AEM Accepted Manuscript Posted Online 16 October 2020
Appl. Environ. Microbiol. doi:10.1128/AEM.01773-20
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Genome editing of the anaerobic thermophile *Thermoanaerobacter ethanolicus* using thermostable Cas9

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ABSTRACT *Thermoanaerobacter ethanolicus* can produce acetate, lactate, hydrogen and ethanol from sugars resulting from plant carbohydrate polymer degradation at temperatures above 65°C. *T. ethanolicus* is a promising candidate for thermophilic ethanol fermentations due to both the pentose and hexose utilization. Although an ethanol balance model in *T. ethanolicus* has been developed, only a few physiological or biochemical experiments regarding the function of important enzymes in ethanol formation have been carried out. To address this issue, we developed a thermostable Cas9 based system for genome editing of *T. ethanolicus*. As a proof of principle, three genes including thymidine kinase gene (*tdk*), acetaldehyde-alcohol dehydrogenase gene (*adhE*) and redox sensing protein gene (*rsp*) were chosen as editing targets, and these genes were edited successfully. As a genetic tool, we tested the gene knock-out and a small DNA fragment knock-in. After optimization of the transformation strategies, 77% genome editing efficiency was observed. Furthermore, our results *in vivo* revealed that redox sensing protein (RSP) play a more improtant role to regulate energy metabolism, including hydrogen production and ethanol formation. The genetic system provides us an effective strategy to identify genes involved in biosynthesis and energy metabolism.

KEYWORDS: *Thermoanaerobacter*; *Thermoanaerobacter ethanolicus*; Thermophiles; Thermostable Cas9; Genome editing; Biofuel

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IMPORTANCE Interest in thermophilic microorganisms as emerging metabolic engineering platforms to produce biofuels and chemicals has surged. Thermophilic microbes for biofuels have been attracted great attention, due to their tolerance of high temperature and wide range of substrate utilization. Based on the biochemical experiments of previous investigation, the ethanol formation was controlled via transcriptional regulation and influenced by the relevant properties of specific enzymes in *T. ethanolicus*. Thus, there is an urgent need to understand the physiological function of these key enzymes, which requires genetic manipulations such as deletion or overexpression of genes encoding putative key enzymes. Here we developed a thermostable Cas9-based engineering tool for gene editing in *T. ethanolicus*. Thermostable Cas9 based genome-editing tool may further be applied to metabolically engineer *T. ethanolicus* to produce biofuels. This genetic system is an important expansion to the genetic tool box of anaerobic thermophile *T. ethanolicus* strain.

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Interest in thermophilic microorganisms as emerging metabolic engineering platforms to produce bio-based fuels and chemicals has surged in recent years (1-3). Biofuels has been considered as the safest and sustainable energy resources. Thermophilic microbes for biofuel have been attracted a great deal of attention in the last years, due to their tolerance of high temperature and wide range of substrate utilization (1, 4).

In line with the lack of a genetic tool, serious pathway engineering in thermophilic strict anaerobes remains challenging. Establishment of genetic techniques has become a foundation for further genetic modification of thermophilic microbes (3, 5). Many strategies have been proposed in the literature to establish thermophilic genetic techniques for generating recombinant strains (6-8). However, genetic tools for thermophiles are still relatively scarce (5, 8, 9). Genetic manipulation of thermophilic bacteria still suffered from some unsatisfied barriers due to their low transformation efficiency of cells and instability of the plasmids (3, 10). The lack of a powerful genome manipulation technique has impeded the full exploration and exploitation of thermophilic microbes.

Thermoanaerobacter species can produce acetate, lactate, hydrogen and ethanol from sugars resulting from plant carbohydrate polymer degradation at temperatures above 65°C (7, 11-13). Members of the genus *Thermoanaerobacter* have a number of potential advantages in ethanol fermentation. For example, some *Thermoanaerobacter* strains can ferment hexose and pentose for biofuels (14). Members of the genus *Thermoanaerobacter* can tolerate pretreated biomass hydrolysates (15). Interest in *Thermoanaerobacter ethanolicus* as potential advantages in ethanol fermentation has surged. However, the practical application of this biocatalytic process is still hindered by the low ethanol tolerance and relatively low final ethanol concentrations. The lack of a powerful genome manipulation technique has impeded the full exploration and exploitation of *Thermoanaerobacter* species (10).

In resent years, progresses have been made in molecular mechanism of ethanol metabolism (13) and ethanol tolerance (12). The ethanol titer of fermentation in *T.*

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ethanolicus JW200 was controlled via transcriptional regulation or a modification on some specific enzyme properties (13). The ethanol productivity and tolerance could be improved simultaneously by overexpressing alcohol dehydrogenase or sigma 24 (12). Thus, there is an urgent need to understand the mechanism of ethanol formation, which requires a genetic manipulation, such as deletion or overexpression of genes encoding key enzymes.

Current progresses on genetic and metabolic engineering of *Thermoanaerobacter* species have been reported in literatures (7, 9, 16, 17). Most genetic engineering of *Thermoanaerobacter* species have focused on gene deletion based on drug-resistant selection marker genes, or auxotrophic marker genes (9, 16). A new markerless gene deletion and integration system was described for *T. ethanolicus* using a thymidine kinase (Tdk) mutant and high-temperature kanamycin (Htk) marker (9). Furthermore, several methods to genetically transform *Thermoanaerobacter* species have been reported in the literature, such as: natural genetic competence, ultrasound-mediated DNA transformation and electro-transformation (18, 19). The early reports have shown that *Thermoanaerobacter* strains supposedly believed to be difficult to transform can potentially employ natural competence for gene transfer (19). A thermostable kanamycin resistance marker and a thermostable gram-positive origin of replication have also been reported in previous studies (19-21). Although basic genetic tools are available for *Thermoanaerobacter* species (9, 16), it is necessary to explore more efficient tools, which enable full exploration and exploitation of ethanologenic *Thermoanaerobacter* species.

CRISPR-Cas9 has been exploited as a genome engineering tool for both eukaryotes and prokaryotes (22, 23). Cas9 from *Streptococcus pyogenes* (SpCas9) was the best characterized and most widely employed SpCas9 for genome engineering (22). However, SpCas9-based editing tool for obligate thermophiles is not yet available as the Cas9 from *S. pyogenes* is not active at elevated temperatures (5). In the recent published report (24), a method based on Type I or Type III endogenous CRISPR-Cas systems for genome editing was developed in the thermophilic archaeon *Sulfolobus islandicus*. It has previously been suggested that Type II CRISPR-Cas systems are

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limited to a few taxa of mesophilic bacteria (24). Recently, three thermostable Cas9 proteins from thermophilic bacteria, *Geobacillus stearothermophilus*, *Geobacillus thermodenitrificans* T12 and *Acidothermus cellulolyticus* have been characterized (25-27). The thermostable Cas9 from *Geobacillus* is active *in vitro* between 20 and 70°C, and the thermostable Cas9 has been applied for *in vivo* genome editing in the thermophile *Bacillus smithii* ET 13831 and *Clostridium thermocellum* at 55°C (25, 28).

T. ethanolicus JW200, of an extreme thermophilic, non-spore-forming anaerobic bacterium, was designated as a type strain for its thermophilic ethanol fermentation platform, where its growth temperature occurred between 37 and 78°C (11). With this fermentation platform, a thermostable Cas9 genome-editing tool was then developed in thermophile *T. ethanolicus* at 65°C and its applicability of the thermostable Cas9 from *G. stearothermophilus* strain GS27 for genome editing of the anaerobic thermophile *T. ethanolicus* was finally to be described and evaluated.

RESULTS AND DISCUSSION

Construction of *Escherichia coli-T. ethanolicus* shuttle expression vector pBlu10-Htk

In order to develop an effective heterologous expression system in anaerobic thermophile *T. ethanolicus*, a shuttle expression vector pBlu10-Htk was successfully constructed (GenBank: MN843970). The shuttle expression vector pBlu-Htk contains many essential elements, including a thermostable Gram-positive origin of replication (29), a thermostable kanamycin resistance gene (Htk) (20), a pUC origin of replication, an ampicillin resistance marker from pBlueScript II SK(+), a kanamycin promoter from pMU423, a Shine-Dalgarno sequence (SD), multiple clone sites (MCS) and a terminator (Fig. 1). In previous investigations, the existing and reported genetic engineering efforts were primarily based on the approach of gene deletions in *T. ethanolicus* (9, 17) with only a few reports of gene expression, or over expression in *T. ethanolicus* (18).

