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运动发酵单胞菌限制–修饰系统缺失突变株的构建及其性质分析*

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摘 要 低遗传转化率阻碍了产乙醇模式菌——运动发酵单胞菌的遗传或分子生物学操作. 本研究将运动发酵单胞菌菌株ZM4的5个限制–修饰系统相关基因失活, 构建了5个限制–修饰系统突变菌株. 研究结果表明: 在突变株Zmmrr和Zm1933中, 甲基化穿梭表达质粒pBBR1MCS-tet的电转化率分别提高了17倍和2倍, 而限制修饰基因ZMO0575的失活则明显降低了甲基化修饰质粒和非甲基化质粒的转化率. 较之其它3个突变株, 突变株Zmmrr和Zm1933具有更高的遗传稳定性. 发酵实验结果进一步表明这些限制–修饰系统突变株并未显著改变运动发酵单胞菌的主要性质, 例如细胞生长、葡萄糖利用率和乙醇产量等. 研究运动发酵单胞菌的限制–修饰系统将有助于构建适合于分子遗传操作的基因工程菌株. 图5 表3 参37

关键词 运动发酵单胞菌; 限制–修饰系统; 基因敲除; 电转化; 乙醇发酵
CLC Q933 : TK63

Construction and Characterization of Restriction-Modification Deficient Mutants in *Zymomonas mobilis* ZM4*

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Abstract Low transformation efficiency is an obstacle to genetic or molecular manipulations in ethanologen *Zymomonas mobilis*. In the present study, 5 defective strains were constructed in *Z. mobilis* strain ZM4 by inactivating restriction-modification (R-M) system candidate genes. Inactivation of ZMO0028 (*mrr*) and ZMO1933 significantly improved electroporation efficiency by 17 folds and 2 folds when ZM4 was transformed with the methylated plasmid DNA. Disruption of ZMO0575 significantly decreased the transformation efficiency when transformed with both methylated and unmethylated plasmid DNAs. In comparison with other mutants, Zmmrr and Zm1933 displayed high stability. Furthermore, fermentation results showed that R-M mutants did not significantly alter the major bacterial traits such as growth, glucose utilization and ethanol yield. In conclusion, R-M systems in *Z. mobilis* were investigated in this study, and the characterization of those R-M genes contributed to creating engineering strains suitable for genetic and molecular manipulations. Fig 5, Tab 3, Ref 37

Keywords *Zymomonas mobilis*; restriction-modification systems; gene knockout; electroporation; ethanol fermentation
CLC Q933 : TK63

1 Introduction

Zymomonas mobilis has emerged as a promising microorganism model for its good capability of producing ethanol as well as other valuable chemicals [1-4]. To meet industrial or research needs, researchers have been modifying *Z. mobilis* via genetic or molecular methods, such as, heterologous genes have been introduced in *Z. mobilis* to improve its performances [5-7]. Furthermore, genomic information is greatly helpful to understand this microorganism on omic levels [8-13]. Lee *et al.* analysed metabolic network in ZM4, and then introduced pentose metabolic pathway to enlarge carbon source, or constructed knockout of certain pathway for helping produce succinic acid [9]. To date conversion of lignocellulose into bioethanol has been paid more attention [14-15], thus

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creating robust engineered strains with outstanding fermentation capability becomes vital to achieve the goal.

However, lack of genetic and molecular tools, especially the poor genetic transformation efficiency has tempered studies and applications of *Z. mobilis*. As early as mid-1980's, Browne tried to introduce plasmid into *Z. mobilis* but the transformation efficiency was low [16]. Most recently, Dong *et al*. (2011) reported a series of novel vectors which improved the bacterial transformation efficiency and stability [17]. Although mechanisms of transformation mediation still remain to be elucidated, studies on the anti-restriction protein Ocr (e.g., commercial product TypeOne Restriction Inhibitor, Epicentre Company) provided a possible way to increase the transformation efficiency [18-19]. Other findings have also demonstrated that suppression of endonuclease activity contributed to maintaining genomic integrity after homologous recombination, and that methylation of plasmid DNAs led to higher transformation efficiency when the non-replicative plasmid was introduced into the bacterium [20-21]. These evidences implied that R-M systems in host cells are likely to be involved in uptake and integration of heterologous DNAs.

Previously, Kerr *et al*. (2011) reported that knockout of certain R-M genes were able to increase transformation efficiency of the replicative plasmids in *Z. mobilis* [22]. However, the functions of other hypothetical R-M system genes in *Z. mobilis*, especially their influences on transformation efficiency as well as on growth and ethanol yield of the bacterium, were not sufficiently understood. In the present study, 5 R-M gene knockout mutants were constructed and their effects on the transformation efficiency were investigated along with their stability. Then major traits including growth, glucose consumption and ethanol yield were analyzed.

