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Construction of an Unmarked *Zymomonas mobilis* Mutant Using a Site-Specific FLP Recombinase

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Summary

Flippase expression was carried out in *Zymomonas mobilis* strain ZM4. The FRT-flanked selection marker gene was first integrated into the ZM4 chromosome by homologous recombination. The *Saccharomyces cerevisiae flp* gene was then introduced under the control of the ZM4 *gap* gene promoter (P*gap*, encoding glyceraldehyde-3-phosphate dehydrogenase) or the λ bacteriophage cI_{857}-p_R contained in the broad-host-range cloning vector pBBR1-MCS-2. This study demonstrated that *flp* was expressed and that the deletion frequency of the FRT-flanked marker gene was very high (approx. 100 %). In addition, the *flp* gene expression vector could be conveniently removed from the resulting unmarked *Z. mobilis* mutants by serially transferring the cells three times into antibiotic-free medium, thereby establishing an efficient method for constructing unmarked *Z. mobilis* mutants.

Key words: *Zymomonas mobilis*, homologous recombination, flippase expression, transformation, unmarked mutants

Introduction

Zymomonas mobilis is a Gram-negative bacterium that has become an attractive ethanologen that can be used in the development of cost-effective ethanol production. *Z. mobilis* can also produce additional high-value chemicals such as sorbitol, levan or phenylacetylcarbinol (*1*), and has attracted additional interest due to its unusually high membrane steroid content (*2*). Since *Z. mobilis* can be used for alcoholic fermentation in pulque, palm wine, beer, *etc.* (*3,4*), it is considered safe for humans, which makes it suitable for use in large-scale biotechnological endeavors. Unfortunately, because *Z. mobilis* strains are inherently resistant (conferred by several indigenous plasmids) to a variety of antibiotics (*5,6*), genetic manipulation of *Z. mobilis* strains remains limited. Among the diverse transformation protocols proposed for *Z. mobilis*, electroporation is the most effective method (*7–13*); however, transformation efficiencies vary greatly depending on the plasmids and strains used.

The FLP-FRT site-specific recombination system from *Saccharomyces cerevisiae* is a powerful and efficient tool for carrying out high-throughput genetic analysis of bacteria in the postgenomic era (*14*). Combined with other effective recombination methods, *e.g.* phage λ Red recombineering (*15,16*), it offers a wide variety of applications for use in bacteria, including the construction of unmarked mutations or site-specific integrations of heterologous DNA into the chromosome (*14*). However, there have been no reports about the use of this approach in *Z. mobilis*.

Similar to other bacteria, genetically modified *Z. mobilis* strains are increasingly being used to define the genomic structure and function of various biotechnological applications (*4,10–13*). The construction of these strains frequently requires gene targeting, including chromosomal gene deletions/mutations and chromosomal insertions of heterologous DNA segments that require antibiotic resistance markers, which are a common option

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for selectable markers in genetic manipulation of *Z. mobilis* strains (*12*,*13*). Currently, gene targeting in *Z. mobilis* is based on random transposition or/and *rec*A homologous recombination mainly by electrotransformation (*12, 13*,*17*,*18*). Therefore, in this study, we focused on testing the effects of the FLP-FRT site-specific recombination system in *Z. mobilis* and developed an effective and rapid method for generating knockout and recycle marker genes for unmarked *Z. mobilis* mutants by electrotransformation. We selected *Z. mobilis* ZM4 strain for this analysis since ZM4 has an excellent ethanol-producing performance and has been the first *Z. mobilis* strain to have its genome sequenced (*19*).

Materials and Methods

Bacterial strains and growth conditions

E. coli TOP10 strain (Invitrogen, Life Technologies, Grand Island, NY, USA) was used for plasmid construction and for recovering plasmids from *Z. mobilis* ZM4 (ATCC 31821) transformants. *E. coli* strain JM110 (Invitrogen) was used for preparing the plasmids transformed into ZM4. TOP10 and JM110 were routinely grown in Luria-Bertani broth at 37 °C. Antibiotics were used when required at the following concentrations (in μg/mL): tetracycline 10, chloramphenicol 25, and kanamycin 50.