Expression of thermostable Cas9 in *T. ethanolicus* JW200

The ability to express thermostable Cas9 gene in *T. ethanolicus* is of critical

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importance for genome editing with the CRISPR-Cas9 systems. In recent published reports, the thermostable Cas9 proteins were characterized (25-27), where the thermostable Cas9 DNA cleavage activity could maintain a maximum detectable activity up to 70°C (25, 26). With this reported characterization, a thermostable Cas9 protein can be selected for a genome editing manipulation towards *T. ethanolicus*.

In line with the lack of well-characterized promoters in *T. ethanolicus*, a set of pBlu10-Htk-based plasmids containing the thermostable Cas9 gene from *G. stearothermophilus* strain GS27 under the control of different promoters were constructed. A native constitutive S-layer promoter **(**P_{slay}**)** and a kanamycin promoter (P_{kan}) from pMU423 were also selected. Plasmid pBlu10-Slay-Cas9 containing the thermostable Cas9 gene was under the control of S-layer promoter (P_{slay}). Plasmid pBlu10-Htk-Cas9 was constructed to tandem express Htk and thermostable Cas9 under the control of kanamycin promoter (P_{kan}) from pMU423.

Notably, when plasmid pBlu10-Slay-Cas9 was transformed into *T. ethanolicus* JW200 cells, the transformation rate was significantly declined by approximately 13-fold when compared to the plasmid pBlu10-Htk that lacks the thermostable Cas9 gene (Fig. 2). As a matter of fact, the same result was also observed when plasmid pBlu10-Htk-Cas9 was transformed into *T. ethanolicus* JW200 (Fig. 2). The transformation rate was significantly declined as expressing thermostable Cas9. The nucleotide sequence of this thermostable Cas9 gene was recorded as 3264 bp. However, the size of the shuttle expression vector pBlu10-Htk was messured as 5756 bp. Since a larger plasmid tends to transform at a less efficiency, a loss of efficiency would be surely demonstrated when plasmid size was almost increased by 1.5 times after adding the Cas9 gene. Another potential explanation for this result is that Cas9 toxicity may very likely present.

It has been reported that the toxic effect to the host caused by uncontrolled Cas9 protein expression was addressed by regulating the expression of Cas9 by an inducible promoter (30). However, only a few inducible promoters have been successfully developed in *Sulfolobus solfataricus*, *Sulfolobus acidocaldarius*, *Caldicellulosiruptor bescii* and *Thermococcus kodakarensis* (31-34). To date, no

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inducible promoter derived from *T. ethanolicus* was found, and only a few of promoter sequences from *Thermoanaerobacter* have been identified (35-37). Some native constitutive promoters were used for thermostable Cas9 gene expression. As a case, a genome-editing tool for *B. smithii* ET 138 was developed (25), where the expression of thermostable cas9 gene was under the control of the native xylL promoter (P_{xylL}).

Thus, to futher confirm the function of this proposed thermostable Cas9 based system for genome editing of *T. ethanolicus* associated with a native constitutive S-layer promoter **(**P_{slay}**)**, the following three unique genes were then selected to verify its validity and efficiency in gene editing with this new system.

Case one: thermostable Cas9-based *tdk* gene deletion in the anaerobic thermophile *T. ethanolicus* JW200

In our experimental design, the thymidine kinase gene (*tdk*) was chosen in this evaluation as the first target gene for deleting as described in materials and methods using Kanamycin selection. Previous research has shown that the Tdk mutant colonies can be selected from the 5-fluoro-2´-deoxyuridine (FUDR) agar plate because Tdk can convert FUDR to fluoro-dUMP (F-dUMP), which will inhibit ThyA convert dUMP to dTMP in nucleic acid metabolism (9).

In the CRISPR/Cas9 system, the cleavage of the target DNA requires a protospacer adjacent motif (PAM) (25, 26). Previous reports have shown that the complete PAM sequence for thermostable Cas9 from *G. stearothermophilus* was determined at 5´-NNNNCRAA-3´, and its optimal spacer length was measured at 21–22nt (26). Based on the thymidine kinase (*tdk*) gene sequence (GenBank: AEYS01000001.1), a PAM sequence in the *tdk* gene was determined, and then, 21nt *tdk* gene-targeting spacer was further designed (Fig. 3). Joining the tracrRNA to the crRNA using a GAAA tetraloop to generate a single-guide RNA (sgRNA) as described by Harrington (26) was also designed.

The synthesized sequence of 21nt *tdk* gene-targeting spacer followed by sgRNA was subcloned into the shuttle expression vector pBlu10-Htk, and the resulting vectors were then referred as pBlu10-sgT. The phosphate acetyltransferase promoter

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(P_{pat}) from *T. ethanolicus* was selected for the expression of 21nt *tdk* gene-targeting spacer along with sgRNA. The plasmid pBlu10-P-sgT was then successfully constructed, which contained 21nt *tdk* gene-targeting spacer along with sgRNA expressing module under the control of promoter P_{pat}.

Furthermore, the plasmid pBlu10-S-P-sgT was then constructed, which contained two essential elements, the thermostable Cas9 gene expression element under the control of promoter P_{slay}, 21nt *tdk* gene-targeting spacer along with sgRNA expressing module under the control of promoter P_{pat}, but no homologous recombination template for repairing.

When plasmid pBlu10-S-P-sgT was transformed into *T. ethanolicus* cell, a further decline of the transformation rate was observed compared to the transformation rate of pBlu10-Slay-Cas9 or pBlu10-Htk-Cas9 (Fig. 2). These results showed that the expression of thermostable Cas9, 21nt *tdk* gene-targeting spacer along with the sgRNA in cell could generate double-stranded breaks (DSBs) in genes. If not repaired, a breakage of DSBs will eventually result in a lethal phenomenon. In general, the DSBs can be repaired by non-homologous end joining in most eukaryotes. However, in most prokaryotes DSBs would be repaired through homologous recombination repair (HRR) that was mediated by a DNA repair template (30).

Thus, a thermostable Cas9 assisted homologous recombination (HR) strategy for genome editing in *T. ethanolic*us was developed. In order to use a minimum of genetic parts, a single plasmid approach was followed. A donor-*tdk* DNA fragment was then inserted into the above vector pBlu10-S-P-sgT. The resulting plasmid pBlu10-S-P-sgT-Tdk contained three essential elements, *tdk* donor DNA, 21nt *tdk* gene-targeting spacer followed by sgRNA expressing module, and the thermostable Cas9 gene expression element.

The plasmid pBlu10-S-P-sgT-Tdk was then transformed into *T. ethanolicus* JW200 to provide a DNA editing template for deleting 297 bp of partial *tdk* gene.

When plasmid pBlu10-S-P-sgT-Tdk was transformed into *T. ethanolicus* JW200 cells, the transformation efficiency was truly enhanced when compared to the plasmid pBlu10-S-P-sgT that lacked homologous recombination template (Fig. 2). Clearly, the

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higher transformation efficiency can be observed primarily because the template for homology-directed-repair would help alleviate toxicity caused by Cas9 cutting the *tdk* gene. However, the lower transformation efficiency was also observed when compared to the plasmid pBlu10-Slay-Cas9 or pBlu10-Htk-Cas9 due to the low efficiency of homologous recombination (Fig. 2).

As our design, colonies were picked up from the solid medium supplemented with 300 mg/L kanamycin. The picked colonies were then analyzed by PCR amplification for both the wild type target and its mutated gene using primers T5 and T6. As shown in Fig. 3A, the primer T5 for screening was located in the upstream of homologous fragment. Thus, only the DNA fragment could be selectively amplified from the genomic DNA template, but not from the plasmid pBlu10-S-P-sgT-Tdk.The amplified PCR products were analyzed by agarose gel electrophoresis as shown in Fig 3B. Two colonies were clean Δ*tdk* mutants (Fig. 3B, Line T1 and 6), and one colony was a mixed wild type/Δ*tdk* genotypes (Fig. 3B Line T2).

