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BIOTECHNOLOGY

Identification and Investigation of Autolysin Genes in *Clostridium saccharoperbutylacetonicum* Strain N1-4 for Enhanced Biobutanol Production

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ABSTRACT Biobutanol is a valuable biochemical and one of the most promising biofuels. *Clostridium saccharoperbutylacetonicum* N1-4 is a hyperbutanol-producing strain. However, its strong autolytic behavior leads to poor cell stability, especially during continuous fermentation, thus limiting the applicability of the strain for long-term and industrial-scale processes. In this study, we aimed to evaluate the role of autolysin genes within the *C. saccharoperbutylacetonicum* genome related to cell autolysis and further develop more stable strains for enhanced butanol production. First, putative autolysin-encoding genes were identified in the strain based on comparison of amino acid sequence with homologous genes in other strains. Then, by overexpressing all these putative autolysin genes individually and characterizing the corresponding recombinant strains, four key genes were pinpointed to be responsible for significant cell autolysis activities. Further, these key genes were deleted using CRISPR-Cas9. Fermentation characterization demonstrated enhanced performance of the resultant mutants. Results from this study reveal valuable insights concerning the role of autolysins for cell stability and solvent production, and they provide an essential reference for developing robust strains for enhanced biofuel and biochemical production.

IMPORTANCE Severe autolytic behavior is a common issue in *Clostridium* and many other microorganisms. This study revealed the key genes responsible for the cell autolysis within *Clostridium saccharoperbutylacetonicum*, a prominent platform for biosolvent production from lignocellulosic materials. The knowledge generated in this study provides insights concerning cell autolysis in relevant microbial systems and gives essential references for enhancing strain stability through rational genome engineering.

KEYWORDS autolysis, *Clostridium*, biobutanol, fermentation, CRISPR-Cas9, biofuel

Biobutanol (*n*-butanol) produced from low-value renewable resources through fermentation with solventogenic clostridia attracts great interest as a potential biofuel source and biochemical feedstock with various applications (1). However, currently, there are still various limitations for biobutanol production, which often lead to low titer, yield, and productivity and prevent the process from being economically

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competitive. *Clostridium saccharoperbutylacetonicum* N1-4 is a hyperbutanol-producing strain, which can produce much higher butanol levels than other well-known clostridial type strains (including *C. beijerinckii* NCIMB 8052 and *C. acetobutylicum* ATCC 824) in regular batch fermentations under similar conditions (2–4). However, it is often observed that the cells of *C. saccharoperbutylacetonicum* N1-4 experienced severe cell lysis at the end of the fermentation. In addition, *C. saccharoperbutylacetonicum* N1-4 often has unstable performance for acetone, butanol, and ethanol (ABE) production during continuous fermentation.

Autolysis activity is known in many *Clostridium* species primarily due to the existence of autolysins. They are responsible for the hydrolysis of different components in the cell wall; based on the different hydrolytic activity on different functional groups of the peptidoglycan, the autolysins are classified into different groups (5). They also play roles in processes such as cell motility, cell separation, cell elongation, peptidoglycan maturation, cell wall turnover, germination, sporulation, and induced lysis (6). Spontaneous autolysis leads to significant loss of cell biomass, and no obvious stationary phase could be observed during the cell growth of solventogenic clostridia (7). Induced autolysis (accompanied by sporulation) could also occur due to chemical-induced stresses, such as stresses from oxygen, acetate, butyrate, and butanol (8).

Autolysins genes have been reported in several solventogenic clostridial strains, such as *lyt*-1 (from *C. acetobutylicum* P262), CA_C0554 (from *C. acetobutylicum* ATCC 824), (9, 10), SMB_G2359, and SMB_G3117 (from *C. acetobutylicum* DSM 1731) (5). CA_C0554 was reported to play an important role in sporulation (7), and the disruption of SMB_G3117 led to a significant increase in the cell biomass (5). Some *N*-acetylmuramidases have also been isolated from extracellular components of *C. saccharoperbutylacetonicum* (ATCC 13564) (11).

In this work, we hypothesize that the identification and deletion of autolysin genes cannot only help to sustain cell biomass and increase the butanol production but also increase the stability and recyclability of the culture cells and thus enhance the performance for long-term continuous fermentations. In this study, we identified the putative autolysin genes in *C. saccharoperbutylacetonicum* N1-4 based on the homologous analysis using BLAST. Then, we overexpressed all 16 putative autolysin genes individually in *C. saccharoperbutylacetonicum* and, by characterizing these recombinant strains, identified four key genes to be responsible for significant cell autolysis activities. Furthermore, we deleted these key autolysin genes using our customized CRISPR-Cas9 system. Further characterization of the mutants through fermentation revealed valuable insights into the effects of autolysins on cell stability and solvent production.

RESULTS AND DISCUSSION

Identification of the autolysin genes. Based on the information about autolysin genes available in the literature, the putative autolysin genes were identified in *C. saccharoperbutylacetonicum* N1-4 (Table 1), which were grouped into four families. It could be noticed that the muramidase-encoding genes in *C. saccharoperbutylacetonicum* account for most of the putative autolysin genes.

Figure 1 shows the growth curve of the 16 strains containing the putative autolysin gene. All the recombinant strains showed decreased growth compared to the control strain, suggesting that all the autolysin genes likely incurred the autolytic activity in the corresponding strain. The gray area on the profile represents the difference in cell growth between the recombinant strain and the control strain. The data were analyzed using repeated-measures analysis of variance (ANOVA). The *P* value is shown on the top of each profile in Fig. 1. For easier comparison, the curves were split at 36 h. For all the curves, no significant difference in the cell growth (the recombinant strain versus the control strain) was noticed during the exponential phase (up to 36 h), but significant differences (at 90% confidence level; $\alpha < 0.1$) were observed in profiles (with the strains PJB24, PJB26, PJB29, and PJB30) during the stationary and death phases (after 36 h). From the four genes, 24, 26, 29, and 30, two (gene 24 and gene 29) of them have

TABLE 1 Sixteen putative autolysin genes identified in *C. saccharoperbutylacetonicum* N1-4

