 0.3 g/liter (a 14.3% increase compared to the wild type). In addition to acetate and butyrate, in a similar manner, lactate was also produced (the maximum was ≥ 1.6 g/liter) and reassimilated in all the strains (Fig. 2F). Deletion of *pta* or *buk* led to

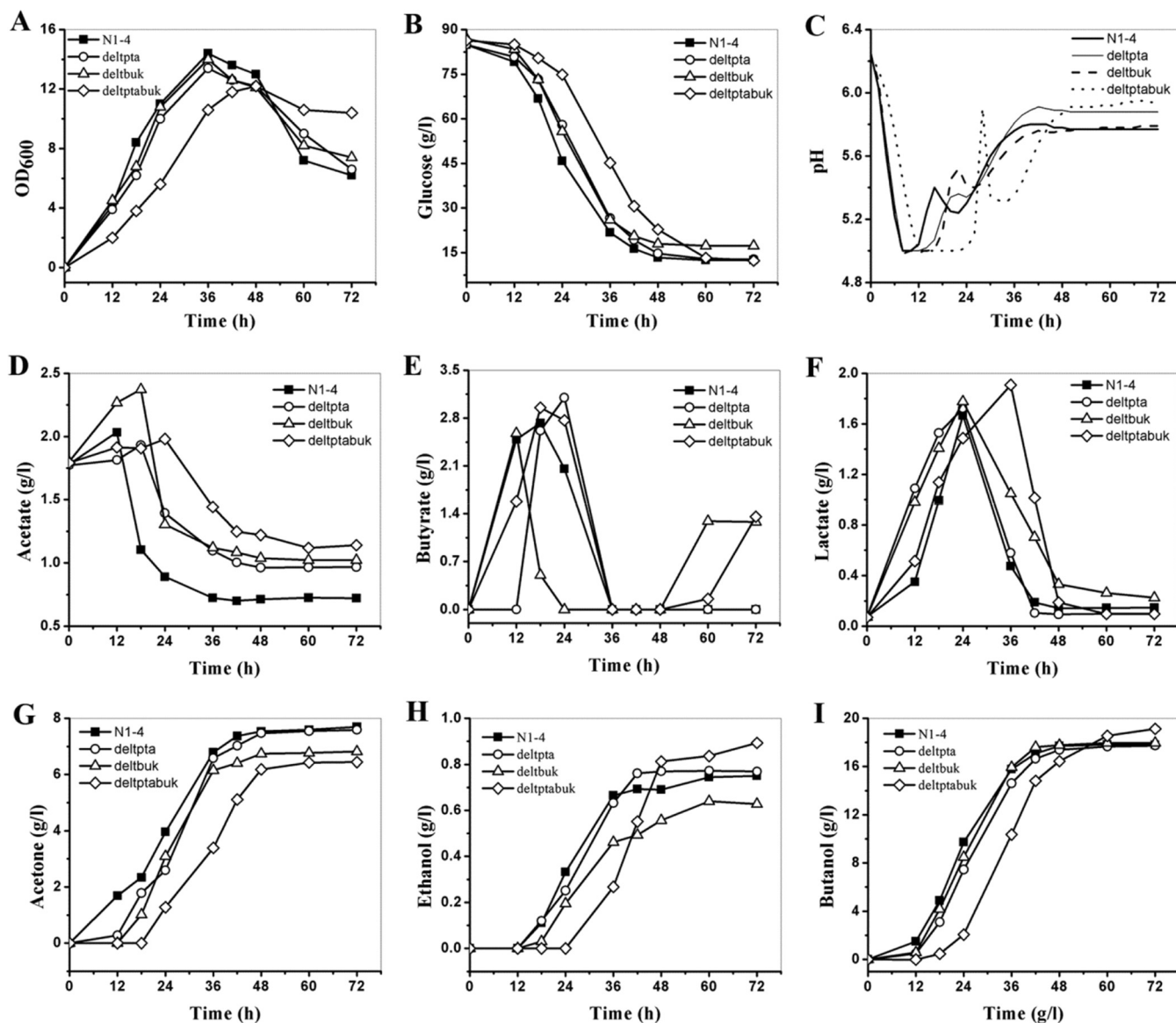


FIG 2 Batch fermentation profiles of the *C. saccharoperbutylacetonicum* mutants compared to the wild type in P2 medium. (A) Cell growth; (B) glucose consumption; (C) pH profiles; (D) acetate production; (E) butyrate production; (F) lactate production; (G) acetone production; (H) ethanol production; (I) butanol production. N1-4, *C. saccharoperbutylacetonicum* N1-4; deltpa, *C. saccharoperbutylacetonicum* deltpa; deltbuk, *C. saccharoperbutylacetonicum* deltbuk; deltpabuk, *C. saccharoperbutylacetonicum* deltpabuk. Fermentation was carried out in replicates, and results from one batch are reported here as representative.

