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Full Length Research Paper

Comparison of the electro transformation of plasmids and plasmid stability between *Zymomonas mobilis* ZM4 and CP4

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Four different plasmids were electro transformed into *Zymomonas mobilis* ZM4 and CP4, two important ethanol-producing strains. The results showed that the best source strain for preparing plasmids was the transformed host strain itself, and *Escherichia coli* JM110 as the source strain could yield significantly higher transformation efficiencies than Top10. The optimal recovery time of transformed ZM4 or CP4 cells to obtain maximum number of transformants and highest transformation efficiency was 11 h for pZB21-mini, pZB21 and pZA22, but 24 or 20 h for pBBR1MCS-2. The optimal electric field strength for pZB21-mini was 13.25 kV /cm in ZM4 and 14.0 kV /cm in CP4.But for pZA22 and pBBR1MCS-2, it was 11.75 kV /cm in ZM4 and 12.5 kV /cm in CP4; for pZB21, also 12.5 kV /cm in CP4.These plasmids were shown to be more stable in ZM4 than in CP4 by serial transfer to antibiotic-free medium and the 3 plasmids were more stable than pBBR1MCS-2. The results will help to support the genetic and biotechnological research of *Z. mobilis* by providing information about some of the most important factors that influence the transformation of ZM4 and CP4, and also providing insights into the similarities and differences in their restriction-modification (R-M) systems.

Key words: *Zymomonas mobilis*, plasmids, electroporation, transformation, plasmid stability

INTRODUCTION

Zymomonas mobilis is a gram-negative bacterium and an attractive and important ethanologen for cost-competitive ethanol production. ZM4 (ATCC31821) and CP4 (NRRL B-14023) are the important *Z. mobilis* strains, which were isolated from sugar cane juice. ZM4 was derived from CP4 and was proved to have better ethanol-producing performance (Rogers et al., 1982; Yablonsky et al., 1988; Joachimsthal et al., 1999; Lawford et al., 2001). Thus, ZM4 was the first to be genome-sequenced among the *Z. mobilis* strains, the results of which have provided insights into many of its characteristics (Seo et al., 2005; Smith, 2007).

Although, many papers have been reported about *Z. mobilis*, current knowledge about the molecular biology and genetic engineering methods of ZM4 and CP4 is still limited (Okamoto and Nakamura, 1992; Jeon et al., 2002; Jeon et al., 2005; Seo et al., 2005; Smith, 2007; Rogers et al., 2007; Hauser et al., 2009; Yang et al., 2010). In addition, some papers have reported differences between ZM4 and CP4, but they have generally concentrated on their ethanol-producing performance and biological characteristics related to the ethanol-producing performance (Rogers et al., 1982; Joachimsthal et al., 1999; Lawford et al., 2001). There was also a study on the difference in cryptic plasmids of ZM4 and CP4 by analysis of the profiles and restriction digest patterns of the plasmids (Yablonsky et al., 1988). As for the methods of genetic manipulation, the electrotransformation of plasmids in ZM4 or CP4 has been reported, but the electroporation data are still limited and incomplete (Okamoto and Nakamura, 1992; Jeon et al., 2002; Jeon et al., 2005). Further, no paper has reported the

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Table 1. Bacterial strains and plasmids used.

Strains/plasmids	Essential properties	Origin/references
Z. mobilis CP4 (NRRL B-14023)	Wild strain	Yablonsky et al.,1988
Z. mobilis ZM4 (ATCC31821)	Wild strain, Genome-sequenced	Rogers et al.,1982 EMBL:AE008692
E.coli Top10	F⁻ *mcr*A Δ(*mrr-hsd* RMS-*mcr*BC) ϕ80 *lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*Δ139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G	Invitrogen
E. coli JM110	*rps*L (Str^r) *thr leu thi*-1 *lac*Y *gal*K *gal*T *ara ton*A *tsx dam dcm sup*E44 Δ(*lac-pro*AB) [F´ *tra*D36 *pro*AB *lac*I^q ZΔM15]	Invitrogen
pZB21-mini	Shuttle vector, *tet*, 3082bp	In our lab
pZB21	Shuttle vector, *amp*, *cml*, *tet*, 5930bp	Zou et al.,2006
pZA22	Shuttle vector, *cml*, *tet*, 6994bp	Misawa et al.,1986
pBBR1MCS-2	broad-host-range plasmid, *kan*, 5144bp	Kovach et al.,1995, GenBank No U02374

