Improved *n*-Butanol Production from *Clostridium cellulovorans* by Integrated Metabolic and Evolutionary Engineering

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ABSTRACT *Clostridium cellulovorans* DSM 743B offers potential as a chassis strain for biomass refining by consolidated bioprocessing (CBP). However, its *n*-butanol production from lignocellulosic biomass has yet to be demonstrated. This study demonstrates the construction of a coenzyme A (CoA)-dependent acetone-butanol-ethanol (ABE) pathway in *C. cellulovorans* by introducing *adhE1* and *ctfA-ctfB-adc* genes from *Clostridium acetobutylicum* ATCC 824, which enabled it to produce *n*-butanol using the abundant and low-cost agricultural waste of alkali-extracted, deshelled corn cobs (AECC) as the sole carbon source. Then, a novel adaptive laboratory evolution (ALE) approach was adapted to strengthen the *n*-butanol tolerance of *C. cellulovorans* to fully utilize its *n*-butanol output potential. To further improve *n*-butanol production, both metabolic engineering and evolutionary engineering were combined, using the evolved strain as a host for metabolic engineering. The *n*-butanol production from AECC of the engineered *C. cellulovorans* was increased 138-fold, from less than 0.025 g/liter to 3.47 g/liter. This method represents a milestone toward *n*-butanol production by CBP, using a single recombinant clostridium strain. The engineered strain offers a promising CBP-enabling microbial chassis for *n*-butanol fermentation from lignocellulose.

IMPORTANCE Due to a lack of genetic tools, *Clostridium cellulovorans* DSM 743B has not been comprehensively explored as a putative strain platform for *n*-butanol production by consolidated bioprocessing (CBP). Based on the previous study of genetic tools, strain engineering of *C. cellulovorans* for the development of a CBP-enabling microbial chassis was demonstrated in this study. Metabolic engineering and evolutionary engineering were integrated to improve the *n*-butanol production of *C. cellulovorans* from the low-cost renewable agricultural waste of alkali-extracted, deshelled corn cobs (AECC). The *n*-butanol production from AECC was increased 138-fold, from less than 0.025 g/liter to 3.47 g/liter, which represents the highest titer of *n*-butanol produced using a single recombinant clostridium strain by CBP reported to date. This engineered strain serves as a promising chassis for *n*-butanol production from lignocellulose by CBP.

KEYWORDS *Clostridium*, adaptive laboratory evolution, consolidated bioprocessing, metabolic engineering, *n*-butanol

Due to the unsustainability of oil resources and global environmental deterioration caused by the excessive use of fossil fuels, considerable attention has recently been focused on lignocellulose biorefinery using a fermentation process for various chemicals and fuels (1). A promising fermentation product as a substitute for petroleum

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is butanol, which can be used as both an industrial commodity and a gasoline substitute (2). Therefore, attention has focused on the production of butanol from various lignocellulosic feedstocks by microbial fermentation. The traditional process involves the synthesis of cellulases, saccharification, and hexose/pentose co-utilization (3, 4). Alternatively, consolidated bioprocessing (CBP) can combine all these processes within one step, which would decrease the cost of capital investment for cellulase production and, thus, offer the potential of economical production of butanol at industrial scale (5). However, no natural CBP-enabling microorganisms or microbial consortia with the ability to produce butanol are currently available (6, 7).

The combination of both cellulolytic and butanol-producing phenotypes within a single microorganism is an appealing challenge. A number of explorations have attempted to engineer *Escherichia coli* or *Saccharomyces cerevisiae* strains that directly produce *n*-butanol/isobutanol from lignocellulose. Achieved advances have been recently reviewed (8–11). However, only a very low butanol titer could be obtained (8). An alternative strategy is to develop native cellulolytic or solventogenic clostridia as an initial microbial chassis (12). Several key milestones have already been accomplished in overexpressing cellulase-encoding genes or the *in vivo* assembly of chimeric minicellulosome in solventogenic clostridia (13–15); however, these recombinant strains are still not capable of efficient growth on lignocellulose. In contrast, introducing a butanol metabolic pathway into native cellulolytic clostridia represents a more meaningful progress toward the realization of CBP. The engineered *Clostridium thermocellum* has been reported to produce 5.4 g/liter of isobutanol from cellulose via a hybrid keto acid pathway (16). Similarly, other anaerobic cellulolytic bacteria, such as *Clostridium cellulolyticum* and *Clostridium cellulovorans* (12, 17, 18), have also been suggested to have the potential to produce butanol by CBP after metabolic engineering.

C. cellulovorans DSM 743B is an anaerobic, cellulolytic mesophile that can directly produce butyric acid as the main metabolic product from lignocellulose (19, 20). The complete sequencing and annotation of the genome deepened the understanding of both the plant cell wall degradation system and the metabolic network in *C. cellulovorans* (21). Studies on nuclease sequence analysis, cellulosome component properties, exoproteome profiles, and crystal structures of key cellulases have also achieved significant progress (22–25). Other breakthroughs on *C. cellulovorans* include genetic system development and genetic tool verification, such as ClosTron and the CRISPR/Cas system, which provide a valuable foundation for metabolic engineering (6, 12, 26, 27). As a proof of concept, Yang et al. overexpressed an aldehyde/alcohol dehydrogenase (*adhE2*) in *C. cellulovorans*, which enabled the recombinant strain to produce 1.42 g/liter *n*-butanol within 252 h (28). This represented the first metabolic engineering exemplified in *C. cellulovorans*; however, several other approaches remain unexplored, such as complicated genetic modification, *n*-butanol tolerance evolution, and process control optimization, which may promote the strain development of *C. cellulovorans*. In a previous study, Wen et al. developed a twin clostridial consortium in which *C. cellulovorans* DSM 743B was comprehensively engineered to promote the butyrate supply for *Clostridium beijerinckii* NCIMB 8052 to achieve *n*-butanol production (6). However, the ability of *C. cellulovorans* to produce *n*-butanol was not exploited.

In this study, metabolic engineering and adaptive laboratory evolution were integrated to explore the *n*-butanol production of *C. cellulovorans* (pure culture) by CBP, as shown in Fig. 1. A coenzyme A (CoA)-dependent acetone-butanol-ethanol (ABE) pathway was introduced into *C. cellulovorans* to direct carbon flux from butyrate to *n*-butanol. Then, a novel evolutionary approach was developed to improve the *n*-butanol tolerance of *C. cellulovorans*, which may boost its *n*-butanol production potential. Finally, the ABE pathway was reconstructed in the evolved strains. The obtained strains produced far more butanol than unevolved recombinant strains.