Corresponding plasmid for overexpression	Gene ID in N1-4	Homologous gene (strain)	Reference strain	% query	% identity
Endopeptidases					
pPJB15	CSPA_RS26630	*ydiL* (BSU06010)	*B. subtilis* 168	37	33
pPJB16	CSPA_RS03200	*ydiL* (BSU06010)	*B. subtilis* 168	56	26
	None identified	*ypbD* (BSU23010)	*B. subtilis* 168		
	None identified	*yyaK* (BSU40830)	*B. subtilis* 168		
Glucosaminidase					
pPJB17	CSPA_RS08050	*lytD* (BSU35780)	*B. subtilis* 168	3	48
	None identified	*lytD* (SMB_G2359)	*C. acetobutylicum* DSM 1731		
N-acetyl-alanine amidases					
	None identified	*cwlJ* (BSU02600)	*B. subtilis* 168		
pPJB18	CSPA_RS01160	*ykvT* (BSU13820)	*B. subtilis* 168	85	41
		sleB (BSU22930)	*B. subtilis* 168	68	56
		sleB (SMB_G3117)	*C. acetobutylicum* DSM 1731	36	44
pPJB19	CSPA_RS21780	*sleB* (SMB_G3117)	*C. acetobutylicum* DSM 1731	16	37
pPJB20	CSPA_RS09105	*sleB* (SMB_G3117)	*C. acetobutylicum* DSM 1731	20	28
pPJB21	CSPA_RS06245	*sleB* (SMB_G3117)	*C. acetobutylicum* DSM 1731		52
Muramidases					
pPJB22	CSPA_RS00240	*ykuG* (BSU14071)	*B. subtilis* 168	5	41
pPJB23	CSPA_RS18890	*lyc* (CA_C0554)	*C. acetobutylicum* ATCC 824	56	41
pPJB24	CSPA_RS13245	*lyc* (CA_C0554)	*C. acetobutylicum* ATCC 824	67	36
pPJB25	Cspa_c38900	*lyc* (CA_C0554)	*C. acetobutylicum* ATCC 824	56	41
pPJB26	CSPA_RS11880	*lyc* (CA_C0554)	*C. acetobutylicum* ATCC 824	56	37
pPJB27	CSPA_RS15280	*lyc* (CA_C0554)	*C. acetobutylicum* ATCC 824	58	38
pPJB28	CSPA_RS03880	*lyc* (CA_C0554)	*C. acetobutylicum* ATCC 824	60	34
pPJB29	Cspa_135p00690	*lyc* (CA_C0554)	*C. acetobutylicum* ATCC 824	57	34
pPJB30	CSPA_RS24880	*lyc* (CA_C0554)	*C. acetobutylicum* ATCC 824	53	26

already been deleted in the prophage-deficient strains Δ1234 and Δ12345 prepared before (12). Therefore, we decided to delete the other two genes (gene 26 and gene 30) in N1-4 wild-type, Δ1234, and Δ12345 strains in order to further evaluate their function related to cell autolysis.

Recently, we obtained the N1-4-C strain by curing the endogenous megaplasmid of the N1-4 strain (13). N1-4-C could produce slightly higher solvents than the mother strain N1-4; however, a clear mechanism behind this was not identified. Based on the results here, the overexpression of gene 29 (Cspa_135p00690), which is located on the megaplasmid, could incur significant autolytic activity (Fig. 1). This might be the reason that N1-4-C could produce more solvents than N1-4 by eliminating this important autolysin gene through plasmid curing. On the other hand, gene 24 is contained in a putative prophage P2 (12) (see Table 2) on the N1-4 chromosome and is probably employed by the phage to enter into the lytic cycle.

Effects of the deletion of autolysin genes on butanol fermentation. Clean single deletion of gene 26 and gene 30 based on the three strains (N1-4, Δ1234, and Δ12345) was obtained (Fig. 2). The double deletion of both gene 26 and gene 30 in the same host was unsuccessful within any of these three mother strains despite numerous attempts. Although the reason behind this is not clear, autolysin genes are known to be related to essential metabolism such as cell division and sporulation (6, 14). This warrants further investigation in the future.

Small-scale batch fermentations were carried out in serum bottles. As shown in Fig. 3, the deletion of gene 26 in N1-4 and Δ12345 led to increased production of cell biomass and reached a higher maximum optical density at 600 nm (OD_{600}) than the mother strain (16.8 in Δ26 versus 14.6 in wild type and 18.8 in Δ12345Δ26 versus 17.6 in Δ12345). While in Δ1234Δ26, the cell growth reached a similar maximum OD_{600} as the mother strain. In both Δ1234Δ26 and Δ12345Δ26, the OD_{600} remained at higher

