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RecET recombination system driving chromosomal target gene replacement in *Zymomonas mobilis*

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ABSTRACT

Background: Zymomonas mobilis is a Gram-negative microaerophilic bacterium with excellent ethanol-producing capabilities. The RecET recombination system provides an efficient tool for direct targeting of genes in the bacterial chromosome by PCR fragments.
Results: The plasmids pSUZM2a-RecET and pSUZM2a-RecE588T were first developed to co-express RecE or RecE588 and RecT for homologous recombination. Thereafter, the PCR fragments of the tetracycline resistance marker gene flanked by 60 bp of *adhA* (alcohol dehydrogenase I) or *adhB* (alcohol dehydrogenase II) homologous sequences were electroporated directly into ZM4 cells harboring pSUZM2a-RecET or pSUZM2a-RecE588T. Both *adhA* and *adhB* were replaced by the tetracycline resistance gene in ZM4, yielding two mutant strains, *Z. mobilis* ZM4 Δ*adhA* and *Z. mobilis* ZM4 Δ*adhB*. These two mutants showed varying extent of reduction in ethanol production, biomass generation, and glucose metabolism. Furthermore, enzyme activity of alcohol dehydrogenase II in *Z. mobilis* ZM4 Δ*adhB* exhibited a significant reduction compared to that of wild-type ZM4.
Conclusion: This approach provided a simple and useful method for introducing mutations and heterologous genes in the *Z. mobilis* genome.

1. Introduction

Zymomonas mobilis is a Gram-negative and microaerophilic bacterium with excellent ethanol-producing capabilities owing to the presence of the unique Entner–Doudoroff (ED) pathway [1,2]. In addition, *Z. mobilis* is of considerable interest for industrial applications since it consumes and processes sugars rapidly, exhibits low biomass production and high ethanol tolerance, and does not require controlled addition of oxygen during fermentation [3,4]. However, the fermentable carbohydrate substrates of *Z. mobilis* are limited to simple sugars such as glucose, fructose, and sucrose, which seriously hinder its application in industry [4]. Advancements in complete genome sequencing of *Z. mobilis* strains [3,4,5], DNA microarray analyses [6], and other molecular biology studies [7], have enhanced the understanding of *Z. mobilis* gene function and genome structure, and provided new strategies for various biotechnological applications [8]. Recent studies have focused on the genetic manipulation of *Z. mobilis* strains using plasmid vectors, expression systems, and gene knockouts for expanding its spectrum of fermentable substrates [9,10]. Genetic engineering of *Z. mobilis* has produced strains that are able to use xylose, starch, and cellulose as substrates [11,12,13,14]. Therefore, chromosomal gene manipulation in *Z. mobilis* is required for further improving its fermentation capability.

A number of gene replacement methods are used to engineer bacterial chromosomal genes. Among these methods, the RecET recombination system-based "ET cloning" developed by Zhang et al. [15,16,17] provides an efficient tool for directly targeting genes in the bacterial chromosome by PCR fragments. This method utilizes homologous recombination mediated by RecE and RecT. The former is a 5′-3′ exonuclease, and the latter is a DNA annealing protein. Using RecET recombination, double-stranded DNA (dsDNA) or single-stranded DNA (ssDNA) with short flanking sequences (30–60 bp) homologous to the target site are introduced into cells expressing RecET. The dsDNA is digested by RecE to produce single-stranded overhangs, which are subsequently bound by RecT, and a homologous recombination event follows at the target site. Compared to the frequently-used suicide plasmid-based chromosomal gene inactivation or FLP-FRT site-specific recombination in *Z. mobilis* [18,19], the RecET recombination system does not require a large number of vector constructions, and is easier and labor-saving. The utilization of RecET recombination has been reported in many microorganisms [20,21,22].

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More recently, scientists have successfully cloned a 19 kb gene cluster into an *Escherichia coli* plasmid [23], suggesting that ET recombination was also an efficient tool for engineering secondary metabolite biosynthetic pathways. However, reports about the use of RecET recombination in *Z. mobilis* are lacking. In this study, we introduced RecET-encoding genes in *Z. mobilis* using an *E. coli–Z. mobilis* shuttle expression vector, and promoted efficient homologous recombination between the ZM4 genomic loci and PCR fragments, which resulted in direct gene knock-out. These findings suggested that the RecET recombination system could be used as a powerful tool for genetic manipulations in *Z. mobilis*.