As a genetic tool, the function of a gene knock-out and knock-in for a small DNA fragment is essential. In our design, the partial sequence of *tdk* gene (297 bp) was knocked out. Meanwhile, the gene knock-in has also been tested, where a sequence 5´- GATATCTCTGTTCTCGAGC-3´ contained restriction enzyme sites *EcoR* V and *Xho* I was inserted into the genome. DNA sequencing results were indeed confirmed at the partial deletion in *tdk* gene and the insertion of restriction enzyme sites of *EcoR* V and *Xho* I (Supplemental material, Fig. S1).

Case two: thermostable Cas9-based *adhE* gene deletion in the anaerobic thermophile *T. ethanolicus* JW200

In the previous investigation (13), the biochemical and physiological properties of the alcohol dehydrogenase (adhE and adhB) in *T. ethanolicus* JW200 were analyzed. The adhE, as a bi-functional alcohol dehydrogenases, played some important roles in ethanol formation (13, 17). In order to investigate the physiological function of adhE, *adhE* gene was then chosen as the second target gene for deleting. Based on the *adhE* gene sequence (GenBank: AEYS01000007.1), a thermostable Cas9 PAM site (5´-NNNNCRAA-3´) in *T. ethanolicus* was determined, and then, the 21nt *adhE*

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gene-targeting spacer was further designed (Supplemental material).The plasmid pBlu10-S-P-sgE-adhE for the deletion of the partial *adhE* gene from the genome of *T. ethanolicus* JW200 was then successfully constructed (Fig. 4), where three essential elements, *adhE* donor DNA, 21nt *adhE* gene-targeting spacer followed by sgRNA expressing module, and the thermostable Cas9 gene expression element were all presented in the vector pBlu10-S-P-sgE-adhE.

The resultant plasmid pBlu10-S-P-sgE-adhE was then transformed into *T. ethanolicus* JW200 to provide a DNA editing template for deleting 232 bp of partial *adhE* gene. Colonies appeared on the solid medium with 300 mg/L kanamycin sulfate were selected. The picked colonies were analyzed by PCR amplification for both the wild type target gene and its mutated gene using primers E5 and E6 (Table 1). Similarly, the primer E5 used for screening was located in the upstream of *adhE* donor DNA fragment. Thus, only the DNA fragment could be selectively amplified from the genomic DNA template, but not from the plasmid pBlu10-S-P-sgE-adhE. As shown in Fig. 4D, three out of 12 colonies presented in the solid medium with kanamycin sulfate were clean Δ*adhE* mutants (Fig. 4D, Line E1, 6 and 8). However, other three different colonies were determined as a mixed wild type/Δ*adhE* genotypes (Fig. 4D, Line E2, 7 and 9). DNA sequencing results were indeed confirmed at the partial deletion in *adhE* with a small DNA fragment knock-in, where a sequence 5´-CATATG-3´ with restriction enzyme sites *Nde* I was successfully inserted into the genome (Supplemental material, Fig. S2). In addition, the fermentation profiles of Δ*adhE* mutant were shown in Table 3 and Table 4. It has been indicated that the ethanol production of Δ*adhE* strain was only observed at a very low level, while the hydrogen production of Δ*adhE* strain was increased (Table 3, 4). The same similar observations were also reported by Zhou, where the ethanol production was profoundly influenced by *adhE* (17).

The genome editing efficiency could be influenced by several factors, such as Cas9 protein expression and the efficiency of homologous recombination repair (5). The promoter P_{slay} or P_{kan} used to drive the expression of thermostable Cas9 was not sufficiently controllable, where the low deletion efficiency was observed. As a

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gram-positive anaerobic thermophile, *T. ethanolicus* is more difficult to be engineered than that of genetically tractable hosts, such as *E. coli* (9).

Previous studies have shown that the deletion efficiency could be further improved by extending time for the thermophile to perform homologous recombination (5). Thus, two different strategies (A & B) were actually to be proposed and conducted in our explorations. At first, we tried the transformation strategy A: 1) Hungate anaerobic tubes contained cultures and plasmids were incubated at 65°C without kanamycin sulfate for 6 to 8 h; 2) After that transformation cultures were plated on a solid medium with 300 mg/L kanamycin sulfate, and further incubated at 65°C. Colonies were analyzed by a bacterial colony PCR, where the low deletion efficiency was indeed observed.

To improve the efficiency, the transformation strategy B was then designed as the followings: 1) Tubes contained cultures and plasmids were incubated at 65°C without kanamycin sulfate for 6 to 8 h; 2) After that up to 200 μL transformation cultures were mixed with 3 mL liquid medium containing 300 mg/L kanamycin sulfate, and further incubated at 65°C until cells were grown; 3) Cultures were then plated on a solid medium without kanamycin sulfate, and further incubated at 65°C. Colonies were also analyzed by a bacterial colony PCR. Compared to that transformation strategy A, the editing efficiency using transformation strategy B was actually improved, where about 77% genome editing efficiency at the *adhE* locus was confirmed.

Meanwhile, the control plasmids pBlu10-S-P-sg-adhE and pBlu10-sgE-adhE were transformed into *T. ethanolicus* JW200 using strategy B, respectively. The plasmid pBlu10-S-P-sg-adhE included three essential elements, the thermostable Cas9 gene expression element, homologous recombination template for repairing, sgRNA expressing module, but no 21nt *adhE* gene-targeting spacer. Another control plasmid pBlu10-sgE-adhE contained only two essential elements, homologous recombination template for repairing, 21nt *adhE* gene-targeting spacer followed by sgRNA expressing module, but no Cas9 gene expression element. Interestingly, we found that the homologous recombination colonies were also obtained using only the control

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plasmid pBlu10-S-P-sg-adhE that excluded 21nt *adhE* gene-targeting spacer, although the efficiency was quite low. However, homologous recombination colonies were not detected using the control plasmid pBlu10-sgE-adhE that excluded Cas9 gene expression element.

In this report, the thermostable Cas9 based system for genome editing of *T. ethanolicus* significantly reduced the cycling time when compared with SpCas9-based engineering tool for facultative thermophiles (5). Because the traditional Cas9 (such as Cas9 from *S. pyogenes*) is not active at elevated temperatures, the facultative thermophiles editing must combine homologous recombination at an elevated temperatures and SpCas9-based counter-selection at moderate temperatures (5).

In general, it is necessary to cure gene deletion plasmids from edited cells to facilitate the multiple genetic manipulations. There are many reports in the literature describing the strategies of plasmid curing (38). For example, a temperature sensitive replicon was applied to facilitate easy plasmid curing in *Corynebacterium glutamicum* (39). Clearly, this strategy is inappropriate for our thermophile system. In order to cure thermostable Cas9 based gene deletion plasmids from edited cells, engineered cells were cultivated in a fresh medium for multiple passages without kanamycin sulfate before plating on hungate anaerobic culture tube without antibiotics. The resultant colonies were picked and tested for resistance to kanamycin sulfate. Approximately 85% of colonies after third passages were sensitive to kanamycin sulfate, confirming that plasmids were cured.

Case 3: thermostable Cas9-based *rsp* gene deletion in the anaerobic thermophile *T. ethanolicus* JW200

In order to investigate the physiological function of a Rex ortholog termed redox sensing protein (RSP) from *T. ethanolicus* JW200, *rsp* gene was then chosen as the third target gene for deleting. At first, a thermostable Cas9 PAM site (5´-NNNNCRAA-3´) was determined in *rsp* gene. Then, based on the *rsp* gene sequence (GenBank: GU586295.1), 21nt *rsp* gene-targeting spacer was further designed. Finally, the plasmid pBlu10-S-P-sgR-RSP for the deletion of the partial *RSP* gene from the genome of *T. ethanolicus* JW200 was constructed. Here, there

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were three essential elements, *rsp* donor DNA, 21nt *rsp* gene-targeting spacer followed by sgRNA expressing module, and the thermostable Cas9 gene expression element, to be designed in the vector pBlu10-S-P-sgR-RSP.