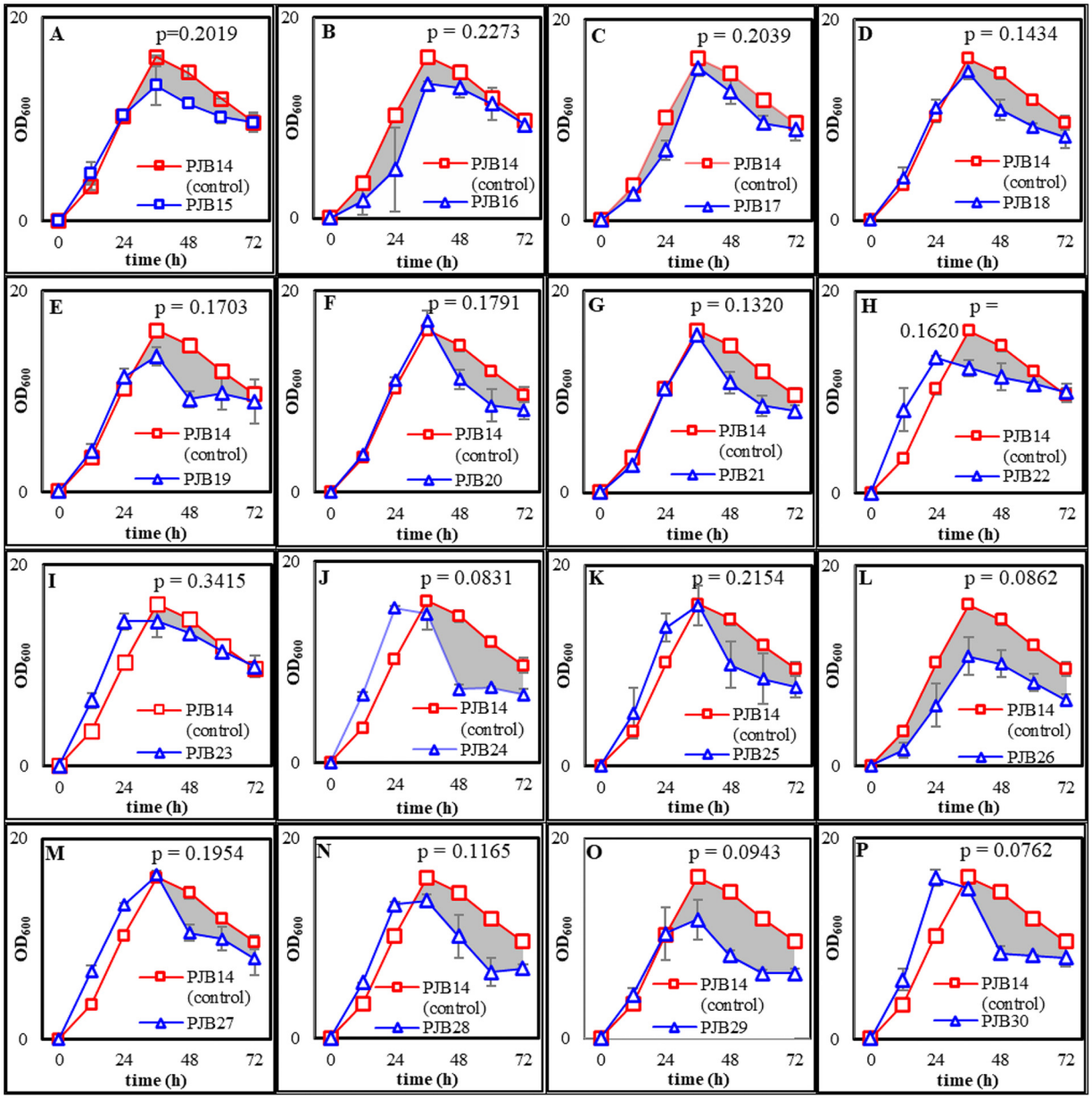

FIG 1 Growth profiles of the recombinant strains with overexpression of the putative autolysin genes compared to the control strain. The *P* value at a 90% confidence level from the repeated-measures ANOVA during the stationary/death phases (36 h to 72 h) has been put at the top of each profile. The error bar represents the standard error at a 95% confidence interval.

levels than their corresponding mother strains in the stationary/death phases, suggesting a reduction in autolysis with the deletion of these autolysis genes. This also confirmed the results that the overexpression of these autolysis genes resulted in a significant decline in cell biomass compared to the control during the stationary/death phases but not during the exponential phase (Fig. 1).

Δ26 produced slightly more butanol (but less acetone) than the wild type, while the butanol production in both Δ1234Δ26 and Δ12345Δ26 was at similar levels as the mother strains (Δ1234 and Δ12345, respectively). On the other hand, the deletion of gene 30 in the wild type (generating the Δ30 strain) led to significantly decreased cell growth and acid crash in the fermentation with much lower solvent production.

TABLE 2 Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference no.
Strain		
E. coli NEB Express	*fhuA2 [lon] ompT gal sulA11 R*(*mcr-73*::miniTn*10*-Tet^s)*2 [dcm] R*(*zgb-210*::*Tn10*-Tet^s) *endA1* Δ(*mcrCmrr*) *114*::IS*10*	New England Biolabs
C. saccharoperbutylacetonicum N1-4 (HMT)	DSM 14923 (ATCC 27021), wild-type strain	DSMZ
N1-4-C	N1-4 strain ΔCsp_135p (with the endogenous plasmid Csp_135p eliminated)	12
PJB14	N1-4 harboring pPJB14 (empty plasmid)	This study
PJB15	N1-4 harboring pPJB15	This study
PJB16	N1-4 harboring pPJB16	This study
PJB17	N1-4 harboring pPJB17	This study
PJB18	N1-4 harboring pPJB18	This study
PJB19	N1-4 harboring pPJB19	This study
PJB20	N1-4 harboring pPJB20	This study
PJB21	N1-4 harboring pPJB21	This study
PJB22	N1-4 harboring pPJB22	This study
PJB23	N1-4 harboring pPJB23	This study
PJB24	N1-4 harboring pPJB24	This study
PJB25	N1-4 harboring pPJB25	This study
PJB26	N1-4 harboring pPJB26	This study
PJB27	N1-4 harboring pPJB27	This study
PJB28	N1-4 harboring pPJB28	This study
PJB29	N1-4 harboring pPJB29	This study
PJB30	N1-4 harboring pPJB30	This study
Δ26	N1-4 strain with CSPA_RS11880 deleted	This study
Δ30	N1-4 strain with the deletion of CSPA_RS24880	This study
Δ1234	N1-4-C with the deletion of the putative prophages P1, P2, P3, P4. P1=(Cspa_c09880–Cspa_c10360), P2=(Cspa_c26510–Cspa_c27350), P3=(Cspa_c36410–Cspa_c36850), P4=(Cspa_c56920–Cspa_c57380)	12
Δ12345	Δ1234 strain with the deletion of bacteriocin P5. P5=(Cspa_c07530–Cspa_c07880)	12
Δ1234Δ26	Δ(CSPA_RS11880)	This study
Δ1234Δ30	Δ(CSPA_RS24880)	This study
Δ12345Δ26	Δ(CSPA_RS11880)	This study
Δ12345Δ30	Δ(CSPA_RS24880)	This study
Plasmid		
pJZ100	Derived from TJ1, used for pPJB14 construction	21
pPJB14	Mother vector for gene overexpression under P_{lac}	This study
pPJB15	pPJB14 derivative, for the overexpression of CSPA_RS26630	This study
pPJB16	pPJB14 derivative, for the overexpression of CSPA_RS03200	This study
pPJB17	pPJB14 derivative, for the overexpression of CSPA_RS08050	This study
pPJB18	pPJB14 derivative, for the overexpression of CSPA_RS01160	This study
pPJB19	pPJB14 derivative, for the overexpression of CSPA_RS21780	This study
pPJB20	pPJB14 derivative, for the overexpression of CSPA_RS09105	This study
pPJB21	pPJB14 derivative, for the overexpression of CSPA_RS06245	This study
pPJB22	pPJB14 derivative, for the overexpression of CSPA_RS00240	This study
pPJB23	pPJB14 derivative, for the overexpression of CSPA_RS18890	This study
pPJB24	pPJB14 derivative, for the overexpression of CSPA_RS13245	This study
pPJB25	pPJB14 derivative, for the overexpression of Cspa_c38900	This study
pPJB26	pPJB14 derivative, for the overexpression of CSPA_RS11880	This study
pPJB27	pPJB14 derivative, for the overexpression of CSPA_RS15280	This study
pPJB28	pPJB14 derivative, for the overexpression of CSPA_RS03880	This study
pPJB29	pPJB14 derivative, for the overexpression of Cspa_135p00690	This study
pPJB30	pPJB14 derivative, for the overexpression of CSPA_RS24880	This study
pYW51	CRISPR-Cas9 mother vector, used to construct plasmid for gene deletions	22
pPJB31	pYW51 derivative for CSPA_RS11880 deletion	This study
pPJB32	pYW51 derivative for CSPA_RS24880 deletion	This study

However, the deletion of gene 30 in either Δ1234 or Δ12345 did not result in a significant phenotype difference in the resultant mutants compared to their mother strains.