the increase of lactate in the mutants, especially in *C. saccharoperbutylacetonicum* deltpabuk, in which lactate reached a peak level of 1.9 g/liter, representing an 18.8% improvement compared to the wild type.

ABE started to be produced in the early exponential phase in all mutants and the wild type (Fig. 2G, H, and I). ABE production from the mutants and the wild type is compared in Table 2. Compared to the wild type, *C. saccharoperbutylacetonicum* deltpa, except for a later initiation for solvent production, demonstrated very similar acetone, butanol and ethanol, production profiles and also similar butanol and ABE yields as well as butanol selectivity (butanol/ABE ratio). *C. saccharoperbutylacetonicum* deltbuk generated an amount of butanol comparable to that for the wild type but decreased production of acetone (by 11.7%) and ethanol (16.7%), resulting in a slight increase in the selectivity (71.3% versus 68.2%). Compared to the wild type, *C. saccharoperbutylacetonicum* deltpabuk produced 15.6% less acetone but 20.8% more etha-

TABLE 2 Summary of fermentation results for the *C. saccharoperbutylacetonicum* mutants compared to the wild-type strain N1-4

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

^aThere was approximately 1.7 g/liter of acetate preadded in the P2 medium.

^bThe reported titers were the maximum values after the solvent production reached a plateau.

^cRatio of butanol to total ABE.

nol. This strain also produced much more butanol (1.3 g/liter more than the wild type, reaching 19.0 g/liter) with a slightly higher butanol yield. The butanol selectivity was also higher (72.4% versus 68.2%).

Fermentation of the mutants in MP2 medium. To exclude the interference of acetate preadded in P2 medium for the fermentation kinetics, MP2 medium was used to carry out further fermentation with the mutants as well as the wild type (Fig. 3). Compared to the fermentation with P2 medium, a longer lag phase of around 12 h in MP2 was observed for the wild type and *C. saccharoperbutylacetonicum* deltbuk, and a prolonged lag phase of around 24 h was observed for both *C. saccharoperbutylacetonicum* deltppta and deltptabuk (Fig. 3A). In the exponential phase, all the mutants grew more slowly and reached much lower maximal ODs than the wild type (Table 2). Corresponding to the slow growth, all mutants consumed glucose more slowly, especially when both the *pta* and *buk* genes were deleted (Fig. 3B). Within 60 h, 77.8 g/liter, 64.6 g/liter, and 69.9 g/liter glucose were consumed by the wild type, *C. saccharoperbutylacetonicum* deltppta, and *C. saccharoperbutylacetonicum* deltbuk, respectively, while in *C. saccharoperbutylacetonicum* deltptabuk, it took 84 h to consume 68.3 g/liter glucose.

As illustrated in Fig. 3C, corresponding well with the long lag phase in cell growth, it took a 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, deltppta, deltbuk, and deltptabuk, respectively). Similarly, the pH was held at 5.0 for a much longer time (10 to 20 h) than in P2 medium before it increased to high levels. Production profiles for acetate and butyrate are demonstrated in Fig. 3D and E. Without presupplemented acetate in MP2, the deletion of the major acetate-producing gene *pta* led to an obvious decrease of acetate production by 14.5% and a significant increase of butyrate production by 52.0% in *C. saccharoperbutylacetonicum* deltppta compared to the wild type, while when the *buk* gene, responsible for butyrate production, was knocked out, a 20.0% decrease of butyrate production and a 175.4% increase of acetate production were observed in *C. saccharoperbutylacetonicum* deltbuk compared to the wild type. In *C. saccharoperbutylacetonicum* deltptabuk, the peak acetate production was comparable to that in the wild type, but the peak butyrate production was 44.0% more than that in the wild type (Table 2). Besides lactate and butyrate, high-level lactate was also detected in all the strains with MP2 (Fig. 3F). Compared with the lactate production in the wild type, improvements of 25.0% and 8.3% were observed in *C. saccharoperbutylacetonicum* deltbuk and deltptabuk, respectively, while *C. saccharoperbutylacetonicum* deltppta demonstrated the highest lactate production, with an 83.3% increase compared to the wild type (Table 2).