The resultant plasmid pBlu10-S-P-sgR-RSP was then transformed into *T. ethanolicus* JW200 using transformation strategy B, as mentioned above, to provide a DNA editing template for deleting 582 bp of partial *rsp* gene. The colonies to be selected were analyzed by PCR amplification for both the wild type target and its mutated gene using primers R5 and R6 (Table 1). Similarly, the primer R5 for screening was located in the upstream of *rsp* donor DNA fragment. Thus, only the DNA fragment could be selectively amplified from the genomic DNA template, but not from the plasmid pBlu10-S-P-sgR-RSP. As shown in Fig 5, all nine colonies contained the Δ*RSP* genotype, where colonies 1, 4, 8 were clean Δ*rsp* mutants, whereas the rest presented a mixed wild type/Δ*rsp* genotype (Fig. 5). As the colony PCR was performed using primers R5 and R6, a small nonspecific band (about 200 bp DNA fragment) was amplified in all samples (Fig. 5). For this reason, two bands were amplified from the clean Δ*rsp* mutants and wild type, while other three bands were amplified from the mixed genotypes.

In recent published report (40), the RSP from *T. ethanolicus* JW200 was characterized, where the mechanism for the regulation of ethanol fermentation by RSP was revealed *in vitro*. Experiments *in vitro* indicated that RSP from *T. ethanolicus* JW200 could bind to the palindromic sequence to repress the transcription of the ethanol fermentation genes *adhA*, *adhB*, and *adhE*, where the transcription of alcohol dehydrogenase gene was repressed by RSP but recovered by adding NADH (40). However, the physiological function of RSP *in vivo* was still unclear.

Here, as our design, the physiological function of RSP *in vivo* was also investigated through gene knock-out. The mutant strain was named as Δ*rsp*. Since RSP sensed the $NADH/NAD^+$ ratio, the effects of ethanol formation by altering intracellular $NADH/NAD^+$ ratio using mannitol or cellobiose as carbon sources at different redox status were further evaluated. It was indicated that the change of a redox level by different reduced carbon sources could affect the hydrogen production, ethanol

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formation and cell growth of wild type JW200, as well as Δ*rsp* mutants (Table 3, 4). As cellobiose has the lower NADH value with respect to mannitol, there was no significant difference in the fermentation profiles between mutant Δ*rsp* strain and the wild type JW200 (Table 3). But a significant difference in the fermentation profile between mutant Δ*rsp* strain and the wild type JW200 has been observed when mannitol was applied as a carbon source (Table 4). The results showed that the mannitol as carbon source would enhance its impact on ethanol formation in Δ*rsp* mutant. An early report has shown that RSP could repress the transcription of the ethanol fermentation genes (40). When *rsp* gene was deleted, the results have shown that the ethanol production of Δ*RSP* was increased slightly when using mannitol as a carbon source if compared with that of the wild type JW200 (39.03 μmols/OD for wt JW200, 46.03 μmols/OD for Δ*rsp*). However, the mechanism determining the distribution of products in thermophilic ethanol fermentation is in general not known. One possibility is that the different alcohol dehydrogenases (adhE, adhB and adhA) may have a different physiological function (13). Previous reports demonstrated that adhE and adhB were responsible for ethanol production, while adhA was responsible for ethanol consumption (13, 17, 41). Another report further presented a more complicated relationship among adhE, adhB and adhA in *Thermoanaerobacter* (17, 42), where the function of adhB and adhA remains unknown and need a further investigation (17). Another possibility in this regard is that the product distribution may be determined by a specific regulatory process. From our fermentation results, the hydrogen production with Δ*rsp* strain was significantly increased by 8.37% when compared with that of wild type JW200 at a case of the cellobiose to be employed as a sole carbon (Table 3). However, when the mannitol was used as the only carbon source in the medium, the hydrogen production of Δ*rsp* strain was then increased by 26.03% when compared with that of the wild type JW200. In addition, the effects on cell growth and the lactate production were also observed when the *rsp* gene was deleted (Table 3, 4).

The RSP of *T. ethanolicus in vivo* may play a more important role in redox cellular balance. The ethanol formation, hydrogen production, as well as its cell growing

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449 status for *T. ethanolicus* could be influenced by RSP. Previous reports have shown
450 that Redox-sensing repressor Rex can sense the changes of NADH/NAD^+ ratio in
451 Gram-positive bacteria (43). The Rex could not only regulate the expression of genes
452 in metabolic pathways (44), but also play an important role in the carbon and energy
453 metabolism (45). The Rex from *Clostridium kluyveri* could bind the promoter regions
454 of numerous genes, which are involved in carbon and energy metabolism, including
455 NADH oxidation, hydrogen production, ATP synthesis (45).

456 In summary, a thermostable Cas9 based system for genome editing of *T.*
457 *ethanolicus* was successfully constructed and evaluated for its validity with three
458 selected genes, *tdk*, *rsp* and the ethanol fermentation gene *adhE*. Low transformation
459 efficiency was remained as a critical factor that could largely affect its gene deletion
460 efficiency, probably due to the thermostable Cas9 toxicity or the low efficiency of
461 homologous recombination in *T. ethanolicus*. After optimization of the transformation
462 strategies, gene deletion efficiency could be significantly improved. Our results have
463 also shown that there were no significant differences for plasmids curing, which three
464 deletion plasmids could be cured conveniently from edited cells after several passages.
465 This novel genetic system can be potentially applied to identify a gene that may
466 involve in a biosynthesis / energy metabolism; and, more importantly, it can also be
467 modified as a vital tool to metabolically engineer *T. ethanolicus* to produce a variety
468 of different biofuels by editing its key enzyme genes. This genetic system is indeed an
469 important expansion and a valuable contribution to the genetic tool box of some
470 anaerobic thermophiles, such as *T. ethanolicus*.

471 **MATERIALS AND METHODS**

472 **Strain and cultivation**

473 *E. coli* JM109 was used for general cloning and cultivated aerobically in Luria–
474 Bertani (LB) medium with ampicillin (100 mg/L) at 37°C. *T. ethanolicus* JW200
475 (DSM 2246) was obtained from Deutsche Sammlung von Mikroorganismen und
476 Zellkulturen (DSMZ) Germany, and grown under strict anaerobic condition. *T.*
477 *ethanolicus* JW200 was cultivated at 65°C in DSMZ medium 122, containing per liter
478 5.0 g cellobiose, 1.3 g $(NH_4)_2SO_4$, 2.6 g $MgCl_2$ $6{\cdot}H_2O$, 1.43 g KH_2PO_4, 1.8 g

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K_2HPO_4, 0.13 g $CaCl_2$ $2{\cdot}H_2O$, 6.0 g Na-β-glycerophosphate, 0.00013 g $FeSO_4$ $7{\cdot}H_2O$, 4.5 g yeast extract, 0.002 g resazurin, 0.5 g L-cysteine-HCl, and 15 g agarose for solid media. Cultures were grown at 65°C under anaerobic conditions. Kanamycin sulfate (300 mg/L) was added to the medium 122 as required.

Construction of the *E. coli-T. ethanolicus* shuttle expression vector pBlu10-Htk

All primer sequences used in this study were listed in Table 1. The plasmids used in this study were listed in Table 2. *E. coli* JM109 was used as a cloning host. *T. ethanolicus* JW200 was used in the genome engineering procedures. Plasmids were constructed using standard molecular biology techniques (46).

The sequence, including a thermostable kanamycin resistance gene (Htk) (20), a thermostable Gram-positive origin of replication (29), a kanamycin promoter from pMU423, multiple cloning sites (MCS), a Shine-Dalgarno (SD) sequence and a terminator, was synthesized by Sangon Biotech Co. Ltd. (Shanghai, China). This synthesized gene was cloned into vector pBlueScript II SK(+) (47) with the following primer pairs pB1, pB2 and pS1, pS2. The resulting vector was referred as *E. coli-T. ethanolicus* shuttle expression vector pBlu10-Htk (GenBank: MN843970) .