Z. mobilis ZM4 was the transformation recipient and anaerobically grown in a glucose-rich medium (RM) containing (in %): D-glucose 2, yeast extract 1, KH_2PO_4 0.2, at pH=6.0, in static cultures at 30 °C. Antibiotics were used when required at the following concentrations (in μg/mL): tetracycline 20, chloramphenicol 100 and kanamycin 310.

Plasmid constructs

The construction of the non-replicating integrating vector pBR328-*ldh*R-FRT-*cat*-FRT-*ldh*L was carried out as reported by Zou *et al.* (*20*). The vector includes a 3.3-kb *Sca*I-*Pst*I DNA fragment of pBR328 and contains two *Ban*II sites located within the *tet* gene sequence (Fig. 1). The FRT-*cat*-FRT cassette was obtained from the pKD3 plasmid (*15*). The *ldh*R and *ldh*L sequences corresponded to the nucleotide sequence between positions 1260034–1261516 and 1261636–1263055, respectively, in the ZM4 genome sequence (*19*) (EMBL:AE008692.2) and were used as the homologous arms. They were amplified by PCR using ZM4 genomic DNA as the template, then sequenced and finally confirmed to contain no mutation (*20*). Thus the targeted genomic region to be deleted was 119 bp.

The *flp* expression vectors pBPF and pBCPF are described in Fig. 1. The pBPF was constructed as follows: the promoter region of the *Z. mobilis gap* gene (P*gap*) was PCR-amplified from ZM4 genomic DNA with primers P7 and P8 (Table 1) and the *flp* gene was PCR-amplified from the pCP20 plasmid (*15*) using primers P9 and P10 (Table 1). The P*gap-flp* fragment was amplified by overlapping the PCR from the former two PCR products with primers P7 and P10. After digestion with *Cla*I and *Xba*I, the fragment was ligated into the *Cla*I-*Xba*I sites of pBBR1 MCS-2 (GenBank: U02374) generating pBPF (*21*). The cI_{857}-p_R-containing 1.6-kb *Eco*RV/*Cla*I fragment of pCP20 was then ligated into the *Eco*RV-*Cla*I site of pBPF resulting in pBCPF.

Molecular manipulation methods, such as PCR, plasmid DNA isolation, restriction enzyme analysis, ligation of DNA fragments and transformation of *E. coli*, were performed as described previously by Sambrook and Russel (*22*).

Table 1. Primers used in this study

Primer names	Essential properties
P1	5′ CGACGATGCATTCTTCTGCC 3′
P2	5′ TTTCACCAGCGTTCTGACCC 3′
P3	5′ ATTCAGGCTGCGCAACTGT 3′
P4	5′ TCGTCAGTTTGTGGAAAGGT 3′
P5	5′ CCCTTGCCAGTGTTTCTTCATT 3′
P6	5′ GGCAAATTTCACGCGGTTTGAA 3′
P7	5′ GGGCAATCGATGTCGATGCCGAGTTG 3′ *Cla*I
P8	5′ ATTGTGGCATGTTTATTCTCCTAACT 3′
P9	5′ GAGAATAAACATGCCACAATTTGGTA 3′
P10	5′ GGTGAGTCTAGAAGAATAGGAACTTCGG 3′ *Xba*I
P11	5′ GCGCGCCTACCTGTGACGGAA 3′
P12	5′ TCATTTAAATGGCGCGCCTTA 3′
P13	5′ CCGCGCGAATTCGTCGAC 3′
P14	5′ GGAGCTGCTTCGAAGTTCCTATACTT 3′
P15	5′ AGGAACTTCGAAGCAGCTCCAGCCTA 3′
P16	5′ CTAGGCGGCCGCAAGCTTGC 3′
P17	5′ AAACCGACCTTTTTCGCC 3′
P18	5′ ATTATCGGTGTTGGTCAT 3′

