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BIOENERGY AND BIOFUELS

Engineering *Clostridium acetobutylicum* with a histidine kinase knockout for enhanced *n*-butanol tolerance and production

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Abstract *Clostridium acetobutylicum* JB200, a mutant strain of *C. acetobutylicum* ATCC 55025 obtained through strain evolution in a fibrous bed bioreactor, had high butanol tolerance and produced up to ~21 g/L butanol from glucose in batch fermentation, an improvement of ~67 % over the parental strain (~12.6 g/L). Comparative genomic analysis revealed a single-base deletion in the *cac3319* gene leading to C-terminal truncation in its encoding histidine kinase (HK) in JB200. To study the effects of *cac3319* mutation on cell growth and fermentation, the *cac3319* gene in ATCC 55025 was disrupted using the ClosTron group II intron-based gene inactivation system. Compared to ATCC 55025, the *cac3319* HK knockout mutant, HKKO, produced 44.4 % more butanol (18.2±1.3 vs. 12.6±0.2 g/L) with a 90 % higher productivity (0.38±0.03 vs. 0.20±0.02 g/L h) due to increased butanol tolerance, confirming, for the first time, that *cac3319* plays an important role in regulating solvent production and tolerance in *C. acetobutylicum*. This work also provides a novel metabolic engineering strategy for generating high-butanol-tolerant and high-butanol-producing strains for industrial applications.

Keywords ABE fermentation · Butanol tolerance · *C. acetobutylicum* · Histidine kinase · Gene knockout · Metabolic engineering

Introduction

Butanol is an important chemical and solvent with wide industrial applications. It has also been recognized as a potential substitute to gasoline and a fuel additive for its higher energy density and superior fuel properties compared to ethanol (Dürre 2007; Kumar and Gayen 2011; Xue et al. 2013). Biological production of butanol through acetone-butanol-ethanol (ABE) fermentation of *Clostridium acetobutylicum* was once the second largest industrial fermentation process in the twentieth century but had lost its competitiveness to petrochemical processes by the 1960s (Jones and Woods 1986). However, with diminishing petroleum resources and rising crude oil prices, biological butanol production from renewable biomass has recently received large attention (Jang et al. 2012a; Lee et al. 2008; Lütke-Eversloh and Bahl 2011; Zhao et al. 2013). Conventional ABE fermentation is severely limited by the end product inhibition caused by butanol (Bowles and Ellefson 1985) and hence suffers from low butanol titer (usually less than 12–14 g/L) (Dürre 1998; Jones and Woods 1986; Zheng et al. 2009), which also negatively affects productivity and increases butanol production cost (Gu et al. 2011). Economic analyses show that butanol separation costs would be cut in half if the final butanol titer is raised from 12 to 19 g/L (Papoutsakis 2008).

Therefore, there have been extensive research efforts aiming at the development of high-butanol-tolerant and high-butanol-producing strains, mainly the species of *Clostridium acetobutylicum* and *Clostridium beijerinckii*, via mutagenesis (Formanek et al. 1997; Jain et al. 1993), metabolic engineering (Harris et al. 2000, 2001; Jang et al. 2012b;

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Nair et al. 1999; Tomas et al. 2003), adaptive evolutionary engineering (Isar and Rangaswamy 2012; Jang et al. 2013), and genome shuffling (Gao et al. 2012; Mao et al. 2010). Random overexpression of genes in a genomic library (Borden and Papoutsakis 2007) and transcriptional analysis have also been employed to identify genes and mechanisms responsible for higher butanol tolerance under butanol stress (Alsaker et al. 2010; Borden et al. 2010; Janssen et al. 2012; Schwarz et al. 2012; Tomas et al. 2004; Wang et al. 2013). However, butanol production and tolerance involved numerous genes in complicated metabolic and regulatory pathways associated with acidogenesis, solventogenesis, sporulation life cycle, quorum sensing, and stress responses (Dürre et al. 2002; Nicolaou et al. 2010), which are still poorly understood. Also, butanol tolerance varies with growth conditions (Zingaro et al. 2013) and increasing a strain's butanol tolerance does not guarantee a higher butanol production (Mann et al. 2012). Although metabolic engineering has made good progress in generating strains with significantly improved butanol production (Lütke-Eversloh 2014), so far the highest butanol-producing strains (up to ~21 g/L) have been generated by traditional random mutagenesis and adaptation under butanol stress (Chen and Blaschek 1999; Yang and Zhao 2013).

Recently, a hyper-butanol-tolerant and hyper-butanol-producing mutant strain, designated as JB200, of *C. acetobutylicum* ATCC 55025 (Jain et al. 1993), which is a biologically pure asporogenic mutant of *C. acetobutylicum* ATCC 4259 (the Weizmann strain, poorly studied in terms of physiology and genetics), was obtained through mutagenesis and adaptation in a fibrous bed bioreactor (Yang and Zhao 2013). Comparative genomic analysis of JB200 and its parental strain revealed seven point mutations in the JB200 genome, which might contribute to its improved butanol tolerance and production (~21 vs. ~12 g/L for ATCC 55025). Among the seven mutations, a single-base deletion in the coding region of the *cac3319* gene causes a large portion (~75 % of the original length) of truncation at the C-terminus of its encoding histidine kinase, a two-component signal transduction protein important for multiple functions in bacteria (Stock et al. 1995; Wolanin et al. 2002). We hypothesize that this mutation plays a role in enhancing butanol tolerance and production of JB200 because Cac3319 has been reported as one of the three orphan histidine kinases involved in the activation of Spo0A (Steiner et al. 2011), which is believed to be the master regulator of both sporulation and solventogenesis in *C. acetobutylicum* (Errington 2003; Paredes et al. 2005).

In this work, the effects of the *cac3319* gene mutation on butanol tolerance and production were studied. To mimic the mutated Cac3319 protein in JB200, the wild-type *cac3319* gene in the parental strain ATCC 55025 was disrupted using the ClosTron group II intron-based gene inactivation system (Heap et al. 2010), and it was designed that the protein product of the knocked-out *cac3319* gene would be similar to the mutated Cac3319 protein in JB200. Compared to its parental strain ATCC 55025, the *cac3319* knockout mutant, designated as HKKO, gave 45 % higher butanol production (up to ~19 g/L) with a 90 % higher productivity due to increased butanol tolerance. For the first time, this study showed that the *cac3319* gene plays an important role in regulating solvent production and tolerance in *C. acetobutylicum*.