The ABE production profiles are shown in Fig. 3G to I and also summarized in Table 2. Compared to the wild type (18.4 g/liter), butanol production was slightly improved

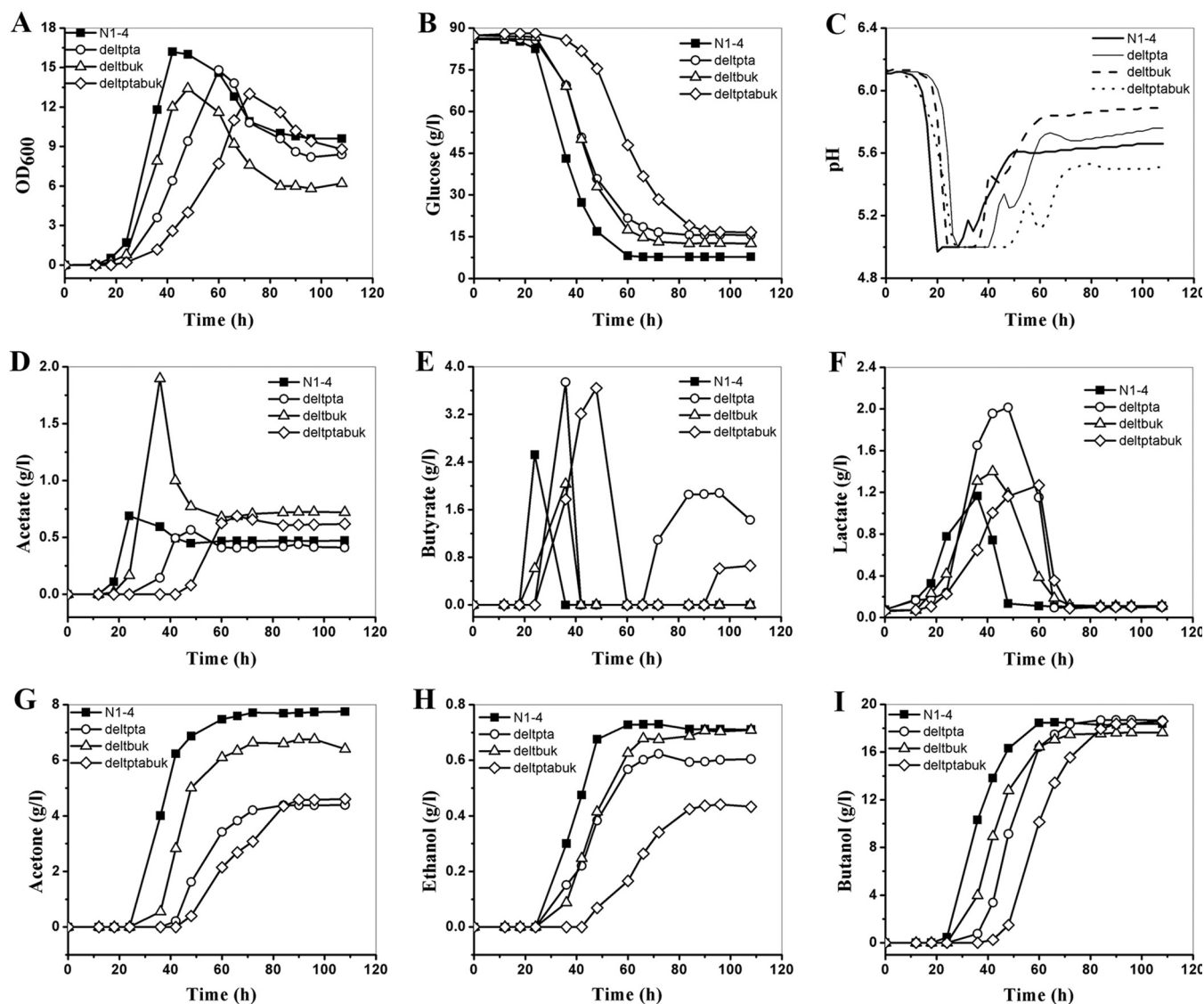


FIG 3 Batch fermentation profiles of the *C. saccharoperbutylacetonicum* mutants compared to the wild type in MP2 medium. (A) Cell growth; (B) glucose consumption; (C) pH profiles; (D) acetate production; (E) butyrate production; (F) lactate production; (G) acetone production; (H) ethanol production; (I) butanol production. N1-4, *C. saccharoperbutylacetonicum* N1-4; deltpa, *C. saccharoperbutylacetonicum* deltpa; deltbuk, *C. saccharoperbutylacetonicum* deltbuk; deltpabuk, *C. saccharoperbutylacetonicum* deltpabuk. Fermentation was carried out in replicates, and results from one batch are reported here as representative.

to 18.7 g/liter and 18.8 g/liter, respectively, in *C. saccharoperbutylacetonicum* deltpa and deltpabuk. However, the butanol yield was improved remarkably by 13.0% in these two mutants, due to their decreased consumption of sugars. The butanol production in *C. saccharoperbutylacetonicum* deltbuk decreased by 0.7 g/liter compared with that in the wild type; however, a butanol yield similar to that in the wild type was still obtained in *C. saccharoperbutylacetonicum* deltbuk, also due to the decreased consumption of sugar. Compared to the wild type, lower levels of acetone production were observed in all three mutants, with 42.9%, 11.7%, and 39.0% decreases in *C. saccharoperbutylacetonicum* deltpa, deltbuk, and deltpabuk, respectively. *C. saccharoperbutylacetonicum* deltbuk generated the same ethanol production as the wild type, but the ethanol production was reduced by 15.2% and 38.9% in *C. saccharoperbutylacetonicum* deltpa and deltpabuk, respectively. The butanol selectivity was improved by ~10% in both *C. saccharoperbutylacetonicum* deltpa and deltpabuk, while that in deltbuk increased slightly.

DISCUSSION