2. Materials and methods

2.1. Bacterial strains, plasmids, and culture conditions

E. coli DH10B was used for cloning *recET* from its genomic DNA. *E. coli* JM109 (*recA supE endA hsdR gyrA relA thi* Δ(*lac-proAB*) F′ *tra* Δ*proAB lacI*^{q} *lacZdM15*) was used for the construction and propagation of the RecET expression plasmid after culturing in Luria-Bertani (LB) medium at 37°C. The wild-type *Z. mobilis* ZM4 (ATCC31821) was used as the host of RecET-mediated homologous recombination. Wild type ZM4 and its mutant derivatives were cultured statically in rich medium (RM) (2% glucose, 1% yeast extract, 0.2% KH_2PO_4, pH 7.0) at 30°C. Agar (1.5%) was added to obtain solid media. Kanamycin (100 μg/mL) was used for *E. coli*, whereas a relatively high kanamycin concentration of 200 μg/mL and 20 μg/mL tetracycline were used for *Z. mobilis* since it is inherently resistant to a variety of antibiotics. The pSUZM2a (5 kb) shuttle expression vector was used for expressing RecE and RecT [24]. This vector contains the kanamycin resistance marker gene from pUC18, an *E. coli* replication origin (Ori E) from pUC18, a *Z. mobilis* replication origin (Ori Z) from the native plasmid pZZM401 of ZM4, and a P_{pdc} promoter of the pyruvate decarboxylase genes (ZMO1360) of ZM4. Therefore, pSUZM2a could be used for continuous protein expression and propagation in both *E. coli* and *Z. mobilis* ZM4 [24]. Plasmid pBR322 was used for cloning the tetracycline resistance gene.

2.2. Manipulation of DNA

E. coli genomic DNA, plasmids, and gel-extracted DNA were prepared using the Omega kit (Omega Bio-Tek, Guangzhou, China). PCR products and DNA bands were separated by electrophoresis on a 1.0% agarose gel. Transformation of *E. coli* was performed using 200-μL aliquots of competent cells prepared using the calcium chloride method. PrimeSTAR Max DNA polymerases were purchased from Takara (Takara Biotechnology, Shanghai, China). Restriction endonucleases and T4 DNA polymerases were purchased from Fermentas (Thermo Scientific, Shanghai, China). DNA sequencing was performed using the dideoxy chain-termination method (TSINGKE Biological Technology, Chengdu, China). Primers used in this study are listed in Table 1.

2.3. Construction of the RecET expression plasmid pSUZM2a-RecET and pSUZM2a-RecE588T

A sequence and ligation-independent cloning (SLIC) method [25,26] was used for constructing pSUZM2a-RecET and pSUZM2a-RecE588T. First, the genomic DNA of *E. coli* DH10B was used as a template for amplifying full length *recET* (the 3′ end of *recE* overlapped by 8 bp with the 5′ end of *recT* in the genome) and truncated *recE588T* (sequence encoding 588 amino acid residues at the 5′-terminus of *recE* was truncated) using primer pairs ET F/ET R and E588T F/E588T R (Table 1). Then, the vector pSUZM2a was amplified using primer pairs pET F/pET R and pE588T F/pE588T R (Table 1), generating the pET and pE588T linear vector, which contained sequences at either ends that were homologous to *recET* and *recE588T*, respectively. After the purification of the PCR products, pET and *recET* or pE588T and *recE588T* were treated with T4 DNA polymerase to generate overhangs, followed by incubation of the linear vectors and the *recET* genes to promote single-strand annealing. Finally, the products were transformed into *E. coli* JM109 and the cells were spread on LB solid medium containing 100 μg/mL of kanamycin. Positive transformants were identified by colony PCR and restriction endonuclease digestion. Finally, *recET* and *recE588T* were individually cloned into pSUZM2a to generate pSUZM2-RecET and pSUZM2-RecE588T, respectively (Fig. 1).

2.4. Transformation of Z. mobilis

Linear PCR fragments or plasmids pSUZM2-RecET and pSUZM2-RecE588T were transformed into *Z. mobilis* ZM4 by electroporation [13]. The *Z. mobilis* ZM4 strain was initially pre-cultured statically in 3-mL RM overnight at 30°C. Fifty milliliters RM was inoculated with 1% pre-cultured cells; the cells were harvested by centrifugation at

Table 1
Oligonucleotide primers used in this study.

Primer	Nucleotide sequences (5′-3′)[a]	Amplification specificity
pET F	TGTAATCGATAATTCAGAGGAATAAAGGTAGCTTGCAGTGGG	Linear pSUZM2a vector homologous with RecET gene
pET R	GGAAGAGTGGTTTTGTGCTCATTGCTTACTCCATATAT	
pE588T F	TGTAATCGATAATTCAGAGGAATAAAGGTAGCTTGCAGTGGG	Linear pSUZM2a vector homologous with RecE588T gene
pE588T R	TTCTACGATTACGGGATCCATTGCTTACTCCATATAT	
ET F	ATATATGGAGTAAGCAATGAGCACAAAACCACTCTTCC	RecET gene
ET R	CCCACTGCAAGCTACCTTTATTCCTCTGAATTATCGATTACA	
E588T F	ATATATGGAGTAAGCAATGGATCCCGTAATCGTAGAA	RecE588T gene
E588T R	CCCACTGCAAGCTACCTTTATTCCTCTGAATTATCGATTACA	
A-F1	GAAAAAAGCTTGGATAGCGGCTTATAGCAACGCCACCTGACGTCTAAGAAAC	Tetracycline resistance gene with a portion of *adhA* gene homologous arms
A-R1	CGTTTTCCCTATATTCGCAAGATGTATGTCTGTTCTGCCAAGGGTTGG	
A-F2	TAGCGATCGCCGAATAGAAGGCATGAGAAGAAAAAAGCTTGGATAGCGG	Tetracycline resistance gene with 60 bp *adhA* gene homologous arms
A-R2	TAACTTTCTGGATCGTAATCGGCTGGCAATCGTTTTCCCTATATTCGCAAG	
B-F1	TGAGAAAACGTCTCGAAAACGGGATTAAAAGCCACCTGACGTCTAAGAAAC	Tetracycline resistance gene with a portion of *adhB* gene homologous arms
B-R1	TGACGGTAGGCTTAATAGCCTGTAAAAATTTGTGTTCTGCCAAGGGTTGG	
B-F2	GGTGATTTTACTCGTTTTCAGGAAAAACTTTGAGAAAACGTCTCGAAAACG	Tetracycline resistance gene with 60 bp *adhB* gene homologous arms
B-R2	TAATAGGCTTTAAATGGCAAATTATTTATGACGGTAGGCTTAATAGCCTG	
GA F	CGCTATGTTGAATATGGGCA	*adhB* gene with up and down stream sequence
GA R	CTCTCAATCCGCTGCCTT	
GB F	AGGCAAAATCGGTAACCACAT	*adhB* gene with up and down stream sequence
GB R	GCGGCTCAAATAAGACGATA	
Tet-in F	TATCGCCGACATCACCGATGGGGAA	Inside Tetracycline resistance gene
Tet-in R	CGAACGCCAGCAAGACGTAGCCCAG	