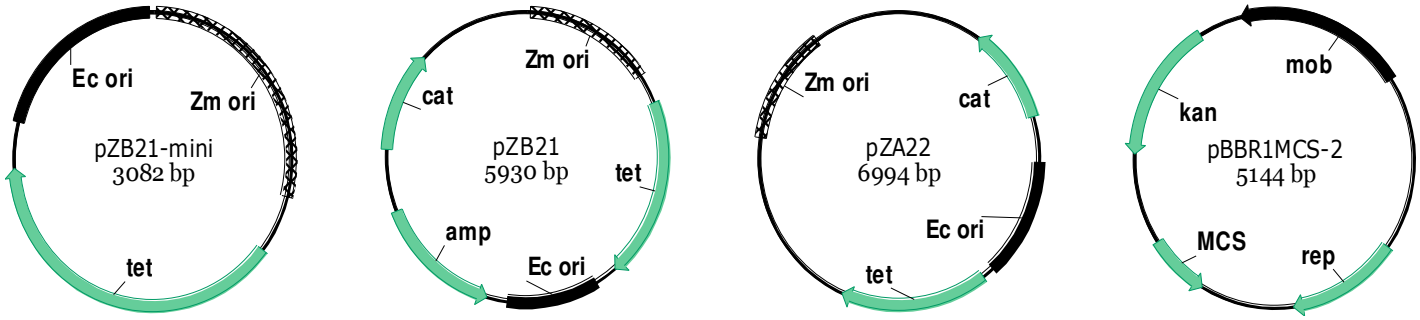

Figure 1. Diagram illustrating plasmids pZB21-mini, pZB21, pZA22 and pBBR1MCS-2. *E. coli* origins of pZB21-mini and pZB21 were from pBR328.*E .coli* origin of pZA22 was from pACYC184. *Z. mobilis* origin was from pZM2. *tet*, tetracycline-resistance gene; *cat*, chloramphenicol-resistance gene; *amp*, ampicillin-resistance gene; *kan*, kanamycin-resistance gene; *mob*, required for plasmid mobilization; *rep*, required for plasmid replication.

comparison of the electrotransformation of plasmids and plasmid stability between ZM4 and CP4 (Okamoto and Nakamura, 1992; Jeon et al., 2002; Jeon et al., 2005).

In this work, *Z. mobilis* ZM4 and CP4 were transformed with four different replicative plasmid vectors and the influences of some key electrical and biological parameters on the transformation efficiency were studied. Further, the plasmid stabilities in the resultant recombinant strains were investigated.

MATERIALS AND METHODS

Bacterial strains, plasmid constructs and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *Z. mobilis* CP4 and ZM4 were transformation recipients and also used for plasmid preparation. *E. coli* strains Top10 and JM110 were used for plasmid preparation and Top10 for recovery of plasmids from *Z. mobilis* CP4 and ZM4 transformants.

In this work, four different plasmids were used pZB21-mini and pZB21 were constructed in our laboratory (Figure 1) (Zou et al., 2006). pZB21 was constructed from pBR328 and included a replicon region of the natural plasmid pZM2 from *Z. mobilis* ATCC10988 (Zou et al., 2006). The *Bsp*T104I and *Nco*I restriction enzyme sites were introduced to the ends of PCR amplified fragment of the region of 22-3095 bp of pZB21. After digestion with *Bsp*T104I and *Nco*I, the fragment was blunted by T4 DNA polymerase and then self-ligated. The resulting recombinant plasmid was named pZB21-mini.

Z.mobilis CP4 and ZM4 were anaerobically grown overnight in a rich medium (RM) (2% D-glucose, 1% yeast extract, 0.2% KH_2PO_4, pH 6.0) in stationary cultures at 30℃ (Okamoto and Nakamura, 1992). Cultures on RM agar plates (1.8% agar) were anaerobically

Table 2. Effect of plasmid source on the transformation efficiency of CP4 and ZM4 with pZB21-mini.