2 Material & Methods

2.1 Bacterial strains and growth conditions

All bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was cultured in Luria-Bertani (LB) medium containing appropriate antibiotics at 37 °C with shaking at 200 r/min. *Z. mobilis* ZM4 (ATCC31821) was cultured in Rich media (RM) [23] at 30 °C without shaking. RM without Mg^{2+} was used for selection of tetracycline (Tet) resistant strains. Antibiotics were added in culture media if required: 100 μg mL^{-1} ampicillin (Amp), 34 μg mL^{-1} Chloramphenicol (Cm) or 15 μg mL^{-1} Tet for *E. coli*; 100 μg mL^{-1} Cm or 20 μg mL^{-1} Tet for *Z. mobilis*.

2.2 Construction of inactivation cassettes of R-M systems in *Z. mobilis*

For gene ZMO0575 or ZMO1934, insertional inactivation plasmids were constructed following the previous description[22] (Fig. 1). Both genes including extra ≤1.0-kb upstream and downstream flank sequences were amplified using *Ex-Taq* DNA polymerase (TaKaRa) from the genomic DNA of *Z. mobilis* ZM4, and then cloned into pGEM-T Easy cloning vector (Promega). The resulting plasmids were named as pGEM-0575 and pGEM-1934, respectively. The *cat* cassette including chloramphenicol resistance gene was amplified from pLysS plasmid (Novagen) using KOD-Plus-Neo DNA polymerase (ToYoBo), and cloned into pJET1.2/blunt cloning vector (Fermentas), with the resulting plasmid named as pJET-cat. Sequentially, the *cat* gene cassette was excised from pJET-cat using certain restriction endonuclease (Table 1) which were introduced at both ends of *cat* cassette, and then inserted into pGEM-0575 or pGEM-1934 digested with the same restriction endonucleases. The insertional inactivation

Table 1 Strains and plasmids used in this study

Strain or plasmid	Characterization	Remarks or references
Strains		
Escherichia coli strain DH5α		Cloning host cell , laboratory stocks
E. coli strain JM110	*dam*$^-$, *dcm*$^-$	Cloning host cell, laboratory stocks
Zymomonas mobilis strain ZM4		Wild type, obtained from CICC, (CICC Nr. 10273)
Z. mobilis strain Zm0028 (Zmmrr)	R-M mutant, ZMO0028:cat	This study
Z. mobilis strain Zm1932	ZMO1932:cat	This study
Z. mobilis strain Zm1933	ZMO1933:cat	This study
Z. mobilis strain Zm1934	ZMO1934::cat	This study
Z. mobilis strain Zm0575	ZMO0575::cat	This study
Plasmids		
pJET1.2/blunt cloning vector		Fermentas
pGEM Easy-T cloning vector		Promega
pBBR1MCS-tet	Replicative broad host plasmid, with tetracycline resistance	The derivate of pBBR1MCS [24]
pLysS	With chloramphenicol resistance	Novagen
pGEM1934-cat	Insert *cat* cassette in ZMO1934	This study
pGEM0575-cat	Insert *cat* cassette in ZMO0575	This study
pJET0028-cat	Replace ZMO0028 with *cat* cassette	This study
pJET1932-cat	Replace ZMO1932 with *cat* cassette	This study
pJET1933-cat	Replace ZMO1933 with *cat* cassette	This study

Table 2 Primers used in the present study

Name	Sequence (5' to 3')	Remarks
For constructed insertional inactivation plasmids		
ZMO1934-F	CTTCGCGGTGGCCCTCAATC	For cloning ZMO1934 and confirming the mutation
ZMO1934-R	TCAACTTGCCCTGATGACCC	For cloning ZMO1934
pLys-BglII-F1934	AGATCTTTGCTTTCGAATTTCTGCCATTC	For cloning *cat* cassette
pLys-BglII-R1934	AGATCTTGACGGAAGATCACTTCGCAG	For cloning *cat* cassette and confirming the mutation
ZMO575-F	CGCTTTTCTCGTGAGCCATC	For cloning ZMO0575 and confirming the mutation
ZMO575-R	TTTCATCAAT GCCTTGCCCT	For cloning ZMO0575
pLys-*Xba* Ⅰ-F575	TCTAGATTGCTTTCGAATTTCTGCCATTC	For cloning *cat* cassette
pLys-*Xba* Ⅰ-R575	TCTAGATGACGGAAGATCACTTCGCAG	For cloning *cat* cassette and confirming the mutation
For fused PCR to construct R-M gene replacing plasmids		
zmrr f1	ATTTGCCAGAATTGCTGACCG	For amplifying 5' region of *zmrr*
Zmrr fused r1	TGCTACGCCTGAATAAGTGATACGGCGACAGAATAACAGACAGG	
Zmrr fused f2	ACAGGGACACCAGGATTTATTTCGGTAGTCTGGTCGGGCATC	For amplifying 3' region of *zmrr*
zmrr r2	AACAAGCGGTCTATTGGTTTCA	
zmrr nest f3	CATTATTGCCGTCAATCGTCC	For confirming the mutation
zmrr nest r3	TGAAGGCGTTCCTCAAGACA	
plys cat f1	TATCACTTATTCAGGCGTAGCA	For cloning *cat* cassette
plys cat r1	AATAAATCCTGGTGTCCCTGT	For cloning *cat* cassette and confirming the mutation
ZM1932 f1	GAAGGGGATGTTCGTCTCAG	For amplifying 5' region of ZMO1932
ZM1932 fused r1	TGCTACGCCTGAATAAGTGATAGTCATTGGCACGACGCTCTT	
ZM1932 fused f2	ACAGGGACACCAGGATTTATTGCTCCGCAAATGACTCCGTG	For amplifying 3' region of ZMO1932
ZM1932 r2	AGCCTGACCCATGCCGATGT	
ZM1932 nest f3	CTTGGCACAGTCGGAAGAGT	For confirming the mutation
ZM1932 nest r3	GCGAACTATCGGACGGTGTT	
ZM1933 f1	TATGACGAAATGAACTCGCACCG	For amplifying 5' region of ZMO1933
ZM1933 fused r1	TGCTACGCCTGAATAAGTGATACGTTTCTGCGATAGACTTTGC	
ZM1933 fused f2	ACAGGGACACCAGGATTTATTATGAAACTGGCTATCTCGGACT	For amplifying 3' region of ZMO1933
ZM1933 r2	GGCATCGGTGGATCAAATT	
ZM1933 nest f3	GCCCGACCACCTACAAGT	For confirming the mutation
ZM1933 nest r3	GGCATCGGTGGATCAAATT	