Further characterization with batch fermentations in bioreactors with pH control was performed for *C. saccharoperbutylacetonicum* N1-4, Δ1234, and Δ1234Δ26. As

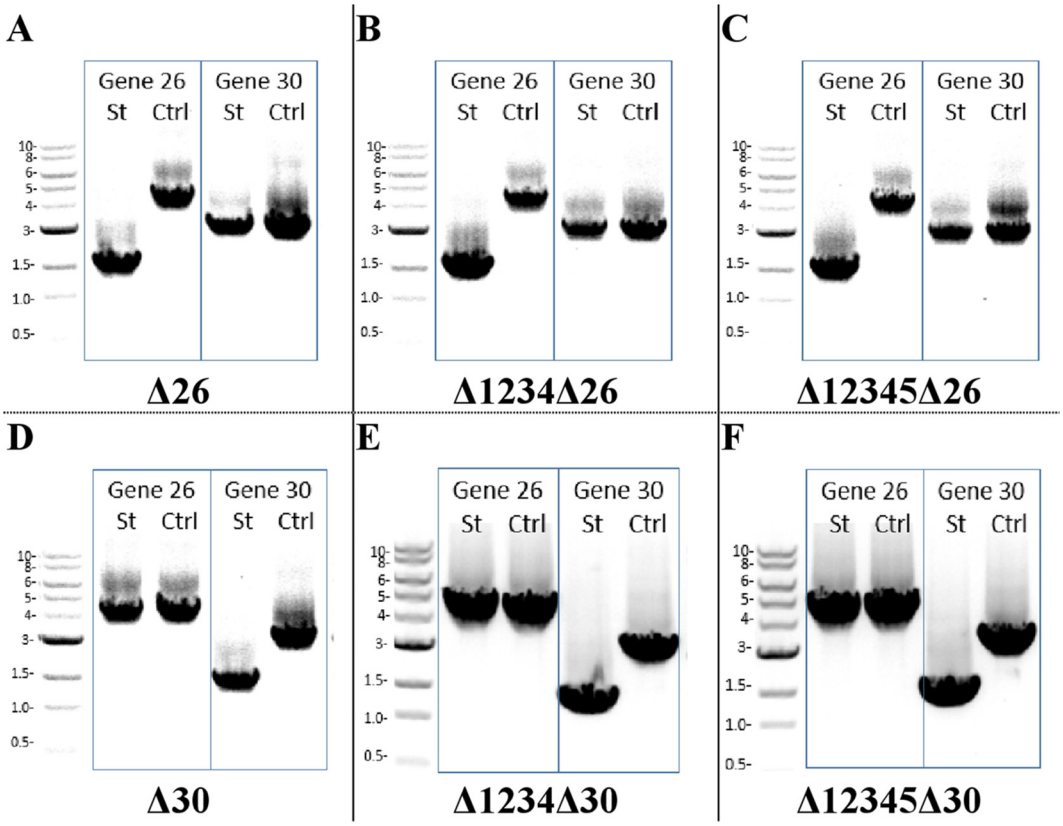

FIG 2 Agarose gel electrophoresis showed the colony PCR results confirming the gene deletion in the mutant strain (St) versus the control strain (Ctrl). (A) Deletion of gene 26 in N1-4; (B) deletion of gene 26 in Δ1234; (C) deletion of gene 26 in Δ12345; (D) deletion of gene 30 in N1-4; (E) deletion of gene 30 in Δ1234; (F) deletion of gene 30 in Δ12345. The positive mutant for the deletion of gene 26 should have a PCR band of 2,186 bp (versus 4,166 bp in the control), and the positive mutant for the deletion of gene 30 should have a PCR band of 2,125 bp (versus 3,070 bp in the control). The NEB 1-kb DNA ladder was used as the marker, with numbers on the left representing the band length in kilobases.

shown in Fig. 4, overall, Δ1234 and Δ1234Δ26 showed very similar fermentation profiles. Δ1234 and Δ1234Δ26 produced a similar level of butanol, which was slightly higher than the wild type (Δ1234 and Δ1234Δ26 also consumed slightly more glucose than the wild type). It took slightly longer for Δ1234 and Δ1234Δ26 to reach the maximum levels for solvent production and cell growth as well compared to the wild type.

Based on the repeated-measures ANOVA (Table S1 in the supplemental material), during the bottle fermentation, Δ26 produced a very similar level of butanol as the wild type, while all the other strains (excluding Δ30) produced significantly higher butanol levels than the wild type. Δ30 produced significantly lower levels of solvents than the wild type and all the other strains because of the acid crash. For pH-controlled fermentation in the bioreactor, the butanol production in Δ1234 was significantly higher than the wild type, while the butanol production in Δ1234Δ26 showed no significant difference from either the wild type or Δ1234.

Transformation efficiency. During our experiments, we noticed significantly higher transformation efficiency of the Δ12345 strain than either the wild type or the Δ1234 strains. As shown in Table 3, Δ12345 has 6.5- to 15-fold higher transformation efficiency than the wild type or Δ1234. Although more research is needed to completely understand this phenomenon, our results suggest that the deletion of the prophage genome P5, or the combined deletion of P5 along with P1, P2, P3, and P4, might have an effect of improving the DNA transformation efficiency of the strain (12). It warrants further investigation of the effect of the deletion of autolysin gene(s) on DNA transformation efficiency in future studies.