Construction of Cas9 expression plasmids

The thermostable Cas9 gene from *G. stearothermophilus* strain GS27 (GenBank: NZ_LUCR01000150) was synthesized by Sangon Biotech Co. Ltd. (Shanghai, China). This synthesized Cas9 gene was cloned into vector pET-28a(+) (48) between *Xho* I and *Xba* I restriction endonuclease sites. The resulting vector was referred as pET-28a(+)-Cas9.

The thermostable Cas9 gene was subcloned into shuttle vectors pBlu10-Htk (GenBank: MN843970) between *Xho* I and *Xba* I restriction endonuclease sites. The PCR amplification was carried out by using pET-28a(+)-Cas9 as template, with the following primers pCas1 and pCas2. PCR was carried out using high-fidelity PrimerSTAR HS DNA polymerase (TaKaRa, Dalian, China). Each cycle had 10 s at 98°C, 15 s at 57°C and 3 min 30s at 72°C. PCR products were purified using the QIAquick PCR purification kit and followed by digestion with restriction enzyme *Xho* I and *Xba* I. The resulting vector was referred as pBlu10-Htk-Cas9 (Table 1). The

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509 expression of the thermostable Cas9 was under the control of kanamycin promoter
510 (P_{kan}) from pMU423.
511 In order to express the thermostable Cas9 under the control of S-layer promoter
512 (P_{slay}), PCR amplification was carried out by using pBlu10-Htk-Cas9 as template,
513 with the following primers pCas3 and pCas4.The S-layer promoter sequence
514 (GenBank: CP033580.1) was amplified by using *T. ethanolicus* JW200 genomic DNA
515 as template, with the following primers pSlay1 and pSlay2. PCR products were
516 purified using the QIAquick PCR purification kit and followed by digestion with
517 restriction enzyme *EcoR* I and *EcoR* V. The resulting vector was referred as
518 pBlu10-Slay-Cas9 (Table 1).
519 **Construction of single-guide RNA (sgRNA) expression plasmids**
520 The tracrRNA and crRNA using a GAAA tetraloop to generate a sgRNA was
521 described by Harrington (26). Based on the Thymidine kinase (*tdk*) sequence
522 (GenBank: AEYS01000001.1), 21nt *tdk* gene-targeting spacer followed by the
523 sgRNA was synthesized by Sangon Biotech Co. Ltd. (Shanghai, China).This
524 synthesized sequence was cloned into vector pET-28a(+) between *Xho* I and *Xba* I
525 restriction endonuclease sites. The resulting vector was referred as pET-28a(+)-sgT.
526 The 21nt *tdk* gene-targeting spacer followed by sgRNA was subcloned into the shuttle
527 expression vector pBlu10-Htk between *Xho* I and *Xba* I restriction endonuclease sites.
528 The PCR amplification was carried out by using pET-28a(+)-sgT as template, with
529 the following primers psgRNA1 and psgRNA2. The resulting vector was referred as
530 pBlu10-sgT.
531 In order to express the 21nt *tdk* gene-targeting spacer alone with the sgRNA under
532 the control of phosphate acetyltransferase promoter (P_{pat}), PCR amplification was
533 carried out by using pBlu10-sgT as template with the following primers p1 and
534 p2.The promoter Pat sequence was amplified by using *T. ethanolicus* JW200 genomic
535 DNA as template, with the following primers pPat1 and pPat2. The resulting vector
536 was referred as pBlu10-P-sgT.
537 **Donor DNA construction**
538 For construction of *tdk* gene deletion substrates, The 5´ and 3´ flanking fragments

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were amplified from *T. ethanolicus* JW200 genomic DNA via PCR with primers T1 and T2 (Table 1). The amplified fragments were ligated into pBlueScript II SK(+) vector. The resulting vector was referred as pBlu-Tdk. In addition, in order to insert a small DNA fragment, PCR amplification was carried out by using pBlu-Tdk as template with the following primers T3 and T4. The PCR products were ligated to generate donor DNA sequence: donor-*tdk*. The resulting vector was then referred as pBlu-Tdk$_{297}$. The donor-*tdk* contained 5´ and 3´ flanking fragments and an insertion sequence with the restriction enzyme site *Xho* I and *EcoR* V.

For construction of *adhE* gene deletion substrates, The 5´ and 3´ flanking fragments were amplified from *T. ethanolicus* JW200 genomic DNA via PCR with primers E1 and E2 (Table 1). The amplified fragments were ligated into pBlueScript II SK(+) vector. The resulting vector was referred as pBlu-adhE. In order to insert a small DNA fragment, PCR amplification was carried out by using pBlu-adhE as template with the following primers E3 and E4. The PCR products were ligated to generate donor DNA sequence: donor-*adhE*. The resulting vector was then referred as pBlu-adhE$_{232}$. The donor-*adhE* contained 5´ and 3´ flanking fragments and an insertion sequence with the restriction enzyme site *Nde* I.

For construction of *rsp* gene deletion substrates, The 5´ and 3´ flanking fragments were amplified from *T. ethanolicus* JW200 genomic DNA via PCR with primers R1 and R2 (Table 1). The amplified fragments were ligated into pBlueScript II SK(+) vector. The resulting vector was referred as pBlu-RSP. PCR amplification was carried out by using pBlu-RSP as template with the following primers R3 and R4. The PCR products were ligated to generate donor DNA sequence: donor-*rsp*. The resulting vector was then referred as pBlu-RSP$_{582}$. The donor-*rsp* contained 5´ and 3´ flanking fragments.

Construction of Thermostable Cas9 based gene deletion plasmids

The tdk gene was targeted by thermostable Cas9 based gene deletion system. The Cas9 gene expression element, including S-layer promoter, SD sequence, Cas9 gene and terminator sequence, was amplified from pBlu10-Slay-Cas9 via PCR with primers pSlay1 and pB3 (Table 1). PCR amplification was carried out using

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high-fidelity PrimerSTAR HS DNA polymerase (TaKaRa, Dalian, China). Each cycle had 10 s at 98°C, 15 s at 57°C and 4 min at 72°C. The PCR fragments were phosphorylated and subcloned into pBlu10-P-sgT with primers pPat1 and pSC1. The resulting vector was referred as pBlu10-S-P-sgT.

The *tdk* donor DNA was amplified from pBlu-Tdk$_{297}$ via PCR with primers T1 and T2. *tdk* donor DNA fragments were phosphorylated and subcloned into pBlu10-S-P-sgT with primers pSC1 and pSlay1. The resulting vector was referred as pBlu10-S-P-sgT-Tdk.

The *adhE* gene was targeted by thermostable Cas9 based gene deletion system. Based on *adhE* sequence (GenBank: AEYS01000007.1), 21nt *adhE* gene-targeting spacer was amplified from pBlu10-S-P-sgT via PCR with primers pgE1 and pgE2. The resulting vector was referred as pBlu10-S-P-sgE.

The *adhE* donor DNA was amplified from pBlu-adhE$_{232}$ via PCR with primers E1 and E2. The *adhE* donor DNA fragments were phosphorylated and subcloned into pBlu10-S-P-sgE with primers pSC1 and pSlay1. The resulting vector was referred as pBlu10-S-P-sgE-adhE.

Meanwhile, the control plasmid pBlu10-S-P-sg-adhE was constructed by PCR amplification using pBlu10-S-P-sgE-adhE as template with the following primers pPat2 and pC1. The plasmid pBlu10-S-P-sg-adhE contained Cas9 gene expression element, *adhE* donor DNA, and sgRNA, but no 21nt *adhE* gene-targeting spacer.

The control plasmid pBlu10-sgE-adhE was constructed by PCR amplification using pBlu10-S-P-sgE-adhE as template with the following primers E1 and pC2. The plasmid pBlu10-sgE-adhE contained 21nt *adhE* gene-targeting spacer, sgRNA and *adhE* donor DNA, but no Cas9 gene expression element.