Materials and methods

Bacterial strains, plasmids, and primers

Bacterial strains, plasmids, and the PCR primers used in this study are listed in Table 1. *C. acetobutylicum* ATCC 55025 was the parental strain used in this study. JB200 (ATCC PTA-12215) was derived from ATCC 55025 through adaptive mutagenesis in a fibrous bed bioreactor (Yang and Zhao 2013). *E. coli* DH10B was used for vector construction and amplification, and *E. coli* TOP10 bearing pAN2 was used for methylation of pMTL007- and pMTL82151-derived plasmids. All strains were maintained frozen in 15 % glycerol at −80 °C. All PCR primers were synthesized by Invitrogen (Carlsbad, CA).

Cell growth and culture media

Unless otherwise noted, all *C. acetobutylicum* strains were cultured in clostridial growth medium (CGM, containing 2 % glucose) or on CGM agar (Hartmanis and Gatenbeck 1984) supplemented, when necessary, with thiamphenicol (30 μg/mL) or erythromycin (50 μg/mL) at 37 °C anaerobically. Culture media in serum bottles were purged with nitrogen to reach anaerobiosis and then sterilized by autoclaving at 121 °C, 15 psig, for 30 min. All CGM agar plates were deoxygenated by overnight incubation in an anaerobic chamber (Forma Scientific, Marietta, OH). *E. coli* strains were grown aerobically at 37 °C in Luria-Bertani (LB) media supplemented, when necessary, with chloramphenicol (25 μg/mL) or tetracycline (20 μg/mL).

DNA and plasmid isolation, manipulation, and transformation

Genomic DNA and plasmids were prepared using QIAGEN Genomic-tip and Genomic DNA Buffer Set, and QIAprep Spin Miniprep Kit (Qiagen, Germantown, MD), respectively. Purification of DNA fragments was carried out using NucleoSpin Gel and PCR Clean-up Kit (MacCherey-Nagel, Bethlehem, PA). All restriction enzymes were supplied by New England Biolabs (NEB, Ipswich, MA) and used according to the manufacturer's instructions. DNA cloning was performed using In-fusion HD cloning kit from Clontech

Table 1 Strains, plasmids, and PCR primers used in this study

Strain/plasmid/primer	Relevant characteristics	Reference/source
Strains		
C. acetobutylicum ATCC 55025	ATCC 4259 asporogenic mutant	ATCC
JB200	ATCC 55025 mutant by mutagenesis	Yang and Zhao (2013)
HKKO	ATCC 55025 *cac3319* mutant	This work
HKKO(cac3319)	Complemented ATCC 55025 *cac3319* mutant	This work
HKKO(pMTL)	HKKO containing empty plasmid pMTL82151 (control)	This work
E. coli DH10B	*mcrA* Δ(*mrr-hsdRMS-mcrBC*) *recA1*	Invitrogen
E. coli TOP10	*mcrA* Δ(*mrr-hsdRMS-mcrBC*) *recA1*	Invitrogen
Plasmids		
pAN2	*Φ3tI*, *p15a ori*, Tet^R	Heap et al. (2007)
pMTL007	ClosTron plasmid for targeted gene knockout	Heap et al. (2007)
pMTL007C-E2::cac3319-364s	ClosTron plasmid retargeted to *C. acetobutylicum* *cac3319* gene; Cm^R	DNA 2.0
pMTL82151	*Clostridium* modular plasmid used for complementation and control; Cm^R	Heap et al. (2009)
pMTL82151::cac3319	*cac3319* complementation plasmid	This work
Primers	Sequence (5′→3′)	
hkkoFor	CATACCATTTTTAATTGCCTTAGC	This work
hkkoRev	TGCATACGTCTATAGCTCCAAC	This work
hkFor	GAAACAGCTATGACCGCGGTCTTTAC TAATCCGAGTTAGTAT	This work
hkRev	CGACTCTAGAGGATCCGCTTGATTAT TATTGTTCCA	This work
M13F	TGTAAAACGACGGCCAGT	This work
M13R	GGAAACAGCTATGACCGC	This work
probeFor	AACTTCGCGACTCATAGAATTATTTCC	This work
probeRev	GTTGGGAGTATTCCTTACCATTTAAGC	This work

(Mountain View, CA). PCR amplifications used for cloning were carried out using the Phusion High-Fidelity PCR kit (NEB), and PCR amplifications used for bacterial colony screening were carried out using *Taq* 2× Master Mix (NEB). Transformation of *E. coli* and agarose gel electrophoresis were performed according to standard procedures. Plasmids were transferred to *C. acetobutylicum* by electrotransformation (Mermelstein and Papoutsakis 1993) in the anaerobic chamber. Prior to transformation, plasmid DNA was purified from *E. coli* TOP10 cells harboring pAN2. This plasmid contained the Φ3T I methyltransferase gene of *Bacillus subtilis* phage Φ3T, which protected plasmid DNA from restriction by *C. acetobutylicum* Cac824I endonuclease (Heap et al. 2007).

Construction of cac3319 knockout mutant HKKO

Group II intron retargeting technique (Heap et al. 2007, 2010) was used to construct the *cac3319* knockout mutant of ATCC 55025 (HKKO). An intron targeting and design tool available at http://clostron.com was used to generate the intron targeting region. First, the *cac3319* gene sequence was input as a query sequence, and all the potential target sites were predicted by the tool using the Perutka method (Perutka et al. 2004). Then, the best target site for the disruption of *cac3319* was selected. The 364/365s target site (which means that the intron is to be inserted between the 364th and 365th base of the original *cac3319* gene) sequence (AAAAATTTAGATTTTGCCTT GGTTCTAATTGTTCTGAAAAATATA) was selected because the generated protein product would best mimic the mutated Cac3319 protein in JB200 (see Fig. S1 in Supplemental Materials). After entering the selected target site sequence, the intron targeting region was generated (see Fig. S2 in Supplemental Materials) and used to modify the mobile group II intron to insert into the selected target site in *cac3319*. The designed intron targeting region was then constructed synthetically and cloned into the ClosTron plasmid pMTL007C-E2 by DNA 2.0 Inc. (Menlo Park, CA) to form the intron retargeted plasmid pMTL007C-E2::cac3319-364s. Following the transformation of the retargeted ClosTron plasmid into *C. acetobutylicum*, the *cac3319*-disrupted mutant was screened according to the reported protocol (Heap et al. 2010). Putative integrants were obtained on the basis of acquisition of erythromycin resistance. Colony PCR screens were undertaken on a random selection of putative integrants

to verify that the retargeted intron had inserted into the desired position in the *cac3319* gene. The gene-specific primers (hkkoFor and hkkoRev) flanking the intron insertion site were used to distinguish between the wild-type and intron-interrupted genes during PCR screening. One positive integrant was isolated and restreaked to screen for loss of the plasmid-encoded thiamphenicol resistance (Heap et al. 2010). The genomic DNA of the selected plasmid loss integrant was subjected to further confirmation by PCR, using the same pair of primers (hkkoFor and hkkoRev). Finally, the PCR fragments generated were subjected to nucleotide sequence analysis (Plant-Microbe Genomics Facility at the Ohio State University) to ascertain that the insertion had occurred at the desired position. Southern hybridization analysis was also performed to ensure that only a single insertion of the intron was present in the genome of HKKO.