C. saccharoperbutylacetonicum N1-4 is well known as a hyper-butanol-producing microorganism and was used for commercial butanol production in Japan from the 1960s until the shutdown of the butanol production facilities (11, 12, 49). As the type strain of the species *C. saccharoperbutylacetonicum*, strain N1-4 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 (50, 51). *C. saccharoperbutylacetonicum* (N1-4) has the largest genome (6.7 Mb) among all the solventogenic clostridia characterized so far (49). It also has a 136-kb megaplasmid which contains no genes apparently related to solvent formation. This is very different from the case for *C. beijerinckii*, which does not have a plasmid, and *C. acetobutylicum*, which contains a 192-kb megaplasmid whose loss leads to strain degeneration (52). DNA transformation in *C. saccharoperbutylacetonicum* is difficult, possibly demonstrating a methylome very different from those previously reported (53–55). An amenable transformation protocol was reported very recently (21). On the other hand, *C. saccharoperbutylacetonicum* demonstrates very robust cell growth (reaching an OD₆₀₀ of up to 16.0 as observed in this study, which has never been seen in batch fermentation with either *C. beijerinckii* or *C. acetobutylicum*), a very high butanol production capability, and high butanol tolerance and selectivity. It also has a comparatively low sporulation frequency and can perform ABE fermentation using a wide range of carbohydrates (12, 51, 56). However, compared with other ABE-producing species such as *C. acetobutylicum* and *C. beijerinckii*, the study of the genomics and genetics of *C. saccharoperbutylacetonicum* is lagging. To elucidate the complex fermentation metabolism in *C. saccharoperbutylacetonicum* and further develop improved strains for solvent production, the development of a versatile genetic engineering system is highly desirable. In this study, an efficient genome editing toolkit was developed based on the CRISPR-Cas9 system, paving the way for efficient genomic engineering in *C. saccharoperbutylacetonicum*. Based on this system, single and double knockout mutants with mutations related to the acid production pathways were generated.