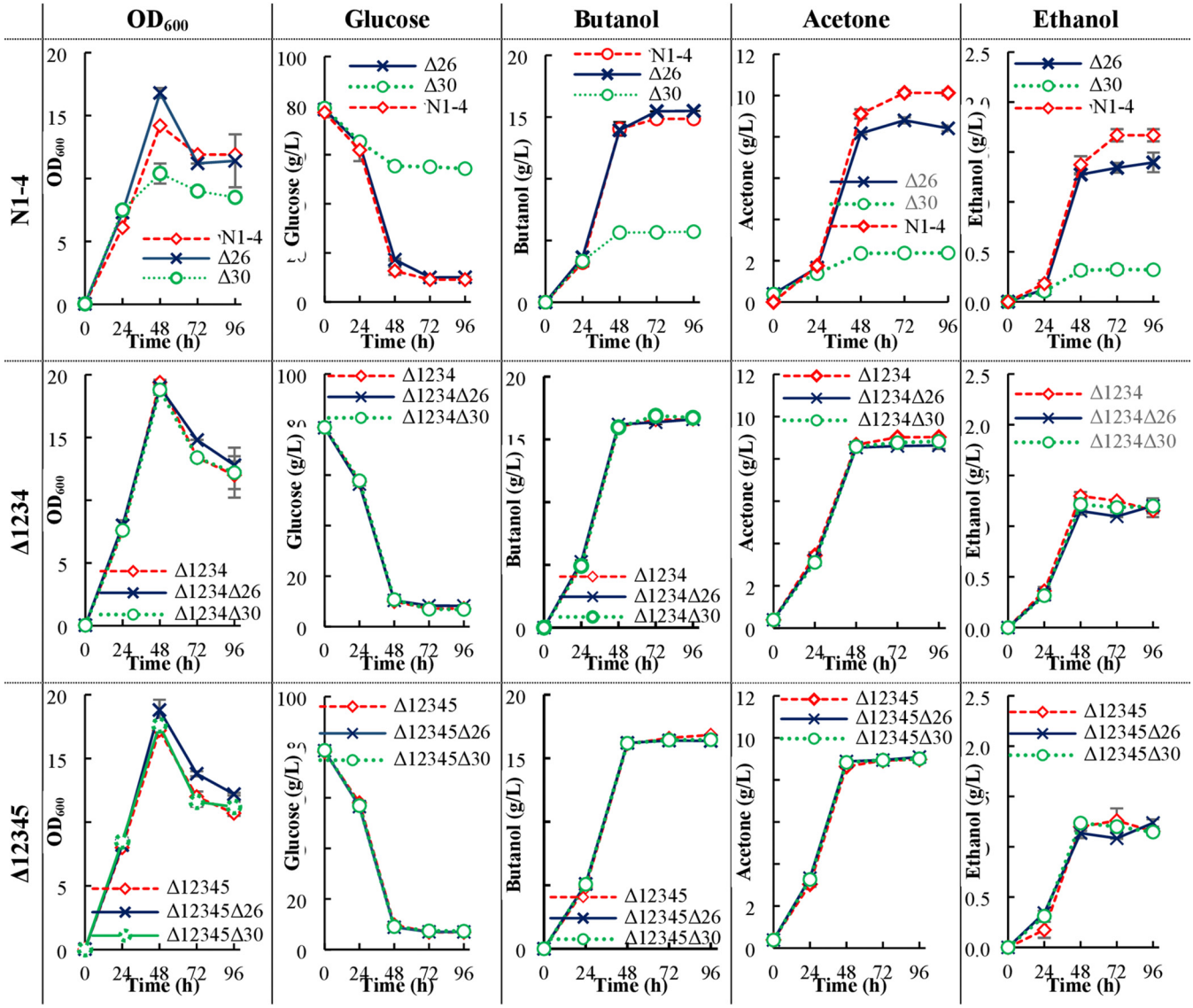

FIG 3 Profiles of serum bottle fermentation using the gene 26 and gene 30 single-deletion mutants compared to the mother strains (*C. saccharoperbutylacetonicum* N1-4, Δ1234, and Δ12345, respectively). The error bar represents the standard error at a 95% confidence interval.

The development of the CRISPR-Cas9 system for genome editing in *Clostridium* has opened up broad possibilities for utilizing these strains as desirable platforms for biochemical production. However, Cas9 protein has high toxicity to the host cells due to its endonuclease activity; when it is expressed under a strong constitutive promoter, it is difficult to transform the CRISPR-Cas9 plasmid into the cells (no transformants could be obtained) (15). The expression of Cas9 under an inducible promoter is a feasible strategy to address this issue, as we do in our CRISPR-Cas9 system. In such a strategy, the genetic manipulation is achieved through homologous recombination first without the expression of Cas9, and then Cas9 is induced to express as a selection pressure for the positive mutant against the unedited mother cells (15). Even so, in many cases, the transformation efficiency is still low (compared to the control plasmid that does not contain Cas9) due to the leaked activity of the inducible promoter and thus toxicity of Cas9 to the cells. More complex systems, such as riboswitches, have been designed to reduce Cas9 toxicity during the transformation and increase expression during the selection for genome editing (16). However, such systems are often difficult to design and implement, and the efficiency is also varied. Alternatively, other researchers have utilized nickase-Cas9, which makes single-strand breakage and induces lower toxicity to the host, but such a strategy