RESULTS

Heterologous *adhE1* introduction enables *C. cellulovorans* to produce *n*-butanol. According to the prediction of KEGG, a complete CoA-dependent butanol synthesis

FIG 1 Integrated metabolic and evolutionary engineering of *Clostridium cellulovorans* DSM 743B for *n*-butanol production from AECC by CBP. A CoA-dependent acetone-butanol-ethanol (ABE) pathway composed of *adhE1* and *ctfA-ctfB-adc* (red font) from *C. acetobutylicum* ATCC 824 was constructed in *C. cellulovorans*. In parallel, *C. cellulovorans* was grown in medium with a serial enrichment of *n*-butanol to evolve and reinforce its butanol tolerance. To maximize butanol production, metabolic and evolutionary engineering were combined, using the evolved strain as a host for metabolic engineering.

pathway exists in *C. cellulovorans*. However, only less than 0.025 g/liter of *n*-butanol can be detected in broth (29). Low endogenous alcohol/aldehyde dehydrogenase activity was confirmed to be a bottleneck for *n*-butanol production (28). It has been reported that *adhE1* overexpression (encoding an alcohol/aldehyde dehydrogenase in *Clostridium acetobutylicum*) can restore the *n*-butanol production to wild-type levels in the *C. acetobutylicum* M5 strain (a nonsporulating, non-solvent-producing mutant without the pSOL1 megaplasmid containing alcohol/aldehyde dehydrogenase and the acetone-formation genes) (30, 31). Accordingly, this study attempted to modify the *n*-butanol pathway in *C. cellulovorans* by introducing alcohol/aldehyde dehydrogenase genes.

There are two homologous *adhE* genes: *adhE1* (CA_p0162, located in the *sol* operon *orfL-adhE1-ctfA-ctfB*) and *adhE2* (CA_p0035, a monocistronic operon) in the *C. acetobutylicum* ATCC 824 strain (32, 33). The effect of overexpressing either of these two genes on *n*-butanol production was evaluated (Fig. 2A). ZQW02 (743B/pXY1-*P_{thi}-adhE2*) produced 0.37 g/liter *n*-butanol with alkali-extracted, deshelled corn cobs (AECC) as the sole carbon source within 108 h. However, the titer of *n*-butanol produced by ZQW03 (743B/pXY1-*P_{thi}-adhE1*) reached 1.63 g/liter, which is almost 4-fold that achieved by ZQW02. The butyraldehyde dehydrogenase activity and the *n*-butanol dehydrogenase activity in ZQW03 were 88.2% and 152% higher than activities in ZQW02 and 7.97- and

FIG 2 Overexpression of *adhE1* enabled *C. cellulovorans* to produce more *n*-butanol than overexpression of *adhE2*. (A) Effects of *adhE1* and *adhE2* introduction on butanol production. (B) Comparison of butyraldehyde dehydrogenase activities and butanol dehydrogenase activities between the ZQW02 and ZQW03 strains. (C and D) Fermentation profiles of ZQW01 and ZQW03, respectively, with AECC as the sole carbon source. The data in panels A and B are the means and standard deviations of three replicates (***, $P \leq 0.001$; **, $P \leq 0.01$; t test).

13.6-fold higher than those in ZQW01 (743B/pXY1- P_{thl}) (as a control), which indicates why the *n*-butanol output by ZQW03 was higher than that by ZQW02 (Fig. 2B).

Then, the fermentation progress of ZQW03 was explored in a 3-liter bioreactor with ZQW01 as a control (Fig. 2C and D; see also Fig. S1 in the supplemental material). Less butyrate was accumulated in the broth by ZQW03 than by ZQW01. Moreover, *n*-butanol synthesis by ZQW03 coincides with butyrate accumulation, and butyrate hardly decreased in the fermentation anaphase, indicating that butyrate was almost not reassimilated by ZQW03 itself. Within 108 h, ZQW03 produced 1.64 g/liter *n*-butanol and 0.635 g/liter ethanol from 37.2 g/liter AECC with a residual of 5.52 g/liter butyrate and 8.78 g/liter total sugars (mainly pentose) in the broth. These results represent an acetone-uncoupled *n*-butanol production process by CBP, which indicates that *C. cellulovorans* has the potent to produce biofuel mixtures with a high *n*-butanol ratio, as previously suggested (34, 35).

Butyrate reassimilation by overexpressing *ctfAB-adc* to drive *n*-butanol production. According to the working hypothesis, *n*-butanol titer and yield may be further promoted by reassimilating and transferring the butyrate accumulated by ZQW02 and ZQW03 to *n*-butanol. In *C. acetobutylicum*, it has been reported that the bifunctional BK-I encoded by *bukI* (CA_c3075) not only catalyzes butyrate formation but also is responsible for the uptake of butyrate without simultaneous production of acetone (36, 37). Therefore, *bukI* from *C. acetobutylicum* 824 was overexpressed coupled with *adhE1* in *C. cellulovorans*. However, the *n*-butanol output of ZQW07 (743B/pXY1- P_{thl} -*buk*- P_{thl} -*adhE1*) did not noticeably change, while the residual butyrate increased by 32.6% (Fig. 3A). This indicates a much higher efficiency of butyrate synthesis than the reassimila-

FIG 3 Overexpressing *ctgAB-adc* in ZQW03 dries *n*-butanol production by reassimilating butyrate. (A) Effects of *ctgAB-adc* and *bukI* introduction on *n*-butanol production and butyrate reassimilation. (B to D) Batch fermentation profile of ZQW05 with AECC as the sole carbon source. The data in panel A are the means and standard deviations of three replicates (**, $P \leq 0.01$; *t* test).

tion in *C. cellulovorans*. This further suggests that the knockout of endogenous *buk* in *C. cellulovorans* may help to direct the butyrate flux to *n*-butanol formation; however, a *C. cellulovorans* mutant deficient in *buk* (*Clocel_3674*, encoding butyrate kinase) could not be generated using ClosTron even with long-term screening.

Alternatively, a routine strategy for butyrate reassimilation in *C. acetobutylicum* and *C. beijerinckii* was used under solventogenesis. The strategy of overexpressing genes involved in the CoA transferase encoded by *ctfA-ctfB* (*CA_p0163-0164* or *Cbei_3833-3834*) has been successfully verified in *C. acetobutylicum*, *Clostridium tyrobutyricum*, and *C. beijerinckii* (6, 38, 39). Both the *adhE1* and *ctfA-ctfB* genes located in *C. acetobutylicum*'s *sol* operon were introduced, followed by its adjacent *adc* gene (*CA_p0165*, with native promoter) (33), since there are no known genes involved in the acetoacetate metabolism of *C. cellulovorans*. Interestingly, the heterologous ABE (acetone-butanol-ethanol) pathway worked very well in ZQW05 (743B/pXY1-*P_{thi}-adhE1-P_{thi}-ctfAB-adc*). Compared to ZQW03, the *n*-butanol output of ZQW05 was enhanced by 49.3%, while residual butyrate and acetate decreased by 13.2% and 17.3%, respectively (Fig. 3A). It is worth noting that the overexpression of *ctfAB-adc* (without *adhE1*) in wild-type *C. cellulovorans* showed no improvement of *n*-butanol production. This indirectly supports the hypothesis that low alcohol/aldehyde dehydrogenase activity maybe a bottleneck for the *n*-butanol synthesis in wild-type *C. cellulovorans*.