[a] The underlined sequences represent the homologous sites used construction of plasmids by SLIC method.

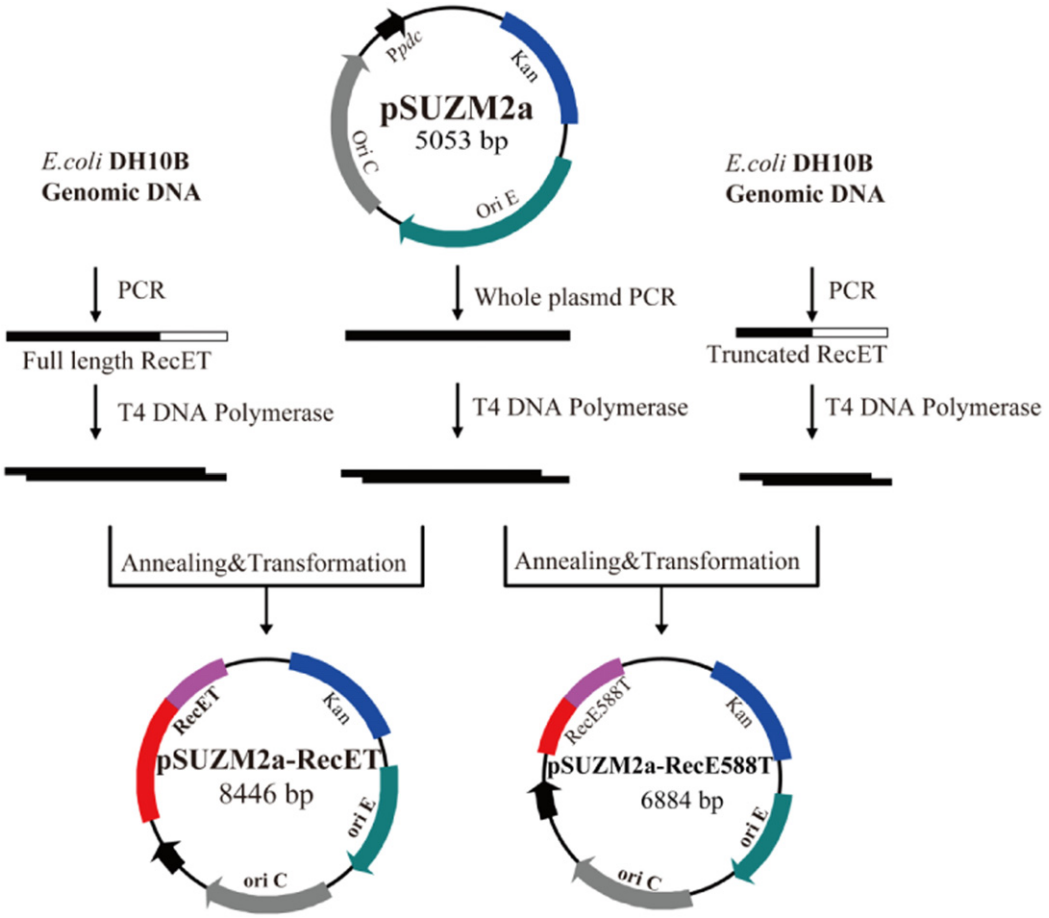

Fig. 1. Construction strategy of RecET expression plasmids in *Zymomonas mobilis* strain by SLIC.

4000 rpm for 10 min at 4°C when optical density $(OD)_{600nm} \approx 0.35–0.4$. Cells were washed thrice with 10% ice-cold glycerol (v/v) and finally resuspended in 1-mL ice-cold glycerol. An aliquot (100 μL) of the cell suspension was then transferred to a 0.2-cm electroporation cuvette, and 1 μg of plasmid DNA or 600-ng DNA fragment was added. A Bio-Rad gene pulser was used with the following conditions: 200 Ω, 50 μF, and 2.5 kV. After pulsing of the cell suspension, 3-mL RM was added to the cells, which were then incubated at 30°C for 3 h (recovery time was 16 h for electroporation of PCR fragments). Thereafter, 200-μL recovery culture was plated onto RM agar plates supplemented with corresponding antibiotics and incubated at 30°C for 3–4 d. Positive transformants were identified by colony PCR with corresponding primers.