constructs were designated as pGEM0575-cat and pGEM1934-cat, respectively (Table 1). The sequences of primers used in this study are shown in Table 2.

Inactivation cassettes of gene ZMO0028 (*mrr*), ZMO1932, ZMO1933 were constructed using fusion PCR strategy as described previously [25] (Fig. 1). Up-/downstream flanking regions (≤1 kb) of each gene were amplified using ZM4 genomic DNA as template. The up-/downstream fragments of corresponding gene were fused with the *cat* gene cassette through fusion PCR. Amplicons were cloned in pJET1.2/blunt cloning vector and selected on LB medium supplemented with Cm. The inactivation constructs were designated as pJETmrr-cat, pJET1932-cat, pJET1933-cat, respectively (Table 1).

All 5 R-M inactivation plasmids were electroporated into ZM4 using the modified method as described previously [22, 26]. Briefly, 100 to 500 ng plasmid DNAs were used for electroporation (Xcell Gene Pulser, Bio-Rad). After pulsing at 16 kV/cm, 25 μF and 200 Ω, 1 mL of RM was added into the electroporated solutions and then incubated at 30 °C for 6 h. Finally, the cells were plated and selected on RM plates supplemented with Cm until colonies were visible (≤3 d).

2.3 Confirmation of R-M deficiency in *Z. mobilis*

Two pairs of primers were used to confirm disruption of the each R-M gene in *Z. mobilis* transformants: one pair was used to amplify the region between upstream and downstream of the corresponding gene (see nest primer pairs in Table 2, e.g., zmmrr nestf3 and Zmmrr nestr3); the other to amplify the region including upstream of the gene and *cat* expression cassette (see remarks in Table 2).

2.4 Genetic transformation efficiency of R-M mutants

To check the electroporation efficiency of the R-M mutants and how the modification of the plasmid DNA could affect the transformation, pBBR1MCS-tet, a derivate from pBBR1MCS-2[24] was prepared from *E. coli* DH5α (dam^+dcm^+) or JM110 (dam^-dcm^-) and introduced into each R-M mutant as well as ZM4, respectively. Electroporation efficiency was presented by the colony forming units (CFUs) on selective plates when 1 μg plasmid DNA was introduced. The calculating formula was described previously [22]: CFU/μg^{-1} DNA= $(C_p/T_p) \times (V_t/V_p)$; where C_p is the colony number counted on selective plates; T_p is the total amount of plasmid DNA (μg) used here; V_t is the total transformation volume (μL); and V_p is the volume (μL) plated.

2.5 Stability test of R-M mutants

Single colony of each R-M mutant strain was inoculated in RM containing Cm and cultivated for up to 24 h ($D_{600\ nm}$≤2.0).