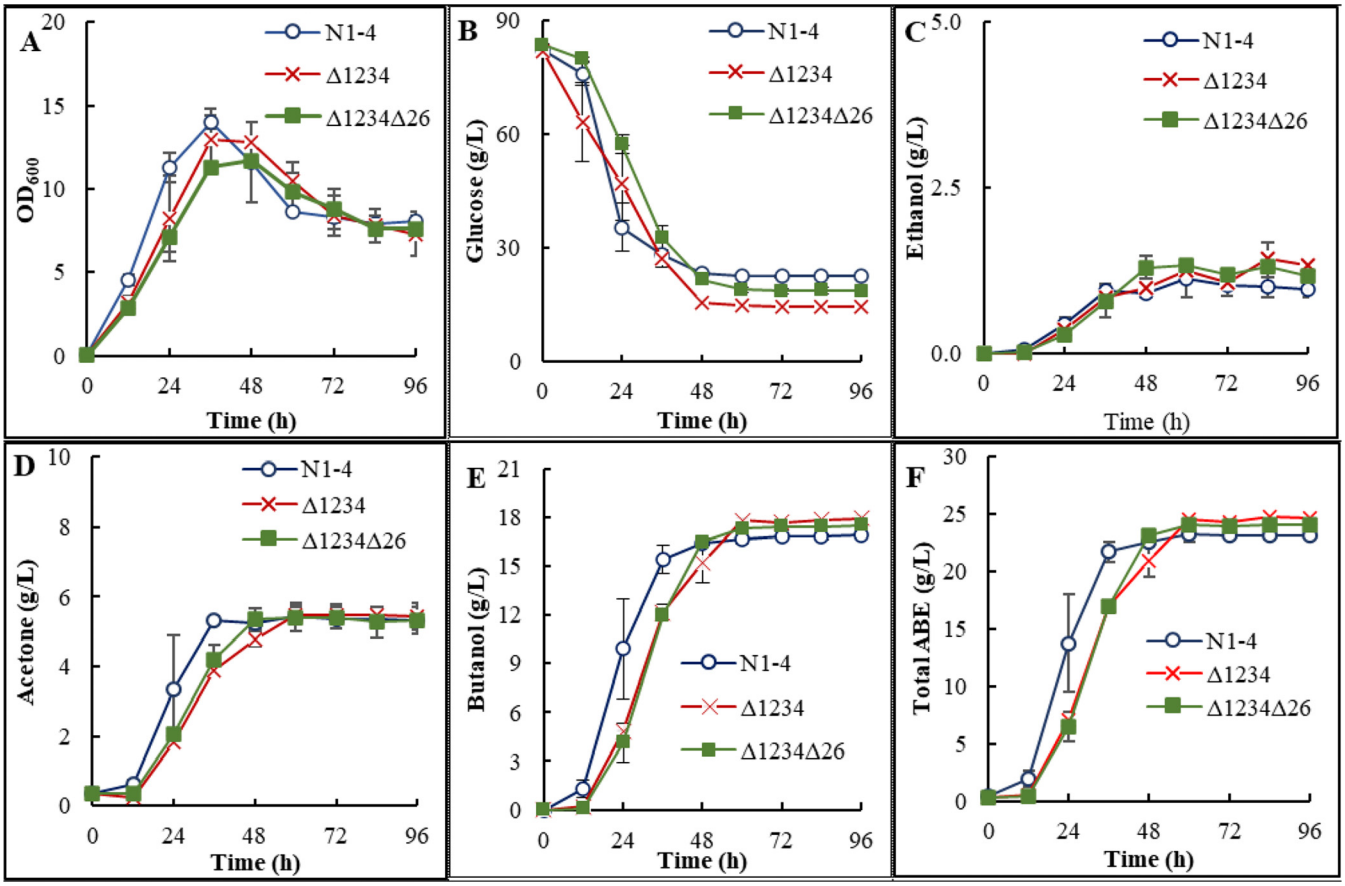

FIG 4 Profiles of batch fermentation in bioreactors with pH controlled for Δ1234Δ26 compared to Δ1234 and the wild type (N1-4). The error bar represents the standard error at a 95% confidence interval.

would often sacrifice the selection efficiency of Cas9 (17). On the other hand, the Cas12a protein (Cpf1) makes double-strand breakage but with 5 bp distal, reducing the toxicity to some extent, and thus can also be used for genome editing with decent efficiency (18). In all cases, reliable transformation efficiency is still the key to success for genome editing in most *Clostridium* strains. Although in *Clostridium saccharoperbutylacetonicum*, the transformation efficiency is generally decently high, low transformation efficiency is still observed in some cases, for example, for the insertion of genes of large sizes using the CRISPR-Cas9 system. In a broader sense, the results we demonstrated here would provide an insightful reference for enhancing DNA transformation efficiency in other *Clostridium* strains and other microorganisms with underdeveloped DNA transformation protocols.

Effects of the deletion of autolysin genes on cell morphology. We checked the cell morphology of Δ26 and Δ30 compared to the wild-type mother strain (N1-4) under a microscope throughout the fermentation process. As shown in Fig. S1, at 96 h of the fermentation (row 2 in Fig. S1), when N1-4 (as well as Δ30) demonstrated obvious au-

TABLE 3 Transformation efficiency

	Transformation efficiency (CFU/μg) in host strain:		
Plasmid	Wild type	Δ1234	Δ12345
pPJB31 (N1-4)	200	192	1,300
pPJB32 (N1-4)	101	271	1,500
pPJB31 (Δ30)	236	329	1,100
Avg (±SE)	179 ± 57	264 ± 56	1,300 ± 163

tolysis, the cells of Δ26 were still in good clostridial shape with the regular sporulation. This is consistent with our results concerning the cell growth and solvent production: compared to N1-4, Δ26 grew to a higher maximum OD and produced slightly higher butanol level (Fig. 3), while Δ30 demonstrated acid crash and showed decreased cell growth compared to the wild type.

In this study, we identified the key autolysin genes responsible for the autolysis in the hyperbutanol-producing strain *C. saccharoperbutylacetonicum* N1-4, which provides an essential reference for the further development of robust strains for industrial fermentation. Peptidoglycan recycling is a metabolic process by which bacteria reutilize their cell wall biomass within one generation during vegetative growth. Peptidoglycan recycling has been extensively reported in Gram-negative bacteria (19). Recently, it has been revealed that peptidoglycan recycling also occurs in some Gram-positive bacteria; although it is not essential for the vegetative growth of Gram-positive bacteria, it provides a benefit for the long-term survival in the stationary phase (20). It warrants further investigation of the peptidoglycan turnover and recycling in *C. saccharoperbutylacetonicum* and other *Clostridium* strains.

MATERIALS AND METHODS

Reagents and strains. Phanta HS Super-Fidelity DNA polymerase and ClonExpress MultiS One Step cloning kit from Vazyme Biotech (Nanjing, China) were used for PCR cloning and plasmid construction through DNA assembly, respectively. *Taq* DNA polymerase from Green Mountains Biosystems (Ann Arbor, MI) and LongAmp *Taq* from New England BioLabs, Inc. (Ipswich, MA) were used for colony PCR (cPCR) to confirm the mutation. BtgZI and NotI restriction enzymes were obtained from New England BioLabs, Inc. All polymerases and restriction enzymes were used following the manufacturers' protocols.

All the strains used in this study are listed in Table 2. *Escherichia coli* NEB Express (New England BioLabs, Inc.) was used for plasmid propagation. *E. coli* was cultivated in Luria-Bertani (LB) broth or LB agar plates supplemented with 100 μg/ml carbenicillin when needed. *C. saccharoperbutylacetonicum* N1-4 (HMT) (DSM 14923) obtained from DSMZ (Braunschweig, Germany), and phage-deficient strains *C. saccharoperbutylacetonicum* Δ1234 and Δ12345 were routinely cultivated in tryptone-glucose-yeast extract (TGY) medium containing 30 g/liter of tryptone, 20 g/liter of sucrose, 10 g/liter of yeast extract, and 1 g/liter of L-cysteine, or on TGY agar plates, in an anaerobic chamber at 35°C. We supplemented 30 μg/ml clarithromycin (Cla) and/or 40 mM lactose (TGYL) when necessary. SMP buffer used for electrotransformation is composed of 270 mM sucrose, 1 mM $MgCl_2$, and 7 mM Na_2HPO_3/NaH_2PO_3 (pH adjusted to 6.5 and filter sterilized). *Clostridium* strains were stored at −80°C in glycerol stocks (20%). The stock was inoculated into TGY at 5% inoculum, and the culture was incubated at 35°C overnight to prepare the seed culture for DNA transformation or fermentation.

Plasmid construction. All the plasmids used in this study are listed in Table 2. All the primers used in this study are listed in Table 4. pJZ100 (21) was used as the mother vector to construct pPJB14. The P_{lac} promoter was cloned from plasmid pYW51 using primers YW2583 and YW2584 and then inserted between the ApaI and BtgZI sites of pJZ100 to replace the original P_{thl} promoter, generating pPJB14.

Sixteen putative autolysin-encoding genes (CSPA_RS26630, CSPA_RS03200, CSPA_RS08050, CSPA_RS01160, CSPA_RS21780, CSPA_RS09105, CSPA_RS06245, CSPA_RS00240, CSPA_RS18890, CSPA_RS13245, Cspa_c38900, CSPA_RS11880, CSPA_RS15280, CSPA_RS03880, Cspa_135p00690, and CSPA_RS24880; numbered as autolysin gene 15 to 30, respectively) from *C. saccharoperbutylacetonicum* N1-4 were identified using the NCBI BLAST tool based on the homologous amino acid sequences from previously identified autolysin-encoding genes in related strains (5). The DNA fragment of the putative autolysin gene was cloned individually using PCR with the set of primers (Table 4) and the genomic DNA of the N1-4 strain as the template. pPJB14 was digested with BtgZI, and then the DNA fragment of each autolysin gene was inserted to obtain the plasmid (pPJB15 to pPJB30; Table 2) for overexpression purposes.