Host strain	CP4				ZM4			
Strain for preparing plasmids	Top10	JM110	CP4	ZM4	Top10	JM110	CP4	ZM4
Transformants /µg DNA	84	3128	125580	912	245	9014	9915	92027
Ratio of transformants /µg DNA	1	37.2	1495.0	10.9	1	36.8	40.5	375.6
Ratio of transformants /µg DNA		1	40.1	0.3		1	1.1	10.2

Cultures OD_{600nm} = 0.40; Field strength, 13.25 kV /cm; recovery time, 14 h; plasmid added, 1 µl (100 ng /µl).

incubated at 30℃ for 2 to 4 days. *E. coli* was grown in LB medium (1% tryptone, 1% NaCl and 0.5% yeast extract), with shaking at 190 rpm at 37℃. Solid media were obtained by adding 1.8% (w/v) agar. Antibiotics, when needed for genetic selections or plasmid maintenance, were added at the following concentrations: tetracycline (Tc) 17.5 µg /ml for *Z. mobilis* and 15 µg /ml for *E. coli*; kanamycin (Km) 310 µg /ml for *Z. mobilis* ZM4, 250 µg /ml for *Z. mobilis* CP4 and 50 µg /ml for *E. coli*.

Plasmid preparation and PCR

When used for the source of plasmid DNA test, pZB21-mini or pBBR1MCS-2 was extracted from 4 host strains, namely, Top10, JM110, CP4 and ZM4 with the plasmid extract kit (Wizard Plus Midipreps DNA Purification system, Promega).The 4 preparations of each plasmid were kept as standardized as to its quantity and quality as possible by the same conditions and manipulations, for example, the similar electrophoretic band pattern, transformation efficiency into DH5α and concentrations with each other. Finally, all preparations were controlled at the concentration of 100 ng /µl.

When used for the recovery time of transformed ZM4 or CP4 cells and electric field strength test, pZB21-mini, pZB21, pZA22, and pBBR1MCS-2 were all prepared from JM110 with the plasmid extract kit (Wizard Plus Midipreps DNA Purification system, Promega) at the final concentration of 500 ng /µl.

General molecular biology procedures were performed according to standard protocols (Ausubel et al., 1999; Sambrook and Russel, 2001). All colony PCR reactions were performed in a TGradient Thermocycler (Biometra,Germany) using *Taq* (Takara) and/or *Pfu* (Takara) DNA polymerases.

Electroporation protocol

Cultures of *Z. mobilis* at early-log phase (optical density at 600 nm,OD_{600}=~0.40) were centrifuged, washed then resuspended in sterile ice-cold 10% glycerol at $1/100^{th}$ of culture volume (100×) to yield final 10^{10} cells /ml concentrations. Freshly prepared competent cells were divided into 120 µl aliquots and plasmid extracts were added. The 2 mm-gap cuvette (Bio-Rad) was kept on ice for 2 min, then placed between electrode plates of the electropulser Micropulser (Bio-Rad) and an appropriate pulse was applied. An aliquot of the suspension was mixed with 0.8 ml of preheated RM and kept for 3 to 28 h at 30℃. At the end of this incubation, the cells were appropriately diluted or concentrated in RM medium and plated on RM selective agar plates. Colonies appearing on RM selective agar plates after 48 to 96 h at 30℃ were replicated onto the same selective agar plates. Transformants from the replica plates were further characterized by colony PCR, plasmid isolation, electrophoretic characterization, and the extracted plasmids were transformed back into *E. coli* Top10 by chemical transformation. Transformation efficiency was calculated as the number of Tc- or Km- resistant transformants per µg of plasmid DNA added. The control samples without DNA also underwent the same protocol, but they did not undergo an electroporating pulse.

Plasmid stability

Cells of *Z. mobilis* harboring exogenous plasmids were grown to the stationary phase in RM medium containing appropriate antibiotics. Aliquots of each culture were inoculated at 1% (v/v) into antibiotic-free RM medium, and then cultured at 30℃ for 16 h, corresponding to the stationary phase. They were serially transferred to the antibiotic-free medium twelve times at 16 h internals. At the end of the third, sixth, ninth and twelfth culture, appropriate dilutions of the culture were spread on antibiotic-free RM agar plates and over 100 colonies appearing on RM agar plates after 48 to 96 h at 30℃ were replicated onto the selective agar plates. The colonies formed on the selective agar plates were further characterized by colony PCR, plasmid isolation, electrophoretic characterization, and the extracted plasmids were transformed back into *E. coli* Top10 by chemical transformation. The proportion of plasmid-carrying cells was determined by counting the positive colonies formed on the selective agar plates.