Southern hybridization analysis

The intron-specific probe (see Fig. S3 in Supplemental Materials) designed to hybridize to the inserted intron of HKKO was generated by PCR using plasmid pMTL007C-E2::cac3319-364s as template and primers probeFor and probeRev (see Table 1). Genomic DNA (~2 μg) of ATCC 55025 and HKKO were digested overnight with the restriction enzyme *Eco*RV, respectively, followed by agarose gel (0.8 %) electrophoresis. DIG-labeled SPP1 DNA cleaved with *Eco*RI (Roche, Mannheim, Germany) was used as molecular weight marker on the gel. Transfer was performed with Amersham Hybond™-N+ nylon membrane (GE healthcare, Buckinghamshire, UK) using a vacuum blotter (Model 785, Bio-Rad, Hercules, CA) according to the manufacturer's instructions. The membrane was then baked at 80 °C for 30 min under vacuum to fix the DNA. Probe labeling and hybridization were carried out using DIG high prime DNA labeling and detection starter kit II (Roche, Mannheim, Germany).

Construction of complementation mutant

To confirm that the phenotypic changes observed in the *cac3319* knockout mutant (HKKO) were attributed to the intron insertion, a complementation mutant was constructed. A 1686-bp fragment encompassing the *cac3319* gene, its upstream promoter, and its downstream sequence were amplified using primers hkFor and hkRev. The fragment was cloned into *Clostridium* modular plasmid pMTL82151 (Heap et al. 2009) between *Sac*II and *Bam*HI sites to generate plasmid pMTL82151::cac3319. The complementation plasmid was first transformed into *E. coli* TOP10 cells containing pAN2 for methylation and then purified and transformed into HKKO to generate the complementation mutant HKKO(cac3319). The pMTL82151 empty vector was also introduced into HKKO to generate a control strain HKKO(pMTL). These mutants were confirmed by colony PCR using M13F and M13R primers (see Table 1) flanking the multiple cloning sites in plasmid pMTL82151.

Fermentation kinetics studies

Batch fermentation kinetics was studied in a 250-ml stirred-tank bioreactor containing 150 mL of P2 medium at 37 °C with pH controlled at >5.0 by adding 20 % ammonium hydroxide. Unless otherwise noted, the P2 medium contained ~100 g/L glucose, 2.2 g/L ammonium acetate, 1 g/L yeast extract, 0.5 g/L KH_2PO_4, 0.5 g/L K_2HPO_4, mineral salts (0.2 g/L $MgSO_4{\cdot}7H_2O$, 0.01 g/L $MnSO_4{\cdot}H_2O$, 0.01 g/L $FeSO_4{\cdot}7H_2O$, 0.01 g/L NaCl), and vitamins (1 mg/L *p*-amino-benzoic acid, 1 mg/L thiamine and 0.01 mg/L biotin). The bioreactor was inoculated with 7.5 mL of a seed culture (5 % of the total fermentation culture volume), which was prepared in a serum bottle containing 50 mL of CGM, inoculated with 0.2 mL of 15 % glycerol-CGM stock culture of a specific strain, and incubated at 37 °C for ~16 h. Samples were taken at regular intervals to monitor cell growth, glucose consumption, and production of acids and solvents.

Butanol tolerance assay

Butanol tolerance of *C. acetobutylicum* was investigated and compared for various strains. Cell growth was monitored by measuring the optical density at 600 nm (OD_{600}) in serum tubes each containing 10 mL CGM and butanol at an initial concentration of 0 to 20 g/L. Each tube was inoculated with a freshly prepared seed culture (at 5 % *v*/*v*) and incubated at 37 °C for ~8 h, with OD_{600} measurements at every 2 h. The specific growth rates at various initial butanol concentrations were estimated from the OD_{600} data. Each condition was studied with triplicate tubes.

Analytical methods

Cell density in the bioreactor was analyzed by measuring optical density (OD) of the cell suspension at 600 nm using a spectrophotometer (UV-16-1, Shimadzu, Columbia, MD). After removing the bacterial cells by centrifuging at 13,000×*g* for 5 min, the clear fermentation broth was analyzed for sugar and product concentrations. Glucose was measured using YSI model 2700 Select Biochemistry Analyzer (Yellow Springs, OH). Acetone, butanol, ethanol, acetic acid, and butyric acid were measured with a gas chromatograph (GC, Shimadzu GC-2014) equipped with a flame ionization detector and a 30-m fused silica column (0.25-μm film thickness and 0.25-mm ID, Stabilwax-DA). Nitrogen was used as the carrier gas at 1.47 mL/min (linear velocity 35 cm/s). Details about the GC analysis can be found elsewhere (Jiang et al. 2014).

Statistical analysis

Unless otherwise noted, batch fermentation kinetics for each strain was studied in triplicate, and the sample means with standard errors are reported. Student's *t* test analysis was performed using JMP software with the significance level α=0.05.