The *rsp* gene was targeted by thermostable Cas9 based gene deletion system. Based on *rsp* sequence (GenBank: GU586295.1), 21nt *rsp* gene-targeting spacer was amplified from pBlu10-S-P-sgT via PCR with primers pgRSP1 and pgRSP2. The resulting vector was referred as pBlu10-S-P-sgR.

The *rsp* donor DNA was amplified from pBlu-RSP$_{582}$ via PCR with primers R1 and R2. The *rsp* donor DNA fragments were phosphorylated and subcloned into

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pBlu10-S-P-sgR with primers pSC1 and pSlay1. The resulting vector was referred as pBlu10-S-P-sgR-RSP.

Transformation of *Thermoanaerobacter ethanolicus* JW200

Transformation of *T. ethanolicus* was performed via a natural competence protocol as described by Shaw (19). Natural competence transformations were conducted in an anaerobic chamber by inoculation of 3 mL medium with 50 µL cultures and 500 ng plasmids in hungate anaerobic culture tube. The tubes were then incubated at 65°C for 6 to 8 h. Up to 200 µL transformation cultures were mixed with 3mL liquid medium at 65°C containing 300 mg/L kanamycin sulfate. For selection of mutants, up to 200 µL transformed culture was mixed with 3 mL medium with 2.5 % (w/v) agar supplemented with 300 mg/L kanamycin sulfate followed by plating on hungate anaerobic tube, and further incubated at 65°C. Several colonies were analyzed by a bacterial colony PCR to confirm that the desired mutation was present.

Plasmid curing

The method of plasmid curing was described by Liu (38). In order to remove the thermostable Cas9 based gene deletion plasmids from edited cells, the edited colony was inoculated into 3 mL of medium without kanamycin sulfate and cultivated at 65°C for 15 h. The culture was serially transferred into fresh medium with an inoculum size of 0.01% (v/v) for several times without antibiotics before plating on hungate anaerobic culture tube. Colonies were confirmed as cured by determining their sensitivity to kanamycin sulfate.

Sanger sequencing and data analysis

The PCR products were cloned into pMD18-T vector using high-fidelity Ex-Taq DNA polymerase (TaKaRa, Dalian, China) and were sequenced by Sangon Biotech Co. Ltd. (Shanghai, China).

Analytical techniques

Fermentation products: acetate, lactate and ethanol were analyzed high pressure liquid chromatography (HPLC) (Shimadzu, Kyoto, Japan) with an Aminex HPX-87H column (Bio-Rad, Hercules, CA). The column was eluted at 50°C with 0.25 g/L H_2SO_4 at a flow rate of 0.4 mL/min. Acetate, lactate and ethanol were detected by a

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RID-10A refractive-index detector (Shimadzu).

Hydrogen was determined by gas chromatography (GC7900, Tianmei, Shanghai) with a TDX 01 80/100 mesh column using a thermal conductivity detector and nitrogen carrier gas.

SUPPLEMENTAL MATERIAL

SUPPLEMENTAL FILE

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (grant numbers 31300088, 31772529), the National Key R&D Program of China (2018YFE0107100) and Doctoral Scientific Research Foundation of Jiangsu University (Grant No.10JDG117)

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809 Figure legends

810 Fig. 1 Maps of shuttle vectors pBlu10-Htk. Ampr: apramycin resistance cassette;
811 Thermo ori: a thermostable Gram-positive origin of replication; Kanr: a thermostable
812 kanamycin resistance marker (Htk); pUC ori: the pUC origin of replication; MCS:
813 multiple clone site; RBS: Ribosome-binding site.

814

815 Fig. 2 The transformation efficiency of plasmids
816 A: pBlu10-Htk; B: pBlu10-P-sgT; C: pBlu10-Htk-Cas9; D: pBlu10-Slay-Cas9; E:
817 pBlu10-S-P-sgT; F: pBlu10-S-P-sgT-Tdk. Three replicates were performed for each
818 DNA sample.

819

820 Fig. 3 Thermostable Cas9-based partial *tdk* gene deletion and insertion
821 A: Schematic overview of design and screening for editing *tdk* gene
822 B: Agarose gel electrophoresis showing the resulting products from genome-specific
823 PCR on colonies from the thermostable Cas9-based *tdk* deletion process from the
824 genome of *T. ethanolicus* JW200. Two out of 8 colonies (Line T1, 6) were clean Δ*tdk*
825 mutants, and Line T2 was mixed wild type/Δ*tdk* genotype. About 1200 bp fragment
826 was amplified from wild type JW200 genomic DNA via PCR with primers T5 and T6
827 (Control: Line C). As 297 bp of partial *tdk* gene was deleted, about 800 bp fragment
828 was amplified from Δ*tdk* genomic DNA via PCR with primers T5 and T6 (Line T1
829 and 6). Lanes: M, DNA marker; C, control, PCR amplification was carried out by
830 using wild type JW200 as template; T1, T2 and 1-6, PCR amplification was carried
831 out using bacterial colony as template.
832 C: Partial sequence of *tdk* gene, 21nt *tdk* gene-targeting spacer: 5´-ATGTGGC
833 AATCCTGCTA CTCG-3´; PAM sequence: 5´-NNNNCAAA-3´.

834

835 Fig. 4 Thermostable Cas9-based partial *adhE* gene deletion and insertion
836 A: Schematic overview of design and screening for editing *adhE* gene;
837 B: Plasmid pBlu10-S-P-sgE-adhE was transformed into *T. ethanolicus* JW200;
838 C: Colonies appeared on the agar anaerobic tubes with 300 mg/L kanamycin sulfate;

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D: Agarose gel electrophoresis showing the resulting products from genome-specific PCR on colonies from the thermostable Cas9-based *adhE* deletion process from the genome of *T. ethanolicus* JW200. Three out of 12 colonies (Line E1, 6 and 8) were clean Δ*adhE* mutants. Other three different colonies (Line E2, 7 and 9) had a mixed wild type/Δ*adhE* genotype. About 1400 bp fragment was amplified from wild type JW200 genomic DNA via PCR with primers E5 and E6 (Control: Line C). As 232 bp of partial *adhE* gene was deleted, about 1180 bp fragment was amplified from Δ*adhE* genomic DNA via PCR with primers E5 and E6 (Line 6, 8). Lanes: M, DNA marker; C, control, PCR amplification was carried out by using wild type JW200 as template; E1, E2 and 1-10, PCR amplification was carried out using bacterial colony as template.

Fig. 5 Thermostable Cas9-based partial *rsp* gene deletion

Agarose gel electrophoresis showing the resulting products from genome-specific PCR on colonies from the thermostable Cas9-based *rsp* deletion process. Colonies (Line 2, 3, 5, 6, 7, 9) were mixed wild type/Δ*rsp* genotype, the others (Line 1, 4, 8) were clean Δ*rsp* mutants. About 1700 bp fragment was amplified from wild type JW200 genomic DNA (Control: Line C1 and C2). As 582 bp of partial *rsp* gene was deleted, about 1100 bp fragment was amplified from Δ*rsp* genomic DNA. Since the colony PCR was performed using primers R5 and R6, a small nonspecific band (about 200 bp DNA fragment) was amplified in all samples including the negative control wild type JW200 and Δ*rsp* mutants. For this reason, three bands were amplified from the mixed genotypes. Lanes: M, DNA marker; C1 and C2, control, PCR amplification was carried out using wild type JW200 as template; 1-9, PCR amplification was carried out using bacterial colony as template.