2.5. Gene knock-out of adhA and adhB

A selectable marker attached to a short homology arm was required for targeting the chromosome of *Z. mobilis* ZM4. Here, we selected the tetracyclin resistance (*tet*^{r}) gene as the selectable marker as it was one of the few antibiotics that affected *Z. mobilis*. Thereafter, we selected two alcohol dehydrogenase genes, *adhA* (ZMO1236) and *adhB* (ZMO1596) located on the ZM4 chromosome, as target genes to be knocked-out using RecET-mediated homologous recombination. Two pairs of long oligonucleotides were designed for PCR amplification of every target gene to attach the 60-bp short homology arms flanked by the selectable marker (*tet*^{r} gene flanked by *adhA* homology arms). In the first PCR amplification using primers A F1/A R1, *tet*^{r} plus 30-bp long homology arms was amplified from pBR322, and the products were used as templates for amplification using primers A F2/A R2 in the second PCR. Finally, 60-bp homology arm-flanked *tet*^{r} was generated (Fig. 2). Linear PCR products of *tet*^{r} knock-out were purified for prompting homologous recombination in *Z. mobilis* harboring pSUZM2-RecET and pSUZM2-RecE588T; wild-type ZM4 was used as a control. The *Z. mobilis* mutants isolated on RM solid medium containing tetracycline were further identified by PCR using various combinations of primers to eliminate false positives.

2.6. Plasmid curing

After integration of *tet*^{r} into the selected chromosomal sites, pSUZM2-RecET or pSUZM2-RecE588T was no longer required for strain growth and application. Therefore the expression plasmids were cured using the following procedure. Individual ZM4 mutant colonies harboring pSUZM2-RecET or pSUZM2-RecE588T were inoculated in 3-mL RM antibiotic-free medium at 37°C (cultivation at elevated temperature). One percent of the cultures were transferred to fresh RM when log phase cultures started producing large amounts

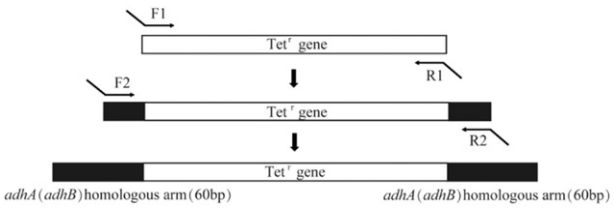

Fig. 2. Schematic representation of the amplify of *tet*^{r} gene used for gene knock-out for the first PCR, using plasmid pBR322 as template, to attach about 30-bp homologous arms on both sides of *tet*^{r} gene; the second round of PCR using above PCR products as template, complete the full 60-bp homologous arms.

of gas. This step was repeated for 20 generations, and final cultures were plated on tetracycline-containing RM solid after dilution. To verify whether the heterogeneous plasmids were still present in *Z. mobilis*, colonies appearing on tetracycline-plus RM agar plates were inoculated in RM solid medium containing 200 μL/mL of kanamycin as well as in the antibiotic-free medium as control. Colonies that grew on the antibiotic-free medium but not on the kanamycin-containing medium were considered as plasmid cured strains. Plasmid cured strains were further identified by PCR using primer pairs ET F/ET R or E588T F/E588T R (Table 1).

2.7. Growth and ethanol production

Wild type and mutant *Z. mobilis* were grown in 100-mL RM for 42 h at 30°C, and the glucose content in the growth medium was increased to 10%. After every 3 h, 3-mL aliquots were aspirated from the fermentation liquor for detecting biomass formation, and production of reducing sugar and ethanol. Cell growth was determined from the OD_{600} values. The concentrations of reducing sugar and ethanol were determined using a liquid chromatogram (LC) (Agilent 1200) equipped with a Hi-plex column, with 5-mM H_2SO_4 as the mobile phase. Column operating temperature of 35°C and a flow rate of 0.6 mL/min were used. Fermentations data of every transformant was repeated thrice.

2.8. ADH assays

To explore the expression of *adhB* in *Z. mobilis adhB* knock-out strains, alcohol dehydrogenase activity (reflected by the amount of NADH produced) was spectrophotometrically estimated at 340 nm. *adhB* knock-out strains were used for preparation of crude cell extracts. *Z. mobilis* cells from 1-mL fermentation liquor were washed with cold distilled water twice and re-suspended in 200-μL ice-cold 50-mM sodium pyrophosphate buffer (pH 8.8). The re-suspended cells were disrupted by sonication, and then the cell debris was removed by centrifugation at 12,000 rpm for 10 min at 4°C. The supernatant of the cell extract was used for determining enzymatic activity and protein concentration after appropriate dilution. Each assay reaction mixture for the oxidation reaction (1 mL) contained 750 μL of 50-mM sodium pyrophosphate buffer, 100 μL of fresh 15-mM β-NAD^+ (Sigma-Aldrich), 100 μL of 95% ethanol, and 50 μL of cell extract prepared as described previously. The enzyme reaction was carried out at 30°C in 1-cm cuvettes, and the reaction was followed by measuring the increase in absorbance at 340 nm for 10 min. One unit of enzyme activity (U) was defined as the amount of enzyme catalyzing the production of 1 mmol of NADH per minute under these conditions. Total protein concentration of each sample was assessed using the BCA protein assay kit (Vazyme).