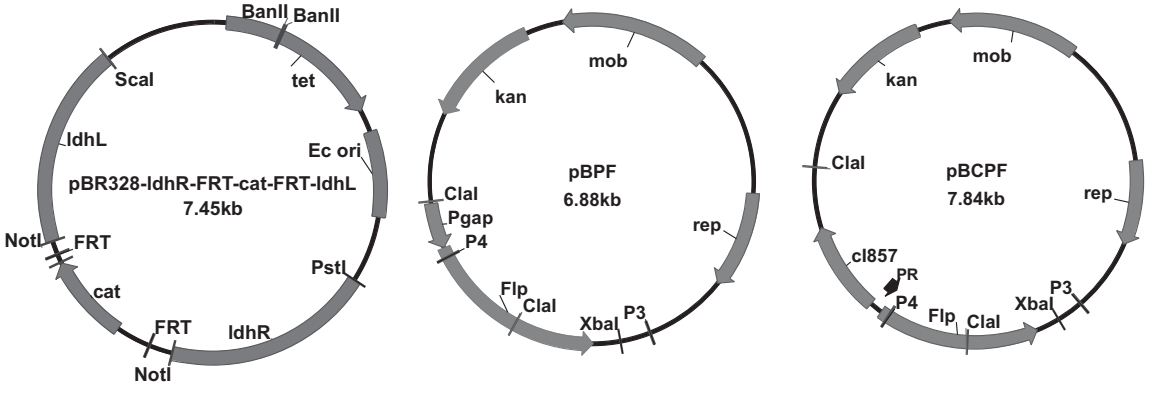

Fig. 1. Schematic illustration of the plasmids pBR328-*ldh*R-FRT-*cat*-FRT-*ldh*L, pBPF and pBCPF

Transformation of Z. mobilis

The general electroporation protocol used was as follows: ZM4 or mutant strains were grown to early log phase ($A_{600\ nm}$=~0.4) and cultures were centrifuged, washed and then resuspended in 1:100 dilution in 10 % glycerol in sterile water to a final concentration of 10^{10} cells/mL. Bacterial cells were then divided into 120-μL aliquots and no more than 3 μL of plasmid extracts from *E. coli* JM110 were added. The plasmid/cell suspension was transferred to chilled 0.2-cm gap cuvettes (Bio-Rad, Hercules, CA, USA) and kept on ice for 2 min, placed between the Micro-Pulser™ electrode plates (Bio-Rad) and appropriate field strength was applied, which varied with different DNA molecules. The capacitance and resistance of the Micro-Pulser were kept constant at 25 μF and 200 Ω, respectively. RM at 0.8–1.0 mL preheated at 30 °C was used as the recovery medium with recovery times between 3 and 18 h at 30 °C. At the end of this incubation, the cells were appropriately diluted or concentrated in RM medium and plated on antibiotic-containing selective RM agar (RM medium plus 20 g/L of Bacto™ agar). Colonies grown on RM selective agar plates after 48–96 h at 30 °C were replated onto new selective agar plates and further characterized.

pBR328-*ldh*R-FRT-*cat*-FRT-*ldh*L was first linearized with the *Ban*II and diluted to a concentration of 1 μg/μL. A volume of 3 μL was used at a field strength of 13.25 kV/cm. The colonies grown on selective agar plates (RM+chloramphenicol) were identified by successive plating onto RM+tetracycline, RM+chloramphenicol, and RM agar plates, then confirmed by PCR, sequencing, and assessed for ethanol and lactic acid production when grown in glucose-containing media.

For pBPF and pBCPF, the DNA concentration was controlled at 300 ng/μL, and 3 μL were used at a field strength of 11.75 kV/cm. The colonies grown on selective agar plates (RM+kanamycin) were identified by successive plating onto RM+chloramphenicol, RM+kanamycin, and RM agar plates, then verified by PCR and extracted plasmids were transformed back into *E. coli* TOP10.

Southern blot analysis

Genomic DNA samples of ZM4 strain and its mutants were isolated using Wizard® Genomic DNA Purification Kit (Promega, Fitchburg, WI, USA) following the manufacturer's instructions and digested with *Bam*HI. Hybridization probe labelling and colour detection with NBT/BCIP were performed using a DIG-High Prime DNA Labeling and Detection Starter Kit I (Roche Applied Science, Indianapolis, IN, USA) following the manufacturer's instructions. Three different probes marked as 1, 2 and 3 were used separately to detect the *cat* gene, the scar which was left behind in place of disrupted *ldh*A gene after excising marker gene, the DNA region to be deleted on the chromosome of ZM4 mutants. The corresponding template DNA for probe labelling was amplified by PCR with primer pairs P11/P12, P13/P16 and P17/P18 (Table 1) from *Ban*II-digested pBR328-*ldh*R-FRT-*cat*-FRT-*ldh*L, PCR products described below and ZM4 genomic DNA, respectively.