First, we evaluated the CRISPR-Cas9 system that was customized for *C. beijerinckii* for genome engineering in *C. saccharoperbutylacetonicum* (37). The *pta* deletion mutant was obtained in the fourth generation of subculture with a mutation rate of 18.75%, and the *buk* deletion mutant was obtained in the seventh generation of subculture with a mutation rate of 6.25% (12.5% for *buk* deletion in generating the *pta buk* double deletion mutant). The varied efficiency in deleting different genes indicated that the CRISPR-Cas9 genome engineering efficiency could be affected by different target sites and homology arm sequences, as previously reported (46). Notwithstanding, the efficiency was much lower than that observed when the same system was used for genome engineering in *C. beijerinckii* (in that case, generally nearly a 100% mutation rate was observed for various genome engineering purposes) (26). This result was not surprising considering the differences between these two strains. Obviously, this customized high-efficiency genome engineering system for *C. beijerinckii* does not function well in *C. saccharoperbutylacetonicum*.

As indicated by previous reports (26, 57), 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 DSB. gRNA expression is especially essential for the functionality of the whole system (45, 46). Therefore, we further set out to identify a more powerful promoter for gRNA expression. The *veg* promoter of the Gram-positive *B. subtilis* (P_{veg_b}) has been employed for efficiency of gRNA expression in *B. subtilis* (47). Therefore, we decided to evaluate its efficiency for gRNA expression in *C. saccharoperbutylacetonicum*; meanwhile, the endogenous hypothetical *veg* promoter in *C. saccharoperbutylacetonicum* (P_{veg_c}) was also selected. Additionally, the *E. coli* promoter P_{J23119} has proven to be effective for gRNA expression in *E. coli* and recently in solventogenic *C. acetobutylicum* and *C. beijerinckii* (33, 38, 48, 58). Therefore, this

promoter was also selected to drive the gRNA expression in this study. As shown in Table 1, by following the same procedure to obtain the *pta* deletion mutant, it was determined that the order of the promoters for efficient gRNA expression (and thus 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 TGYE without serial transfers as done in *C. beijerinckii* (26). 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 could be achieved if we did a careful selection of the P_{vegC} sequence to be used for gRNA expression (especially, the ribosome binding site [RBS] was likely redundant and might have a negative impact since gRNA is a noncoding RNA). It has been reported that the strong expression of gRNA is essential for the functionality of the CRISPR-Cas9 system (45, 46). Along this line, in this study, we provided solid data to verify that the achievement of potent gRNA expression is the critical step to develop a high-efficiency CRISPR-Cas9 system for genome engineering.

As reported for other *Clostridium* strains (44, 59), the inactivation of the *pta* gene did not completely eliminate the acetate formation, indicating that additional acetate-forming pathways might exist in *C. saccharoperbutylacetonicum*. It needs to be pointed out that the acid production pathways in many of the previously characterized mutants were disrupted with TargeTron-based intron insertion approaches (43, 44). It is suspected that residual activities of the enzymes encoded by disrupted genes can still exist in the mutant. In this study, clean and complete deletion of the primary acid production genes was obtained using the CRISPR-Cas9 system; the corresponding acid production in the mutant was not eliminated either. In the fermentation with P2 medium, the deletion of *pta* resulted in a slight decrease of acetate production in *C. saccharoperbutylacetonicum* *deltpta* and *deltptabuk* (Fig. 2D and Table 2). Since ATP is the fundamental demand for cell growth and other metabolisms (60) and more ATP per glucose can be generated through the acetate formation pathway than through the butyrate formation pathway, the deletion of *pta* imposed a severe metabolic burden on the cells (61). To compensate for the energy loss and avoid a significant decline in overall cell growth, more carbon flow was directed to butyrate formation, leading to a >7% increase of butyrate production in both *C. saccharoperbutylacetonicum* *deltpta* and *deltptabuk*. Similarly, the deletion of the *buk* gene led to a 14.3% increase of acetate production in *C. saccharoperbutylacetonicum* *deltbuk* and a slight decrease of butyrate production. Production of butyrate was still observed in *C. saccharoperbutylacetonicum* *deltbuk* and *deltptabuk*, suggesting the existence of other active butyrate formation pathway genes. Besides *buk* (GenBank accession no. [AGF54071.1](#)), which was deleted in this study, there are two other butyrate kinase-encoding genes in the *C. saccharoperbutylacetonicum* N1-4 genome, which are denoted butyrate kinase 1 (WP_015392233.1) and butyrate kinase 2 (WP_015395384.1). Further study is in progress in our lab to evaluate the contributions of these three butyrate kinases to butyrate production in this microorganism.