Furthermore, the fermentation progress of ZQW05 was investigated in a 3-liter bioreactor (Fig. 3B to D). Within 108 h, ZQW05 produced 2.27 g/liter *n*-butanol and 0.712 g/liter ethanol from 38.7 g/liter AECC. This was not a typical two-phase fermentation of acidogenesis followed by solventogenesis as in solventogenic clostridia (40). *n*-Butanol production coincides with butyrate accumulation during the first 72 h, after

FIG 4 Adaptive laboratory evolution improved *n*-butanol tolerance of *C. cellulovorans*. (A) The evolution process of ZQW10. ZQW10 was subcultured in medium supplemented with stepwise enrichment of butanol (3, 6, 9, and 12 g/liter) to evolve and strengthen its *n*-butanol tolerance. (B) Comparison of *n*-butanol tolerance levels of mutant strains derived from ZQW10, ZQW20, and ZQW30. Due to evolution, ZQW23, ZQW25, ZQW33, and ZQW35 can tolerate higher concentrations of *n*-butanol than ZQW13 and ZQW15. In addition, the *n*-butanol tolerance levels of ZQW33 and ZQW35 were superior to those of ZQW23 and ZQW25.

which acetone was also produced. Simultaneously, acetate and butyrate decreased by 8% and 5.1% from their peak titers, which were inferred to be reassimilated by the engineered *C. cellulovorans* strain harboring *ctfA-ctfB* genes (Fig. 3B). During the fermentation anaphase, AECC degradation, total sugar utilization, and *n*-butanol production almost ceased. This finding was in agreement with the results of Yang et al. when their engineered *C. cellulovorans* strain was cultured with glucose or cellobiose as the sole carbon source (28).

Adaptive laboratory evolution to improve the *n*-butanol tolerance of *C. cellulovorans*. Butanol toxicity is a main barrier for solvent production in *Clostridium* (mainly butanol) with a high titer since it may cause microbial growth inhibition and cell death (41). Improvement in butanol tolerance sometimes leads to an increase in butanol production (42). The cellular responses and molecular mechanisms that can overcome butanol stress in *Clostridium* are complex and not yet fully understood (43). Laboratory-adaptive evolution has been shown to be an efficient strategy for the improvement of butanol tolerance (44, 45). In this study, two evolutionary approaches were adapted to boost the *n*-butanol tolerance of *C. cellulovorans* to improve *n*-butanol production.

Wild-type ZQW00 (743B) and recombinant ZQW10 (743B $\Delta spo0A$) were chosen as parent strains for parallel adaptive evolution experiments. Both were cultured in medium supplemented with a serial enrichment of *n*-butanol (from 3 g/liter to 12 g/liter) to promote evolution. As expected, the culture concomitantly adapted to these intensified conditions. It took 17, 15, and 22 days for ZQW10 to evolve so that it could successfully resist 3 g/liter, 6 g/liter, and 9 g/liter of butanol, respectively (Fig. 4A); however, for ZQW00, it took 11, 11, and 25 days, respectively (Fig. S2). The offspring of ZQW10 needed about 29 days to achieve a stable biomass above 1.0 (optical density at 600 nm [OD₆₀₀]), while ZQW00 could not further evolve in medium with added 12 g/liter of *n*-butanol (Fig. 4A and Fig. S2).

After four rounds of evolution over a total of 83 days, the resulting evolved ZQW10 and ZQW00 strains were harvested and spread on plates that were supplemented with 12 g/liter and 9 g/liter *n*-butanol (because the evolved ZQW00 cannot grow well in medium with 12 g/liter of *n*-butanol), respectively. The phenotype of several evolved colonies and selected strains ZQW30 (743B $\Delta spo0A$ *11), derived from ZQW10, and ZQW20 (743B*4), derived from ZQW00, were tested for further assessment and engineering (Fig. S3). With an initial 12 g/liter of *n*-butanol in the medium, the 48-h biomass of ZQW30 achieved 45.2% of that cultured in blank Clocel medium (without *n*-butanol) (6, 49), while ZQW10 did not show noticeable growth. The *n*-butanol tolerance levels of

FIG 5 The dendrogram of genetically modified *C. cellulovorans* strains in this study.

mutants derived from ZQW00, ZQW10, ZQW20, and ZQW30 were evaluated (Fig. 4B and Fig. S4). In general, the strains derived from ZQW30 can resist more butanol than those derived from ZQW20. Since ZQW35 can resist more butanol than ZQW25, ZQW35 was selected for subsequent exploration (Fig. 4B).

spo0A inactivation seems to contribute to strain evolution but weakens AECC degradation by ZQW10 and ZQW30 (Fig. S5). This may be because the *spo0A*-deficient mutants were prone to cell lysis (46, 47). The *spo0A* gene was complemented using plasmid-based expression, driven by a *thl* promoter (48) in ZQW10 and ZQW30. Both ZQW11 (743B $\Delta spo0A$ /pXY1- P_{thl} -*spo0A*) and ZQW31 (743B $\Delta spo0A$ *11/pXY1- P_{thl} -*spo0A*) restored their AECC degradation abilities to a level similar to that of the wild-type (Fig. S5). Accordingly, *spo0A* complementation was implemented in all mutants derived from ZQW10 and ZQW30, and *spo0A* complementation did not strongly affect *n*-butanol tolerance (Fig. S4).

Combination of metabolic and evolutionary engineering to improve *n*-butanol production. We decided to include more advantageous genetic modifications as described above in the evolved strains toward a further improvement of *n*-butanol production. The metabolic profiles were compared among mutants derived from different parent strains (Fig. 5 and 6A). The *n*-butanol output of ZQW33 (743B $\Delta spo0A$ *11/pXY1- P_{thl} -*adhE1*- P_{thl} -*spo0A*) and ZQW35 (743B $\Delta spo0A$ *11/pXY1- P_{thl} -*adhE1*- P_{thl} -*ctfAB-adc*- P_{thl} -*spo0A*) increased by 52.2% and 50.5% compared to the levels of ZQW13 (743B $\Delta spo0A$ /pXY1- P_{thl} -*adhE1*- P_{thl} -*spo0A*) and ZQW15 (743B $\Delta spo0A$ /pXY1- P_{thl} -*adhE1*- P_{thl} -*ctfAB-adc*- P_{thl} -*spo0A*), respectively. This indicates that the reinforced *n*-butanol tolerance improved *n*-butanol production.

Batch fermentation in a 3-liter bioreactor was performed to investigate the metabolic profile of ZQW35. During the first 36 h, no obvious phenotype difference was found between strains before and after evolution. However, AECC degradation, butyrate reassimilation, and *n*-butanol production improved during the fermentation anaphase, suggesting relatively higher cell viability of ZQW35 than of ZQW05. During 96 h, ZQW35 produced 3.02 g/liter *n*-butanol and 0.777 g/liter ethanol from 40.2 g/liter AECC with 4.19 g/liter of residual butyrate (Fig. 6B to D). Compared to levels of ZQW05, the *n*-butanol production increased by 33%, and the residual butyrate decreased by 19.8%.