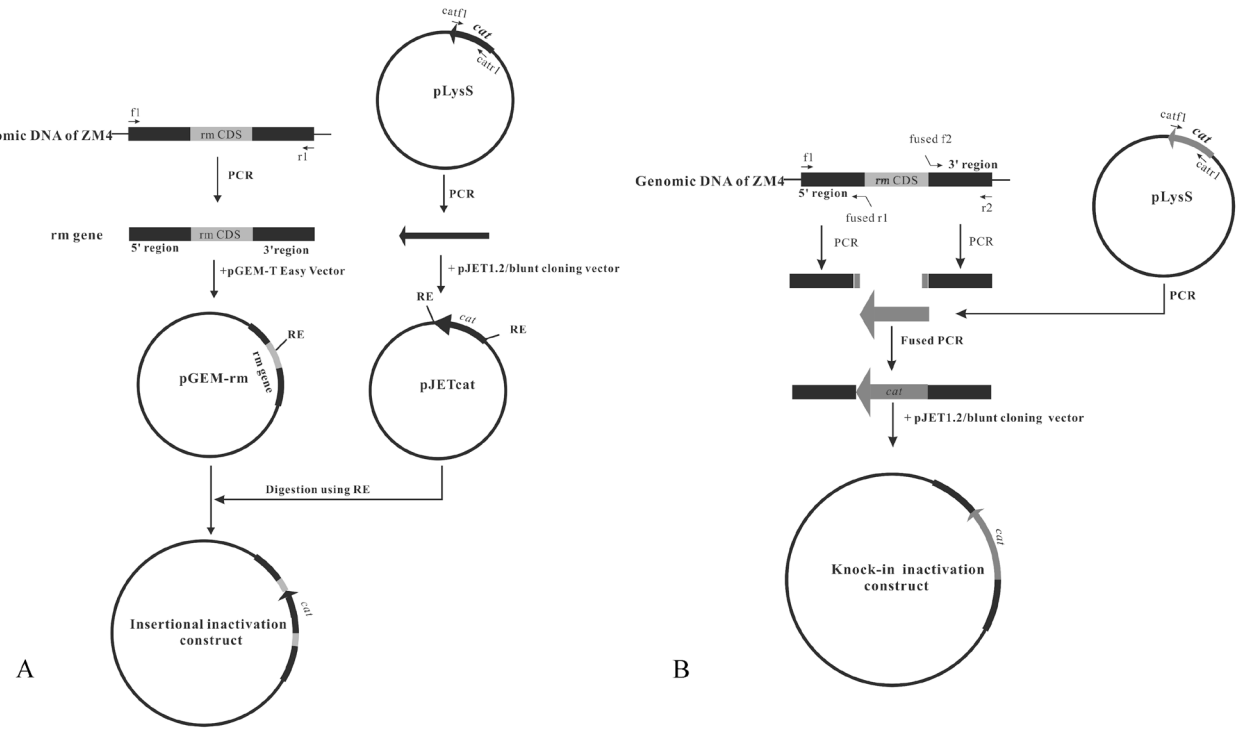

Fig. 1 Schematics of constructing R-M gene inactivation plasmids in *Z. mobilis*

pGEM-rm indicates the cloning plasmids in which RM-encoding genes were inserted. "rm" indicates the genes encoding restriction-modification systems. A: flow diagram of constructing insertional inactivation plasmids for *Z. mobilis*. B: flow diagram of creating knock-in inactivation plasmids for *Z. mobilis*. Details were described in Material & Methods

Then 10 μL of this start cultures was transferred in 1 mL of RM without antibiotics and grew for 24 h. The continuous transfer-growth lasted for 10 d in total. Then the cultures were diluted by 10^{-6} folds and 100 μL of the diluted cells were dispensed on RM plates with or without Cm in parallel. The stability was measured by formula: $(C_{s10}/C_{n10}) \times 100\%$, where C_{s10} and C_{n10} represented the colony number formed on RM selective and unselective medium after 10^{th} round transfer, respectively.

2.6 Fermentation analysis of R-M mutants

Single colony was inoculated in 50 mL of RM and grown for totally 24 h. Then 1 mL of the culture was sampled every 4 h to measure major traits including growth, glucose utilization rate and ethanol yield. Cell growth was represented by the cell density at $OD_{600\ nm}$ (Multi Scanner Spectrometer, Thermo). Glucose utilization, represented by the residual glucose concentration in cultures, was determined using Advanced Bio-Scan 871 Ions Chromatography (Metrohm, Switzerland) with 0.1 mol/L sodium hydroxide as mobile phase at a flow rate of 1 mL/min. Ethanol was assayed using GC122 gas chromatography with a glass column (0.26×200 cm) filled with Porapak Type QS (80-100 mesh, Waters, Milford, MA), with butyl acetate added as inner reference.

The fermentation performance of mutant Zm1933 was further analyzed at high concentrations of glucose. The culture was grown in 100 mL of RM with 100 g L^{-1} or 200 g L^{-1} glucose for 72 h. One milliliter of the cultures were sampled every 12 h to measure the above-mentioned traits, with ZM4 as control. All experiments above were duplicated at least twice and mean values were used for publication.

3 Results

3.1 R-M system mutagenesis in ZM

Database searching revealed 11 putative R-M candidate genes in ZM4 (Table 3). Five of them were successfully interrupted by the recombination strategy, which was confirmed by PCR (Fig. 2). Using nest primer pairs, expected sizes of amplicon of ZMO1934, ZMO0575, ZMO1932, ZMO1933, ZMO0028 (*mrr*) in WT were obtained, which were ≤4.0, 2.0, 2.8, 4.0 and 2.3 kb, respectively. In contrast, corresponding amplified fragments in defective strains were ≤4.5, 3.0, 2.7, 3.1 and 2.4 kb, respectively. To further confirm that *cat* gene had been integrated into the genome, a second primer pairs, which comprised forward primer located in 5' region of corresponding R-M gene and reverse primer of *cat* cassette, were employed to amplify upstream region of each corresponding R-M gene and *cat* expression cassette. Expectedly, the DNA fragments in the mutants ranged from 0.9 kb to 2.4 kb while no amplicon was obtained in WT (Fig. 2). The amplicons obtained using