In MP2 medium without preadded acetate, long lag phases were observed in all the mutants as well as in the wild type (Fig. 3A), indicating a positive effect of acetate on cell growth, as also reported previously (62, 63). The mutants showed lower growth rates than the wild type (Table 2); the disruption of acid production pathways might lead to the decline in energy generation and thus the metabolic burden on cell growth (59). In MP2 medium with the elimination of the interference from the preadded acetate, *C. saccharoperbutylacetonicum* *deltpta* clearly demonstrated reduced acetate production and enhanced butyrate production to compensate for the ATP generation. Similarly, the *buk* deletion in *C. saccharoperbutylacetonicum* *deltbuk* led to an apparent

decrease of butyrate production and a significant increase of acetate production to balance the ATP generation. These results confirmed that the deleted *pta* (GenBank accession no. [AGF55073.1](#)) and *buk* (GenBank accession no. [AGF54071.1](#)) are the primary acid formation pathway genes for acetate and butyrate production, respectively, although other annotated homologous genes exist. A comprehensive transcriptomic analysis is desirable to further enhance the genome annotation and assess the function of each specific gene (64, 65).

As reported previously, lactate dehydrogenase in solventogenic clostridia shows remarkable activities only under certain growth conditions, such as iron limitation (66). However, we observed that the wild-type *C. saccharoperbutylacetonicum* N1-4 produced 1.6 g/liter lactate (at the peak level) in P2 medium and 1.2 g/liter in MP2 medium, which was even more than the production of acetate, while under similar conditions, less than 0.2 g/liter lactate was produced by *C. beijerinckii* 8052 (44) and less than 0.1 g/liter lactate was observed in *C. acetobutylicum* ATCC 824 (66). Therefore, the lactate formation pathway may be more active in *C. saccharoperbutylacetonicum* N1-4. Moreover, there are six genes annotated as lactate dehydrogenase genes in *C. saccharoperbutylacetonicum*; the action of these genes and the mechanism for lactate production and reutilization in *C. saccharoperbutylacetonicum* need to be further elucidated. Similar to the results in *C. beijerinckii* (44), deletion of *pta* or *buk* led to the improvement of lactate production; especially in the *pta* deletion mutant, the lactate production was increased by 83.3% compared to that of the wild type in MP2 medium. It was reported that the lactate formation pathway can serve as a less efficient method for energy generation and NADH oxidation when the pathway for the disposal of protons and electrons by the generation of molecular hydrogen is inhibited (44). The increase of lactate production may help mitigate the decreased ATP production in the mutants.

In P2 medium, the deletion of the *buk* gene in *C. saccharoperbutylacetonicum* *deltbuk* slightly increased the butanol production and butanol yield while significantly decreasing the production of acetone (by 11.7%) and ethanol (by 16.7%), resulting in a higher butanol selection. In *C. saccharoperbutylacetonicum* *deltptabuk*, the acetone production was decreased by 15.6%, and the butanol production was improved by 1.3 g/liter, reaching 19.0 g/liter. Therefore, a much higher butanol selectivity (72.4%, compared to 68.2% in the wild type) was observed. The butanol production reported here represents one of the highest ever reported from batch fermentations for biobutanol production (43). However, a significant improvement in solvent production in the *deltptabuk* strain compared to the wild type was not observed, as previously reported in the mutant of *C. acetobutylicum* with the same homologous genes disrupted (43). 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/liter (67). Butanol tolerance is considered one of the major bottlenecks in improving butanol production in the host. 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* compared to that from the wild type. Furthermore, the butanol selectivity was also improved by ~10% in *C. saccharoperbutylacetonicum* *deltpta* and *deltptabuk* 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) (44), fermentation conditions can be further optimized for the mutants to achieve more desirable solvent production.