Results

Generation of cac3319-disrupted mutant HKKO

In this study, the 364/365s target site sequence was selected to generate the *cac3319*-disrupted mutant HKKO. By cloning the synthesized intron targeting region into the intron RNA-encoding plasmid pMTL007C-E2, the intron was retargeted to specifically interact with the target site and then inserted into the gene of interest (*cac3319*) together with ~2.0 kb of its downstream sequences (see Fig. 1a). Totally, four positive integrants were identified among nine randomly selected putative integrants from ~300 colonies, which had erythromycin resistance, through colony PCR screening (Fig. 1b). Insertion of the retargeted intron at the desired position in the genome of the selected positive integrant was further verified by a combination of PCR amplification (Fig. 1c), DNA sequencing (see Fig. S4 in Supplemental Materials), and Southern hybridization (Fig. 1d). The sequencing result showed that the retargeted group II intron had inserted into the 364/365s target site exactly as predicted by the Perutka algorithm in the HKKO mutant, which should have mutated Cac3319 protein similar to the one found in JB200 (see Fig. S1 in Supplemental Materials). The intron insertion in the chromosome was unique as evidenced in the Southern hybridization analysis (Fig. 1d). The effects of *cac3319* mutation on the phenotype of HKKO mutant were evaluated by studying its batch fermentation kinetics and growth tolerance to butanol as compared to its parental strain and JB200, which are discussed below.

Fermentation kinetics of various strains

Figure 2 shows typical batch fermentation kinetics of ATCC 55025, HKKO, and JB200. It is noted that all batch fermentations were performed with pH controlled at >5.0, with the

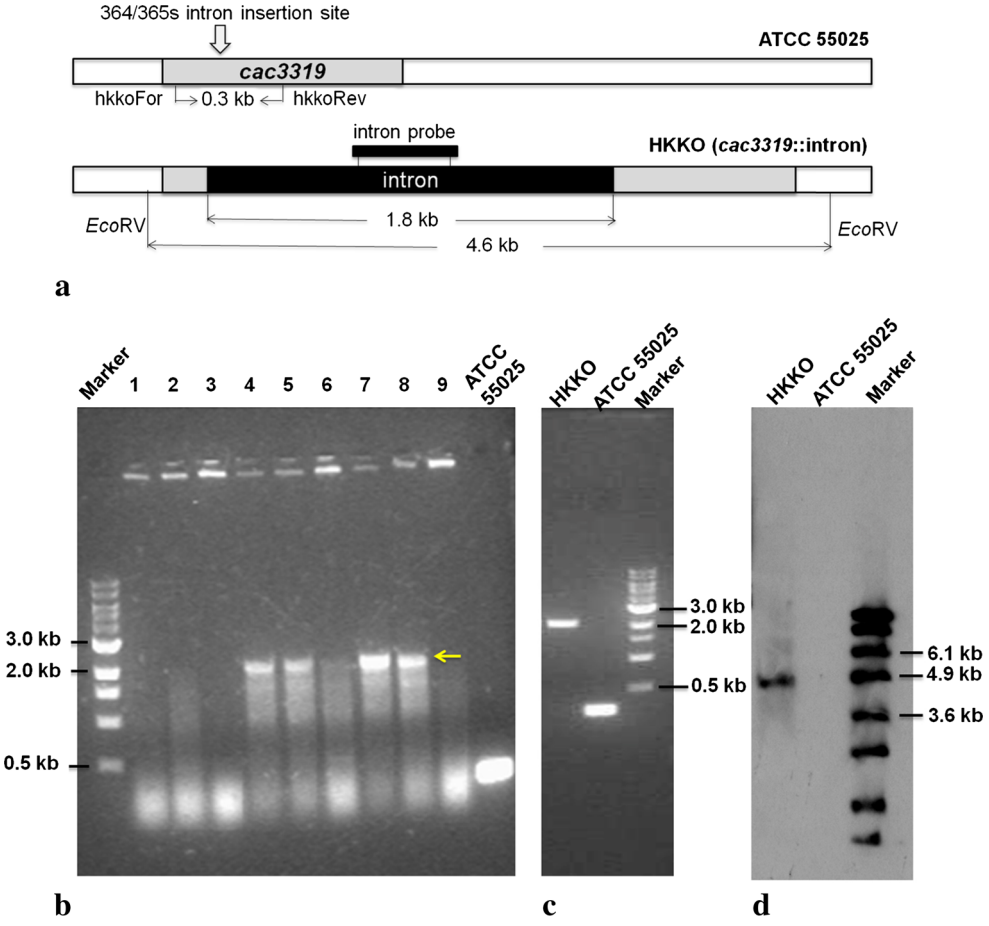

Fig. 1 Construction of *cac3319*-disrupted mutant *C. acetobutylicum* HKKO. **a** Schematic illustration of the intron insertion site of *cac3319* and the expected disrupted *cac3319* in the chromosome. **b** Colony PCR for screening of mutants with disrupted *cac3319* with intron insertion using primers hkkoFor and hkkoRev flanking the insertion site. ATCC 55025 genome was used as a control, which gave a PCR fragment of 0.3 kb. Only positive clones had a PCR fragment of ~2.1 kb (no. 4, 5, 7, and 8). Colony no. 8 was selected for further verification. **c** PCR verification of the HKKO mutant with disrupted *cac3319* with genomic DNA. **d** Southern blotting for the confirmation of unique intron insertion on the chromosome. HKKO gave a single band with the expected size of 4.6 kb, while the negative control (ATCC 55025) gave no band