The PCR products PCR1 and PCR2, corresponding to the nucleotide sequence of the upper and lower part of the scar, respectively, were first amplified from *Ban*II-digested pBR328-*ldh*R-FRT-*cat*-FRT-*ldh*L with primer pairs P13/P14 and P15/P16, respectively. Then the template DNA for probe 2 was amplified by overlapping PCR from the former PCR1 and PCR2 with primers P13 and P16.

Growth, ethanol and lactate production

Fresh late log phase cultures grown at 30 °C in RM were diluted in 100 mL of medium containing (in %): D-glucose 6, yeast extract 1, and KH_2PO_4 0.2, pH=6.0, to an initial $A_{600\ nm}$ of 0.020±0.004. Samples were periodically removed to measure the cell growth. The concentrations of glucose, ethanol and lactate were determined from sample supernatants using a Waters high-performance liquid chromatograph and an Aminex HPX-87H column (Bio-Rad) with 4 mM H_2SO_4 (65 °C, 0.6 mL/min) as the mobile phase. All data were obtained from at least three representative measurements.

Results and Discussion

The electroporation data reported for *Z. mobilis* ZM4 are limited (*10,11*) and the efficiency was shown to be very low in our preliminary experiments. In order to improve the transformation and integration efficiency in ZM4 by electroporation, we first performed extensive evaluation and optimization of various parameters by using the broad host range vector pBBR1MCS-2 and three shuttle plasmids that could replicate in ZM4 (unpublished data). The general transformation protocol finally proposed was shown in Materials and Methods. In this protocol, two key parameters, the field strength and the amount of used DNA molecules, varied with different DNA molecules and need to be further investigated. The investigated levels ideal for the following pBR328-*ldh*R-FRT-*cat*-FRT-*ldh*L, pBPF and pBCPF transformations were also shown in Materials and Methods.

We found that the size of the transformed DNA molecule significantly influences its transformation efficiency in ZM4, which was again proved by the data below on pBBR1MCS-2, pBPF and pBCPF plasmid transformations in this study. Therefore, we selected the *cat* gene (639 bp ORF) as the marker one for constructing the ZM4 mutants *via* a double homologous recombination event.

Disruption of the ldh gene in Z. mobilis ZM4 by homologous recombination

A region on the *Z. mobilis* CP4 or ATCC 39676 chromosome possesses a high degree of homology to nucleotide positions 1261084–1262079 of ZM4 (*19*) (EMBL:AE008692.2), and this region encodes a putative D-lactate dehydrogenase-encoding gene (*ldh*A) responsible for the synthesis of D-lactic acid, which leads to decreased ethanol yields (GenBank: L09650.1) (*23–25*). Due to strain similarities at this locus, it was hypothesized that the nucleotide sequence at positions 1261084–1262079 in the ZM4 genome also possessed an *ldh* gene (ZMO1237) and that ethanol production would benefit from its disruption. To this end an integrating vector designated pBR328-*ldh*R-