RESULTS

In preliminary experiments, transformations of *Z. mobilis* CP4 and ZM4 with pZB21-mini, pZB21 and pZA22, pBBR1MCS-2 by electroporation were all successful, showing that those plasmids could replicate in the 2 *Z. mobilis* strains. At the same time, their transformation efficiencies were all found to be greatly influenced by the source of the plasmid, recovery time, electrical field strength, and so on. Thus, these parameters were selected for comparative study of the transformation of CP4 and ZM4. All data were from a representative experiment that was repeated at least 3 times.

Source of plasmid DNA

As shown in Tables 2 and 3, the transformation efficiency of CP4 varied according to the plasmid source in the following order from the highest to the lowest: CP4 > JM110 > ZM4 > Top10; the transformation efficiency of ZM4 varied according to the plasmid source in the following order: ZM4 > CP4 > JM110 > Top10. Thus, the best source for preparing the plasmids was the host strain being transformed itself and this resulted in the highest transformation efficiency.

Table 3. Effect of plasmid source on the transformation efficiency of CP4 and ZM4 with pBBR1MCS-2.

Host strain	CP4				ZM4			
Strain for preparing plasmids	Top10	JM110	CP4	ZM4	Top10	JM110	CP4	ZM4
Transformants /μg DNA	60	3169	7302	1203	56	2212	2600	15027
Ratio of transformants /μg DNA	1	52.8	121.7	20.1	1	39.5	46.4	268.3
Ratio of transformants /μg DNA		1	2.3	0.4		1	1.2	6.8

Cultures OD_{600} = 0.40; Field strength, 11.75 kV /cm; recovery time, 20 h; plasmid added, 2.5 μl (100 ng /μl).

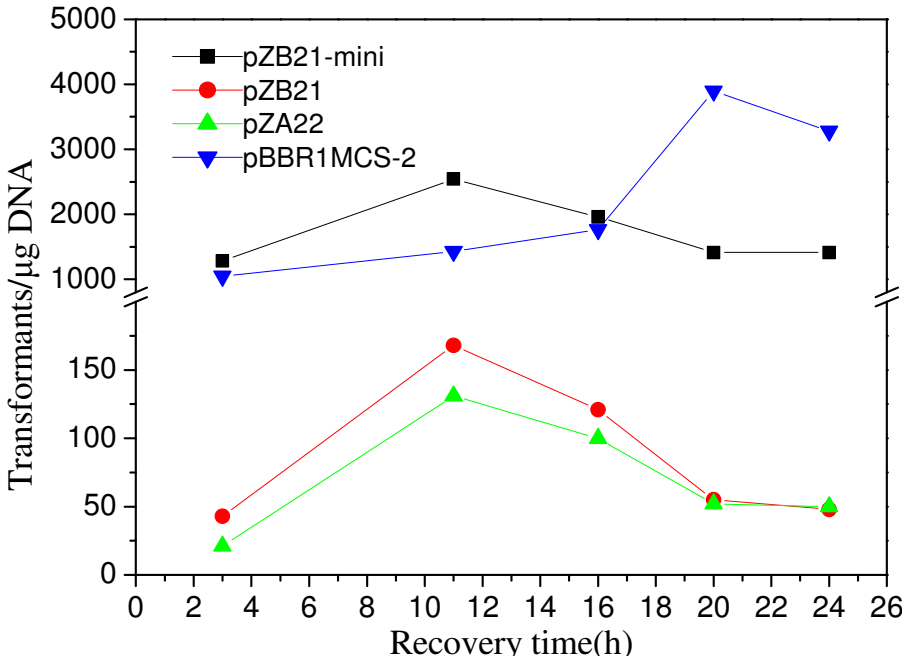

Figure 2. Effect of recovery time of transformed cells on transformation efficiency of CP4 with pZB21-mini, pZB21, pZA22 and pBBR1MCS-2.CP4 cultures OD_{600}=0.40. 1 μl (500 ng /μl). The field strength: 12.5 kV /cm. An aliquot of the suspension was plated at different recovery time. Their maximum transformation efficiencies were 2,542, 168,131 and 3,896 transformants /μg DNA, respectively.