A two-stage pH control strategy was proposed to coordinate native cellular growth (pH of approximately 7.0) (28, 29) and cellulase production with the heterologous pathway of *n*-butanol synthesis and butyrate reassimilation (pH 4.5 to 6.0) in *C. cellulovorans* (33, 40, 49). This further improved *n*-butanol production. In such a strategy, pH was controlled at 7.0 during the first 48 h and then decreased to 6.0. In anaerobic shaken flasks, the *n*-butanol production of ZQW13, ZQW33, ZQW15, and ZQW35 increased by 22.4%, 17.3%, 19.1%, and 13.3%, respectively. Simultaneously, their butyrate secretion levels decreased by 16.3%, 5%, 23.4%, and 15.6%, respectively (Fig. 7A).

FIG 6 Improved butanol tolerance led an enhancement in *n*-butanol production. (A) Effects of laboratory-adaptive evolution on *n*-butanol production. Mutants ZQW33 and ZQW35 that were derived from evolved strain ZQW30 produced much more *n*-butanol than the mutants ZQW13 and ZQW15 that were derived from ZQW10. (B to D) Metabolic profile of batch fermentation of ZQW35 with AECC as the sole carbon source. The data in panel A are the means and standard deviations of three replicates (***, $P \leq 0.001$; *t* test).

A two-stage pH control strategy was implemented in a 3-liter bioreactor to maximize the butanol output of ZQW35 (Fig. 7B to D). Compared to the fermentation behavior shown in Fig. 6B, both the *n*-butanol accumulation and butyrate reassimilation clearly accelerated between 48 and 72 h but almost ceased after 84 h. The batch fermentation can be stopped 12 h earlier than that in the experiment shown in Fig. 6B. During 84 h, ZQW35 produced 3.47 g/liter *n*-butanol and 0.875 g/liter ethanol from 43.1 g/liter AECC, with 3.76 g/liter butyrate and 9.53 g/liter residual total sugars (mainly xylose and arabinose) in the broth (Fig. 7B to D). The *n*-butanol production increased by 14.9%, and the *n*-butanol productivity increased by 31.3% compared to that obtained under a control of constant neutral pH. This supports a beneficial two-stage pH control strategy. The *n*-butanol titer and productivity here were 2.44-fold and 7.32-fold higher than the levels obtained in a previous study (28).

DISCUSSION

In the present study, metabolic engineering and adaptive laboratory evolution were integrated to improve the *n*-butanol production of *C. cellulovorans* DSM 743B from AECC, a low-cost and renewable agricultural waste. This represents an application of a previous study of genetic tools of *C. cellulovorans* DSM 743B in CBP-enabling microbial chassis development (6).

To enable *C. cellulovorans* DSM 743B to produce *n*-butanol, the CoA-dependent ABE pathway, including *adhE1* and *ctfAB-adc* genes from *C. acetobutylicum* ATCC 824, were introduced. The metabolically engineered strains showed increased butanol output compared to results in the study of Yang et al., who overexpressed only *adhE2* in *C. cellulovorans* and eventually achieved 1.42 g/liter *n*-butanol production from cellulose (28). It is interesting that ZQW03 (743B/pXY1-P_{thl}-*adhE1*) exhibited higher *n*-butanol

FIG 7 Two-stage pH control strategy to improve *n*-butanol production further. (A) Effects of two-stage pH control strategy on *n*-butanol and butyrate production. (B to D) Metabolic profile of batch fermentation of ZQW35 using a two-stage pH control strategy with AECC as the sole carbon source. The data in panel A are the means and standard deviations of three replicates (***, $P \leq 0.001$; **, $P \leq 0.01$; *t* test).

output, butyraldehyde dehydrogenase activity, and butanol dehydrogenase activity than ZQW02 (743B/pXY1-*P_{thi}-adhE2*) (Fig. 2B). The higher butanol dehydrogenase activity in ZQW03 may be attributed to *C. cellulovorans* itself. Since *C. cellulovorans* possesses several putative butanol dehydrogenase-encoding genes (*Clocel_1140*, *Clocel_1949*, *Clocel_4197*, *Clocel_2402*, and *Clocel_3817*), it is likely that one of them might be induced by the butyraldehyde generated by the heterogenous AdhE1. A similar phenomenon has been demonstrated in *C. acetobutylicum* under solventogenesis, with the exception that the butanol dehydrogenases expressed in *C. acetobutylicum* are NADPH dependent (32).

Previous studies confirmed Spo0A as a transcriptional regulator that is closely related to sporulation, solvent production, and butanol tolerance in *Clostridium* (50, 51). It plays a central role by mainly regulating sporulation-specific sigma factors, solvent genes, and heat shock protein genes (52). Overexpression of *spo0A* can enhance the solvent tolerance of *Clostridium*, thus increasing its solvent yield; however, this leads to the early onset of sporulation (50, 51).

We hypothesized that there may be further molecular mechanisms that lead to stress resistance in *C. cellulovorans*, such as several two-component systems or membrane transport proteins, that are not entirely dependent on *spo0A* (53, 54). When *spo0A* is knocked out, attenuated, or mutated, other mechanisms may be activated or reinforced for the strain to respond to butanol stress. Accordingly, two parallel evolutionary approaches that use the *spo0A*-knockout strain ZQW10 and wild-type ZQW00 as original strains were investigated and compared.

The evolved *spo0A*-deficient strain ZQW30 was demonstrated to tolerate 12 g/liter of butanol after four rounds, or a total of 83 days, of domestication. This implied putative

molecular mechanisms independent of *spo0A* in *C. cellulovorans* that resist such high butanol concentrations. Comparative analysis of genome resequencing, transcriptional analysis, and functional genomics studies of ZQW30, ZQW20, and ZQW00, coupled with subsequent genetic and biochemical validation experiments, are currently being conducted to investigate the underlying molecular mechanism that is responsible for the higher *n*-butanol resistance in ZQW20 and ZQW30. The evolutionary approach based on the *spo0A*-knockout strain offers a new perspective for the exploration of diversities of stress response mechanisms in *Clostridium*.

In addition, *spo0A* inactivation helps to filter false positives benefiting from *spo0A*-related stress mechanisms, which leads to a more efficient and robust adaptive laboratory evolution. The inactivation of *spo0A* weakens the stress adaptability of *Clostridium*, which means that *Clostridium* is unable to survive (or resist butanol) by forming spores or by regulating *spo0A* expression levels (thus regulating target genes) (52). These circumstances drive the strain to either evolve or die. As shown in Fig. S3, far more survivors derived from ZQW10 than those derived from ZQW00 have real butanol tolerance phenotypes. Therefore, ZQW10 evolved more smoothly (or better) than ZQW00, and the *n*-butanol tolerance levels of the strains derived from ZQW30 (evolved ZQW10) were generally superior to those derived from ZQW20 (evolved ZQW00) (see Fig. 5; see also Fig. S4 in the supplemental material).