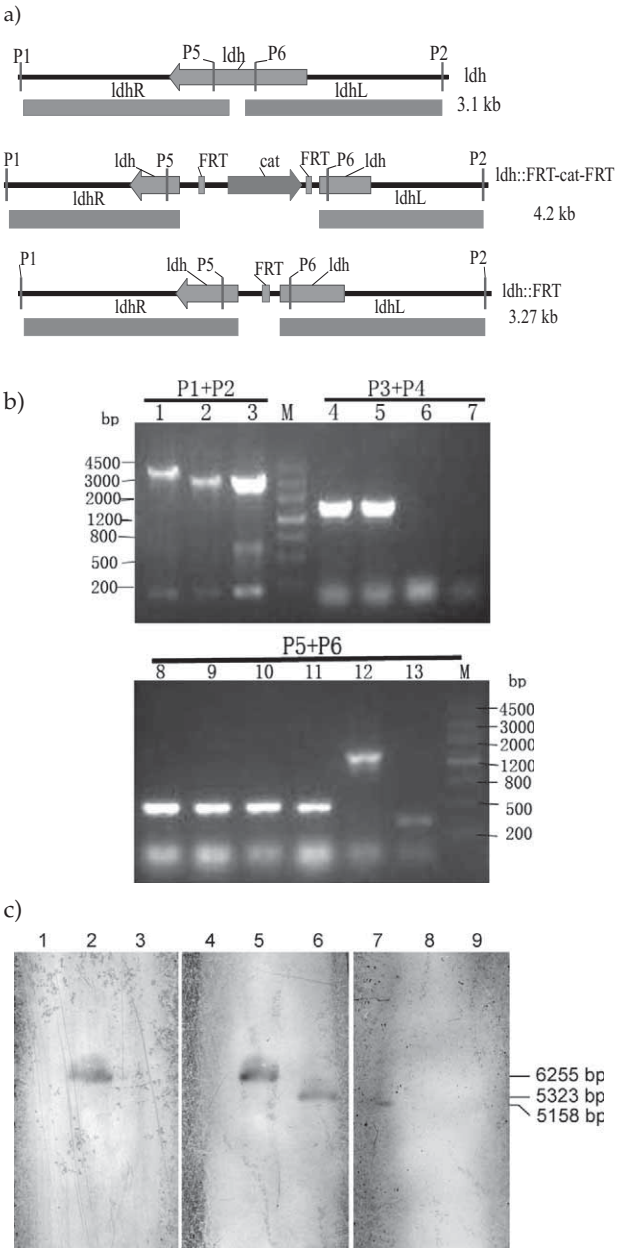

Fig. 2. Construction of the unmarked *ldh* mutant gene using broad--host-range *flp* recombination: a) the schematic representation of the *ldh* region in the chromosome of the strains ZM4 (wt), ZM4 (*ldh*::FRT-*cat*-FRT) and ZM4 (*ldh*::FRT) shows the location of the oligonucleotides used for PCR analysis; b) PCR amplification products obtained using primers P1+P2 (which are external to *ldh*R and *ldh*L) generated fragments of 4.16, 3.23 and 3.06 kb for ZM4 (*ldh*::FRT-*cat*-FRT) (lane 1), ZM4 (*ldh*::FRT) (lane 2) and ZM4 (wt) (lane 3), respectively. PCR-amplified products using primers P3+P4 generated the 1.61-kb fragment for colonies containing pBPF (lane 4) or pBCPF (lane 5), but not for strains ZM4 (*ldh*::FRT) (lane 6) and ZM4 (wt) (lane 7). Products amplified using P5+P6 yielded fragments of 0.49 kb for strains ZM4 (*ldh*::FRT)(pBPF) (lane 8), ZM4 (*ldh*::FRT)(pBCPF) (lane 9) and ZM4 (*ldh*::FRT) (lanes 10 and 11), 1.42 kb for ZM4 (*ldh*::FRT-*cat*-FRT) (lane 12) and 0.32 kb for ZM4 (wt) (lane 13); c) Southern blot analysis with genomic DNA digested with *Bam*HI from ZM4 and ZM4 mutants. Lanes 1, 4 and 7: ZM4 (wt); lanes 2, 5 and 8: ZM4 (*ldh*::FRT-*cat*-FRT); lanes 3, 6 and 9: ZM4 (*ldh*::FRT); lanes 1–3: probe 1, lanes 4–6: probe 2, lanes 7–9: probe 3. The sizes of hybridization fragments in *Bam*HI--digested genomic DNA were 6255 bp for ZM4 (*ldh*::FRT-*cat*-FRT), 5323 bp for ZM4 (*ldh*::FRT) and 5158 bp for ZM4 (wt)

-FRT-*cat*-FRT-*ldh*L was constructed and transformed into ZM4 cells to disrupt *ldh* by homologous recombination. The colonies resistant to chloramphenicol were all sensitive to tetracycline and they were further identified by PCR with the primers P1+P2 and P5+P6 (shown in Table 1 and Fig. 2a). Agarose gel electrophoresis of the PCR products is shown in Fig. 2b. Southern blot analysis with genomic DNA is shown in Fig. 2c. DNA sequencing of PCR products amplified using primers P1 and P2 further confirmed integration of the FRT-*cat*-FRT cassette into the ZM4 genome, as shown in Fig. 2a. The transformation efficiency to obtain the strain ZM4 (*ldh*::FRT-*cat*-FRT) was about 5 transformants per μg of DNA.