Autolysin genes 26 and 30 (corresponding to plasmids pPJB26 and pPJB30, respectively) were selected to delete in *C. saccharoperbutylacetonicum* N1-4, Δ1234, and Δ12345 using our customized CRISPR-Cas9 genome editing system (15, 22). Plasmid pYW51 was used as the mother vector to construct the plasmid for gene deletion (Table 2). To delete gene 26, the small RNA (sRNA) (sCbei_5830) promoter fused with the 20-nucleotide (nt) guiding sequence (5′-GACTCTCCATTAATAGTAATCC-3′) was amplified from *C. beijerinckii* 8052 genomic DNA using primers YW5190 and YW484 and inserted into the BtgZI site of pYW51, generating an intermediate vector. Further, two homology arms (~1 kb in length for each) flanking at both sides of the open reading frame (ORF) of gene 26 were amplified from *C. saccharoperbutylacetonicum* genomic DNA with primer pairs of YW5191/YW5247 and YW5193/YW5194, respectively, and then inserted into the NotI site of the intermediate vector mentioned above, generating pPJB31 for the deletion of gene 26. Similarly, for targeting on gene 30 for the gene deletion, the fragment containing the sRNA promoter and 20-nt guiding sequence (5′-ACTGGTATCCCATAACTTCT-3′) was amplified with primers YW5201 and YW484 and then inserted into the BtgZI site of pYW51, generating an intermediate vector. One ~1-kb homology arm sequence upstream of gene 30 was amplified using primers YW5197/YW5249, and another ~1-kb homology arm sequence downstream of gene 30 was amplified using primers YW5250/

TABLE 4 List of primers used in this study

Primer	Sequence	Cloning purpose[a]
YW2583	CTAAAACTgaattgattgggcccttatatacttggtttatttacttg	For cloning P_{lac} and insertion into pPJB14
YW2584	GAATGTGAACTTGTATAttattacagtCATCGCtatatatattcatt GCGATGgatatatcatttcagccctcctgtgaaattg	
YW2585	caatttcacaggagggctgaaATGATAAATGATTTTGAAC	Insertion of CSPA_RS26630 for pPJB15 construction
YW2586	gcgaatgtgaacttgtataCTAATTATTTAATACTTTCATTAC	
YW2587	caatttcacaggagggctgaaTTGAAGTCTGAAAGCGAATT	Insertion of CSPA_RS03200 for pPJB16 construction
YW2588	CTTGTTGCGAATGTGAACTTGTATActatctaaaacttcctt	
YW2589	caatttcacaggagggctgaaATGAAAAAAAGATTATTATC	Insertion of CSPA_RS08050 for pPJB17 construction
YW2590	gcgaatgtgaacttgtataTTATTTAATTAATGATCCATC	
YW2591	caatttcacaggagggctgaaATGAAAAAGAAATGTAGTAT	Insertion of CSPA_RS01160 for pPJB18 construction
YW2592	gcgaatgtgaacttgtataTTATGCTTTAATTTTGAAAAATG	
YW2593	atttcacaggagggctgaaTTGAAAGGAAGAACTCTAAT	Insertion of CSPA_RS21780 for pPJB19 construction
YW2594	gcgaatgtgaacttgtataTTAATCTACCAAAGTAATCC	
YW2595	caatttcacaggagggctgaaATGTTTAAAGTAAAGAAAG	Insertion of CSPA_RS09105 for pPJB20 construction
YW2596	gcgaatgtgaacttgtataTTATCCCTCTAAAATTATTTTTC	
YW2597	caatttcacaggagggctgaaGTGGATAATTTTAACATATAC	Insertion of CSPA_RS06245 for pPJB21 construction
YW2598	gcgaatgtgaacttgtataTTATAATATTATACAAATCAACC	
YW2599	caatttcacaggagggctgaaATGCCTGAAATAGGTAGT	Insertion of CSPA_RS00240 for pPJB22 construction
YW2600	gcgaatgtgaacttgtataTTAGTAATAAAATTTTGGTATTCCTC	
YW2601	caatttcacaggagggctgaaGTGAAAATGAAAAAGAAAAT	Insertion of CSPA_RS18890 for pPJB23 construction
YW2602	gcgaatgtgaacttgtataCTATCTTAGAATATCCTGTG	
YW2603	caatttcacaggagggctgaaATGAAAGGTATAGATGTAAG	Insertion of CSPA_RS13245 for pPJB24 construction
YW2604	gcgaatgtgaacttgtataTTATTTTGTTTCTATTGAATAGA	
YW2605	caatttcacaggagggctgaaATGAAAAAGAAAATAACTAT	Insertion of Cspa_c38900 for pPJB25 construction
YW2606	gcgaatgtgaacttgtataCTATCTTAGAATATCCTGTG	
YW2607	caatttcacaggagggctgaaATGAATATTAAAAAGAAATTTG	Insertion of CSPA_RS11880 for pPJB26 construction
YW2608	gcgaatgtgaacttgtataTTAGTTTAAAATGTCTTGAG	
YW2609	caatttcacaggagggctgaaATGAAAGGTTTAGATGTAAG	Insertion of CSPA_RS15280 for pPJB27 construction
YW2610	gcgaatgtgaacttgtataTTAACATTTTTCTATAGGTAAGA	
YW2611	caatttcacaggagggctgaaATGAAAAATATAAAAGGAATAG	Insertion of CSPA_RS03880 for pPJB28 construction
YW2612	gcgaatgtgaacttgtataCTAAGATAGTTTAGTTGCAAC	
YW2613	caatttcacaggagggctgaaATGTCATTAATTACTGGAATAG	Insertion of Cspa_135p00690 for pPJB29 construction
YW2614	gttgcgaatgtgaacttgtataTTATTTTTCTAAAGTATTTTTAGTTGC	
YW2615	atttcacaggagggctgaaATGCAAGACAAGAATCC	Insertion of CSPA_RS24880 for pPJB30 construction
YW2616	gttgcgaatgtgaacttgtataTTACAAATTTACTTTTGATCG	
YW32	GTTTTCCCAGTCACGACGTT	For cPCR confirmation of overexpression plasmids
YW33	TTGCTGCTCATGCAGATGAT	
YW484	aaagttaaaagaagaaaatagaaatAtaatctttaatttgaaaagatttaag	For cloning of P_{sRNA} + "20NT," used with YW5290 for pPJB31 construction and with YW5201 for pPJB32construction
YW5190	TTGCTATTTCTAGCTCTAAAACgactctccattaatagtaatcc ATGGTGGAATGATAAGGG	For cloning of P_{sRNA} + "20NT" with YW484 for pPJB31 construction
YW5201	TTGCTATTTCTAGCTCTAAAACactggtatcccataacttct ATGGTGGAATGATAAGGG	For cloning of P_{sRNA} + "20NT" with YW484 for pPJB32 construction
YW5191	gtgatatgactaataattaCTGATGCGTATGATGCAATG	For cloning recombination arm upstream ORF of gene 26
YW5247	CCACTATGTTGTAATTAGAATCcatattatttgggaagcatatc	
YW5193	TAAACAGAATTTAATATTAatttttatctccttagtac	For cloning recombination arm downstream ORF of gene 26
YW5194	cactagtaaccatcacactgATACTTAATTATATTATGAAG	
YW5195	GAGAGATGGGATGGAAGTGG	For cPCR detection of gene 26 deletion
YW5196	CCAAAAATATCCCACCATGG	
YW5197	gtgatatgactaataattaACTACTGTCCCATTTATGG	For cloning recombination arm upstream ORF of gene 30
YW5249	gcgaatttcttccataatcCATAAATCCAACTGTACCG	
YW5250	cacggtacagttggatttatgGATTATGGAAGAAATTCGC	For cloning recombination arm downstream ORF of gene 30
YW5200	atccactagtaaccatcacactgCAGGAGTAACTTGTGTTATG	
YW5203	CAACCGTTCTAGGTCCGAC	For cPCR detection of gene 30 deletion
YW5204	CAAATCCAGTTACGCCTCATC	