Table 3 R-M candidate genes in ZM4[a]

No.	Locus	Description	R-M type	Remarks
1	ZMO0028	Restriction endonuclease	Type Ⅳ methyl-directed restriction enzyme	
2	ZMO1005	DNA methylase N-4/N-6 domain protein	Orphan methyltransferase, subtype *β*	
3	ZZM4_0148	DNA adenine methylase	Type II, subtype α	Located at plasmid pZZM405
4	ZZM4_0160	N6-adenine DNA methyltransferase, M subunit	Type I, subtype γ	Located at plasmid pZZM405
5	ZMO1934	N-6 DNA methylase	Type I	
6	ZMO1759	Conserved hypothetical protein	Type IV	
7	ZZM4_0163	Site-specific deoxyribonuclease, HsdR family	Type I	Located at plasmid pZZM405
8	ZZM_0161	Restriction modification system DNA specificity domain protein	Unclear	Located at plasmid pZZM405
9	ZMO1932		Type I (R subunit) [27]	
10	ZMO1933		Type I (S subunit) [27]	
11	ZMO0575			

[a]R-M system gene candidates were collected from the REBASE (http://rebase.neb.com/rebase.html), Microbesonline (http://www.microbesonline.org/cgi-bin/genomeInfo.cgi?tId=264203) and *Z. mobilis* ZM4 genome database (http://cmr.jcvi.org/cgi-bin/CMR/GenomePage.cgi?org=ntzm01)

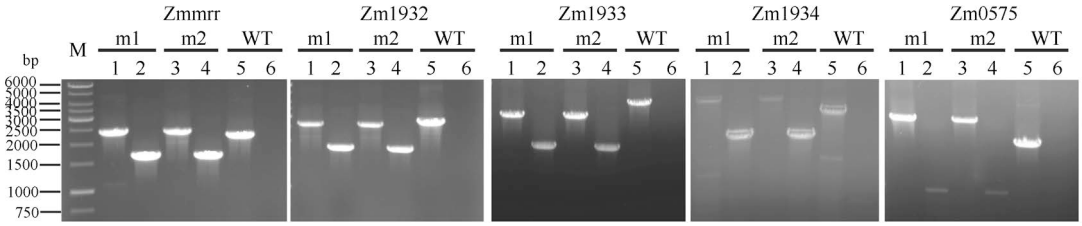

Fig. 2 Confirmation of R-M defective mutants in *Z. mobilis* by PCR

M, GeneRuler 1 kb DNA ladder (Fermentas); m1 & m2: 2 strains from each mutant were chosen to confirm the gene inactivation; WT, wild type strain ZM4. Lane 1, 3, 5: PCR amplicons using nest primer pairs; lane 2, 4, 6: PCR amplicons using forward primer at 5' regions of corresponding R-M gene and reverse primer of *cat* gene cassette

nest primer pairs were sequenced and the results confirmed the inactivation of R-M genes (results not shown).

3.2 Stability of R-M mutants

After 10-d continuous growth under no selective pressure, each mutant R-M gene still conferred the resistance to 100 mg L^{-1} Cm. However, the resistant capabilities were in variety among the five mutants. Zm1932 had the highest survival rate, with its CFU numbers on RM with or without the antibiotic keeping comparable; Zm1933 and Zmmrr had moderate survival percentages, 70% and 56% of colonies surviving under the antibiotic pressure, respectively; however, Zm1934 and Zm0575 had poor survival in the selective medium, only 7% and 20% of colonies surviving under the antibiotic pressure, respectively. By contrast, when re-supplemented with the antibiotic after 10-d sub-growth under no selective pressure, all the 5 mutants in liquid medium under selective pressure were able to grow again though their growth was more slowly than that under continuous selective pressure. These results indicated that the selective pressure was required to keep robust growth of these mutants, and mutant Zmmrr and Zm1933 were fairly stable to some extent in genetics.

3.3 Various effects of R-M system mutagenesis on genetic transformation efficiency

Inactivation of ZMO1933 and ZMO0028 (*mrr*) significantly improved the transformation efficiency of ZM4 when methylated pBBR1MCS-tet isolated from *E. coli* strain DH5α was introduced into cells (Fig. 3). The electroporation efficiency of this methylated DNA in ZM4 was 150 CFUs μg^{-1} DNA, while 400 and 2 600 CFUs μg^{-1} DNA in Zm1933 and Zmmrr, in which the transformation efficiency increased by up to 2 folds and 17 folds, respectively. In contrast, the transformation efficiency of the methylated DNA significantly decreased to only 24 CFUs μg^{-1} DNA when gene ZMO0575 was inactivated. No remarkable influence of inactivation of ZMO1932 and ZMO1934 on the transformation efficiency was observed (correspondingly 120 and 240 CFUs μg^{-1} methylated DNA).