Growth rate, ethanol and lactate production results from ZM4 (*ldh*::FRT-*cat*-FRT) grown in 6 % glucose are shown in Fig. 3 and Table 2. The growth of ZM4 (*ldh*::FRT-*cat*-FRT) was similar to that of other tested strains, but the lactate production was lower than that of the control ZM4 strain.

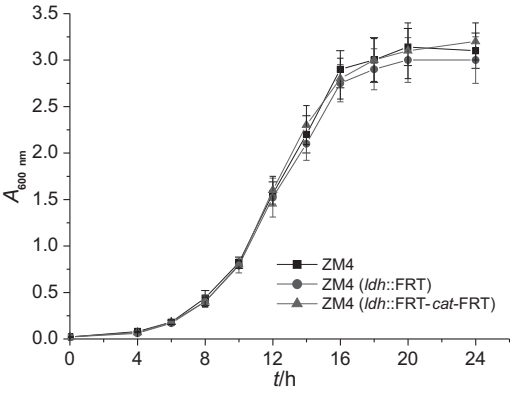

Fig. 3. Growth curves of three strains cultured in RM media containing 6 % glucose (by mass per volume). Respective cultures were grown in RM media at 30 °C and measurements at $A_{600\ nm}$ were taken at the indicated times. The data are expressed as the mean values±SE (standard error) of three observations per time point

In addition, we serially transferred the ZM4 (*ldh*::FRT-*cat*-FRT) cultures at 1 % (by volume) to the antibiotic-free RM medium twelve times at 16-hour intervals. The appropriate dilutions of the twelfth culture were spread on antibiotic-free RM agar plate. Over 100 colonies appearing on the RM agar plates were identified as mentioned above. All tested colonies were resistant to chloramphenicol, sensitive to tetracycline and FRT-*cat*--FRT cassette integrated. This result further indicated the stability of the ZM4 (*ldh*::FRT-*cat*-FRT) strains.

In vivo marker excision

In order to eliminate possible negative effects of the marker gene and also re-use it in another gene deletion, we constructed the *flp*-carrying vectors pBPF and pBCPF. To date there have been no reports describing the use of a conditionally replicating vector in *Z. mobilis* and we too were unsuccessful in transforming ZM4 with either pCP20 or pKD46 (*15*). Furthermore, endogenously or exogenously induced gene expression systems reported for *Z. mobilis* were not ideal, partly due to their relatively

Table 2. Ethanol and lactate production (after 12 h) from media containing 6 % glucose*

Strains	*w*(glucose)	*w*(ethanol)	Ethanol yield per glucose consumed	*w*(lactate)	Lactate yield per glucose consumed
	g/100 g	g/100 g	g/g	g/100 g	g/g
ZM4	2.82±0.05	1.26±0.04	0.405	0.026±0.004	0.008
ZM4 (*ldh*::FRT-*cat*-FRT)	2.86±0.06	1.27±0.04	0.412	0.016±0.002	0.005
ZM4 (*ldh*::FRT)	2.80±0.06	1.31±0.04	0.417	0.012±0.003	0.004

*glucose concentration for the three strains at 0 h was (5.94±0.07) g per 100 g of medium

high base line expression levels before induction (*26–28*). By analyzing a series of parameters that influenced the transformation efficiency of replicative plasmids in ZM4, we thought that pBBR1MCS-2 should be suitable as candidate *flp* vector because: (*i*) the transformation efficiency of pBBR1MCS-2 was high ($6{\cdot}10^3$ transformants per μg of DNA, unpublished data) and could easily be transformed into ZM4, (*ii*) pBBR1MCS-2 could be easily removed from ZM4 because it was relatively unstable in antibiotic-free medium (*29*), and (*iii*) the vector contains the *kan* marker gene (795 bp ORF), different from the *cat* marker gene integrated into the ZM4 chromosome. In order to confirm *flp* expression in *Z. mobilis* (which had not been reported previously), we first selected the constitutive and strong promoter P*gap* of the glyceraldehyde 3-phosphate dehydrogenase-encoding gene (*gap*) from ZM4 (*30*,*31*). In order to achieve tighter regulation of *flp* expression in *Z. mobilis*, we also selected the λ bacteriophage cI_{857}-p_R inducible repressor.