[a]"20NT," 20-nt CRISPR protospacer (guiding sequence).

YW5200. The two homology arms were then inserted into the NotI site of the intermediate vector as mentioned above, generating pPJB32 for the deletion of gene 30.

Plasmid transformation and mutant screening. *Clostridium* cells were electroporated using our previously published protocol (22), with 2.0 kV (1 kV/mm), 25 μF, and 300 Ω; SMP (instead of glycerol) was used as the washing buffer.

For the recombinant strain for gene overexpression (bearing plasmids pPJB15 to pPJB30,

respectively), single colonies were picked for cPCR to confirm the existence of the plasmid using primers YW32/YW33. The confirmed positive recombinant strain was then inoculated into the TGY-Cla$^+$ liquid medium for cultivation. When the culture was grown to early exponential phase, glycerol stock was prepared for each culture and stored at −80°C for later use. For obtaining the mutant strain with gene deletion (for the deletion of gene 26 or gene 30), single colonies from transformants were picked and inoculated into 2 ml TGY liquid medium. When the culture grew up (at the exponential phase), 50 μl was taken and spread onto TGYL-Cla$^+$ agar plates to induce the Cas9 activity for mutant selection. The mutation was confirmed using cPCR with primers flanking upstream and downstream of the targeted homologous recombination region (YW5195/YW5196 and YW5203/YW5204 for confirming the deletion of gene 26 and gene 30, respectively; Table 4). Then, the identified positive mutants were cultured and subcultured in liquid TGY (for 1 to 2 generations) for plasmid curing. The curing of the plasmid was confirmed through replica plating of the colonies onto both TGY and TGY-Cla$^+$ agar plates (the ones that could grow on the TGY plate but not on the TGY-Cla$^+$ plate were deemed the potential ones with plasmid cured). The mutation in the identified clean mutant (with plasmid cured) was further confirmed by cPCR.

Fermentation. Small-scale fermentations were carried out in 250-ml serum bottles with a working volume of 100 ml for the nine strains N1-4, Δ26, Δ30, Δ1234, Δ1234Δ26, Δ1234Δ30, Δ12345, Δ12345Δ26, and Δ12345Δ30. The fermentation broth containing 80 g/liter glucose, 6 g/liter tryptone, and 2 g/liter yeast extract in addition to the P2 medium was prepared and sterilized through autoclaving before starting the fermentation. P2 medium composition was reported before (23). The culture was grown in TGY medium in an anaerobic chamber at 35°C until the OD_{600} reached ~0.8. The culture was then inoculated at a 5% ratio (vol/vol) to the fermentation broth to initiate the fermentation. The fermentation in the serum bottles was carried out in an orbital shaker at 150 rpm and 30°C. Samples were taken every 24 h for analysis.

The strain harboring the plasmid for overexpression of the autolysin gene (pPJB15 to pPJB30, as shown in Table 1) was characterized through fermentation in 500-ml bioreactors (GS-MFC; Shanghai Gu Xin Biological Technology Co., Shanghai, China) under anaerobic conditions. The strain containing the empty plasmid (pPJB14) was used as the control. The fermentation broth containing 80 g/liter glucose, 6 g/liter tryptone, 2 g/liter yeast extract, and 13.7 g/liter lactose in addition to the P2 medium was prepared and autoclaved before the fermentation. We also supplemented 30 mg/liter clarithromycin to the fermentation to help the culture maintain the plasmid. The fermentation was started with 5% inoculum from preculture (which was prepared by growing the strain in TGY medium supplemented with 30 mg/liter clarithromycin). The pH was controlled at >5.0 using 4 M NaOH throughout the fermentation. The temperature was kept at 30°C. Samples were taken every 12 h for analysis. Each fermentation was carried out in at least two replicates.

Larger-scale batch fermentations were carried out in replicates in BioFlo 115 benchtop bioreactors (New Brunswick Scientific Co., Enfield, CT) with a working volume of 1.5 liters for Δ1234 and Δ1234Δ26 (with the wild-type strain as the control). The fermentation broth containing 80 g/liter glucose, 6 g/liter tryptone, and 2 g/liter yeast extract in addition to the P2 medium was prepared and sterilized before the fermentation. The fermentation was carried out at an agitation of 50 rpm, 30°C, with the pH controlled at >5.0 (using 4 M NaOH). Samples were taken every 12 h for analysis.

Analytical procedures. Concentrations of glucose, butanol, acetone, and ethanol were determined on a high-performance liquid chromatographer (Agilent 1260 series; Agilent Technologies, Santa Clara, CA, USA) with a refraction index detector and Varian MetaCarb 87H column (set at 25°C). Aqueous 5 mM H_2SO_4 at a flow rate of 0.6 ml/min was used as the mobile phase. Cell optical density (OD_{600}) was quantified with a cell density meter (Ultrospec 10; Biochrom Ltd., Cambridge, England).

Microscopy. Cell culture was grown in P2 medium using 60 g/liter sucrose as the substrate. At specific time points, cell culture samples were harvested by centrifugation, washed twice, and resuspended in distilled water. Cell morphology was examined using an Olympus BX53F upright microscope (phase contrast mode) equipped with an Olympus DP73 camera (Olympus Corporation, Shinjuku-ku, Japan).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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