In this respect, the inactivation of genes involved in sporulation, such as *spo0A* in *C. cellulovorans*, provides a novel and reliable approach for adaptive laboratory evolution toward enhanced butanol tolerance of *Clostridium*, which may be extended to the study of lignocellulosic hydrolysate resistance.

The reinforced *n*-butanol tolerance indeed improved the *n*-butanol production from 2.27 g/liter of ZQW05 to 3.02 g/liter of ZQW35 (Fig. 3B and 6B). However, the toxicity of 3 g/liter *n*-butanol no longer significantly affected the fermentation phenotype of mutants derived from ZQW20 and ZQW30 (Fig. 4B). This provides an indication of why butanol outputs did not differ strongly between ZQW35 and ZQW25 (743B*4/pXY1-*P_{thi}*-*adhE1*-*P_{thi}*-*ctfAB*-*adc*) or between ZQW33 and ZQW23 (743B*4/pXY1-*P_{thi}*-*adhE1*) (Fig. 6A and Fig. S6). These results imply that *n*-butanol inhibition can limit the *n*-butanol output of the unevolved *C. cellulovorans* strain; however, this seems not to be the essential determinant for the achievement of high *n*-butanol titers.

The integration of metabolic and evolutionary engineering offered a stepwise promotion of *n*-butanol output (Table S1 and Fig. S7), which accelerated the overall development of a *C. cellulovorans* CBP-enabling microbial chassis for increased *n*-butanol production using lignocellulose or glucose as the sole carbon source. The *n*-butanol production of *C. cellulovorans* using AECC could be enhanced by 138-fold, from less than 0.025 g/liter to 3.47 g/liter (Fig. 8). Among CBPs for *n*-butanol production with a single recombinant clostridium strain using various lignocellulosic biomasses, the presented results represent significant progress in *n*-butanol titers and productivity (Table 1). In addition, the evolved strains may offer indications for new *n*-butanol tolerance mechanisms or target genes for reverse metabolic engineering. The engineered *C. cellulovorans* strain offers a promising microbial chassis for *n*-butanol production from lignocellulose by CBP.

However, the *n*-butanol titer and ratio in this study still offer room for improvement. As shown in Fig. 7B and D, a residual of 3.76 g/liter of butyrate and 6.16 g/liter of xylose, which should ideally have been converted to butanol, remained in the broth at the end of fermentation (84 h). To either avoid or decrease butyrate production, endogenous *buk* knockout was attempted in *C. cellulovorans* using ClosTron. However, no mutant of *C. cellulovorans* with inactivated *buk* was obtained. This may be due to an inherent limitation of group II intron technology, the inactivation efficiency of which is gene specific (or dependent) (55, 56). In other studies, the *buk* gene in *C. acetobutylicum* ATCC 824 has been successfully inactivated using in-frame deletion methods; consequently, *buk* inactivation directed carbon flux from butyrate to butanol (57, 58). A similar approach may work for *C. cellulovorans*. With regard to xylose, metabolic engineering is ideally suited for *C. cellulovorans* to alleviate carbon catabolite

FIG 8 Integrated metabolic and evolutionary engineering enhanced *n*-butanol production of *C. cellulovorans* from AECC.

repression (CCR) and promote butanol conversion. The reason for this is that insufficient uptake and utilization of xylose lead to slow growth and excessively long fermentation. Strategies such as overexpression of xylose symporter (XylT) and inactivation of xylose operon transcriptional repressor (XylR) have proven successful in *C. beijerinckii* and *C. tyrobutyricum* (48, 59).

Similar to the achievements reported for other clostridia, ABE pathway engineering (32, 58), pentose metabolic engineering (48, 59), cellulase engineering (13), and understanding the resistance mechanism of inhibitors (41, 42, 53) will likely promote the further development of the *C. cellulovorans* platform more effectively.

MATERIALS AND METHODS

Strains, plasmids, primers, and strain cultivation. All bacterial strains, plasmids, and primers used in this study are listed in Tables 2 and 3 and Fig. 5. *Escherichia coli* DH5 α and ER2275 were aerobically cultivated at 37°C in liquid or solidified Luria-Bertani (LB) medium, which was composed of 5g/liter of yeast extract (AngelYeast Co., Ltd., China), 10 g/liter of tryptone (AngelYeast Co., Ltd., China), and

TABLE 1 Comparison of *n*-butanol production with single recombinant clostridium strain by CBP

Strain	Recombinant strategy	Substrate	Titer (g/liter) ^a	Productivity (g/liter/h)	Reference
<i>C. acetobutylicum</i> ATCC 824	Overexpression optimization of chimeric cellulosomal operons	Crystalline cellulose	ND	ND	15
	Grafting carrier modules at heterologous cellulases' N termini	Crystalline cellulose	ND	ND	60
	Integrating synthetic cellulosomal operons into genome using allele-coupled exchange	Cellohexaose	ND	ND	14
	Anchoring the recombinant cellulosome to the cell surface using the native sortase system	Untreated wheat straw	ND	ND	13
<i>C. cellulolyticum</i> ATCC 35319	Introducing CoA-dependent pathway	Crystalline cellulose	0.12	0.00025	17
<i>C. cellulovorans</i> DSM 743B	Overexpressing <i>adhE2</i> from <i>C. acetobutylicum</i> ATCC 824	Crystalline cellulose	1.42	0.0056	28
	Integrated metabolic and evolutionary engineering	Corn cobs (alkali extracted)	3.47	0.0413	This work

^aND, not determined.