Plasmids pBPF and pBCPF were successfully transformed in ZM4 (*ldh*::FRT-*cat*-FRT) using different recovery times of 3, 11 and 15 h, but the transformation efficiency of pBPF was markedly higher than that of pBCPF (up to 10^2 transformants per μg of DNA compared to the pBCPF efficiency of only 10 transformants per μg of DNA). In addition, we observed that all colonies on initial selective agar plates (RM+kanamycin) containing pBPF or pBCPF were also chloramphenicol-sensitive and deficient in *cat*, that is, they were either ZM4 (*ldh*::FRT) (pBPF) or ZM4 (*ldh*::FRT) (pBCPF) strains. Results of agarose gel electrophoresis of PCR products are shown in Fig. 2b. Southern blot analysis with genomic DNA is shown in Fig. 2c. DNA sequencing of PCR products amplified using primers P1 and P2 further confirmed deletion of the *cat* gene from the ZM4 mutant, as shown in Fig. 2a.

These data demonstrated that the *flp* gene was expressed from P*gap* and cI_{857}-p_R in *Z. mobilis*. Normally, cI_{857}-p_R can be induced at 42 °C, resulting in gene expression in *E. coli*; however, in *Z. mobilis*, the *flp* was adequately expressed under the non-inducible conditions at 30 °C, suggesting that neither cI_{857}-p_R may be capable of mediating stringent control of gene expression in *Z. mobilis*.

Flp expression vector elimination and production of an unmarked mutant

We inoculated pBPF- or pBCPF-containing colonies grown on initial RM+kanamycin plates into liquid RM medium and transferred the resultant cultures at 1 % (by volume) twice. Then, respective cultures were restreaked on RM plates and colonies were identified by the same methods as described above. About 50 % of the identified colonies were kanamycin- and chloramphenicol-sensitive and did not contain either pBPF or pBCPF, that is, they were already ZM4 (*ldh*::FRT) strains.

The growth rate, as well as ethanol and lactate production of the unmarked mutant ZM4 (*ldh*::FRT) strain cultivated in the presence of 6 % glucose are described in Fig. 3 and Table 2. The growth of ZM4 (*ldh*::FRT) was also similar to that of other tested strains. Among the three tested strains, the ethanol yield of the control ZM4 was the lowest and its lactate production highest, but their difference was not significant as expected.

There were some possible reasons for this. First, the precise function of *ldh* (ORF ZMO1237) in ZM4 remains unknown and the knowledge about genes responsible for lactate production in *Z. mobilis* is still very limited (*19*, *23–25*). Although a tetracycline-resistant, putative D-*ldh*-negative mutant of ATCC 39676 had been constructed, this mutant was reported to produce about 50 % less lactic acid than its parent strain under permissive and comparable culture conditions (*25*), which is similar to the findings in our study. In addition, its lactate dehydrogenase (LDH) activity has not been assayed (*25*). Preliminary LDH assay with pyruvate on crude cell extracts (*32*) showed that LDH activities of the mutants were up to 80–95 % of ZM4 activity. Thus, deeper investigation of the function of *ldh* is needed. Second, although another ORF ZMO0256 in ZM4 had also been uncovered by annotation of the genome to be relevant to lactate, it has still not been confirmed experimentally (*19*). Its function and relationship with *ldh* (ZMO1237) also require further study. Last, lactate production in *Z. mobilis* had been shown to be very complicated and affected by many factors, resulting in conflicting findings in the literature on the subject of reduced ethanol yield due to lactic acid production (*3*,*5*,*23–25*). The regulation mechanism of lactate production in *Z. mobilis* needs to be explored in depth, which would also help a detailed investigation of the difference between the mutants and the parent strain under more comparable and variable culture conditions.

Conclusions

The above data demonstrated that unmarked *Zymomonas mobilis* mutants could be constructed by transforming the pBPF or pBCPF plasmids into *Z. mobilis* mutants possessing a chromosomally FRT-flanked marker gene, followed by the identification of colonies on RM plates, and by three rounds of serial transfers of transformed colonies into liquid cultures. This method would also enable to recycle marker genes in *Z. mobilis* strains, which are naturally resistant to a variety of antibiotics. It is efficient, rapid, and useful for studying and developing nov-

el biotechnological applications in *Z. mobilis* and has the potential of generating efficient ethanol-producing bacteria. In addition, this study is a preliminary experimental investigation of the function of formerly putative *ldh*A gene in ZM4 by means of mutant strain construction.

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