The transformation efficiency with the unmethylated plasmid extracted from *E. coli* JM100 was also investigated (Fig. 3). The transformation efficiency using the unmethylated plasmid DNA into *Z. mobilis* was much lower than using the methylated DNA. For instance, introducing unmethylated pBRR1MCS-tet into ZM4 resulted in a nearly 7-fold decrease of transformation efficiency (only 20 CFUs μg^{-1}DNA). However, interruption of ZMO0028 (*mrr*), ZMO1933 and ZMO1934 still improved the transformation efficiency when the unmethylated plasmid was introduced, and the corresponding transformation efficiencies were 580, 180 and 120 CFU μg^{-1} DNA, respectively. The transformation efficiency was noticed to increase by nearly 30 folds in Zmmrr. The other 2 mutants, ZMO1932 and ZMO0575, did not exert positive effects on the transformation efficiency with the unmethylated DNA.

These results suggested that the influences of R-M genes on the genetic transformation efficiency were in variety, and ZMO0028 was demonstrated to significantly improve electroporation efficiency with either methylated or unmethylated DNAs (Fig. 3).

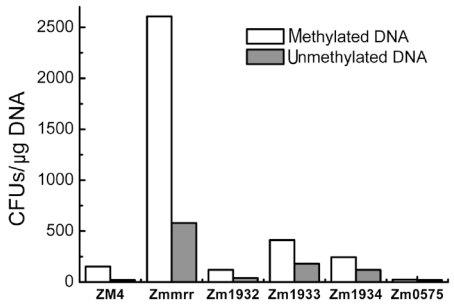

Fig. 3 Electroporation efficiencies of R-M defective mutants in *Z. mobilis*

3.4 Growth and fermentation analysis of R-M defective mutants

The fermentation at the concentration of 20 g L^{-1} glucose showed that R-M defective mutants did not exert dramatic influences on the phenotype. In R-M mutants as well as ZM4, glucose was quickly consumed during early fermentation stage and exhausted during 8-12 h, resulting in trace amounts of glucose remained when the fermentation finished (less than 100 mg L^{-1}, Fig. 4a-f). Although growing profiling of mutant Zm1934 and Zm0575 was different from that of other mutants and WT (Fig. 4e-f), the maximal cell densities of all the 5 mutants and WT reached nearly 4.0 at $OD_{600\ nm}$ when the fermentation was completed. These results demonstrated that the biomass accumulation of each strain was comparable, which indicated that interruption of R-M genes had no deteriorative effect on the cell growth.

Ethanol yield of each mutant showed no significant differences in comparison with WT (Fig. 4). The maximal ethanol yield of WT reached 9 g L^{-1}, while the ethanol yields of mutants, up to 7 g L^{-1}, were slightly lower than the WT (Fig. 4b-f).

To shed light on the effects of high concentration of glucose on the growth and ethanol yield, Zm1933, which displayed relatively high stability among the R-M mutants tested, was grown in RM containing either 100 or 200 g L^{-1} glucose. As shown in Fig. 5a, increasing concentrations of glucose led to declined growth of Zm1933 and ZM4. Compared with the fermentation at 20 g L^{-1} glucose, moderate concentration of