TABLE 2 Strains and plasmids used in this study

Strain or plasmid ^a	Description or genotype ^b	Source or reference(s)
Strains		
<i>E. coli</i> strains		
DH5α	Commercial transformation host	Gibco-BRL, Life Technologies
ER2275	Strain used to premethylate plasmids for electron-transformation in <i>C. acetobutylicum</i> ATCC 824 and <i>C. cellulovorans</i> DSM 743B	New England Biolabs
<i>C. acetobutylicum</i> strains		
ATCC 824	Wild type	ATCC
ZQW00	DSM 743B; wild type	DSMZ
ZQW01	743B/pXY1- <i>P_{thl}</i>	6
ZQW02	743B/pXY1- <i>P_{thl}</i> - <i>adhE2</i>	This study
ZQW03	743B/pXY1- <i>P_{thl}</i> - <i>adhE1</i>	This study
ZQW04	743B/pXY1- <i>P_{thl}</i> - <i>ctfAB-adc</i>	This study
ZQW05	743B/pXY1- <i>P_{thl}</i> - <i>adhE1-P_{thl}</i> - <i>ctfAB-adc</i>	This study
ZQW06	743B/pXY1- <i>P_{thl}</i> - <i>bukI</i>	This study
ZQW07	743B/pXY1- <i>P_{thl}</i> - <i>bukI-P_{thl}</i> - <i>adhE1</i>	This study
ZQW11	743B/pXY1- <i>P_{thl}</i> - <i>adhE1-P_{thl}</i> - <i>spo0A</i>	This study
ZQW10	743B <i>spo0A</i> ::intron	6
ZQW11	743B <i>spo0A</i> ::intron/pXY1- <i>P_{thl}</i> - <i>spo0A</i>	6
ZQW13	743B <i>spo0A</i> ::intron/pXY1- <i>P_{thl}</i> - <i>adhE1-P_{thl}</i> - <i>spo0A</i>	This study
ZQW15	743B Δ <i>spo0A</i> ::intron/pXY1- <i>P_{thl}</i> - <i>adhE1-P_{thl}</i> - <i>ctfAB-adc-P_{thl}</i> - <i>spo0A</i>	This study
ZQW20	743B*4; evolved strain from wild-type ZQW00	This study
ZQW23	743B*4/pXY1- <i>P_{thl}</i> - <i>adhE1</i>	This study
ZQW25	743B*4/pXY1- <i>P_{thl}</i> - <i>adhE1-P_{thl}</i> - <i>ctfAB-adc</i>	This study
ZQW30	743B Δ <i>spo0A</i> *11; evolved strain from wild-type ZQW10	This study
ZQW31	743B Δ <i>spo0A</i> *11/pXY1- <i>P_{thl}</i> - <i>spo0A</i>	This study
ZQW33	743B Δ <i>spo0A</i> *11/pXY1- <i>P_{thl}</i> - <i>adhE1-P_{thl}</i> - <i>spo0A</i>	This study
ZQW35	743B Δ <i>spo0A</i> *11/pXY1- <i>P_{thl}</i> - <i>adhE1-P_{thl}</i> - <i>ctfAB-adc-P_{thl}</i> - <i>spo0A</i>	This study
Plasmids		
pANS1	Φ37I gene, p15A origin; Spec ^r	61
pXY1- <i>P_{thl}</i>	Amp ^r , MLS ^r , pCB102 ori, ColE1 origin, <i>Thl</i> (CA_c2873) promoter region of <i>C. acetobutylicum</i> ; <i>E. coli</i> - <i>C. cellulovorans</i> shuttle vector for expressing genes in <i>C. cellulovorans</i>	62
pXY1- <i>P_{thl}</i> - <i>adhE1</i>	Derived from pXY1- <i>P_{thl}</i> with the <i>adhE1</i> (CA_p0162) gene from <i>C. acetobutylicum</i> overexpression	This study
pXY1- <i>P_{thl}</i> - <i>adhE2</i>	Derived from pXY1- <i>P_{thl}</i> with <i>adhE2</i> (CA_p0035) gene from <i>C. acetobutylicum</i> overexpression	This study
pXY1- <i>P_{thl}</i> - <i>ctfAB-adc</i>	Derived from pXY1- <i>P_{thl}</i> with <i>ctfAB-adc</i> cassette (CA_p0163-0164-0165) from <i>C. acetobutylicum</i> overexpression	This study
pXY1- <i>P_{thl}</i> - <i>adhE1-P_{thl}</i> - <i>ctfAB-adc</i>	Derived from pXY1- <i>P_{thl}</i> with <i>adhE1</i> gene and <i>ctfAB-adc</i> cassette from <i>C. acetobutylicum</i> overexpression	This study
pXY1- <i>P_{thl}</i> - <i>bukI</i>	Derived from pXY1- <i>P_{thl}</i> with <i>bukI</i> (CA_C3075) gene from <i>C. acetobutylicum</i> overexpression	This study
pXY1- <i>P_{thl}</i> - <i>bukI-P_{thl}</i> - <i>adhE1</i>	Derived from pXY1- <i>P_{thl}</i> with <i>bukI</i> and <i>adhE1</i> gene from <i>C. acetobutylicum</i> overexpression	This study
pWJ1- <i>spo0A</i>	Derived from pWJ1 for intron insertion in <i>C. cellulovorans spo0A</i> gene (<i>Clocel</i> _1943) at 586/587 nt	6, 63
pXY1- <i>P_{thl}</i> - <i>spo0A</i>	Derived from pXY1- <i>P_{thl}</i> with <i>C. cellulovorans spo0A</i> gene expression (complementation)	This study
pXY1- <i>P_{thl}</i> - <i>adhE1-P_{thl}</i> - <i>spo0A</i>	Derived from pXY1- <i>P_{thl}</i> with <i>adhE1</i> gene from <i>C. acetobutylicum</i> overexpression and <i>C. cellulovorans spo0A</i> gene complementation	This study
pXY1- <i>P_{thl}</i> - <i>adhE1-P_{thl}</i> - <i>ctfAB-adc-P_{thl}</i> - <i>spo0A</i>	Derived from pXY1- <i>P_{thl}</i> with <i>adhE1</i> gene and <i>ctfAB-adc</i> cassette from <i>C. acetobutylicum</i> overexpression, and <i>C. cellulovorans spo0A</i> gene complementation	This study

^aATCC, American Type Culture Collection, USA; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany.

^bSpec^r, spectinomycin resistance; Amp^r, ampicillin resistance; MLS^r, macrolide-lincosamide-streptogramin resistance; pCB102 ori, Gram-positive origin of replication from *Clostridium butyricum*; Thl, thiolase.

10 g/liter of NaCl with 1.5% agar (wt/vol) if necessary. *C. cellulovorans* DSM 743B was cultivated and evolved in Clocel medium (6, 49). The Clocel solidified medium contained only 1.0% (wt/vol) agar. Previously published protocols were employed for both the cultivation and genetic modification of *C. cellulovorans* DSM 743B (6). All stock cultures were maintained in 25% glycerol and frozen at −80°C.

DNA manipulation and vector construction. All DNA sequencing and oligonucleotide primers syntheses were conducted by Shanghai RuiDi Biological Technology Co., Ltd. (Shanghai, China). Previously described standard procedures were used to accomplish all recombinant DNA manipulations,