Recovery time of transformed cells

More recovery time will generally lead to higher transformation efficiency because of multiplication of the initial transformants. The generation time of CP4 or ZM4 is about 2 h (data unpublished). But it was observed that the number of total transformants was often low or even zero especially for pZB21 and pZA22 in ZM4 when 2 h recovery time was used. At the same time, it was also observed that the number of total transformants reached maximum at some recovery time and then decreased. So the recovery time to obtain maximum number of total transformants was tested. Figures 2 and 3 show that the corresponding transformation efficiency of pZB21-mini, pZB21, and pZA22 into CP4, or pZB21-mini and pZA22 into ZM4 was highest at 11 h. The transformation efficiency of pBBR1MCS-2 was highest at 20 h for CP4 and 24 h for ZM4. Thus, the recovery time to obtain maximum number of transformants and highest transformation efficiency also varied with plasmids. These results may be related to the type of origin or replication in the plasmid.

Electric field strength

As shown in Figures 4 and 5, the optimal electric field strength of pZB21-mini, pZB21, pZA22, and pBBR1MCS-2 with CP4 as the transformation host was 14.0, 12.50, 12.50, and 12.50 kV /cm, respectively; and the optimal electric field strength of pZB21-mini, pZA22, and pBBR1MCS-2 with ZM4 as the transformation host was 13.25, 11.75, and 11.75 kV /cm, respectively. This indicates that optimal electric field strength was

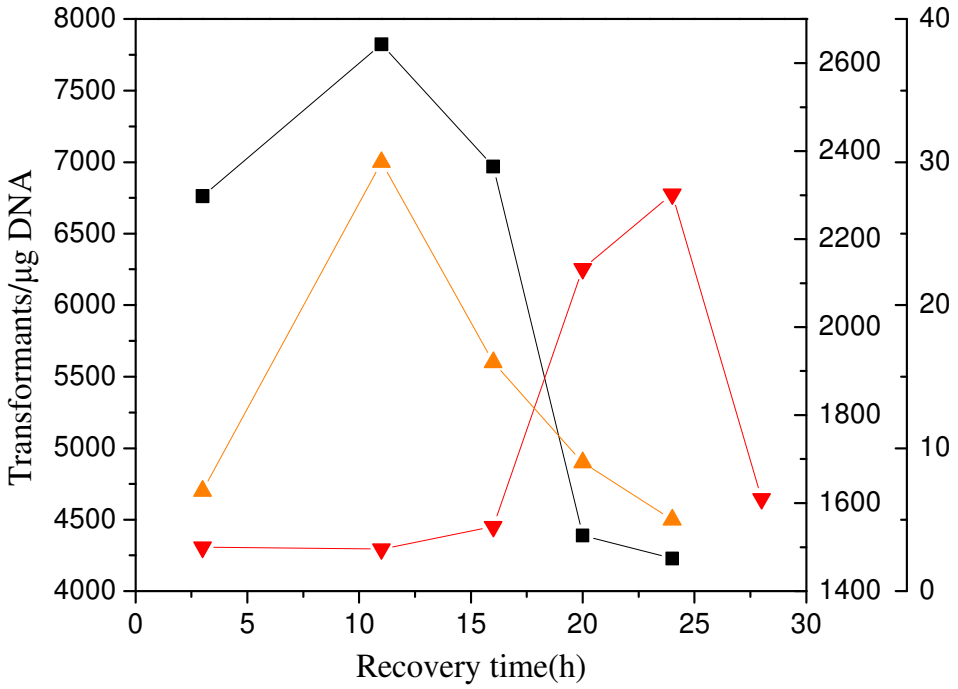

Figure 3. Effect of recovery time of transformed cells on transformation efficiency of ZM4 with pZB21-mini, pZA22 and pBBR1MCS-2. ZM4 cultures OD_{600}=0.40. 1 μl (500 ng /μl). The field strength: 12.5 kV /cm. An aliquot of the suspension was plated at different recovery time. (■) pZB21-mini, (▲) pZA22, (▼) pBBR1MCS-2.Their maximum transformation efficiencies were 7,824, 30 and 2,302 transformants /μg DNA, respectively.