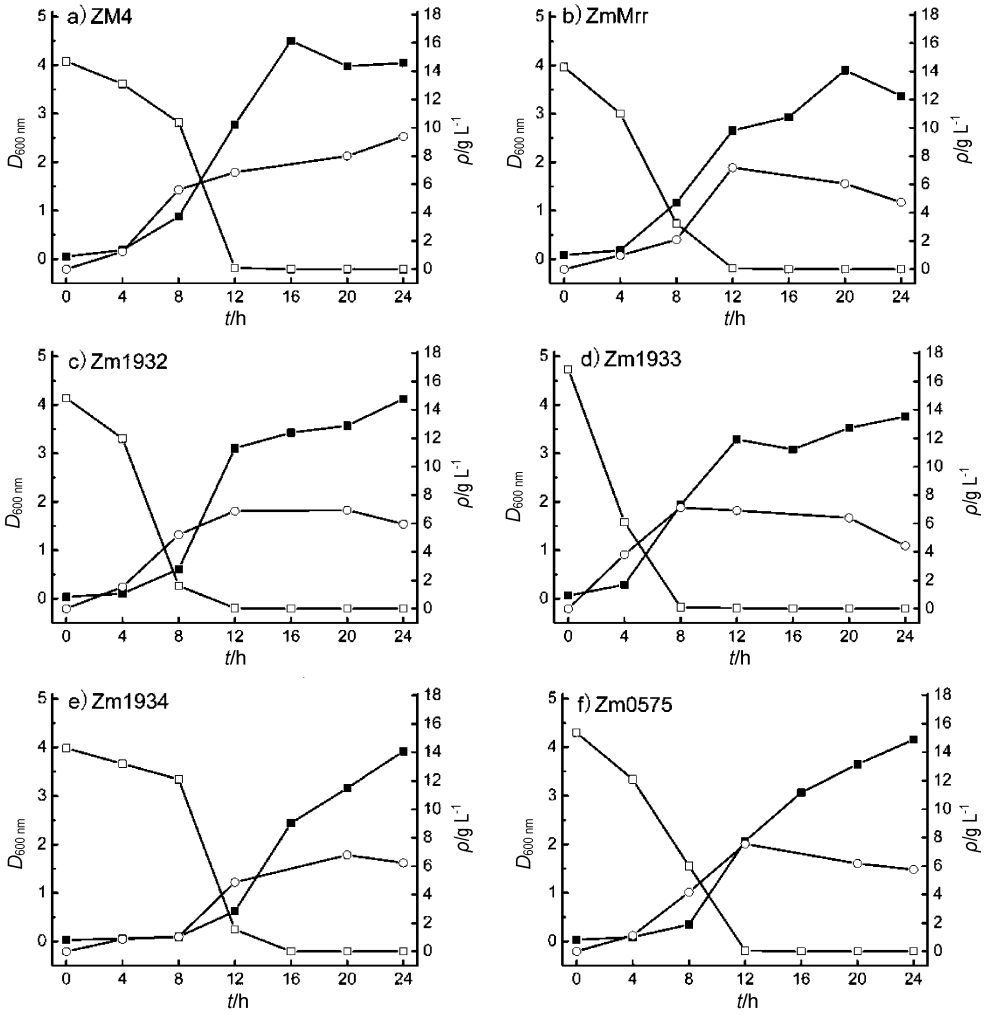

Fig. 4 Fermentation at 20 g L^{-1} glucose of R-M defective mutants

Close square: Cell density ($D_{600\ nm}$); open square: residual glucose amount in the culture; open circle: ethanol yield

glucose (100 g L^{-1}) affected the growth of Zm1933 more than that of ZM4, while higher concentration of glucose (200 g L^{-1}) suppressed both.

High concentration of glucose also caused delayed glucose utilization. Both ZM4 and Zm1933 consumed most carbon source after 24 -h fermentation using 100 g L^{-1} glucose, 12 h delayed than with fermentation using RM. Whereas a 36-h delay occurred when 200 g L^{-1} glucose was used (Fig. 5b-c), both strains almost exhausted glucose at 48 h in this case, which suggested that R-M mutants might follow the same consuming trend as WT.

Fig. 5d showed that ethanol yield coupled neither with the cell growth nor glucose utilization rate at high concentrations of glucose. Interestingly, the ethanol production presented different profiling between the mutant (Zm1933) and WT during the fermentation with 100 g L^{-1} glucose, whereas the glucose utilizing profiling of both strains showed difference with fermentation using different glucose concentration. No matter how different the strains showed during the fermentation, the maximal ethanol production of the mutant Zm1933 finally raised to the same level (nearly 50 L^{-1}) as WT using either 100 or 200 g L^{-1} glucose (Fig. 5).

4 Discussion

Studies on genetic or molecular biology are growing in *Z. mobilis* [28-29]. However, in order to deeply understand and utilize this ethanologen in industry, more genetic modification strains are required. A prerequisite is to develop high and stable transformation/recombination tools. In the present study, several R-M mutants were created, aiming at investigating the impacts of R-M systems on genetic transformation or integration of foreign DNA as well as the phenotype.

Annotations by REBASE database revealed 11 putative R-M genes in the genome of ZM4 (http://rebase.neb.com/cgi-bin/reb_get.pl), and they were classified into type Ⅰ, Ⅱ, Ⅳ and Ophan M. We initially tried to clone all of them, but unfortunately only 5 of them finally got to be inactivated. Inactivation of the R-M system genes caused various effects on genetic transformation of replicative plasmid in *Z. mobilis*. For instance, defect of ZMO0028 (*mrr*) and ZMO1933 was able to increase the electroporation efficiency, whereas defect of ZMO0575 decreased the efficiency; however, interruption of ZMO1932 did not influence electroporation with either the methylated DNA or the unmethylated DNA, thus causing doubt about its nature as an R-M system gene. On the other hand, R-M defective mutants tested in this research showed different transformation efficiencies with methylated plasmid DNAs, although these efficiencies were much lower than unmethylated DNAs. The reasons for influencing the transformation efficiency might be the characteristics of R-M systems. Table 3 showed that ZMO1933 and ZMO1934 belong to type I R-M systems, ZMO1934 encoding M subunit while ZMO1933 encoding S subunit. In this study, the depletion of ZMO1933 improved increased transformation efficiency, possibly because the mutation of R subunit encoded by ZMO1933 led to incorrect recognition of foreign DNA sequence. Consequently the foreign DNAs could not be excised by R