TABLE 3 Primers used in this study

Primer name	Sequence (5'–3')	Description
EBS universal	CGAAATTAGAAACTGCGTTCAGTAAAC	Targetron primer ^a
spo0A-586/587a-IBS	CCGCTCGAGATAATATCTTACTTTCTTACAGGTGCGCCAGATAGGGTG	spo0A Targetron primer ^a
spo0A-586/587a-EBS1d	AGATTGTACAATGTGGTATACAGATAAGCTTACAGCTTAACCTTCTTTGT	spo0A Targetron primer ^a
spo0A-586/587a-EBS2	TGAACGCAAGTTTCTAATTCGGTTAAAGTCGATAGAGAAAGTGCT	spo0A Targetron primer ^a
spo0A_447-467	CTGTGAATCTAAGCCAGTAG	Forward primer inside spo0A from base 447 to 467
spo0A_743-764	TGTGTTACCTTTTGATGTATG	Reverse primer inside spo0A from base 743 to 764
spo0A F	TAGGAGGTAGTTAGAGGATCCCATGGAATTCACAAATCAAAATAAGTG	Forward primer for gene Clocl_1943 clone from 743B chromosome and assembly in plasmid pXY1-P _{thr} -spo0A
spo0A R	CGACGGCCAGTGAAATCCCTTAACCTTACTTTGTTTTTAAAGTC	Reverse primer for gene Clocl_1943 clone from 743B chromosome and assembly in plasmid pXY1-P _{thr} -spo0A
adhE1 F	AAATTAGGAGGTAGTTAGAGGATCCCATGAAAGTCACACAGTAAAGGAATTAGAT	Forward primer for gene adhE1 clone from <i>C. acetobutylicum</i> genome and assembly in plasmid pXY1-P _{thr} -adhE1
adhE1 R	GACGTTGTAACACGACGGCCAGTGAATCCCTTAAGGTTGTTTTTAAACAATTATA	Reverse primer for gene adhE1 clone from <i>C. acetobutylicum</i> genome and assembly in plasmid pXY1-P _{thr} -adhE1
adhE2 F	ATTAGGAGGTAGTTAGAGGATCCCATGAAAGTTACAAATCAAAAGAACTAAAAACA	Forward primer for gene adhE2 clone from <i>C. acetobutylicum</i> genome and assembly in plasmid pXY1-P _{thr} -adhE2
adhE2 R	GTTGTAACACGACGGCCAGTGAATCCCTTAAATGATTATATAGATATCCTTAAGT	Reverse primer for gene adhE2 clone from <i>C. acetobutylicum</i> genome and assembly in plasmid pXY1-P _{thr} -adhE2
bukI F	ATTAGGAGGTAGTTAGAGGATCCCATGTATAGATTACTAATAATCAATCCTGGCTC	Forward primer for gene bukI clone from <i>C. acetobutylicum</i> genome and assembly in plasmid pXY1-P _{thr} -bukI
bukI R	CGTTGTAACACGACGGCCAGTGAATCCCTTATTGTTATCTTCTAGCTTTCTCTCC	Reverse primer for gene bukI clone from <i>C. acetobutylicum</i> genome and assembly in plasmid pXY1-P _{thr} -bukI
bukI F	ATTAGGAGGTAGTTAGAGGATCCCATGTATAGATTACTAATAATCAATCCTGGCTC	Forward primer for gene bukI clone from <i>C. acetobutylicum</i> genome and assembly in plasmid pXY1-P _{thr} -bukI
P _{thr} -adhE1-bukI R	TTATTATTTTATCAATATATTTTGTAAAAATTTATTTGATTCCTTAGCTTTTCTCTC	Reverse primer for gene adhE1 clone from <i>C. acetobutylicum</i> genome and assembly in plasmid pXY1-P _{thr} -bukI-P _{thr} -adhE1
bukI-P _{thr} -adhE1 F	GAAGAAAAAGCTAAGGAATACAAATAATTTTAAACAAATATATTGATAAAAAATAAA	Forward primer for cassette P _{thr} -adhE1 clone from pXY1-P _{thr} -adhE1 and assembly in plasmid pXY1-P _{thr} -bukI-P _{thr} -adhE1
adhE1 R	GACGTTGTAACACGACGGCCAGTGAATCCCTTAAGGTTGTTTTTAAACAATTATA	Reverse primer for cassette P _{thr} -adhE1 clone from pXY1-P _{thr} -adhE1 and assembly in plasmid pXY1-P _{thr} -bukI-P _{thr} -adhE1
ctfAB-adc F	ATTAGGAGGTAGTTAGAGGATCCCATGAACCTCTAAAAATAATTAGATTGAAAAATTT	Forward primer for cassette ctfAB-adc clone from <i>C. acetobutylicum</i> genome and assembly in plasmid pXY1-P _{thr} -ctfAB-adc
ctfAB-adc R	GACGTTGTAACACGACGGCCAGTGAATCCCTAAGTTTATATAAATCTATTATGCAGAA	Reverse primer for cassette ctfAB-adc clone from <i>C. acetobutylicum</i> genome and assembly in plasmid pXY1-P _{thr} -ctfAB-adc
adhE1 F	AAATTAGGAGGTAGTTAGAGGATCCCATGAAAGTCACACAGTAAAGGAATTAGAT	Forward primer for gene adhE1 clone from <i>C. acetobutylicum</i> genome and assembly in plasmid pXY1-P _{thr} -ctfAB-adc
P _{thr} -ctfAB-dhE R	TATTATTTTATCAATATATTTTGTAAAAATTAAGGTTGTTTTTAAACAATTATA	Reverse primer for gene adhE1 clone from <i>C. acetobutylicum</i> genome and assembly in plasmid pXY1-P _{thr} -ctfAB-adc
adhE-ctfAB-adc F	TATAAATTGTTTTTAAAAACAACCTTAATTTTTTAAACAAATATATTGATAAAAAATAA	Forward primer for gene adhE1 clone from pXY1-P _{thr} -ctfAB-adc and assembly in plasmid pXY1-P _{thr} -ctfAB-adc
ctfAB-adc R	GACGTTGTAACACGACGGCCAGTGAATCCCTAAGTTTATATAAATCTATTATGCAGAA	Reverse primer for cassette P _{thr} -ctfAB-adc clone from pXY1-P _{thr} -ctfAB-adc and assembly in plasmid pXY1-P _{thr} -ctfAB-adc
adhE1 F	AAATTAGGAGGTAGTTAGAGGATCCCATGAAAGTCACACAGTAAAGGAATTAGAT	Forward primer for gene adhE1 clone from <i>C. acetobutylicum</i> genome and assembly in plasmid pXY1-P _{thr} -spo0A
P _{thr} -spo0A-adhE1 R	TATTATTTTATCAATATATTTTGTAAAAATTAAGGTTGTTTTTAAACAATTATA	Reverse primer for gene adhE1 clone from <i>C. acetobutylicum</i> genome and assembly in plasmid pXY1-P _{thr} -adhE1-P _{thr} -spo0A

(Continued on next page)

TABLE 3 (Continued)