TABLE 3 Strains and plasmids used in this study

Strain or plasmid	Characteristics ^a	Source or reference
Strains		
<i>E. coli</i> DH5 α	F ⁻ <i>endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20 ϕ80dlacZΔM15 Δ(lacZYA-argF)U169 hsdR17(r_K⁻ m_K⁺) λ⁻</i>	NEB
<i>C. beijerinckii</i> NCIMB 8052	ATCC 51743	ATCC
<i>C. saccharoperbutylacetonicum</i> 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 <i>ori</i> Amp ^r Erm ^r Plac::Cas9 gRNA	26
pYW34-pta	pYW34 P _{sRNA} ::20-nt gRNA targeting <i>pta</i>	This work
pYW34-deltpta	pYW34-pta::homology arm sequences	This work
pYW34-buk	pYW34 P _{sRNA} ::20-nt gRNA targeting <i>buk</i>	This work
pYW34-deltbuk	pYW34-buk::homology arm sequences	This work
pSHW1-pta	pYW34 P _{vegB} ::20-nt gRNA targeting <i>pta</i>	This work
pSHW2-pta	pYW34 P _{vegC} ::20-nt gRNA targeting <i>pta</i>	This work
pSHW3-pta	pYW34 P _{J23119} ::20-nt gRNA targeting <i>pta</i>	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

^aP_{sRNA}, small RNA promoter from *C. beijerinckii*; P_{vegB}, *veg* promoter from *B. subtilis*; P_{vegC}, hypothetical *veg* gene promoter from *C. saccharoperbutylacetonicum*; P_{J23119}, J23119 promoter from *E. coli*.

MATERIALS AND METHODS

Bacterial strains, growth conditions, plasmids, and oligonucleotides. All strains and plasmids used in this study are listed in Table 3, and DNA primers are listed in Table S1 in the supplemental material. *E. coli* strain DH5 α was used for DNA cloning. It was grown aerobically at 37°C in Luria-Bertani (LB) medium supplemented with 100 μ g/ml of ampicillin (Amp) as needed. *C. saccharoperbutylacetonicum* N1-4 was grown in an anaerobic chamber (N₂-CO₂-H₂ with a volume ratio of 85:10:5) at 35°C in tryptone-glucose-yeast extract (TGY) medium containing 30 g/liter of tryptone, 20 g/liter of glucose, 10 g/liter of yeast extract, and 1 g/liter of L-cysteine (44). TGY supplemented with 40 mM lactose (TGYL) was used to induce the Cas9 expression. When appropriate, 25 μ g/ml of erythromycin (Erm) was added to either TGY or TGYL to make the TGYE or TGYLE medium, respectively, for *C. saccharoperbutylacetonicum* cultivation.

Plasmid construction. Plasmid pYW34 (26) carrying 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 the *pta* (GenBank accession no. AGF55073.1) and *buk* (GenBank accession no. 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 BtgZI site of pYW34 with Gibson Assembly, generating pYW34-pta. To achieve the desirable homologous recombination around the *pta* ORF, two homology arms (1,076 bp and 1,078 bp) flanking at both sides of the *pta* ORF were amplified from *C. saccharoperbutylacetonicum* genomic DNA with primers YW1035 and YW1036 and primers YW1037 and YW1038, respectively, and then these two fragments were fused together with primers YW1039 and YW1040 through splicing by overlapping extension PCR (SOE-PCR), and inserted into the NotI site of pYW34-pta, generating pYW34-deltpta.

Similarly, for targeting on *buk*, the fragment containing the sRNA promoter and 20-nt guiding sequence (5'-GGAATGCTTAAGCCAGTAGA-3') was amplified with primers YW484 and YW1053 and then inserted into the BtgZI site of pYW34, generating pYW34-buk. One 1-kb homology arm sequence upstream of the start codon (ATG) of *buk* was amplified using primers YW949 and YW950, and another 1-kb homology arm sequence downstream of the stop codon (TAA) of *buk* was amplified using primers YW951 and YW952. The whole 2-kb homology arm was then obtained through SOE-PCR with primers YW949 and YW952 and inserted into the NotI site of pYW34-buk, generating the plasmid pYW34-deltbuk.