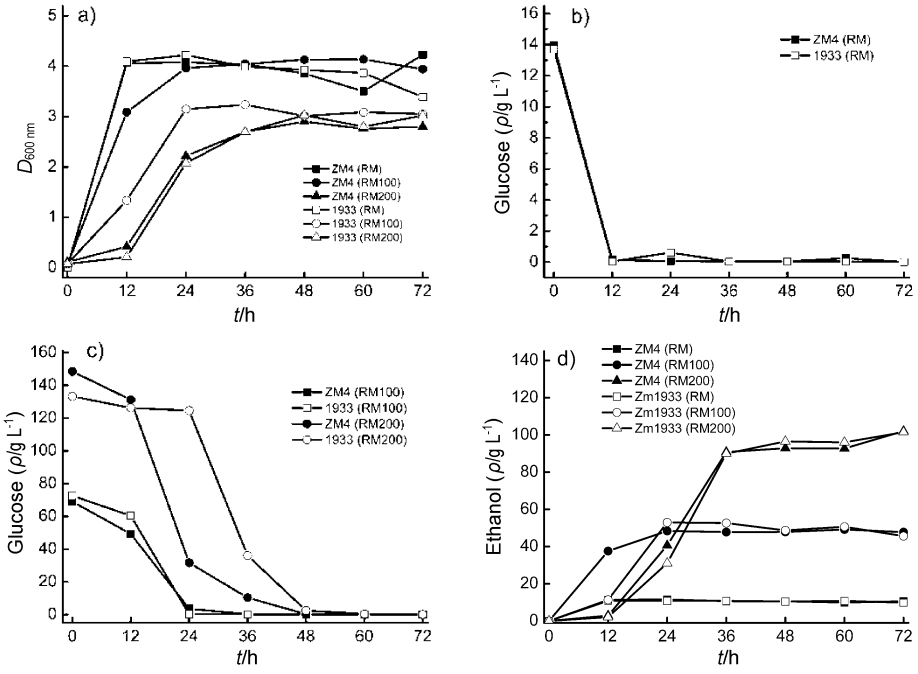

Fig. 5 High glucose fermentation in *Z. mobilis*

a) Growth of ZM4 and Zm1933; b) Glucose utilization during fermentation using 20 g L^{-1} glucose; c) Glucose utilization during fermentation at high concentration of glucose; d) Ethanol yield of ZM4 and Zm1933 under different concentration of glucose. RM: fermentation in RM medium; RM100: RM medium with glucose at concentration of 100 g L^{-1}; RM200: RM medium with glucose at concentration of 200 g L^{-1}

subunit of type I system, and more intact foreign DNAs ensured increased transformation efficiency. M subunit functions in DNA modification (*e.g.*, methylation) and inactivation of ZMO1934 probably caused native as well as foreign DNAs to be methylated incorrectly. Therefore, the unmethylated DNAs from JM110, a *dcm* and *dam*- deficient strain, were prevented from recognition and destroy in mutant Zm1934. Gene ZMO0028 encodes for type IV R-M system, this system specifically recognized and then excised methylated DNAs, thereby its inactivation improved the transformation efficiency of methylated DNAs. So far it is still not clear which type of R-M system ZMO0575 belongs to. Unlike other R-M systems, by contrast, its inactivation affected on the transformation of methylated rather than unmethylated DNAs. In *Helicobacter pylori*, disruption of *Hpy*C1I R-M system was reported to make modified DNAs susceptible to digestion by R-M systems [30], implying that ZMO0575 might work in a similar way in *Z. mobilis*. The different transformation efficiencies indicated that these R-M genes functions in various ways in *Z. mobilis*. Although several documents provided clues, the mechanisms how R-M systems regulate introduction of foreign DNA remain unknown. For example, Blakely *et al*. (2006) suggested unmodified DNA in the bacterial cells was protected by either CIpXP-mediated degradation of type I R-M systems or constitutive expression of *lar* gene in a Rac defective prophage [20]. Zheng *et al*. (2010)also reported that a *mrr*-like and modification-dependent restriction endonuclease, *Msp*JI, has a unique character to cut small DNA fragment containing modified sites out from genomic DNA [31]. Given the R-M systems to bind and modify DNA and then to impose restriction to DNA sequence, it was supposed that the inactivation of R-M systems could possibly alleviate/enhance restriction activity in the host, and then modulate recognition of modified DNA sequence or induce expression of regulatory pathway to protect/damage those foreign DNAs. Another phenomenon remaining to be investigated in the future is various stabilities of the tested R-M mutants. However, lack of knowledge on R-M system regulatory network is a major obstacle to understand why some R-M mutants were more stable in genetics than others. In the present study, results from transformation efficiency and stability test indicated that mutants Zmmrr and Zm1933 might serve as good host strains to performance genetic manipulations.

This study suggested that single gene activation of R-M systems did not influence main phenotypes in *Z. mobilis*. According to previous researches, *glc* operon was revealed to be responsible for the performance of the bacterium at high concentration of glucose [32-33]. Enzymes such as pyruvate decarboxylase, alcohol dehydrogenase, and the genes (pathways) involved in the metabolism of glucose or controlling redoxiation of intracellular oxidation stress have been reported to be responsible for the ethanol yield [34-37]. There was no evidence that R-M systems could influence these main traits in *Z. mobilis*, although the inactivation mutants of the R-M systems did have slight influences on the growth, glucose utilizing and ethanol yield.

5 Conclusion

In summary, some R-M defective mutants like Zmmrr and Zm1933 could serve as promising hosts to investigate genetic or molecular events in *Z. mobilis,* because of their genetic stabilities, improved genetic transformation efficiency as well as the minor influences on cell phenotypes. Further studying on the R-M system will contribute not only to exploiting more genetic or molecular manipulating tools, but also to understanding gene functions in *Z. mobilis*.

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