3. Results and discussion

3.1. Construction of RecET expression plasmids pSUZM2a-RecET and pSUZM2a-RecE588T

We constructed two pSUZM2a-based shuttle expression vectors in this study. Cao et al. [23] reported that the first 588 amino acid of RecE were dispensable for its function, and therefore, RecE could be truncated at its N terminus. We used a PCR-based approach to eliminate the first 1762 bp of *recE* and constructed mutant *recE*, which encoded the truncated RecE588T. The recombination efficiencies of the full length RecET and the truncated RecE588T were compared. SLIC was used to insert *recET* and *recE588T* downstream to the P_{pdc} promoter in pSUZM2a (Fig. 1 and Fig. 3a). Agarose gel analyses of PCR and restriction enzyme digestion of pSUZM2a-RecET (8.4kbp) and pSUZM2a-RecE588T (6.8kbp) (Fig. 3b and Fig. 3c), indicated the successful construction of these two recombinant plasmids. After propagation in *E. coli* JM109, pSUZM2a-RecET and pSUZM2a-RecE588T were extracted and transferred to *Z. mobilis* ZM4 by electroporation, yielding two transformed strains named ZM4-ET and ZM4-E588T.

3.2. Construction of adhA (ΔadhA) and adhB (ΔadhB) knock-out strains

Tet^r cassettes targeting the chromosomal *adhA* and *adhB* of *Z. mobilis* were amplified using two rounds of PCR and transformed in the host strain ZM4-ET, ZM4-E588T, and ZM4 (control), respectively.

Approximately 600 ng of *tet*^{r} DNA was used for electroporation, and after 4 d of incubation, the *tet*^{r} knock-out strains were readily selected from plates containing both kanamycin and tetracycline. The authenticity of the *tet*^{r} knock-out and target gene knock-out mutants were confirmed using PCR with multiple primers in various locations of the target genes (Fig. 4a). Agarose gel electrophoresis of PCR products showed the expected results (Fig. 4b and Fig. 4c). Furthermore, sequencing of PCR products using primer pair GA F/GA R (genomic DNA of *adhA* knock-out mutant as template) or GB F/GB R (genomic DNA of *adhB* knock-out mutant as template) further confirmed that the mutants were created successfully (Fig. 5) upon replacement of the target *adhA* (1.1 kb) or *adhB* (1.5 kb) with *tet*^{r} by RecET-mediated homologous recombination. These two mutants were named as *Z. mobilis* ZM4 Δ*adhA* and *Z. mobilis* ZM4 Δ*adhB*, respectively.

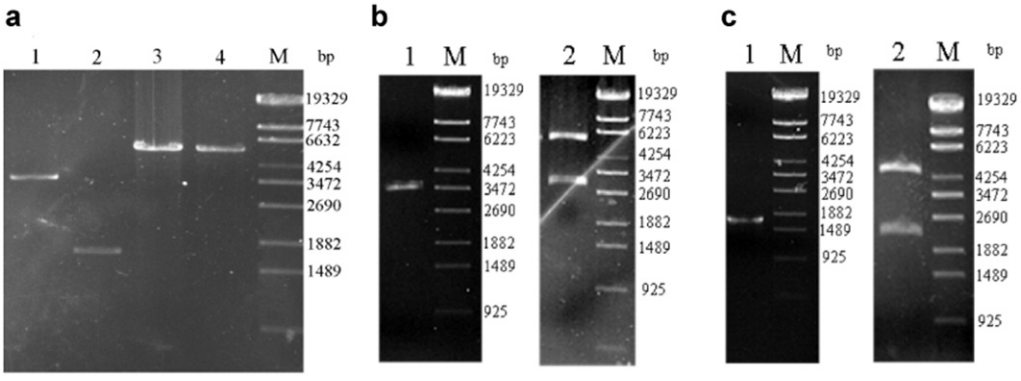

Fig. 3. PCR fragments and identification of RecET expression plasmids. (a) Lanes 1 and 2 represent RecET and RecE588T gene cloned from *E. coli* DH10B genomic DNA; Lanes 3 and 4 represent linear pSUZM2a PCR products with sequences homologous to RecET and RecE588T gene, respectively. (b) Lane 1, PCR identification of pSUZM2a-RecET by using primer pair ET F/ET; lane 2, pSUZM2a-RecET digested by *Eco*RI and *Nco* I. (c) Lane 1, PCR identification of pSUZM2a-RecE588T by using primer pair E588T F/E588T; lane 2, pSUZM2a-RecE588T digested by *Eco*RI and *Nco* I. M: λ-*Eco*T14 I digest DNA Marker.