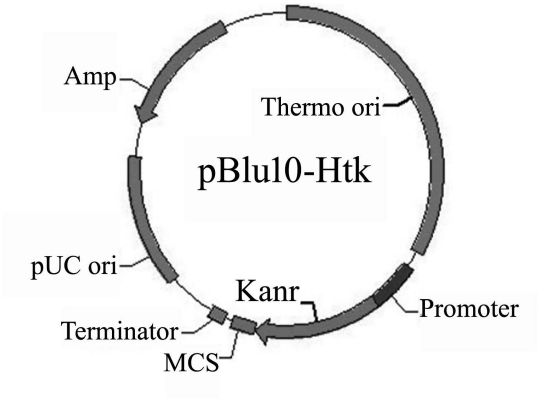
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pBlu10-Htk Multiple Cloning Site Region:

TGACATTAAG AAAGGTGGTT TTTATGCTCGAGGAGCTCGGTACCGGATCCTCTAGATAGT

RBS *Xho* I *Sac* I *Kpn* I *BamH* I *Xba* I

ACCTAGATTTAGATGTCTAAAAAGCTTTTTAGACATCTAATCTTTTCTGAAGTACATCCG

Terminator

CAACTGTCCA TACTC

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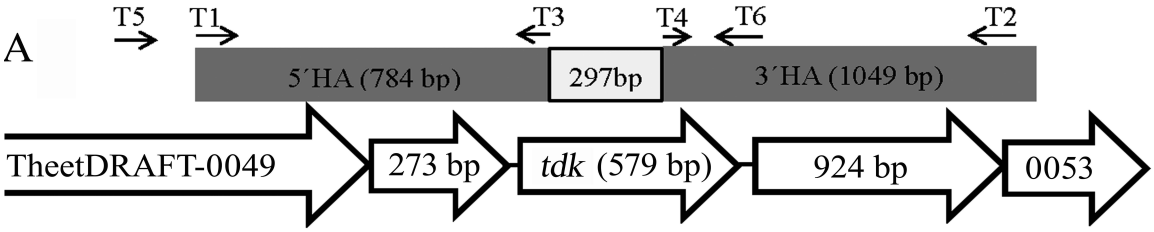
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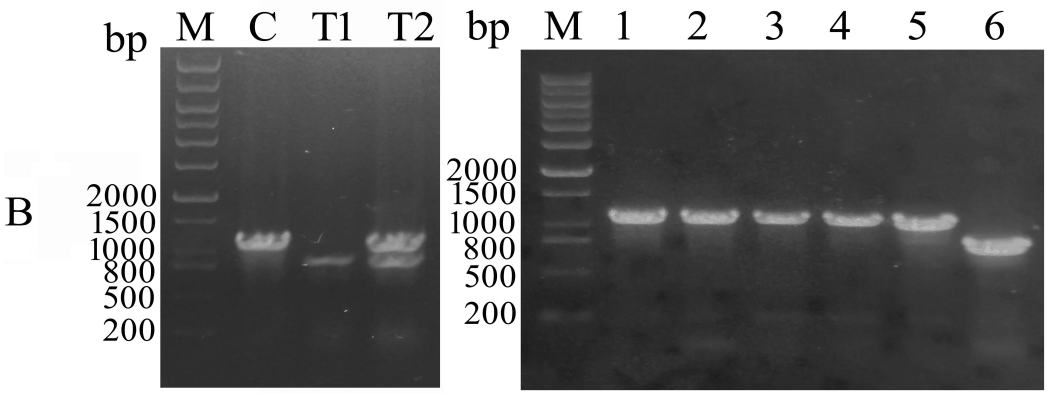
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A



B



C

5′ .....AAGAGAAATTGCAGATCTAGGCAAAAGAGTCATTTGTGCTGGCTTAGATATGGAC
TTTAGGGGGGAACCTTTTGGCCCTACTCCTGATATAATGGCAATTGCGGAGTCTGTAGAT
AAACTGACGGCTATATGTGTGAAATGTGGCAATCCTGCTACTCGCACTCAAAGGCTTATC
AATGGGAAACCTGCAAAATACGATGACCCAATCATTTTAGTAGGGGCCCATGAAACCTAT
GAAGCAAGGTGTCGAAAATGCCATGAAGTTCCTCGT.....3′

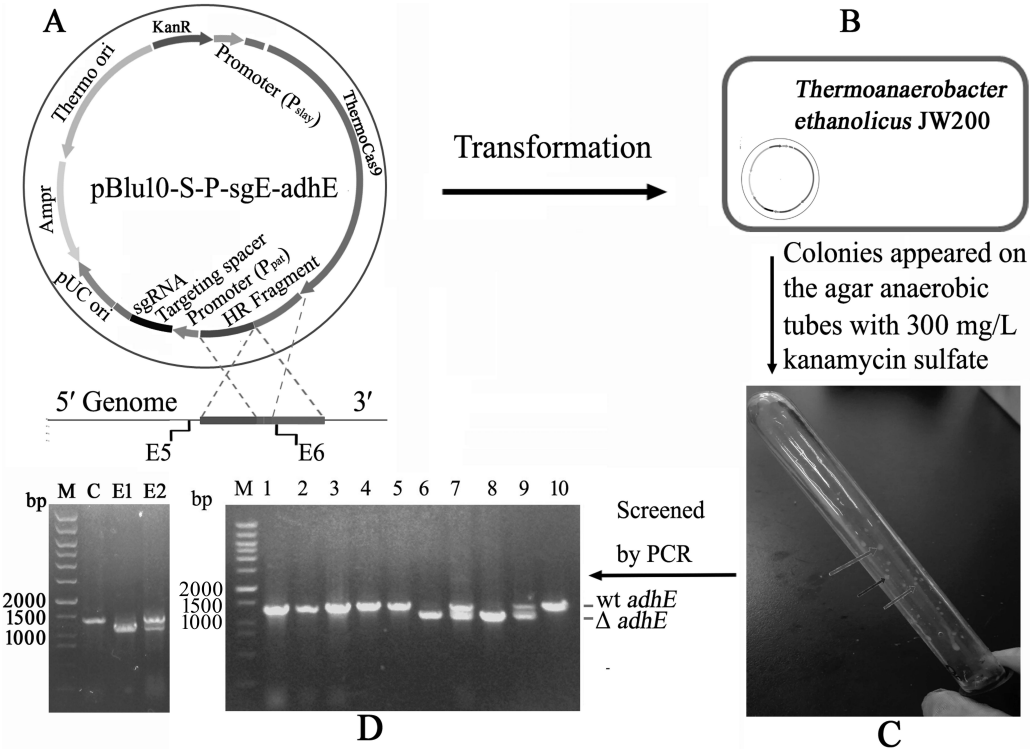
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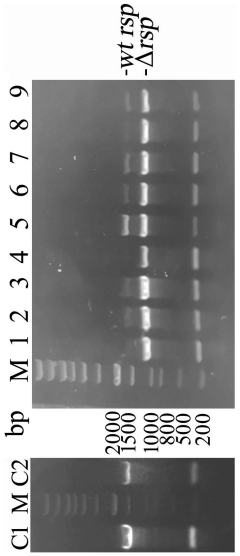
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Table 1 List of oligonucleotides used in this study

Oligonucleotide	Sequence (5′ → 3′)	Purpose
pB1	CAACCCTATCTCGGTCTATTC	PCR amplification
pB2	AGAACATGTGAGCAAAAGGCC	PCR amplification
pS1	AATTGACAAAGTTTTCTATTTG	PCR amplification of the synthesized gene
pS2	GAGTATGGACAGTTGCGGATG	PCR amplification of the synthesized gene
pCas1	GTGCTCGAGAGATACAAAATCGGCCTTG	PCR amplification of Cas9 gene
pCas2	GGGTCTAGATCAATCACGAGTTGATTGTAAC	PCR amplification of Cas9 gene
pCas3	GGTGAATTCCATTAAGAAAGGTGGTTTTTATG	PCR amplification of Cas9 gene and plasmid full sequence
pCas4	ATGAGATACAAAATCGGCCTTGATATC	PCR amplification of Cas9 gene and plasmid full sequence
pSlay1	TTTGAATTCGTGAGATACCCTTGGAACAG	PCR amplification of S-layer promoter
pSlay2	TTTGATATCACAGTCAATCCTCCTCCTTG	PCR amplification of S-layer promoter
psgRNA1	GTGCTCGAGTAGTACCTAGATTTAGATGTC	PCR amplification of 21nt *tdk* gene-targeting spacer and sgRNA
psgRNA2	CCCTCTAGATGCGCAAGGATGGGG	PCR amplification of 21nt *tdk* gene-targeting spacer and sgRNA
p1	TTTGAATTCGAGTATGGACAGTTGC	PCR amplification
p2	ATGTGGCAATCCTGCTACTCG	PCR amplification
pPat1	TTTGAATTCGCGAAATTTTCAGCTGTAGAG	PCR amplification of Pat promoter
pPat2	GGGGATATCTTTAATTTTATCACTATAATATTG	PCR amplification of Pat promoter
T1	GTACAAGAAAAGAAGAATTGC	PCR amplification of Homology Arm of *tdk*
T2	TTGTCTAGACAAACAGTAAGCAAGGAGCAG	PCR amplification of Homology Arm of *tdk*
T3	GTTCTCGAGCATTGCGATAACCTCTGTGTCTTC	PCR amplification of donor DNA sequences of *tdk*
T4	AGAGATATCCCATGAAGTTCCTCGTACATAG	PCR amplification of donor DNA sequences of *tdk*
T5	AGAACGAAGGATATTCCCTGC	For screening
T6	GGTTTTCTTCATCTCTACACC	For screening