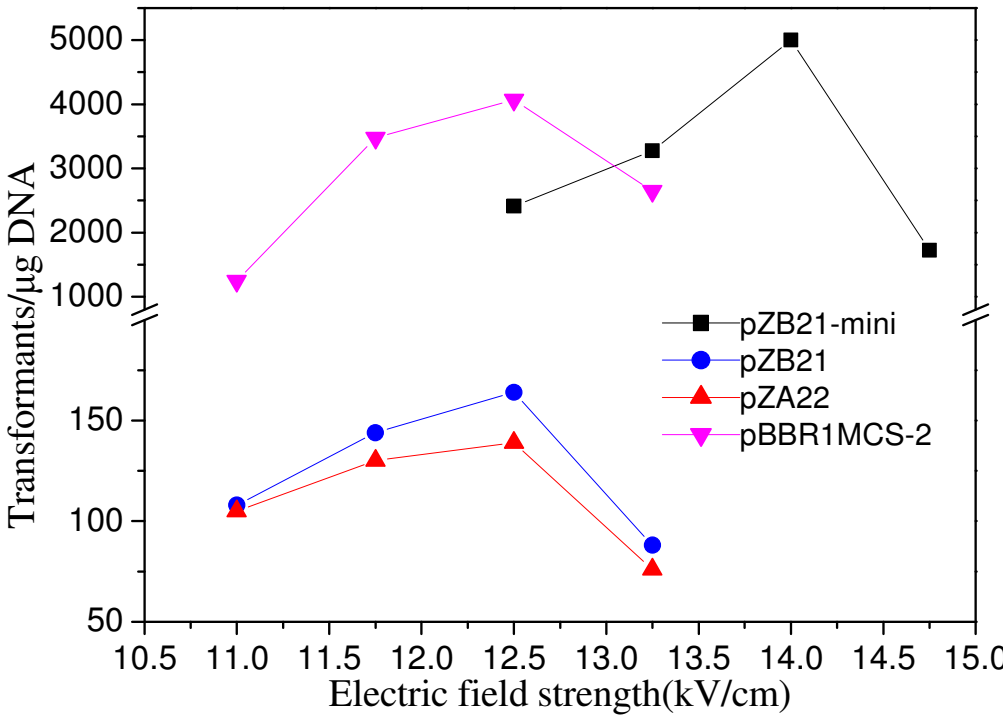

Figure 4. Effect of electric field strength on transformation efficiency of CP4 with pZB21-mini, pZB21, pZA22 and pBBR1MCS-2. CP4 cultures OD_{600}=0.40.1 μl (500 ng /μl). Recovery time:11 h, 11 h, 11 h, 20 h. Their maximum transformation efficiencies were 5,002, 164,139, and4,069 transformants /μg DNA, respectively.