To improve the genomic editing efficiency in *C. saccharoperbutylacetonicum* using the CRISPR-Cas9 system, other strong constitutive promoters were evaluated for gRNA expression (targeting the same 20-nt sequence in *pta* as described above), including the *veg* promoter from *B. subtilis* (P_{vegB}) (47), the hypothetical *veg* (CSPA_RS02900) gene promoter (P_{vegC}) from *C. saccharoperbutylacetonicum*, and the *E. coli* promoter P_{J23119} (48). The P_{vegB} promoter (65 bp) from *B. subtilis* with the 20-nt guiding sequence was amplified using primer pairs YW1340-YW1342 and YW1341-YW1342 in two steps and inserted into the BtgZI and NotI sites of pYW34, generating pSHW1-pta. The P_{vegC} promoter fused with the 20-nt guiding sequence was amplified from *C. saccharoperbutylacetonicum* N1-4 with primers YW1042 and YW1043 and

inserted into the BtgZI site of pYW34, generating pSHW2-*pta*, while the P_{J23119} promoter fused with the 20-nt guiding sequence was amplified using primer pairs YW1338-YW1342 and YW1339-YW1342 in two steps and inserted into the BtgZI and NotI sites of pYW34, generating pSHW3-*pta*. Subsequently, the fragment containing the two homology arm sequences for *pta* deletion was inserted into the NotI site of pSHW1-*pta*, pSHW2-*pta*, and pSHW3-*pta*, generating pSHW1-deltpta, pSHW2-deltpta, and pSHW3-deltpta, respectively.

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

DNA transformation and mutant screening. Transformation of *C. saccharoperbutylacetonicum* N1-4 was carried out with electroporation following the protocol for the transformation of *C. beijerinckii* as described by Wang et al. with minor modifications (44). *C. saccharoperbutylacetonicum* N1-4 was cultivated anaerobically at 35°C until the optical density of the culture at 600 nm (OD_{600}) reached 0.8 to 1.0. The cells were harvested through centrifugation at $4,200 \times g$ at 4°C for 10 min. The cell pellets were washed once with the same volume (as the original cell culture) of ice-cold 15% glycerol and then resuspended in 1/20 volume of ice-cold 15% glycerol. Immediately, 1.0 μ g of plasmid DNA was added to 400 μ l of competent cells, and then the mixture was transferred into a precooled 0.2-cm electroporation cuvette. Electroporation was carried out at a voltage of 1,500 V, a capacitance of 25 μ F, and a resistance of 300 Ω using a Gene Pulser Xcell electroporation system (Bio-Rad Laboratories, Hercules, CA) which was connected to the anaerobic chamber. Subsequently, the cells were transferred into 1.6 ml of TGY medium and recovered anaerobically at 35°C (generally for 10 to 12 h until signs of cell growth were observed). The recovered cells were spread onto TGYE agar plates and incubated at 35°C for about 24 h under anaerobic conditions until colonies appeared.

Following the outgrowth on selective plates, single colonies were picked randomly for colony PCR (cPCR) using primers YW880 and YW881 to confirm the presence of plasmid. The transformants were then incubated in TGYE liquid medium and subcultured successively to permit efficient homologous recombination (37). Starting from the second generation, while the culture continued to be transferred in TGYE medium, it was also transferred into TGYE liquid medium for the induction of Cas9 expression and further spread onto TGYE plates when necessary (that is, when double bands from both the mutant and the wild type were detected through cPCR with TGYE liquid culture as the template). Mutants were screened through cPCR using primers (YW1044 and YW1045 for detecting *pta* deletion and 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 and not to the homology arms on the plasmids).

Due to the existence of plasmids harboring homologous sequences within the mutant strain, vector integration events (VIE) could occur during the bacterial genome engineering (26). To detect VIE, pairs of primers (one annealing to the plasmid and the other annealing to the chromosome) were designed. Specifically, primer pairs YW1044-YW200 and YW847-YW1045 were used for detecting VIE of pYW34-deltpta both 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 (see Fig. S2 in the supplemental material).

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 (44, 68). After around five cycles of subculturing, the culture was spread onto TGY plates to form colonies. cPCR was performed to confirm the curing of plasmids using primers YW880 and YW881. Colonies lacking PCR products were spread onto TGYE plates to further confirm the loss of plasmids. Finally, cPCR was conducted to further verify the existence of the desirable mutation.

Fermentation. Batch fermentation was performed in BioFlo 115 benchtop bioreactors (New Brunswick Scientific Co., Enfield, CT) with a working volume of 1.5 liters. P2 medium or modified P2 (MP2) medium (44) was used as the fermentation medium, and 80 g/liter glucose was used as the sole carbon source. To generate an anaerobic condition, oxygen-free nitrogen was sparged through the fermentation broth starting several hours before the inoculation until the cell culture initiated its own gas production.

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

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

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.00233-17>.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

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