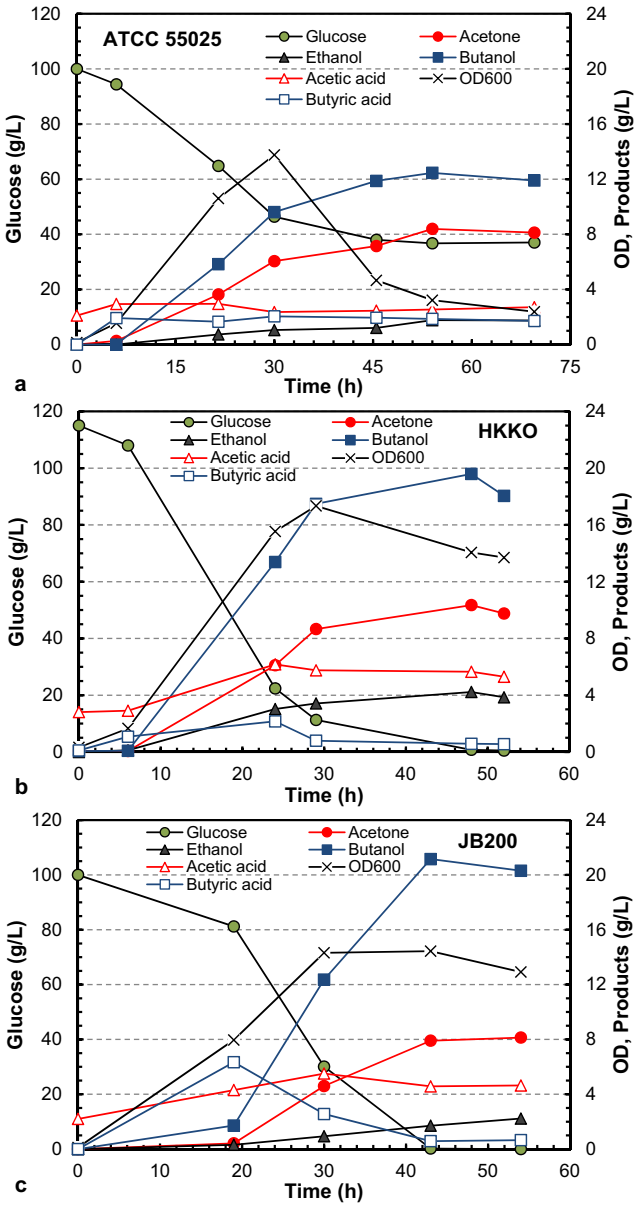

Fig. 2 Batch fermentation kinetics of *C. acetobutylicum* ATCC 55025 wild type and mutants HKKO and JB200 in stirred-tank bioreactor with pH controlled at >5.0. **a** ATCC 55025. **b** HKKO. **c** JB200

actual pH fluctuated around 5.4 (data not shown). As expected, the fermentation of glucose by *C. acetobutylicum* showed the typical biphasic ABE fermentation kinetics: acidogenesis with acetate and butyrate production initially and then solventogenesis with acetone, butanol, and ethanol as main products. For ATCC 55025, cell growth stopped when butanol concentration reached ~10 g/L and the fermentation stopped at ~12 g/L butanol although there was still plenty of glucose present in the medium (Fig. 2a). Clearly, the fermentation by ATCC 55025 was severely limited by butanol toxicity, which caused rapid cell autolysis as indicated by the sharp decrease in the cell density during the stationary phase, a common phenomenon observed in ABE fermentation by solventogenic clostridia (Allcock et al. 1981; Croux et al. 1992). It is noted that both acetate and butyrate stayed at a relatively stable level of 2 to 3 g/L after their initial production throughout the fermentation.

Compared to ATCC 55025, HKKO grew faster to a higher cell density and produced ~50 % more butanol, reaching ~19 g/L (Fig. 2b), which was comparable to, although slightly lower than that (~21 g/L) produced by JB200 (Fig. 2c). Both HKKO and JB200 reassimilated most of the butyric acid produced during the acidogenesis phase to form butanol in the solventogenesis phase, while the concentration of acetic acid reached a plateau after the transition from acidogenesis to solventogenesis, indicating that acid reassimilation in HKKO and JB200 favored the butyric acid to butyryl-CoA pathway.

The increased butanol production by HKKO and JB200 might be attributed to the mutation in *cac3319*, which was verified with the complementation mutant HKKO(cac3319). Similar to ATCC 55025, HKKO(cac3319) also produced only ~12 g/L butanol (Fig. 3a), which was significantly lower than that (~16 g/L) produced by the control strain HKKO(pMTL) (Fig. 3b). Clearly, the complementation of HKKO with its native *cac3319* overexpressed in pMTL82151 restored the

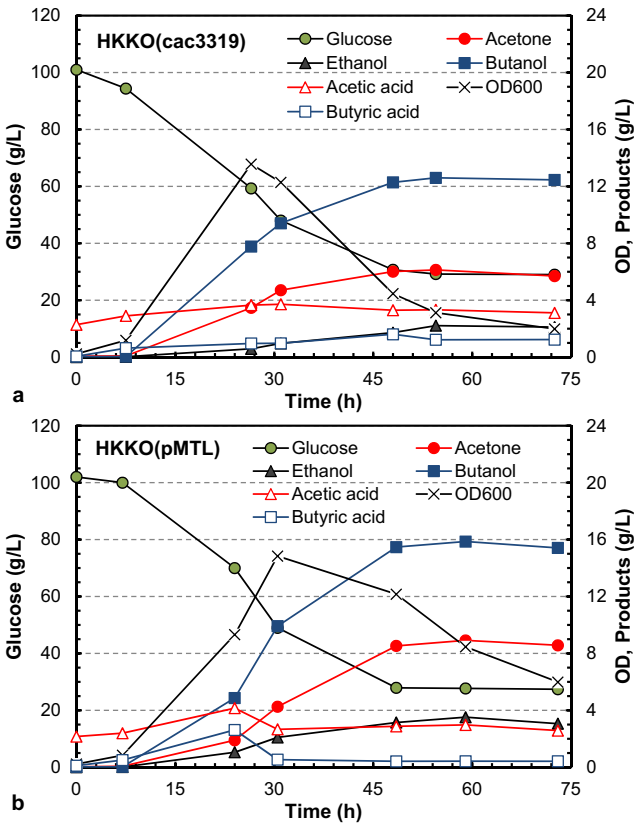

Fig. 3 Batch fermentation kinetics of *C. acetobutylicum* ATCC 55025 mutants HKKO(cac3319) and HKKO(pMTL) in stirred-tank bioreactor with pH controlled at >5.0. The fermentation kinetics was studied with the medium containing 30 μg/mL thiamphenicol. **a** HKKO(cac3319). **b** HKKO(pMTL)

phenotype of ATCC 55025, confirming that the disruption of *cac3319* was indeed the cause of the observed phenotype of HKKO. It is noted that HKKO(pMTL) produced significantly less butanol as compared to HKKO, probably because of the additional metabolic burden imposed by carrying the plasmids for antibiotic resistance, resulting in more cell autolysis and earlier termination of solventogenesis. Nevertheless, HKKO(pMTL) produced ~30 % more butanol than ATCC 55025.