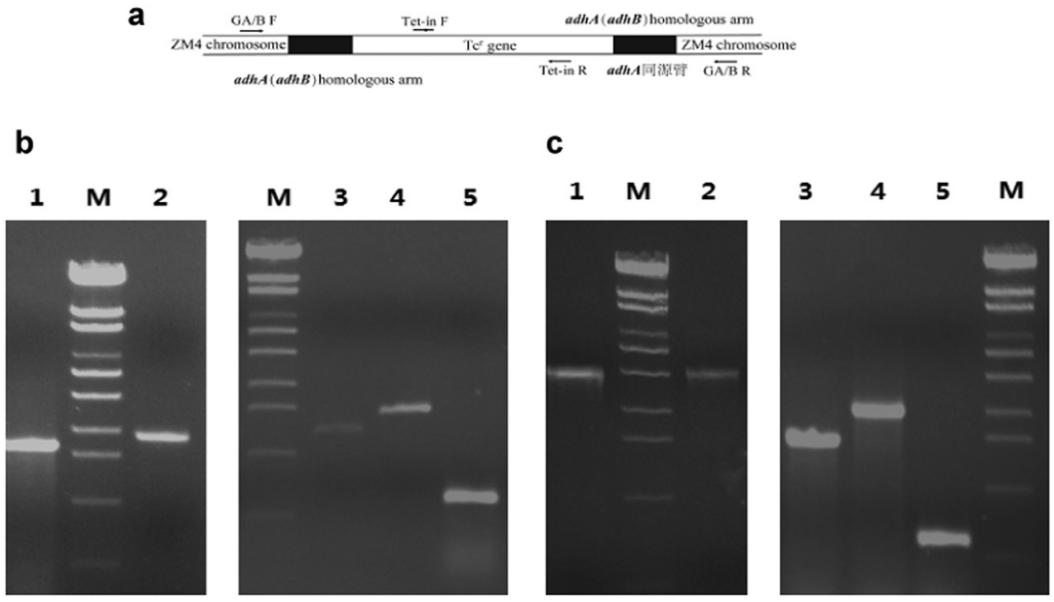

Fig. 4. Confirmation of *adhA* or *adhB* knock-out mutants by PCR. (a) Schematic illustration of the locations of primers is used in PCR; (b) agarose gel electrophoresis of the PCR products of *adhA* knock-out mutants. Lanes 2, 3, 4, 5: primers GA F/GA R, tet-in F/GA R, GA F/tet-in R, tet-in F/tet-in R for ZM4 *adhA* mutant, respectively. Lanes 1: primers GA F/GA R for wild-type ZM4; (c) agarose gel electrophoresis of the PCR products of *adhB* knock-out mutants. Lanes 2, 3, 4, 5: primers GB F/GB R, tet-in F/GB R, GB F/tet-in R, tet-in F/tet-in R for ZM4 *adhB* mutant, respectively. Lanes 5, 6, 7, 8: same primers above for wild-type ZM4.

3.3. Efficiency of the RecET recombination system

A previous report showed that the RecET recombination reaction in *E. coil* could be used for efficient gene cloning [15]. Seventy million colonies were obtained using 0.2-μg PCR product with 60-bp arms on selection medium, more than 95% of which were positive clones. In contrast, 6 ± 2 *tet*^{r} transformants were obtained in this study. This is similar to the result obtained using the site-specific FLP recombinase for the construction of a *Z. mobilis* mutant [26], in which only 5 transformants were obtained per μg of DNA. Therefore, we varied the homology arm lengths (0, 100, 150, and 200 bp) and used different amounts (0.5, 1, 5, and 10 μg) of PCR products for electroporation in the host strain; however, no significant changes were observed in the numbers of *tet*^{r} transformants.

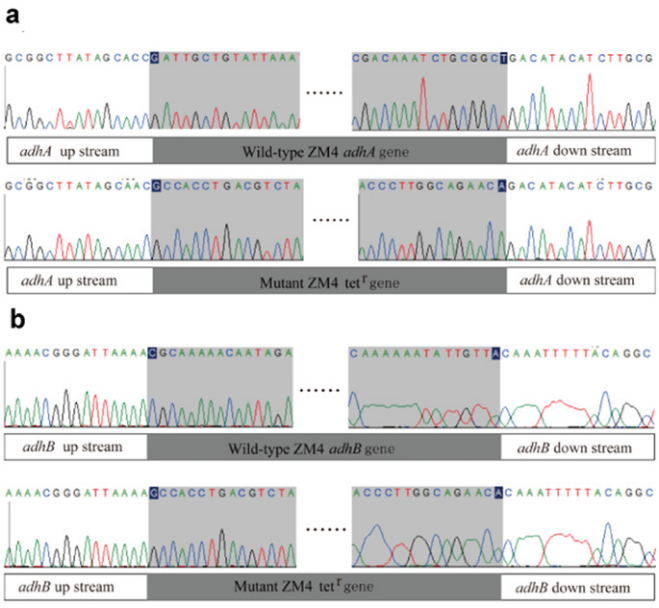

Fig. 5. Sequence analysis of *adhA* (a) and *adhB* (b) knock-out mutants at target locus. Shaded areas showing the sequences of target or *tet*^{r} gene in the wild-type ZM4 and the mutant.