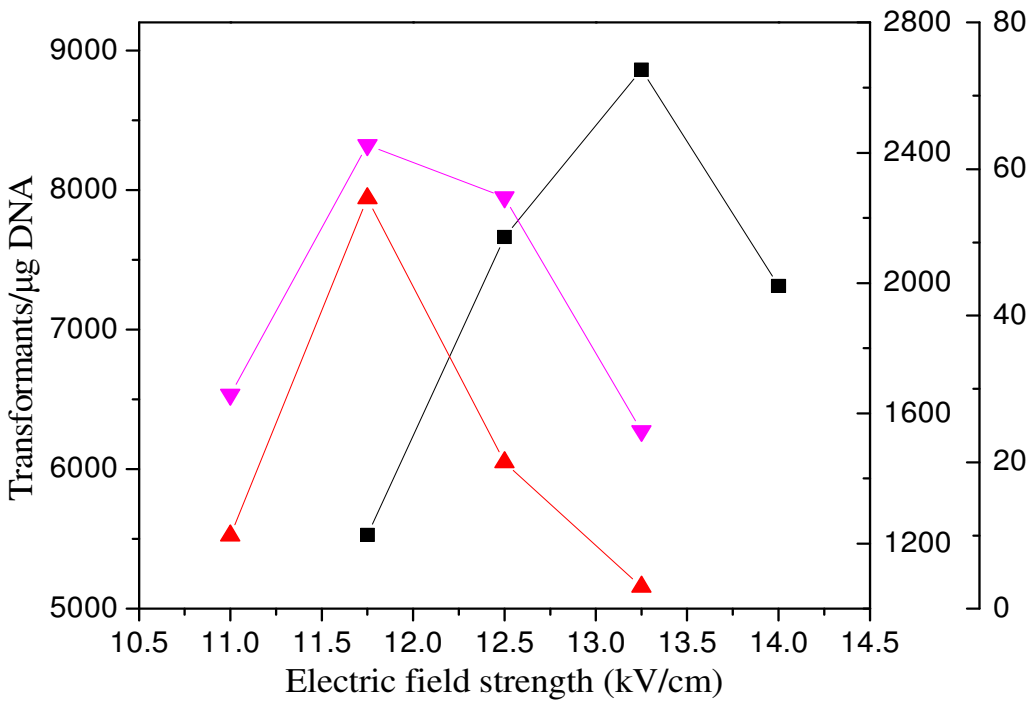

Figure 5. Effect of electric field strength on transformation efficiency of ZM4 with pZB21-mini, pZA22 and pBBR1MCS-2. ZM4 cultures OD_{600}=0.42.1 μl (500 ng /μl).Recovery time:11 h, 11 h, 24 h. (■) pZB21-mini, (▲) pZA22, (▼) pBBR1MCS-2. Their maximum transformation efficiencies were 8,861, 56, and 2,424 transformants /μg DNA, respectively.

influenced by both the kind of plasmids and the host cells. The optimal electric field strength generally decreased with increasing size of the plasmids, which was consistent with the results reported by Szostková and Horáková (Szostková and Horáková, 1998). In addition, the optimal electric field strength was generally lower with ZM4 as the host than with CP4 as the host.

Plasmid stability

Plasmid stability of the recombinant strain is significantly important for its utilization in the industry. As shown in Figure 6, plasmid stability varied with the kind of plasmids and host cells. The 4 plasmids were all more stable in ZM4 than in CP4. The 3 shuttle vectors, namely, pZB21-mini, pZB21, and pZA22, were all more stable than the broad-host-range vector pBBR1MCS-2. Further, the stability of pZB21-mini was marginally higher than that of pZB21 or pZA22, possibly because it is smaller than the others. The generation time of CP4 or ZM4 is about 2 h (data unpublished).So one transfer implies at least 5 generation times and the final generation times were over 60.

DISCUSSION

Hitherto, no paper has reported on what makes ZM4 significantly different from CP4 despite the fact that ZM4 is derived from CP4 (Yablonsky et al., 1988). Knowledge about their differences will help us to understand more deeply and further utilize the unique model of rapid catabolism and inefficient energy conversion; and the ability to rapidly and efficiently produce ethanol from simple sugars in *Z. mobilis*.

This study has revealed some differences in optimal parameters for the electro transformation between ZM4 and CP4 and it will support their genetical and biotechnological comparative research by providing information on suitable vectors and a reliable, reproducible procedure for introducing DNA into strains. On the other hand, these results have also provided an insight into a part of their genetic differences expressed as differences in the transformation efficiencies and stability of the plasmids.

In this study, the host cells, replication origin, size of the plasmids, and source of the plasmids were shown to be the most important factors that influence the transformation frequency. In contrast, the physical and electrical parameters such as the electrical field strength and recovery time are minor factors that influence the transformation efficiencies of the different plasmids.