Primer name	Sequence (5'–3')	Description
adhE1-P _{thr} -spo0A F	TATAAATTGTTTTTAAAAACAACCTTAATTTTTTAACAAAATATATTGATAAAAAATAATA	Forward primer for cassette P _{thr} -spo0A clone from pXY1-spo0A and assembly in plasmid pXY1-P _{thr} -adhE1-P _{thr} -spo0A
spo0A R	CGACGGCCAGTGAATTCCTTAACCTTACTTTGTTTTTAAGTC	Reverse primer for cassette P _{thr} -spo0A clone from pXY1-spo0A and assembly in plasmid pXY1-P _{thr} -adhE1-P _{thr} -spo0A
adhE1 F	AAATTTAGGAGGTAGTTAGAGGATCCCATGAAAGTCACAACAGTAAAGGAATTAGAT	Forward primer for gene adhE1 clone from <i>C. acetobutylicum</i> genome and assembly in plasmid pXY1-P _{thr} -adhE1-P _{thr} -ctfAB- <i>adc</i> -P _{thr} -spo0A
P _{thr} -ctfAB-adhE1 R	TATTATTTTATCAATATATTTTGTAAAAAATTAAAGGTGTTTTTTAAAAACAATTTATA	Reverse primer for gene adhE1 clone from <i>C. acetobutylicum</i> genome and assembly in plasmid pXY1-P _{thr} -adhE1-P _{thr} -ctfAB- <i>adc</i> -P _{thr} -spo0A
adhE1-ctfAB- <i>adc</i> F	TATAAATTGTTTTTAAAAACAACCTTAATTTTTTAACAAAATATATTGATAAAAAATAATA	Forward primer for cassette P _{thr} -ctfAB- <i>adc</i> clone from pXY1-P _{thr} -ctfAB- <i>adc</i> and assembly in plasmid pXY1-P _{thr} -adhE1-P _{thr} -ctfAB- <i>adc</i> -P _{thr} -spo0A
P _{thr} -spo0A-ctfAB- <i>adc</i> R	TATTATTTTATCAATATATTTTGTAAAAAATAAGTTTATATAAATCTATTATGCAGAA	Reverse primer for cassette P _{thr} -ctfAB- <i>adc</i> clone from pXY1-P _{thr} -ctfAB- <i>adc</i> and assembly in plasmid pXY1-P _{thr} -adhE1-P _{thr} -ctfAB- <i>adc</i> -P _{thr} -spo0A
ctfAB- <i>adc</i> -P _{thr} -spo0A F	TTCTGCATAATAGATTATATAAACTTATTTTAAACAAAATATATTGATAAAAAATAATA	Forward primer for gene Clocl _L 1943 clone from 743B chromosome and assembly in plasmid pXY1-P _{thr} -adhE1-P _{thr} -ctfAB- <i>adc</i> -P _{thr} -spo0A
spo0A R	CGACGGCCAGTGAATTCCTTAACCTTACTTTGTTTTTAAGTC	Reverse primer for gene Clocl _L 1943 clone from 743B chromosome and assembly in plasmid pXY1-P _{thr} -adhE1-P _{thr} -ctfAB- <i>adc</i> -P _{thr} -spo0A

^aDesigned by online tools (www.clostron.com).

including genomic DNA preparation, DNA fragment amplification, digestion, ligation, transformation, colony PCR, plasmid extraction, and identification (6).

The construction of the Clostron knockout plasmid pWJ1-*spo0A* derived from pWJ1 and all overexpression (or complementation) plasmids derived from pXY1-*P_{thi}* was conducted according to previously published standard procedures (6).

Generation and screening of genetically modified strains. Electrocompetent cell preparation and electro-transformation of *C. cellulovorans* DSM 743B (here referred to as 743B) were conducted using previously described procedures (6). Positive transformants were screened and identified by colony PCR. Specially, for transformants that were generated from the electro-transformation of pWJ1-*spo0A*, PCR products of the desired mutants were approximately 0.9 kb longer than those of the wild type due to insertion of the group II intron (6). Clostron knockout plasmids were cured by repeated subculture on plates without erythromycin until a single colony that was sensitive to erythromycin had been isolated (6). Figure 5 shows the derivative relationship of all genetically modified *C. cellulovorans* strains in this work.

Butanol tolerance evolutionary engineering and growth tolerance assay. Evolutionary engineering was accomplished by four stages with a stepwise increment of *n*-butanol (3, 6, 9, and 12 g/liter) supplemented to the medium. ZQW10 (743B Δ *spo0A*) and ZQW00 (743B) were grown in tubes with 10 ml of liquid medium in an anaerobic chamber (Thermo Forma, Inc., Waltham, MA, USA) at 37°C. Cultures were inoculated to an OD₆₀₀ of 0.3, grown for 24 h, and then harvested by centrifugation (3,000 × *g*, 2 min) for fresh inoculation. Evolution in medium with higher *n*-butanol concentrations was not launched unless an OD₆₀₀ of 1.0 was achieved within 24 h. Samples from the last batch were spread on solidified medium for clone selection.

A growth assay was used to evaluate the *n*-butanol tolerance of *C. cellulovorans*. The genetically modified or evolved *C. cellulovorans* strains were inoculated in medium with various concentrations of *n*-butanol (0, 3, 6, 9, and 12 g/liter) and grown for 48 h. During this time, their OD₆₀₀ values were monitored and compared.

Batch fermentation of *C. cellulovorans* with constant pH and two-stage pH control. Alkali-extracted corn cobs (AECC) were manufactured according to a previously described protocol, including alkali extraction, neutralization, thorough washing, and drying. The particles varied from a 30- to 40-mesh size (0.45 to 0.60 mm). Cellulose, hemicellulose, and lignin contents were 69.8%, 27.4%, and 1.47% (wt/wt), respectively (6). All batch fermentations with glucose or AECC as the sole carbon source for phenotype comparison (expressed as bar charts) were conducted in 500-ml anaerobic shaken flasks (customized from Guxin Biotech Co., Ltd., Shanghai, China) with a 400-ml working volume. All batch fermentations for the study of the metabolic progress (expressed as line charts) were conducted in a 3-liter BioFlo 110 bioreactor (New Brunswick Scientific Co., Inc., NJ, USA) with a 1.6-liter working volume. The pH was controlled via automatic addition of 5 N NaOH, while temperature and stirring speed were kept at 37°C and 150 rpm, respectively. The medium was supplemented with 15 mg/liter erythromycin for mutants that harbored pXY1 or pWJ1 series plasmids. Samples were collected in an anaerobic chamber at regular intervals, and enzyme activity and biomass, as well as substrate and product concentration, were analyzed. The inoculum size of *C. cellulovorans* DSM 743B was 10% (vol/vol) if not indicated otherwise (49).

Analysis. A DU730 spectrophotometer (Beckman Coulter) was used to monitor cell growth on glucose at an optical density of 600 nm (OD₆₀₀). The cell mass on AECC particles and the AECC concentration were calculated according to previously reported procedures (49). Volatile solvents and organic acids (ethanol, *n*-butanol, acetone acetate, and butyrate) were determined by gas chromatography (7890 A; Agilent, Wilmington, DE, USA), while lactate, monosaccharides, and disaccharides were measured by a high-performance liquid chromatography (HPLC) system (1200 series; Agilent, Wilmington, DE, USA) (6). The assay of butyraldehyde dehydrogenase and butanol dehydrogenase activities in ZQW01, ZQW02, and ZQW03 were accomplished according to the protocol of Yang et al. except that the crude cell extract was obtained using a French press (Constant Systems Limited, UK) (28). The carbon recovery of C₂, C₃, and C₄ products (acetate, ethanol, lactate, butyrate, *n*-butanol, and acetone) was calculated as previously described (6).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.02560-18>.

SUPPLEMENTAL FILE 1, PDF file, 0.6 MB.

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