Several explanations can account for the above result. First, the strong restriction modification (R-M) system of the ZM4 host cells may significantly reduce the transformation efficiency [27]. Kerr et al. [27] showed that inactivation of a putative R-M gene, *ZMO0028*, increased the transformation efficiency 60-fold when unmethylated plasmid DNA was used. Therefore, heterogeneous DNA may be recognized by the R-M system and undergo subsequent nuclease digestion; thus, increasing the amount of PCR products did not improve homologous recombination. Second, expression of RecET or RecE588T from pSUZM2a was not detected by SDS-PAGE analysis. However, the reporter gene encoding GFP was successfully expressed from this vector, indicating that the expression of RecET may be minimal, and therefore, beyond the limit of detection of our assay, in ZM4. The low levels of RecET resulted in insufficient production of the linear DNA required for homologous recombination. Interestingly, all the transformants obtained from tetracycline selective medium were positive recombinants, and there was no significant difference in the recombination efficiencies of RecET and RecE588T, which confirmed that the 5′ → 3′ exonuclease activity was preserved in the truncated RecE.

3.4. Effect of gene knock-out on growth, ethanol production, and enzyme activity

Growth rate, ethanol production, and reducing sugar utilization of wild-type ZM4 and the mutants were analyzed on RM containing 10% glucose. The RecET expression plasmids in Δ*adhA* and Δ*adhB* were cured in antibiotic-free medium, and the strains showing tetracycline resistance and kanamycin sensitivity were named as *Z. mobilis* ZM4 Δ*adhA*-1 and *Z. mobilis* ZM4 Δ*adhB*-1. These strains possess identical genetic background as the wild-type ZM4 except for the presence of *tet*^{r} in the chromosome. Inactivation of *adhA* impaired the growth capacity of the Δ*adhA*-1 strain, such that the strain developed a lag period of more than 3 h (Fig. 6). Maximum ethanol production, accumulated biomass, and glucose uptake rates were lower than those of the wild-type ZM4 control. A prolonged lag period occurred in *Z. mobilis* ZM4 Δ*adhB*-1; especially, the maximum ethanol accumulation

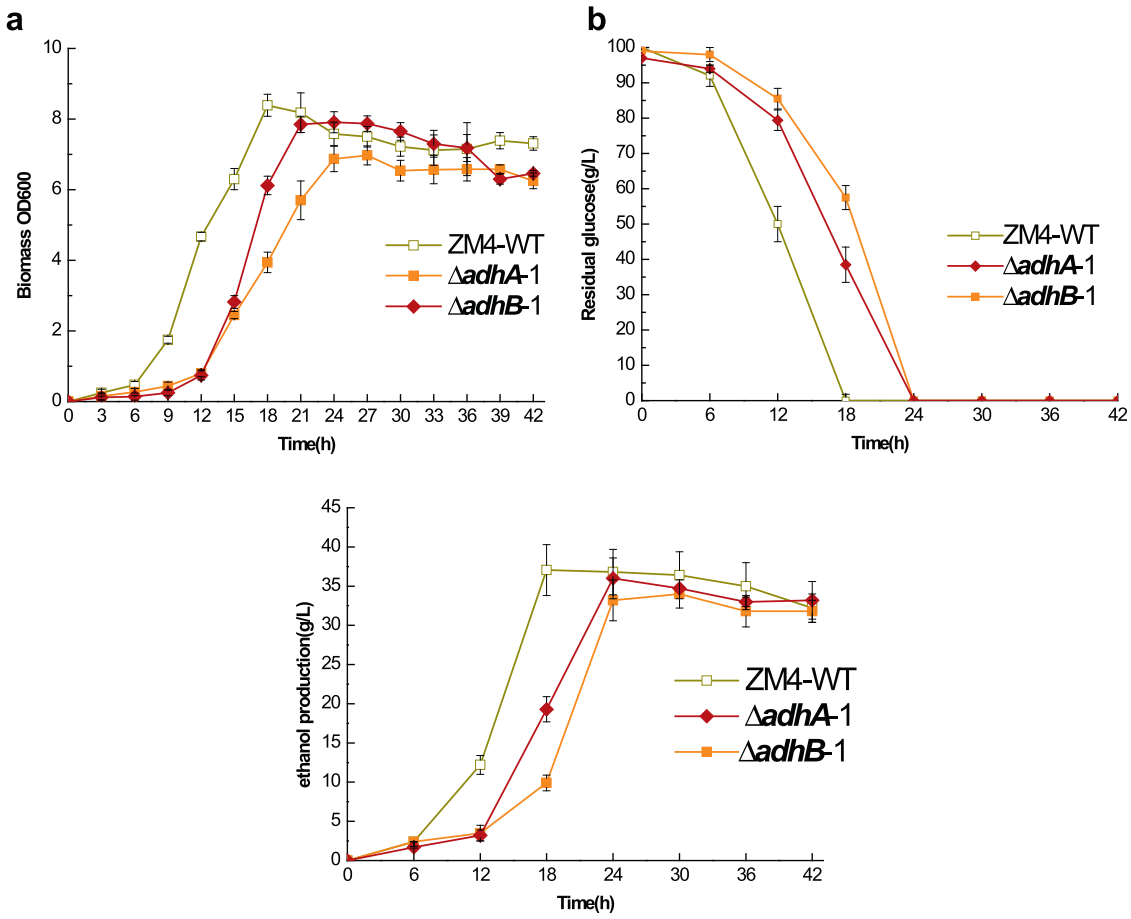

Fig. 6. Characterization of ZM4 and mutant strains under fermentative conditions in 100 g/L of glucose. Growth curves (a), residual glucose (b) and ethanol production (c). Error bars represent standard deviation.