Table 2 summarizes the fermentation kinetics of JB200, ATCC 55025, HKKO, HKKO(cac3319), and HKKO(pMTL). Among them, HKKO had the highest specific growth rate (0.16±0.01 h^{-1}) and butanol productivity (0.38±0.03 g/L h), whereas JB200 had the highest butanol product titer (20.3±1.8 g/L) and yield (0.23±0.01 g/g). While the difference in the specific growth rate among these five strains was not statistically significant, both HKKO and JB200 with a mutated *cac3319* produced 45–61 % more butanol (18.2±1.3 and 20.3±1.8 g/L, respectively, vs. 12.6±0.2 g/L for ATCC 55025) at a much higher productivity (0.38±0.03 and 0.33±0.07 g/L h, respectively, vs. 0.20±0.02 g/L h for ATCC 55025) compared to their parental strain. All strains gave a similar butanol yield of ~0.2 g/g glucose consumed, except for HKKO(cac3319) with a significantly lower yield of 0.17±0.01 g/g, which might be caused by the additional metabolic burden for carrying the plasmid for antibiotic resistance and the overexpression of Cac3319. The additional metabolic burden would require more energy and thus reduce the metabolic product yield. The butanol to total solvent ratio for all five strains was also similar at ~60 % (*w*/*w*), typical of ABE fermentation with *C. acetobutylicum*. These results suggested that the improved butanol production in HKKO and JB200 might be attributed to improved butanol tolerance, which will be further discussed later in this paper, rather than increasing the carbon flux toward butanol biosynthesis over acetone or ethanol production.

Effects of cac3319 mutation on cell growth and butanol production

Figure 4 compares cell growth and butanol and butyrate production in batch fermentations by different strains of *C. acetobutylicum*. In general, HKKO(cac3319) and ATCC 55025, both had unmutated *cac3319*, showed similar growth and fermentation kinetics, with the onset of faster cell autolysis at ~30 h (8–10 g/L butanol) and cease of fermentation at ~50 h (~12 g/L butanol). On the other hand, JB200, HKKO, and HKKO(pMTL) with mutated *cac3319* all showed less cell autolysis and were able to produce much more butanol (16–21 g/L). It is clear that *cac3319* plays an important role in regulating cell growth and butanol production by *C. acetobutylicum*. Interestingly, all strains with the mutated *cac3319* showed rapid reassimilation of butyric acid, which was produced in the acidogenesis phase, in the solventogenesis phase, whereas the strains with unmutated *cac3319* maintained a relatively stable butyric acid level, after initial production, for the entire fermentation period (Fig. 4c). Apparently, the mutation in *cac3319* also increased butyrate production in the acidogenesis phase and the reassimilation of butyrate in the solventogenesis phase, especially for JB200.

Being an asporogenic strain, ATCC 55025 cells died and lysed under butanol stress. When *cac3319* was disrupted, the resulting mutant HKKO exhibited increased growth, reduced autolysis, and accelerated butanol production to reach a higher butanol titer. When the wild-type *cac3319* gene was reintroduced into HKKO, the resulting mutant HKKO(cac3319) exhibited a similar phenotype to that of ATCC 55025. These results suggest that the *cac3319* product downregulates or inhibits cell growth and solvent (butanol) production by *C. acetobutylicum*, which has never been reported before.

Butanol tolerance

Among the three solvents (acetone, butanol, and ethanol) produced in ABE fermentation, butanol is the most toxic to

Table 2 Comparison of *C. acetobutylicum* JB200, ATCC 55025, HKKO, HKKO (cac3319), and HKKO (pMTL) in batch fermentation in stirred-tank bioreactor with pH controlled at >5.0

Strain	Sp. growth rate (h^{-1})	Butanol			Total solvent		
		Titer (g/L)	Yield (g/g)	Productivity (g/L h)	Titer (g/L)	Yield (g/g)	Productivity (g/L h)
JB 200	0.14±0.02	20.3±1.8	0.23±0.01	0.33±0.07	33.3±2.5	0.38±0.05	0.54±0.08
ATCC 55025	0.15±0.01	12.6±0.2	0.20±0.01	0.22±0.02	21.6±1.4	0.34±0.02	0.38±0.06
HKKO	0.16±0.01	18.2±1.3	0.20±0.02	0.38±0.03	31.0±2.8	0.34±0.04	0.65±0.06
HKKO(cac3319)	0.13±0.03	12.8±0.3	0.17±0.01	0.22±0.02	21.2±0.4	0.29±0.01	0.35±0.04
HKKO(pMTL)	0.14±0.00	15.7±0.3	0.19±0.03	0.30±0.05	28.2±0.2	0.34±0.06	0.53±0.08

Data shown are mean±SD (n=3)

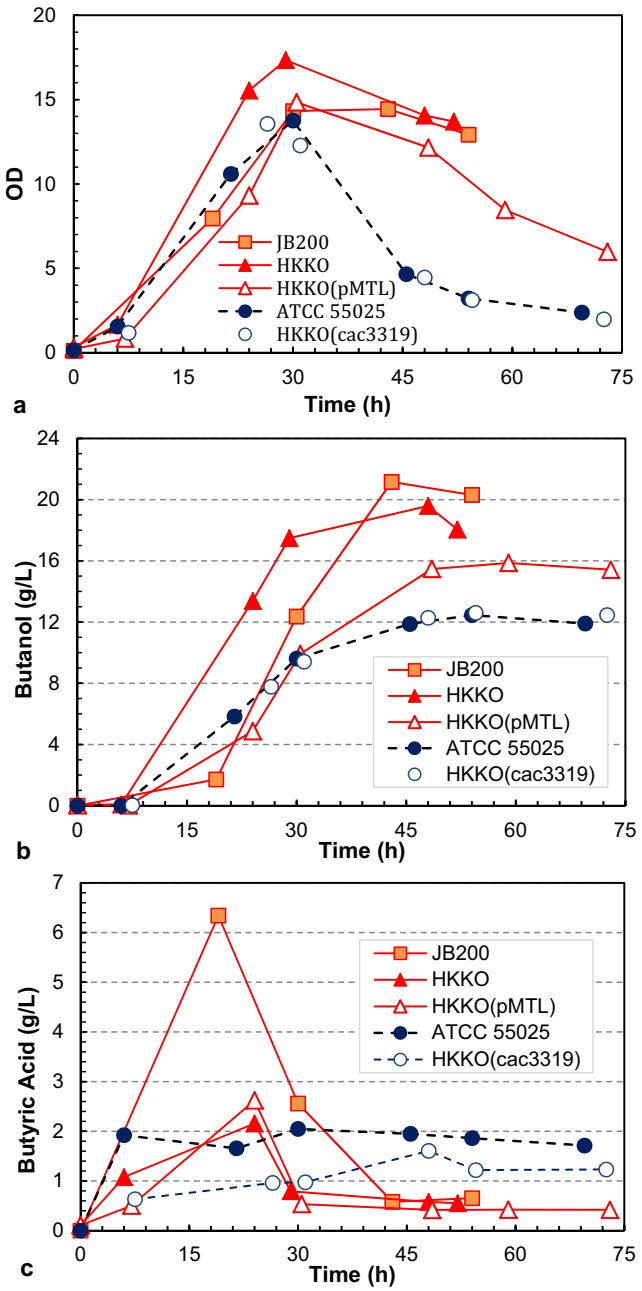

Fig. 4 Comparison of cell growth and butanol and butyrate production in batch fermentations by *C. acetobutylicum* ATCC 55025, HKKO, JB200, HKKO(cac3319), and HKKO(pMTL). **a** Growth kinetics. **b** Butanol production kinetics. **c** Butyric acid production kinetics