The data shown in Tables 2 and 3 would best exemplify the complicated relationships among the 3 factors, namely, the host cells, replication origin and size of the plasmids, and source of the plasmids. For a specific plasmid, the source strain as well as the transformed host

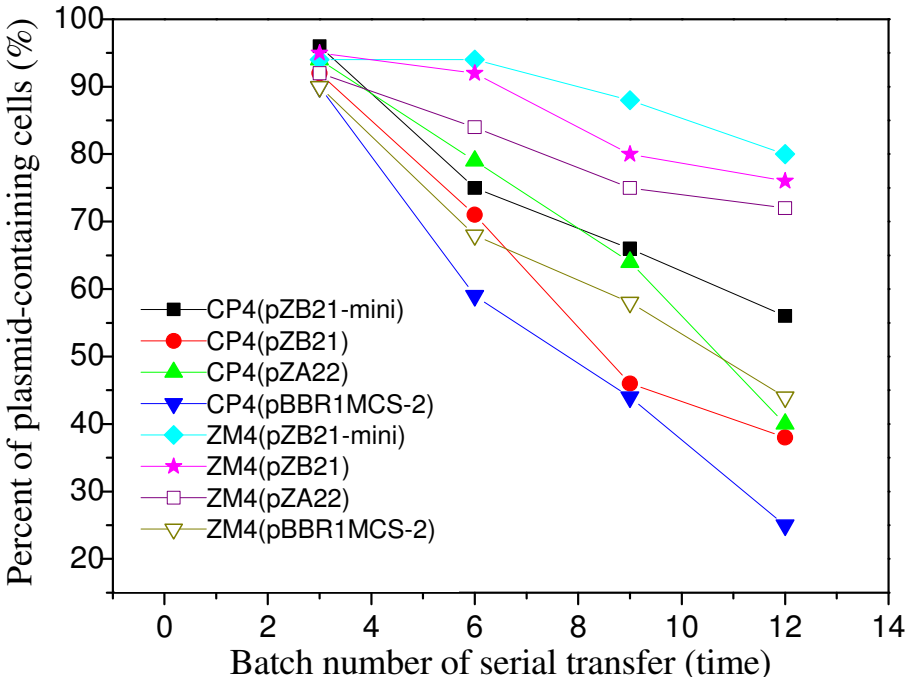

Figure 6. The stability of plasmids in CP4 and ZM4.

strain means a specific kind of genetic restriction-modification (R-M) system. The transformation efficiencies of the plasmids in the transformed host strain decrease while the differences in the restriction-modification (R-M) system between the source strain and the transformed host strain increase. Thus, the R-M systems in ZM4 and CP4 have been proved to differ significantly by the fact that the transformation efficiencies of pZB21-mini or pBBR1MCS-2 prepared from ZM4 greatly differed from those of pZB21-mini or pBBR1MCS-2 prepared from CP4 regardless of the identity of the transformed host strain. In addition, it seems that the degree of R-M of pZB21-mini from ZM4 by CP4 was much higher than that of pZB21-mini from CP4 by ZM4, because the ratio of 125,580 to 912 transformants /μg DNA is 137.7 and the ratio of 92,027 to 9,915 transformants /μg DNA is 9.3 (shown in Table 2). However, this is not suitable for pBBR1MCS-2, since the ratio of 7,302 to 1,203 transformants /μg DNA is 6.1 and the ratio of 15,027 to 2,600 transformants /μg DNA is 5.8 (shown in Table 3).

On the other hand, JM110 as the source strain has been shown to yield significantly higher transformation efficiencies of plasmids than Top10 regardless of the kind of plasmids used and the host strain transformed. This implies that CP4 and ZM4 both may have strict methyl-dependent R-M systems for exogenous DNA since JM110 is *dam* and *dcm* deficient. The results of the ZM4 genome sequencing predicted the existence of *mrr* restriction system gene, and its DNA sequence homology with the *mrr* gene identified from CP4 is high (more than 97%). The *mrr* gene of CP4 encodes a methyl-dependant restriction endonuclease ZmCP4Mrr (Phillips, 2005).

The transformation efficiencies of pZB21-mini, pZB21, pZA22, and pBBR1MCS-2 prepared from JM110 were shown to vary with the tansformed host cells, replication origin, and size of plasmids. Interestingly, plasmid stability was shown to be mainly influenced by the same factors.

In conclusion, on the basis of the comparative analysis of optimal parameters of plasmids electro transformation in CP4 and ZM4, some of the similarities and differences of the genetic R-M systems between CP4 and ZM4, which mainly cause transformation difficulties, have been demonstrated. All of this enables a more detailed insight into the biotechnological properties of this important industrial bacterium. Moreover, manipulation of the genes will be feasible, allowing improved production of fuel ethanol and higher value products.

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