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E1	AGAAAAAACAGAAGTGAAAGAAAC	PCR amplification of Homology Arm of *adhE*
E2	AATGGATCCTATGCATGAAGCATTGTGCATC	PCR amplification of Homology Arm of *adhE*
E3	ATGCAAGTATTGGACTTAGCTTTTC	PCR amplification of donor DNA sequences of *adhE*
E4	ATGGAAGGCATAAAAAGATGTGAAG	PCR amplification of donor DNA sequences of *adhE*
E5	TGCCTAACTTATTACAAGAAAG	For screening
E6	GGTGGAACTCTGAACCATTTC	For screening
pSC1	AATGGATCCATCCTCGAGCATAAAAACCACC	PCR amplification
pSC2	TATAGGCCTAGCGCCAAGAAATAGTAGTAC	PCR amplification
pgE1	GTGTATAAAGATCTTAATTTTATCACTATAATATTG	PCR amplification
pgE2	AGTCAAAGATTGTCATAGTTCCCCTGAGAAATC	PCR amplification
pC1	AAGGATTAAAAGGTCATAGTTCCCCTGAGAAATC	PCR amplification
pC2	GTTACAATCAACTCGTGATTG	PCR amplification
R1	AAAACTGCAGAGACAAATTAGGTCCTTCTATAG	PCR amplification of Homology Arm of *adhE*
R2	CCGCTCGAGTCCTCCATATTCCTTTGGATATG	PCR amplification of Homology Arm of *adhE*
R3	AATAGCCATTGATACTATAGTCTT	PCR amplification of donor DNA sequences of *rsp*
R4	CGCGGATCCAGATTAAATGAAGAAGAGCTC	PCR amplification of donor DNA sequences of *rsp*
R5	GTTAAGTAATTTAAGTGAACC	For screening
R6	AGAGCTCTTCTTCATTTAATC	For screening
pgRSP1	GAAATACGCTATCTTAATTTTATCACTATAATATTG	PCR amplification
pgRSP2	TTCTAGAGAATGTCATAGTTCCCCTGAGAAATC	PCR amplification

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Table 2 List of plasmids used in this study

Plasmid	Features	References
pBluescript II SK(+)	*E. coli* cloning vector	[39]
pBlu10-Htk	The *E. coli- Thermoanaerobacter ethanolicus* shuttle expression vector	This work
pET-28a(+)-Cas9	Cas9 gene between *Xho* I and *Xba* I sites in pET-28a(+)	This work
pBlu10-Htk-Cas9	*Cas9 gene* between *Xho* I and *Xba* I sites in pBlu10-Htk	This work
pBlu10-Slay-Cas9	Thermostable Ca9 expression under the control of promoter S-layer	This work
pET-28a(+)-sgT	21nt *tdk* gene-targeting spacer and sgRNA between *Xho* I and *Xba* I sites in pET-28a(+)	This work
pBlu10-sgT	21nt *tdk* gene-targeting spacer and sgRNA between *Xho* I and *Xba* I sites in pBlu10-Htk	This work
pBlu10-P-sgT	21nt *tdk* gene-targeting spacer and sgRNA expressing module under the control of Pat promoter	This work
pBlu-Tdk	Containing 5´ and 3´ flanking fragments and the Full-length *tdk* gene	This work
pBlu-Tdk$_{297}$	Containing 5´ and 3´ flanking fragments and partial *tdk* segments and the insertional restriction enzyme site *Xho* I and *EcoR* V	This work
pBlu-adhE	Containing 5´ and 3´ flanking fragments the Full-length *adhE* gene	This work
pBlu-adhE$_{232}$	Containing 5´ and 3´ flanking fragments, partial *adhE* segments and the insertional restriction enzyme site *Nde* I	This work
pBlu10-S-P-sgT	Containing Cas9 gene expression element, 21nt *tdk* gene-targeting spacer and sgRNA expressing module under the control of Pat promoter	This work
pBlu10-S-P-sgT-Tdk	Containing Cas9 gene expression element, 21nt *tdk* gene-targeting spacer, sgRNA expressing module and *tdk* donor DNA	This work
pBlu10-S-P-sgE	Containing Cas9 gene expression element, 21nt *adhE* gene-targeting spacer and sgRNA expressing module under the control of Pat promoter	This work
pBlu10-S-P-sgE-adhE	Containing Cas9 gene expression element, 21nt *adhE* gene-targeting spacer, sgRNA expressing module under the control of Pat	This work

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	promoter, and *adhE* donor DNA	
pBlu-RSP	Containing 5´ and 3´ flanking fragments the Full-length *RSP* gene	This work
pBlu-RSP$_{582}$	Containing 5´ and 3´ flanking fragments, partial *rsp* segments and the insertional restriction enzyme site *Nde* I	This work
pBlu10-S-P-sgR	Containing Cas9 gene expression element, 21nt *rsp* gene-targeting spacer and sgRNA expressing module under the control of Pat promoter	This work
pBlu10-S-P-sgR-RSP	Containing Cas9 gene expression element, 21nt *rsp* gene-targeting spacer, sgRNA expressing module and *RSP* donor DNA	This work
pBlu10-S-P-sg-adhE	Containing Cas9 gene expression element, sgRNA expressing module and *adhE* donor DNA, but no *adhE* gene-targeting spacer.	This work
pBlu10-sgE-adhE	Containing 21nt *adhE* gene-targeting spacer, sgRNA expressing module and *adhE* donor DNA, but no Cas9 gene expression element	This work

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Table 3 Fermentation profile of wild type JW200, Δ*rsp* and Δ*adhE* mutants using cellobiose as substrates

Strains	Lactate (μmol/mL)	Acetate (μmol/mL)	Hydrogen (μmol/mL)	Ethanol (μmol/mL)	Maximal OD_{600}
JW200	18.32±0.88	3.21±0.46	10.75±1.29	13.71±0.21	1.24±0.15
Δ*rsp*	18.31±0.59	3.29±0.25	11.65±0.55	10.14±0.84	1.04±0.2
Δ*adhE*	8.85±0.41	11.39±0.79	30.55±1.23	1.11±0.04	0.67±0.16

The fermentation was performed in a 5 ml culture of prereduced medium containing 0.45% yeast extract with a starting pH of 7.0, and under an atmosphere of N_2 at 65°C. Three replicates were performed for each sample.

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Table 4 Fermentation profile of wild type JW200, Δ*rsp* and Δ*adhE* mutants using mannitol as substrates

Strains	Lactate (μmol/mL)	Acetate (μmol/mL)	Hydrogen (μmol/mL)	Ethanol (μmol/mL)	Maximal OD_{600}
JW200	11.19±0.1	0.05±0.02	20.51±0.11	20.53±0.64	0.526±0.08
Δ*rsp*	15.22±0.7	0.1±0.06	25.85±2.12	19.61±2.1	0.426±0.11
Δ*adhE*	1.21±0.25	8.81±1.3	33.22±3.1	0.16±0.15	0.32±0.09

The fermentation was performed in a 5 ml culture of prereduced medium containing 0.45% yeast extract with a starting pH of 7.0, and under an atmosphere of N_2 at 65°C. Three replicates were performed for each sample.

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