cells (Jones and Woods 1986), and therefore, butanol tolerance is a critical factor affecting cell's ability to produce butanol. The effect of butanol concentration in the culture medium on cell growth was studied for ATCC 55025, HKKO, and JB200, and the results are shown in Fig. 5. Among the three strains studied, JB200 had the highest butanol tolerance (up to 16 g/L, Fig. 5c) followed by HKKO (up to 14 g/L, Fig. 5b), whereas ATCC 55025 stopped growth under the challenge of 12 g/L butanol (Fig. 5a). Compared to their parental strain ATCC 55025, both HKKO and JB200 had higher specific growth rates and lower autolysis (death) rates at comparable butanol levels (Fig. 5d), consistent with the batch fermentation kinetics showing that cell autolysis was triggered at a higher butanol level and at a much lower rate for JB200 and HKKO (Fig. 2). It is noted that most of known (wild-type) solventogenic clostridia strains cannot grow in the presence of over ~12 g/L butanol (Yu et al. 2011; Gu et al. 2011). The increased butanol tolerance in HKKO and JB200 is thus attributed to the mutation in *cac3319*, which may play an important role in the regulation of sporulation (Steiner et al. 2011) and stress responses of *C. acetobutylicum*.

Discussion

In solventogenic clostridia, biphasic fermentation of glucose is a common feature. The first phase (acidogenic phase) occurs concomitantly with exponential growth and produces mainly acetate and butyrate. The accumulation of these acids causes a drop in the culture pH and induces solventogenesis. During the second or solventogenic phase, the culture enters the stationary phase while solvents (acetone, butanol, and ethanol) are produced and the acids produced previously are reassimilated, resulting in rising pH. The stationary-phase events also include granulose (a glycogen-like polymer) accumulation, sporulation, and autolysis. It is widely accepted that both solventogenesis and sporulation are regulated by the master regulator Spo0A and are therefore closely coupled to each other (Alsaker et al. 2004; Paredes et al. 2005; Ravagnani et al. 2000; Sullivan and Bennett 2006). In *C. acetobutylicum*, the effect of Spo0A on solvent formation is a balancing act in regulating sporulation versus solvent gene expression: inactivation of *spo0A* severely blocked spore and solvent formation, while strains with overexpressed Spo0A showed early expression of solventogenic genes but failed to produce more solvents, due to an accelerated and enhanced sporulation process (Harris et al. 2002). The activated form of Spo0A, Spo0A~P, acts as a transcription factor and initiates sporulation and solventogenesis by binding the genes involved in the processes (Ravagnani et al. 2000). Recent studies on sporulation initiation in *C. acetobutylicum* suggested two pathways for Spo0A activation; one depends on a histidine kinase encoded by *cac0323*, and the other involves two histidine kinases encoded by *cac0903* and *cac3319*, respectively (Steiner et al. 2011). Sporulation was severely blocked when any one of these three genes was disrupted. On the other hand, disruption of *cac0437*, encoding a kinase-like protein, which deactivates Spo0A, led to hyper-sporulated mutants (Steiner et al. 2011).

In contrast to the initiation of endospore formation, initiation of solventogenesis in *C. acetobutylicum* is poorly