was detected only after 27 h of fermentation, and the yield was 33.3 ± 0.3 g/L, which was less than 36.2 ± 0.25 g/L of *Z. mobilis* ZM4 Δ*adhA*-1 and 37.4 ± 0.26 g/L of wild-type ZM4. In other words, *Z. mobilis* ZM4 Δ*adhB*-1 strain showed lower biomass, decreased specific rate of glucose utilization and lower ethanol yield compared to those of the wildtype or Δ*adhA*-1strain. This indicated that the *adhB* knock-out caused a more serious decrease in growth characteristics than *adhA*.

The estimation of ADH activity in Δ*adhB*-1 during the entire fermentation process demonstrated that the Δ*adhB*-1 strain was severely deficient in ADH II activity in the initial stage or last phase compared to the wild type strain ZM4 (Fig. 7). This indicated that knock-out of *adhB* impaired the circulation of NAD^+ and NADH, and less NADH production was detected in cytoplasmic extracts.

The high ethanol yield and ethanol tolerance of *Z. mobilis* may be due to the presence of two metal-dependent and NAD^+-dependent isoenzymes, namely, ADH I (*adhA*) and ADH II (*adhB*). These two ADHs play a key role in both ethanol production and the regeneration of NAD^+ in the fermentative pathway [28,29]. ADH I is a zinc-bearing alcohol dehydrogenase of the group I ADHs. It is essential for reducing acetaldehyde to produce ethanol in the early stage of anaerobic fermentation. In contrast, the iron-containing ADH II is required at later stages of fermentation when the high concentration of the accumulated ethanol gradually hinders the ADH I-catalyzed reduction of acetaldehyde to ethanol instead of catalyzing the formation of ethanol and NAD^+ in the forward reaction to provide 90% of the NADH to the respiratory chain. Therefore, the ADH II-catalyzed reaction is an important regulator of the NADH flux from the Entner–Doudoroff glycolytic pathway to the respiratory chain. Spectrophotometric measurement of the ADH II activity at 340 nm showed a serious decline (not disappearance) in NADH production in the *adhB* knock-out strain, implying the presence of alternative pathways for the maintenance of redox reaction equilibrium and cell growth.

In addition, ADH II deficiency in the ZM6 strain (*adhB::kan*^{r} mutant) showed three-fold increase of catalase activity, higher levels of acetaldehyde, elevated hydrogen peroxide excretion from aerobically

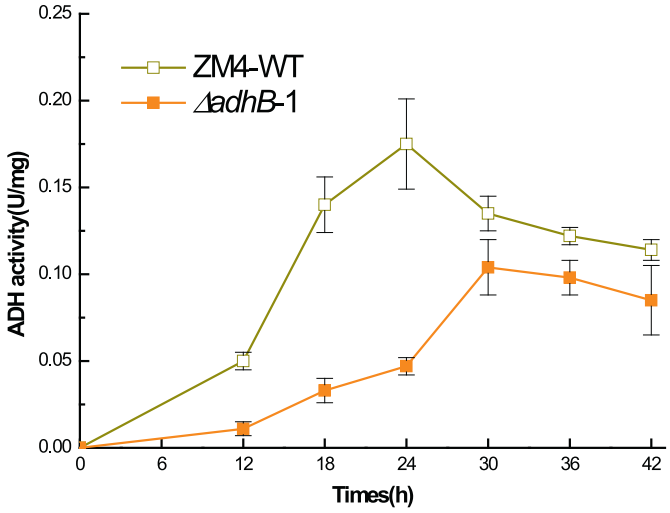

Fig. 7. Alcohol dehydrogenase activities of the wild-type ZM4 and mutant Δ*adhB*-1 strains. Error bars represent standard deviation.

cultured cells, and low levels of biomass concentration, intracellular NADH, and ethanol yield. These evidences suggest that ADH II plays a significant role in the stabilization of NADH pools.

4. Conclusion

We developed two *Z. mobilis* knock-out mutants of chromosomal genes, namely, *adhA* and *adhB*, using the RecET homologous recombination system developed in this study. The successful application of this system demonstrated that it is a powerful tool for the genetic manipulation of *Z. mobilis*. Only few *Z. mobilis* strains are used industrially since a high proportion of *Z. mobilis* genes have no annotated function. The RecET recombination system could be used in future for understanding the function of unknown genes, introducing heterologous genes responsible for metabolizing alternative carbon source, increasing ethanol yield, and for the production of other value-added bio-products.

Conflict of interest statement

There are no conflicts of interest.

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