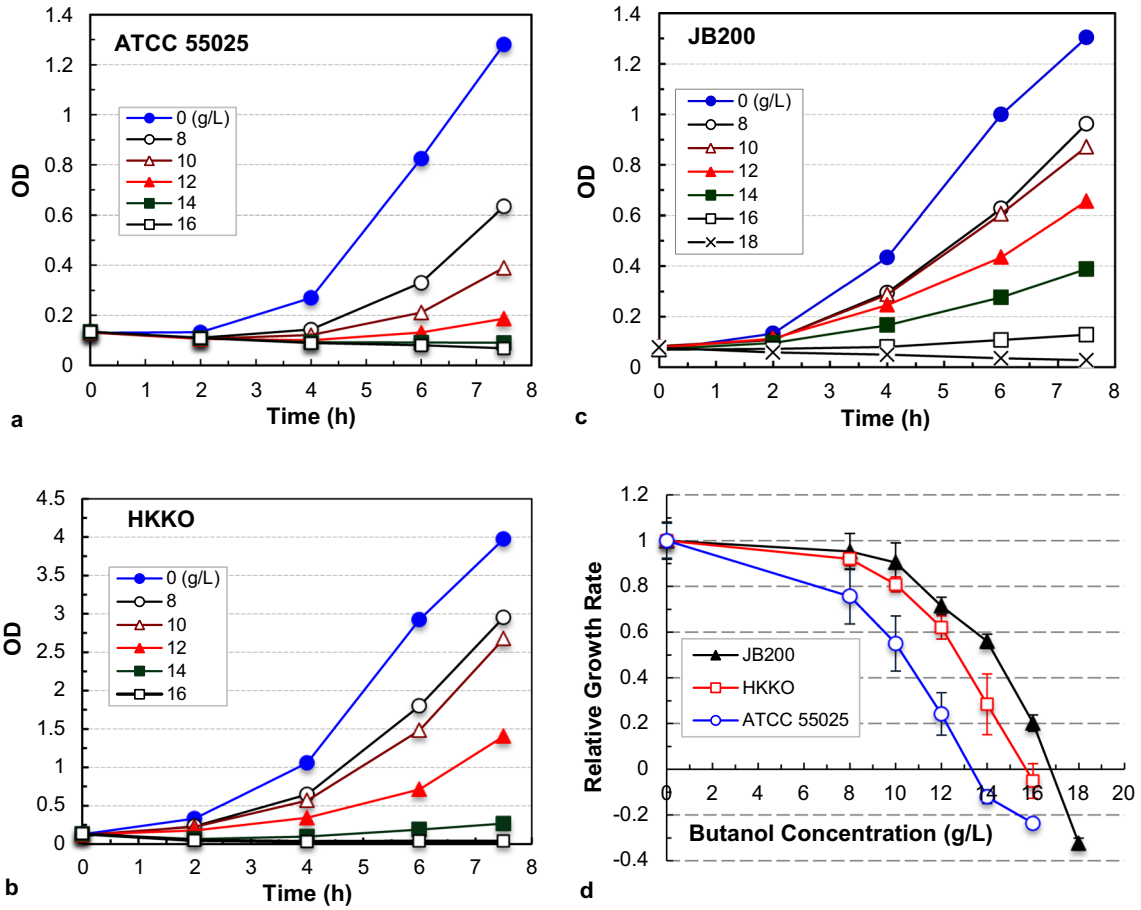

Fig. 5 Effects of butanol on cell growth of *C. acetobutylicum* ATCC 55025, HKKO, and JB200. **a** Growth kinetics of ATCC 55025 at various initial butanol concentrations. **b** Growth kinetics of HKKO at various initial butanol concentrations. **c** Growth kinetics of JB200 at various initial butanol concentrations. **d** Effects of butanol on the specific growth rates of various strains. Relative specific growth rates are shown with the specific growth rate at 0 g/L butanol being 1.0 for each strain. The specific growth rate was estimated from the time course data from the slope of the semi-logarithmic plot of OD vs. time. Data shown are means with standard deviations (error bars) from triplicate experiments

understood. Because of the toxic nature of the produced solvent (butanol), which induces a series of cellular responses, the regulation of solvent formation is much more complex than sporulation. As revealed in several transcriptional studies, more than 1000 genes could be differentially expressed under butanol stress (Janssen et al. 2012; Schwarz et al. 2012; Wang et al. 2013). It is thus difficult to pinpoint the key genes directly affecting butanol tolerance and production in *C. acetobutylicum*. Despite the importance of Spo0A in regulating sporulation and possibly solventogenesis, the impact of the orphan histidine kinases on solvent formation remains unexplored. For the first time, we showed that the *cac3319* gene plays an important role in regulating solvent production and tolerance in *C. acetobutylicum*. Our discovery was based on the comparative genomic analysis of a hyper-butanol-tolerant and hyper-butanol-producing mutant strain JB200 of *C. acetobutylicum* ATCC 55025. Compared with the wild-type *cac3319* gene in ATCC 55025, the mutant *cac3319* gene in JB200 has a frameshift mutation in the coding region, causing an early stop codon; therefore, only 112 amino acids at the N terminus were retained (total 445 amino acids in the wild-type protein). According to the Cac3319 protein annotation (NCBI Reference Sequence: NP_349911.1), the protein sequences containing the histidine kinase core were completely missing due to the mutation, so the mutant Cac3319 protein in JB200 was supposed to be inactive. This was verified by introducing the pMTL82151 vector containing the mutant *cac3319* gene of JB200 into ATCC 55025 and the resulting mutant showing no apparent improvement in solvent production (data not shown). To further investigate the function of the *cac3319* gene in *C. acetobutylicum*, we constructed one *cac3319*-disrupted mutant (HKKO) using ClosTron mutagenesis technology, with the retargeting group II intron designed such that an early stop codon could be obtained after the intron inserted into the desired position of *cac3319* and that the generated protein product would best mimic the mutated Cac3319 protein in JB200. Our results showed that butanol tolerance and production increased more than 50 % when

there was no functional Cac3319, suggesting that Cac3319 exerted an inhibitory effect on or downregulated butanol production. Previous studies have shown that Cac3319, in cooperation with other histidine kinases, is involved in the initiation of sporulation in *C. acetobutylicum* by directly controlling the phosphorylation status of Spo0A (Steiner et al. 2011). Our results suggest that sporulation and solventogenesis in *C. acetobutylicum* are not necessarily linked, consistent with the finding in a transcriptional study showing that expression of sporulation genes was largely unaffected by butanol stress (Alsaker et al. 2010). We thus speculate the existence of a Spo0A-independent signal transduction mechanism for inducing/stimulating solventogenesis, which might be suppressed by Cac3319 under normal fermentation conditions.

Butanol production is embedded in a very complex network, including sporulation, quorum sensing, and pH and redox controls (Dürre et al. 2002), and butanol tolerance is not the only factor affecting butanol production. Overexpressing a major heat-shock protein (GroESL) in *C. acetobutylicum* ATCC 824 increased its *n*-butanol tolerance and production to 17 g/L (Tomas et al. 2003). However, another study showed that overexpressing genes encoding stress proteins (*groESL*, *grpE*, and *htpG*) increased butanol tolerance, but butanol production decreased (Mann et al. 2012). Key genes involved in the acidogenesis and solventogenesis have also been explored through metabolic engineering (Cooksley et al. 2012), and one study showed that butanol production by *C. acetobutylicum* could be greatly enhanced to 18.9 g/L by reinforcing the direct butanol-forming route (Jang et al. 2012b). Classical mutagenesis and adaptation have been most successful in enhancing butanol tolerance and production to reach the highest butanol titer of ~21 g/L so far (Zhao et al. 2013); however, mutants obtained from these methods may be genetically unstable, which means that the mutations are easy to reverse, resulting in strain degeneration. In this study, an engineered strain of *C. acetobutylicum* HKKO with enhanced butanol tolerance and production was obtained by knocking out a histidine kinase encoded by *cac3319* on its chromosome, which provides a novel metabolic engineering strategy for industrial *n*-butanol production. Compared to other mutants constructed through adaptation or metabolic engineering, HKKO is relatively simple to construct via the ClosTron gene knockout system and is stable. Furthermore, since HKKO bears no heterologous plasmid, there is little negative effect from genetic engineering on cell growth (see Fig. 2), and no antibiotic is needed to maintain the mutation during fermentation. Finally, the *n*-butanol titer (18.2±1.3 g/L) and productivity (0.38 g/L h) in batch fermentation from the *cac3319*-disrupted mutant HKKO are among the highest from metabolically engineered *C. acetobutylicum* strains.

In conclusion, *cac3319* plays an important role in regulating solvent production and tolerance in *C. acetobutylicum*. Batch fermentation kinetics studies confirmed that butanol production and tolerance could be greatly improved by disrupting *cac3319*, although how Cac3319 is involved in regulating solvent formation and tolerance remains unclear. The results from this study shed light on the complex regulation network of butanol tolerance and biosynthesis, and the metabolic engineering strategy used in this work also provides a novel approach for generating high-butanol-producing strains for industrial applications.

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